The Effects of Eye Cosmetics on the Ocular Surface and Tear Film

A thesis submitted to Cardiff University for the degree of Doctor of Philosophy

Alison Yuk San Ng

School of Optometry and Vision Sciences

Cardiff University

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Supervisors:

Professor Christine Purslow, Dr Katharine Evans and Professor Rachel North

DECLARATION

This work has not been submitted in substance for any other degree or award at this or any other university or place of learning, nor is being submitted concurrently in candidature for any degree or other award.

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Abstract

Eye cosmetics usage in the UK is commonplace. Despite its popularity, there is a lack of published literature exploring the ocular effects of eye cosmetic usage. The influence of eye cosmetics upon symptoms of dry eye and contact lens discomfort has been suggested but these links have not been established. Consequently, this thesis aims to establish any link between ocular comfort with eye cosmetic usage. This was achieved by conducting a survey which showed the use of eye cosmetics, particularly eyeliner, significantly reduced ocular comfort. Amongst cosmetic users, contact lens wearers did not experience significantly reduced ocular comfort compared to non-contact lens wearers.

Eye care practitioners often report observations of eye cosmetics contaminating the tear film, even when the products are applied to peri-ocular skin, although these reports are anecdotal. This thesis demonstrates that cosmetic pencil eyeliner migrates most readily and maximally contaminates the tear film when applied along the inner lid margin. After two hours post-application, contamination of the tear film from pencil eyeliner was negligible.

This finding led to a subsequent study which examined the clinical and immunological responses of the ocular surface following migration of cosmetic pencil eyeliner. Clinically, eyeliner pencil did not appear to induce signs of ocular surface inflammation. However seven consecutive days of eyeliner application along the inner lid margin increased lipid layer thickness and dry eye symptoms compared to when the eyeliner was applied to peri-ocular eyelid skin. Subclinical signs of ocular surface inflammation were investigated by examining the concentration of inflammatory cytokines, IL-6 and IL-8, in tear fluid. A small reduction of cytokine concentration was exhibited after one day of eyeliner use although concentrations returned to near baseline levels after seven days of use. In conclusion, pencil eyeliner is safe to use and does not appear to induce clinical or subclinical signs of inflammation when used for up to seven days consecutively. The causes of increased dry eye symptoms are undetermined and the longer term effects of eyeliner application remain unknown.

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Who would have thought that I would be writing my acknowledgments on a cold, damp, Saturday afternoon with a mug of tea and a bagel? If writing this thesis has taught me nothing else, it has taught me how to make a fantastic cup of tea and how to procrastinate. Anyhow, I digress.

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"When you are a Bear of Very Little Brain, and you Think of Things, you find sometimes that a Thing which seemed very Thingish inside you is quite different when it gets out into the open and has other people looking at it"

- A.A. Milne, Winnie the Pooh

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List of Abbreviations

↑	Increase
ACLW	Adapted contact lens wear
ADDE	Aqueous deficient dry eye
BAC	Benzalkonium chloride
CL	Contact lens(es)
CLARE	Contact Lens Associated Red Eye
CLPU	Contact lens peripheral ulcer
CLW	Contact lens wear
CN	Cranial nerve
CRT	Corneal refractive therapy
CV	Coefficient of variation
DE	Dry eye
DED	Dry eye disease
DTS	Dysfunctional tear syndrome
ECL	Electrochemiluminescent
EDE	Evaporative dry eye
EL	Eyeliner
ELI	Eyeliner applied along the mucocutaneous junction
ELISA	Enzyme linked immunosorbent assay
ELO	Eyeliner applied on peri-ocular skin
EU	European Union
EW-RGP	Extended wear rigid gas permeable
EW-SiH	Extended wear silicone hydrogel
FLN	Sodium fluorescein
ICC	Intraclass correlation
IFN-γ	Interferon-γ
Ig	Immunoglobulin
IL	Interleukin
LCMS	Liquid chromatography mass spectrometry
LE	Left eye
LG	Lissamine green
LLT	Lipid layer thickness
MALT	Mucosal-associated lymphoid tissue

MCJ	Mucocutaneous junction
MG	Meibomian gland
MGD	Meibomian gland dysfunction
MS	Mass spectrometry
MU	Cosmetic users
NACLW	Non-adapted contact lens wear
NCLW	Non-contact lens wear
NITBUT	Non-invasive tear break-up time
NMU	Non-cosmetic users
OCT	Optical coherence tomography
OD	Optical density
OS	Ocular surface
OSDI	Ocular Surface Disease Index
PBS	Phosphate buffered saline
PCA	Principal component analysis
PMN	Polymorphonuclear leukocyte
RB	Rose bengal
RE	Right eye
RLU	Relative light units
rpm	Revolutions per minute
SCCS	Scientific Committee on Consumer Safety
SD	Standard deviation
TBUT	Tear break-up time
TF	Tear film
Th	T-helper
TLC	Thin layer chromatography
TNF-α	Tumour necrosis factor-α
TTT	Tear thinning time
UK	United Kingdom
UV	Ultra-violet

1   Literature Review: Introduction to ocular surface anatomy and eye cosmetics

1.1   Introduction

The use of eye cosmetics has been documented from as early as 4000BC (Draelos, 2001). The use of kohl in African, Middle Eastern and South Asian tribes and communities held a spiritual role to ward off evil spirits as it was considered that they entered through vulnerable openings such as the eyes (Mahmood *et al.*, 2009).

In Western society, women and men judge eye cosmetic use as a factor in facial attractiveness (Mulhern *et al.*, 2003). The European cosmetics market is valued at over €72billion and is the largest producer and consumer of cosmetics (Colipa, 2012). Market research by Mintel report 70% of women use eye cosmetics (Mintel, 2012) and found a 38% increase in eye cosmetic sales since 2004 (Mintel, 2010). There is an increasing trend of teenagers and under-13 year olds electing to use eye cosmetics (Mintel, 2009). Similarly, market research has indicated that one of the highest users of eye cosmetics is amongst women in fulltime employment, and with retirement ages rising, the number of cosmetic users is set to increase within an older age group (Mintel, 2013).

Despite the high prevalence of usage, there is little published data which explores the clinical signs and ocular effects of eye cosmetics. Dry eye symptoms arising from extrinsic factors such as the use of contact lenses are well known (Lemp *et al.*, 2007). While the influence of regular eye cosmetic use upon dry eye symptoms has been suggested (Guillon and Maissa, 2005; Wolkoff, 2008), it has not been established.

This thesis sets out to investigate the prevalence of eye cosmetic usage in a UK population and examine the impact cosmetic usage has upon measures of ocular comfort. Following this, the thesis will explore the pattern of contamination of tear film when eye cosmetics are applied with close proximity to the ocular surface. Lastly, this thesis investigates the short- and long-term clinical and biochemical impact of using cosmetic pencil eyeliner.

1.2   Introduction to ocular surface anatomy: structure and function

Maintenance of a stable, regular precorneal tear film is paramount for optimum retinal image quality (Rieger, 1992; Rolando *et al.*, 1998; Liu *et al.*, 2010) in addition to ocular surface health. This is achieved by the combined production, spreading and drainage of an optimal tear fluid.

The concept of the "lacrimal functional unit" was devised to illustrate the relationship between the ocular surface, tear film and lacrimal glands in healthy individuals and during inflammatory events arising from dry eye conditions (Stern *et al.*, 2004b). The lacrimal functional unit comprises of the ocular surface (including the cornea, conjunctiva, meibomian glands), the main and accessory lacrimal glands and the interconnecting neural innervation (Beurerman *et al.*, 2004).

1.2.1   The Conjunctiva

The conjunctiva is the most superficial mucous membrane of the eye, continuous with the epidermis of the skin at the lid margin and corneal epithelium at the limbus. It can be divided into three regions: palpebral, bulbar and fornical conjunctivae (Figure 1.1).
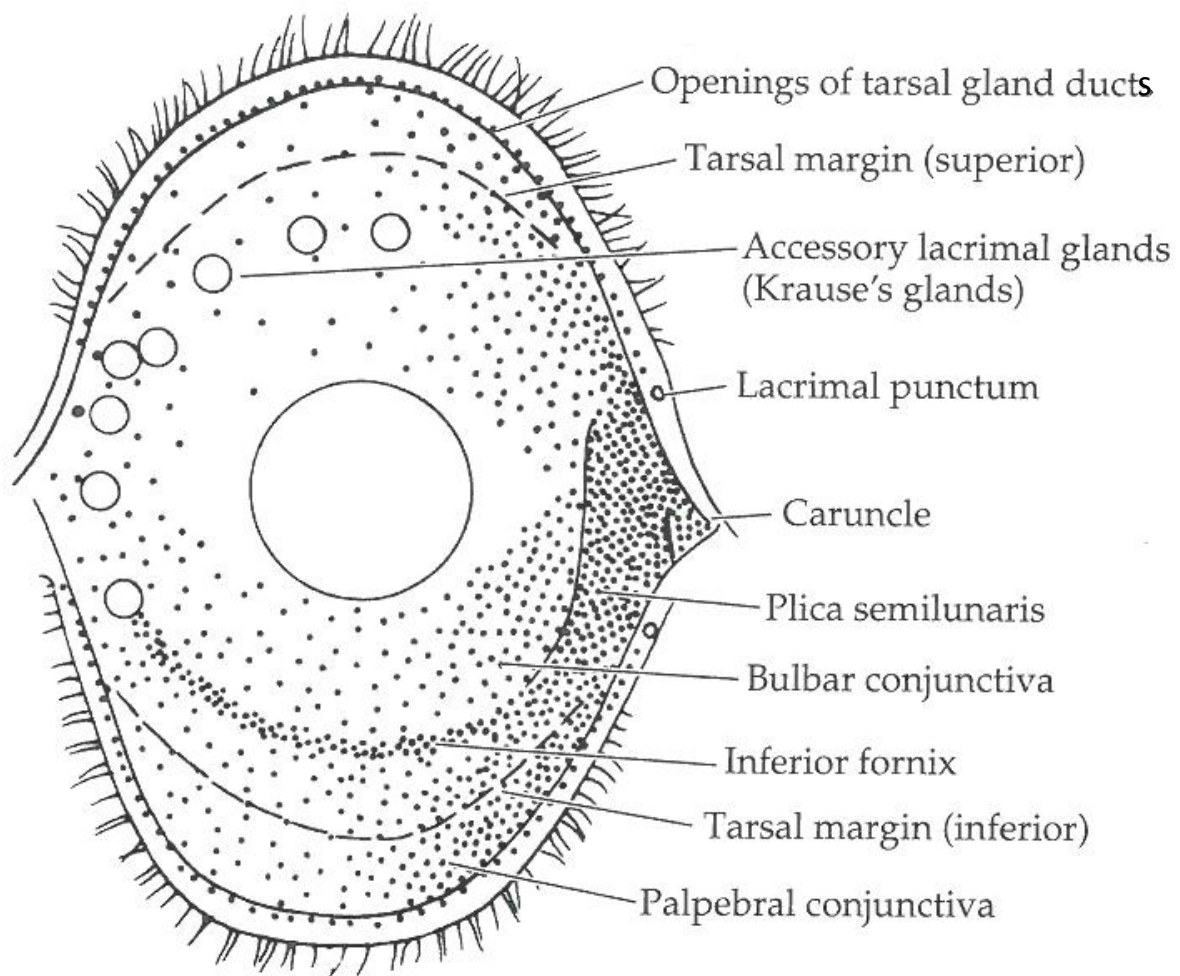
Figure 1.1 Conjunctival anatomy illustrating the regions of the conjunctiva and associated glands, where the small black dots represent mucin-secreting goblet cells (Oyster, 1999b)

There are three principal functions of the conjunctiva:

- Goblet cells, in the conjunctival epithelium, secrete gel mucin into the tear film to lubricate the ocular surface and to alleviate any mechanical friction between the interaction of the eyelids and the palpebral conjunctiva with the blinking mechanism

- Immunocompetent cells are found in the conjunctival epithelium and lamina propria, providing an immunological defence mechanism for the ocular surface (Kikkawa *et al.*, 2003)

- The conjunctiva contains accessory lacrimal glands, the glands of Krause and Wolfring, which maintains homeostatic balance of the tear film by the absorption and secretion of water and electrolytes (Dartt, 2002).

The conjunctival epithelium is two to three cell layers thick (Kikkawa *et al.*, 2003) and overlies a basement membrane which separates conjunctival vasculature. The surfaces of the cells comprising the epithelial layer have multiple microvilli and microplicae to enable the mucous layer of the tear film to adhere and improve the wettability and smoothness of the ocular surface. The conjunctiva forms a main part of the ocular surface immunity. The conjunctival epithelium utilises pattern-recognition receptors which readily identify and respond to microbial antigens which may make contact (Knop and Knop, 2011). Conjunctival epithelial cells secrete antimicrobial proteins in addition to soluble inflammatory mediators to induce inflammation. The conjunctiva is classified as mucosal-associated lymphoid tissue (MALT) as lymphoid tissue is abundant in the lamina propria, the loose connective tissue beneath the conjunctival epithelium (Knop and Knop, 2011). This layer of the conjunctiva contains the vascular network and immune lymphoid tissue, consisting of lymphocytes and accessory leukocyte populations (plasma cells, macrophages, granulocytes, mast cells and dendritic cells). These cells produce and secrete a range of immunoglobulins which hold key anti-inflammatory roles, preventing infection (Knop and Knop, 2011).

There is some controversy regarding the source of conjunctival stem cells. Some evidence points to conjunctival stem cells located in the fornix (Lavker and Sun, 2003) while other research has found them to be scattered in foci throughout the conjunctiva (Pellegrini *et al.*, 1999). The mucocutaneous junction has also been identified as a source of conjunctival stem cells by several other investigators (Pe'er *et al.*, 1996; Wirtschafter *et al.*, 1999; Kikkawa *et al.*, 2003). Conjunctival epithelial stem cells are thought to be bipotent – the differentiation from a conjunctival keratinocyte to conjunctival goblet cell arises from a common progenitor (Pellegrini *et al.*, 1999)

1.2.2   The Cornea

Since the cornea accounts for two thirds of the refractive power of the eye, retinal image quality depends on the clarity and regularity of this transparent, avascular tissue. The cornea is made up of five distinctive layers.

Epithelium — Collagen types IV, VII, IX, XVI
Fibronectin
Heparan sulfate

Bowman's membrane — Proteoglycans:
Keratan sulfate
Dermatan sulfate/Chondroitin sulfate

Stroma — Collagen:
Types I and VI (major)
Types III and V (minor)

Keratocyte

Descemet's membrane
Endothelium — Collagen types IV and VIII
Laminin
Fibronectin
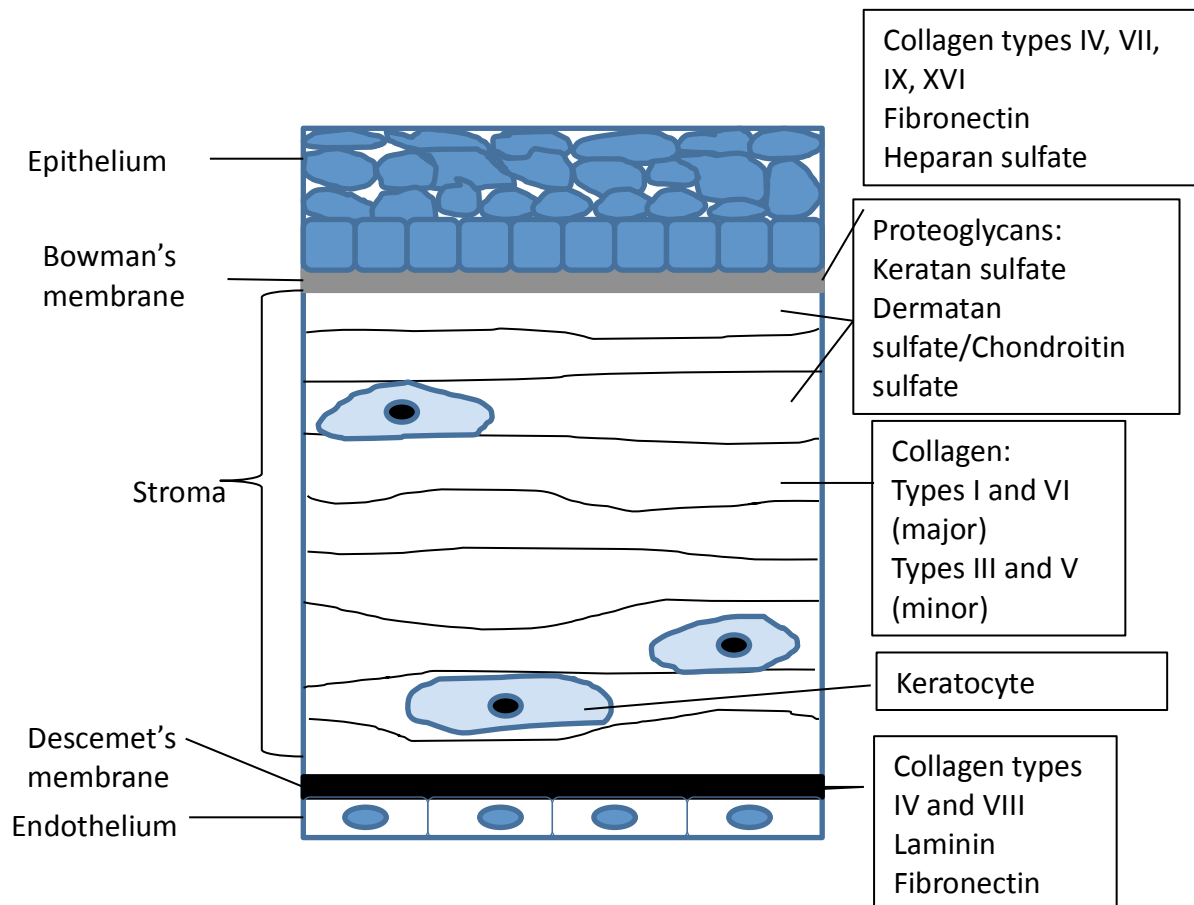
Figure 1.2 Diagram illustrating corneal layers & the different proteins within each layer (not drawn to scale). Adapted from (Forrester *et al.*, 2008)

1.2.2.1   Corneal epithelium

The corneal epithelium is continuous with the conjunctival epithelium at the limbus and represents less than 10% of total corneal thickness. The epithelium is approximately five to six cells thick (~60μm) increasing to 10 cells thick at the peripheral corneoscleral junction

(Snell and Lemp, 1998). The epithelial layer prevents foreign material entering the deeper corneal layers and also acts as a smooth, refractile surface.

Like conjunctival epithelial cells, the uppermost superficial two to three layers of non-keratinised squamous cells are covered with fine microvilli and microplicae. These structures assist with attaching the tear film to the cornea in addition to maximising surface area contact with the tear film. The most superficial layer of the non-keratinised squamous cells are attached to each other by tight junctions, restricting the penetration of small molecules, ions and water into deeper corneal layers. Cell morphology changes through the deeper epithelial layers, changing from wing cells in the suprabasal layer to tall columnar cells in the basal layer to which Bowman's layer is attached. The basal cells of the epithelial layer are derived from limbal stem cells and are responsible for generation of new epithelial cells which migrate to the corneal surface.

The epithelium undergoes a constant cycle of shedding and regeneration of the superficial layer. Complete turnover takes between 7-10 days. Basal cells in the limbal epithelium undergo mitosis to produce daughter cells known as transient amplifying cells (TAC). These cells migrate centripetally to the basal layer of the corneal epithelium where they divide and begin to move anteriorly, differentiating as they migrate. Differentiated cells are shed from the superficial corneal surface and so the cycle of shedding and renewal continues. The proliferation of basal cells and centripetal migration of daughter cells is equal to the loss of cells from the corneal surface (Thoft and Friend, 1983).

1.2.2.2   *Bowman's membrane*

Bowman's membrane is a tough, 12μm thick acellular basement membrane of interwoven collagen fibrils and proteoglycans which protects the stroma beneath. Penetration through Bowman's membrane results in irreversible damage to the regularly arranged collagen fibres of the stroma, resulting in corneal scarring.

1.2.2.3   *Stroma*

The stroma accounts for 90% of total corneal thickness and is largely comprised of collagen fibrils (Types I, III and V) and glycosaminoglycans (GAGs). Collagen accounts for 70% of the weight of a dry cornea (Nishida, 1997). 2-3% of corneal stroma consists of keratocytes which

begin the synthesis of collagen molecules. Collagen molecules arrange themselves into collagen fibrils which rearrange into larger structures of collagen fibres, between 22.5 and 35mm in diameter (Nishida, 1997). Collagen fibril arrangement is in the form of lamellae containing fibrils parallel to each other and to the corneal surface across the entire cornea. 200-300 lamellae are stacked, lying adjacent to each at angles between 0 - 90° provides the cornea with strength and elasticity. Corneal transparency is entirely dependent on the narrow width and regularity of the spacing between collagen fibrils; fluid accumulation within the stroma will disrupt this spacing, degrading transparency (Meek and Boote, 2004).

Interwoven between the collagen fibrils are GAGs which bind to core proteins, forming proteoglycans. GAGs are long chain polysaccharides and hold a negative charge, making them bind readily to positively charged ions such as sodium. This encourages water uptake into the extracellular matrix. Due to their biochemical structure, GAGs are able to absorb and retain large amounts of water. Deturgescence is regulated by the endothelial ion pumps.

1.2.2.4   Descemet's membrane

Descemet's membrane acts as a tough basement membrane for the corneal endothelium and is about 6-12μm thick (increasing with age), located posterior to the stroma. This membrane is made up of Type IV collagen and others, where the fibres are arranged in a regular lattice-like formation, making it structurally tough and elastic and more resistant to corneal perforation (Oyster, 1999a). The spaces between the lattice-formation are filled with fibronectin, a protein which aids the adherence of the membrane to the anterior stroma and posterior endothelium. Descemet's membrane ends abruptly at the corneal margin and becomes continuous with Schwalbe's line in the trabecular tissue within the anterior chamber (Snell and Lemp, 1998).

1.2.2.5   Corneal endothelium

The corneal endothelium is a single layer of cells, approximately 5μm in thickness, responsible for the regulation of fluid and solute levels in the stroma to maintain corneal transparency. The posterior surface of the endothelium is in contact with the aqueous

humour, from which glucose and amino acids move into the cornea to maintain cellular function.

1.2.2.6   Corneal innervation

The cornea is densely innervated and it is estimated that there are between 315 000 and 630,000 nerve endings across the entire corneal surface (Muller *et al.*, 2003). The corneal nerves arise from the ophthalmic division of the trigeminal nerve (cranial nerve (CN) V) by long ciliary nerves which enter the cornea in the deep peripheral stroma. From here, the 2000 nerves move anteriorly and radially towards the centre of the cornea where the some branches form the subepithelial plexus. The nerves continue to move anteriorly, piercing Bowman's layer to form the subbasal epithelial nerve plexus, innervating the basal epithelial cell layer before terminating in the superficial epithelial layers. The nerve fibres lose their myelination immediately as they pass into the cornea.

Each nerve fibre has 100-200 nerve endings in the epithelium which mostly terminate in the superficial epithelial layer. They are protected from direct stimulation by the tight junctions of the epithelial cells and the overlying tear film. Any loss of the superficial epithelial layer exposes nerve endings. Corneal nerve fibres are predominantly polymodal nociceptors which respond to mechanical, thermal and chemical stimuli resulting in the perception of pain (Edelhauser and Ubels, 2003). These fibres have the lowest threshold for mechanical stimulation (Belmonte and Giraldez, 1981) which further indicates their importance in protective mechanism of the cornea.

1.2.3 Eyelids

The eyelids form an integral part of the lacrimal functional unit. The eyelids: protect the ocular surface from foreign objects, spreads the tear film across the ocular surface, and maintains a smooth tear film by blinking to remove debris. Besides their mechanical function, the eyelids also deliver fundamental constituents to the tear film.

Palpebral fissure size depends upon upper and lower tarsal plate positioning, made rigid by tarsal plates. Tarsal plates are made of tough, collagenous connective tissue in which meibomian glands are embedded. A cross section of the upper eyelid is illustrated in Figure 1.3.
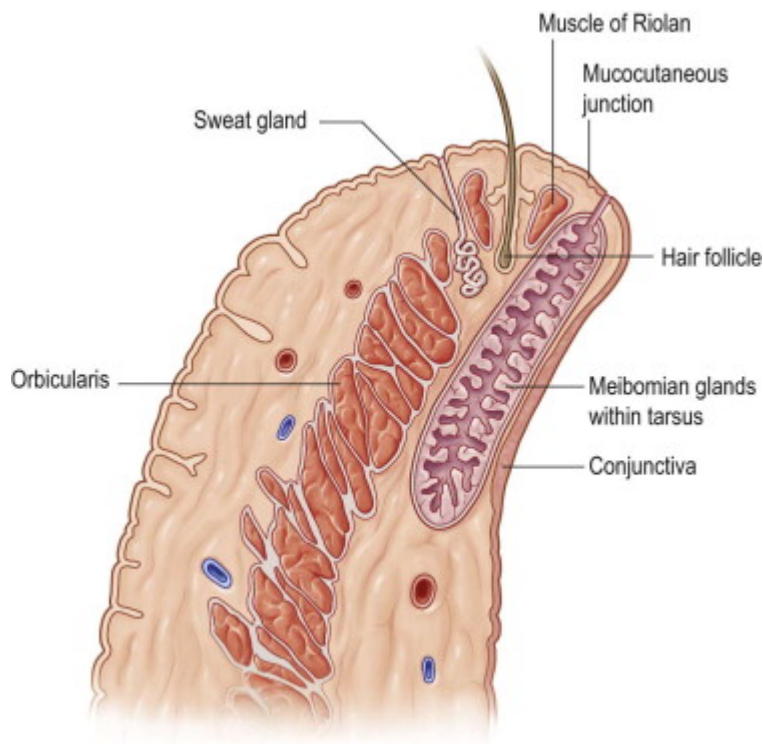
Figure 1.3 Cross section of upper eyelid (from: (Lemke and Lucarelli, 1998)

1.2.3.1 Meibomian glands and accessory glands

Meibomian glands (MGs) secrete meibum which contributes to the lipid phase of the tear film to reduce tear film evaporation. The accessory glands of Zeis are sebaceous glands and the Moll glands are sweat glands (Griepentrog and Lucarelli, 2003). There are 30-40 and 20-25 individual MG present in a single row along the upper and lower eyelid respectively (Bron

et al., 2004), each measuring 1mm wide and 3-12mm in length (Mathers, 2004b). MGs are holocrine glands with multiple grape-like clusters of lipid-synthesising acini which are connected by fine ductules to the main duct (Figure 1.4a). Acini are filled with secretory lipid-forming cells (meibocytes) which, upon maturation, disintegrate at the point of moving from the acinus to the ductile to release meibum (Knop *et al.*, 2011). Acini constantly secrete meibum. During blinking, the orbicularis muscle and the muscles of Riolan within the lids encourage the movement of meibum along the central duct (Figure 1.4b). Blinking also allows the spread of meibum from the inferior tear meniscus across the aqueous tear film with the upward lid movement during the blink phase (Doane, 1980; Ong and Larke, 1990; Foulks, 2007).
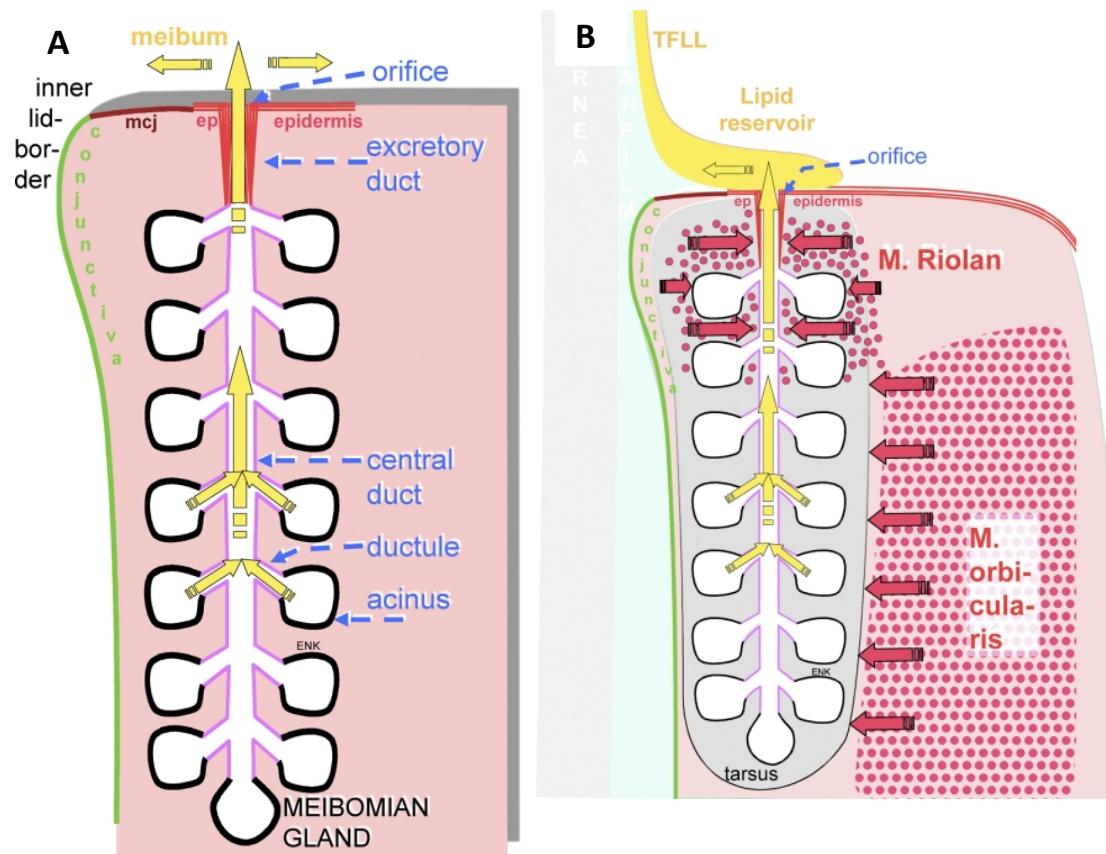
Figure 1.4 (A)Cross section of a meibomian gland and (B) surrounding musculature, the orbicularis muscle and muscle of Riolan. Images from (Knop *et al.*, 2011)

MG secretion is influenced by neuronal and vascular changes (McCulley and Shine, 2004). The influence of hormones on MG function is also well recognised. MG acini have been found to have androgen and oestrogen receptors (Rocha *et al.*, 2000; Wickham *et al.*, 2000) indicating that these hormones play key roles in meibum production and/or composition (McCulley and Shine, 2004). Androgens target acinar epithelial cells, encouraging the production of proteins that increase synthesis and secretion of lipids (Sullivan *et al.*, 2000). Women with Sjögren's syndrome are androgen-deficient which is considered to be a contributing factor in the pathogenesis of evaporative dry eye in these groups of patients (Sullivan *et al.*, 2002). An alteration in lipid composition of meibum and an increase in tear film debris have been documented in cases where human males are taking anti-androgen therapy for prostate cancer (Krenzer *et al.*, 2000; Sullivan *et al.*, 2002) providing further evidence of the role of androgens in MG function.

1.2.4   Main lacrimal gland and accessory lacrimal glands

The lacrimal gland is a tubuloacinar gland, located in the superior temporal angle of the orbit, consists of the orbital lobe and palpebral lobe. The orbital lobe is approximately the size of an almond and is located within the lacrimal fossa of the frontal bone, emptying its contents through 2-8 ducts into the conjunctival sac at the upper temporal fornix. The palpebral lobe is half the size of the orbital lobe and is located beneath the orbital lobe, under the conjunctiva, and has 6-10 excretory ducts (Beurerman *et al.*, 2004). The ducts of the orbital lobe pass through the palpebral lobe thus removal of the palpebral lobe results in a complete lack of lacrimal gland secretion.

Similar to the conjunctiva, the lacrimal glands also form part of MALT family within the human body hence the presence of lymphocytes and other immunocompetent cells such as eosinophilic cells, macrophages and plasma cells in the interstitial tissue. Acinar and ductal epithelial cells of the lacrimal glands secrete water, electrolytes, proteins and mucous into the tear film (Beurerman *et al.*, 2004). The ducts are supplied with a rich capillary network for fluid adsorption.

The main lacrimal gland secretes tears during reflex tearing. Neural innervation of the lacrimal system is served by the trigeminal nerve (CN V) which provides the main afferent pathway, the facial nerve (CN VII) which provides the main efferent pathway, and the

cervical sympathetic nerve fibres. Reflex tear secretion can arise either from stimulation of the cornea, conjunctiva, skin or nose or from central sensory nervous system such as in response retinal stimulation by a bright light (Craig, 2002). Parasympathetic and sympathetic nerves form the efferent part of the reflex arc. VIP-ergic and cholinergic parasympathetic nerves control electrolyte, water and protein secretion and innervate the acinar cells, duct cells, myoepithelial cells and blood vessels. Sympathetic nerves innervate the blood vessels and may have some involvement with the myoepithelial cells (Craig, 2002).

The accessory lacrimal glands of Wolfring and Krause are located in the upper tarsal conjunctiva and the conjunctival fornices respectively. These glands are well innervated by parasympathetic nerves making complete isolation for research more difficult (Lucarelli *et al.*, 2003). In the animal model, secretions from accessory lacrimal glands is sufficient to maintain optimum healthy ocular surface function (Maitchouk *et al.*, 2000).

1.2.5   The tear film

The tear film is a complex, dynamic layer overlying the corneal surface. The tear film lubricates the palpebral and bulbar conjunctival surfaces to maintain ocular comfort in addition to biochemically protecting the ocular surface from infection and disease (Craig, 2002). The interaction of the tear film with the eyelids helps to mechanically flush away foreign particles, cellular debris and allergens. By creating a smooth, regular corneal surface, the optics of the eye and retinal image quality is improved. Insufficient quantity or poor quality of the tear film can result in dry eye, characterised by symptomatic and clinical ocular surface changes (Craig, 2002).

1.2.5.1   Tear film structure

The precorneal tear film, illustrated in Figure 1.5, has classically been described as a trilaminar structure overlying the cornea about 7µm thick, comprising of a lipid layer, aqueous layer and a mucin layer (Wolff, 1946; Holly and Lemp, 1977). The structure is based upon a lipid and mucin layer sandwiching a bulky aqueous layer. However the trilaminar structure has been disputed (Rolando and Zierhut, 2001).
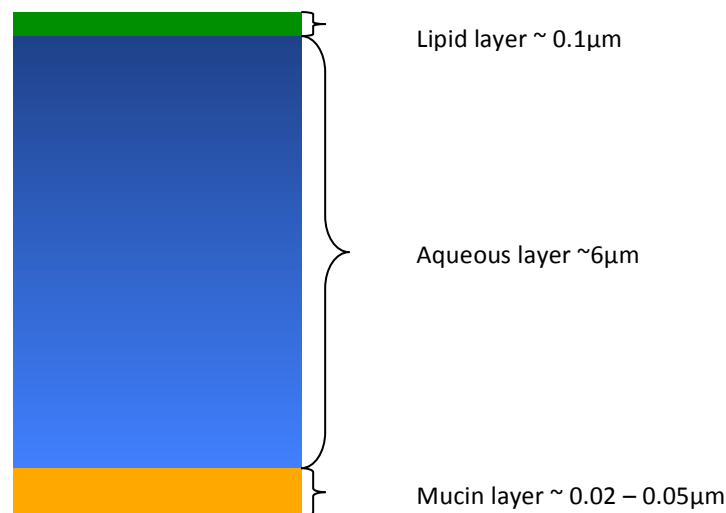
Figure 1.5 Classic trilaminar structure of the precorneal tear film (not drawn to scale)

1.2.5.2 Lipid Layer

The lipid layer is the outermost superficial layer of the tear film. Predominately derived from the MG, it serves an important function in reducing the rate of evaporation of the tear film and maintaining tear film stability. Tear evaporation increases four-fold when the lipid layer is minimal or not confluent (Craig and Tomlinson, 1997), resulting in hyperosmolarity of the tear film causing increased ocular surface damage and inflammation, in addition to symptoms of discomfort (Foulks, 2007; Suzuki *et al.*, 2010).

Meibum secreted by the MG is composed of several lipid classes (Table 1.1). It is liquid at lid temperature (between 33-37°C) (Butovich, Millar and Ham, 2008) and solidifies immediately at room temperature (Butovich, 2009). The melting range of meibum is between 30-34°C and is dependent upon its total composition of individual lipids as each lipid has a different melting point. Melting points can range from 9°C (fatty acids) to 148°C (cholesterol) (Butovich *et al.*, 2008). The application of heat reduces meibum viscosity which aids secretion (Nagymihalyi, Dikstein and Tiffany, 2004). Butovich *et al.* (2010) found changes in temperature had a strong impact on the stability of meibomian films formed from human meibum *in vitro*. The authors proposed that if the corneal temperature drops below 26.4°C (which can occur if ambient temperature is maintained at -20°C), a temperature which is

lower than the melting point of meibum, the functional properties of meibomian films could alter (Butovich, Arciniega and Wojtowicz, 2010).

Table 1.1 Major lipid composition of normal human meibum. Adapted from (Butovich *et al.*, 2008)

Lipid	Range (%)
Wax ester	13 – 68
Sterol ester	8 – 39
Triglycerol	2 – 43
Hydrocarbon	1 – 38
Free fatty acids	0 – 24
Diester	2 – 18
Polar lipids	0 – 16
Alcohols	0 – 5
Cholesterol	0 – 5

There is much interest in comparing the composition of meibum secretions to the tear lipid layer. In normal human tears, it is well established that human meibum and whole tear samples differ in lipid composition, particularly with the proportions of wax esters and phospholipids (Green-Church *et al.*, 2011). Table 1.2 summarises the range of lipids found in the tear lipid layer. The variations in and range in values reported in Table 1.1 and Table 1.2 is likely due to variations in analytical techniques, in addition to natural biological variations. Furthermore, Table 1.1 refers specifically to meibum whereas Table 1.2 refers to tear lipids.

Table 1.2 Lipids detected in human tear fluid, from (Rantamaki *et al.*, 2011)

Lipid class	Mean±SD concentration (µM)	Equivalent % of identified lipids
Phosphatidylcholine (PC)	20.9±10.1	70
Phosphatidylethanolamine (PE)	5.2±0.1	18
Triglyceride (TG) [non-polar]	1.5±0.3	5
Ceramide	0.9±0.2	3
Sphingomyelin	0.9±0.1	3
Phosphatidyl-serine (PS)	0.1±0.0	-

Non-polar esters and sterol esters compose the thick, anterior aspect of the lipid layer in contact with air which minimises aqueous evaporation. This layer contributes 92% lipid layer structure (McCulley and Shine, 2003). A layer of polar lipids, 2-3 molecules thick (Shine and McCulley, 2003) lies between the non-polar lipid layer and aqueous mucin layer with their hydrophobic tails pointing towards the non-polar layer and hydrophilic heads pointing towards the aqueous-mucin layer. This relatively thin layer contributes the remaining 5-15% lipid layer thickness (Green-Church *et al.*, 2011). Phospholipids are dominant in the thin polar layer where their surfactant properties aid the stability of the thicker non-polar layer over the aqueous-mucin layer.

Polar lipids, phosphatidylcholine and phosphatidylethanolamine, make up 88% of total identified lipids in their human tear film samples (Rantamaki *et al.*, 2011). The exact quantity of phospholipids is highly variable between studies due to methodological differences (e.g. lipid collection techniques, meibum contamination, techniques and instruments used in analysis) (Green-Church et al., 2011). For example, Campbell and colleagues *s*truggled to find any phospholipids at the lower limits of detection using liquid chromatography mass spectrometry and thin layer chromatography (Campbell, Griffiths and Tighe, 2011). However, the general consensus is that the presence of phospholipids is

essential for effective spreading and stability of the lipid layer over the underlying aqueous layer (Butovich, 2008; Campbell *et al.*, 2011; Rantamaki *et al.*, 2011).

Proteins arising from the aqueous-mucin layer are increasingly being considered as a major part of the lipid layer. The outer lipid layer is thought to be populated with proteins as studies have shown small volumes of major tear proteins are able to penetrate meibomian lipid films and phospholipid films (Butovich *et al.*, 2008). Since proteins are surface active, they readily populate the air-liquid interface. Proteins compete with meibomian lipids for available surface space, resulting in the proteins either being able to penetrate or attach to the lipid layer. These proteins undergo irreversible denaturation to prevent their return to the aqueous-mucin layer (Green-Church *et al.*, 2011). The processes of intercalation and adsorption of proteins alters the solubility of lipids, thereby reducing the surface tension of water and changing the physical properties of the tear film. The detailed structure of the tear film is presented in Figure 1.6.
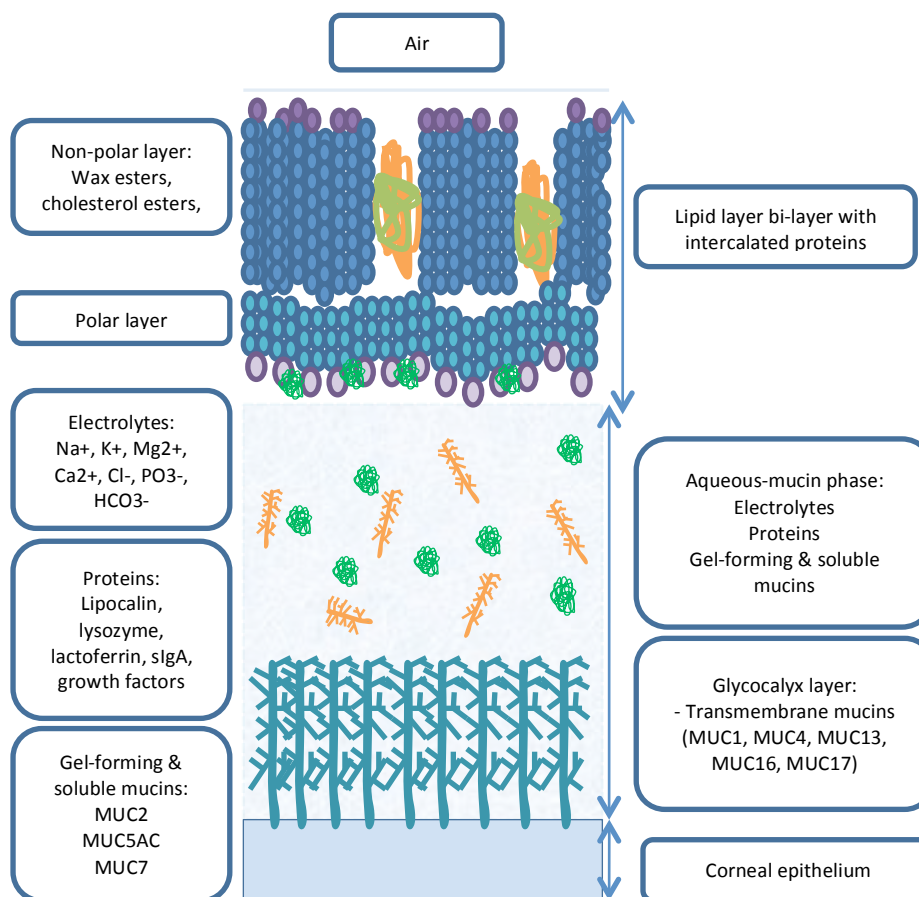
Figure 1.6 Schematic diagram illustrating lipid layer organisation, not drawn to scale (adapted from (Butovich *et al.*, 2008)

1.2.5.3 Aqueous phase

The aqueous phase represents over 98% of the overall tear film and is supplied by the main lacrimal gland and accessory glands of Krause and Wolfring located in the conjunctiva. This layer is rich in metabolites, electrolytes, proteins, growth factors and helps maintain a healthy ocular surface by providing nutrional and immunological mechanisms.

Approximately 90 different types of molecules are found in the aqueous phase of the tear film (Zhou and Beuerman, 2012). Glucose, urea and oxygen are some of the molecules found in the tear film to support the metabolism of the corneal epithelium. Although most of the glucose utilised by the corneal epithelium is derived from the aqueous humour, human tear glucose concentration ranges between 0-5.7mM, with more invasive tear collection techniques yielding greater glucose concentrations (Baca, Finegold and Asher, 2007).

Electrolytes balance tear film osmolarity and promote barrier function recovery when ocular surface cells become compromised (Nelson, 1999). Electrolytes present in the tear fluid include sodium, potassium, magnesium, calcium, chloride, bicarbonate and phosphate ions and are similar in concentration to serum (Gilbard, 1994).

Table 1.3 Major proteins found in the human tear film (taken from: Craig (2002) Structure and function of the preocular tear film.*The Tear Film: Structure, Function and Clinical Examination*. London: Butterworth-Heinemann)

Lacrimal protein	Serum protein
Lysozyme	Albumin
Lactoferrin	Transferrin
Secretory IgA	IgG
Lipocalin	IgM

Table 1.3 lists some of the major proteins found in the aqueous phase and their source. Lysozyme accounts for 20-40% of total tear protein content and is the most alkaline protein in the tear film (Craig, 2002). Lysozyme effectively binds to peptidoglycans of the bacterial

cell walls, enzymatically breaking bacteria down. It is most effective against gram-positive bacteria. Although lysozyme is found in other structures of the human body, such as saliva, it is not found in high enough concentrations to be able to act effectively compared to the concentration in tear fluid.

Lactoferrin serves a bacteriostatic mechanism of action by binding to iron molecules necessary for bacterial replication. It further provides a bactericidal action by binding to lipopolysaccharides found on gram-negative bacteria cell walls.

Immunoglobulins are antigen-specific antibodies, differentiated into isotypes according to their function and action. The human tear film has high levels of IgA and this antibody is considered as the first line of defence. IgA is secreted by the lacrimal gland and stimulated by androgens (Lucarelli *et al.*, 2003). Serum proteins (e.g. IgE, G and M) are found in higher concentrations following ocular trauma or inflammatory events due to leakage from conjunctival vessels. Concentrations of IgA, G and M are elevated in conditions such as blepharoconjunctivits, herpes keratitis and acute follicular conjunctivitis (Garg, 2002). IgE concentrations are elevated during atopic events.

In addition to facilitating the spread and stability of tears, lipocalin also has a role in removing meibomian lipids that contaminate the corneal surface (Gasymov *et al.*, 2005) and exhibits antimicrobial properties (Redl, 2000; Fluckinger *et al.*, 2004).

Smaller signalling proteins (chemokines and cytokines) are also in abundance in tear fluid. Over 1500 proteins have been identified in human tear fluid arising from the lacrimal gland, meibomian glands, goblet cells and accessory lacrimal glands (Zhou and Beuerman, 2012). These proteins have a variety of cellular signalling roles, from cellular trafficking to cellular differentiation. Although present in the healthy tear film, patients suffering from dry eye are found to have increased levels of pro-inflammatory mediators such as interleukin (IL)-1(α and β), IL-6, IL-8 which induce a cascade of inflammatory events on the ocular surface (Nakamura, Sotozono and Kinoshita, 1998; Solomon *et al.*, 2001; Pflugfelder, Stern and Beuerman, 2004). Growth factors present in the tear film stimulate cellular growth, proliferation, maturation, differentiation and movement of cells. Epithelial growth factor (EGF), hepatocyte growth factor (HGF) and transforming growth factor (TGF)-α and TGF-β

have been found to be secreted by the lacrimal gland and hold important roles in corneal wound healing (Nelson, 1999).

1.2.5.4 Mucin phase

The mucin phase is the innermost layer of the tear film which helps bind the tear film to the microvilli on conjunctival and corneal epithelial cells. Goblet cells in the conjunctival epithelium secrete gel-forming mucins which form the mucous layer of the tear film. The distribution of goblet cells in the conjunctiva is illustrated in Figure 1.7.
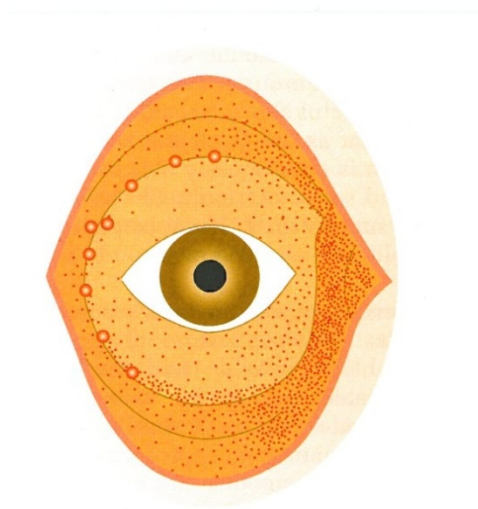
Figure 1.7 Illustration of conjunctival goblet cell distribution, represented by small dots. Larger dots represent the accessory lacrimal glands of Krause (Lawrenson, 2002)

Goblet cells are shaped like goblets with their small basal regions at the level of the basal conjunctival epithelium and their large apices adjacent to the tear film (Lucarelli *et al.*, 2003). Mucins are stored as secretary granules in the apical region and are secreted by stimulation from conjunctival and corneal sensory nerves. Mucin secretion is controlled by an apocrine mechanism where all the secretary granules are emptied once the cell has received the appropriate signal (Dartt, 2002).

Stratified squamous conjunctival and corneal epithelial cells secrete a transmembrane mucin, MUC1, to form a glycocalyx. This acts as an anchoring layer to which a mucus gel

layer adheres. The glycocalyx is fundamental in rendering the ocular surface hydrophilic to enable the spreading of the aqueous phase of the tear film (Rolando and Zierhut, 2001).

The non-Newtonian viscoelastic properties of the tear film result from gel-forming mucins. Mucins allow variable viscosity of the tear fluid depending on the shear rate delivered by the eyelids. This ensures any drag and potential subsequent trauma is minimised during blinks and allows the tear fluid to overcome gravity when the eyes are open (Kikkawa *et al.*, 2003). MUC5AC, is a large gel-forming soluble mucin and is the largest component of the mucin phase. MUC5AC forms networks of long chained polymers in conjunction with secretory mucins MUC2, 7 (Johnson and Murphy, 2004). This maintains tear film stability between blinks, smoothes imperfections of the corneal surface and minimises trauma during blinking (Rolando and Zierhut, 2001). Gel-forming mucins prevent foreign debris and pathogens from reaching epithelial surfaces by engulfing them into a mucous thread which then moves to the drainage pathway at the lower puncta by blinking (Ramamoorthy and Nichols, 2008). There is also evidence that sIgA and antimicrobial proteins such as lysozyme aggregate within a mucous thread, therefore mucins facilitate the trapping of pathogens for biochemical clearance (McDermott, 2011).

Corneal damage arises if mucus adheres to itself or to damaged corneal epithelium which may results from a lack of aqueous tears, damage to epithelial cells or glycocalyx, or contamination of mucus by lipids and desquamating cells (Sharma, 1993). Contrary to the trilaminar tear film structure, there is increasing evidence for a two-layer structure. The proposed architecture involves a biphasic lipid layer that lies above a gel consisting of aqueous and mucins mixed together, in which mucin concentration is greatest at the level of the corneal epithelium, gradually decreasing towards the level of the lipid layer (Rolando and Zierhut, 2001).

1.3 Maintenance of the tear film

Tear volume is dependent upon the rate of production and the rate of drainage and evaporation (Tomlinson, Trees and Occhipinti, 1991). Seventy to ninety percent of the tear volume is found within the marginal tear strips (Mishima *et al.*, 1966). The remainder covers the cornea and bulbar conjunctiva, and beneath the eyelids between the palpebral and bulbar conjunctiva in the conjunctival fornices. In normal individuals, tear volume is about

7µl (Mishima *et al.*, 1966; Scherz, Doane and Dohlman, 1974). More recently, calculations of tear volume using anterior eye optical coherence tomography (OCT) imaging has found a total volume of 2.5µl (Palakuru, Wang and Aquavella, 2007).

