Matrix metalloproteinase-9 in contact lens-related corneal erosions

Author:
Markoulli, Maria

Publication Date:
2011

DOI:
https://doi.org/10.26190/unsworks/15295

MATRIX METALLOPROTEINASE-9 IN CONTACT LENS-RELATED CORNEAL EROSIONS

MARIA MARKOULLI
MOptom GradCertOcTher FBCLA

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
Brien Holden Vision Institute
Sydney, Australia

August, 2011

COPYRIGHT AND AUTHENTICITY STATEMENTS

COPYRIGHT STATEMENT

AUTHENTICITY STATEMENT

CERTIFICATE OF ORIGINALITY

'I hereby declare that this submission is my own work and to the best of my knowledge it contains no materials previously published or written by another person, or substantial proportions of material which have been accepted for the award of any other degree or diploma at UNSW or any other educational institution, except where due acknowledgement is made in the thesis. Any contribution made to the research by others, with whom I have worked at UNSW or elsewhere, is explicitly acknowledged in the thesis.

I also declare that the intellectual content of this thesis is the product of my own work, except to the extent that assistance from others in the project's design and conception or in style, presentation and linguistic expression is acknowledged.'

Signed ……..

Date ……..

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my supervisors, Associate Professor Eric Papas, Dr Nerida Cole and Professor Brien Holden. This PhD has been an incredible journey and all the richer for having Eric as my supervisor and mentor. Words cannot express my gratitude for his support and insight over the last few years. I am ever grateful for the brainstorming sessions and for encouraging me to think outside the square and consider all possible avenues. Thank you also to my co-supervisor Nerida for the much needed guidance and reassurance in the field of biochemistry, and for always being available to help. Thanks also go to my co-supervisor Brien who has had faith in me and supported my career path from day one. His work and passion for what he does is an inspiration. It has been truly an honour and a privilege to work with these wonderful people.

The research reported in this thesis would not have been possible without the funding provided by the Australian Government through the Australian Postgraduate Award, and the funding provided by the Brien Holden Vision Institute (BHVI). The award of the William C. Ezell fellowship by the American Optometric Foundation (sponsored by the George and Jill Mertz Foundation in 2009 and CIBA VISION™ in 2010) provided encouragement that, as Mike May said at the 2009 American Academy of Optometry meeting, “there is always a way”. The Cornea and Contact Lens Society of Australia, the Tear Film and Ocular Surface society, the Graduate Research School of University of New South Wales (UNSW) and the Optometric Vision Research Foundation-Maki Shiobara Foundation have similarly supported my work, and for this I am truly grateful.

The biggest part of the journey is the people you meet who inspire, motivate and teach, each in their own way. Dr Michele Madigan is one such person who taught me to “think like a hemidesmosome” along with the art of zymography. Her adventurous take on science and advice have been invaluable. I would also like to thank Professor Robert Sack for his advice early on; Vivienne Miller for keeping me enrolled and reviewed; Denise and Robyn Lawler and Dr Lewis Williams for welcoming me to R2 in the final phases of my writing.

I would also like to acknowledge the institutes that supported my work: the BHVI Clinical Research and Trials Centre for allowing me to use their clinic rooms during my overnight studies; the BHVI biochemistry laboratory for use of their facilities; i-media for their help with conference posters; the L.V.Prasad Eye Institute for allowing me to observe their contact lens clinic in 2008; the team at Minomic International Ltd for their help with differential gel electrophoresis; the Bioanalytical Mass Spectrometry Facility at UNSW for their aid with mass spectrometry; and CIBA VISION™ Australia who kindly donated solution for the clinical study.

The studies presented in this thesis would not have been possible without the study participants who donated their time and tears, and endured contact lens wear and sleeping on air mattresses, all in the name of science. Thank you one and all.

I have made some truly wonderful friendships on the way, and each of these friendships has lessened the load and made the journey more enjoyable. Cathleen, you were right, a PhD is much like “driving with the hand break on”. Your support has kept me going and your dedication to your work has been motivational. Thank you mein kumpel. Eric, thank you for helping me put things into perspective and being a helping hand throughout the most difficult times. My lab buddies: Kalika, Ben and Sharon, thank you for the endless supply of smarties, ingenious trouble-shooting skills and patience, respectively! Thanks also go to: Andrea, for endless discussions on tear collection and the encouragement; Ravi for always knowing that special Microsoft shortcut that could help me save time. To my fellow postgraduate students Nic, Jaya, Krupa, Negar, Eon, Moneisha, Nag and Roopa and my friends Jess, Brian, Judy and Jennie, thank you for your friendship.

Lastly, but most importantly, I want to thank my family whose thoughts and prayers have propelled me forward. Thanks go to my extended family of aunts, uncles and cousins for their support. Without question, I owe this thesis to my mum Militsa and my grandparents who gave so much for me and instilled in me the importance of education and contributing to the community. Their unconditional love has helped me maintain perspective during this long, but very worthwhile journey.

DEDICATION

To my mum Militsa,

my γιαγιά Μαρούλλα

and

my παππού Νικόλα

ABSTRACT

A corneal erosion is created by a full thickness loss of corneal epithelium. They are painful, increase the risk of infection, and can lead to discontinuation of contact lens wear. Understanding their aetiology would assist in managing this important adverse event.

In non-contact lens wearers, a collagen degrading enzyme known as matrix metalloproteinase-9 (MMP-9) has been found to be upregulated in the corneas of those prone to erosions. MMP-9 is thought to weaken corneal adhesion complexes, contributing to the cascade of events leading to erosions. This thesis investigates the effect of contact lens wear on MMP-9 and its regulators in the tear film.

In this study, a flush tear collection technique was first validated against basal and reflex tear collection. This permitted rapid and repeatable retrieval of the major proteins in the same proportions as occurs in basal tears.

Tears were collected from healthy, non-contact lens wearers to establish the diurnal variation of MMP-9 and its regulators. Eye-closure resulted in a 200-fold increased expression of MMP-9 compared to only a 3- and 5-fold increase in its regulators TIMP-1 and NGAL, respectively. After the first night of extended contact lens wear, MMP-9 increased significantly compared to baseline and daily wear, with no change in its regulators. This increase in MMP-9 returned to baseline after one month.

Differential gel electrophoresis was used to gauge the effect of extended wear on the tear proteome. Changes were identified in serum albumin, immunoglobulin A and zinc α2-glycoprotein, while other major proteins were largely unaffected.

The tears of three contact lens wearers who developed erosions were compared to normals. A greater MMP-9:TIMP-1 ratio was found upon awakening, both at baseline and after the first night of contact lens wear compared to normals. Elevated NGAL at baseline that further increased after the first night of extended wear was also found in one participant. The tear proteome profile also changed significantly after the first night of extended wear in one participant.

These studies demonstrated increased expression of MMP-9 in response to extended contact lens wear. This increase may predispose the cornea to adverse events during the early stages of extended wear.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

TERMINOLOGY AND ABBREVIATIONS

A	ampere
ADAMTS	a disintegrin and metalloprotease with thrombospondin motifs
ANOVA	analysis of variance
APMA	4-aminophenyl-mercuric acetate
Baseline	before lens wear
BCA	bicinchoninic acid assay
BHVI	Brien Holden Vision Institute
BSA	bovine serum albumin
CD147	cluster of differentiation 147
CLPU	contact lens peripheral ulcer
D	dioptre
Da	dalton
DIGE	differential gel electrophoresis
Dk/t	oxygen transmissibility
DMF	dimethylformamide
DTT	dithiolthreitol
DW	daily wear
EDTA	disodium ethylene-diaminetetra-acetic acid
ELISA	enzyme-linked immuno assay
EM	expectation maximisation
EMMPRIN	extracellular matrix metalloproteinase inducer
EW	extended wear
FDA	Food and Drug Administration
g	gravitational force
HCl	hydrochloric acid
HRP	horseradish peroxidase
H_2SO_4	sulfuric acid
ICAM	intercellular adhesion molecule
IFN	interferon

IgA	immunoglobulin A
IL	interleukin
IPG	immobilised pH gradient
kDa	kilodalton
LASIK	laser-assisted *in situ* keratomileusis
M	molar
mg	milligrams
MK	microbial keratitis
mL	millilitres
µL	microlitres
µm	micrometres
mM	millimolar
MMP	matrix metalloproteinase
MPa	megapascals
MS	mass spectrometry
MT-MMP	membrane-type matrix metalloproteinase
MW	molecular weight
NaCl	sodium chloride
ng	nanograms
NGAL	neutrophil gelatinase-associated lipocalin
NH_4HCO_3	ammonium bicarbonate
nm	nanometres
OD	right eye
OS	left eye
OU	both eyes
pg	picograms
pH	measure of acidity or basicity
pI	isoelectric point
pmol	picomole
ppm	parts per million
RGP	rigid gas permeable lenses

SCX	strong cation ion exchange column
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEAL	superior epithelial arcuate lesion
TBP	tributylphosphine
TEMED	tetramethylethylenediamine
TGF	transforming growth factor
TIMP	tissue inhibitor of metalloproteinases
TMB	tetramethylbenzidine
TNF	tumour necrosis factor
TPC	total protein content
UNSW	The University of New South Wales
V	volts
v/v	volume per volume
VCAM	vascular adhesion molecule
VHrs	volt-hours
VIHEC	Vision CRC and Institute for Eye Research Human Ethics Committee
W	watts

CHAPTER 1 LITERATURE REVIEW

1.1 INTRODUCTION

Contact lenses are worn by an estimated 140 million people worldwide[1] and are a valuable alternative to spectacle wear and refractive surgery. Their uses range from refractive error correction and myopia control[2] to therapeutic applications.[3] Safe contact lens wear is therefore of paramount importance.

Despite the advent of new, high oxygen permeability, silicone hydrogel materials, contact lenses continue to cause adverse events which range in severity from asymptomatic micro-infiltrates,[4] to the fortunately rare microbial keratitis (MK).[1, 5, 6] Several of these adverse events can result in significant morbidity to the wearer[7] as well as interruption or discontinuation of contact lens wear.[8]

Corneal erosions are one of the adverse events that occur in contact lens wear,[9-13] with the associated discomfort and disruption to lifestyle having the potential to cause significant patient anxiety. Although not yet proven, a hypothetical link has been made between corneal erosions and MK.[13] The risk associated with the presence of a corneal erosion is that the natural barrier of the cornea to infection is no longer intact, hence allowing the entry of pathogens. Because of this, erosions may have significance that extends beyond their own occurrence. It is therefore important to be clear on what erosions are, how to differentiate them from other adverse events and their aetiology. In non-contact lens wearers, erosions are thought to result from defective anchoring of the epithelium to the stroma.[14-16] Understanding the mechanisms that underpin corneal erosion formation in contact lens wear is fundamental to establishing means by which they might be prevented. Currently these causes are unknown.

This chapter reviews the phenomenon of corneal erosions in contact lens wear, including their differentiation from other contact lens-related adverse events. It then considers current knowledge of the mechanisms underlying their formation both with and without contact lens wear, including the role of collagen degrading enzymes known as matrix metalloproteinases (MMPs). Finally, the aims and hypotheses of the thesis are introduced.

A publication has stemmed from this literature review in *Contact Lens & Anterior Eye*[17] (presented here with permission from the journal) as well as two presentations (Appendix B: Publications and presentations).

1.2 CORNEAL EROSIONS

1.2.1 Definition and clinical appearance

A corneal erosion is a full thickness detachment of epithelium in a localised and well-circumscribed region of the cornea[18-20] that can occur either with[4, 10, 11, 21, 22] or without contact lens wear.[18-20] The reference to 'full thickness' in the definition of an erosion relies on inferences drawn from its appearance[10] and the abnormal epithelial basement membrane histology typical of non contact lens-related recurrent erosions.[14, 23] To date however, there have been no studies confirming that this is a consistent clinical feature in contact lens wear.[20] Clinically, an erosion is usually observed as an absolute defect that stains with sodium fluorescein (Figure 1.1). Alternatively, an area where the epithelium is detached, but still remains adherent at its borders may be seen[24] (Figure 1.2). Presumably, this represents an earlier stage in development and is analogous to a blister of the skin where the raised epidermis at the centre of the lesion eventually sloughs off to expose the underlying dermis.

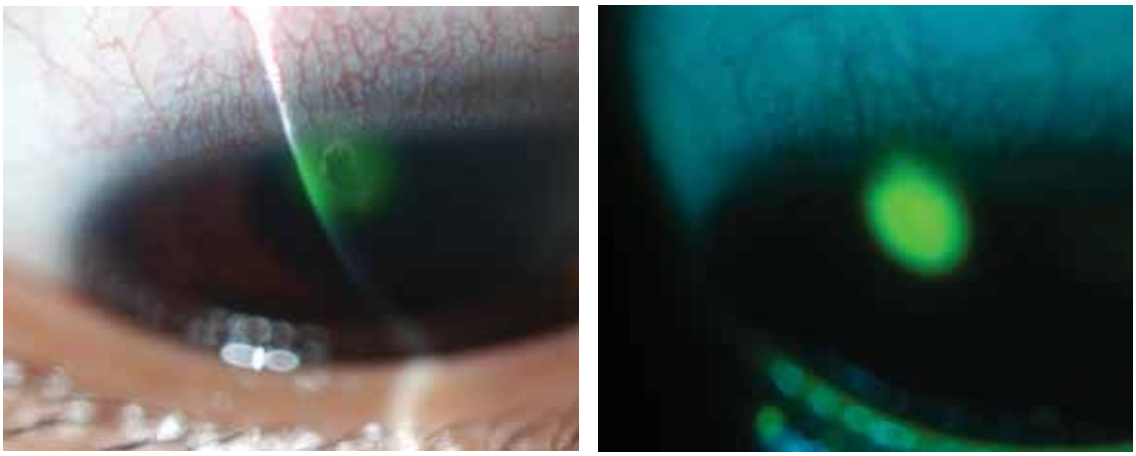


Figure 1.1: A contact lens-related corneal erosion as an absolute epithelial defect.

This erosion was photographed with white light (LEFT) and with cobalt blue light and a Wratten #12 filter (RIGHT) after the instillation of sodium fluorescein to reveal stromal glow. Image courtesy of the Brien Holden Vision Institute Clinical Research and Trials Centre, Sydney, Australia.

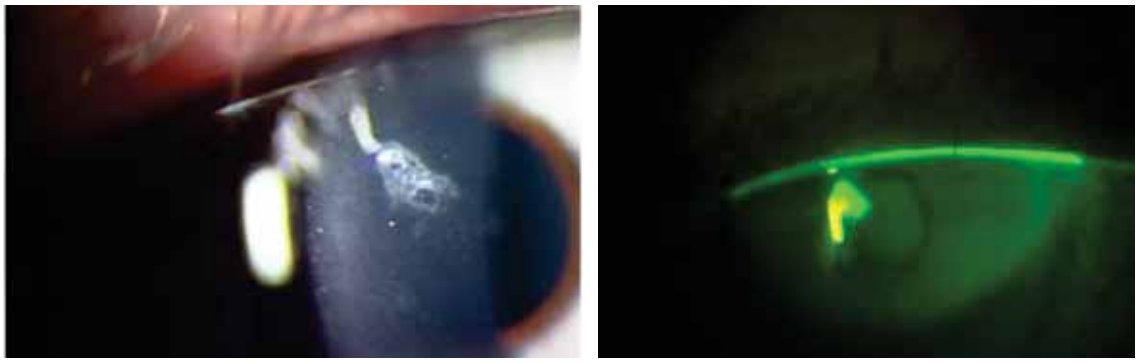


Figure 1.2: A contact lens-related corneal erosion where the epithelium is detached, but still remains adherent at its borders.

This erosion was photographed with white light (LEFT) and with cobalt blue light and a Wratten #12 filter (RIGHT) after the instillation of sodium fluorescein.

1.2.2 Size and location

The size of erosions that occur either with or without contact lens wear may range from 0.1 mm in diameter, to macro-erosions that cover large areas of the cornea.[18, 25] Regardless of contact lens wear status, erosion appearance is described with the same common features, suggesting similar aetiology. Observing with fluorescein and a Wratten #12 filter reveals immediate stromal glow (Figure 1.1, right and Figure 1.2, right), suggesting that the full thickness of the epithelial barrier has been breached. In cases where the epithelium is still loosely adherent, (Figure 1.2 the lesion will appear elevated). When the defect is absolute (Figure 1.1), fluorescein will both pool in, as well as stain, the floor of the hollow area, which most likely is basement membrane.

Erosions occur in all areas of the cornea and multiple lesions can present at the same time.[25] It has been found that 87.5% localise inferiorly,[19] and regardless of contact lens wear, the most common location is close to the midline, just below the pupil.[20, 25] This is also the region where basement membrane changes are commonly found to occur.[26]

1.2.3 Associated signs and symptoms

While it is not unusual for erosions to occur in the absence of accompanying symptoms,[13] limbal and bulbar hyperaemia may be evident in the affected sector and photophobia and tearing are commonly experienced.[19, 20] Importantly, there is no mucopurulent discharge, a useful differentiating factor with respect to corneal

infection.[10] A delayed inflammatory response has been reported in some cases.[10] Vision loss is thought to be associated with the resultant epithelial irregularities, the result being astigmatism and abnormal tear break-up.[20, 27] Meibomian gland dysfunction has also been reported to be common in non-contact lens wearers with recurrent corneal erosions.[28]

When contact lenses are present, wearers may experience significant foreign body sensation, particularly upon awakening if in extended wear (EW).[25] Alternatively, there may be a sharp pain upon contact lens removal, followed by a foreign body sensation that is exacerbated by blinking. Contact lenses in some cases may also be difficult to remove, possibly indicating decreased movement or even binding, due to a reduced tear film thickness,[29, 30 31] or due to a hypotonic shift in tear osmolarity [32]especially if worn during sleep.

1.2.4 Incidence

Contact lens-related corneal erosions have been reported with both hydrogel and silicone hydrogel materials.[9-11, 13, 21, 22] Rates in daily wear (DW) are very low, with one study with 558 participants and 2271 participant months reporting 0.04 events per 100 participant-months,[9] which equated to only a single case. A second study conducted over 7 years with 6,130 participants reported 0.01-0.05% per visit,[13] with daily disposable contact lenses being free of events.[13] Although these two studies report on the incidence using difference measures, both indicate a low rate in DW. In EW, erosions were found to occur much more frequently at 0.6-2.6% of visits overall.[13] Silicone hydrogels were found to have a higher rate (0.95-1.68% of visits) than conventional hydrogels at (0.05-0.35% of visits).[13] Sixty-eight percent of erosions in this study occurred within the first three months of contact lens wear adaptation.[13]

1.2.5 Association with infection

MK is a rare occurrence in healthy eyes, with contact lens wear posing the highest relative risk.[33] The incidence of infection has been estimated as 11.9 per 10,000 wearers in DW silicone hydrogels as opposed to 25.4 per 10,000 wearers in EW.[5] This excess risk continues to be a major limiting factor for the EW modality.[34]

While a direct link between contact lens-related erosions and progression towards MK has not been proven, only one case ever having been reported where multiple central corneal "lesions" coalesced into a central ulcer,[12] there is circumstantial evidence suggesting that such may be the case.[33, 35] Calculation of the theoretical rates at which corneal erosions occur concurrently with contact lenses that are contaminated with gram-negative bacteria, shows that this is higher with EW, at 24.8-60.0 per 10,000 people, than in DW at 1.6-16.4 per 10,000 people.[13] Interestingly, these rates are comparable with the frequency of MK in both situations,[5] the implication being that both a corneal erosion and bacterial contamination need to be present for infection to occur. Worthy of note in this analysis is that none of the erosions in the series reported were associated with gram-negative contact lens contamination.[13]

The risk associated with the presence of a corneal erosion, particularly in contact lens wear, is that the natural barrier of the cornea to infection is no longer intact, hence allowing the potential for entry of pathogens and inflammatory cells. The avid binding of *Pseudomonas aeruginosa* to injured or traumatised corneal epithelium has been previously established.[36] The presence of the basal lamina has been found to be necessary to prevent bacterial entry[37] whilst extensive superficial staining has been found to be insufficient for bacterial penetration.[38] Vigilance in the presence of a corneal erosion is therefore prudent to prevent consequences such as infection,[39] although it is clear that other protective mechanisms, such as antimicrobial factors in the tears are also protective.[38, 40]

1.2.6 Prognosis and recurrence

Contact lens-related corneal erosions usually heal within several hours[25] (Figure 1.3) to one to two days of contact lens discontinuation[10] and do so with no scarring. Careful observation of the patient with a corneal erosion is necessary to ensure that inflammation or infection does not result.[10]

When corneal wounding occurs, as in the case of an erosion, the wound healing process primarily targets the re-establishment of continuity of the epithelium covering the cornea and later the attachment of this to the basement membrane.[41] During migration, cell-cell adhesion is retained with the desmosomes so that cells move as a

sheet rather than as individual cells.[42] Hemidesmosomes which adhere the basal epithelial cells to the basement membrane (Section 1.2.8.1.1), however, are lost from cells actively moving to cover a wound.[41, 42] Migrating epithelium is therefore not as adhesive as unwounded epithelium.[42] As complete adherence to the underlying basement membrane may not occur for a few days,[43] no contact lens wear is advised until the area surrounding the erosion settles and secure adhesion is regained.

Although there are reports of recurrences of corneal erosions in contact lens wear,[25] data are not currently available on the rate of recurrence. In non-contact lens wearers, where erosions are recurrent, the median frequency of the attacks is every 60 days[18] with 24% occurring weekly, while 51% occur monthly.[18] The recurrence of corneal erosions following trauma has been estimated as 1:150.[18] Recurrences are most common upon awakening, this being described in 76% of patients.[20]

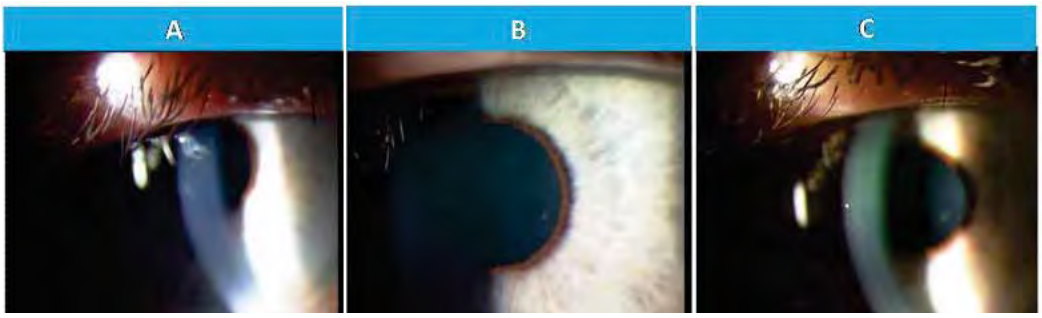


Figure 1.3: Time lapse of the corneal wound healing process for a contact lens wearer presenting with corneal erosion.

The patient presented complaining of difficulty removing her contact lens. A: Central corneal erosion immediately after contact lens removal; B: Appearance after three hours shows almost complete wound closure; C: Appearance of erosion after seven hours suggests a healed erosion with overlying epithelial staining.

1.2.7 Differential diagnosis

The following section reviews contact lens-related differential conditions and their distinction from corneal erosions in contact lens wear.

1.2.7.1 Microbial keratitis

An important differential of corneal erosions is MK, which is an ocular emergency. This is characterised by progressive excavation of the epithelium, Bowman's layer and

stroma with infiltration and necrosis of tissue.[4] Unlike corneal erosions, clinical signs include mucopurulent discharge, satellite lesions, lid oedema and conjunctival chemosis along with anterior chamber reaction and occasionally a hypopyon. The location will most likely be central and paracentral[33] but can occur anywhere, with a size greater than 1 mm in size. Without aggressive treatment, this worsens despite contact lens removal and the pain will be increasing and quite severe. Patients will present with progressive pain, photophobia, swelling and discharge.[33] In a study by Keay et al., 25% of patients with MK were found to have had previous episodes requiring urgent attention such as ocular trauma and foreign bodies.[33] Rigorous treatment with fortified antibiotics, for example, tobramycin 1.3% and cefazolin 5%, along with cycloplegia and analgesia are indicated. These will resolve with a corneal scar and an irregular cornea with vision loss if the scar is in the visual axis.

1.2.7.2 Contact lens peripheral ulcer

A contact lens peripheral ulcer (CLPU) is a round anterior stromal focal infiltrate with diffuse infiltration[44] and overlying corneal staining and delayed stromal glow. Unlike erosions, CLPUs represent an inflammatory reaction of the cornea that has been associated with gram-positive colonisation of the contact lens.[45] The condition is characterised by focal excavation of epithelium, infiltration and necrosis of anterior stroma.[4] Bowman's layer however remains intact.[44] Bulbar and limbal redness will be present, along with the symptoms of watering and pain[44] but a CLPU will not cause the mucopurulent discharge or the photophobia seen in MK. Lid oedema and chemosis are not usually present, however there may sometimes be an anterior chamber reaction. The location, as the name suggests, will usually be peripheral. CLPU sufferers experience immediate relief on contact lens removal and resolution can be within seven days[4, 45] although the majority take two to three weeks.[4] Unlike erosions, cases of CLPU will most often resolve with a bulls eye scar.[4, 44] Along with cessation of contact lens wear, topical antibiotics are prescribed occasionally as prophylaxis, while regular saline rinses combined with cold compresses and analgesia are used to alleviate symptoms.[4] It is hypothesised that the initial epithelial break occurs either due to epithelial injury or due to dissolution of the epithelial layers by biochemical factors such as leukotriene B4.[44] The condition may recur with contact lens wear.[4]

1.2.7.3 Superior epithelial arcuate lesions

Superior epithelial arcuate lesions (SEALs) are raised linear or arcuate excavations that occur in the superior cornea during contact lens wear, in the region covered by the upper eyelid. Forty eight percent of cases will have coalescent staining, 35% stromal glow and 50% infiltrates.[46] In 39% of cases there will be a foreign body sensation, while 35% will be asymptomatic.[46] Treatment consists of temporary discontinuation of contact lens wear and ocular lubricants. Prophylactic antibiotics are prescribed in severe cases. Resolution occurs within 1-9 days, taking longer if there are infiltrates present.[46] Although there is generally no scar, 50% recur.[46]

1.2.7.4 Punctate epithelial staining and solution induced corneal staining

There has been some confusion regarding the definition of corneal erosions with the term 'punctate epithelial erosion' occasionally used to describe diffuse, epithelial damage that has a punctate appearance when stained with sodium fluorescein (Figure 1.4). An example of punctate staining is that seen in solution toxicity to contact lens care solutions.[47] This kind of presentation affects only the uppermost layers of the epithelium and does not merit designation as 'erosion' which typically extends to the deeper layers, often exposing the basement membrane and resulting in immediate stromal glow (Figure 1.1).

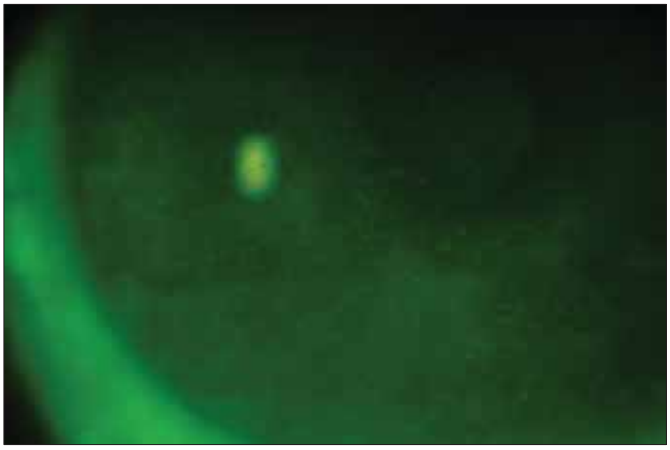


Figure 1.4: Solution induced corneal staining, also described as punctate staining.

This affects the superficial layers of the epithelium and differs from corneal erosions which extend to the deeper layers and result in stromal glow (Figure 1.1). Photo courtesy of the Brien Holden Vision Institute Clinical Research and Trials Centre.

1.2.8 The normal cornea

In order to understand the aetiology of contact lens-related corneal erosions, it is important to be clear on normal corneal physiology, and how the healthy corneal epithelium maintains adhesion to the underlying stroma.

The cornea is the major refractive surface of the eye and through its transparency enables light to be transmitted to the retina. In order to maintain this transparency, the cornea needs to ensure its integrity and avascularity and also maintain its organised structure.

The cornea is chiefly comprised of three distinct tissue layers (Figure 1.5): A stratified epithelium separated by the basal lamina from the collagenous stroma followed by the endothelium which is attached to the stroma by Descemet's membrane, a specialised thickened basal lamina.[48]

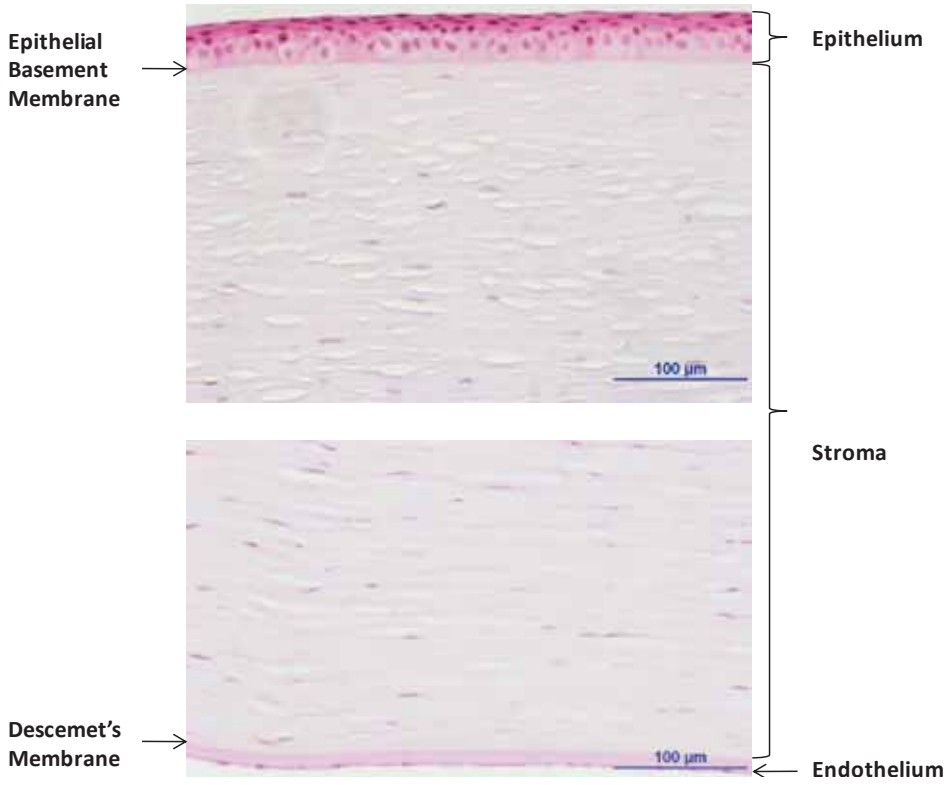


Figure 1.5: Light micrograph image of transverse sections of a human cornea.

Images courtesy of Dr Michele Madigan, SaveSight Institute, Sydney, Australia.

1.2.8.1 The epithelium

The corneal epithelium is a 50-µm, multi-layered physical barrier that forms a protective tight junctional barrier to prevent net decreases of fluid transport, electrolytes and macromolecules[49] out of the stroma while also functioning to prevent the entry of pathogens.[50] In order to do this, it must maintain an unbroken layer of cells to cover the corneal surface and it must also maintain tight adherence to the epithelial basement membrane and Bowman's membrane[41] which separate it from the stroma.

The basal cells form the bottom-most layer of the epithelium, and it is this proliferative layer which differentiates into intermediate, or wing cells and superficial cells,[49] and is desquamated five to seven days later.[42] Cells along the basement membrane are therefore constantly making and breaking their adhesions to the basement membrane (hemidesmosomes).[42] The superficial cells are semi-permeable and highly resistive owing to the presences of tight junctions and zonulae occludens.[50]

1.2.8.1.1 The corneal adhesion complexes

The corneal epithelium has two major mechanisms for cell-to-cell adherence and for adherence to its substrate:

1. Direct molecular interaction of receptors with ligands located either within the membranes of the epithelial cells or in the extracellular matrix.[42] Cell-to-cell and cell-to-matrix adhesion is mediated by three major families: N-cell adhesion molecules (CAMs),[51] cadherin family and the integrins.[42]

2. Through adhesive junctions:

    a. Cell-to-cell adhesive junction with desmosomes: These link intermediate or keratin filaments on the cytoplasmic face of cell through dense plaques along the cell membrane to a similar structure on the opposing cell membrane.[42] These are particularly prevalent along the wing cell layer and more sparse along the basal cells and apical cells.[42]

b. Cell-matrix adhesion junction: Intermediate filaments insert into an electron-dense plaque along the membrane. From this, anchoring filaments extend through the lamina lucida to the lamina densa of the basement membrane and provide a connection between the hemidesmosomes and the underlying stroma.[42] Opposite the lamina densa, anchoring fibrils composed of type VII collagen insert from the stromal side, forming an intertwining network in the anterior 2 μm of the stroma and in Bowman's membrane.[42] Anchoring fibrils then insert into anchoring plaques[42] among the stromal collagen fibrils.[52] Anchoring plaques are amorphous electron-dense structures composed of type VII and type IV collagen.[52, 53] All these factors are termed the adhesion complex (Figure 1.6).[42, 54]

Epithelial integrity may be compromised in the case where adhesion complexes are compromised. It should be noted that in the normal cornea adhesions are so strong that mechanical scraping results in fracture of the basal cell rather than separation at the interface of the epithelium and the basement membrane,[23, 41] suggesting that the whole adhesion complex, from hemidesmosomes through to the basement membrane, through to the anchoring fibres, may work as a unit.[23] It is of considerable interest, therefore, that in erosion formation, as in diabetic corneas,[52] or corneas with epithelial basement membrane dystrophies,[20, 39] these adhesions can be broken.[52]

Collagens are the major structural components of the extracellular matrix and are vital to tissue architecture and cellular processes including adhesion and migration.[55] These collagens interact with extracellular matrix proteins and it is this interaction which is important for normal processes such as development, growth, tissue remodelling as well as pathological processes.[55] Of the 28 types of collagen,[55] collagen type IV is the most abundant structural basement membrane component but is not required for basement membrane formation, only for basement membrane maintenance. It also mediates cell adhesion through binding to integrin and non-integrin receptors.[55] Collagen type VII is epithelial specific and is the largest collagen as well as the main structural component of the anchoring fibrils adhering the basement membrane to the stroma.[55] Any disturbance to the collagen within the adhesion complexes may therefore affect the adhesion of the corneal epithelium.

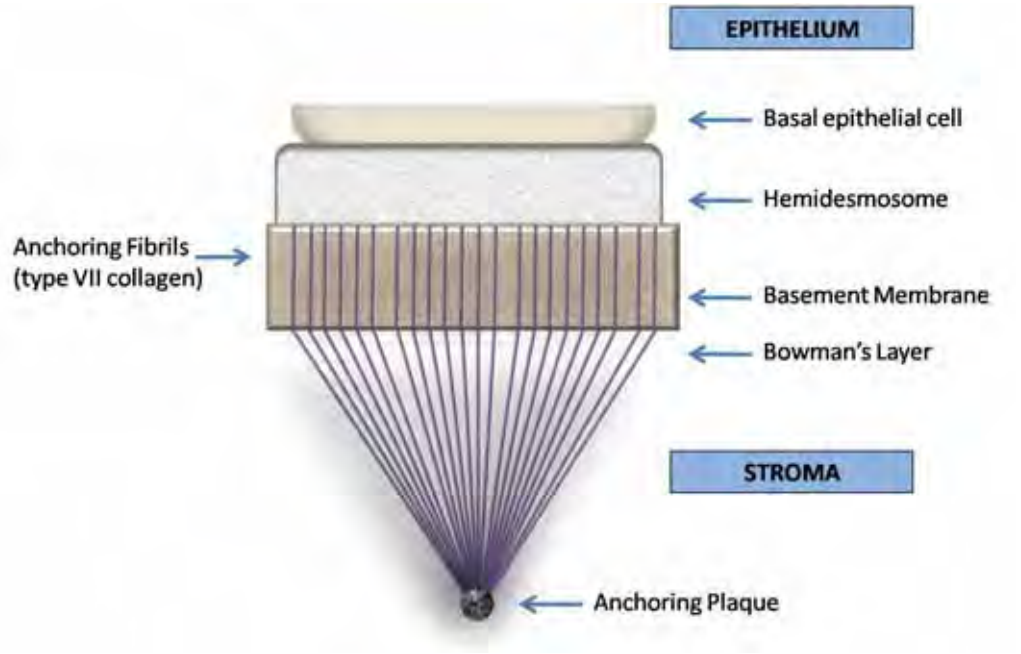


Figure 1.6: A schematic of the corneal adhesion complex.

Anchoring filaments extend through the basement membrane and emerge as anchoring fibrils in the stromal side, forming an intertwining network in the anterior 2 µm stroma.[42] These anchoring fibrils insert into anchoring plaques. All these factors are termed the 'adhesion complex'.[42] Type VII collagen is the anchoring fibril collagen, its domain being in the lamina densa and in the anchoring plaques.[23, 42] Adapted by Dr Cathleen Fedtke from a diagram by Dr Michele Madigan.

1.2.8.2 Bowman's membrane and the stroma

Bowman's membrane or layer consists of collagen fibrils closely but randomly packed into a layer which is not sharply differentiated from the underlying stroma and has most recently been measured as 17.7 ± 1.6 µm in the centre.[56]

The stroma is a densely packed connective tissue with relatively few cells. It is composed of more than 200 sheets of regularly layered fibrillar collagen sheets as well as keratocytes which produce and remodel these collagens.[57] The alignment of the collagen fibres in the stroma largely determines the optical uniformity of the cornea. This organisation is affected by the amount of water in the stroma which is determined by the pumping activity of the endothelium and the barrier function of the epithelium.[49]

1.2.8.3 Descemet's membrane and the endothelium

Descemet's membrane is the structure-less basement membrane lining the inner stroma, followed by a monolayer of endothelial cells. The endothelium can be seen as a mosaic of hexagonal cells under high magnification with the slit-lamp biomicroscope, and functions as a barrier to fluid moving into the cornea and actively pumps fluid out of the corneal stroma and into the anterior chamber. It is this metabolic activity of the endothelium which is responsible for controlling stromal oedema. Decompensation of the endothelium could result in stromal thickening and epithelial bullae formation and consequently, corneal erosion.[57]

1.2.9 Aetiology of contact lens-related erosions

The mechanisms that result in the corneal adhesion complexes (Figure 1.6) being overwhelmed by the action of the contact lens to produce an erosion, are yet to be understood. According to the Collins dictionary,[58] erosion is "the wearing away of rocks and other deposits on the earth's surface by the action of water, ice, wind, etc". Thus in geology the process is a gradual one. For the cornea however, the sudden appearance of well-circumscribed, full-thickness epithelial loss suggests that any causative external force is acting more abruptly. Possibly there is also a more gradual, accompanying internal process that weakens the corneal adhesions such that they subsequently 'give way' under suitable provocation, such as may occur for example, with a bound contact lens. The following reviews the currently known causes of corneal erosions, with particular focus on the mechanisms hypothesised to be associated with contact lens wear.

1.2.9.1 The bound contact lens

Contact lens adhesion to the cornea has been hypothesised as a possible aetiology for erosion formation.[21, 59] The suggestion is that when a contact lens becomes bound to the cornea, as commonly happens during overnight wear,[60, 61] an attachment forms between an area of epithelium and the contact lens surface. When the eye opens and blinking is resumed, this attachment creates a mechanically induced corneal erosion by pulling a 'plug' of epithelium away from its surrounds as contact lens motion recommences.[21] Gillbard et al. postulated that a reduction in tear osmolarity in

combination with a reduced post-lens tear film increases contact lens-cornea adhesion, resulting in contact lens binding and corneal erosions.[59] The post-lens tear film has been shown to reduce to half its thickness within half an hour of eye closure.[30] A reduction in post-lens tear film thickness could contribute to a reduction in lens movement[29] which in turn may result in contact lens binding, as well as an accumulation of inflammatory debris, contributing to inflammatory events[21, 62] and possibly erosion formation. While no studies are known to have tested these hypotheses of erosion formation, it has been demonstrated that mechanical removal of the epithelium in healthy corneas requires significant effort.[23] Any suitable contact lens-cell adhesion would thus need to be quite tenacious to be effective and it is unclear how such a bond might develop *in vivo*. Concurrent destabilisation of the epithelial adhesion complexes thus seems to be necessary so that the traction forces created by the contact lens motion would be sufficient to precipitate an erosion, suggesting that multiple factors may be contributing.

1.2.9.2 Contact lens thickness and water content

Very thin, high water content lenses have been associated with erosions and corneal staining.[11, 63] In work done in the 1980's, one approach to avoid overnight oedema was to use very thin (22-60 μm) high water content hydrogel contact lenses.[11, 64] These thin, high water content lenses caused lesions in the central and in the central inferior cornea which were described as erosions. As their location coincided with the thinnest part of the contact lens and where the tear film was deemed to be thinnest and least stable, the mechanism proposed was that contact lens dehydration exacerbated thinning of the post-contact lens tear film resulting in mechanical damage to the epithelial surface. The fact that lesions were less severe in situations of high humidity lent support to this theory.[11, 63] Contrarily, a recent study has found no association between corneal staining and either high water content contact lenses or contact lens dehydration.[65] Similarly, no association was found between corneal staining and silicone hydrogels, suggesting that these lens types are protective by virtue of the increased oxygen supply to the cornea.[65]

1.2.9.3 Bacterial load

The observation that a protease derived from the bacterium *P. aeruginosa* causes epithelial erosions when injected into rabbit corneas[66, 67] has led to the suggestion that an increased bacterial load may be an important factor in the pathology of the condition. Contact lenses isolate the epithelium from the defence mechanisms of blinking, tear flow and the antimicrobial agents in tears.[68, 69] This reduced flow and exchange of tears under the contact lens may contribute to an increased concentration of microorganisms and their toxins, hence augmenting their impact on the ocular surface.[70] *P. aeruginosa* is the most common cause of keratitis during contact lens wear[7, 36, 71] and has many factors which help it initiate and maintain infection.[71] One of these is an ability to produce proteases capable of degrading the epithelial adhesion complexes. Okamoto et al. showed that bacterial proteinases, such as *P. aeruginosa* elastase, *Vibrio cholerae* proteinase, and thermolysin, activate latent collagen degrading enzymes known as matrix metalloproteinases (MMPs) and hence contribute to matrix degradation.[72] Additionally, Tang et al. demonstrated that *P. aeruginosa* small protein (PASP) cleaves corneal collagen to cause erosion-like defects. Bacterial proteinases thus appear able to contribute to, or even initiate tissue damage and this interference with the integrity of the connective tissue exacerbates inflammation. The formation of erosions seems to be one manifestation of this activity.

1.2.9.4 Reduced epithelial density

Madigan and Holden have shown that the epithelium of hydrogel contact lens wearing eyes is easier to remove than non-contact lens wearing eyes.[73] This effect was associated with a reduction in the number of hemidesmosomes, which in turn was due to reduced epithelial density under the long-term hypoxic conditions prevailing during the study. Hypothetically, conditions such as these could contribute to erosion formation by increasing the chances of epithelial cleavage anterior to the basement membrane.[23]

1.2.9.5 Contact lens modulus

Modulus is described as the force per unit area required to compress the contact lens material by a given amount.[74] The high modulus of the first generation silicone

hydrogel contact lenses has been associated with mechanical complications such as SEALs and contact lens palpebral conjunctivitis, mucin balls and corneal moulding effects.[74] Efron and Morgan postulate that the benefit of increased oxygen transmissibility is offset by the increased modulus of the older generation silicone hydrogels.[75] They suggest that this is due to sub-clinical trauma induced by the eyelid-lens interaction.[75] This hypothesis is given further impetus by the findings of Stapleton et al. who found an incidence of 1.9% of presumed MK in conventional soft contact lenses during DW as compared to an incidence of 11.9% in DW with silicone hydrogel contact lenses,[5] as well as the higher rate of erosions found in silicone hydrogels compared to conventional hydrogels found by Willcox et al.[13]

1.2.10 Aetiology of non contact lens-related erosions

Corneal erosions are known to also occur in non-contact lens wearers and this is well reported in the literature.[18-20, 39, 76-79] A review of the aetiology of corneal erosions in the absence of contact lens wear may provide a background on which to base the hypotheses of the underlying mechanisms resulting in erosions in contact lens wear.

1.2.10.1 Corneal dystrophies

One study found that 59% of patients with recurrent corneal erosions have a concurrent form of superficial dystrophy.[20] These dystrophies result in a deficient epithelial basement membrane, the absence or abnormality of hemidesmosomes and loss of anchoring fibrils,[18] which, in turn, reduce the adhesion of the epithelium to the basement membrane. These corneal epithelial basement membrane dystrophies include map-dot-fingerprint dystrophy and Meesman's[80] as well as the stromal dystrophies of granular and lattice dystrophy.[20, 80, 81]

Epithelial basement membrane dystrophy, or map-dot-fingerprint dystrophy, is the most common dystrophy associated with erosions,[20, 39] and is usually bilateral and frequently asymmetric. Clinically, it is characterised by epithelial microcysts which give the 'dot' appearance, subepithelial ridges which give the 'fingerprint' appearance and map-like sub-epithelial patches which give the 'map' appearance[14] (Figure 1.7). When viewed histologically or *in vivo* with the confocal microscope, the basement membrane

is irregular,[15] with protrusions into the epithelium[82] attributed to a thickened and abnormal basement membrane.[14, 82, 83] Intraepithelial microcysts are also seen.[14, 15, 82]

Figure 1.7: Epithelial basement membrane dystrophy in a patient with an active erosion.

Map-dot-fingerprint dystrophy (LEFT) in the same patient with an active erosion (MIDDLE). Confocal microscopy revealed irregular basement membrane protruding into the epithelium (RIGHT).

1.2.10.2 Trauma and laser-assisted *in situ* keratomileusis (LASIK)

The most frequent trigger of recurrent corneal erosions is initial minor trauma to the corneal surface, for example, with a fingernail or paper cut.[20] Thermal and chemical injuries or herpetic keratitis are other causes.[77] Interestingly, deeper injuries such as foreign bodies embedding or stromal laceration do not result in recurrent corneal erosions.[81] The presumptive cause of trauma-related recurrent corneal erosions is due to the formation of defective adhesion complexes after the initial injury, and hence a failure of the epithelium to regain tight adhesion to the underlying stroma.[16] *In vivo* confocal microscopy has revealed activated keratocytes in the epithelium of those with trauma-induced recurrent corneal erosions, suggestive of a chronic inflammatory response.[84] Anterior stromal puncture, one of the surgical treatment methods of recurrent erosions is advocated to promote epithelial adhesion by creating a subepithelial scar[85, 86] and hence allowing for new connective tissue to be created. This provides a substrate to which the epithelium adheres.[86]

Corneal epithelial adhesion abnormalities following laser-assisted *in situ* keratomileusis (LASIK) have also been reported.[87-89] In LASIK, the close proximity between the head of the microkeratome and the corneal epithelial surface increases the risk of epithelial trauma.[88] Corneal epithelial adhesion abnormalities following LASIK were studied by Kenyon et al.[89] who found epithelial defects in 51 corneas of the 500 consecutive eyes

examined.[89] The authors surmise that the development of epithelial defects is dependent on the thickness of the corneal flap, with more erosions developing with the thicker flap.[89] Corneas with epithelial basement membrane dystrophy exhibited similar qualities to those that developed epithelial defects following LASIK.[88, 89] These authors also suggest that the bilaterality of the epithelial defects in some people could suggest that some people are predisposed to epithelial dysadhesion despite being asymptomatic and unidentifiable by slit-lamp biomicroscopy,[89] a hypothesis also put forward by others.[87]

1.2.10.3 Bullous keratopathy

Erosions are also known to occur as a result of bullous keratopathy. Bullous keratopathy occurs following intraocular surgery complications and following trauma and inflammatory and dystrophic diseases which cause functional failure to the endothelial cells.[90] Zhu and colleagues analysed the corneas of 13 patients suffering with bullous keratopathy following surgery and found that all diseased corneas showed epithelial abnormalities such as thickening and epithelial bullae.[90]

1.2.10.4 Age-related changes

The basement membrane is known to thicken with age either by continued membrane deposition, or by membrane duplication.[91] The increasing thickness of the membrane in some cases exceeds the anchoring fibril length, effectively blocking the linkage between the anchoring fibrils and Bowman's membrane,[91] presumably this being the mechanism underpinning the propensity to erosions in this age group.

1.2.10.5 Systemic conditions

1.2.10.5.1 Ocular rosacea

Corneal erosions have been associated with ocular rosacea,[91, 104] with up to 15% of patients with the condition thought to suffer from recurrent corneal erosions.[77] In this condition the skin, cheeks, nose and forehead are involved with persistent erythema, papules, pustules, hypertrophic sebaceous glands and telangiectasia.[92] Ocular rosacea also includes blepharitis, meibomitis, chalazia, styes, hyperaemia, peripheral corneal vascularisation, sub-epithelial infiltrates and gross ulcerations.[92] The ocular surface

disease in rosacea has been associated with the elevated pro-inflammatory cytokine interleukin-1α (IL-1α) and the MMPs.[93] Tetracycline has been shown to be effective in treating the condition[92] by virtue of its inhibitory effect on IL-1α and MMPs [94]

1.2.10.5.2 Diabetes

Diabetes has been linked to corneal erosion formation.[77, 95, 96] Similarly, decreased tear production, increased corneal thickness, reduced corneal sensation,[96] neurotrophic ulceration and persistent epithelial defects are also seen in diabetes.[52] In studies comparing diabetic corneas to those of non-diabetic controls, diabetic corneas were found to have a multilaminar, irregular and thickened basement[52, 96] with significantly less penetration of the anchoring fibrils from the deepest layer of the basal lamina to the stroma when compared to the controls.[52] This may explain the defective adhesion of the diabetic epithelial basal lamina to the underlying stroma, as the duplicated basal lamina could act as a barrier to the penetration of the anchoring fibrils.[52]

1.2.10.5.3 Alport syndrome

Alport syndrome is a genetic disease of basement membranes[55] and is characterised by progressive glomerulopathy as well as sensorineural hearing loss. It has been associated with anterior lenticonus and retinal flecks[97] as well as recurrent corneal erosions in 20% of people with the condition. [97]

1.2.10.5.4 Epidermolysis bullosa

Epidermolysis bullosa[98] is characterised by repeated muco-cutaneous blistering after minor trauma[55] and can also result in corneal erosions.[99, 100] Excess collagen degradation has been associated with this blistering phenomenon,[98] resulting in recurrent breakdown of the adhesions between the dermis and the epidermis of the skin, resulting in skin blisters. Its association with corneal erosions[99, 100] is presumably due to the collagen degradation of the adhesion complexes. The condition also affects other organs, including the oral, gastrointestinal and respiratory tracts.[55]

1.2.10.6 Increased expression of collagen degrading enzymes

An increased expression of collagen degrading enzymes known as MMPs has been associated with corneal erosions in non-contact lens wearers.[43, 78, 93, 101, 102] These

enzymes function to maintain and remodel the tissue architecture[101, 103] and are so-called because of their ability to degrade the structural proteins of the extracellular matrix.[104] The MMPs have been implicated in diverse physiological processes including embryonic development,[105, 106] tissue morphogenesis and wound repair, as well as pathological processes including inflammatory diseases, the autoimmune blistering disease epidermolysis bullosa,[104] rheumatoid arthritis[104,107] the pathophysiology of traumatic brain damage,[108, 109] and the progression of cancer[104] due to the breakdown of the structural barriers.[104]

In the cornea, the controlled presence of MMPs is important in maintaining homeostasis. Their uncontrolled production however, can have collagen degrading effects,[101] and theoretically, this might contribute to erosion formation.[78] MMP-9 is the primary matrix-degrading enzyme produced by basal corneal epithelial cells and neutrophils[110] and is known to be active against the major components of the epithelial basement membrane such as collagen type VII.[111] As the fibrils responsible for anchoring the basement membrane to the stroma are composed of type VII collagen, this activity may result in a weakening of these attachments, contributing to the cascade of events that cause corneal erosions.[78, 93 81] In agreement with this finding, reduced levels of collagen type VII in eyes with recurrent corneal erosions has also been described,[23] suggestive of the cleavage plane being at the level of the collagen type VII anchoring fibrils.[23] During erosion formation, MMP-9 has also been found to cleave the ectodomain of α6β4 integrin, one of the membrane components of hemidesmosomes.[43] It is of note that upregulated levels of MMP-9 have been associated with other epithelial defects such as those seen in corneal ulceration,[48,110] ocular rosacea,[112] thermal injuries,[113] dry eye[114] and pterygia.[115]

MMP-9 can be inhibited, and hence regulated, by binding to molecules such as tissue inhibitors of metalloproteinases-1 (TIMP-1) or non-specific inhibitors such as α_2-macroglobulin.[116] The ratio of MMP to TIMP is an important indicator of how well the potential for matrix degradation is regulated at any particular time and delayed wound healing or collagen degrading effects may ensue when the balance becomes disturbed.[117] Some treatment strategies for corneal erosions are thus directed at inhibiting MMP-9 so as to prevent the degradation of the basement membrane

components.[77, 112] Whether MMP-9 contributes to contact lens-related corneal erosions is yet to be established. Due to the significance of MMPs and their role in non contact lens-related corneal erosion formation, the following section reviews MMP's.

1.3 MATRIX METALLOPROTEINASES

Matrix metalloproteinases (MMPs), also called matrixins, were first described by Gross and Lapiere in 1962 during the rapid phase of collagen removal in the tail of metamorphosing tadpoles.[118] They are so-called because of their dependence on metal ions for catalytic activity and their ability to degrade the structural proteins of the extracellular matrix.[104]

MMPs are endopeptidases, that is, proteases which split the peptide bonds within the protein substrate.[119] Endopeptidases are classified according to their active site, and include the serine, cysteine, aspartic and metallo-proteases. Proteases are also known as proteinases, proteolytic enzymes or peptidases and collectively they are all hydrolases as they hydrolyse the peptide bond of proteins.[119]

Each MMP consists of:

- a signal peptide
- a pro-peptide domain
- a catalytic domain (active)
- a zinc-binding domain
- a C-terminal hemopexin-like domain[120] (except for MMP-7 and MMP-26[51])

All MMPs are secreted as inactive pro-enzymes or zymogens.[105] In order to cleave the extracellular matrix, they require activation. Another agent is required to cleave the pro-peptide conformation, hence contributing to the cascade that activates the enzyme. These zymogens lose peptides of approximately 10 kDa upon activation.[105] In this active form, the enzyme is now capable of degrading its substrate, as well as being open to binding by its inhibitors, the TIMPs, if sufficient TIMPs are present. The pro-enzyme is also capable of forming these complexes with the TIMPs.[121]

Table 1.1: Classification of MMPs.

MMP Classification	MMP names	Action
Collagenases	MMP-1, MMP-8 and MMP-13	Cleave interstitial collagens I, II and III.[123] [124] Upregulated by interleukin1-β (IL1-β) and tumour necrosis factor-α (TNF-α).[125] MMP-1 degrades types I, II, III and X collagens, type I gelatin, the antiprotease α1-antitripsin.[126]
Gelatinases	MMP-2 and MMP-9	Degrade gelatin, collagen types III, IV, V, VII, X and XI, elastin, laminin, fibronectin, and proteoglycan, hence contributing significantly to extracellular matrix turnover.[51] MMP-2: Expressed by stromal keratocytes and less so in the epithelium and is upregulated during wound healing.[48] MMP-9: is activated by cytokines and transforming growth factor-β (TGF-β)[124]
Stromelysins	MMP-3, MMP-10 and MMP-11	Digest collagen IV and fibronectin.[123, 127] Upregulated by IL1-β and TNF-α. MMP-3 and -10 are known to degrade proteoglycans, laminin, fibronectin and collagens and to activate MMP-1, - 2, -8, -13.[128]
Matrilysins Also called minimal-domain MMPs	MMP-7 and MMP-26	Digest fibronectin and gelatin; lack the C-terminal hemopexin-like domain.[123] These are the smallest of the MMPs having only the pro-peptide and catalytic domains.[129]
Membrane-type MMPs (MT-MMPs)	MMP-14, MMP-15, MMP-16, MMP-24, MMP-17 and MMP-25	Digest gelatin, fibronectin, laminin; can activate pro-MMP-2;[127] cell-surface bound and are involved in pericellular activities.[101]
Heterogeneous group	MMP-12, MMP-19, MMP-20, MMP-21, MMP-23, MMP-27 and MMP-28	Cleave substrates such as elastin and aggrecan.[123] MMP-12 is expressed in macrophages.[122]

1.3.1 Classification of MMPs

There are at least 23 MMPs in humans[122] and these can be grouped into sub-families depending on their substrate specificity: collagenases, gelatinases, stromelysins, matrilysins, membrane-types (MT-MMPs) and other MMPs (Table 1.1).[120]

1.3.2 MMP-9

In the cornea, the controlled presence of MMPs is important in maintaining homeostasis. Their uncontrolled production, however, can have collagen degrading effects.[101] MMP-2 and MMP-9 are the two main MMPs found in the cornea and in the tear film. MMP-2 is synthesised by corneal keratocytes[116, 130] and by the fibroblasts, macrophages, endothelial and epithelial cells.[131] MMP-9, also known as gelatinase B, is the largest member of the MMP family and is the primary matrix-degrading enzyme produced by basal corneal epithelial cells and neutrophils.[110, 132, 133] In order for neutrophils to reach the point of inflammation as part of their role in the first line of defence, they can release MMP-9,[51] presumably to allow their chemotaxis.[51] MMP-9 is known to degrade the major components of the epithelial basement membrane such as collagen type VII (Table 1.1)[111] and impede re-epithelialisation of the cornea.[112]

MMP-9 has seven domains:

- a signal peptide
- a pro-peptide domain (lost on activation)
- a domain of the active enzyme (containing the cysteine residue involved in activation)
- a putative zinc-binding domain
- an intervening fibronectin-like domain
- a serine-, threonine-, and proline-rich collagen type V domain[51]
- a C-terminal hemopexin-like domain which helps determine the substrate specificity.[105] This terminal is also important for TIMP binding.[51]

The active enzyme and zinc-binding domains form the ‘active site’ and hence are the responsible for the degradation.[51]

MMP-9 is produced as a mixture of monomers and homodimers.[51] Epithelial cells secrete the inactive pro-MMP-9 complexed with TIMP-1 while stimulated neutrophils cells secrete pro-MMP-9 complexed with neutrophil-gelatinase associated lipocalin (NGAL, section 1.3.3.5.1).[134, 135] In the open eye tear film, Sack and colleagues found that MMP-9 is of mixed origin, that is, of both basal epithelium and neutrophil origin. In the closed eye environment however, they found an exponential increase of pro-MMP-9 with the majority of this complexed with NGAL as opposed to TIMP-1, indicating an increased concentration of neutrophils.[135]

1.3.3 Regulation of MMP-9

The integrity of the extracellular matrix is maintained by a balance, or a dynamic equilibrium, between the synthesis and degradation of matrix components. An imbalance in this equilibrium may result in disease where greater levels of inhibitors result in fibrosis while greater levels of proteases result in destruction. Expression of most MMPs is usually low, however it is increased when extracellular matrix remodelling is required.[51] Complete inhibition of MMP-9, although attractive due to its collagen degradative effects, may result in corneal opacification due to ineffective remodelling of the extracellular matrix, as demonstrated in a mouse model.[136]

Regulation is tight and complex and occurs at several different levels:

1. Gene transcription
2. Cell storage of MMP-9[51] – regulates MMP-9 secretion
3. Activation of pro-MMP-9 after secretion
4. Inhibition by TIMP-1
5. Regulation by other mechanisms

1.3.3.1 Gene transcription

MMP regulation occurs primarily at the level of transcription.[101] MMP gene expression is regulated by numerous stimulatory and suppressive factors that influence multiple signalling pathways.[104] MMP-1 for example can be induced by its own substrate, collagen type I, and repressed once it has cleaved that substrate. MMP-9 produced by

neutrophils is transcripted only in the later stages of neutrophil development and is stored in granules.[51]

Cytokines appear to be among the natural regulators of MMPs. Girard et al. showed that IL-1 treatment can alter the expression of MMPs, inducing the expression of collagenase, stromelysin, MMP-2 and pro-MMP-9.[124] Transforming growth factor-β (TGF-β) is thought to stimulate deposition of the extracellular matrix through the secretion of matrix products.[124] Both these cytokines are important in wound healing. Other cytokines such as tumour necrosis factor-α (TNF-α), IL-18 and interferon-γ (IFN-γ) have also been found to stimulate MMP-9 production.[137]

1.3.3.2 Secretion

Once MMPs become translated, most of them are secreted. However, some MMPs such as MMP-8 and -9 are under secretory control.[104] These are synthesised by differentiating granulocytes in the bone marrow, stored in circulating neutrophils and released when the neutrophils are activated.[104]

1.3.3.3 Activation

MMPs are secreted in the inactive form. The cysteine residue in the pro-peptide domain forms a bond with the zinc in the catalytic domain, and in this way the MMP is inactive. For activation to occur, this bond must be disrupted,[123] referred to as the 'cysteine switch'. The bond is replaced by a water molecule, resulting in a catalytic zinc ion and hence an intermediate active enzyme.[123] The pro-domain of the MMP is further removed, reducing the molecular mass of the MMP and yielding an enzyme that is now completely active. MMP-9 is 92 kDa when inactive. The conversion from pro-enzyme form to active form results in a decrease in molecular weight due to the proteolytic cleavage from the N-terminal portion of the proteins.[124] The active MMP-9 containing the C-terminal hemopexin domain is 82 kDa, while the C-terminally truncated version of active MMP-9 is 65 kDa.[51]

In vitro, pro-MMP-9 can be activated by chemical and physical agents such as low pH, heat and the organomercurials such as 4-amino-phenylmercuric acetate, or APMA.[138] *In vivo*, MMPs are generally activated by other proteinases such as plasmin and

MMP-3.[114] The MT-MMPs complexed with TIMP-1, for example can also activate MMP-9.[51] Similarly, MMP-7, MMP-26, MMP-1, MMP-13, MMP-3, MMP-2, MMP-10 have all been reported in a review by Van den Steen et al. as being part of the activation network of MMP-9.[51]

Upregulated levels of cytokines and lymphocyte infiltration are associated with host response to inflammation. IL-1β is a proinflammatory cytokine associated with this response.[139] In addition to upregulating vascular adhesion molecules and activating phagocytosis, IL-1β is also involved in activating chemokines in order to recruit neutrophils into the cornea.[139] IL-1β has also been shown to induce MMP-9 expression.[139]

Extracellular matrix metalloproteinase inducer, or EMMPRIN/CD147, is a glycoprotein expressed on the corneal epithelial surface[140] and is known to modulate MMP expression during wound healing through cell-cell interactions.[140] CD147 is normally expressed in corneal epithelial and endothelial cells, and keratocytes, the retinal pigment epithelium, some retinal layers, as well as in tears,[141] aqueous[56] and vitreous humor.[142] Altered CD147 expression is suggested to be associated with retinal pigment epithelium dysfunction,[143] and it is also reported to be involved in the pathogenesis of corneal ulceration,[140, 144] keratoconus,[145, 146] and in glaucoma-related optic neuropathy.[147] CD147, has also been shown to increase MMP-9 and is being targeted for its possible therapeutic potential, as its inhibition would potentially inhibit the pathological excess of MMPs and hence excess degradation.[140]

1.3.3.4 Inhibition by tissue inhibitors of metalloproteinases (TIMPs)

After activation, MMP-9 is regulated by inhibition or degradation. TIMPs form 1:1 non-covalent complexes with MMPs, hence blocking access of substrates to the MMP catalytic site. Both the active and pro-enzyme form are able to bind to TIMP-1.[116, 121]

Four TIMP proteins have been found in humans, with TIMP-1 having the greater affinity for MMP-9.[51] TIMP-1 is an inducible protein while TIMP-2 is a constitutive protein. TIMP-3 is in the extracellular matrix and TIMP-4 is found in cardiac tissue. The TIMP-1 gene transcription is induced by pro-inflammatory cytokines such as IL-1, IL-6,

TNF-α. TIMP-1 binds to activated MMP-1, activated MMP-3 and both activated and latent MMP-9.[148]

For the pro-enzyme complex, the C-terminal domains of both molecules are involved.[121] Although the purpose of the pro-MMP-9:TIMP-1 complex is unknown, it has been suggested that this complex stabilises the zymogen.[121] Roderfield and colleagues[121] showed that pro-MMP-9 is already complexed to TIMP-1 intracellularly, in the golgi apparatus, suggesting that even after secretion as a complex, TIMP-1 may still be able to interfere with the activation of pro-MMP-9 by other MMPs by virtue of its free N-terminal.[121] The active MMP-9 complex occurs between the N-terminal of TIMP-1 and the active site of MMP-9.[51, 121] TIMP-2 also inhibits MMP-9 but to a much lesser extent than TIMP-1.[51] The MMP:TIMP complex can dissociate to yield enzyme and active TIMP-1.[105, 149]

MMP-3 acts as an activator of MMP-9 through its interaction with TIMP-1. When MMP-3 concentration is lower than that of TIMP-1, MMP-3 is inhibited and hence does not activate MMP-9.[51] When the concentration of MMP-3 exceeds that of TIMP-1, TIMP-1 is displaced from MMP-9 to MMP-3 and MMP-9 is completely activated.[51]

In addition to inhibiting MMPs, TIMPs also have growth factor and antiangiogenic properties, independent of MMP activity,[150] and TIMP-1 also has anti-apoptotic effects[151]

Therapeutic intervention for diseases associated with upregulated MMP levels is targeted at TIMP concentrations.[152] Paterson and colleagues compared the effect of recombinant TIMP-1 in reducing corneal ulceration and perforation after alkali injury in the rabbit model.[153] This was compared to a synthetic inhibitor of MMPs.[153] The recombinant TIMP-1 was found to exhibit better re-epithelialisation suggesting that the eyes treated with this develop more effective preservation of the extracellular matrix to support the epithelium.[153]

1.3.3.5 Regulation by other mechanisms

1.3.3.5.1 Neutrophil gelatinase-associated lipocalin (NGAL)

An additional protein, NGAL has been also found to complex with MMP-9,[135] although its role is to protect MMP-9 from degradation, rather than to inhibit it.

