Changes in tear film biochemistry in end of day ocular discomfort with and without contact lenses

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Changes in tear film biochemistry in end of day ocular discomfort with and without contact lenses

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A thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

School of Optometry and Vision Science
The University of New South Wales, Sydney, Australia

And
Vision Cooperative Research Centre, Sydney, Australia

And
Brien Holden Vision Institute, Sydney, Australia

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An estimated 30% of contact lens (CL) wearers suffer from discomfort which is more common and aggravated in the evening. This work examined changes in tear film proteins and lipids, especially those previously associated with non-contact lens dry-eye or inflammation, for their diurnal variation and their association with comfort during CL wear. Comfort decreased at the end of the day, and this was exacerbated by CL wear. Simultaneous quantification of five proteins (lysozyme, lactoferrin, lipocalin 1, prolactin-induced protein and proline rich 4) in 2.5 microlitres of tears was achieved using a novel method based on selected reaction monitoring (SRM) mass spectrometry. The concentrations of prolactin-induced protein, proline rich protein 4, resolvin D1 and C3a are reported for the first time. Concentrations of lysozyme and lactoferrin, secretory phospholipase A2, bradykinin, prostaglandins, cysteinyl leukotrienes, resolvin D1, phosphatidylcholines, lysophosphatidylcholines, sphingomyelins, phosphatidylserines, cholesterol esters, triacylglycerides, wax esters and free cholesterols did not change during the day, during CLs wear and they were not associated with CL comfort. C3 and C3a levels decreased during the day from morning to evening, implying that the classical complement cascade was not associated with discomfort. An end of day decrease in phosphatidylethanolamines and an increase in the concentration of its breakdown product, lysophosphatidylethanolamines, in normal tears was established in these studies. Tear levels of cholesterol esters increased but only during CL wear. The changes in concentration of these lipids were unrelated to comfort. Lipocalin 1 and proline rich protein 4 increased over the day. An increase in prolactin-induced protein (PIP) was associated with end of day ocular discomfort in lens wear and no lens wear but did not change when comparing asymptomatic and symptomatic CL wearers. Leukotrienes B_4 level was higher in evening tears of contact lens wearers compared to non-lens wearers and its level was higher in symptomatic CL wearers compared to asymptomatic lens wearers. Overall, the results of these studies suggest that CL-related discomfort is not related to changes in many proteins or lipids in the tear film, but certain components such as PIP and LTB_4 may be partly related to comfort during CL wear.

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From the deep of my heart: thank you all.

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TERMINOLOGY/ABBREVIATIONS/CHEMICAL UNITS

abreviation	Description
µg	microgram
µL	microliter
C	complement
CE	cholesterol esters
CE	cholesterol esters
CHO	Cholesterol
CID	collisional induced dissociation
CL	contct lens wear
COX	cyclooxygenase
$CysLT_1$	called cysteinyl leukotrienes receptor 1
$CysLT_2$	called cysteinyl leukotrienes receptor 2
Da	Dalton
EGF	epidermal growth factor
EGFR	epithelial growth factor receptor
EIA	enzyme immuno assay
ELISA	Enzyme-Linked Immunosorbent Assay
ESI	Electrospray Ionisation
fmol	femtomol
GC/MS	Gas Chromatography Mass Spectrometry
GCDFP-15	gross cystic disease fluid protein 15
HETE	hydroxyeicosatetraenoic acid
HMWK	high-momecular-weight kininogen
HPETE	hydroperoxyeicosatetraenoic acid
HPLC	High Performance Liquid Chromatography
HREA	the Human Research Ethics Advisory Panel

ICATs	isotopecoded affinity tags
IFN	interferons
Ig	Immunoglobulins
IL	interleukins
iTRAQ	isobaric tag for relative and absolute quantitation
LOX	lipoxygenase
LPC	lysophosphatidylcholines
LPE	lysophosphatidylethanolamines
LT	leukotriene
LTB4	Leukotriene B4
LTC4	Leukotriene C4
LTD4	Leukotriene D4
LTE4	Leukotriene E4
m/z	The combination of mass to charge ("transition")
MALDI	Matrix Assisted Laser Desorption/Ionisation Proteolysis
MMP	Matrix metalloproteinase
mRNA	messenger ribonucleic acid
MS	Mass spectrometry
MW	Molecular weight
nCL	non-contact lens wearers
NCL	no contact lens wear
ng	nanogram
OHFA	(O-acyl)-ω-hydroxy fatty acids
PAGE	polyacrylamide gel electrophoresis
PC	phosphatidylcholines
PE	phosphatidylethanolamines
pg	picogram
PG	prostaglandin

PIP	Prolactin-inducible protein
PL	phospholipids
PMN	polymorphonuclear cells
PRP	Lacrimal proline rich protein
PS	phosphatidylserines
Q	quadrupole
Q1	First quadrupole
Q2	Second quadrupole
Q3	Third quadrupole
RvD1	resolvin D1
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SDS-PAGE	standard deviation
SELDI-TOF	Surface-enhanced laser desorption/ionisation time of flight
SEM	standard errors of mean
sIgA	secretory immunoglobulin A
SM	sphingomyelins
sPLA2	secretory phospholipase A2
SPSS	Package for the Social Sciences
SRM	Selected Reaction Monitoring
SS	Sjögren's syndrome
TAG	triacylglycerides
TG	Triglycerides
TGF-b	transforming growth factor-b
TLC	Thin Layer Chromatography
TNFR2	receptors for tumour necrosis factor-α
TNF-α	tumour necrosis factor-α
TXA_2	Thromboxane A2
WE	wax esters

Chapter 1: Introduction and background

1.1 Introduction

For almost half a century soft contact lenses (CLs) have been used for correction of refractive errors[1] with an estimated 120 million wearers world-wide.[2] CLs provide a wider field of view, reduced aberration in the peripheral fields and less limitation in the movement of the eyes, therefore a more natural vision compared to spectacles, especially in high ametropia,[2] where the spectacle frame and the optics of the lenses can limit the wearer's field of clear view and the minification of images caused by the lenses can reduce quality of vision. Landmark advances in lens designs and materials have been achieved in recent years. Although, thousands of new lens wearers choose CLs as their preferred method for correction of ammetropia, the current rate of CL wear is five times less than the global estimated population of people who need and can afford contact lenses.[2] This results from approximately one quarter of CL wearers eventually discontinuing CL wear[3] mainly due to symptoms of dryness and discomfort.[3-12] CL-induced discomfort may be mediated by components that exist in or are released into tears during the day, rather than the characteristics or imperfections of CL materials or surfaces. By elucidating the possible differences of tear components in comfortable and uncomfortable eyes, we may be able to contribute to finding new avenues for improvement of end of day comfort, thereby reducing the number of drop-outs from lens wear.

1.2 Thesis aim and structure

This thesis presents an examination of diurnal variation of the tear film for a broad spectrum of components within the scope of dry-eye or inflammation and association of each component with subjective comfort ratings in CL wear and no lens wear to provide a better understanding of the underlying causes of end of day CL-induced discomfort. To achieve this aim ***Chapter 1***: provides an introduction to the tear film structure, tear film lipids and proteins and t role in tear film stability and ocular surface health. This chapter reviews the current literature to understand the role of selected components and provides a summary of what is currently known about the level of lipids and proteins in CL wear and CL-related inflammation and dryness. A brief discussion is provided on methods used to identify tear proteins and lipids. The literature is also reviewed for CL-related discomfort and changes in the ocular surface and tear biochemistry. ***Chapter 2***: investigates the role of diurnal changes of selected inflammatory mediators on end of day ocular discomfort with and without CL wear (study 1). ***Chapter 3***: determines the diurnal changes of arachidonic acid and eicosapentaenoic acid derivatives in CL wearers (study 2). ***Chapter 4***: introduces a new methodology for quantification of specific proteins in tears. This technique, called selected reaction monitoring mass spectrometry, is used to quantify proteins in very small volume of tear samples (study 3). ***Chapter 5***: determines diurnal changes of the selected proteins in CL wearers by using selected reaction monitoring mass spectrometry and association of the levels of measured proteins with subjective ocular comfort(study 4). ***Chapter 6***: investigates the association between subjective ocular comfort ratings and changes in the concentration of selected biochemical factors in tears of symptomatic and asymptomatic CL wearers (study 5). ***Chapter 7***: investigates the

possible changes in tear lipids of lens wearers during the day and association of the levels of measured lipids with ocular discomfort (study 6). ***Chapter 8***: summarises and discusses the results and limitations of this work and recommends future studies that can be conducted to extend this research.

1.3 Tear film

In humans, the layer of liquid coating the front part of the non-lens wearing eye is known as the pre-corneal tear film. Tears are released from the lacrimal, accessory, goblet and meibomian glands (**Figure 1:1**). Normal tears have a mean pH value similar to that of the plasma which is regulated at about 7.1 - 8.0.[13] Normal (basal) tear volume is around 5 - 10 μL,[14, 15] and the secretion rate is about 1.2 - 3.4 μL per minute,[14, 16, 17] with a total turnover rate of tears at approximately 16% per minute.[14] The thickness of the pre-corneal tear film ranges between 3 and 40 μm, depending on the method used for measurement.[18-22] Although, using a non-invasive method, the thickness of the tear film was evaluated at about 40 μm in one study,[22] these measurements have not yet been confirmed by any other study.[23, 24] More importantly this thickness of tear film has not been supported by any other study. There are four types of tears: basal, reflex (secreted following eye irritation), closed-eye tears and emotional tears (produced during emotional stress). The four tear types vary in composition and the differences are partly caused by the rate of lacrimation in each type.[25, 26] The level of total protein, for example, is significantly lower in emotional tears compared to basal and reflex tears,[27] and tear immunoglobulin A (IgA) has its highest level in closed eyes compared to basal and reflex tears.[28, 29] The composition of tear proteins may also vary depending on

sampling (tear collection) method.[30] Tear samples are frequently collected using either a microcapillary tube[31-33] or a Schirmer paper strip.[34-36] Tear film quality and composition also depends on fine regulatory mechanisms affected by neural and hormonal influences which control the secretions of the lacrimal acinar cells and also a slower type of secretion from plasma cells via the epithelium.[37, 38]

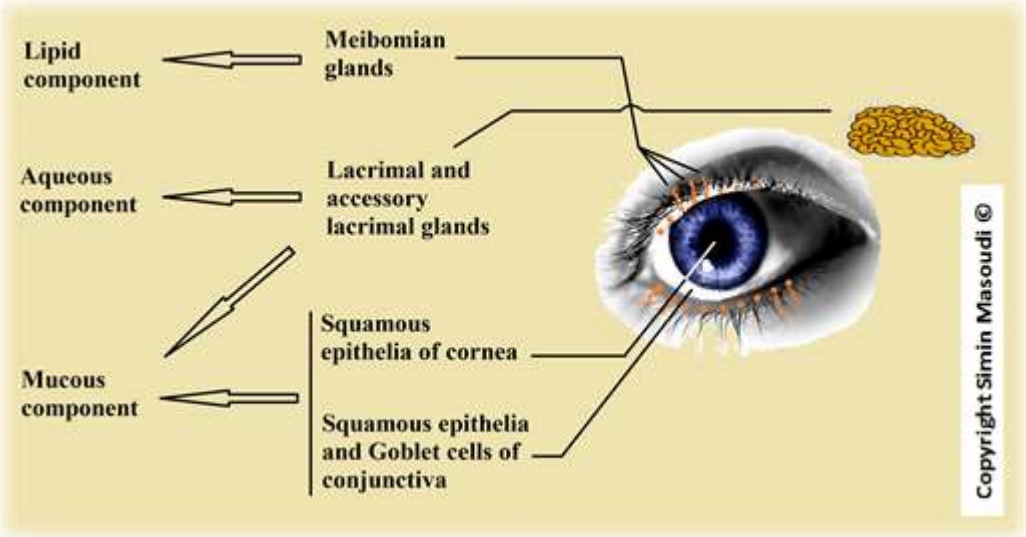

Figure 1:1 Schematic depiction of major tear film components and the glands producing them.

1.3.1 Tear film structure, function and composition

Different structural models have been suggested for the tear film. These models are still under discussion but it is generally accepted that human tears have three basic layers: an anterior lipid layer, a middle aqueous layer and a deeper mucin layer rich in glycoproteins and glycocalyx (**Figure 1:2**).[39-41] Each of these three layers serves important roles in eye health and normal vision. The tear film's main functions are lubrication of the eyelids to facilitate the blinking action, resurfacing of the ocular

surface for improved optical clarity, nourishing of the cells at the ocular surface as well as providing physical and immune protection against infection.[42-45] Thus, to maintain a healthy ocular surface it is important to maintain a stable production of tears as well as a regular turnover of the fluid. The composition of tear film has been partially elucidated, however in view of the dynamic nature of tears and the methods used for tear collection, studies may yield different results. For instance, the concentration of albumin, IgG[30] and antioxidants[46] are higher when tears are collected by Schirmer paper filters compared to capillary tubes. Also, Secretory IgA levels are higher in non-stimulated than in stimulated tears.[29] It is accepted that the main components of tears are water, lipids, proteins, mucins, electrolytes, salts, peptides and metabolites.[47-50] Therefore, it could be claimed that a healthy ocular surface is reliant on maintaining a stable and thick tear film and that any change in the tear film in terms of composition, structure, stability and volume could cause ocular discomfort.

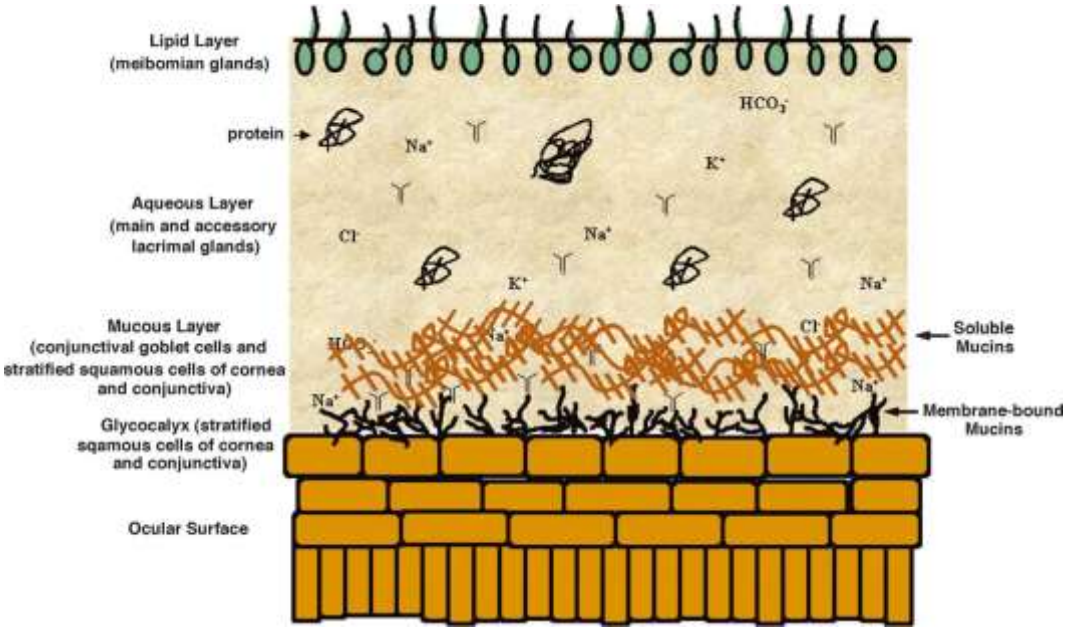

Figure 1:2 Schematic depiction of tear film demonstrating mucin, aqueous and lipids as three main layers of tear film (schematic drawing courtesy of Hodges and Dartt, Int. Rev. Cytol Regulatory Pathways in Lacrimal Gland Epithelium, 2003).

1.3.2 Tear lipids

The tear lipid layer is produced by the meibomian glands which are located in the eyelid margin.[51] Meibum, the product of meibomian glands, facilitates even spreading of lipids over the tear film.[19, 52] The tear lipid layer is about 50 - 100 nm thick[53, 54] and spreads over the aqueous layer of tear film with each blink. The melting temperature of tear lipid is around 19 - 32°C.[55] A stable lipid layer is essential to lowering the surface tension of tears over the ocular surface[56] and hence maintaining a stable tear film. The lipid layer also plays an important role in structural integrity of the tear film as it prevents tear evaporation[57] and helps with maintaining a smooth optical surface at the front of the eye.[19] The secretion of meibomian glands is controlled by rich innervation of para-sympathetic and sympathetic nerve fibres[37, 58, 59] and hormones[60] but the role of the nerves in secretion of these glans is unknown. The tear lipid layer can be

divided into a polar lipid (inner) layer and a non-polar lipid (neutral, outer) layer (**Figure 1:3**).[61] The non-polar lipid component of the tear film is a mixture of wax esters, cholesterol esters, free fatty acids, diesters and sterol esters,[62] while the polar layer consists of phospholipids (phosphatidylcholine, phosphatidylethanolamine, sphingophospholipids),[63] ceramides, cerebrosides, sphyngomyelins, triglycerides and omega-acylhydroxy fatty acids.[61, 64] Sufficient amounts of each of these lipids are essential for the effective functioning of this layer.[61, 65, 66] Increased levels of non-polar lipids, the ratio of non-polar lipids to phospholipids, and saturation level of the lipid components can increase tear film stability by increasing its elasticity.[67] Meibomian gland dysfunction can be the main cause of lipid abnormalities; however other factors such as lipid peroxidation and lipolysis or a combination of these factors can affect the functionality of this layer.[68, 69] Although the effect of CL wear on meibomian glands needs to be investigated further[70] studies have reported alterations of the lipid composition in lens wear.[71-73] It has been found that a CL can act as a foreign body and partition the tear lipid layer[74] while changing the level of some of the tear components. Glasson *et al.* found that intolerant CL wearers have significantly more secretory phospholipase A2 ($sPLA_2$) in their tears than tolerant wearers.[75] Also, the authors have reported increased concentrations of two small aldehydes, by-products of lipid peroxidation degradation in the tears of intolerant CL wearers.[75] Nichols *et al.* reported that in patients with ocular dryness related to CL wear, the pre-lens lipid layer tended to be thinner than that of subjects without dry eye.[76] A number of reports are available regarding the amount of lipid deposition on CLs as related to lens material[77-79] and one study showed a correlation between CL deposition and ocular comfort.[80] The major tear lipids are discussed in the next section.

Flush and reflex tears contain much lower levels of most lipid components than basal tears.[81] Most research on the effect of method of tear collection on lipid levels were performed on meibum rather than tears. Spatula (hard and soft expression),[82] microcapillary tubes,[83] Schirmer's test strips[2, 84] and acetate absorbent filters[85] are the most common methods used for collection of meibum and tear lipids. Each of these techniques has advantages and disadvantages. For example, microcapillary tubes are well tolerated by human donors, but provide the smallest volume of samples.[86] The Schirmer's test strip technique is considered the safest way of collecting tears but obtains enough lipids just for some techniques such as HPLC–MS.[84, 86] Collection methods such as brush or cytology micro-brush collection with immediate extraction can result in detection of different lipid classes.[87] Sample handling and storage are also very important in studying tear lipids.[88]

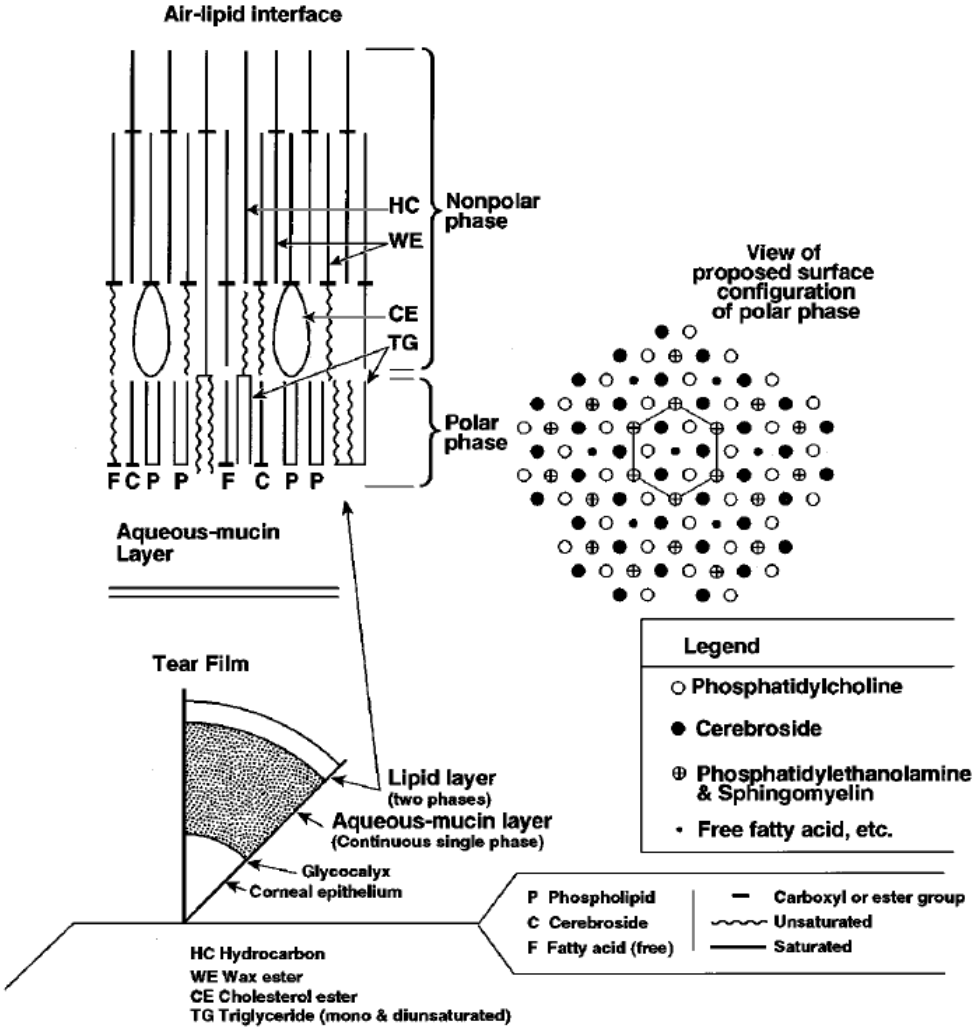

Figure 1:3 Proposed model of tear film lipid layer. Proteins are thought to be interspersed in the lipid layer (Courtesy of McCulley J.P. - The Lipid Layer, The Outer Surface of the Ocular Surface Tear Film, 2001, Bioscience Reports, peer-reviewed open access journal).

1.3.2.1 Non-polar lipids

Cholesterol

It is hypothesised that free cholesterol (CHO) plays an important role in tear stability[89] by forming a layer at the air-water interface.[90] The concentration of cholesterol in human tears is approximately 15 ± 6 µg/g.[91] A recent study reported that the concentration of free cholesterol in meibum is less than 2% of total lipids.[92]

Cholesterol levels vary along with reduced tear film stability in conditions such as keratoconjunctivitis sicca,[93] Sjögren's syndrome and chronic blepharitis.[93, 94] Furthermore, one study demonstrated that the concentration of cholesterol in the tears of CL wearing subjects was less than that of the normal eye.[95] Tear cholesterol deposition on CLs ranges between 2 and 37 µg per lens[96, 97] depending on the type of CL worn.[98]

Triglycerides

Triglycerides (TGs) are esters produced from the combination of glycerol and a short carbon chain, less than twenty carbon molecules (C20), saturated or unsaturated fatty acids.[99] TGs are hydrolysed by lipoprotein lipase, resulting in release of fatty acids and monoglycerides. These products are used by cells in metabolic reactions.[100, 101] No report was found in the literature about concentration of TGs in tears of dry eye and non-dry eye individual and their association with comfort.

Wax esters and cholesterol esters

Wax esters (WE) and cholesterol esters (CE) constitute most of the human tear lipids[237, 271] (43 ± 4% and 39 ± 3% respectively).[102] WEs contain primarily a monounsaturated fatty acid (C18:1),[103] oleic acid.[82, 104] These fatty acids are esterified to long chain saturated alcohols (C 18:0 to C 28:0). The esterified fatty acids in tears are mostly made up of eighteen carbon chains (C18) which contain no double chemical bonds (18:0).[103] The WE with the lowest concentration in tears has four double bounds (18:4).[103] CE is formed by linking a fatty acid chain (such as C16, C20) to cholesterol. It has been suggested that when fatty acids are deficient, CEs can substitute their

functions[105] and though WEs can supplement a triglyceride deficit, they can never replace triglyceride functions entirely.[61] A positive association has been demonstrated between the level of cholesterol esters and CL-induced symptoms and dryness.[106, 107] No report was found in the literature about tear levels of WEs and CE.

1.3.2.2 Polar lipids

Phospholipids

In general, structurally phospholipids (PLs) consist of hydrophilic head groups (such as diglycerides with a phosphate group) and hydrophobic tails (long fatty acids).[108-110] These lipids, in conjunction with other polar lipids help with maintaining a low surface tension on the ocular surface and are crucial to stability of the tear film.[56] The PL concentration in tears is higher than that in meibum suggesting that the meibomian glands are not the exclusive source of PLs.[102] Tear PL are involved in interactions between the aqueous layer and the non-polar lipids to help them overlay the ocular surface.[19, 51, 61, 66, 74, 111] There are at least two known mechanisms for regulation of tear levels of phospholipids, one is by tear lipocalin binding phospholipids[112-114] and the second is by secretory phospholipase A_2 ($sPLA_2$) hydrolysing phospholipids at the sn-2 position.[115] It has been shown that in CL-related dryness the concentration of PLs change, possibly due to the action of $sPLA_2$.[75] In humans, PLs make up to 12 ± 7% of the total tear lipids.[102] The average level of PLs in tears during CL wear is approximately 174 ± 36 µg/mL which differs significantly between lens types. PL concentration in CL wear is lower than its level in tears of non-CL wearers.[78] These results support an earlier study showing an increase in the ratio of non-polar to polar

lipids during CL wear.[116] Patients with chronic blepharitis but no symptoms of dryness had a concentration of PL similar to that of normal subjects, while patients with chronic blepharitis in conjunction with dry eye had lower concentration of PL.[117] All these findings suggest that PLs have an important role in ocular dryness and discomfort symptoms.

Phosphatidylethanolamines

Phosphatidylethanolamines (PEs) can be synthesised by adding ethanolamine to the diglyceride group of PL, hence PEs are one of the subtypes of phospholipids.[118] PE represent 0.80 to 1.99% of tear lipids,[102, 119] while phosphatidylethanolamines along with phosphatidylcholines together comprise 88 ± 6% of all polar lipids.[120] Maintaining the balance between PE and other phospholipids is critical for tear film stability; anionic changes in tear pH can change this balance.[121] PE levels decrease in chronic belpharitis and dry eye disease.[117] PE can deposit on CLs.[122]

Phosphatidylcholines

Phosphatidylcholines (PCs) are another class of phospholipids that are synthesised by methylation of the amine in phosphatidylethanolamines.[123] PCs have choline as a head group. PCs fatty acids can be saturated or unsaturated. PCs make up 1.6 to 3.0% of total tear lipids.[102, 119] PCs can deposit on CLs.[77]

Phosphatidylserine

Phosphatidylserine (PS) has also been found in tears. Its concentration has been reported to be between 0.3 and 0.6% of total lipid measured in tears.[119, 120] As yet, there is no report on PS in relation to discomfort and dry eyes.

Sphingomyelin

Sphingomyelin (SM) usually consists of sphingosine as a head group and a fatty acid.[124] Sphingomyelin is one of the major polar lipids present in meibum.[65] The total concentration of SM in tears is 5 ± 1 pmol/μL[77] which comprises 1.1 ± 0.6% of total tear lipids.[102] A study of deposits of phospholipids on CLs showed a significant effect of CL composition on SM deposition.[77]

1.3.2.3 Arachidonic acid derivatives

Prostaglandins

Stimuli such as microbes or their products and CL wear result in an increase in pro-inflammatory mediators in the tears and elicit ocular surface inflammation.[125-127] As part of a complex inflammatory response, lipid inflammatory mediators participate in the corneal response to injury and infection.[128, 129] Arachidonic acid derivatives play a key role in this response. Arachidonic acid can be freed from phospholipids by $sPLA_2$.[130] The enzymes cyclooxygenase and peroxidase can release prostaglandin H2 from arachidonic acid[131-133] which is an upstream product for prostaglandins (PG).[134-137] These mediators are formed and serve in the initiation and termination of acute

inflammation, in addition to the transition from acute to chronic inflammation.[138] The level of PGE_2 is increased in the tears of dry eye patients.[139] The level of prostaglandins also increases after mechanical irritation and injury,[140, 141] as well as in corneal inflammation.[142]

Leukotrienes

Leukotrienes (LTs) are a family of inflammatory mediators that result from oxidation of arachidonic acid by 5-lypoxygenase enzyme.[143] They can be secreted by polymorphonuclear leukocytes, macrophages, and mast cells that are stimulated by activators such as bacterial peptides or immune complexes.[144] The presence of leukotriene B_4 (LTB_4), a potent activator of polymorphonuclear cells (PMN),[145] has been demonstrated in tears of inflamed eyes (herpes simplex virus infection of the rabbit cornea).[146] The same mediator is produced in a range of conditions where the inflammatory response is proposed to be involved such as in closed-eye tears,[147] in normal CL wear[127] as well as during acute ocular surface inflammation associated with CL wear.[148] An increase of LTC4, LTD4, and LTE4 in dry eye disease and other ocular inflammatory diseases can cause excess secretion of mucus by stimulating conjunctival goblet cells.[149]

12-hydroxyeicosatetraenoic acid

Some arachidonic acids are converted into hydroxyeicosatetraenoic acid (HETEs) (mainly 12-HETE) by epoxygenase in the epithelium, endothelium, and

stroma of cornea in response to injury.[150-152] Previous studies suggest that 12-HETE's concentration can rapidly increase after injury even before infiltration of PMNs into the cornea,[150-153] and can be chemotactic and chemokinetic factors for leukocytes[154] and aid cell growth and differentiation.[155, 156] According to Mieyal,[157] elevated levels of 12-hydroxyeicosatetraenoic acid (12(R)-HETrE) are observed in most types of inflammatory reactions of the anterior eye. It is expressed in a time-dependant manner during inflammation, while its inhibition leads to reduction of inflammation.[125, 158]

1.3.2.4 Resolvins and protectins

During the last decade two new families of compounds, namely resolvins and protectins, were identified in humans[159-161] Eicosapentaenoic and docosahexaenoic acids (polyunsaturated fatty acids) function as precursors to these compounds. Studies have shown that these fatty acids are essential for ocular health.[160, 162] Neuroprotectin D_1 can protect neural systems[163] in different inflammatory episodes such as stroke[164] and Alzheimer's disease.[165] Resolvins D_1 and E_1 promote the resolution of inflammatory exudates,[159, 160, 166] to allow for resolution of inflammation back to the non-inflamed levels and to maintain tissue homeostasis.[138] Resolvins and protectins can remove cytokines from the inflamed tissue,[167] and reduce neutrophil trafficking and regulate reactive oxygen species.[159, 160] These omega-3-derived lipid mediators respond to inflammation in a time-dependent manner, thereby giving the process gradual progression and resolution.[166, 168, 169] Resolvins can reduce corneal angiogenesis and cytokine production.[149, 170] Resolvin D_1 can block the effect of cysteinyl leukotrienes on the conjunctiva by activating specific receptors on conjunctival goblet cells to prevent

increase of calcium ions.[149] Abnormal regulation of resolvin D_1 appears to be associated with persistent tissue inflammation.[140, 141, 168] The level of resolvin D_1 and its diurnal variations in tears are reported for the first time in this thesis.

1.3.3 Tear proteins

More than 1500 different proteins have been identified in normal tear fluid.[171] Tear proteins are usually hydrophilic by nature and are electrically charged. The intrinsic characteristics of these proteins and their structure give the tear fluid a high degree of complexity.[172, 173] Thus, the tear film's characteristics in part depend on tear protein combinations. Changes in the protein profile of the tear film may reflect changes in the health of the ocular surface.[174] Basal tears contain 2 to 12 mg/mL of total protein.[26, 27, 29, 175-177] Total protein level does not change between basal and reflex tears but increases in closed eye tears.[178, 179] Major tear proteins include lysozyme, lactoferrin, secretory immunoglobulin A (sIgA), serum albumin, lipocalin and lipophilin,[180-182] which are involved in the ocular surface defence and tear stability.[183] Tear proteins secretion is controlled by different mechanisms. For example, secretion of transferrin is regulated by nerves,[184] but secretion of secretory IgA is controlled by endocrine impact of lacrimal glands.[185] Thus the amount of these two proteins in reflex and stimulated tears will differ; as only one protein will be increased by neural stimulation. Tear film stability can be increased by enhancing protein secretion from the lacrimal gland.[186] Some of these proteins reportedly change in concentration in dry eye conditions[187] and in CL-induced dryness.[188] Studies have shown that the concentration of prolactin-induced protein increased, and proline rich protein decreased in CL wear.[188]

Glasson *et al.* demonstrated that tear samples collected from symptomatic CL wearers contained significantly higher levels of lipocalin and lipases than tolerant wearers.[75] Changes were not evident for total protein content, lysozyme, lactoferrin, or sIgA when tears of tolerant and intolerant CL wearers were compared.[189, 190] In dry eye patients, tear samples had reduced levels of prolactin-induced protein, lipocalin 1, lactoferrin and lysozyme.[174]

A summary of studies investigating tear proteins in CL wear, dry eye or CL wear related dryness is presented in **Table 1:1**.

Below is a brief overview of the proteins investigated in this thesis. In this thesis we try to probe tears for some proteins with high and low concentrations in tears especially those which have been studied in both CL wear and dry eye. We hypothesise that proteins identified in tears as markers of dry eye show similar changes in CL-induced dry eye (Sections 1.3.3.1 to 1.3.3.9).

Table 1:1 Proteins that have been shown to change in dry eye, CL wear or CL-related dry eye.