1.3.1   Neural regulation of tear production

Tear production is regulated by a feedback system maintained by a reflex loop which incorporates the lacrimal functional unit and brainstem with additional inputs from the nasolacrimal system and emotional centres of the brain (Gaffney *et al.*, 2010), illustrated in Figure 1.8. In the waking state, afferent impulses arising from an exposed ocular surface regulates tear flow and increased irritation generates greater lacrimal secretion (Gaffney *et al.*, 2010). Minor stimulation can increase tear film turnover by up to 500% (Jordan and Baum, 1980).
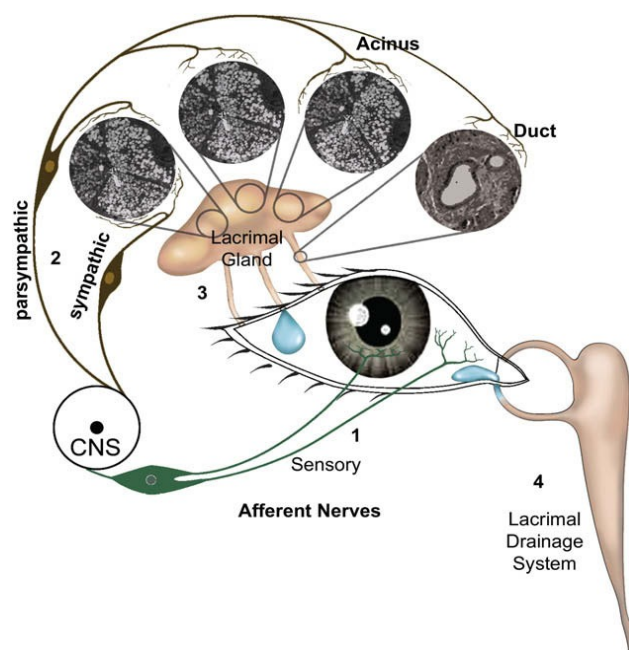
Figure 1.8 Illustration of the sympathetic and parasympathetic neural innervation of the lacrimal functional unit. Image from (Dartt, 2009)

1.3.2   Blinking

The interaction of the orbicular and levator muscles of the eyelids facilitate the spreading of tears and reconstruction the disrupted tear film due to evaporation (Rolando and Zierhut,

2001). Blinking also plays an important role in the drainage of tears, the removal of debris from the ocular surface and the meibum secretion.

Blink rates vary between 8 to 12 blinks per minute (Carney and Hill, 1982; Abelson and Langelier, 2006). The control of the blinking is not fully understood; one hypothesis suggests that the sensation or irritation that occurs during surface drying and tear break up stimulates blinking (Collins *et al.*, 1989). This is further supported by studies which show patients that suffer from dry eye have increased blink rates compared to healthy individuals (Yap, 1991; Al-Abdulmunem, 1999). This is thought to be an adaptation to overcome reduced tear film stability (Johnson and Murphy, 2004). Human infants blink at a rate of 3-4 blinks per minute which suggests their tear film is far more stable and protects against evaporation more effectively than the adult tear film (Mathers, 2004a).

1.3.3   Tear production

Basal tearing, tear flow occurring in quiet room conditions in the absence of desiccating or other stresses to the exposed ocular surface, is produced by the accessory lacrimal glands and corneal and conjunctival epithelial cells (Dartt, 2002) at a rate between 1 -2µl/minute (Mishima, 1965; Jordan and Baum, 1980). A more recent publication suggests average tear flow is closer to 1µl/minute in normals (Tomlinson, Doane and McFadyen, 2009). The tear turnover rate in normals is approximately 16% per minute (Tomlinson *et al.*, 2009). Basal tearing is influenced by a variety of factors including the use of topical anaesthetics (Jordan and Baum, 1980) and temperature (Parra *et al.*, 2010).

Topical and systemic medications known to cause lacrimal gland hyposecretion include: high doses of oral diuretics, antihistamines, tricyclic antidepressants, anti-cholinergic drugs, beta-blockers and anti-hypertensive drugs (Jaanus, 1992; Craig, 2002). Decreased ocular surface sensitivity following contact lens use, ocular surface inflammation, corneal surgery, herpetic keratitis and diabetes also leads to reduced aqueous production by the lacrimal glands (Johnson and Murphy, 2004).

1.3.4   The lacrimal drainage system

Tears are drained away by the nasolacrimal system, by the process of evaporation and by conjunctival absorption. The main route of tear elimination is through the nasolacrimal system (Figure 1.9).
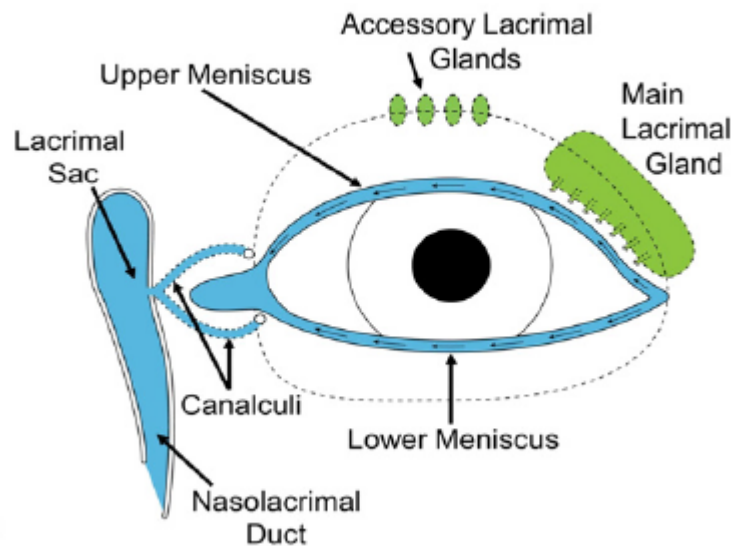
Figure 1.9 Tear flow and drainage (Gaffney *et al.*, 2010)

Tears are directed to the puncta, situated medially on both the upper and lower eyelid, approximately 0.5-1.0mm in diameter and are the openings of the canaliculi. In 90% of individuals the superior and inferior canaliculi merge to form the common canaliculus, which leads to the lacrimal sac. The inferior canaliculus collects four times as much tear flow than the superior canaliculus due to gravity (Craig, 2002). Tears exit the superior puncta and canaliculus by capillary attraction. The movement of tears to the puncta, canaliculi and finally the lacrimal sac is driven by blinks (Figure 1.10).

Prior to blink
System fully open

During closing phase
Puncta meet and close
Canaliculi and sac are compressed
Fluid moves into duct

During reopening
Canaliculi and sac reexpand
Relative negative pressure
  develops in sac

As blink ends
Puncta reopen
Fluid flows into puncta and
  canaliculi
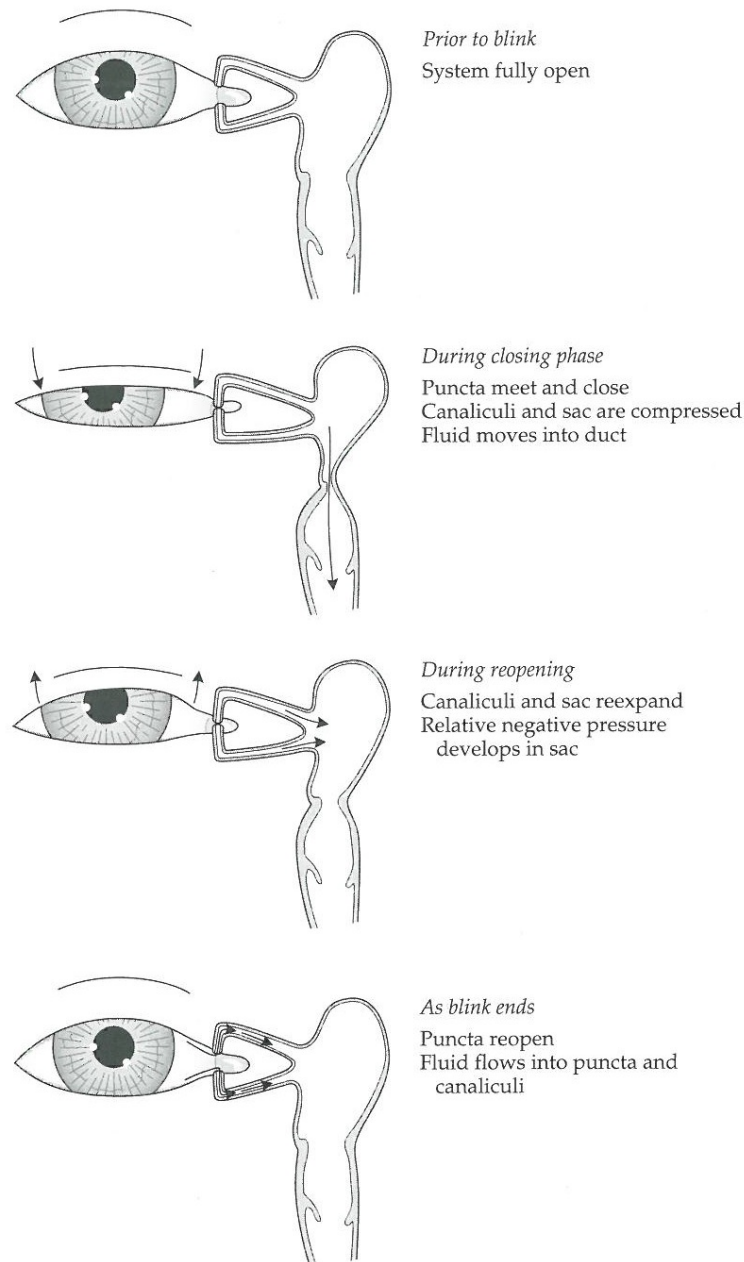
Figure 1.10 Illustration of tear fluid drainage. Image from (Oyster, 1999b)

During a blink tear flow is directed nasally towards the puncta; the rapid closing of the temporal aspect of the palpebral aperture further facilitates tear drainage (Craig, 2002). At the start of a blink with the lids open, the canaliculi are filled with tear fluid drawn in from the previous blink. The action of the lids when half closed causes the puncta to become occluded by the lid margins and the canaliculi and lacrimal sac begin to be squeezed. Upon complete closure, the canaliculi are completely compressed and all the fluid contained

within them has been forced into the sac. When the lids begin to open and reach the halfway point, pressure on the canaliculi is released however the puncta remain occluded. Finally at the end of the blink, the lids are fully open freeing the puncta; the canaliculi and sac return to their normal status and tear fluid is drawn into the puncta leading to the canaliculi. Approximately 2.0µl of tears is drained per blink and increases when gravity acts in the direction of tear flow into the nasolacrimal duct (Sahlin and Chen, 1997).

1.3.5   Tear film stability

The human tear film does not remain stable for long periods of time and ruptures when blinking is prevented (Guillon, 2002). Several theories regarding tear film instability exist.

The first theory involves the migration and subsequent contamination of lipids across the mucous layer as the tear film thins by evaporation after a blink. Once sufficient lipid from the outer layer of the tear film has contaminated the inner mucous layer, the mucous becomes hydrophobic thus no longer supporting the aqueous phase resulting in tear film rupture (Holly, 1973). Two problems arise with this model; firstly it is assumed that lipids can pass across the tear film however lipids have low solubility and diffusivity and absorb within the normal range of break-up times. Secondly the theory fails to model why reduced break-up times are exhibited in patients with mucous and ocular surface abnormalities (Johnson and Murphy, 2004).

A second theory suggested that it is the rupture of the mucous layer, due to the van der Waals dispersion forces acting on the mucous layer, that causes exposure of the aqueous layer to make contact with the hydrophobic corneal epithelium (Sharma and Ruckenstein, 1985). This causes the aqueous phase to break, bringing the lipid layer into contact with the epithelium. The van der Waal forces on their own are not great enough to cause tear film disruption; additional factors such as evaporation, tear drainage and disruption of the mucous layer by contaminants results in tear destabilisation.

An additional model proposed that the hydrostatic pressure in the concave tear menisci is negative, thus as the tear menisci form during the upward blink phase, additional tear fluid is drawn from the pre-ocular tear film, eventually causing separation of the forming tear film (King-Smith *et al.*, 2004). This break between the tear menisci and the preocular tear

film readily be observed following the instillation of the diagnostic dye, sodium fluorescein. Furthermore, the effect of gravity in this model causes tear thinning to occur more readily closer to the upper lid than the lower lid (Braun and Fitt, 2003).

1.3.6   Tear film evaporation

Tear film evaporation is reduced by 80-90% in the normal eye by the presence of lipids in the tear film (Tomlinson and Khanal, 2005). Ten to twenty percent of secreted tears is lost by evaporation in normal conditions (Mishima *et al.*, 1966) which represents a small loss of water, which the lacrimal gland readily replaces in healthy individuals. The average evaporative loss is 0.14±0.07µl/min in normal eyes (Tomlinson *et al.*, 2009). This increases in patients with dry eye and differs between different types of dry eye, as shown in Table 1.4.

Table 1.4 Evaporation rates for the normal eye, dry eyes (as a group), aqueous deficient dry eye (ADDE) and evaporative dry eye (EDE) (Tomlinson *et al.*, 2009)

	Evaporation ($x10^{-7}g/cm^2/sec$)	Evaporation (µl/min)
Normal	13.54 ± 6.52	0.14 ± 0.07
All dry eye	21.05 ± 13.96	0.21 ± 0.13
ADDE	17.91 ± 10.49	0.17 ± 0.10
EDE	25.34 ± 13.80	0.26 ± 0.16

Factors such as temperature and air humidity are directly proportional to tear evaporation (Tomlinson *et al.*, 2009). Evaporative loss increases with palpebral aperture width and is increased in up gaze (Cho *et al.*, 2000; 2007). Tear film evaporation rates are higher in older age groups and in females (Guillon and Maissa, 2010) which correlates well with the increased prevalence of dry eye in older females. In addition to MG drop-out (Mathers, 1993), lipid volume and lipid layer thickness, indicators of MG function, also correlate with tear evaporation (Mathers and Daley, 1996; Craig and Tomlinson, 1997).

The application of a contact lens to the eye has also been shown to increase evaporation (Hamano, 1981; Cedarstaff and Tomlinson, 1983; Nichols and Sinnott, 2006), which leads to increased reports of dryness symptoms (Nichols *et al.*, 2005; Nichols and Sinnott, 2006).

Evaporation is not consistently related to the water content of the lens and water loss by dehydration of the lens makes only a small contribution to the increase in evaporation from lens wear (Cedarstaff and Tomlinson, 1983). However, the use of an old contact lens increases evaporation compared to the use of a new contact lens (Mathers, 2004a).

1.4   Dry eye syndrome

The revised definition of dry eye disease (DED) by the International Dry Eye Workshop (DEWS) is as follows: "*…a multifactorial disease of the tears and ocular surface that results in symptoms of discomfort, visual disturbance, and tear film instability with potential damage to the ocular surface. It is accompanied by increased osmolarity of the tear film and inflammation of the ocular surface*" (Lemp *et al.*, 2007). The increased prevalence of symptoms related to dryness is greater in contact lens wear and it has been implicated that the use of eye cosmetics can also increase dryness symptoms (Guillon and Maissa, 2005; Wolkoff, 2008).

The prevalence of DES varies between 5.5-33.7%, depending on which epidemiological study is analysed as the definition of DED differs between studies (Lemp *et al.*, 2007). There is increased prevalence of DED with age, in postmenopausal women and patients with autoimmune diseases (Moss, Klein and Klein, 2008; Gayton, 2009).

DEWS maintain a classification of two major classes of dry eye – aqueous deficient dry eye (ADDE) and evaporative dry eye (EDE) (Figure 1.11). EDE is the most prevalent form of dry eye disease (Albietz, 2000; Lemp *et al.*, 2012). Lemp *et al.* (2012) found 86% of dry eye patients in their cohort were classified to have an element of evaporative dry eye. Additionally, ADDE and EDE are not mutually exclusive – 36% of patients in the same study by Lemp *et al.* (2012) had a combination of ADDE and EDE.
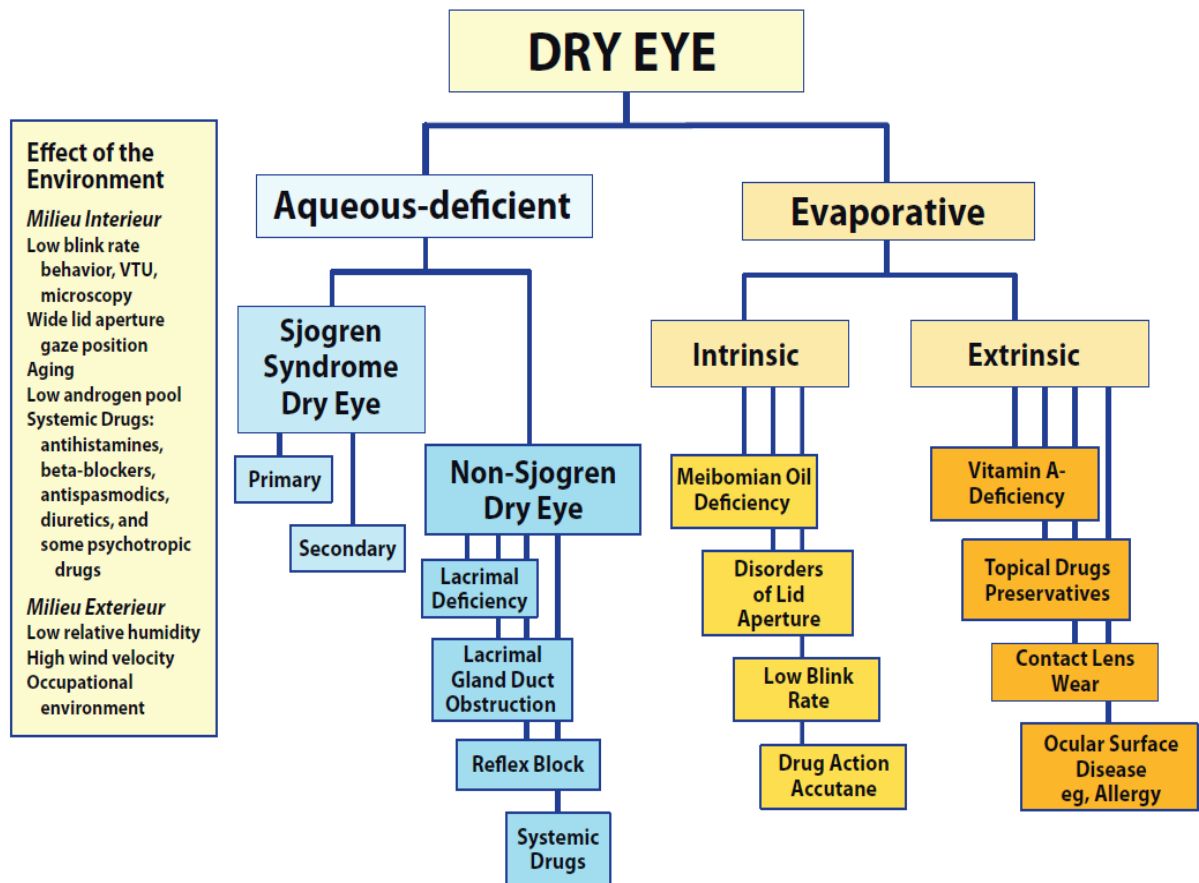
Figure 1.11 Major aetiological causes of dry eye (Lemp *et al.*, 2007)

1.4.1   Aqueous deficient dry eye (ADDE)

ADDE arises from a reduction in lacrimal gland secretion and results in tear hyperosmolarity. The tear film evaporates at a normal rate however, it is evaporating from a reduced aqueous pool (Lemp *et al.,* 2007). This leads to an imbalance of electrolytes in the tear film, causing hyperosmolarity and initiating a cascade of inflammatory events. ADDE is further categorised into Sjögren's syndrome dry eye and non-Sjögren's syndrome dry eye.

Sjögren's syndrome dry eye most frequently presents secondary to systemic autoimmune connective tissue diseases such as rheumatoid arthritis, systemic lupus erythematosus, and mixed connective tissue disease. Lacrimal gland atrophy secondary to lymphocytic infiltration results in gland hyposecretion; together with inflammatory mediators produced within the lacrimal gland, a cycle of ocular surface inflammation results.

The term non-Sjögren's syndrome dry eye is used in cases where ADDE is due to lacrimal dysfunction in the absence of systemic autoimmune features of Sjögren's syndrome dry eye. Non-Sjögren's syndrome dry eye may arise from lacrimal gland deficiencies, obstruction of the lacrimal gland ducts and reflex hyposecretion.

1.4.2 Evaporative Dry Eye (EDE)

Evaporative dry eye (EDE) is caused by excessive tear evaporation from the ocular surface in the presence of normal lacrimal secretion. The DEWS report ascribes the origins of EDE to arise from intrinsic and extrinsic factors (Figure 1.11) (Lemp *et al.*, 2007). Meibomian gland dysfunction, blepharitis, poor lid/globe apposition and low blink rate contribute to the intrinsic influences of EDE. Ocular surface disorders, contact lens wear, ocular surface disease and allergic conjunctivitis contribute to the extrinsic influences of EDE although intrinsic and extrinsic causes are not mutually exclusive.

1.4.2.1 Meibomian gland dysfunction (MGD)

The International Workshop on Meibomian Gland Dysfunction (MGD) defines MGD as "*…a chronic, diffuse abnormality of the meibomian glands, commonly characterised by terminal duct obstruction and/or qualitative/quantitative changes in the glandular secretion. It may result in the alteration of the tear film, symptoms of eye irritation, clinically apparent inflammation and ocular surface disease*" (Nichols *et al.*, 2011). MGD is one of the primary causes of EDE (Bron and Tiffany, 2004; Tong *et al.*, 2010).

The International MGD workshop classified MGD into hypo- and hyper-secretory states and obstructive states, (Figure 1.12). In obstructive MGD, blockages to MG duct terminals cause reduced meibum delivery to the tear film. Hyposecretory MGD is the term reserved for reduced meibum delivery in the absence of obvious obstruction. In hypersecretory MGD, excessive volumes of meibum is released in a response to palpation of the lid margin in the absence of active gland inflammation or structural gland changes (Nelson *et al.*, 2011). Reduced or poor quality meibum delivery to the tear film ultimately causes tear instability and increased evaporation of the tear film.
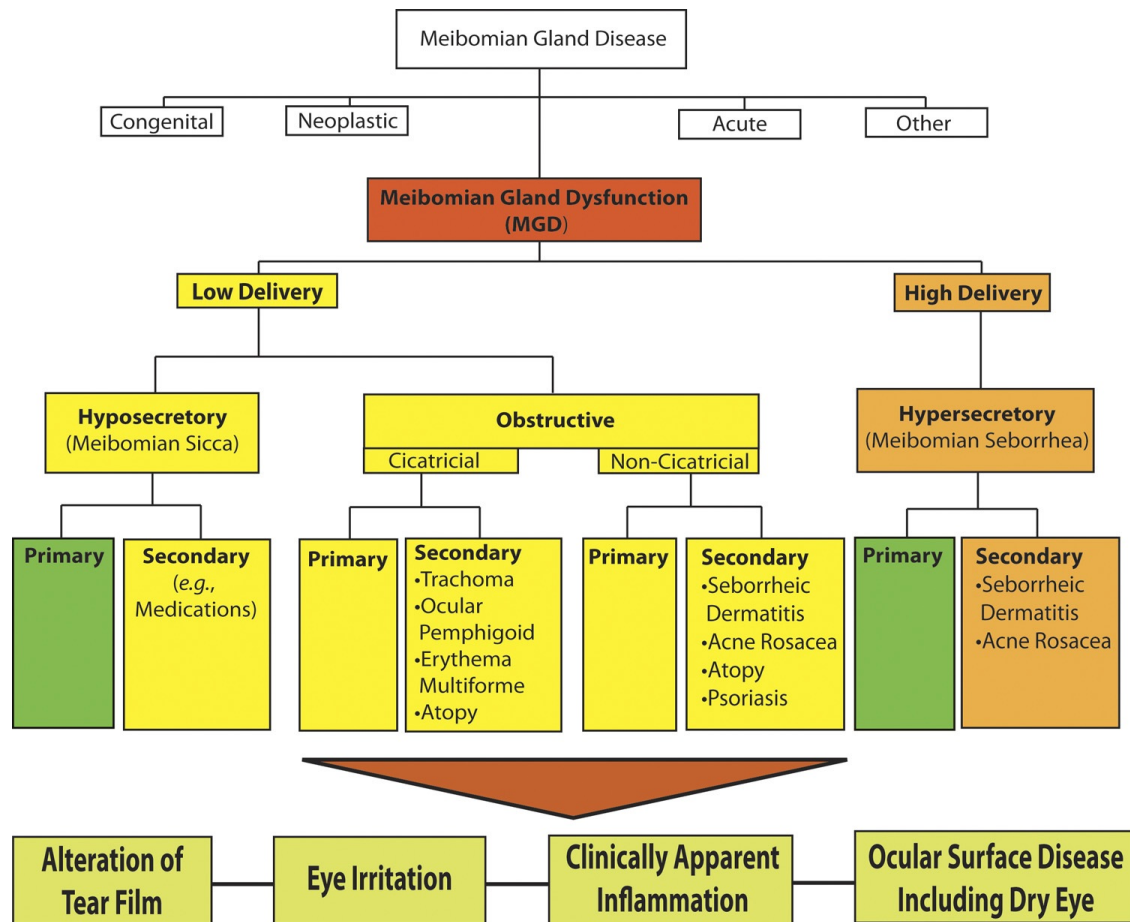
Figure 1.12 The classification and causes of MGD, defined by the International Workshop of MGD (Nichols *et al.*, 2011)

MGD is often associated with inflammatory changes of the lid margins, meibomian orifices and meibum delivery or composition (Foulks and Bron, 2003). These changes have been attributed to a reduction in tear stability, loss of lubrication and damage to the ocular surface epithelium (Goto *et al.*, 2003b; 2007). Dermatological conditions such as seborrheic dermatitis and acne rosacea can affect the MG due to their involvement with sebaceous glands (Foulks and Bron, 2003).

Wax and cholesterol esters are the main lipids in meibum secretion and biochemical differences in the composition and distribution of these lipids may have an impact on the efficiency of secretion (Joffre *et al.*, 2008), causing obstructive MGD. Meibum in patients suffering from MGD show lower levels of triglycerides, cholesterol esters and unsaturated fatty acids (Green-Church *et al.*, 2011). This compositional change is responsible for the

increased melting point and, in conjunction with hyperkeratinisation of the terminal duct, results in subsequent stagnation of meibum within the duct.

The effect of lid flora on meibum composition has been linked with tear film stability. Bacteria such as *Staphylococcus aureus* and *Propionibacterium acnes* are present in healthy individuals although bacteria numbers are significantly greater in active inflammation of the MG. These bacteria have been shown to produce lipases and esterases which break down tear film lipids (Green-Church *et al.*, 2011). Additionally, lid flora releases free fatty acids and mono- and triglycerides (Dougherty and McCulley, 1986) which hydrolyses lipids at the lid margins. This results in the production of meibomian foam, a common clinical sign in MGD patients, which may be a source of irritation (Dougherty and McCulley, 1986).

1.5   Clinical signs and tests to determine ocular surface health and integrity

1.5.1   Patient history and reported symptoms

The aetiology of symptoms reported by patients suffering from abnormal ocular surface conditions is not well understood however its occurrence suggests the stimulation of nociceptors innervating the corneal surface (Lemp *et al.*, 2007). Factors which may cause this include: tear hyperosmolarity, tear film break up during the inter-blink period, shear-stress between the lids and ocular surface, reduced expression of mucins at the ocular surface, the presence of inflammatory mediators at the ocular surface and hypersensitivity of nociceptive sensory nerves (Lemp *et al.*, 2007).

DES symptoms vary in type and severity and there is poor correlation between clinical signs and symptoms (Nichols, Nichols and Mitchell, 2004; Fuentes-Paez *et al.*, 2011; Cuevas *et al.*, 2012). Symptoms often vary throughout the day, usually worsening in the evening (Begley *et al.*, 2003; Chalmers and Begley, 2006). Reported symptoms include (Nichols, 2006):

Ache	Pain
Blurring (fluctuating)	Photophobia
Burning	Scratchiness
Conjunctival redness	Soreness
Dryness	Sticky tears
Discomfort (irritation)	Stinging
Foreign body sensation	Swollen, red eyelids
Grittiness	Tiredness
Itching	Watery eyes
Ocular fatigue	

Dry eye questionnaires are instruments commonly used amongst a battery of tests used for the diagnosis of dry eye. There are vast variations in questionnaire designs. Some questionnaires have been validated for use in clinical trials since there is increasing emphasis on patient-reported outcomes (Guillemin *et al.*, 2012), particularly as the disease poorly correlates with the clinical signs. There are advocates that suggest DES should be

managed primarily according to symptoms (Behrens *et al.*, 2006). Questionnaires may establish the risk factors, frequency, intensity and environmental triggers of dry eye symptoms. A number of dry eye questionnaires have been designed and used in clinical practice and clinical trials to diagnose dry eye, evaluate symptoms or monitor treatment however there is not one universally accepted questionnaire which accomplishes this (Nichols, 2006). A summary of the questionnaires used for screening is shown in Table 1.5.

Table 1.5 Summary of dry eye questionnaires, adapted from (Nichols, 2006; Guillemin *et al.*, 2012)

Survey	Questionnaire summary	Use
Dry Eye Epidemiology Project (DEEP) (Oden *et al.*, 1998)	Specifically developed for screening DE patients in an epidemiological study. 19 questions based on symptoms and associated factors	Screening tool for DE patients in epidemiological studies
McMonnies Dry Eye Questionnaire (McMonnies, 1986)	14 questions based on symptoms and risk factors	Screening for possibility of DE and assess risk factors
Ocular Surface Disease Index (OSDI) (Schiffman *et al.*, 2000)	12-item questionnaire assessing symptoms of ocular irritation and the impact on vision-related tasks. Efficient in classifying dry eye severity. Based on symptoms experienced in the past week.	Screening survey has been used extensively in clinical trials, developed by Allergan Inc. (Irvine, CA)
Dry Eye Questionnaire (DEQ)/DEQ-5 and Contact Lens Dry Eye Questionnaire (CLDEQ)/CLDEQ-8 (Begley *et al.*, 2001; Nichols *et al.*, 2002)	DEQ is a 23-question symptom, associated factors and self-diagnosis survey based on symptoms in the past week. A shorter version (DEQ-5) developed consists of 5 questions only. CLDEQ developed, based upon DEQ, consists of 36 questions to screen for DE symptoms in CL wear specifically. A shorter version (CLDEQ-8) consists of 8 questions only.	Screening questionnaire used to assess frequency, severity and impact of symptoms. CLDEQ used in clinical trials when refitted with different CLs
Standard Patient Evaluation of Eye Dryness Survey (SPEED) (Korb *et al.*, 2005)	20 questions based on symptom severity and frequency at 3 different time points (at the visit, within the past 72 hours and within the past 3 months)	Screening questionnaire used to assess symptoms and monitor change
Symptom Assessment in Dry Eye (SANDE) (Schaumberg *et al.*, 2007)	Two questions in visual analogue scale format assessing the frequency and severity of DE symptoms. Can be used for follow-up visits to monitor effectiveness of therapy	Questionnaire useful for monitoring changes in symptoms over time

1.5.2   Ocular surface staining

The use of diagnostic dyes (sodium fluorescein, lissamine green and rose bengal) on the ocular surface aids the assessment of ocular surface integrity that often becomes compromised due to tear film instability and hyperosmolarity. Ocular surface staining aids not only with diagnosis but allows the clinician to determine disease severity and efficacy of treatment and interventions (Bron, Evans and Smith, 2003). When sodium fluorescein (FLN) is used, the intensity and fluorescence of the dye over the ocular surface gives an indication of the thickness of the tear film. The degree of uniformity of the dye indicates ocular surface integrity. Thinning of the tear film will show reduced fluorescence and a black spot or line indicative of a break in the tear film.

While sodium fluorescein (FLN) is used extensively in clinical ophthalmology for anterior eye examinations, the mechanisms of corneal and conjunctival staining are poorly understood. It was initially considered that FLN pools in indentations and between the intercellular gaps which may arise from corneal and conjunctival epithelial disruption. However due to persistent corneal staining following FLN instilled in rabbit corneas (Wilson, Ren and Laurent, 1995), this suggests corneal staining also may be due to FLN uptake by epithelial cells. Furthermore, cornea epithelium has been shown to be permeable to FLN *in vitro* (Ward, Walker and Dimitrijevich, 1997) and *in vivo* (McNamara *et al.*, 1998).

In contrast to FLN, rose bengal (RB) and lissamine green (LG) selectively stain damaged areas of the ocular surface in which the tear film is discontinuous, staining dead cells and cells with an immature glycocalyx (Argueso *et al.*, 2006). LG is now the preferred choice of dye over RB as it is better tolerated by patients and gives comparable results to RB in the evaluation of ocular surface integrity (Korb *et al.*, 2008; Machado, Castro and Fontes, 2009).

The volume of diagnostic dye administered can influence the determination of ocular surface damage. A large, uncontrolled dose of FLN can over-saturate the epithelium, masking true staining because of fluorescein quenching; conversely, the visibility of staining with RB or LG is highly dose-dependent (Bron *et al.*, 2003). The use of yellow barrier filters enhances contrast during observations, further increasing sensitivity of FLN staining (Ousler *et al.*, 2005). Furthermore, the timing between instillation of the diagnostic dye to evaluation of the ocular surface is critical, although this depends on tear volume and tear

turnover rate when using fluorescein. For LG, the ocular surface ought to be evaluated 1-4 minutes after instillation of 10µl of the dye (Ousler *et al.*, 2005).

1.5.3 Tear break-up time (TBUT)

Tear film instability is one of the core mechanisms of DES (Lemp *et al.*, 2007). The TBUT, the duration taken for the tear film to rupture before a subsequent blink and for the appearance of dry spots on the cornea to appear, is a common test for assessing the stability of the tear film and can be conducted using invasive and non-invasive techniques.

1.5.3.1 Fluorescein (FLN) TBUT

The FLN TBUT is defined as the time taken between the last complete blink and the first appearance of a dry spot or disruption in the tear film (Bron *et al.*, 2007). The instillation of FLN disrupts the tear film, making this technique invasive, thus typical TBUT results are shorter than non-invasive techniques. The diagnosis for DES is a TBUT of less than 10 seconds (Lemp and Hamill, 1973) however other studies have used a cut-off of less than 5 seconds when smaller volumes of fluorescein (e.g. 5µl of 2% fluorescein) have been used (Abelson *et al.*, 2002). Incomplete or partial blinking will cause break up over the inferior portion of the ocular surface (Guillon, 2002); tear break up repeatedly restricted to the same area may arise from a superficial epithelial defect (Lemp, Dohlman and Holly, 1970).

The repeatability of FLN TBUT measurements is influenced by the volume, concentration and method of administered FLN, the time between instillation and measurement, instructions given to the patient regarding blinking and eye opening, the method of observation and the use of barrier filters (Abelson *et al.*, 2002; Johnson and Murphy, 2005). Therefore, standardisation of this procedure is essential.

1.5.3.2 Non-invasive TBUT

Non-invasive TBUT (NITBUT) measures tear film stability by recording the time taken for an image of a regular pattern projected onto the corneal surface to distort or break-up following a full blink. This technique minimises reflex tearing and other variables related to repeatability issues encountered with fluorescein TBUT. The observations made in NITBUT

tests are representative of local tear thinning, rather than tear film rupture at that point, thus it is the tear thinning time (TTT) which is being measured.

The TTT can be measured using several instruments. Observing the mires on a one-position keratometer or placido-disc topographer can be used to determine TTT, which showed better reproducibility and lower variability than seen for fluorescein TBUT measurements (Madden, Paugh and Wang, 1994). Commercially available instruments such as the Keeler Tearscope (Keeler, Windsor, U.K.) allow direct observation of the tear lipid layer and tear thinning. The instrument projects cold white light onto the cornea, allowing observation of the tear lipid in specular reflection. A grid insert can be placed within the illuminated inner surface of the instrument thus indirectly observing a distortion or break in the grid pattern when the tear film thins (Mengher *et al.*, 1985).
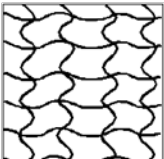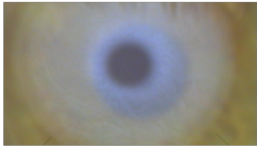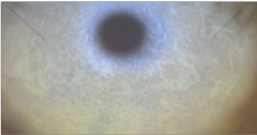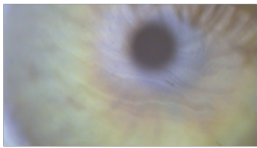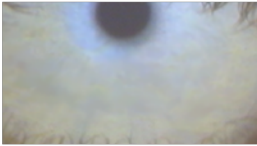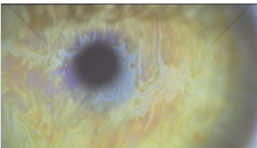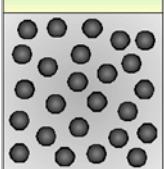
Measurements of NITBUT are usually greater than FLN TBUT; one study reports NITBUT to be between 35.6 and 44.7 seconds in healthy subjects depending on the instrument used (Madden *et al.*, 1994).

1.5.4   Tear lipid layer observation

As previously described, a sufficient tear lipid layer is paramount in preventing evaporation of the tear film. Lipid layer thickness (LLT) has been shown to correlate well with symptoms in patients with severe dry eye (Blackie *et al.*, 2009). Interferometers allow visualisation of the tear lipid layer and provide a qualitative and semi-quantitative approach to LLT by interpretation of coloured fringes which occur due to a change in refractive indices between the lipid and aqueous-mucin interface.

The use of interferometery in clinical practice has been limited due to the lack of commercially available devices. The Keeler Tearscope can provide a direct view of the lipid layer by specular reflection (Figure 1.13). Equivalent LLT have been calculated using computational techniques, based on the coloured hues from the lipid layer images generated using the Kowa DR-1 interferometer (Kowa Co., Nagoya, Japan) (Goto *et al.*, 2003a). These values have been adopted in the Lipiview Ocular Science Interferometer (Blackie *et al.*, 2009).

Figure 1.13 The grading of the tear lipid layer using the Keeler Tearscope, including equivalent estimated lipid layer thickness and prevalence (Keeler Tearscope Instruction Manual)

Patients presenting with open meshwork lipid layer patterns are likely to present with symptoms of dry eye due to an inadequate lipid layer. However patients with coloured fringes may also be symptomatic due to inadequate lipid layer spread (Keeler Tearscope Instruction Manual).

1.5.5 Tear quantity

Invasive tests such as the Schirmer test II (with local anaesthesia) and phenol red thread test are used to examine tear quantity. The wetting length of a Schirmer strip or phenol red thread placed into the inferior fornix gives a measure of tear quantity. Performing the

Schirmer test without anaesthesia (Schirmer I test) assesses tear flow from reflex tearing. This has been shown to have low sensitivity and high specificity in the diagnosis of ADDE (Lucca, Nunez and Farris, 1990) and has poor repeatability (Nichols, Mitchell and Zadnik, 2004).

Since 75-90% total tear fluid volume is found in the tear menisci (Holly, 1985), examination of the tear menisci is a good indicator of tear quantity. A non-invasive technique, tear meniscus height analysis shows good sensitivity and specificity in the diagnosis of ADDE (Shen *et al.*, 2009). Video meniscometry measures the radius of tear film menisci which is one digital, non-invasive method in diagnosing ADDE (Yokoi and Komuro, 2004). More recently, the use of anterior eye OCT (optical coherence tomography) has allowed precise measurement of tear meniscus height and subsequent calculations of tear volumes using associated mathematical algorithms (Mainstone, Bruce and Golding, 1996; Palakuru *et al.*, 2007; Shen *et al.*, 2009).

1.5.6   Tear osmolarity

Tear fluid hyperosmolarity is considered a core mechanism driving the cycle of DES, causing ocular surface inflammation and damage, and initiation of compensatory events in dry eye. Tear osmolarity is determined by the quantity of solutes and electrolytes present in the tear film. Tear hyperosmolarity may occur if the aqueous evaporates from an already insufficient tear volume, if the aqueous evaporates excessively, or a combination of both causes. Normal tears have an osmolarity of approximately 300mOsm/L (Gilbard, 1994). The validated cut-off level for hyperosmolarity for dry eye diagnosis is 316mOsm/L (Tomlinson *et al.*, 2006) and has been shown to be the most sensitive marker in diagnosis dry eye diesease severity in of a battery of clinical tests (Sullivan *et al.*, 2010; Suzuki *et al.*, 2010).

Tear osmolarity tests are considered the gold standard in dry eye diagnosis (Farris, 1994; Bron *et al.*, 2007) and has become more accessible with commercially-available devices. The Advanced Instruments Model 3100 Tear Osmometer (Advanced Instruments, Inc., Norwood, Massachusetts) and Tearlab Osmolarity System (OcuSense, Inc., San Diego, California, USA) are two "lab-on-a-chip" tests which require a 50 nanolitre sample of tears, making it a suitable clinical test to use with aqueous deficient patients. These instruments have been

shown to yield repeatable results (Yildiz *et al.*, 2009) with good correlation to dry eye severity (Versura, Profazio and Campos, 2010).

1.6   Eye cosmetics

Cosmetic formulation and applications have evolved over the centuries and modern day Western cosmetic products are designed to meet consumer demands. Recent market research has shown that 80% women have used face and eye cosmetics in the last 12 months and 87% women under the age of 55 use eye cosmetics (Mintel, 2013).

A summary of eye cosmetic formulations produced by COPLIA (Guidelines realised in collaboration with The European Association of Poison Centres and Clinical Toxicologists) can be found in Appendix I.

Eye cosmetics can be classified into eyelash cosmetics and eyelid cosmetics depending upon the indicated use and site of application. Mascara and eyeliner are classified as eyelash cosmetics and eye shadows and concealers are classified as eyelid cosmetics. Three of the most popular eye cosmetics (Mintel, 2013) (mascara, eye shadow and eyeliner) will be discussed below in detail.

1.6.1   Mascara

One of the most popular eye cosmetic products (Mintel, 2013), mascara is applied to the eyelashes to darken and elongate the lashes, giving the appearance of larger eyes. Mascara is available in two formulations – cake and liquid (Draelos, 2001), with the latter remaining the most readily available form.

Cake mascara contains soap and pigments compressed into a cake, which is applied to the base of the lashes using a water-moistened brush. This formulation is more readily tolerated by patients with sensitive skin, however as it is not a water-resistant product, it can easily smudge with tearing or perspiration (Draelos, 2001). Liquid mascara is contained in a tube with an applicator wand to which a brush is attached which comprises the lid of the product. The design of the applicator brush is almost as important as the formulation of the mascara itself, with newer applicators separating lashes more effectively to create longer-

looking lashes (Mintel, 2013). Liquid mascara is further differentiated into water-based and solvent-based mascaras.

Water-based mascara comprises of waxes, pigments and resins dissolved in water (Draelos, 2001). Increased wax is added to the formulation to make the product water-resistant, and adding polyvinylpyrollidone (PVP) resins decreases smudging during wear (Draelos, 1995a; O'Donoghue, 2000). Water-based mascara is easily contaminated by bacteria therefore contains preservatives, usually parabens, to inhibit their growth in the product. Solvent-based mascara is waterproof. Pigments and waxes are added to petroleum distillate which gives it good resilience but is difficult to remove with water alone; the use of oil-based or cream cosmetic removers will facilitate removal. Fewer preservatives are required in a petrolatum-based solvent formulation as it creates an inhospitable bacterial breeding environment. Consequently, this formulation is more sensitising than some other formulations discussed. Further developments of mascara manufacture has seen the launch of hybrid mascaras in the cosmetics market which have a short drying time of a water-based mascara but provides the waterproof lash separation desired by many users (Draelos, 2001).

1.6.2   Eyeliner

Eyeliner defines the margins of the lids and is used to accentuate and shape the appearance of the eyes (Mulhern *et al.*, 2003). It is applied close to the lashes, either outside of the eyelid or behind the lash line, along the inner lid margin. The exact application of the product is usually dependent on fashion trends (Figure 1.14).
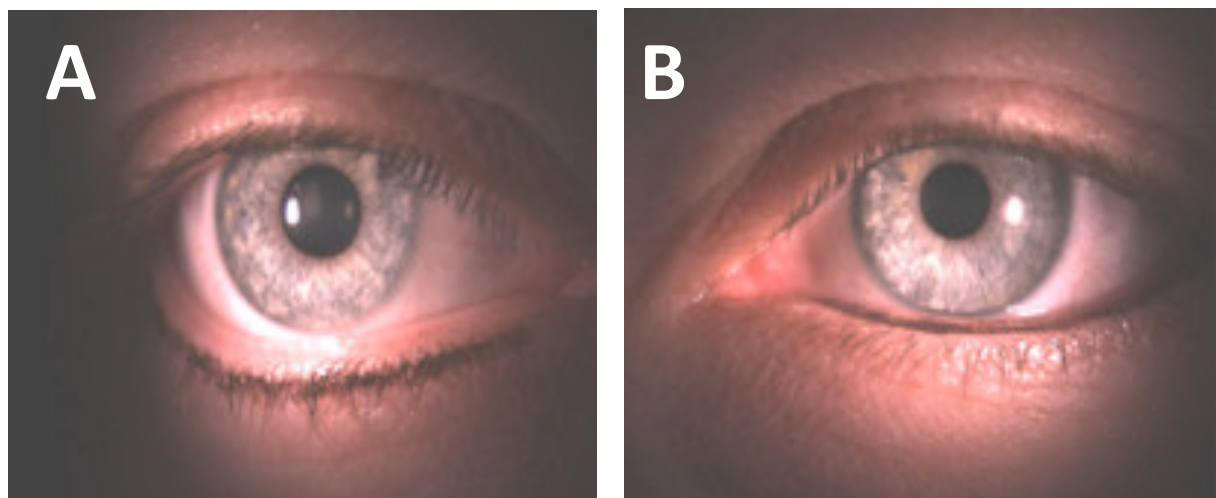
Figure 1.14 Eyeliner applied outside the lash line (A) and within the lash line (B)

Eyeliner is available in three forms: cake, liquid and pencil. The formulation of cake eyeliner is similar to powder eye shadow with added surfactants to help form a paste mixed with water. It is applied in a similar way as cake mascara. Cake eyeliner has been replaced by the popularity of liquid eyeliners which contain pigments suspended in a water-soluble latex base (Draelos, 2001). One of the key ingredients in liquid eyeliners is a viscosity-controlling agent to thicken the product, suspend pigments and enable smooth application of the product (Orecchinoi, 1994). The high water content of liquid eyeliner leaves it open to easy bacterial and fungal contamination and growth thus the use of preservatives is essential (Orecchinoi, 1994).

Pencil eyeliners consist of waxes combined with pigments, mineral or vegetable oils and lanolin derivatives formulated into a rod, encased in wood. Although pencil eyeliners carry the same risks of bacterial and fungal contamination as eye cosmetics, re-sharpening the pencil removes exposed eyeliner thus reducing contamination (Draelos, 2001).

1.6.3   Eye shadow

Eye shadows are applied to the eyelids to increase prominence of the eyes and the use of contrasting colours can enhance eye colour (Mulhern *et al.*, 2003). Eye shadows are available in a variety of formulations: pressed powders, creams, emulsions, anhydrous creams and pencils.

Powder eye shadows are one of the most popular formulations given the ease of application using a dry soft sponge-tipped applicator or brush. Cream and stick eye shadows are often comprised of pigments in an emollient, such as petrolatum, lanolin or cocoa butter. Cream eye shadows contain some water compared with anhydrous eye shadows that are free from water. Anhydrous formulations are waterproof however they often have a short wear time as the product tends to migrate into the eyelid folds, particularly for those with oily skin types or extra skin folds of the eyelids (Draelos, 1995b). Anhydrous sticks and pencils remain a popular choice and are composed of pigments based in petrolatum with added waxes to hold the consistency required for formation into a rod. The pencil formulation is encased in wood and the product is applied by rubbing the pencil across the lid. This formulation tends to be less creamy than its counterparts.

1.7   Documented ocular effects of eye cosmetics

Although eye cosmetics have been around for centuries, there are few reports of the ocular side-effects of their use. Those that have been reported are summarised in this next section.

1.7.1   Increased risk of eye infections

There is an increased risk of bacterial or fungal infections if contaminated eye cosmetic products are used. Normal commensal ocular flora include *Haemophilus species (sp*.), *Staphylococcus sp*., *Corynebacterium sp*., *Pneumococcus sp*. and *Streptococcus sp*. and are commonly found around the skin of the eyelids. They are not usually harmful however under certain conditions, bacteria can breed within cosmetic products and subsequently cause eye infections (Pack *et al.*, 2008).

Most eye cosmetics contain preservatives which inhibit bacterial growth within the product. One paper reports there must be more than 5000 organisms/mm in order to hinder the function of preservatives in liquid eyeliner (Wilson *et al.*, 1971). Repeated use of eye cosmetics by multiple users (e.g. testers at cosmetic counters) increases exposure to contaminants and can inoculate the product with bacteria (Dawson and Reinhardt, 1981). Contamination occurs at a much slower rate for products with only a single user (Pack *et al.*, 2008).

Bacterial presence within a product is proportional to the amount of use and the age of the product (Wilson, Julian and Ahearn, 1975), particularly as the effectiveness of preservatives diminishes with age (Bhadauria and Ahearn, 1980). After 3 months of use, microbial presence was found in over 30% of tested mascaras although microbial contamination is a characteristic of individual users (Pack *et al.*, 2008). The labelling of cosmetic products by manufacturers of products for sale in the European Union is governed by the European Cosmetics Regulation ((EC) No. 1223/2009). This states that cosmetic products must provide a symbol (Figure 1.15) indicating the length of time the product remains useable after opening, known as "the period after opening".
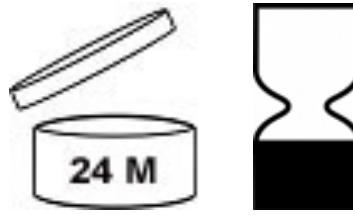
Figure 1.15 Symbols commonly found on cosmetic products. The "open jar" symbol (left) will contain the number of months (denoted by the letter M) the product remains usable after opening. The egg-timer symbol (right) will contain a "best-before" or expiry date.

1.7.2   Risk of mechanical trauma

Corneal trauma with mascara applicator wands is well documented (Wilson and Ahearn, 1977; Reid and Wood, 1979; Wilson *et al.*, 1990). Some users of liquid mascara separate clumped eyelashes using a straight pin, a technique which has led to corneal trauma (Wilson *et al.*, 1990). Corneal trauma gives microbes an immediate entry route to cause minor to severely sight-threatening infections. There are a number of papers where patients have suffered *Pseudomonas aeruginosa* infections secondary to mascara wand injuries where the mascara was contaminated with the bacterium following dilution with tap water (Wilson and Ahearn, 1977; Reid and Wood, 1979; Wilson *et al.*, 1990).

1.7.3   Toxicity

Kohl products, derived from lead compounds, are frequently used in Asia, Africa and the Middle East (Coroneo, Rosenberg and Cheung, 2006). In these cultures, it is applied to the eyes of infants to prevent evil spirits from entering into the child and is also used to prevent glare effects by applying it to the inner rim of the eyelid (behind the lash line) (Ullah *et al.*, 2010). Eyelid skin is very thin and allows for the easy penetration or absorption of chemicals (O'Donoghue, 2000). One study found levels of lead in the blood of infants aged between 6-16 months to be 62% greater than those infants to whom no kohl was applied (Nir *et al.*, 1992). The regular use of kohl has been shown to significantly elevate lead concentration in the blood and lower haemoglobin levels (Al-Ashban, Aslam and Shah, 2004). Additionally the deposition of lead and other heavy metals in the epidermis leads to peri-ocular hyperpigmentation in kohl users (El Safoury, El Fatah and Ibrahim, 2009).

1.7.4   Allergic responses

It is estimated that 12% of cosmetic reactions occur on the eyelid, 4% of which is due to eye cosmetic use (Draelos, 2001). Common allergens are perfume constituents and preservatives (Meyandier, Meyandier and Mark, 1994). Agents known to cause allergic contact dermatitis responses in susceptible users are summarised in Appendix II.

Eyelash cosmetics tend to cause a band of dermatitis along the lash line while eyelid cosmetics will affect the whole eyelid (Meyandier *et al.*, 1994). Eye cosmetics such as waterproof eye shadows and mascaras may require specialised cleansers to remove the product which may contain surfactants that can cause an adverse reaction in conjunction with extra mechanical action used to remove the cosmetic product (Draelos, 1995b).