NGAL, also known as lipocalin 2, siderocalin, 24p3 and uterocalin, is a 25 kDa protein[134, 154] covalently bound to MMP-9 from human neutrophils.[51, 155] It exists as a monomer, a homodimer and a 135 kDa disulfide bond-linked heterodimer with MMP-9.[134, 156] NGAL belongs to the lipocalin family which bind and transport hydrophobic molecules such as prostaglandins, retinoids, arachidonic acid and hormones. Through their role as transport vehicles, lipocalins can influence a variety of cellular functions.[115] NGAL also has a bacteriostatic effect by depleting small iron-binding molecules known as siderophores synthesised by bacteria to acquire iron from their surroundings.[157, 158] An additional role of NGAL is to protect cells against oxidative stress.[159] Paradoxically, NGAL can also act as a biomarker of disease by virtue of its ability to protect MMP-9 from proteolytic degradation.[154] It is thought to be a potential biomarker of acute kidney injury following renal transplantation.[155, 158, 160] In systemic and urinary tract infection NGAL is increased, potentially due to an increase in neutrophil concentration.[155] Tumours expressing NGAL have increasing growth rates as well as increased levels of MMP-9.[156] This suggests that the increased expression of NGAL found in breast cancer may protect MMP-9 from degradation and therefore upregulate the dissolution of Bowman's layer as driven by MMP-9,[161] resulting in progression of the tumour.[156] NGAL has also been found to be elevated in the cardiac microvasculature in hypoxic conditions.[162]

It is not yet known what role NGAL plays in the tear film. In tears, lipocalins can reduce tear evaporation by increasing the solubility of lipids.[163] It is thought that NGAL is a potential stabiliser of MMP-9[51] and has been show to accompany an MMP-9 increase in uveitis.[164, 165] In eyes with pterygyia, NGAL has been shown to be 2.4-fold upregulated,[166] consistent with the elevated levels of MMPs in these eyes.[115] This co-localisation of MMPs and NGAL has been described as a factor increasing the

basement membrane degradation in pterygium formation. Pro-MMP-9 complexes with NGAL have been found in the open and closed eye tear film.[135]

1.3.3.5.2 Non-specific inhibitors

Natural proteinase inhibitors include α_2-macroglobulin. These irreversibly trap activated MMP so that the complex can be removed by the receptor.[104]

Synthetic inhibitors regulate MMP activity and include disodium ethylene-diaminetetra-acetic acid (EDTA) as a chelating agent of calcium and zinc, as MMP activity is dependent upon the presence of calcium.[105]

1.3.4 Physiological functions of MMP-9

MMPs play an important role in modulating apoptosis, angiogenesis, cell migration, wound healing, tissue remodelling and inflammation in all connective tissues.[135] By virtue of its role in remodelling the extracellular matrix, a temporal increase in MMP-9 has been associated with brain development,[167] as well as development of the liver, the bronchial epithelium and the thyroid gland.[51] Temporal expression of MMP-9 has also been found during embryo development,[168] post-partum uterine involution, cervical dilation and remodelling of the endometrial tissues during the menstrual cycle.[51]

1.3.5 Pathological functions of MMP-9

The imbalance of MMP-9 with its regulators may contribute to damage, whether it is due to excess or inadequate levels of MMP-9 and consequently, excess or inadequate extracellular matrix breakdown. When the regulatory mechanisms fail, inflammation, infection, degeneration and malignancy may result.

MMPs play a role in the invasion, metastasis, and angiogenesis processes during cancer formation.[104] Their ability to degrade the components of the extracellular matrix contributes to the capability of tumours to metastasise. For this to occur, the tumour needs to break away, breach the surrounding stroma and basement membrane and hence enter the circulation and spread to the next point.[105, 169]

MMP-9 in inflammation may act directly on the tissue or indirectly through the inflammatory cascade. Tissue stroma is maintained by a balance between extracellular matrix deposition and degradation.[110] In conditions such as rheumatoid arthritis and atherosclerosis, this balance appears to be lost, leading to excess degradation and eventual tissue destruction.[110] An imbalance in MMP:TIMP has also been associated with osteoarthritis.[170]

MMP-9 is upregulated in bullous pemphigoid, an autoimmune blistering disease[104] as well as with the formation and rupture of intracranial and aortic aneurysms.[104] Lehoux et al. have shown that MMP-9 seems to contribute to early hypertensive vascular remodelling[171] and is associated with vascular distensibility.

An upregulation of MMP-9 has also been associated with cerebral ischaemia.[172] Bauer and colleagues found that hypoxia increases MMP-9 activity in the cerebral microvasculature. This increase augments the hypoxia-induced vascular leakage, as was demonstrated by the reduced hyperpermeability following inhibition of MMP-9.[172] By virtue of its inhibitory effect on MMP-9, TIMP-1 has been found to be neuroprotective. [173]

In the eye, MMPs have been associated with the ageing macula, through their effect on Bruch's membrane.[174] Bruch's membrane is an extracellular matrix and is particularly responsible for the movement of nutrients and waste products between retinal photoreceptors and the choroidal blood supply.[174] MMPs are secreted by the retinal pigment epithelium and the choroidal endothelium[174] and they mediate the breakdown of the extracellular matrix. In the ageing macula, an increase in the thickness of Bruch's membrane along with deposition of abnormal extracellular matrix material have been implicated in the age-related macular degeneration disease process.[175] Active forms of MMP-2 and -9 have been found to be minimal in the ageing macula.[174] Increased levels of TIMP-3 suggest that this ratio impacts on the extracellular matrix turnover and hence results in the thickened Bruch's membrane seen in age-related macular degeneration.[174, 176] This is an area considered to be of future therapeutic potential.

1.3.6 MMP-9 and the ocular surface

MMP expression plays a role both in the long and short-term in corneal wound healing. In the short-term there is a peak in MMP-9 activity during the healing of epithelial wounds.[101] MMPs are essential for migration of cells through the extracellular matrix and the removal of collagen which was denatured during injury. This is important, because partially degraded collagen molecules will not bind very well, resulting in a weakened basement membrane. MMP-9 synthesis is en-sync with basement membrane degradation; there is a rapid increase in expression initially, this tapering over a period of a few weeks.[101] MMP-2, MMP-3 and MMP-1[117] on the other hand are mainly seen in the stroma and increase gradually over many months of matrix remodelling.[101] This process must be carefully controlled by TIMPs to prevent the MMPs from degrading the intact, functional matrix. Over-expression of MMP-9 leads to failure to re-epithelialise and chronic corneal ulcerations.[101] Improved basement membrane integrity has been found when MMP-9 was inhibited.[101] The excessive synthesis of MMP-9 therefore turns a normal situation of basement membrane remodelling into membrane dissolution.[48]

Fini et al. through their work suggest that gelatinases contribute to the epithelial defect which ultimately results in corneal ulceration,[110] from the initial epithelial defect formation, right through to the resolution of the ulcer and cell repair.[48] Stromal ulceration is initiated by defective wound healing.[48] In cases such as diabetic ulceration the reformation of strong epithelial-stromal adhesive structures may not occur due to the presence of duplications of the basement membrane,[48] resulting in recurrent epithelial breakdown.[48] The presence of inflammation is also a characteristic of corneal ulcers. The third process common in corneal ulcers is the dependence on specific enzymatic mechanisms with reactivity against corneal stromal collagens.[48]

MMP-9 has been implicated in diverse pathological functions in the ocular surface and some of these are presented in Table 1.2. MMP-9 has been detected in both tears[111, 177, 178] and corneal epithelial culture supernatant[112] and in tears has been a measure of the success of treatment.[179] There has been an observed correlation between tear film levels of MMP-9 and the progression of peripheral ulcerative keratitis in patients with

rheumatoid arthritis.[133] Elevated levels of latent MMP-9 have also been found in the tears of those with blepharitis, allergic eye disease, dry eye,[93, 114, 180] active vernal keratoconjunctivitis[111] and conjunctivochalasis.[181] Lema et al. analysed the tears of patients with keratoconus and found elevated levels of IL-6 and TNF-α. MMP-9 was similarly elevated.[178] These results contribute to the school of thought that degraded extracellular matrix could be a factor in the corneal ectasia seen in keratoconus.[178] MMPs have also been implicated in the progression of pterygia. The pterygium cells invading over Bowman's layer show an elevated level of MMP-1, -2, -9 and -13 which may be causing the dissolution of Bowman's layer.[115, 182] Fibroblast activation may also result from this, contributing to the cleavage of fibrillar collagen in Bowman's layer by the production of MMP-1.[182] A minor invasion of the pterygium head, as compared to more aggressive progression, has been correlated with higher levels of TIMP-1.[183]

A greater concentration of MMP-9 has been found in the tears of patients with the inflammatory skin condition, rosacea.[93] People with this condition are also known to be more likely to develop corneal vascularisation, epithelial basement membrane dystrophy and recurrent corneal epithelial erosions (Section 1.2.10.5.1).[93] These patients also show a higher concentration of the pro-inflammatory cytokine IL-1α which has been associated with activating MMPs such as the collagenases and gelatinases.[93, 139] This has been associated with the chain of events which result in rosacea-related ocular surface disease, such as corneal erosions.

As described in section 1.2.10.6, elevated levels of MMP-2 and -9 have been found in the tears of those with recurrent corneal erosions, and unlike normal corneas, both active MMP-2 and MMP-9 have been found.[78, 93, 102, 112] Doxycycline has been found to inhibit MMP-9 activity and has been found to be effective in preventing erosion recurrences.[77, 184] Together these findings support the rationale that these MMPs may contribute to the breakdown of the corneal adhesion complexes which ultimately culminates in corneal erosion.

Table 1.2: Studies relating to MMP-9 in the anterior eye.

Reference	Tissue examined	Condition	Outcomes
Fini and Girard, 1990[110]	Corneal cell culture (rabbit)	Normal cornea	▪ Epithelial cells predominantly produce pro-MMP-9 ▪ Stromal fibroblasts synthesise pro-MMP-2
Di Girolamo et al., 1996[185]	Aqueous humour (human and rabbit)	Uveitis	▪ Increased pro-MMP-9 in uveitis aqueous humour ▪ No change in TIMP-1 levels compared to controls ▪ MMP-3 identified in uveitis aqueous humour
Di Girolamo et al., 1997[125]	Necrotising scleritis tissue (human)	Scleritis	▪ Localised MMP-3, MMP-9 and TIMP-1 in necrotising scleral tissue ▪ MMP-3 and -9 and TIMP-1 are regulated by interleukin (IL)-1α and tumour necrosis factor (TNF)-α
Barro et al., 1998[186]	Tears (human)	Corneal transplant failure	▪ Elevated active and pro-MMP-9 compared to successful grafts ▪ MMP-9:NGAL complex found in tears of normals
Afonso et al., 1999[93]	Tears (human)	Ocular rosacea	▪ Only pro-MMP-9 detected in normals ▪ Both active and pro-MMP-9 detected in ocular rosacea ▪ MMP-9 activity correlated with IL-1α concentration
Kernacki et al., 1999[187]	Cornea (mouse)	*Pseudomonas Aeruginosa*-induced corneal ulceration	▪ After infection, TIMP-1 expression protects against further degradation
Garrana et al., 1999[78]	Corneal debridement samples (human)	Recurrent corneal erosions	▪ Elevated MMP-2 found in human epithelia affected with recurrent corneal erosions
Sobrin et al., 2000[112]	Tears and cultured corneal epithelial cells (human)	Ocular rosacea	▪ Increased MMP-9 compared to normals ▪ Increased TIMP-1 compared to normals ▪ No difference in the MMP-9:TIMP-1 ratio between ocular rosacea and normals ▪ MMP-3 found in ocular rosacea only; activated pro-MMP-9 ▪ Doxycycline reduced the activity of MMP-9
Smith et al., 2001[133]	Tears, cultured corneal and conjunctival epithelial cells (human)	Peripheral ulcerative keratitis	▪ Pro-MMP-9 and a species of MMP-9 at 116 kDa were found ▪ Lower TIMP-1 levels were found compared to controls

Reference	Tissue examined	Condition	Outcomes
Dursun et al., 2001[77]	N/A (human)	Recurrent corneal erosions	▪ Doxycycline and corticosteroids were effective in providing resolution and preventing recurrences of corneal erosions
Kumagai et al., 2002[188]	Tears (human)	Vernal keratoconjunctivitis	▪ Both pro-MMP-9 and active MMP-9 were found in the tears of patients with vernal keratoconjunctivitis compared to only the latent form in the tears of normals
Sakimoto et al., 2004[189]	Tears, cultured corneal and conjunctival epithelial cells (human)	Ocular surface disease	▪ Both the latent and active forms of MMP-9 were present in the tears of those with ocular surface disease such as Mooren's ulcer, corneal perforation and thermal burns ▪ Only the latent form was present in the tears of normals
Lema and Durán, 2005[178]	Tears (human)	Keratoconus	▪ Elevated levels of interleukin-6, TNF-α and MMP-9 in tears of patients with keratoconus
Pflugfelder et al., 2005[190]	Cultured human corneal epithelial cells and mouse model	Experimentally induced dry eye	▪ Increased MMP-9 found in dry eye ▪ Administration of active MMP-9 increases epithelial permeability to fluorescein ▪ This increase is due to the detachment of the apical epithelial cells induced by the administration of MMP-9 in dry eye ▪ MMP-9 disrupts the tight junctions of epithelial cells, promoting cell detachment
John-Aryankalayil et al., 2006[166]	Human corneal and limbal tissues	Pterygium	▪ A 2.4-fold increase in NGAL was found compared to normals
Rohini et al., 2007[191]	Cornea, tear and serum (human)	Fungal keratitis	▪ MMP-9 and MMP-8 were significantly elevated compared to normals ▪ Insufficient corresponding increase in TIMP-1
Holopainen et al., 2009[192]	Tears (human)	Climatic droplet keratopathy	▪ Elevated levels of pro-MMP-2 and -9 were found compared to controls ▪ No active MMP-2 or -9 was found in either group
Chotikavanich et al., 2009[114]	Tears (human)	Dry eye	▪ MMP-9 concentration of 8.39 ± 4.70 ng/mL in the normals compared to 381.24 ± 142.83 ng/mL in the dry eye group
Pal-Ghosh et al., 2011[43]	Rat corneal epithelium	Recurrent corneal erosions	▪ MMP-9 was found to cleave the ectodomain of α6β4 integrin, one of the membrane components of hemidesmosomes.

1.4 LITERATURE ON CONTACT LENS-RELATED CORNEAL EROSIONS AND MMP-9

To date, there have been no papers published on the role of MMP-9 in contact lens-related corneal erosions. However, there have been four papers published and one conference paper presented on MMP-9 and contact lenses.

Lema and colleagues compared the levels of IL-6, TNF-α, the cell adhesion molecules intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1) and MMP-9 in four groups: 20 patients with keratoconus wearing rigid gas permeable contact lenses (RGPs), 28 keratoconus patients without RGPs, 20 myopic RGP wearers and 20 myopic subjects without contact lenses.[193] They found a significant increase in IL-6, TNFα, ICAM-1, VCAM-1 in keratoconus patients wearing contact lenses compared to no contact lens wear, and a non-significant difference in MMP-9 (66.5 and 93.4 ng/mL respectively).[193] In normal patients wearing RGPs, the levels of MMP-9 were significantly increased compared to controls (12.9 *vs.* 6.1 ng/mL) as were TNF-α levels.[193] In another study, Pannebaker et al. found significantly greater levels of MMP-1 in the tears of people with keratoconus, both with and without RGP wear, when compared to the group of normal RGP wearers (34.4 ± 84.4, 3,483.4 ± 3,881.0 and no detection, respectively.[194]

Zhang and colleagues measured the levels of MMP-9 in the corneas of rats after wearing high and low oxygen permeability contact lenses for two weeks. No differences were found.[195]

Hadassah and colleagues evaluated the effectiveness of collagen bandage contact lenses on corneal healing in the case of corneal erosions, ulcers, dry eyes and corneal lesions.[196] They found significant levels of MMP-2 and -9 initially which reduced significantly after seven days of contact lens wear, a factor which they attribute to corneal healing in the presence of a collagen bandage contact lens.

Papas et al. conducted a pilot study on five people wearing orthokeratology contact lenses.[197] They found an increased amount and activity of MMP-9 in the eye wearing the contact lens compared to the non-contact lens wearing eye. Moreover, both the

amount and activity of MMP-9 increased with length of contact lens wear. The authors suggest that this increase may contribute to the epithelial cell migration that occurs in orthokeratology.

To date, no papers have been published on the impact soft contact lens wear has on MMP-9 expression. Similarly, it is not known whether different wear modalities and lens types influence this expression and whether MMP-9 is a factor in contact-lens related corneal erosions.

1.5 RATIONALE FOR RESEARCH

The aetiology of contact lens-related corneal erosions is not well understood. The association of erosions in non-contact lens wearers with MMP-9 suggests that MMP-9 may also be a factor in contact lens wear. It is not currently known how soft contact lens wear affects the levels of MMP-9 in the ocular tear film or indeed whether these levels are related to the corneal erosions which sometimes ensue.

1.6 THESIS AIMS

This thesis aims to explore the effect of contact lens wear on the diurnal profile of MMP-9 and its associated proteins in the human tear film and relate this to corneal erosions.

It is feasible that contact lens wear affects other proteins which may contribute to erosion formation. Hence, a further aim of this thesis is to identify changes in the tear proteome associated with contact lens wear and how these changes compare with those how develop erosions.

The results of these studies will provide a greater understanding of the tear protein changes associated with contact lens wear and the mechanisms by which adverse events such as erosions occur. Furthermore, this understanding may help underpin future research into preventative mechanisms and means by which to predict those at risk of adverse events such as corneal erosions.

1.7 THESIS HYPOTHESIS

Increased levels of MMP-9 have been associated with the occurrence of corneal erosions in non-contact lens wearers.[43, 93, 101, 102] Additionally, contact lenses are known to alter the levels of a number of proteins in the tear film.[198-201] Based on this information, the hypothesis to be tested is that contact lens wear is associated with increased expression of MMP-9 which predisposes the cornea to erosion as a result of increased degradation of the extracellular matrix and the adhesion complexes.

1.8 THESIS OVERVIEW

This thesis describes the outcomes of these investigations as follows:

Chapter 2 describes the validation of a flush tear collection technique. As the subtle changes leading to corneal erosion development in the otherwise healthy eye are not identifiable with slit-lamp biomicroscopy, the more sensitive approach of tear analysis to detect changes in proteins such as MMP-9 was used in this thesis. A simple method for collection of representative tear samples was therefore validated.

Chapter 3 uses this flush tear collection method to establish the diurnal variation of MMP-9 and its associated factors, TIMP-1 and NGAL, in the tears of healthy normals.

Chapter 4 describes the effect of contact lens wear in the DW and EW schedules on the diurnal levels of MMP-9, TIMP-1 and NGAL.

Chapter 5 evaluates the impact of contact lens wear on the tear proteome as a whole through the use of a broad spectrum technique known as differential gel electrophoresis (DIGE) in order to establish other changes associated with contact lens wear which may contribute to erosion formation.

Chapter 6 applies the knowledge obtained from healthy normals during their adaptation to contact lens wear as established in Chapter 4 and Chapter 5 and compares this to those with contact lens-related corneal erosions.

Chapter 7 summarises and discusses the findings and limitations of this work and recommends future studies to extend the findings.

CHAPTER 2 THE VALIDATION OF THE FLUSH TEAR COLLECTION TECHNIQUE

2.1 OVERVIEW

Chapter 1, Section 1.6 introduced the aims of this thesis to explore the biochemical changes in the tear film during contact lens wear which may contribute to the development of corneal erosions. In order to effectively measure these changes, it is necessary to ensure that the technique used to collect tears and their constituent proteins is appropriate. One limitation of many tear collection techniques is the inadequate volume that typically results. One way around this is to use a technique known as 'flush' collection. In the following sections a standardised version of this method is described and validated for both basal and reflex collection. This method is used in the subsequent chapters of this thesis.

The work presented in this chapter has been published in *Current Eye Research*[202] and is presented here with permission from the journal (Confirmation Number: 10287500). This work has also been presented as a poster at the *International Society for Eye Research* in Montreal, July 2010 (Appendix B: Publications and presentations).

2.2 INTRODUCTION

As the pre-ocular tear film is critical to the integrity of the ocular surface, assessment of its biochemical characteristics presents a valuable means of studying this region of the eye in both health and disease. Examples include the use of tear fluid analysis to determine changes contributing to altered corneal integrity in keratoconus[203] and diabetes,[204] establish differences between the open and, closed eye environments[205] and assess the effect of contact lens wear on the ocular surface.[201, 206-209] For this reason, tear fluid analysis forms the basis of the studies presented in this thesis.

Tear samples can be obtained from human participants in several ways, but drawbacks are associated with each approach. For example, tears can be absorbed into Schirmer strips resting on the lower eyelid and subsequently eluted for biochemical analysis.[102, 186, 210, 211] Although simple to perform, this procedure tends to collect cellular, as well as secreted, proteins and the physical presence of the strip can cause mechanical

stimulation of the corneal and conjunctival epithelium. As a result, the composition of the collected sample may differ from that in pure basal secretion.[212-214]

A common alternative is to use microcapillary tubes to draw tears from the reservoir within the conjunctival sac, usually at the fornices. While generally less invasive than Schirmer strips, attempts to collect basal tears with this method can be tedious at best; particularly where there is a paucity of flow, as often occurs in dry eye or when the eyes are opened after awakening. It is also challenging to avoid inadvertent contact with the lids, and/or ocular surface and thus induce reflex tearing.

An option is to simply provoke or utilise reflex tearing as a means of providing increased tear volume at a faster rate. This is itself an inherently variable endeavor that is difficult to perform with consistency. In addition to volume change, the procedure of inducing reflex tears also yields a change in the protein spread, with the main regulated proteins of the lacrimal gland, such as lysozyme, lactoferrin and tear lipocalin, remaining constant, while others such as immunoglobulin A and G, albumin and fibronectin change dramatically.[215, 216]

A number of workers[184, 217-224] have attempted to make the collection process less demanding by sampling tears after first flooding the eye with saline. The idea is that the increased fluid volume will be much easier, and quicker, to coax into a microcapillary tube but will still contain the same moieties as normal tears, albeit at a reduced concentration.

This assumption has only been partially verified. One-dimensional gel electrophoresis has shown that similar major proteins appear in samples collected by the 'flush' method as are in basal tears,[218] but total protein measures appear to be more variable when compared to reflex tears.[15, 221] Part of the reason for this may be that the technique used in these studies involved multiple instillations of saline. In addition to sample dilution, this might well have triggered reflex tearing and therefore contributed to the variability.

It is also noteworthy that the technique itself is not applied in any standard fashion. Among the studies conducted to date, both the number and volume of saline

instillations have varied among the various groups reporting on its use.[184, 217-222, 224] These technical issues cast doubt on the validity of the flush method as a means of providing tear samples that are truly representative of the fluid existing within the conjunctival sac.[225] Additionally, the behaviour of the less abundant protein species in flush tear collection is not known. With this in mind a standard method of flush tear collection is proposed based on the single application of a controlled volume of saline.

2.3 AIMS

The aims of this chapter are to describe a standardised flush technique and the efforts to validate it. This was achieved by establishing test-retest repeatability, and comparing the amount and variety of proteins contained in flush samples with those in basal and reflex secretions obtained conventionally. Both major and less abundant protein species were evaluated to give as broad a picture as possible of the differences between techniques.

2.4 HYPOTHESIS

Flush tear collection can collect a greater volume of tears compared to basal and reflex tear collection without affecting the variety and proportion of proteins collected.

2.5 METHODS

2.5.1 Ethics approval

This study was approved by the institutional ethics committee of the University of New South Wales (UNSW, approval number 084055 and 084056). Informed consent was obtained from 16 non-contact lens wearers (11 female, 5 male) according to the tenets of the Declaration of Helsinki 1975 as amended in 2000 (Appendix A: Participant information statement and consent forms). All participants were aged between 18 and 46 years of age (30 ± 7 years). Participants were healthy and were not taking medications for any general health conditions. A requirement of the study was that the participants were either non-contact lens wearers or non-habitual lens wearers who had not worn contact lenses in the month preceding the study. Participants with pre-existing ocular irritation or with active corneal infection or any active ocular

disease were excluded from the study. White light examination was conducted prior to tear collection. Exclusion criteria also included any systemic disease that may affect ocular health, any systemic or topical medications up to 12 weeks prior to and during the trial that could affect ocular physiology, as well as previous corneal refractive surgery. Pregnant or lactating women were excluded from the study.

2.5.2 Study design

Tears were collected six times from each participant by the same investigator, MM, experienced in the technique. Each of the basal, reflex and flush methods described below was used on two occasions in random order allowing at least 24 hours between collections. At each visit, tears were obtained from both eyes and were not pooled, the right eye always being collected first. A new microcapillary was used for each eye. Collection times ranged between 10:45 AM and 16:35 PM, however, the majority were between 12:00 PM and 2:00 PM.

2.5.3 Tear collection method

2.5.3.1 Basal tear collection

Basal tears were collected using a 10 μL microcapillary tube (Blaubrand® intraMark, Wertheim, Germany). Collection time was limited to a maximum of two minutes which was deemed long enough to enable adequate collection, but short enough to avoid stimulation of reflex tears.[226] Participants tilted their head towards the side of collection while looking in the opposite direction. The microcapillary tube rested in the lateral tear meniscus. Care was taken to minimise the touch to the bulbar conjunctiva or the lid margin to avoid the risk of stimulation. Participants were allowed to blink during the procedure, after the microcapillary tube was withdrawn. Collection was paused if any reflex tearing was suspected. A fresh microcapillary tube was used for each eye. Tear collection rate was monitored for all samples by noting the time and volume collected. The sample was expelled from the microcapillary tube using a pump into a siliconised polypropylene microcentrifuge tube of 0.65 mL capacity, (Sigma-Aldrich, Steinheim, Germany) and placed on ice until processing within the hour.

2.5.3.2 Reflex tear collection

Reflex tears were collected as for basal tears except that the sneeze reflex was stimulated by gently inserting a sterile cotton bud into the nasal passages to induce tear production.[227] Collection took place for approximately one minute to enable sufficient volume to be obtained.

2.5.3.3 Flush tear collection

Flush tears[202] were collected by instilling a single, 60 µL drop of non-preserved, unit dose sterile saline (sodium chloride injection 0.9%, AstraZeneca, North Ryde, Australia) into the inferior palpebral fold using an Eppendorf pipette (Figure 2.1). Care was taken not to touch the eye with the pipette tip. Participants were then instructed to gently close their eyes and rotate them twice. Fluid was then immediately collected by microcapillary as before, with a limit of one minute being imposed to avoid reflex tearing.[226]

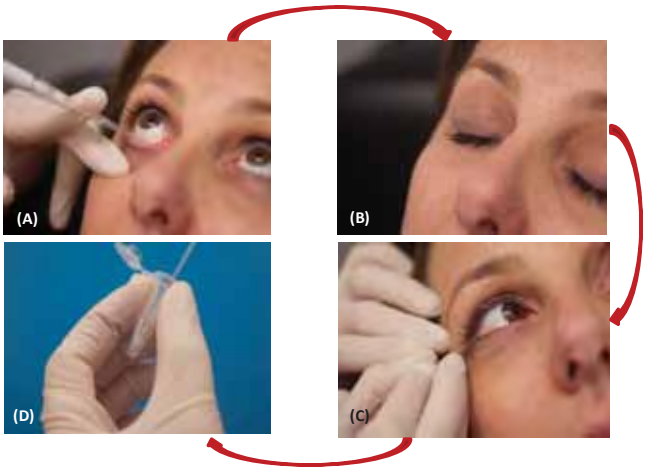


Figure 2.1: Example of the procedure for flush tear collection.

(A) A 60 µL drop of sterile saline is inserted into the eye
(B) The participant closes and rolls eyes around and then opens the eyes
(C) The tears are collected using a microcapillary tube
(D) The tears are expelled into a tube for analysis

2.5.4 Treatment of tear samples

After collection, samples were centrifuged at 1,145 g force for 20 minutes at 4 °C to remove cellular debris. The supernatants were collected and stored in two aliquots in

siliconised polypropylene microcentrifuge tubes (Sigma-Aldrich, Steinheim, Germany) at -80 °C. One aliquot was used for total protein content (TPC) and the other for immunoglobulin A (IgA) and any further analysis. The samples were analysed within one month of tear collection. No protein extraction solvent was applied prior to running the samples.

2.5.5 Total protein content

Total protein content (TPC) was determined using the bicinchoninic acid method (BCA) and using Pierce reagents and flat-bottom Nunc-F Maxisorp 96-well microplates (Thermo Fisher Scientific, Rochester, NY, USA). Serial dilutions of bovine serum albumin (BSA, Sigma Life Science, Sigma Aldrich Inc, Canada) were used as standard. This was loaded in triplicate starting at 2 mg/mL down to 0.1 mg/mL in MilliQ water and 10 μL was added to each well. Tear samples were loaded at a 1:10 dilution where volume allowed. No samples were pooled. Tears were analysed in duplicate and a 10 μL volume was added to each well. Solution A (BCA Protein Assay Reagent, Pierce, Thermo Fisher Scientific, Rochester, NY, USA) and Solution B (BCA Protein Assay Reagent B; Pierce, Thermo Fisher Scientific, Rochester, NY, USA) were combined in respective volumes of 20 mL and 0.4 mL. This mixture was then added at a volume of 200 μL per well. The optical density was read at 595 nm on the Spectrafluor Plus, Tecan Multifunction Microplate reader using the X-Fluor 4 software (NY, USA) after 30 minutes and a standard curve generated using the BSA as a reference (Figure 2.2).

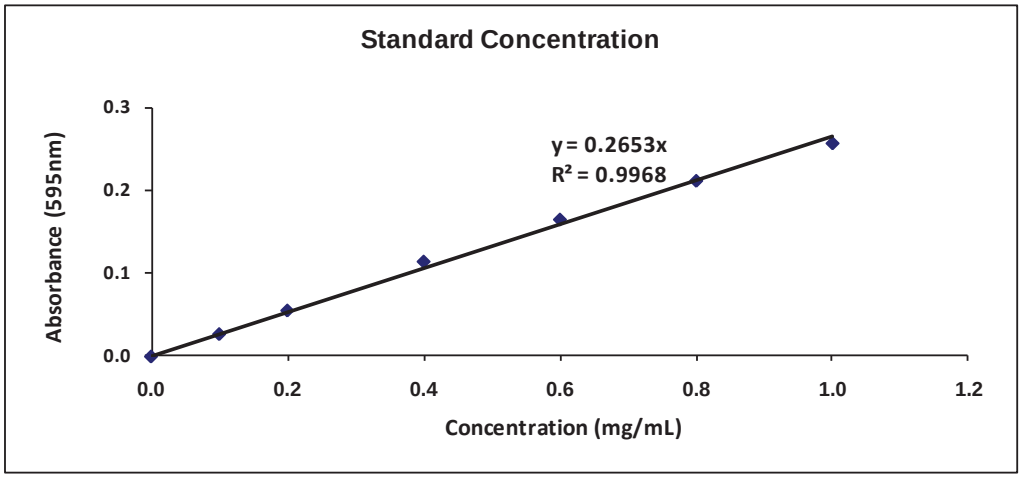


Figure 2.2: Example of a linear curve obtained during total protein content analysis using the X-Fluor 4 software (NY, USA).

2.5.6 Immunoglobulin A analysis

Immunoglobulin A (IgA) concentration was determined using sandwich enzyme-linked immunosorbent assay (ELISA) with the Bethyl Human IgA Quantitation kit (Montgomery, Texas) following the manufacturer's instructions. Wells of Nunc-F Maxisorp 96-well microplates (Thermo Fisher Scientific, Rochester, NY, USA) were coated with 100 μL of the primary antibody. The antibody was allowed to bind overnight at room temperature for tear samples of the first eight participants and for three hours for the tear samples of the second group of eight participants. Tear samples were not pooled. The plates were washed three times with wash buffer (50 mM Tris, 0.14 M sodium chloride (NaCl), 0.05% Tween20 (Sigma-Aldrich, Steinheim, Germany), pH 8.0). The wells were then blocked with 200 μL blocking solution containing 50 mM Tris, 0.14 M NaCl, 1% BSA (pH 8.0) for 30 minutes. The blocking solution was then removed and the wells washed three times with the wash buffer. A volume of 100 μL of tears diluted in sample diluent (50 mM Tris, 0.14 M NaCl, 1% BSA, 0.05% Tween 20, pH 8.0) were loaded at 1:3,000 dilutions in duplicate. These were allowed to incubate for one hour and the wells were then washed five times with the wash buffer. A volume of 100 μL of horseradish peroxidase (HRP) detection antibody was added to each well in a dilution of 1:20,000. This was incubated for one hour after which it was removed and the wells were aspirated with wash buffer. Tetramethylbenzidine (TMB) peroxidase substrate was added to each well in a volume of 100 μL and stopped with 2 M sulfuric acid (H_2SO_4) after 30 minutes incubation. The optical density was then measured using the Spectrafluor Plus, Tecan Multifunction Microplate reader using the X-Fluor 4 software (NY, USA) at 450 nm. Each plate included seven dilutions of the standard in duplicate from which the standard curve was generated. The mean absorbance of the blank wells was subtracted from the mean of each sample and each standard in order to determine the final absorbance.

2.5.7 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Tear sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using the Laemmli system[228] followed by scanning densitometry was performed as a means of qualitative analysis of the major tear proteins of the basal and flush tears of five

non-contact lens wearing participants. The basal and flush tears of four healthy contact lens wearers were also analysed as described in Section 2.5.2. These four participants met the study selection criteria (Section 2.5.2) but were contact lens wearers of more than one month duration. No restriction was placed on contact lens type or wear schedule.

2.5.7.1 Buffer System

SDS-PAGE analysis was performed on the Bio-Rad Mini-Protean® Tetra Cell (Bio-Rad, Hercules, CA, USA) in a Laemmli discontinuous buffer system[228] which incorporates buffers of different pH and composition to generate a voltage gradient. Tear proteins were separated using a homogenous 12% gel (40% acrylamide/bis, 1.5 M Tris-HCl pH 8.8, 10% SDS, tetramethylethylenediamine (TEMED), 10% ammonium persulfate) and a 4% stacking gel (40% acrylamide/bis, 0.5 M Tris-HCl 6.8, 10% SDS, TEMED, 10% ammonium persulfate) cast in-laboratory. The larger pore size and pH of the stacking gel concentrate the proteins before they enter the resolving gel while the smaller pore size of the resolving gel separates the proteins according to their molecular size, acting like a sieve. Upon loading, the tear samples were run at 200 V for approximately one hour in a running buffer (Tris base, glycine, 10% SDS, pH 8.3).

2.5.7.2 Standard and sample preparation

Individual tear samples were mixed with SDS-PAGE loading buffer (0.5 M Tris-HCl, pH 6.8, 10% SDS, 0.5% bromophenol blue, glycerol, β–mercaptoethanol) and denatured by heating at 100 °C for five minutes. The presence of SDS denatures proteins and gives them a net negative charge and a uniform charge-to-mass ratio, while β-mercaptoethanol is used as a reducing agent for disulfide bonds to eliminate protein secondary structure so that the proteins can be separated according to their mass/size. A fixed mass of 10 μg was added to each well as calculated from the TPC. A Precision Plus Protein™ Standard (Bio-Rad, Hercules, CA, USA) was run in parallel to the samples as a marker.

2.5.7.3 Image analysis

Gels were fixed and then stained with Coomassie Blue R250 and were imaged using the Bio-Rad GS 800 scanner (Bio-Rad, Hercules, CA, USA). Lane profiles were analysed using Quantity One software (Bio-Rad, Hercules, CA, USA). One gel was performed for each of five participants.

2.5.8 Mass spectrometry

2.5.8.1 Liquid chromatography mass spectrometry

Liquid chromatography mass spectrometry (MS) was conducted at the Bioanalytical Mass Spectrometry Facility at UNSW on three replicates of each sample type, all from the same participant, in order to gauge whether the same spectrum of proteins was identifiable across tear collection methods. Tear samples were not pooled. Samples were reduced with 10 mM dithiolthreitol (DTT) in 50 mM ammonium bicarbonate (NH_4HCO_3) for 30 minutes at 37 °C, followed by alkylation with 25 mM iodoacetamide in 50 mM ammonium NH_4HCO_3 for 30 minutes at 37 °C. Finally, the samples were digested with trypsin at 2 ng/mL in 20 mM NH_4HCO_3 overnight, for approximately 14 hours at 37 °C. The following morning, 1% formic acid was added to each of the digested samples for 10 minutes, followed by the addition of acetonitrile for 10 minutes. The supernatant was transferred to a new tube and evaporated to dryness. The dried samples were re-suspended in 0.05% heptafluorobutyric acid and 1% formic acid in water and injected on a reverse phase C18 column coupled with an LTQ-FT Ultra mass spectrometer (Thermo Fisher Scientific, Rochester, NY, USA).

2.5.8.2 Mascot analysis

All MS/MS samples were analysed using Mascot software (Matrix Science, London, UK).[229] Mascot was set up to search the National Centre for Biotechnology Information database (unknown version, 7686184 entries) using the digestion enzyme trypsin. Mascot was searched with a fragment ion (MS/MS) mass tolerance of 0.40 Da and a parent ion (MS) tolerance of 4.0 ppm. Oxidation of methionine, iodoacetamide derivative of cysteine, acrylamide of unknown and acrylamide adduct of cysteine were specified in Mascot as variable modifications. Scaffold (version Scaffold 2, Proteome

Software Inc., Portland, Oregon, USA) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 80% probability as specified by the Peptide Prophet algorithm.[230] Protein identifications were accepted if they could be established at greater than 80% probability and contained at least one identified peptide. Protein probabilities were assigned by the Protein Prophet algorithm.[231] Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

2.5.9 Statistical analysis

Normality was tested using the Shapiro-Wilk test due to sample size less than 50. Repeated measures analysis of variance (ANOVA), with Bonferroni corrected Student's t-tests post-hoc, was used to compare the three collection methods. Sphericity was verified with Mauchly's test. A 95% confidence level ($p < 0.05$) was applied. SPSS 16.0 GP (Chicago, USA) was used for the analysis. Results are reported as mean ± standard deviation (SD).

2.5.9.1 The use of expectation maximisation for missing variables

Missing data due to inadequate volume were replaced in PASW (PASW statistics, version 18.0, Chicago, USA) using an expectation maximisation (EM) procedure, substituting zeros when negative values were generated.

2.5.9.2 Bland-Altman analysis

Agreement between assay duplicates, eyes and days were evaluated using Bland-Altman analysis.[232] The differences between the two variables were plotted against their mean and limits of agreement (mean ± two SDs) and coefficients of repeatability (1.96 x the SD of mean difference between replicates)[233] were calculated.

2.6 RESULTS

2.6.1 Normality

The Shapiro-Wilk test indicated that when normality was tested separately for eyes and days collected, each method had one out of the four sampling groups not normally distributed for TPC (p = 0.005, 0.001 and 0.03 for basal, reflex and flush respectively). When eyes and days were averaged for each method, all data were normally distributed ($p > 0.05$).

For IgA concentration, only one occasion for the basal tears was not normally distributed (p = 0.04).

2.6.2 Tear collection

There were no significant differences between right and left eyes (F = 0.84, p = 0.38), collection days (F = 0.90, p = 0.36) or participant gender (F = 0.84, p = 0.45) in terms of collection rate for any of the methods. Accordingly, values for each participant across the two days and for each eye were averaged. Table 2.1 shows the mean volume, and collection rates for each method (n = 16). Both volume and collection rate were highest for the flush tears. All the collection rates were significantly different between methods (F = 66.56, $p < 0.05$).

Table 2.1: Group mean ± SD of collected volumes and collection rates.

Standard collection times were two minutes for basal and one minute for reflex and flush tears (n = 16). All the collection rates were significantly different between methods (p < 0.05). Volume was significantly greater for flush tears (p < 0.05).

Collection Method	Volume (μL, mean ± SD)	Collection rate (μL/minute, mean ± SD)
Basal	8.6 ± 11.8	4.6 ± 6.7
Reflex	14.6 ± 11.6	13.9 ± 11.1
Flush	27.2 ± 11.7	25. 7 ± 12.4

2.6.3 Repeatability

2.6.3.1 Assay variability

For each assay, samples were tested in duplicate in order to check for the repeatability of the technique for each sample type. The Bland-Altman limits of agreement and coefficients of repeatability for each sample type are shown in Table 2.2 for the TPC assays and in Table 2.3 for the IgA assays. The greatest assay variability was 1.22 mg/mL for the TPC assay (basal tears) and 0.08 mg/mL for the IgA assay (basal tears). Values greater than this were considered to be true differences between samples rather than a result of experimental variability.

Table 2.2: Limits of agreement and coefficients of repeatability for the total protein content (TPC) assay duplicates.

Tear Type	Mean of differences	SD of differences	Limits of agreement	Coefficient of repeatability
Basal	-0.33	0.61	-1.53 to 0.87	1.22
Flush	-0.16	0.22	-0.59 to 0.27	0.44
Reflex	-0.39	0.47	-1.3 to 0.53	0.94

Table 2.3: Limits of agreement and coefficients of repeatability for the immunoglobulin A (IgA) assay duplicates.

Tear Type	Mean of differences	SD of differences	Limits of agreement	Coefficient of repeatability
Basal	0.00	0.04	-0.08 to 0.08	0.08
Flush	-0.01	0.03	-0.07 to 0.05	0.06
Reflex	0.00	0.03	-0.06 to 0.06	0.06

2.6.3.2 Day-to-day and eye-to-eye repeatability

Repeated measures ANOVA showed no difference between eyes or days for any of the methods, whether for TPC (F = 2.62, p = 0.13 and F = 1.62, p = 0.22, for eyes and days respectively), IgA concentration (F = 3.76, p = 0.07 and F = 0.76, p = 0.40, eyes and days respectively) or their ratio (F = 0.09, p = 0.77 and F = 2.60, p = 0.13, eyes and days respectively).

Coefficients of repeatability[232] were calculated for each method as between replicates taken from the two eyes of each participant and also on different days. These are shown in Table 2.4 along with the limits of agreement.

Table 2.4: Limits of agreement and coefficients of repeatability between eyes and days for each tear collection method for total protein content (TPC) and immunoglobulin A (IgA).

			Basal	Reflex	Flush
Limits of Agreement	TPC (mg/mL)	Days	-3.19 to 7.55	-7.49 to 6.97	-3.49 to 4.15
		Eyes	-6.35 to 5.61	-7.66 to 6.22	-3.97 to 3.05
	IgA (mg/mL)	Days	-0.46 to 0.24	-0.48 to 0.44	-0.36 to 0.33
		Eyes	-0.35 to 0.61	-0.39 to 0.35	-0.12 to 0.38
Coefficient of Repeatability	TPC (mg/mL)	Days	5.38	7.24	3.83
		Eyes	5.98	6.94	3.50
	IgA (mg/mL)	Days	0.58	0.93	0.69
		Eyes	0.68	0.75	0.49

2.6.4 TPC and IgA analysis

As there were no significant differences between eyes or days for either TPC or IgA, data were averaged prior to comparison between collection methods. Table 2.5 summarises the outcome. As expected, TPC was highest for basal and least for flush tears. The latter were significantly less concentrated than both basal and reflex tears (p = 0.001 and 0.008, respectively).

IgA concentration in basal tears was significantly greater than in both reflex (p = 0.002) and flush tears (p < 0.001). However, when expressed as a percentage of TPC, the amount of IgA was identical in basal and flush samples (p = 1.00). Concentrations were normalised against TPC so as to compensate for the differences in volume.

Table 2.5: Group mean ± SD for total protein content (TPC), immunoglobulin A (IgA) concentration and IgA as a percentage of TPC for basal (B), reflex (R) and flush (F) tears (n = 16) with associated *p* values.

	Basal	Reflex	Flush	*p* value		
				B-R	R-F	B-F
TPC (mg/mL)	7.14 ± 2.22	6.01 ± 2.11	3.79 ± 1.51	0.15	0.001	0.008
IgA concentration (mg/mL)	1.04 ± 0.29	0.64 ± 0.26	0.65 ± 0.23	<0.001	1.00	0.002
IgA percentage (%)	19.8 ± 14.9	11.4 ± 3.9	19.8 ± 8.7	0.15	0.02	1.00

2.6.5 SDS-PAGE

Figure 2.3 shows the SDS-PAGE patterns for basal and flush tears (representative) from one participant. Only basal and flush tears were compared as these were found to be

proportionately similar in the previous protein analysis of IgA (Section 2.6.4). Lane profile analysis exhibited the presence of all major protein bands in both basal and flush tear samples, the molecular weight (MW) of these being consistent with those known for lactoferrin, albumin, IgA, lipocalin and lysozyme.[234] The gel band intensities were profiled in order to identify regions of differences. In each case the same peaks were present for both basal and flush samples.

2.6.5.1 SDS-PAGE of tears from contact lens wearers

Prior to using the flush technique in contact lens wearers in Chapter 4 and Chapter 5, flush tears were compared to basal tears in order to confirm that the same proteins were collected in the presence of a contact lens. Figure 2.4 is a representative gel of the basal and flush tears of one contact lens wearer. The same proteins were identified with both the basal and flush technique as can also be seen by the common peaks shown in Figure 2.4.

2.6.6 Mass spectrometry

In order to give a broader picture of the less abundant proteins in tear samples, mass spectrometry was performed on three replicates from each tear collection method, all from the same participant, according to the above protocol. The results are shown in Table 2.6. As a means of showing commonality between the three replicates for each method, Venn diagrams were constructed as shown in Figure 2.5. From these it can be seen that basal sampling was the most consistent as 26 out of the total 29 proteins detected were present in all three replicates. Consistency was slightly reduced for both the flush and reflex methods which repeatedly returned 18 out of 27 and 18 out of 29 proteins respectively. This may be a result of the greater volume of flush and reflex tears diluting the lower abundance proteins so that they are not consistently detected. Taking only the consistently returned proteins for each method, a further Venn diagram was constructed to examine the distribution of these moieties between techniques. This is shown in Figure 2.6 where it can be seen that 15 proteins were common to all three sampling groups. These 15 are the more abundant proteins and are indicated with asterisks in Table 2.6. Comparing flush and basal collection showed 17 common proteins, the same number as for reflex and basal.

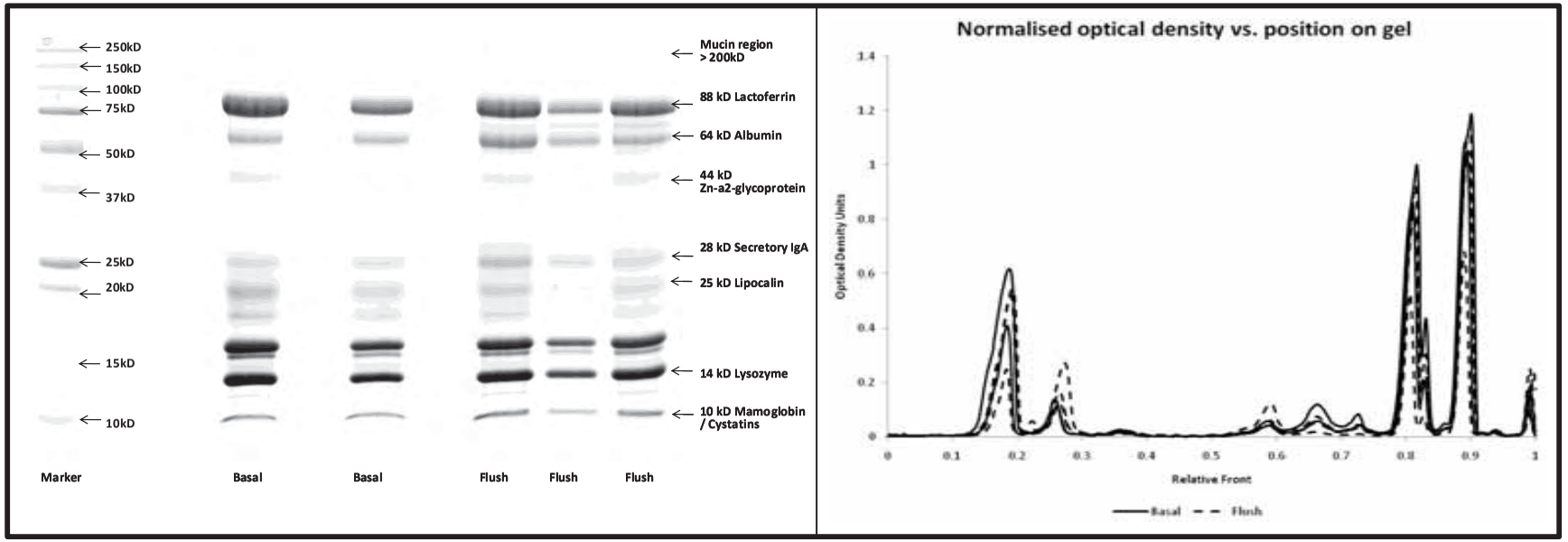


Figure 2.3: Representative one-dimensional gel of Coomassie Blue-stained proteins in basal and flush tears in a non-contact lens wearer (LEFT) and graphs of the corresponding normalised optical density versus the relative front of the gel (RIGHT).

LEFT: Lane one is the molecular weight marker (MW) and each of the five subsequent lanes include 10 µg of protein load.

RIGHT: Each peak corresponds to a protein of the same molecular mass. The same peaks were identified in all samples.

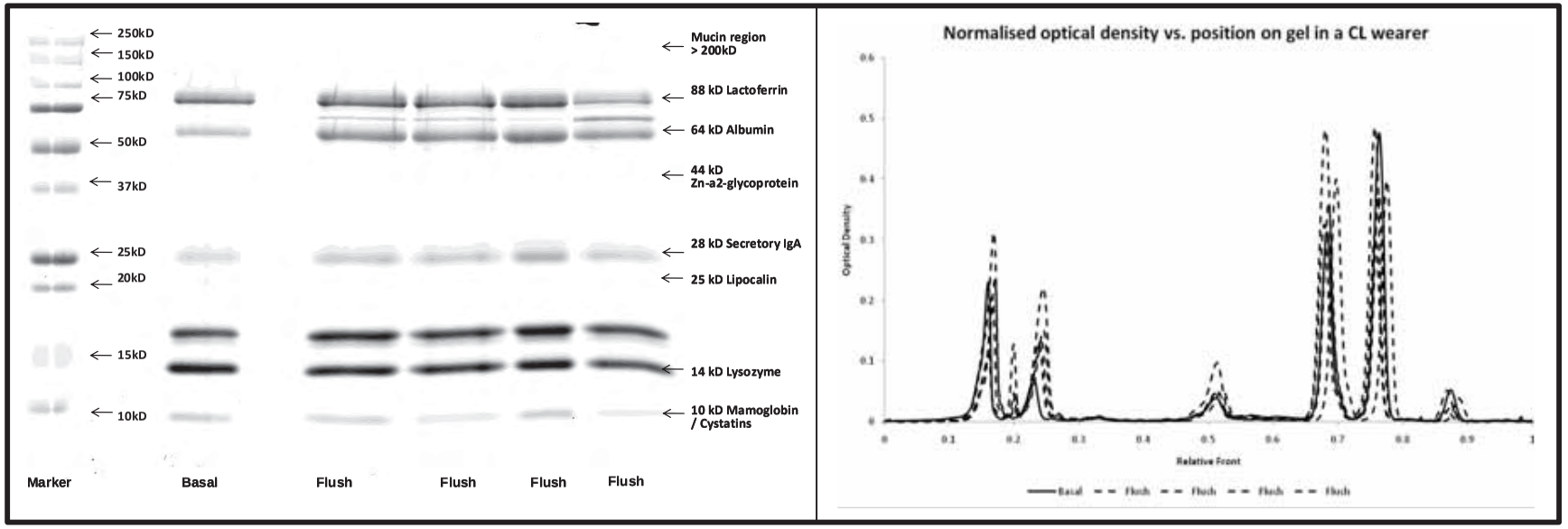


Figure 2.4: Representative one-dimensional gel of Coomassie Blue-stained proteins in basal and flush tears of a contact lens wearer (LEFT) and graphs of the corresponding normalised optical density versus the relative front of the gel (RIGHT).

LEFT: Lane one is the molecular weight (MW) marker and each of the five subsequent lanes includes 10 µg of protein load.

RIGHT: Each peak corresponds to a protein of the same molecular mass. The same peaks were identified in all samples.

Table 2.6: Proteins in the basal, reflex and flush tears of one participant as identified by mass spectrometry.

Asterisks (*) indicate proteins captured in all replicates and collection methods. Gray boxes highlight the proteins not identified in that sample. Percentage coverage refers to the percentage of peptides of that protein found.

			Flush replicates			Reflex replicates			Basal replicates		
			1	2	3	1	2	3	1	2	3
Identified proteins (32)	Accession number	Molecular weight	Percent coverage								
Lactoferrin*	gi|186833	78 kDa	54%	56%	54%	56%	42%	60%	51%	53%	65%
Lipocalin*	gi|4504963	19 kDa	65%	62%	62%	62%	52%	62%	62%	62%	62%
The complex formed between MHC-like zinc α2-glycoprotein alpha2-glycoprotein and prolactin inducible protein*	gi|145579641	14 kDa	70%	70%	75%	70%	44%	75%	81%	70%	75%
Mutant human lysozyme substituted at the surface positions*	gi|12084272	15 kDa	55%	65%	58%	71%	58%	64%	64%	65%	60%
Unnamed protein product	gi|34527233	53 kDa	9%	14%	13%	15%	15%	11%	15%	18%	15%
Polymeric immunoglobulin receptor*	gi|238236	83 kDa	11%	8%	9%	10%	9%	10%	27%	24%	16%
Cystatin s*	gi|4503109	16 kDa	54%	49%	41%	62%	20%	62%	49%	62%	49%
Proline rich 4*	gi|1050983	15 kDa	12%	28%	22%	22%	22%	28%	22%	22%	22%
Lacritin precursor*	gi|15187164	14 kDa	17%	30%	24%	30%	30%	23%	24%	24%	24%
Secretoglobin*	gi|4505171	11 kDa	40%	32%	32%	59%	32%	59%	68%	59%	68%
Proline rich, lacrimal 1*	gi|116805344	27 kDa	12%	12%	12%	12%	12%	12%	12%	7%	12%
Unnamed protein product	gi|189053131	19 kDa	39%	44%	44%	26%	0%	39%	39%	39%	39%
Immunoglobulin J chain*	gi|114319027	20 kDa	15%	11%	15%	27%	23%	15%	33%	27%	23%
Apolipoprotein	gi|178855	49 kDa	15%	7%	0%	7%	0%	7%	21%	13%	17%
Lipophilin a*	gi|5729907	10 kDa	10%	10%	10%	10%	10%	22%	23%	22%	10%
Proline rich 4 (lacrimal) isoform 1	gi|154448882	17 kDa	0%	10%	10%	10%	10%	10%	10%	10%	10%
Ig alpha-2 chain C region*	gi|70058	37 kDa	14%	4%	4%	14%	14%	14%	4%	4%	14%
Contribution of hydrophobic residues to the stability of human lysozyme*	gi|157832585	15 kDa	22%	22%	22%	22%	22%	22%	22%	22%	22%

			Flush replicates			Reflex replicates			Basal replicates		
			1	2	3	1	2	3	1	2	3
Identified proteins (32)	Accession number	Molecular weight	Percent coverage								
Ig L-chain V-region	gi|27552515	23 kDa	7%	7%	0%	7%	7%	0%	20%	20%	7%
Ig kappa chain V-III (KAU cold agglutinin)	gi|106586	23 kDa	9%	0%	0%	9%	0%	9%	18%	26%	9%
Solution structure of W60g mutant of human beta2- macroglobulin	gi|158431179	12 kDa	10%	20%	10%	10%	0%	10%	0%	0%	0%
Structural changes of the active site cleft and different saccharide binding modes in human lysozyme	gi|1065033	15 kDa	22%	0%	22%	0%	0%	22%	22%	0%	22%
Human tear lipocalin von ebners gland protein	gi|56554584	18 kDa	10%	10%	10%	10%	0%	10%	10%	10%	10%
Ig A alpha1 bur	gi|223099	51 kDa	5%	0%	5%	5%	5%	5%	5%	5%	5%
Ig A H	gi|229537	51 kDa	0%	0%	0%	1%	1%	1%	1%	3%	0%
Phospholipase A2	gi|119615316	12 kDa	0%	0%	0%	0%	0%	9%	26%	9%	26%
Unnamed protein product	gi|194384278	33 kDa	4%	4%	0%	4%	0%	4%	4%	4%	4%
Deleted in malignant brain tumors 1, isoform CRA_A	gi|119569693	112 kDa	1%	1%	0%	1%	0%	1%	0%	1%	1%
Mesothelin or CAK1 antigen precursor	gi|1145724	69 kDa	0%	0%	0%	0%	0%	0%	4%	0%	0%
Immunoglobulin alpha heavy chain variable region	gi|62871224	14 kDa	0%	0%	14%	0%	0%	0%	14%	14%	14%
Secretory IgA1	gi|146387599	64 kDa	0%	0%	0%	0%	0%	0%	5%	5%	5%
Human cystatin c	gi|14278690	13 kDa	0%	0%	0%	0%	0%	22%	0%	0%	0%

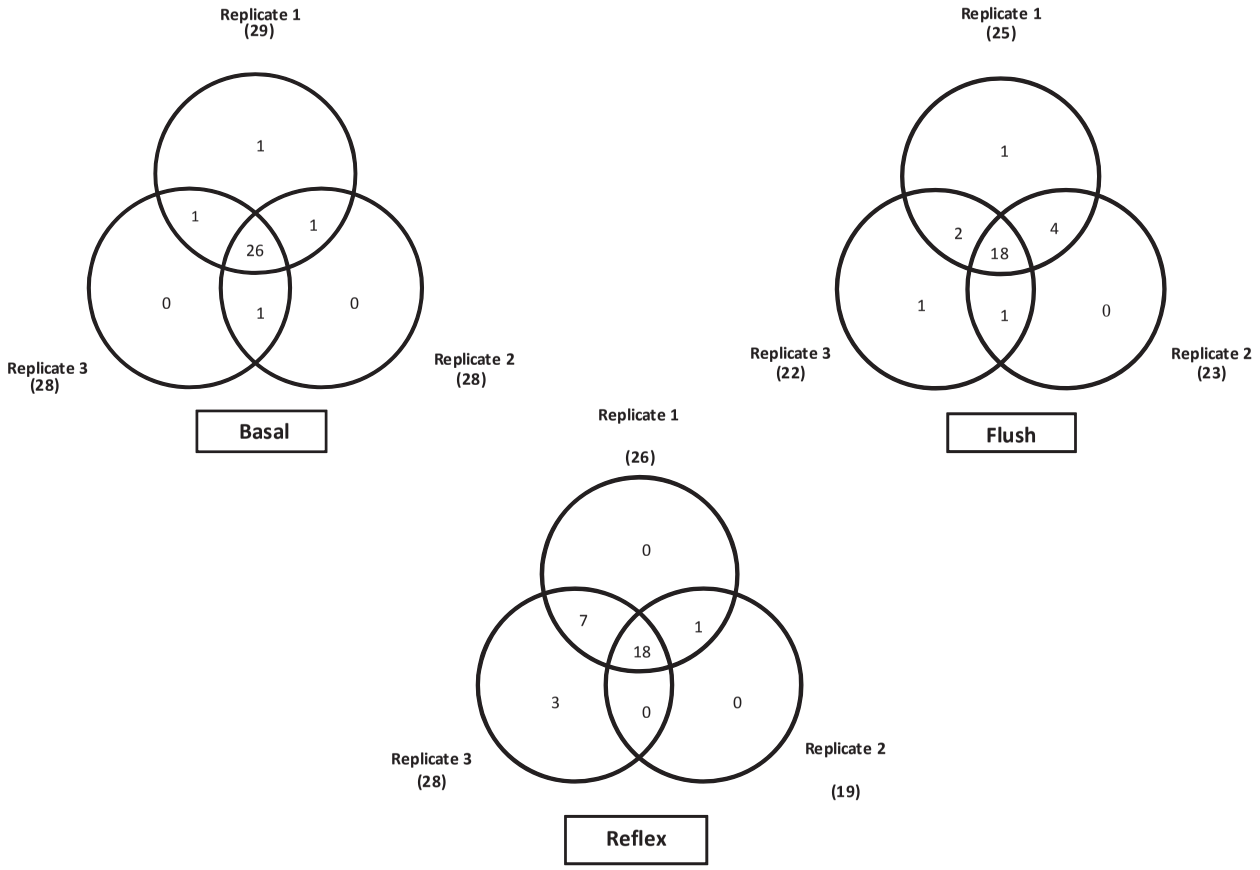


Figure 2.5: Venn diagrams indicating commonality of protein species among three replicates each of basal, reflex and flush tear samples.

The number in brackets indicates the total number of proteins found in that replicate. Replicates 1 and 2 were taken on the same day, from the right and left eyes respectively, while replicate 3 was taken from the right eye on a second day.

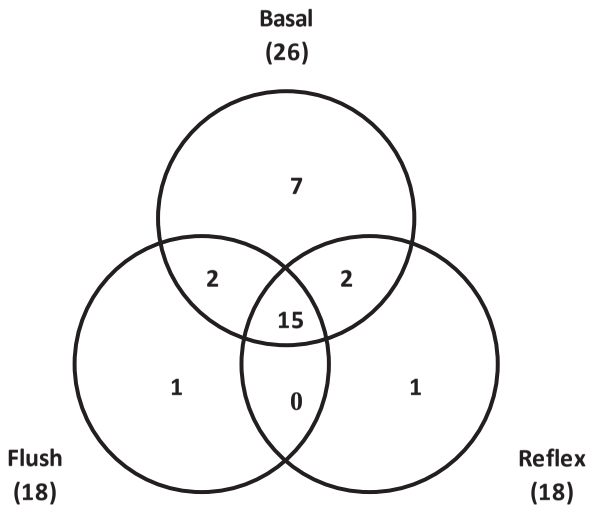


Figure 2.6: Venn diagram showing commonality among those proteins consistently captured by each collection method (basal, reflex and flush).

The numbers in brackets indicate the total number of proteins consistently captured in each of three replicates of the same sampling method.

2.7 SUMMARY AND DISCUSSION

When performing tear collection the aim is to obtain a true representation of the status of the tear film at that point in time. In conducting the present study the purpose was to establish if a standardised flush method of tear collection would permit such a view to be obtained more easily than the traditional approach of collecting basal secretions, while maintaining adequate repeatability between replicate events.

2.7.1 Volume and flow-rate

The rapidity of the flush technique is obvious from Table 2.1, where it can be seen that increasing the volume of fluid in the conjunctival sac allows a standard 10 μL sample to be collected in less than 30 seconds, on average. This is at least four times faster than traditional basal sampling and, even taking into account the need for an additional saline instillation step, provides a less challenging and more comfortable procedure for participants, not to mention investigators.

2.7.2 Validation

Of course, speed is of no value unless the contents of the sample maintain fidelity with those of the original which, in this instance, are best represented by basal secretions. To evaluate the extent to which this was achieved, flush samples were compared with basal tears from the same participants, using a number of indicators.

2.7.2.1 Variety of proteins detected

SDS-PAGE was used to show that the capture of major proteins was essentially the same between the two methods (Figure 2.3) a similar finding shown by others.[210, 218, 235] Similarly, the presence of a contact lens did not change the capture of the major proteins, as shown in Figure 2.4. To add further weight to these results the status of a wider range of protein moieties using mass spectrometry was also examined. The Venn diagrams in Figure 2.5 show that consistency of proteins collected between replicates was similar for reflex and flush, but both were reduced relative to the basal sampling method. Thus where basal sampling consistently returned 26 proteins, reflex and flush produced only 18. However, as can be seen from Figure 2.6, fifteen of these consistent

proteins were common to all the techniques and these were identified as being the more abundant species (asterisked in Table 2.6). It should be noted that basal tears returned seven species not regularly found by the reflex or flush methods. Basal tears therefore appear to consistently collect a greater number of the less abundant proteins compared to reflex and flush tears, and a similar amount to that found by Green-Church et al.[210] Reflex and flush essentially return the same proteins.