Protein	Dry eye	CLDE*	ICLW*	CLW*
Lysozyme	D[174, 187]		NC[189]	
Proline-rich protein 3	D[187]			
Nasopharyngeal carcinoma-associated proline-rich protein	D[187]			
α -1-Antitrypsin	I[187]			
Proline-rich protein 4	D[187]	D[188]		
Calgranulin A	I[174, 187]			
3,700 Da (not named)	D[187]			
3,916 Da (not named)	I[187]			
α-enolase	I[174]			
α -1-acid glycoprotein 1	I[174]			
S100 A8 (calgranulin A)	I[174]			
S100 A9 (calgranulin B)	I[174]			
S100 A4	I[174]			
S100 A11 (calgizzarin)	I[174]			
Prolactin-inducible protein	D[174]	I[188]		
Lipocalin	D[174]		I[75]	
Lactoferrin	D[174]		NC[189]	
ß-2 microglobulin		D[188]		
Lacritin		D[188]		
Secretoglobin 1D1		D[188]		
Secretoglobin 2A2		I[188]		
Serum albumin		I[188]		
Glycoprotein 340		I[188]		
$sPLA_2$			I[64]	
Secretory Immunoglobulin A				D[191]
High-molecular-weight kininogen				I[192]

*Contact lens wear (CLW), contact lens-induced dry eye (CLDE), intolerant contact lens wearers (ICLW), decreased (D), increased (I), not changed (NC).

1.3.3.1 Lactoferrin

Lactoferrin, an iron-binding monomeric glycoprotein with an apparent molecular weight of about 80,000 Da, is produced by the lacrimal glands[193] and makes up to 25% of the protein in human tears.[194, 195] The presence of lactoferrin in human

tears has been established using immunochemical techniques.[29, 196] The concentration of this protein in human tears is between 0.8 to 6.3 mg/mL.[175, 195, 197, 198] Lactoferrin protects the ocular surface against bacteria and possesses bacteriostatic properties due to its ability to make certain metals (such as iron) unavailable to microorganisms.[199-204] Lactoferrin helps with wound healing as studied on human limbal epithelial cells in the cornea[205, 206] and functions as a strong inhibitor of the classical complement system.[207] This is achieved through blocking of C3 convertase production[195, 201] that inhibits complement mediated red blood cell lysis.[208] The ability Lactoferrin's to inhibit complement (C) activity implies that in addition to its antimicrobial properties, an anti-inflammatory role may be expected for this molecule.[201] Additionally, lactoferrin has an affinity for binding to proteins,[209] and can penetrate into lipid layers at much higher rates compared to lipocalin.[210] These findings again would suggest that lactoferrin is an important component of the nonspecific host defence system of tears and the external eye. Its deposition on CLs has been previously demonstrated.[211]

1.3.3.2 Lysozyme

Lysozyme is the most abundant anti-microbial protein in tears[182] and comprises up to 20 - 40% of total tear protein content.[202, 203] The mean lysozyme content of tears in normal subjects is about 0.7 - 3.0 mg/mL[175, 189, 212, 213] and remains constant in basal, closed and reflex tears. Lysozyme's molecular weight is approximately 17,000 Da. This protein is produced by the lacrimal gland.[214] The concentration of lysozyme in tears reaches its highest in the age group 21 to 40 years, and decreases with age thereafter.[213] This protein has been found only in tears of humans and some primates but not in some

other species.[215] Its level in tears is higher than in other body fluids.[216, 217] The level of this protein increases between 9 and 12 o'clock pm and decreases dramatically from midnight to 3 am.[218] A murine study has shown that lysozyme inhibits bone collagenase.[219] Lysozyme deposition on and into CLs has been identified in a number of studies.[220-222]

1.3.3.3 Lipocalin

The lipocalins are a family of proteins which transport small hydrophobic molecules such as lipids.[113] Lipocalins with a molecular weight of approximately 20,000 Da,[223, 224] make up to 15 - 33% of tear protein[225] and with a reported concentration of between 1.06 to 2.51 mg/mL,[23, 72] have the second highest concentration of proteins in human tears.[26, 29, 212] Lipocalins site of production is predominantly the lacrimal glands where it is packaged into granules together with lysozyme and lactoferrin before being secreted into the tear fluid.[226] It is believed that these three proteins can interact together.[227] Lipocalin 1 being multifunctional and the principal lipid-binding protein in tears can solubilise a large amount and different forms of lipids by binding and/or releasing lipids to adjust tear lipid composition.[228] Tear lipocalin can also increase the wettability of the ocular surface and maintain optical clarity.[56, 113, 229, 230] It is involved in transportation of vitamins and helps with maintaining the integrity of the tear film.[231] This protein is increased in the tears of intolerant CL wearers.[75]

1.3.3.4 Proline rich protein

The lacrimal proline rich protein (PRP) family[232, 233] has a molecular weight of approximately 15000 Da. Members of this family contain high percentage of the amino acid proline (21 - 27%),[234] and an almost similar amount of acidic and basic amino acids. Despite the antimicrobial function and the mucosal defence mechanism suggested for PRPs in saliva,[235-237] their function in tears is yet to be established. Earlier studies failed to detect proline-rich protein in tears using immunoassay-based methods, even though there was evidence of large amounts of messenger ribonucleic acid (mRNA) in lacrimal tissue.[232, 233] However, lacrimal proline-rich protein has been detected in the human tear fluid by mass spectroscopy techniques,[235] but so far no study has measured the exact amount of PRPs in tears. Semi-quantitative studies have shown that the level of one of these proteins known as proline rich protein 4 decreased in tears of dry eye patients and uncomfortable CL wearers.[174, 188]

1.3.3.5 Prolactin-induced protein

Prolactin-inducible protein (PIP), also known as gross cystic disease fluid protein 15 (GCDFP-15), is a low molecular weight (~17,000 Da) protein. It has been isolated from saliva, tears, sweat, seminal plasma, the sub-mucosal gland of the lung, as well as amniotic fluid.[238, 239] PIP secretion can be regulated by androgens.[239-241] PIP messenger ribonucleic acid (mRNA) responds to human prolactin and growth hormone in a time- and dose-dependent manner.[239] PIP can regulate water transportation. A study has linked prolactin-induced protein to osmoregulation of tears through regulation of aquaporin 5.[242] A murine model of Sjögren's syndrome (SS) demonstrated that

prolactin-induced protein caused abnormal distribution of aquaporin 5 by binding to it. Aquaporin 5 is linked with rapid water transportation from the apical membrane of the lacrimal gland.[243] Thus PIP may be involved in induction of severe dry eye conditions in SS patients.[244] In Sjögren's syndrome, aquaporin 5 is stored in the cytoplasm of the lacrimal glands suggesting that aquaporin 5 trafficking from cytoplasm to membrane is impaired in these patients.[243] PIP may be involved in regulation of the immune system[245, 246] by binding to lymphocyte receptors (immunoglobulin G and CD4-T).[245, 247] PIP also strongly binds to streptococci as well as other types of bacteria, thus perhaps participating in non-immune defence mechanisms against pathologic bacteria.[248, 249] The level of this protein increased in tears of patients with ocular dryness related to CLs.[188]

1.3.3.6 Secretory Immunoglobulin A

Immunoglobulins (Ig), also known as antibodies, are glycoproteins[250] that are produced by a specific type of lymphocyte called plasma cells. Immunoglobulins can occur in two forms, a soluble form that is secreted from the cell, and a membrane-bound form that is attached to the surface of a plasma cell and is called a surface immunoglobulin. The latter facilitates the activation of these cells.[251, 252] Five immunoglobulins (IgA, IgD, IgE, IgG and IgM) have been found in the mammalian body. Each performs different roles, and helps direct the appropriate immune response for different types of foreign agents in different ways such as by neutralisation of the agent.[253-257] Several studies have been reported on immunoglobulin levels in tears of normal people.[258-261] Immunoglobulin A (IgA) is the main immunoglobulin found in mucous secretions including tears and plays a critical role in mucosal immunity.[260, 262-

[264] IgA can be found in a dimeric form called secretory IgA (sIgA) which can protect the immunoglobulin from being degraded by proteolytic enzymes.[265, 266] IgA can inhibit the inflammatory effects of other immunoglobulins.[267] The effect of IgA on the activation of the complement system is controversial but it seems that this protein is a poor activator of this system.[268] sIgA is produced locally as a result of stimulation of mucosal membranes and not by leakage of this immunoglobulin from plasma.[264, 269] Secretory immunoglobulin A level in basal tears varies between subjects[26, 218] (0.54 to 2.80 mg/mL)[176, 177, 197, 270] and is reduced significantly in stimulated tears (0.06 to 0.38 mg/mL)[178, 212, 271] while it is increased in closed eye tears (3 to 10 mg/mL).[191, 212] Although some studies indicate that the level of sIgA is significantly reduced in CL wear[270, 272-274] other studies have reported no change during lens wear.[275, 276] IgA deposits have been identified on CLs.[211]

1.3.3.7 Secretory phospholipase A2

Phospholipases are a large family of enzymes that hydrolyse phospholipids and release diacylglycerols, lysophospholipids and fatty acids. Phospholipases consist of cytosolic and secretory forms. The main types of these enzymes are A, B, C and D, based on their catalytic reactions.[277] Phospholipase A has two subgroups: A1 and A2,[278, 279] the latter can commonly be found in mammalian tissues.[280, 281] Secretory phospholipase A2 ($sPLA_2$) facilitates and increases cleavage of the second carbon group of glycerol (specifically sn-2 acyl)[282, 283] to release arachidonic acid which can be converted to inflammatory mediators such as prostaglandins and leukotrienes.[284, 285] Additionally, $sPLA_2$ can cleave lipid peroxidation products which have an important

role in host defence.[286, 287] In the human tear film, $sPLA_2$ is secreted by the lacrimal glands and the goblet cells of conjunctival epithelia.[214, 288, 289] It is found in high concentrations in tears of normal subjects, ranging between 1.4 μg/mL[288] and 54.5 ± 33.9 μg/mL,[290] and is proposed to participate in the innate immune defence against microbial infection in the ocular surface.[291, 292] Elevated levels are also reported in tears of patients with external inflammatory disease[293] and dry eye.[293, 294] These findings suggest that $sPLA_2$ may play a role in the inflammatory responses of the ocular surface. Moreover, a higher expression of this enzyme has been demonstrated in the tears of intolerant CL wearers when compared to tolerant lens wearers.[75] Several studies have reported deposition of $sPLA_2$ on hydrogel CL materials.[295, 296]

1.3.3.8 The complement system (C3 and C3a)

The complement system consists of a group of proteins that originate from hepatocytes,[297] tissue macrophages,[298-300] blood monocytes[301] and epithelial cells[302] to help the immune system fight pathogens.[303] These proteins are usually inactive and are activated by proteases including members of the complement family. The terminal stage of the activation of this system can result in enhanced antibody-dependent phagocytosis, chemotactic migration of macrophages and neutrophils, cell adhesion, lysis of foreign cells,[304] release of hydrolytic enzymes, and the formation of arachidonic acid metabolites and active oxygen species.[305] Activation results in the production of the cell-killing membrane attack complex.[306] The three pathways that can activate complement system are the classical, alternative and lectin.[307] All these pathways produce the protease C3 convertase.[308-310] Activation of C3 then generates C3a and C3b,

and the C3 convertase can cause cleavage in other molecules to activate other proteins in this pathway.[311] C3a has anaphylatoxic and chemotactic activites[312] and degranulates mast cells.[313, 314] C3a activation of mast cells releases histamine, causes contraction of smooth muscles, and increases permeability of capillaries.[313] C3 and C3a can cause an anaphylatoxic reaction and can function as potent inflammatory mediators in the humeral system.[315] Activation of the complement system in the ocular surface can cause inflammation and tissue damage as in other body parts. Conversion of C3 to C3a is regarded as diagnostic for activation of the complement system in response to injury or immunological response.[207, 208, 316, 317] C3 has been detected in both open and closed eyes conditions.[207]

An *in vivo* study demonstrated that the corneal cells are highly resistant to the complement system, due to very high expression of a powerful complement regulatory protein (CD59) in the corneal epithelium.[318] Also, CD59 deposition has been detected on CL.[319] However, it has been demonstrated that the complement system is active in tears and its level is significantly higher in closed eye conditions.[207] Deposition of C3 has been reported on CLs.[208]

1.3.3.9 The kinin-kallikrein system

The kinin-kallikrein system is a group of proteases comprising of plasma and tissue kininogenases. The plasma and tissue kininogenases differ in terms of molecular weight, substrate specificity, immunological characteristic, type of kinin released[320, 321] and function.[322, 323] kininogenases are found in glandular cells, neutrophils and biological fluids,[324] and can release vasoactive peptides.[325] In humans prekallikrein is

secreted by hepatocytes[326] and when affected by kininogenase enzymes can produce high and low molecular weight kininogen which are multifunctional proteins that act as cofactors for coagulation and inflammation and also can protect cells from damage.[325] Plasma kallikrein forms bradykinin from high molecular weight kininogen.[327] Bradykinin is a peptide containing 9 amino acids.[328] It functions as a peripherally acting inflammatory mediator[329, 330] which can produce a delayed burning sensation and cause swelling and pain.[331, 332] Bradykinin participates in a wide range of proinflammatory functions and stimulates cell proliferation.[192, 333] mRNAs of kallikrein (a low molecular weight kininogen) as well as bradykinin receptors have been detected in the eye suggesting that kallikrein and bradykinin may act in an autocrine or paracrine fashion.[334, 335] High molecular weight kininogen increases in tears of CL wearers.[192]

1.3.3.10 Other proteins of potential importance in CL-induced dry eye

Beside the major tear proteins discussed so far, tears contain a variety of potential inflammatory mediators including a range of different cytokines and interleukins (IL). Some of these mediators such as VEGF,[336] IL-6, IL-8, IL-1β and hepatocyte growth factor, granulocyte-macrophage colony-stimulating factor (GM-CSF)[127, 148, 337] have been detected in inflammatory diseases related to CL wear. The level of interleukins (IL)-2, IL-4, IL-5, IL-6, IL-10, interferon (IFN)-γ, tumour necrosis factor (TNF)-α, IL-8, IL-1α and IL-1β are higher in tears of dry eye patients.[338-340] Not all of these can be found normally in tears; IL-6 has been measured during CL wear but was not detected in non-CL wearers.[31] There are also a wide range of anti-inflammatory mediators such as soluble IL-6 receptor, IL-2 receptor, epithelial growth factor receptor (EGFR), tumor

necrosis factor receptor 2 (TNFR2).[339, 341, 342] Matrix metalloproteinases (MMPs) is another group of proteins studied in CL wear[343, 344] and dry eye.[339] MMPs are a family of zinc-dependent peptidases which maintain and remodel the tissue architecture.[345] Human tears contain some of the members of this family (e.g. MMPs 1, 2, 3 8, 9, 10)[346-351] but they are mostly inactive[346, 352, 353] or show very low activity in tears.[354, 355] The level of MMP-9 (gelatinase B) increases in the tears of neophyte CL wearers.[343] Peroxidases are another group of important tear proteins functioning as enzymes essential for control of inflammatory conditions.[356, 357] Histamine, the decarboxylated form of the amino acid histidine, is present in basal tears. Its highest level has been recorded in closed eye conditions but its concentration remained unchanged during asymptomatic lens wear.[358] Neuromediators such as substance P, calcitonin gene–related peptide, neuropeptide Y, vasoactive intestinal peptide and nerve growth factor have been evaluated in dry eye and eye inflammation.[359, 360] Another tear protein is albumin which is a serum protein derived from the ocular surface vessels.[361] Albumin concentration is low in normal conditions [362] but increases quite dramatically in ocular surface trauma[30] and dry eye.[363]

1.4 Introduction to proteomics and lipidomics techniques

Different methods have been used to study proteins and lipids in biological samples (such as body fluids). Tear fluid can be a useful matrix for research and for diagnosis of eye diseases[364-366] as well as systemic diseases such as diabetes[367] and cancer.[368] Although collection of this biofluid is relatively easy, tear protein analysis is highly dependent on the tear collection method. For example, absorption methods such

as filter paper or sponges contain a higher proportion of ocular surface cellular proteins.[369] As a result, using a standard protocol for tear collection is important. Another challenge in tear fluid biomarker research is the difficulty associated with availability and collection of large volumes of samples required for testing. Frequently, sample volumes are very small and testing requires extremely sensitive and reliable techniques for analysis to ensure reproducible results. Also, studies have proved that the number of proteins identified in human tears vary widely depending on the technique used.[171, 370, 371]

A variety of techniques have been employed for analysis of tear proteins including enzyme linked immunosorbent assay (ELISA),[29, 195] one dimensional gel electrophoresis (1D-gel),[32, 372] 2D-gel,[246, 373, 374] protein arrays,[375] and mass spectrometry methods such as liquid chromatography–mass spectrometry (LC-MS),[376] LC-MS/MS,[180, 370, 377] surface-enhanced laser desorption/ionisation (SELDI),[187, 378-380] matrix assisted laser desorption ionisation (MALDI)[235, 381] liquid chromatography matrix assisted laser desorption ionisation (LC-MALDI),[377] isobaric tags for relative and absolute quantitation (iTRAQ) technology,[382-384] two-dimensional nano-LC nano-electrospray ionisation dual mass spectrometry (nano-ESI-MS/MS),[385] and high performance liquid chromatography mass spectrometry (HPLC-MS).[386, 387]

Different methods also have been used to measure tear lipids and their deposition on CLs. Some of these methods include: fluorescein spectrophotometry performed in conjunction with thin layer chromatography (TLC),[388] high performance liquid chromatography (HPLC) and oxidative methods,[389] enzyme-linked immunosorbent assay (ELISA),[148] gas chromatography mass spectrometry (GC/MS) in

conjunction with TLC and HPLC,[117] chip-based nano-electrospray ionisation tandem mass spectrometry,[102] electrospray ionisation tandem mass spectrometry (ESI-MS/MS),[77] direct insertion/electron ionisation-mass spectrometry (DI/EI-MS).[390] TLC is often combined with other analytical techniques, such as GC and gas liquid chromatography (GLC) is the most informative technique of detection and identification of tear lipid analysis which provides precise information on lipids molecular masses and fragmentation patterns.[88]

Below is a brief overview of the methods used in this thesis.

1.4.1 Enzyme-Linked Immunosorbent Assay (ELISA)

Enzyme immunoassays are techniques that have been used for decades to quantify analytes in a variety of samples including tears.[75, 358, 391] The most commonly used type of this assay is the enzyme-linked immunosorbent assay (ELISA). This method is based on antigen-antibody interactions. Immobilised antibodies are used to capture specific analytes with subsequent detection of these captured analytes by a second antibody that is linked to an enzyme. Addition of the substrate of enzyme is used to produce a colour change in the substrate. The concentration of colour then is measured by a spectrophotometer.[392] The advantage of ELISA to other immunoassay techniques, such as multiplex bead array assay, is that it specifically detects just one analyte, therefore the chance of cross-reactivity is very low and its sensitivity is much higher.[393] Also, ELISA can detect and quantify almost any analyte for which a purified antibody is accessible while multiplex assays are not available for detection of some analytes.[393]

1.4.2 Mass spectrometry

Mass spectrometry is an analytical technique that provides qualitative and quantitative information about analytes based on mass.[394, 395] The ion source, the mass analyser, and the detector are the three main sections of the mass spectrometer.[396] Based on the state of the sample (solid, liquid or gas), a number of ionisation techniques can be used to turn a portion of a sample into ions. The ions then can be transported by a magnetic or electric field to the mass analyser. The produced ions can then be extracted based on their mass-to-charge ratio and be moved onto a detector. The detector calculates the abundance of each ion by the number of times that ion hits a sensitive surface.

Two common ionisation techniques that are usually used to analyse liquid biological samples are electrospray ionisation (ESI) and matrix-assisted laser desorption/ionisation (MALDI).[397] Time of flight, orbitrap and quadrupole mass analysers are the most frequently used mass analysers for biomolecular analysis. If two or more of them are used in one mass spectrometer, the resultant analytical system is called tandem mass spectrometry (MS/MS). The method in this thesis used a common form of the mass analyser which is triple quadrupole mass spectrometer with three consecutive quadrupole stages. The first acts as a mass filter to transmit particular ions to the second quadrupole, a collision chamber, wherein the ion can be broken into fragments. The third quadrupole also acts as a mass filter, to transmit a particular fragment ion to the detector. So, a triple quadrupole acts as a mass-selection filter.

Common chromatographic techniques combined with mass spectrometry are gas chromatography-mass spectrometry (GC-MS) and liquid chromatography mass

spectrometry (LC-MS) that are used to separate different compounds. Column chromatography is one of the methods of separation and purification of both solids and liquids based on distribution of components in a mixture between a fixed (stationary) and a moving (mobile) phase and differential adsorption of substances by an adsorbent. The method functions for separation of components on nanogram up to kilogram scales. The main advantage of column chromatography is the relatively low cost and disposability of the stationary phase used in the process.[398, 399] The main difference between the two types of the chromatography is that in GC-MS the mobile phase is gas and in LC-MS the mobile phase is liquid, usually a mixture of water and organic solvent, instead of gas.[400]

The advances in quantitative mass spectrometry in the last decade and use of synthetic stable isotope labeled compounds in conjunction with mass spectrometry technology has become the method of choice for quantification of a broad range of molecules and complex mixtures in clinical research.[401]

1.4.2.1 Selected reaction monitoring mass spectrometry (SRM)

Selected reaction monitoring is a method of analysis with a triple quadrupole mass spectrometer (QQQ MS) (**Figure 1:4**) to detect multiple analytes such as peptides in complex mixtures in a single run.[401-403] Multiple mass analysers (mass filters) help to detect selected analytes with very high accuracy.

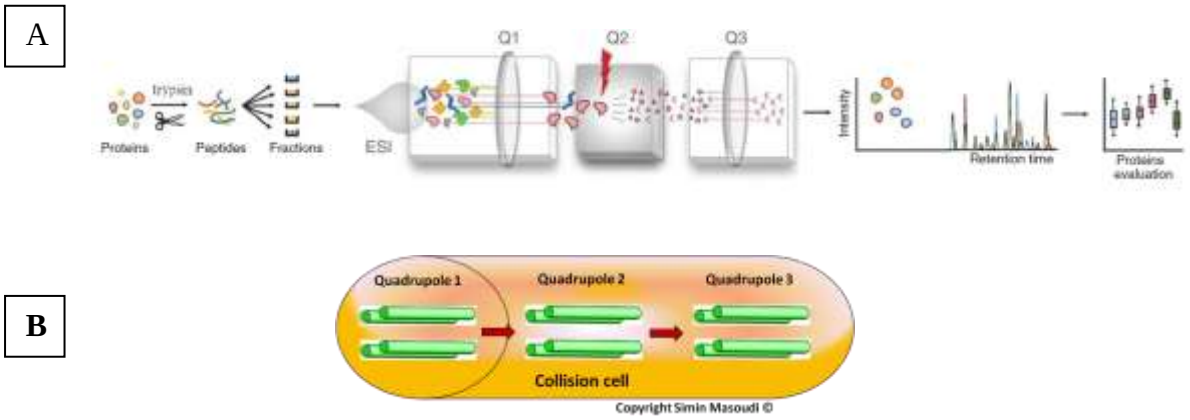

Figure 1:4 SRM analysis on QQQ MS. A) Several analytes are coeluting from the chromatographic system. In the first quadrupole one specific analyte is selected, and then it is transferred to Q2 where it is fragmented to generate product ions. The product ions are then selected by Q3 among all the product ions released from Q2. Finally, the selected product ions are measured by the detector (Drawing courtesy of Picotti P. with modifications - Selected reaction monitoring–based proteomics 2012) B) Triple quadrupole in mass spectrometer.

The first quadrupole (Q1) selects the desired ionised analyte (parent ion); the second quadrupole (Q2) fragments parent ions via collision-induced dissociation (CID) energy and the third quadrupole (Q3) is used to monitor the (one or more) fragment ion(s). The combination of mass to charge (m/z) in the first and third quadrupole is called "transition". Since the ionizing efficiency of parent ions and also fragmentation efficiency can be different,[404] at least two ions from a single protein are needed to generate positive and reliable results. Use of radio labelled peptides as internal standards[405, 406] helps to quantify peptides.[407] These standards are chemically identical to native peptides but different in mass have similar chromatographic elution, ionisation efficiency and relative distribution of fragment ions. The ratio of native peptides to known amounts of the internal standard can be used to quantify the native peptides. By detecting specific SRM transitions, this method can strictly target a predetermined set of

peptides. For this purpose, proteotypic peptides that present good MS responses and uniquely identify the targeted protein need to be determined.[408] Then, fragmented ions that provide optimal signal intensity in the mass spectrometer for the targeted peptide are selected. Quantification for each peptide is based on calculation of the peak height for the heavy (radio labelled) transition to that of the light (non-radio labelled) transition. The average concentration of at least two peptides for each protein will represent the concentration of the selected protein. The sensitivity of this method allows quantification of analytes below femtomole (fmol) levels. This method has been used to quantify drug analytes,[409] hormones[410] and proteins in blood.[411] In this thesis, SRM-MS is introduced for the first time as a method for quantification of multiple proteins in very small volumes of tear samples (Chapter 4:).

1.5 CL wear and ocular discomfort

It has been argued that CLs may induce subclinical changes to the ocular surface, just as superficial foreign bodies would.[412] Up to 75% of the lens wearing population[10, 413-415] are marginally successful or even unsuccessful in lens wear due to various symptoms of discomfort[6] more commonly occurring in the evening.[7, 10] Some people can wear lenses comfortably just for few hours (comfortable wearing time) but not feel comfortable with their lenses for the entire day (total wearing time). [6, 414] CL wearers have significantly more ocular symptoms than non-wearers.[8, 416, 417] Symptoms of discomfort may include ocular fatigue, itchiness, dryness, irritation, and scratchiness and signs such as redness.[418] The dry eye symptoms described by CL wearers are similar to those experienced by non-lens-wearers, but CL wearers experience higher

frequency and intensity of dryness with increased wear duration.[414] Frequent replacement of CL for daily wear or removing lenses and replacing them with new ones during the day can improve (but not eliminate) ocular discomfort.[6, 419] However this may not influence end of day discomfort.[420] Symptom relief occurs after removing lenses,[413] and therefore, CL wear-related dryness is a temporary condition which seems to be different from dry eye.[413] However, underlying reasons for CL-related discomfort may differ from those of the dry eye patient where a predisposition for dry eye may be the cause of discomfort and symptoms might not relief upon removal of lenses. CL wear alters the tear film structure, function and component concentrations in numerous ways.[127, 148, 190, 421-425] It can divide tear film into pre- and post-lens tears[426, 427] (**Figure 1:5**) and reduce tear film thickness,[428, 429] especially the pre-lens tear film.[427, 430] CL wear can alter the spread of the lipid layer[431] and its stability,[432] affecting the tear break up time[433] and possibly increasing tear evaporation,[434] which can be associated with increased symptoms of discomfort.[424, 435-441] Furthermore, a disturbed tear film[442] in conjunction with environmental conditions such as low humidity or high temperature can increase tear evaporation,[433, 443] resulting in tear hyperosmolarity and ocular surface inflammation as well as dryness.[444-446] In addition, CLs have the potential to lower oxygen transmission to the ocular surface and occasionally cause epithelial disruption.[6] CLs can increase the risk of ocular surface inflammation and infection.[447] CLs also can change the ocular temperature,[448] tear turnover rate[190] and tear volume.[449, 450] These factors, together or alone, can be the possible causes of ocular discomfort. A number of studies have addressed the underlying causes of CL-induced discomfort and an association seems to exist between ocular discomfort related to CL wear and changes in the tear film.[75, 190] However, some authors have argued that these findings may have

been influenced by end of day ocular or general physical fatigue common between non-lens wearers and lens wearers.[451]

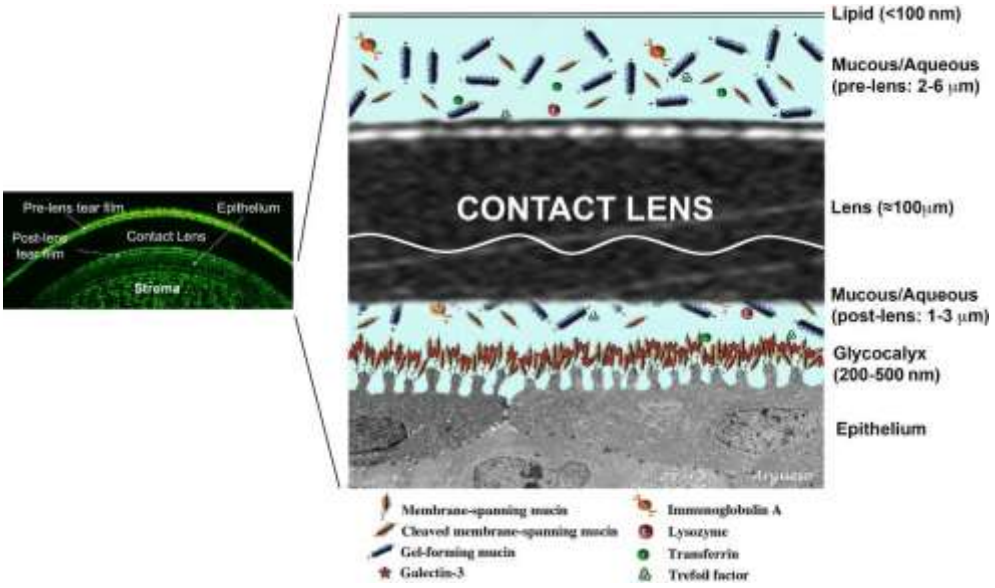

Figure 1:5 Contact lenses divide the tear film into pre- and post-contact lens layers (courtesy of Craig J.P. *et al*. TFOS International workshop on contact lens discomfort, the tear film subcommittee, 2013).

Ocular surface inflammation has been detected by cytological methods in patients with symptoms of discomfort but only a small number of subjects showed clinically significant evidence.[35] Previous tear analyses have shown that physiological properties including pH levels,[452, 453] osmotic pressure[454] and inflammatory mediators vary diurnally while wearing CLs.[26, 455] The mechanisms underlying these events have been assumed to cause adverse responses to CLs[31] and induce discomfort and dryness.[412] In the current literature, there are no studies attempting to establish an association between changes in the ocular comfort and level of pH, osmotic pressure and inflammatory mediators.

1.6 Assessment of ocular comfort

Although CLs may cause inflammation and induce symptoms of dryness,[444, 456-459] these symptoms have not been regarded as a disease. Therefore, presenting a definition for CL discomfort is still a significant challenge. The report of TFOS dry eye workshop in 2007 has listed discomfort as one of the main symptoms of dry eye.[460] Factors such as CL material,[6, 461-463] lens care products,[462, 464, 465] lens wear regimens[466, 467] and environmental factors (climate, allergens, humidity, temperature) can affect the contact lens material hydration.[468, 446] These factors can induce reversible changes to the eye which may worsen or ameliorate symptoms.[469] Previous reports have shown that factors such as (inherent, age, sex, and race, as well as ocular and systemic disease) are contributing factors to CL-induced discomfort.[3, 470, 471] Over the recent decade, a number of questionnaires have been designed to measure ocular discomfort but many were not designed specifically for CL patients and still the clinical significance of scores for some of these remain unclear.[472, 473] Moreover, the question types used in the questionnaires and the systems and scales used for rating of symptoms vary between studies, rendering comparison of the results a difficult task.[474-476] A number of questionnaires have been developed to specifically assess CL-related dry eye.[7, 10] The McMonnies-Ho questionnaire and the Contact Lens Dry Eye Questionnaire (CLDEQ) and its short version (CLDEQ-8) are the most commonly used questionnaires.[473, 477] CLDEQ has been recognised as an efficient screening survey for identification of CL-related dry eye. CL-related dry eye correlates better with the scores from the CLDEQ-8.[457, 478] Also, assessment of ocular comfort is most often performed using subjective ratings as an indication of ocular comfort levels.[479-483] Traditionally, CL comfort scores are recorded on ordinal scales such as using the typical 1 to 100 scale (1 is extremely

uncomfortable and 100 extremely comfortable), probably because of easier collection and analysis of data compared to other methods. However, the feeling of comfort (or discomfort) might be a complex psychological process that subjects might not be able to categorise into a word or a number.[484] Although visual analogue scales have been shown to be the most accurate of all commonly used grading scales in assessment of CL wearing comfort,[485] their accuracy has not been determined because there is no accurate method available for comparison. CL wear-induced discomfort has been defined as a 30% decrease of subjective ratings for comfort (increased dryness) after six hours of lens wear.[189, 435] One study indicated that five units of change in comfort level are most likely to show clinically significant symptoms.[486] Further complicating comfort assessment is the fact that assessing patients' eye comfort by questionnaires often produces different results from those obtained from subjective ocular ratings. Further research is clearly needed in this field.

1.7 Thesis Overview

It is evident from the literature that wearing CLs can change the level of certain biochemical factors in tears. This thesis investigates the change in tear proteins and lipids and their association with ocular discomfort during the day with CL wear and no lens wear.

1.7.1 Thesis hypothesis

- Hypothesis 1: Ocular comfort changes are associated with higher levels of inflammatory mediators in tears with and without lens wear as has been demonstrated in dry eyes.
- Hypothesis 2: Selected reaction monitoring method can detect and quantify tear proteins simultaneously in very small volume of tear samples.
- Hypothesis 3: Proteins identified in tears as markers of dry eye show similar changes in CL-induced dry eye.
- Hypothesis 4: Tear film protein and lipid levels are different between symptomatic and asymptomatic lens wearers at the end of the day.
- Hypothesis 5: CL-induced dry eye is caused by changes in the level of tear lipids as has been demonstrated in dry eye patients.

1.7.2 Thesis aims

1. To elucidate changes to inflammatory mediators C3, C3a, $sPLA_2$, bradykinin, LTB_4 (Study 1), cysteinyl leukotrienes (C_4, E_4 and $D_{4)}$, prostaglandins and resolvin D_1 (Study 2) with and without CL wear and their association with ocular discomfort.