1.7.5   Conjunctival pigmentation

Frequent and long-term use of mascara and eyeliner has been documented to cause conjunctival pigmentation ranging from diffuse pigmentation of the tarsal conjunctiva and conjunctival fornices to discrete, punctate deposits (Sugar and Kobernick, 1966; Donaldson, 1969; Platia, Michels and Green, 1978; Hidayat *et al.*, 1997).

1.7.6   "Masses" in the lacrimal system and conjunctiva

There are several cases where patients who were frequent users of mascara and kohls were found to have obstructions within the lacrimal system caused by accumulation of eye cosmetics. Hidayat *et al.* (1997) documented 10 patients who suffered abnormal pigmentation of the conjunctiva and lacrimal sac due to kohl use: eight suffered obstruction of lacrimal canaliculi and/or common lacrimal canaliculus; three suffered from dacryocutaneous fistulas; seven had black pigmentation of the lacrimal sac and surrounding anatomy. Black pigmentation of the lacrimal sac resembled a malignant melanoma with the absence of a mass and the biopsies revealed the pigment to be surrounded by a chronic inflammatory infiltrate and a high lead content in the tissue.

A similar reported case involves a 50-year old female was referred for investigation of an elevated mass on her bulbar conjunctiva, adjacent to her cornea. Histological examination of the mass showed it consisted of conjunctival epithelium with multiple dark particles,

likened to mascara; malignant cells were absent and the mass was subsequently termed a "mascaroma" (Shields *et al.*, 2005).

1.7.7   Changes in the tear lipid layer

Two studies have suggested that the use of cosmetics has a negative impact upon the tear lipid layer and the presence of foam at the inner canthus. Cosmetics have been suggested to be one major cause of lipid layer destabilization (Lozato, Pisella and Baudouin, 2001).

A series of studies examining foam located in the outer canthus of the eyelids has been conducted by Norn (Norn, 1963; Norn, 1987b1987a). Foam was described to be a mixture of sebum and meibum which, with repeated blinks, causes air bubbles to form in the lateral canthus of the eye which (Norn, 1963; Norn, 1987b). It has been shown that females using eye cosmetics had a lower incidence of foam at the inner canthus (Franck and Skov, 1989). It was hypothesised that the compounds in the cosmetics may bind to the lipids comprising foam, therefore reducing the amount of stable foam globules (Franck and Skov, 1989).

1.8   Legal standards of cosmetic manufacture

The European Commission define cosmetics as "*any substance or preparation intended to be placed in contact with the various external parts of the human body (epidermis, hair system, nails, lips and external genital organs) or with the teeth and the mucous membranes of the oral cavity with a view to exclusively or mainly cleaning them, perfuming them, changing their appearance and/or correcting body colours and or/protecting them or keeping them in good condition*". Therefore colour cosmetics, such as eye cosmetics, form only a small category within the broader definition of a cosmetic product.

The manufacture of cosmetics in the European market is regulated by the European Cosmetics Regulation ((EC) No. 1223/2009) which ensures that all cosmetics products are safe and cause no harm to human health. This newest regulation includes revisions adopted in 2009 to take into account the latest technological developments covering the use of nanomaterials. The primary purpose of the Cosmetics Regulation is human safety. Annex II of the European Cosmetics Regulation ((EC) No. 1223/2009) currently lists over 1300 substances that are prohibited in the use of cosmetics. There are strict guidelines on substances restricted to concentration limits and "positive-lists" which indicate only certain colorants, preservatives and UV filters can be used in cosmetics, outlined in Annexes III, IV, VI and VII respectively.

The UK has revised the Cosmetic Products Enforcement Regulations 2013 to reflect the European framework. UK regulations state that each cosmetic product must undergo a safety assessment performed by a duly qualified professional before it is placed on the market which must be conducted by one of the following: a pharmacist, medical practitioner, chartered biologist or a chartered chemist. No other person is authorised to carry out or take responsibility for safety assessments of cosmetic products. Despite many products labelled as "hypoallergenic, safe for sensitive skin, allergy tested, dermatologist tested, ophthalmologist tested, clinically tested, non-irritating, non-sensitising" there are currently no standard testing methodologies for such claims (Draelos, 2001).

1.8.1 Testing the eye irritation of cosmetic products

All cosmetic ingredients and products need to be tested to ensure it is safe for consumers to use through its intended purposes and in cases of accidental exposure to the eye. The testing of any finished cosmetic products on animals within the EU was banned from 11th September 2004. The testing ban on ingredients or combinations of ingredients applied from 11th March 2009. In the absence of alternative test methods, results of animal tests performed before 2009 can still be used to support the safety of the cosmetic product.

Until these bans were enforced, the Draize eye test was the international gold standard for assessing eye irritation (Wilhelmus, 2001). Rabbits are often the animals of choice for this test. The test material is applied to the conjunctival sac of the animal and the physiological responses of the cornea, iris and conjunctiva are scored accordingly after assessing the extent of injury. The Draize test became a very applicable test for many products, allowing the identification of severely irritant and corrosive substances and testing for moderate eye irritation potential. However, the test has been criticised as it is very painful to animals and the scoring is open to subjectivity resulting in low inter-laboratory reproducibility (Wilhelmus, 2001). Furthermore, there are differences in sensitivity to the substances tested in animals and man.

Amendments to the Draize eye test have been suggested and implemented to reduce the pain and allow for higher recovery rates of animals. Firstly, the low volume eye test uses one-tenth of the test material compared to the conventional Draize test and is applied to the rabbit's cornea, without holding the eyes shut after instillation (Cormier *et al.*, 1996). Secondly, reserving the Draize test for the final stage of assessment when all other tests produce a negative result has also reduced the amount of animal testing (Eskes *et al.*, 2004). However, implementing this only allows assessment of mild to moderate irritants and also does not eliminate the need for an *in vivo* test.

1.8.2 *In vivo* assessment of irritation

In vivo eye irritation assays are assessed by topical application of the test material to the ocular surface however the duration of exposure of the test material is unknown and can vary between animals. Exposure to deeper cell layers depends on the speed and ability of a

chemical to penetrate and fix to surface layers before being flushed away. The time taken for cells to manifest cytotoxicity, the level at which substances become toxic to cells, is not equal to the exposure time required for cells to be injured and necrotise (Eskes *et al.*, 2004). For example, reactive chemicals such as bleach show delayed onset of toxicity thus the evaluation of injury is pertinent after one day of exposure to the ingredient.

In the Draize test, the depth of corneal injury in the first three hours of chemical exposure is predictive of the eventual degree and duration of ocular lesions in rabbits (Eskes *et al.*, 2004). The degree of corneal damage arising from increasing levels of irritants is summarised in Table 1.6.

Table 1.6 Depth of corneal injuries sustained from exposure to increasing irritants (summarised from (Eskes *et al.*, 2004)

Degree of irritancy	Depth of corneal injury
Slight irritants	Superficial corneal epithelium
Mild to moderate irritants	Primarily corneal epithelium & superficial stroma
Severe irritants	Deep stroma or full stromal depth

1.8.3 Developments of alternative methods to animal testing

Six major validation and evaluation studies took place between 1991 and 1997 to determine alternatives to the Draize test (Cormier *et al.*, 1996) in preparation for phasing out animal activity in ingredient and product testing. Alternative methods to the Draize test include using: isolated organs, chorioallantoic membranes, human corneal epithelial models, cell-based cytotoxicity assays and cell-function based assays. These methods will be briefly described (Eskes *et al.*, 2004):

1.8.3.1 *Isolated organs*

Corneas from isolated eyes, typically of bovine, porcine, chicken or rabbit origin, derived from slaughterhouses or from animals used for other toxicological testing, are subjected to exposure to test materials. Properties such as corneal opacity, permeability, hydration and

thickness are examined in addition to further screening of each individual corneal layer. Histological examination is conducted as an endpoint to the test, and the depth of injury of the test material is compared to that in a reference material.

1.8.3.2 Organotypic methods

Chorioallantoic membranes (CAM) have been used to model the side effects of mild to severe irritants on conjunctival tissue. CAM are derived from fertilised chicken eggs and testing occurs on the ninth day of embryonation when nerve tissue and pain perception has not yet developed. The test material is placed directly onto a CAM and observed for signs of haemorrhage, lysis and coagulation within five minutes. Variations of tests using CAM exist, including observations of hyperaemia and ghost vessels, and the use of trypan blue staining to quantify CAM destruction.

1.8.3.3 Human corneal epithelium models

The EpiOcular^TM corneal model (MatTek, Ashland, MA, USA) and SkinEthic *in vitro* human corneal epithelium (HCE) model (SkinEthic, Nice, France) are currently the most promising methods available for achieving animal replacement. The EpiOcular^TM assay consists of human-derived keratinocytes cultured in a serum-free medium. The cells differentiate to form a multi-layer structure, similar to that of human corneal epithelium, thus providing a good construct for assessing cell damage resulting from mild to moderate irritants. The SkinEthic HEC model consists of immortalised human corneal epithelial cells cultured on a polycarbonate substrate to form epithelial tissue, allowing for tests including the release of inflammatory cytokines and gene expression.

1.8.3.4 Cell based cytotoxicity methods

The neutral red uptake, neutral red release and red blood cell haemolysis assays are tests which look at cytotoxicity at a cellular level. These tests tend to have limited potential in determining moderately and severely irritating substances in isolation however, can prove useful when used in conjunction with a battery of other tests

1.8.3.5   Cell function based assays

In the fluorescein leakage test (FLT), adherent cell cultures are grown to form tight junctions and desmosomes, mimicking corneal epithelium barrier function, on inserts that separate the medium into two compartments. Following exposure to the test substance, cell damage is assessed by measuring the permeability of sodium fluorescein from the upper compartment to the lower compartment using spectrophotometric methods. The FLT can also be used to monitor cellular recovery after exposure to the test substance.

Other cell function based assays measure irritation potential indirectly. Cultured cells release acidic metabolic products; a silicon microphysiometer can measure pH changes of the culture medium, during and after the administration of test substances which is repeated until cells no longer release any acidic metabolites.

1.8.3.6   The use of human subjects

A range of tests have been validated and approved for testing the safety of ingredients and finished products before they become available to consumers. The rigorous tests conducted under tightly controlled environments ensure European Cosmetic Regulations are met and cosmetic products are safe and cause no harm to human health. *In vitro* test results are not always consistent with human clinical subjective and objective measurements (Burdick *et al.*, 2003) although there is a lack of literature available to support this. This is often due to ethical considerations required for using human subjects (White *et al.*, 2010). Products that present at the stage of testing on human subjects will have established toxological profiles and no pre-existing concerns prior to application. They are also only tested on a small number of subjects to establish skin and mucous membrane compatibility (White *et al.*, 2010).

One published study conducted on human subjects documents examiners provocatively instilling neat formulations of liquid cosmetics, mascara and powder eye shadow directly into the inferior fornix of the eyes of human subjects (Gao and Kanengiser, 2004). This was performed to mimic accidental ocular contamination which may occur in real-life situations and observe subsequent clinical changes. Post-instillation evaluation tests were conducted at intervals of: 30 seconds, 5 minutes, 60 minutes, 120 minutes and 24 hours. Fluorescein

staining was scored significantly higher following powder eye shadow instillation than the other eye cosmetic products. Mascara and powder eye shadow showed the highest scores of subjective irritation 30 seconds after instillation, however symptoms dropped markedly after 5 and 15 minutes respectively.

No *in vivo* human safety testing is conducted during the early developmental phases of cosmetic manufacture and any *in vivo* human outcomes predominately rest on consumer satisfaction.

1.9   Summary of literature

All cosmetics in the European market have undergone vigorous tests to ensure they are safe for human use. Many of the papers documenting the side-effects of eye cosmetic use were published prior to 2000 and refer to dated formulations of products. Furthermore, the toxicity-related studies refer to formulations of kohl commonly available in the Middle East and Africa. Besides the increased risk of eye infections, there is still little knowledge of the ocular effects of modern, Western cosmetic formulations. With continuous investment into research and development of cosmetic formulations to meet current day technology and consumer demands, there is little suggestion that long-term use of eye cosmetics poses any detrimental changes to ocular health.

The prevalence of dry eye has been shown to be greater in women in epidemiological studies; potentially partly due to increased cosmetic use amongst the female population (Lozato *et al.*, 2001; Wolkoff, 2008), amongst other factors. Contact lens patients often report symptoms of dryness, of which eye cosmetic use has been suggested to play a role (Lozato *et al.*, 2001; Guillon and Maissa, 2005; Craig *et al.*, 2013), however there is a lack of evidence-based research to prove this.

1.10 Aims and hypotheses

The aims of this thesis are to:

- Examine the impact of eye cosmetic usage on ocular comfort
- Investigate the migration of cosmetic products that are applied externally around the eyes (step 1 in Figure 1.16)
- Determine the clinical effects of eye cosmetics on the ocular surface and tear film (step 2 in Figure 1.16)
- Examine the biochemical effects resulting from eye cosmetics application (step 3 in Figure 1.16)
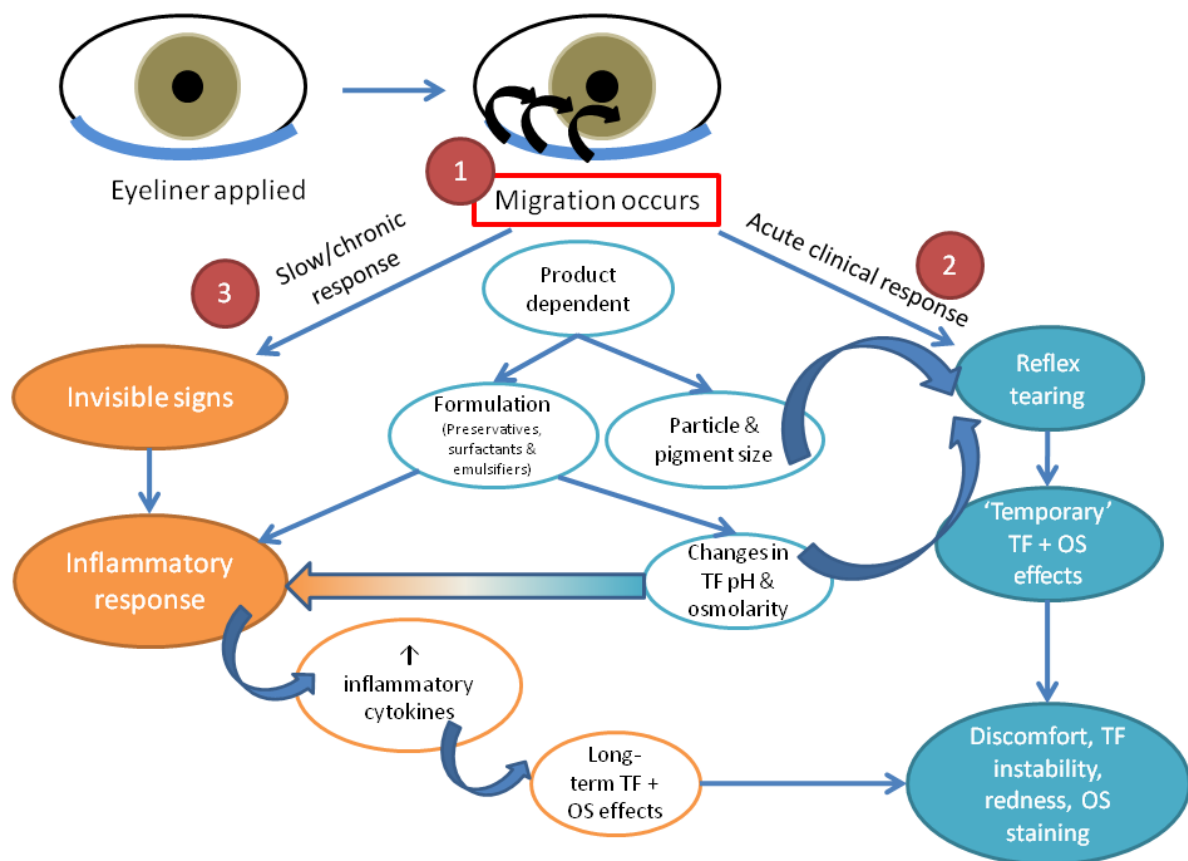
Figure 1.16 The hypothesis cascade (TF = tear film; OS = ocular surface)

The hypotheses include:

- Ocular comfort is greater in those that do not use eye cosmetics

- Cosmetic products applied to the peri-ocular skin migrate – this can be detected clinically and sub-clinically (illustrated in steps 2 and 3 in Figure 1.16)

- The effects of cosmetic product migration compromises the ocular surface and tear film (illustrated in step 2 in Figure 1.16)

- The migration of eye cosmetics onto the ocular surface can induce ocular surface inflammation which may not manifest immediately, unlike the clinical signs (illustrated in step 3 in Figure 1.16)

2 A survey of eye cosmetic usage and associated ocular comfort

This chapter forms the basis of a first study for this thesis. A selection of the results has been published in a peer-reviewed journal which can be found in the Appendix (Ng *et al.*, 2012).

2.1 Introduction

Colour cosmetics encompass: face cosmetics (including foundations, blushers, pressed and loose powders), eye cosmetics (including eye shadows, eyeliners, eyebrow pencils and mascara), lip cosmetics (including lipstick, lip glosses and lip pencils) and nail cosmetics (including nail varnishes, hardeners and strengtheners) (Mintel, 2013). Since 2001, the market research company, Mintel, annually report the trends and consumer opinions in colour cosmetic products in the UK. In the 2013 report, 30% of the colour cosmetics market is dominated by eye cosmetics (Mintel, 2013). While market research provides some information about the popularity and motivation behind the use of eye cosmetics, there is no published data that explores the eye cosmetic use with relation to reported clinical ocular effects. The cessation of contact lens wear has been attributed to dry eye and discomfort (Young, 2004; Richdale *et al.*, 2007; Nichols *et al.*, 2013). The intrinsic relationship between these issues with the influence of external factors such as regular eye cosmetic use has been suggested (Lozato *et al.*, 2001; Guillon and Maissa, 2005; Craig *et al.*, 2013) but not well established.

An important first study for this thesis was to examine cosmetic usage in a UK population so that the impact of potential issues uncovered by subsequent experiments in this thesis would be appreciated. This was achieved by using an online survey.

2.1.1 Aims

The primary aims of this cosmetics survey included establishing:

- The eye cosmetic products commonly used by the population
- The habits of eye cosmetic users
- The differences in global comfort and perceived comfort between cosmetic and non-cosmetic users

- The opinions and implications surrounding the usage of eye cosmetics

The survey also aimed to compare the responses given by contact lens wearers (CLW) and non-contact lens wearers (NCLW) and establish any differences in cosmetic habits, perceived comfort and opinions between these groups.

2.2   Methods

The survey was designed to be completed online using the survey software provider, Bristol Online Survey (University of Bristol, Bristol, UK) and hosted on the Cardiff University network. A copy of the survey is included at the end of this section between pages 59-64 (Figure 2.1 - Figure 2.6).

The survey comprised of 23 questions, divided into four distinct sections:

- Section 1: establish demographics and baseline data including gender, age, spectacle and contact lens (CL) use, eye drop use, allergies and eye sensitivity.

- Section 2: assign all respondents with a global comfort score using Ocular Surface Disease Index (OSDI; Allergan Inc., Irvine, California). The OSDI questionnaire was selected to represent global comfort of respondents as it has been shown to be a reliable and valid instrument for measuring and classifying the severity of dry eye syndrome (Schiffman *et al.*, 2000). Upon completion of the questionnaire, a score from 0-100 is assigned where a greater score indicates greater dry eye symptoms. Scores greater than 15 are indicative of clinically diagnosed dry eye (Schiffman *et al.*, 2000).

- Section 3: determine the use of eye cosmetics amongst respondents, including the frequency of use and the products of choice. Respondents were also asked to rate their perceived comfort of their eyes on an average day when cosmetics were and were not used, using a scale from 0 to 10 where 0 represented "very uncomfortable" and 10 represented "very comfortable".

- Section 4: collect the opinions of respondents on six statements regarding the effects of eye cosmetic use.

The survey was reviewed by a focus group, consisting of staff and students within the School of Optometry and Vision Sciences in addition to naïve subjects, and was reviewed on a

further 2 occasions following revisions. The survey was launched on 14th February 2011 and all staff and students holding a Cardiff University email address received an email inviting their participation in the survey. All responses were anonymous.

Cosmetics questionnaire

Welcome

Thank you for taking the time to complete this survey about eye make-up. You do not have to be a make-up wearer to take part. This survey is run by the School of Optometry & Vision Sciences at Cardiff University.

This survey will aim to find out about your eyes, the type of eye make-up you may use and how comfortable your eyes normally feel. It will also ask what you think about eye make-up. It should not take more than 10 minutes to complete.

The questionnaire is anonymous so please answer the questions as honestly as possible.

If you have any questions please get in touch using the contact details below.

Yours sincerely,

Alison Ng
Postgraduate Researcher
School of Optometry and Vision Sciences
Cardiff University
Maindy Road
Cardiff
CF24 4LU

029 2070556

Email: NgA@cardiff.ac.uk

My supervisor is Dr Christine Purslow, and you can email her at: PurslowC@cardiff.ac.uk

Data Protection

For the purposes of this survey Cardiff University is the data controller. All data collected in this survey will be held securely by the survey software provider (Bristol University) under contract and then retained by the School of Optometry, Cardiff University in accordance with the Data Protection Act (1998). Data from the survey, including answers to questions where personal details are requested, will only be used by the School of Optometry for research purposes.

Cookies, personal data stored by your Web browser, are not used in this survey.

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https://www.surveys.cardiff.ac.uk/cosmetics   25/07/2011 15:24:17

Figure 2.1 Page 1 of the Online Eye Cosmetics Survey

Cosmetics questionnaire

1. What is your gender?

- ○ Male
- ○ Female
- ○ Prefer not to say

2. How old are you?

[   ]

3. What is your area of study/work?

[Select an answer   ▼]

If you selected Other, please specify:

[   ]

4. What is your main occupation?

[Select an answer   ▼]

If you selected Other, please specify:

[   ]

5. Do you wear spectacles?

○ Yes ○ No

6. Do you use contact lenses?

○ Yes ○ No

    a. Do you use soft contact lenses or hard (rigid gas permeable) contact lenses?
- ○ Soft contact lenses
- ○ Hard (rigid) gas permeable contact lenses

    b. How often do you use contact lenses?

    [Select an answer   ▼]

    c. How often do you replace your contact lenses?

    [Select an answer   ▼]

    If you selected Other, please specify:

    [   ]

7. Do you use any eye drops to make your eyes feel more comfortable?

○ Yes ○ No

    How often do you use these drops?

    [Select an answer   ▼]

    If you selected Other, please specify:

    [   ]

8. Do you suffer from hay fever or eye allergies?

○ Yes ○ No

9. Do you consider yourself to have sensitive eyes?

○ Yes ○ No

Figure 2.2 Page 2 of the Online Eye Cosmetics Survey

60

10. On average, how often do you rub your eyes?

Select an answer ▾

11. Have you experienced any of the following **during the last week**?

	All of the time	Most of the time	Half of the time	Some of the time	None of the time
a. Eyes that are sensitive to light?	○	○	○	○	○
b. Eyes that feel gritty	○	○	○	○	○
c. Painful or sore eyes?	○	○	○	○	○
d. Blurred vision?	○	○	○	○	○
e. Poor vision?	○	○	○	○	○

12. Have problems with your eyes limited you in performing any of the following **during the last week**?

	All of the time	Most of the time	Half of the time	Some of the time	None of the time	N/A
a. Reading?	○	○	○	○	○	○
b. Driving at night?	○	○	○	○	○	○
c. Working with a computer or bank machine (ATM)?	○	○	○	○	○	○
d. Watching TV?	○	○	○	○	○	○

13. Have your eyes felt uncomfortable in any of the following situations **during the last week**?

	All of the time	Most of the time	Half of the time	Some of the time	None of the time	N/A
a. Windy conditions?	○	○	○	○	○	○
b. Places or areas with low humidity (very dry)?	○	○	○	○	○	○
c. Areas that are air conditioned?	○	○	○	○	○	○

14. Do you use eye make-up?

○ Yes ○ No

How often do you wear eye cosmetics?

Select an answer ▾

15. On an average day, please tick all the products that you apply **around the eyes**:

If you do not use make-up, or any particular type, please select "I do not use this product"

	Average number of days per week

Figure 2.3 Page 2 of the Online Eye Cosmetics Survey (continued)

	0	1	2	3	4	5	6	7	I do not use this product
a. Under-eye concealer or highlighter	O	O	O	O	O	O	O	O	O
b. Primer or foundation applied around the eyelids	O	O	O	O	O	O	O	O	O
c. Mascara	O	O	O	O	O	O	O	O	O
d. False eyelashes	O	O	O	O	O	O	O	O	O
e. Liquid eyeliner - *outside* the lash line *(see Figure 1)*	O	O	O	O	O	O	O	O	O
f. Liquid eyeliner - **within** the lash line *(see Figure 2)*	O	O	O	O	O	O	O	O	O
g. Pencil eyeliner - *outside* the lash line *(see Figure 1)*	O	O	O	O	O	O	O	O	O
h. Pencil eyeliner - **within** the lash line *(see Figure 2)*	O	O	O	O	O	O	O	O	O
i. Powder eye shadow	O	O	O	O	O	O	O	O	O
j. Cream eye shadow	O	O	O	O	O	O	O	O	O

Figure 1 - shows eyeliner applied **outside** the lash line
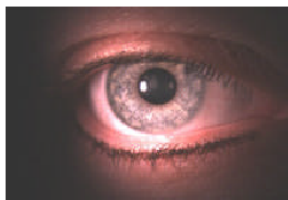
Figure 2 - shows eyeliner applied **within** the lash line

16. Do you touch up or reapply your eye make-up throughout the day?

If you do not wear eye make-up, please select N/A (Not Applicable)

○ Yes ○ No ○ N/A

17. If you were allowed to use only one eye make-up product, which would it be?

If you do not wear eye make-up, please select N/A (Not Applicable)

Select an answer ▾

Figure 2.4 Page 2 of the Online Eye Cosmetics Survey (continued)

62

18. When do you replace your eye makeup?

If you do not wear eye make-up, please select N/A (Not Applicable)

	When the product runs out	As directed by the packaging	When the product becomes contaminated or spoiled	At regular timed intervals (e.g. monthly)	I do not replace it	Not applicable (N/A)	Other (please specify)
a. Under-eye concealer or highlighter	☐	☐	☐	☐	☐	☐	☐
b. Primer or foundation around the eyelids	☐	☐	☐	☐	☐	☐	☐
c. Mascara	☐	☐	☐	☐	☐	☐	☐
d. False eyelashes	☐	☐	☐	☐	☐	☐	☐
e. Liquid eyeliner	☐	☐	☐	☐	☐	☐	☐
f. Pencil eyeliner	☐	☐	☐	☐	☐	☐	☐
g. Powder eye shadow	☐	☐	☐	☐	☐	☐	☐
h. Cream eye shadow	☐	☐	☐	☐	☐	☐	☐

19. Have you ever stopped wearing eye make-up for any of the following reasons?
(select all that apply)

☐ Skin allergy (your skin reacted)
☐ Eye allergy (your eyes reacted)
☐ Mild eye irritation
☐ Eye infection
☐ A healthcare professional told me to
☐ I have never stopped wearing make-up for any reasons like these
☐ I do not wear eye make-up
☐ Other *(please specify)*:

20. Do you remove your eye makeup before going to sleep?

If you do not wear eye make-up, please select N/A (Not Applicable)

○ Always
○ Usually
○ Rarely
○ Never
○ N/A

21. On the scale from 0 - 10, how would you rate the comfort of your eyes on an **average day that you use eye make-up**?

0 = very uncomfortable, 10 = very comfortable

○0 ○1 ○2 ○3 ○4 ○5 ○6 ○7 ○8 ○9 ○10 ○I do not wear eye make-up

22. On the scale from 0 - 10, how would you rate the comfort of your eyes on an **average day that you *do not* use eye make-up**?

0 = very uncomfortable, 10 = very comfortable

○0 ○1 ○2 ○3 ○4 ○5 ○6 ○7 ○8 ○9 ○10 ○I do not wear eye make-up

Figure 2.5 Page 2 of the Online Eye Cosmetics Survey (continued)

23. Please rate your agreement or disagreement with each of the following statements

	Strongly DISAGREE	Disagree	No opinion	Agree	Strongly AGREE
a. Wearing eye make-up has no influence on the comfort of my eyes	○	○	○	○	○
b. Wearing eye make-up is good for my eyes	○	○	○	○	○
c. Wearing eye make-up has a detrimental effect on the health of my eyes	○	○	○	○	○
d. Eye make-up ends up in my eyes by the end of the day	○	○	○	○	○
e. I would not leave the house without applying some type of eye make-up	○	○	○	○	○
f. My eyes will be just as healthy in 20 years time whether or not I wear eye make-up	○	○	○	○	○

This is the end of the questionnaire.

Please click the CHECK ANSWERS & CONTINUE button to review your answers and submit your completed questionnaire.

Continue >

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Figure 2.6 Page 2 of the Online Eye Cosmetics Survey (continued)

2.2.1 Analysis of completed surveys

Data obtained from the survey was downloaded as a Microsoft Excel worksheet (Microsoft. Microsoft Excel. Redmond, Washington: Microsoft, 2007) from Bristol Online Survey on 18th April 2011. OSDI scores were calculated within Microsoft Excel using the information provided by respondents in Section 2 with the equation

$$OSDI = \frac{(Sum\ of\ scores\ X\ 25)}{Number\ of\ questions\ answered}$$

Statistical analysis of data was performed using Microsoft Excel 2007 and SPSS 16.0 for Windows (SPSS Inc., Chicago, IL). Data was tested for normality using a Kolmogorov-Smirnov test. Since the data were not normally distributed, non-parametric statistics were applied. Data analysis for categorical data and continuous variables was conducted using the χ^2 test, and the Mann-Whitney U test or Kruskal-Wallis test (where appropriate), respectively. Two-sided p-values<0.05 were considered significant for all statistical tests. Data are presented as median values with interquartile (IQ) ranges in parentheses.

Where boxplots are presented, circles (o) represent outliers between one and a half to three times greater than the IQ range and asterisks (*) represent outliers three times greater than the IQ range.

2.3 Results

These data was examined to find the demographics of respondents. As a proportion of male respondents reported using cosmetics, this data was analysed separately and not compared to female data beyond the demographics analysis due to considerably fewer numbers. The frequency of eye cosmetics use were examined and correlated with ocular comfort scores. Specifically, the relationship between eyeliner use and related comfort scores is explored in greater detail due to the close proximity of the application of these products with the ocular surface. The data is then re-examined with respect to contact lens wear (CLW) and non-contact lens wear (NCLW). Lastly, the opinions of cosmetic users to questions relating to the perception of the potential ocular effects of eye cosmetic use were explored.

2.3.1 Survey demographics and overview of cosmetics use

The survey was completed by 1462 respondents. The median age of respondents was 25 (IQ range 21-35 years); 1360 (93%) were female and 102 (7%) were male.

Table 2.1 summarises the population demographics.

Table 2.1 Population demographics

	Total cohort
n	1462
M : F	102 : 1360
Age (years)	25 (IQ range 21-35)
Eye drop use	337 (23%)
Sensitive eyes	478 (33%)
Hay fever or eye allergies	540 (37%)

There were 1297 respondents (89%) that reported use of eye cosmetics. 1206 (93%) of cosmetic users were female; 91 male respondents reported wearing eye cosmetics.

Figure 2.7 shows the frequency of cosmetic usage by respondents. 83% all respondents reported using eye cosmetics at least 3-5 times per week.
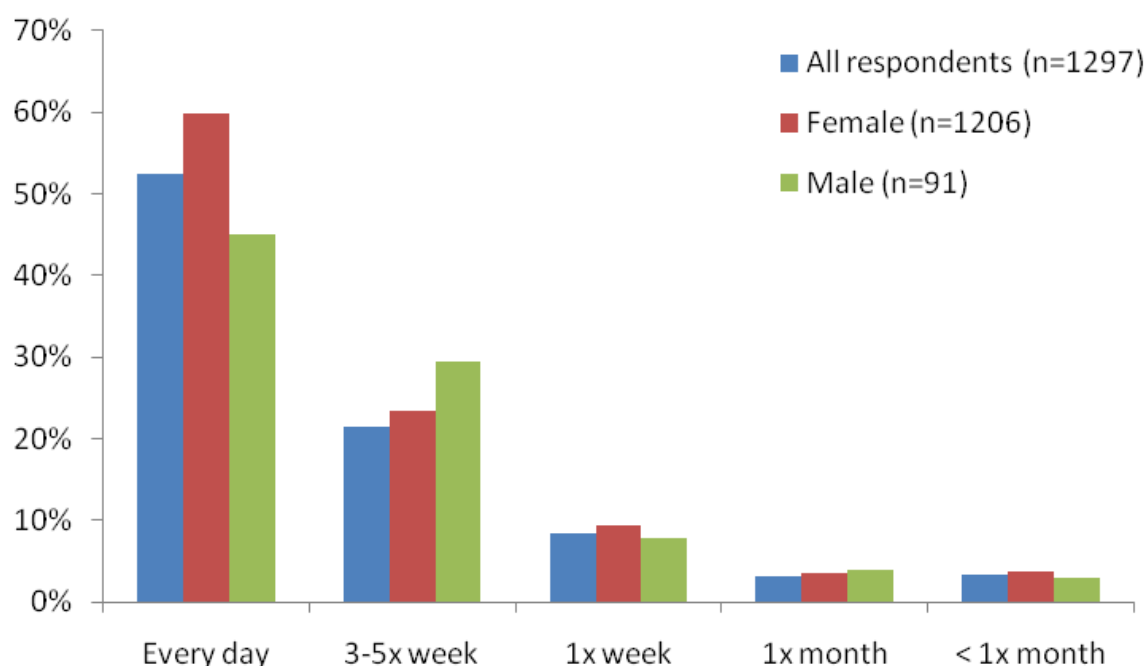
Figure 2.7 Frequency of eye cosmetics use amongst respondents of the cosmetics survey

Regular usage of cosmetic products was defined as use of the product three or more (≥3) times a week. The most regularly used cosmetic products applied around the eyes were mascara (70%), pencil eyeliner (50%) and foundation (45%), as shown in Table 2.2. In male users, the most regularly used products were mascara (69%), pencil eyeliner (49%) and foundation (40%).

Table 2.2 Frequency of regular product use

Product	% of all cosmetic users
Mascara	70
Pencil eyeliner	50
Foundation	45
Concealer	36
Powder eye shadow	24
Liquid eyeliner	17
Cream eye shadow	4
False lashes	1

2.3.2   Perceived ocular comfort with and without eye cosmetics

Eye cosmetic users were asked to rate on an ordinal scale of 0-10 the perceived ocular comfort of their eyes on days that eye cosmetics are applied and not applied, where 0 = very uncomfortable eyes and 10 = very comfortable eyes. Figure 2.8 illustrates the distribution of perceived ocular comfort scores. The median perceived ocular scores were greater without cosmetics (9 vs. 8 respectively). A Wilcoxon signed rank test showed respondents were significantly more comfortable when cosmetics were not used (p<0.001).
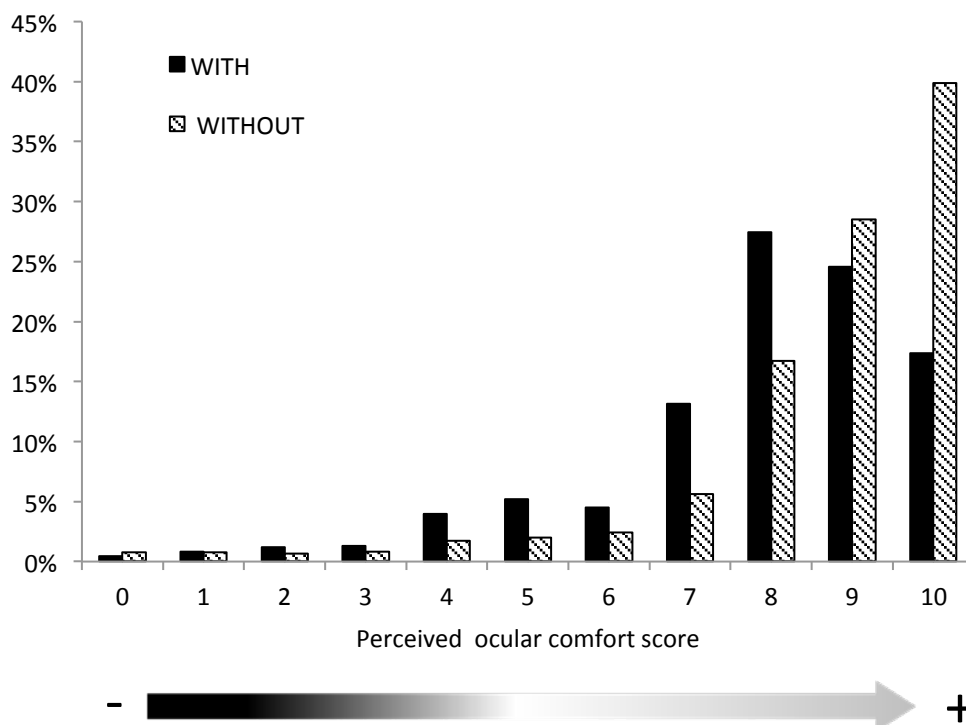
Figure 2.8 Distribution of perceived comfort scores of cosmetic users that responded to the cosmetics survey (n=1297)

2.3.3   Relationship between OSDI scores and cosmetics usage

The median OSDI score (reflecting symptoms of ocular dryness in the previous week) for the total cohort was 10.4 (IQ range 4.2-16.7), with a range of scores between 0-85.4. In the total cohort, 418 respondents (29%) exceeded a score of 15, the validated cut-off for dry eye diagnosis (Schiffman *et al.*, 2000). In the small number of male respondents, the median OSDI score was 8.3 (IQ range 4.2-14.1).

Although eye cosmetic users indicated greater dry eye symptoms with higher OSDI values compared to non-users (10.4, IQ range 4.2-16.7 vs. 8.3, IQ range 2.1-14.6 respectively), this difference failed to reach statistical significance (Mann-Whitney U test, p=0.065), as illustrated in Figure 2.9.
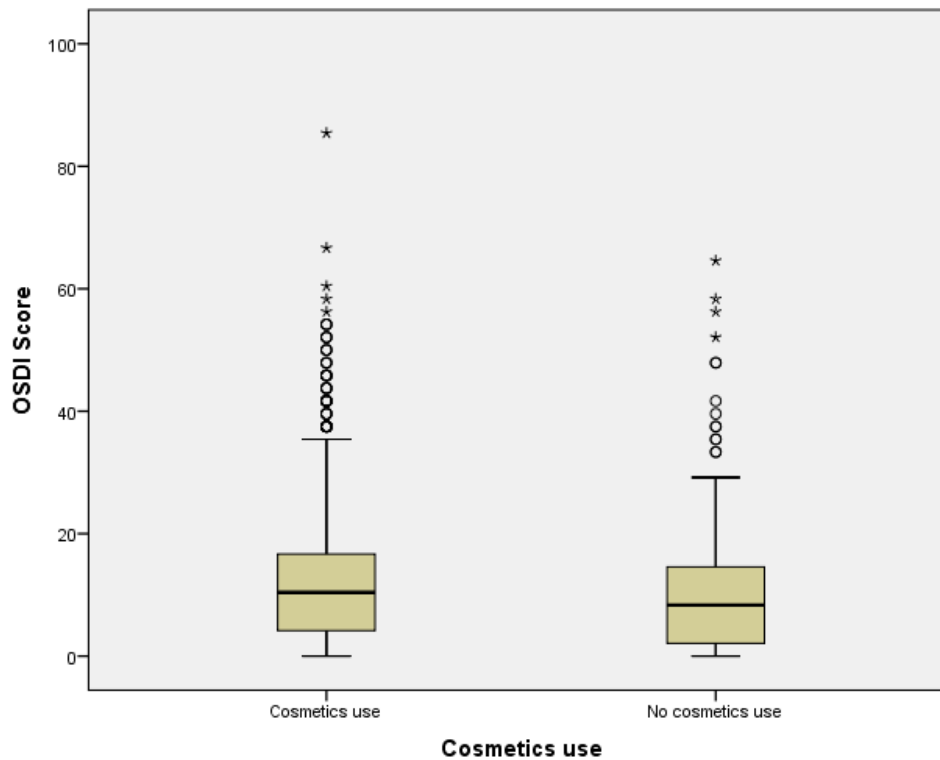
Figure 2.9 OSDI comparison for cosmetic users (n=1297) and non-cosmetic users (n=165) in the cosmetics survey

The data set of cosmetic users was further explored for differences in median OSDI scores according to frequency of cosmetics use, summarised in Figure 2.10. There was no apparent trend in median OSDI scores with increasing frequency of cosmetics use, and there was no significant difference in median OSDI scores between the groups (Kruskal-Wallis test, p=0.922).
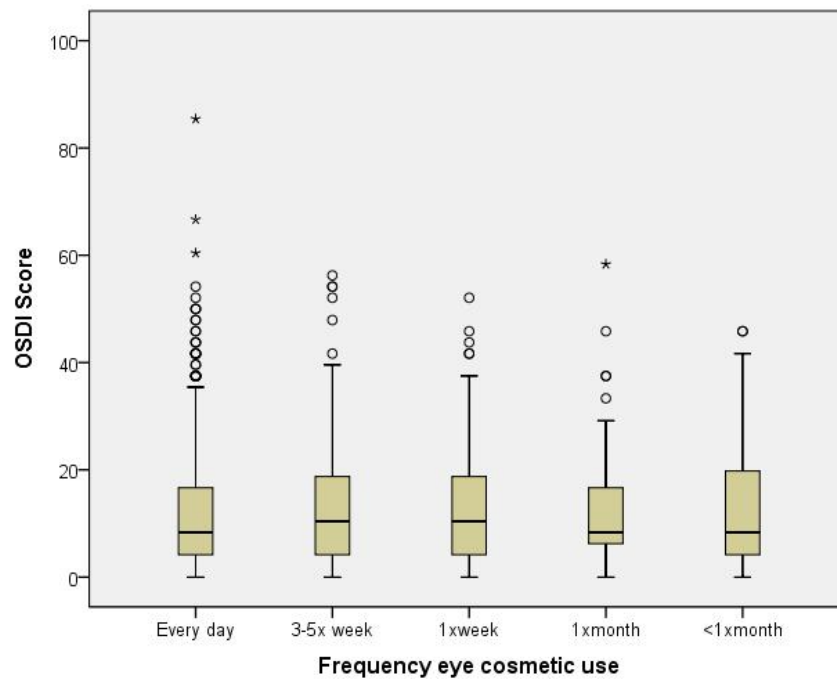
Figure 2.10 Median OSDI scores according to frequency of cosmetics use amongst users of cosmetics (n=1297)

2.3.3.1   The relationship between OSDI scores and regular product usage

Analysis of the median OSDI score of cosmetic users was conducted for each cosmetic product comparing regular (≥3 times a week) or light (<3 times a week) usage with Mann-Whitney U tests. Table 2.3 shows a summary of the results.   The numbers of respondents who were regular users of false lashes was comparatively small, therefore no comparison was made. There was no statistical difference in median OSDI scores between regular and light users of any other product.

Table 2.3 Median (IQ in parentheses) OSDI scores for regular and light usage of each cosmetic product

	Concealer	Foundation	Mascara	Powder eye shadow	Cream eye shadow	Eyeliner	False lashes
Regular users (≥3x)							
n	472	586	909	447	51	616	11
Median OSDI	10.4 (4.2-18.8)	10.4 (4.2-16.7)	10.4 (4.2-16.7)	8.3 (4.2-18.8)	10.4 (6.3-17.7)	10.4 (4.2-17.2)	12.5 (4.2-22.9)
Light users (<3x)							
n	327	317	350	659	446	522	345
Median OSDI	10.4 (4.2-17.7)	10.4 (6.3-18.8)	10.4 (4.2-18.8)	10.4 (4.2-16.7)	10.4 (4.2-18.8)	8.3 (4.2-16.7)	12.5 (6.3-12.8)
P-value (Mann-Whitney U test)	0.927	0.176	0.489	0.487	0.962	0.159	-

The survey was designed to explore eyeliner use according to product type and application position, and this analysis is summarised in Table 2.4. Median OSDI scores indicated reduced comfort in the eyeliner-using group (10.4, IQ 4.2-16.7) compared to the non-using group (8.3, IQ 2.1-16.7), which was significantly different (Mann-Whitney U test, p=0.007, Figure 2.11). OSDI scores were similar irrespective of the position of eyeliner application (within the lash line vs. outside the lash line), and the type of eyeliner applied (pencil vs. liquid) as shown in Figure 2.12.

Table 2.4 Comparison of OSDI scores according to eyeliner use, eyeliner formulation and application method (ELI = eyeliner applied within the lash line; ELO = eyeliner applied outside the lash line)

	n	Median (IQ) OSDI score	P-value (Mann-Whitney U test)
Any eyeliner use			
- Eyeliner use	1082	10.4 (4.2-16.7)	0.007
- No eyeliner use	215	8.3 (2.1-16.7)	
ELI product comparison			
- Pencil ELI	505	10.4 (6.3-16.7)	0.400
- Liquid ELI	27	14.6 (4.2-21.9)	
ELO product comparison			
- Pencil ELO	513	10.4 (4.2-16.7)	0.480
- Liquid ELO	163	10.4 (4.2-18.8)	
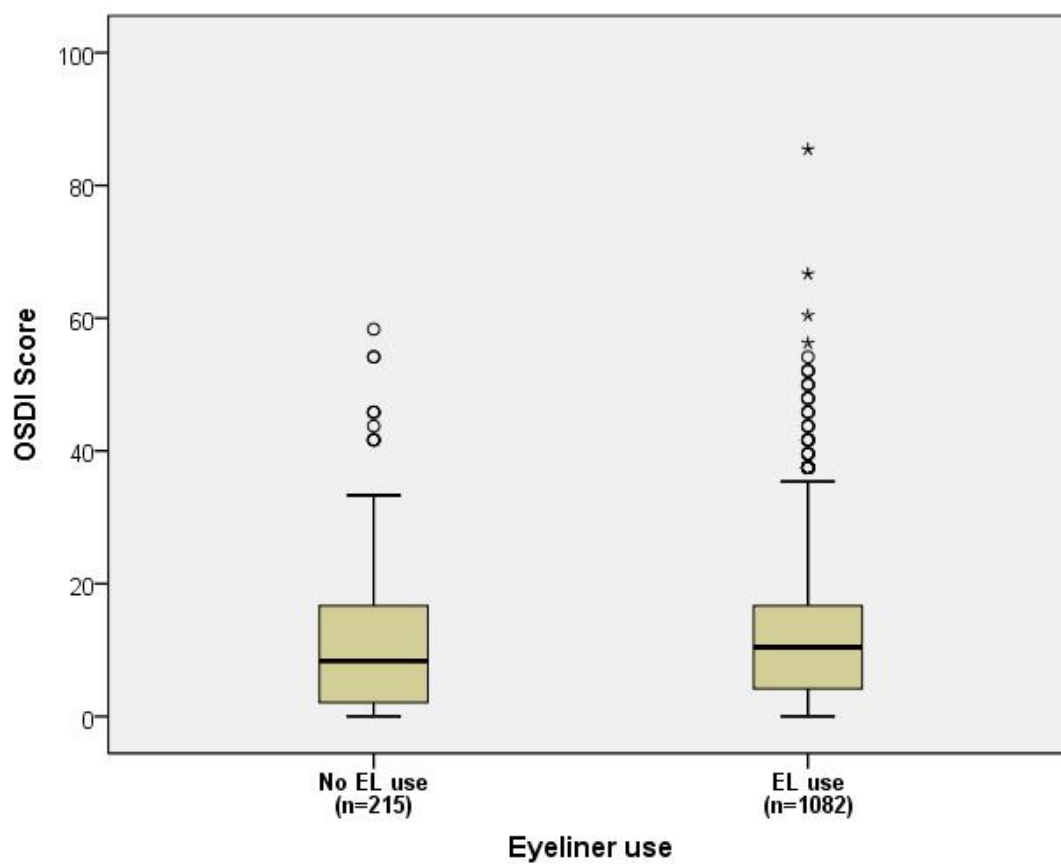
Figure 2.11 OSDI scores of respondents in the cosmetics survey that reported they did not use eyeliner vs. the respondents that reported usage of eyeliner (p=0.007)
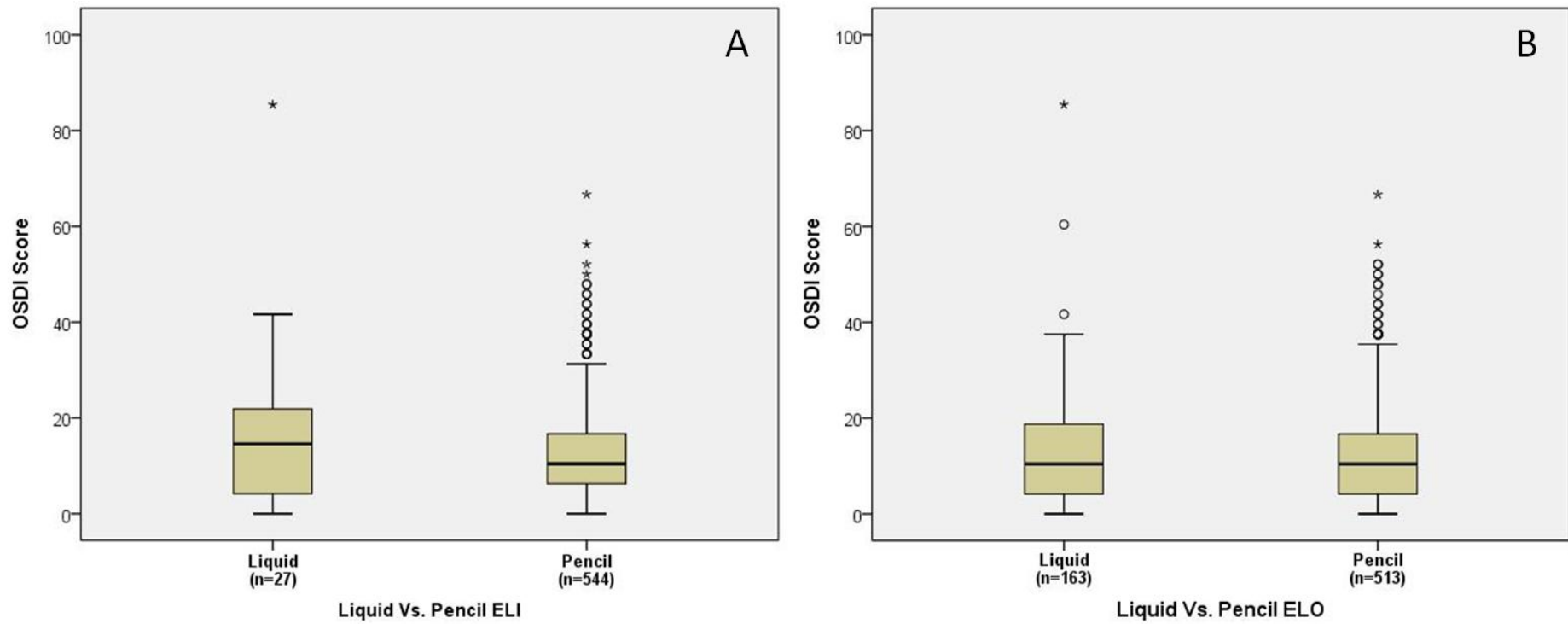
Figure 2.12 OSDI scores of respondents that used eyeliner according to eyeliner formulation (liquid vs. pencil) with ELI application (p=0.400) (A) and ELO application (p=0.480) (B)

2.3.4   Contact lens wear and eye cosmetics

The data from the total cohort was segregated by contact lens (CL) wear to compare perceived ocular comfort and OSDI scores in CL wearers (CLW) compared to non-contact lens wearers (NCLW).

Of the total cohort, 466 respondents were CLW and 996 were NCLW. Table 2.5 shows a comparison of the demographics between these groups. A greater proportion of CLW (35%) reported the use of eye drops to improve ocular comfort compared with NCLW (17%). When asked if respondents considered themselves to have sensitive eyes, 34% CLW and 32% NCLW felt they had sensitive eyes. There was a slightly higher proportion of CLW reporting ocular allergy (41%) compared with the NCLW group (37%).