2.7.2.2 Protein concentration

To investigate the behaviour of protein concentration, both total protein and IgA were measured. The latter was chosen as a representative protein because its concentration is known to alter as a result of ocular stimulation,[236] making it a reasonable indicator of the potential for the flush method to be invasive, relative to basal collection. Both total protein and IgA concentrations were reduced in the flush samples, as would be expected due to the increased volume. However, their relative amount was the same in flush and basal samples, as was indicated by the IgA:TPC ratio (Table 2.5). Taking this observation together with those discussed earlier, provides good evidence that flush collection produces a sample that, while of low absolute concentration, largely mirrors basal tears both in terms of the most abundant proteins captured and their relative amounts.

2.7.2.3 Repeatability

Various investigators have reported high levels of variability when using the flush approach.[220, 221] It seems likely that this performance has been influenced by a lack of consistent methodology. In particular, the use of different numbers of multiple saline instillations between studies and even sampling events[218, 220, 221] is far from ideal. Using a single, controlled volume of saline appears to largely solve this problem as, in terms of both total protein and IgA concentrations, repeatability for the flush method in the current study was at least as good as that for basal collection, and better than for reflex tears. In contrast, the Schirmer technique has been shown to variably affect the plasminogen levels in tears compared to basal tears[214] and to collect 53 unique proteins compared to basal tear collection.[210]

2.8 OUTCOMES OF CHAPTER 2

In cases where less abundant proteins need to be identified consistently across samples, basal sampling remains the most suitable approach, as it collects a larger range of such moieties. In most other circumstances however, the standardised method of flush tear collection outlined above can be considered as an alternative to basal tear collection. It permits repeatable retrieval of largely the same range of the more abundant proteins, in the same proportions as basal tears, but is quicker, and thus less disturbing for participants and more convenient for investigators. This method is therefore used as the tear collection technique in the subsequent chapters.

CHAPTER 3 THE DIURNAL VARIATION OF MMP-9 AND ITS ASSOCIATED FACTORS IN THE TEARS OF HEALTHY NON-CONTACT LENS WEARERS

3.1 OVERVIEW

Chapter 2 established the suitability of the flush tear collection technique to allow for adequate tear volume to be collected without altering the overall protein profile. Chapter 3 applies this tear collection technique to the study of the diurnal variation of matrix metalloproteinase-9 (MMP-9) and its associated factors in the tear film of healthy non-contact lens wearers. MMP-9 has been associated with conditions such as recurrent corneal erosions[78] and corneal ulcers.[48] It is not known what role, if any, this enzyme plays in contact lens wear and in the erosions that occasionally result. This chapter establishes the baseline levels of MMP-9 and its associated factors in human tears. With this understanding, the effect of contact lens wear will be determined in Chapter 4. Furthermore, the diurnal variation reported here allows for comparisons to be made with those who develop erosions during contact lens wear in Chapter 6.

The work presented in this chapter has been accepted for publication in *Investigative Ophthalmology & Vision Science.* These findings were also presented at the Association for Research in Vision and Ophthalmology conference in 2010, the British Contact Lens Association conference in 2010, and the Cornea and Contact Lens Society of Australia meeting in 2010 (Appendix B: Publications and presentations).

3.2 INTRODUCTION

MMPs are a family of collagen degrading endopeptidases whose function is to maintain and remodel the tissue architecture.[101, 103] In the cornea, the controlled presence of MMPs may be important in maintaining homeostasis; however their uncontrolled production could have collagen degrading effects. MMP-9 is the primary matrix-degrading enzyme produced by basal corneal epithelial cells[110] and neutrophils[135] and is known to degrade the major constituents of the epithelial basement membrane such as collagen type VII.[111] As the fibrils responsible for anchoring the basement membrane to the stroma are composed of type VII collagen,

an increase in this activity may result in a weakening of these attachments, contributing to the cascade of events that result in corneal erosion.[93,78]

Regulation of matrix degradation is controlled by the ratio of MMP-9 to its inhibitor, tissue inhibitor of metalloproteinase-1 (TIMP-1) and an imbalance in this ratio caused by a relative excess of MMP-9 may indicate the potential for delayed wound healing and collagen degrading effects.[117] On the other hand, MMP-9 can be protected from degradation by another protein known as neutrophil gelatinase-associated lipocalin (NGAL). NGAL has been suggested to be a major contributor to the 'closed eye' tears and is known to complex with pro-MMP-9 when it is produced by neutrophils.[135] By preventing MMP-9 from degradation, NGAL protects the activity of MMP-9, hence perpetuating its degrading effects. Hence the activity of MMP-9 is a delicate balance between its inhibitor TIMP-1 and its protector, NGAL.

Conditions such as corneal erosions and infections, as well as contact lens-related adverse events, are exacerbated upon waking, suggesting that degrading activity is upregulated overnight. Thus it is essential to understand how MMP-9, TIMP-1 and NGAL vary as a function of the diurnal cycle in healthy non- contact lens wearers.

3.3 AIMS

The studies described in this chapter set out to establish:

1. the eye-to-eye variability in MMP-9 and TIMP-1 and the influence of eye-order
2. the day-to-day variability of MMP-9, TIMP-1 and NGAL
3. the diurnal variation of MMP-9, TIMP-1 and NGAL expression in the flush tears of healthy non-contact lens wearers
4. the influence of gender and age on these protein levels
5. the association with clinical signs

3.4 HYPOTHESES

The closed eye ocular tear film is known to exist in a state of sub-clinical inflammation.[237] As MMP-9 is majorly sourced from neutrophils,[135] and as neutrophils increase in concentration during sleep,[238] the hypotheses of this chapter therefore are

that MMP-9 increases during sleep and that this increased expression is balanced by its inhibitor in order to control the level of collagen degradation.

3.5 METHODS

3.5.1 Ethics approval

This study was approved by the institutional ethics committee of the University of New South Wales (UNSW) for Study 1 (approval numbers: 084055 and 084056) and the VIHEC human ethics committee with ratification from UNSW for Study 2 (approval number 10/09 and HREC 10244). The tenets of the Declaration of Helsinki 1975 as amended in 2000 were followed and Good Clinical Practice was adhered to. Informed consent was obtained from 46 participants (27 females, 19 males) aged between 18 and 46 years of age (mean 28 ± 6 years) after explanation of the nature and possible consequences of the study (see Appendix A: Participant information statement and consent forms). Participants were recruited from the general university population and through approved email notices.

3.5.2 Sample size calculation

Sample size was determined so that the study group could subsequently be split into four groups in Chapter 4. Approximately seven participants in each group were required in order to demonstrate a statistically significant difference at 95% confidence and with 80% power, of 400 ± 300 ng/mL in MMP-9 concentration based on a previous study.[197]

3.5.3 Inclusion criteria

Participants were healthy and were not taking any medications for general health conditions. A requirement of the study was that the participants were either non-contact lens wearers or non-habitual contact lens wearers who had not worn contact lenses in the month preceding the study. The specific criteria are listed as follows. Participants enrolled in the trial must:

- be able to read and comprehend English and give informed consent as demonstrated by signing a record of informed consent

- be aged between 18 and 46 years
- be willing to comply with the wearing and clinical trial visit schedule as directed by the investigator
- have ocular health findings considered to be "normal" and which would not prevent the participant from safely wearing contact lenses
- have vision correctable to at least 6/12 in each eye with contact lenses

3.5.4 Exclusion criteria

Participants enrolled in the trial must NOT:

- have worn contact lenses in the month preceding enrolment
- be habitual contact lens wearers
- have pre-existing ocular irritation that would preclude contact lens fitting
- have an ocular condition that may preclude safe wearing of contact lenses
- have an active corneal infection or any active ocular disease that would affect wearing of contact lenses
- need concurrent category S3 and above ocular medication up to 12 weeks prior to and during the trial
- have any systemic disease including diabetes (self-reported) that may affect ocular health. Conditions such as systemic hypertension do not automatically exclude prospective participants
- take any systemic or topical medications up to 12 weeks prior to and during the trial that will affect ocular physiology or the performance of the contact lenses
- have had eye surgery within 12 weeks immediately prior to enrolment for this trial
- have undergone corneal refractive surgery
- have any ocular injury or condition (including keratoconus and herpes keratitis) of the cornea, conjunctiva or eyelids that would affect the wearing of contact lenses
- have contraindications to contact lens wear

- be currently enrolled in another clinical trial or have participated in a clinical trial within the previous two weeks
- be pregnant or lactating

3.5.5 Study design

Two studies were conducted as follows:

3.5.5.1 Study 1

For the first 37 participants sample size was determined so that the study group could subsequently be split into four groups of at least seven participants in Chapter 4. A baseline visit was conducted between 11 AM and 2 PM ('midday') whereby informed consent was obtained and baseline measurements were taken prior to tear collection from both eyes. Participants were asked to stay overnight in the clinic and tears were collected again before sleep and immediately upon awakening.

Tears collected from each eye were stored and analysed separately so that eye-to-eye variability could be determined. Tears were analysed for total protein content (TPC), MMP-9 and TIMP-1 concentrations. Due to insufficient tear volume, NGAL was subsequently measured in Study 2.

3.5.5.2 Study 2

Study 2 with nine participants was conducted in order to measure NGAL, perform zymography (Section 3.6.9.9) and differential gel electrophoresis (Chapter 5). This group of nine participants was not split into further groups. Tears from both eyes were collected and pooled to allow for sufficient volume. In order to ensure that the results from Study 2 were comparable to those of Study 1, the same variables as Study 1 were measured, that is, TPC, MMP-9 and TIMP-1. Additionally, four of these nine participants had also taken part in Study 1.

This group of nine participants had their tears collected from both eyes at two time-points: at midday and upon awakening. Each time-point was performed on two separate occasions in order to establish the normal day-to-day variability within the diurnal cycle.

3.5.6 Clinical techniques

The clinical techniques used at each time-point are outlined below in Table 3.1. The order of tests was arranged to minimise the impact on the ocular surface prior to tear collection. Visual acuity and evaluation of the cornea and conjunctiva without fluorescein were conducted prior to tear collection, while fluorescein evaluation and lid eversion were conducted following tear collection.

Table 3.1: The clinical techniques performed at each time-point.

'Midday 1'and 'midday 2' refer to the first and second midday visit conducted in Study 2. Only one midday visit was conducted in Study 1 (midday 1). The 'before sleep' visit was only conducted in Study 1.

Procedures	Midday	Before sleep	Upon awakening
Visit window	Between 11 AM and 2 PM	Before sleep, same day as 'midday'	Immediately upon awakening, morning after 'midday'
Informed consent	Y (midday 1 only)	N	N
Meet inclusion/exclusion criteria	Y (midday 1 only)	N	N
Demographics	Y (midday 1 only)	N	N
History at baseline	Y (midday 1 only)	N	N
Updated history/any medical problems	Y (midday 2 only)	Y	Y
Baseline information: - spectacle refraction - visual acuity - keratometry	Y (midday 1 only)	N	N
Corneal topography	N	N	N
Symptoms and problems	Y	Y	Y
Wear time	N	N	N
Visual acuity	Y	N	N
Slit-lamp biomicroscopy: Redness, contact lens fit Evaluation of cornea and conjunctiva including fluorescein Upper tarsus (fluorescein on indication)	Y	Y	Y
Fundoscopy (superfield lens)	Y (midday 1 only)	N	N
Photos/video	*	*	*
Flush tear collection	Y	Y	Y
Adverse event data	Y**	Y**	Y**
Visit summary	Y	Y	Y

Y = Yes, required information, N = No, not required
** At optometrists discretion*
*** If adverse event detected at time of visit*

3.5.6.1 Visual acuity and slit-lamp biomicroscopy

Visual acuity was measured at each visit using computer letter charts.[239] Slit lamp biomicroscopy (Zeiss SL-120, Carl Zeiss Meditech, Jena, Germany) was performed according to Table 3.1. Bulbar and limbal redness, palpebral redness and roughness as well as corneal and conjunctival staining were assessed in order to exclude any pre-existing conditions at baseline. Corneal and conjunctival staining were conducted with fluorescein (Fluorets ophthalmic strips, 1 mg, Chauvin Pharmaceuticals, Essex, UK) and a Wratten #12 filter (Kodak, Los Angeles, USA). Retroillumination was used to examine the cornea for the presence of subtle abnormalities. The order of tests was such to minimise the impact on the ocular surface prior to tear collection. As such, visual acuity and evaluation of the cornea and conjunctiva without fluorescein were routinely conducted prior to tear collection while fluorescein evaluation and lid eversion were routinely conducted following tear collection.

3.5.6.2 Grading scales

Clinical grading was conducted by a concordant optometrist (MM) using the Brien Holden Vision Institute grading scales[240] (Appendix C: Brien Holden Vision Institute grading scales).

3.5.6.3 Tear collection

Flush tears[202] as described in Chapter 2, Section 2.5.3.3, were collected from both eyes using a 10 μL microcapillary tube (Blaubrand® intraMark, Wertheim, Germany) at each visit. This method has also been validated for MMP-9 measurement.[241] The samples from each eye were stored separately for Study 1 and pooled for Study 2. A limit of one minute was imposed to avoid reflex tearing.[226] Midday, before sleep and upon awakening visits were conducted consecutively. For the 'upon awakening' visits, participants slept overnight in the clinic. They were awakened and instructed not to open their eyes as they were lead to the clinic chair. Upon instruction, the participants were asked to open their eyes and flush tear collection commenced immediately under dim lighting. All samples were collected by the same investigator (MM). The order of eyes for tear collection was randomised.

3.5.6.3.1 Tear sample treatment

After collection, samples were centrifuged at 1,145 g for 20 minutes at 4 °C to remove cellular debris. The supernatants were collected and stored in siliconised polypropylene microcentrifuge tubes (Sigma-Aldrich, Steinheim, Germany) at -80 °C in three aliquots for Study 1 and five aliquots for Study 2.[242] One aliquot was used for total protein content (TPC) analysis and the other two were used for MMP-9 and TIMP-1 analysis. The additional two aliquots for Study 2 were used for NGAL analysis (Section 3.5.7.4) and zymography (Section 3.5.7.5) as well as differential gel electrophoresis (Chapter 5).

3.5.7 Laboratory techniques

3.5.7.1 Total protein content

The diurnal variation of TPC has been previously reported [226, 243, 244] and was measured in this study, first as a confirmation that the results presented here reflect those previously published, and second, because a knowledge of TPC was necessary for zymography (Section 3.5.7.5) and the proteomic analysis performed in Chapter 5.

TPC was therefore determined using the bicinchoninic acid method (BCA) and using Pierce reagents and flat-bottom Nunc-F Maxisorp 96-well microplates (Thermo Fisher Scientific, Rochester, NY, USA) as per Chapter 2, Section 2.5.5. Each sample was identified by a number.

3.5.7.2 MMP-9 assay

Total MMP-9 concentration was determined using sandwich enzyme-linked immunosorbent assay (ELISA) with the RnD Systems Inc DuoSet kit (Minneapolis, MN, USA). This assay recognises the 92 kDa latent and 83 kDa active forms of MMP-9. From communication with the manufacturers, it is apparent that this assay also detects MMP-9 complexed with both TIMP-1 and NGAL. The manufacturer's guidelines state that TIMP-1 interferes at concentrations greater than 1.56 ng/mL. Other MMPs are not known to cross-react or interfere when tested at 50 ng/mL. The sensitivity of the MMP-9 assay is less than 0.156 ng/mL according to the RnD Quantikine kit.

The assay was carried out according to the manufacturer's directions. Briefly, Nunc-F Maxisorp 96-well microplates (Thermo Fisher Scientific, Rochester, NY, USA) were coated with 100 μL of the capture antibody overnight at room temperature. The plates were washed three times with wash buffer (0.05% Tween20, Sigma-Aldrich, St. Louis, MO, USA) and then blocked with 200 μL reagent diluent (1% bovine serum albumin, BSA, Sigma Aldrich Inc, Canada) for a minimum of one hour. The blocking buffer was then removed and the wells washed three times with the wash buffer. Tear samples were diluted in reagent diluent in 1:25 for the 'midday' and 'before sleep' tears and 1:2,000 dilutions for the 'upon awakening' tears. Duplicate samples (100 μL) were incubated for two hours before washing the plate three times with the wash buffer. A volume of 100 μL of detection antibody was added to each well. This was incubated for two hours after which it was removed and the wells were washed with wash buffer. Streptavidin-horseradish peroxidase (HRP) was added for 20 minutes and then washed out. Tetramethylbenzidine (TMB) liquid substrate system for ELISA (Sigma-Aldrich, MO, USA) was added to each well in a volume of 100 μL and stopped with 50 μL of 2 M sulphuric acid (H_2SO_4) after 20 minutes of incubation. The optical density was then measured at 540 nm and subtracted from 450 nm, to account for any optical imperfections of the plate, using SkanIT® RE for MSS 2.4.2 software (ThermoScientific, Vantaa, Finland), and assay blanks were subtracted from each sample or standard. Supplied standards were used to generate a standard curve ranging from 2,000 pg/mL to 15.6 pg/mL using a four-parameter logistic fit. Where samples were below the detection limits of the standard curve, they were allocated a value of zero.

3.5.7.3 TIMP-1 assay

The concentration of TIMP-1 was determined using ELISA with the RnD Systems Inc DuoSet kit (Minneapolis, MN, USA). This kit recognises natural and recombinant TIMP-1. The manufacturer's guidelines state that there is no cross-reactivity or interference with MMP-1, -2 or -3 or TIMP-2 when tested at 50 ng/mL. MMP-9 is known to interfere at concentrations greater than 100 ng/mL in the RnD Quantikine kit. The sensitivity of the quantikine kit (DTM100 RnD Systems Inc, Minneapolis, MN, USA) is reported by the manufacturer as less than 0.08 ng/mL.

Wells of Nunc-F Maxisorp 96-well microplates (Thermo Fisher Scientific, Rochester, NY, USA) were coated with 100 μL of the capture antibody. The antibody was allowed to bind overnight at room temperature. The plates were washed three times with wash buffer (0.05% Tween20, Sigma-Aldrich, St. Louis, MO, USA). The wells were then blocked with 200 μL reagent diluent (1% BSA) for a minimum of one hour. The blocking buffer was then removed and the wells were washed three times with the wash buffer. A volume of 100 μL of tears was analysed in duplicate, diluted in reagent diluent in 1:200 for the 'midday' tears and 1:1,000 dilutions for the 'upon awakening' tears. These were allowed to incubate for two hours and the wells were then washed three times with the wash buffer. A volume of 100 μL of detection antibody was added to each well. This was incubated for two hours after which it was removed and the wells were washed with wash buffer. Streptavidin-HRP was added for 20 minutes and then washed out. TMB liquid substrate system for ELISA (Sigma-Aldrich, MO, USA) was added to each well in a volume of 100 μL and stopped with 50 μl of 2 M H_2SO_4 after 20 minutes of incubation. The optical density was then measured at 540 nm and subtracted from 450 nm using SkanIT® RE for MSS 2.4.2 software (ThermoScientific Vantaa, Finland). Each plate included seven dilutions of the standard in duplicate starting at 2,000 pg/mL from which the standard curve was generated (four-parameter logistic). The mean absorbance of the blank wells was subtracted from the mean of each sample and each standard in order to determine the final absorbance.

3.5.7.4 NGAL assay

NGAL concentration was determined using ELISA with the RnD Systems Inc 'human Lipocalin-2/NGAL' DuoSet kit (DY1757, Minneapolis, MN, USA). This kit measures natural and recombinant human NGAL. The manufacturer's guidelines state that there is no cross-reactivity or interference with human lipocalin-1 or MMP-9 when tested at 50 ng/mL. A 0.3% cross-reactivity of recombinant human MMP-9/NGAL complex was also shown in the quantikine kit. The sensitivity of the quantikine kit is reported by the manufacturer as less than 0.012 ng/mL.

Wells of Nunc-F Maxisorp 96-well microplates (Thermo Fisher Scientific, Rochester, NY, USA) were coated with 100 μL of the capture antibody. The antibody was allowed to

bind overnight at room temperature. The plates were washed three times with wash buffer (0.05% Tween20, Sigma-Aldrich, St. Louis, MO, USA). The wells were then blocked with 200 μL reagent diluent (1% BSA) for a minimum of one hour. The blocking buffer was then removed and the wells washed three times with the wash buffer. A volume of 100 μL of tears was analysed in duplicate, diluted in reagent diluent in 1:400 for the 'midday' tears and 1:1,000 dilutions for the 'upon awakening' tears. These were allowed to incubate for two hours and the wells were then washed three times with the wash buffer. A volume of 100 μL of detection antibody was added to each well. This was incubated for two hours after which it was removed and the wells were washed with wash buffer. Streptavidin-HRP was added for 20 minutes and then washed out. TMB liquid substrate system for ELISA (Sigma-Aldrich, MO, USA) was added to each well in a volume of 100 μL and stopped with 50 μL of 2M H_2SO_4 after 20 minutes of incubation. The optical density was then measured at 540 nm and subtracted from 450 nm using SkanIT® RE for MSS 2.4.2 software (ThermoScientific Vantaa, Finland). Each plate included seven dilutions of the standard in duplicate starting at 2,000 pg/mL from which the standard curve was generated (four-parameter logistic). The mean absorbance of the blank wells was subtracted from the mean of each sample and each standard in order to determine the final absorbance. Only the tears of the participants in Study 2 were analysed for NGAL due to limited volume.

3.5.7.5 Gelatin zymography

Because MMP-9 is present in various forms, it is important to distinguish the active forms from the pro-forms or the complexed forms. Zymography was conducted in order to establish the profile of these in the tear film at midday and upon awakening.

Zymography is the most sensitive and widely used technique for the detection of MMP-9,[51] with a detection limit of 32 pg.[245] Zymography identifies MMPs by their molecular weight and degradation of substrate. The incorporation of gelatin substrate in polyacrylamide gels allows for the identification of MMP-9 and -2, in both the pro-enzyme and active form, by the degradation of the gelatin substrate along with their molecular weight.[123, 246, 247] This is possible because the pro-enzymes are activated *in situ* by the denaturation-renaturation process and autocatalytic

cleavage.[247] Coomassie Blue is used to stain the gel and proteolytic activity is detected as clear bands against a blue background.[247] Other MMPs, such as MMP-1 and -3, can also be detected, but at a much lower sensitivity than MMP-9 and -2 as gelatin is not the appropriate substrate.

3.5.7.5.1 Sample preparation

A mass of 10 µg of each tear sample was added to 5 µL of loading buffer (Novex Tris Glycine sodium dodecyl sulfate (SDS) sample buffer (2x), Invitrogen, Carlsbad CA, USA) which is denaturing but non-reducing and the volume brought up to 12 µL with MilliQ water. The samples were incubated for 30 minutes at room temperature. The SDS allows the latent MMP form to unfold, hence denaturing the enzymes and rendering them inactive. The absence of β-mercaptoethanol means that the conditions are non-reducing. The SDS also causes the separation of MMP-9:TIMP-1 complexes.[247]

3.5.7.5.2 MMP-9 Standard preparation

A concentration of 50 ng/mL of MMP-9 standard (RnD Systems, Minneapolis, MN, USA) was activated by incubating with 1 mM 4-aminophenyl-mercuric acetate[138] (APMA, Sigma-Aldrich, Germany) at 37 °C for one hour. A 1 µL volume of this was then added to a 5 µL volume of 2x sample buffer and topped up to a volume of 12 µL with MilliQ water. This was incubated for 30 minutes at room temperature.

3.5.7.5.3 Running gels

Precision Plus Protein™ Dual Colour Standard marker, Bio-Rad, Hercules CA, USA) was loaded into the first lane of each gel as a molecular weight marker. A volume of 10 µL of marker, activated MMP-9 standard and tear samples were loaded into 10% polyacrylamide gels containing 1 mg/mL gelatin (10% Zymogram (1 mg/mL gelatin) gels from Invitrogen, Carlsbad, CA, USA). Electrophoresis was run at a constant voltage of 120 V for 3.5 hours in running buffer (Glycine, Tris base and 1% SDS) at 4 °C using the Invitrogen Novex Mini-Cell XCell SureLock™ Electrophoresis cell (Carlsbad, CA, USA).

3.5.7.5.4 Renaturing gel

Following electrophoresis, the gels were renatured with 2.5% Triton®X-100 (Astral-Scientific, Amresco, OH, USA) for one hour on the rotary shaker at room temperature. The resolved proteins are renatured by exchange of SDS with the non-ionic detergent Triton®X-100. This way, proteolysis is postponed until the sample proteins have been resolved into bands of concentrated activity.[247]

3.5.7.5.5 Developing gel

The renaturing solution was decanted and the gels were equilibrated with 1x development buffer (Novex Zymography developing buffer 10x from Invitrogen, Carlsbad, CA, USA) for 30 minutes at room temperature on the rotary shaker. This was then decanted and a fresh solution of the developing buffer was added. The gels were then incubated at 37 °C for 16 to 20 hours (overnight).

3.5.7.5.6 Staining gel

After the overnight incubation, the developing buffer was decanted and the gels were stained with 0.2% filtered Coomassie Blue for two hours. Following the staining, the gels were destained with fresh 30% ethanol/10% acetic acid solution for 15, 30 and 60 minutes respectively so that bands of proteolytic activity were visible. Coomassie Blue staining of the gel revealed the areas degraded by the MMPs as white areas against a blue background.

3.5.7.5.7 Inhibition by EDTA

Disodium ethylene-diaminetetra-acetic acid (EDTA), which is known to inhibit MMPs, was added to a separate renaturing and developing buffer. After electrophoresis, the final well containing an identical sample to that in the third well, was cut off and renatured in buffer containing 20 mM EDTA, followed by incubation in developing buffer containing EDTA. These bands should have no proteolytic activity upon staining and were used as controls.

3.5.7.5.8 Activation by APMA

4-aminophenyl-mercuric acetate (APMA) is known to activate the 92 kDa zymogen.[138] In order to confirm the activity of MMP-9, one sample on each gel was activated with 1 mM APMA and incubated for one hour at 37 °C.

3.5.7.5.9 Drying of the gels

The gels were incubated for one hour in drying solution (40% methanol, 5% glycerol) and then placed between two sheets of cellophane in drying frames on a mini-gel drying system (Dryease; Invitrogen, Carlsbad, USA). These were then dried upright in a fume cupboard overnight.

3.5.7.5.10 Image analysis

The dried gels were scanned with the GS-800 scanner (Bio-Rad, Hercules CA, USA) and images were analysed using Quantity One (Bio-Rad, Hercules, CA, USA). The identification of MMPs was achieved by comparison against known molecular weight standards and inclusion of an MMP-9 standard. For densitometric analysis, the colours of the scan were inverted so that MMPs were visualised as dark bands against a white background. A densitometric profile was generated for each lane.

3.5.8 Statistical analysis

Normality was tested using the Shapiro-Wilk test. Repeated measures analysis of variance (ANOVA) was used to compare data between time-points. Mauchly's test was used to verify the sphericity assumption. Where main effects were significant, Student t-tests with Bonferroni correction were used post hoc. PASW version 18.0 (Chicago, USA) was used for the analysis. Results are reported as mean ± standard deviation (SD). Analysis was conducted to establish whether there was a difference between eyes with Study 1 (Section 3.6.6) and days with Study 2 (Section 3.6.7).

3.5.8.1 Bland-Altman analysis

The degree of agreement between replicates taken at the same time-point but on different days and from both eyes at each time-point was evaluated using Bland-Altman analysis.[232] The differences between the two replicates were plotted

against their mean and coefficients of repeatability (SD of the mean differences multiplied by 1.96) were calculated.

3.5.8.2 The use of expectation maximisation for missing variables

In some cases it was not always possible to have measurements due to insufficient sample volume. In those cases where data were missing, in order to permit the ANOVA to proceed without wasting data, replacement was made using expectation maximisation (EM) and any negative values generated by the process were replaced with zero. In cases where the levels of the protein measured were not detectable, these samples were allocated a value of zero.

3.5.8.3 Correlation analysis

Pearson's correlation was used where both variables were quantitative and Spearman's correlation was used when one of the two variables were categorically ordinal.

3.6 RESULTS

3.6.1 Study enrolment

A total of 46 participants were enrolled in this study, four of whom participated in both Study 1 and Study 2 (Section 3.5.5 and Figure 3.1). One participant discontinued after a successful baseline visit and this was from Study 1. Table 3.2 and Table 3.3 show the study enrolment and attendance for Study 1 and Study 2, respectively.

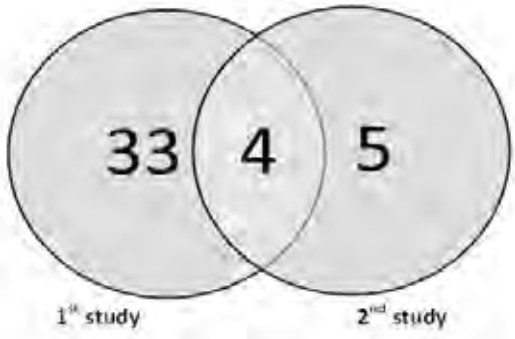


Figure 3.1: A Venn Diagram of the sample size of the 2 studies.

The 1st study had a total of 37 participants and the 2nd study a total of 9 participants. Four of these participants were common to both studies

Table 3.2: Study enrolment and attendance for Study 1 (n = 37).

Visits	Midday	Before sleep	Upon awakening
Study enrolment			
Participants enrolled in study	37		
Study Progress			
Number attended this visit	37	36	36
Number missed this visit	0	0	0
Permanent discontinuations at this visit or prior to next visit	1	0	0
Cumulative discontinuations in study	1	1	1

Table 3.3: Study enrolment and attendance for Study 2 (n = 9).

'Midday 1'and 'midday 2' refer to the first and second midday visit conducted in Study 2. Similarly, 'upon awakening 1' and 'upon awakening 2' refer to the first and second overnight visit.

Visits	Midday 1	Upon awakening 1	Midday 2	Upon awakening 2
Study enrolment				
Participants enrolled in study	9			
Study Progress				
Number attended this visit	9	9	9	9
Number missed this visit	0	0	0	0
Permanent discontinuations at this visit or prior to next visit	0	0	0	0
Cumulative discontinuations in study	0	0	0	0

3.6.2 Demographics

Table 3.4 shows the mean age ± SD and gender demographics while Figure 3.2 displays the spread of ethnicities in the study group.

Table 3.4: Mean age ± SD and gender demographics of study participants.

Mean Age ± SD	Number of males	Number of females	Total number of participants
28 ± 6 years	19	27	46

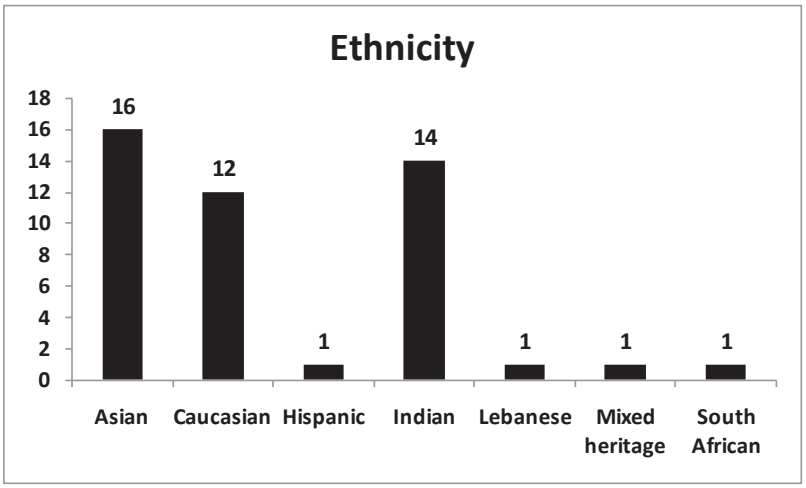


Figure 3.2: A frequency histogram showing the spread of ethnicities in the study.

3.6.3 Clinical variables

Table 3.5, Table 3.6 and Table 3.7 present the clinical findings.

Table 3.5: Refractive error and keratometry at baseline (mean ± SD).

N = 90 eyes (refractive error and keratometry data were missing for one participant.)

Variables	Mean ± SD
Refractive error - sphere (D)	-1.81 ± 2.29
Refractive error - cylinder (D)	-0.60 ± 0.49
Keratometry - flat (D)	42.53 ± 1.58
Keratometry - steep (D)	43.34 ± 1.62

Table 3.6: Extent of corneal staining (mean ± SD) at each visit as graded with the Brien Holden Vision Institute scale.[240]

'Midday 1'and 'midday 2' refer to the first and second midday visit conducted in Study 2. Similarly, 'upon awakening 1' and 'upon awakening 2' refer to the first and second overnight visit. Only one 'midday' and 'upon awakening' visit was conducted in Study 1: 'midday 1' and 'upon awakening 1'. N = 46 for midday 1 and n = 9 for 'upon awakening 1', 'midday 2' and 'upon awakening 2'.

Visit	Area 1	Area 2	Area 3	Area 4	Area 5
Midday 1	0.0 ± 0.2	0.0 ± 0.3	0.0 ± 0.0	0.1 ± 0.3	0.2 ± 0.4
Before Sleep					
Upon Awakening 1	0.1 ± 0.5	0.1 ± 0.3	0.0 ± 0.0	0.1 ± 0.2	0.2 ± 0.4
Midday 2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.2	0.0 ± 0.0
Upon Awakening 2	0.1 ± 0.3	0.1 ± 0.3	0.1 ± 0.3	0.2 ± 0.4	0.2 ± 0.4

Table 3.7: Clinical variables at each visit as graded with the Brien Holden Vision Institute scale.[240]

'Midday 1'and 'midday 2' refer to the first and second midday visit conducted in Study 2. Similarly, 'upon awakening 1' and 'upon awakening 2' refer to the first and second overnight visit. Only one 'midday visit' and' upon awakening' visit was conducted in Study 1: 'midday 1' and 'upon awakening 1'.

Variables	Visit	N	Mean	SD
Blepharitis (0-4, 0.5)	Midday 1	46	0.7	0.6
	Before sleep			
	Upon awakening 1			
	Midday 2	9	0.9	0.8
	Upon awakening 2			
Meibomian gland dysfunction (0-4, 0.5)	Midday 1	46	0.9	0.7
	Before sleep			
	Upon awakening 1			
	Midday 2	9	1.1	0.8
	Upon awakening 2			
Limbal redness (0-4, 0.5)	Midday 1	46	1.1	0.4
	Before sleep	22	1.0	0.1
	Upon awakening 1	30	1.4	0.5
	Midday 2	9	1.2	0.5
	Upon awakening 2	8	1.8	0.4
Bulbar redness (0-4, 0.5)	Midday 1	46	1.2	0.4
	Before sleep	22	1.1	0.3
	Upon awakening 1	30	1.4	0.5
	Midday 2	9	1.3	0.6
	Upon awakening 2	8	2.0	0.4
Palpebral redness (0-4, 0.5)	Midday 1	46	1.1	0.4
	Before sleep			
	Upon awakening 1			
	Midday 2	8	1.6	0.5
	Upon awakening 2			
Tarsal abnormalities (0-4, 0.5)	Midday 1	46	1.0	0.4
	Before sleep			
	Upon awakening 1			
	Midday 2	8	1.4	0.4
	Upon awakening 2			
Conjunctival staining (0-4, 0.5)	Midday 1	46	0.1	0.3
	Before sleep			
	Upon awakening 1			
	Midday 2	9	0.0	0.0
	Upon awakening 2			
Microcysts (0-4, 0.5)	Midday 1	46	0.0	0.0
	Before sleep			
	Upon awakening 1			
	Midday 2	9	0.0	0.0
	Upon awakening 2			

Variables	Visit	N	Mean	SD
Striae/oedema (0-4, 0.5)	Midday 1	46	0.0	0.0
	Before sleep			
	Upon awakening 1			
	Midday 2	9	0.0	0.0
	Upon awakening 2			
Infiltrates (0-4, 0.5)	Midday 1	46	0.0	0.0
	Before sleep			
	Upon awakening 1			
	Midday 2	9	0.0	0.0
	Upon awakening 2			

3.6.4 Adverse events

No adverse events occurred during these measurements. The participants in this study proceeded to contact lens wear (Chapter 4). The data of any participants who experienced an adverse event during contact lens wear were removed from the subsequent analysis in order to give a true representation of the healthy profile of the proteins analysed. As there were four participants who developed adverse events with contact lens wear (Chapter 4, Section 4.6.3), the sample size for the protein analysis was therefore 34 for Study 1 and 8 for Study 2, giving a total of 42 participants. The data of these four participants with adverse events are reported separately and compared to those of normals in Chapter 6.

3.6.5 Normality

Test of normality indicated that not all data were normally distributed. Because repeated measures ANOVA, which was used for the subsequent analysis, is a robust statistical technique against departures from normality[248] and the sample size was greater than 30 for all variables (except those involving NGAL), this violation does not cause major problems for interpretation. ANOVA analysis was therefore conducted.

3.6.6 Eye-to-eye repeatability and the effect of randomisation

3.6.6.1 Tear flow-rate

In this study, the order of eyes for tear collection was randomised. To establish whether the collection order influenced the outcome, flow-rate was analysed

according to order of eyes collected, that is, the first eye compared to the second eye rather than right versus left. On that basis, the first eye yielded a significantly greater flow-rate than the second (F = 13.07, p = 0.001). When right eyes were compared directly to left eyes, this effect was removed and the eyes were no longer significantly different (F = 0.99, p = 0.33). This validated the randomisation approach. Table 3.8 shows the limits of agreement and repeatability coefficients for tear flow-rate between eyes at each visit when eye order is randomised.

Table 3.8: Difference in tear flow-rate between eyes when randomised.

Limits of agreement and coefficients of repeatability for each visit (n = 34).

Visit	Mean of differences (µL/minute)	SD of differences (µL/minute)	Limits of agreement (µL/minute)	Coefficient of repeatability (µL/minute)
Midday	6.5	15.7	-24.8 to 37.8	30.7
Before sleep	-3.1	14.0	-31.0 to 24.9	27.4
Upon awakening	1.2	18.2	-35.2 to 37.6	35.7

3.6.6.2 TPC, MMP-9 and TIMP-1

Eye-to-eye variability was established for the concentrations of TPC, MMP-9 and TIMP-1 at each time-point. This was first established by comparing the eyes in terms of order collected, that is, the first eye collected compared to the second eye and then by comparing right and left eyes after incorporating randomisation.

The eyes were significantly different 'upon awakening' for TPC and TIMP-1, the first eye collected being greater than the left eye (18.1 ± 10.1 versus 13.1 ± 9.1 mg/mL and 275.2 ± 156.4 versus 216.3 ± 124.4 ng/mL respectively, p values shown in Table 3.9). When right and left eyes were compared so that randomisation was incorporated, this effect was no longer apparent (p values shown in Table 3.10).

Table 3.9: *T*-statistics and *P* values for differences between eyes for each variable when analysed according to the order collected (first eye versus second eye, n = 34).

The bold italicised values indicate significant differences between eyes.

Visit	TPC	MMP-9	TIMP-1
Midday	t = 0.74, p = 0.47	t - -0.03, p = 0.98	t = 0.72, p = 0.48
Before sleep	t = -0.16, p = 0.88	t = -1.91, p = 0.06	-
Upon awakening	***t = 2.75, p = 0.009***	t = 1.40, p = 0.17	***t = 2.52, p = 0.02***

Table 3.10: *T*-statistics and *P* values for differences between eyes for each variable when analysed according to right eye versus left eye, incorporating randomisation, n = 34.

Visit	TPC	MMP-9	TIMP-1
Midday	$t = 0.37$, $p = 0.71$	$t = -1.18$, $p = 0.25$	$t = 0.49$, $p = 0.63$
Before sleep	$t = 0.24$, $p = 0.81$	$t = 0.39$, $p = 0.70$	-
Upon awakening	$t = -0.10$, $p = 0.92$	$t = 0.47$, $p = 0.64$	$t = 0.64$, $p = 0.53$

Coefficients of repeatability[233] and limits of agreement were calculated at each time-point and for each protein analysed. This was conducted between replicates taken from the two eyes of each participant when randomised (Table 3.11). The coefficient of repeatability for MMP-9 'upon awakening' indicated a substantial amount of variability between eyes at this time-point.

Because no significant difference was found between eyes when they were randomised for either TPC, MMP-9 or TIMP-1, for the remainder of the analysis eyes were averaged and repeated measures was performed on this.

Table 3.11: Difference in protein concentration between eyes when randomised.

Limits of agreement and coefficients of repeatability for each visit, n = 34.

Visit	Variable	Mean of differences	SD of differences	Limits of agreement	Coefficient of repeatability
Midday	**TPC (mg/mL)**	0.1	1.9	-3.7 to 3.9	3.7
	MMP-9 (ng/mL)	-2.7	11.5	-25.8 to 20.3	22.6
	TIMP-1 (ng/mL)	2.8	44.8	-86.8 to 92.4	87.8
Before sleep	**TPC (mg/mL)**	0.2	3.0	-5.9 to 6.2	5.9
	MMP-9 (ng/mL)	0.1	10.3	-20.5 to 20.7	20.2
Upon awakening	**TPC (mg/mL)**	-0.1	12.0	-24.2 to 23.9	23.6
	MMP-9 (ng/mL)	253.6	2395.8	-4,537.9 to 5,045.2	4,695.7
	TIMP-1 (ng/mL)	-1.6	153.9	-309.4 to 306.2	301.6

3.6.7 Day-to-day repeatability

3.6.7.1 Tear flow-rate

In Study 2 the 'midday' and 'upon awakening' visits were repeated so as to determine the test-retest repeatability at the same time-point. For each of these visits the flow-rate data from the eyes were averaged before assessing the day-to-day repeatability. Visits were not significantly different (F = 0.39, p = 0.55) nor was there a difference between days at the same time-point (F = 0.29, p = 0.87). Table 3.12 shows the limits of agreement and the coefficient of repeatability for tear flow-rate when repeated on two separate occasions for each time-point.

Table 3.12: Difference in tear flow-rate between days at the same time-point.
Limits of agreement and coefficients of repeatability for each visit, n = 8.

Visit	Mean of differences (µL/minute)	SD of differences (µL/minute)	Limits of agreement (µL/minute)	Coefficient of repeatability (µL/minute)
Midday	4.0	5.5	-7.0 to 15.0	10.8
Upon Awakening	-4.8	9.3	-23.4 to 13.8	18.2

3.6.7.2 TPC, MMP-9, TIMP-1 and NGAL

Of the proteins measured, only NGAL at 'midday' was significantly different between days (Table 3.13). Table 3.14 lists the coefficients of repeatability along with the limits of agreement between replicates taken on two days for each protein. The coefficients of repeatability suggest that for this population of eight participants, 'upon awakening' results in a significant amount of variability day-to-day for MMP-9 and NGAL.

Table 3.13: *T*-statistics and *P* values for differences between days for each variable, n = 8.
The bold italicised values indicate significance.

Visit	TPC	MMP-9	TIMP-1	NGAL
Midday	t = -0.03 p = 0.98	t = -1.16 p = 0.29	t = -1.66 p = 0.14	***t = -2.50*** ***p = 0.04***
Upon awakening	t = 0.32 p = 0.75	t = 1.57 p = 0.16	t = 1.41 p = 0.20	t = 1.10 p = 0.31

Table 3.14: Difference in protein concentration between days.

Limits of agreement and coefficients of repeatability for each visit, n = 8.

Visit	Variable	Mean of differences	SD of differences	Limits of agreement	Coefficient of repeatability
Midday	**TPC (mg/mL)**	-0.1	1.0	-2.2 to 1.9	2.0
	MMP-9 (ng/mL)	-5.7	13.9	-33.5 to 22.1	27.3
	TIMP-1 (ng/mL)	-28.2	69.3	-166.7 to 110.3	135.7
	NGAL (ng/mL)	-729.2	852.9	-2,434.9 to 976.5	1,671.6
Upon awakening	**TPC (mg/mL)**	0.0	4.3	-8.6 to 8.6	8.4
	MMP-9 (ng/mL)	64.2	1,134.8	-2,205.4 to 2,333.8	2,224.2
	NGAL (ng/mL)	599.5	2,890.9	-5,182.3 to 6,381.4	5,666.2
	TIMP-1 (ng/mL)	45.5	96.3	-147.2 to 238.1	188.8

As the days were not significantly different, except for NGAL, for the remainder of this chapter days were averaged and repeated measures ANOVA was performed on this.

3.6.8 Difference between studies

3.6.8.1 Tear flow-rate

Because four of the eight participants in Study 2 had also participated in Study 1, this gave the opportunity to confirm that tear flow-rate was not significantly different between the two studies ($F = 4.383$, $p = 0.13$). The limits of agreement and coefficient of repeatability between studies at the same time-point for these four participants are shown in Table 3.15.

Table 3.15: Difference in tear flow-rate between studies at the same time-point for the four participants who took part in both Study 1 and Study 2.

Limits of agreement and coefficients of repeatability for each visit.

Visit	Mean of differences µL/minute	SD of differences µL/minute	Limits of agreement µL/minute	Coefficient of repeatability µL/minute
Midday	3.9	11.5	-19.2 to 26.9	22.6
Upon Awakening	16.5	14.2	-11.8 to 44.8	27.7

The coefficients of repeatability for eyes (Table 3.8), days (Table 3.12) and studies (Table 3.15) are comparable, suggesting that flow-rate is a repeatable variable.

3.6.8.2 TPC, MMP-9 and TIMP-1

The four participants who took part in both Study 1 and Study 2 enabled determination of whether levels of TPC, MMP-9 and TIMP-1 were comparable between studies. There were no significant differences between studies (Table 3.16).

Table 3.16: *T*-statistics and *P* values comparing total protein content (TPC), MMP-9 and TIMP-1 for the two studies, n = 4.

Visit	TPC	MMP-9	TIMP-1
Midday	t = -1.10, p = 0.35	t = -0.40, p = 0.72	t = 1.26, p = 0.30
Upon awakening	t = -0.94, p = 0.42	t = -1.24. p = 0.31	t = -0.29, p = 0.79

Table 3.17 shows the repeatability coefficients for the four participants who took part in both Study 1 and Study 2. The eyes were averaged for Study 1 and the days were averaged for Study 2 and the coefficients of repeatability and limits of agreement were calculated. In doing this, a greater repeatability was obtained than when comparing eyes (Table 3.11) or days (Table 3.14) except for MMP-9 at 'midday'.

Table 3.17: Difference in protein concentration between studies, n = 4.

The eyes were averaged from Study 1 and the days were averaged from Study 2.

Visit	Variable	Mean of differences	SD of differences	Limits of agreement	Coefficient of repeatability
Midday	**TPC (mg/mL)**	-0.3	0.5	-1.2 to 0.7	0.9
	MMP-9 (ng/mL)	-7.0	34.6	-76.2 to 62.3	67.8
	TIMP-1 (ng/mL)	16.1	25.6	-35.0 to 673.3	50.1
Upon awakening	**TPC (mg/mL)**	-2.1	4.6	-11.3 to 7.0	8.9
	MMP-9 (ng/mL)	-829.6	800.4	-2430.4 to 771.2	1568.8
	TIMP-1 (ng/mL)	-10.0	70.2	-150.4 to 130.3	137.5

With this confirmation that the two studies were comparable for these four participants, and eyes and days were not different, the eyes and days were averaged and the common visits from the two studies were combined.

3.6.9 Diurnal variation

3.6.9.1 Tear flow-rate

Because protein concentration can be influenced by tear flow-rate, the factor of flow-rate was considered prior to establishing protein concentration (Table 3.18). The flow-rates were not significantly different between 'midday' and 'upon awakening' ($p = 0.82$). The flow-rate 'before sleep' was significantly greater than that at both the 'midday' ($p = 0.048$) and 'upon awakening' ($p = 0.02$) visits.

Table 3.18: The diurnal variation of the tear flow-rate.

The tear collection flow-rate from the two studies was combined following confirmation that flow-rate did not vary between eyes (when randomised), days or studies. Brackets denote pairs of statistically significant visits ($p < 0.05$).

Visit	Flow-rate (mean ± SD, µL/minute)
Midday (n = 42)	22.8 ± 10.8
Before Sleep (n = 34)	27.9 ± 17.1
Upon Awakening (n = 42)	22.4 ± 12.6

3.6.9.2 TPC

Table 3.19 lists the TPC concentrations at each visit for each study and when the studies were combined. In Study 1 concentrations of TPC were significantly greater 'upon awakening' than both 'midday' and 'before sleep' ($p < 0.001$). 'Midday' and 'before sleep' visits were not significantly different ($p = 0.1$) There were no significant differences between genders ($F = 0.15$, $p = 0.70$). Likewise, for Study 2, TPC was again significantly greater upon awakening compared to midday ($p = 0.02$) and was not significantly different between males and females ($F = 0.04$, $p = 0.86$). When the studies were combined, TPC was again significantly greater upon awakening than at midday ($p > 0.001$) and there was no significant differences between genders ($F = 0.03$, $p = 0.86$).

Table 3.19: The diurnal variation of the total protein content (TPC) in the tear film.

Brackets denote pairs of statistically significant visits ($p < 0.05$).

	Visit	TPC (mean ± SD, mg/mL)
Study 1 (n = 34)	Midday	3.3 ± 1.2
	Before sleep	4.6 ± 3.6
	Upon awakening	15.8 ± 8.6
Study 2 (n = 8)	Midday	2.8 ± 1.8
	Upon awakening	12.4 ± 6.7
Combined (n = 42)	Midday	3.2 ± 1.3
	Upon awakening	15.2 ± 8.5

3.6.9.3 MMP-9

For Study 1, the concentration of MMP-9 was significantly greater 'upon awakening' compared to both 'midday' and 'before sleep' ($p > 0.001$) while the latter two visits were not significantly different ($p = 1.00$). The concentration of MMP-9 was not different between genders ($F = 2.89$, $p = 0.10$). For Study 2, the 'midday' and 'upon awakening' time-points were significantly different ($p = 0.03$) and gender again was not a factor ($F = 3.08$, $p = 0.13$). When the studies were combined, the 'midday' and 'upon awakening' time-points were significantly different ($p < 0.001$) and there was no difference between gender ($F = 3.67$, $p = 0.06$). The concentrations are summarised in Table 3.20.

Table 3.20: The diurnal variation of MMP-9 in the tear film.

Brackets denote pairs of statistically significant visits ($p < 0.05$).

	Visit	MMP-9 (mean ± SD, ng/mL)
Study 1 (n = 34)	Midday	11.3 ± 15.1
	Before sleep	8.7 ± 12.0
	Upon awakening	2,350.4 ± 2,005.3
Study 2 (n = 8)	Midday	3.7 ± 9.4
	Upon awakening	594.7 ± 771.4
Combined (n = 42)	Midday	9.4 ± 14.2
	Upon awakening	2,058.9 ± 1,958.3

3.6.9.4 TIMP-1

TIMP-1 was measured as the main inhibitor of MMP-9 (Section 1.3.3.4). The concentrations are summarised in Table 3.21. This was not measured 'before sleep'. For Study 1, the concentration of TIMP-1 was significantly greater upon awakening

compared to that at midday (p < 0.001). Once again, there were no significant differences between genders (F = 2.18, p = 0.15). For Study 2, TIMP-1 was significantly greater upon awakening compared to before sleep (p = 0.001) and was not significantly different between genders (F = 0.68, p = 0.44). When the two studies were combined, TIMP-1 concentrations were significantly greater upon awakening compared to midday (p < 0.001) with no difference between genders (F = 1.53, p = 0.23).

Table 3.21: The diurnal variation of TIMP-1 in the tear film.

Brackets denote pairs of statistically significant visits (p < 0.05).

Study	Visit	TIMP-1 (mean ± SD, ng/mL)
Study 1 (n = 34)	Midday	72.7 ± 38.0
	Upon awakening	243.0 ± 114.0
Study 2 (n = 8)	Midday	95.8 ± 37.0
	Upon awakening	222.8 ± 65.8
Combined (n = 42)	Midday	73.4 ± 39.7
	Upon awakening	243.9 ± 111.3

3.6.9.5 Ratio of MMP-9:TIMP-1

The ratio of MMP-9:TIMP-1 provides a measure of how much of the MMP-9 measured is potentially inhibited by TIMP-1. These ratios are summarised in Table 3.22. In Study 1, repeated measures ANOVA on the ratio of MMP-9:TIMP-1 indicated that the ratio 'upon awakening' was significantly greater than that at 'midday' (p > 0.001). Results were not significantly different between genders (F = 2.55, p = 0.12). Likewise in Study 2, the ratio of MMP-9:TIMP-1 was significantly greater 'upon awakening' compared to 'midday' (p = 0.01) and gender was not significantly different (F = 4.95, p = 0.07). When the studies were combined, the ratio of MMP-9:TIMP-1 was again significantly greater 'upon awakening' than at 'midday' (p < 0.001) and gender was not significantly different (F = 2.76, p = 0.10).

Table 3.22: The diurnal variation of MMP-9:TIMP-1 in the tear film.

Brackets denote pairs of statistically significant visits ($p < 0.05$).

Study	Visit	Ratio of MMP-9:TIMP-1
Study 1 (n = 34)	Midday	0.4 ± 0.3
	Upon awakening	14.8 ± 17.4
Study 2 (n = 8)	Midday	0.04 ± 0.10
	Upon awakening	2.3 ± 2.5
Combined (n = 42)	Midday	0.3 ± 0.4
	Upon awakening	12.6 ± 13.4

3.6.9.6 NGAL

NGAL was significantly elevated 'upon awakening' compared to 'midday' (3,559.0 ± 1,948.7 ng/mL and 733.3 ± 533.6 ng/mL respectively, $p < 0.001$, Figure 3.7) with no significant differences between genders ($F = 0.87$, $p = 0.39$).

3.6.9.7 Ratio of MMP-9:NGAL

The ratio of MMP-9:NGAL upon awakening was significantly greater than that at midday (0.01 ± 0.04 and 0.14 ± 0.13, $p = 0.003$, Figure 3.8). There were significant differences between genders, with males having significantly greater ratios upon awakening than females and the reverse at midday ($F = 6.11$, $p = 0.048$).

3.6.9.8 Clinical correlations

Correlation analysis did not show any correlation between the diurnal change in clinical values and the diurnal change in the proteins measured.

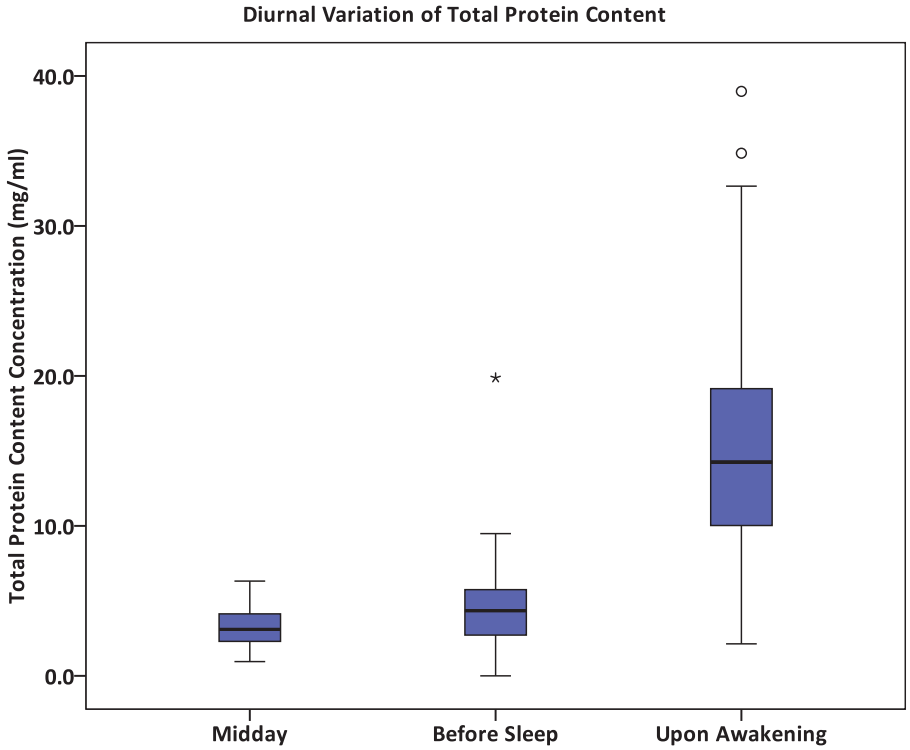


Figure 3.3: The diurnal variation of the total protein content in the tear film.

'Upon awakening' was significantly greater than the other two visits ($p < 0.001$, $n = 42$ at 'midday' and 'upon awakening' and $n = 34$ 'before sleep').

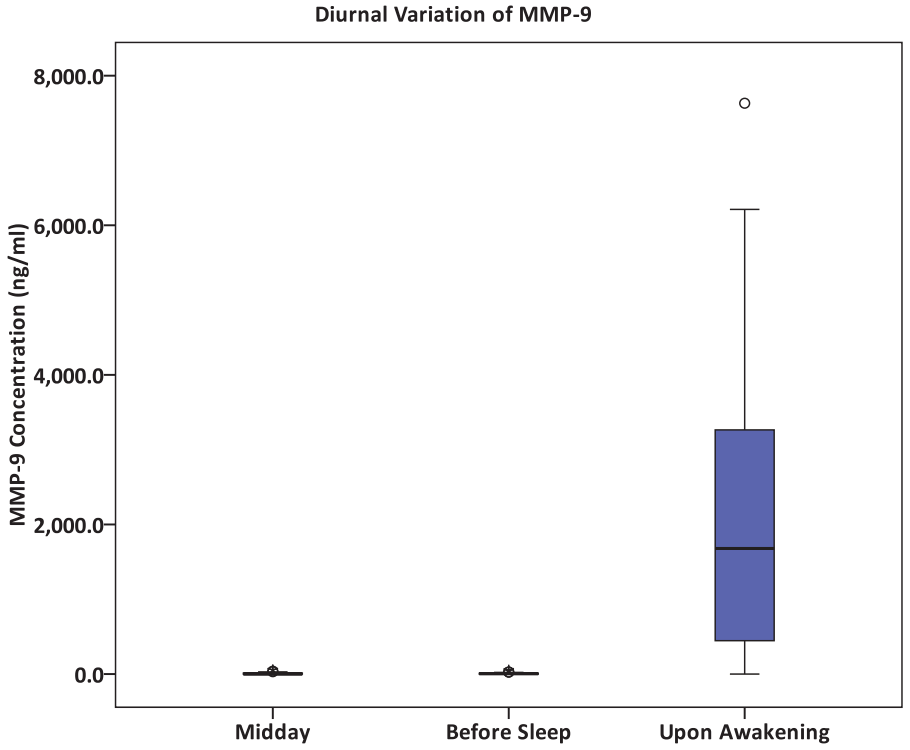


Figure 3.4: The diurnal variation of MMP-9 in the tear film.

'Upon awakening' was significantly greater than the other two visits ($p < 0.001$, $n = 42$ and at 'midday' and 'upon awakening' and $n = 34$ 'before sleep').

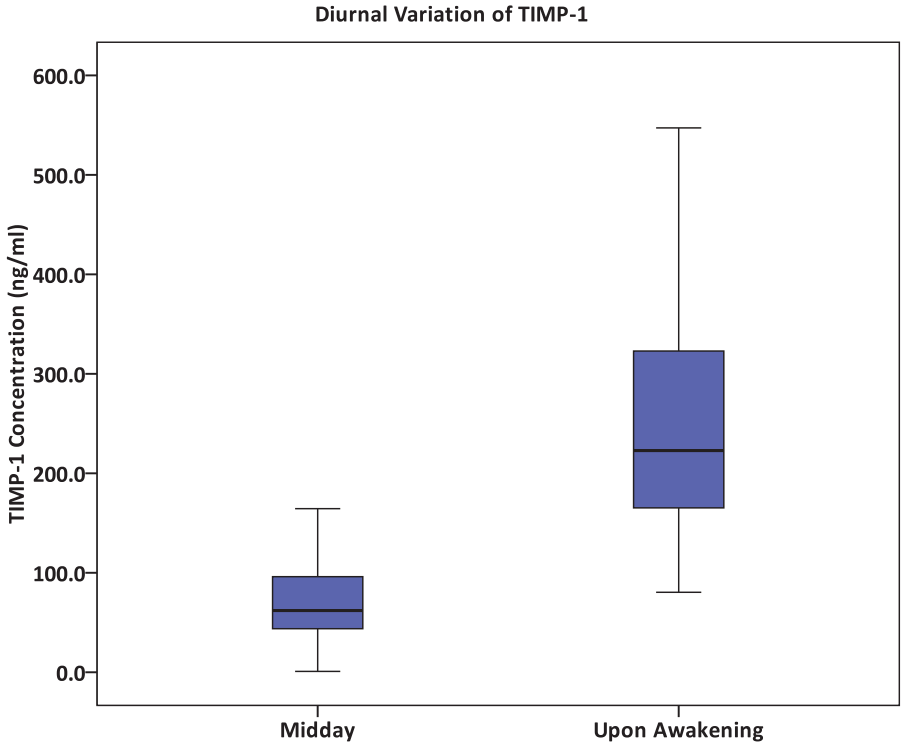


Figure 3.5: The diurnal variation of TIMP-1 in the tear film.

Upon awakening was significantly greater than at midday ($p < 0.001$, $n = 42$).

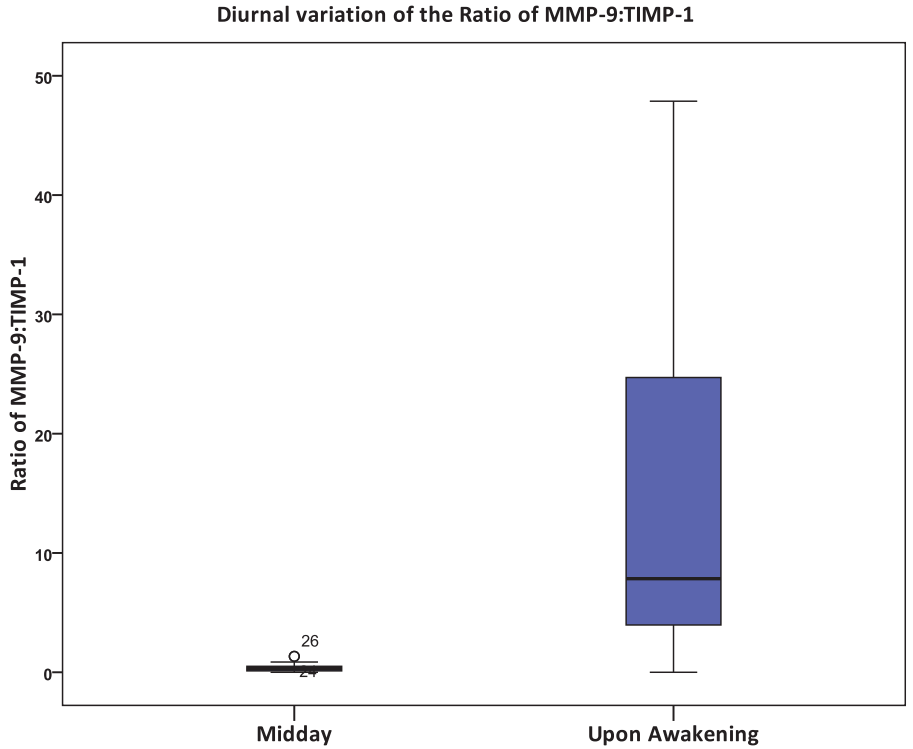


Figure 3.6: The diurnal variation of the ratio of MMP-9:TIMP-1 in the tear film.

Upon awakening was significantly greater than at midday ($p < 0.001$, $n = 42$).

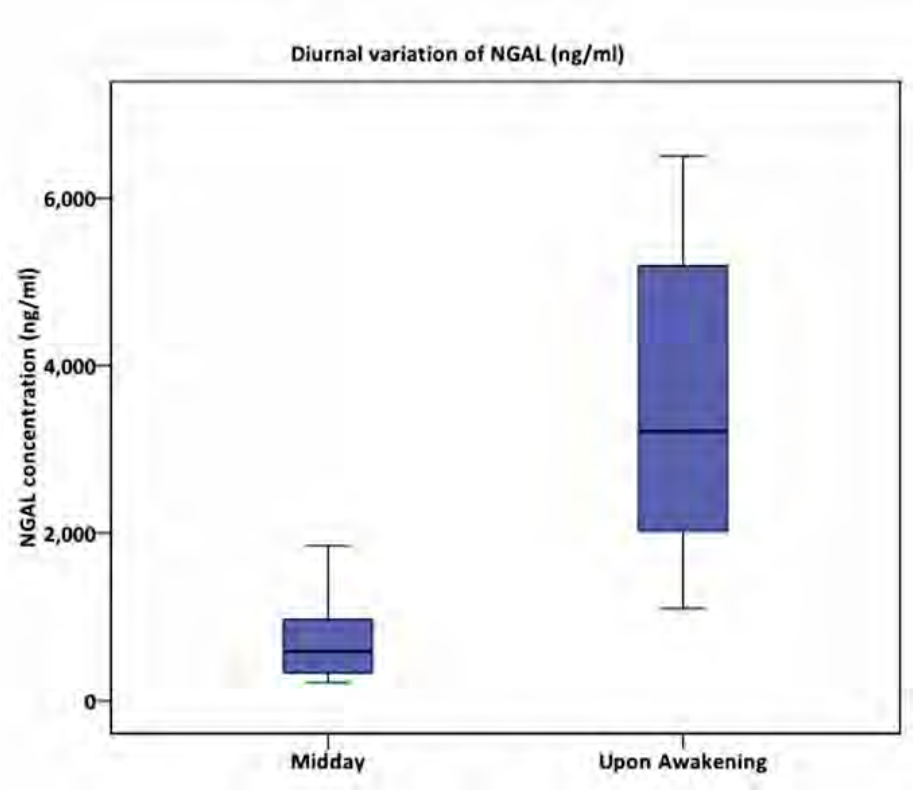


Figure 3.7: The diurnal variation of NGAL in the tear film.