2. To develop a new approach for accurate identification and quantification of several individual proteins in tears using Selected Reaction Monitoring (SRM). (Study 3)

3. To quantify the amount of lysozyme, lactoferrin, lipocalin 1, prolactin-induced protein and proline rich protein 4 during the day with and without CLs and elucidate their effect on ocular comfort. (Study 4)

4. To analyse whether the level of measured proteins and arachidonic acid derivatives differ between symptomatic and asymptomatic lens wearers. (Study 5)

5. To analyse non-polar and polar tear lipids and quantify their levels in the morning and evening with and without CLs and to determine any correlation between tear lipid levels and ocular comfort. (Study 6)

Elucidating differences in biochemical factors of tears with and without contact lens wear may be helpful to find the underlying reasons of contact lens discomfort.

Here, a brief overview of the pre-ocular tear film is given to provide an introductory understanding of the pre-ocular tear film and its interaction with CLs.

Chapter 2: Inflammatory mediators and contact lens-induced discomfort

2.1 Overview

Dry eye disease is associated with instability of tears and inflammation of the ocular surface.[463, 464] Contact lens wear itself can cause inflammation on the ocular surface.[75, 127, 148, 487] This study aims to investigate diurnal changes in the concentration of certain inflammatory mediators in tears and association of these mediators with subjective comfort ratings with and without CL wear (Study 1). We hypothesise that ocular comfort changes are associated with higher levels of C3, C3a, $sPLA_2$, bradykinin, sIgA and LTB_4 in tears with and without lens wear.

2.2 Introduction

Discontinuation of CL wear mostly occurs due to ocular discomfort.[3, 5, 9, 10] Discomfort can be associated with sensations such as itchiness, dryness, irritation, and scratchiness and signs such as redness.[189] These symptoms have been associated with dry eye conditions in non-lens-wearers.[488] Contact lens mechanical factors[489] (material,[490] wettability,[491] design[492]) and lens fit[493] are among the factors that may cause contact lens discomfort. According to Nichols *et al.*, CL wearers are more likely than non-lens wearers to experience intensified symptoms of dryness towards the end of the day.[414] It has been also demonstrated that CL-induced discomfort can be improved (but not eliminated) by removing lenses and replacing them with new ones during the day.[494] Based on these finding, it appears that CL-induced discomfort may be mediated by

components existing or released into the tears during the day, rather than the characteristics or imperfections of CL materials or surfaces. Also, the levels of these components may vary in tears at the end of the day, when discomfort is at its most intense.

As part of a complex inflammatory response, lipid inflammatory mediators participate in corneal responses to injury and infection.[129] The presence of leukotriene B_4 (LTB_4), a lipid product of the 5-lipoxygensae metabolic pathway of arachidonic acid metabolism and a potent activator of polymorphonuclear cells,[145] has been demonstrated in closed-eye tears,[147] during CL wear[127] as well as during acute ocular surface inflammation associated with CL wear.[148] The lipid-related protein secretory phospholipase A_2 ($sPLA_2$) is an enzyme secreted by the lacrimal glands and conjunctival goblet cells.[214, 288, 289] It is present in relatively high concentration in tears of normal subjects.[288, 290] Elevated levels of $sPLA_2$ have been reported in tears of patients with external inflammatory disease,[293] dry eye patients,[293, 294] and CL intolerant individuals.[75]

Bradykinin is known to mediate pain elsewhere in the body[331] and participates in a wide range of proinflammatory responses. Its precursor, high molecular weight kininogen, is increased in tears of CL wearers,[192] but its role in CL comfort has not been investigated. C3, and its breakdown product C3a can function as potent mediators of acute inflammation.[315] C3 has been detected in both open and closed eye tears.[207]

Several studies have reported secretory immunoglobulin A as the predominant immunoglobulin in tears. It is involved in ocular surface defence,[191, 260, 495, 496] but there are no reports on its relationship to CL comfort. The concentration of this protein varies

based on the type of tears collected; it is lowest in reflex tears[497] and highest in closed eye tears.[26, 191, 212] There are no reports regarding the role of this protein in CL-related discomfort.[191] The present study investigates association between subjective ocular comfort ratings and diurnal changes in tear concentrations of C3, C3a, $sPLA_2$, bradykinin, sIgA and LTB_4 with and without CL wear.

2.3 Methods

2.3.1 Ethics approval

The clinical trial was conducted in accordance with the guidelines of the Declaration of Helsinki and Australian National Health and Medical Council regulations. The protocol was approved by the Institutional Ethics Committee of the Vision Cooperative Research Centre and Brien Holden Vision Institute and all subjects signed informed consent prior to examination.

2.3.2 Participants

This study was performed as a prospective, open-label daily wear, single group two-staged (with and without CLs) investigation of tear composition and its relationship with subjective responses of comfort in neophytes and experienced CL wearers. Participants were recruited from the participant population at the investigational site by way of a general email and also were recruited from the general population by way of approved advertising by the Ethics Committee on community notice boards. A total of

45 participants (3 neophyte and 42 experienced CL wearers) with no ocular or systemic disease were enrolled in this trial (please see section 3.3.6 for sample size calculation). As inclusion criteria, subjects were required to be 18 years old and above, be current contact lens wearers, having "normal" ocular health findings not preventing the participant from safely wearing contact lenses, have visual acuity correctable to at least 6/12 (20/40) or better in each eye with contact lenses, be able to demonstrate ability to collect tear samples on their own using equipment provided. Patients with pre-existing ocular irritation, injury of the cornea, conjunctiva or eyelids, active corneal infection or any active ocular disease were excluded from the study. Slit-lamp examination was performed to exclude any corneal pathology prior to enrolment. Participants consisted of 29 females and 16 males, with mean age of 28.0 ± 9.3. The trial duration for each participant was approximately 3 weeks.

2.3.3 Comfort rating scale

Since the study was performed by collecting tear samples by the subjects at home, objective methods[498] or extensive questionnaires[474-476] were not used for assessment of comfort at the time of tear collection. Participants were asked to rate the comfort of their eyes prior to tear collection on a 1 – 100 scale where 1 was extremely uncomfortable and 100 extremely comfortable.[438, 486]

2.3.4 Tear sample collection

Volunteers were asked to avoid wearing CLs for a minimum of 7 days (washout period) before commencing tear collection. In Stage 1 no contact lenses (NCL) were worn and in Stage 2 participants were dispensed with Etafilcon A lenses (1-DAY Acuvue Moist, Johnson and Johnson Vision Care, Jacksonville, FL, USA). Subjects were trained to collect their own tears with a glass micro capillary tube (BLAUBRAND® intraMARK, Wertheim, Germany) for several practice sessions to ensure that subjects could consistently collect more than 2 µL of tears without causing reflex tearing. Tears were collected twice a day from one eye or both eyes for 7 - 10 consecutive days by the method described before.[26] Briefly, tears were collected with a glass capillary tube from inferior cul-de-sac without stimulating reflex tearing for a maximum of 10 µL at each time of tear collection from both eyes and stored in the same microcentrifuge Eppendorf Tubes® (Hamburg, Germany) but different tubes were used for morning, evening, CL and NCL tears. A new tube was used to store tears at each time point of tear collection. Participants were asked to wear their lenses for at least 6 hours and no maximum time of lens wear was set (until bed time). The first collection was performed in the morning during the first hour after waking (sample 1) and the second collection, in the evening before going to bed (sample 2). Alternatively for sample 2, participants would collect their tears at any time beyond 6 hours of lens wear if experiencing discomfort. It was not possible to determine which category sample 2 fell into (discomfort or end of day). At each collection time participants reported subjective comfort. In Stage 2 tear collections were performed prior to CL insertion in the morning and prior to CL removal in the evening. No minimal volumes were designated. Participants were advised to store the tear samples in their freezer and bring

the samples in an ice container every day to the laboratory as soon as tear samples were taken out of the freezer. Upon receipt, the samples were kept frozen at -80 °C. **Figure 2:1** demonstrates tear sample collection timeline.

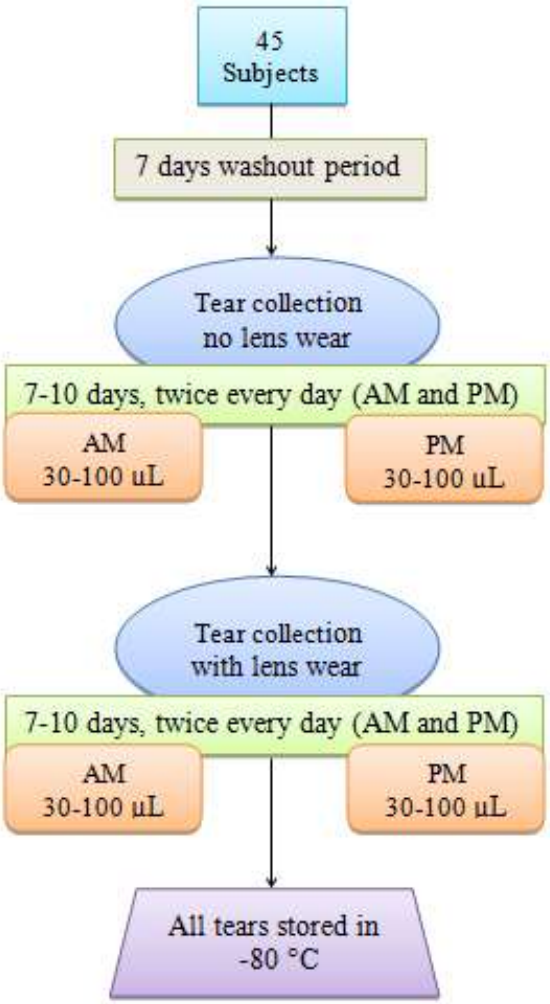

Figure 2:1 The diagram illustrates tear sample collection timeline. Tears were collected from one eye or both.

2.3.5 Tear sample preparation

After each patient finished collecting all samples and before conducting the experiments on the tears collected by each individual in each stage and time point, samples were pooled together regardless of subjective comfort rating to produce a total of four samples for each individual (**Table 2:1**). The volume of tears collected from patients varied from one to the other and ranged between 30 - 100 µL. The rationale for this method was to obtain large volumes of samples and also reduce intra-individual variability. Then samples were centrifuged at 5000 g for 10 minutes at 4 °C to remove any cellular debris before starting experimentation.

Table 2:1 A total of 4 large volume samples were produced for each individual.

Stage	Time
NCL	AM
	PM
CL	AM
	PM

2.3.6 Tear sample collection for mediator variability studies

Experienced tear donors (ranging in age from 24 - 30 years), without any history of eye diseases, were selected (two experienced CL wearers and one non-lens wearer) to collect their own tears at approximately the same time of day (mid-day) for four days as described above. Tears collected on each day for each individual were stored separately at -80 °C and then used to check for day to day variation of mediators.

To ensure that the variation was not caused by inter-individual differences and that any possible variation or lack of variation found in results was only due to changes of LTB_4 between days; an attempt was made to choose subjects as close as possible in terms of age and ocular response. Hence, only three subjects were selected for this part of investigation.

2.3.7 Tear assays

Tears were assayed for C3, C3a and sIgA using commercially available enzyme linked immunosorbent assay (ELISA) kits and LTB_4, bradykinin and $sPLA_2$ enzyme immunoassay (EIA) kits according to manufacturers' protocols (see **Table 3:1** for details of kits). Each sample was diluted (see **Table 2:2** for dilution factors) and sample concentration for each mediator was calculated from the corresponding linear correlation produced using known concentrations of standard antigen *vs* absorbance. For all mediators the limit of detection (LOD) was used based on the manufacturers' assay manuals (**Table 3:1**). Based on the preliminary experimentations the level of these mediators in tear samples were above the LODs presented in **Table 2:2** and at least 40 µL of tears were needed to complete these experiments.

Table 2:2 Assay kits and limits of detection.

Mediator	Kit source (manufacturers)	Tear dilution factor	Limit of detection for each mediator
C3	AssayPro (St. Charles, MO, USA)	100	0.5 µg/ml
C3a	BD Biosciences (San Jose, Ca, USA)	200	7 pg/ml
LTB_4	Assay Designs (Ann Arbor , MI, USA)	10	5.6 pg/ml
$sPLA_2$	Cayman Chemicals (Ann Arbor, MI, USA)	100,000	15.6 pg/ml
Bradykinin	Phoenix Pharmaceuticals (Belmont, CA, USA)	5	10 pg/ml
sIgA	Bethyle laboratories (Montgomery, TX, USA)	50,000	7.8 ng/ml

2.3.8 Statistical analysis

Sample size calculation was performed based on significant paired difference of 1 ± 2 log transformed unit of $sPLA_2$ 80% power, at the 5% level of significance. A total of 40 subjects were required to complete the study. Data was analysed using the IBM® SPSS® Statistical Research Methods and Social Science Statistics (Armonk, New York). Results are expressed as mean ± standard deviation (SD). Since the data were not normally distributed tear component data was log transformed for the purpose of statistical analysis. The comfort ratings and concentrations of tear components and their changes were analysed using linear mixed models with subject random intercepts. The model was tested for the effect of lens wear, time and the interaction of lens and time. Diurnal changes were compared between stages (CL *vs* NCL) using paired t-test. Level of significance was set at 5%. Also, an initial analysis comparing the effect of sampling days (days 1, 2, 3 and 4) was carried out for LTB_4 using linear mixed model analysis.

2.4 Results

2.4.1 Effect of contact lens wear and time on ocular comfort

Four subjects out of 45 discontinued the study prior to final study visit (because of time, job conflict and other reasons) so were not included in the analysis. Comfort ratings, measured on a 1 - 100 scale, declined from the time of first tear collection (AM) to the time of second tear collection (PM), (**Table 2:3**). The decline occurred for both CL and NCL wear, although it was more pronounced during CL wear (p = 0.001) (**Table 2:4**).

Table 2:3 Comparison of diurnal variations of comfort (CL *vs* NCL).

Variable	Stage	N	AM Mean ± SD	PM Mean ± SD	CL Group *p*-value	Time *p*-value
Comfort (Average)	NCL	41	89.1 ± 10.2	84.2 ± 12.6	0.001	0.001
	CL	40	89.0 ± 10.1	76.7 ± 15.2		

One subject did not complete comfort rating during CL wear.

Table 2:4 Comparison of diurnal variations of comfort (CL *vs* NCL).

Comfort	NCL (AM-PM)	CL (AM-PM)	Paired Difference	*p*-value
	Mean ± SD	Mean ± SD	Mean ± SD	
(PM - AM)	-5.3 ± 10.0	-11.9 ± 12.2	6.6 ± 9.5	0.001

2.4.2 Tear levels of mediators

Table 2:5 shows the level of mediators in tears with and without CL wear at the two time points of tear collection (AM and PM). Although bradykinin and $sPLA_2$ levels did not change ($p > 0.05$) the concentrations of LTB_4, C3, C3a and sIgA dropped by the end of the day with or without CL use ($p < 0.01$). Contact lens wear alone did not affect the concentration of the mediators ($p > 0.05$). The only difference between the CL and NCL groups was the concentration of LTB_4 which was significantly higher in CL wear in the evening ($p = 0.03$).

Table 2:5 Tear mediators by time point and lens wear status (n = 41).

Mediator	CL Group	AM	PM	*p*-value	
		Mean ± SD	Mean ± SD	CL *vs* NCL	AM *vs* PM
Bradykinin (ng/ml)	NCL	8.61 ± 5.02	7.55 ± 3.46	0.70	0.17
	CL	8.83 ± 5.50	7.89 ± 5.75		
C3 (µg/ml)	NCL	82.64 ± 81.35	57.49 ± 50.06	0.43	0.001
	CL	92.21 ± 81.20	67.65 ± 70.87		
C3a (µg/ml)	NCL	3.60 ± 2.18	2.10 ± 1.42	0.35	0.001
	CL	3.61 ± 2.29	2.44 ± 2.08		
C3a / C3	NCL	0.01 ± 0.00	0.01 ± 0.00	0.34	0.005
	CL	0.01 ± 0.01	0.00 ± 0.00		
LTB_4 (ng/ml)	NCL	0.43 ± 0.10	0.35 ± 0.11	0.03	0.001
	CL	0.46 ± 0.12	0.40 ± 0.10		
sIgA (mg/ml)	NCL	6.66 ± 1.22	1.72 ± 1.61	0.59	0.001
	CL	5.19 ± 5.90	2.04 ± 2.50		
$sPLA_2$ (µg/ml)	NCL	11.44 ± 8.01	11.50 ± 10.00	0.09	0.84
	CL	9.36 ± 6.57	11.42 ± 10.53		

In this study, 3 of 41 subjects were neophyte CL wearers. The general trend of changes in mediators during the day and in between stages was the same in neophytes as in experienced lens wearers. The only exception was the concentration of LTB_4 which was higher in neophytes both with and without lenses (overall concentration of LTB_4 in experienced CL; 0.40 ± 0.13 *vs* neophyte CL 0.56 ± 0.12; $p = 0.02$; mean ± SD).

To investigate the day-to-day variability of mediators, tear samples were analysed separately (without pooling). Based on the volume of samples used earlier while measuring other mediators selected for this study, it was determined that at least 35 µL of tears would be needed to measure the variability of all mediators. In view of limitation of tear sample volume, only LTB_4 was analysed here as this mediator in the current study had a higher level in the CL wear group. To ensure that subjects were as close as possible in terms of age and ocular response, only three subjects were selected for this part of investigation (for details see Section 2.3.7). The analysis of LTB_4 over four consecutive days showed that there was no significant difference between the levels of LTB_4 for subjects on different days ($p = 0.89$) (**Table 2:6**). Because of limitation in sample volume, it was not possible to measure the day to day concentrations for other mediators.

Table 2:6 The mean level of tear LTB_4 for each day (n = 3).

Day	Mean	SD	*p*-value
1	0.57	0.09	0.89
2	0.51	0.12	
3	0.69	0.14	
4	0.45	0.16	

2.4.3 Association of ocular comfort and tear mediator concentration

There were no associations between mediator concentration or C3a/C3 ratio and comfort ratings in the morning or evening ($p > 0.05$) (**Table 2:7**).

Table 2:7 Correlations between Comfort Drop & Proteins/Lipids Change (in log values).

Lipid / Protein	CL Group	Pearson's Correlation	*p*-value
C3	NCL	0.08	0.63
	CL	0.02	0.89
C3a	NCL	-0.12	0.43
	CL	-0.04	0.83
C3a/C3	NCL	-0.01	0.90
	CL	-0.02	0.78
LTB_4	NCL	0.07	0.69
	CL	0.14	0.50
sIgA	NCL	0.03	0.72
	CL	0.15	0.37

2.5 Discussion

This study has demonstrates that ocular comfort decrease from morning to evening, and the decrease is more pronounced with CL wear. This decline in ocular comfort has been well documented in the literature.[453, 488, 499] Previous tear analysis studies have shown that physiological properties including pH levels,[452] osmotic pressure[454] and inflammatory mediator levels vary diurnally.[26, 455] We hypothesised that the decline in ocular comfort in the evenings would correlate with measurable increase in concentration of inflammatory mediators in the tear film. Bradyknin and $sPLA_2$ did

not change under the study conditions ($p > 0.05$), but the absolute concentration of LTB_4, sIgA, C3, and C3a dropped by the end of the day in both groups ($p < 0.01$). However, the change of sIgA, C3, and C3a over time (AM *vs* PM) was similar for both CL and NCL groups, indicating that CL wear had no effect on diurnal changes of these mediators. Conversely, despite the diurnal reduction of LTB_4 in both CL and NCL groups its level remained higher in the CL wearing group (CL *vs*. NCL, $p = 0.03$), indicating that CL wear may have influenced LTB_4 concentration. This finding is consistent with the results of previous studies that showed LTB_4 concentration was higher after 8 hours of sleep in experienced CL wearers compared to non-lens wearers.[148] Also, the concentration of LTB_4 was higher in neophyte lens wearers when compared to experienced CL wearers. This finding confirms the results of a previous study by Thakur *et al*.[127] Measurement of LTB_4 concentration in consecutive days did not show any significant difference, suggesting that no biological variations exist between days for this mediator. LTB_4 is a lipid product of the 5-lipoxygensae metabolic pathway of arachidonic acid metabolism and the findings of this study may be indicative of activation of the arachidonic acid metabolic pathway during adaptation to CL wear. This may require further investigation of tear levels of other metabolites of this pathway under similar experimental conditions.

Since the total amounts of tears collected were highly variable, the total amount of tears was not measured and all values were expressed as concentrations rather than total amount of mediator. However, the concentration of sIgA in tears for each subject was analysed to ensure that tear collection did not result in high levels of reflex tearing, as the concentration of this protein decreases upon reflex tearing.[212] The concentration in tears collected in the morning (**Table 2:5**) is consistent with levels reported in basal

tears.[500] The effect of CL wear on levels of sIgA in tears is controversial, with some studies finding no effect,[271, 275, 276] and other studies showing a reduction in its concentration during CL wear.[191, 270, 274] The current data shows that during daily disposable wear of Acuvue Moist lenses there was no reduction in sIgA in tears compared to non-lens wear. The concentration of sIgA was reduced in tears collected in the evening, indicating that these samples might contain more reflex than basal tears, or that production of sIgA decreases during the day. Despite this finding, there was no effect of CL wear on the concentration of sIgA. In order to be able to detect changes in comfort (practical relevance), a drop of at least 5 units is needed.[486] In this study, 43.8, 20.0, 12.5, 7.5 and 16.3 percent of patients recorded less than 5, 5 - 10, 10 – 15, 15 - 20 and >20 units of comfort reduction from morning to evening accordingly. No association was found between comfort and tested mediators. The small overall reduction in comfort may have precluded the study from demonstrating a relationship between reduction in comfort and specific mediators. Also, reduction in the level of measured mediators from morning to evening rejected our hypothesis. This may suggest using an alternative subject group and/or less comfortable contact lens types may be helpful to further test these hypotheses.

2.6 Conclusion

The present study demonstrates that the concentration of C3, C3a, LTB_4 and sIgA change diurnally. However, the absolute level of these mediators in tears was not associated with the decrease in comfort seen during CL wear.

Chapter 3: Diurnal changes of lipid inflammatory mediators

3.1 Overview

Having established that the level of leukotriene B_4 was higher in CL wear compared to no lens wear in Chapter 2:, this chapter investigated whether any alterations in the level of other arachidonic acid or eicosapentaenoic acid derivatives were detectable during the day or any difference in their levels was evident between CL and no CL wear. We hypothesised that ocular discomfort during lens wear is related to higher levels of pro-inflammatory mediators and decreased of anti-inflammatory mediators during the day. Therefore, the aim of this study is to elucidate changes to inflammatory mediators cysteinyl leukotrienes (C_4, E_4 and D_4), prostaglandins and resolvin D_1 with and without CL wear from morning to evening and their association with ocular discomfort (Study 2). We hypothesise that ocular comfort changes are associated with higher levels of these mediators in tears with and without lens wear.

3.2 Introduction

Inflammation is part of the innate immunity[501] that protects tissues against harmful stimuli causing injury.[502] However, inflammation on the ocular surface can lead to microvascular leakage[503, 504] and visual disturbances.[505] CLs interact mechanically with the corneal surface and modify the physiological processes in the corneal tissue,[452-454] often resulting in inflammatory events. These corneal inflammatory events have been attributed to mechanical, allergic and microorganism stimuli,[125-127] however the

pathogenesis remains unexplained. Contact lens wear may be asymptomatic or may result in foreign body sensation and even ocular pain.[506]

Previous authors have investigated the changes in the level of inflammatory mediators in tears with CLs.[127, 358, 507] These changes may lead to reduced corneal function,[508-510] and dryness.[4] Lipids and lipid-derived mediators play critical regulatory roles in a variety of cellular functions, including inflammation.[511] Arachidonic acid present in the cell membranes can be released from cells that are activated by mechanical trauma or stimulated by chemicals such as bradykinin and cytokines.[512] Prostaglandins (PGs) and leukotrienes (LTs) are arachidonic acid metabolites that take part in numerous homeostatic biological functions and can escalate a cycle of inflammation and irritation.[513, 514] Leukotrienes, 5-lipoxygenase pathway products, are produced by polymorphonuclear leukocytes, macrophages, mast cells and epithelial cells after stimulation by, for example, bacterial peptides or immune complexes.[144, 515] Ocular epithelial cells can generate leukotrienes in response to bacterial lipopolysaccharide.[516] Leukotrienes can cause conjunctival hypersensitivity, increase microvascular permeability,[517, 518] miosis and secretion of mucus.[149, 519, 520] Leukotrienes also act as eosinophil chemo-attractants.[521-523] LTC_4, LTD_4, LTE_4, are cysteinyl leukotrienes and can initiate an anaphylactic reaction to pathogens[524, 525] by activation of two receptors $CysLT_1$ and $CysLT_2$.[526, 527] The activation of these receptors may result in smooth muscle contraction, oedema and damage to the mucus layer.[528, 529]

In contrast, prostaglandins (PG) are derived from the cyclooxygenase (COX) pathway. Prostaglandins have proinflammatory roles and are produced by most cell types in the body.[134] Animal studies have demonstrated that in some tissues such as vesicular glands, prostaglandin E concentration was higher while in other tissues such

as the lung and the brain, prostaglandin F dominates.[530] PGs have different physiological and pharmacological actions, such as lowering blood pressure (PGE_1), causing fever (PGE_2) or luteolytic effects ($PGF_{2\alpha}$).[531-533] They can elicit hypersensitivity (allergic) responses by increasing the sensitivity of pain receptors in peripheral inflammation.[534-536] It has been assumed that these mediators are formed and serve in the initiation and termination of acute inflammation, in addition to the transition from acute to chronic inflammation.[138] In the eye, $PGF_{2\alpha}$ mediates smooth muscle contraction[537] and reduction of intraocular pressure.[538] The level of these mediators increase in mechanical irritation and injury.[140, 141] Increased levels of PGE_2 have been reported in tears of dry eye patients[54] and after cataract surgery.[139, 539] **Figure 3:1** demonstrates the arachidonic acid pathway and its derivatives.

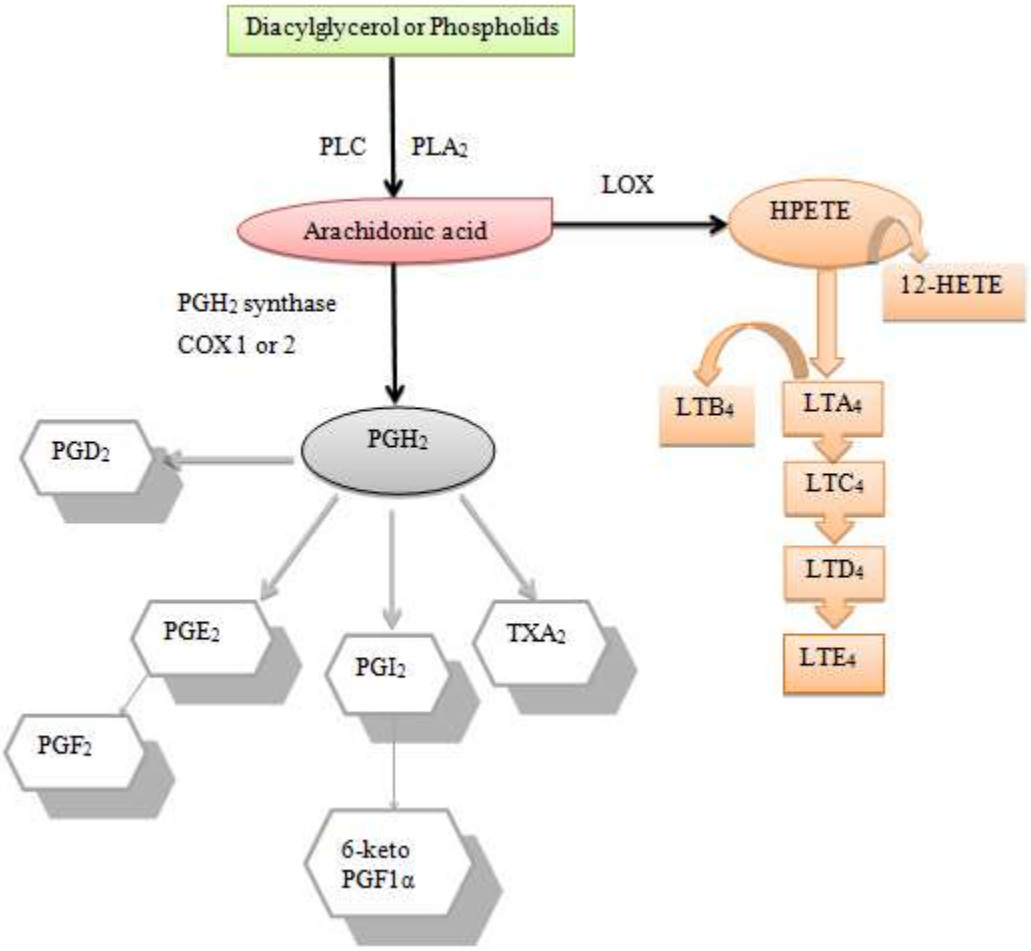

Figure 3:1 Arachidonic acid is released from membrane phospholipids through the action of phospholipase A2 or C (PLA2 or C). The cyclooxygenase **(**COX) pathway produces various prostaglandins (PG) and Thromboxane A2 (TXA_2), and the lipoxygenase (LOX) pathways produces LTs (leukotrienes) and HETEs (hydroxyeicosatetraenoic acids). HPETE is the abbreviation for hydroperoxyeicosatetraenoic acid.

The presence of these mediators in inflammation can sometimes be excessive[540] and damage body tissues, therefore they need to be controlled.[541, 542] Resolvins (omega-3-derived lipid mediators) are synthesised from eicosapentaenoic acid and can promote resolution of inflammation[159, 160, 166] to help the tissue return to the non-inflamed state and to maintain homeostasis.[543] The role of resolvins as anti-inflammatory mediators has been demonstrated in animal models.[160, 544, 545] Resolvin D_1 (RvD_1) can block neutrophil trafficking,[546] promotes phagocytosis by human macrophages[547] and

regulates production of reactive oxygen species.[159, 160, 548] Production of resolvins is a distinct stage in the progression of inflammation as well as its resolution.[166, 168, 169] Abnormalities in the regulation of resolvins appear to result in prolonged episodes of inflammation in tissues and autoimmune reactions.[168]

The principal goal of this study was to determine diurnal changes of prostaglandins, cysteinyl leukotrienes and resolvin D_1 in tears with and without CLs to find out their possible association with CL discomfort at the end of day (aim 1). Because of the limited volume of tear samples and the possible low concentration of each of these mediators in tears which would require higher sample volumes for measurement, screening tests (described in Section 3.3.3) were chosen for analysis of tear prostaglandins (combined E_2, F_1, $F_{1\alpha}$ and $F_{1\beta}$) and cysteinyl leukotrienes (combined D_4, E_4 and C_4).

3.3 Materials and methods

3.3.1 Participants

This study was performed as a prospective, open-label daily wear, single group two-staged investigation of tear composition with and without CLs in experienced CL wearers. Ethics approval was obtained through the Human Research Ethics Advisory Panel (HREA) of the University of New South Wales (UNSW), Sydney, Australia. All procedures in this study were conducted in compliance with the tenets of the Declaration of Helsinki. All subjects signed an informed consent form before enrolment in the study. A new group of participants with total number of 30 and with no ocular or

systemic disease were enrolled in this trial. Participants consisted of 19 females and 11 males, with mean age of 29.1±7.3. The trial duration for each participant was approximately 3 weeks.

3.3.2 Tear sample collection and rating of ocular comfort

The tear sample collection and preparation methods have been described in Chapter 2: (2.3.3, 2.3.4 and 2.3.5). Briefly, participants collected their own tears twice daily using a glass capillary tube, in the morning during the first hour after waking and in the evening before going to bed. The duration of lens wear was at least 6 hours. Subjects were trained to collect their own tears during several practice sessions to ensure that they could consistently collect more than 2 µL of tears without causing reflex tearing and stop tear collection if reflex tearing occurs. Subjects collected tears for 3 - 4 days. No CLs were worn during stage 1 and 1-DAY ACUVUE® MOIST® lenses (Johnson and Johnson Vision Care, Jacksonville, FL, USA) were worn during stage 2. Lenses were worn on a daily disposable basis and no lens care solution was used in this study. Subjective ratings of comfort were also recorded just before tear collection on a scale of 1 - 100 with an anchor at 100 (extremely comfortable).

3.3.3 Tear assay

Mediator concentrations were measured in tear samples using resolvin enzyme immunoassay (EIA) kit, prostaglandin screening EIA kit and enzyme immunoassay for the cysteinyl leukotrienes concentration. The screening EIA kits for prostaglandins

measured prostaglandins E_2, F_1, $F_{1\alpha}$ and $F_{1\beta}$ simultaneously. With cysteinyl leukotrienes, each EIA kit measured leukotrienes D_4, E_4 and C_4 simultaneously. All kits were obtained from Cayman Chemicals (MI, USA). The detection limits were 15 pg/mL for resolvin D_1 and 29 pg/mL for prostaglandins. The antiserum used for the leukotriene assay had a cross-reactivity of 100% with LTC_4 and D4 and 67% with LTE_4. The detection limit was 13 pg/mL. Experiments were conducted according to the manufacturer's protocols. Each sample was diluted in the ratio of 1:99 (v/v) to measure prostaglandins and leukotrienes and to the ratio of 1:49 (v/v) to measure resolvin D_1. Total volumes of 8 µL of tears were needed for these experiments. Accurate sample concentration for each mediator was calculated from the corresponding standard curve produced using known concentrations of standard antigen *vs* absorbency.

3.3.4 Tear sample collection for studying the day to day variability of mediators

Tears were collected from three experienced tear donors at the same time of day (as described in section 2.3.6) to check for the repeatability of leukotrienes levels when comparing day to day measurements for each individual and when comparing findings between individuals.