Table 2.5 Comparison of demographics between CLW and NCLW

	CLW	NCLW
n	466 (32%)	996 (68%)
M : F	26 : 440	76 : 920
Age (median years)	25 (21-32)	24 (20-35)
Eye drop use	164 (35%)	173 (17%)
Sensitive eyes	156 (34%)	322 (32%)
Hay fever or eye allergies	189 (41%)	370 (37%)

The majority of CLW were soft lens wearers (96%); almost half of these subjects wore CLs every day (Table 2.6).

Table 2.6 CLW demographics

CL type	n (% of CL users)
Soft CL	446 (96%)
RGP	20 (4%)
Freq CL use	
Every day	208 (45%)
3-5x week	99 (21%)
Once a week	78 (17%)
Once a month	33 (7%)
< Once a month	48 (10%)
CL replacement	
Daily disposable	164 (35%)
Every 2 weeks	43 (9%)
Monthly	189 (41%)
3-6 months	23 (5%)
Annually	12 (3%)
Other	35 (8%)

The use of eye cosmetics in CLW and NCLW is summarised in Table 2.7 and shows similar proportions of CLW and NCLW using eye cosmetics.

Table 2.7 Comparison of eye cosmetic use amongst CLW and NCLW

	CL wearers	NCL wearers
Eye cosmetic use	408 (88%)	871 (87%)
No eye cosmetic use	58 (12%)	125 (13%)

The frequency of eye cosmetics usage is shown in Figure 2.13 and shows 84% of CLW and NCLW reported eye cosmetic usage at least 3-5 times a week. Furthermore, Figure 2.14 shows CLW and NCLW share similarities in the most regularly used cosmetic products. Both groups indicate mascara (71%), foundation (45%/46% in CLW/NCLW respectively) and concealer (35%/38% CLW/NCLW respectively) were the most regularly applied products.
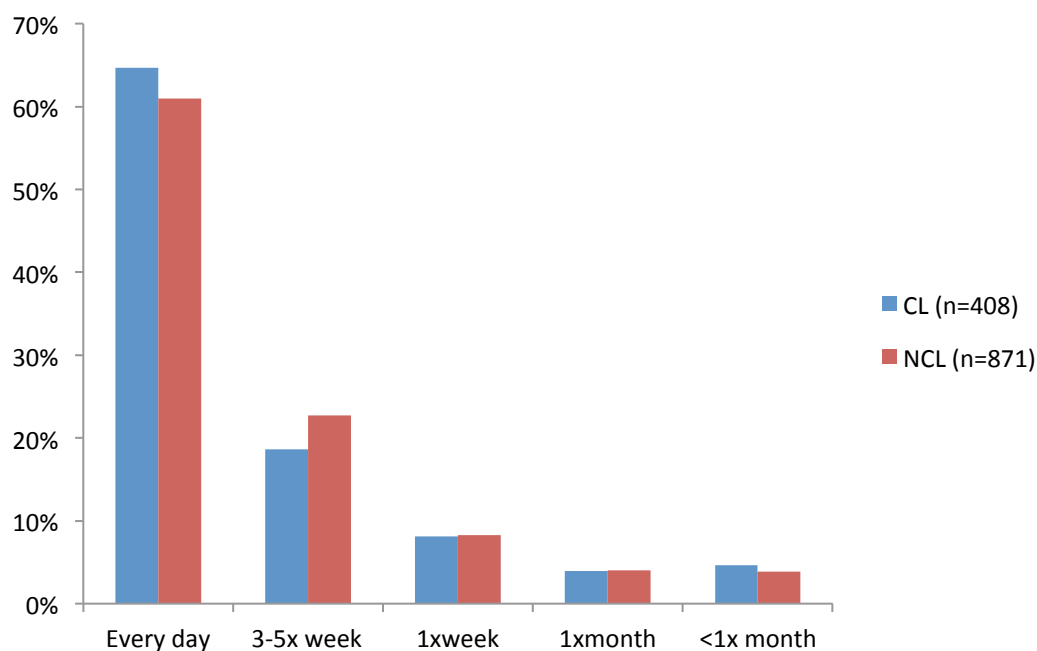
Figure 2.13 Frequency of cosmetics amongst CLW and NCLW groups
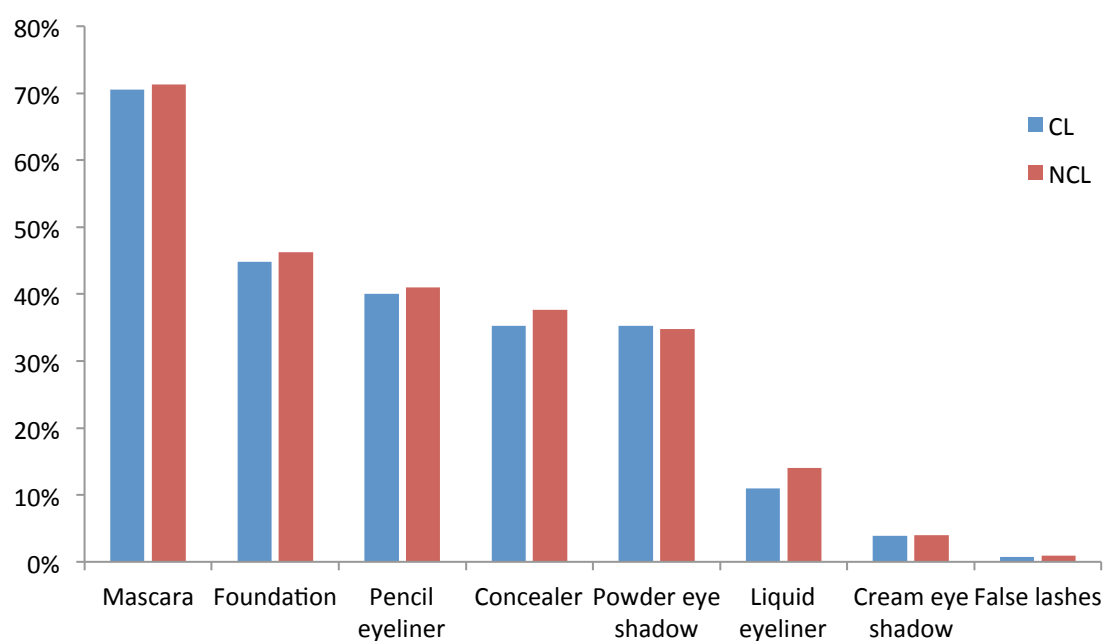
Figure 2.14 Percentage of CLW and NCLW regularly using each type of eye cosmetic product

2.3.5　Ocular comfort with and without eye cosmetics amongst CLW

Figure 2.15 illustrates the distribution of perceived ocular comfort scores in NCLW and CLW. In both groups, the median perceived ocular comfort score was greater without cosmetics

compared to when cosmetics were used, with scores of 9 and 8 respectively. Respondents in both groups were significantly more comfortable on days when eye cosmetics were not applied (Wilcoxon signed rank test, p<0.001).
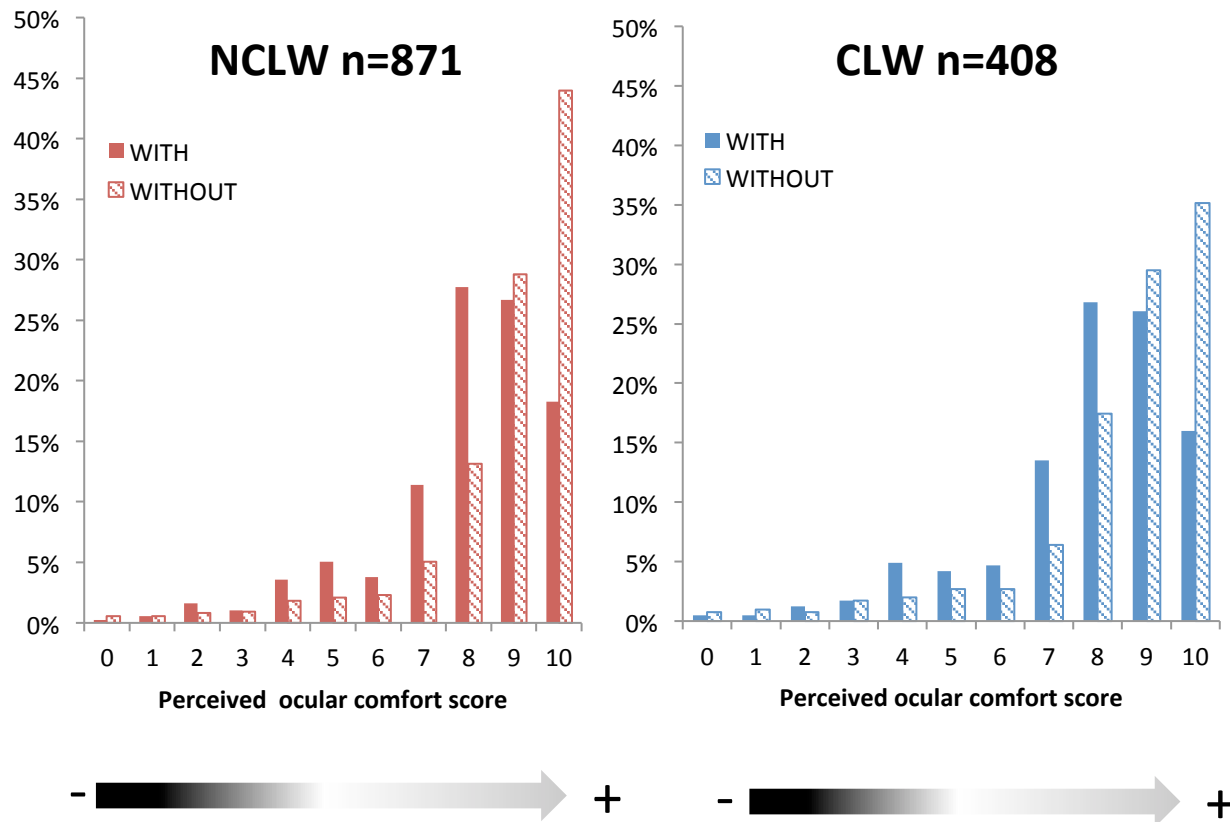
Figure 2.15 Graph of perceived ocular comfort in NCLW and CLW on an average day when cosmetics were and were not applied (percentage of respondents)

2.3.6 The relationship between OSDI scores and CLW in cosmetics users

The median OSDI score for the CLW group was 8.3 (IQ range 4.2-16.7), with a range of 0-66.7, and 135 respondents (33%) exceeding a score of 15. Similarly, the median OSDI score for the NCLW group was 8.3 (IQ range 4.2-16.7), with a range of 0-85.4, with 283 respondents (32%) exceeding a score of 15. The difference in OSDI scores between CLW and NCLW was not statistically different (Mann-Whitney U test, p=0.762).

Figure 2.16 illustrates the difference in OSDI scores according to eye cosmetic use and CLW. Median OSDI values were the same amongst cosmetic users and non-users in the NCLW

group (8.3, IQ 2.1-14.6 in non-users vs. 8.3 IQ 4.2-16.7 in cosmetic users). Although median OSDI values were slightly greater for non-cosmetic users in the CLW group (9.4, IQ 2.1-14.6 in non-users vs. 8.3, IQ 4.2-16.7 in cosmetic users), the difference did not reach statistical significance (Mann-Whitney U test, p=0.396).
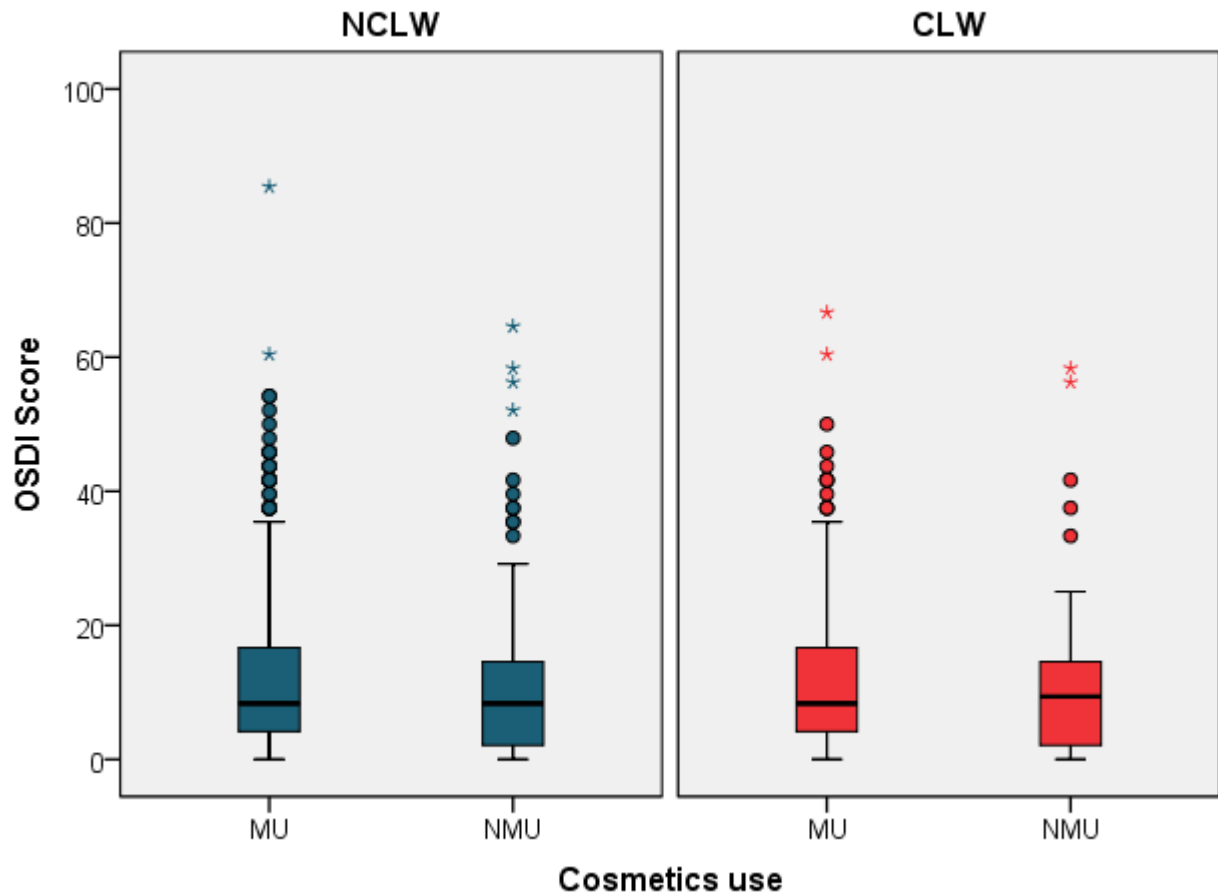
Figure 2.16 Comparison of OSDI values in MU and NMU amongst NCLW and CLW.

The data set of cosmetic users was further explored for differences in OSDI scores according to frequency of cosmetics use (see full statistical analysis in Appendix III). There was no apparent trend in median OSDI scores with increasing frequency of cosmetic use in CLW and NCLW. OSDI scores ranged 5.2-10.4 and 6.3-10.4 in CLW and NCLW respectively in frequency categories "Every day/3-5 times per week/Once a week/Once a month/Less than once a month".

2.3.6.1 The relationship of OSDI scores and regular product usage in CLW and NCLW

Analysis of the median OSDI score of users was conducted for each cosmetic product according to regular (≥3 times a week) or light (<3 times a week) usage and comparisons were made using Mann-Whitney U tests.

Table 2.8 and Table 2.9 show the results for the CLW group and NCLW respectively.

Table 2.8 Median (IQ in parentheses) OSDI scores for regular and light usage of each cosmetic product in CLW

Regular users (≥3x)							
	Concealer	**Foundation**	**Mascara**	**Powder eye shadow**	**Cream eye shadow**	**Eyeliner**	**False lashes**
n	114	183	288	144	16	197	3
Median OSDI	11.5 (6.3-16.7)	10.4 (5.2-18.8)	10.4 (4.2-16.7)	8.3 (4.2-19.3)	7.3 (5.7-15.1)	10.4 (6.3-18.8)	22.9 (22.9-39.6)
Light users (<3x)							
n	144	101	128	229	152	185	117
Median OSDI	9.4 (4.2-20.3)	12.5 (6.3-20.8)	10.4 (4.2-22.9)	10.4 (6.3-16.7)	12.5 (6.3-20.8)	10.4 (6.3-18.8)	10.4 (6.3-18.8)
p-value (Mann-Whitney U test)	0.559	0.128	0.384	0.238	0.364	0.736	-

In the CLW group, there were no statistical differences in median OSDI scores between regular and light users of each product.

Table 2.9 Median (IQ in parentheses) OSDI scores for regular and light usage of each cosmetic product in NCLW

Regular users (≥3x)							
	Concealer	**Foundation**	**Mascara**	**Powder eye shadow**	**Cream eye shadow**	**Eyeliner**	**False lashes**
n	328	403	621	303	35	419	8
Median OSDI	8.3 (4.2-18.8)	8.3 (4.2-16.7)	8.3 (4.2-16.7)	8.3 (4.2-16.7)	10.4 (6.3-18.8)	10.4 (4.2-16.7)	7.3 (2.1-12.5)
Light users (<3x)							
n	213	216	222	430	294	337	228
Median OSDI	10.4 (4.2-16.7)	8.3 (4.2-16.7)	8.3 (4.2-16.7)	8.3 (4.2-16.7)	10.4 (4.2-18.2)	8.3 (4.2-16.7)	8.3 (4.2-16.7)
p-value (Mann-Whitney U test)	0.814	0.518	0.886	0.925	0.586	0.037	-

In the NCLW group, there was a statistically significant difference between median OSDI scores in regular and light eyeliner users (light users were more comfortable, p=0.037) however no other products reached statistical significance. Further analysis of OSDI scores amongst eyeliner users in CLW and NCLW were conducted according to where the product was applied and the type of product that was used, as shown in Table 2.10.

In the CLW group, median OSDI scores were significantly greater in the eyeliner-using group (10.4, IQ 4.2-18.8) compared to the non-using group (7.3, IQ 2.1-14.1) (Mann-Whitney U test, p=0.026). This finding was not reflected in the NCLW group, where there was no difference in the median or IQ range of OSDI scores in eyeliner and non-eyeliner using respondents. In both CLW and NCLW, there was a tendency for greater median OSDI scores in liquid eyeliner users compared with pencil, irrespective of the location of application. However the number of users of liquid eyeliner was very small compared with the users of pencil eyeliner.

Table 2.10 Comparison of OSDI scores in CLW and NCLW according to eyeliner use, eyeliner formulation and application method (ELI = eyeliner applied within the lash line; ELO = eyeliner applied outside the lash line)

		n	Median (IQ) OSDI score	p-value (Mann-Whitney U test)
CLW	Any eyeliner use			
	- Eyeliner use	318	10.4 (4.2-18.8)	0.026
	- No eyeliner use	90	7.3 (2.1-14.1)	
	ELI			
	- Pencil ELI	149	10.4 (6.3-18.8)	0.440
	- Liquid ELI	6	18.8 (5.7-28.6)	
	ELO			
	- Pencil ELO	163	10.4 (4.2-16.7)	0.854
	- Liquid ELO	35	10.4 (4.2-20.8)	
NCLW				
	Any eyeliner use			
	- Eyeliner use	377	8.3 (4.2-16.7)	0.978
	- No eyeliner use	494	8.3 (4.2-16.7)	
	ELI			
	- Pencil ELI	379	10.4 (4.2-16.7)	0.847
	- Liquid LELI	14	13.5 (1.0-19.8)	
	ELO			
	- Pencil ELO	518	10.4 (4.2-16.7)	0.991
	- Liquid ELO	107	8.3 (4.2-18.8)	

2.3.7 Opinions on the effects of eye cosmetic use

In the survey, respondents were asked to rate their agreement to six statements relating to the use of eye cosmetics. Amongst cosmetic users, the relationships between OSDI and perceived ocular comfort scores with agreement or disagreement with the statements were also explored. In the analysis of perceived ocular comfort scores, the mean difference in scores on days when cosmetic were and were not used was calculated i.e.

Mean Difference in Comfort scores = Score on a day **WITH** cosmetics – Score on a day **WITHOUT** cosmetics

This analysis was conducted to see if a relationship between the mean difference in perceived comfort scores and the response type to the statement existed. A positive integer represents reported comfort to be better with than without cosmetics use; the reverse is true for negative integers.

2.3.7.1 *Wearing eye make-up has no influence on the comfort of my eyes*

38% of respondents strongly disagreed or disagreed with the statement compared with 49% that strongly agreed or agreed (Figure 2.17).

Wearing eye make-up has no influence on the comfort of my eyes

88
6%

235
16%

460
32%

486
33%

193
13%

■ Strongly disagree ■ Disagree ■ No opinion ■ Agree ■ Strongly agree
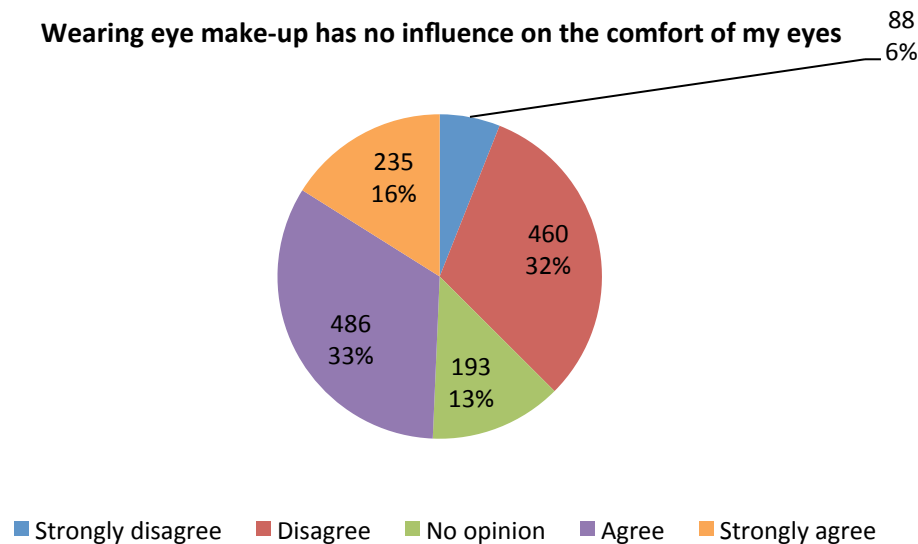
Figure 2.17 Responses given by total cohort to the statement "Wearing eye make-up has no influence on the comfort of my eyes"

The proportion of responses given cosmetic users (MU) and non-cosmetic users (NMU) are shown in Figure 2.18. A χ² test showed a significant difference between the opinions given by MU and NMU (p<0.001) to the statement "Wearing eye make-up has no influence on the comfort of my eyes", with a greater proportion of NMU expressing "No opinion".

Wearing eye make-up has no influence on the comfort of my eyes
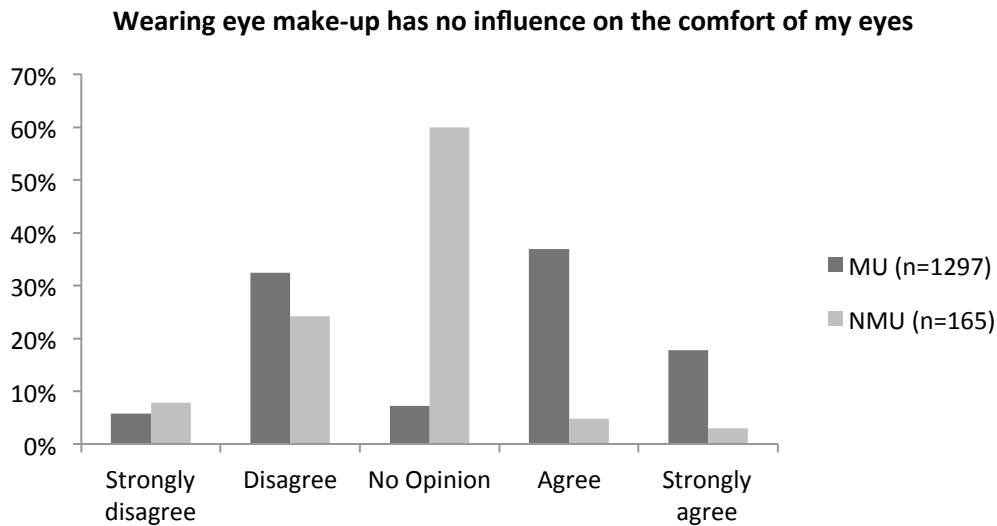
Figure 2.18 Comparison of MU and NMU responses for the statement "Wearing eye make-up has no influence on the comfort of my eyes"

Median OSDI scores were explored according to agreement of the statement in cosmetic users. Figure 2.19 shows respondents in agreement with the statement were significantly less symptomatic than those in disagreement with the statement (6.3, IQ 4.2-14.6 vs. 12.5, IQ 6.3-20.3 respectively, Kruskal-Wallis test, p<0.001). A post-hoc Mann-Whitney U-test indicated a statistically significant difference in OSDI scores between respondents in disagreement to those in agreement with the statement (p<0.0001).
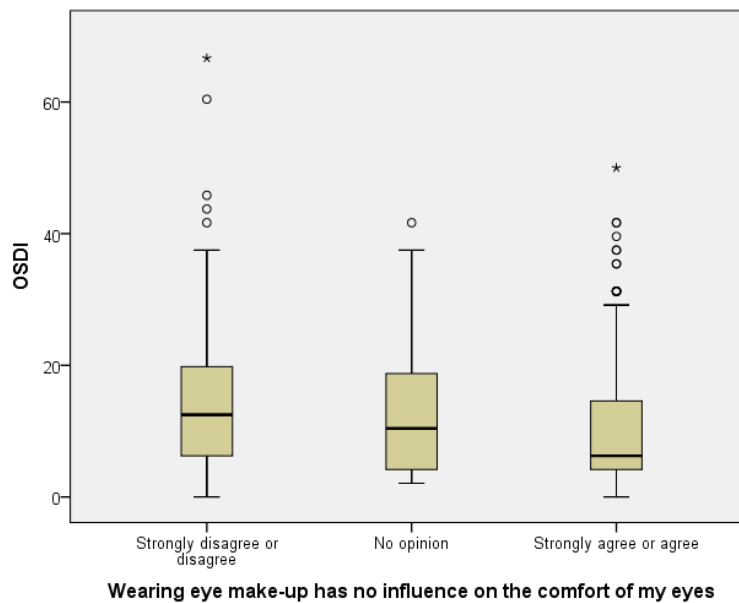
Figure 2.19 OSDI scores to the statement "Wearing eye make-up has no influence on the comfort of my eyes" in cosmetic users

Figure 2.20 shows increasing mean difference in perceived comfort scores with increasing disagreement with the statement. Respondents with the greatest difference in comfort scores between MU and NMU situations logically felt eye cosmetic use had an influence on ocular comfort. The difference in scores between each response group reached statistical significance (Kruskal-Wallis test, p<0.001). Post-hoc Mann-Whitney U-tests indicated a significant difference in the mean difference in perceived comfort scores between the group in disagreement with the statement and the group in agreement with the statement (p<0.0001) and also with the group in disagreement statement with the group with no opinion (p<0.05).
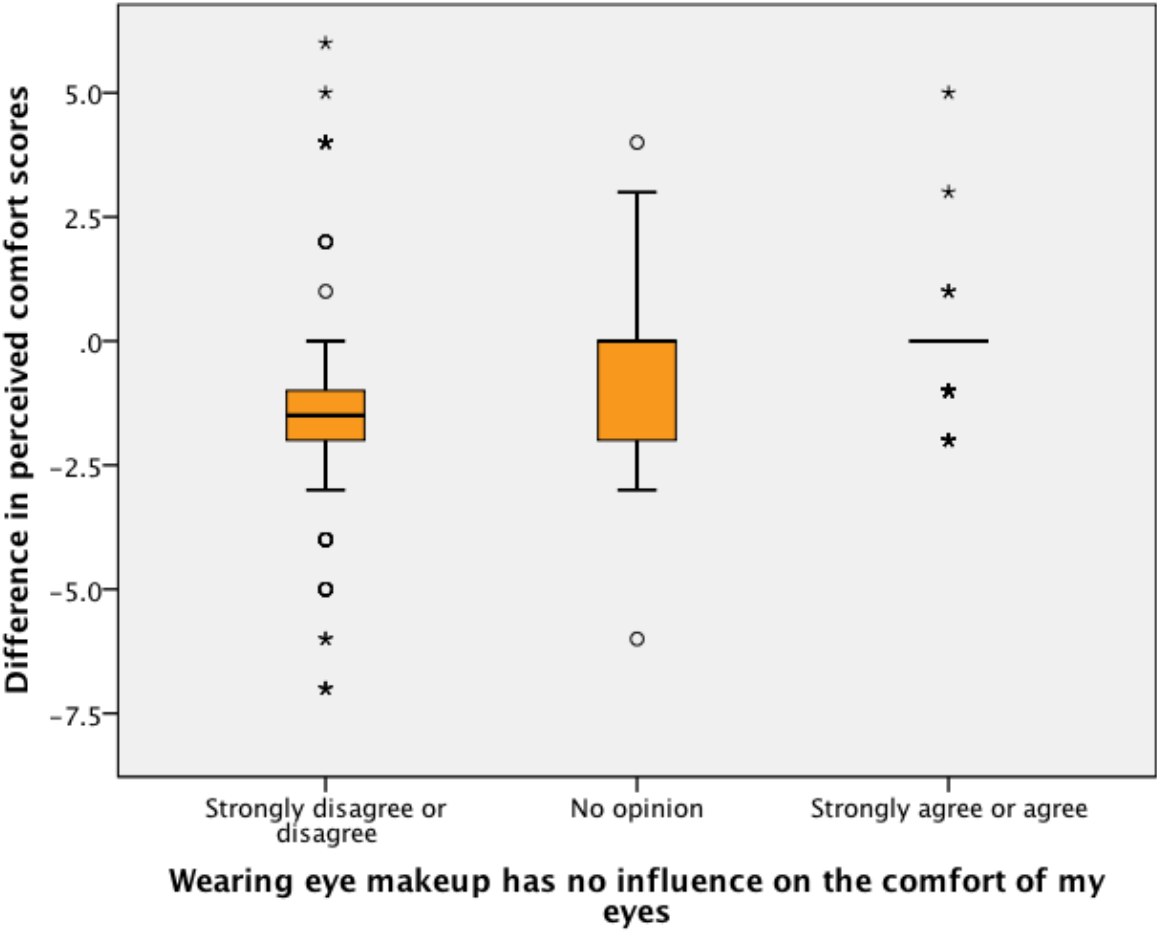
Figure 2.20 Difference in perceived ocular comfort scores for responses to "Wearing make-up has no influence on the comfort of my eyes"

2.3.7.2   Wearing eye make-up is good for my eyes

65% respondents strongly disagreed or disagreed with the statement compared to 2% that agreed (Figure 2.21). No respondents strongly agreed with the statement.

Wearing make-up is good for my eyes
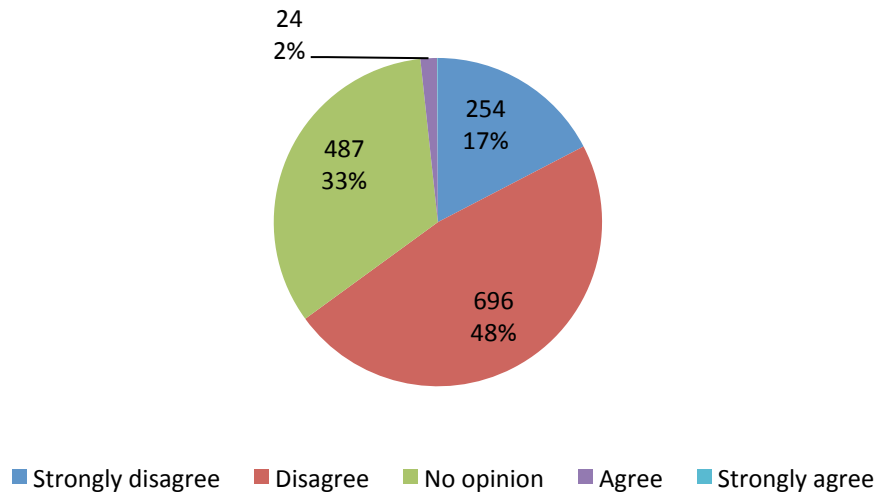
Figure 2.21 Responses given by total cohort to the statement "Wearing eye make-up is good for my eyes"

The proportion of responses given by MU and NMU are shown in Figure 2.22. 51% of MU disagreed with the statement whereas 51% of NMU expressed "No opinion" to the statement. A χ² test showed a significant difference between the opinions expressed by MU and NMU (p<0.001) to the statement "Wearing make-up is good for my eyes". Due to violation of the assumptions of the χ² test (3 cells had an expected count less than 5) the p-value was obtained using the Monte Carlo exact test at a 99% confidence interval for 10,000 samples.

Wearing make-up is good for my eyes
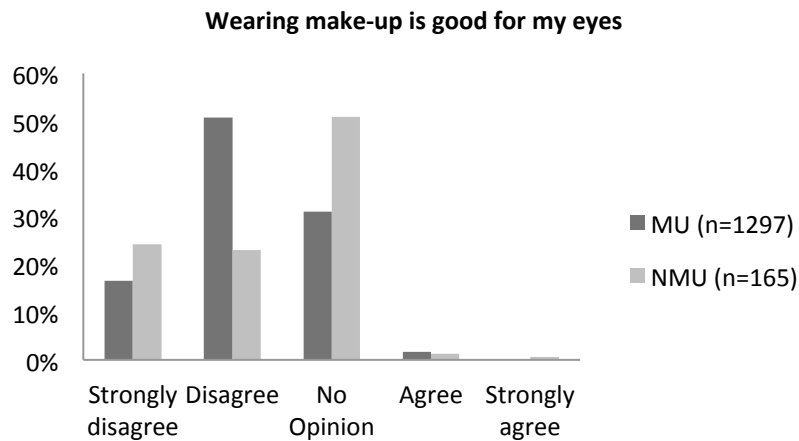
Figure 2.22 Comparison of MU and NMU responses for the statement "Wearing eye make-up is good for my eyes"

The median OSDI score was compared within each response type given to the statement "Wearing make-up is good for my eyes" in cosmetic users. Respondents in disagreement with the statement had the higher median OSDI scores (i.e. lowest comfort) compared to respondents in agreement (10.4, IQ 4.2-18.8 vs. 5.2, IQ 2.1-19.8 respectively) however this did not reach statistical significance (Kruskal-Wallis test, p=0.154).

The difference between perceived ocular comfort scores, shown in Figure 2.23 shows respondents that agreed with the statement reported improved ocular comfort when using eye cosmetics compared to the non-using state. The reverse is true for those that disagree or strongly disagree with the statement and the difference between groups is significantly different (Kruskal-Wallis test, p<0.001). Post-hoc Mann Whitney U-tests were conducted and showed significant differences in the perceived comfort score measures between the respondents that disagreed with the statement to those with no opinion (p<0.0001) and to those in agreement (p<0.001) with the statement. Additionally, there was a significant difference in perceived comfort score measures between the respondents with no opinion to the statement and those in agreement with the statement (Mann-Whitney U-test, p<0.005).
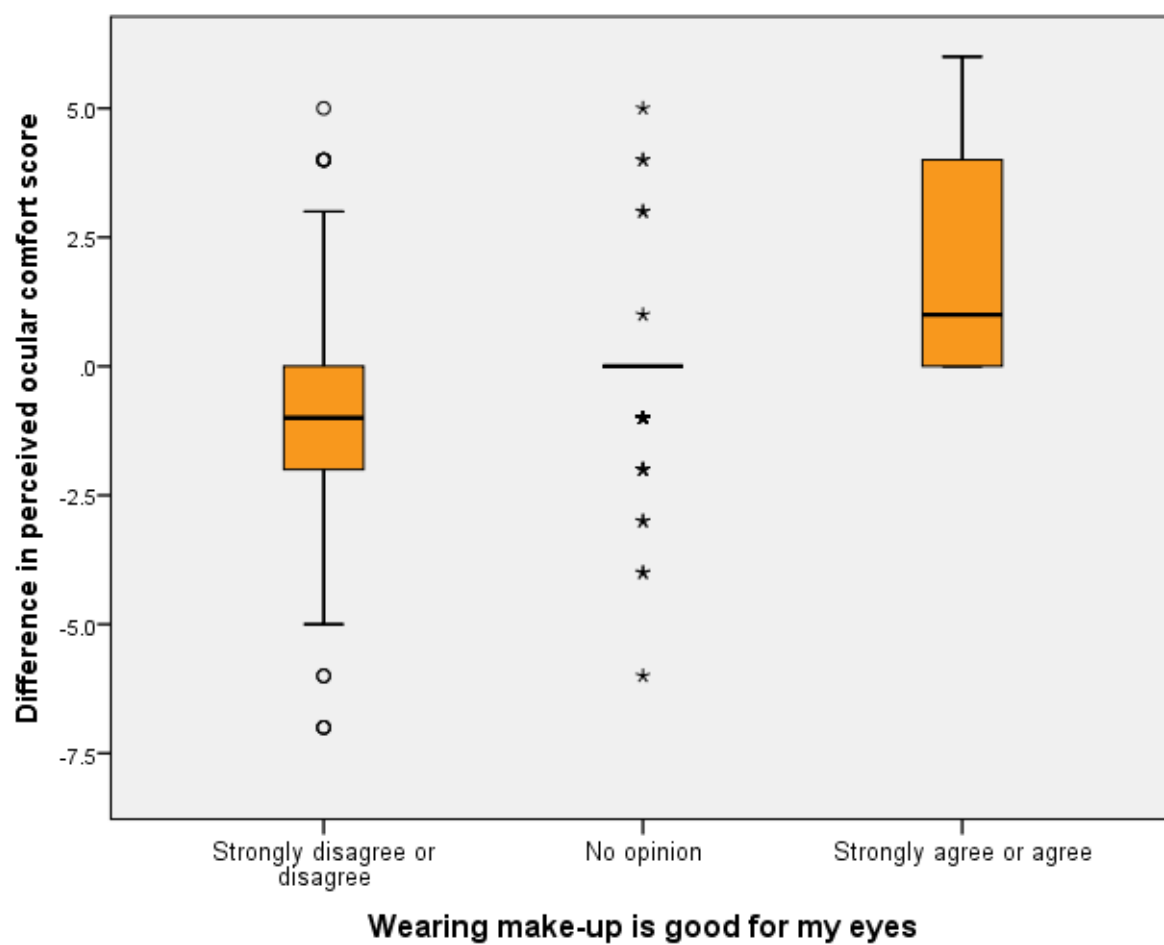
Figure 2.23 Difference in perceived ocular comfort scores for responses to "Wearing make-up is good for eyes"

2.3.7.3 Wearing eye make-up has a detrimental effect on the health of my eyes

40% of respondents disagreed or strongly disagreed with the statement compared with 18% that agreed or strongly agreed (Figure 2.24).

Wearing eye make-up has a detrimental effect on the health of my eyes

21
1%

96
7%

486
33%

614
42%

245
17%

■ Strongly disagree ■ Disagree ■ No opinion ■ Agree ■ Strongly agree
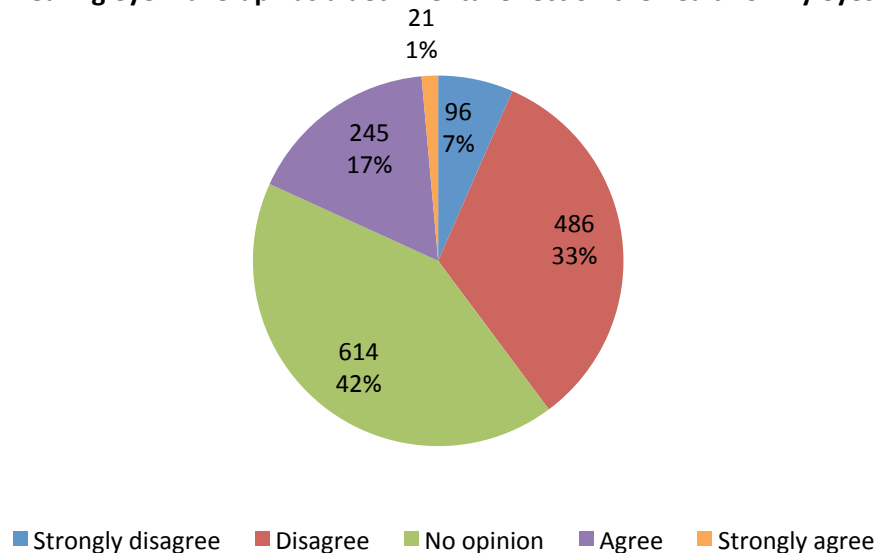
Figure 2.24 Responses given by total cohort to "Wearing eye make-up has a detrimental effect on the health of my eyes"

The responses given by MU and NMU are shown in Figure 2.25. NMU had a tendency to agree with the statement whereas MU tended to disagree with the statement. A χ^2 test was conducted and showed a significant difference between the opinions given by MU and NMU ($p<0.001$). Due to violation of the assumptions of the χ^2 test (1 cell had an expected count less than 5) the p-value was obtained using the Monte Carlo exact test at a 99% confidence interval for 10,000 samples.

Wearing eye make-up has a detrimental effect on the health of my eyes
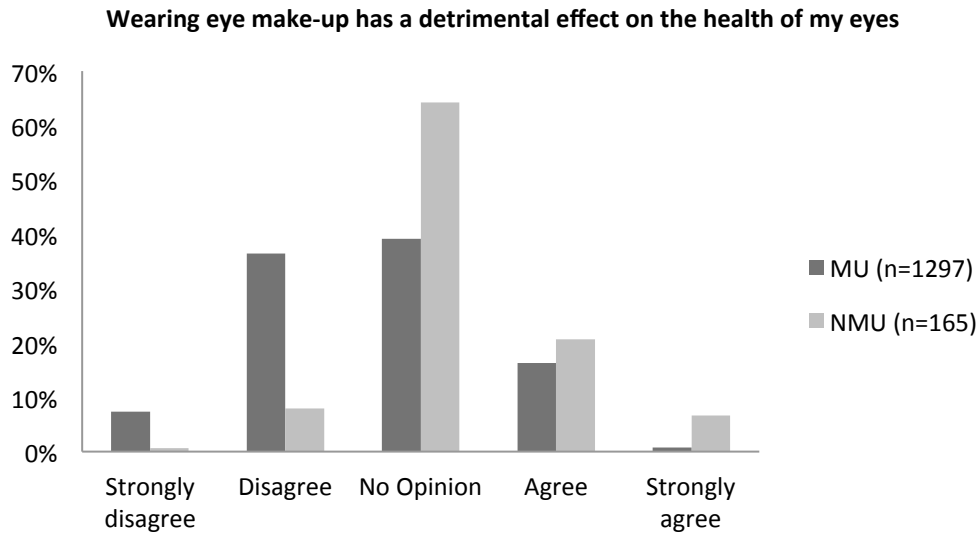
Figure 2.25 Comparison of MU and NMU responses to "Wearing eye make-up has a detrimental effect on the health of my eyes"

The median OSDI score was compared within each response category to the statement "Wearing make-up is good for my eyes" in cosmetic users. Figure 2.26 shows respondents in agreement with the statement had the highest mean OSDI scores (i.e. lowest comfort) however, the difference between all three responses was not statistically different (Kruskal-Wallis test, p=0.183).
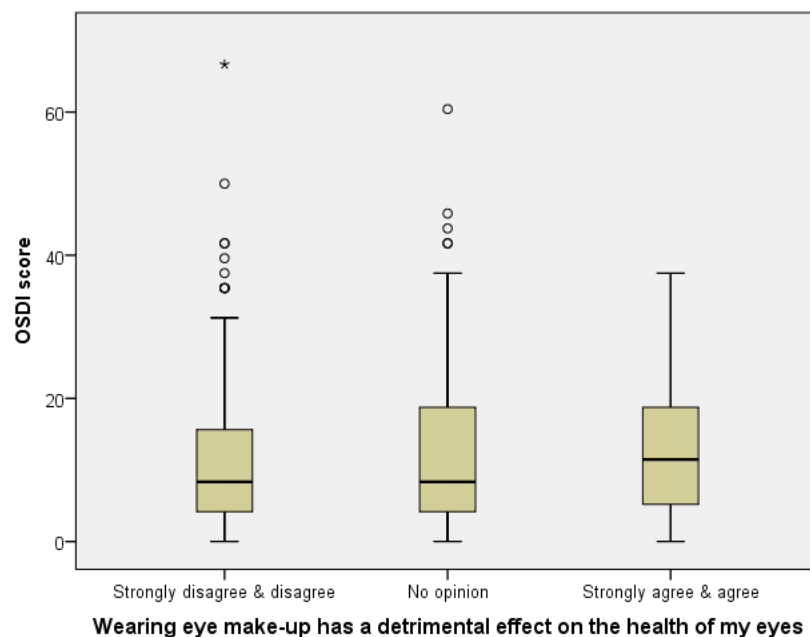
Figure 2.26 Mean OSDI scores according to responses to "Wearing eye make-up has a detrimental effect on the health of my eyes" in MU

The respondents in agreement with the statement reported the greatest reduction in perceived ocular comfort when cosmetics were used (Figure 2.27), which was significantly different (Kruskal- Wallis test, p<0.001). Post-hoc Mann Whitney U-tests were conducted and showed significant differences in the perceived comfort score measures between the respondents that disagreed with the statement to those that agreed (p<0.0001) with the statement. Additionally, there was a significant difference in perceived comfort score measures between the respondents with no opinion to the statement and those in agreement with the statement (Mann-Whitney U-test, p<0.001).
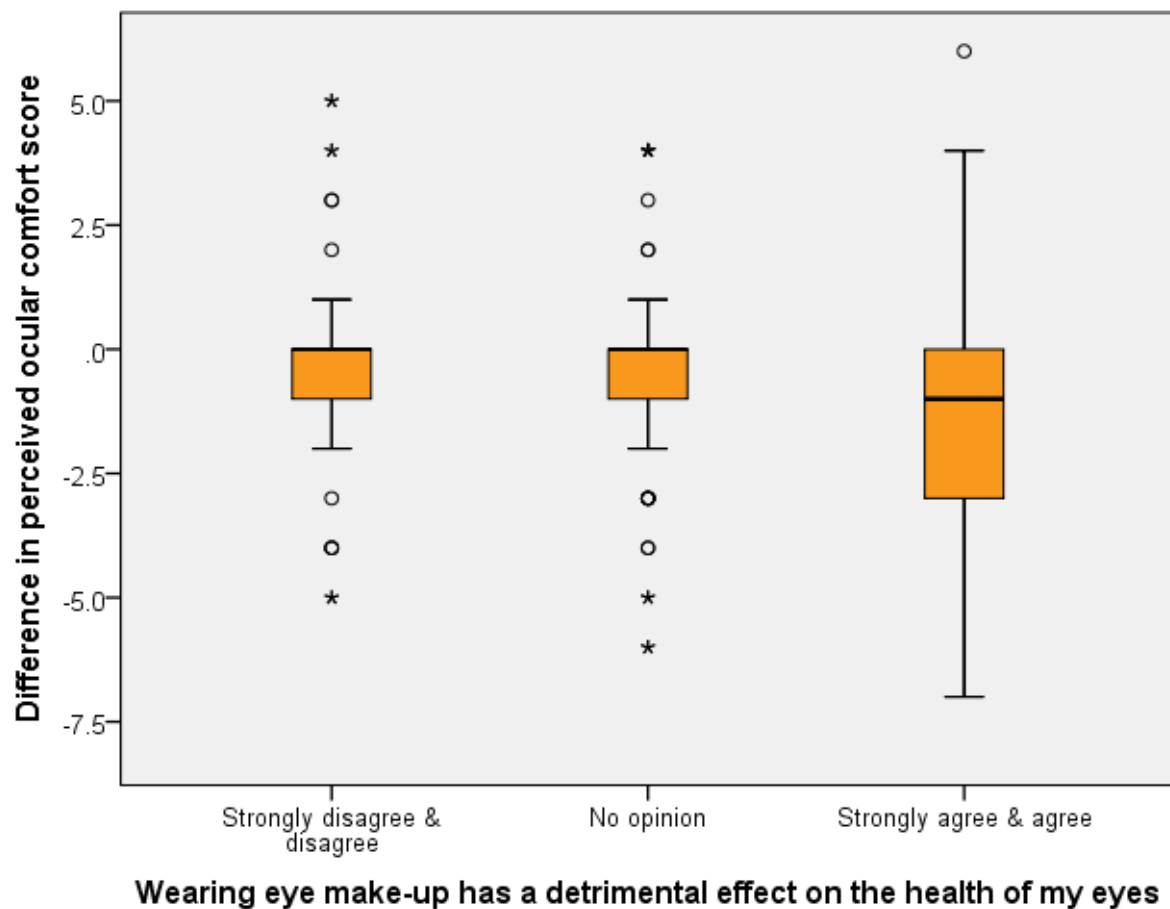
Figure 2.27 Difference in perceived comfort scores for responses to "Wearing eye make-up has a detrimental effect on the health of my eyes"

2.3.7.4 My eyes will be just as healthy in 20 years' time whether or not I wear eye make-up

20% of respondents strongly disagreed or disagreed compared with 34% of respondents that strongly agreed or agreed with the statement that long term ocular health is unaffected by eye cosmetic use (Figure 2.28).

My eyes will be just as healthy in 20 years time whether or not I wear eye make-up

■ Strongly disagree	■ Disagree ■ No opinion ■ Agree ■ Strongly agree
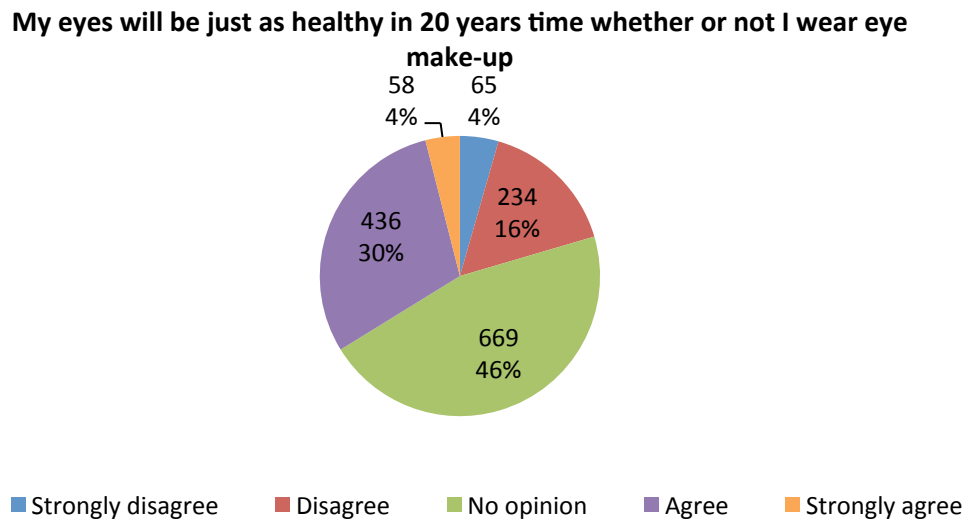
Figure 2.28 Responses given by total cohort to the statement "My eyes will be just as healthy in 20 years' time whether or not I wear eye make-up"

The responses given by MU and NMU are shown in Figure 2.29. A χ² test showed a significant difference between the opinions expressed by MU and NMU (p<0.001) to the statement "My eyes will be just as healthy in 20 years' time whether or not I wear eye make-up". 33% of MU agreed with the statement compared with 6% of NMU. 62% of NMU expressed "No opinion" to the statement compared with 44% of MU.