'Upon awakening' NGAL was significantly elevated compared to 'midday' ($p < 0.001$, $n = 8$).

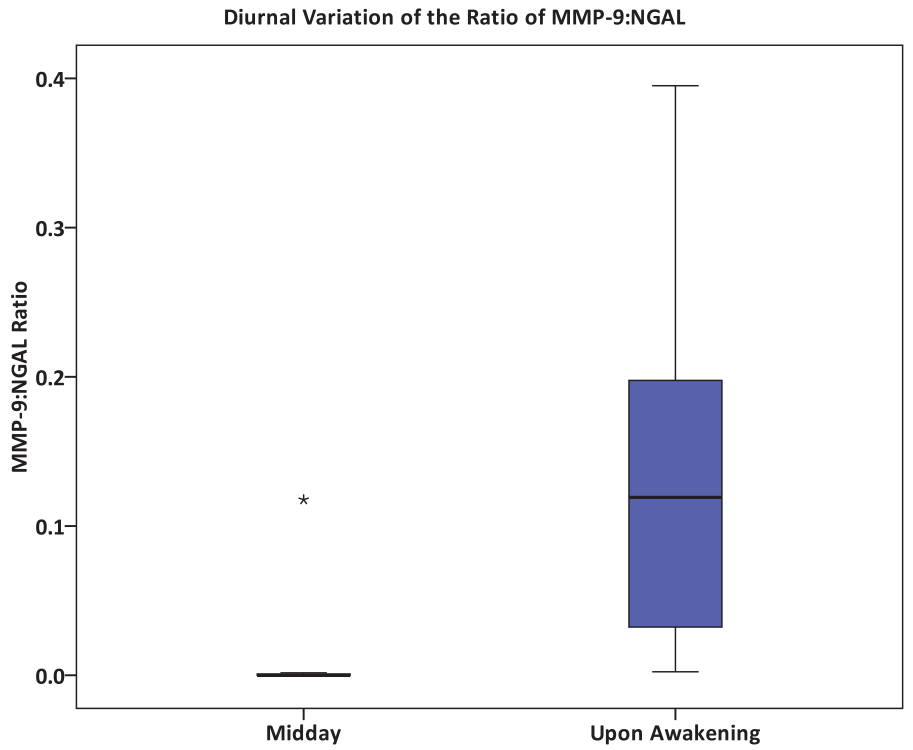


Figure 3.8: The diurnal variation of the MMP-9:NGAL ratio in the tear film.

'Upon awakening' was significantly greater than 'midday' with NGAL exceeding the concentration of MMP-9 at both time-points ($p = 0.003$, $n = 8$).

3.6.9.9 Zymography

Figure 3.9 shows a Coomassie Blue-stained gel of tear samples of two non-contact lens wearing participants at 'midday' and 'upon awakening'. Each sample was added in both a 5- and 10-µg protein mass. The 10 µg mass was chosen for subsequent gels as a means of better discerning bands. The clear bands against the stained gelatin background are areas of gelatinolytic activity.[247] Overnight closure resulted in prominent bands at 92, 135 and >200 kDa (lanes 2-5, Figure 3.9). These bands are consistent with those previously identified by others and correspond to pro-MMP-9, NGAL or α_2-macroglobulin complexes not dissociated by SDS, and a dimer of MMP-9.[186, 188, 249, 250] At midday (lanes 6-9, Figure 3.9), faint bands were detected at 135 kDa and 92 kDa, corresponding to the MMP-9 complex and pro-MMP-9 respectively. The difference between the 'upon awakening' lanes and the 'midday' lanes is consistent with the diurnal variation of MMP-9 measured with ELISA in Section 3.6.9.3 .

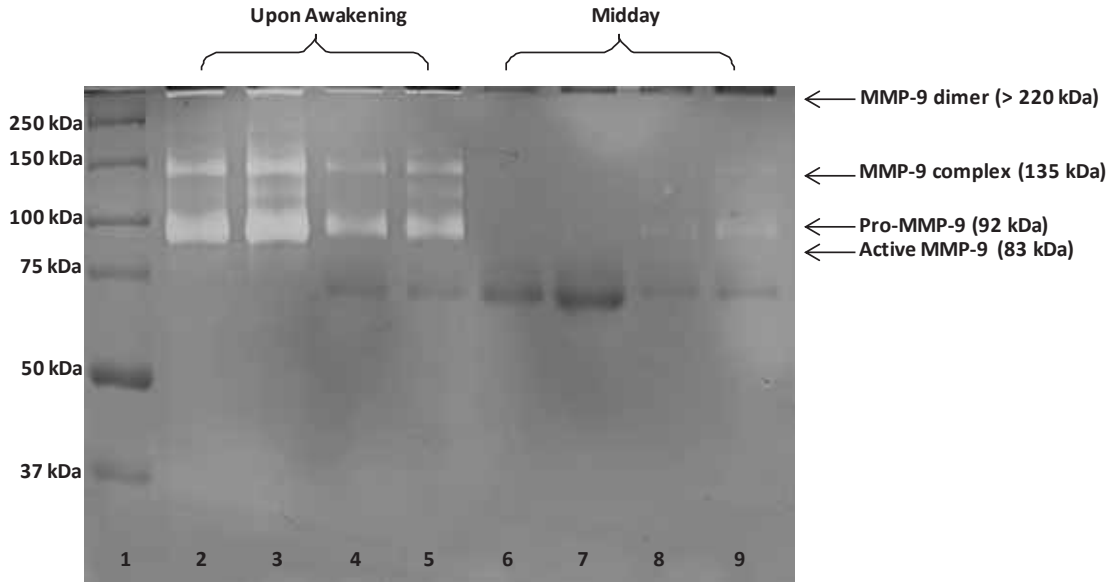


Figure 3.9: Coomassie Blue-stained zymogram of tears 'upon awakening' and at 'midday'.

Lane 1 is the molecular weight marker and lanes 2 and 3 are 'upon awakening' tears of one participant with a 5- and 10-µg load respectively, while lanes 4 and 5 are those of a second participant, also 'upon awakening' tears with a 5- and 10-µg load respectively. Lanes 6 and 7 are the 'midday' tears of one participant with a 5- and 10-µg load respectively, and lanes 8 and 9 are the 'midday' tears of a second participant, also with a 5- and 10-µg load.

3.7 SUMMARY AND DISCUSSION

3.7.1 Summary of results

This chapter investigated the diurnal variation of MMP-9, its inhibitor TIMP-1 and its regulator NGAL in the healthy tear film. A 200-fold up-regulation in MMP-9 concentration levels was found upon awakening compared to the levels at midday and before sleep. This 200-fold increase in total MMP-9 upon awakening was not proportional to the five-fold increase in TPC or the three-fold increase in TIMP-1. The ratio of MMP-9 to TIMP-1 was found to be 0.3 at midday, indicating an excess of TIMP-1 during open eye periods. This ratio was significantly reversed by 40-fold upon awakening, with more MMP-9 than TIMP-1 present in the tear film. NGAL exceeded the concentration of MMP-9 both at midday and upon awakening.

3.7.2 Variability within a normal population

In this chapter, eye-to-eye variability was established by comparing the concentration of the proteins measured from the first eye to those of the second eye (Table 3.9) prior to randomisation. The first eye collected was found to have a significantly higher concentration than the second eye upon awakening for two of the three proteins compared. This difference could potentially be attributable to some reflex tearing occurring following eye opening, hence reducing the concentration measured in the second eye collected. Alternatively, by the time the second eye is measured, (there was approximately two minutes between eyes), the concentration levels are already starting to return to midday levels. When randomisation was incorporated, this effect was no longer apparent (Table 3.10), a factor that was mirrored by the tear collection flow-rate. Tear flow-rate was significantly greater in the first eye collected, the reverse of that expected in view of the higher concentration in the first eye. This phenomenon highlights the importance of randomising eyes and factoring tear flow-rate in studies involving tear collection.

The coefficients of repeatability presented in Table 3.11, Table 3.14 and Table 3.17 show that considerable variability was present in the levels of all the measured proteins both within subjects and between measurement occasions. This behaviour

suggests that within the normal population the concentration of MMP-9 varies substantially, particularly upon awakening.

No difference was found between genders for the proteins measured.

3.7.3 MMP-9 in the diurnal tear film

MMP-9 is a vital but potentially destructive enzyme by virtue of its ability to degrade corneal collagen. Control of this activity is thus critical to maintaining corneal health. When the eyes are open, there is little cause for concern as, in agreement with previous studies,[9-11, 14, 29-31] this data suggest that MMP-9 concentrations are negligible and significantly exceeded by those of the inhibitor molecule, TIMP-1. The potential for proteolytic activity is thus inhibited and hence the corneal epithelium and basement membrane are protected from degradation.

During sleep however, this situation changes dramatically as MMP-9 concentration increases roughly 200-fold, while TIMP-1 barely triples. The shift in the balance from TIMP-1 to MMP-9 dominance, which may be a downstream effect of increased neutrophil activity,[135, 226] suggests that during sleep there may be an increased propensity for basement membrane degradation. In reality however, such tissue damage was not evident among the participants in this study, nor does it occur overnight in the general population. Accordingly it seems likely that other mechanisms are in place to protect the ocular surface at this time. Zymography indicates that the MMP-9 in the closed eye tear film is in the zymogen form rather than the active form (Figure 3.9), suggesting that this may be one mechanism. Additionally, the dimeric form of MMP-9 detected at > 220 kDa does not differ in terms of enzymatic activity from its monomeric form at 92 kDa. When MMP-9 is present in excess of that which can be inhibited by TIMP-1, it forms dimers. This dimer however, is much more stable and is activated with lower efficiency by MMP-3,[251] potentially being another regulatory mechanism against excess extracellular matrix degradation.[249] It may be that MMP-9 is also being inhibited by other proteins such as the non-specific inhibitor α_2-macroglobulin, an inhibitor known to be present in the closed eye.[252] The band identified by zymography at 135 kDa is thought to be a complex of pro-MMP-9 to either α_2-macroglobulin or NGAL. NGAL, unlike TIMP-1 and α_2-macroglobulin, is

thought to protect MMP-9 from degradation, rather than inhibit it, hence increasing its activity. NGAL increases greatly in the presence of epithelial injury and inflammation.[156, 160] In this chapter, NGAL was found to exceed the concentration of MMP-9, both at midday and upon awakening, a finding that was in agreement with previous studies.[135] Sack et al. found significant levels of both TIMP-1 and NGAL complexed to MMP-9 suggesting that MMP-9 is of mixed origin in the open eye.[135] In the closed eye, they found that the majority of MMP-9 was complexed to NGAL, indicative of the greater contribution by neutrophils and suggestive of the sub-clinical inflammatory state of the closed eye.[135] The apparent protective role of NGAL to MMP-9 suggests that during sleep, their complex enables MMP-9 to act on the extracellular matrix. This diurnal variation may be necessary to allow for increased cell desquamation during sleep. The diurnal variation of TIMP-1 is such to counteract the actions of MMP-9 sufficiently to allow matrix remodelling while preventing excess degradation. This period, however, may also be the period where the cornea is most vulnerable to any disturbances, such as those associated with extended wear contact lenses.

3.7.4 Implications of findings

3.7.4.1 Corneal erosions and conditions exacerbated upon awakening

Many conditions such as recurrent corneal erosions and corneal ulcers are known to be exacerbated by eye closure.[20] Due to its action on collagen and hence the anchoring complexes of the corneal epithelium, MMP-9 could contribute to the increased incidence of recurrent erosions by virtue of its diurnal variation, particularly when the regulatory mechanisms are not controlled.[78, 253] The diurnal profile of MMP-9 and its associated factors could also indicate why the epithelium is particularly prone to erosion and infection with overnight contact lens wear,[5, 13] as well as corneal ulceration.[187,48, 93, 133]

3.7.4.2 Treatment of ocular surface conditions

An understanding of the diurnal profile of MMP-9 could be used to provide clinical advice in the form of the best treatment times in cases of corneal inflammation,

ulceration or recurrent erosions. As there is a significant increased expression of MMP-9 during sleep, it may be best to treat ocular surface conditions vigorously before sleep and immediately upon awakening in order to reduce the increase in MMP-9 that naturally occurs overnight. The lack of correlation between clinical signs of redness and MMP-9 in this study suggests that even without the clinical signs of redness, the concentrations of MMP-9 could still be significant.

3.7.4.3 Glaucoma medication

Finally, it may be that these results also have implications for certain topical therapies, in particular the use of prostaglandin analogues. These compounds are used for the treatment of glaucoma and work by increasing the concentration of MMP-9[254] as a means of degrading ciliary muscle extracellular matrix and increasing uveo-scleral outflow.[255] Concurrent increased MMP-9 concentrations have also been found in the tear film,[256] increasing the potential for corneal damage as has been found in both animal[257] and human studies.[258] Despite this, only one study has reported on the occurrence of erosions in those being treated with prostaglandins.[258] It may then be advisable to adjust the instillation timing for prostaglandin drops to avoid the periods before sleep and immediately upon awakening, particularly for those already prone to corneal erosions.

3.7.5 Limitations of study

A limitation of this study was that no anti-proteolytic agents were used for storing samples. A recent study by Tarr and colleagues has shown that storage of blood plasma in similar conditions to this study, without anti-proteolytic agents, does not affect the stability of MMP-9 and TIMP-1, even after a period of three years.[259] This finding is opposed to the previous results of Rouy et al, who showed a decrease in active MMP-9 after storage under similar circumstances.[260] Future work assessing the stability of MMP-9 in tear samples, both with and without anti-proteolytic agents would establish whether anti-proteolytic agents are necessary to prevent MMP degradation during storage.

Participants with diabetes were excluded from this study. This exclusion was based on self-report which allows for the possibility of undiagnosed diabetes. In diabetes, increased MMP-9 has been associated with poor wound healing of foot ulcers,[261] whilst CD 147, a transmembrane protein known to regulate MMPs, has been shown to increase in expression in diabetes, and also increase MMP-9.[262] Additionally, a recent study has shown that MMP-9 is elevated in the retinas and microvessels of rats with diabetic retinopathy.[263] It is not known whether undiagnosed diabetes could have influenced the results of this study.

3.8 OUTCOMES OF CHAPTER 3

Chapter 3 has shown that there is a substantial diurnal variation of MMP-9 and its associated factors in tear film of healthy non-contact lens wearers, a variation greater than that used to estimate the study sample size.[197] Concentrations of MMP-9 are negligible during the day and completely inhibited by TIMP-1. Upon awakening MMP-9 increases 200-fold, an increase that is not completely inhibited by TIMP-1. This diurnal change, along with the presence of NGAL which protects MMP-9 from degradation, suggests that the closed eye is an environment conducive to extracellular matrix remodelling. The fact that we typically do not see corneal damage upon awakening suggests that other regulatory mechanisms are active.

As contact lenses are in close proximity to the tear film and the corneal epithelium, it is hypothesised that contact lens wear causes an increase in MMP-9, particularly when worn on an extended wear schedule. An understanding of the normal diurnal variation established in this chapter allows the exploration of how this profile is impacted by contact lens wear in Chapter 4.

CHAPTER 4 THE EFFECT OF CONTACT LENS WEAR ON THE MMP-9 DIURNAL PROFILE

4.1 OVERVIEW

The study described in Chapter 3 demonstrated the diurnal profile of matrix metalloproteinase-9 (MMP-9) and its associated factors in the healthy, non-contact lens wearing eye and established that MMP-9 expression is 200-fold greater upon awakening. This dramatic shift during sleep may be necessary for the regeneration that occurs overnight. The effect of contact lens wear on this profile is unknown. To explore this in this chapter, the same group of participants from Chapter 3 were fitted with contact lenses in either a daily wear (DW) or extended wear (EW) schedule. Their tears were collected during the first day of contact lens wear and after one month of wear and analysed for concentrations of MMP-9, tissue inhibitor of metalloproteinases-1 (TIMP-1) and neutrophil gelatinase-associated lipocalin (NGAL).

The work presented in this chapter was presented at the Tear Film and Ocular Surface meeting in 2010, the Cornea and Contact Lens Society of Australia meeting 2010 and the British Contact Lens Association meeting in 2011 (Appendix B: Publications and presentations).

4.2 INTRODUCTION

The diurnal variation of MMP-9, as demonstrated in Chapter 3, suggests that during sleep, the cornea is an environment conducive to extracellular matrix remodelling. This is also the period when the cornea is more prone to erosion events both with[25] and without[20, 264] contact lens wear. In non-contact lens wearers susceptible to corneal erosion, an increase in MMP-9 expression has been found.[93, 101, 102] In this thesis it is hypothesised that the erosions that occur in contact lens wear are in part due to a contact lens-related increase in MMP-9, hence weakening the epithelial adherence to the stroma and making the cornea more susceptible to erosion (Chapter 1, Section 1.7). In this chapter the hypothesis tested is that contact lens wear is associated with an increase in MMP-9.

Contact lens-related corneal erosions have been reported with both hydrogel and silicone hydrogel materials.[10, 11, 21, 22] Of the two studies reporting rates of erosions in DW, estimated rates equated to 0.04 per 100 participant-months[9] (one case only) and 0.01-0.05% of visits[13] respectively. No events were seen with daily disposable use.[13] In EW, erosions have been found to occur at a greater rate, occurring in 0.6-2.6% of visits, with silicone hydrogels having the higher rate (0.95-1.68% of visits compared to 0.05-0.35% of visits for hydrogels).[13] Sixty-eight percent of erosions in this study occurred within the first three months of contact lens wear,[13] suggesting that, as with microbial keratitis,[5] the adaptation process is a factor.

In this chapter, the effect of EW and DW on MMP-9 compared to no contact lens wear (as established in Chapter 3), as well as the change with adaptation is explored.

4.3 AIMS

This study therefore aims to explore:

1. how early EW and DW of contact lenses influences the diurnal cycle of MMP-9 and other related proteins in the tear film
2. how adaptation to EW and DW of contact lenses influences the diurnal cycle of MMP-9 and other related proteins in the tear film
3. whether contact lens type plays a role in the MMP-9 profile

4.4 HYPOTHESIS

In accordance with previously established biochemical changes associated with EW[206, 209, 244] as compared to DW[68, 193, 201] as well as the greater risk of erosion,[13, 25] infection and inflammation[1, 5] associated with EW, the hypothesis of this chapter is that contact lens wear increases the expression of MMP-9 in the tear film when worn on an EW schedule when compared to the baseline profile and the profile in DW.

4.5 METHODS

4.5.1 Ethics approval

This study was a continuation of the study presented in Chapter 3. As with Chapter 3, this study was approved by the institutional ethics committee of the University of New South Wales (UNSW) and the VIHEC human ethics committee with ratification from UNSW and followed the tenets of the Declaration of Helsinki 1975 as amended in 2000. Informed consent was obtained from the same participants as in Chapter 3, Section 3.5.1.

4.5.2 Sample size calculation

Sample size was determined to permit stratification into four groups. Sample size was determined based on a previous study which used equivalent techniques to those used in this study,[197] such that approximately seven participants in each group were required in order to demonstrate a statistically significant difference of 400 ± 300 ng/mL in MMP-9 concentration at a confidence level of 95% power of 80%.

4.5.3 Inclusion and exclusion criteria

The same participants from Chapter 3 advanced to contact lens wear in this study and the same inclusion and exclusion criteria were set (Sections 3.5.3 and 3.5.4). Participants were healthy and were not taking any medications for any general health conditions. A requirement of the study was that the participants were either non-contact lens wearers or non-habitual contact lens wearers who had not worn lenses in the month preceding the study. Pregnant or lactating women were excluded from the study.

4.5.4 Study design

This was a prospective, bilateral, open label clinical study where the same participants from Chapter 3 were randomised using a computer-generated randomisation table to wear either lotrafilcon B (O_2OPTIX™, CIBA VISION™, Duluth, Atlanta, USA) or senofilcon A (ACUVUE® OASYS™, Johnson & Johnson Vision Care, Rochester, NY, USA) with the choice of extended wear (EW) or daily wear (DW) for one month (Figure 4.1).

O_2OPTIX™ was chosen for its higher modulus, while ACUVUE® OASYS™ was chosen for its lower modulus, to determine whether contact lens type impacted on the MMP-9 profile.

Figure 4.1 demonstrates the study sample size allocation. One participant from the DW group in ACUVUE® OASYS™ crossed over to the EW group, also in ACUVUE® OASYS™, following a one month washout period (Figure 4.1). Four of the participants in the DW group also participated in the EW phase one year later with O_2OPTIX™ (Study 2, Figure 4.1). Study 2 mirrored Study 1 except that the visit 'before sleep' was excluded (Figure 4.2 and as described in Chapter 3, Section 3.5.5.2).

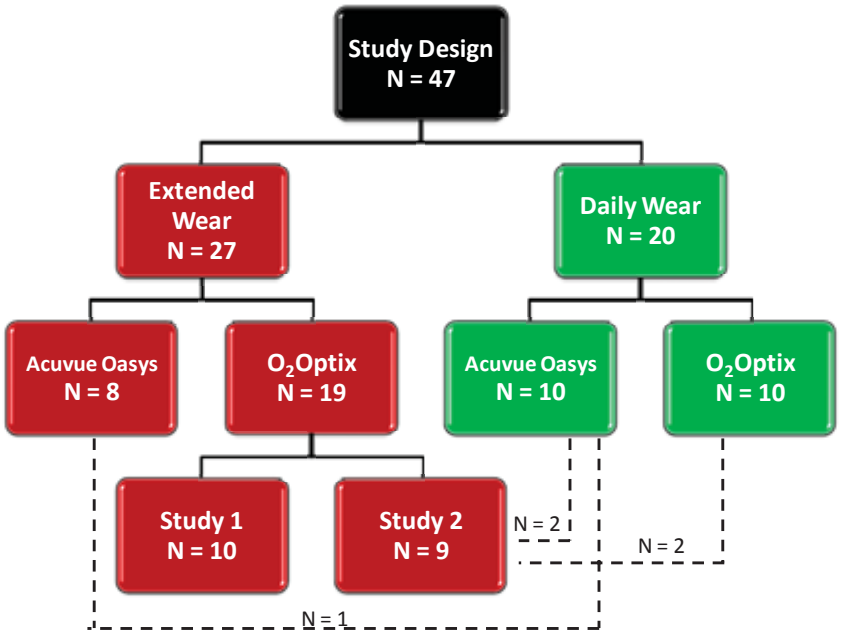


Figure 4.1: Study sample size allocation.

A total of 47 participants were randomised to ACUVUE® OASYS™ or O_2OPTIX™, with the choice of wear schedule. The dotted lines refer to the number of participants who crossed over from daily wear (DW) to extended wear (EW). One participant wore Acuve Oasys for one month on a DW schedule and after a one month washout period wore ACUVUE® OASYS™ in EW. Four participants from the DW group also participated in the EW group following a one year gap.

4.5.4.1 Visit schedule

Following the baseline visits, as described in Section 3.5.5, participants attended for a contact lens fit and a one month visit, where tears were collected at midday, before

sleep and upon awakening (Figure 4.2). A one week visit was included as a safety check. For Study 2 (Figure 4.1), the 'before sleep' visit was excluded, as indicated by the asterisks in Figure 4.2.

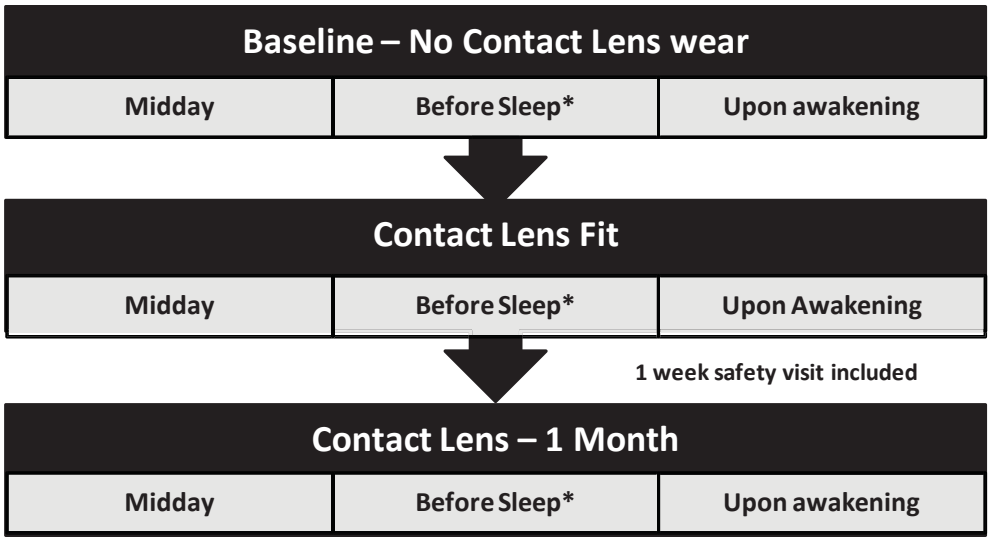


Figure 4.2: The visit schedule for all participants (n = 47).

The asterisk (*) highlights the 'before sleep' visits which were not conducted during Study 2 (n = 9). For the extended wear (EW) group, the 'upon awakening' visit at the 'contact lens fit' was the first night of continuous wear, while for the daily wear (DW) group, this was without contact lens in situ. Similarly, at the 'one month' visit, the 'upon awakening' visit for the EW group involved continuous overnight contact lens wear and for the DW group no contact lenses were worn.

4.5.4.2 Instructions to participants

4.5.4.2.1 General advice

All participants were instructed on contact lens handling (including the ability to tell if a contact lens was inside-out), insertion and removal, as well as maintenance of contact lenses. Moreover, the participants were advised on the signs and symptoms of adverse events and were issued with an after-hours contact number to contact should these occur. Participants were also advised to wear the contact lenses on a one month replacement schedule. Contact lenses were disinfected daily for the DW group and upon removal for the EW group. General hygiene as expected for contact lens wear, such as washing and drying of hands prior to removal, insertion or handling of contact

lenses was advised. Participants were taught contact lens insertion and removal at the contact lens fit 'midday' visit (Figure 4.2).

4.5.4.2.2 Wear regime

The EW group was instructed to wear the contact lenses a minimum of five days and nights per week for up to one month of wear (with five days leeway). The DW group was instructed to wear the contact lenses a minimum of five days per week for six hours per day with no maximum time provided the contact lenses were not worn overnight.

4.5.5 Contact lenses and solutions

Participants were randomised to either O_2OPTIX™ (CIBA VISION™ corporation, Duluth, Atlanta, USA) or ACUVUE® OASYS™ (Johnson & Johnson Vision Care, Rochester, NY, USA, Table 4.1) for Study 1, while all nine participants in Study 2 were allocated to O_2OPTIX™. The randomisation was allocated using randomisation.com (seed 10074 for the EW group and seed 6092 for the DW group).

Participants were supplied with sufficient contact lenses (including a spare pair) and contact lens care product to enable them to maintain wearing schedules between study visits as per protocol for the duration of the study.

Table 4.1: Contact lens parameters.

Parameter	O_2OPTIX™	ACUVUE® OASYS™
Manufacturer	CIBA VISION™	Johnson & Johnson Vision Care
Material	lotrafilcon B	senofilcon A
Power range	+6.00 to -10.00 D	+8.00 to -12.00 D
Diameter (mm)	14.2	14.0
Base Curve used (mm)	8.6	8.4
Water content (%)	33	38
Centre Thickness at -3.00 D (mm)	0.08	0.07
Dk/t at -3.00 D	138	147
Elastic Modulus, MPa	0.83	0.49
FDA group	1	1

Abbreviations: D, dioptre; DK/t, oxygen transmissibility; FDA, Food and Drug Administration

The contact lens care product used in the study was AOSept® Plus, sometimes referred to as Clear Care® (CIBA VISION™, Duluth, Atlanta, USA, Table 4.2). It is used to clean

and disinfect contact lenses, but the solution cannot be placed directly in the eye as it contains 3% hydrogen peroxide. The solution must be used in conjunction with a case supplied containing a platinum disc which catalyses the reaction converting hydrogen peroxide into oxygen and water over a six hour period. The surfactant cleaner contained in AOSept® Plus cleans the contact lens via the agitation caused by oxygen released during breakdown of the hydrogen peroxide. The reason AOSept® Plus was chosen is because it has been associated with reduced solution induced corneal staining and hence infiltrative events[47] as compared to preservative based multipurpose solutions.[9]

Unit-dose sterile 0.9% unpreserved saline (sodium chloride injection 0.9%, AstraZeneca, North Ryde, Australia) was supplied for rinsing purposes or as an in-eye lubricant as required.

Table 4.2: Care solution components.

Contact lens solution	Company	Composition	Description of use
AOSept® Plus	CIBA VISION™	Hydrogen peroxide 3%, phosphonic acid, sodium chloride, a phosphate buffer system, pluronic surfactant	Multipurpose disinfecting solution for contact lenses

4.5.6 Clinical techniques

After completing the baseline visits (Table 3.1), participants proceeded to contact lens wear. The clinical techniques used at each time-point at the contact lens fit, the one week visit and the one month visit are outlined below in Table 4.3, Table 4.4 and Table 4.5, respectively. The order of tests was such to minimise the impact on the ocular surface prior to tear collection. As such, visual acuity and evaluation of the cornea and conjunctiva without fluorescein were conducted prior to tear collection while fluorescein evaluation and lid eversion were conducted following tear collection as per Table 4.3, Table 4.4 and Table 4.5.

Visual acuity and slit-lamp biomicroscopy was performed as per Section 3.5.6.1. Subjective refraction was performed at the baseline visit (Table 3.1) to measure

sphere, cylinder and axis for each person so that the appropriate contact lens power could be prescribed.

Table 4.3: The clinical techniques at each time-point for the contact lens fit.

Procedures	Midday	Before sleep	Upon awakening
Visit window	Between 11 AM and 2 PM	Before sleep, same day as 'midday'	Immediately upon awakening, morning after 'midday'
Informed consent	N (obtained previously, Table 3.1)	N	N
Meet inclusion/exclusion criteria	N	N	N
Demographics	N	N	N
History at baseline	N	N	N
Updated history/any medical problems	Y	Y	Y
Baseline information: - spectacle refraction - visual acuity - keratometry	N	N	N
Corneal topography	N	N	N
Symptoms and problems	Y	Y	Y
Wear time	N	N	N
Visual acuity	Y	N	N
Slit-lamp biomicroscopy: Redness, contact lens fit Evaluation of cornea and conjunctiva including fluorescein Upper tarsus (fluorescein on indication)	Y	N	Y
Photos/video	*	*	*
Contact lens fitting	Y	N	N
Contact lens returns	N	N	N
Tear collection	Y	Y	Y
Adverse event data	Y**	Y**	Y**
Visit summary	Y	Y	Y

Y = Yes, required information, N = No, not required

* *At optometrists discretion*

** *If adverse event detected at time of visit*

Table 4.4: The clinical techniques at the one week visit and in the case of unscheduled visits.

Procedures	One week visit	Unscheduled visits/ adverse events
Visit window	7 days ± 3 days from the contact lens fit, between 11 AM and 2 PM	N/A
Informed consent	N	N
Meet inclusion/exclusion criteria	N	N
Demographics	N	N
History at baseline	N	N
Updated history/any medical problems	Y	Y
Baseline information: - spectacle refraction - visual acuity - keratometry	N	*
Corneal topography	N	N
Symptoms and problems	Y	Y
Wear time	Y	Y
Visual acuity	Y	Y
Slit-lamp biomicroscopy: Redness, contact lens fit Evaluation of cornea and conjunctiva including fluorescein Upper tarsus (fluorescein on indication)	Y	Y
Photos/video	*	*
Aseptic contact lens removal	N	*
Lid and conjunctiva swabs	N	*
Contact lens fitting	Y	N*
Contact lens returns	N	N
Tear collection	Y	Y
Adverse event data	Y**	Y
Visit summary	Y	Y

Y = Yes, required information, N = No, not required

* *At optometrists discretion*

** *If adverse event detected at time of visit*

Table 4.5: The clinical techniques at each time-point at the one month visit.

Procedures	Midday	Before sleep	Upon awakening
Visit window	Between 11 AM and 2 PM	Before sleep, same day as 'midday'	Immediately upon awakening, morning after 'midday'
Informed consent	N	N	N
Meet inclusion/exclusion criteria	N	N	N
Demographics	N	N	N
History at Baseline	N	N	N
Updated History/any medical problems	Y	Y	Y
Baseline information: - spectacle refraction - visual acuity - keratometry	N	N	N
Corneal topography	N	N	N
Symptoms and Problems	Y	Y	Y
Wear time	Y	N	N
Visual acuity	Y	N	N
Slit-lamp biomicroscopy: Redness, contact lens fit Evaluation of cornea and conjunctiva including fluorescein Upper tarsus (fluorescein on indication)	Y	N	Y
Photos/video	*	*	*
Contact lens fitting	Y	N	N
Contact lens returns	Y	N	N
Tear collection	Y	Y	Y
Adverse event data	Y**	Y**	Y**
Visit summary	Y	Y	Y

Y = Yes, required information, N = No, not required
** At optometrists discretion*
*** If adverse event detected at time of visit*

4.5.6.1 Assessment of contact lens fit

All quantitative measures of centration, movement and lag were made using a graticule eyepiece. At 16x slit-lamp magnification, the measurement limit of the graticule is 0.1 mm.

Primary gaze movement was measured as the change in position of the contact lens edge after a blink, while the participant is fixating in the primary gaze.

Contact lens tightness was assessed using the 'push-up' test. The inferior contact lens edge was pushed up by digitally manipulating the lower lid. Tightness was rated as a percentage using the following guidelines: 100% = 'contact lens cannot be moved';

0% = 'contact lens held in place by lid tension and falls from cornea as soon as lower lid is moved prior to push-up'; and 40% - 50% = 'optimum: easy and smooth movement when pushed with the lower lid. The contact lens moves with the lid and does not resist movement.'

4.5.6.2 Adverse event procedures

Adverse events were categorised according to the definitions set by the Brien Holden Vision Institute.[4] An optometrist (MM) was present or on call for the duration of the study. Participants were advised to inform the optometrist immediately of any event not normal, for example, unusual redness, pain or irritation. At the clinic, the eye was briefly assessed using a slit-lamp biomicroscope under low illumination to ascertain the nature of the condition, if any. If an adverse event occurred, the appropriate procedures were conducted, including referral for medical treatment and swabs, if necessary (Table 4.4). Any adverse events were followed until complete resolution to the reasonable satisfaction of the participant and the optometrist.

4.5.6.3 Tear collection and tear sample treatment

Flush tears[202] were collected from both eyes at midday, before sleep and immediately upon waking, the order of eyes being randomised. This was performed as per Chapter 2, Section 2.5.3.3, at the baseline, contact lens fit and one month visits as per Figure 4.2. Tears were centrifuged and aliquoted as per Section 3.5.6.3.1.

4.5.7 Laboratory techniques

4.5.7.1 Total protein content

Total protein content (TPC) was determined using the bicinchoninic acid method (BCA) and using Pierce reagents and flat-bottom Nunc-F Maxisorp 96-well microplates (Thermo Fisher Scientific, Rochester, NY, USA) as per Chapter 2, Section 2.5.5.

4.5.7.2 MMP-9 assay

Total MMP-9 concentration was determined using sandwich enzyme-linked immunosorbent assay (ELISA) with the RnD Systems Inc DuoSet kit (Minneapolis, MN, USA) as per Section 3.5.7.2.

4.5.7.3 TIMP-1 assay

TIMP-1 was determined using ELISA with the RnD Systems Inc DuoSet kit (Minneapolis, MN, USA) as per Section 3.5.7.3.

4.5.7.4 NGAL assay

NGAL was determined using ELISA with the 'human Lipocalin-2/NGAL' RnD Systems Inc DuoSet kit (DY1757, Minneapolis, MN, USA) as per Section 3.5.7.4. NGAL was not measured for the DW schedule due to limited tear volume.

4.5.7.5 Zymography

Zymography was conducted as per Section 3.5.7.5. The tears 'upon awakening' from six participants wearing O_2OPTIX™ in EW were compared at baseline, after the first night of EW and after one month of adaptation.

4.5.8 Statistical analysis

Normality was tested using the Shapiro-Wilk test. Repeated measures analysis of variance (ANOVA) was used to analyse the visits. Mauchly's test was used to verify the sphericity function. Where main effects were significant, Student t-tests with Bonferroni correction were used post hoc. PASW version 18.0 (Chicago, USA) was used for the analysis. Results are reported as mean ± standard deviation (SD). The relationship between protein concentrations at various time-points was investigated using Pearson's correlation coefficient.

4.5.8.1 The use of expectation maximisation for missing variables

In some cases it was not always possible to have measurements due to inadequate sample volume. In those cases where data were missing, in order to permit the ANOVA to proceed, replacement was made using expectation maximisation (EM) and any negative values generated by the process were replaced with zero. In cases where concentration levels were not detectable, these samples were allocated a value of zero. Because it was shown that right and left eyes were not significantly different in Section 3.6.6, the right and left eye of each participant were averaged for this analysis.

4.6 RESULTS

4.6.1 Study enrolment

A total of 47 participants were enrolled in the study. Table 4.6 and Table 4.7 show the number of participants enrolled and the number who completed each phase of the study for both EW and DW. Table 4.8 and Table 4.9 display the study discontinuations for either of the two wear modalities.

Table 4.6: Study enrolment and attendance for the extended wear group.

Study indicators	Baseline	Contact lens fit	One week visit	One month visit
Participants enrolled in study	27			
Number attended this visit	27	26	25	23
Number missed this visit	0	0	0	0
Permanent discontinuations at this visit or prior to next visit	1	1	2	0
Cumulative discontinuations in study	1	2	4	4

Table 4.7: Study enrolment and attendance for the daily wear group.

Study indicators	Baseline	Contact lens fit	One week visit	One month visit
Participants enrolled in study	20			
Number attended this visit	20	19	19	18
Number missed this visit	0	0	0	0
Permanent discontinuations at this visit or prior to next visit	1	0	1	0
Cumulative discontinuations in study	1	1	2	2

Table 4.8: Discontinuations in the extended wear group.

Participant Number	Visit	Reason for discontinuation
PhD2008-002-018	Baseline (before sleep)	Participant choice
PhD2008-002-011	Contact lens fit (before sleep)	Participant unhappy with vision
PhD2008-002-004	One week visit	Erosion formation and discomfort
PhD2010-004	Unscheduled (13 days after the one week visit)	Erosion formation and discomfort

Table 4.9: Discontinuations in the daily wear group.

Participant Number	Visit	Reason for discontinuation
PhD2008-003-007	One week visit	Participant choice – contact lens discomfort
PhD2008-003-015	Unscheduled (phonecall prior to contact lens fit)	Participant opted to discontinue due to recent onset illness

4.6.2 Demographics

Table 4.10 presents the mean age ± SD for the DW and EW groups as well as the balance of males and females in each group.

Table 4.10: Study demographics for the extended wear (EW) and daily wear (DW) groups.

Study group	Age Mean ± SD	Males	Females	Total number of participants
EW	27 ± 7 years	14	13	27
DW	29 ± 6	6	14	20

4.6.3 Adverse events

There were four adverse events in this study – three corneal erosions and one contact lens peripheral ulcer. The data of these participants who experienced an adverse event were excluded from the subsequent analysis. Their data are presented in Chapter 6 as a comparison with the normal profile established in this chapter. The total sample size for the subsequent analysis was 43, that is, 18 for the DW group and 25 for EW group.

4.6.4 Clinical variables

Table 4.11 and Table 4.12 present the clinical variables at each visit.

Table 4.11: Extent of corneal staining (mean ± SD) in each corneal area as graded with the Brien Holden Vision Institute scale.[240]

EW = extended wear, DW = daily wear.

Visit	Group	Area 1	Area 2	Area 3	Area 4	Area 5
Baseline – Midday	EW	0.0 ± 0.1	0.1 ± 0.3	0.0 ± 0.0	0.1 ± 0.3	0.2 ± 0.5
	DW	0.1 ± 0.2	0.0 ± 0.2	0.0 ± 0.0	0.1 ± 0.3	0.1 ± 0.3
Baseline – Upon Awakening	EW	0.1 ± 0.5	0.1 ± 0.3	0.0 ± 0.0	0.1 ± 0.2	0.2 ± 0.4
	DW					
Contact Lens Fit – Midday	EW	0.0 ± 0.2	0.0 ± 0.1	0.0 ± 0.1	0.1 ± 0.3	0.2 ± 0.5
	DW	0.0 ± 0.0	0.0 ± 0.2	0.0 ± 0.0	0.1 ± 0.2	0.2 ± 0.4
Contact Lens Fit – Upon Awakening	EW	0.1 ± 0.4	0.2 ± 0.4	0.1 ± 0.3	0.1 ± 0.2	0.3 ± 0.5
	DW	0.1 ± 0.3	0.1 ± 0.3	0.1 ± 0.2	0.0 ± 0.2	0.3 ± 0.5
1 Month – Midday	EW	0.1 ± 0.3	0.0 ± 0.1	0.0 ± 0.0	0.2 ± 0.4	0.3 ± 0.5
	DW	0.0 ± 0.0	0.0 ± 0.2	0.0 ± 0.0	0.1 ± 0.3	0.4 ± 0.6
1 Month – Upon Awakening	EW	0.1 ± 0.4	0.0 ± 0.2	0.0 ± 0.1	0.1 ± 0.3	0.4 ± 0.6
	DW	0.0 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.6

Table 4.12: Clinical variables at each visit for the extended (EW) and daily wear (DW) groups (mean ± SD) as graded with the Brien Holden Vision Institute scale.[240]

Variables	Visit	EW	DW
Blepharitis (0-4, 0.5)	Baseline – Midday	0.8 ± 0.6	0.6 ± 0.5
	Baseline – Upon Awakening		
	Contact Lens Fit – Midday	0.8 ± 0.6	0.5 ± 0.5
	Contact Lens Fit – Upon Awakening		
	1 Month – Midday	0.9 ± 0.5	0.4 ± 0.4
	1 Month – Upon Awakening		
Meibomian gland dysfunction (0-4, 0.5)	Baseline – Midday	0.9 ± 0.7	0.8 ± 0.7
	Baseline – Upon Awakening		
	Contact Lens Fit – Midday	1.0 ± 0.6	0.6 ± 0.5
	Contact Lens Fit – Upon Awakening		
	1 Month – Midday	1.1 ± 0.6	0.6 ± 0.5
	1 Month – Upon Awakening		
Limbal redness (0-4, 0.5)	Baseline – Midday	1.2 ± 0.4	1.0 ± 0.3
	Baseline – Upon Awakening	1.4 ± 0.5	1.3 ± 0.3
	Contact Lens Fit – Midday	1.1 ± 0.5	1.0 ± 0.1
	Contact Lens Fit – Upon Awakening	1.8 ± 0.6	1.3 ± 0.3
	1 Month – Midday	1.1 ± 0.6	1.1 ± 0.4
	1 Month – Upon Awakening	1.5 ± 0.4	1.1 ± 0.4
Bulbar redness (0-4, 0.5)	Baseline – Midday	1.3 ± 0.5	1.1 ± 0.3
	Baseline – Upon Awakening	1.5 ± 0.6	1.3 ± 0.4
	Contact Lens Fit – Midday	1.2 ± 0.5	1.2 ± 0.2
	Contact Lens Fit – Upon Awakening	2.0 ± 0.6	1.4 ± 0.4
	1 Month – Midday	1.1 ± 0.6	1.1 ± 0.4
	1 Month – Upon Awakening	1.5 ± 0.5	1.3 ± 0.3
Palpebral redness (0-4, 0.5)	Baseline – Midday	1.1 ± 0.4	0.9 ± 0.5
	Baseline – Upon Awakening		
	Contact Lens Fit – Midday	1.1 ± 0.3	1.0 ± 0.3
	Contact Lens Fit – Upon Awakening		
	1 Month – Midday	1.3 ± 0.4	1.1 ± 0.3
	1 Month – Upon Awakening		
Tarsal abnormalities (0-4, 0.5)	Baseline – Midday	1.1 ± 0.4	0.9 ± 0.3
	Baseline – Upon Awakening		
	Contact Lens Fit – Midday	1.1 ± 0.4	1.0 ± 0.1
	Contact Lens Fit – Upon Awakening		
	1 Month – Midday	1.2 ± 0.3	1.0 ± 0.3
	1 Month – Upon Awakening		
Conjunctival staining (0-4, 0.5)	Baseline – Midday	0.1 ± 0.3	0.2 ± 0.3
	Baseline – Upon Awakening		
	Contact Lens Fit – Midday	0.1 ± 0.2	0.1 ± 0.2
	Contact Lens Fit – Upon Awakening	0.9 ± 0.5	0.2 ± 0.3
	1 Month – Midday	1.1 ± 0.5	0.7 ± 0.4
	1 Month – Upon Awakening	1.0 ± 0.4	0.3 ± 0.4

Variables	Visit	EW	DW
Microcysts (0-4, 0.5)	Baseline – Midday	0.0 ± 0.0	0.0 ± 0.0
	Baseline – Upon Awakening		
	Contact Lens Fit – Midday	0.0 ± 0.0	0.0 ± 0.0
	Contact Lens Fit – Upon Awakening	0.0 ± 0.0	0.2 ± 1.0
	1 Month – Midday	0.0 ± 0.1	0.0 ± 0.0
	1 Month – Upon Awakening	0.0 ± 0.2	0.1 ± 0.8
Striae/Oedema (0-4, 0.5)	Baseline – Midday	0.0 ± 0.0	0.0 ± 0.0
	Baseline – Upon Awakening		
	Contact Lens Fit – Midday	0.0 ± 0.0	0.0 ± 0.0
	Contact Lens Fit – Upon Awakening	0.0 ± 0.0	0.0 ± 0.2
	1 Month – Midday	0.0 ± 0.0	0.0 ± 0.0
	1 Month – Upon Awakening	0.0 ± 0.0	0.0 ± 0.0

4.6.5 Normality

Normality checks were performed and reported in Chapter 3, Section 3.6.5.

4.6.6 Confirmation of uniformity of groups at baseline

Prior to performing the analysis, a one-way ANOVA was conducted to confirm that the groups were not statistically different at baseline, hence ensuring that any differences found in subsequent analysis was in fact due to the factors being tested, that is, contact lens wear, wear modality and contact lens type. Where the assumption of homogeneity was violated, robust tests of equality of means were used (Brown-Forsythe).

When the groups that were subsequently allocated to DW and EW were compared at baseline, that is, prior to contact lens wear, none of the measured proteins were statistically different as shown in Table 4.13. Similarly, when each variable at baseline was compared between the groups according to the contact lens type to which they were subsequently allocated, no difference was found (Table 4.13).

The groups were then further split according to the wear schedule and contact lens type. In this way, MMP-9 upon awakening prior to contact lens wear, was significantly different between the four groups (p = 0.02, Table 4.13). Specifically, the group wearing ACUVUE® OASYS™ in EW was statistically greater than both the DW group in ACUVUE® OASYS™ (p = 0.04) and the O_2OPTIX™ group in EW (p = 0.02) as shown in Figure 4.3.

Age was not statistically different between the four groups (p = 0.78).

As the four groups differed for MMP-9 upon awakening at baseline, for the subsequent analysis when comparing the four groups according to contact lens type and wear schedule, this was conducted by comparing the change from baseline at the same time-point.

Table 4.13: ***P* values when comparing the extended wear (EW) and daily wear (DW) groups at baseline, the contact lens types at baseline, and when split into four groups according to contact lens type and wear schedule.**

The bold italicised values indicate significant differences

Variable	*P* value (EW *vs.* DW)	*P* value (ACUVUE® OASYS™ *vs.* O_2OPTIX™)	*P* value (contact lens type and wear schedule = four groups)
TPC – Midday	0.46	0.32	0.51
TPC – Upon Awakening	0.70	0.52	0.12
MMP-9 – Midday	0.13	0.92	0.41
MMP-9 – Upon Awakening	0.80	0.28	*0.02*
TIMP-1 – Midday	0.77	0.06	0.30
TIMP-1 – Upon Awakening	0.55	0.58	0.74

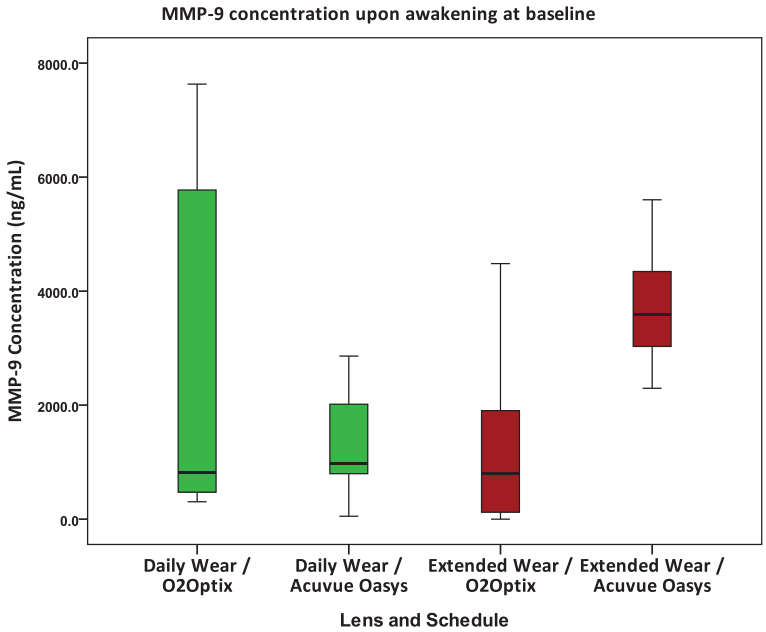


Figure 4.3: MMP-9 concentration at baseline 'upon awakening' for each group.

The group that was later fitted with ACUVUE® OASYS™ in extended wear had significantly greater baseline levels of MMP-9 than both the daily wear group in ACUVUE® OASYS™ (p = 0.04) and the O_2OPTIX™ group in extended wear (p = 0.02).

4.6.7 The diurnal variation in contact lens wear

Primary analysis with repeated measures ANOVA for each of the variables measured was initially conducted with the in-subject factors of *day* and *time-point,* where *day* refers to either the baseline, contact lens fit, or one month visits and *time-point* refers to either midday, before sleep, or upon awakening. *Wear schedule, gender* and *contact lens type* were included as with-in subject factors. Scrutiny of the output indicated significant interaction between the levels of *wear schedule* (that is EW versus DW) and its interactions with *day* and *time-point*. No significant interactions were found for *gender*. For the subsequent analysis, *gender* was therefore excluded and subsequent repeated measures ANOVA was conducted for both *wear schedules* for each of the variables measured.

4.6.7.1 TPC

Table 4.14 lists the TPC concentration at each time-point for the DW and EW schedules. For the DW group, the diurnal profile did not vary statistically, either at the contact lens fit or at the one month visit when compared to the baseline profile (that is, there was no interaction between *time-point* and *day*, F= 0.82, p = 0.45). Similarly for the EW group, the TPC was not significantly different (F = 0.55, p = 0.58). In both DW and EW, the contact lens types did not differ as there was no interaction between *time-point* and *contact lens* (F = 1.89, p = 0.19 and F = 0.78, p = 0.39 respectively).

Table 4.14: The diurnal variation of the total protein content (TPC, mean ± SD) at baseline, the contact lens fit and after one month.

The diurnal profile did not vary statistically from baseline at any visit (p = 0.45 for extended wear and 0.58 for daily wear). N = 18 for the daily wear group and n = 25 for the extended wear group, except for 'before sleep' where n = 17.

Day	Time-point	Extended wear (mg/mL)	Daily wear (mg/mL)
Baseline	Midday	3.1 ± 1.3	3.4 ± 1.4
	Before Sleep	5.5 ± 4.7	3.8 ± 2.0
	Upon awakening	14.8 ± 8.4	15.8 ± 8.8
Contact lens fit	Midday	2.3 ± 1.5	2.8 ± 1.9
	Before Sleep	3.9 ± 2.1	3.0 ± 1.8
	Upon awakening	15.3 ± 8.9	13.4 ± 6.8
One Month	Midday	3.0 ± 1.9	2.8 ± 2.1
	Before Sleep	4.0 ± 2.2	3.1 ± 2.4
	Upon awakening	13.7 ± 9.2	12.8 ± 7.5

4.6.7.2 MMP-9

Table 4.15 lists the MMP-9 concentration at each time-point for the DW and EW schedules. For the DW group, the diurnal profile did not vary statistically from baseline, either at the contact lens fit, or at the one month visit (that is, there was no interaction between *day* and *time-point*, $F = 0.51$, $p = 0.61$).

For the EW group there was a significant interaction between *day* and *time-point* ($F = 6.88$, $p = 0.002$). Specifically, at one month, the midday concentrations of MMP-9 were significantly greater than those at baseline and at the contact lens fit ($p = 0.03$ and 0.009 respectively). Furthermore, the concentration of MMP-9 upon awakening after the first night of EW, was significantly greater than that at baseline ($p = 0.02$) but not significantly different to the concentration at one month upon awakening ($p = 0.08$). The baseline and one month visits upon awakening were not significantly different ($p = 0.63$). As demonstrated in Figure 4.7, the variance upon awakening after the first night of EW was much greater than that at baseline and than the DW group at the same time-point (Figure 4.6).

Table 4.15: The diurnal variation of MMP-9 (mean ± SD) at baseline, the contact lens fit and after one month of contact lens wear.

Brackets denote pairs of statistically significant visits at the same time-point ($p < 0.05$). N = 18 for the daily wear group and n = 25 for the extended wear group, except for 'before sleep' where n = 17.

Day	Time-point	Extended wear (ng/mL)	Daily wear (ng/mL)
Baseline	Midday	6.6 ± 9.9	13.2 ± 18.3
	Before Sleep	10.7 ± 14.7	6.7 ± 8.7
	Upon awakening	2,123.3 ± 1,762.8	1,969.4 ± 2,252.3
Contact lens fit	Midday	4.8 ± 9.8	3.9 ± 6.3
	Before Sleep	3.4 ± 5.7	3.1 ± 6.8
	Upon awakening	3,598.7 ± 3,229.1	2,373.0 ± 2,091.6
One month	Midday	16.9 ± 19.6	12.2 ± 20.6
	Before Sleep	19.5 ± 13.8	14.2 ± 14.3
	Upon awakening	2,408.2 ± 1,376.1	2,584.7 ± 1,774.2

The two contact lens types did not differ at any of the visits for either the DW or EW schedule.

For the EW group, there was a strong positive correlation between the concentration of MMP-9 upon awakening at baseline and at the contact lens fit ($r = 0.66$, $p < 0.001$)

and the one month visit (r = 0.78, p < 0.001). The contact lens fit and one month visit also correlated upon awakening (r = 0.68, p < 0.001). For the DW group, no correlation was present between the baseline levels upon awakening at the contact lens fit (r = 0.16, p = 0.54) but a moderate correlation was found at the one month visit (r = 0.53, p = 0.02).

4.6.7.3 TIMP-1

Table 4.16 lists the concentrations of TIMP-1 at each time-point for the DW and EW schedules. For the DW group, the profile did not vary statistically between the midday visits (p = 0.10), but upon awakening, the contact lens fit was significantly greater than the baseline (p = 0.02). For the EW group, the diurnal profile did not vary statistically, from baseline, either at the contact lens fit, or at the one month visit (p = 0.17).

There was a moderate correlation between the baseline upon awakening and that at the contact lens fit (r = 0.51, p = 0.03) for the DW group, while there was no such correlation for the EW group.

Table 4.16: The diurnal variation of TIMP-1 (mean ± SD) at baseline, the contact lens fit and after one month of contact lens wear.

Brackets denote pairs of statistically significant visits (p < 0.05). N = 18 for the daily wear group and n = 25 for the extended wear group.

Day	Time-point	Extended wear (ng/mL)	Daily wear (ng/mL)
Baseline	Midday	75.0 ± 36.0	71.2 ± 45.2
	Upon awakening	235.1 ± 88.4	256.2 ± 139.0
Contact lens fit	Midday	56.5 ± 44.3	85.1 ± 88.9
	Upon awakening	320.4 ± 278.6	396.6 ± 221.7
One month	Midday	63.7 ± 60.5	111.5 ± 90.2
	Upon awakening	283.0 ± 129.5	350.1 ± 144.2

4.6.7.4 Ratio of MMP-9:TIMP-1

Table 4.17 lists the MMP-9:TIMP-1 ratios at each time-point for the DW and EW schedules. A ratio less than 1 indicates an excess of TIMP-1, while a ratio greater than 1 indicates an excess of MMP-9. For the DW group, the diurnal profile did not vary statistically from baseline, either at the contact lens fit, or at the one month visit (that is, there was no interaction between *time-point* and *day*, F = 2.66, p = 0.09).

For the EW group, the ratio at midday after one month of contact lens wear was significantly greater compared to midday at baseline and midday at the contact lens fit (p = 0.004 and 0.007 respectively). The midday ratio at baseline and the contact lens fit were not significantly different (p = 1.00). The shift in the ratio from TIMP-1 dominance before contact lens wear at midday (ratio of 0.7 ± 0.2) to MMP-9 dominance after a month of contact lens wear (ratio of 1.2 ± 1.4) reinforces the increased expression of MMP-9 in EW that is not matched by TIMP-1.

Upon awakening for the EW group, the ratio was significantly greater after the first night of contact lens wear than the one month visit (p = 0.048) but not different to baseline.

As with the concentration of MMP-9 for the EW group, the ratio upon awakening at the baseline visit correlated significantly with that at the contact lens fit (r = 0.72, p < 0.001) and the one month visit (r = 0.72, p < 0.001). There were no correlations for the DW group.

Table 4.17: The diurnal variation of the ratio of MMP-9:TIMP-1 (mean ± SD) at baseline, the contact lens fit and after one month of contact lens wear.

Brackets denote pairs of statistically significant visits (p < 0.05). N = 18 for the daily wear group and n = 25 for the extended wear group.

Day	Time-point	Extended wear (ng/mL)	Daily wear (ng/mL)
Baseline	Midday	0.7 ± 0.2	0.5 ± 0.4
	Upon awakening	13.2 ± 13.4	11.8 ± 13.7
Contact lens fit	Midday	0.2 ± 0.3	0.3 ± 0.2
	Upon awakening	18.6 ± 19.9	5.4 ± 4.7
One month	Midday	1.2 ± 1.4	2.1 ± 3.5
	Upon awakening	10.4 ± 7.7	11.4 ± 9.0

4.6.7.5 NGAL

Table 4.18 lists the NGAL concentration at each time-point for the EW schedule. At midday, the contact lens fit was significantly less than the one month concentrations of NGAL (p = 0.01) but not significantly different to baseline (p = 0.055). NGAL was not significantly different upon awakening for any of the three days (p = 0.80).

The visits upon awakening did not correlate. The midday visit at the contact lens fit, however, correlated with the one month upon awakening visit (r = 0.76, p = 0.03).

Table 4.18: The diurnal variation of NGAL (mean ± SD) at baseline, the contact lens fit and after one month.

Brackets denote pairs of statistically significant visits (n = 8, p < 0.05).

Day	Time-point	Extended wear (ng/mL)
Baseline	Midday	733.3 ± 533.6
	Upon awakening	3,559.0 ± 1,948.7
Contact lens fit	Midday	192.6 ± 59.6
	Upon awakening	4,369.1 ± 1,667.8
One month	Midday	351.9 ±136.9
	Upon awakening	4,036.8 ± 1,889.1

4.6.7.6 Ratio of MMP-9:NGAL

Table 4.19 lists the MMP-9:NGAL ratios at each time-point and day for the EW group. The diurnal profile did not vary statistically from baseline, either at the contact lens fit, or at the one month visit (p = 0.95).

There was a strong correlation between the baseline ratio at midday and that at the same time-point at the contact lens fit (r = 0.99, p < 0.001). The ratio upon awakening at baseline correlated with the same time-point at the contact lens fit (r = 0.84, p = 0.01) and the one month visit (r = 0.78, p = 0.02).

Table 4.19: The diurnal variation of the ratio of MMP-9:NGAL at baseline, the contact lens fit and after one month at each time-point (n = 8).

Day	Time-point	Extended wear (ng/mL)
Baseline	Midday	0.01 ± 0.04
	Upon awakening	0.14 ± 0.13
Contact lens fit	Midday	0.07 ± 0.18
	Upon awakening	0.21 ± 0.18
One month	Midday	0.09 ± 0.13
	Upon awakening	0.21 ± 0.33

4.6.7.7 Clinical associations

There was no correlation between MMP-9 and bulbar redness or limbal redness upon awakening when considered as the change between the baseline and the contact lens fit (r = 0.36, p = 0.15 and r = 0.27, p = 0.10 respectively). Similarly, there was no correlation between clinical signs and TPC or TIMP-1.

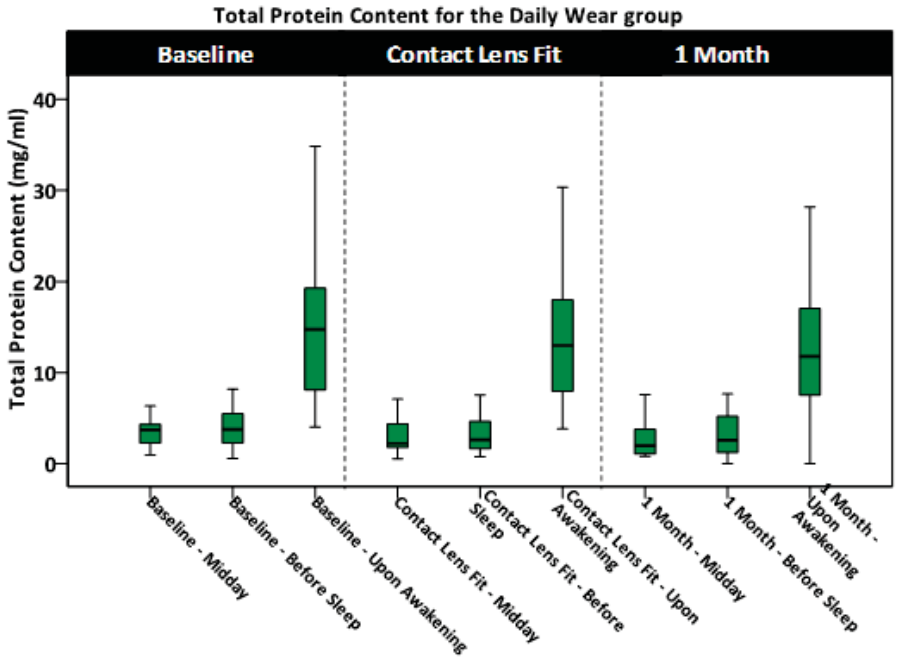


Figure 4.4: Total protein content for the daily wear group.

The diurnal profile did not vary statistically, either at the contact lens fit or at the one month visit when compared to the baseline profile (p = 0.45, n = 18).

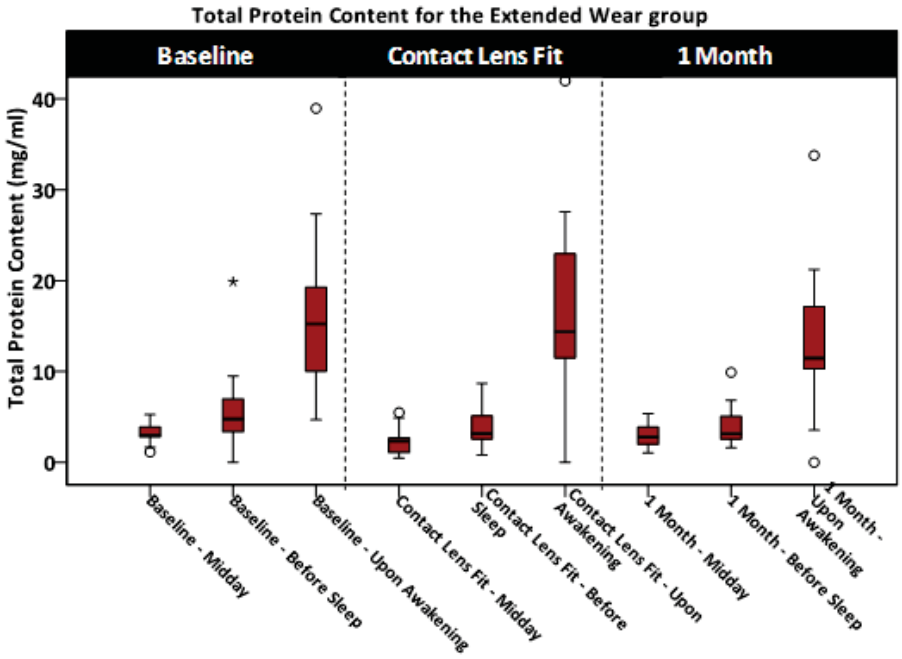


Figure 4.5:Total protein content for the extended wear group.

The diurnal profile did not vary statistically, either at the contact lens fit or at the one month visit when compared to the baseline profile (p = 0.58, n = 25 except before sleep where n = 17).