3.3.5 Statistical analysis

A preliminary experiment indicated that 30 subjects were required to show a significant paired difference of 1 ± 2 log transformed unit of prostaglandins in the

morning and evening in CL wear with a 95% confidence and 80% power. The IBM® SPSS® Statistical Research Methods and Social Science Statistics (Armonk, New York) was used to evaluate lipid levels and the correlation coefficients between continuous variables. The comfort ratings and concentrations of tear lipids and their changes were analysed using linear mixed models. Pearson's correlation coefficients were computed to examine the association of the mediators and ocular comfort. The results were expressed as mean ± standard error of the mean (SEM). An initial analysis compared the effect of stage (CL *vs* NCL) for each lipid class. The factors involved in the model, namely stage and sampling time (morning *vs* evening) were considered as repeated effects within subject group. A *p*-value of < 0.05 was considered statistically significant. Initial analysis comparing the effect of sampling days (days 1, 2, 3 and 4) for cysteinyl leukotrienes was carried out using linear mixed model analysis.

3.4 Results

3.4.1 Effect of contact lens wear and time on ocular comfort

Comfort ratings, measured on a 1 - 100 scale, declined from morning to evening (**Table 3:1** and **Figure 3:2**). The decline occurred both with and without CL wear, but in this study there was no difference in comfort level between the two stages ($p = 0.10$).

Table 3:1 1 - 100 comforts rating comparing AM to PM (n = 30).

		AM		PM		**Stage (CL *vs* NCL) *p*-value**	**Time *p*-value**
	Stage	Mean	Std.	Mean	Std.		
Comfort	NCL	85.23	17.68	80.31	17.32	0.10	0.001
	CL	84.45	18.93	75.20	18.12		

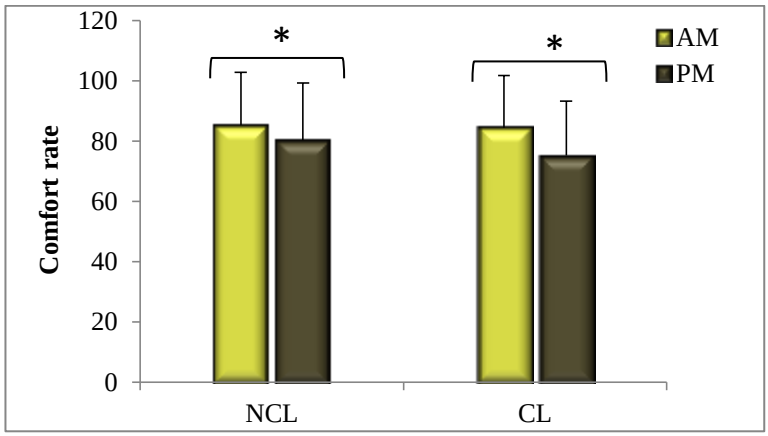

Figure 3:2 Diurnal variation of ocular comfort (AM vs PM, $p = 0.001$; CL vs NCL, $p = 0.10$) (n = 30).

Table 3:2 shows the level of mediators measured in this study and **Figure 3:3** demonstrates the trend of changes for these mediators during the day. The mean concentration of prostaglandins, leukotriene $C_4/D_4/E_4$ and resolvin D_1 measured were 10.69 ± 1.28 ng/mL, 8.70 ± 0.38 ng/mL and 1.61 ± 0.14 ng/mL respectively. No significant differences were found between the morning and evening levels of these mediators and CL wear did not affect their levels during the day ($p > 0.05$;). In just the CL stage the concentration of prostaglandins showed an apparent higher level during the day but this did not reach statistical significance (9.47 ± 0.91 AM *vs* 14.10 ± 4.86 PM; $p = 0.28$).

Table 3:2 The level of arachidonic acid and eicosapentaenoic acid derivatives in AM and PM with and without CL (CL *vs* NCL), n = 30. *p*-values are based on logarithmic transformation calculation.

Mediator	CL Group	Mean ± SEM (ng/mL)		*p*-value*	
		AM	PM	CL *vs* NCL	AM *vs* PM
Leukotrienes	NCL	8.77 ± 0.61	8.94 ± 0.95	0.59	0.81
	CL	8.13 ± 0.60	8.96 ± 0.85		
Prostaglandins	NCL	9.31 ± 0.88	9.89 ± 0.98	0.98*	0.48
	CL	9.47 ± 0.91	14.10 ± 4.86		
Resolvin D_1	NCL	1.93 ± 0.33	1.50 ± 0.30	0.54	0.44
	CL	1.59 ± 0.38	1.32 ± 0.16		

* *p*-value > 0.05 with and without logarithmic transformation calculations.

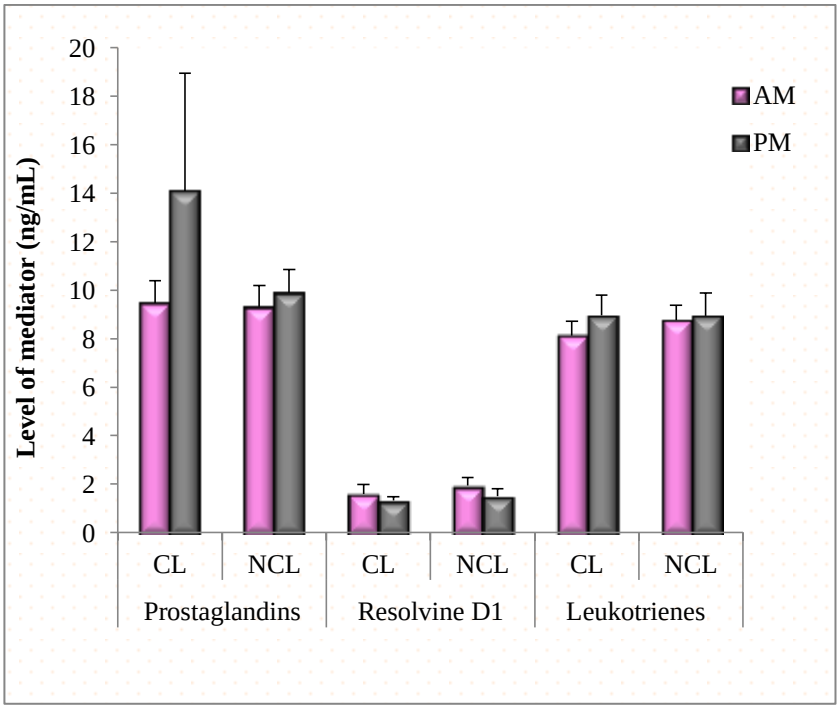

Figure 3:3 Diurnal variation of prostaglandins, leukotrienes and resolvin D_1 with and without contact lenses. Data are mean ± SEM (AM *vs* PM and CL *vs* NCL, $p > 0.05$).

Because of limited sample volumes the day to day variability of cysteinyl leukotrienes only was evaluated. The results of this analysis showed no difference

between the concentrations of this mediator between days. **Table 3:3** demonstrates the mean ± SEM of this mediator in tears collected by 3 subjects over 4 days.

Table 3:3 Day to day variability of cysteinyl leukotrienes (n = 3).

day	Mean	SEM	*p*-value
1	8.08	1.83	0.46
2	8.96	1.83	
3	7.86	2.05	
4	8.79	1.25	

3.4.2 Association between tear proteins and ocular comfort rate

Although the ocular comfort dropped at the end of day both with and without CLs (**Table 3:1**), no association was found between ocular subjective ratings and the level of any of the mediators (**Table 3:4**).

Table 3:4 Association of concentration of mediators and ocular comfort.

Mediator	Pearson's Correlation	Sig. (2-tailed)
Prostaglandins	-0.10	0.28
Resolvin D_1	0.07	0.42
Leukotrienes	-0.04	0.73

3.5 Discussion

This study investigates the role of arachidonic acid and eicosapentaenoic acid derivatives in ocular discomfort with CLs. The levels of prostaglandins (E_2, F_1, $F_{1\alpha}$ and $F_{1\beta}$), cysteinyl leukotrienes (D_4, E_4 and C_4), and resolvin D_1 were measured in tears

collected in the morning and evening from one group of subjects with and without CL wear. This is the first time that resolvin D_1 has been quantified in tears. Resolvin D_1 is one of the mediators important in ceasing acute inflammation to help tissue and organs return to homeostasis. It mainly prevents neutrophil infiltration into inflamed areas and blocks pro-inflammatory activity of LTB_4.[549] The concentration of these mediators increases during inflammation.[23, 138, 548, 550] The level of resolvin D_1, prostaglandins and cysteinyl leukotrienes remained unchanged during the day and CL wear had no effect on the level of these mediators. The lack of change of these mediators during the day and absence of association with comfort level rejects our hypothesis that changes in the levels of these mediators are associated with end of day ocular discomfort. In the current study the morning subjective comfort levels (1 – 100, with 100 being the most comfortable) in CL and NCL wearers were found to be 84.45 ± 8.93 and 85.23 ± 17.68 (mean ± std). This finding is comparable to those reported earlier in lens wear[435, 438, 551] while there is no preceding information about morning subjective comfort rating in no lens wear. The comfort ratings decreased from morning to evening in both CL wear and NCL wear stages but in contrast to our other studies there was no difference in end of day comfort between no-lens wear and lens wear groups. This may suggest that the group of experienced CL wearers who participated in these studies may have been experienced discomfort in non-lens wearing stage and the results may not be representative of general CL wearing population. This suggestion can be supported by differences found in end of day comfort between wearing and non-wearing lens stages, available in the literature[453, 552, 553] as well as our own studies presented in Chapters 2, 5 and 7. Therefore it might be worthwhile to repeat similar studies using a larger number of subjects with overt discomfort such as in individuals with dry eye or CL intolerance.

The method of measurement of prostaglandins and leukotrienes involved using EIA screening kits measuring a number of mediators simultaneously. Some of these mediators have been measured in different settings previously. The level of LTC_4 has been reported to be between 0.07 and 0.19 ng/ml in tears of healthy subjects (without CL wear).[554, 555] The concentration of PGE_2 in dry eye patients and healthy control groups with no history of ocular or systemic disease was 2.72 ± 3.42 ng/ml *vs.* 0.88 ± 0.83 ng/ml (mean ± SD).[139] These values were obtained using nano-liquid chromatography tandem mass spectrometry. Although, in our study four prostaglandins were measured simultaneously using immunoassay methods and the previous results are substantially lower than the findings of our study, the measured prostaglandis may not be directly comparable to our measured levels; still they can offer some interpretation by way of comparison.

Contact lens wear-induced discomfort has been documented as 30% decrease in subjective ratings for comfort after six hours of lens wear.[189, 435] In this study comfort dropped by 30% in about 7% of subjects during the day. So, a limitation of this study may be that the subjects recruited for the purpose of these trials may not be representative of the general CL wearing population. Also, The concentrations of these mediators in tears are very low and therefore high volumes of samples will be required to measure them. Further data on the level of prostaglandins in symptomatic CL wearers may need to be obtained to clarify the role of these mediators in ocular discomfort in CL wear. Other lens types may need to be included in trials to verify these findings. Moreover, it is possible that other molecules involved in the inflammatory process and/or changes in the level of other compounds such as proteins are involved in mechanisms of CL-related discomfort.

3.6 Conclusions

This study was performed in a group of volunteers in two subsequent stages without and with CLs using enzyme immunoassay methods. Cysteinyl leukotrienes, prostaglandins and resolvin D_1 had no diurnal variation with or without CLs in tears. No correlation was found between the levels of these mediators and ocular comfort. Therefore the initial hypothesis of association between prostaglandins, leukotrienes and resolvin D_1 with ocular discomfort is rejected.

Chapter 4: Quantification of tear proteins using selected reaction monitoring

4.1 Overview

The aim of this chapter is to design and develop selected reaction monitoring (SRM) mass spectrometry for quantification of tear proteins. We hypothesised that this technique can be used to quantify a range of proteins in small volume samples.

4.2 Introduction

CLs can function as superficial foreign bodies and can alter the tear film[556, 557] and thus activate mechanisms leading to CL-related adverse events,[31] resulting in ocular discomfort and dryness.[412,188] The mechanisms underlying these events are unknown but might involve changes in the levels of tear proteins similar to those documented in dry eye conditions.[187]

A variety of methods for measuring tear proteins have been described, including enzyme-linked immunosorbent assay (ELISA)[75, 212] and two-dimensional SDS-PAGE analysis (2-DE).[558] The results of such studies depend on antibody cross-reactivity and gel mobilities, and frequently need to be verified by alternative methods such as protein sequence analysis. Thus relatively large quantities of protein are required to achieve reliable results.[559] Other methods, aiming at multidimensional protein identification, have also been employed.[560, 561] Whether these alternative methods can be reliably applied to clinical samples is still not clear. In view of the increasing interest in measuring protein levels in tears, an improved method is needed that resolves the

drawbacks of previous techniques and allows reliable and repeatable quantification of proteins in such complex mixtures.

Recent studies have described mass spectrometry (MS) as a general method for quantification of proteins in human fluids.[405, 562-566] Proteomics and transcriptional profiling methods have been implemented by other researchers to examine the tear proteome[174, 187, 188] and have resulted in identification of a number of candidate protein markers for detection of diseases such as cancer[567, 568] or dry-eye.[174] Complex humeral fluids other than the tear fluid, including serum and urine have been subjected to quantification by selective reaction monitoring (SRM).[569, 570] For samples with high and intermediate protein abundance, the SRM method is highly sensitive and can simultaneously quantify up to 40 proteins[571] while maintaining high mass accuracy in protein profiling. Additionally, using the SRM method, quantitative mass spectrometric analysis of endogenous human peptides can be performed against internal standards. Synthetic peptides labelled with stable isotopes are used for this purpose. These isotopes are identical in chemical structure to the corresponding endogenous peptides despite their difference in mass, rendering the preparations of standards reliable. Thus, the SRM methodology can be proposed as a potentially useful experimental tool for studies aimed at identification of proteins in human tears and their changes during ocular disease such as dry eye or during CL wear.

Previous SRM quantification of complex humeral fluids involved derivatisation and depletion of proteins and peptides, requiring additional purification steps.[572, 573] In view of the limited availability of tear samples and taking into consideration that only 5 - 10 μL of tears are available for collection at every attempt, the main aim of this study

was to establish whether the SRM method could be used for simultaneous quantification of proteins in very small sample volumes of human tears without requiring prior purification of each individual protein component. In order to successfully establish the assays, a limited number of proteins described in the literature as potential markers of dry eye[174] were selected to evaluate accuracy, precision, linearity, limit of detection, and quantification of the SRM method.

4.3 Method

4.3.1 Material and chemicals

Dithiothreitol, iodoacetamide, recombinant human lysozyme and lactoferrin (≥ 90% purity as indicated by suppliers) and Trizma® Base were obtained from Sigma Aldrich (St. Louis, MO). Recombinant human lipocalin-1 was obtained from R&D systems (Minneapolis, MN). Sequencing grade modified trypsin was purchased from Promega (San Luis Obispo, CA). Urea was obtained from VWR International Ltd (Poole, UK). Acetonitrile high performance liquid chromatography (HPLC) gradient grade, ammonium bicarbonate, heptafluorobanzile, acetone and formic acid were purchased from Merck (Darmstadt, Germany). Deionised water was prepared on a cartridge-deioniser from Milli-Q (Millipore; Billerica, MA).

4.3.2 Subjects

All studies were approved by human ethics committee of the University of New South Wales and followed the Declaration of Helsinki. All subjects signed written informed consent before commencing the study. All experiments were performed on aliquots of tear samples collected from seven normal volunteers (male = 1, female = 6). Subjects had no ocular disease and were either CL wearers (n = 3) or NCL wearers (n = 4).

4.3.3 Tear collection

Subjects were trained to use capillary tubes for tear collection in the morning and evening for 7 - 10 days using a non-invasive method (minimal or no eye irritation) described elsewhere.[26] In this study collection of tear samples was performed twice a day only to collect larger volumes of samples rather than comparing any two time points together. Briefly, each subject tilted his or her head to one side and gently applied the tip of a 10 µL glass microcapillary tube (BLAUBRAND® intra MARK, Wertheim, Germany) with rounded edges to the corner of the eye (lateral canthus). A maximum of 10 µL tears were collected at each time of tear collection from both eyes (overall tear samples varied between subjects and was 60-100 µL) and were expelled into 0.5 mL eppendorf tubes (Scientific Specialities Inc., CA) using a small manual rubber pump. Subjects were asked to store samples in a -20 °C home freezer and transport them on ice to the university laboratory facility, where the samples were stored at -80 °C prior to processing.

4.3.4 Tear processing method

The total volume of tear samples collected by each subject was expected to reach 35 – 100 µL. No minimum volumes were designated for sample collection. The first 10 µL of samples donated by each individual were stored separately for comparison of results in later stages of the study. The remaining samples were pooled together to obtain one large sample for each individual for the consistency of results in all method development experiments. The tear samples were centrifuged at 1000 g for 10 minutes at 4 °C to remove debris or cells. The supernatants were aliquoted and stored at -80 °C for subsequent use. Tears were processed as described elsewhere for plasma with modifications.[574] Briefly, for each experiment 2.5 µL of the pooled sample was diluted 1:1 (v/v) with deionised water and 5 µL of 8 M urea, 300 mM Tris, pH 7.8 were added. Urea was used as a denaturant and proteins were reduced with 0.5 µL dithiothreitol (100 mM), kept for 30 minutes at 37 °C. Sufficient reagents were prepared for the complete study so as to minimise the variance produced by such a procedure. Cysteine residues were alkylated by 0.5 µL iodoacetamide (200 mM) in water to obtain 20 mM concentration in the samples and the samples were incubated for 30 minutes at 37 °C in the dark. Ice cold acetone was added to the sample to the ratio of 4:1 (v/v). The mixture was incubated at -20 °C for 1 hour. The samples were centrifuged in a 5840R centrifuge (Eppendorf AG, Hamburg, Germany) at 4000 g for 15 minutes at 4 °C. After centrifugation, the supernatant was discarded. The pellet was collected by decanting; the acetone was removed by air drying for 10 minutes and non-fractionated tear proteins were reconstituted in 2.5 µL of 100 mM ammonium bicarbonate buffer pH 8.50. Trypsin (200 ng/µL) was added to achieve a 1:50 enzyme-to-substrate ratio (w/w) on the assumption that ~10 µg/µL total proteins were present in tears[29, 195] and the mixture

was incubated at 37 °C for 17 hours to produce a mixture of tryptic peptides. Samples were acidified to pH 2.0 by adding 0.05% heptafluorobanzile in 0.1% formic acid to stop digestion. For quantitation of proteins in the samples, a concentration-balanced mixture of 10 isotopically labelled internal standard peptides (50% acetonitrile in 0.1% (v/v) formic acid) was added to each digested tear sample at a ratio of 1 volume of peptides mixture to 8 volumes of acidified tear digest.

4.3.5 Selection of signature peptides

Five tear proteins described in the literature as potential markers of dry eye disease (lactoferrin, lysozyme, lipocalin 1, proline rich protein 4 and prolactin-induced protein)[174, 187, 188] were studied using purely *in-silico* methods. The FASTA[575] formatted sequences of target proteins were directly pasted into the Skyline program v1.5 (MacCoss Lab, University of Washington, Seattle, USA) which generated a full list of peptides and corresponding product ions (Y-ions) and the results were compared against the human National Institute of Standards and Technology (NIST) spectral library. Tear samples were run on a nano-flow liquid chromatography mass spectrometer (LTQFT Ultra, Thermo Electron, Bremen, Germany); the results were compared against the Mascot search engine to identify the more frequently observed tryptic peptides in the MS/MS analysis.[576-578] By using the combined information of the previous two steps, a list of peptides ending in C-terminal lysine (K) or arginine (R), containing 5 - 15 amino acids were selected for analysis. Selection of each peptide was based on the signal strength, intensity of electrospray ionization process and chromatographic peak shape (symmetric peak with a narrow width). Peptides containing methionine and cysteine or

those with two basic amino acids (KK, RR, or KR) at the N or C terminus were excluded from experiments.[579] To select appropriate transitions, the list of transitions was saved as a file (in the csv format) and exported into the 4000 quadrupole-linear ion trap mass spectrometer (Qtrap, Applied Biosystems, USA). The double charged and singly charged fragment ions (first and third quadrupoles) were selected based on their signal intensity and the ability to discriminate target peptides from the rest of the peptides present in the sample. Transitions with lowest coefficient of variation (CV) were chosen as the best transitions. These were mostly the ones with highest abundance and were used to quantify proteins in tear samples. To ensure that the peptides selected using this procedure were sequence-unique to each of the proteins of interest; the Basic Local Alignment Search Tool (BLAST) was used to perform a homology search against the National Center for Biotechnology Information (NCBI) database. Skyline v1.5 (an open source program) was used to analyse the files generated by SRM which automatically generated the chromatogram, retention time and peak area for each peptide and its corresponding product ions.

4.3.6 Chromatography and mass spectrometry instrumentation

Instrumentation used for chromatography included a nano-LC Ultimate 3000 HPLC (Dionex, Amsterdam, Netherlands) and Switchos and Famos autosampler (LC-Packings, Amsterdam, Netherlands). One microliter of tear digests were concentrated and desalted onto a micro C18 pre-column (500 µm x 2 mm, Michrom Bioresources, Auburn, CA) with H_2O:CH_3CN (98:2, 0.05 % trifluoroacetic acid) at 15 µL/min for 4 minutes. The pre-column (Valco 10 port valve, Houston, TX), which was connected via

a fused silica capillary (Upchurch Scientific, Oak Harbor, WA), was automatically switched into a nano-flow liquid chromatography (LC) system.[580] Solvents used in chromatography were H_2O:CH_3CN (98:2, 0.1% formic acid; Solvent A) and H_2O:CH_3CN (36:64, 0.1% formic acid; Solvent B). Samples were eluted at ~300 nl/min over 30 min. SRM analyses were performed on a 4000 Qtrap. The dual mass spectrometry (MS/MS) system consisted of an ion spray (voltage 2.4 kV), curtain gas flow of 12 µL/min and nebulising gas flow of 5 µL/min with positive ion mode and was controlled by Analyst 1.5 software (Applied Biosystems) and interface heater temperature set at 150°C. Collision energies (CE) were optimised as described before (CE = 0.043 m/z + 4.756)[581] for maximum transmission of individual "signature peptides".[582] A total of 60 SRM transitions (3 per peptide) were monitored during an individual sample analysis. Instrument parameters remained unchanged for each unlabelled/labelled pair. To ensure that maximum specificity was achieved with samples as complex as human tears, SRM transitions were acquired at unit resolution in the first and third quadrupoles. Dwell times of 25 or 50 ms were used for selected transitions and cycle times did not exceed 1 second.

4.3.7 Unlabelled and labelled peptides (internal standards)

Following selection of the signature peptides (the dominant ions in collision-induced dissociation fragmentations)[583] uniformly isotope-labelled stable peptides containing C-terminal [^{13}C, ^{15}N]-lysine or [^{13}C, ^{15}N]-arginine and corresponding normal (non-isotope labelled peptides) were purchased from Sigma Aldrich (St. Louis, MO). Individual synthetic unlabelled peptide stocks of 1 nmol/µL and labelled peptide stocks

of 10 pmol/µL were prepared in 0.1% formic acid and/or 30 - 50% acetonitrile in 0.1% formic acid (v/v) (based on hydrophobicity of the peptide). To investigate the purity of the labelled and un-labelled peptides and to confirm their homology with the corresponding endogenous forms of the tear peptides, all purchased peptides were analysed by amino acid analysis (AAA). AAA was performed by the manufacturer for the labelled peptides and by the Australian Proteome Analysis Facility (Sydney, Australia) for the unlabelled peptides. To reflect the AAA values, stock dilutions were performed to 0.1 nmol/µL with 30% acetonitrile in 0.1% formic acid. The synthetic peptides also were used to optimise the mass spectrometry analysis parameters.

4.3.8 Calibration curves of synthetic peptides

Serial dilutions of synthetic [$^{12}C/^{14}N$] peptides were generated by diluting unlabelled standard peptides to final concentrations of 1, 5, 25, 125, 250, 500, 1000 fmol/µL in the presence of 50 fmol/µL of the labelled peptides. The ratio of the peak areas of the unlabelled to labelled peptides was plotted against the concentration of the corresponding unlabelled peptides. Limits of detection (LOD) and quantification (LOQ) were determined using a previously reported technique.[574, 584] Optimal LOD and LOQ were achieved for each peptide by performing three independent identical experiments. The 95th percentile of the blank samples was used as the limit of blank (LOB) and was calculated as $mean_b + {}_{t1-\alpha} \times SD_b$ where $mean_b$ and SD_b are the mean and standard deviation of the blank sample. The LOD was calculated using the following formula ($LOD = LOB + C_\beta \times SD_s$) where SD_s is the standard deviation of the lowest level spike.[574] The LOQ was assumed to be three times the LOD.[573] To determine the effect

of matrix on the linearity of the peptide dilutions, the same procedure was carried out in the presence of digested tears. Tear samples were diluted (1:50 in 25% acetonitrile in 0.1% (v/v)) before spiking the unlabelled standard peptides to final concentrations of 1 to 1000 fmol/µL adding 50 fmol/µL signature peptide [$^{13}C/^{15}N$] into the tears. Blank runs of digested tears with labelled peptides provided estimates of chemical background levels in the absence of signature peptide peaks. Furthermore, an estimate of carryover was determined by running a series of gradient HPLC washout runs. The relative intensities of all the three transitions were identified and background correction was applied uniformly throughout the study. A seven-point standard curve (a plot of response against known concentration) was produced for each of the three transitions of each peptide to evaluate the linearity of the SRM measurement across the range of spiked peptides for each experiment. All experiments were repeated three times. Experimentally measured concentrations of the spiked peptides were plotted against the theoretical concentration values (all in molar units) to find the rate of recovery of each peptide. To ensure the method covered a sufficient range for measurement of peptide levels in unknown samples, the combination of linear regression with response factor [(peak area ratio - y intercept)/concentration)] plots were tested to identify analyte concentrations that did not respond in a linear manner for each peptide. To ensure that the sample dilution matched the concentration range of the standards, dilutions of a tryptic digest of the pooled tear samples were prepared to a range of 2 - 18 fold and were spiked with 50 fmol/µL of all internal standards. Three replicate LC-SRM/MS analyses of each sample dilution were performed. The concentration of each of the 10 peptides in the pooled sample was calculated by dividing the concentration of each

peptide by that of the corresponding internal standard, averaged for three identical but independently performed experiments.

4.3.9 Quantification of tear protein concentrations in tear samples

To assess the general applicability of the labelled peptide as an internal standard for quantification of selected tear proteins, aliquots of pooled tear samples were processed for analysis (as described above). The concentration of endogenous proteins (µg/µL) in tear samples was deduced using the peak area ratio between the endogenous and labelled peptides (50 fmol/µL). Labelled internal standard peptides were added post digestion. Replicate analyses of the spiked tear samples provided estimates of assay precision.

4.3.10 Reproducibility of SRM and protein recovery rate

Human tears and equal concentrations of commercially available lactoferrin, lysozyme and lipocalin proteins (10, 5, 2.5, 1.25 µg/µL) were digested separately with trypsin by the procedure described above (proline rich 4 and prolactin-induced protein were not commercially available). To simulate biological duplicates, each concentration point was digested in duplicate prior to adding the protein digest mixture to diluted tear digests. Internal standards for each peptide were prepared in a similar manner. All LC-SRM/MS runs were performed in duplicate (technical replicates). Digestion of recombinant proteins served as a control for digestion efficiency across diminishing concentration points. To assess the concentration of purchased recombinant proteins,

LavaPep total protein assay (Star Scientific, Sydney, NSW, Australia) was performed according to the manufacturer's instructions. Comparison of measured and the nominal spiked protein concentration values was recorded as protein recovery rate.

4.3.11 Reproducibility of acetone precipitation

Accuracy of experimentally-prepared protein concentrations can be affected by acetone precipitation rates[572, 585] which may vary between samples. The potential effect of this confounding factor was examined with pooled tear samples and calculation of the CVs of all 10 peptides. Three independent *in vitro* pooled tears were measured on three consecutive days and each sample was run in duplicate.

4.3.12 Comparison of protein concentration determined by SRM-MS and Immunoassay in tears

The concentration of lactoferrin in the tears of participants was confirmed by ELISA (Hycult biotech, Uden, The Netherlands) according to the manufacturer's instructions.

4.4 Results

4.4.1 Signature peptides

The list of target proteins, their "signature peptides", three sets of mass-to-charge ratio (m/z) of transitions (first and third quadrupole) per peptide and their retention times in the SRM assay are presented in **Table 4:1**.

Table 4:1 Target proteins and their signature peptides.

Identifier	Protein Name	Peptide Sequence	Abbreviation	Purity (%)	Precursor M/z Z(Q1,Q2)= 2	Product M/z (Q3) Z (Q3) = 1	Fragmentation	The best fragment ion type	Retention Time
gi|186833	Lactoferrin	DGAGDVAFIR	lac-DGAG	> 95	510.76	777.43, 605.34, 506.31	y7, y5, y4	y7	28.7
		DGAGDVAFIR		98	515.76	787.43, 615.39, 516.32	y7, y5, y4	y7	28.3
		FQLFGSPSGQK	lac-FQLF	> 95	598.31	920.48, 807.40, 660.33	y9, y8, y7	y9	32.1
		FQLFGSPSGQK		99	602.32	928.50, 815.41, 668.35	y9, y8, y7	y9	32.1
gi|1335210	Lysozyme	ATNYNAGDR	lys-ATNY	> 95	491.22	695.31, 532.25, 418.21	y6, y4, y5	y4	15.5
		ATNYNAGDR		98	496.23	705.32, 542.26, 428.21	y6, y4, y5	y4	15.4
		STDYGIFQINSR	lys-STDY	> 95	700.84	934.51, 764.41, 617.34	y8, y6, y5	y5	33
		STDYGIFQINSR		98	705.85	944.52, 774.41, 627.35	y8, y6, y5	y5	33
gi|50960143	Lipocalin-1	NNLEALEDFEK	lip-NNLE	> 95	661.32	1093.54, 980.46, 851.42	y9, y8, y7	y8	32.6
		NNLEALEDFEK		99	665.32	1101.56, 988.47, 859.43	y9, y8, y7	y8	32.6
		GLSTESILIPR	lip-GLST	> 95	593.35	698.46, 498.34, 272.17	y6, y4, y2	y2	32.5
		GLSTESILIPR		99	598.35	708.46, 508.35, 282.18	y6, y4, y2	y2	32.4
gi|116642853	Prolactin-inducible protein	TYLISSIPLQGAFNYK	pro-indu-TYLI	> 95	907.99	1324.69, 1037.54, 699.35	y12, y9, y6	y9	38.5
		TYLISSIPLQGAFNYK		99	912	1332.70, 1045.56, 707.36	y12, y9, y6	y9	38.5
		FYTIEILK	pro-indu-FYTI	> 95	513.8	879.52, 716.46, 615.41	y7, y6, y5	y6	35.8
		FYTIEILK		98	517.8	887.53, 724.47, 623.42	y7, y6, y5	y6	35.8
gi|4504963	Proline rich protein 4	QLSLPR	pro-QLSL	> 95	357.22	472.29, 385.26, 272.17	y4, y3, y2	y2	26.1
		QLSLPR		97	362.22	482.30, 395.26, 282.18	y4, y3, y2	y2	26.1
		FPSVSLQEASSFFR	pro-FPSV	> 95	801.4	1357.67, 1084.54, 971.46	y12, y9, y8	y12	40
		FPSVSLQEASSFFR		96	806.41	1367.68, 1094.55, 981.47	y12, y9, y8	y12	40

Bold face type indicates labelled peptide internal standard with labelled amino acid in red. The product ions and fragments are colour-coordinated.

4.4.2 Linear Response of SRM Quantitation

Excellent linear responses ($R^2 > 0.99$) were obtained for 7 of the 10 peptides (**Figure 4:1)**. The peptides that showed lower linear responses (TYLISSIPLQGAFNYK, FYTIEILK and FPSVSLQEASSFFR) still showed acceptable R^2 values (0.9425 - 0.9881). Addition of light synthetic peptides to digested tears showed high intensity and peak area ratios for the range of concentrations used indicating that these peptides responded well and could be used in this assay. **Figure 4:2** shows the chromatographs of NNLEALEDFEK from lipocalin 1 as an example. Also, the relative ratios of the transitions in both solutions agreed closely indicating that there was little interference from the matrix in the MS channels. **Figure 4:3** shows the extracted ion chromatograms of FQLFGSPSGQK peptide of lactoferrin as an example.