My eyes will be just as healthy in 20 years time whether or not I wear eye makeup
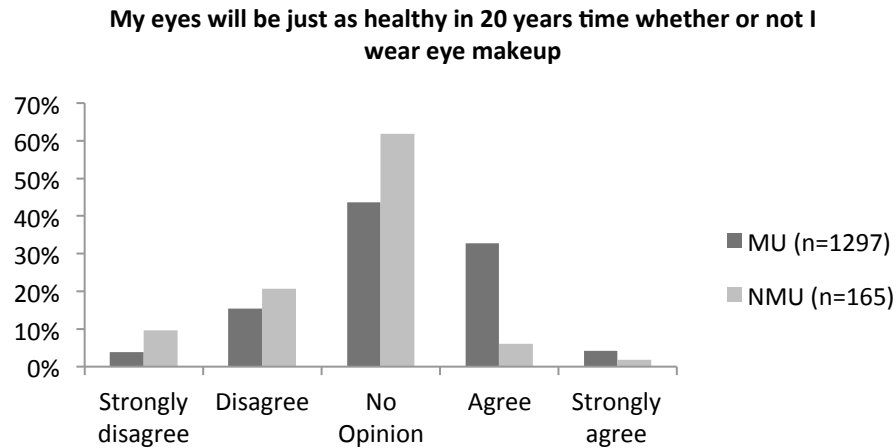
Figure 2.29 Comparison of MU and NMU responses for the statement "My eyes will be just as healthy in 20 years' time whether or not I wear eye make-up"

Figure 2.30 shows median OSDI scores of cosmetic users were greater (i.e. lower comfort) in the group of respondents in disagreement with the statement (8.3, IQ 4.2-16.7) compared with the group in agreement (6.3, IQ 2.1-18.8) however the difference between groups was not statistically significant (Kruskal-Wallis, p=0.763).
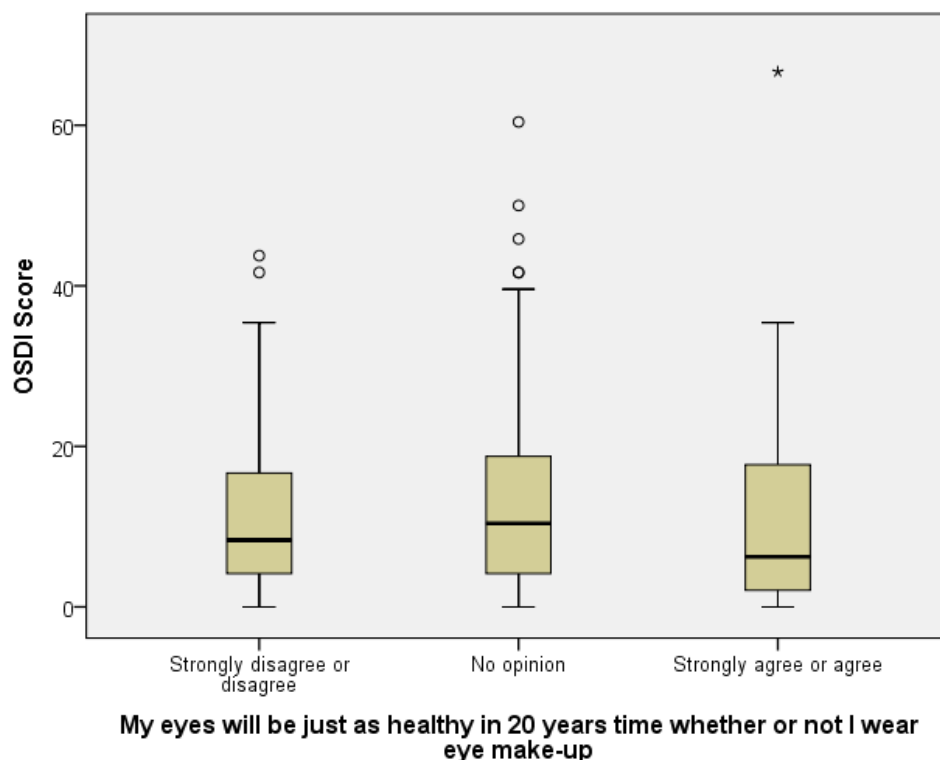
Figure 2.30 OSDI scores to responses to the statement "My eyes will be just as healthy in 20 years' time whether or not I wear eye make-up" in MU

Figure 2.31 shows differences in perceived comfort were greatest in the respondents in disagreement with the statement. The difference between groups was significantly different (Kruskal-Wallis, $p<0.001$). Post-hoc Mann Whitney U-tests were conducted and showed significant differences in the perceived comfort score measures between the group of respondents that disagreed with the statement to those that agreed ($p<0.001$) with the statement.
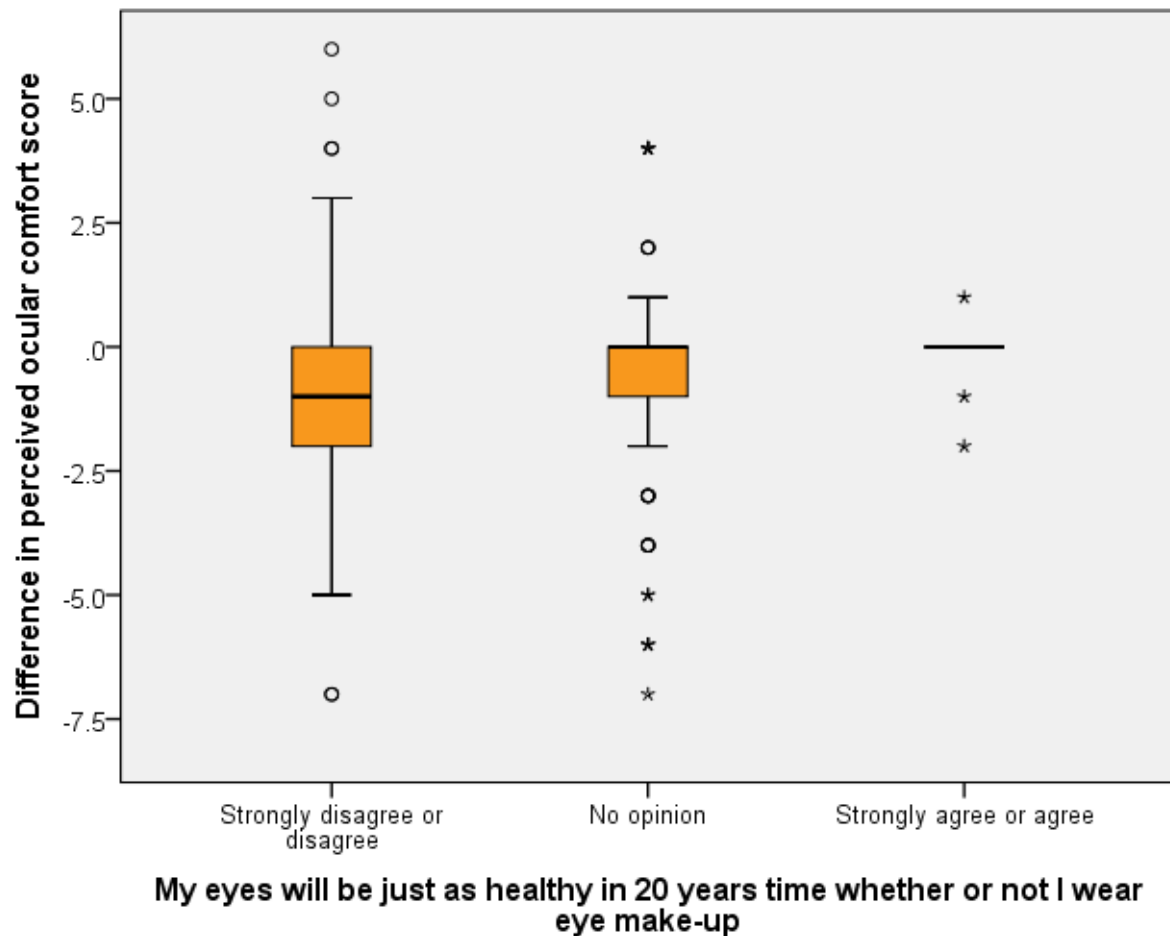
Figure 2.31 Difference in perceived comfort scores for responses to "My eyes will be just as healthy in 20 years' time whether or not I wear eye make-up"

2.3.7.5 Eye make-up ends up in my eyes by the end of the day

Due to the nature of the statement "Make-up ends up in my eyes by the end of the day", data analysis was conducted on MU only. 44% of MU agreed with the statement compared with 45% of users that disagreed (Figure 2.32).

Eye make-up ends up in my eyes by the end of the day

67
5%

118
9%

461
36%

139
11%

512
39%

■Strongly disagree ■Disgaree ■No opinion ■Agree ■Strongly agree
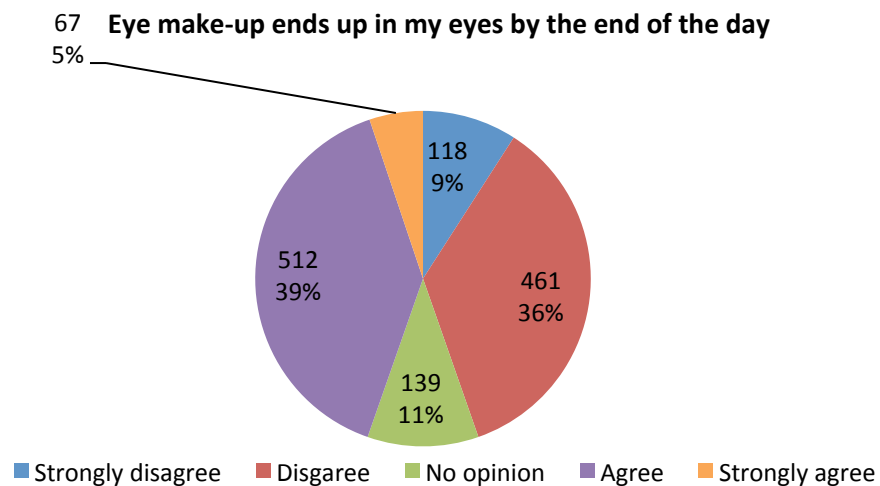
Figure 2.32 Responses given by MU to the statement "Eye make-up ends up in my eyes by the end of the day"

Figure 2.33 shows MU in agreement with the statement had slightly higher median OSDI score (i.e. lower comfort) compared with those in disagreement with the statement. The difference in OSDI scores between all three groups was statistically significant (Kruskal-Wallis test, $p<0.05$). Post-hoc Mann Whitney U-tests showed a statistically significant difference in OSDI scores only between the respondents in disagreement to the statement and respondents in agreement with the statement ($p<0.001$).
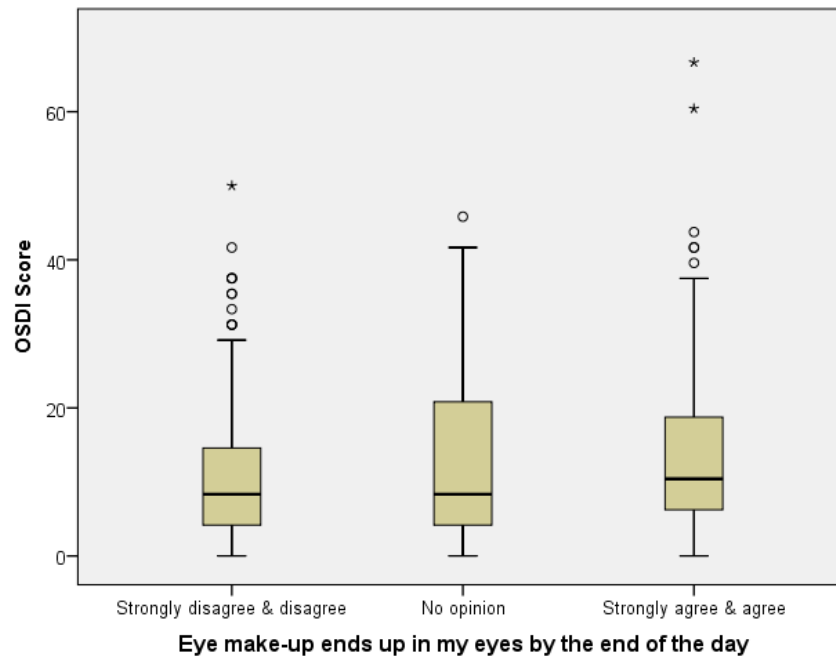
Figure 2.33 OSDI scores to responses to the statement "Eye make-up ends up in my eyes by the end of the day" in MU

Figure 2.34 shows respondents in agreement with the statement had worse comfort when wearing cosmetics compared with that disagreed. The difference in perceived ocular comfort scores was statistically significant (Kruskal-Wallis, $p<0.001$). Post-hoc Mann Whitney U-tests showed a statistically significant difference in comfort scores only between the respondents in disagreement to the statement and respondents in agreement with the statement ($p<0.0001$).
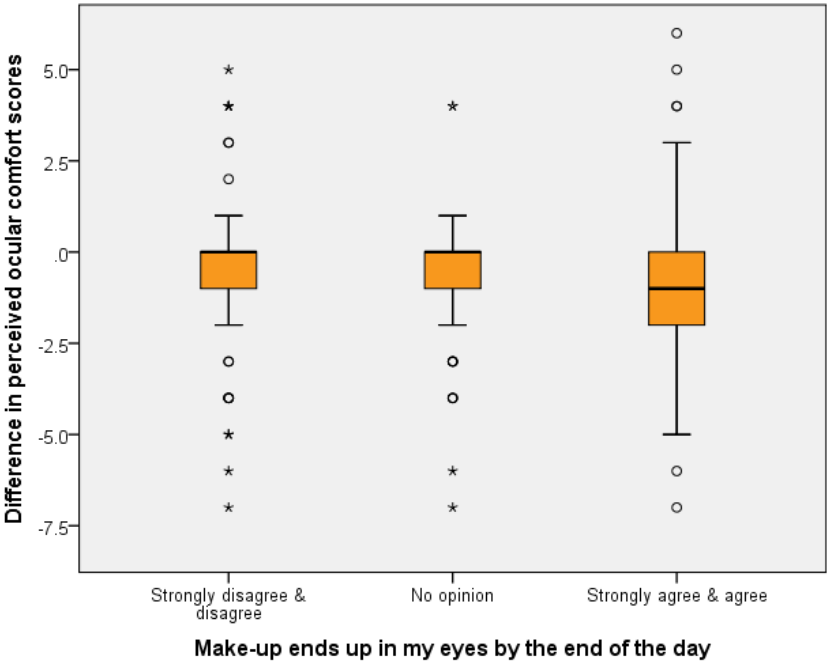
Figure 2.34 Difference in perceived comfort scores for responses to "Eye make-up ends up in my eyes by the end of the day"

2.3.7.6 *I would not leave the house without applying some type of eye make-up*

Due to the nature of the statement "I would not leave the house without applying some type of eye make-up", data analysis was conducted on MU only. 42% of MU agreed with the statement compared with 55% of users that disagreed (Figure 2.35).

I would not leave the house without applying some type of eye make-up
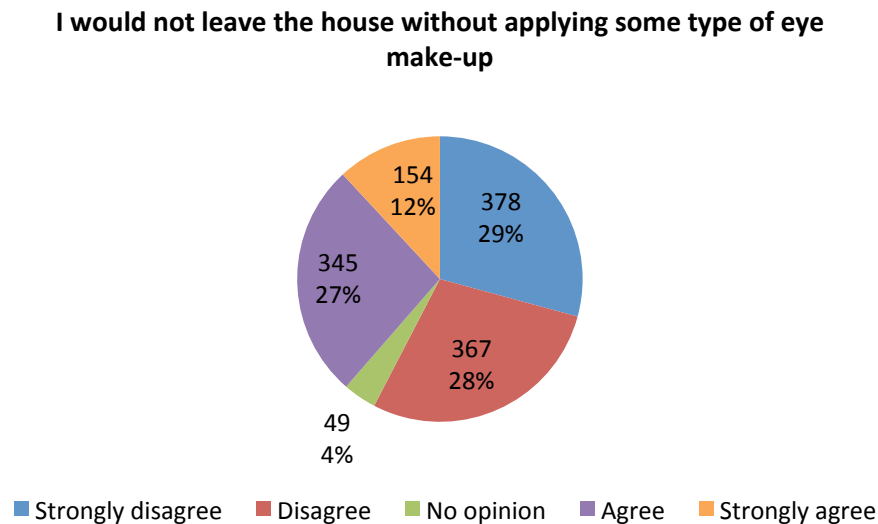
Figure 2.35 Responses given by MU to "I would not leave the house without applying some type of eye make-up"

Figure 2.36 shows respondents in disagreement with the statement were users that experienced greater amounts of perceived discomfort cosmetic use compared to those that agreed with the statement which was significantly different (Kruskal-Wallis, $p<0.001$). Post-hoc Mann Whitney U-tests were conducted and showed significant differences in the perceived comfort score measures between those in disagreement to those in agreement ($p<0.0001$) with the statement. Additionally, there was a significant difference in perceived comfort score measures between the respondents that disagreed with the statement and those with no opinion to the statement (Mann-Whitney U-test, $p<0.005$).
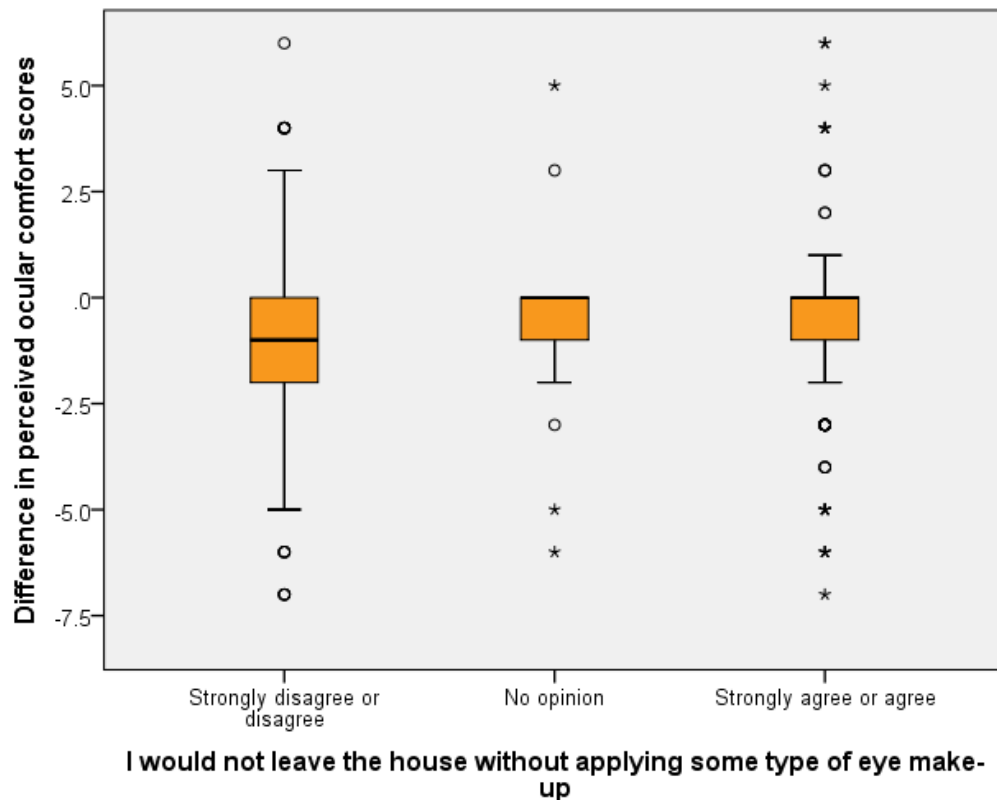
Figure 2.36 Difference in perceived ocular comfort scores for responses to "I would not leave the house without applying some type of eye make-up"

2.3.8   Summary of results

- The most popular types of cosmetics products were mascara, pencil eyeliner and foundation. The choices of products used amongst CLW and NCLW were similar.

- Perceived ocular comfort was significantly reduced with cosmetics usage. This was apparent in CLW and NCLW.

- OSDI scores were greater amongst eye cosmetic users although this did not reach statistical significance. In CLW, non-users had slightly greater OSDI scores than cosmetic users.

- No trends were identified with the frequency of cosmetic usage and OSDI scores.

- Negative perceptions of eye cosmetics were significantly associated with reduced comfort during cosmetics use.

2.4   Discussion

It is well established that contact lens wearers often report greater levels of dryness and discomfort than non-lens wearers (Guillon and Maissa, 2005) however, there is little evidence that the application of cosmetics around the eyes may further influence reported comfort levels.

The motivation behind completing the survey may be greater in cosmetic users than non-users due to increased relevance and existing interest. This is reflected when comparison between the number of respondents in each group is made and the female dominance in the total cohort. The results from this survey reflect data published in recent market research reports. The three most popular cosmetic products used in this population were mascara, pencil eyeliner and foundation. This supports market research findings, which showed mascara to be the popular eye cosmetic product, most likely due to the ease of application (Mintel, 2013). When examining the data with respect to CLW, there were no significant differences between the products used in the CLW compared to the NCLW. This finding indicates that CLW does not seem to affect the habits or popularity of eye cosmetic usage.

This survey examined the impact of cosmetic usage upon ocular comfort using two measures: perceived ocular comfort scores and using the validated OSDI questionnaire. Perceived ocular comfort was found to be significantly more comfortable when cosmetics were not used. This was apparent in the CLW group and NCLW group. Although the median OSDI score was slightly higher in the cosmetics-using group compared with non-users, the difference was small and was not significant. Within the CLW group, OSDI scores were slightly greater in non-cosmetic users than cosmetic-users and were also not significant. There may an element of self-selection of eye cosmetic use amongst CLW. It is well established the CL discomfort is common cause for the cessation of CL usage (Nichols *et al.,* 2013). The motivation for CL usage is interesting although beyond the scope of this thesis. However, one motivating factor for CL usage is for cosmesis, particularly amongst females (Gupta and Naroo, 2006). This overlap in eye cosmetics and CL usage for decorative purposes may be one reason why CLW may continue to use eye cosmetics, even if a reduction in ocular comfort is experienced. Furthermore, it may be the case that CLW who

do not use eye cosmetics (who already have greater dry eye symptoms given their higher OSDI scores) are less inclined to use eye cosmetics in fear of further exacerbating discomfort. The reverse may hold true for cosmetics users who may have higher tolerance to changes in ocular comfort and therefore can use eye cosmetics more frequently. It is important to note that the difference between OSDI scores in light and regular use of all cosmetic products is small and were not statistically significant. Additionally, the cohort in this study is predominantly healthy, with only 29% of the total cohort having scores greater than 15 which is indicative of dry eye (Schiffman *et al.*, 2000). The magnitude of difference between light and regular users may be greater in a cohort with a greater number of dry eye respondents. The OSDI questionnaire has been shown to be a valid and reliable tool with good sensitivity and specificity in distinguishing normal subjects and patients with dry eye disease (Schiffman *et al.*, 2000). The use of the OSDI questionnaire may not be the most appropriate measure of ocular comfort for this study as it is based upon symptoms of dry eye, rather than irritation.

No trends were identified with increased frequency of cosmetics usage within the total cohort or when the data were compared in the CLW and NCLW group. When explored further, the data from this survey suggests that using eyeliner seems to cause an increase in dry eye symptoms. This was apparent in the total cohort and also amongst CLW. However, the increase in dry eye symptoms was not evident when exploring specifically the type of eyeliner formulation used (pencil vs. liquid) and the region of application (within or outside the lash line). A separate study has shown that 17% of normal subjects and 64% dry eye subjects reported symptoms of "soreness, scratchiness, dryness or burning" following cosmetic use (Guillon and Maissa, 2005). The same study found 26% soft CLW suffering from dry eye reported the same symptoms with cosmetics use.

The potential origins of discomfort arising from cosmetics usage are multi-factorial. The data from the survey showed 37% of respondents suffered hay fever or ocular allergy. This question was added to the survey as it was thought that respondents with allergy may be more hypersensitive to eye cosmetics. The prevalence of ocular allergy in one UK survey was found to be 8% (Wolffsohn *et al.*, 2011). The seemingly higher prevalence of ocular allergy in online cosmetics survey may be due to the lack of differentiation between seasonal allergic

conjunctivitis and seasonal allergic rhinitis, both terms which fall under the familiar term "hay fever" (Bilkhu, Wolffsohn and Naroo, 2012). Cosmetic products applied with close proximity to the ocular surface may inadvertently migrate onto the ocular surface. This may occur as a result of manual transfer or contraction of fine peri-ocular musculature (MacKeen *et al.*, 1998). Once cosmetic products have reached the tear film, an alteration of pH and tear osmolarity may cause reduced tear film stability and subsequent discomfort. Preservatives may cause irritation to the ocular surface (Malik and Claoue, 2012), in a similar way they irritate peri-ocular skin. Additionally, pigments and particles suspended in cosmetic products may cause foreign body sensation due to their particle size. In the development of ophthalmic pharmaceutical preparations, it has been recommended that drug particle sizes do not exceed 10µm to minimise eye irritation (Chowhan, Lang and Missel, 2012). Lipid deposits arising from cosmetics are often seen in CLW (Tlachac, 1994; Tsukiyama *et al.*, 2010). These deposits often affect contact lens wettability, resulting in discomfort and a reduction in visual acuity (Lorentz and Jones, 2007). The type of deposits attracted to the surface is dependent on the polymer properties of the lens materials; some silicone hydrogel contact lens materials are more prone to lipid spoliation than conventional hydrogel lenses (Carney, Nash and Sentell, 2008). While this survey did not explore the type of CL materials used by respondents, the increased trend of silicone hydrogel CL prescribing by eye care practitioners (Morgan *et al.*, 2012) means that there may be increased prevalence of cosmetic product deposition on CLs. Users that applied liquid eyeliner within the lid margin appeared to be less comfortable than pencil eyeliner users although this difference was not significant. Liquid and pencil eyeliner formulations differ: pencil eyeliners can contain up to 70% waxes, oils and fats combined with cosmetic colorants; liquid eyeliners comprise of a combination of film forming agents, waxes, emollients and viscosity controlling agents in which cosmetic colorants are added (Colipa, 2000). Variations in product formulation may be part of the reason why a difference between OSDI scores in liquid and pencil eyeliner users exists.

All cosmetic products manufactured for sale in Europe must undergo rigorous safety assessments before they are placed on the market to ensure that the product does not cause harm to human health. Lead toxicity and changes in conjunctival pigmentation are well documented complications arising from the use of kohl commonly used in Indian and

Middle Eastern cultures (Hidayat *et al.*, 1997; Al-Ashban *et al.*, 2004; Hardy *et al.*, 2006; El Safoury *et al.*, 2009) however there is little published literature which discusses the long term side effects of Western eye cosmetic use. There are several reported cases where the use of mascara and eyeliner has resulted in increased conjunctival pigmentation, ranging from diffuse pigmentation of the tarsal conjunctiva and conjunctival fornices to discrete, punctate deposits (Sugar and Kobernick, 1966; Donaldson, 1969; Platia *et al.*, 1978) although it is important to note that these publications are dated in light of modern cosmetic product formulations. More recently there have been reported cases of accumulation of cosmetic products within the lacrimal system and ocular surface which have resembled melanomas (Shields *et al.*, 2005; Ciolino, Mills and Meyer, 2009; Clifford, Jeffrey and Maclean, 2011). However it would appear the reported incidence of these unusual circumstances are relatively rare compared with the reported incidence of allergic contact dermatitis around the eyelids following cosmetics use, which is approximately 4% (Adams and Maibach, 1985). Therefore it is unsurprising that 42% and 46% of respondents expressed "No opinion" to the statements "Wearing eye make-up has a detrimental effect on the health of my eyes" and "My eyes will be just as healthy in 20 years' time whether or not I wear eye make-up" respectively.

Although only 2% of respondents agreed with the statement "Wearing eye make-up is good for my eyes" compared with 65% that disagreed with the statement, 83% of cosmetic users still continue to use eye cosmetics at least 3-5 times a week. Interestingly the cosmetic users that agreed with the statement also showed improved perceived ocular comfort scores when cosmetics were used. The trends of perceived ocular comfort score and responses to the statements "Wearing make-up has no influence on the comfort of my eyes", "Wearing make-up is good for my eyes" and "My eyes will be just as healthy in 20 years' time whether or not I wear eye make-up" were similar, where respondents in disagreement to these statements had the greatest difference in perceived ocular comfort scores and greater OSDI scores. This conforms to logic and suggests the questionnaire was completed reliably by respondents.

It is important to acknowledge that the statistical analysis in this chapter has not accounted for Bonferroni error which may arise from multiple comparisons, particularly in sections

2.3.3.1 and 2.3.6.1. Future statistical analysis could take into account the potential for Type I error by utilising a Bonferroni correction (α-value divided by the number of comparisons made).

The results show that a combination of the habits and type of cosmetics products used in the cohort is vast and varied. Not one respondent in the survey only used one type of cosmetic product so the OSDI scores are likely to be influenced by the use of a combination of products. Using eye cosmetics affects perceived comfort in CLW and NCLW. Despite these findings and respondent's opinions largely showing negative attitudes towards the link between eye cosmetic usage and ocular health, over 80% respondents reported using at least eye make-up 3-5 times a week. The following chapters will explore how the use of eye cosmetics interacts with the tear film and ocular surface. Firstly, proof of how eye cosmetics applied to peri-ocular skin migrate and contaminate the tear film will be examined. Following this proof of concept chapter, the clinical and immunological aspects arising from controlled eye cosmetics application will be explored.

3   Proof of eyeliner migration

3.1   Introduction

Before examining the clinical effects of any migration of cosmetic products to the ocular surface and tear film, it is important to establish that migration does occur. This chapter explores the proof of concept about passive product migration from the ocular adnexa to the tear film and ocular surface.

The European Commission's Scientific Committee on Consumer Safety has estimated the approximate exposure level to eyeliner is 0.005g, covering an area of $3.2cm^2$, assuming the product is applied to both eyelids and each line is approximately 1mm wide (White *et al.*, 2010). Conventional eye cosmetic products have the potential to migrate onto the ocular surface and into the tear film, behaviour that has been anecdotally reported during routine anterior eye examinations by eye care professionals (Tsukiyama *et al.*, 2010). In non-contact lens wearers, slit lamp evaluation of the tear film can show fine particles and pigments derived from eye cosmetics suspended in the tear film. Also, contact lens wearers are often seen to have pigments and lipid components of eye cosmetics deposited on the lenses (Tsukiyama *et al.*, 2010). The desire for colour cosmetics to reside for longer periods of time on the skin has driven the formulation of "long-lasting" cosmetic products (Mintel, 2013).

3.1.1   The influence of peri-ocular musculature

Cosmetics found in the tear film may result from simple migration, arising from poor application technique or from eye rubbing. However inadvertent, passive migration of products from the peri-ocular skin into the tear film has been attributed to the muscles of Riolan which lie beneath eyelid skin (MacKeen *et al.*, 1998). Considered to be anatomically separate from the pre-tarsal portion of the orbicularis oculi, the muscles of Riolan are divided into two regions (Figure 3.1): one anterior to the tarsal plate (pars ciliaris) and one posterior to the meibomian glands (pars subtarsalis) (Lipham, Tawfik and Dutton, 2002). The fine vertically aligned muscle fibres lie adjacent to the lid margins keep the eyelashes appropriately orientated (MacKeen *et al.*, 1998; May *et al.*, 2000) and are thought to allow

small vertical movements of peri-ocular skin, thus facilitating the migration of products applied around the eyes towards the tear film (MacKeen *et al.*, 1998).
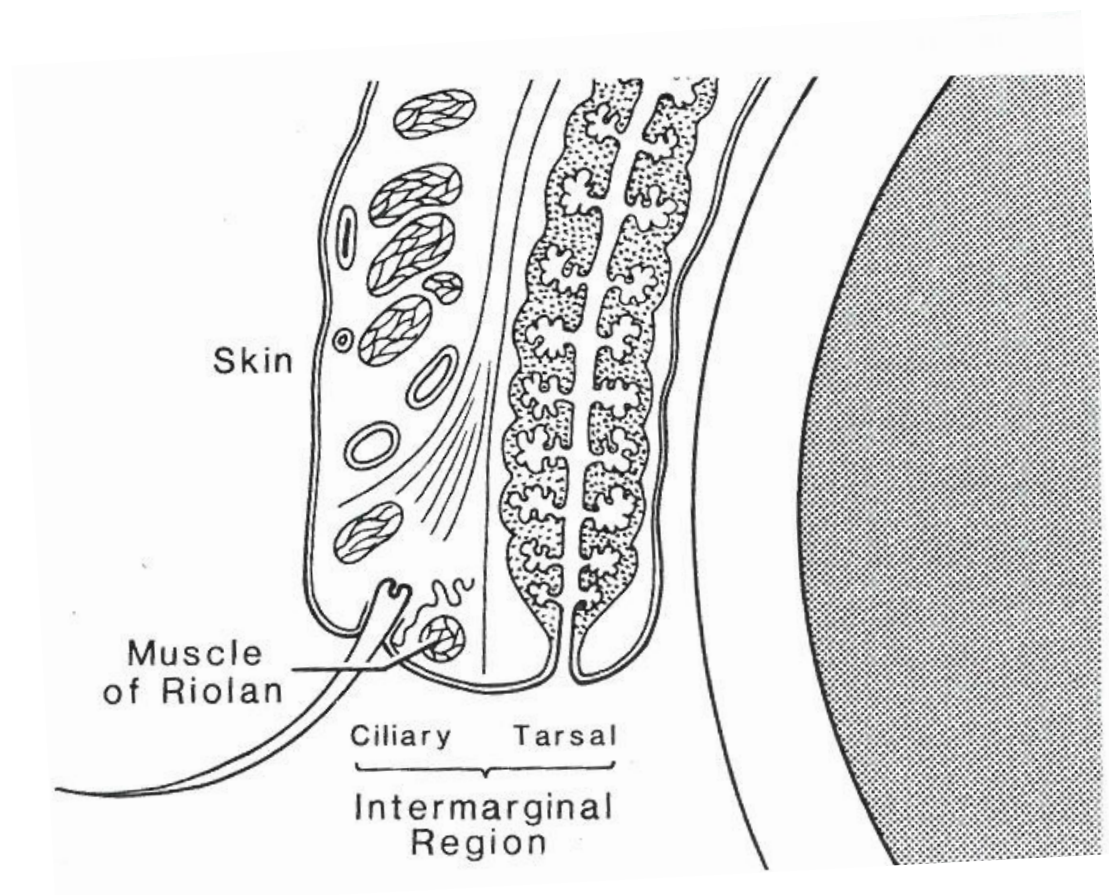
Figure 3.1 Cross section of the lid margin. The muscle of Riolan controls the anterior portion the eyelid margins

3.1.2   The migration of drugs and fluorescent markers

Several reports demonstrate the movement of particles across the mucocutaneous junction (MCJ), driven by the development of alternative methods of ocular drug delivery which avoid direct instillation of the drug onto the eye (MacKeen *et al.*, 1998).

A glycerol-petrolatum ointment containing pilocarpine applied to the lower eyelid margin has been shown to cause a reduction in intraocular pressure (MacKeen, Roth and Doane, 1996). It was hypothesised that this effect was a result of the migration of the drug-lipid vehicle combination across the lid margin. A second study by the same authors employed the application of a 3mm line of calcium carbonate ointment between 6 o'clock and the lateral canthus of dry eye subjects. This resulted in a decrease in dry eye signs and

symptoms which the authors attributed to the migration of ointment into the tear film. (MacKeen *et al.*, 1998).

The studies using pilocarpine and calcium carbonate ointments used secondary outcome measures to prove the hypothesis of ointments migrating across the MCJ. Later studies visualised this migration process by using ointments which were a different colour, or by the addition of fluorescent markers. For example, zinc oxide ointment (though not an ophthalmic preparation) applied to the lower lateral lid skin was used to demonstrate the movement of ointments applied to the peri-ocular skin as the white preparation was easily visible (MacKeen *et al.*, 1998). Later studies involved the addition of 10% sodium fluorescein (FLN) to a glycerol-petrolatum ointment applied at several locations along the margin of the lower lid. Both the zinc oxide and FLN ointments demonstrated visible migration from their original application points within one minute. In the application of the FLN preparation, fluorescence was visible for over 15 minutes post-application (MacKeen *et al.*, 1998).

Tsubota *et al.* performed a similar study by adding 1% FLN to petrolatum ointment which was then applied to the lower eyelids of four subjects (Tsubota *et al.*, 1999). Objective fluorometric evaluation indicated the presence of FLN in the tear film 30 minutes post-application, with fluorescence still evident six hours later. Goto *et al.* formulated equal volumes of hydroxyethyl cellulose gel and 10% FLN solution, which they described as CPM (cosmetic product material). This was applied by a beautician along peri-ocular skin (at different distances from the upper and lower lid MCJ) and along the inner eyelid margin (Goto *et al.*, 2010). The migration of CPM when applied at different distances from the upper and lower lid margins was compared. The results showed greater and quicker migration occurred where CPM was applied closest to the ocular surface. Additionally, when examining the effects of increasing tear volume, CPM migrated significantly quicker following the instillation of saline eye drops.

The movement of foreign substances across lid margins has been exploited in the formulation of liposomal spray treatments for dry eye syndrome. Such a spray is administered 10cm away from closed eyes. It is estimated that 0.11±0.01ml of liposomal spray is delivered by a single application to the lid surface (Craig *et al.*, 2010), covering a surface area of 50cm^2 (Lee *et al.*, 2004; Chowhan *et al.*, 2012). The movement of liposomes

from the lids onto the ocular surface has been likened to the penetration of eye drops to the ocular surface when applied to the medial canthus of closed lids, which is well documented (Smith, 1991; Loewenstein *et al.*, 1994; Alster *et al.*, 2000; Dausch *et al.*, 2006). Considering the typical blink cycle, with the eyes closed, liposomes which have migrated from the lids into the lipid "reservoir" at lid margins will initially mix with the natural lipids secreted from the meibomian glands. It is not until the open eye phase of the blink cycle does the lipid layer spread upwards across the ocular surface, drawing the aqueous phase with it. The application of liposomal spray has been shown to enhance tear film stability and improve lipid layer patterns for up to 90 and 60 minutes post-application respectively (Craig *et al.*, 2010). Again, this proves migration via a secondary measure related to tear film characteristics rather than primary proof.

While Goto's study proves to be a model of the migration of products applied to peri-ocular skin, the mechanism of migration is only proven for hydrophilic hydroxyethyl cellulose gel combined with FLN, and not for everyday cosmetic products available to consumers *per se*. Indeed, the authors justify the formulation of their own CPM to avoid brand bias. It can also be argued that the conclusions from the studies by MacKeen *et al.,* Tsubota *et al.* and Craig *et al.* which utilise contaminating treatments with FLN provide limited understanding of the mechanisms of eye cosmetic migration. FLN is a highly charged molecule and the diffusion of FLN is greater in gels than in water (Casalini *et al.*, 2011). Furthermore, with the difference in molecular weight between FLN and the excipients, the FLN tracer may migrate quicker than the vehicle or the active ingredients in an ointment. Therefore, it is possible that the conclusion of such studies do not tell us about the migration of the excipients such as hydroxyethyl cellulose or glycerol-petrolatum, but only of the charged FLN tracers in the ointments. It has been suggested that in order to determine the migration and residency time of ointments, covalent binding of compounds with radioactive tracers could be more effective (Greaves, Wilson and Birmingham, 1993; Meadows *et al.*, 2002). Using radioactive tracers has its practical disadvantages – a radio-isotope pharmacy is required to bond radioactive isotopes to the ointment excipients.

More recently, quantum dots have been used to visualise the dynamics of a normal tear film (Khanal and Millar, 2010). Quantum dots (qdots) are highly fluorescent nano-sized semiconductors encapsulated with an outer shell that absorbs light in the ultraviolet and

deep blue regions of the electromagnetic spectrum. Qdots can be manufactured to encompass a range of nano-sizes which dictates the spectral emission. They have been used in cell biology for immunohistochemical imaging by conjugating qdots to proteins and small molecules (Biju *et al.*, 2008). The outer coating of qdots can be tailored to make the particle hydrophilic or lipophilic, depending upon the intention of use (Khanal and Millar, 2010). Figure 3.2 shows a diagrammatic structure of a qdot.

Core nanocrystal determines colour

Inorganic shell determines brightness & stability

Organic coating can be made hydrophilic or lipophilic to optimise for conjugation with biomolecular coat
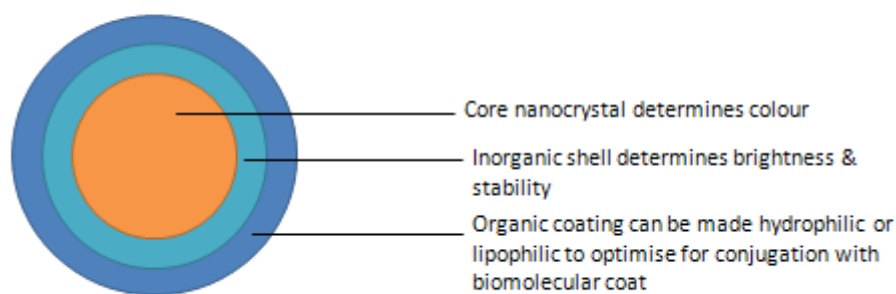
Figure 3.2 Diagram of the composition of a quantum dot (qdot)

Khanal & Millar applied 20-25nm sized lipophilic qdots solution to the lower eyelid margin of seven subjects between the meibomian gland openings and eyelashes (Khanal and Millar, 2010). The fluorescence and migration of qdots were observed with a slit lamp and cobalt blue filter. With this technique, the authors were able to observe the exact migration of qdots before and after a blink. The dispersion and incorporation of qdots into the outer layer of the tear film only occurred after a blink and the authors noted they moved in a similar pattern to the naturally occurring particles already present in the tear film. Although the qdots remained on the eyelid margins for over two hours after application, their fluorescence in the tear film was no longer detected after this time.

The behaviour of lipophilic qdots may be most similar to that of lipophilic waxes commonly used in eyeliner pencil formulations compared to other studies examining the migration of ointment and solutions. However, many of the existing conclusions about migration of ointments applied to peri-ocular skin are limited to the vehicle or marker used in each study. Furthermore, there is no published work concerning the migration of cosmetic products, other than anecdotal reports.

3.1.3 Methods of observing particles in the tear film

Qdots are a relatively recent way to examine contaminants in the tear film and tear film kinetics. Naturally occurring particles in the tear film have previously been studied to analyse tear film kinetics (Berger and Corrsin, 1974; Owens and Phillips, 2001; Varikooty, Keir and Simpson, 2012). The origins of these particles are not established but are thought to be cellular debris and dust from the environment (Owens and Phillips, 2001; Khanal and Millar, 2010). These particles were thought to be greater than 0.1µm in size as they protrude out of the lipid layer (Norn, 1979; Owens and Phillips, 2001) and more recently, have been calculated to be 7µm^2 in size (Varikooty *et al.*, 2012). Two important studies which have examined the velocity and spread of these naturally occurring particles are summarised in Table 3.1. Both studies utilised commercially available slit lamps and filmed tear film dynamics using a video camera attached to one slit lamp ocular.

Table 3.1 Summary of experimental set-ups used to examine naturally occurring particles in the tear film

	Owens and Philips (2001)	**Varikooty *et al.* (2012)**
Method of image capture	Video camera (Sony Handicam CCD-TR3-3E) attached to slit lamp ocular. Video output to a television screen	CCD video camera (Sony DXC-C33) mounted on a slit lamp
Frame capture rate	25Hz (25 frames per second)	30 frames per second
Magnification	Slit lamp magnification x20 however total magnification (from cornea to television screen) x130	Slit lamp magnification x8
Illumination	Kept to a minimum to avoid reflex tearing	170 lux (the minimal intensity that enabled a high particle contrast for optimum visualisation)
Slit lamp angle of illumination	40°	30°
Slit lamp beam width	1 x 2mm (horizontal x vertical)	15mm
Position of slit lamp beam	Inferior temporal to pupil margin	N/A (central cornea observed)
Filming duration	30 – 60 seconds	10 seconds
Method of image processing	Measured distance travelled across television screen in 0.04s (1/25Hz) by individual particles; precision of screen measurement = 1mm (equivalent to 8µm on the eye)	ImageJ software with the particle tracking plug-in customised to track the position of highly reflective particles through successive frames during one inter-blink interval

3.1.4   Aims and hypotheses

The aim of this study was to investigate, record and quantify the migration of a conventional eye cosmetic pencil when applied to peri-ocular skin in two different locations: outside the lash line (ELO) and behind the lash line (ELI).

The primary hypothesis of this study was that eyeliner applied in the peri-ocular region does migrate passively into the tear film. Secondarily, eyeliner applied within the lash line is likely to migrate more readily and contaminate the tear film compared with eyeliner applied outside the lash line (ELO). Furthermore, the amount of particles observed on the pre-ocular tear film at the end of the recording time will be similar after both methods of eyeliner applications.

3.2   Methods

Three female subjects (median age 26 years, range 26-30 years) were recruited from students and staff at the School of Optometry and Vision Sciences, Cardiff University. The study was approved by the Research Ethics Committee at the School of Optometry and Vision Sciences, Cardiff University and was in accordance with the regulations of the Declaration of Helsinki. Informed consent was obtained from all subjects prior to commencing the study.

3.2.1   Exclusion criteria

Subjects were excluded from the study if they: were pregnant, exhibited any ocular or systemic pathology known to affect the tear film or ocular surface or if their initial examination revealed abnormal ocular findings; described a history of ocular trauma, infection or inflammation within the previous three months; reported previous allergic responses following the use of eye cosmetics.

3.2.2   Study design

All subjects were instructed to attend on a day where no eye cosmetics were worn for at least the previous 12 hours. Subjects were initially examined by slit lamp biomicroscopy to establish eligibility for the study and to ensure no remaining eye cosmetics were evident on the peri-ocular skin or eyelashes. Suitable subjects were randomly assigned one of two application conditions: eyeliner applied behind the lash line (ELI) or applied outside the lash line (ELO). The examiner applied the eyeliner to ensure standardisation. The eyeliner was applied to both upper and lower lid of the right eye only, as illustrated in Figure 3.3.
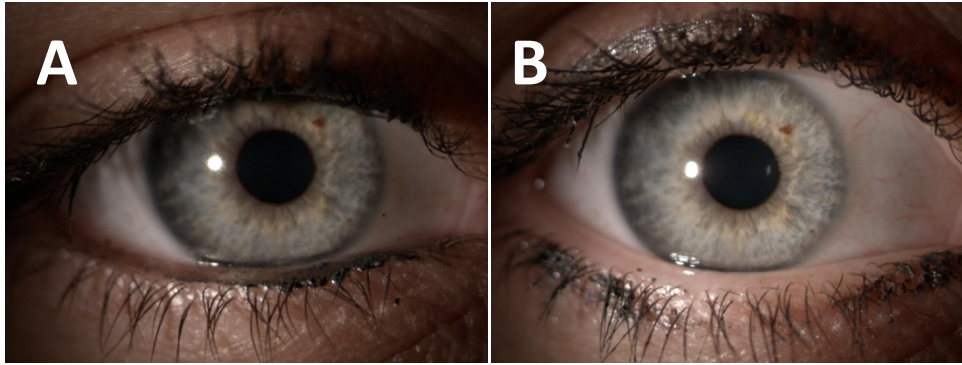
Figure 3.3 Images illustrating ELI (A) and ELO (B) application

A soft eyeliner pencil (Avon Glimmerstick Liqui-glide Graphite, Avon, UK) was selected for this study. The smooth, soft consistency was desirable for this study as this ensured successful application on participants. The eyeliner pencil was also highly pigmented and contained a greater quantity of glitter particles than other eyeliner pencils which was a desirable feature to aid migration identification. The ingredients of this pencil eyeliner can be found in Appendix IV. Following eyeliner application to the right eye only, the central region of the cornea and overlying tear film was immediately filmed using a digital video slit lamp (Bon 75-SL DigiPro3 HD, Bon, Germany) for 30 seconds, focussed on the precorneal tear film and the glitter particles suspended within it. Subjects were requested to maintain fixation in the primary position and to blink at their normal rate and pressure. The precorneal tear film was filmed again at the following time points for 30 second intervals: 5, 10, 15, 20, 25, 30, 60, 120 and 240 minutes post-eyeliner application. In between filming, subjects were advised to relax and were instructed not to touch the face.

Subjects underwent a minimum 24 hour wash out period prior to repeating the study in the alternative eyeliner position at the same time of day under the same room conditions.

3.2.3 Slit lamp videotaping set up and image processing

The slit lamp (Bon 75-SL DigiPro3 HD, Bon, Germany) was set up to X16 magnification with the slit beam width 9mm (H), 5mm (W) and the angle of illumination 55°. The slit lamp frame capture rate was fixed at 8.2 frames per second by the slit lamp manufacturer. The beam was placed temporal to the pupil on Purkinje image I. The slit lamp videos were processed using ImageJ software (ImageJ, version 1.47u) (Abramoff, Magalhães and Ram, 2004). This analysis process will now be discussed.

Each 30 second video clip produced a series of 210 images. The clip was imported into ImageJ as a stack of images. This stack of images was converted to 8-bit greyscale (Figure 3.4). The stack was screened to remove any frames that contained full or partial blinks. A region of interest (ROI) was manually selected which would contain the Purkinje image across all frames without selecting areas of image quality drop-off. This ROI was cropped from the stack of images (Figure 3.5). The background was subtracted using the "Subtract background" function, setting the rolling ball to 5 pixels (Figure 3.6). This step was performed to remove the Purkinje image from each frame (Figure 3.7). The maximum threshold was set using the frame that contained maximum particle spots by observation. This threshold was applied for all frames within one video file (Figure 3.8). The "Analyze particles" function was used to count the number of pigment particles present in each frame. The settings selected to analyse the particles are as follows: size 0-10px^2, circularity 0-1.00 (Figure 3.9). The results generated from the "Analyze particles" function were exported into Microsoft Excel (Microsoft. Microsoft Excel. Redmond, Washington: Microsoft, 2007) for data analysis.

3.2.4   Data analysis

The mean number of particles per time point was calculated from the data imported into Microsoft Excel for each subject. The mean and standard deviation of number of particles visible at each time point per subject was calculated.