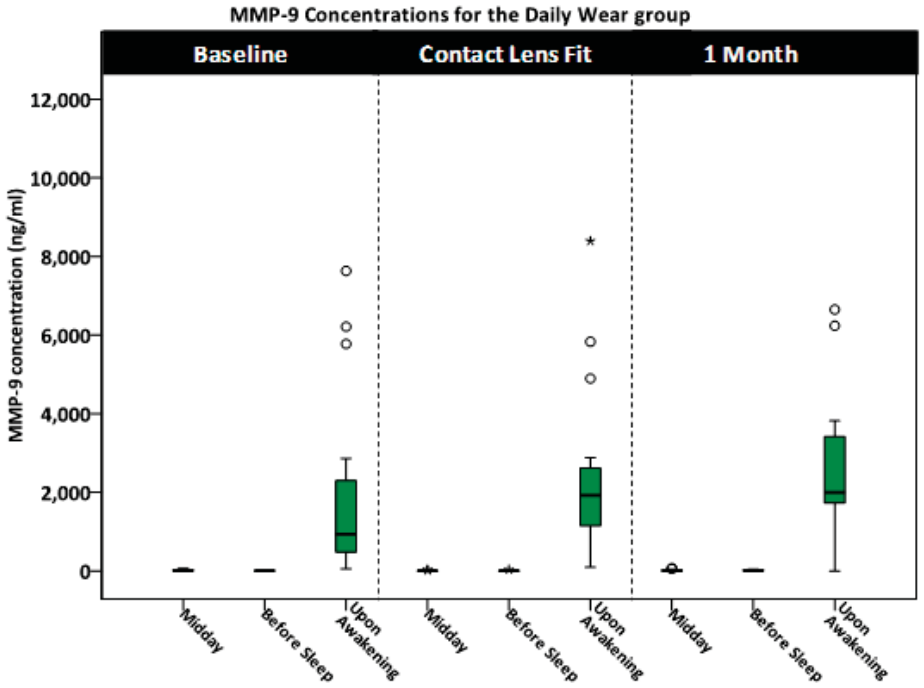


Figure 4.6: MMP-9 concentration for the daily wear group.

The diurnal profile did not vary statistically from baseline, either at the contact lens fit, or at the one month visit (p = 0.61, n = 18).

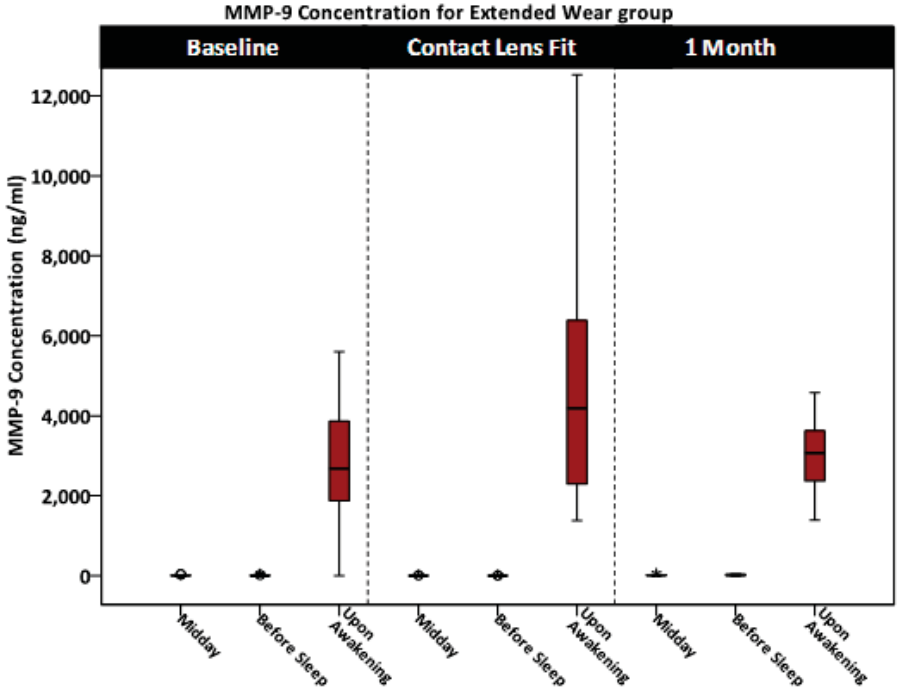


Figure 4.7: MMP-9 concentration for the extended wear group.

After the first night of extended wear, MMP-9 was significantly greater than baseline (p = 0.02) but not significantly different than one month (p = 0.08). The baseline and one month visits were not significantly different (p = 0.63, n = 25 except before sleep where n = 17).

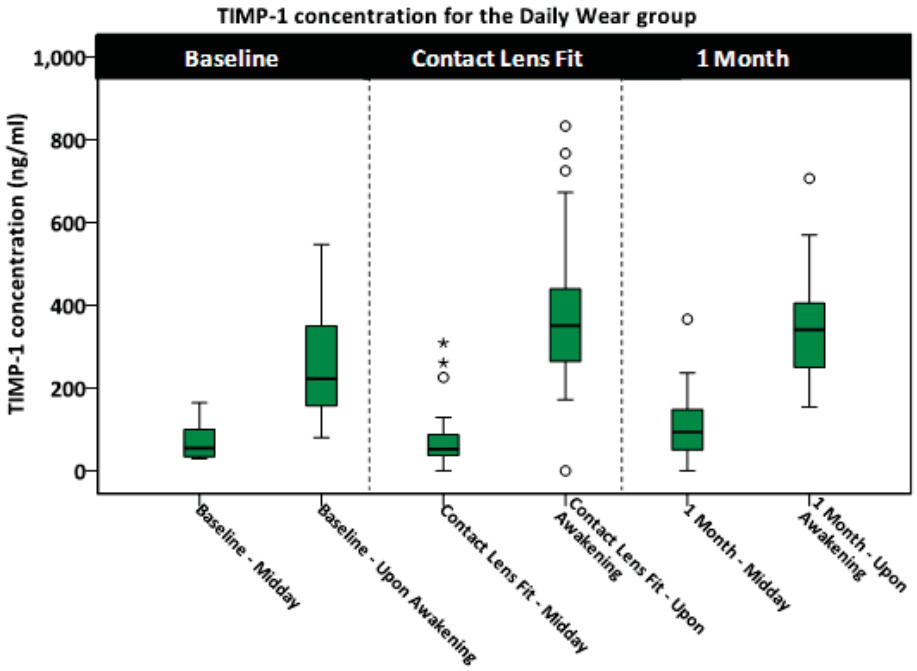


Figure 4.8: TIMP-1 concentration for the daily wear group.

Upon awakening, the contact lens fit was significantly greater than the baseline (p = 0.02, n = 18).

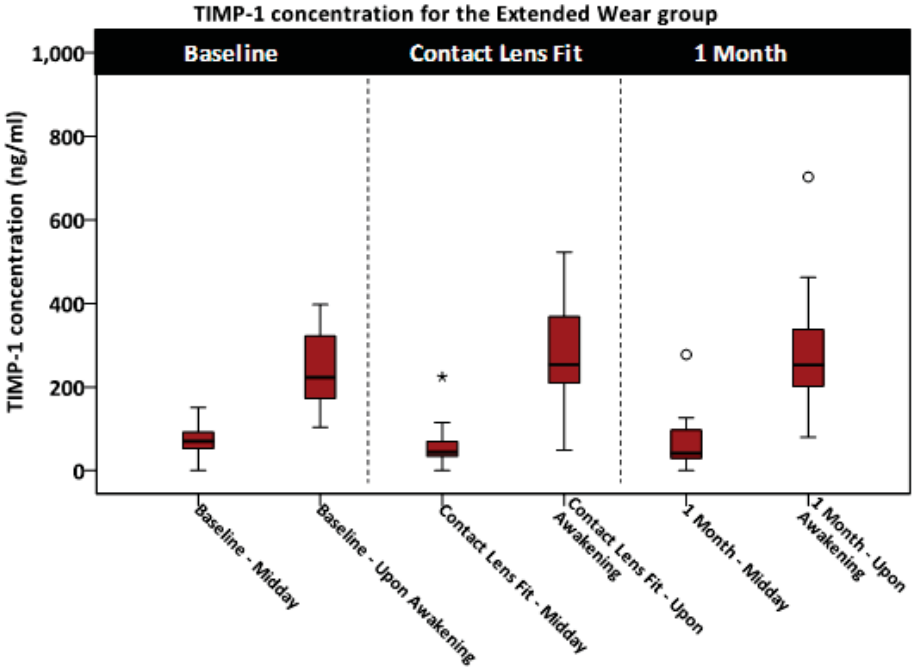


Figure 4.9: TIMP-1 concentration for the extended wear group.

The diurnal profile did not vary statistically, from baseline, either at the contact lens fit, or at the one month visit (p = 0.17, n = 25).

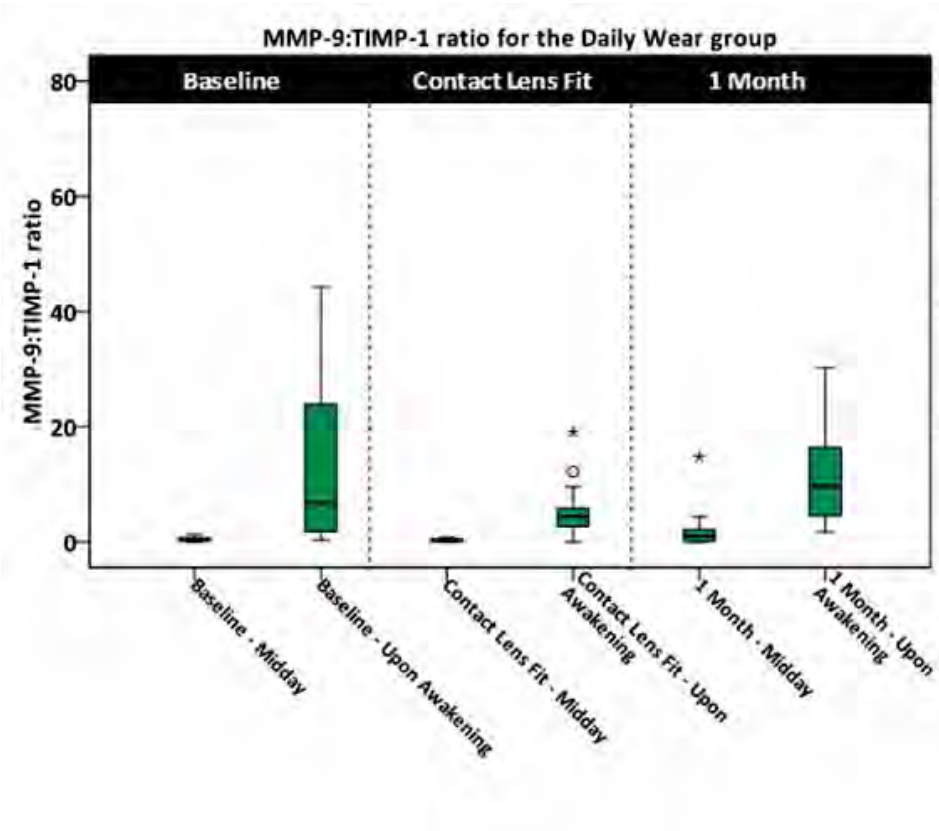


Figure 4.10: Ratio of MMP-9:TIMP-1 for the daily wear group.

The diurnal profile did not vary statistically from baseline, either at the contact lens fit, or at the one month visit (p = 0.09, n = 18).

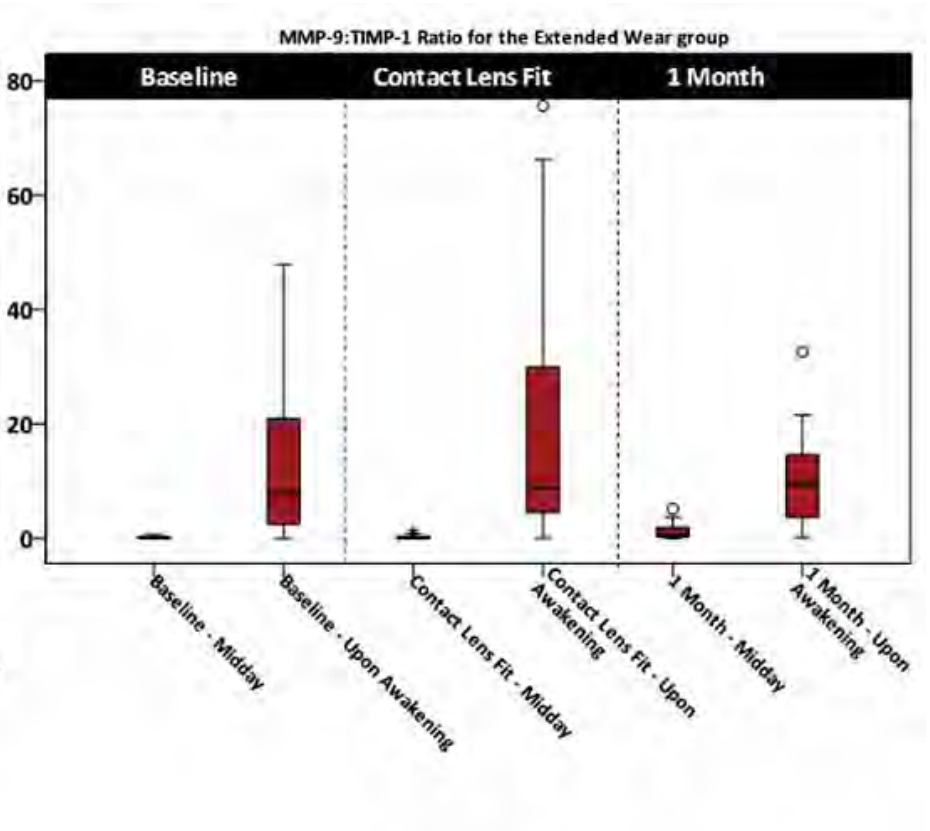


Figure 4.11: Ratio of MMP-9:TIMP-1 for the extended wear group.

Upon awakening, the ratio was significantly greater after the first night of contact lens wear than the one month visit (p = 0.048, n = 25).

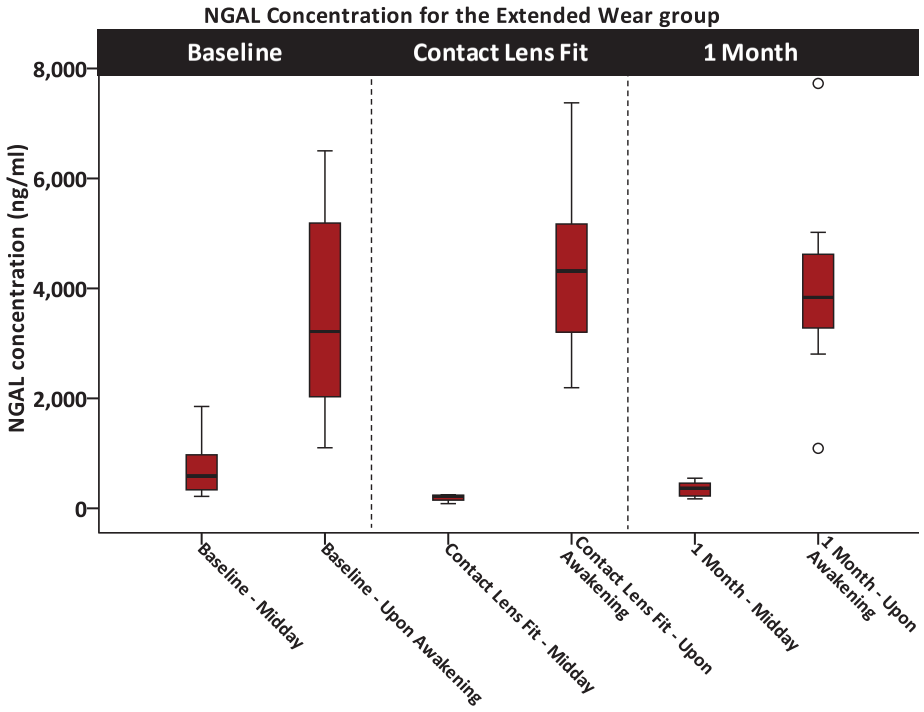


Figure 4.12: NGAL concentration for the extended wear group.

NGAL was measured only in the extended wear group, Study 2. NGAL was not significantly different upon awakening for any of the three days (p = 0.80, n = 8).

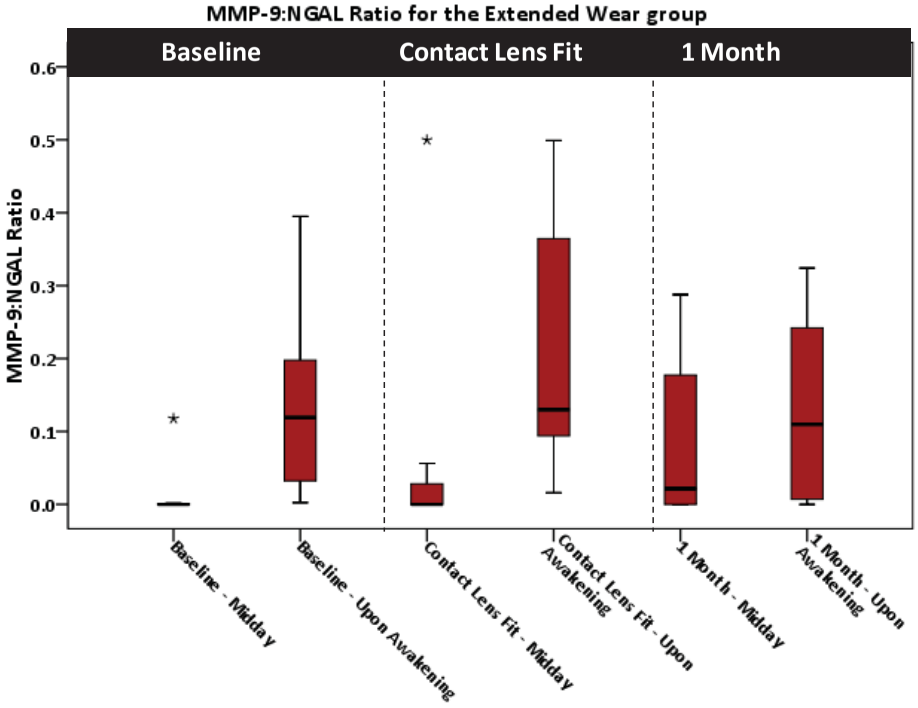


Figure 4.13: Ratio of MMP-9:NGAL for the extended wear group.

The diurnal profile did not vary statistically from baseline, either at the contact lens fit, or at the one month visit (p = 0.95, n = 8).

4.6.7.8 Zymography

Because significant changes were shown in EW upon awakening, zymography was performed only on the tears collected upon awakening from six EW participants, all wearing O_2OPTIX™. Figure 4.14 displays the representative tears of two participants at baseline, after the first night of EW and after a month of adaptation. Bands were detected at 92, 135 and >200 kDa at all visits for all participants. These bands are consistent with those previously identified by others and correspond to pro-MMP-2, pro-MMP-9, NGAL or α_2-macroglobulin complexes not dissociated by SDS, and a dimer of MMP-9.[186, 188, 249, 250] One participant showed significant levels of MMP-9 after the first night of EW (Figure 4.14) which extended beyond the 72, 92 kDa band and into the 83 kDa band, and one participant showed this at the one month visit. Densitometry however, was unable to discern two separate bands in the region of 92 and 83 kDa corresponding to pro- and active MMP-9 (not shown). The proportions of all species of MMP-9 increased with contact lens wear.

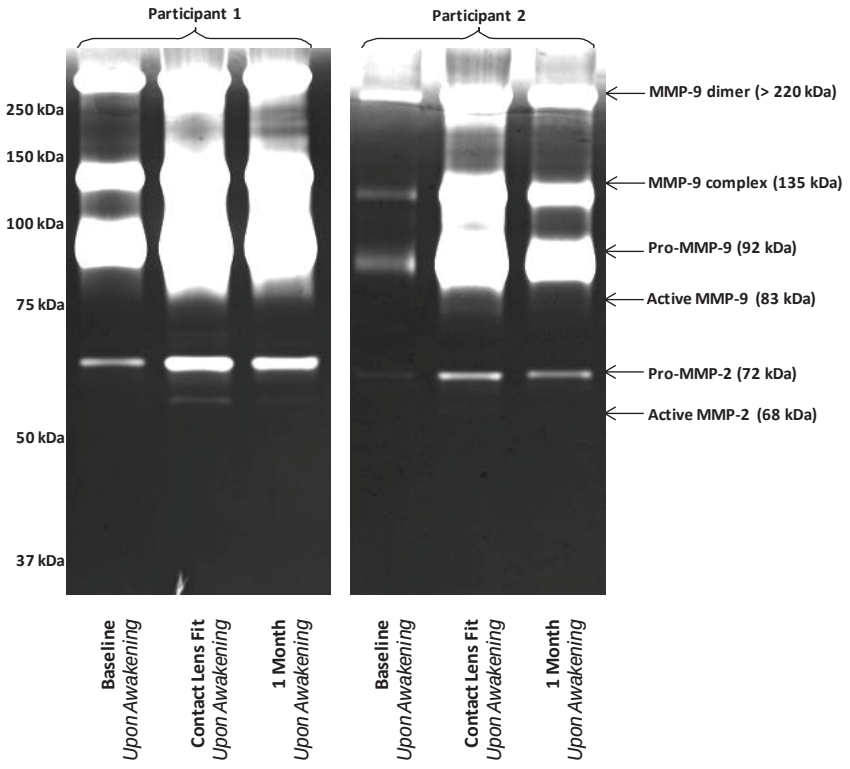


Figure 4.14: The zymograms of two non-contact lens wearers adapting to extended wear with O_2OPTIX™.

Each tear sample was collected upon awakening. A 10 µg protein load was added to each lane.

4.7 SUMMARY AND DISCUSSION

4.7.1 Summary of results

Extended contact lens wear has been associated with a greater incidence of microbial keratitis[5] and corneal erosions,[13] conditions which in non-contact lens wear have been linked to elevated levels of MMP-9.[43, 48, 101, 133, 223] As EW of contact lenses is a major risk factor in these events, this study measured the impact of contact lens wear on the baseline levels of MMP-9 and the proteins with which it complexes (as established in Chapter 3). As further confirmation of the effect of EW, the DW modality was also compared to baseline.

The changes to the baseline profile of MMP-9 were negligible with DW, supportive of the minimal risk DW appears to have on the ocular surface (Figure 4.8 and Table 4.15). Moreover, after the first day of DW, TIMP-1 increased significantly upon awakening, resulting in a significantly reduced MMP-9:TIMP-1 ratio compared to both baseline and EW (Figure 4.10 and Table 4.17). Although the significance of this increase is not known, it may be that in DW the contact lens acts as a stabilising influence. This increase in TIMP-1 expression after a day of contact lens wear may be a protective response of the ocular surface to the presence of the contact lens. Further study is needed to understand this response.

The profile in EW, however, differed significantly to that of the baseline and DW profile. Specifically, after the first night of overnight wear, a significant increase in the expression of MMP-9 was found (Figure 4.7 and Table 4.15), while there was no significant change in TIMP-1 or NGAL (Figure 4.9 and Figure 4.12, respectively). Zymography revealed that while all species of MMP-9 increased in proportion, there was no evidence of the active form (Figure 4.14). By the one month visit, the EW profile approached that of baseline upon awakening.

4.7.2 Extended contact lens wear disturbs the MMP-9 diurnal profile

This is the first study to report on the impact of silicone hydrogel contact lenses on the diurnal variation of MMP-9. A study by Lema et al. found that in normal patients wearing rigid gas permeable contact lenses, the levels of MMP-9 were significantly

increased compared to controls (12.9 ng/mL versus 6.1 ng/mL),[193] a result that was in agreement with the trend of increasing MMP-9 found in orthokeratology[197] and the significant increased expression found in this study with EW. An advantage of the study presented in this chapter is the repeated measures design, allowing an exploration of the impact of contact lens wear on the neophyte.

The disturbance to the MMP-9 profile observed during EW as compared to DW is in accordance with previously established biochemical changes associated with EW[206, 209, 244] as compared to DW[68, 193, 201] as well as the greater risk of erosion,[13, 25] infection and inflammation[1, 5] associated with EW. The imbalance of MMP-9 by TIMP-1, as is evident from the greater ratio after the first night (Figure 4.11), suggests that at this stage of contact lens wear, the cornea may have a greater propensity for collagen degradation and hence be at greater risk of erosions. Zymography however confirms that although there is an increased expression of MMP-9 with EW, this does not appear to be the active form in this population of healthy contact lens wearers. The greater variability found in the MMP-9 concentration after the first night of EW (Figure 4.7) intimates that contact lens wear impacts the population in variable ways, with those at the upper extreme being potentially more at risk of the collagen degrading effects of imbalanced MMP-9. The comparison with the DW group, reinforces that sleeping in contact lenses is indeed the causative factor disturbing the equilibrium of the tear film. Chapter 6 will compare the activity in this group of normals to those who develop adverse events in contact lens wear in order to establish whether there is a difference in MMP-9 expression.

MMP-9 is regulated by other MMPs, such as MMP-3[265, 266] and other cytokines such as TNF-α.[267] Any factor that affects these other MMPs and cytokines will also influence MMP-9. Sack and colleagues showed that the majority of MMP-9 during sleep in the tear film is sourced from neutrophils,[135] which also increase during sleep.[244] Furthermore, Thakur and Willcox found that neophytes had significantly greater numbers of neutrophils compared to non-contact lens wearers, while experienced wearers had the least.[209] It may be that the increased expression of MMP-9 with initial EW is a result of this greater number of neutrophils.[209] Moreover, the fact that TIMP-1 concentrations in this study did not change from baseline, is in agreement with the

understanding that TIMP-1 is not produced by neutrophils and hence will not increase in circumstances where MMP-9 increases as a result of neutrophil release. [135] The insignificant increase in NGAL in this study is surprising considering the known association with MMP-9 released by neutrophils and NGAL.[135] The observed power of the NGAL analysis according to the ANOVA was 0.30 and may suggest that a greater sample size is necessary to measure changes in NGAL.

An additional hypothesis as to the reason why MMP-9 increases with contact lens wear is that EW may be a sub-clinical form of corneal injury, resulting in a cellular response. MMP-9 is known to increase with wound healing.[50] When this mechanism over-reacts, it may result in excess MMP-9 and hence the increased expression seen in EW.

4.7.2.1 Adaptation restores the MMP-9 profile to baseline levels

After a month of contact lens wear, MMP-9 concentration upon awakening was no longer different to the concentration at baseline, suggesting that the ocular surface was adapting to EW, a finding that is in agreement with the reduced number of neutrophils found by Thakur and Willcox in experienced daily wearers proceeding to EW for the first time.[209] How quickly this adaptation happens is not known.

The levels of MMP-9 during the day after one month of continuous wear, however, was significantly greater than that at baseline, and greater than the levels of TIMP-1, shifting the tear film balance from TIMP-1 dominance to MMP-9 dominance. A study of longer duration would establish whether the MMP-9:TIMP-1 ratio remains that way in EW, or whether there is a return to baseline.

4.7.3 The influence of gender and contact lens type

Two contact lens types were used in this study: the higher modulus O_2OPTIX™ and the lower modulus ACUVUE® OASYS™ (Table 4.1). Modulus was compared because of the known role it plays in mechanical adverse events such as superior epithelial arcuate lesions.[9] In this study no difference was found between the contact lens types, suggesting that the change seen is the same regardless of the contact lens type and that the over-ruling factor, therefore, is wear schedule.

4.7.4 Changes to MMP-9 do not correlate with clinical signs

The lack of correlation of MMP-9 concentrations with the clinical signs of bulbar and limbal redness during contact lens wear was in agreement with the lack of correlation found at baseline (Section 3.6.9.8), suggesting that redness cannot be used as a predictor of elevated concentration of MMP-9 in the tear film. Similarly, Carnt et al. found that limbal redness was not a predictor of corneal infiltrates, reinforcing that corneal changes are not always reflected by signs of redness of the conjunctiva.[47]

4.7.5 Implications of findings

While the levels of MMP-9 baseline are not only tolerated, but presumably required for the extracellular matrix turnover, the concentrations with contact lens wear, if unbalanced, may contribute to pathology, particularly if combined with a bacterial load. This is supported epidemiologically by the greater incidence of erosions in EW and during adaptation[13] and the greater incidence of microbial keratitis in EW and during adaptation.[5, 6] It may be that the people most prone to these conditions are those with greater levels of MMP-9 during contact lens wear, which could therefore dissolve the epithelial basement membrane and hence result in epithelial detachment.[112] The strong correlation between MMP-9 upon awakening at the baseline visit with that of the contact lens fit and the one month visit upon awakening suggests that a higher level of MMP-9 at baseline is predictive of higher levels with EW.

In view of the increased expression of MMP-9 in the initial phase of EW, followed by the subsequent adaptation, vigilance with regards to the signs and symptoms of erosions and infection may be advised both to practitioners and their patients during the adaptation period. This advice extends also to patients wearing bandage contact lenses for the first time. Those wearing bandage contact lenses often do so due to an already compromised cornea and further collagen degradation may be detrimental. The initial increase in MMP-9 in EW is a factor to be considered by practitioners and, potentially, the use of an inhibitor such as doxycycline may be advised.[184] The consistency of the MMP-9 profile in DW with the baseline profile in this study, and the findings by Thakur and Willcox that experienced contact lens wearers in DW when allocated to EW had lower levels of neutrophils than neophytes,[209] suggests that a

period of DW should be advised prior to proceeding to EW. This assertion requires confirmation with a study exploring the effect of initial DW on the levels of MMP-9 prior to proceeding to EW. A further study exploring the effect of initial DW followed by EW on the adverse event rate, particularly the erosion rate, would help answer the question as to whether a period of DW is necessary prior to proceeding to EW, and if so, how long this phase should be.

4.7.6 Limitations of study

A limitation of this study was that although participants were randomised to contact lens type, they were not randomised to the wear schedule. It may be argued that there may be an inherent difference in the group of people who choose the DW schedule as opposed to the EW schedule, for example, in terms of compliance. In order to overcome this, the groups were compared at baseline according to their chosen wear schedule, to establish any differences prior to contact lens wear (Section 4.6.6).

An additional limitation of this study is that it was conducted for a period on only one month. According to a study by Willcox and his colleagues,[13] the majority of erosions occur within the first three months of contact lens wear. Ideally, a study following a group of neophytes as they adapt to contact lens wear over a period of at least three months may provide greater insights into the adaptation process and its association with corneal erosions.

4.7.7 Future work

Chapter 6 explores the profiles of participants who developed adverse events including corneal erosions and a contact lens peripheral ulcer in order to compare these profiles to those of healthy normal contact lens wearers as established in this chapter.

The knowledge that contact lens wear in EW increases MMP-9 suggests that this may be an area to target for the prevention of adverse events such as erosions. There may be a means by which to prevent this increase in contact lens wear, whether it be through increasing the concentrations of TIMP-1 in the tear film in the form of an eye drop, or adding a collagen like substance to the contact lens matrix to act as a competitive substrate.[268]

4.8 OUTCOMES OF CHAPTER 4

Chapter 4 demonstrated that in the neophyte, EW appears to initially disturb the tear film homeostasis with an increase in MMP-9 expression upon awakening compared to baseline and DW, regardless of the contact lens type. There is no corresponding increase in TIMP-1 or NGAL. These levels return to baseline after one month suggesting adaptation.

MMP-9 is part of a network of proteins and is therefore unlikely to be the only protein impacted by contact lens wear. Chapter 5 therefore explores the changes in the tear proteome using a broad spectrum technique known as differential gel electrophoresis.

CHAPTER 5 THE EFFECT OF CONTACT LENS WEAR ON THE TEAR PROTEOME

5.1 OVERVIEW

Chapter 4 explored the impact of contact lens wear on the diurnal variation of matrix metalloproteinase-9 (MMP-9) and identified a significant increase in its expression after the first night of extended wear (EW), with a return to baseline after one month. MMP-9 however is part of a complex network of cytokines and MMPs and is therefore unlikely to be the only change with contact lens wear. An understanding of the impact of contact lens wear on the proteome as a whole is important to the understanding how contact lens wear results in adverse events such as corneal erosions. Chapter 5 therefore employs a broad spectrum protein analysis technique known as differential gel electrophoresis (DIGE) to establish the differences in the proteome during the adaptation to extended wear (EW) and the extent of these differences. This is followed by mass spectrometry in order to identify the proteins that differed. An understanding of this profile in healthy neophytes as they adapt to contact lens wear allows for the comparison to those who develop corneal erosions, a comparison which will be made in Chapter 6.

The DIGE work presented in this chapter was conducted at the Minomic International Ltd laboratories under the kind guidance of Dr Belinda Schiller and Dr Anna Fitzgerald. The mass spectrometry was performed by the Bioanalytical Mass Spectrometry Facility at the University of New South Wales. The work presented here has been accepted for publication in *Optometry & Vision Science* and was also presented as a poster at the Association for Research in Vision and Ophthalmology meeting in 2011 (Appendix B: Publications and presentations).

5.2 INTRODUCTION

The 'proteome' is the composition of all the proteins expressed by the genome of an organism in a tissue or in biological fluids, such as serum, urine and tears.[269] This proteome will vary between tissues, disease states and with environmental changes. Chapter 5 explores how the healthy tear proteome is altered by extended contact lens

wear and Chapter 6 compares these profiles to those prone to contact lens-induced corneal erosions.

Proteomics has been used to identify the major proteins whose abundance and profile differs across two or more related samples (biomarkers).[269] Biomarkers can be measured and can act as indicators of normal or pathological processes and also change in response to intervention.[269] In knowing which biomarkers are altered during contact lens wear, candidates which may contribute to adverse events can be identified. Moreover, in the case of contact lens-related adverse events such as corneal erosions (Chapter 6), biomarkers could have utility in determining the aetiology and in monitoring the effect of intervention or preventative mechanisms.[155]

Proteomics involves three steps: protein separation, protein identification and protein quantitation. The method chosen for this chapter was differential gel electrophoresis (DIGE), a modification of two-dimensional gel electrophoresis which is widely used as a means of separating proteins,[270] and then further characterised by mass spectrometry.

Two-dimensional gels separate proteins based on their isoelectric points in the first dimension (Section 5.5.5.4), and then according to their apparent size in the second dimension (Section 5.5.5.5). Protein spots of interest can be excised from gels and then digested with trypsin or other proteases (Section 5.5.5.11).[271] These digests can then be subjected to a range of mass-spectrometric techniques (Section 5.5.6). By comparing spot differences and intensities from different samples, changes in the level of expression of individual proteins can be detected and quantified, thus permitting the identification of biomarkers.[271] One limitation of traditional comparative two-dimensional gel electrophoresis is the high level of gel-to-gel variation in spot patterns.[270] The difficulty in reproducing spot patterns on different gels makes it difficult to distinguish true biological variation from experimental variation. DIGE allows the detection and quantitation of differences between samples resolved on the same gel or across gels, when linked by an internal standard.[272] Unlu and colleagues first modified two-dimensional gels by pre-labelling the samples with two cyanine dyes, allowing the two samples to be run on the same gel.[272] Three mass (500 Da) and charge-matched spectrally resolvable fluorescent dyes (Cy2, Cy3, and Cy5) are used to

label a control (the internal standard) and two different protein samples *in vitro* prior to electrophoresis.[272] The internal standard is run on each gel of the experiment and is used to normalise protein abundance measurements across multiple gels by measuring the protein spot of each biological sample as a ratio against the same spot of the internal standard, and hence comparing this to the same spot for another biological sample. This allows quantitation and minimises experimental variation.[273]

This fluorescence-based detection has a dynamic range of five orders of magnitude and the dye sensitivity is capable of detecting 0.25-0.95 ng of sample,[274] compared to a dynamic range of one order for Coomassie Blue-stained gels[271] and two orders of magnitude for silver staining.[275, 276]

DIGE has been previously used in cancer research,[277, 278] and recently has been used to compare between the tears of contact lens wearers with and without dry eye,[235] between the tears of males and females,[279] between the corneal epithelial and stromal profiles in keratoconus compared to normals,[280] and between the nuclei in age-related nuclear cataracts and normal crystalline lenses.[281] It has not been used with flush tears or to determine the impact of contact lens wear on the tear proteome.

In Chapter 5, DIGE and mass spectrometry are used to observe the changing proteome during the early and adaptive phases of extended contact lens wear. As the major changes found in the MMP-9 profile in Chapter 4 were in the tears 'upon awakening', and due to the limited tear volume, Chapter 5 will predominantly evaluate this time-point.

5.3 AIMS

This study aims to establish:

1. The technical variability of the DIGE technique with flush tears (Section 5.6.1)
2. The biological variability of the tear proteome in terms of
    a. Day-to-day repeatability (Section 5.6.2.1)
    b. Diurnal variation (Section 5.6.2.2)
3. The impact of extended contact lens wear on the tear proteome (Section 5.6.3)

5.4 HYPOTHESIS

Based on the findings in Chapter 4 of increased expression in MMP-9 during EW, and previous reports of biochemical changes associated with contact lens wear,[199, 208, 282-285] including increased expression of some proteins, and decreased expression of others,[199] the hypothesis of this chapter is that the tear proteome in EW will differ from that in healthy non-contact lens wearers. It is hypothesised that there will be an increase in proteins such as albumin due to serum leakage during extended contact lens wear, while there will be a decrease in other proteins such as secretoglobin.[199]

5.5 METHODS

5.5.1 Study design

The tears analysed in this study were from Study 2 presented in Chapter 3 and Chapter 4 (Figure 4.1). Accordingly, this was a prospective, bilateral, open-label clinical study, where the tears from eight healthy participants were analysed using DIGE. All participants wore O_2OPTIX™ (Table 4.1) in the EW modality according to the schedule described in Figure 4.2. Only the tears from the EW group were analysed as this is the group whose protein profile changed from baseline in terms of MMP-9 concentration (Section 4.6.7.2) and this is the wear schedule associated with the greater incidence of infection[5] and erosion.[13]

The tears of one participant with an adverse event in Study 2 were excluded from this analysis and are presented in Chapter 6.

5.5.2 Sample size calculation

The tears of eight healthy participants were analysed, a sample size similar to that used by others[281 235, 274] and based on a sample size calculation of power of 0.8, significance level of 0.05, differential expression in the same protein spot between samples of 3.6 and random variation of 4.4 (based on the preliminary results of Section 5.6.2).[286]

5.5.3 Clinical techniques

Clinical procedures were conducted as per Table 3.1 for baseline, Table 4.3 for the contact lens fit, Table 4.4 at the one week safety visit and Table 4.5 at the one month visit. Flush tears[202] were collected from both eyes as described in Chapter 2 (Section 2.5.3.3) at the baseline visits, contact lens fit and after one month of contact lens wear. In order to allow for adequate volume, the tears were pooled from right and left eyes. Tears were centrifuged and aliquoted as per Section 3.5.6.3.1.

5.5.4 Total protein content

Total protein content (TPC) was determined as per Section 3.5.7.1 and the results of this are reported in Sections 0 and 4.6.7.1.

5.5.5 Differential gel electrophoresis

The fluorescent dyes used were CyDye™ DIGE Fluors for Ettan DIGE (minimal labelling, GE Healthcare UK Limited, Amersham Place, Buckinghamshire, UK). Figure 5.1 outlines the DIGE procedure.

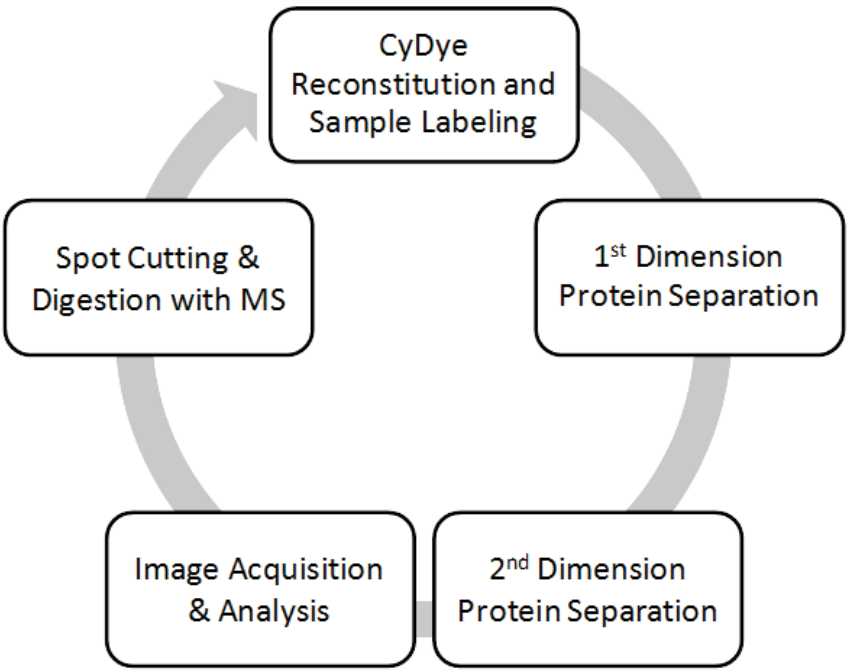


Figure 5.1: The procedures involved in differential gel electrophoresis

5.5.5.1 Experimental study design

The following technical and biological analyses were performed:

5.5.5.1.1 Technical variability

- The differences between the three CyDyes when used with flush tear samples (Section 5.6.1.1): CyDyes label the lysine residues so that 1-2% of the total protein is labelled.[271, 274, 287] As these dyes do not differ in molecular weight and charge, the same protein labelled with any dye migrates to the same point.[287] In order to confirm this for flush tear samples, a single sample labelled with each of the three dyes Cy2, Cy3 and Cy5 was electrophoresed on the same gel, and results are shown in Section 5.6.1.1.
- The gel-to-gel repeatability when using the same CyDye (Section 5.6.1.2): The same sample was loaded onto three different gels following the same preparation and using the same dye (Cy3). The gels were electrophoresed at the same time under the same running conditions and with the same batch of gels.

5.5.5.1.2 Biological variability

- The day-to-day repeatability of the proteome at two distinct time-points:
  - Midday (Section 5.6.2.1.1): Samples were collected from the same eye of the same participant on three different occasions.
  - Upon awakening (Section 5.6.2.1.2): Samples were collected from four participants on two occasions 'upon awakening'.
- The diurnal variation of the tear proteome (Section 5.6.2.2):
  - Tears collected from three participants prior to contact lens wear at midday were compared to those upon awakening.

5.5.5.1.3 The impact of extended contact lens wear on the tear proteome

Samples were randomised to either Cy3 or Cy5 labelling in each gel. Cy2 was always used for the internal standard. For eight participants, the tears 'upon awakening' were compared at baseline, the first night of contact lens wear and after one month of EW. As additional confirmation, the same sample from one individual was run on two separate gels using the same CyDye (Section 5.6.3.1).

5.5.5.2 CyDye reconstitution

One millimolar stock solutions of the CyDyes were prepared in anhydrous dimethylformamide (DMF, Sigma Aldrich, Steinheim, Germany) and stored at -20 °C until use. These stock solutions were diluted 1:5 in DMF to a final concentration of 200 pmol/μL.[280]

5.5.5.3 Sample labelling

All labelling reactions and all subsequent procedures were performed with aluminium foil covering the samples so as to limit the exposure of the fluorescent dyes to light. In order to prepare the samples, the pH was checked and adjusted with Tris if necessary to bring to a pH of 8-9. The decision was taken not to precipitate the samples in order to minimise sample loss.

Samples were randomised to either the Cy3 or Cy5 dye using randomisation.com (seed 21077). A volume of 1 μL of CyDye per 50 μg of protein (half labelling – 200 pmol of dye per 50 μg of protein)[280] was added and this was centrifuged and left on ice in the dark for 30 minutes. To this, 1 μL of 10 mM lysine was added to stop the reaction and this was left on ice in the dark for 10 minutes. At this stage the samples could be covered in aluminium foil and stored in -80 °C until processing.

The internal standard was prepared by combining 25 μg of protein from each biological sample in the study and labelling this with 1 μL of Cy2 per number of gels to be run.[287] In this way, every protein from each sample was represented in the internal standard[287] and this was run on every gel. The same volume of lysine was then used to stop the reaction (1 μL per gel to be run).

At the time of running the gel, for each gel, the two samples, 50 μg each (one with Cy3 and one with Cy5) and 50 μg of internal standard were added to 200 μL of the MiPrepF solution (Minomic International Ltd) as used by others.[288] After 20 minutes, 5 μL of tributylphosphine (Bio-Rad ReadyPrep TBP Reducing Agent, Bio-Rad, Hercules, CA, USA) was added to this. After 30 minutes, 10 μL of 40% acrylamide (Bio-Rad, Hercules, CA, USA) was added to this mixture for 20 minutes.

5.5.5.4 First dimension protein separation

The first dimension in two-dimensional gel electrophoresis is isoelectric focusing in which proteins are separated according to their net charge irrespective of their mass.[269] The principle is that electrophoresis is carried out in a pH gradient allowing each protein to migrate to an isoelectric point (pI), that is, the point at which its pI value is equivalent to the surrounding pH and its net charge is zero.[269]

For each gel, passive rehydration of 11 cm pH 4-7 immobilised pH gradient (IPG) strips (Ready Strip™ IPG, Bio-Rad, Hercules, CA, USA) was performed in 200 μL of pre-hydrated MiPrepF for six hours. The strips were then focused using the IPGPhor-II Isoelectric Focusing System (GE Healthcare UK Limited, Amersham Place, Buckinghamshire, UK) using cup-loading (two cups per strip, GE Healthcare UK Limited, Amersham Place, Buckinghamshire, UK) and moistened electrode strips (four per strip, GE Healthcare UK Limited, Amersham Place, Buckinghamshire, UK) as shown in Figure 5.2. Once mineral oil was added to the ceramic manifold (Figure 5.2), the strips were focused for a minimum of 14 hours using a six-step isoelectric focusing: 100 V for one hour, 300 V for two hours, 1,000 V for two hours, 2,500 V for one hour, 7,500 V for eight hours and then 100 V until off. A level of 63,000 VHrs was considered optimum. In cases where the level was below this, the strips were focused for a further two hours at 2,500 VHrs in order to surpass the desired volt hours. The IPGPhor-II was covered at all times with aluminium foil to prevent the entry of light.

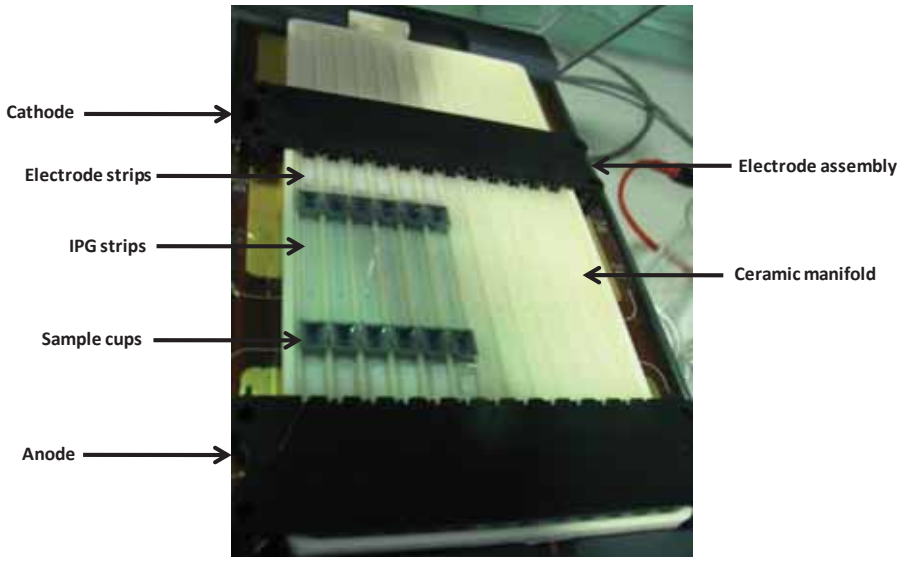


Figure 5.2: First dimension protein separation.

The strips were focused using an IPGPhor-II Isoelectric Focusing System (GE Healthcare UK Limited, Amersham Place, Buckinghamshire, UK) using cup-loading (two cups per strip) and moistened electrode strips.

5.5.5.5 Reduction and alkylation

Following isoelectric focusing, the strips were drained of any excess oil and placed in the -80 °C freezer for one hour. After one hour, each strip was reduced (6 M urea, 2% sodium dodecyl sulfate (SDS), 20% glycerol, 65 mM dithiolthreitol (DTT) in Tris/HCl buffer) for 15 minutes and then alkylated (6 M urea, 2% SDS, 20% glycerol, 2.5% acrylamide in Tris/HCl buffer) for a further 15 minutes at room temperature.

5.5.5.6 Second dimension protein separation

The second dimension separates proteins according to their molecular mass irrespective of their charge. Exposure of denatured proteins to SDS binds stoichiometrically to the polypeptide backbone and carries a large negative charge.[269] This charge exceeds any intrinsic charge carried by the proteins allowing separation on the basis of molecular size alone.[269]

Following reduction and alkylation the strips were then moved to the wells of pre-cast gels (Criterion Tris-HCl Gel, 8–16%, IPG+1 well, 11 cm IPG strip, 13.3 x 8.7 cm, Bio-Rad, Hercules, CA, USA). A volume of 800 μL 0.5% blue agarose was added to the wells prior

to addition of the strip. A molecular weight marker was added to each gel (Precision Plus Protein® Unstained Standards, Bio-Rad, Hercules, CA, USA). Tris-glycine buffer was added to both the chambers and the gels were electrophoresed at 200 V, 3 A, 300 W, 4 °C for one hour.

5.5.5.7 Image acquisition

As two differently-labelled samples and the internal standard were multiplexed onto each gel, each sample was detected with the appropriate excitation and emission wavelengths summarised in Table 5.1.

Table 5.1: The scanning laser and emission filter wavelength for each of the CyDyes (GE Healthcare UK Limited, Amersham Place, Buckinghamshire, UK).

	Cy2	Cy3	Cy5
Scanning laser (nm)	488	532	633
Emission filter	520 (band pass 40)	580 (band pass 30)	670 (band pass 30)

The gels were scanned using Bio-Rad Molecular Imager Fx and External Laser Molecular Imager Fx and the Quantity One®, version 4.6.3, Molecular Imager Pharos FX software (Bio-Rad, Hercules CA, USA). Samples were scanned at high intensity for the validation and then medium intensity for the remaining gels. Where medium intensity was saturated (Cy3 scans), the gels were re-scanned at 40% PMT.

5.5.5.8 Image analysis

Progenesis SameSpots (nonlinear dynamics, UK) version 3. was used for the image analysis as used by others,[289] courtesy of Minomic International Ltd. Due to the presence of an internal standard in each gel, the gels were aligned so that the same spots could be compared within and between gels.

5.5.5.8.1 Image quality control

Progenesis SameSpots software (nonlinear dynamics, UK) was used immediately following scanning to ensure that each gel was suitable for analysis, by ensuring that image intensity and the dynamic range captured was suitable. Progenesis initially performs 'Image QC' on all images to ensure that the image is not saturated and the intensity level is appropriate.

In this analysis, the Cy3 samples were occasionally saturated or at low dynamic range when scanned at 'medium' intensity. The Cy3 gels were therefore scanned both at medium intensity and at 40% PMT. Once the QC analysis was performed, the best of the two scans was chosen for the analysis. No problems were found for either the Cy2 or the Cy5 scans at medium intensity.

5.5.5.8.2 DIGE set-up and reference image selection

The DIGE gels were grouped according to the gel number and automatic alignment was performed using the Cy2 gel as reference image gel.

5.5.5.8.3 Alignment

The gels were aligned by positioning vectors over the major common proteins to ensure alignment. The pink spots shown in Figure 5.3 belong to the reference gel (the internal standard) and the green spots correspond to the gel being aligned. By aligning with the same reference gel, all gels are therefore aligned with each other. In each case, the spots were manually aligned. Once optimal alignment was achieved, automatic vectors were added. Each gel was checked so that any inappropriate vectors were rejected.

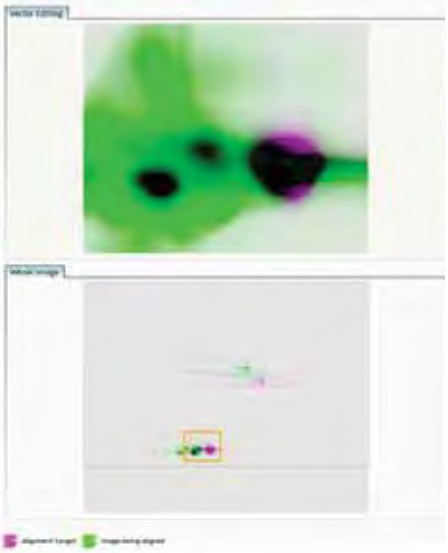


Figure 5.3: Alignment of gels using the internal standard as the gold reference.

The pink spots belong to the reference gel and the green spots belong to the gel being aligned. The top image is a magnified view of the spots being aligned and the bottom image is the two gels viewed overlapping and unaligned.

5.5.5.9 Statistical analysis

The spot ratios obtained from matched gels across repeats were averaged[274] and ANOVA was conducted by the Progenesis SameSpots software on each gel spot, with a *p* value of less than 0.05 denoting statistical significance. Protein abundance is expressed as a normalised ratio relative to spots from the internal standard. The 'fold' difference between samples is calculated from this.

5.5.5.10 Coomassie Blue staining

DIGE minimal labelling labels approx 5% of proteins. The remaining 95% are unlabelled and therefore may be analysed with mass spectrometry following Coomassie Blue staining. After scanning, the gels were fixed in 10% ethanol/7% acetic acid for one hour and then stained with Coomassie Blue overnight. The next day they were destained with 10% ethanol/7% acetic acid for a minimum of one hour and then stored in 1% acetic acid until analysis with mass spectrometry.

5.5.5.11 Spot cutting

The protein spots that were found to be significantly different in terms of normalised volumes and fold difference were identified using the Progenesis SameSpots software and were cut out and stored in an Eppendorf tube overnight prior to digestion and identification with mass spectrometry.

5.5.6 Mass spectrometry

Mass Spectrometry was conducted at the Bioanalytical Mass Spectrometry Facility (BMSF) at UNSW. The spots were destained in 25 mM ammonium bicarbonate (NH_4HCO_3) and acetonitrile (ACN). Samples were reduced with 10 mM dithiolthreitol (DTT) in 50 mM Ammonium Bicarbonate (NH_4HCO_3) for 30 minutes at 37^{o}C, followed by alkylation with 25 mM iodoacetamide (IA) in 50 mM Ammonium Bicarbonate (NH_4HCO_3) for 30 minutes at 37^{o}C. Finally, the samples were digested with trypsin at 2 ng/mL in 20 mM Ammonium Bicarbonate (NH_4HCO_3) overnight, for approximately 14 hours at 37^{o}C. The following morning, 1% formic acid (FA) was added to each of the digested samples for 10 minutes, followed by the addition of acetonitrile (ACN) for 10 minutes. The supernatant was transferred to a new tube and evaporated to

dryness. The dried samples were re-suspended in 0.05% Heptafluorobutyric anhydride (HFBA) and 1% formic acid (FA) in water.

Nano-Liquid chromatography (nano-LC) was performed using an Ultimate 3000 HPLC and autosampler system (Dionex, Amsterdam, Netherlands). Samples were injected into a fritless nano column (75µ x ~10cm) containing C18 media (5µ, 200 Å Magic, Michrom) manufactured according to Gatlin.[32] Peptides were eluted using a linear gradient according to the conditions in TABLE 1, over 50 minutes, at a flow rate of 0.3µL/min. Mobile phase A consisted of 0.1% Formic Acid in H2O, while mobile phase B consisted of ACN:H_2O (8:2) with 0.1% Formic Acid.

High voltage (1,800 V) was applied to a low volume tee (Upchurch Scientific) and the column tip positioned ~ 0.5 cm from the heated capillary (T=250°C) of a LTQ FT Ultra (Thermo Electron, Bremen, Germany) mass spectrometer. Positive ions were generated by electrospray and the LTQ FT Ultra operated in data dependent acquisition mode (DDA). A survey scan m/z 350-1750 was acquired in the FT ICR cell (Resolution = 100,000 at m/z 400, with an accumulation target value of 1,000,000 ions). Up to the 6 most abundant ions (>3,000 counts) with charge states > +2 were sequentially isolated and fragmented within the linear ion trap using collisionally induced dissociation with an activation q = 0.25 and activation time of 30 ms at a target value of 30,000 ions. M/z ratios selected for MS/MS were dynamically excluded for 30 seconds.

Peak lists of MS/MS data were generated using Mascot Daemon/extract_msn (Matrix Science, London, England, Thermo) and were entered into the search program Mascot version 2.1 (http://www.matrixscience.com) and searched against human (Homo sapiens) proteins in the NCBI protein database (August 2010). Precursor tolerances were 4 ppm and product ion tolerances were ± 0.4 Da. Modifications accounted for are Acrylamide (C), Carbamidomethyl (C), and Oxidation (M), enzyme specificity was trypsin, and one missed cleavage was possible. No decoy database was employed.

5.6 RESULTS

5.6.1 Technical variability of the DIGE technique

5.6.1.1 CyDye comparison

DIGE detected 205 protein spots for the same sample when labelled with either Cy3 or Cy5 when normalised against the Cy2 sample. Of these spots, 6% (13 spots) were found to have an equal spot volume when normalised against Cy2, while 17% (35 spots) had a greater than 2-fold difference between Cy3 and Cy5 (range of 2-4.4 fold).

Of the 192 spots that were not equal between the Cy3 and Cy5, 85% (170 spots) had a greater normalised volume for the Cy5 sample. All spots had a normalised volume of 0.2 or more. A normalised volume of less than 0.2 in the subsequent analysis was regarded as an absent spot.

The samples were subsequently randomised to Cy3 or Cy5 for Sections 5.6.2 and 5.6.3. A difference of greater than 4.4-fold was considered to be outside of experimental variability when comparing single samples.

5.6.1.2 The gel-to-gel variability

When the same sample was loaded on three different gels, using the same CyDye (Cy3), DIGE detected 139 protein spots for gel 1, 126 for gel 2 and 133 for gel 3. Missing spots were based on a normalised volume of 0.2 or less as established in Section 5.6.1.1.

Of these spots, 22% (30 spots) were found to have a less than 1.5-fold difference when normalised against Cy2, 40% (55 spots) had a fold difference between 1.5 and 1.9 while 38% (54 spots) had a fold difference of 2 or more (range of 2-3.6 fold).

From the comparison of the same sample run on the same gel with the three CyDyes (Section 5.6.1.1) and the same sample run on three gels using the same CyDye (Section 5.6.1.2), a fold of greater than 4.4 was deemed to be a biological difference rather than an experimental difference when comparing single samples, and a normalised volume of 0.2 or less was considered as an absent spot.

5.6.2 Biological variability of the tear proteome

5.6.2.1 The repeatability of the tear proteome

5.6.2.1.1 Midday

DIGE detected 233 protein spots for day 1, 232 for the day 2 and 239 for day 3. Out of the total 247 spots identified across all three days, seven (2%) were found to have a greater than 4.4-fold difference (range of 4.9-7) and 57% of the spots (141 spots) had less than a 2-fold difference from each other.

5.6.2.1.2 Upon awakening

When the 'upon awakening' tears collected from four participants between two nights were compared, 308 spots were found and were common for both nights. Only one spot had a greater than 4.4-fold difference between the two nights and this was not significant (p = 0.72). Eight spots (3% of the proteome) were significantly different between the two nights, the fold difference ranging between 1.4 to 2.1.

5.6.2.2 Diurnal variation of the tear proteome

DIGE detected 257 protein spots in the midday tears and 298 spots in the 'upon awakening' tears of three participants (representative in Figure 5.5). Figure 5.4 shows the commonality of the two time-points. There were 10 spots that were significantly greater at midday (p < 0.05), although only six of these had a fold greater than 4.4 (range of 4.7-8.9-fold) and one was absent 'upon awakening'. In contrast, 85 spots (29%) were significantly greater 'upon awakening', 31 of which had a fold difference greater than 4.4 (range 4.7-9.4) and 42 which were completely absent at midday. The spots which were both significantly different and more than 4.4-fold different (Figure 5.5) were cut and identified with mass spectrometry. These are listed in Table 5.2.

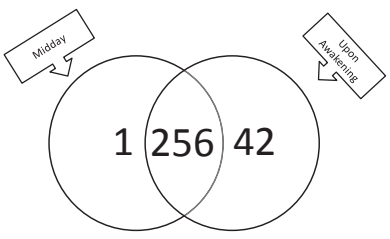


Figure 5.4: Venn diagram showing commonality of the tear proteome between midday and upon awakening.

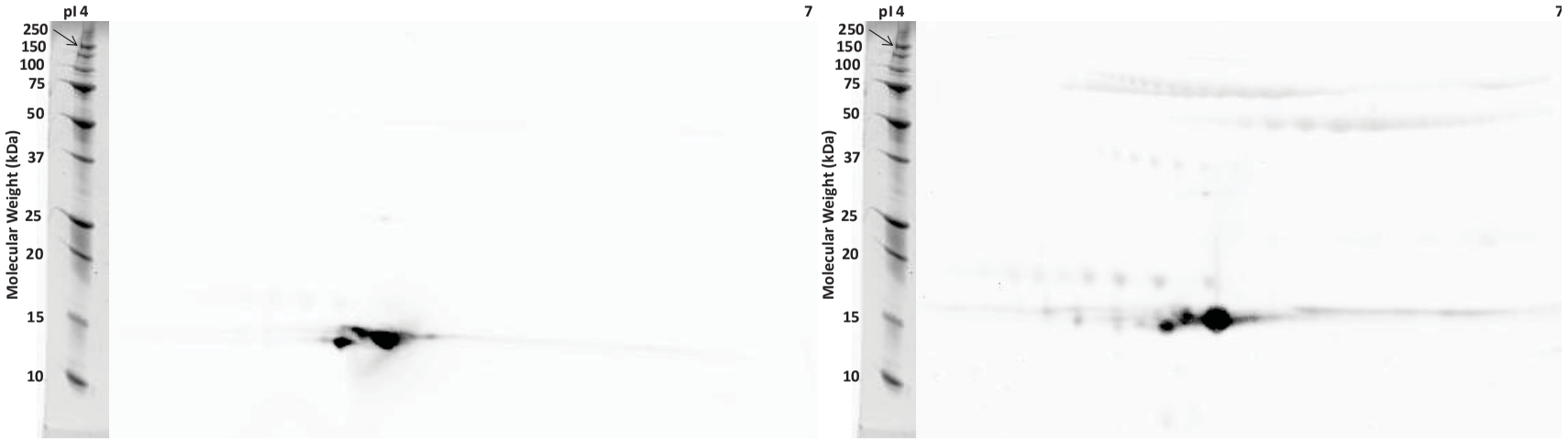


Figure 5.5: The tear proteome at midday (LEFT) and upon awakening (RIGHT) of one participant.

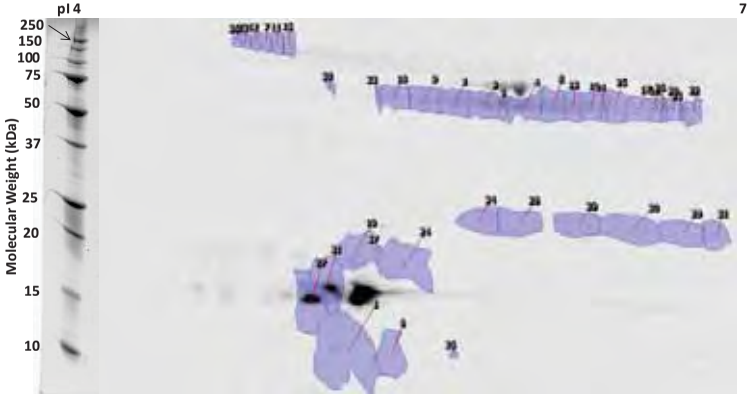


Figure 5.6: The spots identified by Progenesis SameSpots (nonlinear dynamics, UK) as being both significantly different between midday and upon awakening ($p < 0.05$) and greater than 4.4-fold different.

These spots were cut and analysed with mass spectrometry. The identifying number corresponds to the rank assigned in Table 5.2

Table 5.2: The spots identified by Progenesis SameSpots (nonlinear dynamics, UK) as being both significantly different between midday and upon awakening ($p < 0.05$) and greater than 4.4-fold different were cut and identified by mass spectrometry.

Protein abundance is expressed as a normalised ratio relative to spots from the internal standard. The 'fold' difference between samples is calculated from this.