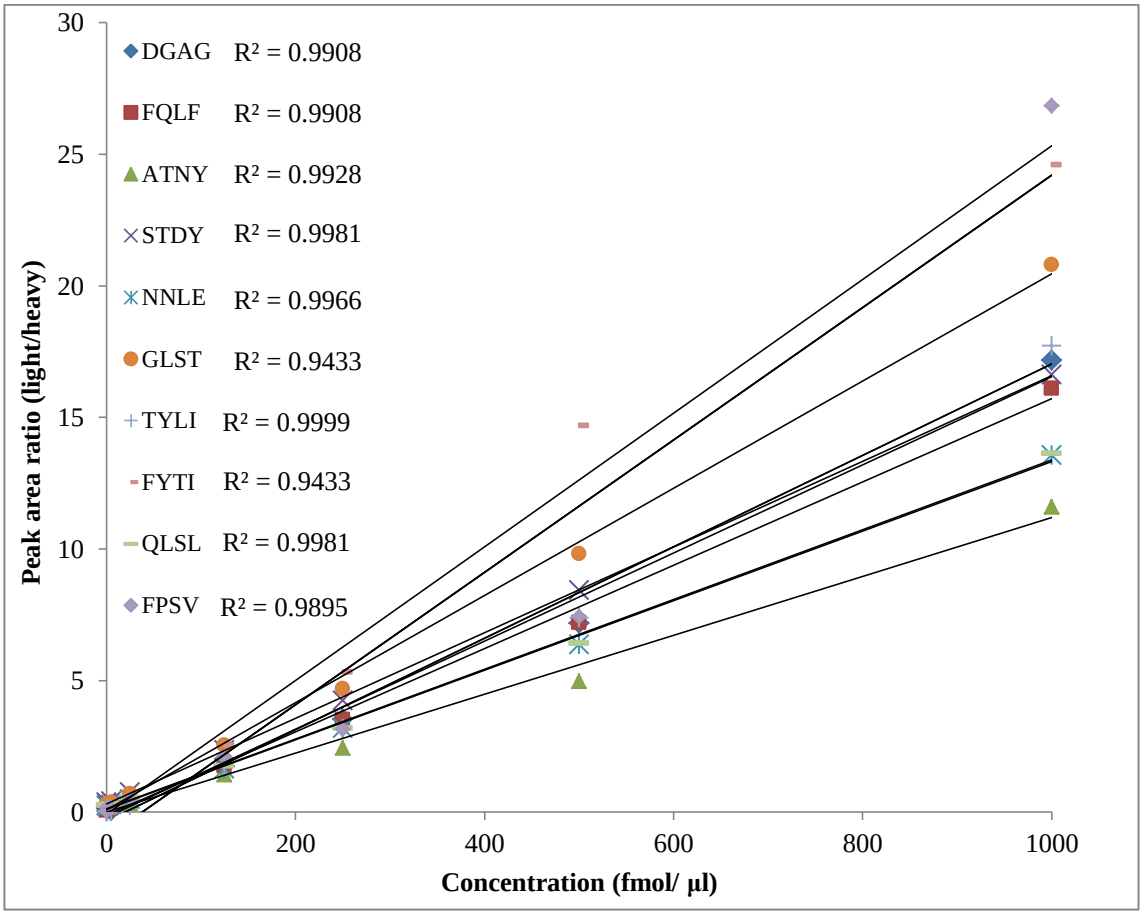

Figure 4:1 Concentration curves and corresponding R2 values for all ten signature peptides in solvent (0.1% formic acid to 50% formic acid in acetonitrile, v/v) over the range of 1 – 1000 fmol.

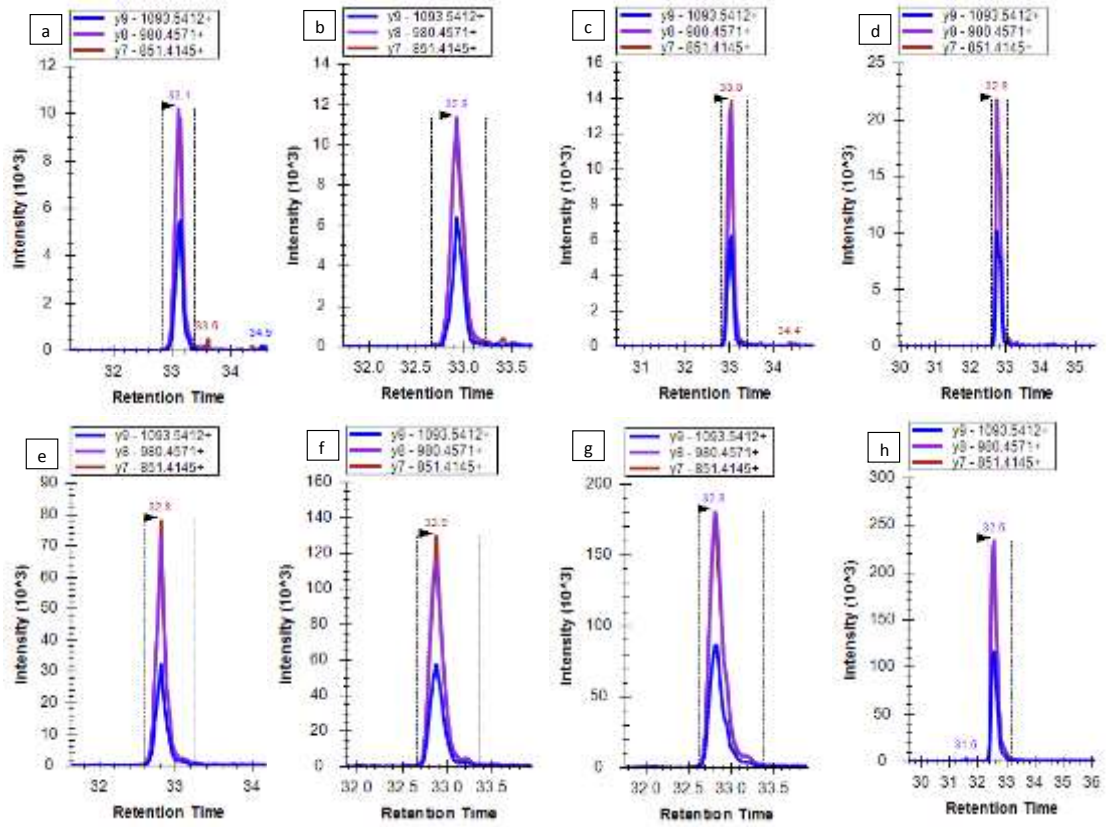

Figure 4:2 Lipocalin 1 NNLEALEDFEK peptide chromatographs. a) Diluted digested tears; addition of high synthetic peptides (1, 5, 24, 125, 250, 500 and 1000 fmol/mL; b-h) to digested tears showed higher intensity and peak area ratio for the range of concentrations indicating that these peptides responded well and can be used in this assay.

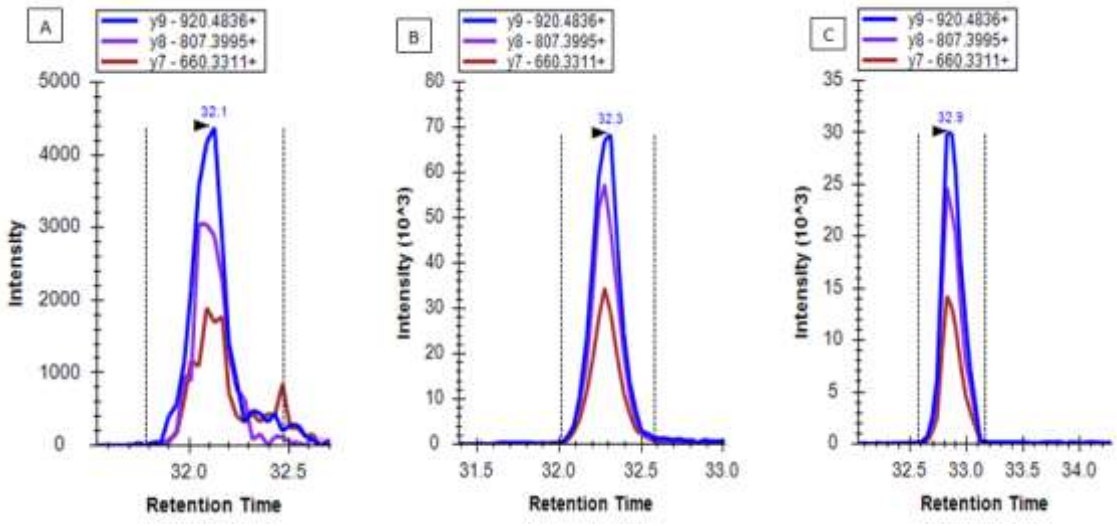

Figure 4:3 Extracted ion chromatograms monitored for the FQLFGSPSGQK peptide of lactoferrin presented as and example to show the lack of matrix interference present in the MS channel. A) blank matrix (no material derived from the sample), B) pure standard material (25 fmol/µL), C) tears with the 1 fmol concentration of a lacto-FQLF peptide.

4.4.3 Limit of detection and quantification

The limit of detection (LOD) for the ten selected peptides was between 0.53 - 8.96 pg/ml and limit of quantification (LOQ) was 1.59 - 26.88 pg/µL, when peptides were added to the diluted tears (**Table 4:2**). Of note is the fact that each of two signature peptides for each protein could yield a different LOQ for their respective proteins.

4.4.4 Reproducibility and precision of SRM assays

Table 4:3 shows a compilation of response curves for the ten peptides plotted on a linear-linear scale of experimentally determined concentrations against theoretical concentrations of the target analyte and provides an overview of recovery of the peptides. The decrease/increase in the slopes of the response curves might result from

the complexity of protein digestion. The LOQ estimations across peptides (**Table 4:2**) and coefficients of variation (CVs) of 1.3 - 15.1% indicated good reproducibility. Protein recovery rates during protein digestion and sample preparation, determined in trypsinised digests, were 75.0%, 60.4% and 79.8% for lactoferrin, lysozyme and lipocalin-1, respectively. The results of CVs of the selected peptides after acetone precipitation are presented in **Table 4:3**. For eight of the 10 peptides the CVs were ≤ 18% whereas only two peptides had a CV between 21 - 24%.

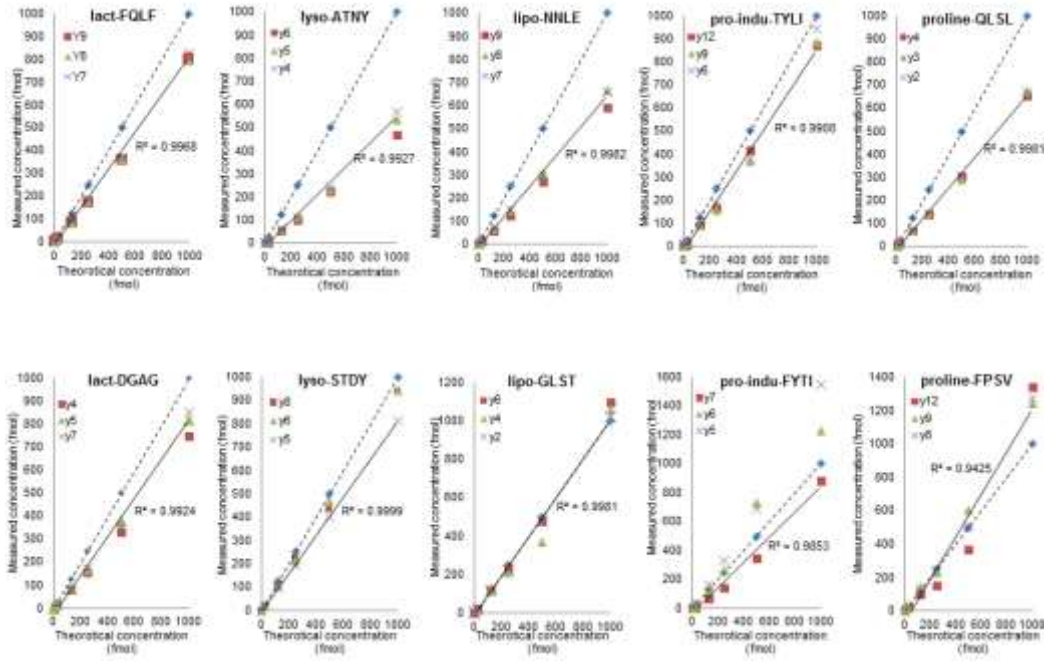

Figure 4:4 Reproducibility of linear calibration curve slopes for 10 selected peptide standards spiked in tears. The seven point curve for each peptide display the concentration curves for the detection of each peptide in selected proteins. The R square has been provided only for the best transition (see Tables 1 and 2). Comparison of the plots demonstrates good linearity, with most of the slopes falling close to the diagonal, stitched lines (theoretical slope = 1), and good agreement between the three transitions at each concentration point.

Table 4:2 LOD/LOQ for target peptides in healthy human pooled tear samples

Protein	Signature peptide	Molecular Mass (KDa)	Fragmentation	Recovery (%)	linear slope	CV (%)	LOD (pg/µL)	LOQ (pg/µL)
Lactoferrin	DGAGDVAFIR	78	y7	84	0.992	10	5.48	16.45
	FQLFGSPSGQK		y9	79	0.996	1.5	6.42	19.27
Lysozyme	ATNYNAGDR	16	y4	56	0.992	6.3	3.46	10.37
	STDYGIFQINSR		y5	82	0.999	3.5	8.96	26.88
Lipocalin-1	NNLEALEDFEK	19	y8	66	0.998	5.5	5.24	15.73
	GLSTESILIPR		y2	102	0.995	1.4	5.9	17.71
Prolactin-induced protein	TYLISSIPLQGAFNYK	16	y9	87	0.988	15.1	6.51	19.53
	FYTIEILK		y6	127	0.985	2.8	0.53	1.59
Proline rich protein 4	QLSLPR	16	y2	68	0.988	9.5	4.13	12.4
	FPSVSLQEASSFFR		y12	127	0.943	1.3	0.81	2.44

CVs were calculated from all replicates for each peptide using a single transition at 5 fmol concentration which was close to LOD for most of the peptides.

Table 4:3 The CVs of 10 selected peptides by acetone precipitation.

Protein	Peptide	CV (%)
Lactoferrin	DGAG	7.7
	FQLF	23.1
Lysozyme	ATNY	21.5
	STDY	17.2
Lipocalin 1	NNLE	14.2
	GLST	13.9
Prolactin-induced protein	TYLI	17.5
	FYTI	11.3
Proline rich protein 4	QLST	10.7
	FPSV	12.3

4.4.5 Quantification of selected proteins in tears

The concentrations of selected tear proteins using SRM-MS were as follows: lactoferrin at 1.20 ± 0.77 μg/μL, lysozyme at 2.11 ± 1.50 μg/μL, lipocalin 1 at 1.75 ± 0.99 μg/μL, prolactin-induced protein at 0.09 ± 0.06 μg/μL and proline rich protein 4 at 0.80 ± 0.50 μg/μL. **Table 4:4** shows the comparison of the results of the concentration of lactoferrin by the SRM and ELISA methods for seven tear samples. Using a paired t test, there were no differences between ELISA (1.18 ± 0.46 μg/μL) and SRM (1.22 ± 0.63 μg/μL) results ($p < 0.05$).

Table 4:4 Comparison of lactoferrin concentration measured by SRM and ELISA method.

Sample No.	Lactoferrin (µg/µL)	
	ELISA	SRM
1	1.44	1.47
2	0.77	0.68
3	1.59	1.1
4	1.46	1.95
5	0.46	0.57
6	0.92	0.65
7	1.63	2.1

4.5 Discussion

The tear film is a unique fluid which contains a wide range of proteins. Using nano-HPLC-MS/MS, de Souza *et al.*,[370] identified 491 proteins in the tear film of one individual. Zhou *et al.*,[171] using various fractions of tears and nano-reverse phase HPLC-MS/MS, identified 1543 proteins in tears collected from 4 healthy non-CL wearers, with 714 proteins being present in all samples. A large number of these proteins have an established role in physiological maintenance of the ocular surface[195, 269] but some might be involved in various pathological conditions and tear film abnormalities. The tear film composition is known to change in response to various pathological conditions.[269, 294] Hence, individual tear film components have been proposed as suitable biomarkers for diseases.[174, 187, 188, 586] However, in view of the

limited availability of tear samples, new methods need to be developed in order to facilitate quantification of multiple tear components using small volume tear samples.

This study examines selected reaction monitoring as a technique for quantification of proteins in small-volume human tear samples. A major part of the study was focused on developing the tear processing method directed at increasing digestion efficiency and protein recovery rate. Particular attention was paid to simplification of the tear processing method. The experimental method described here detected and quantified the concentration levels for five proteins. Our results for lactoferrin, lysozyme and lipocalin are consistent with findings from previous ELISA studies,[29, 75, 175, 587] and we confirmed this for lactoferrin in the current study. Tear concentrations of proline rich protein 4 (0.80 ± 0.50 μg/μL) and prolactin-induced protein (0.09 ± 0.06 μg/μL) are reported here for the first time.

Quantification of proteins in this study was performed in tear digests using two signature peptides per selected protein as quantitative factors and stable isotope-labelled variant of the same peptides as internal standards. Labelled peptides were also used for normalisation of the natural peptide peaks using a modification of earlier described methods.[588] In this method we used precipitation to separate tear proteins from the remaining components of the matrix. An attempt was made to reduce false-positive responses in the mass spectroscopy and chromatography methods by comparing signals for each individual peptide in tear samples with those obtained from pure standards. The comparisons yielded highly similar results suggesting accuracy of the findings. Optimised sample dilution rates were used in these studies to avoid system saturation. Still, in general, poor chromatography technique and inevitable attenuation of MS

signals as well as software related errors in detection of signal peaks might influence accuracy of quantification in peptide SRM-MS.[589]

The results of this study confirm earlier findings[579] suggesting that equimolar concentration of peptides chosen for quantification of each protein (paired signature peptides) have different MS detectability. The lack of consistency in performance of paired signature peptides could be caused by several factors including incomplete trypsin digestion,[571] loss of peptides[560] and unequal ionization rate of individual peptides.[574] It is recommended that at least two signature peptides be used for quantification of each protein so that an average of the two can be used for quantification.

In conclusion, these experiments used 10 stable internal standard peptides to develop rapid, specific, and sensitive SRM assays to quantify the levels of five human tear proteins. This method requires some sample preparation but no protein purification or use of antibodies, and has linearity across a broad range of protein/peptide concentrations. Further work will be required to expand the established panel of isotopically labelled peptides to include additional proteins. The expanded assay would potentially serve as a valuable investigative tool for exploration of tear proteins in various tear-related studies and the effect of diseases on the tear matrix. This method can potentially be used to target relevant proteins in a range of applications, making this a very specific investigational tool.

Chapter 5: Diurnal variation of tear proteins and ocular discomfort

5.1 Overview

The aim of this study is to quantify diurnal variations of lactoferrin, lysozyme, lipocalin 1, proline rich protein 4 and prolactin-induced protein in human tears with and without CLs and to determine their association with ocular comfort levels while testing for repeatability of these changes. We hypothesised that changes in the concentration of these proteins is associated with changes in ocular comfort and that these changes are not simply due to day to day variation of these proteins.

5.2 Introduction

Proteins are one of the main components of human tears and are secreted from lacrimal and accessory glands. There are more than 700 different proteins in the normal tear fluid,[171] with the total protein concentration estimated at between 2 to 12 mg/mL in basal tears.[26, 27, 29, 175-177] Frequently, their concentrations vary with tear flow rate[212] and tear method collection method. [30] Tear proteins contribute to ocular defence against microbial invasion.[590] Changes in their profile may render the tear film unstable[19, 56] which in turn leads to changes in the health of the ocular surface[174] and discomfort.[591] CLs can interact with the ocular

It is known that CLs can affect tear proteins in two different ways. Firstly, lenses can absorb tear proteins[295, 592] and secondly, they can alter the secretion levels of specific proteins into tears.[220, 369, 593-595] The mechanisms underlying these events are yet

unknown but surface and alter the tear film.[412, 556, 596] Therefore, CLs may activate mechanisms leading to CL-related adverse events[31] and ocular discomfort and dryness.[412,188] These symptoms have been reported in between half to three quarters of lens wearers [9, 553] specifically at the end of the day.[435] Since most contact lens discomfort complaints appear to result from CL-related dryness, the process might involve changes in the levels of tear proteins similar to those documented in dry eye conditions.[187] Tear samples collected from dry eye patients have shown reduced levels of prolactin-induced protein, lipocalin-1, lactoferrin and lysozyme.[174] Nichols *et al.* found that the concentration of prolactin-induced protein increased, while that of proline rich protein decreased in CL-related dryness, but not in normal CL wearers.[188] Glasson *et al.* demonstrated that tear samples collected from symptomatic CL wearers contained significantly higher levels of lipocalin and $sPLA_2$ than tolerant wearers.[75, 189] To further understand the drivers of end of day CL-related discomfort, it may be helpful to compare alterations of protein levels in the tears of CL wearers. The aim of this study was to use quantitative proteomics, by using selected reaction monitoring (SRM) mass spectrometry to investigate the association of subjective ocular comfort ratings and diurnal changes in tear concentration of lactoferrin, lysozyme, lipocalin 1, proline rich protein 4 and prolactin-induced protein with and without CL wear.

5.3 Methods

5.3.1 Participants

The protocol was designed in accordance with the guidelines of the Association for Research in Vision and Ophthalmology and was approved by the Institutional Ethics Committee of the University of New South Wales. This study was a single group, two-staged investigation. Thirty experienced CL wearers (20 females and 10 males, mean age 28.3 ± 9.2) were enrolled and signed informed consent (please see section 6.3.8 for sample size calculation).

5.3.2 Tear sample collection and comfort rating scale

The procedure has been described in Chapter 2: (2.3.3, 2.3.4 and 2.3.5). Briefly, participants were trained to collect their own basal tears from either eye twice a day, in the morning and in the evenings, when their eyes were symptomatic or just before going to bed if there was no sign of discomfort. In stage 1, CLs were not worn. In stage 2, participants were dispensed with 1-DAY ACUVUE® MOIST® lenses (Johnson and Johnson Vision Care, Jacksonville, FL, USA). Subjective ratings of comfort were also recorded just before tear collection on a scale of 1 - 100 with an anchor at 100 (extremely comfortable). Lenses were worn on a daily disposable basis and no lens care solution was used in this study.

5.3.3 Tear sample collection for studying the variability of proteins

To compare the day to day variations of the selected proteins in tears, four experienced tear donors (ranging in age from 28 to 32 years), without any history of eye diseases, were selected (three experienced CL wearers and one non-lens wearer) to collect their own tears for 5 consecutive days (days 1, 2, 3, 4 and 5). Tear collections were done at the same time of the day (at about mid-day) and individual samples were stored separately (were not pooled) at -80 °C until tested.

5.3.4 Materials and chemicals

Materials and chemicals were used as described earlier (Section 5.3.1).

5.3.5 Tear processing

Before the main experiment, the tear samples collected in each day were added together based on stage and time point of collection (four pooled samples for each individual: morning and evening without CL wear, morning and evening with CL wear) (2.3.3 and 2.3.4). Samples (2.5 µL) were diluted with 2.5 µL deionised water. Five microlitres of 8 M urea and 0.5 µL dithiothreitol (100 mM) were added and kept for 30min in 37 °C. Cysteine residues were alkylated by 0.5 µL iodoacetamide and after incubation for 30 min at 37 °C in the dark, ice cold acetone was added to the sample to the ratio of 4:1 (v/v). The mixture was incubated at -20 °C for 1 hr. Then, samples were

centrifuged at 10,000 rpm for 15 min at 4 °C and the supernatants were discarded. The acetone was removed by air drying for 10min and non-fractionated tear proteins were reconstituted in 2.5 µL of 100 mM ammonium bicarbonate buffer (pH 8.50). On the assumption that 10 µg/µL total protein was present in each pooled tear sample,[29-195] trypsin (200 ng/µL) was added to achieve a 1:50 enzyme-to-substrate ratio (w/w) and the mixture was incubated at 37 °C for 17 hr to produce a mixture of tryptic peptides.

5.3.6 Quantitative analysis of LC-SRM/MS

The following method has been described in sections 4.3.5, 4.3.6, 4.3.8. Briefly, two tryptic peptides were chosen for each protein and synthesised stable isotope-labeled and unlabelled amino acids were purchased from Sigma Aldrich (St. Louis, MO). Calibration standards were generated where the unlabelled peptides were diluted to final concentrations of 1, 5, 25, 125, 250, 500, 1000 fmol/µL in the presence of a fixed amount of 50 fmol/µL of the labeled peptides. The ratio of the peak areas of the unlabelled to labelled peptides (y-axis) was plotted against the concentration of the corresponding unlabelled peptides (x-axis). To determine the concentration of the endogenous proteins, tear digests were diluted 1:8 (v/v) with 25% acetonitrile in 0.1% formic acid containing labeled peptides at an equimolar concentration to a final concentration of 50 fmol/µL as internal standards. Chromatography was performed using an Agilent 1100 nano-flow LC system (Agilent Technologies). Next, all samples were analysed by nano-LC-SRM/MS on a 4000QTrap mass spectrometer (Applied Biosystems, Framingham, MA). Data analysis was performed using Skyline program v1.5 (MacCoss Lab, University of Washington, Seattle, USA) software. The concentration of each of the two endogenous peptides representing each tear protein

(µg/µl) was deduced by calculation of the peak area ratio between the endogenous peptides and the relative internal standards and the equation of the relative standard curve. Then the following equation was used to deduce the concentration of each peptide in µg/µL [(concentration of peptide per fmol/µL) x (overall dilution factor) x (protein molecular weight)/1x10^9]. An average of two peptide concentrations for each protein in each pooled tear sample was recorded. The limit of quantification for all peptides was ≤ 20 pg/µL.

5.3.7 Total protein content

To measure tear proteins concentrations independently from tear flow total protein content of each sample was determined using LavaPep total protein assay (Fluorotechnics, Gladesville, NSW, Australia) utilising bovine serum albumin as standard according to the manufacturer's instruction.[372]

5.3.8 Statistical analysis

Sample size was calculated (yielding 30) based on pilot experiments on tear samples collected in the morning and evening to show a significant, paired difference of 0.5 mg/mL of lipocalin 1 with 80% power, at the 5% level of significance. Lipocalin was chosen as the most abundant protein among the proteins of interest and because there are differences in lipocalin levels between asymptomatic and symptomatic lens wearers.[75] The IBM® SPSS® Statistical Research Methods and Social Science Statistics (Armonk, New York) was used to evaluate protein levels and relationship between continuous variables. Diurnal changes were compared between lens wear

stages using the paired t-test. Quantitative data (paired) were reported as mean ± standard deviation (SD). The comfort ratings and concentrations of tear proteins and their changes were analysed using linear mixed models with subjects as random intercepts (redefining time and stage with dependency for each subject) and stage (CL vs NCL) and time (AM vs PM) as repeated effect factors within subjects (controlling the covariance structure of the time and stage for a single subject). Therefore, the mixed model would determine whether significant differences exist among morning *vs* evening in CL wear *vs* NCL among randomly chosen patients. Analysis of variance (ANOVA) tests were used to determine the differences in tear protein levels among the five days.

5.4 Results

5.4.1 Effect of contact lens wear and time on ocular comfort

Comfort ratings, measured on a 1 - 100 scale, reduced from morning to evening, (**Table 5:1**). The reduction occurred both with and without CL wear, although it was greater with CL wear (PM, CL *vs* NCL $p = 0.02$).

Table 5:1 1 - 100 comfort rating comparing AM to PM.

	Stage	N	Mean ± SD		*p*-value	
			AM	PM	CL *vs* NCL	AM *vs* PM
Comfort (Avg)	NCL	30	88.33 ± 12.51	84.95 ± 12.59	0.02	0.001
	CL	29	88.01 ± 14.27	79.46 ± 16.47		

5.4.2 Concentration of proteins in tears

Table 5:2 shows the level of each individual protein in tears with and without CL wear at the two time points (AM and PM). Prolactin induced protein showed a significant higher level in the evening, both with and without CL ($p < 0.016$). However, wearing CLs did not affect the concentration of prolactin-induced protein or any of the other proteins tested. The levels of lactoferrin, lysozyme, lipocalin 1 and proline rich protein 4 did not change during the day. After normalizing the concentration of each protein to total protein in each tear sample the level of prolactin-induced protein remained higher in the evening but the difference between lipocalin 1 and proline rich protein 4 from morning to evening became statistically significant, whereas for lactoferrin and lysozyme, diurnal changes remained insignificant. The level (mean ± SD) of total protein (mg/mL) in the morning was 17.83 ± 7.90 in CL and 18.36 ± 7.51 in NCL; while its level in the evening was 14.67 ± 7.68 in CL *vs* 15.01 ± 6.65 in NCL (AM *vs* PM, $p = 0.006$; CL *vs* NCL, $p > 0.05$). **Table 5:3** shows the mean level of tear proteins for each day (n = 4). It demonstrates that there is no significant difference between the levels of these tear proteins over the period of five days ($p > 0.05$).

Table 5:2 Tear proteins concentrations (mg/mL) in AM *vs* PM and changes in paired differences of proteins in contact lens and no contact lens wear (n = 30).

Protein	Stage	Mean ± SD (mg/mL)		*p*-value		Mean ± SD (ratio to total protein)		*p*-value	
		AM	PM	CL *vs* NCL	AM *vs* PM	AM	PM	CL *vs* NCL	AM *vs* PM
Lactoferrin	NCL	1.14 ± 0.57	1.14 ± 0.81	0.62	0.67	0.06 ± 0.05	0.07 ± 0.04	0.75	0.16
	CL	1.16 ± 0.57	1.13 ± 0.71			0.05 ± 0.05	0.07 ± 0.09		
Lysozyme	NCL	2.02 ± 1.32	2.13 ± 1.36	0.60	0.40	0.11 ± 0.10	0.13 ± 0.10	0.26	0.12
	CL	2.10 ± 1.77	2.17 ± 1.55			0.13 ± 0.13	0.14 ± 0.15		
Lipocalin 1	NCL	1.65 ± 0.77	1.86 ± 1.03	0.91	0.28	0.08 ± 0.05	0.10 ± 0.07	0.52	0.02
	CL	1.75 ± 1.13	1.76 ± 1.03			0.08 ± 0.06	0.10 ± 0.11		
Prolactin-induced protein	NCL	0.08 ± 0.04	0.09 ± 0.05	0.81	0.02	0.005 ± 0.005	0.006 ± 0.006	0.53	0.01
	CL	0.08 ± 0.06	0.09 ± 0.05			0.005 ± 0.007	0.007 ± 0.010		
Proline rich protein 4	NCL	0.82 ± 0.60	0.86 ± 0.45	0.22	0.33	0.04 ± 0.02	0.05 ± 0.03	0.82	0.002
	CL	0.73 ± 0.48	0.80 ± 0.43			0.03 ± 0.02	0.05 ± 0.04		

Table 5:3 The mean level of tear proteins for each day (n = 4).

Tear protein	Day	Mean mg/ mL	SD	Time (*p*-value)
Lactoferrin	1	1.04	0.77	0.46
	2	1.21	0.60	
	3	1.23	0.53	
	4	1.92	1.91	
	5	1.16	0.68	
Lysozyme	1	0.61	0.40	0.96
	2	0.59	0.23	
	3	0.67	0.31	
	4	0.62	0.42	
	5	0.54	0.25	
Lipocalin 1	1	1.56	1.20	0.89
	2	1.42	0.87	
	3	1.77	1.10	
	4	1.63	1.24	
	5	1.27	0.71	
Prolactin-induced protein	1	0.15	0.11	0.93
	2	0.14	0.07	
	3	0.18	0.13	
	4	0.16	0.12	
	5	0.14	0.09	
Proline rich protein 4	1	1.14	0.83	0.85
	2	1.01	0.47	
	3	1.35	0.90	
	4	1.16	0.69	
	5	0.98	0.59	

5.4.3 Association between tear proteins and ocular comfort

Table 5:4 shows the relationship between tear proteins and subjective comfort rating. In particular, the change in concentration of prolactin-induced protein weakly

correlated with changes in ocular comfort during the day (p = 0.025, r = -0.294) (**Figure 5:1**) although it may not be clinically meaningful (r < 0.3). No relationship was found between concentrations of other proteins and changes in comfort during the day.

Table 5:4 Correlations between comfort changes (AM - PM) and changes in the level of proteins from AM to PM.

Protein	Pearson Correlation	*p*-value
Lactoferrin	-0.056	0.678
Lysozyme	-0.107	0.423
Lipocalin-1	-0.178	0.182
Prolactin-induced protein	-0.294	0.025
Proline rich protein 4	-0.228	0.086

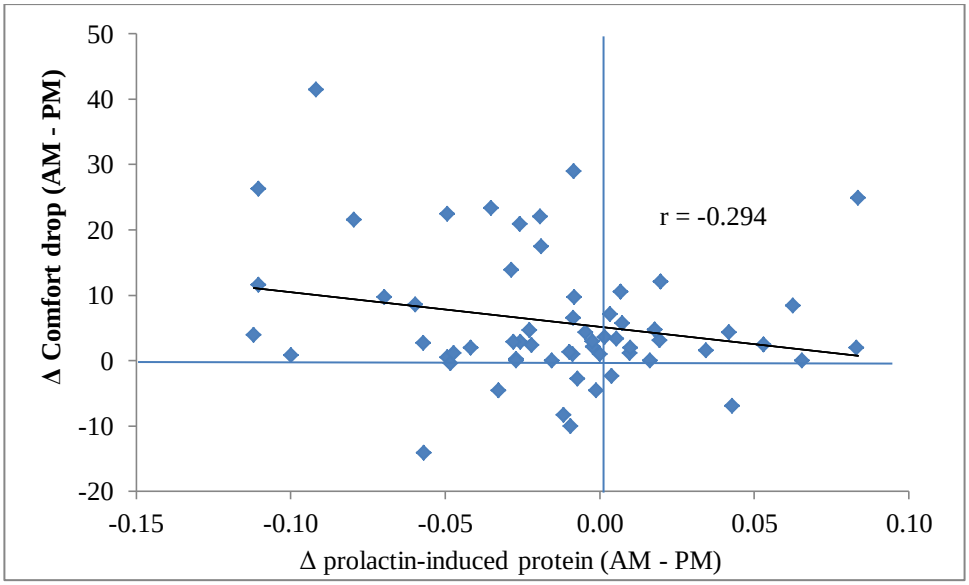

Figure 5:1 Scatter plots representing the relationship between the difference in the level of prolactin-induced protein and changes in ocular comfort level from AM to PM (p = 0.025) (n = 30). Larger differences in the concentration of PIP show greater discomfort reported by subjects.

Table 5:5 shows whether there was any difference between the concentrations of each of the measured proteins in the two time points of tear collection with and without CLs.

Table 5:5 Differences in concentration of tear proteins: Findings in morning and evening with and without CLs (n = 30).