Figure 3.4 Converting the stack of images into 8-bit greyscale
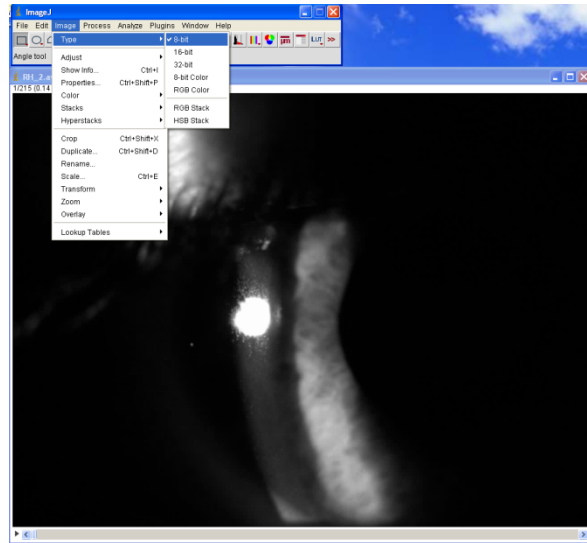
Figure 3.5 Region of Interest (ROI) cropped from stack of images
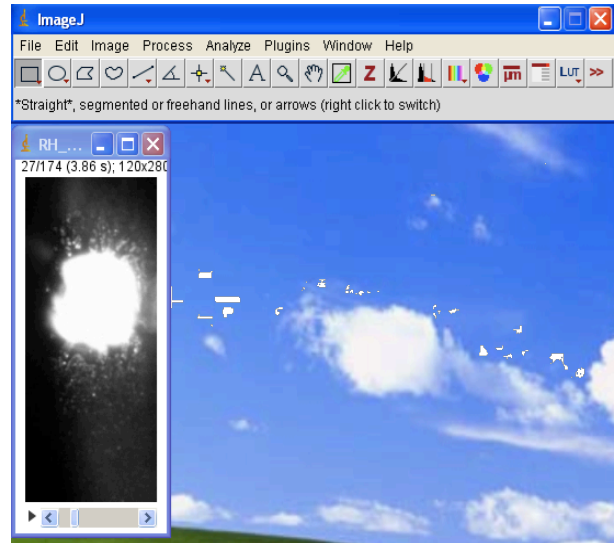
Figure 3.6 Selecting "Subtract Background"
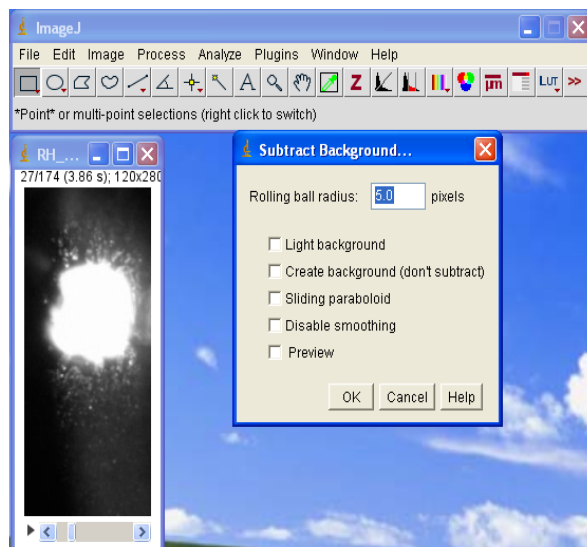
Figure 3.7 Resultant image stack appearance after "Subtract Background" function has been applied
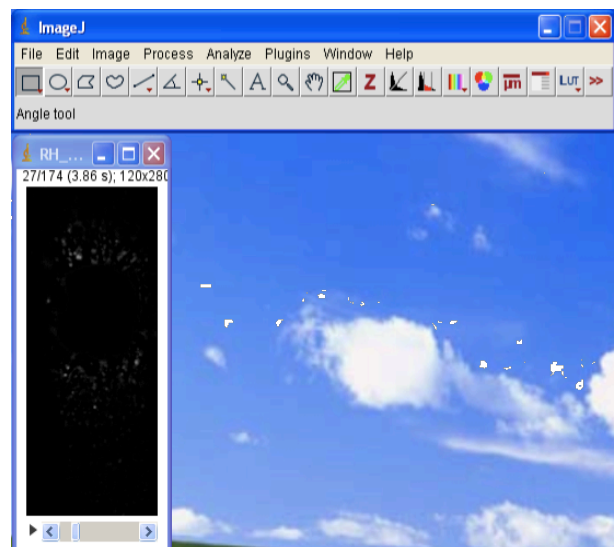
Figure 3.8 Selecting a threshold to apply to the stack of images. Red dots highlight particles which were counted by the software.
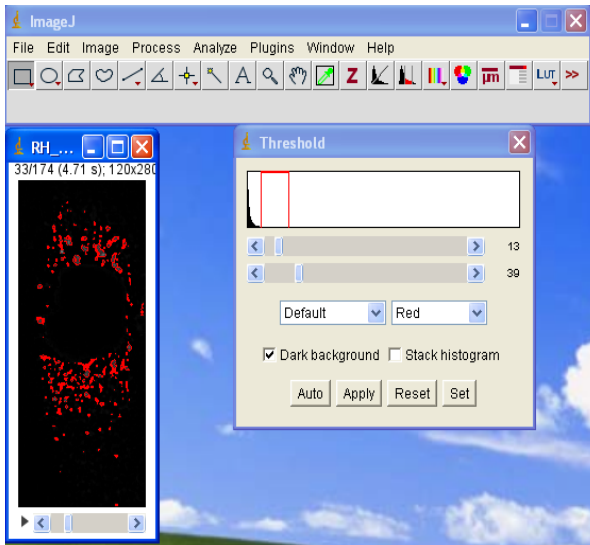
Figure 3.9 Application of the "Analyze Particles" function
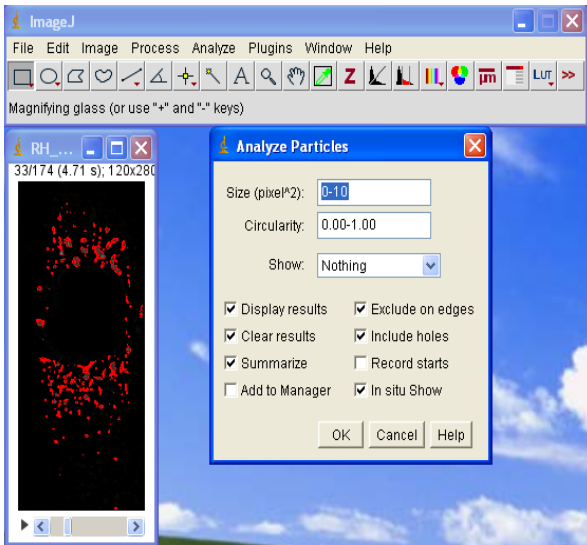
3.3   Results

Table 3.2 shows slit lamp images of ELI and ELO for the subject RH at baseline and four different time points of the study.

Table 3.2 Images for ELI and ELO at four different time points of the study for subject RH
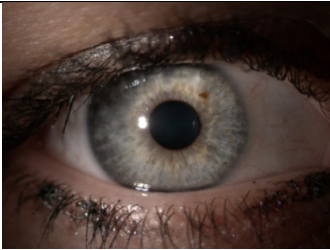
	ELI	ELO
Baseline		
Immediate post-eyeliner		
30 minutes		
60 minutes		
120 minutes		

Table 3.3 shows the number of particles observed in each of three participants over the time course in the ELO and ELI applications.

Table 3.3 Quantity of particles identified in the pre-ocular tear film of three subjects at nine time points after ELI and ELO application (* = results based on one static image only as recording function was not enabled for this observation)

	Time point	Number of particles		
		Subject		
		RH	**TC**	**FN**
ELI	**Baseline**	24±12	2±2	8±5
	Immediate post- eyeliner	126*	116±32	80±30
	5 minutes	227±96	50±15	27±21
	10 minutes	288±61	51±29	19±14
	15 minutes	163±91	24±9	23±13
	20 minutes	87±48	22±9	15±9
	25 minutes	121±41	29±12	10±7
	30 minutes	155±62	30±10	9±5
	60 minutes	89±30	11±5	6±4
	120 minutes	30±15	8±4	5±3
ELO	**Baseline**	6±4	7±3	3±1
	Immediate post- eyeliner	9±7	9±5	3±2
	5 minutes	40±20	17±6	3±2
	10 minutes	27±12	11±5	2±2
	15 minutes	31±19	8±5	2±1
	20 minutes	76±31	10±6	12±5
	25 minutes	73±28	11±6	7±5
	30 minutes	85±26	4±3	7±4
	60 minutes	34±21	8±4	1±1
	120 minutes	10±9	6±3	3±2

Figure 3.10 shows the eyeliner collected in the inner canthus of one participant after 30 minutes after ELO application.

Figure 3.10 Image of Avon eyeliner collected in the inner canthus (circled) of one participant after ELO application

Figure 3.11, Figure 3.12 and Figure 3.13 shows the number of particles observed in the tear film in three participants over 120 minutes following ELI and ELO application. The graphs indicate that ELI application results in a gradual increase in particles in the tear film from immediate application, with subject RH reaching maximal number of particles by 10 minutes post-application (Figure 3.11). Subjects TC and FN exhibited a steady decline in detectable particles after 5 minutes post-application, returning to baseline values by 120 minutes (Figure 3.12 and Figure 3.13, respectively). Subject RH exhibited a steady decline in detectable particles after 10 minutes post-application however gradually increased between 20-30 minutes before decreasing again.

The number of particles observed in the tear film in the same participants on a separate occasion with the eyeliner applied to peri-ocular skin (ELO) is also shown in each of the graphs. Figure 3.11 to Figure 3.13 shows the subjects reached maximal number of particles in the tear film at different times: RH had the maximum quantity of particles in the tear film at 30 minutes post-application compared with FN at 20 minutes. TC had the maximum quantity of particles in the tear film at 5 minutes post-application.

The maximum number of particles in the tear film was greater in the ELI condition compared with the ELO condition, as indicated in the graphs and in Table 3.3. For subject RH, the maximum number of particles in ELI was 288 compared with 85 in ELO (a 30%

difference). For subjects TC and FN, the difference in maximum particles between ELI and ELO is in the order of 15%. Furthermore, the time taken to reach the maximal quantity was less in the ELI condition compared with ELI.
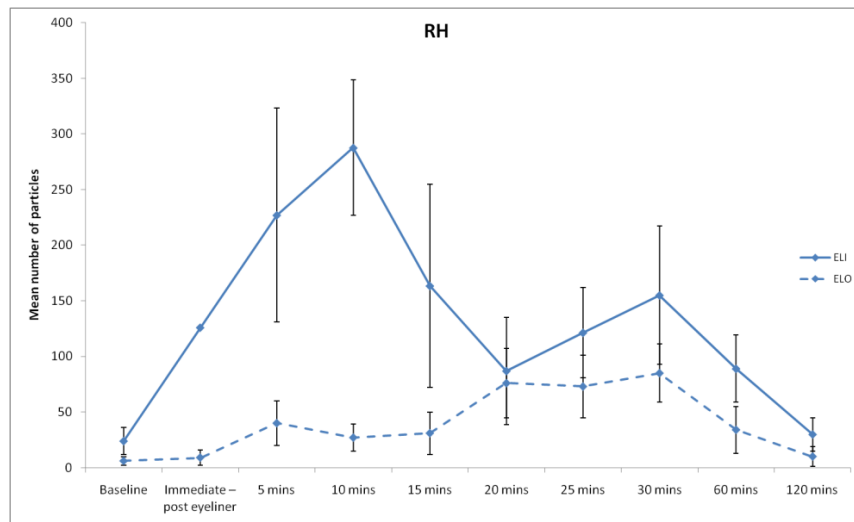
Figure 3.11 Number of particles after ELI and ELO application for subject RH (error bars = standard deviation)
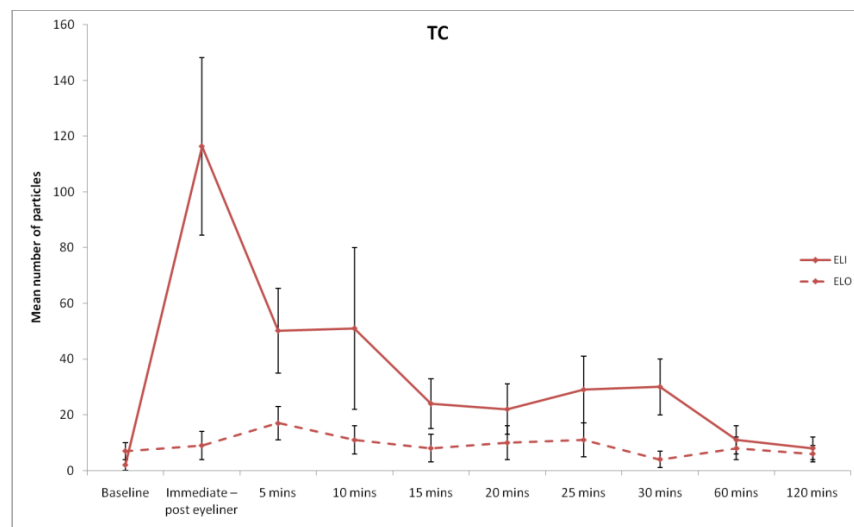
Figure 3.12 Number of particles after ELI and ELO application for subject TC (error bars = standard deviation)
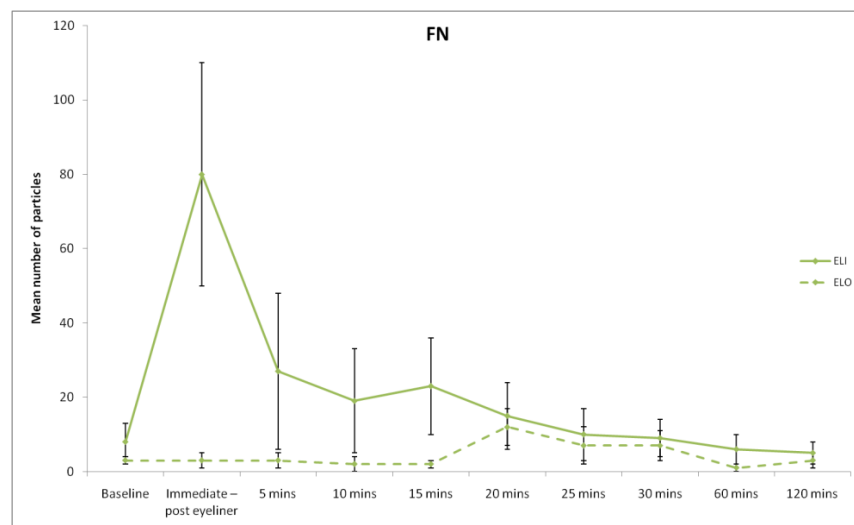
Figure 3.13 Number of particles after ELI and ELO application for subject FN (error bars = standard deviation)

3.4   Discussion

The results of this study have proved for the first time that migration of cosmetic pencil eyeliner the tear film occurs when eyeliner is applied to lid margin skin. This was achieved directly by counting the number of glitter particles suspended in the tear film. The migration of the cosmetic pencil particles occurred more readily when applied behind the lash line (ELI application). This is in accordance with the results derived by Goto *et al.* who found the contamination of the tear film with their bespoke CPM was significantly quicker and greater when the product was applied along the inner eyelid compared with the same product applied 2mm away from the eyelid margins (Goto *et al.*, 2010).

The application of the cosmetic pencil eyeliner was controlled by the examiner. However, vast variations in the maximum quantity of particles suspended in the tear film occurred in both eyeliner conditions. Historically, it has been considered that the momentary touch between upper and lower eyelids during the blink cycle facilitates the spread of lipids secreted by the meibomian glands (MGs). Recently, two studies have challenged this by showing how the keratinised portions of the upper and lower lid do not touch upon full, complete blinks (Korb, Blackie and McNally, 2013; Pult, Riede-Pult and Murphy, 2013). In fact, the upper lid "overshoots" the lower lid, a term classified as "overblink" by Pult *et al.* (2013). It is therefore unlikely that the migration of eyeliner is facilitated by the mechanical action of the upper and lower lid touching upon blinks. To observe eyeliner materials that migrated from the lid margin into the tear meniscus and pre-ocular tear film, the materials must first enter the tear lipid layer. This migration may have been facilitated by meibomian oil with which they have been mixed during application, more readily so in the ELI application.

It is interesting to note that cosmetic pencil eyeliner applied to the peri-ocular skin (ELO) also migrated across the lid margin to contaminate the tear film. When applied in this manner, the migration was slower and the quantity of glitter in the tear film was up to 30% less than ELI application. Goto's CPM applied 2mm from the eyelid margin has been shown to migrate and contaminate the tear film, although the quantity of contamination is significantly less than if the product is applied closer to eyelash line (Goto *et al.*, 2010). Additionally, the movement of liposomes across closed eyelids has previously been

demonstrated which also suggests lipids can readily move into the tear film (Craig *et al.*, 2010; Korb *et al.*, 2013). The exact means of how this may occur is uncertain and currently the muscles of Riolan have been ascribed to advance the migration of such particulate matter (MacKeen *et al.*, 1998). The warmth of the eyelids may also aid the mobility of lipid products as the lipid approaches its melting point. One recent study found external eyelid temperature to be 36°C in healthy individuals without lid margin disease (Purslow, 2013). For example, derivatives of coconut oil commonly used in many eye cosmetics, including pencil eyeliner, have a low melting point between 28-30°C (Rowe *et al.*, 2012) which means upon application onto eyelid skin at 36°C, the product would certainly melt.

At the final time point of observation (2 hours post-application), the quantity of particles detected in the tear film of all participants had returned to baseline values. The eyeliner ingredients have two potential routes of exit: via the lacrimal drainage system or outwardly back onto surround lid skin and lashes. This would mostly be determined by the solubility of the components of the eyeliner pencil. Lipid-soluble ingredients and associated glitter particles that migrated from the lid margin and were confined to the tear lipid layer may have diffused outwardly onto the lid skin and lashes as they have no access to the lacrimal puncta due to the hydrophilic nature of the mucosal tissue. This has been shown by Khanal and Millar by using lipophilic qdots (Khanal and Millar, 2010). Any water-soluble eyeliner ingredients which may have breached the tear lipid layer would mix with the aqueous phase and eventually drain via the puncta. This has been evidenced by one paper which documented seven patients that presented with black pigmentation of the lacrimal sac and surrounding anatomy after several years of eyeliner usage (Hidayat *et al.*, 1997). Particulate matter, carried by the aqueous-mucin phase, may be scavenged passively by mucous gel might be seen gathered at the nasal canthus prior to subsequent loss via the nasolacrimal system, as shown in Figure 3.10.

The ocular surface retention time of ointment is greater than eye drops (Greaves *et al.*, 1993) and varies according to its viscosity (Tong *et al.*, 2012). However, studies examining the retention time of eye drops often use duration of the mode of action as an outcome measure, which is not necessarily the same as retention time. For example, pilocarpine is effective up to six hours after initial instillation (Hopkins and Pearson, 1998). The study of pilocarpine ointment applied to the external eyelid margin found a reduction in intraocular

pressure up to 8 hours after initial application (MacKeen *et al.*, 1998) although migration of this ointment over the lid margin may not have been continuous up to this time point. Petrolatum ointment mixed with sodium fluorescein as a marker and applied to the external lid margin has been shown to maintain tear film fluorescence up to six hours after initial application (Tsubota *et al.*, 1999). However, as already discussed, as the sodium fluorescein was not covalently bonded to the petrolatum ointment, this may merely illustrate fluorescein migration over the lid margin over this duration. Therefore, the current studies of ointments applied to peri-ocular skin in the literature do not sufficiently reveal their retention time on the ocular surface. Although not an ointment, one study has examined the retention time of a castor oil emulsion eye drop instilled directly onto the ocular surface using high performance liquid chromatography. The study found traces of the eye drop was detectable in the tear film four hours after instillation (Maissa *et al.*, 2010). It is anticipated that different eye cosmetics will have unique retention times on the ocular surface due to variations in formulations and subsequent viscosities.

Strictly speaking, the pattern of migration observed in this small study applies to this product on these subjects, and was observed only in a Caucasian population. Eyelid anatomy has been shown to be different in various ethnicities. For example, Asians do not typically exhibit the upper eyelid crease, unlike those of Caucasian descent. Besides epicanthal folds and absent or shortened supratarsal crease, Asian eyelashes often demonstrate lash ptosis which may be due to difference in the orbicularis oculi, muscles of Riolan and tarsal plate (Malik *et al.*, 2007; Lee, Ahn and Kim, 2013). The distinct anatomical differences between Caucasian and Asian upper eyelids may indeed affect the pattern of cosmetic eyeliner migration and warrants further investigation.

In conclusion, this study has shown pencil eyeliner migrates and occurs most readily when it is applied behind the lash line. While migration of eyeliner following the application to the peri-ocular skin does occur, the migration is comparatively slower and the contamination is less. The migration of eyeliner applied around the eyelid margins has the potential to have an impact upon the tear film and ocular surface. Since the bulk of common pencil eyeliners are formulated of waxes and lipids, short- and long-term clinical tear film and ocular surface changes may occur after their use. The next chapter presents the results from a study exploring the clinical implications arising from cosmetic pencil eyeliner use.

4   The effects of eyeliner migration across the eyelid margin: clinical outcomes

4.1   Introduction

Preparations of eye cosmetics contain a range of preservatives, surfactants and emulsifiers. These ingredients may irritate the ocular surface, in a similar way that they might irritate peri-ocular skin in susceptible people (Malik and Claoue, 2012). Pigments and particles suspended in cosmetic may cause a foreign body sensation and/or tear film instability. Indeed it is recommended that particle sizes in preparations of ophthalmic pharmaceutical agents should be no larger than 10µm to minimise eye irritation (Chowhan *et al.*, 2012). The method of application of eye cosmetics is often dictated by fashion trends and personal preference. Products such as eyeliner appear to have greater potential to readily contaminate the tear film as they are often applied along peri-ocular skin at the lash line or within the lash line (Figure 4.1)
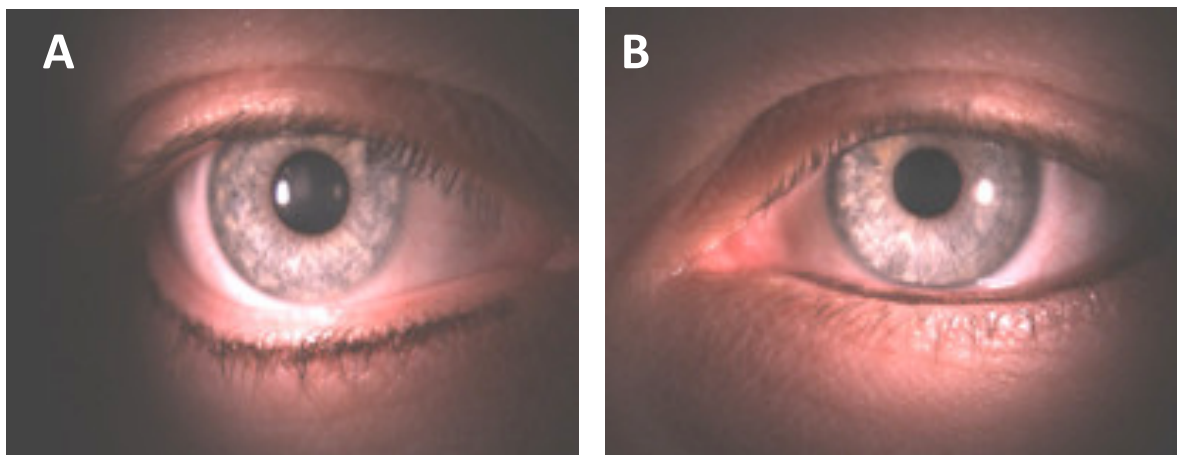
Figure 4.1 Eyeliner applied outside the lash line (A) and within the lash line (B)

In fact, the notion of tear film contamination following kohl application was exploited in ancient civilisations as it was believed to protect and treat eye ailments and provide glare relief (Mahmood *et al.*, 2009).

Eyeliner pencils are one of the most popular formulations of eyeliner, composed of natural or synthetic waxes, oils and fats, combined with pigments and formulated into rods and encased with wood (Draelos, 2001). Conjunctival pigmentary changes have been

documented resulting from long-term use (i.e. over several decades) (Pao, Murchison and Eagle, 2012); however these reports have indicated eyeliner use in conjunction with other eye cosmetics. The consequences of the interaction of eyeliner with the tear film are unknown.

The ingredients typically used in pencil eyeliner formulations are summarised in Table 4.1.

Table 4.1 The maximum permissible weight/volume (w/v) for each ingredient in eyeliner formulations as directed by Colipa (The European Cosmetics Association) Frame formulations for the EU. The frame formulations outlines the ingredients, maximum levels and functions of each ingredient per cosmetic product, were written to assist poison centres in administrating the correct treatment.

Ingredient(s)	Example of ingredient	Maximum levels (%w/v)
Waxes, oils and fats	Ozokerite Carnauba Hydrogenated vegetable oil	70
Cosmetic colorants, colour additives	Pearlescent agents	50
Silicones, volatile silicones	Cyclopentasiloxane	50
Fillers	Talc	30
Polymers, resins	Nylon	20
Surfactants	Polysorbate 60 PEG-6 Sorbitan stearate	5
UV filters	Zinc oxide Titanium dioxide	5
Additional ingredients	Vitamins Plant extracts	5
Preservatives, antimicrobials, antioxidants	Parabens	1
Parfum		0.3

Waxes and oils form the largest component of eyeliner pencils. Cosmetic pencils need to be able to remain solid at room temperature but need to be able to glide onto the skin upon application and maintain residence for long periods of time. Optimal combinations of natural and synthetic waxes and oils are used to formulate the lipid component of eyeliner pencils to this desired consistency (Schlossman, 2001).

The interaction of these ingredients with the ocular surface and tear film remains largely unknown. Although regulated eye cosmetic products should cause no harm or morbidity to

the ocular surface (the potential toxicity of individual components have been tested), mild undesirable effects may go undetected or unreported. Many consumers simply choose to stop using sensitising products from their cosmetic routine.

Chapter 2 describes the morphology of the tear lipid layer as two-layered structure consisting of non-polar and polar lipids. The fundamental role of the lipid layer is to prevent tear evaporation. A study by Craig and Tomlinson demonstrated a four-fold increase in tear evaporation when the lipid layer is minimal or inconsistent (Craig and Tomlinson, 1997). Increased tear evaporation can result in evaporative dry eye (EDE) and current treatment strategies aim to reduce evaporation by increasing and stabilising the lipid layer. These treatments will briefly be discussed.

4.1.1 Modifying the tear lipid layer by increasing meibum

There is good evidence that the application of heat and expression of the meibomian glands (MG) improves meibum delivery to the tear film (Goto *et al.*, 2002a; Olson, Korb and Greiner, 2003; Arciniega *et al.*, 2011). The International Workshop on Meibomian Gland Dysfunction (MGD) advises the use of eyelid warming with moderate to firm massage and expression of MG secretion in Stage 2 of MGD (Geerling *et al.*, 2011).

Simple digital expression of the MG after heat application to the eyelids is an effective way of temporarily reducing tear evaporation rate for up to 48 minutes (Arciniega *et al.*, 2011). The Lipiflow System (Morrisville, NC, USA) builds upon conventional digital expression and is a sophisticated medical device licensed for practitioner use. The instrument provides heat directly to the palpebral surfaces of the upper and lower eyelids simultaneous to applying pulsatile pressure to the outer eyelid surfaces. Prospective studies have shown that one single 12 minute treatment has yielded improved symptoms, MG secretion and tear stability for up to nine months post-treatment (Korb and Blackie, 2010; Friedland *et al.*, 2011; Greiner, 2012). These studies have shown by addressing the problem of the tear lipid layer by treating the MG, there is an improvement in clinical signs and symptoms.

4.1.2 Manipulating the lipid layer via the application of oil-based products

As the management of dry eye syndrome has become more targeted, the introduction of products that specifically target the lipid layer, via application into or around the eye, may offer insight into how the components of eyeliner may mix with the tear film. The mixing of lipid-based dry eye treatments, used to treat patients with an inadequate lipid layer in evaporative dry eye disease, may mimic the effects of the lipid components of pencil eyeliner mixing with the tear film. The literature describes two methods of lipid administration: direct (emulsion eye drops and ointments applied along the inner eyelid) and indirect (ointments applied to external skin and sprays applied to closed eyes).

Direct instillation of oil-in-water emulsion eye drops upon tear stability have been investigated using a formulation of 2% castor oil and 5% polyoxyethylene castor oil emulsifier in distilled water (Goto *et al.*, 2002b). Significant improvements were found in lipid layer thickness (LLT), tear evaporation, ocular surface staining, TBUT and meibomian orifice obstructions after administering the formulation six times daily for two weeks compared to a saline placebo in 20 dry eye subjects. In a separate study, a significant reduction in tear film evaporation and improvement in lipid film quality was found after using 1.25% castor oil emulsion eye drops three times a day for 30 days compared with hypromellose (Khanal *et al.*, 2007). Supportive work by Maïssa *et al.* detected castor oil in tear samples up to four hours after a single instillation of the same formulation of eye drops (Maissa *et al.*, 2010). These studies have shown that the use of castor oil in formulations of eye drops can directly target and change the lipid layer composition. Castor oil is highly polar lipid with a hydrophilic and hydrophobic group thus assisting the interaction of polar oil with the natural polar layer of the tear film and the adjacent aqueous-mucin phase (Goto *et al.*, 2002b). The longer residency time of lipid-enhancing treatments on the ocular surface reflects this interaction, unlike aqueous-targeting treatments which have a relatively short residency time.

The direct application of 2mm of ofloxacin (antibiotic) ointment along the lower inner eyelid margin of dry eye subjects was shown to have significant benefits in treating dry eye. The ointment base consisted of a mix of polar and non-polar lipids (liquid paraffin, white

petrolatum and purified lanolin) and the authors hypothesised that the non-polar lipid base improved clinical outcome measures (Goto *et al.*, 2006). However, it is also possible that the antibiotic may have had beneficial therapeutic effect in the treatment of MGD (Goto *et al.*, 2006; Geerling *et al.*, 2011).

Indirect application of lipid treatments has also shown to be beneficial in the treatment of EDE. As described in Chapter 4, the application of calcium carbonate ointment from 6 o'clock to the lateral canthus of the external lower lid margin improved dry eye symptoms when used twice a day for a period of three months (MacKeen, Roth and Doane, 1996; MacKeen *et al.*, 1998; Tsubota *et al.*, 1999). The migration of this ointment over the mucocutaneous junction was proven by contaminating petrolatum ointment with sodium fluorescein and observing tear film fluorescence with a fluorometer. Fluorescein was evident in the tear film up to six hours after the initial application of the petrolatum ointment (Tsubota *et al.*, 1999).

Liposomal sprays applied to closed lids has repeatedly shown improvements in lipid layer thickness, tear film stability and subjective reports of comfort (Lee *et al.*, 2004; Dausch *et al.*, 2006; Craig *et al.*, 2010; Pult, Gill and Riede-Pult, 2012). Polar lipids maintain the appropriate surface tension between the upper non-polar layer and the lower aqueous-mucin layer to aid the spread and stability of the tear film (Dausch *et al.*, 2006; Green-Church *et al.*, 2011). The phospholipid, phosphatidycholine, is a major polar lipid constituent in the human tear film and in the liposomal sprays, is derived from purified soy-lecithin (Lee *et al.*, 2004). Lipid layer thickness has been shown to be increased for up to 60 minutes post-application of liposomal sprays (Craig *et al.*, 2010). Interestingly, liposomal sprays formulated from isoflavonoids (also a derivative of soy which does not contain any phospholipid) worsened tear film stability and decreased ocular comfort (Pult *et al.*, 2012).

Chapter 3 established the proof of concept of migration of substances from the adnexa into the tear film. Malik and Claoué hypothesise that lipophilic substances initially diffuse through the tear film lipid layer, and since lipid-based substances are insoluble in the aqueous-mucin phase, they collect beneath the polar layer of the (inner) lipid layer (Malik and Claoue, 2012). Chapter 3 illustrated conventional pencil eyeliner, when applied around the eyelid margins, eventually migrates across the lid margin to contaminate the tear film

although there are no investigations in the literature that has examined the effects of the interaction of lipid-based pencil eyeliner on tear film parameters.

4.1.3   Aims and hypotheses

The principal aim of this study is to investigate the clinical changes following the application of pencil eyeliner in two different methods: within the lash line (ELI) or outside the lash line (ELO). It is hypothesised that this study will examine the "acute" response within the hypothesis cascade (see Figure 4.2).

Chapter 3 showed eyeliner applied within the lash line (ELI) is more likely to migrate into the tear film. Therefore it is hypothesised this method of application is more likely to be associated with changes to the ocular surface. Conversely, eyeliner applied outside the lash line (ELO) will also migrate but at a slower rate and as a result, the changes may be less immediate or marked. As pencil eyeliner is predominantly oil/wax-based, it is also hypothesised the maximal clinical influence will be on tear film stability and quality.
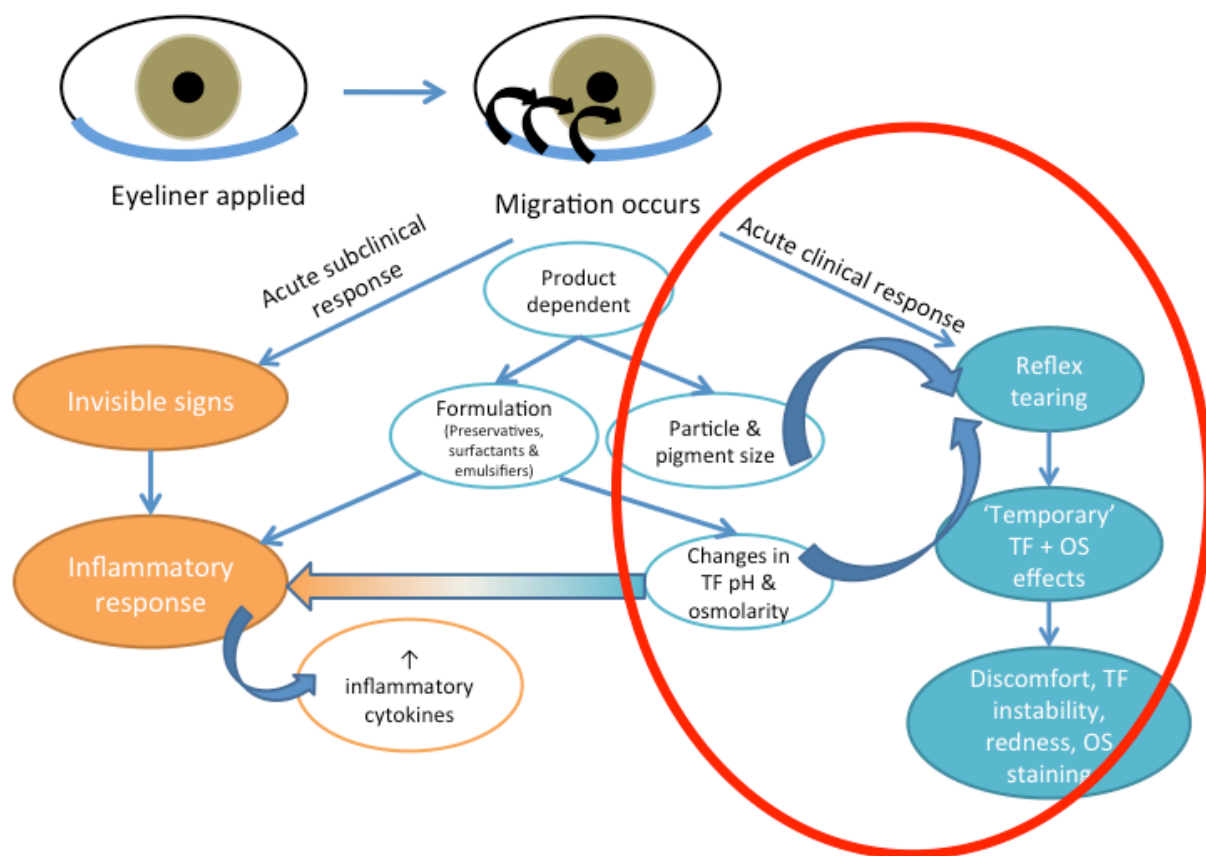
Figure 4.2 The hypothesis cascade for this study. Circled is the pathway which formulates the basis of investigations for this chapter

4.2 Methods

24 female subjects (median age 22 years, range 18-39 years) were recruited from students and staff at the School of Optometry and Vision Sciences, Cardiff University. The study was approved by the Research Ethics Committee at the School of Optometry and Vision Sciences, Cardiff University and was in accordance with the regulations of the Declaration of Helsinki. Informed consent was obtained from all subjects prior to commencing the study.

4.2.1 Exclusion criteria

Subjects were excluded from the study if they: were pregnant or breast-feeding; described or exhibited any ocular or systemic pathology known to affect the tear film or ocular surface or if their initial examination revealed relevant abnormal ocular findings; described a history of ocular trauma, infection or inflammation within the previous three months; reported previous allergic responses following the use eye cosmetics; were regular contact lens wearers (≥3 days per week).

For inclusion, subjects who were pre-existing users of eye cosmetics were required to cease use of these products for five consecutive days prior to participation of the study.

4.2.2 Study design

A randomised, controlled, crossover study was conducted.

A baseline visit established the subject's suitability for the study. Suitable subjects were randomly assigned to one of two "conditions": eyeliner applied behind the lash line (ELI) or applied outside the lash line (ELO). Subjects were supplied with one black pencil eyeliner (MaxFactor Kohl pencil 020 Black, Procter & Gamble, UK) for their personal use and were also instructed to take care and avoid the eye area when applying moisturisers and other facial care products. The MaxFactor pencil eyeliner was selected due to the MaxFactor brand placement in the global cosmetics market. Procter & Gamble dominate 8% colour cosmetic UK market share, comparable with brands such as Avon and Estée Lauder (8% and 9% market share, respectively) (Mintel, 2012).Subjects were instructed to apply the eyeliner daily in that manner for seven days and to refrain from using any other eye cosmetics and contact lens wear. All subjects were also provided with eye cosmetic remover (Simple Eye

Make-up Remover, Simple Health & Beauty Ltd, UK) and instructed in the use of this product, by dispensing onto cotton wool pads to remove eyeliner at the end of the day. Simple Eye Make-up Remover was selected for this study as it has been demonstrated that this particular product does not alter lipid layer patterns nor disrupt tear film stability (Pearce, Harvey-Brown and Higginson, 2010). The ingredients in each of the supplied products are shown in Table 4.2.

Table 4.2 Ingredients listed in the pencil eyeliner and eye cosmetic remover supplied to subjects

MaxFactor Kohl pencil 020 Black (Procter & Gamble, UK)	Simple Eye Make-up Remover (Simple Health & Beauty Ltd, UK)
Hydrogenated Palm Oil	Aqua,
Hydrogenated Coco-Glycerides	Poloxamer 184
Caprylic/Capric Triglyceride	Butylene Glycol
Hydroxylated Lanolin	Ceteth-20
Synthetic Beeswax	PEG-20 Glyceryl Laurate
Dimethicone	Laureth-23
Stearalkonium Hectorite	Panthenol
Propylparaben	Disodium Phosphate,
Propylene Carbonate	Calcium Disodium
Tocopherol	EDTA
Ascorbyl Palmitate	Chlorhexidine Digluconate
Lecithin	Sodium Methylparaben
Glyceryl Stearate	Sodium Phosphate
Glyceryl Oleate	Citric Acid
Citric Acid	
[+/- CI 75470, CI 77000, CI 77007, CI 77019, CI 77491, CI 77492, CI 77499, CI 77510, CI 77742, CI 77891]	

Subjects attended the laboratory six times. They were reviewed one and seven days after eyeliner application. Subjects underwent a one-week washout period before commencing the second eyeliner application. The order of procedures conducted and parameters examined on each visit is summarised in Table 4.3.

Table 4.3 Procedures conducted on at each visit

	Visit 1 (Baseline)	Visit 2 (Day 1 EL_1)	Visit 3 (Day 7 EL_1)	Visit 4 (Washout check – 7 days after EL_1 ceased)	Visit 5 (Day 1 EL_2)	Visit 6 (Day 7 EL_2)
Screening & consent	x					
Visual acuity	x	x	x	x	x	x
OSDI questionnaire	x		x	x		x
Ocular comfort scale	x	x	x	x	x	x
Slit lamp examination grading ocular surface redness	x	x	x	x	x	x
Tear film assessment	x	x	x	x	x	x
Tear sample collection	x	x	x	x	x	x
Corneal & conjunctival staining check	x	x	x	x	x	x

Best corrected logMAR visual acuity (VA) was recorded on all visits and the Ocular Surface Disease Index (OSDI) questionnaire (Schiffman *et al.*, 2000) to grade dry eye severity was completed by all subjects on Visits 1, 3, 4, and 6. Subjects were also asked to subjectively rate their level of comfort on a scale of 1 to 10 (10 indicating maximum comfort).

The anterior eye and ocular surface was examined via slit lamp biomicroscopy (Takagi SM-30, Takagi Ophthalmic Instruments Europe Ltd., Manchester, UK) with x16 magnification and graded to 0.1 intervals using the Efron Grading Scale for Contact Lens Complications (Efron, 2004). Tear film stability (non-invasive break-up time, NITBUT) and lipid layer thickness (LLT) was assessed using a Tearscope (Keeler, Windsor, UK). LLT was graded according to the manufacturer's guidelines, summarised in Table 4.4.

Table 4.4 Summary of Tearscope grading system for lipid layer patterns (Guillon and Guillon, 1994; Guillon, 1998)

Lipid layer pattern	Approximate lipid layer thickness (nm)	Prevalence
Open meshwork	~13-50	15%
Closed meshwork	~13-50	14%
Wave/Flow	~50-70	29%
Amorphous	~80-90	19%
Coloured fringes	~90-180	17%
Globular	>200	6%

A sample of tears was collected by placing a 10µl glass microcapillary (Kimble Chase, NJ, USA) against the inferior temporal tear meniscus avoiding contact with the lower lid and conjunctiva to prevent reflex tearing and contamination of eyeliner with close proximity to the lower lid margin. Tear collection was limited to 5 minutes, or when a 10µl sample was collected. Tear samples were delivered into Eppendorf tubes and immediately stored at -80°C for later analysis (see Chapter 5). Corneal staining was assessed using sodium fluorescein (Fluoret, Chauvin). Conjunctival staining was assessed using lissamine green (HUB Pharmaceuticals, LLX, CA) and graded according to the Oxford Grading Scheme (Bron *et al.*, 2003; Bron *et al.*, 2007).

4.2.3   Statistical analysis

Prior to the main analysis of the effects of the intervention, the correlation between right (RE) and left (LE) eyes was examined for bulbar redness, NITBUT, LLT and conjunctival staining using Pearson correlation on data collected from seven day results. Significant correlation between eyes was observed for all these variables (0.883>r>0.989; p<0.0001). Thus, the measured values used for each analysis were an average of the RE and LE of subjects (Newcombe and Duff, 1987).

These data were tested for normality using the Shapiro-Wilk test. Following statistical advice, data for all variables were not normally distributed (p<0.001) but for all continuous

variables parametric statistics were still applied as the sample of results are representative of a normal population (Bland and Altman, 2009). Upon further data examination, non-parametric statistics were applied and no differences were detected in any variable when compared with the parametric statistic. Each variable was examined for period interaction and carry-over effects using paired t-tests and no were effects detected (see Appendix V for this data). Paired t-tests were conducted for all parameters comparing ELI and ELO after one day and seven days of intervention. Analysis of the ordinal data (perceived comfort scores and LLT) was conducted using non-parametric statistics (Wilcoxon signed-ranks test). Two-sided p-values<0.05 were considered significant for all statistical tests.

4.3   Results

The short-term (1 day) and long-term (7 day) results are both presented. Comments and subjective responses from participants were also collated and can be found in Appendix VI.

4.3.1   Short term changes within one day (D0 to D1)

Table 4.5 summarises the findings at the one day (D1) check point.

Table 4.5 Summary of results following 1 day of eyeliner use. Values are shown as means and SD in parentheses unless otherwise specified. Paired samples t-tests were used to analyse the data.

	ELO	ELI	ELO minus ELI	P value (paired samples t-test)
Bulbar Redness				
Temporal				
Baseline	0.47 (0.12)	0.49 (0.25)	0.02 (0.24)	0.734
1 day	0.49 (0.18)	0.52 (0.18)	0.02 (0.29)	0.793
p-value (baseline vs. 1 day)	*0.598*	*0.334*		
Nasal				
Baseline	0.47 (0.14)	0.49 (0.23)	0.02 (0.22)	0.673
1 day	0.49 (0.18)	0.50 (0.23)	-0.01 (0.30)	0.865
p-value (baseline vs. 1 day)	*0.512*	*0.744*		
NITBUT				
Baseline	11.8 (5.7)	10.9 (4.3)	1.0 (4.8)	0.333
1 day	12.5 (5.5)	11.7 (5.9)	0.2 (7.5)	0.897
p-value (baseline vs. 1 day)	*0.480*	*0.321*		
LLT (median & IQ range)				
Baseline	3.25 (3-4)	3.00 (3-4)	0.25	0.112
1 day	3.00 (3-4)	3.00 (3-4)	0.00	0.525
p-value (baseline vs. 1 day)	*0.888*	*0.254*		
Conjunctival staining				
Temporal				
Baseline	0.07 (0.23)	0.07 (0.19)	0.00 (0.26)	1.000
1 day	0.10 (0.29)	0.05 (0.15)	-0.05 (0.45)	0.575
p-value (baseline vs. 1 day)	*0.649*	*0.647*		
Nasal				
Baseline	0.05 (0.16)	0.11 (0.26)	-0.06 (0.24)	0.198
1 day	0.19 (0.38)	0.17 (0.38)	0.02 (0.53)	0.471
p-value (baseline vs. 1 day)	*0.063*	*0.418*		
Corneal staining				
Baseline	0.03 (0.11)	0.00 (0.00)	0.03 (0.11)	0.185
1 day	0.05 (0.12)	0.04 (0.20)	0.01 (0.13)	0.818
p-value (baseline vs. 1 day)	*0.394*	*0.328*		
Perceived ocular comfort (median & IQ range)				
Baseline	9 (8-10)	9 (8-10)	0 (-1-1)	0.971
1 day	9 (7-10)	9 (8-10)	0 (0-1)	0.846
p-value (baseline vs. 1 day)	*0.227*	*0.601*		

Bulbar redness tended to increase during eyeliner use (both ELO and ELI), but these changes were small (grade difference in the order of 0.02) and not clinically nor statistically significant from baseline nor each other (p=0.793).

Similarly there were small changes in conjunctival staining nasally and temporally after both eyeliner interventions. There was a four-fold increase in nasal conjunctival staining after 1 day ELO application (grade 0.05 to 0.19) although this was not statistically significant. There was an increase in nasal conjunctival staining after 1 day ELI application from baseline (grade difference 0.06) however these changes were neither clinically or statistically significant when compared to baseline measurements. Differences in temporal conjunctival staining were smaller than that detected in the nasal conjunctiva and did not reach statistical significance (Figure 4.3). In both interventions, corneal staining increased from baseline however were not significantly different to each other (p=0.818).
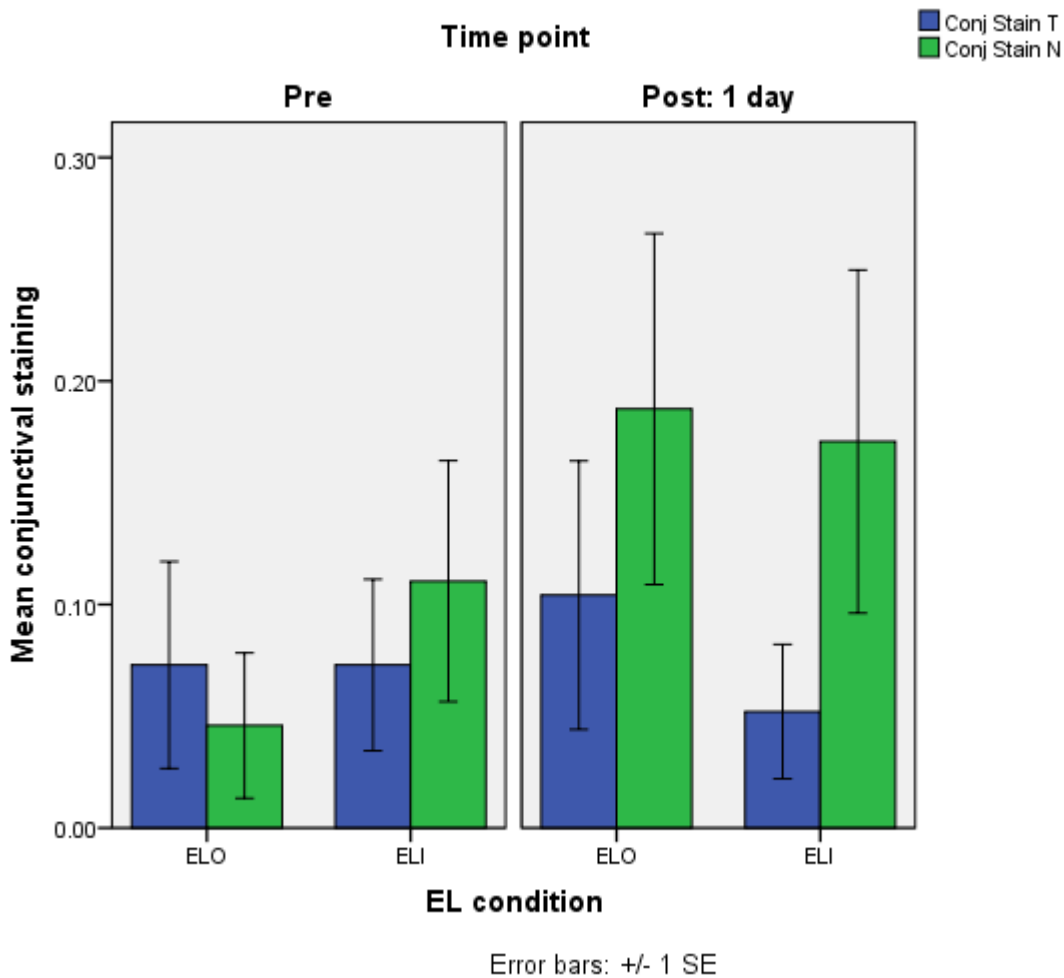
Figure 4.3 Mean conjunctival staining at baseline (pre) and following 1 day ELO and ELI

Whilst there was a trend for increased tear film stability in both methods of eyeliner application (0.7s improvement ELO, 0.8s improvement ELI), the comparison of these changes did not reach statistical significance from baseline nor each other (p=0.897). Additionally, LLT remained similar across both interventions. Perceived ocular comfort remained unchanged following eyeliner use in both conditions.

4.3.2 Long term changes after seven days (D0 to D7)

Table 4.6 summarises the results following at the seven day time point (D7).

Table 4.6 Summary of results following 7 days of eyeliner use. Values are shown as means and SD in parentheses, unless otherwise specified. Paired samples t-tests were used to analyse the data. Boxes shaded in grey indicate p<0.05.

	ELO	ELI	ELO minus ELI	P value
Bulbar Redness				
Temporal				
Baseline	0.48 (0.13)	0.49 (0.25)	0.01 (0.23)	0.858
7 days	0.48 (0.13)	0.53 (0.20)	-0.03 (0.19)	0.380
p-value (baseline vs. 7 day)	*0.734*	*0.322*		
Nasal				
Baseline	0.48 (0.15)	0.48 (0.16)	0.00 (0.05)	1.000
7 days	0.49 (0.18)	0.50 (0.23)	0.01 (0.29)	0.904
p-value (baseline vs. 7 day)	0.646	0.572		
NITBUT				
Baseline	11.8 (5.7)	10.9 (4.3)	1.0 (4.8)	0.334
7 days	11.3 (5.6)	11.3 (5.7)	1.0 (7.2)	0.511
p-value (baseline vs. 7 day)	*0.626*	*0.648*		
LLT (median & IQ range)				
Baseline	3 (3-4)	3 (3-4)	0	0.112
7 days	3 (3-4)	4 (3-4)	1	0.032
p-value (baseline vs. 7 day)	*0.151*	*0.034*		
Conjunctival staining				
Temporal				
Baseline	0.07 (0.23)	0.07 (0.19)	0.00 (0.26)	1.000
7 days	0.02 (0.10)	0.01 (0.05)	-0.01 (0.27)	0.852
p-value (baseline vs. 7 day)	*0.233*	*0.056*		
Nasal				
Baseline	0.05 (0.16)	0.11 (0.26)	-0.06 (0.24)	0.198
7 days	0.06 (0.22)	0.22 (0.44)	-0.16 (0.52)	0.154
p-value (baseline vs. 7 day)	*0.778*	*0.302*		
Corneal Staining				
Baseline	0.03 (0.11)	0.00 (0.00)	0.03 (0.11)	0.185
1 day	0.06 (0.13)	0.09 (0.24)	-0.03 (0.21)	0.411
p-value (baseline vs. 1 day)	*0.185*	*0.057*		
Perceived ocular comfort (median & IQ range)				
Baseline	9 (8-10)	9 (9-10)	0 (-2-1)	0.971
7 days	9 (7-10)	8 (7-9)	1 (0-1)	0.362

p-value (baseline vs. 7 day)	0.030	0.002		
OSDI				
Baseline	5.93 (7.27)	6.11 (8.06)	-0.17 (5.85)	0.886
7 days	6.07 (7.59)	8.27 (8.48)	-2.21 (5.12)	0.046
p-value (baseline vs. 7 day)	0.912	0.099		

No significant changes were seen in bulbar redness, tear film stability or OSDI score after seven days of either method of eyeliner application. After seven days ELI, temporal conjunctival staining appeared to reduce compared to baseline (p=0.056), but this change was not mirrored nasally (p=0.302), as illustrated in Figure 4.4. Corneal staining increased after seven days ELI (grade difference 0.09) which approached statistical significance (p=0.057).

Analysis of LLT indicated that whilst the ELO intervention elicited no change, the ELI intervention was associated with an increased grade in thickness categories that was statistically significant (p=0.032). Additionally, LLT was significantly thicker after seven days of ELI application compared with ELO application (p=0.032).