Rank	ID	Protein name	Accession number	Mascot Score	Mass (Da)	Peptide hits	ANOVA (p)	Fold	Midday	Upon Awakening
									Mean normalised volume from DIGE	
1	6	Prolactin-inducible protein precursor	gi|4505821	278	16562	13	0.02	13.7	10.68	0.78
2	257	Ig A alpha1 Bur	gi|223099	733	50581	25	0.003	9.4	0.08	0.70
3	265	Ig A alpha1 Bur	gi|223099	704	50581	26	0.005	9.3	0.07	0.69
4	252	Serum albumin	gi|62113341	266	69039	8	0.006	9.2	0.08	0.71
5	279	Ig A alpha1 Bur	gi|223099	525	50581	16	0.007	9.1	0.08	0.72
7	152	Polymeric immunoglobulin receptor precursor	gi|31377806	829	83232	27	0.002	8.6	0.11	0.95
8	245	Ig A H	gi|229537	552	51076	19	0.008	8.5	0.08	0.66
9	251	Ig A alpha1 Bur	gi|223099	601	50581	18	0.004	8.3	0.08	0.68
10	258	Ig A alpha1 Bur	gi|223099	625	50581	22	0.008	7.8	0.09	0.70
11	153	Polymeric immunoglobulin receptor precursor	gi|31377806	382	83232	11	0.002	7.8	0.12	0.95
12	151	Polymeric immunoglobulin receptor precursor	gi|31377806	635	83232	20	0.002	7.8	0.12	0.95
14	256	Ig Aalpha1 Bur	gi|223099	629	50581	21	0.005	7.6	0.09	0.67
15	146	Transmembrane secretory component	gi|238236	673	83262	17	0.001	7.6	0.13	0.97
17	250	Not identified					0.006	7.2	0.09	0.68
18	253	Ig A alpha1 Bur	gi|223099	636	50581	18	0.005	7	0.09	0.64
19	381	Not identified					0.007	6.9	10.05	1.45
20	242	Not identified					0.005	6.7	0.10	0.65
21	248	Ig Aalpha1 Bur	gi|223099	476	50581	12	0.008	6.6	0.11	0.71
22	237	Ig A1 Bur	gi|229585	577	73331	19	0.003	6.3	0.10	0.65
23	156	Polymeric immunoglobulin receptor precursor	gi|31377806	517	83232	13	0.005	6.3	0.16	0.98
25	246	Not identified					0.008	6.2	0.11	0.68

Rank	ID	Protein name	Accession number	Mascot Score	Mass (Da)	Peptide hits	ANOVA (*p*)	Fold	Midday	Upon Awakening
									Mean normalised volume from DIGE	
26	241	Not identified					0.004	6.2	0.11	0.66
27	404	Not identified					0.031	6.2	11.94	1.94
28	368	Ig A L	gi|229536	322	22767	15	0.006	6.1	0.09	0.57
29	369	Ig lambda chain	gi|106653	194	24474	4	0.001	5.8	0.14	0.78
30	370	IgA	gi|229536	408	22767	15	<0.001	5.8	0.09	0.54
31	400	Lipocalin-1 precursor	gi|4504963	474	19238	15	0.023	5.7	11.12	1.95
32	365	Immunoglobulin lambda light chain	gi|6467839	190	22767	5	0.005	5.7	0.14	0.78
33	254	Ig A alpha1 Bur	gi|223099	563	50581	16	0.013	5.7	0.10	0.58
34	367	Ig A L	gi|229536	394	22767	16	0.012	5.6	0.11	0.59
35	487	Not identified					0.046	5.6	9.68	1.74
36	158	Transmembrane secretory component	gi|238236	326	83262	8	0.003	5.5	0.19	1.05
38	260	Ig Aalpha1 Bur	gi|223099	480	50581	13	0.009	5.4	0.13	0.71
39	372	Bence-Jones protein	gi|350476	319	22662	12	0.002	4.7	0.11	0.51

Individual ions scores > 42 indicate identity or extensive homology (p<0.05). Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.

5.6.3 Changes in the tear proteome in response to extended wear

DIGE was used to determine differences in the individual tear protein abundance upon awakening at baseline, after the first night of extended contact lens wear and after one month of adaptation to EW (Figure 5.7 and Figure 5.8). DIGE detected 311 protein spots at baseline, which were also present after the first night of contact lens wear and after one month, 15 of which were identified as being statistically different (5% of the proteome, Figure 5.7). Of these, five spots differed between baseline and the first night of EW and 10 differed between baseline and one month. Of the differences between baseline and the first night of EW, all were greater at baseline. Of the differences between baseline and the one month, six were greater at one month and four were greater at baseline. The spots which were different ($p < 0.05$) were cut and mass spectrometry was performed on these. The results are listed in Table 5.3.

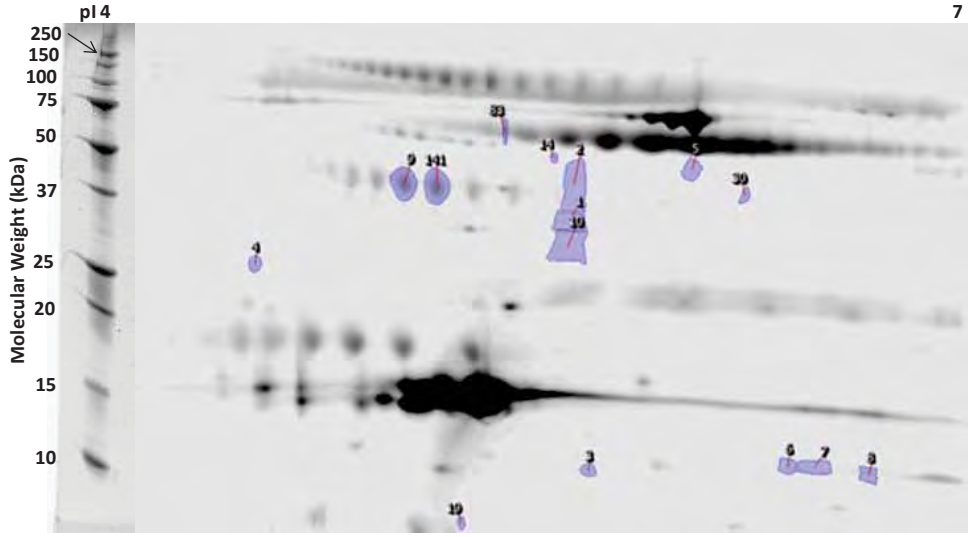


Figure 5.7: The spots identified by the Progenesis SameSpots (nonlinear dynamics, UK) to be significantly different from baseline with contact lens wear.

The number corresponds to the rank assigned in Table 5.3.

5.6.3.1 Repeatability within the study

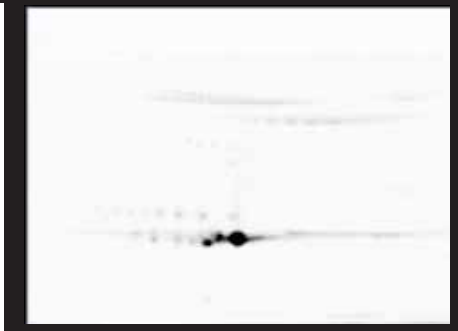
One sample from baseline upon awakening was run on two separate gels (the third and the fourth gels) but under the same running conditions and with the same CyDye (Cy3) in order to check for repeatability. Only one spot had a fold difference greater than 4.4 (4.5-fold).

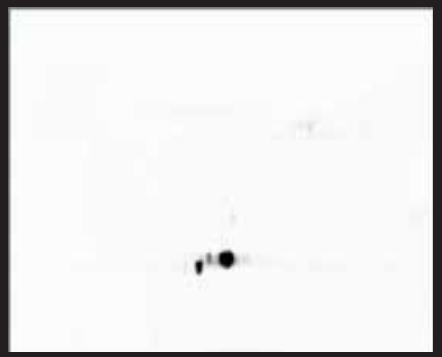
Table 5.3: The spots identified by Progenesis SameSpots (nonlinear dynamics, UK) as being significantly different between visits ($p < 0.05$) were cut and identified by mass spectrometry.

Protein abundance is expressed as a normalised ratio relative to spots from the internal standard. The 'fold' difference between samples is calculated from this.

Rank	ID	Protein name	Accession number	Mascot Score	Mass (Da)	Peptide hits	ANOVA (*p*)	Fold	Baseline *Upon awakening*	Contact lens fit *Upon awakening*	One month *Upon awakening*
									Mean normalised volumes from DIGE		
1	328	NOT IDENTIFIED	Not identified				0.008	7.6	0.95	0.72	5.47
2	294	NOT IDENTIFIED	Not identified				0.03	4.8	1.38	1.01	4.88
3	476	NOT IDENTIFIED	Not identified				0.006	2.7	0.43	0.68	1.15
4	360	NOT IDENTIFIED	Not identified				0.04	2.6	2.68	1.38	3.57
5	295	NOT IDENTIFIED	Not identified				0.04	2.4	1.01	1.08	2.48
6	469	NOT IDENTIFIED	Not identified				0.03	2.0	1.75	0.86	1.06
7	470	NOT IDENTIFIED	Not identified				0.01	1.8	1.55	0.82	0.84
8	472	NOT IDENTIFIED	Not identified				0.03	1.7	1.62	1.07	0.96
9	303	zinc α2-glycoprotein	gi|38026	494	34714	18	0.04	1.6	0.5	0.42	0.32
10	344	NOT IDENTIFIED	Not identified				0.055	6.2	1.29	1.23	7.61
19	513	NOT IDENTIFIED	Not identified				0.02	2.7	1.05	1.54	2.87
14	293	serum albumin	gi|62113341	556	69039	15	0.046	3.1	0.86	0.97	2.66
30	310	NOT IDENTIFIED	Not identified				0.03	2.2	1.35	1.96	2.99
83	254	Ig A alpha1 Bur	gi|223099	552	50581	14	0.048	1.9	0.85	1.12	0.67
116	471	NOT IDENTIFIED	Not identified				0.049	1.5	1.61	1.05	1.20
141	305	zinc α2-glycoprotein	gi|38026	275	34714	10	0.077	1.5	0.55	0.45	0.38

Individual ions scores > 42 indicate identity or extensive homology (p<0.05). Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.

Participant	Baseline	First night of contact lens wear	One month contact lens wear
1			
2			
3			

Participant	Baseline	First night of contact lens wear	One month contact lens wear
4			
5			
6			

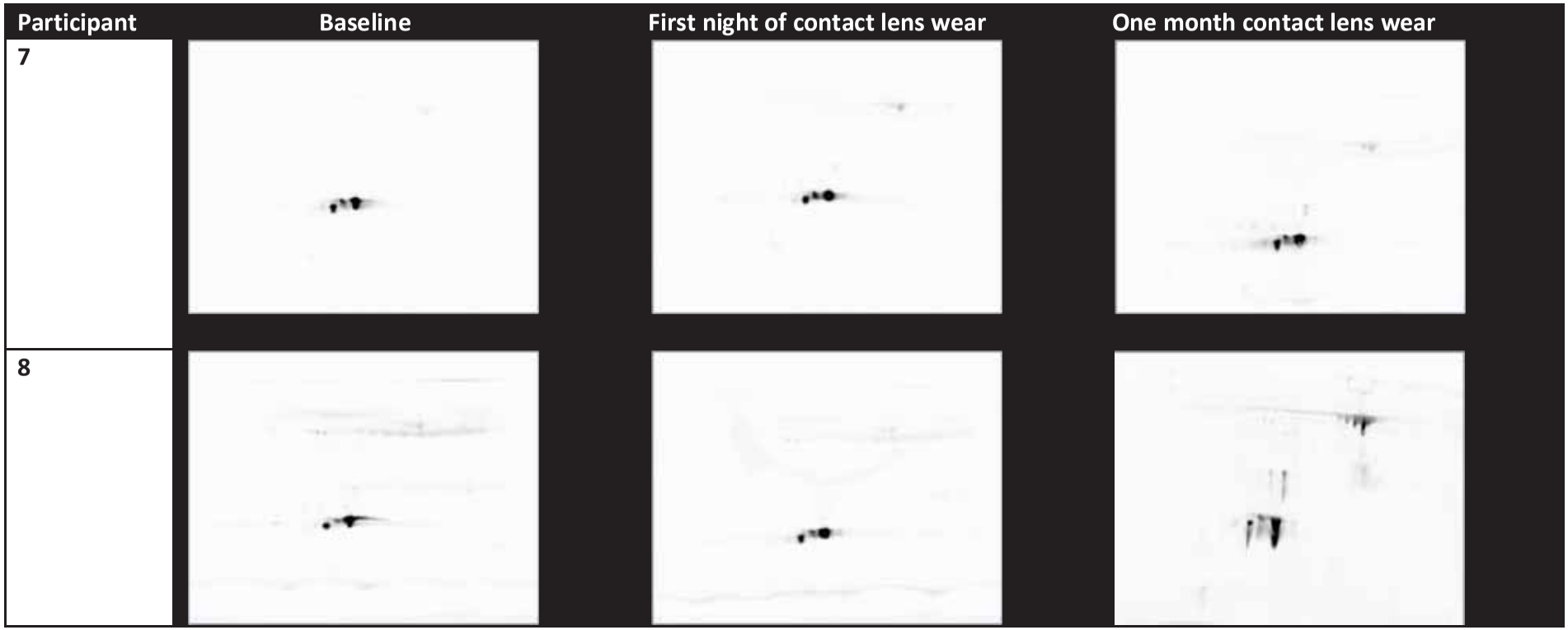


Figure 5.8: The gels of each participant 'upon awakening' as they proceed from no contact lens wear at baseline, to neophytes after the first night of contact lens wear, through to experienced contact lens wear after one month in the extended wear schedule.

5.7 SUMMARY AND DISCUSSION

5.7.1 Summary of results

This study first established the technical variability of the DIGE technique using flush tears by comparing the labelling efficacy of the three CyDyes. Both Cy3 and Cy5 identified the same proteins relative to Cy2 with the highest fold-difference being 4.4. This was subsequently set as the maximum fold difference attributable to experimental variability when comparing single gels. The reproducibility of the healthy, non-contact lens wearing tear proteome, as well as its diurnal variation was then determined. The proteome was found to vary by up to 3% at the same time-point, with a significant increase in protein spots found between midday (257) and upon awakening (298). With this background, the changes in the tear proteome in response to EW of contact lenses were determined. Fifteen proteins spots differed significantly out of the 311 spots identified during contact lens wear. Of these, the greatest differences were found between baseline and one month of EW.

5.7.2 Technical variability of DIGE use with flush tears

A major obstacle in proteomics is that of technical and biological variation.[142] The technical variability includes the repeatability between gels and is a factor that DIGE attempts to overcome with the inclusion of an internal standard against which all gels are normalised.

This chapter established that the upper range of the CyDye variation within and between gels was 4.4-fold when used with flush tears, with Cy5 giving the greater normalised volume. These findings are consistent with those determined by others for mouse liver homogenates,[274] and skeletal muscle.[290] The allocation of CyDye to individual samples was therefore randomised for the remainder of the study to minimise bias, and a 4.4-fold difference was considered to be the limit above which variation exceeded experimental variability. The limit of detection was determined to be at the normalised volume of 0.2 in these experiments and this level was considered to be the cut-off below which spots were considered to be absent.

5.7.3 Biological variability of the flush tear proteome

When the tears of four participants were compared across two occasions upon awakening, 308 spot were identified, of which one had a fold difference greater than 4.4, although not significant, while eight spots were significantly different but with a fold difference less than 4.4. These findings suggest that the variability of the tear proteome at the same time-point is largely repeatable.

The diurnal variation of the tear proteome produced much greater differences consistent with the total protein content of the tear film at midday and upon awakening, as well as the diurnal variation of MMP-9, TIMP-1 and NGAL reported in Chapter 3 and the findings of others.[226]

5.7.4 Number of proteins found with DIGE

The number of proteins found in the tear film is a matter of much contention and is dependent on the technique used as well as the tear collection technique and the time of day collected. In one study, mass spectrometry following one-dimensional gel electrophoresis identified up to 491 proteins in human day tears,[291] whilst on the lower end, other groups have identified between 54 and 60 proteins using various mass spectrometric techniques.[292, 293] Using two-dimensional gels Herber and colleagues found 166 protein spots in the basal tears of normals.[294] In work done by Green-Church et al., 40 unique proteins were identified in tears with several proteins being observed multiple times at different molecular weight regions.[210] The authors attribute this to post-translational modifications or the formation of protein homopolymers of lower molecular weight proteins or protein complexes not denatured.[210] These authors also observed higher molecular weight proteins at lower molecular weight regions in the gel and attribute this to the degradation due to storage or tear proteases.[210] In this chapter 257 proteins were found at midday and 298 were 'upon awakening', in agreement with previous studies.[181]

5.7.5 The impact of extended wear on the tear proteome

In order to examine the effects of contact lens wear on the tear proteome, the proteome without contact lens wear upon awakening was compared to that after the

first night of overnight wear and after one month of continuous wear for eight participants. The EW schedule was chosen because this is where the greatest difference was found in the MMP-9 profile (Section 4.6.7.2) and this is also the wear schedule associated with the greatest risk of adverse events.[5] Due to limited tear volume only the tears 'upon awakening' were analysed, specifically because this is where the greatest difference was found for MMP-9 after the first night of EW (Section 4.6.7.2).

The changes found with EW were only slightly greater than those attributable to normal variability at the same time-point (Section 5.6.2.1.2). Of the 311 spots identified, 15 were significantly different ($p < 0.05$), but only three were identified with mass spectrometry, possibly due to a low volume. Of the proteins identified, zinc α2-glycoprotein was found to decrease with contact lens wear, albumin was found to increase three-fold and immunoglobulin A (IgA) was found to increase with initial EW and then reduce after one month. Zinc α2-glycoprotein is a multifunctional protein, secreted in body fluids[295] including tears[222, 265] and plays a role in lipid mobilisation and degradation.[295] The reduction of zinc α2-glycoprotein in contact lens wear suggests a reduction in lipid degradation. In contrast, an increase in lipid degradation has been found in intolerant contact lens wearers compared to tolerant contact lens wearers,[284] hence influencing the stability of the tear film. The decrease in zinc α2-glycoprotein found in this study suggests that in this group of asymptomatic contact lens wearers, lipid degradation upon awakening is reduced with EW. It may be that the reduction in the tears is attributed to an increased deposition on the contact lens surface[296] or due to the stagnation of the closed eye tear film in contact lens wear. The increase in albumin found in this study may be due to the increased permeability of the blood-tear barrier induced by contact lens wear[297] and hence serum leakage from the conjunctival capillaries. Albumin is a 66 kDa serum protein known to deposit on contact lens materials[298] and its greater concentration in the tear film has been related to increased contact lens deposition and increased bacterial adhesion.[299] The initial increase found in tear IgA has also been reported in a study on orthokeratology contact lenses,[198] while a second study on soft contact lenses has reported a decrease

in this protein.[201] The role of IgA in the tears is thought to prevent bacterial binding to epithelial cells.[300]

The increase found in protein S100 A8, and the decrease in lysozyme found in soft contact lens wearers by Kraman et al. using microarrays was not replicated in this study.[199]

5.7.6 Limitations of DIGE

As with many proteomic techniques, DIGE is subject to human error. The analysis process is dependent on 100% spot alignment which involves substantial user editing when using the software and also in deciding on what constitutes a spot and whether the spot should be split or merged with a neighbouring area. This human judgement may contribute to errors in the number of spots identified

Additionally, as gels must be post-stained with Coomassie Blue prior to mass spectrometry, and as Coomassie Blue is less sensitive than the CyDyes, some proteins may not be detectable from the Coomassie Blue-stained gel.[276]

Another limitation of the DIGE technique is the ineffective analysis of the low abundance proteins due to the wide range of protein abundance levels.[261] As a result, multiple proteomic techniques are necessary in order to completely identify the tear proteome. A study of epithelial and stromal profiles in keratoconus corneas found nano-ESI-LC-MS(MS)^2 to be superior to DIGE as it identified a greater number of proteins,[280] while another study used tandem mass spectrometry to identify proteases and their inhibitors in the tears.[291] A technique other than mass spectrometry-related approaches is the protein array[205] which consists of analytical protein chips containing arrays of antibodies and similar capture antibodies.[269] This technique however is limited by the number of antibodies available. Hence, proteomic techniques are complementary and future work may involve the use of multiple techniques in order to gain a more comprehensive understanding of the effect of contact lens wear on the tear proteome.

The absence of MMP-9 in these gels is surprising and may be a result of the limited dynamic range of this technique compared to immunoassays, and the presence of

MMP-9 in low levels compared to the high abundance proteins identified here.[210] It may be that the presence of MMP-9 is masked by the higher abundance of other species. Additionally, the use of detergents and reducing agents during DIGE following the first dimension may render proteases inactive, and hence affect the ability to detect these in their functional form.[301] A study by Tian et al of the pancreatic juice protein extracts of normals and people with pancreatic cancer, identified a 3.5-fold difference in MMP-9 using DIGE and MS, and further confirmed this using western blots.[302] In view of this finding, the absence of MMP-9 in the gels presented in this chapter is surprising considering the high concentration measured upon awakening, and the two-fold difference reported in Chapter 4 between baseline and the first night of EW. Additional work is required to establish the reason for this discrepancy.

The effect of storage on DIGE results is not known. However, based on the work by SItaramamma and colleagues, storage of tears for up to four months at -70 °C following centrifugation does not affect protein concentration.[303] Further work is required to establish the effect of storage on the tear proteome as analysed with DIGE.

5.7.7 Future work

As some biologically important differences were found in the tear proteome during the adaptation to EW, confirmatory measurements need to be performed in the future such as immunoassay of the different proteins[304] in prospective clinical studies targeted at analysing these specific differences.[305]

5.8 OUTCOMES OF CHAPTER 5

Chapter 5 evaluated the DIGE technique for flush tears and established the repeatability of the tear proteome. As with Chapter 3, the diurnal variation of the tear proteome was determined and a substantial increase in both protein numbers and normalised volume was found. When the proteome 'upon awakening' was compared at baseline, after the first night of contact lens wear and after a month of EW, the proteome did not change significantly, with only a 5% change in the tear proteome. Of the proteins identified by mass spectrometry, zinc α2-glycoprotein was found to decrease with contact lens wear, albumin was found to increase three-fold and

immunoglobulin A (IgA) was found to increase with initial EW and then reduce after one month.

This chapter, along with Chapter 4 have therefore established the impact of extended contact lens wear on the tear profile. With this understanding, Chapter 6 therefore compares these profiles of uneventful contact lens wear to those with corneal erosions.

CHAPTER 6 MMP-9 AND THE TEAR PROTEOME IN CONTACT LENS-RELATED CORNEAL EROSIONS

6.1 OVERVIEW

As reviewed in Chapter 1, matrix metalloproteinase-9 (MMP-9) has been found to be increased in expression in the corneas and tears of non-contact lens wearers prone to erosions.[102, 112] In order to establish whether contact lens wearers who develop erosions also have elevated levels of MMP-9, Chapter 3 first established the normal diurnal variation of MMP-9 in the tear film, and Chapter 4 demonstrated that this increases significantly with initial extended wear (EW). The tear proteome was explored in Chapter 5, and changes were identified in serum albumin, immunoglobulin A and zinc α2-glycoprotein with EW. During the course of these studies, three participants experienced an erosion event and one participant presented with a contact lens peripheral ulcer (CLPU). In Chapter 6 their individual MMP-9 levels are compared to the group of normals presented in Chapter 3 and Chapter 4. Moreover, the tear proteome of one participant with an erosion event is compared to that in uneventful EW as established in Chapter 5.

6.2 AIMS

This chapter aims to:

1. compare the levels of MMP-9 and its associated factors in uneventful contact lens wear to those who develop adverse events in contact lens wear.
2. compare the tear proteome of one participant who developed a corneal erosion in extended contact lens wear to that in uneventful contact lens wear.

6.3 HYPOTHESES

Based on the higher MMP-9 levels in non-contact lens wearers with erosions,[93, 102] the hypothesis is that people who develop erosions during contact lens wear have an increased expression of MMP-9, and neutrophil gelatinase-associated lipocalin (NGAL) compared to normals. Additionally, the hypothesis that the tear proteome differs between normal wearers and those who develop erosions will also be tested.

6.4 METHODS

6.4.1 Participants

As shown in Table 6.1, four adverse events occurred during the studies presented in Chapter 3 and Chapter 4. The data from these participants were excluded from the previous analyses and are presented here as a comparison to uneventful contact lens wear.

Table 6.1: Adverse events in the studies presented in Chapter 3 and Chapter 4.
EW = extended wear; DW = daily wear.

Participant	Schedule	Visit	Eye	Contact lens	Diagnosis
1	EW	Unscheduled (19 days, upon awakening)	OD	O_2OPTIX™	Corneal erosion
2	EW	Contact lens fit (Before sleep)	OS	O_2OPTIX™	Corneal erosion
3	DW	Contact lens fit (Before sleep)	OD	O_2OPTIX™	Corneal erosion
4	DW	One month (Midday)	OS	ACUVUE® OASYS™	Contact lens peripheral ulcer

6.4.2 Study design

The participants presented in this chapter participated in the same study as described in Chapter 3 and Chapter 4. Briefly, flush tears as described in Chapter 2, Section 2.5.3.3, were collected at baseline and participants were then fitted with contact lenses and examined as described in Chapter 4, Section 4.5.6. Participants were given the choice of EW or daily wear (DW) and their tears were collected at the first day of contact lens wear and following one month of adaptation (Figure 4.2). Each time, tears were collected at midday, before sleep and upon awakening. Although tears were collected at the time of the adverse event, tear volume was often limited, hence limiting the analysis possible. These results are not reported in this chapter.

6.4.3 Laboratory techniques

Tears were analysed for total protein content (TPC), MMP-9, tissue inhibitor of metalloproteinase-1 (TIMP-1) and NGAL as described in Section 3.5.7. MMP-9 activity

was ascertained with zymography (Section 3.5.7.5). The tear proteome of one participant with an erosion was compared at baseline upon awakening to that after the first night of EW using differential gel electrophoresis (DIGE), as described in Chapter 5, Section 5.5.5.

6.5 RESULTS

6.5.1 Case studies

6.5.1.1 Participant 1: corneal erosion in the right eye upon awakening after 19 days of EW with O_2OPTIX™

6.5.1.1.1 History

Participant 1 was a 29-year-old Asian male with no previous contact lens experience and no relevant history except for a complaint of occasional dry eyes. At the contact lens fit, Participant 1 was fitted with O_2OPTIX™ in the EW schedule. The assessment of the fit was 'optimal' and the tightness was graded as 47% for both eyes.

6.5.1.1.2 Symptoms

At the one week visit, Participant 1 reported bilateral dryness, worse by the end of the day with difficulty focusing. Slit-lamp biomicroscopy was unremarkable except for mild meibomian gland dysfunction in both eyes, for which lid hygiene was recommended.

After 13 days of extended contact lens wear, Participant 1 presented for an unscheduled visit complaining of dryness, tired eyes and difficulty focusing. Slit-lamp biomicroscopy revealed slight to moderate palpebral redness (grade 2.5) in both eyes with minimal corneal staining in the both eyes (superiorly and inferiorly, extent, depth and type 1).

After 19 days of extended contact lens wear, he presented for a second unscheduled visit complaining of foreign body sensation in the right eye upon awakening, exacerbated with the blink. This eye was now red and photophobic with tearing but no discharge. The participant reported regular use of saline drops due to dryness with contact lens wear.

6.5.1.1.3 Clinical signs at time of event

After 19 days of contact lens wear the participant presented with contact lenses *in situ*. Clinical assessment revealed a back surface round deposit on the contact lens in the right eye. The contact lens was mobile. Corneal assessment revealed an area of erosion at the inferior pupil margin and four adjacent areas of scuffed epithelium (Figure 6.1). The staining was graded as extent 3, depth 4 and type 4, with immediate stromal glow. The surrounding scuffed epithelium had the appearance of blistered epithelium. Bulbar and limbal redness was grade 3.2 in the right eye and grade 2.5 in the left eye. Contact lenses were removed and the participant was asked to rinse with sterile saline every hour. He was reviewed after two and 10 hours and on the following day.

After two hours, the redness had subsided to grade 2.5 bulbar and 2.3 limbal redness in the right eye. The area of scuffed epithelium had healed and the area of eroded epithelium at the inferior pupil margin was now grade 1 in extent, depth and type.

After 10 hours, no staining was present.

At the one-day follow-up visit all signs and symptoms had subsided and the participant was discontinued from the study and advised to avoid EW in the future.

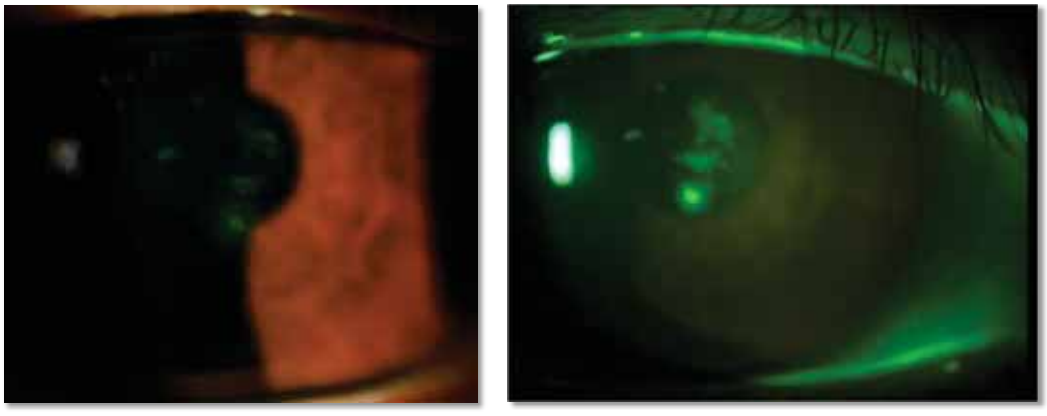


Figure 6.1: Participant 1 presented after 19 days of extended wear with O_2OPTIX™ with a corneal erosion surrounded by areas of scuffed epithelium.

This erosion was photographed with white light (LEFT) and with cobalt blue light and a Wratten #12 filter (RIGHT) after the instillation of sodium fluorescein.

6.5.1.1.4 Protein Analysis

The data for this participant are shown in Sections 6.5.2 to 6.5.6 and are represented with green asterisks. The TPC and MMP-9 concentrations were greater than the normal group mean after the first night of EW, while TIMP-1 concentrations were lower than average at both baseline upon awakening and after the first night of EW. The MMP-9:TIMP-1 ratio, and the concentration of NGAL were greater than average at both baseline upon awakening and after the first night of EW. Zymograms shown in section 6.5.7 show possible active MMP-9 after the first night of EW. DIGE analysis was also performed on the tears of this participant and this is reported in section 6.5.8.

6.5.1.2 Participant 2: corneal erosion in the left eye after the first five hours of wear with O_2OPTIX™

6.5.1.2.1 History

Participant 2 was a 25-year-old Caucasian male with no previous history of contact lens wear. He had a previous history of infiltrates, inflamed pingueculae and episcleritis. Three months prior to the study he had an episode of commotio retinae following a soccer injury.

At baseline he presented with corneal staining nasally and inferiorly in the right eye (extent 2, depth 2 and grade 2 for both areas) and centrally and inferiorly in the left eye (grade 1 for both). There were four faint scars superiorly in the right eye and two supero-nasally in the left eye.

Participant 2 fitted with O_2OPTIX™ and the overall fit was assessed as acceptably tight in the right eye and acceptably loose in the left eye, with a tightness grading of 55% and 45% for right and left eyes respectively.

6.5.1.2.2 Symptoms

The participant did not report any symptoms at the time of the erosion event. On further questioning, he noted that he had tried not to blink very often since the contact lens fit and that he tended to adopt the ‘comfort position’, that is, tilting the head back so as to minimise interaction of the eyelids with the contact lens edge.

6.5.1.2.3 Clinical Signs at the time of the event

After five hours of wear, Participant 2 attended for the 'before sleep' visit (Figure 4.2). At this visit, slit-lamp examination revealed an erosion in the left eye estimated as 0.2 mm wide and 0.4 mm in height (Figure 6.2). The participant was discontinued from contact lens wear in the left eye but proceeded to wear the right contact lens overnight.

The following morning, the staining in the left eye had resolved to reveal an area of negative staining. There were no associated infiltrates or anterior chamber reaction. The participant resumed contact lens wear as DW two days later. He returned for the one week follow-up visit where he was discontinued due to symptoms of itchiness and discomfort after attempting EW for one night.

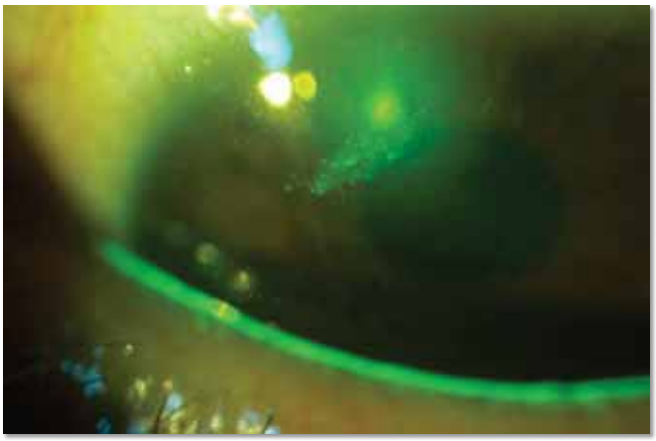


Figure 6.2: Participant 2 presented with a corneal erosion after the first five hours of contact lens wear with O_2OPTIX™.

6.5.1.2.4 Protein analysis

The data for this participant are shown in Sections 6.5.2 to 6.5.6 and are represented with a blue solid circle for the left eye with the erosion and with a blue ring for the right eye which did not experience an event. At baseline, upon awakening, the TPC was greater than the normal group mean for the eye that developed an erosion during contact lens wear. At the time of the erosion event, both TPC and MMP-9 were less than the group mean. Upon awakening, after the first night of EW in the eye without the event, MMP-9 was equal with the group mean. The left eye with the erosion event was without contact lens wear at the same time-point and had MMP-9 levels less than

the group mean. TIMP-1 levels at this time-point were much greater than the group mean for both eyes, and consequently the ratio of MMP-9:TIMP-1 was also less than the group mean. Zymograms shown in section 6.5.7 were unremarkable compared to the group of normals.

6.5.1.3 Participant 3: corneal erosion in the right eye after the first 7.5 hours of wear with O_2OPTIX™

6.5.1.3.1 History

Participant 3 was a 30-year-old Indian male with no history of contact lens wear and a history of viral conjunctivitis 11 years ago. At baseline there was no staining and both corneas were clear. He was subsequently fitted with O_2OPTIX™ as DW. The overall assessment of the fit was graded as optimal for both eyes with a tightness rating of 45% and 43% for the right and left eyes, respectively.

6.5.1.3.2 Symptoms

On contact lens insertion, Participant 3 complained of foreign body sensation which shortly subsided. After 7.5 hours of contact lens wear, the foreign body sensation resumed.

6.5.1.3.3 Clinical Signs at time of the event

After 7.5 hours of contact lens wear, slit-lamp biomicroscopy revealed a corneal erosion in the right eye, estimated as 0.1 mm by 0.1 mm (Figure 6.3). Participant 3 was subsequently discontinued from contact lens wear and re-examined the following morning.

Upon awakening, the area of erosion had resolved, as had the symptoms. He returned for examination two days later and re-commenced DW. He was advised to contact the optometrist should he experience further symptoms. He was reviewed two days later and reported foreign body sensation. No corneal staining or other clinical signs were present. He therefore proceeded with DW for one month.

6.5.1.3.4 Protein analysis

The data for this participant are shown in Sections 6.5.2 to 6.5.6 and are represented with a purple solid square for the right eye with the erosion and with a purple empty

square for the left eye which did not experience an event. At baseline, the TPC was greater than the normal group mean for the both eyes before sleep and upon awakening during contact lens wear. This was also the case upon awakening after the first day of DW for the eye that did not develop an adverse event. MMP-9 upon awakening was much greater than the group mean at all three visits. The TIMP-1 concentration was less than the group mean upon awakening at baseline. After the erosion event, however, TIMP-1 levels upon awakening were greater than the group mean. These TIMP-1 levels reduced after one month of contact lens wear, so that upon awakening they were less than the group mean. The ratio of MMP-9 to TIMP-1 was greater than the group mean upon awakening at all three visits, except for the eye that developed the erosion which was no different to the group mean at the time of the event.

Figure 6.3: Participant 3 presented with foreign body sensation and a small corneal erosion 7.5 hours after being fitted with O_2OPTIX™.

6.5.1.4 Participant 4: CLPU in the right eye after one month of DW in ACUVUE® OASYS™

6.5.1.4.1 History

Participant 4 was a 28-year-old female with a history of retinal laser and occasional contact lens wear, several times a year, the last occasion being two months prior to commencing the study. Slit-lamp biomicroscopy was unremarkable and the participant

proceeded to contact lens wear. She was fitted with ACUVUE® OASYS™ in the DW schedule.

6.5.1.4.2 Symptoms

At the one week visit she complained of occasional blur but was otherwise asymptomatic and slit-lamp examination was unremarkable.

At the one month visit Participant 4 complained of foreign body sensation in the right eye and no contact lens wear the previous week due to a cold.

6.5.1.4.3 Clinical Signs

At the one month visit, slit-lamp examination revealed a CLPU in the left eye with diffuse infiltration surrounding the focal area of infiltration. Fluorescein staining revealed stromal glow (Figure 6.4). Contact lens wear was ceased and the participant was discontinued from the study. She was monitored until resolution and advised to contact the optometrist should there be a change in redness or in discomfort or apparent size of the lesion. She was instructed to use saline rinses on a regular basis.[4]

6.5.1.4.4 Protein analysis

The data for this participant are shown in Sections 6.5.2 to 6.5.6 and are represented with a red solid triangle for the eye that developed the CLPU and with an empty triangle for the eye that was event-free. The TPC was greater than the group mean for the eye with the CLPU at the time of the event upon awakening while the MMP-9:TIMP-1 ratio was less than the group mean at the same time-point.

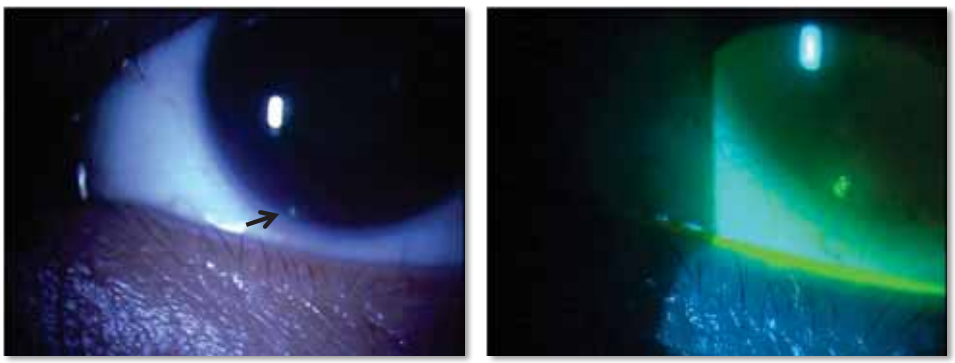


Figure 6.4: Participant 4 presented with a contact lens peripheral ulcer in the left eye (shown with arrow, LEFT) after one month of daily wear with ACUVUE® OASYS™. Stromal glow was evident with sodium fluorescein staining (RIGHT).

6.5.2 TPC

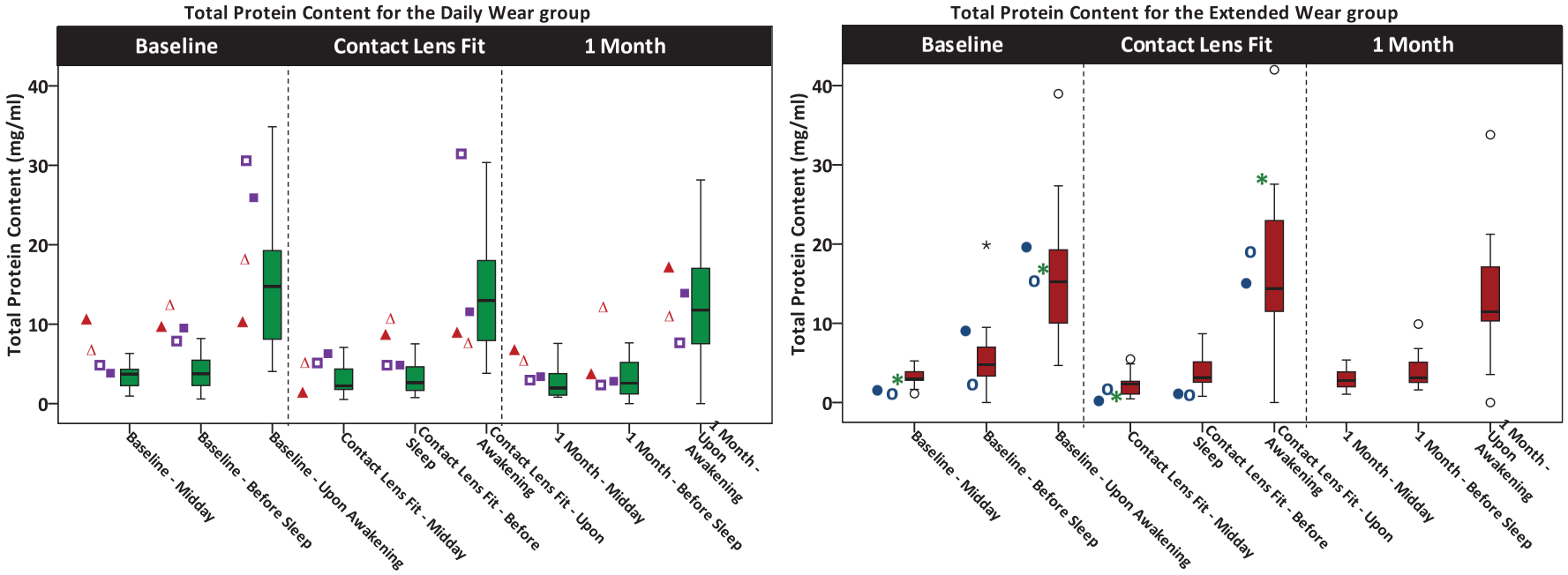


Figure 6.5: The total protein content for each participant graphed as a comparison to the group of normals.

* *Participant 1 (OU pooled) developed a corneal erosion OD after 19 days of extended wear with O_2OPTIX™*
• *Participant 2 OS developed a corneal erosion after five hours of wear with O_2OPTIX™*
O *Participant 2 OD no event*
■ *Participant 3 OD developed a corneal erosion after 7.5 hours of wear with O_2OPTIX™*
□ *Participant 3 OS no event*
▲ *Participant 4 OD developed a contact lens peripheral ulcer after one month of daily wear with ACUVUE® OASYS™*
Δ *Participant 4 OU no event*

6.5.3 MMP-9

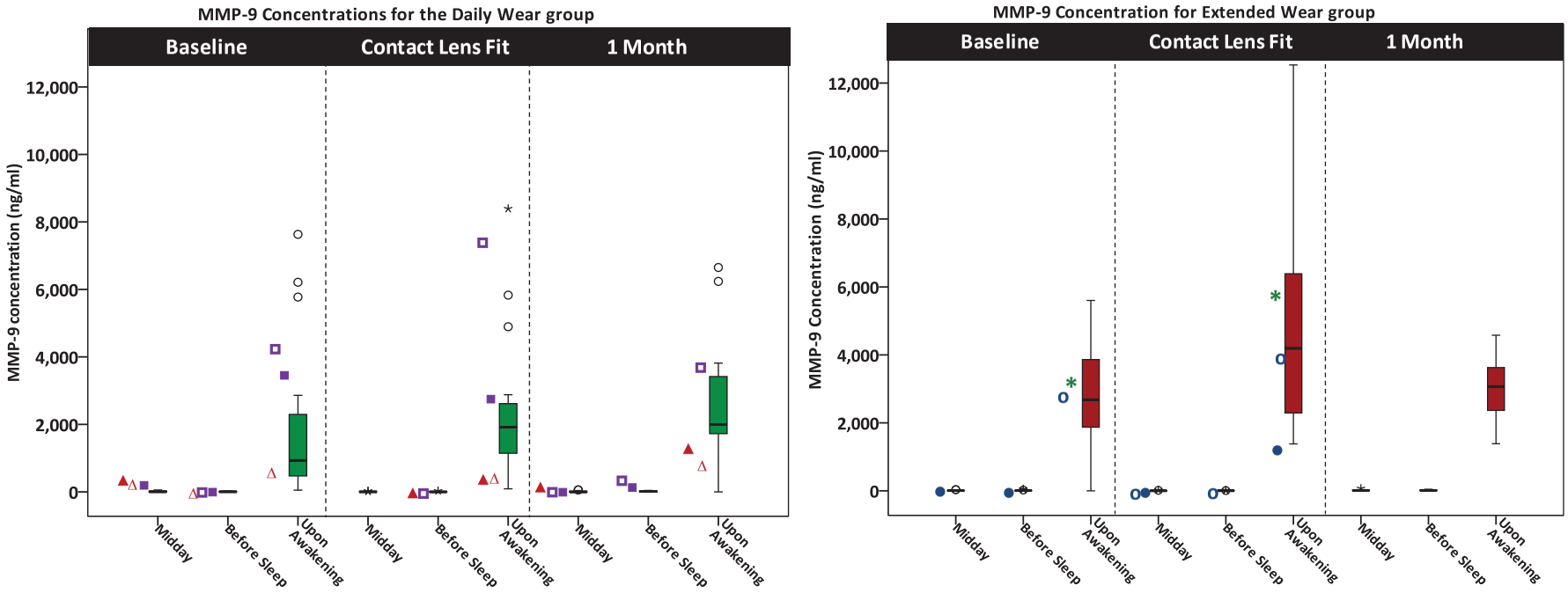


Figure 6.6: The MMP-9 concentration for each participant graphed as a comparison to the group of normals.

* *Participant 1 (OU pooled) developed a corneal erosion OD after 19 days of extended wear with O_2OPTIX™*
• *Participant 2 OS developed a corneal erosion after five hours of wear with O_2OPTIX™*
O *Participant 2 OD no event*
■ *Participant 3 OD developed a corneal erosion after 7.5 hours of wear with O_2OPTIX™*
□ *Participant 3 OS no event*
▲ *Participant 4 OD developed a contact lens peripheral ulcer after one month of daily wear with ACUVUE® OASYS™*
Δ *Participant 4 OU no event*

6.5.4 TIMP-1

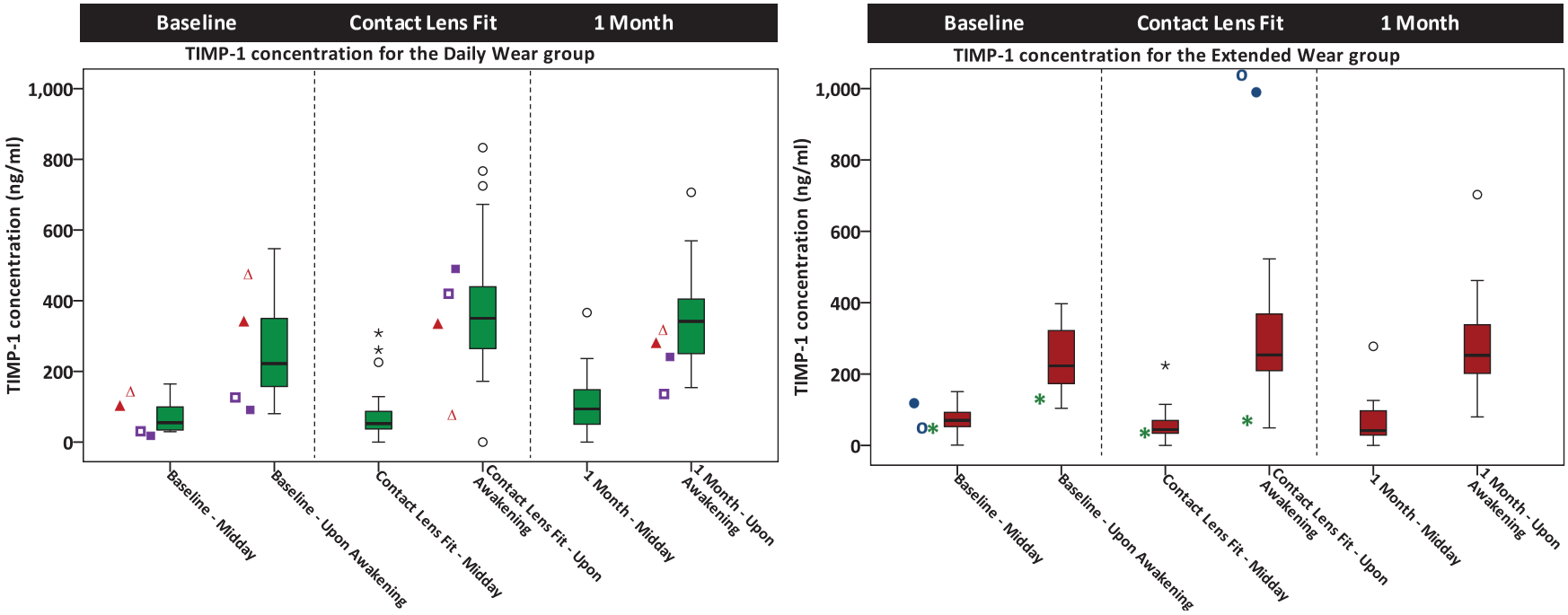


Figure 6.7: The TIMP-1 concentration for each participant graphed as a comparison to the group of normals.

* *Participant 1 (OU pooled) developed a corneal erosion OD after 19 days of extended wear with O_2OPTIX™*
• *Participant 2 OS developed a corneal erosion after five hours of wear with O_2OPTIX™*
O *Participant 2 OD no event*
■ *Participant 3 OD developed a corneal erosion after 7.5 hours of wear with O_2OPTIX™*
□ *Participant 3 OS no event*
▲ *Participant 4 OD developed a contact lens peripheral ulcer after one month of daily wear with ACUVUE® OASYS™*
Δ *Participant 4 OU no event*

6.5.5 Ratio of MMP-9:TIMP-1

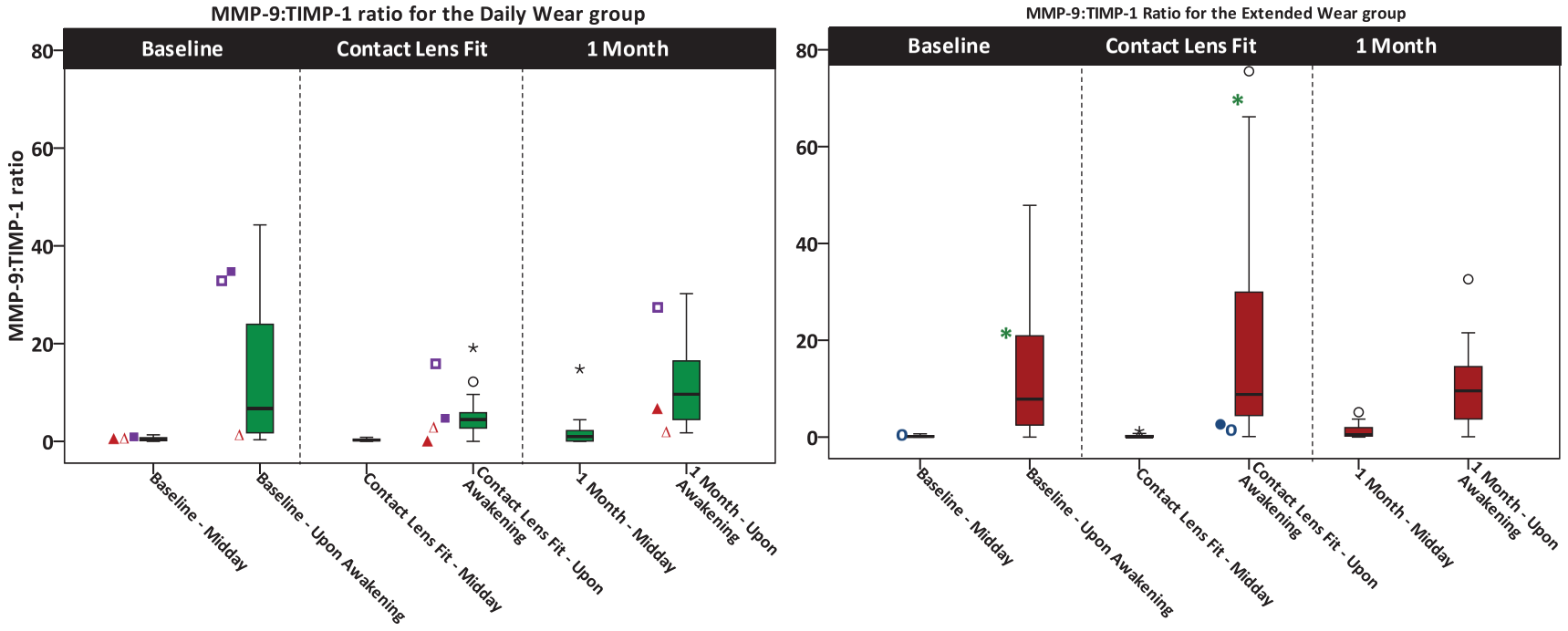


Figure 6.8: The MMP-9:TIMP-1 ratio for each participant graphed as a comparison to the group of normals.

* *Participant 1 (OU pooled)developed a corneal erosion OD after 19 days of extended wear with O_2OPTIX™*
• *Participant 2 OS developed a corneal erosion after five hours of wear with O_2OPTIX™*
O *Participant 2 OD no event*
■ *Participant 3 OD developed a corneal erosion after 7.5 hours of wear with O_2OPTIX™*
□ *Participant 3 OS no event*
▲ *Participant 4 OD developed a contact lens peripheral ulcer after one month of daily wear with ACUVUE® OASYS™*
Δ *Participant 4 OU no event*

6.5.6 NGAL

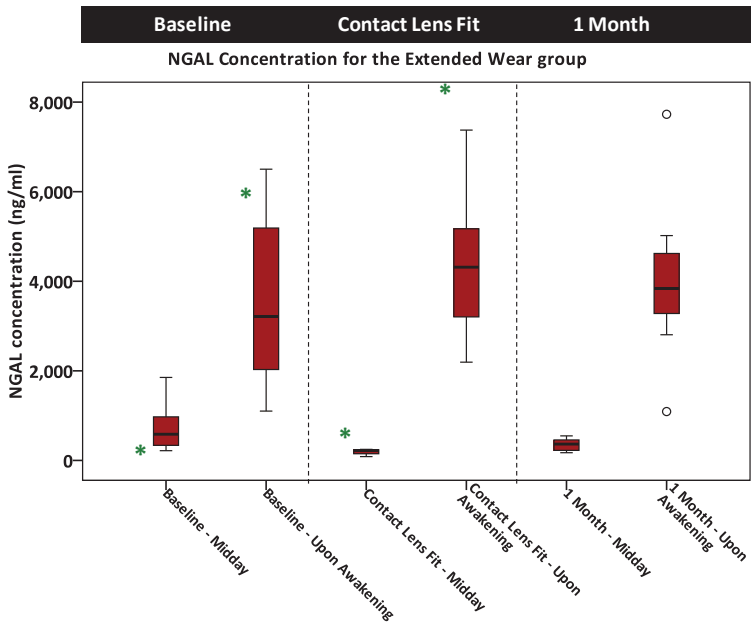


Figure 6.9: The NGAL concentration graphed as a comparison to the group of normals.

* *Participant 1 (OU pooled) developed an erosion after 19 days of extended wear with O_2OPTIX™*

6.5.7 Zymography

Zymograms of the tear samples of the three erosion participants are shown in Figure 6.10. Bands were identified at >200, 135, 83 and 72 kDa corresponding to the MMP-9 dimer, an MMP-9 complex, pro-MMP-9 and and pro-MMP-2 respectively as previously identified by others.[183, 234-238] One of the three erosion participants (Participant 3) showed greater MMP-9 at baseline compared to normals (Chapter 3, Section 3.6.9.9, representative normal gel shown in Figure 6.10). Participant 1 showed significant levels of MMP-9 after the first night of EW which extended beyond the 92 kDa band and into the 83 kDa band. However, densitometry was unable to discriminate separate bands in this region (92 and 83 kDa) corresponding to pro- and active MMP-9, possibly due to overlap of the two species. An additional band at approximately 190 kDa was also identified after the first night of EW for Participant 1. This was also detected by densitometry (data not shown) as a faint band after one month of DW in Participant 3. This has been reported by others in urine samples of cancer patients[249] and identified as a disintegrin and metalloprotease with thrombospondin motifs-7 (ADAMTS-7).

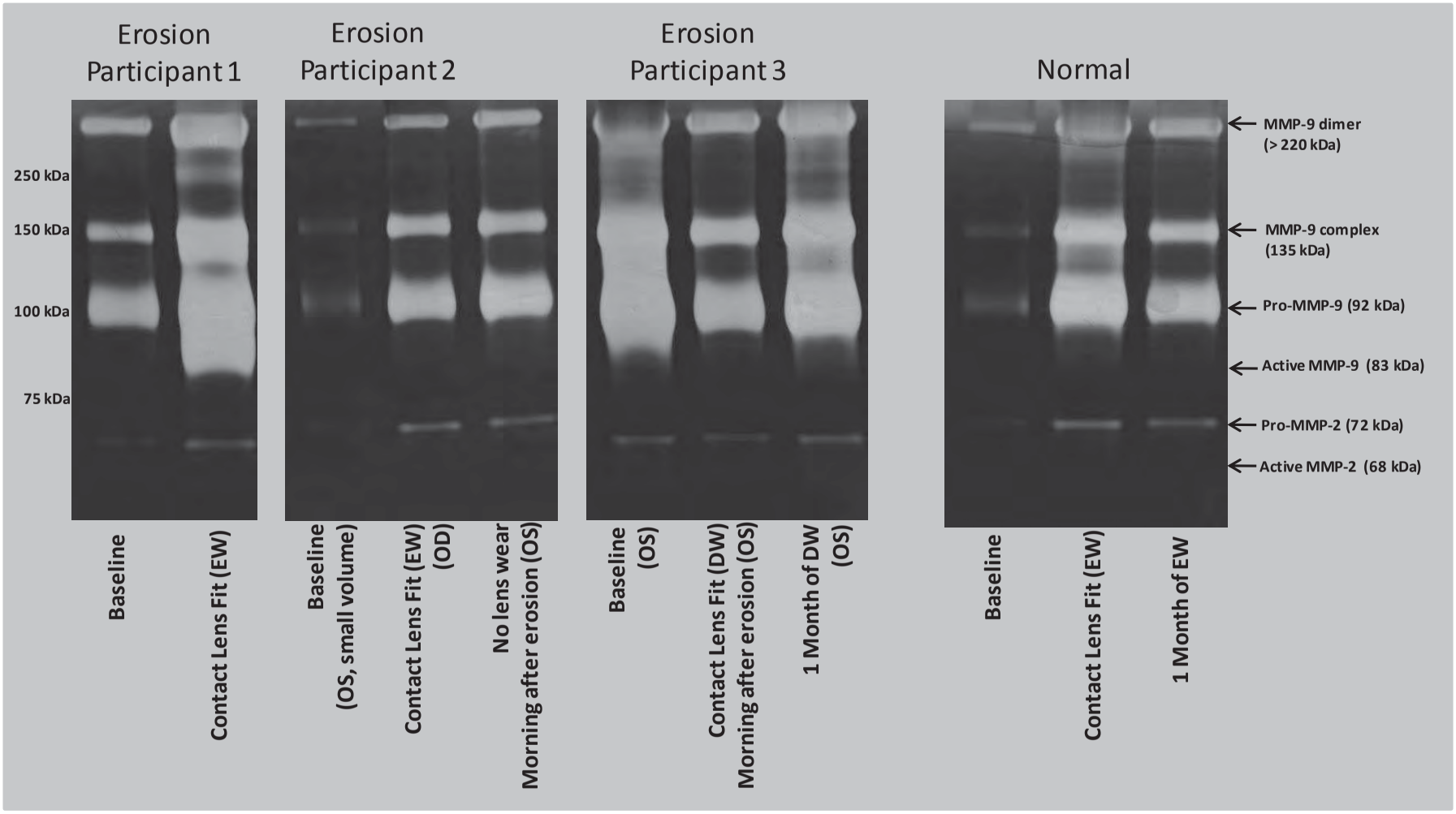


Figure 6.10: The zymograms of each of the three participants who developed corneal erosions in the study compared to a normal participant.

All tears were collected immediately upon awakening. The tears of the right and left eyes were pooled for Participant 1 and for the 'normal' participant presented here. For Participants 2 and 3 eyes were not pooled. The 'normal' tears are from Chapter 4, section 4.6.7.8.

6.5.8 The tear proteome

6.5.8.1 Erosion Participant 1 at baseline compared to the first night of extended wear

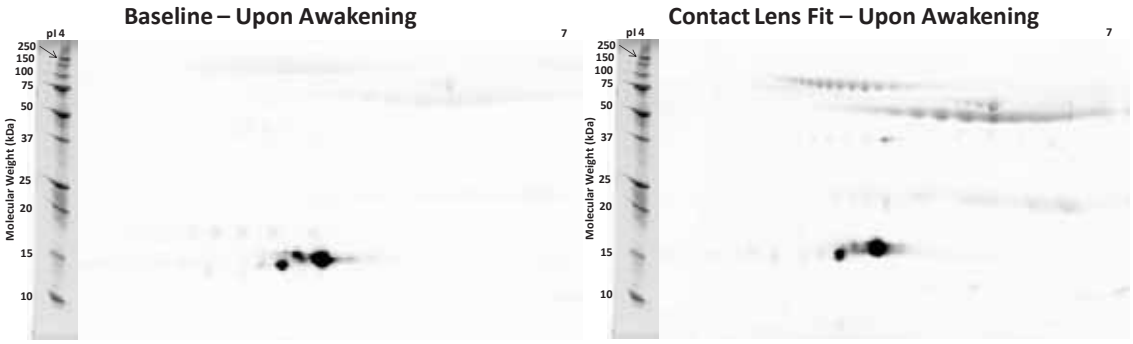


Figure 6.11: The differential gels of Participant 1 upon awakening at baseline and after the first night of extended wear.

After 19 days of EW, this participant developed a corneal erosion.

DIGE analysis of the tears of Participant 1, Figure 6.11, found 25 spots to be more than 4.4-fold different upon awakening after the first night of EW compared to baseline 'upon awakening'. The fold difference between the two visits ranged between 4.4- to 18.8-fold, with 11 spots being greater at baseline and 14 spots being greater after the first night of EW. Of these 25 spots (Figure 6.12), four were identified by mass spectrometry (Table 6.2). In contrast, for the eight normals reported in Chapter 5, no spots were more than 2-fold different between baseline and the first night of contact lens wear (Section 5.6.3).

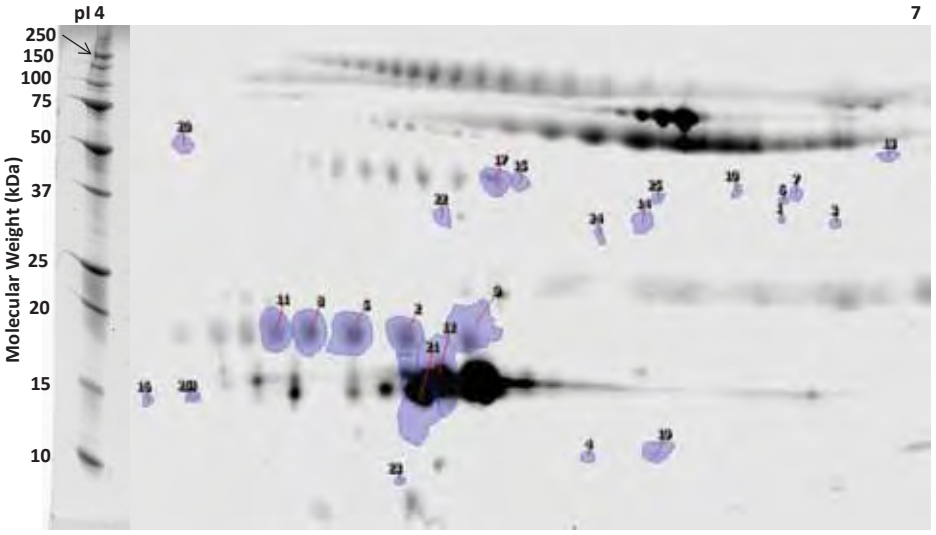


Figure 6.12: The spots identified to be more than 4.4-fold different between baseline upon awakening and after the first night of extended wear.

6.5.8.2 Erosion Participant 1 compared to normals

When the tear proteomes of the erosion participant and the group of normals (Chapter 5, Section 5.6.3) were compared at baseline upon awakening, only one protein spot was greater than 4.4-fold different.

When the tear proteome of the erosion participant was compared to the normal group at the contact lens fit 'upon awakening', 14 spots were more than 4.4-fold different (range 4.6-23.7, Figure 6.13). Nine spots were greater in normalised volume for the erosion participant and four were greater for the group of normals. Eight of these spots were identified with mass spectrometry and these are listed in Table 6.3.

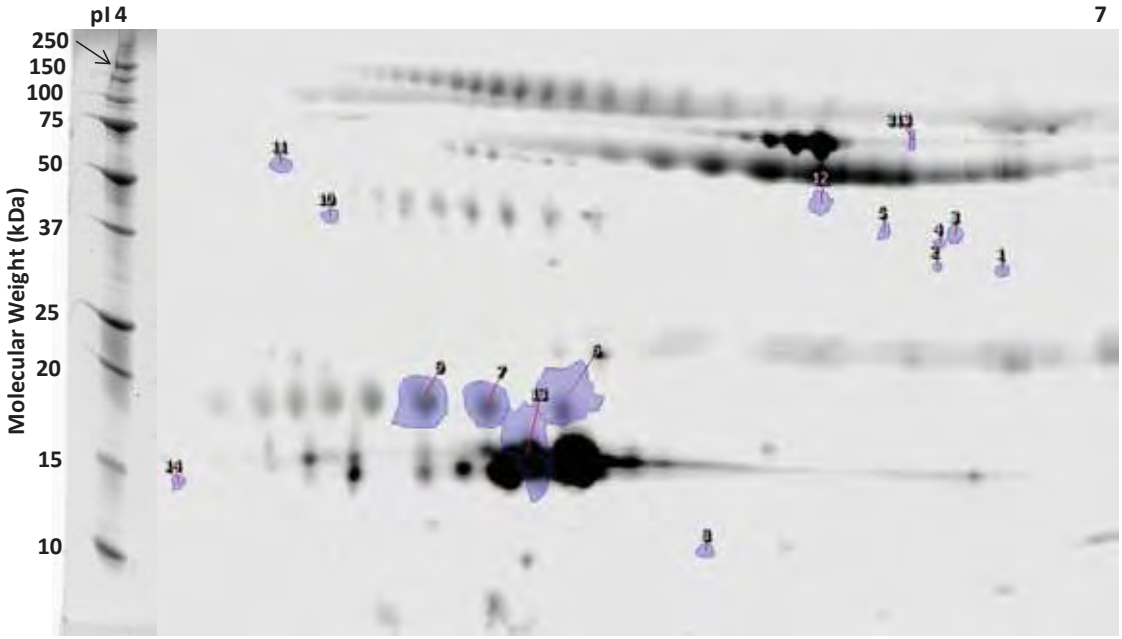


Figure 6.13: The protein spots identified by differential gel electrophoresis to differ between the erosion participant and the group of normals after the first night of extended wear.