Protein	**CL *vs* NCL**	**AM *vs* PM**
Lactoferrin	No	No
Lysozyme	No	No
Lipocalin 1	No	Yes
Prolactin-induced protein	No	Yes
Proline rich protein 4	No	Yes

5.5 Discussion

The present study examines diurnal changes in five tear proteins and subjective ocular comfort ratings in 30 subjects at two different times of day using selected reaction monitoring mass spectrometry. The objective of this study was to quantify the differences in lactoferrin, lysozyme, lipocalin 1, prolactin-induced protein and proline rich protein 4 in tear samples with CL wear and with no CL wear between morning and evening and to find possible associations between the changes in these proteins and ocular discomfort at the end of the day. The results of this study showed that ocular comfort decreased from morning to evening. The decrease in comfort was exacerbated

by CL wear (**Table 5:1**) which confirms the results of previous studies[7, 488] and also the results reported in the earlier chapters of this thesis. The concentrations of lactoferrin, lysozyme, lipocalin 1 and proline rich protein 4 did not change during the day or with CL wear. However, the concentration of prolactin-induced protein was significantly higher in the evening than morning to but did not change during CL wear (**Table 5:2**).

To eliminate the dependence of measured protein concentration values on tear flow, the concentration of these proteins were calculated as the ratio to the total protein of each sample. Following this normalisation, the ratio of lipocalin 1, prolactin-induced protein and proline rich protein 4 showed increases from morning to evening both in CL and NCL wear.[597, 598] After this normalisation the ratio of lipocalin 1, prolactin-induced protein and proline rich protein 4 showed higher level in the evening than morning in both CL and NCL wear (**Table 5:2**). The concentration of lactoferrin, lysozyme and lipocalin reported in this study are consistent with findings from previous ELISA studies,[29, 75, 175, 587] and confirm the results of Chapter 4:. The diurnal variation of lysozyme, lipocalin and lactoferrin has been studied before[26, 218] and the comparison of concentration of lysozyme and lactoferrin in CL wear and no lens wear has been reported earlier.[189, 274] The previous results agree with our results. This is the first time that diurnal variations of these proteins in CL wearers were compared with no lens wear. The results of total protein measured in this study are higher than previous reports.[26, 176, 177] Although, there are a number of reports about the concentration of total protein in tears with only one time point of the day[26, 599] measured, only one study reported the concentration of total protein in tears in the morning and before going to bed. Markoulli *et al.* reported a diurnal variation in total protein in tears.[179] Our results are consistent with their results in the morning and the drop in the evening before going

to bed but the levels in the evening were higher in the current study. The subjects in the previous study were healthy, non-lens wearers and tear collection was performed without CL wear. However, in our study tears were collected while subjects wearing CLs. Additionally, the methods used in these studies were not identical (LavaPep and Bicinchoninic acid method). The difference in tear total protein between the two groups and method can be a subject for further investigation. Previous authors have demonstrated that the concentration of major tear proteins including lactoferrin, lysozyme and lipocalin (in total including all subclasses of this family) remains constant between basal and reflex tear,[26, 28] but total tear proteins levels is significantly lower in reflex tears compared to basal tears.[26, 600] Also, the total protein concentration during the day remains unchanged.[601, 602] We trained subjects to properly collect their own basal tears and cease collection if reflex tearing started. However, to take into account the possibility of reflex tearing, which is very difficult to avoid, we normalised the measured levels of proteins to the total protein of each sample.

5.5.1 Prolactin-induced protein

The concentration of prolactin-induced protein remained higher in the evenings. Prolactin-induced protein has been previously identified in the tear film of CL wearers with dry-eye symptoms, and increases in concentration with dry eye.[188] In the current study we could not find any significant difference in the level of this protein when comparing CL wear to no lens wear. However, for prolactin-induced protein and comfort ratings, normalisation of the evening values by the morning values (Evening values – morning values = Δ comfort or Δ prolactin-induced protein) resulted in a

negative correlation with ocular comfort (**Table 5:4**), *i.e.* larger changes in prolactin-induced protein levels by the end of the day (Δ prolactin-induced protein) were associated with a larger drop in comfort rating (Δ comfort rating). In Table 5:3 (midday tear collection) the level of PIP is twice as that of PIP in Table 5:2 (AM and PM tear collection). The concentration of PIP was higher in midday than morning and evening. The difference in concentration of PIP may be due to the biological changes of this protein during the day. There is limited knowledge regarding the concentration of PIP in the literature and the concentration of this protein in tears is being reported for the first time in this thesis. Further investigation will be required to clarify this matter. A recent study has linked prolactin-induced protein to osmoregulation of tears through regulation of aquaporin 5.[242] Aquaporin 5 is linked with rapid water transport from the apical membrane of the lacrimal gland.[243] The proposed link between prolactin-induced protein and aquaporin 5 may be involved in the induction of severe dry eye conditions[244] as it may participate in down-regulation of aquaporin 5-mediated water transportation from the lacrimal gland, causing a reduction in tear menisci during the day,[603] and a decrease in tear volume in CL-induced dry eye.[604, 605] This can be suggested as one possible mechanism for ocular discomfort.

5.5.2 Lipocalin 1

Relative concentration of lipocalin 1 showed significantly higher levels in the evenings. Increased concentration of lipocalin 1 has been previously reported in intolerant CL wearers (discontinued lens wear) when compared to tolerant CL wearers.[75] In this study the levels of lipocalin 1 increased in the evening but no

difference was found between the concentration of this protein in CL wear and no lens wear and there was no association between eye comfort and concentration of this protein. All subjects in this study were current CL wearers and not intolerant to lens wear, as described by Glasson *et al.*[75] and this may be the reason why we could not find an association between comfort levels and concentration of this protein.

5.5.3 Proline rich protein 4

Relative concentration of proline rich protein 4 to total protein of tear samples also showed higher level in evening in this study but there was no difference between the CL wear and no lens wear conditions. In a previous report a decrease in the level of this protein has been demonstrated in CL-related dry eye (compared to subjects who did not feel any dryness while wearing lenses)[188] and also in dry eye patients.[174] Despite the role suggested for proline-rich proteins in the mucosal defence mechanism,[606-608] their function in tears is yet to be described.[235] More research is needed to find the mechanism of the effect of this protein on ocular discomfort.

The higher levels of relative proline rich protein 4 and lipocalin 1 to total protein in the evening may mean that some of the tear samples were reflex tears and these two proteins levels were different in reflex tears compared to basal tears. Although the change in the concentration of three out of five proteins of interest showed significant higher levels in the evening, further work needs to be done to establish the association of their levels with ocular comfort in symptomatic and asymptomatic lens wearers. As this study was not initially designed to examine this hypothesis, it will be examined in the following chapter.

5.6 Conclusion

The concentration of prolactin-induced protein, lipocalin 1 and proline rich protein 4 was higher in the evening compare to morning and the increase in the level of prolactin-induced protein was associated with a reduction in subjective comfort ratings. However, there were no changes in the concentration of lactoferrin and lysozyme during the day and during CL wear, suggesting the need for further investigation on the concentration of prolactin-induced protein, lipocalin 1 and proline rich protein 4 in symptomatic and asymptomatic lens wearers.

Chapter 6: Tear film in symptomatic and asymptomatic lens wearers

6.1 Overview

In previous chapters, the diurnal variation of a number of tear lipids and proteins including inflammatory mediators was examined. No associations were found between the concentration of these various mediators and subjective ocular ratings of comfort, with the exception of an association between prolactin-induced protein and ocular discomfort. One possible reason might be that there were not enough symptomatic lens wearers enrolled in the studies. Only 5 - 15% of the subjects in the previous studies demonstrated a drop in comfort equal to or more than 30 units.[435, 438] This study aims to investigate the tears of symptomatic and asymptomatic lens wearers (aim 4). We hypothesis that there are differences between the concentrations of prostaglandins, leukotriene B4, cysteinyl leukotrienes, lactoferrin, lysozyme, lipocalin 1, proline rich protein 4 and prolactin-induced protein of symptomatic and asymptomatic lens wearers.

6.2 Introduction

It is known that CLs can change the tear film components[148, 609] and cause ocular discomfort. Discomfort includes symptoms such as ocular fatigue, itchiness, dryness, irritation.[418] The tears of symptomatic CL wearers have been studied by others.[75, 188] In the previous chapters diurnal changes of a range of lipids and proteins were discussed in CL wearing volunteers who mostly did not have severe ocular discomfort. In chapter 2, 3 and 5 discomfort was estimated subjectively. A number of tear components which

based on the literature were expected to show changes in association with reduction of comfort, did not change diurnally or the diurnal changes could not be linked with end of day ocular discomfort. Therefore, a number of previously selected components were investigated in tears of asymptomatic and symptomatic subjects.

Tear components investigated in this chapter were selected primarily based on the literature as described in earlier chapters of this thesis. Priority was given to the components which were confirmed to be involved in dry eye or CL related dryness and discomfort in the literature[75, 174, 187, 188] and those which were indicated by the studies in the current thesis as possible candidates of higher priority. In view of limited availability of samples this investigation was performed as a pilot study.

6.3 Methods

6.3.1 Participants, tear sample collection and comfort rating scale

This was a pilot study to understand whether subjects with high levels of discomfort during lens wear had changes to their tear film constituents. The participants were recruited from clinical records. All participants signed a consent form prior to enrolment in the study. The study was approved by the Institutional Ethics Committee of the University of New South Wales in accordance with the guidelines of the Association for Research in Vision and Ophthalmology. Subjects aged between 18-45 and no eye disease with vision correction is between +6 and -12 dioptres were included in the study. Forty five healthy, adapted CL wearers were enrolled (31 females and 14 males, mean age 29.2 ± 10.2). Of these, 22 had self-described symptoms of dryness and

discomfort with CLs and 23 were asymptomatic. Three of the subjects had dropped out of lens wear before the study commenced (they wore CL for this study) or wore lenses occasionally and had consequent reduction of ocular comfort and wearing time and could tolerate CLs for no more than 5 to 6 hours. The other symptomatic subjects reported that lenses caused them discomfort. The asymptomatic group could wear CLs comfortably with no symptoms and without use of lubricants for one working day or longer. Participants were dispensed with Etafilcon A lenses (1-DAY Acuvue Moist, Johnson and Johnson Vision Care, Jacksonville, FL, USA) and were trained to collect their own tears in the evening as described in Chapter 2: (2.3.4 and 2.3.5) before going to bed or whenever subjects felt discomfort in their eyes (the duration of lens wear was at least 5 hours). Non-stimulated tear samples from both eyes were collected using disposable micro-capillary tubes (BLAUBRAND® intraMARK, Wertheim, Germany) and tears were immediately transferred into Eppendorf Tubes® and stored in the freezer at subject's home prior to transferring to the university laboratory on ice where samples were stored at -80 °C freezer immediately until analysed.

Subjects were asked to rate their ocular comfort on the scale of 1 (very uncomfortable) to 100 (extremely comfortable) in the evening at the time of each tear collection. The duration of the study was 5 days. Lenses were worn on a daily disposable basis (*i.e.* a new lens worn each day) and no lens care solution was used during this study.

6.4 Materials and chemicals

Prostaglandin and cysteinyl leukotrienes enzyme immunoassay (EIA) kits were obtained from Cayman Chemicals (MI, USA) (3.3.3). LTB_4 EIA kit was from Assay Designs (MI, USA). Other materials and chemicals used to measure protein concentrations have been described in Chapter 4: (4.3.1).

6.4.1 Tear processing

Before experimentation, tear samples from each subject were pooled (five samples for each subject and samples volumes ranged between 3- 10 µL). Then samples were centrifuged at 5000 g for 10 minutes at 4 °C to remove any cellular debris. The aqueous was removed and aliquoted into two separate fresh tubes (2.5 µL in one tube for measuring proteins by mass spectrometry and the remainder in another tube to measure prostaglandins, cysteinyl leukotrienes and LTB_4 by EIA). Tear samples were processed to measure proteins immediately after centrifugation and the second aliquot was immediately stored at -80°C.

6.4.2 Biochemical analysis

6.4.2.1 Mass spectrometry assay

Tears were assayed for concentration of proteins using selected reaction monitoring mass spectrometry. The tear processing method has been described in Chapter 4: (4.3.4) and the mass spectrometry method in Chapter 5: (5.3.6).

6.4.2.2 EIA assays

Tears were assayed for prostaglandins, cysteinyl leukotrienes and leukotriene B_4 using EIA kits according to the manufacturer's protocol (described in sections 2.3.7 and 3.3.3).

6.4.3 Statistical analysis

A sample size calculation was performed based on significant paired difference of 1 ± 1 log transformed unit of LTB_4, 80% power at the 5% level of significance. At least 20 subjects in each group were needed to complete the study. The IBM® SPSS® Statistical Research Methods and Social Science Statistics (Armonk, New York) was used to analyse the data. Quantitative data were reported as mean ± standard errors of the mean (SEM). Unpaired t-test was used to compare the differences between the tear components of symptomatic and asymptomatic lens wearers.

6.5 Results

6.5.1 Comfort during lens wear

The average evening comfort level in the subjects was above 70 for the asymptomatic group and equal or below 70 for the symptomatic (**Table 6:1**). Two of the subjects that enrolled as asymptomatic lens wearers at initial enrolment, rated their comfort level with lenses below 70 in the evening and so were included in symptomatic group for the data analysis.

Table 6:1 1 - 100 comfort rating in the evening comparing symptomatic to asymptomatic contact lens wearers.

CL group	N	Mean ± SD	*p*-value
Symp.	24	57.28 ± 12.38	0.001
Asymp.	21	83.96 ± 9.51	

6.5.2 Levels of protein and lipid mediators in tears

The level of each individual protein and lipid in tears of symptomatic and asymptomatic lens wearers in the evening are shown in **Table 6:2**. LTB_4 showed a significant higher level in the evening in symptomatic CL wearers ($p = 0.03$). The levels of lysozyme showed a trend for decrease in the tears of symptomatic subjects at the end of the day. The levels of lactoferrin, lipocalin 1, proline rich 4, prolactin-induced protein, prostaglandins and cysteinyl leukotrienes did not show any trend (*i.e.* $p > 0.1$) change between the comfort groups.

Table 6:2 Protein and lipid concentrations (mg/mL unless otherwise mentioned) and their differences in symptomatic and asymptomatic lens wearers.

Protein / Lipid	CL group	N	Mean	SEM	*p*-value*
Lactoferrin	Symp.	24	0.67	0.10	0.9
	Asymp.	20	0.79	0.17	
Lysozyme	Symp.	24	0.58	0.10	**0.10**
	Asymp.	20	1.73	0.46	
Lipocalin-1	Symp.	24	1.12	0.12	0.34
	Asymp.	20	1.41	0.45	
Prolactin-induced protein	Symp.	24	0.10	0.01	0.79
	Asymp.	20	0.16	0.08	
Proline rich protein 4	Symp.	24	0.75	0.07	0.26
	Asymp.	20	0.74	0.14	
LTB_4 (ng/µL)	Symp.	24	0.32	0.06	0.03
	Asymp.	21	0.17	0.04	
Prostaglandins (ng/µL)	Symp.	20	6.72	2.53†	0.21
	Asymp.	21	2.53	0.46	
Leukotrienes (ng/µL)	Symp.	17	9.11	0.68	0.20
	Asymp.	13	7.97	0.99	

*p-values in red indicate significant difference between groups, in bold indicate a trend ($p > 0.1$) for differences between groups.

†p-value reported based on logarithmic transformed data. Values include outliers, no appreciable change noticed after removal of outliers.

Figure 6:1 demonstrates the comparison of proteins and lipid level in the evening in tears of symptomatic and asymptomatic CL wearers.

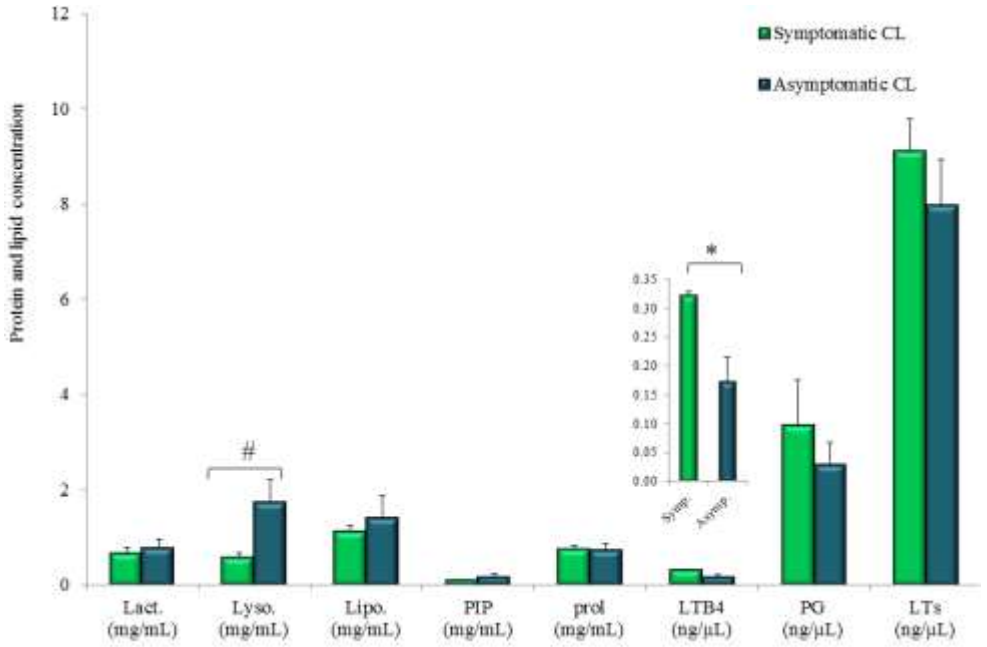

Figure 6:1 Comparison of proteins and lipid levels in tears collected in the evening by symptomatic and asymptomatic contact lens wearers (symptomatic, n = 19; asymptomatic, n = 21). Values are shown as mean ± SEM.* $p < 0.05$; # $p < 0.1$. Lactoferrin (Lacto), Lysozyme (Lyso.), Lipocalin 1 (Lipo.), Prolactin-induced protein (PIP), Proline rich protein 4 (Prol.), Leukotriene B_4 (LTB_4), Prostaglandins (PG), Cysteinyl leukotrienes (LTs).

6.6 Discussion

This study examines the concentration of tear lactoferrin, lysozyme, lipocalin 1, prolactin-induced protein, proline rich protein 4, LTB_4, cysteinyl leukotrienes and prostaglandins in symptomatic and asymptomatic CL wearers. The level of LTB_4 in the evening with CLs in (Study 1) was slightly higher than findings in the current study. The LTB_4 concentration in the symptomatic group was significantly higher than asymptomatic lens wearers. The concentration of proline rich protein 4, prolactin-induced protein, lactoferrin, lipocalin 1 and lysozyme were similar to the findings in

Study 4. Whilst changes for lysozyme concentration did not reach significance (p = 0.1), we might be able to reject that ocular discomfort is related to changes of lysozyme concentration, but since this was a preliminary test we might not be able to reject small effect size. Therefore, it may be sound reason to repeat the study to validate the findings using a large sample size to see if the significance level could be reached.[610] Under this study conditions lysozyme was lower in the symptomatic group. The results of the previous report in this thesis (Chapter 5:) also could not show any diurnal variation for this protein and any significant difference between CL wear and no lens wear. The concentration of lysozyme measured in the current study is consistent with previous reports. Its level is lower in dry eye patients compared to healthy subjects.[174, 187] Carney *et al.* showed that there was no significant difference between the level of lysozyme in CL wear and no lens wear,[198] and Glasson *et al.*[190] found no difference in lysozyme concentration between tolerant and intolerant lens wearers. On the other hand, lysozyme is one of the tear's major proteins that protects ocular surface against infection.[202, 203] Therefore, the results of the current study may mean that in symptomatic lens wearers, when compared to asymptomatic lens wearers, the innate ocular surface defence mechanism may be deficient in lysozyme. Finding whether this deficiency can result in increased susceptibility to ocular infection will depend on future investigation. This would imply that future investigation may include this protein as a candidate for involvement in CL-related discomfort. In future studies at least 37 symptomatic subjects and 37 asymptomatic (control) subjects will be needed to reject the null hypothesis that the level of this protein is equal in these two groups with probability of 80% power at 5% significance.

The elevated level of LTB_4 in symptomatic lens wearers confirms the results from chapter 2 suggesting that this mediator may play a role in CL discomfort even though we could not find any direct correlation between concentration of this mediator and subjective ratings. As LTB_4 plays a role in inflammatory response,[148] these findings indicate that Etafilcon A CLs may cause inflammation in the ocular surface. The level of prostaglandins and cysteinyl leukotrienes measured in this study are the same as the results in Chapter 3: but their concentration were not different in symptomatic and asymptomatic groups.

Glasson *et al.* have reported that tear lipocalin concentration is higher in intolerant CL wearers.[75] The findings of this study do not support the previous report. Glasson *et al.* used ocufilcon D CLs and defined intolerant lens wearers as subjects who had discontinued lens wear due to discomfort symptoms arising in the first 6 hours of lens wear.[189] This is somewhat different from the definition of symptomatic subjects in the current study which was based on subjective ratings of ocular discomfort. Subjects wearing ocufilcon D CLs may have experienced different levels of discomfort compared to Etafilcon A lenses which were used in the current study. Additionally, the difference in CL material may have influenced absorption of tear components into the CL matrix,[593, 611] thereby influencing the tear concentration of some of the molecules.

Investigating the asymptomatic and symptomatic groups for concentration of lactoferrin, there was no difference between the two groups for this protein. As described in Chapter 5:, lactoferrin does not change diurnally and no association was found with comfort ratings in the current investigation. These results confirm previous findings[189] and suggest that lactoferrin may not be involved in CL-related discomfort.

These findings are further supported by the fact that the concentration of lactoferrin reported here is consistent with the previous results.[175, 195, 197, 198] This study indicates that prolactin-induced protein is not associated with ocular discomfort in symptomatic lens wearers, but still its diurnal variation might be associated with end of day ocular discomfort. Inclusion criteria included a positive response to the question, "Do you have any problem or symptoms when wearing contact lenses?" regardless of the frequency or severity, the presence of eye symptoms, verified with response to self-rating of ocular comfort as equal to or less than 70 were assumed as symptomatic lens wear.[433, 436] Using other tools, such as questionnaires or clinical evaluation of ocular surface, could be helpful in assessment of ocular discomfort. Further, studies involving a larger sample size, implementing more complex selection criteria for symptomatic subjects, such as use of improved questionnaires and a variety of lens types will help with verifying these findings and identification of tear components involved in CL-related ocular discomfort.

6.7 Conclusion

This study probed human tears for concentration of lactoferrin, lysozyme, lipocalin 1, proline rich protein 4, prolactin-induced protein, LTB_4, cysteinyl leukotrienes and prostaglandins in symptomatic and asymptomatic CL wearers. The LTB_4 concentration in symptomatic lens wearers was significantly higher in symptomatic CL wearers compared to the asymptomatic group. No other differences were found in the level of tear factors of interest between the two groups.

Chapter 7: Diurnal variations of tear lipids

7.1 Overview

Tear molecular lipid composition was examined in this prospective investigation. This study focused on investigating the concentration of tear lipids in the morning and evening, with and without CLs and the possible association of tear lipid changes with ocular discomfort. We hypothesise that changes in the concentration of polar and non-polar lipids in the tear film in the morning versus evening are associated with ocular discomfort.

7.2 Introduction

Contact lenses divide the tear film into a pre- and post-lens tear film,[426, 427] changing its structure and function.[222, 442, 463, 612] Contact lens wearers are more likely than non-lens wearers to experience symptoms of dryness.[414] One potential cause of dryness in CL-wear is likely to be increased tear evaporation.[445, 460] The lipid layer plays an important role in the structural integrity of the tear film as it prevents tear evaporation[57] and helps maintain a smooth optical surface at the front of the eye.[19, 613] Therefore, one important area of investigation in CLs ocular discomfort becomes the impact of CLs on the lipid layer.

The tear lipid layer is produced predominantly by the meibomian glands and spreads over the aqueous layer of tear film with each blink. Stability, thickness and composition of the lipid layer are essential to lower the surface tension of tears and

facilitate their spreading over the ocular surface.[56] This layer has an internal structure that can be divided into polar (inner layer) and non-polar layers (outer layer).[61] The non-polar lipid component of the tear film is a mixture of wax esters, cholesterol esters, free fatty acids, diesters, triacylglycerol, and sterol esters,[62, 614] while the polar layer consists of phospholipids (phosphatidylcholines, phosphatidylethanolamines, sphingophospholipids),[63] ceramides, cerebrosides, sphingomyelin, triglycerides and omega-acylhydroxy fatty acids.[61, 64] Sufficient amounts of each of these lipids are essential for the effective functioning of this layer.[61, 65, 66]

A few studies have reported alterations of the lipid composition or lipid related proteins during lens wear or with CL intolerance.[74, 75] Glasson *et al.* found that intolerant CL wearers have significantly more secretory phospholipase A2 and lipocalin (a lipid binding protein) in their tears than tolerant wearers.[75] Nichols *et al.* reported that, in patients with ocular dryness related to CL wear, the pre-lens lipid layer tended to be thinner than that of subjects without dry eye.[76] A number of reports are available regarding the amount of lipid deposition on CLs as related to lens material.[77-79] Studies have shown association between tear film deposit on CLs and ocular comfort. [464, 552, 609] In this study we hypothesised that change in tear lipids composition and concentration during the day is associated with ocular discomfort.

7.3 Materials and methods

7.3.1 Chemicals and supplies

High performance liquid chromatography (HPLC) grade methanol, chloroform, methyl-*tert* butyl ether (MBTE), liquid chromatography mass spectrometer (LC-MS) grade ammonium acetate, analytical grade butylated hydroxytoluene (BHT) and D6-cholesterol were obtained from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Phospholipid standards were obtained from Avanti Polar Lipids (Alabaster, Alabama, USA). Cholesterol (≥ 99%) and cholesterol ester standards were obtained from Nu-Chek Prep (Elysian, MN, USA) and triacyglycerol standard from CDN isotopes (Point-Claire, Quebec, Canada). Vials with polytetrafluoroethylene (PTFE)/silicone septa were purchased from Waters (Rydalmere, NSW, Australia), and borosilicate capillary tubing from SDR Clinical Tech (Middle Cove, NSW, Australia).

7.3.2 Ethics approval

Ethics approval was obtained through the Human Research Ethics Advisory Panel (HREA) at the University of New South Wales (UNSW), Sydney, Australia. All procedures conducted in this study were conducted in compliance with the tenets of the Declaration of Helsinki. All subjects signed an informed consent form before enrolment in the study.

7.3.3 Subject selection

Experience soft contact lens wearing subjects who had no ocular diseases and were aged 18-45 with vision correction between +6 and -12 dioptres were recruited for this study.

7.3.4 Tear sample collection and comfort rating scale

Tears were collected from 30 normal experienced lens wearers (13 newly recruited subjects and 17 recruited previously) in two stages (see section 7.3.8 for power calculation). Participants consisted of 21 females and 9 males with a mean age of 30.4 ± 9.5 years. Details for this section have been presented in Chapter 2 (2.3.4 and 2.3.5). Briefly, participants collected their own tears twice daily in the morning during the first hour after waking, and in the evening. Subjects were asked to wear CLs continuously for at least 6 hours. Subjects were trained on several practice sessions to collect their own tears using glass micro capillary tube (BLAUBRAND® intraMARK, Wertheim, Germany) to ensure that they could consistently collect more than 2 µL of tears without causing reflex tearing. No CLs were worn in stage 1 and Etafilcon A lenses (1-DAY Acuvue Moist, Johnson and Johnson Vision Care, Jacksonville, FL, USA) were worn during stage 2 with no time in between the two stages. Lenses were worn on a daily disposable basis and no lens care solution was used in this study. Subjective ratings of comfort were also recorded just before tear collection on a scale of 1 - 100 with an anchor at 100 (*i.e.* subjects did not experience any form of eye discomfort).

7.3.5 Tear sample preparation and lipid extraction

Tear sample preparation has been described in Chapter 2 (2.3.5). 5 µL from each tear sample were transferred to a 300 µL glass-insert HPLC vial (Thermo-Fisher Scientific, Scoresby, Vic). Lipid extraction was performed as described elsewhere.[102, 615] Briefly, 270 µL of a solution containing methanol/MTBE/internal standard mix (6:20:1, v/v/v; 0.01% BHT) was directly added to each vial containing the 5 µL tear sample. Each sample contained a mixture of the following internal standards: 2.4 picomol (pmol) phosphatidylcholine (PC) 19:0/19:0 (number of carbons in fatty acid chain: double bonds in each chain), 2.4 pmol dihydrosphingomyelin 12:0, 1.5 pmol phosphatidylserine (PS) 17:0/17:0, 1.5 pmol phosphatidylethanolamine (PE) 17:0/17:0, 10 pmol of *D5* triacylglycerol (TAG) 16:0/16:0/16:0 and 80 pmol of cholesterol ester (CE) 14:0. The samples were then mixed on an orbital platform shaker for 1 hour, at room temperature. Phase separation was induced by addition of 50 µL of 150 mM aqueous ammonium acetate followed by vortexing. Tubes were centrifuged at 5000 *g* for 5 minutes to complete phase separation. Approximately 200 µL of the upper organic layer was removed to a new 300 µL glass-insert HPLC vial, dried under a stream of nitrogen at 37°C, resuspended in 50 µL methanol:chloroform (2:1 v/v) and stored at -20 °C until analysis. Samples were diluted 3-fold in methanol:chloroform (2:1 v/v) containing 15 mM ammonium acetate prior to mass spectrometric analysis.

7.3.6 Mass spectrometry

All mass spectra were obtained using a chip based nano-electrospray ionization source (TriVersa Nanomate®, Advion, Ithaca, NY, USA) equipped with a hybrid linear ion trap triple quadrupole mass spectrometer (QTRAP® 5500, ABSCIEX, Foster City, CA, USA) according to the method described by Brown *et al.*[102] Ten microlitres of extracted samples were loaded into the mass spectrometer by a pipette tip via nano-electrospray ionization (ESI) chip with an orifice diameter of 4.1 μm. Target lipids and MS scan parameters are shown in **Table 7:1**. A precursor ions list was prepared and experimental conditions for positive and negative ion modes were performed as described previously.[102] Smoothing, identification, removal of isotope contribution from lower mass species, and correction for isotope distribution were included in data analysis with LipidView® (ABSCIEX) software version 1.1. Isotopic ions may have a greater contribution to the total abundance of each ion and peak area of other ions. Quantification was achieved by comparison of peaks with head group–specific internal standards, after correction for isotope contribution.[616] To achieve this, the isotopic ion distribution of each ion was calculated from isotope models and subtracted before subsequent isotope calculations were performed.[616]

Limits of detection (LOD) and quantification (LOQ) were determined using a previously reported statistical method described by Armbruster *et al.*[617] In brief, in each spectrum the total ion count assumed as noise was calculated, and the mean and standard deviation of the noise were used to calculate the LOD (mean +3 x SD) and LOQ (10 x SD). Ions presenting below these limits were excluded from the analysis.

Table 7:1 Target lipid class, ion observed, MS/MS experiment (precursor ion (PI) or neutral loss (NL)), collision-induced dissociation CID energy, and internal standard utilised for MS/MS identification and quantification of lipids in human tear.

Target	Lipid Ion	MS/MS	CID energy	Internal Standard
CE	[M+NH4]+	PI m/z 369.4	25	CE 14:0
TAG (18:1 FA)	[M+NH4]+	NL 299.3	35	D5 TAG 48:0
TAG (16:1 FA)	[M+NH4]+	NL 271.3	35	D5 TAG 48:0
TAG (18:0 FA)	[M+NH4]+	NL 301.3	35	D5 TAG 48:0
TAG (16:0 FA)	[M+NH4]+	NL 273.3	35	D5 TAG 48:0
SM	[M+H]+	PI m/z 184.1	55	DHSM 12:0
PC	[M+H]+	PI m/z 184.1	55	PC 38:0
LPC	[M+H]+	PI m/z 184.1	55	PC 38:0
PE	[M+H]+	NL 141	30	PE 34:0
PS	[M+H]+	NL 185	35	PS 34:0
CHO	[M+H]+	PI m/z 369.4	15	D7 CHO
WE (18:1 FA)	[M+NH4]+	PI m/z 283.3	30	WE 18:1/18:0
WE (16:1 FA)	[M+NH4]+	PI m/z 255.3	30	WE 18:1/18:0
WE (17:0 FA)	[M+NH4]+	PI m/z 271.3	30	WE 16:0/18:0
WE (16:0 FA)	[M+NH4]+	PI m/z 257.3	30	WE 16:0/18:0

In total 155 molecular lipid species were quantified; cholesterol ester (CE), Triacylglycerol (TAG), sphingomyelin (SM), wax esters (WE), free cholesterol (CHO), phosphatidylcholine (PC), lysophosphatidylcholine (LPC), phosphatidylethanolamine (PE) and phosphatidylserine (PS). The numbers in front of each lipid class (e.g. 18:1) demonstrate the number of carbon atoms in the fatty acid chain and the number of double bounds in each chain. Ammonium acetate (+NH4) was added to samples to facilitate ionisation. (Courtesy of Brown *et al.* 2013 with modifications, Reprinted by permission of Association for Research in Vision and Ophthalmology).

7.3.7 Total protein content

LavaPep total protein assay (Fluorotechnics, Gladesville, NSW, Australia) was used to determine the total protein concentration in 2 µL of each sample using bovine serum albumin (BSA) as standard. The tear samples (1:50 dilution) were mixed with

LavaPep working solution (according to the manufacturer's instructions) and the absorbance was measured at 540 nm using a spectrophotometer (Tecan Spectrofluoro Plus; Tecan Group Ltd., Männedorf, Switzerland). The levels of total protein in individual tears were calculated from the standard BSA curve. [618]

7.3.8 Statistical analysis

Descriptive statistics such as averages and standard errors of the mean were used to describe the data. Pilot experiments on significant paired differences of wax esters (1 pmol/µg) and cholesterol esters (0.1 pmol/µg), as major tear lipids, in the morning and evening showed that a minimum of 30 subjects were needed to show a difference at the 5% level of significance and 80% power. The IBM® SPSS® Statistical Research Methods and Social Science Statistics (Armonk, New York) were used to evaluate lipid levels and correlation coefficients between continuous variables. Due to variation of total lipid across tear samples the comparison on a normalized basis was done for each lipid class and species and the results were reported as mole fraction..[102] The comfort ratings and concentrations of tear lipids and their changes were analysed using linear mixed models. The results were expressed as mean ± standard errors of the mean (SEM). The statistical significance of changes in lipids species from morning to evening was evaluated using mixed model analysis. Post hoc Bonferroni correction for multiple comparisons was used. An initial analysis compared the effect of stage (CL *vs* NCL) for each lipid class. The factors involved in the model, namely, stage and sampling time (morning *vs* evening) were repeated effects within subjects. When the interaction of

stage with lipid class was found as significant, lipid species were investigated individually.