Perceived ocular comfort on the 10 point scale was reduced for each intervention with no significant difference in this reduction between conditions (p=0.362), illustrated in Figure 4.5. In contrast, the increase in dry eye symptoms (measured using the OSDI questionnaire) was greatest for ELI compared with ELO (p=0.046) (Figure 4.6).
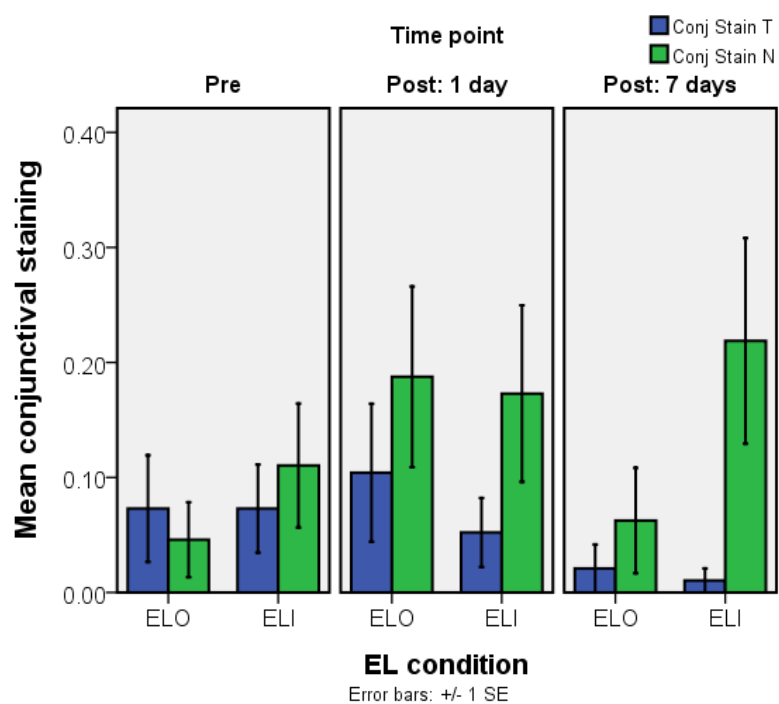
Figure 4.4 Mean conjunctival staining across all three time points (pre-, post 1 day and post-7 days eyeliner application) in both eyeliner conditions, ELO and ELI
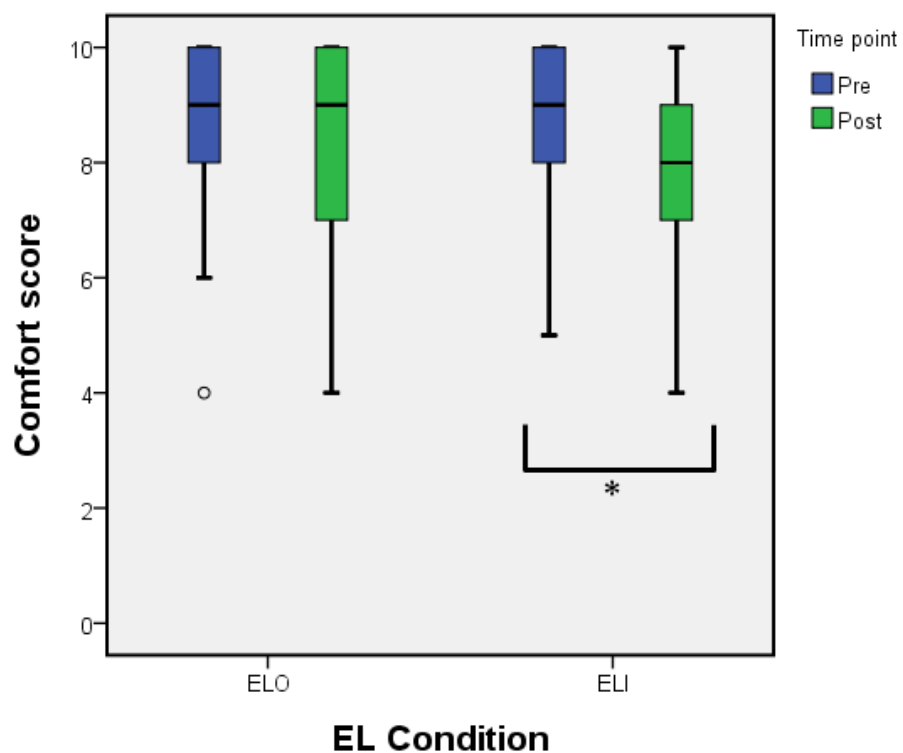
Figure 4.5 Changes in perceived comfort score at baseline and after 7 days of eyeliner use. The asterisk (*) denotes p<0.05. Whiskers represent range of values.
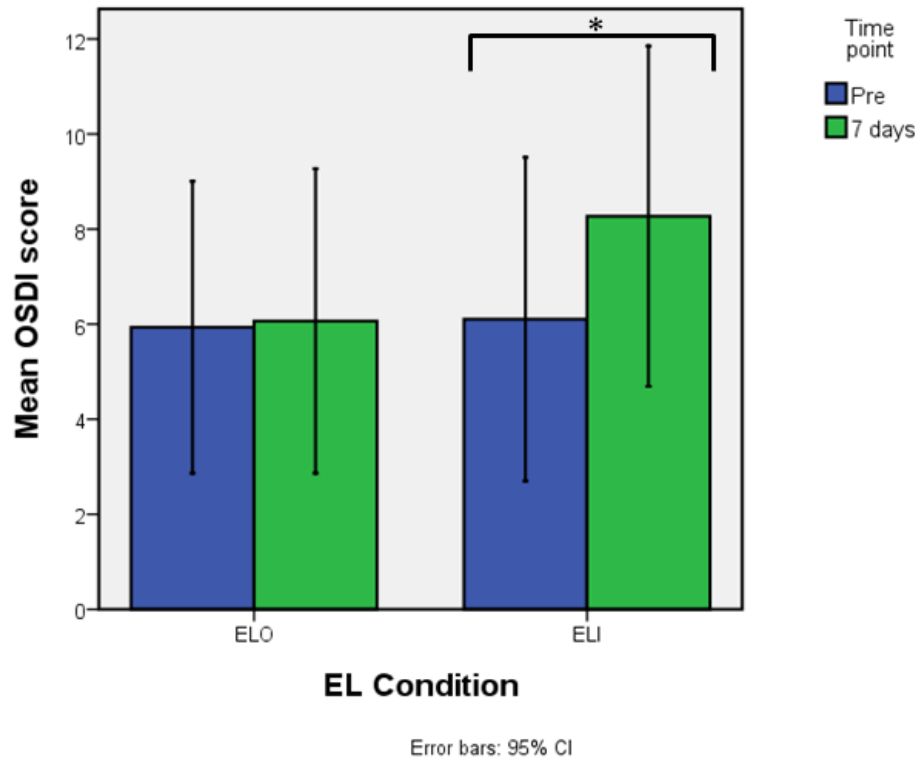
Figure 4.6 Mean OSDI scores at baseline and after 7 days of eyeliner use. The asterisk (*) denotes p<0.05.

4.4   Discussion

The results indicate that any changes that arise from a combination of eyeliner use and removal with eye cosmetic remover after seven days of use in this cohort were small and difficult to detect clinically, particularly when reviewing subjects following one day of eyeliner use. All the parameters showed clinically insignificant fluctuations from baseline measurements after using the eyeliner pencil ELO and ELI at day one.

In this study, short-term use of eyeliner increased nasal conjunctival staining which approached statistical significance. This was most marked for ELO application compared with ELI. Figure 3.10 in Chapter 3 illustrates the collection of pencil eyeliner located in the inner canthus within 30 minutes of eyeliner application. It is possible that eyeliner applied along the peri-ocular skin migrates and combines with mucous in the tears which, while moving towards the nasal lacrimal duct, "brushes" against nasal conjunctival epithelial cells. Staining of the nasal conjunctiva has been shown to be significantly correlated with OSDI scores and dry eye symptomology (Begley *et al.*, 2003; Yoon *et al.*, 2011). Certainly, OSDI and perceived comfort scores significantly increased following seven days ELI application, although nasal conjunctival staining did not reflect this. No significant changes in corneal staining were observed between any visits or methods of eyeliner application. Corneal staining with sodium fluorescein has been shown to be a poorly reflect corneal injury or toxicity (Ward, 2008), whereas lissamine green selectively stains damaged ocular surface epithelial cells or cells unbound with mucin (Hamrah *et al.*, 2011).

Of more significant note is the impact of ELI on lipid layer pattern. Following seven days eyeliner application within the lash line (ELI), there was a significant thickening of the tear film lipid layer. The first five ingredients and the bulk of the eyeliner pencil supplied to subjects in this study comprised of lipids, waxes and their derivatives, namely hydrogenated palm oil, hydrogenated coco-glycerides, caprylic/capric triglyceride, hydroxylated lanolin and synthetic beeswax. The increase in LLT is likely to have been caused by the migration of the eyeliner product into the tear film; with the eyeliner product applied within the lash line and in contact with the tear meniscus, contamination of the tear film is likely to be much greater than if the eyeliner is applied on the peri-ocular skin, as already discussed in Chapter

3 (Proof of Concept). This is reflected in the results, whereby ELI elicited a significant increase in LLT whereas ELO did not.

Changes in LLT have been demonstrated in dry eye treatments directly targeting the lipid layer. Instilling an emulsion eye drop has been shown to restructure the lipid layer (Di Pascuale, Goto and Tseng, 2004). Analysis of tear samples collected four hours after instillation of the castor oil-containing eye drops and detected castor oil in 53% of samples (Maissa *et al.*, 2010). Khanal *et al.* conducted long-term studies with the same eye drop and found repeated application of the drop (three times a day) for 30 days yielded significant increases in LLT. Thus, repeated application of lipid-containing products seems to provide a long term benefit in increasing LLT, thereby reducing tear evaporation (Khanal *et al.*, 2007). The same principle may be applied in the incidence of eyeliner application: any significant improvements in LLT may not be apparent until repeated applications are made due to a cumulative effect, hence why changes are not detectable following only 1 day of use.

The mechanisms for LLT increase may be three fold. The typical (external) eyelid temperature in healthy individuals is 36°C (Purslow, 2013). The formulation of eye cosmetic pencils represent a range of waxes and lipids encompassing a different of melting points to allow for ease of application, soft feel and to remain solid in pencil format (Rabasco Alvarez and González Rodríguez, 2000; Schlossman, 2001). Some lipids, such as hydrogenated coco-glycerides, have melting points close to eyelid temperature (33.5-35.5°C (Wiechers and Souto, 2010)). If such lipids are melted into a liquid state, this may aid the mixing of lipids with the pre-ocular tear film. Furthermore, palm oil and coconut oil derivatives (cocoglycerides) found in many cosmetic products can be broken down by sebum in the skin thereby releasing fatty acids (Lautenschläger, 2004). Beeswax and other lipophilic ingredients may supplement and enhance the existing lipid layer, in much the same way as castor oil emulsion drops behave (Goto *et al.*, 2002b; Di Pascuale *et al.*, 2004; Khanal *et al.*, 2007; Maissa *et al.*, 2010).

Secondly, the application of lipid products along the lid margin may enhance meibomian gland secretion. Several subjects showed signs of irregular residual eyeliner pencil along meibomian gland orifices within two hours post-application, despite applying fully along the lid margin. It is possible that the meibomian gland secretions are dissolving the lipid

structure from the eye pencil application due to natural chemical lipid-lipid interactions. One study has shown a significant improvement in meibomian gland obstruction following instillation of castor oil-based eye drops six times a day for two weeks (Goto *et al.*, 2002b). Another study has also reported improved meibomian gland secretion with homogenised castor oil (Maissa *et al.*, 2010).

Lastly, repeated daily application and removal of eyeliner results in unintended regular palpation of the lid margins. It is important to emphasise that the subjects in this study were supplied with an eyeliner pencil and a make-up remover as a complete package thus the observed outcomes were a result of both products used in the prescribed manner. The application and removal of eyeliner could have "massaged" the lid margins, thus expressing meibum from the MG, in a manner not dissimilar to that advised in MGD therapy (Geerling *et al.*, 2011). Additionally, the method of ELI application meant participants were debriding the inner lid margin on a daily basis for one week which may have effectively debrided any mildly hyperkeratised portions of the lid margins, further freeing meibum delivery to the pre-ocular tear film (Blackie and Korb, 2013).

Although LLT did improve, subjects reported decreased comfort and OSDI scores were significantly elevated after 7 days of eyeliner use. The exact origins of discomfort are unclear. Eyeliner pencil contains both natural and synthetic ingredients however all these individual ingredients, while approved for use in eye cosmetics, may cause disruption to the ocular surface as it may recognise the eye pencil to be a foreign material. More commonly, sensations of irritation arise from changes in pH and osmolality (Baeyens and Gurny, 1997). Specifically, the concentration of ingredients such as sodium lactate and glycols are found to be proportional to the severity of burning and itching sensations (Debbasch *et al.*, 2005). Propylparaben is one of the ingredients listed in the pencil eyeliner. Sodium methylparaben, which has a chemical structure similar to propylparaben, is one of the ingredients in the eye cosmetic remover also given to subjects to remove the eye pencil at the end of each day. Propylparaben and methylparaben are para-hydroxybenzoic acid ester preservatives. They are infrequently used in ophthalmic drug preparations as they are considered as inefficient bacteriostatic agents and slow in their bacterial action (Chowhan *et al.*, 2012). If used in ophthalmic drug preparations, methylparaben can be found in concentrations between 0.1-0.2% (Chowhan *et al.*, 2012), and propylparaben between 0.005-0.01% (Rowe *et al.*,

2012). Para-hydroxybenzoic acid ester preservatives are known to cause ocular irritation and stinging in ophthalmic preparations (Chowhan *et al.*, 2012). While the exact concentrations of these substances are not specified on the labelling of the pencil eyeliner and eye cosmetic remover, it is likely that these substances may be one of many causing the reduction in comfort and increased symptoms of dry eye. Using analytical chemistry techniques such as high-performance liquid chromatography (HPLC) on contaminated tear samples may be one method of determining which eye pencil ingredients have migrated to contaminate and mix with the pre-ocular tear film. It is also important to acknowledge the potential impact of the Hawthorne effect on the results observed in this study. The Hawthorne effect is a phenomenon where the subjective responses of participants are altered according to a variety of interventions due to participants being aware of their observation by experimenters. The Hawthorne effect may be implicated in the outcome of the responses obtained in the perceived comfort scores and the OSDI score.

The natural diurnal variation in bulbar redness is recognised. Bulbar conjunctival redness has previously been demonstrated to be greatest at the start of the day and 2-3 hours after waking (Duench *et al.*, 2007). The subjects in this study were asked to present for assessment 2-4 hours after applying eyeliner in the morning. However, for visits where eyeliner application was not required (e.g. visits 1 and 4 corresponding to baseline and post-washout visits) the assessment time was variable. This difference in time for assessment itself could account for diurnal variations in bulbar conjunctival redness within a subject.

One major limitation of this study is the small sample size. As this was the first study of this kind and no references were identified in the literature, power calculations were difficult to perform as the effect size and variability for each measure was unknown. Given the small clinical changes observed in this study, ideally a larger cohort of participants is required.[1] In this study, participants were subjected to each method of eyeliner intervention for seven days. This duration of use may not be long enough to elicit differences that are clinically

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[1] For example, although there was a four-fold increase in nasal conjunctival staining after 1 day ELO application, this was not statistically significant. Post-hoc power calculations conducted (http://hedwig.mgh.harvard.edu/sample_size/size.html/) have shown 65 subjects would be required to determine a statistically significant change (based on the probability is 80% that the study will detect a treatment difference at a two-sided 0.05 significance level, if the true difference between treatments is 0.5 units).

detectable. Conversely, the formulation of the pencil eyeliner may not elicit any significant negative clinical change in this cohort of healthy controls. Since the pencil eyeliner has undergone manufacturing tests prior to market launch, the product should be safe to use for its intended purpose. During the design of the study, it was anticipated a group of neophytes with no history of eye cosmetic use would be recruited however it became apparent that for the age range of participant recruitment and enrolment, this was not possible.

It is important to acknowledge that the statistical analysis in this chapter has not accounted for Bonferroni error which may arise from multiple comparisons. Future statistical analysis could take into account the potential for Type I error by utilising a Bonferroni correction (α-value divided by the number of comparisons made). Additionally, it is important to acknowledge potential bias in this study. Both the subject and the investigator were aware of the treatments including whether the EL application was ELO or ELI. It was difficult to implement a strategy which might allow either single or double masking but in not doing so, there may have been subsequent potential impact of a lack of masking on the measurements of symptoms (from the subjects) and even the grading of clinical features (by the investigator).

This study has shown that the clinical signs and consequences of eyeliner application, in conjunction with regular usage of eye cosmetic remover, are difficult to detect. Repeated application of ELI increased LLT; however this also resulted in decreased ocular comfort and increased dry eye symptomology. While no clinically significant changes were detected, the decreased ocular comfort may result from responses occurring at a subclinical level. The next chapter will explore the ocular immune response in this study by examining the tear fluid collected from each participant at each visit.

5 The effects of eyeliner migration across the eyelid margin: biochemical outcomes

This chapter reports the biochemical findings of an experiment which parallels the clinical study in Chapter 5, where the clinical findings resulting from eyeliner use is outlined. The investigation into a small aspect of ocular surface immunology was conducted to explore whether subclinical effects of contamination from cosmetic products could be detected, in order to complement the clinical features assessed in the study. The development strategy and optimisation of the analytical techniques used are also described. Firstly, a brief overview of relevant ocular surface immunity will be presented.

5.1 Mechanisms of ocular surface defence

The ocular surface is at the forefront of attack from the surrounding environment and has become well adapted to prevent damage from external stimuli via three main mechanisms: physical processes, chemical processes and immune processes.

1. Mechanical, anatomical and physical processes are the first and foremost means of ocular surface defence. Gross mechanisms of defence include blinking, active tear flow and reflex tearing to an attempt to prevent and clear any pathogens or chemicals (Stapleton, Stretton and Sankaridurg, 2003). At a cellular level, the outermost surface epithelial cells of the conjunctiva and cornea are bound together by tight junctions. This provides an effective barrier to the diffusion of molecules and ions from external sources (McDermott, 2011). Tight junctions between epithelial cells also maintain cell polarity which prevents lateral diffusion of membrane proteins to maintain cell-specific functions. Maintenance of cell polarity also aids the adhesion of the mucin glycocalyx gel which traps foreign debris and pathogens for removal via blinking. The constant desquamation and renewal of ocular surface epithelial cells also limits the ocular surface damage and infection as the outermost superficial cells are sloughed away before invasion into deeper epithelial layers can occur (McDermott, 2011).

2. Non-soluble chemicals found in tear fluid play a major role in maintaining ocular surface defence. Some of the major tear proteins (lysozyme, lactorferrin, sIgA and lipocalin) have already been described in Chapter 1. These large, abundant proteins arising from the

lacrimal gland exhibit antimicrobial properties. Ocular surface epithelial cells also secrete their own antimicrobial proteins (defensins and cathelicidins) which are much smaller proteins, capable of signalling immune and inflammatory cells to the site of infection in addition to stimulating the production of inflammatory cytokines. Complement proteins are soluble chemicals maintain innate immune surveillance and are involved in clearing pathogens by opsonisation, immobilising phagocytes to the necessary site and lysis of pathogens (Gregory, 2001).

3. Immune cells provide a network of cellular defence systems in several capacities. The conjunctiva forms part of the mucosal-associated lymphoid tissue (MALT) thus the presence of immune cells such as lymphocytes and macrophages is to be expected (Hingorani, Metz and Lightman, 1997). The action of immune cells is activated by signalling proteins released by ocular surface cells and present in serum (Stapleton *et al.*, 2003).

Together, these three mechanisms are at the frontline of ocular surface defence, preventing and/or limiting damage, trauma and infection.

5.2   The innate and adaptive immune system

When foreign substances or pathogens enter or contact the ocular surface, the innate (non-specific) defence system is activated, resulting in the removal of pathogen. The first stage is clearing of the pathogen by physical and chemical barriers to prevent infection without inducing inflammation. If this first stage has been breached, the second stage of innate immunity involving cellular and secretory molecules work together to eradicate the pathogen (Gregory, 2001). Immune memory is not built up during the processes that occur under innate immunity, thus the same immune response will be elicited, even if the pathogen has been present previously. In contrast, the specific (adaptive) immune system has built up memory and resistance to previous pathogens. For this to occur, the specific, or adaptive immune system enrols different immune cells to facilitate their defence. The characteristic features of the innate and specific immune systems are summarised in Table 5.1. While these two immune systems have different features, there is increasing evidence that they are not mutually exclusive and work together to maintain optimal ocular defence (Gregory, 2001). The innate system may initially provide the necessary signals to allow the adaptive system to elicit an antibody response and attract leukocytes to the affected site

(Stapleton *et al.*, 2003). For this thesis, the focus will remain on a small aspect of the biochemical behaviour of the innate system.

Table 5.1 Summary of the features of the innate and specific immune system (Stapleton *et al.*, 2003)

	Innate (non-specific)	**Adaptive (specific)**
Features:	No immune memory	Immune memory develops
Immune cells involved:	Phagocytes – neutrophils & macrophages Granulocytes	T-lymphocytes B-lymphocytes Antigen presenting cells
Mediated by:	Cytokines Defensins Complement proteins	Antibodies Complement proteins Cytokines

5.3   Proteins and chemical messengers in tear fluid

More recently, over 1500 proteins have been identified in healthy human tears. Tear proteins can originate from the following main sources (Zhou and Beuerman, 2012):

- those secreted by the lacrimal gland and other glands of the lacrimal functional system (e.g. meibomian, goblet cells, etc.)
- ocular cells or tissue leakage products for homeostatic maintenance or in response to cellular damage
- foreign proteins which may have been released by pathogens

Tear fluid becomes a "reservoir" of cellular markers (Nishida, 2011). Since present in healthy and pathological states, the profile of protein biomarkers in tissues are increasingly being utilised in diagnosis, determination of treatment pathways and monitoring treatment efficacy in different disease states. Large proteins expressed by the lacrimal gland such as lysozyme, lactoferrin and sIgA are dominant in the tear film and well recognised in their bactericidal, bacteriostatic and autoimmune roles. Smaller proteins, such as cytokines and chemokines, are present in much lower concentrations and maintain vital intercellular signalling, with cell differentiating and proliferating functions. These proteins arise from

ocular surface cells and comprise 90% of the normal tear proteome (Zhou and Beuerman, 2012). The profile of these smaller proteins provides a "fingerprint" of the homeostasis of the ocular surface at any one moment in time.

Three stages are crucial in the process of successful innate immunity (Gregory, 2001):

- The response must be immediate
- The response must be amplified
- The pathogens must be cleared

The specific immune system must be activated if the pathogen is not cleared quickly. As the cornea is an avascular tissue and few immune cells are present, the conjunctiva is often the first ocular surface tissue to respond to exposure to allergens, toxins and pathogens. An inflammatory pathway is induced which results in increased blood flow to the conjunctiva (resulting in hyperaemia), increased capillary permeability resulting in fluid leakage and immune cells to the site of injury (Nishida, 2011). Both allergy and infection can induce inflammation. Acute phase responses are activated by the innate immune system and employ a number of cytokines. This process is outlined in Figure 5.1 and Figure 5.2.

Cytokines are proteins that act as chemical messengers which in turn elicit a particular response or a series of responses. They are secreted by immune and non-immune cells such as fibroblasts, epithelial and endothelial cells, located in the conjunctiva and corneal epithelia and stroma in response to trauma, inflammation, the presence of bacterial and allergenic stimuli (Stapleton *et al.*, 2003). Cytokines are delivered by cells to the surrounding environment and bind to high affinity surface receptors to induce the appropriate outcome (McInnes, 2013).
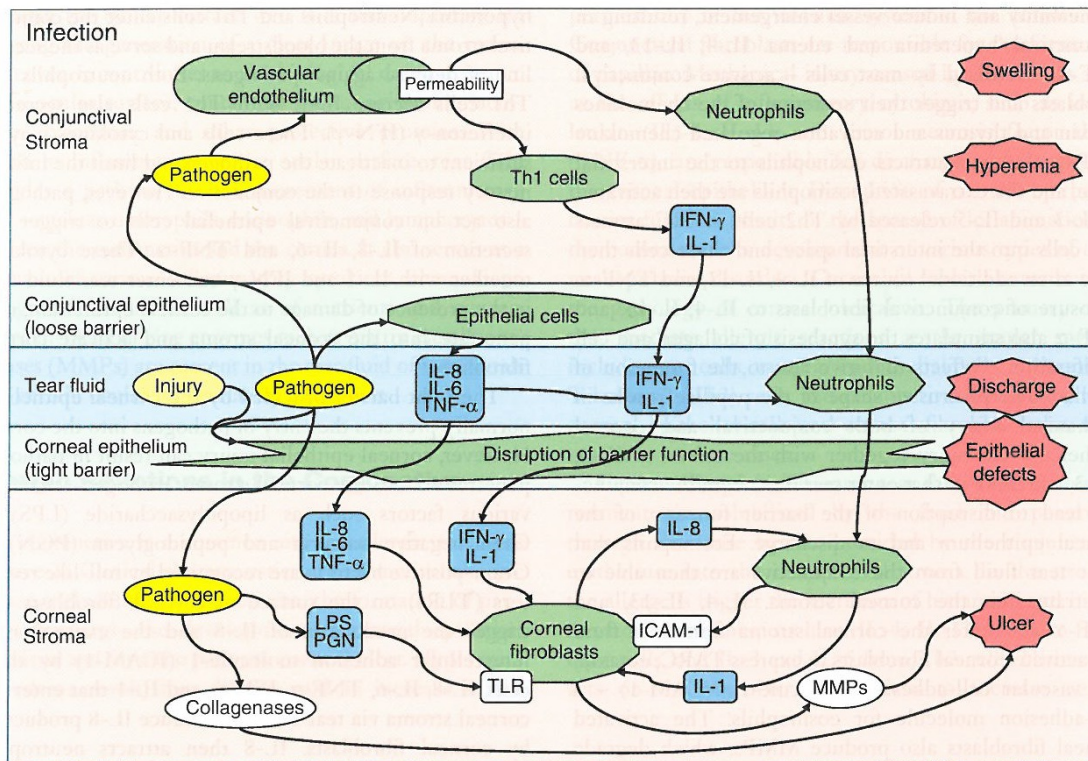
Figure 5.1 The relationship between the clinical signs, cellular and cytokine interactions during ocular surface infections (Nishida, 2011)
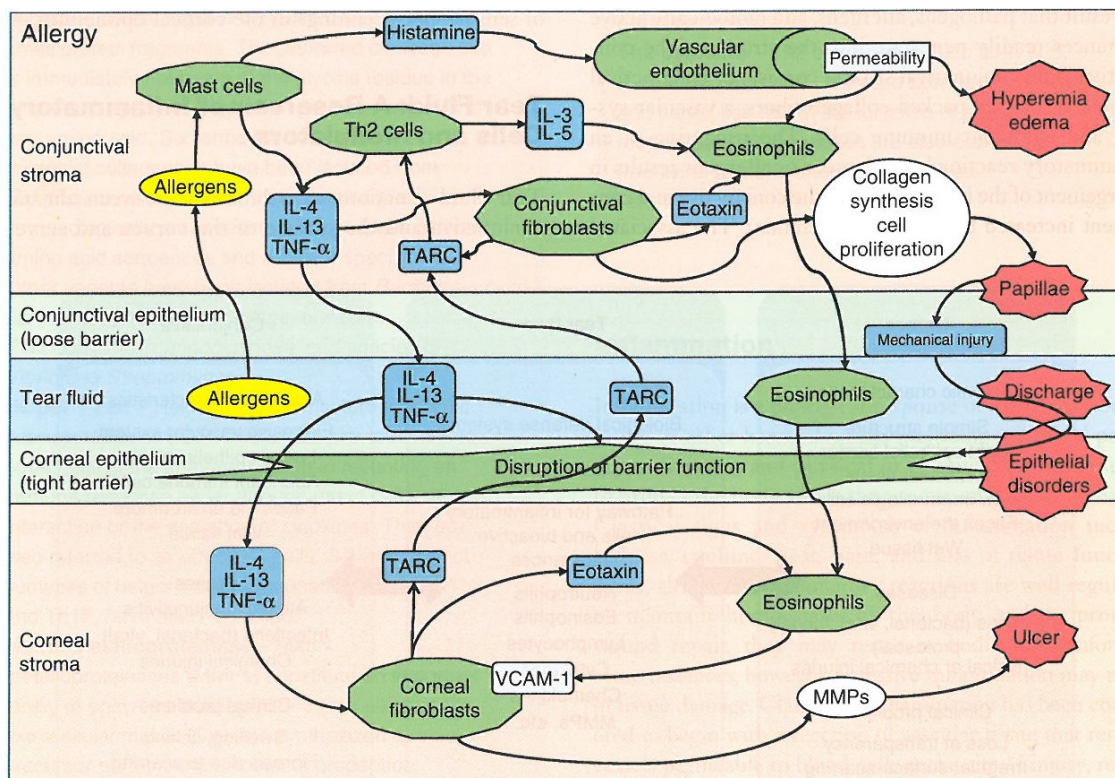
Figure 5.2 The relationship between the clinical signs, cellular and cytokine interactions during ocular surface allergy (Nishida, 2011)

Of the immune cells (T-cells) which do secrete cytokines, the type of cytokine secreted is dependent upon the cell lineage. CD4+ T-helper (Th) cells produce cytokines which recruit and regulate the activity of other immune cells and are characterised by the type of cytokines they produce (Stapleton *et al.*, 2003). CD4+ T-helper subsets include: Th0, Th1, Th2, Th17 and regulatory T-cells. The types of cytokines and their associated functions are summarised in Table 5.2.

Table 5.2 The cytokines and associated functions related to identified T-helper cell subtypes (McInnes, 2013)

T-helper cell subtype	Cytokines	Associated function
Th0	-	Naïve T-cell with unrestricted cytokine profile
Th1	IFN-γ, IL-2	Produced by activation of macrophages to induce local responses. Th1 cells regulate delayed-type hypersensitivity reactions (Type IV)
Th2	IL-4, IL-10	Associated with adaptive immune response. Th2 cells mediate allergic and antibody responses
Th17	IL-17A, IL-22, IL-26	Th17 play a role in a wide range of autoimmune responses previously considered Th1-mediated and implicated in mucosal defence
Regulatory T-cell	TGF-β, IL-10, IL-12	Suppressive function and may control development of autoimmune disease

The secretion of cytokines by Th1 and Th2 cells maintain an equilibrium which influences the overall progression of pathological processes. For example, interferon (IFN)-γ is predominately secreted by Th1 cells and has been found to inhibit cytokine secretion of Th2 cells; similarly, IL-10 secreted by Th2 cells is able to inhibit cytokine secretion by Th1 cells (Hamrah *et al.*, 2004). Since similar cytokines are secreted by a range of immune cells, there

is a degree of overlap between cytokine functions; some cytokines have both pro- and anti-inflammatory properties.

Cytokines have been detected in tear fluid in healthy and pathological eyes. Table 5.3 shows six commonly detected cytokines in tear fluid, describes their functions and mechanisms and clinical manifestations.

Table 5.3 Commonly detected cytokines, their functions and clinical ocular manifestations. (PMN=polymorphonuclear leukocytes, CLW = contact lens wear, CLARE = contact lens associated red eye, DED = dry eye disease)

Cytokine	Function	Additional mechanisms/actions	Clinical ocular manifestations	Origin of cytokine	Approximate tear fluid concentration in normals
IL-1 (α and β)	Important mediator of inflammation and immunity IL-1α; stored in epithelial cells & stromal keratocytes and is released upon cell membrane rupture (Akpek and Gottsch, 2003) IL-1β requires cleavage to become active (MMP-9 activates pre-cursor IL-1β)	Induces IL-6, -8, TNF-α and GM-CSF (granulocyte-macrophage colony-stimulating factor) Stimulates production of MMP enzymes by epithelial and inflammatory cells Present in healthy, human tears (Nakamura *et al.*, 1998)	↑in ocular rosacea, bullous keratopathy, keratoconus, sterile corneal ulcerations (Solomon *et al.*, 2001) and patients using eye drops for glaucoma (Malvitte *et al.*, 2007). Also ↑ in conjunctivochalasis (Acera *et al.*, 2008) Inactive -1β ↓ in Sjögrens syndrome (Solomon *et al.*, 2001)	Lacrimal gland, and ocular surface cells (Stern, Beuerman and Pflugfelder, 2004a)	IL-1α 43pg/ml IL-1β 30pg/ml (Stern *et al.*, 2004a)

Cytokine	Function	Additional mechanisms/actions	Clinical ocular manifestations	Origin of cytokine	Approximate tear fluid concentration in normals
IL-1Ra	Inhibits activities of pro-inflammatory IL-1α and -1β Produced abundantly in inflamed tissues, thought to be an endogenous regulatory mechanism against IL-1 mediated inflammation and tissue damage (Huang *et al.*, 2012)		↑ in dry eye (Solomon *et al.*, 2001; Huang *et al.*, 2012)	Lacrimal gland (Solomon *et al.*, 2001), Corneal and conjunctival epithelium (greatest expression in apical layer of corneal epithelium (Huang *et al.*, 2012)	220-300ng/ml (Huang *et al.*, 2012)
MMP-9 (Matrix Metalloproteinase-9)	Proteolytic enzyme, activated by MMP-3 (Pflugfelder *et al.*, 2004); pro-inflammatory; can be inhibited by TIMP-1 (Markoulli *et al.*, 2012)	Primary matrix-degrading enzyme Plays a role in disrupting corneal epithelial barrier that develops in dry eye and can degrade collagen	↑ in DED, chronic blepharitis, allergic eye disease, and conjunctivochalasis (Stern *et al.*, 2004a; Acera *et al.*, 2008) Diurnal variation (200-fold increase with sleep (Markoulli *et al.*, 2012)) ↑ in CLW (Calonge *et al.*, 2010) and keratoconus (Lema and Duran, 2005)	PMNs and epithelial cells (Stern *et al.*, 2004a)	39ng/ml (Gonzalez-Perez *et al.*, 2012)

Cytokine	Function	Additional mechanisms/actions	Clinical ocular manifestations	Origin of cytokine	Approximate tear fluid concentration in normals
IL-6	Interacts synergistically with IL-1 and TNF-α Stimulates epithelial migration (Gonzalez-Perez et al., 2012) and induces expression of MMPs. Affects production of IgA by B-cells	Present in normal, human tears (Nakamura et al., 1998)	↑in CL wear (Dogru et al., 2011; Gonzalez-Perez et al., 2012; Poyraz, Irkec and Mocan, 2012), DED (Yoon et al., 2007; Lam et al., 2009), chronic blepharitis (Acera et al., 2008), Sjögren's syndrome, keratoconus (Lema and Duran, 2005; Balasubramanian et al., 2012), bacterial conjunctivitis (Fodor et al., 2006), corneal foreign bodies (Fodor et al., 2006), after penetrating keratoplasty (Fodor et al., 2006) and patients using eye drops for glaucoma (Malvitte et al., 2007)	Lacrimal gland, fibroblasts, endothelial cells and keratinocytes (Stern et al., 2004a)	42 pg/ml (Stern et al., 2004a) 2.2pg/ml (Gonzalez-Perez et al., 2012) 130pg/ml (Carreno et al., 2010) 7-10pg/ml in CLW (Poyraz et al., 2012)

Cytokine	Function	Additional mechanisms/actions	Clinical ocular manifestations	Origin of cytokine	Approximate tear fluid concentration in normals
IL-8	Neutrophil chemotaxis & activation; ↑ secretion to IL-1 and TNF-α (Poyraz *et al.*, 2012); Major mediator of inflammatory response	Present in normal, human tears (Nakamura *et al.*, 1998)	↑during sleep (Thakur and Willcox, 2000), CLW (Thakur and Willcox, 2000; Kallinikos, Morgan and Efron, 2006; Calonge *et al.*, 2010; Poyraz *et al.*, 2012), DED (Huang *et al.*, 2012), bacterial conjunctivitis (Fodor *et al.*, 2006), corneal foreign bodies (Fodor *et al.*, 2006), after penetrating keratoplasty (Fodor *et al.*, 2006), CLARE and CLPU (Thakur and Willcox, 1998), keratoconus (Balasubramanian *et al.*, 2012) and in patients using eye drops for glaucoma (Malvitte *et al.*, 2007) Positive correlation with ↑ corneal and conjunctival staining (Huang *et al.*, 2012)	Fibroblasts, endothelial cells, keratinocytes, macrophages, T-cells and neutrophils (Stern *et al.*, 2004a)	107-176pg/ml in CLW (Poyraz *et al.*, 2012) 322pg/ml (Carreno *et al.*, 2010) 602pg/ml (Gonzalez-Perez *et al.*, 2012)

Cytokine	Function	Additional mechanisms/actions	Clinical ocular manifestations	Origin of cytokine	Approximate tear fluid concentration in normals
TNF-α	First wave of cytokines; stimulates cells to produce other factors that amplify inflammatory response (Yoon *et al.*, 2007)		↑ in keratoconus (Lema and Duran, 2005; Balasubramanian *et al.*, 2012) Also ↑ in dry eye but not correlated with severity (Yoon *et al.*, 2007)	Lacrimal gland, ocular surface cells (Stern *et al.*, 2004a)	0.36-1.97ng/ml (Stern *et al.*, 2004a)

5.3.1 Cytokine concentrations in normal tear fluid versus in pathological conditions

Choosing which cytokines to study in disease depends upon the aetiology of the disease, the expected abundance of the cytokine, analytical reliability and association with disease progression. In conditions such as keratoconus, specific cytokines such as IL-4, -6, -10, and TNF-α have been analysed as they are closely associated with chronic inflammation or regulatory processes (Lema and Duran, 2005). However, as previously discussed, one cytokine can trigger a cascade of other cytokines, thus there are often several cytokines implicated within one disease. For example, pro-inflammatory IL-6 is produced in response to IL-1 and Tumour Necrosis Factor (TNF)-α (Lema and Duran, 2005).

Despite their low concentrations, the fine balance of these cytokines is important in influencing a pro- or anti-inflammatory cascade. Small changes may be a pre-cursor to clinically detectable signs and/or symptoms of disease. The potential for tears to be used as diagnostic fluid is increasingly being considered due to their accessibility. This has led to increased investigations into the human tear proteome in normal populations (Nakamura *et al.*, 1998), in various pathologic states such as keratoconus (Lema and Duran, 2005), contact lens associated red eye (CLARE) (Thakur and Willcox, 1998), dry eye (Tishler *et al.*, 1998; Yoon *et al.*, 2007; Acera *et al.*, 2008), allergic eye disease (Uchio *et al.*, 2000; Acera *et al.*, 2008) and in contact lens wear (Thakur and Willcox, 1998; Schultz and Kunert, 2000; Kallinikos *et al.*, 2006; Gonzalez-Perez *et al.*, 2012; Poyraz *et al.*, 2012) (see Table 5.3). The premise that a sample of tear fluid can provide a "snapshot" of the ocular surface health status at any given moment is proving attractive for ocular surface researchers. For example, Huang *et al.* (2012) found that IL-1Ra and IL-8 were the two markers that correlated best with signs of corneal staining. IL-8 has also shown good correlation with symptoms such as itching, burning and bulbar redness, and there is growing evidence to support the use of this marker in evaluating human eye tolerance to mild irritants in cosmetics formulations (Debbasch *et al.*, 2005).

Pro- and anti-inflammatory cytokines are present in normal human tears (Nakamura *et al.*, 1998; Sonoda *et al.*, 2006; Carreno *et al.*, 2010; Zakaria *et al.*, 2012). These studies suggest that IL-6 and -8 are expressed by conjunctival or corneal epithelial cells and not by the

lacrimal gland. Secondly, it also suggests that pro-inflammatory IL-6 and -8 are present in healthy, normal individuals and may have additional roles in ocular surface maintenance and/or homeostasis.

5.4 Analysis of tear fluid

5.4.1 Methods of tear fluid analysis

Tear fluid collection can be a minimally-invasive process with training and careful technique. Techniques adopted for tear collection include microcapillary pipettes, Schirmer strips, cellulose rods, surgical sponges (Acera *et al.*, 2008) and flush mechanisms (Markoulli *et al.*, 2011; Guyette *et al.*, 2013).

It is well recognised that concentrations of the large lacrimal gland proteins IgA, IgG, albumin and fibronectin are decreased in reflexed tear fluid, however other primary lacrimal gland proteins such as lysozyme, lactoferrin, and tear lipocalin remain constant (Fullard and Snyder, 1990; Fullard and Tucker, 1991). The literature surrounding changes in inflammatory cytokines in basal and reflex tear fluid is more variable. Nakamura *et al.* (1998) found a significant decrease in IL-1β, -6 and -8 concentrations between basal and reflex tearing. However, Sonada *et al.* (2006) noted a significant decrease in IL-8 concentration and not IL-1β and -6, despite using the same methods of reflex tear stimulation (nasal stimulation) and tear collection techniques (microcapillary pipettes). The inconsistency in these findings may be attributed to different cytokine analysis techniques employed which will be discussed in the next section. Zakaria *et al.* (2012) explored the differences in cytokine concentration based upon the location of tear collection. The results indicated decreased concentrations of IL-6 and -8 when collecting reflex tears with patients sat upright and further decreased concentration when the patient was supine. These findings highlights that the method of tear collection needs to be standardised.

The primary challenge encountered in tear fluid analysis arises from the limited volume of tear fluid sample obtained, particularly when examining a population with dry eye syndrome. A study in 1966 proposed tear fluid volumes to be on average 7μl in normal subjects (Mishima *et al.*, 1966). More recently, using OCT technology, basal tear fluid volume in the lower tear meniscus has been calculated to be 0.64μl (Palakuru *et al.*, 2007).

The tear fluid volume in the menisci of non-Sjögrens syndrome dry eye patients has also been reported to be significantly reduced (up to 49% compared with healthy subjects) (Shen *et al.*, 2009; Chen *et al.*, 2010; Yuan *et al.*, 2010).

Several biochemical techniques have been adopted in tear fluid biomarker research: enzyme-linked immunosorbent assay (ELISA), multiplex bead assay, electrochemiluminescent (ECL) assays and mass spectrometry. These techniques require varying quantities of tear fluid sample, from as little as 2µl from one patient (Malvitte *et al.*, 2007) to pooling several samples for sufficient tear fluid (Nakamura *et al.*, 1998; Thakur and Willcox, 2000; Kallinikos *et al.*, 2006) to run the assays. These analytical techniques will now be discussed in more detail.

5.4.1.1 *Enzyme-Linked Immunosorbent Assay (ELISA)*

ELISAs have been used commonly to detect and quantify antigens or antibodies in a given sample and has become the "gold standard" in cytokine detection in serum and plasma within a clinical setting (Elshal and McCoy, 2006). ELISAs have high repeatability and sensitivity in detecting antigens. The technique involves immobilising the antigens of interest in a given sample on a fixed well wall or membrane and adding an antibody which binds to the antigen. A second antibody that is linked to an enzyme is usually added which attaches to the antigen-antibody complex. Finally, a substrate that binds with the enzyme is added which causes a measurable colour change or fluorescence to aid identification and quantification. This process is illustrated in Figure 5.3**.**
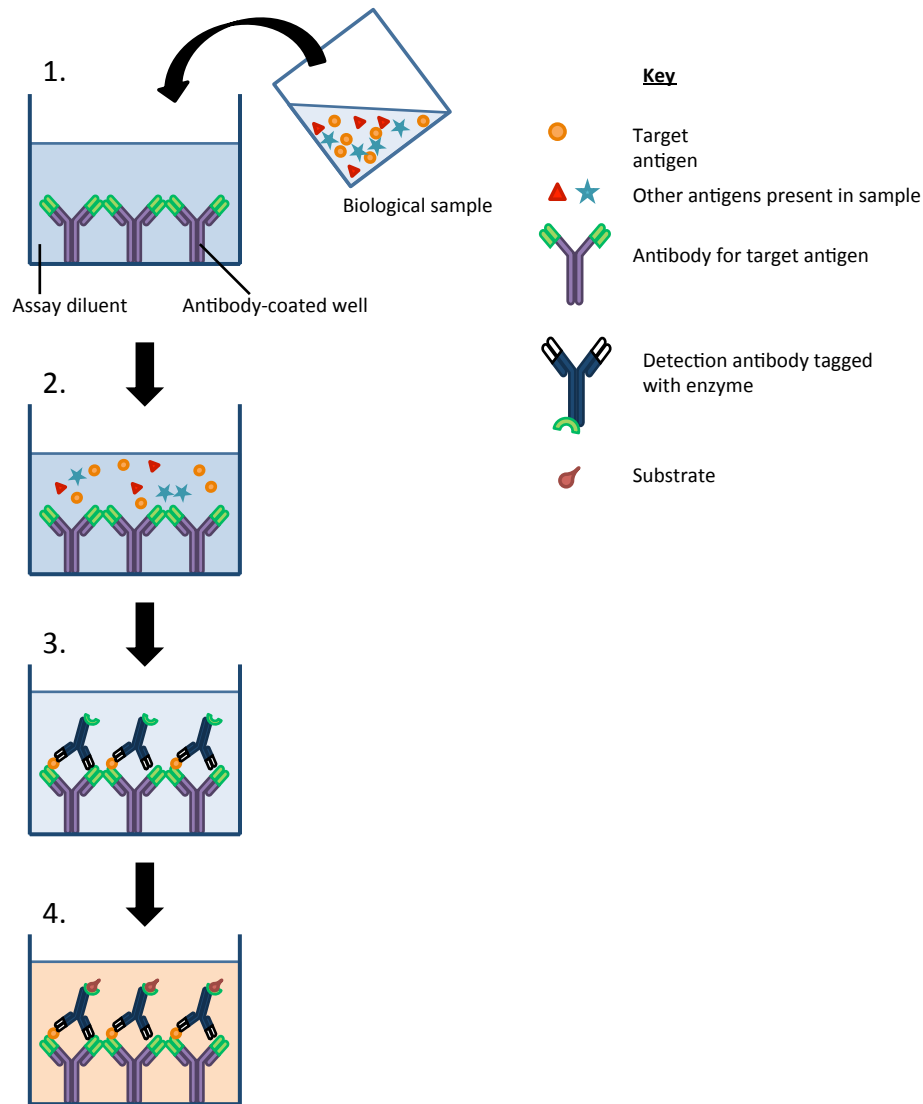
Figure 5.3 Process of an ELISA. (1 & 2) The sample is added to the antibody-coated well and assay diluent. The target antigen binds to the antibody fixed in the well. Any unbound antigens are washed away before (3) adding the detection antibody. The detection antibody, tagged with an enzyme, only binds to the target antigen. (4) Substrate is added to the well which initiates a colour change in colorimetric assays or luminescence in chemiluminescence assays. The optical density of the well is measured.

Cytokines have been successfully determined in tear fluid using ELISA, however sample volumes required to run these biochemical tests are in the order of 100-200µl. As a result, tear samples are often diluted to allow sufficient sample for multiple repeat analyses of

cytokines, or samples from patients are pooled. Furthermore, this limits the detection of several different antigens within one sample.

5.4.1.2   Multiplex bead assays

Multiplex cytometric bead assays (CBA) allow up to 100 cytokines to be simultaneously detected within a single sample of tear fluid with good sensitivity and reproducibility for even low abundant proteins (Elshal and McCoy, 2006; Zhou and Beuerman, 2012). Tear fluid samples between 2-10µl (Malvitte *et al.*, 2007; Zakaria *et al.*, 2012) are sufficient to run a successful assay.

This technique is likened to a combination of ELISA and flow cytometry. Several monoclonal antibodies are conjugated on the surface of different sets of microspheres whereas single antibodies are bound to flat surfaces in 96-well plate ELISAs. Multiplex bead assays use fluorescence to identify and quantify cytokines.

The ability to detect multiple cytokines within one sample is advantageous, particularly when large volumes of tear fluid are difficult to collect. However, this leaves the assay open to cross-reactivity whereby the lack of specificity between some antibody-antigen complexes may result in over- or under-estimation of cytokine concentrations (Elshal and McCoy, 2006). Variability in absolute levels of cytokines within one sample has been found when tested on several different multiplex assays from different manufacturers. While a good correlation between ELISA and multiplex bead array exists, absolute quantitative values are not comparable (Elshal and McCoy, 2006), particularly between manufacturers. The correlation improves and variability is reduced when identical antibodies and diluents are used to compare these two techniques (Elshal and McCoy, 2006).

5.4.1.3   Electrochemiluminescent (ECL) assays

Electrochemiluminescent (ECL) assays utilise highly reactive "reporter" compounds, such as ruthenium, which emit light upon the application of voltage. The principle and process of conducting ECL assays is similar to ELISAs. The antigen of interest within a sample is immobilised in wells lined with a capture antibody containing a working electrode. A second antibody labelled with ECL compounds is added to the well which binds to the antigen-

antibody complex in the well. A voltage is passed through the well to induce the chemiluminescent reaction via oxidation reactions within the wells.

ECL assays are advantageous over ELISA as the assay can be multiplexed, requires a significantly smaller sample volume (25μl), is highly sensitive with a large range of detection (Mathew, Biju and Thapalia, 2005; Subbaraman *et al.*, 2013). "Reporter" compounds are highly stable and only emit light upon the application of voltage. This allows assays to be quantified without the use of extra washing steps (a fundamental aspect in ELISAs), which removes any source of variations in addition to reducing the time and labour required to run the assay (Thompson *et al.*, 2009). Additionally, background noise is minimal as the stimulation is decoupled from the signal for plate-reading (Subbaraman *et al.*, 2013).

This technique has recently been optimised for use in tear fluid analysis. For example, in a panel of nine pro-inflammatory cytokines, six cytokines were successfully detected in human tear fluid in samples as small as 1μl (Subbaraman *et al.*, 2013) making this technique ideal in subjects with ADDE.

5.4.1.4 *Mass spectrometry*

Mass spectrometry (MS) has been used to determine the human tear proteome as it can identify and quantify proteins within complex samples (Zhou and Beuerman, 2012). The technique requires initial sample processing, by ionisation, gel-digestion or chromatography, to separate some of the proteins within the sample prior to undergoing MS. Using ionisation MS techniques, only a few microlitres of tear fluid is required (Zhou and Beuerman, 2012).

MS analyses the sample according to the molecular weight of each protein detected. Various MS techniques exist depending on the molecular weight of the proteins of interest. SELDI-TOF (surface-enhanced laser desorption ionisation – time of flight) MS is best suited to small proteins (mass range 1500 – 30kDa) however these small proteins can be difficult to identify in healthy tear fluid. MALDI-TOF (matrix-assisted laser desorption ionisation-time of flight) MS allows analysis of proteins with molecular weights ranging between 700 – 4000Da and can detect over 100 peptide peaks within one sample, although most of the peaks have not been identified (Zhou and Beuerman, 2012).

As with the other analytical methods discussed, MS requires specific equipment however the technique is quick and relatively simple method to retrieve the profile of proteins within a sample. The results derived from MS provides information about the molecular weight of the proteins and although a method to easily identify specific proteins at each molecular weight peak has not yet been devised (Zhou and Beuerman, 2012).

5.4.2   Aims and hypothesis

The aim of this study was to detect and quantify the levels of pro- and anti-inflammatory cytokines in collected tear fluid samples after the application of pencil eyeliner in a group of volunteers, using ELISA. ELISA was selected as the method of cytokine detection for this study due to the sensitivity of the technique and availability of resources.