Table 6.2: Proteins spots greater than 4.4-fold different between the baseline upon awakening and the first night of extended wear for Participant 1 were identified with mass spectrometry.

Protein abundance is expressed as a normalised ratio relative to spots from the internal standard.

Rank	Protein name	Accession number	Mascot Score	Mass (Da)	Peptide hits	Fold	Erosion Baseline	Erosion contact lens fit
							Mean normalised volumes	
8	Extracellular glycoprotein lacritin precursor	gi|15187164	163	14237	8	12.7	1.47	0.12
11	Extracellular glycoprotein lacritin precursor	gi|15187164	95	14237	5	8.9	1.40	0.16
12	Human tear lipocalin von ebners gland protein	gi|56554584	555	17914	28	8.3	2.44	0.29
21	Lipocalin-1 precursor	gi|4504963	469	19238	19	5.2	2.47	0.48

Table 6.3: Proteins spots greater than 4.4-fold different between the erosion Participant 1 and the group of normals after the first night of extended wear were identified with mass spectrometry.

Protein abundance is expressed as a normalised ratio relative to spots from the internal standard.

Rank	Protein name	Accession number	Mascot Score	Mass (Da)	Peptide hits	Fold	Erosion	Normals
							Mean normalised volumes	
1	Lipocalin-1 precursor	gi|4504963	158	19238	7	23.7	34.59	1.46
6	Extracellular glycoprotein lacritin precursor	gi|15187164	195	14237	4	7	0.13	0.94
8	Fibrinogen beta	gi|223130	54	12720	1	5.5	3.72	0.68
9	Extracellular glycoprotein lacritin precursor	gi|15187164	96	14237	6	5.3	0.10	0.53
10	Apolipoprotein J precursor	gi|178855	241	48772	5	5.3	0.55	2.93
11	Lactoferrin	gi|34412	222	78029	4	5.3	0.33	1.76
13	Human tear lipocalin von ebners gland protein	gi|56554584	555	17914	28	4.8	0.29	1.40
14	Secretory phospholipase A2	gi|443191	102	13909	5	4.6	0.40	1.84

6.6 SUMMARY AND DISCUSSION

6.6.1 Summary of results

Chapter 6 compared the levels of TPC, MMP-9 and TIMP-1 of three study participants who developed corneal erosions and one participant who developed a CLPU to those who wore contact lenses uneventfully as described in Chapter 3 and Chapter 4. The levels of NGAL and the change in the tear proteome were also compared in one erosion participant to the group of normals.

For two participants, the ratio of MMP-9 to its inhibitor, TIMP-1, exceeded the normal ratio at baseline and for one of these participants, the ratio tripled with initial EW upon awakening (Participant 1, Figure 6.8). A high ratio prior to contact lens wear may predispose an individual to erosion in the presence of a contact lens by virtue of the increased potential for collagen degradation combined with the trigger that is the contact lens. Additionally, the difference between baseline upon awakening and the first night of EW may potentially be predictive of erosion risk. The physiological variation evident in the group of normals in Figure 6.8 suggests that other mechanisms are responsible for regulating the effects of MMP-9, as not all those with an elevated ratio developed corneal erosions in this study. The hypothesis that the ratio of MMP-9:TIMP-1 is a marker of those prone to erosions during contact lens wear would require confirmation with a larger study.

In one of the three erosion participants (Participant 3), greater MMP-9 activity was evident upon awakening even before contact lens wear as is evident from the zymogram in Figure 6.10. Participant 1 showed significant levels of MMP-9 after the first night of EW which extended beyond the 92 kDa band and into the 83 kDa band. This more pronounced difference in MMP-9 activity both before contact lens wear and after contact lens wear between this small group who developed erosions and the group of normals supports the suggestion that those prone to adverse events may be distinguishable at the earlier phase of contact lens wear.

An additional species of MMP-9 was identified at 190 kDa in two of the three erosion participants after contact lens wear (Figure 6.10). Although this species has not

previously been described in tears before and was not observed in the group of normals, it has been identified in urine of cancer patients as a disintegrin and metalloprotease with thrombospondin motifs-7 (ADAMTS-7)[249] and is inhibited by α_2-macroglobulin.[306] The ADAMTS family consist of zinc metalloproteinases with at least one thrombospondin repeat. They have been implicated in connective tissue organisation, wound healing, cell migration, angiogenesis and inflammation.[127] Sack and colleagues describe an additional MMP-9 in the range of 116kDa, similar to the other two complexes, raising the point that there may be a third protein which complexes with MMP-9.[135] Future work may consider the confirmation of the presence of this species with a larger study and identification using immunoblotting.

An elevated NGAL concentration at baseline that further doubled after the first night of EW was found in one participant who later developed an erosion. NGAL is known to bind and activate latent MMP-9 and MMP-8 and may therefore upregulate the dissolution of Bowman's layer as driven by MMP-9.[134] It has been used as a biomarker for disease in the kidney by virtue of its protective effect of MMP-9.[158] NGAL however, also has an antimicrobial role by virtue of its iron sequestering activity,[157] and it has also been found to be protective during oxidative stress.[159] Its role in the tear film and ocular surface however is not well understood and the reason for the elevation noted in this participant requires further investigation.

As a whole, the tear proteome changed significantly after the first night of EW in Participant 1, compared to minimal change in the group of normals (Chapter 5). One protein that was found to be much greater in this participant compared to the group of normals after the first night of EW was lipocalin. A two-fold increase in NGAL was also found with contact lens wear for this participant. NGAL belongs to the lipocalin family and potentially may be contributing to the difference in the lipocalin identified by DIGE. Lacritin was found to have decreased expression with contact lens wear in this participant. Lacritin promotes basal tear secretion and is also a growth factor, stimulating human corneal epithelial cell growth.[307] It has previously been found by Nichols and Green-Church to be down-regulated in contact lens-related dry eye[235] and by Koo et al. to be down-regulated in blepharitis.[308] The finding of this study suggests that both reduced tear secretion and reduced epithelial renewal may contribute to

adverse events in contact lens wear, a concept that requires confirmation with a larger study.

The greater difference in the tear proteome at baseline as compared to the first night of EW in the one participant who developed an erosion after 19 days of EW, suggests that in some contact lens wearers EW is associated with a more substantial change in the tear proteome than others. A greater sample size of participants with adverse events may help establish whether this change between baseline and the first night of EW can be predictive of those at risk of erosions, and by association, microbial keratitis.[13]

6.6.2 Implications of findings

The results of this study suggest that elevated MMP-9 levels that are not balanced by their inhibitor TIMP-1 during contact lens wear may be predictive of those likely to proceed to adverse events such as erosions. A greater sample size of contact lens wearers with erosions may help confirm this association. This imbalance may weaken the adhesion of the epithelium to the underlying stroma, and when associated with contact lens wear and other factors such as a bound contact lens, may contribute to erosion development. The elevated NGAL found in the tears of one erosion participant may further exacerbate the actions of MMP-9, by preventing its degradation and hence enabling it to act on the epithelial adhesions. Alternatively, the elevated NGAL may be a response to oxidative stress or bacterial load. When these factors are further combined with bacterial load, which in itself may increase MMPs in the cornea and tears,[67] the risk of a destabilised epithelium, along with the risk of infection is further augmented.

6.6.3 Erosion rate in this study

In the study presented in Chapter 3 and Chapter 4, three of the 46 participants developed erosions, one after a period of EW and the other two after their first 5-7.5 hours of contact lens wear. This incidence appears to be greater than that anticipated.[13] Erosions are rarely seen clinically due to their fast resolution, and they are often diagnosed on the basis of symptoms. The regular visits particularly in the

early phases of wear in this study may explain the higher rate of erosions seen, which otherwise may have resolved following contact lens removal. It may also be that erosions are in fact more common than is reported.[13]

6.6.4 Limitations of study

A limitation of this study is the small sample size of participants with corneal erosions. In view of the rare presentation of erosions and the fast healing rate,[13] the case study approach was taken in this chapter as a means of identifying individual factors likely to predispose contact lens wearers to erosions. A larger sample size would allow confirmation of the results of this chapter. It is possible that some participants could develop erosions even after one month of lens wear.[13] A larger study of longer duration observing erosion rate and measuring MMP-9 and TIMP-1 would contribute greatly to the understanding of whether those with greater expression of MMP-9 in the earlier phases of contact lens wear, or indeed at baseline, are at continued risk of developing corneal erosions.

The tears analysed in this study were collected at the same time-points as the normals presented in Chapter 3 and Chapter 4. Although tears were collected at the time of the adverse event, tear volume was often limited, hence limiting the analysis possible. However, because of the wound healing process that would be in process in the case of an active erosion, the response to contact lens wear is reported here rather than the response at the time of the adverse event.

6.6.5 Future work

In order to test the hypothesis that contact lens wearers at risk of erosion formation can be identified by tear collection during the early phases of contact lens wear, a study to compare the MMP-9:TIMP-1 ratio, NGAL concentration and the tear proteome with DIGE is proposed. In the proposed study, tears will be collected upon awakening at baseline and after the first night of EW in the following groups:

- healthy neophyte controls
- current non-contact lens wearers with a previous erosion event in contact lens wear

- current non-contact lens wearers with a previous microbial keratitis event in contact lens wear

Establishing the biomarkers associated with corneal erosions in contact lens wear could enable these to be used both as predictive tools, as well as incorporated into preventative measures. Sjogren's syndrome[219] and dry eye,[114, 309] for example, have been associated with their biomarkers.

Further studies are also necessary to understand the role of NGAL in the tear film, whether this is indeed protective in the case of oxidative stress, or an indication of greater MMP-9 activity.

6.7 OUTCOMES OF CHAPTER 6

Chapter 6 compared the diurnal profiles of healthy contact lens wearers to a group of three participants who developed corneal erosions during contact lens wear. A greater MMP-9:TIMP-1 ratio at baseline upon awakening that further increased after the first night of EW, along with increased NGAL concentration was found, suggesting that people who develop erosions in contact lens wear have increased collagen degrading activity that is further exacerbated by contact lens wear, a finding that requires confirmation with a larger scale study.

Moreover, a significant change in the tear proteome with DIGE in one participant between baseline and the first night of EW, compared to the minimal change in the case of normals, suggests that the tear proteome responds differently in those prone to erosions. This response may be a predictive tool, a finding that also requires confirmation with a larger study.

CHAPTER 7 SUMMARY AND CONCLUSIONS

7.1 SIGNIFICANCE OF RESEARCH

Corneal erosions in contact lens wear are of significance in the discomfort and disturbance they cause to the contact lens wearer, and in their associated risk with microbial keratitis (MK).[13] Understanding their aetiology is vital to their management and prevention.

In non-contact lens wearers, matrix metalloproteinase-9 (MMP-9) has been found to be upregulated in the corneas and tears of those prone to erosions.[93, 112] MMP-9 is thought to weaken corneal adhesion complexes by virtue of its ability to degrade the constituents of the extracellular matrix.[104] Through this ability, MMP-9 plays an important role in physiological processes such as wound healing, and when not appropriately balanced, in pathological processes such as corneal erosions in the absence of contact lens wear. Whether MMP-9 plays a role in contact lens-related corneal erosions is unknown.

Identifying the impact contact lens wear has on tear proteins such as MMP-9 may be key to understanding the aetiology of erosions and identifying the proteins to target in order to prevent erosion occurrence. The aim of this thesis, therefore, was to establish the effect of contact lens wear on the MMP-9 profile in the tear film, and on the tear proteome as a whole. A further aim of this thesis was to compare these profiles to those who develop corneal erosions in contact lens wear in order to determine differences in their profiles.

7.2 SUMMARY

7.2.1 Validating a technique for rapid and repeatable tear collection

Tear analysis is an important means of measuring the status of the ocular surface by virtue of its proximity to the cornea and conjunctiva and was therefore used in this thesis to determine the effect of contact lens wear on the ocular surface. The tear collection technique needed to allow for adequate volume to be collected with minimal disturbance to the participant and with minimal impact on the composition of

the tear film. For this purpose, Chapter 2 standardised and compared the flush technique, a technique where a saline drop is instilled into the inferior conjunctival sac prior to collection, to the more commonly used basal and reflex tear collection. The flush tear collection method did not influence the composition of the tear film as determined by the proportion of immunoglobulin A (IgA) measured to total protein content (TPC). This method was also found to be a repeatable and rapid means for collecting the major tear proteins. Moreover, it allowed for a larger volume of tears to be collected. Because the subsequent studies involved analysis of multiple proteins from the same sample, and tears were also collected upon awakening, a time-point notorious for the low volume and reflex tearing, the flush method was therefore chosen as the tear collection technique.

7.2.2 MMP-9 increases substantially during sleep

Prior to determining the effect contact lens wear has on the MMP-9 profile, its physiological variability was established in Chapter 3 by looking at the difference between eyes and the difference between days, each at the same time-point. While the coefficient of repeatability was greater upon awakening than it was at midday when comparing days, at both times it was double the average at that time-point. This consideration reveals significant physiological variation, a factor to consider when determining differences.

Chapter 3 further established the diurnal profile of MMP-9 and found that concentrations of MMP-9 are negligible during the day and completely inhibited by tissue inhibitor of metalloproteinases-1 (TIMP-1). During sleep, MMP-9 increases 200-fold, an increase that is not matched by its inhibitor, TIMP-1. Neutrophil gelatinase-associated lipocalin (NGAL) also complexes with MMP-9 and was therefore also measured and found to exceed MMP-9 at all times. NGAL has been previously identified in ‘closed eye’ tears and is known to complex with pro-MMP-9 when it is produced by neutrophils.[135] By preventing MMP-9 from degradation, NGAL protects the activity of MMP-9, hence perpetuating its proteolytic effects. NGAL also has a bacteriostatic effect by sequestering small iron-binding molecules known as siderophores synthesised by bacteria to acquire iron from their host.[157, 158] An

additional role of NGAL is to protect cells against oxidative stress.[159] Its exact role in the tear film and the reason for its diurnal variation is not known.

The diurnal profile of these proteins suggests that the closed eye is an environment conducive to extracellular matrix remodelling. The fact that we do not see corneal damage upon awakening suggests that there are other regulatory mechanisms which prevent excess extracellular matrix degradation, and events such as recurrent corneal erosions may result when these mechanisms fail.

7.2.3 Extended contact lens wear further increases MMP-9 overnight

With this understanding of the normal profile of MMP-9 and its associated factors, Chapter 4 explored the effect of contact lens wear in the extended wear (EW) and daily wear (DW) schedules and changes with adaptation. Although DW did not change the profile, EW caused a significant increase in MMP-9 upon awakening, with no corresponding increase in TIMP-1, suggestive that at this early stage of contact lens wear the cornea may have a greater tendency for collagen degradation and hence be at greater risk of events such as erosions.[112] Although such tissue damage does not occur in the majority of contact lens wearers, the risk of erosion,[13] infection and inflammation[1, 5] is greater both during overnight contact lens wear and during adaptation. The finding of increased MMP-9 expression after the first night of EW suggests that this may be a contributing factor when regulatory mechanisms fail and also reinforces overnight wear as a risk factor for these events.

The increase seen in overnight contact lens wear may be a result of wound healing related to contact lens wear, or may be a result of another factor. However, due to the known role of MMP-9 in basement membrane degradation and the greater association of erosions upon awakening,[25] its increased presence in contact lens wear may increase the risk of adverse events.

7.2.4 Extended contact lens wear influences the tear proteome

In order to gain a broader understanding of the impact of contact lens wear, in Chapter 5 the tear proteome as a whole was evaluated using differential gel electrophoresis (DIGE), a technique used in biomarker research.[277]

The DIGE technique involves the use of three mass and charge-matched dyes (Cy2, Cy3 and Cy5)[272] to label two samples and an internal standard (Cy2) on each gel, the two samples on each gel being normalised against the internal standard. The technique was validated to establish the difference in the labelling of the CyDyes when used with flush tears and the difference between gels when running the same sample. From this validation, a 4.4-fold difference in normalised volume was established as the threshold for when comparing single gels and a fold difference below this was considered to be due to experimental variability. DIGE was then applied to establish the repeatability of the tear proteome and its diurnal variation, the former indicating an overall repeatable proteome at both midday and upon awakening, and the latter indicating a change in up to 29% of the tear proteome during sleep.

With this background, the impact of extended contact lens wear on the tear proteome upon awakening was established. A change in 1% of the proteome was found from baseline to the first night of contact lens wear and a 3% change from baseline compared to one month of EW, suggesting that although the tear proteome does change with initial EW, the change continues even after one month. Future work to quantify the proteins found to vary is necessary to understanding these changes.

7.2.5 Contact lens-related corneal erosions: increased MMP-9:TIMP-1, NGAL, and a disturbed tear proteome

In Chapter 6 the profiles of three contact lens wearers who developed corneal erosions during the course of the study were compared to the established normal values reported in Chapter 3, Chapter 4 and Chapter 5. Although the small sample size presented here did not allow for inferential statistical analysis, a higher MMP-9 concentration upon awakening at both baseline and after the first night of contact lens wear, along with the greater MMP-9:TIMP-1 ratio suggest that MMP-9 may be a factor in the aetiology of these erosions. By virtue of these elevated levels of MMP-9, those prone to erosions may be identifiable at either baseline or after the first night of contact lens wear, a finding that requires confirmation with a larger study.

Of further interest, and worthy of further study, is the greater concentration of NGAL found in one erosion participant, both at baseline and after the first night of contact

lens wear. The role of NGAL in the tear film is not well understood and as discussed earlier, its increased expression may be a result of oxidative stress,[159] or due to an increased requirement for antibacterial activity.[157] Alternatively, an increased in NGAL expression may be an indication of an increase in MMP-9 expression, due to its protective effect on MMP-9. This effect on MMP-9 has identified NGAL as a biomarker for kidney disease[158] and breast cancer[156] and in the tear film may be indicative of extracellular matrix degradation. Further work is necessary to understand the role of NGAL in the ocular surface, both in health and disease.

The zymographic analysis of the three erosion participants indicated that in one participant, after the first night of contact lens wear, a significant increase in activity was found, along with an additional species of MMP-9 at 190 kDa, not found at baseline or in other participants. One of the erosion participants had greater activity at baseline compared to other participants. The increased activity in this group compared to normals suggests that people prone to erosions in contact lens wear, whether it be in DW or EW, may be identifiable by their unusual MMP-9 profile upon awakening before contact lens wear and after the first night of contact lens wear.

MMP-9 and its related proteins are not likely to be the only proteins contributing to the erosion cascade, and this is supported by the proteomic difference at baseline and upon awakening for one erosion participant, compared to the minimal change for the group of normals. This suggests that factors other than MMP-9 may contribute to the process which ultimately leads to erosion formation. An assessment of the proteome upon awakening at baseline and after the first night of contact lens wear may also be a means of predicting those prone to erosions, and by association, MK.[13]

The finding of two erosions on the first day of wear indicates that EW is not the only factor contributing to the process.

7.2.6 Contact lens-related corneal erosions: a multifactorial aetiology

The fact that higher levels of MMP-9 compared to TIMP-1 did not always result in erosions in this study, suggests that the underlying cause of erosions is multifactorial.

Weakened epithelial adherence to the stroma as a result of a contact lens-associated increased expression of MMP-9, combined with a reduced tear film and sudden mechanical action such as rapid eye movement during sleep, eyelid action upon awakening or the regained mobility of a contact lens adhered during sleep,[21, 59-61] may be necessary to result in erosion. Similarly, factors such as contact lens thickness,[11] bacterial load[66, 67] and contact lens modulus[310] may all augment the risk of corneal erosions during contact lens wear. This is analogous to the multifactorial aetiology of MK, where a combination of bacterial load, changed tear immunology as well as exposed stroma is required for infection to occur.[69]

7.3 LIMITATIONS OF RESEARCH

The extracellular matrix remodelling proteins of the ocular surface are comprised of many proteins, including MMP-3, α_2-macroglobulin and ADAMTS-7. Due to the limitation of tear volume, the proteins studied directly were limited to MMP-9, TIMP-1 and NGAL. In order to further explore potential biomarkers related to contact lens-related corneal erosions, DIGE was performed as a means of examining differences in the more abundant proteins. Future work would look at quantifying the differences identified with DIGE, as well as directly studying proteins which contribute to the regulation of MMP-9 such as α_2-macroglobulin and ADAMTS-7, as well as the cytokines such as interleukin-8 (IL-8) and IL-1β and other MMPs such as MMP-3.

The small sample size of erosions was a challenge faced in this thesis. A larger scale study in the future would allow for a stronger comparison of the proteins measured here in contact lens wearers who develop either erosions or MK to the group of normals.

7.4 FUTURE RESEARCH

Based on the knowledge gained in this thesis, it is feasible that MMP-9 contributes to the development of erosions during contact lens wear. Theoretically, a study of the MMP-9 response in a group who previously developed either erosion or MK in contact lens wear may help confirm this finding. Measuring the levels of MMP-9, TIMP-1 and NGAL, and examining the tear proteome in this group upon awakening before contact

lens wear and after one night of EW may help confirm whether these factors do indeed differ between this group and the group of normals shown in Chapter 3, Chapter 4 and Chapter 5. This confirmation would be of predictive and potentially preventative value. Identifying those contact lens wearers likely to develop erosion or infection would allow practitioners to advise against EW or to monitor more frequently during the initial stages of contact lens wear.

It is not known when the adaptation found in this study occurs, as seen with the return of MMP-9 to baseline after a month of contact lens wear. A study measuring MMP-9 upon awakening on a more frequent basis, for example weekly, during the first month of wear may explain how quickly this adaptation happens. Additionally, a study measuring the concentration of MMP-9 after the first night of EW when preceded by a period of DW may help establish whether a period of adaptation in DW prevents the increase in expression after the first night of EW found in this study. This understanding may be translated to advice to practitioners and contact lens wearers, so that a period of DW may be advised prior to proceeding to EW.

Moreover, a means to prevent the increased expression of MMP-9 associated with EW may also be a matter of future research and indeed would have therapeutic potential. The association of MMP-9 with erosions[101] and corneal ulcers[48] would suggest that a contact lens-associated increase in MMP-9 expression may increase the propensity to both. It may be key, therefore, to look into mechanisms by which to reduce the increased expression of MMP-9 during the initial phases of EW. Currently there are bandages designed to reduce the levels of MMPs in wounds by either providing a competitive substrate, for example, Promogran (Johnson and Johnson, Rochester, NY, USA)[311] and Fibracol (Johnson and Johnson, Rochester, NY, USA),[312] or by preventing the synthesis of MMPs, for example DerMax® (Dermagenics Inc, Memphis, TN, USA).[313] A contact lens product with such components could potentially have both therapeutic and preventative effects.

7.5 CONCLUSIONS

This thesis has contributed to the understanding of the effect of contact lens wear on the tear film and, specifically, to the concentrations of MMP-9 and its associated

factors. A significant diurnal variation was found in MMP-9 in the healthy non-contact lens wearing eye, upon awakening being the period of greatest concentration, establishing an environment conducive to extracellular matrix remodelling. Overnight contact lens wear further increased MMP-9, an increase which was not followed by its inhibitor TIMP-1. After a one month period of continuous wear, these proteins returned to baseline, indicative of adaptation. This initial disturbance suggests that initial EW wear may be the most disruptive to the corneal epithelium and the riskiest for erosion development. The higher baseline levels of MMP-9 seen in a small group of contact lens wearers with erosions, along with the increase seen with contact lens wear, suggests an important role for this enzyme in the pathogenesis of contact lens-related corneal erosions, a finding that may be of predictive value and is worth pursuing with further study as it may have significant implications for practitioner prescribing. Moreover, the difference between the tear proteome upon awakening before contact lens wear and after the first night of EW in one contact lens wearer who later developed an erosion suggests that the extent of the proteome changes in the early phases of contact lens wear may also be predictive of erosion risk.

REFERENCES

1. Stapleton F, Keay L, Jalbert I, Cole N. The epidemiology of contact lens related infiltrates. *Optom. Vis. Sci.* 2007;84:257-272.

2. Tarutta E, Chua WH, Young T, et al. Myopia: why study the mechanisms of myopia? Novel approaches to risk factors signalling eye growth- how could basic biology be translated into clinical insights? Where are genetic and proteomic approaches leading? How does visual function contribute to and interact with ametropia? Does eye shape matter? Why ametropia at all? *Optom. Vis. Sci.* 2011;88:404-447.

3. Rubinstein MP. Applications of contact lens devices in the management of corneal disease. *Eye (Lond)* 2003;17:872-876.

4. Sweeney DF, Jalbert I, Covey M, et al. Clinical characterization of corneal infiltrative events observed with soft contact lens wear. *Cornea* 2003;22:435-442.

5. Stapleton F, Keay L, Edwards K, et al. The incidence of contact lens-related microbial keratitis in Australia. *Ophthalmology* 2008;115:1655-1662.

6. Dart JK, Radford CF, Minassian D, Verma S, Stapleton F. Risk factors for microbial keratitis with contemporary contact lenses: A case-control study. *Ophthalmology* 2008;115:1647-1654.

7. Keay L, Edwards K, Naduvilath T, Forde K, Stapleton F. Factors affecting the morbidity of contact lens-related microbial keratitis: A population study. *Invest. Ophthalmol. Vis. Sci.* 2006;47:4302-4308.

8. Richdale K, Sinnott LT, Skadahl E, Nichols JJ. Frequency of and factors associated with contact lens dissatisfaction and discontinuation. *Cornea* 2007;26:168-174.

9. Carnt NA, Evans VE, Naduvilath TJ, et al. Contact lens-related adverse events and the silicone hydrogel lenses and daily wear care system used. *Arch. Ophthalmol.* 2009;127:1616-1623.

10. Dumbleton K. Noninflammatory silicone hydrogel contact lens complications. *Eye Contact Lens* 2003;29(IS):S186-S189.

11. Holden BA, Sweeney DF, Seger RG. Epithelial erosions caused by thin high water content lenses. *Clin. Exp. Optom.* 1986;69:103-107.

12. Zantos SG, Holden BA. Ocular changes associated with continuous wear of contact lenses. *Aust. J. Optom.* 1978;61:418-426.

13. Willcox MD, Naduvilath TJ, Vaddavalli PK, Holden BA, Ozkan J, Zhu H. Corneal erosions, bacterial contamination of contact lenses, and microbial keratitis. *Eye Contact Lens* 2010;36:340-345.

14. Labbe A, Nicola RD, Dupas B, Auclin F, Baudouin C. Epithelial basement membrane dystrophy: Evaluation with the HRT II Rostock Cornea Module. *Ophthalmology* 2006;113:1301-1308.

15. Rosenberg ME, Tervo TM, Petroll WM, Vesaluoma MH. In vivo confocal microscopy of patients with corneal recurrent erosion syndrome or epithelial basement membrane dystrophy. *Ophthalmology* 2000;107:565-573.

16. Tripathi RC, Bron AJ. Ultrastructural study of non-traumatic recurrent corneal erosion. *Br. J. Ophthalmol.* 1972;56:73-85.

17. Markoulli M, Papas E, Cole N, Holden B. Corneal erosions in contact lens wear. *Cont. Lens Anterior Eye* 2011.

18. Heyworth P, Morlet N, Rayner S, Hykin P, Dart J. Natural history of recurrent erosion syndrome - a 4 year review of 117 patients. *Br. J. Ophthalmol.* 1998;82:26-28.

19. Reidy JJ, Paulus MP, Gona S. Recurrent erosions of the cornea: epidemiology and treatment. *Cornea* 2000;19:767-771.

20. Brown N, Bron A. Recurrent erosion of the cornea. *Br. J. Ophthalmol.* 1976;60:84-96.

21. Dumbleton K. Adverse events with silicone hydrogel continuous wear. *Cont. Lens Anterior Eye* 2002;25:137-146.

22. Tseng SS-Y, Yang WW-C, Hsiao JC-J. Bilateral corneal erosion due to retail purchase of unfitted prescription contact lenses: A case report. *Cornea* 2008;27:1179 - 1181.

23. Chen YT, Huang CW, Huang FC, Tseng SY, Tseng SH. The cleavage plane of corneal epithelial adhesion complex in traumatic recurrent corneal erosion. *Mol. Vis.* 2006;12:196-204.

24. Ramamurthi S, Rahman MQ, Dutton GN, Ramaesh K. Pathogenesis, clinical features and management of recurrent corneal erosions. *Eye (Lond)* 2006;20:635-644.

25. Mandathara P, Sankaridurg P, Krishna P, Sangwan V. Corneal erosions with silicone hydrogel lens wear in India: clinical features and outcome. *British Contact Lens Association.* Manchester, United Kingdom; 2011.

26. Werblin TP, Hirst LW, Stark WJ, Maumenee IH. Prevalence of map-dot-fingerprint changes in the cornea. *Br. J. Ophthalmol.* 1981;65:401-409.

27. Wong VW, Chi SC, Lam DS. Diamond burr polishing for recurrent corneal erosions: Results from a prospective randomized controlled trial. *Cornea* 2009;28:152-156.

28. Hope-Ross MW, Chell PB, Kervick GN, McDonnell PJ. Recurrent corneal erosion: clinical features. *Eye (Lond)* 1994;8:373-377.

29. Bruce AS, Mainstone JC. Lens adherence and postlens tear film changes in closed-eye wear of hydrogel lenses. *Optom. Vis. Sci.* 1996;73:28-34.

30. Nichols JJ, King-Smith PE. The effect of eye closure on the post-lens tear film thickness during silicone hydrogel contact lens wear. *Cornea* 2003;22:539-544.

31. Little SA, Bruce AS. Role of the post-lens tear film in the mechanism of inferior arcuate staining with ultrathin hydrogel lenses. *CLAO J.* 1995;21:175-181.

32. Little SA, Bruce AS. Osmotic determinants of postlens tear film morphology and hydrogel lens movement. *Ophthalmic Physiol. Opt.* 1995;15:117-124.

33. Keay L, Edwards K, Naduvilath T, et al. Microbial keratitis: Predisposing factors and morbidity. *Ophthalmology* 2006;113:109-116.

34. Cheng KH, Leung SL, Hoekman HW, Beekhuis WH, Mulder PGH, Geerards AJM. Incidence of contact-lens-associated microbial keratitis and its related morbidity. *Lancet* 1999;354:181-185.

35. Ionides AC, Tuft SJ, Ferguson VM, Matheson MM, Hykin PG. Corneal infiltration after recurrent corneal epithelial erosion. *Br. J. Ophthalmol.* 1997;81:537-540.

36. Ren DH, Petroll WM, Jester JV, Ho-Fan J, Cavanagh HD. The relationship between contact lens oxygen permeability and binding of Pseudomonas aeruginosa to human corneal epithelial cells after overnight and extended wear. *CLAO J.* 1999;25:80-100.

37. Alarcon I, Kwan L, Yu C, Evans DJ, Fleiszig SM. Role of the corneal epithelial basement membrane in ocular defense against Pseudomonas aeruginosa. *Infect. Immun.* 2009;77:3264-3271.

38. Alarcon I, Tam C, Mun JJ, Ledue J, Evans DJ, Fleiszig SM. Factors impacting corneal epithelial barrier function against pseudomonas aeruginosa traversal. *Invest. Ophthalmol. Vis. Sci.* 2010.

39. Das S, Seitz B. Recurrent corneal erosion syndrome. *Surv. Ophthalmol.* 2008;53:3-15.

40. Lee EJ, Evans DJ, Fleiszig SM. Role of Pseudomonas aeruginosa ExsA in penetration through corneal epithelium in a novel in vivo model. *Invest. Ophthalmol. Vis. Sci.* 2003;44:5220-5227.

41. Khodadoust AA, Silverstein AM, Kenyon DR, Dowling JE. Adhesion of regenerating corneal epithelium. The role of basement membrane. *Am. J. Ophthalmol.* 1968;65:339-348.

42. Gipson IK. Adhesive mechanisms of the corneal epithelium. *Acta Ophthalmol. Suppl.* 1992;13-17.

43. Pal-Ghosh S, Blanco T, Tadvalkar G, et al. MMP9 cleavage of the β4 integrin ectodomain leads to recurrent epithelial erosions in mice. *J. Cell Sci.* 2011;124:2666-2675.

44. Holden BA, Reddy MK, Sankaridurg PR, et al. Contact lens-induced peripheral ulcers with extended wear of disposable hydrogel lenses: Histopathologic observations on the nature and type of corneal infiltrate. *Cornea* 1999;18:538-543.

45. Grant T, Chong MS, Vajdic C, et al. Contact lens induced peripheral ulcers during hydrogel contact lens wear. *CLAO J.* 1998;24:145-151.

46. Holden BA, Stephenson A, Stretton S, et al. Superior epithelial arcuate lesions with soft contact lens wear. *Optom. Vis. Sci.* 2001;78:9-12.

47. Carnt N, Jalbert I, Stretton S, Naduvilath T, Papas E. Solution toxicity in soft contact lens daily wear is associated with corneal inflammation. *Optom. Vis. Sci.* 2007;84:309-315.

48. Fini ME, Cook JR, Mohan R. Proteolytic mechanisms in corneal ulceration and repair. *Arch. Dermatol. Res.* 1998;290 Suppl:S12-23.

49. Ko JA, Liu Y, Yanai R, Chikama T, Takezawa T, Nishida T. Upregulation of tight-junctional proteins in corneal epithelial cells by corneal fibroblasts in collagen vitrigel cultures. *Invest. Ophthalmol. Vis. Sci.* 2008;49:113-119.

50. Lu L, Reinach PS, Kao WW. Corneal epithelial wound healing. *Exp. Biol. Med.* 2001;226:653-664.

51. Van den Steen PE, Dubois B, Nelissen I, Rudd PM, Dwek RA, Opdenakker G. Biochemistry and molecular biology of gelatinase B or matrix metalloproteinase-9 (MMP-9). *Crit. Rev. Biochem. Mol. Biol.* 2002;37:375-536.

52. Azar DT, Spurr-Michaud SJ, Tisdale AS, Gipson IK. Decreased penetration of anchoring fibrils into the diabetic stroma - a morphometric analysis. *Arch. Ophthalmol.* 1989;107:1520 - 1523.

53. Gipson IK, Spurr-Michaud SJ, Tisdale AS. Anchoring fibrils form a complex network in human and rabbit cornea. *Invest. Ophthalmol. Vis. Sci.* 1987;28:212-220.

54. Zagon IS, Sassani JW, Ruth TB, McLaughlin PJ. Epithelial adhesion complexes and organ culture of the human cornea. *Brain Res.* 2001;900:205-213.

55. Van Agtmael T, Bruckner-Tuderman L. Basement membranes and human disease. *Cell Tissue Res.* 2010;339:167-188.

56. Tao A, Wang J, Chen Q, et al. Topographical thickness of Bowman's layer determined by ultra-high resolution spectral domain optical coherence tomography. *Invest. Ophthalmol. Vis. Sci.*

57. Mackiewicz Z, Maatta M, Stenman M, Konttinen L, Tervo T, Konttinen YT. Collagenolytic proteinases in keratoconus. *Cornea* 2006;25:603-610.

58. Sinclair JM, Wilkes GA, Krebs WA. *Collins English dictionary*: HarperCollins Publishers; 1998.

59. Gillbard JP, Cohen RG, Baum J. Decreased tear osmolarity and absence of theinferior marginal tear strip after sleep. *Cornea* 1992;11:231-233.

60. Kenyon E, Polse KA, Mandell RB. Rigid contact lens adherence: incidence, severity and recovery. *J. Am. Optom. Assoc.* 1988;59:168-174.

61. Baum JL. The Castroviejo Lecture. Prolonged eyelid closure is a risk to the cornea. *Cornea* 1997;16:602-611.

62. Stapleton F, Dart J, Minassian D. Nonulcerative Complications of Contact Lens Wear: Relative Risks for Different Lens Types. *Arch. Ophthalmol.* 1992;110:1601-1606.

63. Orsborn GN, Zantos SG. Corneal desiccation staining with thin high water content lenses. *CLAO J.* 1988;14:81-85.

64. McNally JJ, Chalmers RL, Payor R. Corneal epithelial disruption with extremely thin hydrogel lenses. *Clin. Exp. Optom.* 1987;70:106-111.

65. Nichols JJ, Sinnott LT. Tear film, contact lens, and patient factors associated with corneal staining. *Invest. Ophthalmol. Vis. Sci.* 2011;52:1127-1137.

66. Marquart ME, Caballero AR, Chomnawang M, Thibodeaux BA, Twining SS, O'Callaghan RJ. Identification of a novel secreted protease from Pseudomonas aeruginosa that causes corneal erosions. *Invest. Ophthalmol. Vis. Sci.* 2005;46:3761-3768.

67. Tang A, Marquart ME, Fratkin JD, et al. Properties of PASP: a Pseudomonas protease capable of mediating corneal erosions. *Invest. Ophthalmol. Vis. Sci.* 2009;50:3794-3801.

68. McClellan K, Cripps AW, Clancy RL, Billson FA. The effect of successful contact lens wear on mucosal immunity of the eye. *Ophthalmology* 1998;105:1471 - 1477.

69. Fleiszig SM. The Glenn A. Fry award lecture 2005. The pathogenesis of contact lens-related keratitis. *Optom. Vis. Sci.* 2006;83:866-873.

70. Choo JD, Holden BA, Papas EB, Willcox MD. Adhesion of Pseudomonas aeruginosa to orthokeratology and alignment lenses. *Optom. Vis. Sci.* 2009;86:93 - 97.

71. Willcox MD. Pseudomonas aeruginosa infection and inflammation during contact lens wear: a review. *Optom. Vis. Sci.* 2007;84:273-278.

72. Okamoto T, Akaike T, Suga M, et al. Activation of human matrix metalloproteinases by various bacterial proteinases. *J. Biol. Chem.* 1997;272:6059-6066.

73. Madigan MC, Holden BA. Reduced epithelial adhesion after extended contact lens wear correlates with reduced hemidesmosome density in cat cornea. *Invest. Ophthalmol. Vis. Sci.* 1992;33:314-323.

74. Steffen R, Schnider C. A next-generation silicone hydrogel lens for daily wear. *Optician* 2004;227:23-25.

75. Efron N, Morgan PB. Are hypoxia or modulus causes of contact lens-associated keratitis? *Clin. Exp. Optom.* 2009;92:329-330.

76. Agrawal P, Kostakis V, Ramsey AS. Recurrent corneal erosion. *Ophthalmology* 2007;114:1953-1954.

77. Dursun D, Kim MC, Solomon A, Pflugfelder SC. Treatment of recalcitrant recurrent corneal erosions with inhibitors of matrix metalloproteinase-9, doxycycline and corticosteroids. *Am. J. Ophthalmol.* 2001;132:8-13.

78. Garrana RMR, Zieske JD, Assouline M, Gipson RK. Matrix metalloproteinases in epithelia from human recurrent corneal erosion. *Invest. Ophthalmol. Vis. Sci.* 1999;40:1266 - 1270.

79. Lowe RF. Recurrent erosion of the cornea. *Br. J. Ophthalmol.* 1970;54:805-809.

80. Weiss JS. Corneal dystrophies: Molecular genetics to therapeutic intervention--Fifth ARVO/Pfizer Ophthalmics Research Institute Conference. *Invest. Ophthalmol. Vis. Sci.* 2010;51:5391-5402.

81. Dua HS, Lagnado R, Raj D, et al. Alcohol delamination of the corneal epithelium: an alternative in the management of recurrent corneal erosions. *Ophthalmology* 2006;113:404-411.

82. Hernandez-Quintela E, Mayer F, Dighiero P, et al. Confocal microscopy of cystic disorders of the corneal epithelium. *Ophthalmology* 1998;105:631-636.

83. Zaidman GW, Hong A. Visual and refractive results of combined PTK/PRK in patients with corneal surface disease and refractive errors. *J. Cataract Refract. Surg.* 2006;32:958-961.

84. Chikama T, Takahashi N, Wakuta M, Morishige N, Nishida T. In vivo biopsy by laser confocal microscopy for evaluation of traumatic recurrent corneal erosion. *Mol. Vis.* 2008;14:2333-2339.

85. McLean EN, MacRae SM, Rich LF. Recurrent erosion. Treatment by anterior stromal puncture. *Ophthalmology* 1986;93:784-788.

86. Watson SL, Barker NH. Interventions for recurrent corneal erosions. *Cochrane Database Syst Rev* 2007;CD001861.

87. Srinivas R, Debraj S, Geetha I, Rajesh F, Prema P. Bilateral epithelial defects after laser in situ keratomileusis. *Indian J. Ophthalmol.* 2005;53:37-42.

88. Ti SE, Tan DT. Recurrent corneal erosion after laser in situ keratomileusis. *Cornea* 2001;20:156-158.

89. Kenyon KR, Paz H, Greiner JV, Gipson IK. Corneal epithelial adhesion abnormalities associated with LASIK. *Ophthalmology* 2004;111:11-17.

90. Zhu SN, Nolle B, Duncker G. Adhesion molecule expression in bullous keratopathy. *Graefes Arch. Clin. Exp. Ophthalmol.* 1996;234 Suppl 1:S159-166.

91. Alvarado J, Murphy C, Juster R. Age-related changes in the basement membrane of the human corneal epithelium. *Invest. Ophthalmol. Vis. Sci.* 1983;24:1015-1028.

92. Jenkins MS, Brown SI, Lempert SL, Weinberg RJ. Ocular rosacea. *Am. J. Ophthalmol.* 1979;88:618-622.

93. Afonso AA, Sobrin L, Monroy DC, Selzer M, Lokeshwar B, Pflugfelder SC. Tear fluid gelatinase B activity correlates with IL-1alpha concentration and fluorescein clearance in ocular rosacea. *Invest. Ophthalmol. Vis. Sci.* 1999;40:2506-2512.

94. Hanemaaijer R, Visser H, Koolwijk P, et al. Inhibition of MMP synthesis by doxycycline and chemically modified tetracyclines (CMTs) in human endothelial cells. *Adv. Dent. Res.* 1998;12:114-118.

95. Akpek EK, Merchant A, Pinar V, Foster CS. Ocular rosacea: patient characteristics and follow-up. *Ophthalmology* 1997;104:1863-1867.

96. Schultz RO, Van Horn DL, Peters MA, Klewin KM, Schutten WH. Diabetic keratopathy. *Trans. Am. Ophthalmol. Soc.* 1981;79:180-199.

97. Rhys C, Snyers B, Pirson Y. Recurrent corneal erosion associated with Alport's syndrome. Rapid communication. *Kidney Int.* 1997;52:208-211.

98. Bauer EA, Eisen AZ. Recessive dystrophic epidermolysis bullosa. Evidence for increased collagenase as a genetic characteristic in cell culture. *J. Exp. Med.* 1978;148:1378-1387.

99. Quinn NB. Management of recurrent corneal erosions in an Infant with epidermolysis bullosa. *American Academy of Optometry*; 2009.

100. Zhuang H, Zhou X, Xu J. A novel method for pachymetry mapping of human precorneal tear film using pentacam with fluorescein. *Invest. Ophthalmol. Vis. Sci.* 51:156-159.

101. Sivak JM, Fini ME. MMPs in the eye: Emerging roles for matrix metalloproteinases in ocular physiology. *Prog. Retin. Eye Res.* 2002;21:1-14.

102. Sakimoto T, Shoji J, Yamada A, Sawa M. Upregulation of matrix metalloproteinases in tear fluid of patients with recurrent corneal erosion. *Jpn. J. Ophthalmol.* 2007;51:343-346.

103. Blanco A, Meloni M, Mazzone M. Re-epithelizing effect of xanthan gum in a model of mechanical injury performed on cultured human corneal epithelium (HCE). *ARVO Meeting Abstracts* 2008;49:3404.

104. Sternlicht MD, Werb Z. How matrix metalloproteinases regulate cell behavior. *Annu. Rev. Cell Dev. Biol.* 2001;17:463-516.

105. Woessner JF, Jr. Matrix metalloproteinases and their inhibitors in connective tissue remodeling. *FASEB J.* 1991;5:2145-2154.

106. Huh MI, Lee YM, Seo SK, et al. Roles of MMP/TIMP in regulating matrix swelling and cell migration during chick corneal development. *J. Cell. Biochem.* 2007;101:1222-1237.

107. Di Girolamo N, Indoh I, Jackson N, et al. Human mast cell-derived gelatinase B (matrix metalloproteinase-9) is regulated by inflammatory cytokines: Role in cell migration. *J. Immunol.* 2006;177:2638-2650.

108. Wang X, Jung J, Asahi M, et al. Effects of matrix metalloproteinase-9 gene knock-out on morphological and motor outcomes after traumatic brain injury. *J. Neurosci.* 2000;20:7037-7042.

109. Vecil GG, Larsen PH, Corley SM, et al. Interleukin-1 is a key regulator of matrix metalloproteinase-9 expression in human neurons in culture and following mouse brain trauma in vivo. *J. Neurosci. Res.* 2000;61:212-224.

110. Fini ME, Girard MT. Expression of collagenolytic/gelatinolytic metalloproteinases by normal cornea. *Invest. Ophthalmol. Vis. Sci.* 1990;31:1779-1788.

111. Leonardi A, Brun P, Abatangelo G, Plebani M, Secchi A. Tear levels and activity of matrix metalloproteinase (MMP)-1 and MMP-9 in vernal keratoconjunctivitis. *Invest. Ophthalmol. Vis. Sci.* 2003;44:3052 - 3058.

112. Sobrin L, Liu Z, Monroy DC, et al. Regulation of MMP-9 activity in human tear fluid and corneal epithelial culture supernatant. *Invest. Ophthalmol. Vis. Sci.* 2000;41:1703-1709.

113. Fini ME, Parks WC, Rinehart WB, et al. Role of matrix metalloproteinases in failure to re-epithelialize after corneal injury. *Am. J. Pathol.* 1996;149:1287-1302.

114. Chotikavanich S, de Paiva CS, Li de Q, et al. Production and activity of matrix metalloproteinase-9 on the ocular surface increase in dysfunctional tear syndrome. *Invest. Ophthalmol. Vis. Sci.* 2009;50:3203-3209.

115. Di Girolamo N, Wakefield D, Coroneo MT. Differential expression of matrix metalloproteinases and their tissue inhibitors at the advancing pterygium head. *Invest. Ophthalmol. Vis. Sci.* 2000;41:4142-4149.

116. Matsubara M, Girard MT, Kublin CL, Cintron C, Fini ME. Differential roles for two gelatinolytic enzymes of the matrix metalloproteinase family in the remodelling cornea. *Dev. Biol.* 1991;147:425-439.

117. Daniels JT, Geerling G, Alexander RA, Murphy G, Khaw PT, Saarialho-Kere U. Temporal and spatial expression of matrix metalloproteinases during wound healing of human corneal tissue. *Exp. Eye Res.* 2003;77:653-664.

118. Gross J, Lapiere CM. Collagenolytic activity in amphibian tissues: A tissue culture assay. *Proc. Natl. Acad. Sci. U. S. A.* 1962;48:1014-1022.

119. Wilkesman J, Kurz L. Protease analysis by zymography: A review on techniques and patents. *Recent Pat. Biotechnol.* 2009.

120. Nagase H, Woessner JF, Jr. Matrix metalloproteinases. *J. Biol. Chem.* 1999;274:21491-21494.

121. Roderfeld M, Graf J, Giese B, et al. Latent MMP-9 is bound to TIMP-1 before secretion. *Biol. Chem.* 2007;388:1227-1234.

122. Visse R, Nagase H. Matrix metalloproteinases and tissue inhibitors of metalloproteinases: Structure, function, and biochemistry. *Circ. Res.* 2003;92:827-839.

123. Snoek-van Beurden PA, Von den Hoff JW. Zymographic techniques for the analysis of matrix metalloproteinases and their inhibitors. *Biotechniques* 2005;38:73-83.

124. Girard MT, Matsubara M, Fini ME. Transforming growth factor-beta and interleukin-1 modulate metalloproteinase expression by corneal stromal cells. *Invest. Ophthalmol. Vis. Sci.* 1991;32:2441-2454.

125. Di Girolamo N, Lloyd A, McCluskey P, Filipic M, Wakefield D. Increased expression of matrix metalloproteinases in vivo in scleritis tissue and in vitro in cultured human scleral fibroblasts. *Am. J. Pathol.* 1997;150:653-666.

126. Parks WC, Mecham RP (eds). *Matrix Metalloproteinases*. First ed. San Diego: Academic Press; 1998.

127. Shiomi T, Lemaître V, D'Armiento J, Okada Y. Matrix metalloproteinases, a disintegrin and metalloproteinases, and a disintegrin and metalloproteinases with thrombospondin motifs in non-neoplastic diseases. *Pathol. Int.* 2010;60:477-496.

128. Barksby HE, Milner JM, Patterson AM, et al. Matrix metalloproteinase 10 promotion of collagenolysis via procollagenase activation: implications for cartilage degradation in arthritis. *Arthritis Rheum.* 2006;54:3244-3253.

129. Clark IM. *Matrix metalloproteinase protocols*. New Jersey: Humana Press Inc.; 2001.

130. Ollivier FJ, Gilger BC, Barrie KP, et al. Proteinases of the cornea and preocular tear film. *Vet. Ophthalmol.* 2007;10:199-206.

131. Lanchou J, Corbel M, Tanguy M, et al. Imbalance between matrix metalloproteinases (MMP-9 and MMP-2) and tissue inhibitors of metalloproteinases (TIMP-1 and TIMP-2) in acute respiratory distress syndrome patients. *Crit. Care Med.* 2003;31:536-542.

132. Fini E, Girard MT, Matsubara M, Bartlett JD. Unique regulation of the matrix metalloproteinase, gelatinase B. *Invest. Ophthalmol. Vis. Sci.* 1995;36:622-633.

133. Smith VA, Rishmawi H, Hussein H, Easty DL. Tear film MMP accumulation and corneal disease. *Br. J. Ophthalmol.* 2001;85:147-153.

134. Kjeldsen L, Johnsen AH, Sengeløv H, Borregaard N. Isolation and primary structure of NGAL, a novel protein associated with human neutrophil gelatinase. *J. Biol. Chem.* 1993;268:10425-10432.

135. Sack RA, Sathe S, Beaton AR, Bogart B, Lew G. Changes in the diurnal pattern of the distribution of gelatinases and associated proteins in normal and pathological tear fluids: Evidence that the PMN cell is a major source of MMP activity in tear fluid. *Adv. Exp. Med. Biol.* 2002;506:539-545.

136. Mohan R, Chintala SK, Jung JC, et al. Matrix metalloproteinase gelatinase B (MMP-9) coordinates and effects epithelial regeneration. *J. Biol. Chem.* 2002;277:2065-2072.

137. Quiding-Jarbrink M, Smith DA, Bancroft GJ. Production of matrix metalloproteinases in response to mycobacterial infection. *Infect. Immun.* 2001;69:5661-5670.

138. Shapiro SD, Fliszar CJ, Broekelmann TJ, Mecham RP, Senior RM, Welgus HG. Activation of the 92-kDa gelatinase by stromelysin and 4-aminophenylmercuric acetate. *J. Biol. Chem.* 1995;270:6351-6356.

139. Xue ML, Wakefield D, Willcox MDP, et al. Regulation of MMPs and TIMPs by IL-1{beta} during corneal ulceration and infection. *Invest. Ophthalmol. Vis. Sci.* 2003;44:2020-2025.

140. Gabison EE, Huet E, Baudouin C, Menashi S. Direct epithelial-stromal interaction in corneal wound healing: Role of EMMPRIN/CD147 in MMPs induction and beyond. *Prog. Retin. Eye Res.* 2009;28:19-33.

141. Gipson IK. The ocular surface: The challenge to enable and protect vision. *Invest. Ophthalmol. Vis. Sci.* 2007;48:4391-4398.

142. Molloy MP, Brzezinski EE, Hang J, McDowell MT, VanBogelen RA. Overcoming technical variation and biological variation in quantitative proteomics. *Proteomics* 2003;3:1912-1919.

143. Daniel M, Rowley K, McDermott R, Mylvaganam A, O'Dea K. Diabetes incidence in an Australian Aboriginal population. An 8-year follow-up study. *Diabetes Care* 1999;22:1993 - 1998.

144. Soderberg S, Zimmet P, Tuomilehto J, et al. High incidence of type 2 diabetes and increasing conversion rates from impaired fasting glucose and impaired glucose tolerance to diabetes in Mauritius. *J. Intern. Med.* 2004;256:37-47.

145. Thomson SE, McLennan SV, Kirwan PD, et al. Renal connective tissue growth factor correlates with glomerular basement membrane thickness and prospective albuminuria in a non-human primate model of diabetes: possible predictive marker for incipient diabetic nephropathy. *J. Diabetes Complications* 22:284-294.

146. Njolstad I, Arnesen E, Lund-Larsen P. Sex differences in risk factors for clinical diabetes mellitus in a general population: a 12-year follow-up of the Finnmark Study. *Am. J. Epidemiol.* 1998;147:49 - 58.

147. Kirwan RP, Fenerty CH, Crean J, Wordinger RJ, Clark AF, O'Brien CJ. Influence of cyclical mechanical strain on extracellular matrix gene expression in human lamina cribrosa cells in vitro. *Mol. Vis.* 2005;11:798-810.

148. Liu Z, Ivanoff A, Klominek J. Expression and activity of matrix metalloproteases in human malignant mesothelioma cell lines. *Int. J. Cancer* 2001;91:638-643.

149. Murphy G, Koklitis P, Carne AF. Dissociation of tissue inhibitor of metalloproteinases (TIMP) from enzyme complexes yields fully active inhibitor. *Biochem. J.* 1989;261:1031-1034.

150. Gomez DE, Alonso DF, Yoshiji H, Thorgeirsson UP. Tissue inhibitors of metalloproteinases: structure, regulation and biological functions. *Eur. J. Cell Biol.* 1997;74:111-122.

151. Lambert E, Boudot C, Kadri Z, et al. Tissue inhibitor of metalloproteinases-1 signalling pathway leading to erythroid cell survival. *Biochem. J.* 2003;372:767-774.

152. Baker AH, Edwards DR, Murphy G. Metalloproteinase inhibitors: Biological actions and therapeutic opportunities. *J. Cell Sci.* 2002;115:3719-3727.

153. Paterson CA, Wells JG, Koklitis PA, Higgs GA, Docherty AJ. Recombinant tissue inhibitor of metalloproteinases type 1 suppresses alkali-burn-induced corneal ulceration in rabbits. *Invest. Ophthalmol. Vis. Sci.* 1994;35:677-684.

154. Bolignano D, Donato V, Lacquaniti A, et al. Neutrophil gelatinase-associated lipocalin (NGAL) in human neoplasias: A new protein enters the scene. *Cancer Lett.* 2010;288:10-16.

155. Endre Z, Westhuyzen J. Early detection of acute kidney injury: Emerging new biomarkers. *Nephrology* 2008;13:91-98.

156. Fernandez CA, Yan L, Louis G, Yang J, Kutok JL, Moses MA. The matrix metalloproteinase-9/neutrophil gelatinase-associated lipocalin complex plays a role in breast tumor growth and is present in the urine of breast cancer patients. *Clin. Cancer Res.* 2005;11:5390-5395.

157. Flo TH, Smith KD, Sato S, et al. Lipocalin 2 mediates an innate immune response to bacterial infection by sequestrating iron. *Nature* 2004;432:917-921.

158. Devarajan P. Review: Neutrophil gelatinase-associated lipocalin: A troponin-like biomarker for human acute kidney injury. *Nephrology* 2010;15:419-428.

159. Roudkenar MH, Halabian R, Bahmani P, Roushandeh AM, Kuwahara Y, Fukumoto M. Neutrophil gelatinase-associated lipocalin: A new antioxidant that exerts its cytoprotective effect independent on Heme Oxygenase-1. *Free Radic. Res.* 2011;45:810-819.

160. Zappitelli M, Washburn K, Arikan A, et al. Urine neutrophil gelatinase-associated lipocalin is an early marker of acute kidney injury in critically ill children: a prospective cohort study. *Crit. Care* 2007;11:R84.

161. Yan L, Borregaard N, Kjeldsen L, Moses MA. The high molecular weight urinary matrix metalloproteinase (MMP) activity is a complex of gelatinase B/MMP-9 and neutrophil

gelatinase-associated lipocalin (NGAL): Modulation of MMP-9 activity by NGAL. *J. Biol. Chem.* 2001;276:37258-37265.

162. Hemdahl A-L, Gabrielsen A, Zhu C, et al. Expression of neutrophil gelatinase–associated lipocalin in atherosclerosis and myocardial infarction. *Arterioscler. Thromb. Vasc. Biol.* 2006;26:136-142.

163. Glasgow BJ, Abduragimov AR, Gasymov OK, Yusifov TN. Tear lipocalin: structure, function and molecular mechanisms of action. *Adv. Exp. Med. Biol.* 2002;506:555-565.

164. Hofmaier F, Hauck SM, Amann B, Degroote RL, Deeg CA. Changes in matrix metalloproteinase network in spontaneous autoimmune uveitis model. *Invest. Ophthalmol. Vis. Sci.* 2011.

165. Bratt T, Ohlson S, Borregaard N. Interactions between neutrophil gelatinase-associated lipocalin and natural lipophilic ligands. *Biochim. Biophys. Acta* 1999;1472:262-269.

166. John-Aryankalayil M, Dushku N, Jaworski CJ, et al. Microarray and protein analysis of human pterygium. *Mol. Vis.* 2006;12:55-64.

167. Canete-Soler R, Gui YH, Linask KK, Muschel RJ. MMP-9 (gelatinase B) mRNA is expressed during mouse neurogenesis and may be associated with vascularization. *Brain Res. Dev. Brain Res.* 1995;88:37-52.

168. Canete-Soler R, Gui YH, Linask KK, Muschel RJ. Developmental expression of MMP-9 (gelatinase B) mRNA in mouse embryos. *Dev. Dyn.* 1995;204:30-40.

169. Lochter A, Galosy S, Muschler J, Freedman N, Werb Z, Bissell MJ. Matrix metalloproteinase stromelysin-1 triggers a cascade of molecular alterations that leads to stable epithelial-to-mesenchymal conversion and a premalignant phenotype in mammary epithelial cells. *J. Cell Biol.* 1997;139:1861-1872.

170. Joronen K, Salminen H, Glumoff V, Savontaus M, Vuorio E. Temporospatial expression of tissue inhibitors of matrix metalloproteinases-1, -2 and -3 during development, growth and aging of the mouse skeleton. *Histochem. Cell Biol.* 2000;114:157-165.

171. Lehoux S, Lemarie CA, Esposito B, Lijnen HR, Tedgui A. Pressure-induced matrix metalloproteinase-9 contributes to early hypertensive remodeling. *Circulation* 2004;109:1041-1047.

172. Bauer AT, Burgers HF, Rabie T, Marti HH. Matrix metalloproteinase-9 mediates hypoxia-induced vascular leakage in the brain via tight junction rearrangement. *J. Cereb. Blood Flow Metab.* 2010;30:837-848.

173. Tejima E, Guo S, Murata Y, et al. Neuroprotective effects of overexpressing tissue inhibitor of metalloproteinase TIMP-1. *J. Neurotrauma* 2009;26:1935-1941.

174. Kumar A, El-Osta A, Hussain AA, Marshall J. Increased sequestration of matrix metalloproteinases (MMPs) in ageing human Bruch's membrane: Implications for ECM turnover. *Invest. Ophthalmol. Vis. Sci.* 2010;51:2664-2670.

175. Karwatowski WS, Jeffries TE, Duance VC, Albon J, Bailey AJ, Easty DL. Preparation of Bruch's membrane and analysis of the age-related changes in the structural collagens. *Br. J. Ophthalmol.* 1995;79:944-952.

176. Hussain AA, Lee Y, Zhang J-J, Marshall J. Disturbed matrix metalloproteinase (MMP) activity of Bruch's membrane in age-related macular degeneration (AMD). *Invest. Ophthalmol. Vis. Sci.* 2011; 52:4459-4466.

177. Holopainen JM, Moilanen JA, Sorsa T, et al. Activation of matrix metalloproteinase-8 by membrane type 1-MMP and their expression in human tears after photorefractive keratectomy. *Invest. Ophthalmol. Vis. Sci.* 2003;44:2550-2556.

178. Lema I, Duran JA. Inflammatory molecules in the tears of patients with keratoconus. *Ophthalmology* 2005;112:654-659.

179. Reviglio VE, Hakim MA, Song JK, O'Brien TP. Effect of topical fluoroquinolones on the expression of matrix metalloproteinases in the cornea. *BMC Ophthalmol* 2003;3:10.

180. Corrales RM, Luo L, Chang EY, Pflugfelder SC. Effects of osmoprotectants on hyperosmolar stress in cultured human corneal epithelial cells. *Cornea* 2008;27:574-579.

181. Acera A, Rocha G, Vecino E, Lema I, Duran JA. Inflammatory markers in the tears of patients with ocular surface disease. *Ophthalmic Res.* 2008;40:315-321.

182. Dushku N, John MK, Schultz GS, Reid TW. Pterygia pathogenesis: Corneal invasion by matrix metalloproteinase expressing altered limbal epithelial basal cells. *Arch. Ophthalmol.* 2008;119:695-706.

183. Tsai Y-Y, Chiang C-C, Yeh K-T, Lee H, Cheng Y-W. Effect of TIMP-1 and MMP in pterygium invasion. *Invest. Ophthalmol. Vis. Sci.* 2010;51:3462-3467.

184. Smith VA, Khan-Lim D, Anderson L, Cook SD, Dick AD. Does orally administered doxycycline reach the tear film? *Br. J. Ophthalmol.* 2008;92:856-859.

185. Di Girolamo N, Verma MJ, McCluskey PJ, Lloyd A, Wakefield D. Increased matrix metalloproteinases in the aqueous humor of patients and experimental animals with uveitis. *Curr. Eye Res.* 1996;15:1060-1068.

186. Barro CD, Romanet JP, Fdili A, Guillot M, Morel F. Gelatinase concentration in tears of corneal-grafted patients. *Curr. Eye Res.* 1998;17:174-182.

187. Kernacki KA, Barrett R, Hazlett LD. Evidence for TIMP-1 protection against P. aeruginosa-induced corneal ulceration and perforation. *Invest. Ophthalmol. Vis. Sci.* 1999;40:3168-3176.

188. Kumagai N, Yamamoto K, Fukuda K, et al. Active matrix metalloproteinases in the tear fluid of individuals with vernal keratoconjunctivitis. *J. Allergy Clin. Immunol.* 2002;110:489-491.

189. Sakimoto T, Shoji J, Kanno H, Sawa M. Gelatinase expression in ocular surface disorders. *Jpn. J. Ophthalmol.* 2004;48:17-22.

190. Pflugfelder SC, Farley W, Luo L, et al. Matrix metalloproteinase-9 knockout confers resistance to corneal epithelial barrier disruption in experimental dry eye. *Am. J. Pathol.* 2005;166:61-71.

191. Rohini G, Murugeswari P, Prajna NV, Lalitha P, Muthukkaruppan V. Matrix metalloproteinases (MMP-8, MMP-9) and the tissue inhibitors of metalloproteinases (TIMP-1, TIMP-2) in patients with fungal keratitis. *Cornea* 2007;26:207-211.

192. Holopainen JM, Serra HM, Sanchez MC, et al. Altered expression of matrix metalloproteinases and their tissue inhibitors as possible contributors to corneal droplet formation in climatic droplet keratopathy. *Acta Ophthalmol.* 2009.

193. Lema I, Duran JA, Ruiz C, Diez-Feijoo E, Acera A, Merayo J. Inflammatory response to contact lenses in patients with keratoconus compared with myopic subjects. *Cornea* 2008;27:758-763.

194. Pannebaker C, Chandler HL, Nichols JJ. Tear proteomics in keratoconus. *Mol. Vis.* 2010;16:1949-1957.

195. Zhang Y, Gabriel MM, Mowrey-McKee MF, Barrett RP, McLellan S, Hazlett LD. Rat silicone hydrogel contact lens model: Effects of high- versus low-Dk lens wear. *Eye Contact Lens* 2008;34:306-311.

196. Hadassah J, Bhuvaneshwari N, Rao U, Sehgal PK. Evaluation of succinylated collagen bandage lenses in corneal healing by the expression of matrix metalloproteinases (MMP-2 and MMP-9) in tear fluid. *Ophthalmic Res.* 2009;42:64-72.

197. Papas EB, Petznick A, Stapleton F, Garrett Q. Involvement of matrix metalloproteinase-9 (MMP-9) and tissue inhibitor of matrix metalloproteinase-1 (TIMP-1) during orthokeratology. *ARVO Meeting Abstracts* 2006;47:117.

198. Choy CK, Cho P, Benzie IF, Ng V. Effect of one overnight wear of orthokeratology lenses on tear composition. *Optom. Vis. Sci.* 2004;81:414-420.