7.4 Results

7.4.1 Effect of CL wear and time on ocular comfort

Comfort ratings, measured on a 1 - 100 scale, declined from the morning to the evening (**Table 7:2**). The decline occurred both with and without CL wear, more so with CL wear (p = 0.031).

Table 7:2 1 - 100 comforts rating comparing AM to PM.

			AM	PM	*p*-value		
	Stage	**N**	**Mean ± SD**	**Mean ± SD**	**Stage**	**Time**	**Interaction of time & stage**
Comfort	NCL	29*	89.2 ± 12.40	82.9 ± 13.21	0.02	0.001	0.03
	CL	30	88.6 ± 14.62	77.1 ± 13.67			

* One of the subjects did not rate her ocular comfort in stage 1 (NCL).

7.4.2 Tear lipid classes

Ten lipid classes, namely phosphatidylcholines (PC), lysophosphatidylcholines (LPC), phosphatidylethanolamines (PE), lysophosphatidylethanolamines (LPE), sphingomyelins (SM), phosphatidylserines (PS), cholesterol esters (CE), triacylglycerides (TAG), wax esters (WE) and free cholesterol (CHO) were quantified in this study. Individual lipid classes were normalised with respect to total lipid and

total protein in each sample. The total concentration of each lipid class is presented in **Table 7:3**.

Table 7:3 The overall concentration of each lipid class normalised to total lipid (%) or protein (per µg) (n = 120).

Lipid	Mean ± SEM	
	(%Total lipids)	(pmol/µg)
LPC	9.28 ± 0.67	0.51 ± 0.04
PC	7.92 ± 0.61	0.49 ± 0.05
SM	3.32 ± 0.17	0.21 ± 0.02
CHO	21.38 ± 0.73	1.41 ± 0.13
CE	22.27 ± 1.04	2.02 ± 0.26
PS	1.90 ± 0.29	0.10 ± 0.01
LPE	3.72 ± 0.26	0.21 ± 0.01
PE	1.03 ± 0.07	0.07 ± 0.01
TAG	3.04 ± 0.40	0.20 ± 0.03
WE	26.12 ± 1.80	2.55 ± 0.33

Figure 7:1 shows the overall level of each individual lipid (collected in the morning and evening with and without CL wear). The data were normalised in two different ways: to the level of total lipids (**Figure 7:1 A**) and total proteins (**Figure 7:1 B**) of each tear sample. The PE and SM percentages in both calculations are the same while CE and WE percentages are lower and LPE, PS, PC, LPC and TAG percentages are higher in comparison to total lipids than total proteins.

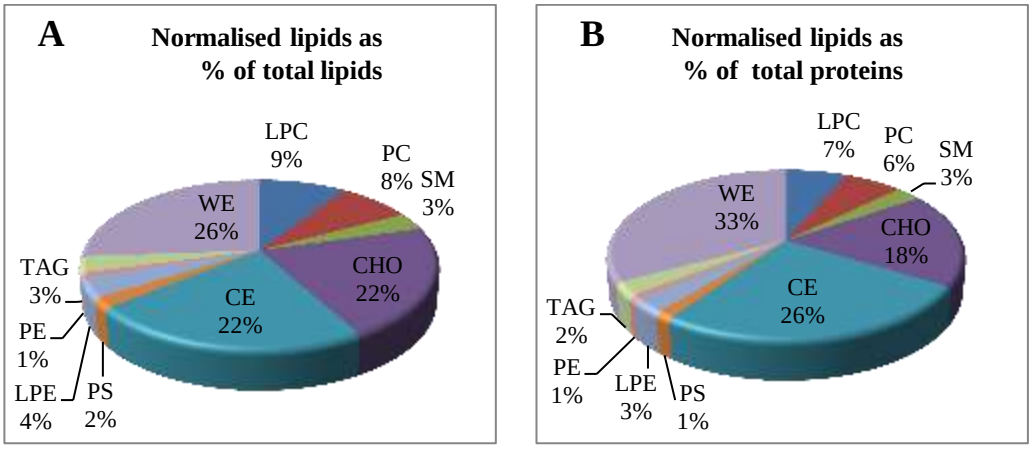

Figure 7:1 Pie charts illustrate the overall lipidomics of different lipid classes of human tears using HPLC/SRM-MS. The concentration of each lipid class normalised to: A) total lipids and B) total proteins of each tear sample. Tear samples were collected by capillary tubes. Values are presented as average of all samples of the study, including morning and evening tears with and without contact lenses (n = 120).

Figure 7:2 demonstrates the differences in tear lipids between CL and NCL wear in the morning and evening. When data were normalised to total lipids significantly more LPE ($p < 0.001$) and less PE ($p < 0.05$) were found in tears in the evening when no CLs were worn. During CL wear the level of CE was significantly higher in the evening compared to morning ($p = 0.02$, AM *vs* PM), and concentration of CE with CL was higher in the evening compared to the same time point when no lenses were worn ($p = 0.02$) (**Figure 7:2 A**). However, when normalisation was done to the total protein level, the levels of PE and LPE were significantly different compared to morning but there were no significant differences between the amounts of lipids in CL wear (**Figure 7:2 B**).

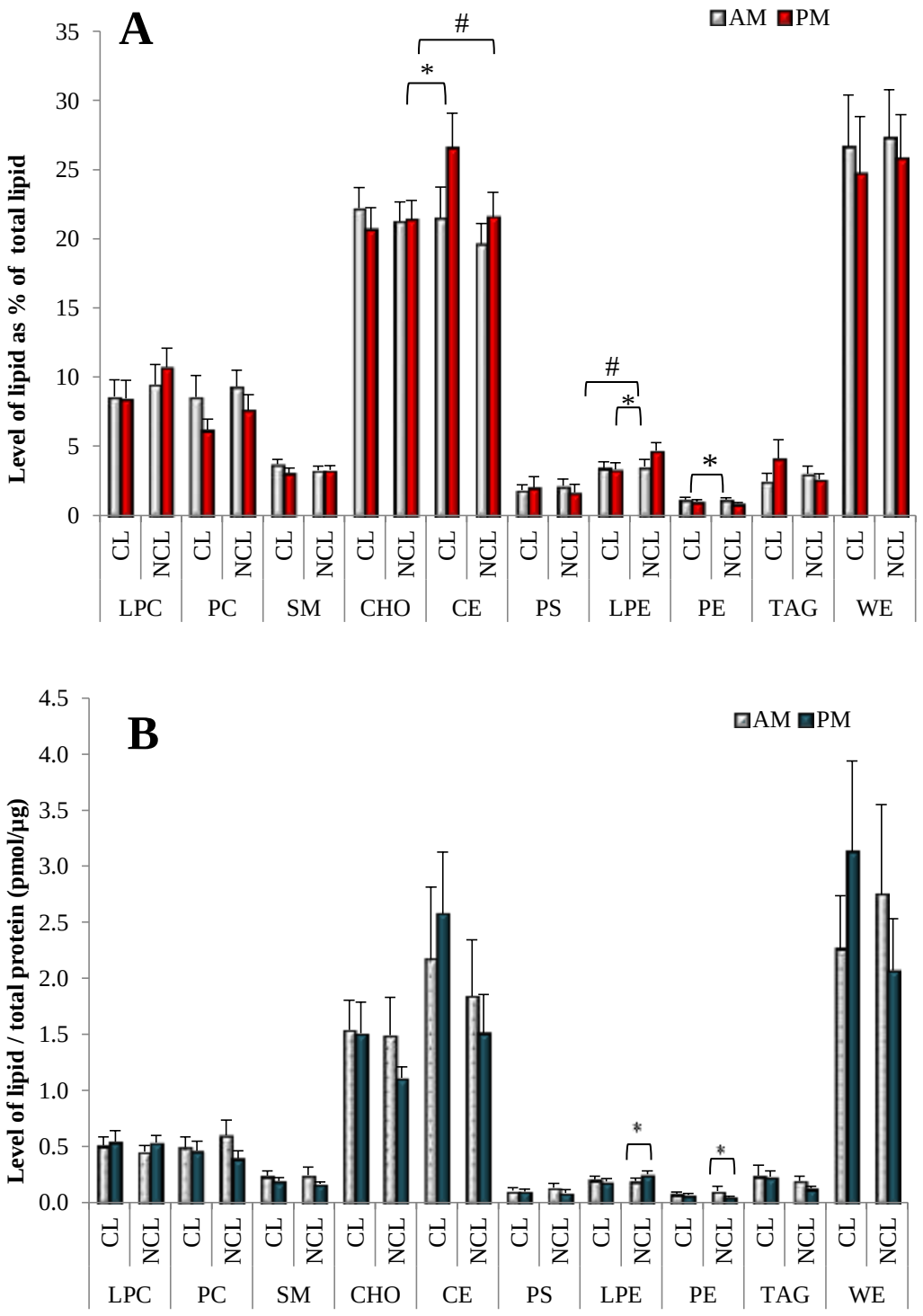

Figure 7:2 Quantitative comparison of lipid profiles in human tears (n = 30). Lipid concentrations have been normalised to A) total lipids, B) total proteins in each sample. Values are shown as mean ± SEM.* $p < 0.05$; AM *vs* PM; # $p < 0.05$; CL *vs* NCL in PM.

7.4.3 Tear lipid species

Molecular speciation of PE and LPE were compared between morning and evening tears collected with no lens wear (**Figure 7:3 A and B**).

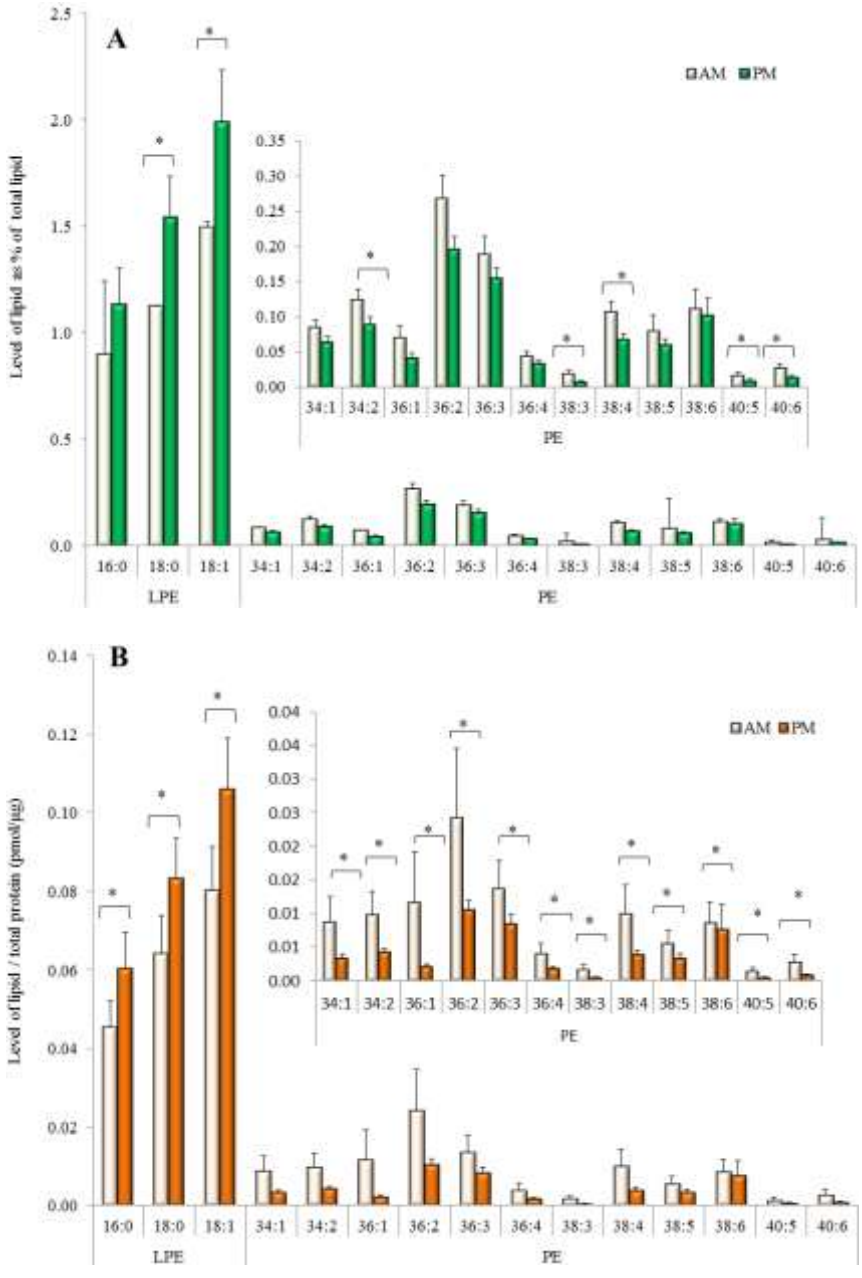

Figure 7:3 Diurnal changes of tear PE species without CLs; normalised to A) total lipids, B) total proteins (n = 30). Values are shown as the mean ± SEM. * $p < 0.05$ morning *vs* evening. The inset graphs show the bars with lower levels in the main graph using a larger scale.

The concentration of two species of LPE (species 18:0 and 18:1) were higher in the evening (mean ± SEM; 18:0, 1.12 ± 0.15% am *vs* 1.54 ± 0.19% pm, $p = 0.013$; 18:1, 1.50 ± 0.22% am *vs* 1.99 ± 0.25% pm, $p = 0.004$). The tear concentration of five of the twelve species of PE (comprising 76.2% of the total PE) decreased from morning to evening (the two most abundant of these species of PE were: 34:2, 0.12 ± 0.01% am *vs* 0.09 ± 0.01% pm, $p = 0.023$; 38:4, 0.11 ± 0.01% am *vs* 0.07 ± 0.01% pm, $p = 0.019$). LPE and PE comprised 4 - 5% of the total lipids. **Table 7:4** provides the concentrations of all LPE and PE species in the morning and evening with NCL. The concentration of other lipid classes and the ratio of PE/LPE did not change over time when no contact lenses were worn.

Table 7:4 LPE and PE molecular lipid, means and standard error of the mean (n = 30) in tears without wearing CL (AM *vs* PM). Lipids were normalised to total lipids (%) or proteins (per µg).

Lipid class	Lipid species	Mean ± SEM (level of lipid as % of total lipid)			Mean ± SEM (pmol/µg)		
		AM	PM	*p*-value	AM	PM	*p*-value
LPE	16:00	0.90 ± 0.15	1.14 ± 0.17	0.073	0.05 ± 0.01	0.06 ± 0.01	0.000
	18:00	1.12 ± 0.15	1.54 ± 0.19	0.013	0.06 ± 0.01	0.08 ± 0.01	0.000
	18:01	1.50 ± 0.22	1.99 ± 0.25	0.004	0.08 ± 0.01	0.11 ± 0.01	0.000
PE	34:01	0.08 ± 0.01	0.06 ± 0.01	0.076	0.01 ± 0.00	0.00 ± 0.00	0.000
	34:02	0.12 ± 0.01	0.09 ± 0.01	0.023	0.01 ± 0.00	0.00 ± 0.00	0.000
	36:01	0.07 ± 0.02	0.04 ± 0.01	0.094	0.01 ± 0.01	0.00 ± 0.00	0.000
	36:02	0.27 ± 0.03	0.20 ± 0.02	0.062	0.02 ± 0.01	0.01 ± 0.00	0.000
	36:03	0.19 ± 0.02	0.15 ± 0.01	0.146	0.01 ± 0.00	0.01 ± 0.00	0.000
	36:04	0.04 ± 0.01	0.03 ± 0.00	0.117	0.00 ± 0.00	0.00 ± 0.00	0.000
	38:03	0.02 ± 0.00	0.01 ± 0.00	0.021	0.00 ± 0.00	0.00 ± 0.00	0.000
	38:04	0.11 ± 0.01	0.07 ± 0.01	0.019	0.01 ± 0.00	0.00 ± 0.00	0.000
	38:05	0.08 ± 0.02	0.06 ± 0.01	0.342	0.01 ± 0.00	0.00 ± 0.00	0.002
	38:06	0.11 ± 0.03	0.10 ± 0.02	0.706	0.01 ± 0.00	0.01 ± 0.00	0.001
	40:05	0.02 ± 0.00	0.01 ± 0.00	0.036	0.00 ± 0.00	0.00 ± 0.00	0.000
	40:06	0.03 ± 0.00	0.01 ± 0.00	0.014	0.00 ± 0.00	0.00 ± 0.00	0.000

Fatty acid chain lengths and lipid unsaturation have been shown in front of each species (*e.g.* 12:0, contains a fatty acid chain of 12 carbons and no double bounds) (the names of species are presented as, number of carbons in fatty acid chain: double bonds in each chain).

Figure 7:4 shows the concentrations of each species of CE in tears of CL wearers when data were normalised to the level of total lipids (no difference was found in the level of CE in the analysis to total protein). The concentrations of fourteen

species of CE were higher in the evening. **Table 7:5** demonstrates the mean ± standard errors of the mean of these species.

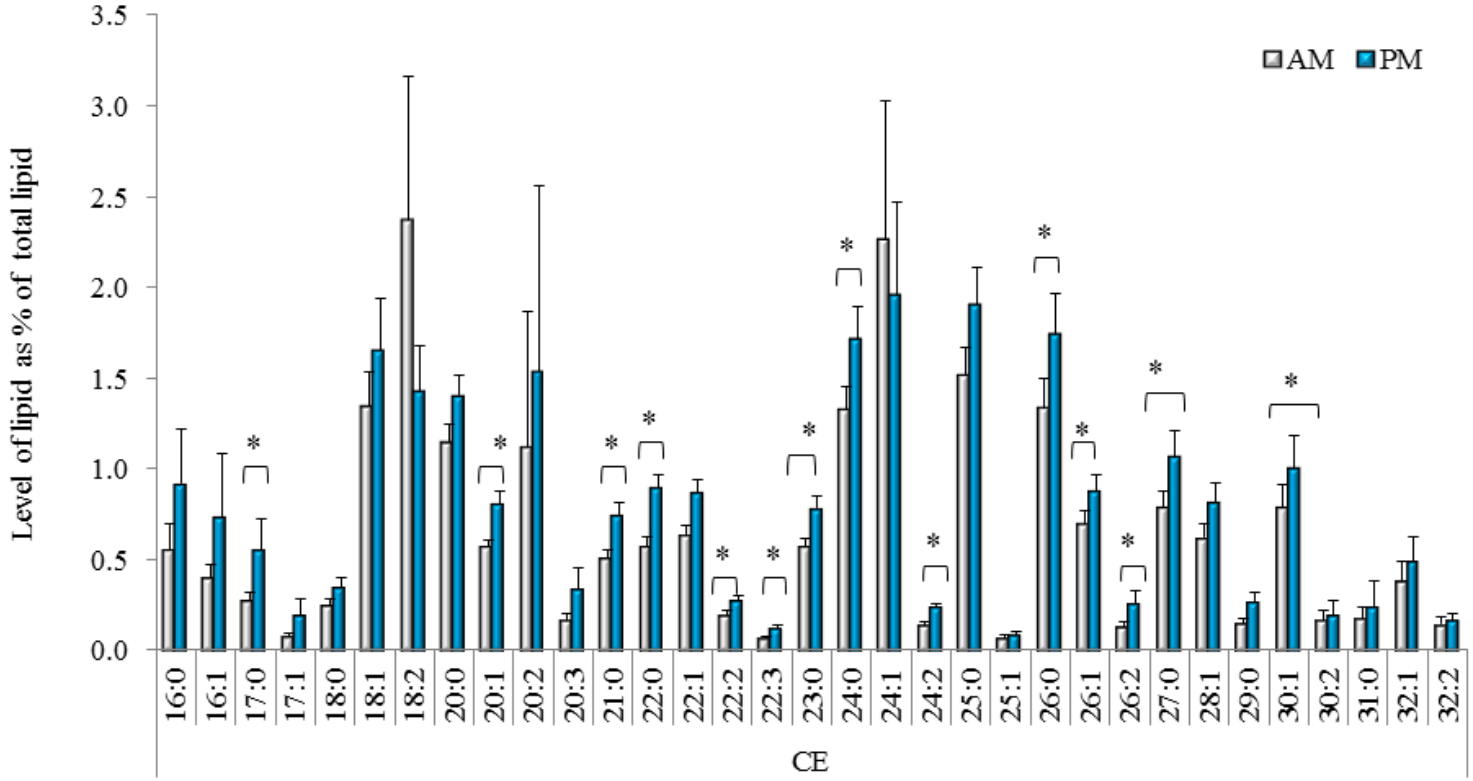

Figure 7:4 Molecular lipid speciation of CE in human tears with CLs (n = 30). The species were normalised to the total of lipids in each sample (mol%). Values are shown as the mean ± SEM. * $p < 0.05$ AM *vs* PM. Ammonium acetate (+NH4) was added to facilitate ionisation.

Table 7:5 Cholesterol esters (CE) in tears during CL wear (n = 30). All measurements are listed as mol% of total lipid. "CE C_n: double bond".

CE species	Mean ± SEM (Level of lipids as % of total lipids)		*p*-value
	AM	PM	
16:00	0.56 ± 0.14	0.92 ± 0.30	0.27
16:01	0.40 ± 0.07	0.74 ± 0.35	0.37
17:00	0.27 ± 0.05	0.56 ± 0.17	0.04
17:01	0.08 ± 0.01	0.19 ± 0.09	0.06
18:00	0.24 ± 0.04	0.34 ± 0.06	0.09
18:01	1.34 ± 0.19	1.65 ± 0.29	0.4
18:02	2.37 ± 0.79	1.43 ± 0.25	0.21
20:00	1.14 ± 0.10	1.40 ± 0.11	0.07
20:01	0.57 ± 0.04	0.80 ± 0.07	0.00
20:02	1.12 ± 0.75	1.54 ± 1.02	0.12
20:03	0.17 ± 0.03	0.34 ± 0.11	0.15
21:00	0.51 ± 0.04	0.74 ± 0.07	0.00
22:00	0.57 ± 0.06	0.89 ± 0.08	0.00
22:01	0.63 ± 0.05	0.87 ± 0.06	0.01
22:02	0.20 ± 0.02	0.27 ± 0.03	0.01
22:03	0.06 ± 0.01	0.12 ± 0.02	0.03
23:00	0.57 ± 0.04	0.78 ± 0.06	0.00
24:00	1.33 ± 0.13	1.71 ± 0.18	0.03
24:01	2.26 ± 0.76	1.95 ± 0.52	0.52
24:02	0.14 ± 0.02	0.24 ± 0.02	0.00
25:00	1.51 ± 0.16	1.90 ± 0.21	0.09
25:01	0.07 ± 0.01	0.09 ± 0.01	0.32
26:00	1.34 ± 0.16	1.74 ± 0.23	0.05
26:01	0.69 ± 0.07	0.88 ± 0.09	0.04
26:02	0.13 ± 0.03	0.26 ± 0.07	0.03
27:00	0.79 ± 0.09	1.06 ± 0.15	0.05
28:01	0.62 ±0.08	0.81 ± 0.11	0.06
29:00	0.15 ± 0.02	0.27 ± 0.05	0.04

CE species	Mean ± SEM (Level of lipids as % of total lipids)		*p*-value
	AM	PM	
30:01	0.79 ± 0.12	1.00 ± 0.18	0.09
30:02	0.17 ± 0.05	0.19 ± 0.09	0.45
31:00	0.18 ± 0.06	0.24 ± 0.14	0.52
32:01	0.38 ± 0.10	0.49 ± 0.14	0.25
32:02	0.14 ± 0.04	0.16 ± 0.04	0.22

The names of species are presented as, number of carbons in fatty acid chain: double bonds in each chain (*ie*. 30:01 means 30 carbons and one double bond exist in fatty acid chain.

The results of the new analysis (exclusion of lipids common between cells and tears) showed that the level of SM in the evening was lower compared to morning when no CLs were worn (**Table 7:6**) and there were no other differences in tear lipids with and without lens wear.

Table 7:6 Comparison of tear lipids normalised to the total lipids of each sample after exclusion of LPE, PE, PS and CHO (n = 30).

Lipid	Study Visit	Mean ± SEM (pmol/µg)		*p*-value	
		AM	PM	AM *vs* PM	CL *vs* NCL
SM	CL	0.21 ± 0.04	0.16 ± 0.02	0.04	0.75
	NCL	0.22 ± 0.07	0.12 ± 0..01		
LPC	CL	1.00 ± 0.56	0.52 ± 0.09	0.44	0.32
	NCL	0.44 ± 0.06	0.51 ± 0.07		
PC	CL	0.37 ± 0.08	0.37 ± 0.06	0.22	0.91
	NCL	0.46 ± 0.10	0.29 ± 0.06		
TAG	CL	0.05 ± 0.01	0.05 ± 0.01	0.83	0.23
	NCL	0.08 ± 0.03	0.06 ± 0.02		
CE	CL	1.82 ± 0.48	1.90 ± 0.59	0.44	0.54
	NCL	1.95 ± 0.69	1.22 ± 0.32		
WE	CL	2.26 ± 0.48	3.13 ± 0.81	0.91	0.59
	NCL	2.74 ± 0.81	2.07 ± 0.46		

To probe for the effect of tear lipids which may have originated from cells of the ocular surface rather than from meibomian glands, the calculations were revised after removing the data for lipids common between tears and internal structures of cells. Thus, values for LPE, PE, PS and CHO[619-623] were excluded from the analysis. Data for WE, CE, PC, LPC and SM were normalised by dividing them by total lipids (LPE, PE, PS and CHO were excluded from total lipids). **Figure 7:5 A** shows the comparison of the lipids in tears measured in this study with the same lipids reported by Brown *et al*.[102] (**Figure 7:5 B)**. Based on this comparison the level of phospholipids in tears in the current study was 15% higher (27% *vs* 12% of total lipids), CE level was 11% less (30% *vs* 41% of total lipids) than Brown *et al* study. The levels of WEs and TAGs were almost the same in both studies.

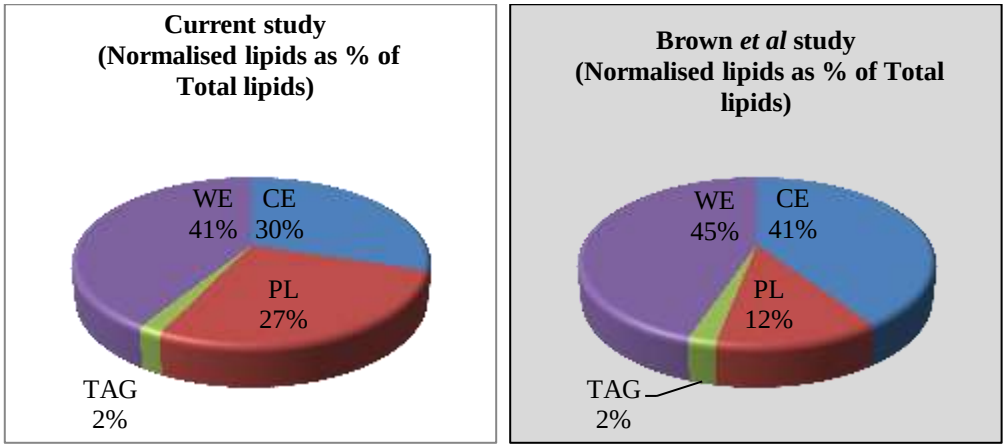

Figure 7:5 Comparison of lipid classes measured in the current study and that of Brown *et al* (2013). Lipid classes measured in the current study after exclusion of lipids common between tears and internal structures of cells (*ie.* PLs and CHO) (n = 30).

Figure 7:6 presents the concentration of each lipid in morning and evening after removing the values for LPE, PE, PS and CHO and **Table 7:7** compares the molecular species of SM during the day.

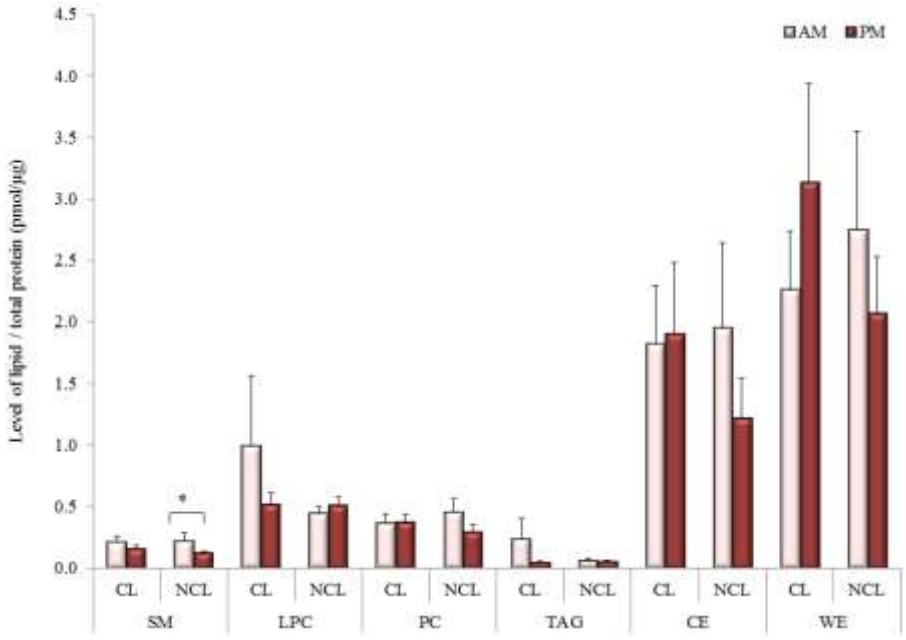

Figure 7:6 Quantitative comparison of tear lipids with and without contact lens wear in the morning *vs* evening. Values are shown as the mean ± SEM (n = 30). * $p < 0.05$.

Table 7:7 Comparison of molecular species of SM during the day with and without contact lenses. Short chain fatty acid lipids (intracellular lipids) have been excluded from the anaylsis (n = 30).

SM species	Study Visit	Mean ± SE (pmol/µg)		*p*-value	
		AM	PM	AM *vs* PM	CL *vs* NCL
32:01	CL	0.01 ± 0.00	0.01 ± 0.00	0.22	0.94
	NCL	0.02 ± 0.00	0.01 ± 0.00		
34:01	CL	0.09 ± 0.02	0.07 ± 0.01	0.04	0.87
	NCL	0.10 ± 0.03	0.06 ± 0.01		
34:02	CL	0.01 ± 0.005	0.01 ± 0.004	0.08	0.82
	NCL	0.01 ± 0.01	0.01 ± 0.00		
40:01	CL	0.02 ± 0.04	0.01 ± 0.01	0.68	0.62
	NCL	0.02 ± 0.05	0.01 ± 0.01		
42:01	CL	0.04 ± 0.01	0.03 ± 0.04	0.07	0.68
	NCL	0.04 ± 0.08	0.02 ± 0.01		
42:02	CL	0.04 ± 0.05	0.02 ± 0.02	0.04	0.72
	NCL	0.04 ± 0.07	0.02 ± 0.01		

The names of species are presented as, number of carbons in fatty acid chain: double bonds in each chain.

7.4.4 Association of tear lipids with comfort rating

No association was found between subjective comfort ratings and the concentration of lipids in any of the normalised data with and without CLs.

7.5 Discussion

The present results indicate that reduction in comfort at night compared to morning in NCL wear was 6.3% while with CL wear it was 11.6% which shows significant decrease in both stages but significantly lower with lens wear. As discussed by others, the reduction in comfort during lens wear might not be entirely related to CLs and could be partly due to general fatigue. [624]

Lipids were probed for differences in their concentrations during the day with and without CLs. In this study, data were normalised to total lipids and total proteins to compare the results of normalisation in two different ways. The results of both methods of analysis identified that the concentration of phosphatidylethanolamines were lower and their breakdown product lysophosphatidylethanolamines were higher in the evening with no lens wear. Other studies have reported that PE increases the stability of the tear film by lowering surface tension[117, 172] and may play an important role in tear film stability.[117, 625] PE may be one of the phospholipids that form an interface between the aqueous layer and hydrophobic lipids[74] due to its ability to bind hydrogen and other polar lipids.[105] The decrease in concentration of PE may cause disruption of the tear lipid structure and its functionality, and increase tear evaporation,[626] shorten tear break up time[172] and consequently increase dryness and discomfort in the eye at the end of day without CLs.

Another finding of this study is higher concentration of CE (one of the major non-polar tear lipids) in the evening while wearing CLs compared to the same time point in non-lens wear. A positive association between increased levels of cholesterol esters and stability of the tear film has been reported.[67, 627] However, the results here did not show an association between CE and subjective comfort. A limited number of CE species showed a change in concentration during the day. In CEs the differences between fatty acid chain length and the number of double bonds in each chain can change their melting point.[628, 629] The concentration of CE was negatively associated with lipid layer thickness[106] which may mean that increasing the concentration of CE

with CLs in the evening may reduce thickness of the tear film, decrease tear break up time. In the present study no correlation was found to ocular discomfort.