199. Kramann C, Boehm N, Lorenz K, et al. Effect of contact lenses on the protein composition in tear film: A ProteinChip study. *Graefes Arch. Clin. Exp. Ophthalmol.* 2011 249:233-243.

200. Sariri R, Khamedi A. Variations in electrophoretic tear protein pattern due to contact lens wear. *J. Chromatogr. A.* 2007;1161:64-66.

201. Stapleton F, Willcox MD, Morris CA, Sweeney DF. Tear changes in contact lens wearers following overnight eye closure. *Curr. Eye Res.* 1998;17:183-188.

202. Markoulli M, Papas E, Petznick A, Holden B. Validation of the flush method as an alternative to basal or reflex tear Collection. *Curr. Eye Res.* 2011;0:1-10.

203. Abalain JH, Dossou H, Colin J, Floch HH. Levels of collagen degradation products (telopeptides) in the tear film of patients with keratoconus. *Cornea* 2000;19:474-476.

204. Lane JD, Krumholz DM, Sack RA, Morris C. Tear glucose dynamics in diabetes mellitus. *Curr. Eye Res.* 2006;31:895-901.

205. Sack RA, Conradi L, Krumholz D, Beaton A, Sathe S, Morris C. Membrane array characterization of 80 chemokines, cytokines, and growth factors in open- and closed-eye tears: Angiogenin and other defense system constituents. *Invest. Ophthalmol. Vis. Sci.* 2005;46:1228-1238.

206. Carney FP, Morris CA, Willcox MD. Effect of hydrogel lens wear on the major tear proteins during extended wear. *Aust. N. Z. J. Ophthalmol.* 1997;25 Suppl 1:S36-38.

207. Glasson MJ, Stapleton F, Keay L, Sweeney D, Willcox MD. Differences in clinical parameters and tear film of tolerant and intolerant contact lens wearers. *Invest. Ophthalmol. Vis. Sci.* 2003;44:5116-5124.

208. Tan M, Thakur A, Morris C, Willcox MD. Presence of inflammatory mediators in the tears of contact lens wearers and non-contact lens wearers. *Aust. N. Z. J. Ophthalmol.* 1997;25 Suppl 1:S27-29.

209. Thakur A, Willcox MDP. Contact lens wear alters the production of certain inflammatory mediators in tears. *Exp. Eye Res.* 2000;70:255-259.

210. Green-Church KB, Nichols KK, Kleinholz NM, Zhang L, Nichols JJ. Investigation of the human tear film proteome using multiple proteomic approaches. *Mol. Vis.* 2008;14:456-470.

211. Li S, Sack R, Vijmasi T, et al. Antibody protein array analysis of the tear film cytokines. *Optom. Vis. Sci.* 2008;85:653-660.

212. Choy CK, Cho P, Chung WY, Benzie IF. Water-soluble antioxidants in human tears: Effect of the collection method. *Invest. Ophthalmol. Vis. Sci.* 2001;42:3130-3134.

213. van Haeringen NJ, Glasius E. The origin of some enzymes in tear fluid, determined by comparative investigation with two collection methods. *Exp. Eye Res.* 1976;22:267-272.

214. van Setten GB, Stephens R, Tervo T, Salonen EM, Tarkkanen A, Vaheri A. Effects of the Schirmer test on the fibrinolytic system in the tear fluid. *Exp. Eye Res.* 1990;50:135-141.

215. Fukuda M, Fullard RJ, Willcox MD, et al. Fibronectin in the tear film. *Invest. Ophthalmol. Vis. Sci.* 1996;37:459-467.

216. Fullard RJ, Tucker DL. Changes in human tear protein levels with progressively increasing stimulus. *Invest. Ophthalmol. Vis. Sci.* 1991;32:2290-2301.

217. Argueso P, Balaram M, Spurr-Michaud S, Keutmann HT, Dana MR, Gipson IK. Decreased levels of the goblet cell mucin MUC5AC in tears of patients with Sjogren syndrome. *Invest. Ophthalmol. Vis. Sci.* 2002;43:1004-1011.

218. Bjerrum KB, Prause JU. Collection and concentration of tear proteins studied by SDS gel electrophoresis. Presentation of a new method with special reference to dry eye patients. *Graefes Arch. Clin. Exp. Ophthalmol.* 1994;232:402-405.

219. Caffery B, Joyce E, Boone A, et al. Tear lipocalin and lysozyme in Sjogren and non-Sjogren dry eye. *Optom. Vis. Sci.* 2008;85:661-667.

220. Ng V, Cho P, Mak S, Lee A. Variability of tear protein levels in normal young adults: Between-day variation. *Graefes Arch. Clin. Exp. Ophthalmol.* 2000;238:892-899.

221. Ng V, Cho P, Wong F, Chan Y. Variability of tear protein levels in normal young adults: Diurnal (daytime) variation. *Graefes Arch. Clin. Exp. Ophthalmol.* 2001;239:257-263.

222. Yasueda S-i, Yamakawa K, Nakanishi Y, Kinoshita M, Kakehi K. Decreased mucin concentrations in tear fluids of contact lens wearers. *J. Pharm. Biomed. Anal.* 2005;39:187-195.

223. Smith VA, Hoh HB, Easty DL. Role of ocular matrix metalloproteinases in peripheral ulcerative keratitis. *Br. J. Ophthalmol.* 1999;83:1376-1383.

224. Spurr-Michaud S, Argueso P, Gipson I. Assay of mucins in human tear fluid. *Exp. Eye Res.* 2007;84:939-950.

225. van Haeringen NJ. Clinical biochemistry of tears. *Surv. Ophthalmol.* 1981;26:84-96.

226. Sack RA, Tan KO, Tan A. Diurnal tear cycle: evidence for a nocturnal inflammatory constitutive tear fluid. *Invest. Ophthalmol. Vis. Sci.* 1992;33:626-640.

227. Solomon A, Dursun D, Liu Z, Xie Y, Macri A, Pflugfelder SC. Pro- and anti-inflammatory forms of interleukin-1 in the tear fluid and conjunctiva of patients with dry-eye disease. *Invest. Ophthalmol. Vis. Sci.* 2001;42:2283-2292.

228. Laemmli UK. Cleavage of structural proteins during the assembly of the head of the bacteriophage. *Nature* 1970;227:680-685.

229. David NP, Darryl JCP, David MC, John SC. Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis* 1999;20:3551-3567.

230. Keller A, Nesvizhskii AI, Kolker E, Aebersold R. Empirical statistical model to estimate the accuracy of peptide identifications made by MS/MS and database search. *Anal. Chem.* 2002;74:5383-5392.

231. Nesvizhskii AI, Keller A, Kolker E, Aebersold R. A statistical model for identifying proteins by tandem mass spectrometry. *Anal. Chem.* 2003;75:4646-4658.

232. Bland JM, Altman DG. Statistical methods for assessing agreement between two methods of clinical measurement. *Lancet* 1986;1:307-310.

233. Institution BS. *Precision of Test Methods, Part 1: Guide for the determination of Repeatability and Reproducibility for a standard test method*. BS 5497, Part 1. London: BSI; 1979.

234. Molloy MP, Bolis S, Herbert BR, et al. Establishment of the human reflex tear two-dimensional polyacrylamide gel electrophoresis reference map: New proteins of potential diagnostic value. *Electrophoresis* 1997;18:2811-2815.

235. Nichols JJ, Green-Church KB. Mass spectrometry-based proteomic analyses in contact lens-related dry eye. *Cornea* 2009;28:1109-1117.

236. Fullard RJ, Snyder C. Protein levels in nonstimulated and stimulated tears of normal human subjects. *Invest. Ophthalmol. Vis. Sci.* 1990;31:1119-1126.

237. Sack RA, Beaton A, Sathe S, Morris C, Willcox M, Bogart B. Towards a closed eye model of the pre-ocular tear layer. *Prog. Retin. Eye Res.* 2000;19:649-668.

238. Wilson G, O'leary DJ, Holden BA. Cell content of tears following overnight wear of a contact lens. *Curr. Eye Res.* 1989;8:329-335.

239. Ehrmann K, Fedtke C, Radic A. Assessment of computer generated vision charts. *Cont. Lens Anterior Eye* 2009;32:133-140.

240. *Cornea and Contact Lens Research Unit. C.C.L.R.U. Grading Scales*. University of New South Wales, Sydney, Australia; 1996.

241. Petznick A, Evans MDM, Madigan MC, Markoulli M, Garrett Q, Sweeney DF. A comparison of basal and eye-flush tears for the analysis of cat tear proteins. *Acta Ophthalmol. (Copenh).* 2011;89:e75-e81.

242. Sitaramamma T, Shivaji S, Rao GN. Effect of storage on protein concentration of tear samples. *Curr. Eye Res.* 1998;17:1027 - 1035.

243. Sack RA, Sathe S, Beaton A. Tear turnover and immune and inflammatory processes in the open-eye and closed-eye environments: Relationship to extended wear contact lens use. *Eye Contact Lens* 2003;29:S80-82.

244. Tan KO, Sack RA, Holden BA, Swarbrick HA. Temporal sequence of changes in tear film composition during sleep. *Curr. Eye Res.* 1993;12:1001-1007.

245. Leber TM, Balkwill FR. Zymography: A single-step staining method for quantitation of proteolytic activity on substrate gels. *Anal. Biochem.* 1997;249:24-28.

246. Birkedal-Hansen H, Taylor RE. Detergent-activation of latent collagenase and resolution of its component molecules. *Biochem. Biophys. Res. Commun.* 1982;107:1173-1178.

247. Hawkes SP, Li H, Taniguchi GT. Zymography and reverse zymography for detecting MMPs, and TIMPs. *Methods Mol. Biol.* 2001;151:399-410.

248. Stevens J. *Applied multivariate statistics for the social sciences*. 2nd ed. New Jersey, USA: Lawrence Erlbaum Associates; 1992.

249. Roy R, Louis G, Loughlin KR, et al. Tumor-specific urinary matrix metalloproteinase fingerprinting: Identification of high molecular weight urinary matrix metalloproteinase species. *Clin. Cancer Res.* 2008;14:6610-6617.

250. Kjeldsen L, Johnsen AH, Sengelov H, Borregaard N. Isolation and primary structure of NGAL, a novel protein associated with human neutrophil gelatinase. *J. Biol. Chem.* 1993;268:10425-10432.

251. Olson MW, Bernardo MM, Pietila M, et al. Characterization of the monomeric and dimeric forms of latent and active matrix metalloproteinase-9. Differential rates for activation by stromelysin 1. *J. Biol. Chem.* 2000;275:2661-2668.

252. Sathe S, Sakata M, Beaton AR, Sack RA. Identification, origins and the diurnal role of the principal serine protease inhibitors in human tear fluid. *Curr. Eye Res.* 1998;17:348-362.

253. Dua HS. Recurrent corneal erosion syndrome. *Surv. Ophthalmol.* 2008;53:664-664.

254. Weinreb RN, Kashiwagi K, Kashiwagi F, Tsukahara S, Lindsey JD. Prostaglandins increase matrix metalloproteinase release from human ciliary smooth muscle cells. *Invest. Ophthalmol. Vis. Sci.* 1997;38:2772-2780.

255. Lindsey JD, Kashiwagi K, Kashiwagi F, Weinreb RN. Prostaglandins alter extracellular matrix adjacent to human ciliary muscle cells in vitro. *Invest. Ophthalmol. Vis. Sci.* 1997;38:2214-2223.

256. Honda N, Miyai T, Nejima R, et al. Effect of latanoprost on the expression of matrix metalloproteinases and tissue inhibitor of metalloproteinase 1 on the ocular surface. *Arch. Ophthalmol.* 2010;128:466-471.

257. Noecker RJ, Herrygers LA, Anwaruddin R. Corneal and conjunctival changes caused by commonly used glaucoma medications. *Cornea* 2004;23:490-496.

258. Costagliola C, Del Prete A, Verolino M, et al. Effect of 0.005% latanoprost once daily on intraocular pressure in glaucomatous patients not adequately controlled by beta-blockers twice daily: A 3-year follow-up. Experience and incidence of side effects in a prospective study on 76 patients. *Graefes Arch. Clin. Exp. Ophthalmol.* 2002;240:379-386.

259. Tarr GP, Williams MJA, Phillips LV, van Rij AM, Jones GT. Seasonal variation and stability of matrix metalloproteinase-9 activity and tissue inhibitor of matrix metalloproteinase-1 with storage at − 80 °C. *Clin. Biochem.* 2011;44:1346-1348.

260. Rouy D, Ernens I, Jeanty C, Wagner DR. Plasma storage at -80 degrees C does not protect matrix metalloproteinase-9 from degradation. *Anal. Biochem.* 2005;338:294-298.

261. Jackson D, Herath A, Swinton J, et al. Considerations for powering a clinical proteomics study: Normal variability in the human plasma proteome. *Proteomics Clin. Appl.* 2009;3:394-407.

262. Bell AW, Deutsch EW, Au CE, et al. A HUPO test sample study reveals common problems in mass spectrometry-based proteomics. *Nat Meth* 2009;6:423-430.

263. Kowluru RA. Role of matrix metalloproteinase-9 in the development of diabetic retinopathy and its regulation by H-Ras. *Invest. Ophthalmol. Vis. Sci.* 2010;51:4320-4326.

264. Laibson PR. Recurrent corneal erosions and epithelial basement membrane dystrophy. *Eye Contact Lens* 2010;36:315-317.

265. Hahn-Dantona E, Ramos-DeSimone N, Sipley J, Nagase H, French DL, Quigley JP. Activation of proMMP-9 by a plasmin/MMP-3 cascade in a tumor cell model. Regulation by tissue inhibitors of metalloproteinases. *Ann. N. Y. Acad. Sci.* 1999;878:372-387.

266. Ramos-DeSimone N, Hahn-Dantona E, Sipley J, Nagase H, French DL, Quigley JP. Activation of matrix metalloproteinase-9 (MMP-9) via a converging plasmin/stromelysin-1 cascade enhances tumor cell invasion. *J. Biol. Chem.* 1999;274:13066-13076.

267. Li DQ, Lokeshwar BL, Solomon A, Monroy D, Ji Z, Pflugfelder SC. Regulation of MMP-9 production by human corneal epithelial cells. *Exp. Eye Res.* 2001;73:449-459.

268. Nagayasu A, Hosaka Y, Yamasaki A, et al. A preliminary study of direct application of atelocollagen into a wound lesion in the dog cornea. *Curr. Eye Res.* 2008;33:727-735.

269. Twyman RM. *Principles of Proteomics*. York, UK: Garland Science/BIOS Scientific Publishers - Taylor & Francis group; 2004.

270. Ong S-E, Pandey A. An evaluation of the use of two-dimensional gel electrophoresis in proteomics. *Biomol. Eng.* 2001;18:195-205.

271. Wu TL, Nedelkov D, Nelson RW. Two-dimensional difference gel electrophoresis. *Methods Mol. Biol.* 2006;328:71-95.

272. Unlu M, Morgan ME, Minden JS. Difference gel electrophoresis: A single gel method for detecting changes in protein extracts. *Electrophoresis* 1997;18:2071-2077.

273. Alban A, David SO, Bjorkesten L, et al. A novel experimental design for comparative two-dimensional gel analysis: Two-dimensional difference gel electrophoresis incorporating a pooled internal standard. *Proteomics* 2003;3:36-44.

274. Tonge R, Shaw J, Middleton B, et al. Validation and development of fluorescence two-dimensional differential gel electrophoresis proteomics technology. *Proteomics* 2001;1:377-396.

275. Larbi NB, Jefferies C. 2D-DIGE: comparative proteomics of cellular signalling pathways. *Methods Mol. Biol.* 2009;517:105-132.

276. Patton WF. Detection technologies in proteome analysis. *J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci.* 2002;771:3-31.

277. Gharbi S, Gaffney P, Yang A, et al. Evaluation of two-dimensional differential gel electrophoresis for proteomic expression analysis of a model breast cancer cell system. *Mol. Cell Proteomics* 2002;1:91-98.

278. Zhou H, Ranish JA, Watts JD, Aebersold R. Quantitative proteome analysis by solid-phase isotope tagging and mass spectrometry. *Nat. Biotechnol.* 2002;20:512-515.

279. Ananthi S, Santhosh RS, Nila MV, Prajna NV, Lalitha P, Dharmalingam K. Comparative proteomics of human male and female tears by two-dimensional electrophoresis. *Exp. Eye Res.* 2011;92:454-463.

280. Joseph R, Srivastava OP, Pfister RR. Differential epithelial and stromal protein profiles in keratoconus and normal human corneas. *Exp. Eye Res.* 2011;92:282-298.

281. Su S, Liu P, Zhang H, et al. Proteomic analysis of human age-related nuclear cataracts and normal lens nuclei. *Invest. Ophthalmol. Vis. Sci.* 2011;52:4182-4191.

282. Callender M, Morrison PE. A quantitative study of human tear proteins before and after adaption to non-flexible contact lenses. *Am. J. Optom. Physiol. Opt.* 1974;51:939-945.

283. Farris RL. Tear analysis in contact lens wearers. *Trans. Am. Ophthalmol. Soc.* 1985;83:501-545.

284. Glasson MJ, Stapleton F, Willcox MDP. Lipid, lipase and lipocalin differences between tolerant and intolerant contact lens wearers. *Curr. Eye Res.* 2002;25:227-235.

285. Lakkis C, Banbury L, Morris C. Secretory IgA in tears and saliva of contact lens wearers. *ARVO Meeting Abstracts*; 2008:4850.

286. Horgan GW. Sample size and replication in 2D gel electrophoresis studies. *J. Proteome Res.* 2007;6:2884-2887.

287. Marouga R, David S, Hawkins E. The development of the DIGE system: 2D fluorescence difference gel analysis technology. *Anal. Bioanal. Chem.* 2005;382:669-678.

288. Zhao Z, Liu J, Wasinger VC, et al. Tear lipocalin is the predominant phosphoprotein in human tear fluid. *Exp. Eye Res.* 2010;90:344-349.

289. Donoghue P, Staunton L, Mullen E, Manning G, Ohlendieck K. DIGE analysis of rat skeletal muscle proteins using nonionic detergent phase extraction of young adult versus aged gastrocnemius tissue. *J. Proteomics* 2010;73:1441-1453.

290. Malm C, Hadrevi J, Bergström SA, et al. Evaluation of 2-D DIGE for skeletal muscle: Protocol and repeatability. *Scand. J. Clin. Lab. Invest.* 2008;68:793-800.

291. de Souza GA, Godoy LM, Mann M. Identification of 491 proteins in the tear fluid proteome reveals a large number of proteases and protease inhibitors. *Genome Biol.* 2006;7:R72.

292. Li N, Wang N, Zheng J, et al. Characterization of human tear proteome using multiple proteomic analysis techniques. *J. Proteome Res.* 2005;4:2052-2061.

293. Zhou L, Beuerman RW, Foo Y, Liu S, Ang LP, Tan DT. Characterisation of human tear proteins using high-resolution mass spectrometry. *Ann. Acad. Med. Singapore* 2006;35:400-407.

294. Herber S, Grus FH, Sabuncuo P, Augustin AJ. Two-dimensional analysis of tear protein patterns of diabetic patients. Electrophoresis 2001;22:1838-1844.

295. Hassan MI, Waheed A, Yadav S, Singh TP, Ahmad F. Zinc α2-glycoprotein: A multidisciplinary protein. Mol. Cancer Res. 2008;6:892-906.

296. Yamada M, Mochizuki H, Kawashima M, Hata S. Phospholipids and their degrading enzyme in the tears of soft contact lens wearers. Cornea 2006;25:S68-S72.

297. Lundh RL, Liotet S, Pouliquen Y. Study of the human blood-tear barrier and the biochemical changes in the tears of 30 contact lens wearers (50 eyes). *Ophthalmologica* 1984;188:100-105.

298. Luensmann D, Jones L. Albumin adsorption to contact lens materials: a review. *Cont Lens Anterior Eye* 2008;31:179-187.

299. Taylor RL, Willcox MD, Williams TJ, Verran J. Modulation of bacterial adhesion to hydrogel contact lenses by albumin. *Optom. Vis. Sci.* 1998;75:23-29.

300. Masinick SA, Montgomery CP, Montgomery PC, Hazlett LD. Secretory IgA inhibits Pseudomonas aeruginosa binding to cornea and protects against keratitis. *Invest. Ophthalmol. Vis. Sci.* 1997;38:910-918.

301. Zhao Z, Russell PJ. Trypsin activity assay in substrate-specific one- and two-dimensional gels: A powerful method to separate and characterize novel proteases in active form in biological samples. *Electrophoresis* 2003;24:3284-3288.

302. Tian M, Cui YZ, Song GH, et al. Proteomic analysis identifies MMP-9, DJ-1 and A1BG as overexpressed proteins in pancreatic juice from pancreatic ductal adenocarcinoma patients. *BMC Cancer* 2008;8:241.

303. Sitaramamma T, Shivaji S, Rao GN. HPLC analysis of closed, open, and reflex eye tear proteins. *Indian J. Ophthalmol.* 1998;46:239-245.

304. Wilkins MR, Appel RD, Van Eyk JE, et al. Guidelines for the next 10 years of proteomics. *Proteomics* 2006;6:4-8.

305. Mischak H, Apweiler R, Banks RE, et al. Clinical proteomics: A need to define the field and to begin to set adequate standards. *Proteomics Clin. Appl.* 2007;1:148-156.

306. Luan Y, Kong L, Howell DR, et al. Inhibition of ADAMTS-7 and ADAMTS-12 degradation of cartilage oligomeric matrix protein by alpha-2-macroglobulin. *Osteoarthr. Cartil.* 2008;16:1413-1420.

307. Wang J, Wang N, Xie J, et al. Restricted epithelial proliferation by lacritin via PKCα-dependent NFAT and mTOR pathways. *J. Cell Biol.* 2006;174:689-700.

308. Koo BS, Lee DY, Ha HS, Kim JC, Kim CW. Comparative analysis of the tear protein expression in blepharitis patients using two-dimensional electrophoresis. *J. Proteome Res.* 2005;4:719-724.

309. Grus FH, Podust VN, Bruns K, et al. SELDI-TOF-MS ProteinChip Array Profiling of Tears from Patients with Dry Eye. *Invest. Ophthalmol. Vis. Sci.* 2005;46:863-876.

310. Efron N, Hollingsworth JG. New perspectives on keratoconus as revealed by corneal confocal microscopy. *Clin. Exp. Optom.* 2008;91:34-55.

311. Veves A, Sheehan P, Pham HT, for the Promogran Diabetic Foot Ulcer S. A randomized, controlled trial of Promogran (a collagen/oxidized regenerated cellulose dressing) vs standard treatment in the management of diabetic foot ulcers. *Arch. Surg.* 2002;137:822-827.

312. Donaghue VM, Chrzan JS, Rosenblum BI, Giurini JM, Habershaw GM, Veves A. Evaluation of a collagen-alginate wound dressing in the management of diabetic foot ulcers. *Adv. Wound Care* 1998;11:114-119.

313. van Rossum M, Vooijs D, Walboomers X, Hoekstra M, Spauwen P, Jansen J. The influence of a PHI-5-loaded silicone membrane, on cutaneous wound healing in vivo. *J. Mater. Sci. Mater. Med.* 2007;18:1449-1456.

APPENDIX A: PARTICIPANT INFORMATION STATEMENT AND CONSENT FORMS

FLUSH STUDY AND ADAPTATION STUDY: EXTENDED WEAR (STUDY 1)

Approval No. 084055

THE UNIVERSITY OF NEW SOUTH WALES AND THE INSTITUTE FOR EYE RESEARCH

PARTICIPANT INFORMATION STATEMENT AND CONSENT FORM

24 HOUR TEAR COLLECTION WITH AND WITHOUT EXTENDED WEAR CONTACT LENSES

Protocol Title:	24 Hour Tear Collection With And Without Extended Wear Contact Lenses
Protocol Number:	PhD2008-002
PI/ICF Amendment Number:	Amendment 6
Investigator:	Maria Markoulli
Contact Number:	Work Hours: 02-9385 7612 or After Hours: 0419 810 121

PARTICIPANT SELECTION AND PURPOSE OF STUDY

You are invited to participate in a study looking at how the tears of the eye vary over 24 hours and how these vary with contact lenses when worn on an extended wear basis for 1 month. We hope to establish the levels of various components in the tears over a 24 hour period both with and without contact lens wear. We further hope to determine whether contact lens wear on an extended wear basis influences this cycle. You were selected as a possible participant in this study because you meet the suitability criteria of not having worn contact lenses in the previous month, not being a habitual contact lens wearer and having normal ocular health.

DESCRIPTION OF STUDY AND RISKS

If you decide to participate, we will perform a baseline examination where your spectacle prescription will be measured and your ocular health will be examined. A small volume of your tears will also be collected as described below. If you are suitable for the study, you will be asked to stay in the Institute for Eye Research (IER) clinic overnight. Your tears will then be collected:

- shortly before you sleep
- first thing upon awakening

You will then be scheduled for the next series of visits where you will be randomised to wear either ACUVUE® OASYS™ or O_2OPTIX™ contact lenses. Both these lenses are commercially available. You will be asked to wear these on an extended wear basis (that is, overnight) for 30 nights for a minimum of 5 days and nights per week. Tears will again be collected:

- before lens insertion
- after lens insertion
- before sleep
- upon awakening

A 1 week visit will be scheduled as a safety check. You will then be asked to return for the following tear collection schedule after 1 month of contact lens wear:

- midday visit
- before sleep
- upon awakening
- 6 hours after awakening.

In total, there will be three overnight visits at the IER clinic. The visit schedule is summarised below:

Visit Type	Abbreviation
Baseline visit – midday visit, no contact lenses	V1
Before sleep – no contact lenses	V2
Upon awakening – no contact lenses	V3
Midday visit – contact lens insertion	V4
Before sleep – contact lens wear	V5
Upon awakening – contact lens wear	V6
1 week – contact lens wear	V7
Midday visit – 1 month contact lens wear	V8
Before sleep – 1 month contact lens wear	V9
Upon awakening – 1 month contact lens wear	V10
6 hrs after awakening – no contact lens wear	V11

Up to the first 10 participants of this study will be asked to participate in a validation study whereby their tears will be collected on 6 occasions prior to commencement of the main study. This will be conducted between 11am and 2pm on 6 separate days. This group may also be asked to collect their own tears upon awakening on two separate occasions. Instructions will be provided to this group.

PROCEDURES
The procedures to be performed are outlined below:

- **Auto-Keratometry:** Automated measurement of the curvature of the eyes. Measured with an auto-refractor / keratometer.
- **Auto-Refraction:** Automated measurement of the spectacle lens prescription required to correct vision. Measured with an auto-refractor / keratometer.
- **Refraction:** Measurement of the spectacle lens prescription required to correct vision. Measured using a phoroptor and/or trial frame and trial lenses.
- **Visual Acuity:** Measurement of the standard of vision achieved with spectacles or contact lenses using standard letter charts. Measurements may be taken with each eye individually or with both eyes.

- **Over-Refraction:** After insertion of the contact lens, the need for lens power alteration is determined by placing a spectacle lens in front of the eye and asking for your feedback. The lens may be placed using trial lenses and a trial frame, or a phoroptor.
- **Slit-Lamp Biomicroscopy:** Contact lens performance and the ocular surface are examined with a specialised microscope called a slit-lamp. The microscope allows the eye to be viewed with white light or a blue light. A non-contact lens may be used with this to assess the back of the eye at the baseline visit.
- **Fluorescein Assessment:** The ocular surface is assessed by instilling a harmless fluorescent dye called "fluorescein". The dye does not feel uncomfortable and is temporary.
- **Lid Eversion:** The upper eye lid is folded back using a cotton bud to allow examination of the under surface.
- **Questionnaire:** Questions assessing comfort, vision, ease of use, handling, problems encountered and satisfaction may be asked verbally at your appointment.
- **Aseptic Lens Removal:** Lens removal is performed using sterile gloves (Ansell, Examtech) so that normal bacteria from the skin do not contaminate the lens. Care is taken to avoid touching the eyelids and eyelashes with the glove whilst removing the lens. Once the lens is removed it is placed directly into a sterile container. Both the saline in the container and the lenses are analysed for bacteria.
- **Tear Samples:** Small volumes of tears, approximately one drop of your tears, are gently drawn into a fine glass tube placed at the edge of the lower eyelid. You may be asked to use a cotton bud to tickle the nasal passages in order to induce reflex tearing. Alternatively, a drop of unit dose sterile saline (AstraZeneca) may be instilled in your eye prior to tear collection.
- **Adverse Response Protocol:** In the event of a problem occurring during lens wear, swab samples may be collected. These are obtained by lightly brushing the eye and lower eyelid to determine the normal levels of bacteria present in the eye. These procedures will be explained to you in detail.

All clinic visits are attended at the IER Clinic, Level 5, North Wing, Rupert Myers Building, Kensington NSW 2033.

RISKS AND PRECAUTIONS

This study involves research, which means that with the products or treatments provided to you there may be risks or consequences that are not currently known or are not foreseeable.

Slight irritation, redness, dryness or watering of the eyes may occur during tear collection.

All lens products have the potential to cause side effects in some individuals. The most serious complication is corneal infection. This is a rare disease (1/500 per year if wearing lenses during sleep and 1/2,500 per year with daily wear). The possible consequences of such an event are severe pain, hospitalisation, corneal scarring and possible loss of vision. Smoking also increases the risk in contact lens wearers.

Other complications which may occur are: inflammation of the eye - such as acute red eye and sterile ulcers; growth of blood vessels into the cornea; temporary decreased vision and/or iritis (inflammation of the colour part of the eye); pain; abrasion (scratching) of the eye; burning or itching (irritation); excessive watering (tearing) of the eyes; unusual eye secretions; redness of eyes; reduced sharpness of vision; sensitivity to light; and/or dryness of eyes.

The disinfecting solution contains 3% hydrogen peroxide, so it is not to be used directly in the eye or for rinsing lenses prior to inserting into the eye as the solution must be neutralised. The solution must be used with the dedicated lens case with neutralising disc and lenses must be soaked for at least 6 hours before inserting on the eye. A burning sensation will be experienced if unneutralised AOSept® Plus comes into contact with the eye.

If you experience any of the above, you should remove your lenses and place in the study solution, AOSept® Plus, in the dedicated lens case and contact the clinic immediately.

If you are pregnant or are planning to become pregnant and have additional questions, please ask the Optometrist.

BENEFITS

Your optometric care, contact lenses and solutions will be provided free-of-charge during the study period. Contact lenses offer improved peripheral (side) vision and the convenience of not wearing spectacles. However, we cannot and do not guarantee or promise that you will receive any benefits from this study. Information learned in this study may benefit others in the future.
Two movie vouchers will be given to you as recognition of your time.

ALTERNATIVE TREATMENTS

Alternative methods of visual correction and contact lens care other than those used in this study are available. These include currently marketed contact lenses, refractive surgery, and spectacles. Your study Optometrist will discuss the risks and benefits of these alternate treatments. However, should these be required, you must purchase them at your own expense from a source other than the IER and in consultation with your primary eye health care professional.

SAFEGUARD

For your safety, you must tell the Optometrist all of your past and present health history; all prescription, over-the-counter, and herbal medications/treatments that you are presently using; if you have any known allergies (for example an allergy to latex) or if you have had any previous reactions to medications; and if you are currently participating 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:

- Use only of lenses and solutions provided by the IER for the assigned duration and mode of wear (i.e. overnight wear);
- Attendance for scheduled visits;
- Reporting of all changes that occur to your health, medications and, in particular, your eyes;
- The return of all lenses and unopened cases and solutions, when deemed necessary by the optometrist; the return of any foils for used lenses;
- To stay overnight at the IER clinic on three separate occasions;
- To be confident in inserting and removing your own lenses.

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

Your study Optometrist will inform you of any significant new findings about the study products and procedures that might develop during the course of this research and that might affect your willingness to continue to participate in the study.

YOUR CONSENT

Your decision whether or not to participate will not prejudice your future relations with the University of New South Wales and the IER. 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 questions, please feel free to ask us. If you have any additional questions later, your Optometrist, Maria Markoulli (phone number 9385 7612), will be happy to answer them.

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

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 Investigator*
_____	_____
(Please PRINT name)	*(Please PRINT name)*
_____	_____
Date	*Date*

THE UNIVERSITY OF NEW SOUTH WALES AND THE INSTITUTE FOR EYE RESEARCH

REVOCATION OF CONSENT

24 HOUR TEAR COLLECTION WITH AND WITHOUT EXTENDED WEAR CONTACT LENSES

Protocol Title:	24 Hour Tear Collection With And Without Extended Wear Contact Lenses
Protocol Number:	PhD2008-002
PI/ICF Amendment Number:	Amendment 6
Investigator:	Maria Markoulli
Contact Number:	Work Hours: 02-9385 7612 or After Hours: 0419 810 121

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 and the Institute for Eye Research.

_____ _____

Signature *Date*

_____

Please PRINT Name

Please send this Revocation of Consent to:

Ms Maria Markoulli
Institute for Eye Research
Level 5, North Wing
Rupert Myers Building
KENSINGTON NSW 2033
Australia

FLUSH STUDY AND ADAPTATION STUDY: DAILY WEAR

Approval No. 084056

THE UNIVERSITY OF NEW SOUTH WALES AND THE INSTITUTE FOR EYE RESEARCH

PARTICIPANT INFORMATION STATEMENT AND CONSENT FORM

24 HOUR TEAR COLLECTION WITH AND WITHOUT DAILY WEAR CONTACT LENSES

Protocol Title:	24 Hour Tear Collection With And Without Daily Wear Contact Lenses
Protocol Number:	PhD2008-003
PI/ICF Amendment Number:	Amendment 6
Investigator:	Maria Markoulli
Contact Number:	Work Hours: 02-9385 7612 or After Hours: 0419 810 121

PARTICIPANT SELECTION AND PURPOSE OF STUDY

You are invited to participate in a study looking at how the tears vary over 24 hours and how these vary with contact lenses when worn on a daily wear basis for 1 month. We hope to establish the levels of various components in the tears over a 24 hour period both with and without contact lens wear. We further hope to determine whether contact lens wear on a daily wear basis influences this cycle. You were selected as a possible participant in this study because you meet the suitability criteria of not having worn contact lenses in the previous month, not being a habitual contact lens wearer and having normal ocular health.

DESCRIPTION OF STUDY AND RISKS

If you decide to participate, we will perform a baseline examination where your spectacle prescription will be measured and your ocular health will be examined. A small volume of your tears will also be collected as described below. If you are suitable for the study, you will be asked to stay in the Institute for Eye Research (IER) clinic overnight. Your tears will then be collected:

- shortly before you sleep
- first thing upon awakening

You will then be scheduled for the next series of visits where you will be randomised to wear either ACUVUE® OASYS™ or O_2OPTIX™ contact lenses. Both these lenses are commercially available. You will be asked to wear these on a daily wear basis for 30 days and for a minimum of 5 days per week, with a minimum of 6 hours per day when they are worn. There is no maximum time to wear lenses provided they are not worn overnight. Tears will again be collected:

- before lens insertion
- after lens insertion
- before sleep

- upon awakening

At the 1 week visit will be scheduled for a safety check. You will then be asked to return for the following tear collection schedule after 1 month of contact lens wear:

- midday visit
- before sleep
- upon awakening
- 6 hours after awakening.

In total, there will be three overnight visits at the IER clinic. The visit schedule is summarised below:

Visit Type	Abbreviation
Baseline visit – midday visit no contact lens	BL – V1
Before sleep – no contact lens	V2
Upon awakening – no contact lens	V3
Midday visit – contact lens insertion	V4
Before sleep – 20 mins after lens removal	V5
Upon awakening – before contact lens wear	V6
One week DW – contact lens wear	V7
Midday visit – 1MDW contact lens wear	V8
Before sleep – 20 mins after lens removal	V9
Upon awakening – no contact lens wear	V10
6hrs after awakening – no contact lens wear	V11

Up to the first 10 participants of this study will be asked to participate in a validation study whereby their tears will be collected on 6 occasions prior to commencement of the main study. This will be conducted between 11am and 2pm on 6 separate days. This group may also be asked to collect their own tears upon awakening on two separate occasions. Instructions will be provided to this group.

PROCEDURES
The procedures to be performed are outlined below:

- *Auto-Keratometry:* Automated measurement of the curvature of the eyes. Measured with an auto-refractor / keratometer.
- *Auto-Refraction:* Automated measurement of the spectacle lens prescription required to correct vision. Measured with an auto-refractor / keratometer.
- *Refraction:* Measurement of the spectacle lens prescription required to correct vision. Measured using a phoroptor and/or trial frame and trial lenses.
- *Visual Acuity:* Measurement of the standard of vision achieved with spectacles or contact lenses using standard letter charts. Measurements may be taken with each eye individually or with both eyes.
- *Over-Refraction:* After insertion of the contact lens, the need for lens power alteration is determined by placing a spectacle lens in front of the eye and asking for your feedback. The lens may be placed using trial lenses and a trial frame, or a phoroptor.

- *Slit-Lamp Biomicroscopy:* Contact lens performance and the ocular surface are examined with a specialised microscope called a slit-lamp. The microscope allows the eye to be viewed with white light or a blue light. A non-contact lens may be used with this to assess the back of the eye at the baseline visit.
- *Fluorescein Assessment:* The ocular surface is assessed by instilling a harmless fluorescent dye called "fluorescein". The dye does not feel uncomfortable and is temporary.
- *Lid Eversion:* The upper eye lid is folded back using a cotton bud to allow examination of the under surface.
- *Questionnaire:* Questions assessing comfort, vision, ease of use, handling, problems encountered and satisfaction may be asked verbally or answered by you directly via computer at your appointment.
- *Aseptic Lens Removal:* Lens removal is performed using sterile gloves (Ansell, Examtech) so that normal bacteria from the skin do not contaminate the lens. Care is taken to avoid touching the eyelids and eyelashes with the glove whilst removing the lens. Once the lens is removed it is placed directly into a sterile container. Both the saline in the container and the lenses are analysed for bacteria.
- *Tear Samples:* Small volumes of tears, approximately one drop of your tears, are gently drawn into a fine glass tube placed at the edge of the lower eyelid. You may be asked to use a cotton bud to tickle the nasal passages in order to induce reflex tearing. Alternatively, a drop of unit dose sterile saline (AstraZeneca) may be instilled in your eye prior to tear collection.
- *Adverse Response Protocol:* In the event of a problem occurring during lens wear, swab samples may be collected. These are obtained by lightly brushing the eye and lower eyelid to determine the normal levels of bacteria present in the eye. These procedures will be explained to you in detail.

All clinic visits are attended at the IER Clinic, Level 5, North Wing, Rupert Myers Building, Kensington NSW 2033.

RISKS AND PRECAUTIONS

This study involves research, which means that with the products or treatments provided to you there may be risks or consequences that are not currently known or are not foreseeable.

Slight irritation, redness, dryness or watering of the eyes may occur during tear collection.

All lens products have the potential to cause side effects in some individuals. The most serious complication is corneal infection. This is a rare disease (1/500 per year if wearing lenses during sleep and 1/2,500 per year with daily wear). The possible consequences of such an event are severe pain, hospitalisation, corneal scarring and possible loss of vision. Smoking also increases the risk in contact lens wearers.

Other complications which may occur are: inflammation of the eye - such as acute red eye and sterile ulcers; growth of blood vessels into the cornea; temporary decreased vision and/or iritis (inflammation of the colour part of the eye); pain; abrasion

(scratching) of the eye; burning or itching (irritation); excessive watering (tearing) of the eyes; unusual eye secretions; redness of eyes; reduced sharpness of vision; sensitivity to light; and/or dryness of eyes.

The disinfecting solution contains 3% hydrogen peroxide, so it is not to be used directly in the eye or for rinsing lenses prior to inserting into the eye as the solution must be neutralised. The solution must be used with the dedicated lens case with neutralising disc and lenses must be soaked for at least 6 hours before inserting on the eye. A burning sensation will be experienced if unneutralised AOSept® Plus comes into contact with the eye.

If you experience any of the above, you should remove your lenses and place in the study solution, AOSept® Plus, in the dedicated lens case and contact the clinic immediately on 02 9385 7516 during work hours or 02 9962 9441(pager) after hours.

If you are pregnant or are planning to become pregnant and have additional questions, please ask the Optometrist.

BENEFITS

Your optometric care will be provided free-of-charge during the study period. Contact lenses offer improved peripheral (side) vision and the convenience of not wearing spectacles. However, we cannot and do not guarantee or promise that you will receive any benefits from this study. Information learned in this study may benefit others in the future. Two movie vouchers will be given to you as recognition of your time.

ALTERNATIVE TREATMENTS

Alternative methods of visual correction and contact lens care other than those used in this study are available. These include currently marketed contact lenses, refractive surgery, and spectacles. Your study Optometrist will discuss the risks and benefits of these alternate treatments. However, should these be required, you must purchase them at your own expense from a source other than the IER and in consultation with your primary eye health care professional.

SAFEGUARD

For your safety, you must tell the Optometrist all of your past and present health history; all prescription, over-the-counter, and herbal medications / treatments that you are presently using; if you have any known allergies (for example an allergy to latex) or if you have had any previous reactions to medications; and if you are currently participating 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:

- Use only of lenses and solutions provided by the IER for the assigned duration and mode of wear (i.e. daily wear);
- Attendance for scheduled visits;

- Reporting of all changes that occur to your health, medications and, in particular, your eyes;
- The return of all lenses and unopened cases and solutions, when deemed necessary by the optometrist. The return of any foils for used lenses;
- To stay overnight at the IER Clinic on three separate occasions;
- To be confident in inserting and removing your own lenses.

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

Your study Optometrist will inform you of any significant new findings about the study products and procedures that might develop during the course of this research and that might affect your willingness to continue to participate in the study.

YOUR CONSENT

Your decision whether or not to participate will not prejudice your future relations with the University of New South Wales and the IER. 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 questions, please feel free to ask us. If you have any additional questions later, your Optometrist, Maria Markoulli (phone number 9385 7612), will be happy to answer them.

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

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 Investigator*
_____	_____
(Please PRINT name)	*(Please PRINT name)*
_____	_____
Date	*Date*

THE UNIVERSITY OF NEW SOUTH WALES AND THE INSTITUTE FOR EYE RESEARCH

REVOCATION OF CONSENT

24 HOUR TEAR COLLECTION WITH AND WITHOUT DAILY WEAR CONTACT LENSES

Protocol Title:	24 Hour Tear Collection With And Without Daily Wear Contact Lenses
Protocol Number:	PhD2008-003
PI/ICF Amendment Number:	Amendment 6
Investigator:	Maria Markoulli
Contact Number:	Work Hours: 02-9385 7612 or After Hours: 0419 810 121

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 and the Institute for Eye Research.

_____ _____

Signature *Date*

_____

Please PRINT Name

Please send this Revocation of Consent to:

Ms Maria Markoulli
Institute for Eye Research
Level 5, North Wing
Rupert Myers Building
KENSINGTON NSW 2033
Australia

ADAPTATION STUDY: EXTENDED WEAR (STUDY 2)

Approval No. 10/09

THE UNIVERSITY OF NEW SOUTH WALES AND THE BRIEN HOLDEN VISION INSTITUTE

POSTGRADUATE STUDENT WORK IN THE BRIEN HOLDEN VISION INSTITUTE CLINICAL RESEARCH AND TRIALS CENTRE

PARTICIPANT INFORMATION STATEMENT AND CONSENT FORM

A CLINICAL STUDY OF THE DIURNAL VARIATION OF THE TEAR PROTEOME DURING THE ADAPTATION TO CONTACT LENSES IN THE EXTENDED WEAR SCHEDULE

Protocol Title:	A clinical study of the diurnal variation of the tear proteome during the adaptation to contact lenses in the extended wear schedule
Protocol Number:	PhD2010-002
PI/ICF Amendment Number:	1
Investigator:	Maria Markoulli
Contact Number:	Work Hours: 0419 810 121 or After Hours: 0419 810 121

PARTICIPANT SELECTION AND PURPOSE OF STUDY

You are invited to participate in a study looking at how the tears of the eye vary over 24 hours and how these vary with contact lenses when worn on an extended wear basis for 1 month. We hope to establish the levels of various components in the tears over a 24 hour period both with and without contact lens wear. We further hope to determine whether contact lens wear on an extended wear basis influences this cycle. You were selected as a possible participant in this study because you meet the suitability criteria of not having worn contact lenses in the previous month, not being a habitual contact lens wearer and having normal ocular health.

DESCRIPTION OF STUDY AND RISKS

If you decide to participate, we will perform a baseline examination where your spectacle prescription will be measured and your ocular health will be examined. A small volume of your tears (approximately one drop) will also be collected as described below. This sample will be analysed for specific components that may be affected during contact lens wear, as part of scientific research. If you are suitable for the study, you will be asked to return in the evening in order to stay in the Brien Holden Vision Institute (Brien Holden Vision Institute) clinic overnight. Your tears will then be collected first thing upon awakening. This will be repeated once more prior to the contact lens fitting visit.

You will then be scheduled for the next series of visits where you will be fitted with O_2OPTIX™ contact lenses which are commercially available. You will be asked to wear these on an extended wear basis (that is, overnight) for 30 nights (for a minimum of 6 days and 5 nights per week). Tears will again be collected as follows:

- before lens insertion
- after lens insertion
- upon awakening

A 1 week visit will be scheduled as a safety check. You will then be asked to return for the following tear collection schedule after 1 month of contact lens wear as follows:

- midday visit
- upon awakening.

In total, there will be four overnight visits at the Brien Holden Vision Institute clinic. The visit schedule is summarised below:

Visit Type	Abbreviation
Baseline visit 1 – Midday, no contact lenses	V1
Baseline visit 1 – Upon awakening – no contact lenses	V2
Baseline visit 2 – Midday, no contact lenses	V3
Baseline visit 2 – Upon awakening – no contact lenses	V4
Contact lens insertion – Midday visit	V5
Contact lens insertion – Upon awakening	V6
1 week – contact lens wear	V7
1 month contact lens wear – Midday visit	V8
1 month contact lens wear – Upon awakening	V9

PROCEDURES

The procedures to be performed are outlined below:

- **Auto-Keratometry:** Automated measurement of the curvature of the eyes. Measured with an auto-refractor / keratometer.
- **Auto-Refraction:** Automated measurement of the spectacle lens prescription required to correct vision. Measured with an auto-refractor / keratometer.
- **Intraocular Pressure:** The measurement of the pressure of the eye using a non-contact probe known as a "tonometer". The non-contact tonometer measures the pressure by applying a quick, gentle puff of air onto the ocular surface.
- **Refraction:** Measurement of the spectacle lens prescription required to correct vision. Measured using a phoroptor and/or trial frame and trial lenses.
- **Visual Acuity:** Measurement of the standard of vision achieved with spectacles or contact lenses using standard letter charts. Measurements may be taken with each eye individually or with both eyes.
- **Over-Refraction:** After insertion of the contact lens, the need for lens power alteration is determined by placing a spectacle lens in front of the eye and asking for your feedback. The lens may be placed using trial lenses and a trial frame, or a phoroptor.
- **Slit-Lamp Biomicroscopy:** Contact lens performance and the ocular surface are examined with a specialised microscope called a slit-lamp. The microscope allows the eye to be viewed with white light or a blue light. A non-contact lens may be used with this to assess the back of the eye at the baseline visit.

- **Fluorescein Assessment:** The ocular surface is assessed by instilling a harmless fluorescent dye called “fluorescein”. The dye does not feel uncomfortable and is temporary.
- **Lid Eversion:** The upper eye lid is folded back using a cotton bud to allow examination of the under surface.
- **Fundoscopy**: Also known as “ophthalmoscopy”, this is the assessment of the health of the retina (the back of the eye) using either a hand held light known as a direct ophthalmoscope, or fundoscopy, a lens held in front of the eye during slit-lamp biomicroscopy. This will be conducted at baseline 1 as part of a complete eye exam.
- **Questionnaire:** Questions assessing comfort, vision, ease of use, handling, problems encountered and satisfaction may be asked verbally at your appointment.
- **Aseptic Lens Removal:** Lens removal is performed using sterile gloves (Ansell, Examtech) so that normal bacteria from the skin do not contaminate the lens. Care is taken to avoid touching the eyelids and eyelashes with the glove whilst removing the lens. Once the lens is removed it is placed directly into a sterile container. Both the saline in the container and the lenses are analysed for bacteria.
- **Tear Samples:** Small volumes of tears, approximately one drop, are gently drawn into a fine glass tube placed at the edge of the lower eyelid. A drop of unit dose sterile saline (AstraZeneca) may be instilled in your eye prior to tear collection to make the procedure easier.
- **Adverse Response Protocol:** In the event of a problem occurring during lens wear, swab samples may be collected. These are obtained by lightly brushing the eye and lower eyelid to determine the normal levels of bacteria present in the eye. These procedures will be explained to you in detail.

All clinic visits are attended at the Brien Holden Vision Institute Clinic, Level 5, North Wing, Rupert Myers Building, Kensington NSW 2033.

RISKS AND PRECAUTIONS

This study involves research, which means that with the products or treatments provided to you there may be risks or consequences that are not currently known or are not foreseeable.

Slight irritation, redness, dryness or watering of the eyes may occur during tear collection.

All lens products have the potential to cause side effects in some individuals. The most serious complication is corneal infection. This is a rare disease (1/500 per year if wearing lenses during sleep and 1/2,500 per year with daily wear). The possible consequences of such an event are severe pain, hospitalisation, corneal scarring and possible loss of vision. Smoking also increases the risk in contact lens wearers.

Other complications which may occur are: inflammation of the eye - such as acute red eye and sterile ulcers; growth of blood vessels into the cornea; temporary decreased vision and/or iritis (inflammation of the colour part of the eye); pain; abrasion

(scratching) of the eye; burning or itching (irritation); excessive watering (tearing) of the eyes; unusual eye secretions; redness of eyes; reduced sharpness of vision; sensitivity to light; and/or dryness of eyes.

The disinfecting solution, AOSept® Plus, contains 3% hydrogen peroxide, so it is not to be used directly in the eye or for rinsing lenses prior to inserting into the eye as the solution must be neutralised. The solution must be used with the dedicated lens case with neutralising disc and lenses must be soaked for at least 6 hours before inserting on the eye. A burning sensation will be experienced if unneutralised AOSept® Plus comes into contact with the eye.

If you experience any of the above, you should remove your lenses and place in the study solution, AOSept® Plus, in the dedicated lens case and contact the clinic immediately.

If you are pregnant or are planning to become pregnant and have additional questions, please ask the Optometrist.

BENEFITS

Your optometric care, contact lenses and solutions will be provided free-of-charge during the study period. Contact lenses offer improved peripheral (side) vision and the convenience of not wearing spectacles. However, we cannot and do not guarantee or promise that you will receive any benefits from this study. Information learned in this study may benefit others in the future.
Two movie vouchers will be given to you as recognition of your time.

ALTERNATIVE TREATMENTS

Alternative methods of visual correction and contact lens care other than those used in this study are available. These include currently marketed contact lenses, refractive surgery, and spectacles. Your study Optometrist will discuss the risks and benefits of these alternate treatments. However, should these be required, you must purchase them at your own expense from a source other than the Brien Holden Vision Institute and in consultation with your primary eye health care professional.

SAFEGUARD

For your safety, you must tell the Optometrist all of your past and present health history; all prescription, over-the-counter, and herbal medications/treatments that you are presently using; if you have any known allergies (for example an allergy to latex) or if you have had any previous reactions to medications; and if you are currently participating 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:

- Use only of lenses and solutions provided by the Brien Holden Vision Institute for the assigned duration and mode of wear (i.e. overnight wear);

- Attendance for scheduled visits;
- Reporting of all changes that occur to your health, medications and, in particular, your eyes;
- The return of all lenses and unopened cases and solutions, when deemed necessary by the optometrist; the return of any foils for used lenses;
- To be confident in inserting and removing your own lenses.

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, VIHEC Secretariat, Level 4, Rupert Myers Building, Gate 14 Barker Street, Kensington NSW 2033 SYDNEY 2052 AUSTRALIA. Any complaint you make will be investigated promptly and you will be informed of the outcome.

FEEDBACK TO PARTICIPANTS

Your study Optometrist will inform you of any significant new findings about the study products and procedures that might develop during the course of this research and that might affect your willingness to continue to participate in the study.

YOUR CONSENT

Your decision whether or not to participate will not prejudice your future relations with the University of New South Wales and the Brien Holden Vision Institute. 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 questions, please feel free to ask us. If you have any additional questions later, your Maria Markoulli (phone number 0419 810 121), will be happy to answer them.

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

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 Investigator*
_____	_____
(Please PRINT name)	*(Please PRINT name)*
_____	_____
Date	*Date*

THE UNIVERSITY OF NEW SOUTH WALES AND THE BRIEN HOLDEN VISION INSTITUTE

POSTGRADUATE STUDENT WORK IN THE BRIEN HOLDEN VISION INSTITUTE CLINICAL RESEARCH AND TRIALS CENTRE

PARTICIPANT INFORMATION STATEMENT AND CONSENT FORM

A CLINICAL STUDY OF THE DIURNAL VARIATION OF THE TEAR PROTEOME DURING THE ADAPTATION TO CONTACT LENSES IN THE EXTENDED WEAR SCHEDULE

Protocol Title:	A clinical study of the diurnal variation of the tear proteome during the adaptation to contact lenses in the extended wear schedule
Protocol Number:	PhD2008-002
PI/ICF Amendment Number:	1
Investigator:	Maria Markoulli
Contact Number:	Work Hours: 0419 810 121 or After Hours: 0419 810 121

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 and the Brien Holden Vision Institute.

_____ _____

Signature *Date*

_____

Please PRINT Name

Please send this Revocation of Consent to:

Maria Markoulli
Brien Holden Vision Institute
Level 5, North Wing
Rupert Myers Building
KENSINGTON NSW 2033
Australia

APPENDIX B: PUBLICATIONS AND PRESENTATIONS

PUBLICATIONS

- **Markoulli M,** Papas E, Nerida Cole, Holden BA, The diurnal variation of matrix metalloproteinase-9 and its associated factors in human tears, accepted for publication in *Investigative Ophthalmology & Vision Science,* 29th January 2012
- **Markoulli M,** Papas E, Nerida Cole, Holden BA, Differential Gel Electrophoresis of the Tear Proteome, accepted for publication in *Optometry & Vision Science,* 25th January 2012
- **Markoulli M,** Papas E, Petznick A, Holden BA, Corneal erosions in contact lens wear, *Contact Lens & Anterior Eye,* 2012, 35:2-8
- **Markoulli M,** Francis IC, Yong J, Jalbert I, Carnt N, Cole N, Papas E**,** A histopathological study of bulbar conjunctival flaps occurring in two contact lens wearers, *Cornea,* 2011, 30:1037-1041
- **Markoulli M,** Papas E, Petznick A, Holden BA, Validation of the flush method as an alternative to basal or reflex tear collection, *Current Eye Research,* 2011, 36:198-207
- Petznick A, Evans MDM, Madigan MC, **Markoulli M,** Garrett Q, Sweeney DF, A comparison of basal and eye-flush tears for the analysis of cat tear proteins, *Acta Ophthalmologica,* 2011, 89:e75-e81
- Sankaridurg P, **Markoulli M,** Lazon de la Jara P, Harmis N, Holden BA, Lid and conjunctival microbiota in children with contact lens wear, *Optometry & Vision Science,* 2009, 86:312-317
- Invited article written for the Australian *Contact Lenses Magazine* on "Corneal erosions – why do they happen and what can we do?" 2009
- Herse P, Hans A, Hill J, Langejans J, **Markoulli M**, The Proview Eye Pressure Monitor: influence of clinical factors on accuracy and agreement with the Goldmann Tonometer, *Ophthalmic & Physiological Optics*, 2005, 25:416-420

ORAL PRESENTATIONS

May 2011 — British Contact Lens Association, Manchester, UK
Paper: **Maria Markoulli,** Eric Papas, Nerida Cole, Brien Holden
From a neophyte to an experienced wearer – how adaptation to contact lens wear changes the composition of the tear film

May 2011 — Southern Regional Congress, Melbourne, Australia
Invited presentation: *Therapeutic applications of soft contact lenses*

November 2010 — Cornea and Contact Lens Society of Australia, Melbourne, Australia
Invited presentation: *How does the ocular surface change during contact lens adaptation?*

August 2010 — The University of New South Wales Faculty of Science competition, Sydney, Australia
Presentation: *Contact Lenses: A friend or a foe?*

May 2010 — British Contact Lens Association, Birmingham, UK
Paper: **Maria Markoulli,** Eric Papas, Brien Holden
Diurnal variation of matrix metalloproteinase-9 in the tear film and its association with clinical signs

November 2009 — Cornea and Contact Lens Society of Australia, Perth, Australia
Invited presentation: *Corneal erosions: Known causes and current treatments*

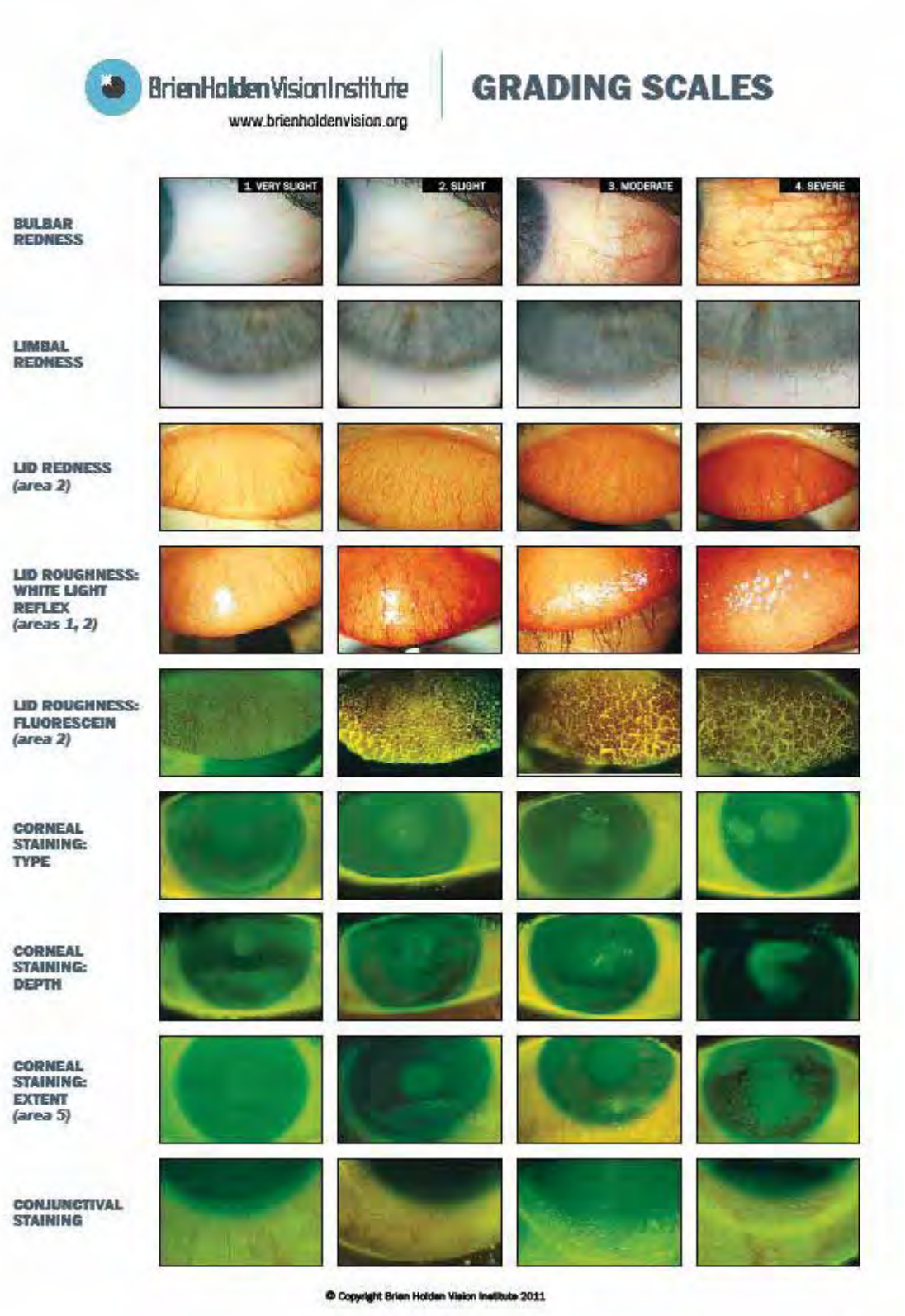
October 2009 — Optometrists' Association Australia, Sydney, Australia
Presentation: *Corneal erosions – Why do they happen and what can we do?*

August 2009: — Dry Eye Conference, Sydney, Australia
Presentation: *Proteins and the ocular surface*

POSTER PRESENTATIONS

May 2011	Association for Research in Vision and Ophthalmology, Florida, USA **Maria Markoulli,** Eric Papas, Nerida Cole, Brien Holden *The adaptation of the tear proteome to extended wear contact lenses*
August 2010	The University of New South Wales Faculty of Science competition, Sydney, Australia *Contact Lenses: A friend or a foe?*
September 2010	Tear Film and Ocular Surface Society, Florence, Italy **Maria Markoulli,** Eric Papas, Nerida Cole, Brien Holden *The effect of contact lens wear on the diurnal profile of matrix metalloproteinase-9 and its inhibitor in the tear film*
July 2010	International Society of Eye Research, Montreal, Canada **Markoulli M,** Papas E, Petznick A, Holden BA *Validation of the flush method as an alternative to basal or reflex tear collection*
May 2010	Association for Research in Vision and Ophthalmology, Florida, USA **Maria Markoulli,** Eric Papas, Brien Holden *Diurnal Variation of MMP-9 in the tear film*

APPENDIX C: BRIEN HOLDEN VISION INSTITUTE GRADING SCALES



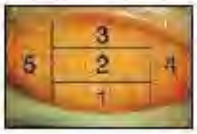
Brien Holden Vision Institute
www.brienholdenvision.org

GRADING SCALES

APPLICATION OF GRADING SCALES

- Patient management is based on how much the normal ocular appearance has changed.
- In general, a rating of slight (grade 2) or less is considered within normal limits (except staining).
- A change of one grade or more at follow up visits is considered clinically significant.

PALPEBRAL CONJUNCTIVAL GRADES



- The palpebral conjunctiva is divided into five areas to grade redness and roughness.
- Areas 1, 2 and 3 are most relevant in contact lens wear.

ADVERSE EFFECTS WITH CONTACT LENSES

CLPC CONTACT LENS PAPILLARY CONJUNCTIVITIS

Inflammation of the upper palpebral conjunctiva

Signs
- Redness
- Enlarged papillae
- Excess mucus

Symptoms
- Itchiness
- Mucus strands
- Lens mislocation
- Intolerance to lenses

INFILTRATES

Accumulation of inflammatory cells in corneal sub-epithelial stroma. Inset: high magnification view

Signs
- Whitish opacity (focal) or grey haze (diffuse)
- Usually confined to 2-3mm from limbus
- Localized redness

Symptoms
- Asymptomatic or scratchy, foreign body sensation
- Redness, tearing and photophobia possible

CLARE CONTACT LENS MORNING RED EYE

An acute corneal inflammatory episode associated with sleeping in soft contact lenses

Signs
- Unilateral
- Intense redness
- Infiltrates
- No epithelial break

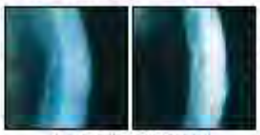
Symptoms
- Wakes with irritation or pain
- Photophobia
- Lacrimation

POLYMEGETHISM

VASCULARIZATION

Vessel extension beyond translucent limbal zone is recorded (mm)

STROMAL STRIAE and FOLDS

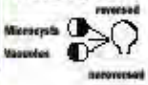


One striae = 5% edema
One fold = 8% edema
(each additional striae or fold indicates 1% more edema)

Record number observed

MICROCYSTS and VACUOLES

Located in epithelium. Identified by side showing brightness.



Record number observed

CORNEAL STAINING GRADES

- Staining assessed immediately after single instillation of fluorescein using cobalt blue light and wratten 12 (yellow) filter over the slit lamp objective.
- The cornea is divided into five areas. The type, extent and depth of staining are graded in each area.

Type
1 Micropunctate
2 Macropunctate
3 Coalescent macropunctate
4 Patch

Extent Description
1 ≤5%
2 6-15%
3 16-30%
4 >30%

Depth Description*
1 Superficial epithelium
2 Deep epithelium, delayed stromal glow
3 Immediate localized stromal glow
4 Immediate diffuse stromal glow

* Based on penetration of fluorescein and slit lamp optic section

EROSION

Full thickness epithelial loss over a discrete area

Signs
- No stromal inflammation
- Immediate spread of fluorescein into stroma

Symptoms
- Can be painful
- Photophobia
- Lacrimation

CLPU CONTACT LENS PERIPHERAL ULCER

Round, full thickness epithelial loss with inflamed base, typically in the corneal periphery which results in a scar. Insets: with fluorescein, scar

Signs
- Unilateral, "white spot"
- Localized redness
- Infiltrates
- Post healing scar

Symptoms
- Varies from foreign body sensation to pain
- Lacrimation and photophobia may occur

INFECTED ULCER

Full thickness epithelial loss with stromal necrosis and inflammation, typically central or paracentral

Signs
- Intense redness
- "White patch" (raised edges)
- Infiltrates
- Epithelial and stromal loss
- Anterior chamber flare
- Conjunctival and lid edema

Symptoms
- Pain, photophobia
- Redness, mucoid discharge
- ↓ VA (if over pupil)