Further probing of these results raised the question of whether the source and origins of these lipids were only the tears or whether cells and cellular materials may be present in the tear samples as a result of freezing and thawing samples before centrifugation. One indication for this latter suggestion is that the concentration of free cholesterol and phospholipids in the current study was higher than reported previously.[34, 86, 119, 390] Phosphatidylethanolamines,[619] lysophosphatidylethanolamines and phosphatidylserines[620] and free cholesterol[621, 622] occur both in tears and as part of cells and their level was high in the tear samples used in this study. After excluding these lipids and some species of other lipid classes that may overlap with lipids in epithelial cells (*e.g.* wax esters and sphingomyelins with fatty acid chains containing less than 20 carbons)[620] and tears, the data were re-analysed and the results showed that the concentration of sphingomyelins dropped in the evening when no CLs were worn but no other changes were found in concentration of other lipid classes with or without lenses. Even after removing those lipids that may have originated from cells shed from the ocular surface. The level of remaining phospholipids was still substantially higher than Brown *et al.*'s report.[102] This may suggest that the normal tear phospholipids mainly originate from the internal cellular structure of the ocular surface. Also, this may suggest that the method of tear collection and a centrifugation step before storage are important when studying tear lipids.

Overall, no association was found between ocular discomfort and changes in the level of tear lipids during the day. This may have been resulted in from symptomatic

lens wearers being not highly represented in the subject pool or it could be that the technique of tear collection and storage may have affected the concentration of tear lipids. Although several studies have investigated the profile of tear lipids in normal and abnormal conditions, little has been done by way of identification of diurnal variation of tear lipids, and their effect on ocular comfort with CLs. The results of this study suggest that there are some diurnal variations in tear lipids with and without CLs. Continued research into other tear lipids can prove helpful. One group of lipids could be (O-acyl)-ω-hydroxy fatty acids (OAHFA) as previous reports suggest that OAHFA has an important role in the stability of the tear film[79] and that its level decreases in dry eye syndrome.[64, 103] .[102]

7.6 Conclusions

Tear film lipids were measured in both NCL and CL wear. The level of sphingomyelins, phosphatidylethanolamine and its breakdown product lysophosphatidylethanolamine showed a diurnal variation in non-lens wear, leaving more than 91 – 94% of measured tear lipids unchanged during the day without CL wear. However, cholesterol esters showed a significant change during the day with CLs. The method of tear collection and data normalisation are key to interpreting the results. No associations were found between diurnal variations of tear lipids and ocular discomfort at the end of the day. Normal tear phospholipids possibly originate from the internal cellular structure of the ocular surface.

Chapter 8: Summary and recommendations

8.1 Summary

For more than 120 million people in the world CLs are the preferred method of correction of vision.[2] This could be for a number of reasons such as physical activities (sports), specific professional visual requirements, recreational purposes or even purely aesthetics and cosmesis. For CL wearers glasses simply are not a viable option. However, according to the literature many CL wearers eventually decide to stop CL wear or resolve to wear them less frequently due to ocular comfort issues.[3-5, 7] As a result, CL drop-outs have been described as one of the main causes why the global number of CL wearers does not grow quickly.[3]

The drop in comfort at the end of day is an important factor in CL wear that many wearers experience.[3, 6] Discomfort has been described as ocular fatigue, itchiness, dryness, irritation, scratchiness and may be associated with ocular redness.[418] Discomfort symptoms associated with CL wear are similar to but more magnified compared to those found in non-lens-wearers.[488] A major hypothesis in this thesis was that the levels of certain tear lipids and proteins, previously identified to change in the tears of dry eye sufferers, change during the day with and without CL wear and that these changes were associated with CL-induced discomfort.[127, 187, 339, 358, 630, 631] Glasson *et al.* have reported significant differences in the tear concentration of lipocalin, $sPLA_2$ and degraded lipids between tolerant and intolerant lens wearers.[75] Nichols *et al.* showed significant differences in the tear concentration of β-2 microglobulin, proline rich protein 4, lacritin, secretoglobin $1D_1$, secretoglobin 2A2, albumin, glycoprotein 340

and prolactin-inducible protein in individuals with CL-related dry eye compared to normal CL wearers.[188] Both of these previous studies reported differences in the levels of these compounds and an association with discomfort during lens wear. However, contrary to the current study, these studies did not monitor diurnal variations of the molecules in relation to ocular comfort.

This chapter summarises and discusses the key findings of previous chapters as well as the limitations of the research and future work. **Figure 8:1** demonstrates the number of subjects participating in each study of the current project. A synopsis of the studied molecules is presented in **Table 8:1**.

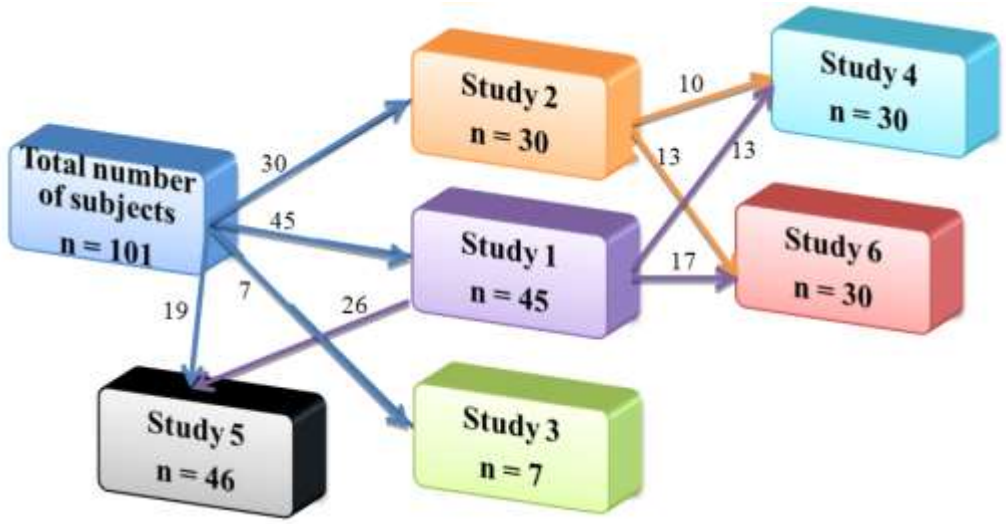

Figure 8:1 The diagram shows the number of subjects participating in each of the studies of this thesis. Numbers between boxes indicates the quantity of subjects that were common between each two studies. Depending on the volume of tear samples collected from each subject, some samples ran out early and the consecutive studies were performed using the remaining samples while recruiting additional volunteers. Therefore, sample volumes determined use of samples in different studies (no preselection).

Table 8:1 List of molecules investigated for this thesis and their changes under study conditions and association with ocular comfort. Significant changes (p-value < 0.05) or positive associations are shown with a tick mark.

Group	Molecule name	AM *vs* PM	CL *vs* NCL	Symptomatic *vs* asymptomatic	Association with comfort
Lipids	Phosphatidylcholines				
	Lysophosphatidylcholines				
	Phosphatidylethanolamines	✓			
	Lysophosphatidylethanolamines	✓			
	Sphingomyelins				
	Phosphatidylserines				
	Cholesterol esters	✓			
	Triacylglycerides				
	Wax esters				
	Free cholesterol				
Proteins	Lactoferrin				
	Lysozyme				
	Lipocalin 1	✓			
	Proline rich 4	✓			
	Prolactin-induced protein	✓			✓
	sIgA	✓			
Inflammatory mediators (Proteins / Lipids)	C3	✓			
	C3a	✓			
	$sPLA_2$				
	Bradykinin				
	LTB_4	✓	✓	✓	
	Prostaglandins				
	Cysteinyl leukotrienes				
	Resolvin D_1				

Components with no diurnal variation (AM *vs* PM)

Tear lipids including polar and non-polar lipids were quantified in this study. Tear lipids are deemed essential for the effective functionality of the tear lipid phase[61, 65, 66] and they are influential in increasing the elasticity of the tear lipid phase, leading to its enhanced stability.[67] In the current investigation, the concentration of phosphatidylcholines, lysophosphatidylcholines, sphingomyelins, phosphatidylserines, cholesterol esters, triacylglycerides, wax esters and free cholesterols did not change during the day and CLs had no effect on their levels. Therefore, these components can probably be excluded from the mechanisms of induction of ocular discomfort.

Lysozyme and lactoferrin are two major tear proteins which together comprise 45 - 65% of total tear protein[194, 195, 202, 203] and are known to change in dry eye.[174, 187] The concentration of these two proteins (measured individually) however, remained unchanged during the day in the current studies. This finding could be expected based on the literature where previous findings suggest stable lysozyme levels in the range of hours of the day investigated here.[218] Lactoferrin is known as an inhibitor for the classical complement system (anti-inflammatory effect)[207] and possesses bacteriostatic properties.[199-204] Lactoferrin binds to proteins[209] and easily penetrates into lipid layers.[210] Lactoferrin and lysozyme function in a manner similar to surface activating agents in the lung;[56, 632, 633] they assist with spreading the lipid phase on top of the aqueous phase of tears. The result of this investigation suggests that end of day ocular discomfort would not involve problems with even distribution of the lipid phase as regulated by lysozyme and lactoferrin.

Secretory phospholipase A2 is involved in the inflammatory response[634] and its increased activity was recorded in dry eye conditions as well as in intolerant CL wearers.[294] The investigations performed for this thesis could not demonstrate any change in the concentration of this enzyme although at this stage we cannot comment about changes in the activity of $sPLA_2$ in our test conditions. Due to the small amount of tears collected, we could not analyse the activity of $sPLA_2$. Whilst the concentration of $sPLA_2$ did not change, the LTB_4 levels, an eventual metabolite of arachidonic acid released from phospholipids by the action of $sPLA_2$, were elevated in CL wear (Chapter 2:). Thus, the mechanisms for a possible inflammatory response may not involve change in concentration of $sPLA_2$ but might be caused by either increased activity of $sPLA_2$ (not tested here) or other mechanisms of activation of arachidonic acid derivatives.

Prostaglandin levels increase following mechanical irritation and injury[140, 141] in corneal inflammation[142] and in the tears of dry eye patients.[139] However, the concentration of prostaglandins E_1, E_2, $F_{1\alpha}$ and $F_{2\alpha}$ did not change in the current study indicating that inflammation and mechanical irritation mediated by these mediators would not be a contributor to end of day ocular discomfort.

According to the literature, cysteinyl leukotrienes (LTC4, LTD4, and LTE4) may be involved in excessive secretion of mucus in dry eye disease and other ocular inflammatory diseases.[149] In the current study cysteinyl leukotrienes concentration did not change suggesting that inflammatory response as mediated by these components is not involved in end of day discomfort. This finding is also supported by resolvin D_1 levels remaining unchanged in this investigation (no diurnal variation with and without CLs), as elevation of resolvin D_1 would indicate the necessity of blocking the

inflammatory effect of cysteinyl leukotrienes[149] to resolve continued production of inflammatory exudates.[159, 160, 166]

Bradykinin is a peptide,[328] a peripherally acting inflammatory mediator[329, 330] that participates in producing delayed burning sensation and swelling and pain.[331, 332] In the current studies, the concentration of bradykinin remained unchanged during the day and during CL wear.

Components with diurnal variation (AM vs PM)

Maintaining the balance between phosphatidylethanolamines (PE) and other phospholipids is critical for tear film stability.[121] PE levels decrease in dry eye disease.[117] The work reported here, demonstrated changes in PE and the product of its hydrolytic breakdown, lysophosphatidylethanolamines (LPE), in normal tears (no lens wear). Lower level of PE at the end of day and higher level of its breakdown product in normal tears suggest that there is a biological cycle for this lipid and that it may have a possible link with end of day ocular discomfort, although this study found no association between their absolute concentrations or ratio and end of day discomfort with contact lenses.

Cholesterol esters are one of the two most abundant tear lipids.[237, 271] A positive association has been demonstrated between the level of cholesterol esters and CL-induced dryness.[106, 107] In the present study, tear levels of cholesterol esters increased (AM *vs* PM) but only during CL wear and the changes were not associated with CL discomfort.

C3 and C3a are potent inflammatory mediators of the humeral system.[315] Their activation on the ocular surface can cause inflammation and tissue damage.[315] Conversion of C3 to C3a is regarded as diagnostic for activation of the complement system in response to injury or immunological response.[207, 208, 316, 317] C3 has been detected in both open and closed eyes (a condition assumed to involve inflammation).[207] In our studies C3 and C3a levels decreased during the day (AM *vs* PM). The ratio C3a/C3 also decreased from morning to evening, which implies that the classical complement cascade was not activated in these studies and hence may have not participated in induction of ocular discomfort.

Leukotriene B_4 (LTB_4) has been quantified in closed-eye tears[147] in normal CL wear[127] as well as in acute ocular surface inflammation associated with CL wear.[148] In our studies, LTB_4 decreased (AM *vs* PM) indicating that in these experiments LTB_4 is perhaps not a main driver of CL comfort at the end of day.

Secretory immunoglobulin A (sIgA) has been detected in tears and plays a critical role in mucosal immunity.[260, 262-264] Earlier reports have documented reduction of sIgA[270, 272-274] or no change in CL wear.[32, 275, 276] In the current studies, tear levels of sIgA decreased in the evening (AM *vs* PM).

Lipocalin 1 is involved in transportation of lipids and other hydrophobic molecules[113] and may be involved in improved wettability of the ocular surface.[45, 97] It is increased in the tears of intolerant CL wearers[75] and in patients with CL-related ocular dryness.[369] [369] Lipocalin 1 increased in these studies (diurnal variation, AM *vs* PM).

Proline rich protein 4 is known to increase in tears of patients with CL-related ocular dryness.[369] Its function in tears yet remains to be described. In our studies, proline rich protein 4 increased during the day.

Prolactin-induced protein may be involved in osmoregulation of tears through regulation of aquaporin 5.[242] The level of this protein is increased in tears of patients with ocular dryness related to CLs.[369] In our studies, prolactin-induced protein increased (AM *vs* PM).

Components with different levels in contact lens wear and no lens wear

Leukotriene B_4 is involved in inflammation. LTB_4 is able to release lysosomal enzymes from neutrophils.[618] It is a potent chemo-attractant produced from leukocytes. Also, it can induce the adhesion of leukocytes enabling them to cross over through the vascular endothelium into the tissue.[635]

In the present study, LTB_4 level was higher in evening tears of contact lens wearers compared to non-lens wearers (Chapter 2:), and its level was higher in symptomatic CL wearers compared to asymptomatic lens wearers (Chapter 7). Therefore, these findings may suggest that inflammation may play a role in ocular discomfort with CLs. These findings confirm those of other researchers in that CL wear may induce inflammation.[127, 358] However, this study did not find any direct association between subjective ocular comfort ratings and changes in the concentration of this mediator from morning to evening. According to the literature, LTB_4 level is higher after 8 hours of sleep when compared to open eye tears.[147] The morning tear sample

collection in these studies was performed during the first hour after waking. Therefore, the morning tear level of LTB_4 recorded in these studies may have been influenced by its levels in closed eye conditions (sleeping overnight). This is the first report of diurnal changes of LTB_4 with and without contact lens wear and it can be suggested that future research should quantify the concentration of this mediator in more than two time points, so as to eliminate the effect of closed eye conditions on day time changes of the mediator.

Components associated with ocular comfort

Prolactin-induced protein concentration was higher at the end of the day and showed association with end of day ocular discomfort (AM *vs* PM). The association held true for both lens wear and no lens wear conditions. Tear volume is known to be reduced in intolerant CL wearers.[189] Prolactin-induced protein is linked to osmoregulation of tears through regulation of aquaporin 5[242] and is proposed to be involved in the induction of severe dry eye conditions[244] through down-regulation of aquaporin 5-mediated water transportation from the lacrimal glands, causing a reduction in tear menisci during the day.[603] Further studies may be required to investigate the mechanisms of action of prolactin-induced protein and its involvement in ocular discomfort.

Another finding related to prolactin-induced protein is that despite the diurnal changes (AM *vs* PM) and association with end of day discomfort established for this protein (Chapter 5:), in the study where asymptomatic and symptomatic CL wearers were

compared, no change in the levels of prolactin-induced protein could be found (Chapter 6:). This finding may have resulted in from the study design. In the study comparing AM with PM changes (Chapter 5:), the association of prolactin-induced protein with discomfort was established only when data were normalised by subtracting morning values for prolactin-induced protein from the evening values for each individual (ΔPIP). To ensure that the measured changes were caused by test conditions rather than any inter-subject variation of this protein[636] normalisation was performed. An association was found between ΔPIP and end of day subjective ratings. The rationale behind this method of analysis was that individuals had highly variable levels of prolactin-induced protein but for each individual an increase in prolactin-induced protein was noted in AM *vs* PM comparisons. This normalisation was not done for the data in Chapter 6: as asymptomatic and symptomatic prolactin-induced protein levels were measured only at one time point. Perhaps an association could be established if ΔPIP would have been measured in three groups of NCL, asymptomatic and symptomatic CL wear. Further investigation will be required in this area.

A summary of novel findings reported in this thesis

- To address the inherent problem of limited availability of tear samples and unavailability of conventional methods of measurement for some tear proteins, (i.e. no commercially available antibodies) a method based on selected reaction monitoring (SRM) mass spectrometry (MS) was developed in this thesis for the first time. This method in conjunction with stable-isotope-labeled tryptic peptides and triple-quadrupole mass spectrometry, quantifies multiple

individual proteins in a single tear sample. Here we report simultaneous quantification of five proteins (lactoferrin, lysozyme, lipocalin 1, prolactin-induced protein (PIP) and proline rich 4) in 2.5 µL of tears.

- The concentrations of proline rich protein 4, prolactin-induced protein, resolvin D_1 and C3a are reported for the first time in this thesis.
- Contact lens-induced discomfort at the end of the day may be associated with higher levels of LTB_4 and PIP in tears.

8.2 Conclusion

Prolactin induced protein seems to be involved in the process of end of day ocular discomfort. The findings reported in this thesis indicate that concentration of this protein changes from morning to evening but is not influenced by CL wear. Elevated levels of LTB_4 in CL wearers (compared to non-lens wearers) and higher concentrations of this mediator in symptomatic lens wearers (compared to asymptomatic) suggest involvement of inflammation mediated through at least one of the arachidonic acid metabolic pathways in lens wear. However, no association was found between the level of LTB_4 and end of day ocular discomfort. Tear levels of proline rich protein 4, prolactin-induced protein, resolvin D_1 and C3a are reported for the first time.

Taken together, the results of these studies suggest that CL-related discomfort is a complex reaction involving a variety of lipids and proteins, including inflammatory mediators.

8.3 Limitations of the project

In this project subjects were trained to collect their own tears as described by other authors.[33, 599, 637] Tear sample collection, storage and transportation to the lab by the participating volunteers may have led to cellular contamination of the samples (bursting cells in the samples) and may have affected the concentration of tear lipids and proteins. This suggestion is supported by earlier reports about appearance of white blood cells in tears collected from the conjunctival sack in the morning.[33, 127, 638-641] Taking into consideration all morning tear samples were collected during the first hour after waking up, white blood cells if not other cell types, may have infiltrated into the tear samples. One conclusion for this study therefore is that the methods of tear collection and centrifugation before storage have a crucial role in study of tear lipids (Chapter 7:). Lactoferrin and lipocalin 1 are mostly secreted by lacrimal glands[193] and there are no reports of production of these proteins by conjunctiva and corneal cells or PMNs. However, lysozyme can be produced by PMNs,[370] therefore cellular breakdown may have affected the concentration of this protein in tears. There is little known about proline rich protein 4 and prolactin-induced protein in tears. Additional studies will be needed to determine the intracellular concentration of these two proteins.

It is already known that tear sample storage and multiple freeze-thaw procedures (more than 6 times) can result in 8 - 10% decrease of protein concentration in closed eye tears.[642] While repeated freeze thaw cycles were avoided during in-house experimentation, collection of tear samples by patients may have influenced the preservation conditions of samples prior to delivery to the laboratory.

The current studies could be expanded by probing tears for cytokines. However, the level of cytokines in tears was being investigated by others in the laboratory during the same time as this research for the thesis was being conducted. These studies used multiplex bead assays to measure the concentration of 27 cytokines, chemokines and growth factors (cytokines) in tears using the same paradigm as the current study (AM vs PM, CL *vs* no CL wear). These studies demonstrated that the multiplex bead assays were un-reliable (low repeatability) for 12 of the cytokines studied, and for the remaining 15 cytokines IL-8 was the only cytokine that changed in both groups during the day without or with lens wear and there was no association between any cytokine and the comfort score with or without lens wear (personal communication with Prof Mark Willcox). This indicates that cytokines, chemokines and growth factors may not be associated with end of the day discomfort during contact lens wear. Although it should be taken into consideration that even under the best tear collection techniques, freezing samples may result in degradation of inflammatory mediators.[643-645]

As explained in Chapter 7: in each stage and time point, samples were pooled together regardless of subjective comfort rating to produce a total of four samples for each individual. This might be looked at as an apparent flaw of the study design because for each subject, samples which were collected with and without sensation of discomfort were pooled together. However, large volumes of tears for each person were needed at each stage and time point so as to quantify all of the molecules of interest. To compensate for this issue, experiments were performed for a selected number of components in symptomatic and asymptomatic subjects (Chapter 6:). An outcome of this project therefore is that in future at least for proteins, components can be quantified in sample volumes as small as 2.5 µL, using the new method described in Chapter 4:.

Effectively the above method of pooling samples together may become unnecessary in future studies.

In all studies of this thesis, except Study 3 and Study 5, subjects participated in both stages of lens wear and no lens wear, thus the study group served as their own control. This study design allowed for better understanding of individual differences rather than the difference of the average between groups and showed causal effect between the levels of selected proteins and lipids in the morning and evening with and without CLs. Although, using the same group in the study helped with the flexibility and low cost of these studies compared to using different groups, the results may not be extrapolated to the general population.

Contact lens discomfort in this study was investigated based on subjective ratings. Use of other tools such as questionnaires or clinical evaluation of ocular surface might be helpful in assessment of ocular discomfort.

Normal subjects were those with no ocular disease and subjects were invited from the general data base of the clinic at the school of optometry and Brien Holden Vision Institute clinics who were interested to participate in the studies and except one the studies (Study 5) no minimal comfort level was designated when recruiting subjects. Based on the literature we expected that at least 1/3 of the subjects would have symptoms of discomfort at the end of day and that they would report a significant drop in ocular comfort level of about 20-30 percent. Only about 12-15% of subjects experienced that level of drop in comfort.

Availability of small volumes of tear samples was the biggest limitation of this study. The volume of tears collected from patients varied from one to another and ranged between 30 - 100 µL while at least 60 µL of tears were needed for various analyses described in this thesis and some of the sample volumes were too small to detect some of the mediators of interest. Although we developed a method to measure tear proteins in very small volumes of tear samples, still it would have been ideal to measure arachidonic acid metabolites (leukotrienes C4, E4, D4 and prostaglandins E and F) as well as resolvins D and E simultaneously using GC-MS so as to save tear samples for measuring more tear chemicals such as other arachidonic acid metabolites including 12-HETE and 12-HETrE. We trialled the method, but it was not possible to attain a suitable limit of detection for any of the selected analytes using this technique. Investigation by mass spectrometry for these mediators would not fit into the timelines of this project for a number of reasons including the time required to extract these lipids from small volumes of tear samples (which are in picomolar levels in tears), the time needed to run each sample, and the potential for variability between runs due to the many manual steps involved in the methodology which could cause analyte loss in each step. Therefore, this part of the work is not reported in this thesis and further screening was performed using EIA kits for leukotrienes and prostaglandins. These kits required larger volumes of samples compared to mass spectrometry, but were advantageous in that EIA kits provided a simple analytical procedure with minimal sample pre-treatment, making them more suitable for analysis of large batches of samples.

8.4 Future directions

In an ideal situation these experiments should be repeated by collecting tear samples at a clinic rather than at home to ensure proper control for tear sample collection and quality of samples (basal tears, no stimulation, proper refrigeration and handling of samples and centrifugation before freezing samples). Evening samples must be collected essentially when volunteers confirm being symptomatic. This is normally later in the day and would be around 8 to 10 pm, presenting difficulties in collecting samples at optimal times. In the current study, subjects were asked to collect evening tears prior to sleeping or when symptoms of dryness or discomfort would occur. For future research, subjects additionally should be asked to mark/separate the samples collected when prompted by discomfort. This may improve differentiation between the tear sample types by level of discomfort.

Also, future investigation replicating the work presented here should be designed so as to take into consideration the potential impact of different CL materials and designs.

SRM method has the potential to analyse more than 100 transitions in a single run.[411] With further work, the technique can potentially be expanded to use for simultaneous quantification of up to 40 proteins in very small volumes of human tears, with implications in tear biomarker identification in diseases such as dry eye.

Additionally, it may be valuable to further investigate the role of (O-acyl)-omega-hydroxy fatty acids (OAHFA) as it has been demonstrated that these lipids may be involved in dry eye conditions.[51, 79]

Another line of future work would be to test the hypothesis that by adding the lipid components identified in this thesis as potentially involved in CL-related discomfort back into the tears, comfort and other clinical parameters will be improved. This type of work may prove highly beneficial as supported by the findings in the literature indicating that lubricating (lipid-containing) drops can improve tear break up time.[646]

Due to higher level of LTB_4 recorded in tears of CL wearers compared to the non-lens wearing group and also its increased concentration in symptomatic CL wearers, arachidonic acid derivatives might be the potential group of inflammatory mediators involved in CL discomfort and other pathways and metabolites of this group may need to be studied further.

The level of lysozyme showed a trend of difference between symptomatic and asymptomatic groups ($p \leq 0.1$). In future studies at least 37 symptomatic subjects and 37 asymptomatic (control) subjects will be needed to reject the null hypothesis that the level of lysozyme is equal in the two groups with probability of 80% power at 5% significance.

Better understanding of diurnal variations of tear proteins and lipids and elucidation of their nature and roles may contribute to the treatment of CL discomfort.

Patient consent form

Approval No (HC12055)

PARTICIPANT INFORMATION STATEMENT AND CONSENT FORM

Contact lenses wear ocular discomfort and changes to tear film components

You are invited to participate in a study examining changes in tear film components with and without contact lens wear. The purpose of this study is to investigate the possible correlation between ocular discomfort and changes in tear composition including inflammatory mediators and lipids over the course of the day. You can participate in this study if you are between 18 and 45 years old, have no ocular disease and your vision correction is between +6 and -12 dioptres and currently use contact lenses.

DESCRIPTION OF STUDY AND RISKS

If you decide to participate, we will perform a baseline screening examination to determine your suitability for this study. You will be trained to collect your own tears using a non-invasive technique and will collect your own tears twice daily. You will be asked to rate your eye comfort on a scale of 1 - 100 at the time of tear collection. Morning tear collection should be within 1 hour after waking. Evening/symptomatic tear collection must be performed prior to sleeping or when symptoms of dryness or discomfort occur.

This study includes two stages:
During the first stage you may wear your own habitual spectacles (no contact lens wear) for maximum 10 days or until at least a total of 60 μL (almost one drop) of tears is collected.

During the second stage you will wear daily disposable contact lenses for a maximum of ten days or until at least a total of 60 μL of tears is collected for this stage. During this stage tear collection should be performed before you start wearing contact lenses in the morning and lenses are removed only after you collect the evening/symptomatic session tears (please note that you must not sleep in the lenses).

PROCEDURES

Collection of tear samples: You will be trained by the investigators of the project to place a fine glass tube (with a rounded edge) at the edge of your lower eyelid to gently draw out a small volume of your tears (equivalent to a very small drop). This training will take place in the Rupert Meyer's building and may last 15 to 30 minutes. A container and a small ice-box will be provided to you to store tear samples in it. You can store multiple tear samples in this container. The storage container should be kept in a freezer for as long as possible. Upon leaving home, you need to deliver the tear samples to Brien Holden Vision Institute (BHVI) within the shortest time period feasible (preferably within three hours).

RISKS AND PRECAUTIONS

Episodes of minor irritation, redness or watering of the eyes may occur during tear collection. These should subside in a few minutes after tear collection. The most potentially serious adverse effect you may

encounter during contact lens wear is microbial keratitis. The risk of infection/inflammation is reported to be 1 - 5 in 10,000 people per year and is a sight threatening condition. It may rarely cause significant pain and hospitalisation. If any persistent irritation or redness of the eyes occurs during the course of study, you should contact Mrs Simin Masoudi on _____. If you are unable to get in contact with us, you should go to the Emergency Clinic of the Sydney Eye Hospital at 8 Macquarie Street, Sydney or contact the Eye Hospital on (02) 9382 7111.

BENEFITS
This study is part of a PhD project designed to yield information which may benefit other contact lens wearers in the future. However, we cannot and do not guarantee or promise that you will receive any direct benefits from this study. While your participation in this study is highly appreciated, to partially compensate for your efforts and time you will be provided with a 20$ gift voucher at the end of the study.

SAFEGUARD
You should inform the investigators if you have ever experienced any allergies to latex or any medications. You should also refrain from participating in this study if you are currently enrolled in any other clinical study. If you are not completely truthful about your health status and treatments, you may be exposing yourself to harm by participating in this study.

By agreeing to participate in this study, you are agreeing to the following:

- Reporting of all changes that occur to your health, medications and in particular your eyes;
- To be confident in inserting and removing your own lenses
- To be confident in your ability to safely and reliably collect your tears.
- To ensure your safety during the periods of lens wear, look at your eyes in a mirror and ask yourself "Do my eyes see well, feel good and look good?" If the answer to any of these is "No" make use of the emergency phone number (9962 9441emergency only) – valid only for the duration of the study.

CONFIDENTIALITY AND DISCLOSURE OF INFORMATION
Any information that is obtained in connection with this study and that can be identified with you will remain confidential and will be disclosed only with your permission, except as required by law. If you give us your permission by signing this document, we plan to publish the results obtained in this study in the form of group data responses at scientific conferences. In any publication, information will be provided in such a way that you cannot be identified.

Complaints may be directed to the Ethics Secretariat, The University of New South Wales, SYDNEY 2052 AUSTRALIA (phone 9385 4234, fax 9385 6648, email ethics.sec@unsw.edu.au). Any complaint you make will be investigated promptly and you will be informed of the outcome.

Feedback to participants
If you indicate that you wish for us to do so by providing your email address below, we will email you a summary of research findings on completion of this study.
Your email address (optional) _____

Your consent
Your decision whether or not to participate will not prejudice your future relations with the University of New South Wales. If you decide to participate, you are free to withdraw your consent and to discontinue participation at any time without prejudice.

If you have any question, please feel free to ask us. If you will have any additional questions later, Mrs Simin Masoudi on _____ and she will be happy to answer them.

You will be given a copy of this form to keep.

PARTICIPANT INFORMATION STATEMENT AND CONSENT FORM (continued)

Contact lenses wear ocular discomfort and changes to tear film components

You are making a decision whether or not to participate. Your signature indicates that, having read the information provided above, you have decided to participate.

.....	.....
Signature of Research Participant	Signature of Witness
.....	.....
(Please PRINT name)	(Please PRINT name)
.....	.....
Date	Nature of Witness

.....
Investigators signature

.....
Date

REVOCATION OF CONSENT

Contact lenses wear ocular discomfort and changes to tear film components

I hereby wish to **WITHDRAW** my consent to participate in the research proposal described above and understand that such withdrawal **WILL NOT** jeopardise any treatment or my relationship with The University of New South Wales.

....
Signature Date

.....
Please PRINT Name

The section for Revocation of Consent should be forwarded to Prof. Mark Willcox, School of Optometry and Vision Science, The University of New South Wales, Sydney 2052

Publications, awards and presentations related to this thesis

Peer reviewed journals

- Simin Masoudi, Ling Zhong, Mark Raftery, Mark Willcox. Quantification of five tear proteins using Selected Reaction Monitoring (SRM) Mass Spectrometry. Invest Ophthalmol Vis Sci 2014 Feb 10;55(2):767-75

Oral Presentations at international conferences

- Diurnal changes of lipid inflammatory mediators in human tears with and without contact lenses. The International Conference on the Tear Film & Ocular Surface: Basic Science and Clinical Relevance (TFOS), Taormina Italy, September 2013

- Diurnal changes of human tear proteins with and without contact lenses and their correlation with ocular comfort. The International Society for Contact Lens Research (ISCLR), Kyoto Japan, August 2013

- The application of Selected Reaction Monitoring method for quantification of tear proteins. Bioanalytical Mass Spectrometry Facility Seminar, 2012, UNSW, Australia (Invited seminar)

Poster and paper presentations

- Simin Masoudi, Fiona Stapleton, Todd Mitchell, Mark Willcox. "Diurnal variations of human tear lipids", ARVO Annual Meeting, Orlando, Florida, USA. May 4 - 8, 2014 (acceptance received)

- Simin Masoudi, Fiona Stapleton, Mark Willcox. "Diurnal changes of lipid inflammatory mediators in human tears with and without contact lenses", the International Conference on the Tear Film & Ocular Surface: Basic Science and Clinical Relevance (TFOS), Taormina Italy, September 2013

- Simin Masoudi, Jennie Diec, Fiona Stapleton, Thomas Naduvilath, Mark Willcox. "Diurnal changes of human tear proteins with and without contact lenses and their correlation with ocular comfort", International Society for Contact lens , Kyoto Japan, Augest 2013

- Simin Masoudi, Ling Zhong, Mark Raftery, Mark Willcox. "Method development for analysis of tear proteins using Multiple Reaction Monitoring", ARVO Annual Meeting, Seattle, Washington, USA. May 5 - 9, 2013

- Simin Masoudi, Zhenjun Zhao, Amali Ariyavidana, Jennie Diec, Pauline Xu, Mark Willcox. "Contact lens wear induced ocular discomfort and changes to inflammatory mediators in human tears" British Contact Lens Association (BCLA), 2012

Scholarships, Awards and Grants

- UNSW Tuition Fee Remission Scholarship (TFR), 2010 - 2014

- Faculty of Science Tuition Fee stipend Scholarship, The University of New South Wales, 2010 - 2014

- Postgraduate Research Support Scheme (PRSS) Award, The University of New South Wales, 2013

- The International Society for Contact Lens Research (ISCLR) student grant, 2013

- Brien Holden Vision Institute Travel Grant, 2013

- Brien Holden Vision Institute Travel Grant, 2014

- The Association for Research in Vision and Ophthalmology Travel Grant, 2014

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