The hypothesis of this study is when migration of eyeliner applied around the eyes occurs, a response may be induced which may not yet yield a clinically detectable change; however, the change may be demonstrated at a subclinical level by examining markers of inflammation since the chemicals in the pencil eyeliner would be recognised as a foreign substance to ocular surface cells. It is hypothesised that this may be a slower process (in comparison to the acute response, see Figure 5.4) therefore any indication of inflammatory responses may not be emphasised until repeated use and subsequent migration of the eye cosmetic has occurred.
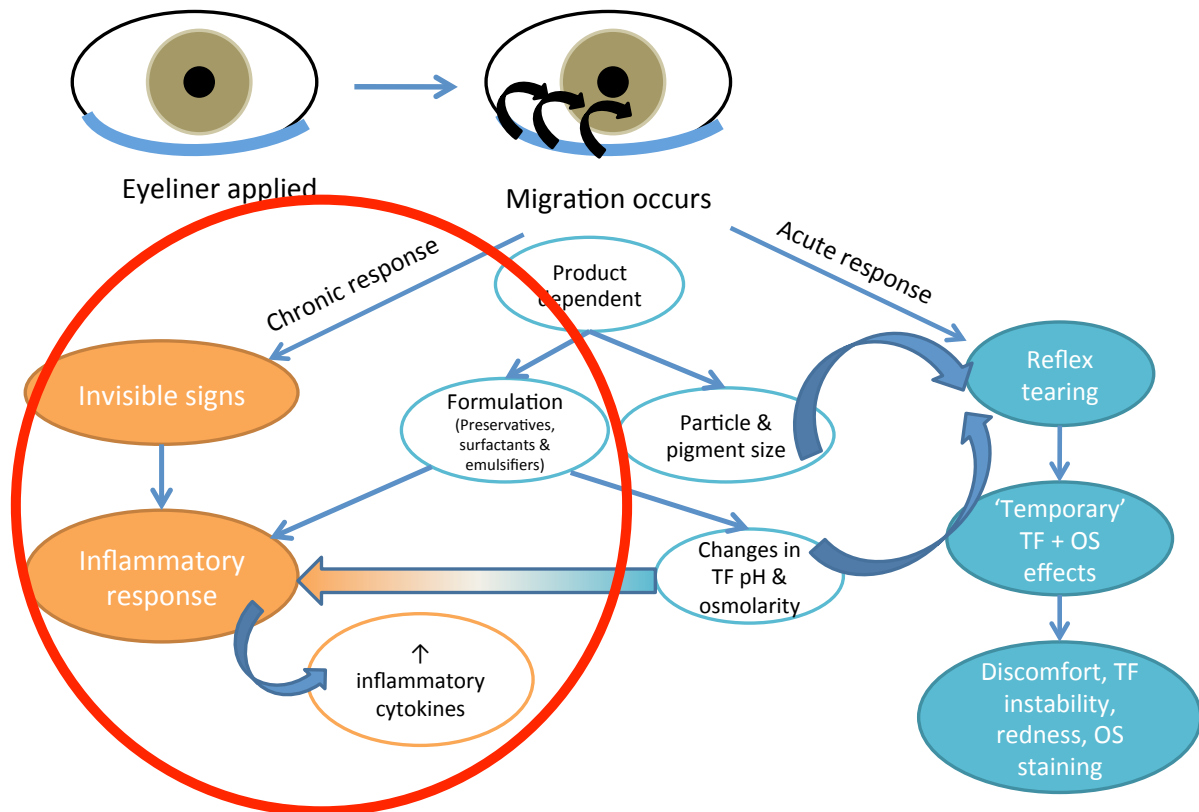
Figure 5.4 The hypothesis cascade for this study, investigating the chronic response to eyeliner usage. Circled is the pathway which formulates the basis of investigations for this chapter.

All eye cosmetics have been screened for safety by industry regulations thus the markers for inflammation are not expected to reach levels described in the literature for pathological conditions. As there are no papers in the literature which document the cytokine levels of inflammatory markers in human tear fluid after the use of any colour-based eye cosmetic products, this chapter will be split into two parts. The first part of this chapter will focus on preliminary work in the form of an optimisation study which ultimately aims to determine the most successful tear fluid dilutions with the selected ELISA kits. The second part of this chapter will focus on using the appropriate dilutions to examine cytokine concentrations in tear fluid collected from subjects during the study, as outlined in Chapter 4.

5.5   Optimisation of tear fluid cytokine detection using ELISA

5.5.1   Introduction

The analysis of tear fluid using ELISA has been documented in the literature. Appendix VII summarises a selection of the literature that has used ELISA to determine inflammatory cytokine concentration in various pathological conditions and interventions (including keratoconus, contact lens users and dry eye patients) and healthy controls. The trend in these values is the concentration of these markers is upregulated under these conditions and interventions compared to healthy controls.

The methodology varies greatly between studies and the normative values of cytokines also display wide variety in the published literature. Discussions with colleagues in the field led to the understanding that it was important to optimise the methodology for analysis for the typical tear fluid volumes collected and the cytokine of interest.

The four inflammatory markers chosen for preliminary and optimisation investigations were IL-1β, IL-1Ra, IL-6 and IL-8. IL-1, -6 and -8 are also known as acute-phase proteins which are, notably, found in plasma in the earliest stages on inflammation and are also detected in tear fluid since they are expressed by conjunctival and corneal epithelia.

5.5.2   Choice of cytokines

This section will provide an overview of the four cytokines selected for the preliminary optimisation work. These cytokines are: IL-1β, IL-1Ra, IL-6 and IL-8.

The pro-inflammatory cytokine IL-1 (agonist IL-1β and antagonist IL-1Ra)

IL-1 is an important mediator of inflammation and is released in response to trauma and injury (McDermott, 2011). The margin between acceptable levels and toxicity arising from elevated levels of IL-1 is very narrow (Dinarello, 1996). IL-1 is responsible for initiating an acute-phase response to inflammation, chemotaxis of inflammatory and effector cells, up-regulating cell adhesion molecules and stimulating neovascularisation (Hamrah *et al.*, 2004). Secreted by macrophages, IL-1 has been found to be up-regulated in a range of inflammatory systemic and localised conditions including septic shock (Solomon *et al.*, 2001), rheumatoid arthritis (Bresnihan, 1999) and periodontitis (Boch, Wara-aswapati and Auron, 2001; Christodoulides *et al.*, 2007). Within the eye, IL-1 has been implicated in the development of corneal transplant rejection, dry eye disease, microbial keratitis, keratoconus and sterile corneal ulceration (Hamrah *et al.*, 2004). The connection to dry eye makes it a logical choice for the purpose of this thesis.

The pro-inflammatory IL-1 family consists of two agonists, IL-1α and IL-1β, and one antagonist, IL-1Ra. Both IL-1 agonists are synthesised as precursor proteins with a molecular mass of approximately 31kDa and cleavage to the mature form involves specific cellular proteases (Dinarello, 1996). In IL-1α, the precursor and mature states are biologically active. However, with IL-1β, the precursor form is minimally biologically active and requires cleavage to the mature 17kDa form to become fully active. The cleavage from precursor to mature IL-1β occurs within cells involving a range of proteases, the most efficient being MMP-9 (Schonbeck, Mach and Libby, 1998). This tightly regulated mechanism seems to suggest that IL-1β can be a potentially harmful and destructive cytokine (Dinarello, 1996). The detection of IL-1β in plasma is typically at lower concentrations than IL-6, IL-8 and TNF-α for several reasons: a significant quantity of pro-IL-1β remains inside cells and IL-1β can also bind to large proteins such as complement, α-2-macroglobulin and soluble type II IL-1 receptor (Dinarello, 1996). Indeed, tear fluid concentration of precursor IL-1β is found to be 12 times greater than mature IL-1β (Solomon *et al.*, 2001). IL-1β can originate from

monocytes, macrophages, dendritic cells or injured epithelial cells (Paulsen *et al.*, 2005) and has been detected in human corneal epithelium. It is important to note that cytokines do not work independently and often the activity of one can induce the action of another. For example, IL-1β can induce the production of IL-6 (Cubitt, Lausch and Oakes, 1995) and IL-8 (Elner *et al.*, 1991).

The "anti-inflammatory" antagonist, IL-1Ra blocks the action of the agonists, IL-1α and −β. IL-1Ra irreversibly binds to IL-1 agonists, but does not induce any intracellular response (Arend *et al.*, 1998). Two forms of IL-1Ra have been found, secreted IL-1Ra (sIL-1Ra) and intracellular IL-1Ra (icIL-1Ra) (Kennedy *et al.*, 1995). sIL-1Ra has a slightly larger molecular weight than its counterpart (22-26kDa) and is produced by corneal epithelial and stromal cells (Kennedy *et al.*, 1995). icIL-1Ra exists in two forms with differing molecular weight. The 18kDa form of icIL-1Ra is produced by stromal cells whereas the 16kDa form is produced by both corneal and epithelial cells (Kennedy *et al.*, 1995). It is hypothesized that the secretory form exists on the ocular surface as it makes this peptide "freely available" without requiring prior cell damage or death to occur to release the intracellular form, thus quickly limiting inflammation, tissue damage and ultimately loss of corneal transparency and vision (Kennedy *et al.*, 1995).

IL-1Ra levels are higher than IL-1β levels (Dinarello, 1996). Levels of IL-1Ra have been demonstrated to correlate with disease severity and in some conditions such as myocardial infarction, post general surgery and in asymptomatic patients with HIV-1 (Dinarello, 1996). This has also been demonstrated in the eye where Huang *et al*. (2012) found IL-1Ra correlated well with clinical signs and severity of dry eye disease. IL-1Ra is found in abundantly in tear samples compared with the IL-1 agonists in normal, healthy subjects (Solomon *et al.*, 2001).

IL-6

IL-6 is produced by monocytes, macrophages, fibroblasts, endothelial cells and keratinocytes in response to pro-inflammatory cytokines, IL-1 and TNF-α (Cubitt *et al.*, 1995). It is a multifunctional cytokine, enabling cellular differentiation by acting on B- and T-cells (Tanaka, Narazaki and Kishimoto, 2012), increasing the production of antibodies and activating lymphocytes. IL-6 also induces the production of acute-phase proteins and is

essential in signalling the early phases of injury or infection (Tanaka *et al.*, 2012). While essential for immune regulation, it is well established that the overproduction of IL-6 can cause autoimmune and inflammatory conditions such as rheumatoid arthritis and Crohn's disease (Kishimoto, 2006). Within the ocular surface, IL-6 is secreted by corneal epithelial cells and keratocytes, although the latter is more responsive to increased IL-6 secretion following exposure to IL-1α (Cubitt *et al.*, 1995).

Since IL-6 is induced by IL-1β, IL-6 has been used as a surrogate marker for IL-1β (Dinarello, 1996). Acera *et al.* (2008) found IL-6 concentration in tear fluid to be significantly higher in patients with chronic blepharitis, in the absence of elevated IL-1β concentration. Dinarello argues that increasing evidence points to IL-6 as being the most appropriate marker in assessing elevated circulation of active IL-1 according to disease severity (Dinarello, 1996). While IL-6 is found to be upregulated in inflammatory states, it does not instigate inflammatory symptoms. Instead, it has been shown to induce high levels of IL-1Ra and suppresses gene expression and synthesis of inflammatory cytokines (Dinarello, 1996).

IL-6 has been demonstrated to be elevated in tear samples from contact lens wearers (Schultz and Kunert, 2000; Gonzalez-Perez *et al.*, 2012; Poyraz *et al.*, 2012), patients with Sjögren's syndrome(Yoon *et al.*, 2007) and dry eye disease (Yoon *et al.*, 2007; Lam *et al.*, 2009; Massingale *et al.*, 2009). IL-6 also correlates well with the clinical signs and symptoms of evaporative dry eye (Enriquez-de-Salamanca *et al.*, 2010).

IL-8

IL-8 is a chemokine which mediates an inflammatory response by inducing neutrophil chemotaxis and activation (Poyraz *et al.*, 2012). IL-8 is secreted by corneal epithelial cells and keratocytes (Cubitt *et al.*, 1993), in addition to monocytes, T-lymphocytes, neutrophils and fibroblasts (Poyraz *et al.*, 2012). Exposure of corneal epithelial cells to IL-1α and TNF-α induces IL-8 synthesis, with IL-1α having the greatest effect (Cubitt *et al.*, 1993). Whilst found in the tears of healthy, normal individuals, it is considered that IL-8 may also be important in ocular surface maintenance or homeostasis (Nakamura *et al.*, 1998).

IL-8 has been shown to be positively correlated with corneal and conjunctival staining (Lam *et al.*, 2009; Enriquez-de-Salamanca *et al.*, 2010) and the severity of dry eye disease (Lam *et*

al., 2009; Massingale *et al.*, 2009; Huang *et al.*, 2012). Additional factors which have been shown to correlate with elevated levels of IL-8 include: silicone hydrogel (Gonzalez-Perez *et al.*, 2012; Poyraz *et al.*, 2012) and conventional contact lens use (Poyraz *et al.*, 2012), corneal refractive therapy (Gonzalez-Perez *et al.*, 2012), corneal neovascularisation (Zakaria *et al.*, 2012), bacterial conjunctivitis (Fodor *et al.*, 2006), foreign body irritation (Fodor *et al.*, 2006) and penetrating keratoplasty (Fodor *et al.*, 2006).

IL-8 has also shown good correlation with symptoms such as itching, burning and bulbar redness, and there is growing evidence to support the use of this marker in evaluating human eye tolerance to mild irritants in cosmetics formulations (Debbasch *et al.*, 2005).

5.5.3 Output measures in ELISA

A general overview of the principle of ELISAs has been described in section 5.4.1.1.

In the final stages of ELISA, an enzyme substrate is added to the well. The amount of substrate bound to the well is directly proportional to the amount of antigen bound to the well and may induce a visible colour change (colorimetric ELISA) or may be colourless (chemiluminescent ELISA). Some ELISA kits are designed as "high sensitivity (HS)" kits that enable the detection of antigens which are expected to be present in samples at low concentrations. These kits utilise an additional amplifier solution which is added to the well after the substrate to amplify the response. In HS kits, a colour change does not occur until this step is completed. A STOP solution may be added to prevent further colour development. Signal measurements are made immediately after the final step of the assay using microplate readers.

For colorimetric ELISAs, the optical density (OD) of each well is measured. The OD values of each well are read at two different wavelengths to account for non-specific emission of light by the microplate itself. In contrast, the intensity of light emitted is measured in chemiluminescent ELISAs in relative light units (RLU) using a microplate luminometer.

5.5.4   Aims for optimisation work

The aims of this preliminary study were:

- To examine which cytokines could be studied, according to the volume of tear fluid collection

- To calculate the dilution factors required to perform the assays

- To establish typical fluid cytokine values in a normal population

5.5.5 Methods

5.5.5.1 Participants

Nine female subjects (median age 25 years, range 23-32 years) were recruited from students at the School of Optometry and Vision Sciences, Cardiff University. The study was approved by the Research Ethics Committee at the School of Optometry and Vision Sciences, Cardiff University and was in accordance with the regulations of the Declaration of Helsinki. Written, informed consent was obtained from all subjects prior to commencing the study.

Subjects were excluded from the study if they: were pregnant or breast-feeding; exhibited any ocular or systemic pathology known to affect the tear film or ocular surface or if their initial examination revealed abnormal ocular findings; described a history of ocular trauma, infection or inflammation within the previous 3 months; reported previous allergic responses following the use eye cosmetics; were regular contact lens wearers (≥3 days per week).

5.5.5.2 Study design

Prior to commencing the study, all subjects underwent a minimum five consecutive day washout period, refraining from all eye cosmetics and contact lens wear.

A baseline visit established the subject's suitability for the study. This visit included a slit lamp examination to ensure no colour cosmetic products were present on the ocular adnexa or suspended in the tear film. Best-corrected visual acuity was measured and a sample of basal tear fluid was collected from each eye using a 10µl glass microcapilliary pipette (Kimble Chase, NJ, USA), placed against the outer third of the inferior tear meniscus with the subject looking supero-nasally. A new microcapilliary pipette was used for each sample. Subjects were reminded to blink normally and care was taken to avoid stimulation of the ocular surface. Total tear collection was limited to five minutes to prevent reflex tearing, discomfort and ocular irritation. The volume of tear fluid collected ranged between 2-10µl per eye. Samples were expelled from the microcapilliary pipettes into 1.5ml Eppendorf tubes and immediately stored at -80°C.

Each subject was supplied with one black pencil eyeliner (MaxFactor Kohl pencil 020 Black, Procter & Gamble, UK) for their personal use. Subjects were instructed to apply the eyeliner daily behind the lash line for seven days (ELI) and to refrain from using any other eye cosmetics and contact lenses during this period. Subjects were also advised to avoid the eye area when applying moisturisers and other facial care products.

To standardise eye pencil removal, each subject was provided with eye make-up remover (Simple Eye Make-up Remover, Simple Health & Beauty Ltd, UK) and instructed on the use of this product by dispensing onto cotton wool pads to remove eyeliner at the end of the day. The list of ingredients in the pencil eyeliner and eye makeup remover can be found in Chapter 4 (Table 4.2).

Subjects attended the clinic for a second visit on the seventh day of eyeliner application. Best corrected visual acuity was measured and samples of tear fluid were collected from both eyes, as described.

5.5.5.3 Cytokine analysis

(a) Calculation of tear fluid dilution factors

Four Quantikine® ELISA kits (R&D Systems Inc., Abingdon, UK) were selected to analyse the concentrations of four different cytokines in the tear fluid samples. Where possible, Quantikine® High Sensitivity kits were selected to allow maximum detection of cytokines which were expected to be at low concentrations in tear fluid. A summary of the selected kits is shown in Table 5.4.

Table 5.4 Summary of R&D systems ELISA kits (source: product inserts). *indicate High Sensitivity (HS) kits

Cytokine	Kit used	Assay range (pg/ml)	Amount of sample required per well (µl)
IL-1β*	Human IL-1β/IL-1F2 Immunoassay, Quantikine® HS ELISA	0.125 – 8	150
IL-1Ra	Human IL-1Ra/IL-1F3 immunoassay, Quantikine®	31 – 2000	100
IL-6*	Human IL-6 immunoassay, Quantikine® HS ELISA	0.16 – 10	100
IL-8	Human IL-8 chemiluminescent, QuantiGlo®	1.6 – 5000	50

A range of dilutions were performed on each sample for each cytokine assay to establish which dilution would be optimum for the capabilities of the ELISA kits. The calculations for dilutions for this preliminary study were based upon from the expected cytokine concentrations in control participants from published current literature and the sensitivity of the kit. The dilutions performed on each assay are summarised in Table 5.5.

Table 5.5 Dilutions performed on tear fluid samples, denoted by (X) of all participants for each assay.

Dilution factor	IL-1β	IL-1Ra	IL-6	IL-8
25x	X		X	
50x	X		X	X
100x	X		X	X
200x	X			X
500x		X	X	X
1000x		X	X	X
5000x		X		

(b) Tear fluid sample preparation

The samples were brought from -80°C to room temperature to thaw. When thawed, each sample was centrifuged at 8000rpm for two minutes.

The samples were sorted according to sample volume. Tear fluid samples from subjects in excess of 10µl enabled all cytokines to be examined at all the appropriate dilution factors (shown in Table 5.5 above) in single runs. Where sample volumes were less than 8µl,

samples were pooled (right and left eye) according to pre- or post-eyeliner intervention per subject. This enabled all four cytokines to be investigated. Once samples were pooled (if necessary), all samples were diluted accordingly with phosphate buffered saline (PBS), mixing well with a vortex mixer each time. Although 34 tear samples were collected from 9 subjects, only 30 samples were usable due to adequate sample volume (15 baseline samples, 15 post-eyeliner intervention samples).

(c)   Performing the assay

Each Quantikine® ELISA kit was supplied with an instruction booklet. The methodologies between kits for performing the assays were similar. Differences in: the duration of each incubation period, the number of washes after the addition of solutions, the addition of amplifier solutions (for High Sensitivity kits) and the wavelengths required to determine the optical density of each well existed between each kit. A general overview of this methodology is summarised.

The reference Standard (supplied with each ELISA kit) was prepared and a serial dilution of the Standard was performed, as per kit instructions. To date, there is no published literature documenting the validation of ELISA for use on tear fluid. Where a R&D ELISA kit supplied two standards as reference to serum or urine (IL-6 and IL-1Ra ELISA kits), the serum calibrator was selected as the expected cytokine concentration is greater in serum than urine which would make the assay more applicable to tear fluid sample.

Assay diluent was added to each well of the 96-well plates supplied. Diluted standards were used for the first two columns in duplicate and tear fluid samples were added to the remaining wells, according to a layout plan of the 96-well plate. The plates were left to incubate for the appropriate amount of time, depending on the cytokine under investigation. While the plates were incubating, the wash buffer (supplied) was prepared and the plate washer was primed once with deionised water and for a second time using the wash buffer.

When the incubation period was over, the plates were washed the specified number of times, depending on the cytokine under investigation. The plates were removed from the plate washer, inverted onto clean paper towels and tapped to remove excess wash buffer,

taking care to ensure the plate did not dry entirely at any point. The conjugate (supplied) was added to each well and left to incubate. During this incubation period, the substrate (supplied) was prepared. Immediately after incubation, the plate was washed again with wash buffer as directed. The substrate was added to each well and incubated for the specified duration. The stop solution (supplied) was added to each well immediately after the substrate-incubation period was complete. The high sensitivity (HS) kits required the addition and incubation of an amplifier solution (supplied) prior to applying the stop solution.

The optical densities of each well were read within 30 minutes of applying the stop solution using a plate reader (FLUOstar Omega, BMG Labtech Ltd., UK). This was set to measure absorbance values at the specified two optical wavelengths. For the IL-8 chemiluminescent ELISA, the plate reader was set to measure luminescence, according to manufacture guidelines. Using plate reading software (MARS Data Analysis Software, BMG Labtech Ltd., UK), a standard curve plotting the optical density (OD) against the concentration of the standard to a four parameter logistic curve-fit (or cubic spline curve fit for the IL-8 chemiluminescent ELISA) was generated.

5.5.6　　Results

The intra-assay coefficient of variation (CV) of each assay plate is summarised in Table 5.6. The CV was calculated using the following equation: $\frac{mean}{standard\ deviation}$ of the OD values obtained from the wells using calibrator standards. All plates had a CV less than 8% which is generally accepted as good precision and low variability (Crowther, 2001).

Table 5.6 Intra-assay CV for all four ELISA cytokine assays

Cytokine	Intra-assay CV (%)
IL-1β	2.82
IL-1Ra	3.94
IL-6	4.16
IL-8	4.41

5.5.6.1　　Cytokine IL-1β

Dilution factors of 1:25, 1:50, 1:100 and 1:200 were used for all samples in the IL-1β assay. 11 baseline samples and 18 post-eyeliner samples were tested. All OD values from the ELISA plate lay at the lower range of the standard curve. IL-1β was not detected in any of the baseline samples. Only four samples (from three subjects) yielded detectable levels of cytokines in the post-eyeliner samples, shown in Figure 5.5. Three out of four results were from samples that had been pooled from RE and LE tear fluid. The mean ± SD concentration of IL-1β (7 days post-eyeliner intervention) was 27±31pg/ml (range 0.86-68pg/ml).
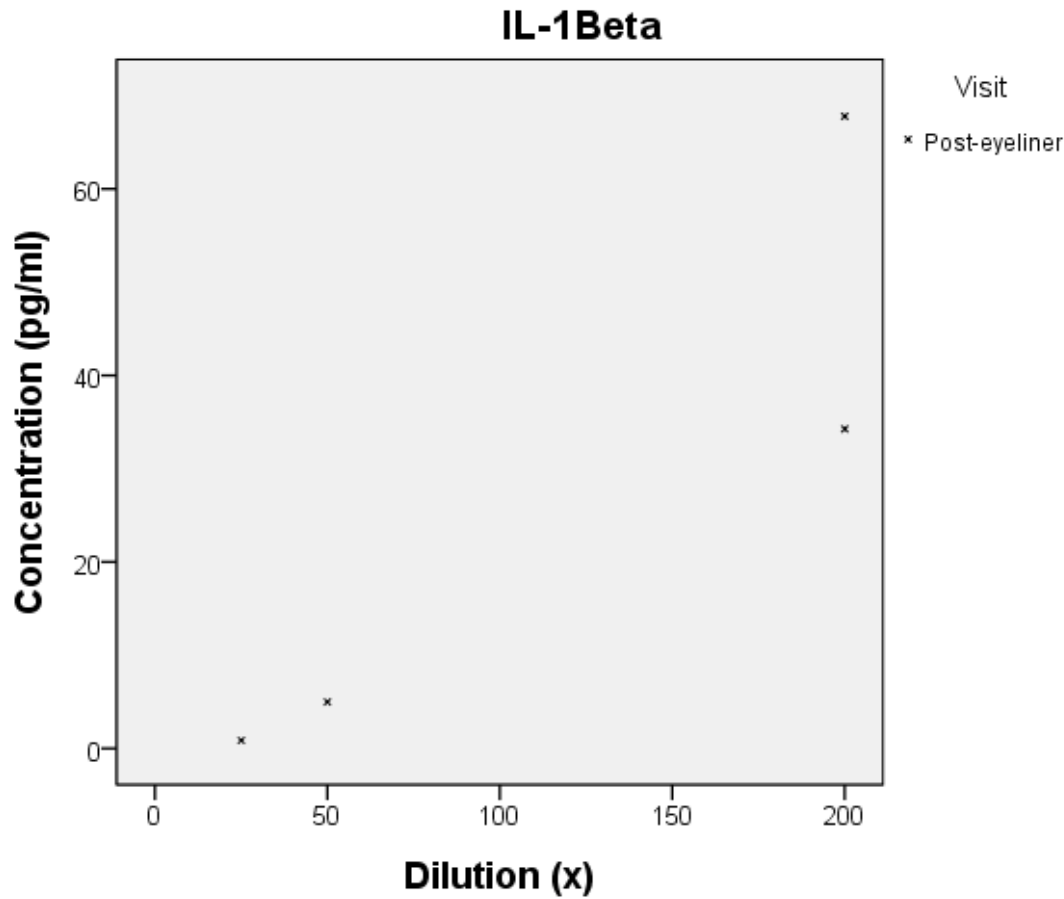
Figure 5.5 Concentration vs. dilution for the IL-1β assay

5.5.6.2   Cytokine IL-1Ra

Dilution factors of 500x, 1000x and 5000x were used for all samples in the IL-1Ra assay. 21 baseline samples and 31 post-eyeliner samples were tested. All OD values from the ELISA plate lay at the lower range of the standard curve. Of the 21 baseline samples, three samples from three subjects yielded detectable IL-1Ra concentrations (mean 95261±61316pg/ml, range 25285 – 139590pg/ml).

Of the 31 post-eyeliner samples, seven samples from six subjects yielded detectable cytokine concentrations which were lower than baseline concentrations (mean 44244±23228pg/ml, range 27100 – 87363pg/ml). Figure 5.6 shows the dilution for most of the detectable samples in this cohort should not exceed 1000x.
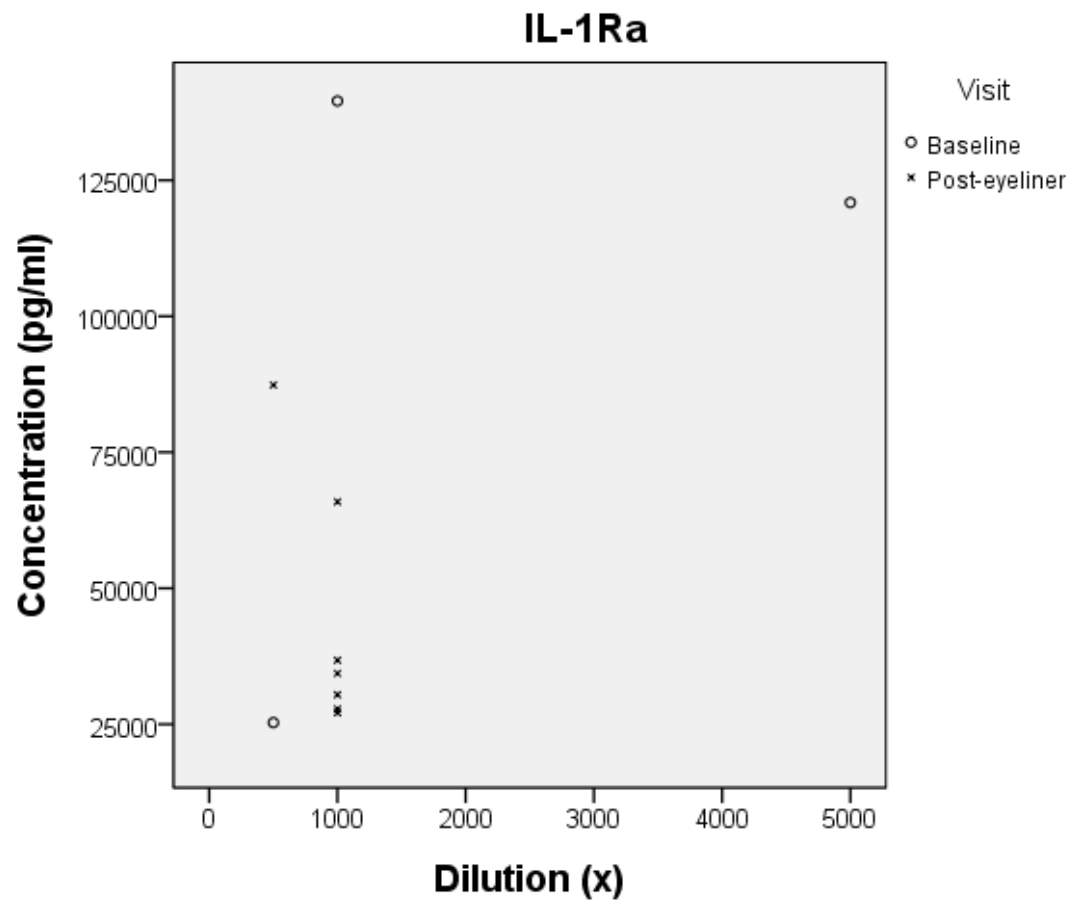
Figure 5.6 Concentration vs. dilution for the IL-1Ra assay

5.5.6.3   Cytokine IL-6

Dilution factors of 25x, 50x, 100x, 500x and 1000x were used for all samples in the IL-6 assay. 27 baseline samples and 30 post-eyeliner samples were tested. All OD values from the ELISA plate fell at the lower range of the standard curve. Of the 27 baseline samples, four samples from four subjects yielded detectable IL-6 concentrations (mean 21.5±20.9pg/ml, range 8.63 – 52.55g/ml), shown in Figure 5.7. Three of these samples were pooled RE and LE samples.

Of the 30 post-eyeliner samples, three samples from three subjects yielded detectable cytokine concentrations which were lower than baseline concentrations (mean 17.5±12.5pg/ml, range 7.5-31.5pg/ml). Two of these samples were pooled RE and LE samples. Figure 5.7 indicates the maximum dilution for IL-6 detection in tear fluid samples for the cohort should not exceed 50x as none of the detectable amounts arose from greater dilutions.
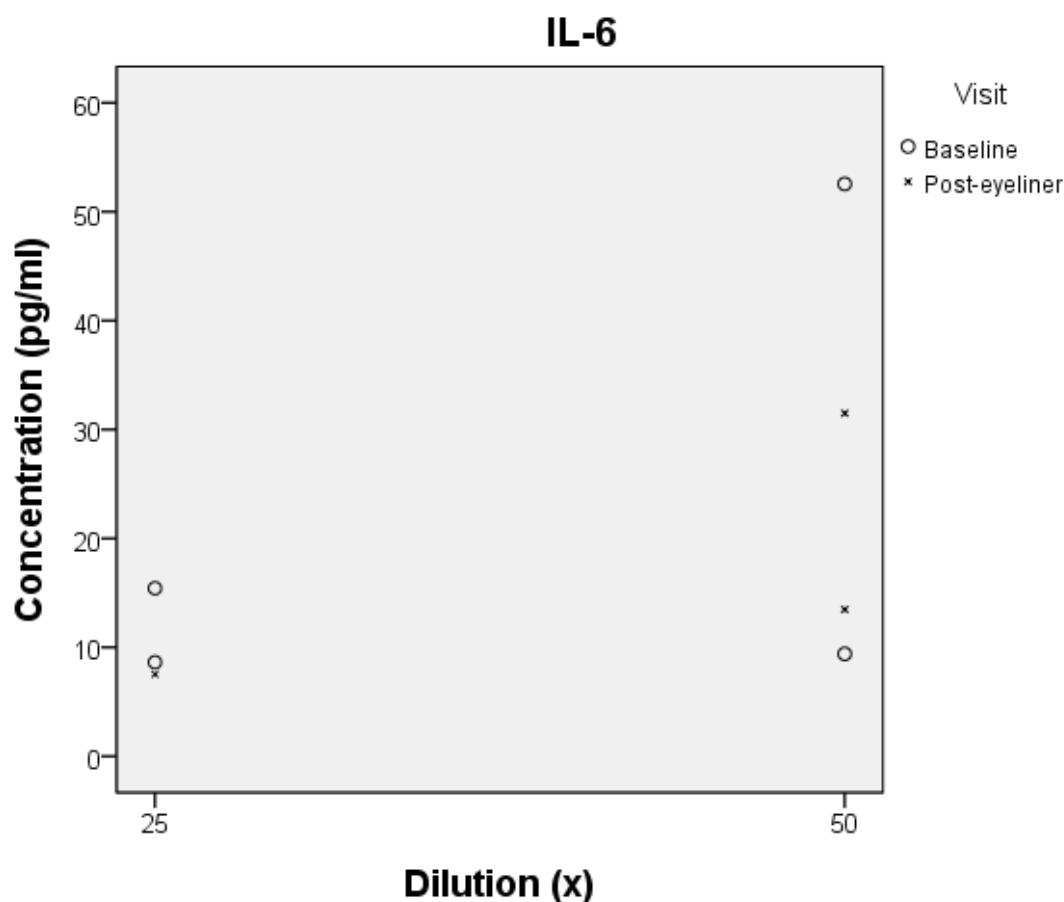
Figure 5.7 Concentration vs. dilution for the IL-6 assay

5.5.6.4   Cytokine IL-8

Dilution factors of 50x, 100x, 200x, 500x and 1000x were used for all samples in the IL-8 assay. 28 baseline samples and 35 post-eyeliner samples were tested. All OD values from the ELISA plate lay at the lower range of the standard curve. Of the 28 baseline samples, five samples from four subjects yielded detectable IL-8 concentrations (mean 222±82pg/ml, range 80 – 278pg/ml). Of these five samples, two samples were pooled RE and LE samples.

Of the 35 post-eyeliner samples, seven samples from four subjects yielded detectable cytokine concentrations which were lower than baseline concentrations (mean 211±98pg/ml, range 80 – 352pg/ml). Of these seven samples, none were pooled. Figure 5.8 shows the maximum dilution for IL-8 detection in tear fluid samples for the cohort should not exceed 100x.
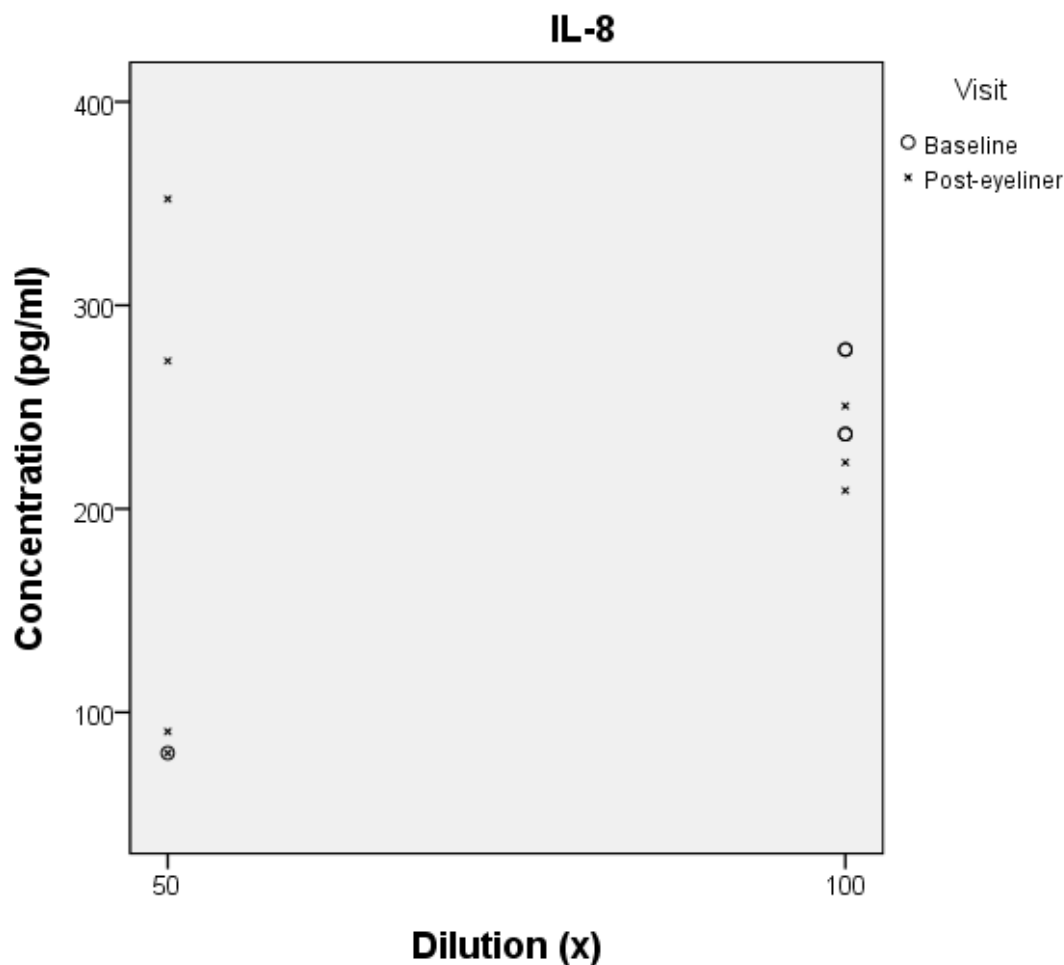
Figure 5.8 Concentration vs. dilution for the IL-8 assay

5.5.6.5   Summary of results

Table 5.7 shows a summary of the mean and range concentrations of the four cytokines detected in tear fluid samples at baseline and after seven days of eyeliner use.

Table 5.7 Summary of cytokine concentrations at baseline and seven days post-eyeliner use

* Indicates the mean concentration for the entire sample set (i.e. including undetectable samples)

Cytokine	EL intervention	Number detectable samples	Mean± SD concentration (pg/ml)	Mean± SD concentration (pg/ml)*	Range concentration (pg/ml)
IL-1β	Baseline	0	-	-	-
	Post-eyeliner	4	27±31	6±17	0.86-68
IL-1Ra	Baseline	3	95261±61316	13609±39278	25285-139590
	Post-eyeliner	7	44244±23228	9991±21483	27100-87363
IL-6	Baseline	4	21.5±20.9	3±11	8.63-52.55
	Post-eyeliner	3	17.5±12.5	2±6	7.5-31.5
IL-8	Baseline	5	222±82	40±92	80-278
	Post-eyeliner	7	211±98	42±95	80-352

5.5.7   Discussion

The purpose of this preliminary study was to determine the appropriate dilution factors of the tear fluid samples to run ELISAs to detect selected pro- and anti-inflammatory cytokines. The results from these preliminary assays indicate several of the cytokines were difficult to detect in these samples. This may be as a result of over-dilution of the tear fluid samples. In an effort to find a maximum and minimum dilution factor for each assay, the mean concentration of each cytokine in control subjects was calculated from the literature. However, from 15 publications which report mean cytokine concentration in normal control subjects Appendix VII, a range of different ELISA assay kits from several manufacturers were used which makes it challenging to draw conclusions about the most useful methodology. According to such publications, the absolute cytokine concentration amongst normal individuals varies greatly. For example, IL-8 concentration in healthy individuals in the literature ranges between 100-24000pg/ml (Thakur and Willcox, 2000; Poyraz *et al.*, 2012).

To limit variability, trends can be observed by using the kits provided by the same manufacturer where antibodies, diluents and stop solutions will be the same (Elshal and McCoy, 2006). However, absolute values should be taken with caution until standardised techniques have been developed specifically for tear fluid analysis (Zhou and Beuerman, 2012).

For some of the samples ran in the assays for this preliminary study, it was observed that not all samples were detectable at the lowest dilution. This is most clearly illustrated in the IL-6 assay where more samples were detectable at 50x dilution compared to the 25x dilution. This may have arisen due to two factors. Firstly, it is possible that the lower dilutions were out of range above the top standard (i.e. the sample was too concentrated). This may have been a possibility as the assay range of the IL-6 assay used in this study was 0.125 – 8pg/ml whereas the mean IL-6 concentration was 17.5±12.5pg/ml. Secondly, cytokines may have only been detectable at the greater dilution due to matrix effects. Matrix effects occur when the sample contains such greater quantities of other proteins and molecules that they begin to interfere with the quality of the assay.

Of the four cytokines analysed in this preliminary study, mature IL-1β generated the lowest detection rate. Only post-eyeliner tear fluid samples contained detectable levels of IL-1β, of

which 75% were samples pooled from RE and LE. The difficulty in detecting this cytokine is not surprising as mature IL-1β is associated with severe, destructive inflammatory events (Dinarello, 1996). The pre-cursor IL-1β is found in relatively high concentrations, 12 times greater than the mature form, however once cleaved into the mature form, a path of destructive inflammatory events is initiated (Solomon *et al.*, 2001). A study examining cytokine concentrations after accidental exposure to household chemicals found mature IL-1β was not significantly elevated compared to control eyes whereas IL-6 levels were significantly greater in injured eyes (Berry and Jeffreys, 2001).

In this optimisation work the concentration of ant-inflammatory IL-1Ra was considerably greater than the other cytokines analysed in tear fluid. Due to the greater concentration of this cytokine, it was still detectable at a dilution of 1000x. A typical concentration of IL-1Ra in tear fluid is often reported in the order of nanograms/ml compared with picograms/ml for the IL-1β, -6 and -8 (see Table 5.3) and is often found 25000 to 40000 times greater than inflammatory IL-1 (Solomon *et al.*, 2001). The seemingly excessive concentration of this antagonistic cytokine reflects the fact only a small increase in pro-inflammatory IL-1 can induce a biologically devastating cascade of inflammation (Solomon *et al.*, 2001). Increased expression of IL-1Ra has been shown in aqueous deficient dry eye patients (Solomon *et al.*, 2001; Huang *et al.*, 2012) and positively correlates with ocular surface staining (Huang *et al.*, 2012).

Previous work has shown that IL-8 concentrations may be a sensitive and reliable human *in vivo* endpoint in the prediction of human eye tolerance to mildly irritating substances (Debbasch *et al.*, 2005). The investigators found IL-8 release to have good correlations with symptoms (itching and burning) and bulbar conjunctival redness (Debbasch *et al.*, 2005). Other studies have shown that IL-8 concentrations are positively correlated with ocular surface staining in dry eye disease (Lam *et al.*, 2009; Enriquez-de-Salamanca *et al.*, 2010).

The difficulty in making comparisons and deriving conclusions between baseline and post-intervention cytokine concentrations in this study exists as very few paired results were obtained. Combined with the lack of duplicated samples at duplicated dilutions in the wells, this makes statistical analysis on this preliminary study difficult. To improve the likelihood in obtaining paired results (i.e. pre- and post-eyeliner comparison), the dilution factors need to

be reduced and repeated samples need to be tested. Since it is difficult to collect greater volumes of tears, it is more practical to assess more pertinent cytokines which may be easily and reliability detected using ELISA.

Therefore, IL-6 and IL-8 were selected to be used in the final study. Although IL-1Ra was detectable in higher concentrations and at lower dilutions, it was decided that the results would only be most useful if the agonist IL-1β would also be analysed since it is the antagonist/agonist ratio that influences the preponderance of inflammatory disease (Arend, 2002). Previous studies have shown ELISA to be repeatable in detecting IL-6 in tear fluid samples and the assay was run successfully in this preliminary study. Additionally, the investigation of IL-6 expression in ocular surface pathologies and dry eye disease is well documented in the literature. IL-8 has also been repeatedly detected using ELISA. Furthermore, the IL-8 ELISA kit used in this preliminary study utilises luminescence as the output measure. This has two main benefits: it enables a wider range of cytokine detection and has improved sensitivity compared to colorimetric ELISA assays (Apfel and Enderle, 2006). The IL-8 assay requires only 50µl sample per well which is also advantageous for small tear fluid volumes.

The optimisation study has shown that any dilutions of tear fluid samples greater than 50x reduces the likelihood of cytokine detection. Thus, the optimal maximum dilution factor for the follow-on study will be 50x for IL-6 and IL-8 assays.

5.6   The detection of inflammatory cytokines in tear fluid following the application of pencil eyeliner

The clinical outcomes from a crossover study in which 27 healthy female subjects applied pencil eyeliner to the peri-ocular skin or within the lash line for seven days with a washout period between conditions are described in Chapter 4. At each visit of this crossover study, tear fluid was collected from each subject for later biochemical analysis. As discussed in section 5.4.2, the aim of this study was to investigate if these subjects exhibited subclinical changes following the eyeliner interventions by analysing tear fluid for inflammatory cytokines IL-6 and IL-8.

Section 5.5.2 provided a general overview of IL-6 and IL-8 cytokines. Table 5.8 provides an overview of IL-6 and IL-8 cytokine values found in the tear fluid of healthy controls and in various pathological conditions or interventions in the literature (a comprehensive version of this table can be found in Appendix VIII. In healthy individuals, the mean concentration of IL-8 is greater than IL-6 (956 ± 1396pg/ml vs. 45 ± 76pg/ml), calculated from averaging the all IL-8 values found in the literature. Table 5.8 shows that the concentration of IL-8 and IL-6 in tear fluid is elevated in pathological eyes and following interventions, such as using glaucoma medications, compared to the control subjects. The method of cytokine detection is also detailed in Table 5.8 as multiplex bead analysis has a tendency to provide higher values of cytokine concentrations.

Table 5.8 Concentration of IL-8 and IL-6 in tear fluid observed in healthy controls, pathological eyes and treatments (assay type specified, MBA = multiplex bead analysis).

	Mean±SD IL-8 concentration (pg/ml)	Mean±SD IL-6 concentration (pg/ml)	Reference(s)
Healthy controls	956±1396, range 5-4600 (ELISA) 2439±5806, range 125-16791 (MBA)	45±76, range 2-22 (ELISA) 273, range 26.5-1000 (MBA)	See Appendix VIII
Pathological eyes & treatments	**Range IL-8 concentration (pg/ml)**	**Range IL-6 concentration (pg/ml)**	
Contact lens wear	107-148000 (ELISA)	3-218 (ELISA)	(Thakur and Willcox, 2000; Gonzalez-Perez *et al.*, 2012; Poyraz *et al.*, 2012)
Contact lens associated red eye	65-420000 (ELISA)	19-320 (ELISA)	(Thakur and Willcox, 1998)
Contact lens peripheral ulcer	0.9-3400 (ELISA)	46-125 (ELISA)	(Thakur and Willcox, 1998)
Penetrating keratoplasty	220 (ELISA)	170 (ELISA)	(Fodor *et al.*, 2006)
Corneal foreign body	203 (ELISA)	109 (ELISA)	
Cataract operation	171 (ELISA)	189 (ELISA)	
Bacterial conjunctivitis:	661 (ELISA)	366 (ELISA)	
Dry eye	245-9730 (ELISA + MBA)	17-1626 (ELISA + MBA)	(Acera *et al.*, 2008; Massingale *et al.*, 2009; Huang *et al.*, 2012; Guyette *et al.*, 2013)
Meibomian gland dysfunction	1657 (MBA)	289 (MBA)	(Lam *et al.*, 2009)
Topical glaucoma treatment	4650 (MBA)	3000 (MBA)	(Malvitte *et al.*, 2007)
Keratoconus	-	5.5-6.7 (ELISA)	(Lema and Duran, 2005; Lema *et al.*, 2009)
Conjunctivochalasis	-	52 (ELISA)	(Acera *et al.*, 2008)
Blepharitis	-	17 (ELISA)	
Allergic eye disease	-	33 (ELISA)	

5.6.1   Methods

The subject demographics, exclusion and inclusion criteria and study design are outlined in Chapter 4.2. Briefly, each subject attended six visits: baseline, 1 day and 7 day post-ELI, washout, 1 day and 7 day post-ELO. At each visit, a sample of tear fluid was collected prior to the application of ocular surface staining agents. The procedures and the order in which they were conducted can be found in section 4.2.4.

5.6.1.1   Tear collection and storage

Basal tear fluid was collected from each eye using a Kimble® 10µl glass microcapilliary pipette, placed along the lower tear meniscus with the subject instructed to look supero-nasally. A new microcapilliary pipette was used for each sample. Subjects were instructed to blink normally and care was taken to prevent stimulation of reflex tearing due to ocular irritation. The time taken to collect tear fluid was limited to five minutes to prevent reflex tearing, ocular irritation and discomfort. The volume of tear fluid samples ranged between 2-10µl per eye.

Samples were expelled from the microcapilliary pipettes into 1.5ml Eppendorf tubes and immediately stored at -80°C until processing of the samples.

5.6.1.2   Tear fluid preparation

268 tear fluid samples were collected from 24 subjects between November 2011 and July 2012. Samples were brought to room temperature, thawed and centrifuged at 8000rpm for two minutes. Due to sample volume loss previously encountered during storage, all samples from RE and LE were pooled (wherever possible) according to visit number and subject, resulting in 123 pooled tear fluid samples for analysis. Pooled tear fluid volumes ranged between 2-15µl.

5.6.1.3   Sample dilution

IL-8 was the priority cytokine due to the wide range of detection and sensitivity of the assay. The smaller sample volume (50µl compared with 100µl for IL-6 assay) required per well of the IL-8 assay was also advantageous given the reduced sample encountered after long-term storage. Furthermore this allowed any pooled sample volume greater than 5µl to be

run in duplicate. The preliminary data indicated the dilution factor for IL-8 and IL-6 should not exceed 100x and 50x respectively. To maximise the potential for cytokine detection, a maximum 25x dilution factor was used, where possible. Table 5.9 summarises the dilution factors and the number of wells used for each assay.

Table 5.9 Dilutions and number of wells per plate per assay. All samples were diluted by 25x except where shaded in grey. Dilution factors of 55x were used for sample volumes of 4- and 6μl on the IL-6 assay to maximum replicates.

Pooled sample volume (μl)	Dilution for IL-8	Number of wells (IL-8)	Dilution for IL-6	Number of wells (IL-6)
2	*30x*	*1*	-	-
3	25x	1	-	-
4	25x	1	*55x*	*1*
5	25x	2	-	-
6	25x	2	*55x*	*1*
7	25x	1	25x	1
8	25x	1	25x	1
9	25x	2	25x	1
10	25x	2	25x	1
11	25x	2	25x	1
12	25x	2	25x	1
13	25x	2	25x	1
14	25x	2	25x	2
15	25x	2	25x	2

5.6.1.4 Performing the IL-8 assay

The ELISA kit used in the preliminary study (Quantiglo® Chemiluminescent Human CXCL8/IL-8, R&D Systems Inc., Abingdon, UK) was selected to analyse the tear fluid samples for IL-8. Due to the number of samples, two 96-well plates were used. After sample preparation, the IL-8 assay was conducted according to the product insert. A brief overview of the procedures is described below.

The supplied reagents and Standards were prepared, according to the manufacturer guidelines. A serial dilution of the supplied Standard was performed. 100µl assay diluent was added to each well, followed by 50µl Standard or sample to the relevant wells according to the layout of the 96-well plate. When all Standards and samples were loaded, the plates were left to incubate at room temperature for two hours on a horizontal orbital microplate shaker set at 500rpm.

Each plate was washed four times with wash buffer (supplied) in a plate autowasher immediately after incubation. After the last wash, the plates were inverted and blotted against dry paper towels to ensure excess wash buffer was removed. 200µl supplied conjugate was added to each well and the plates were covered and left to incubate for three hours at room temperature on the orbital plate shaker. Following this incubation period, the wells were washed again using the described method prior to adding 100µl chemiluminescence reagent to each well. Each plate was left to incubate for a further 10 minutes at room temperature on a worktop protected from the light. The luminescence of each well was determined immediately after incubation using a plate reader (FLUOstar Omega, BMG Labtech Ltd., UK). The relative light units (RLU) of each well were determined using UV emission under the following settings outlined by the manufacturer: 1 minute lag time, 0.5 second/well read time, gain 2000. A standard curve plotting the RLU against the concentration of the IL-8 standard to a cubic-spline fit was generated using plate reading software (MARS Data Analysis Software, BMG Labtech Ltd., UK).

A summary of this process is illustrated in Figure 5.9.

1. Samples prepared & serial dilution of supplied Standard performed

2. 100µl assay diluent added to each well

3. 50µl standard/sample added to each well

4. Plate incubated at room temperature for 2 hours on orbital shaker at 500rpm

5. Plate washed 4 times with wash buffer, inverted and blotted against dry paper towels.

6. 200µl conjugate added to each well

7. Plate incubated at room temperature for 3 hours on orbital shaker

8. Wash plate (see step 5)

9. 100µl chemiluminescence reagent added to each well. Plate incubated for 10 minutes at room temperature, protected from light sources

10. Plate read using microplate reader

Figure 5.9 Summary of IL-8 ELISA procedure using the R&D Systems Quantiglo® Chemiluminescent Human CXCL8 kit

5.6.1.5   *Performing the IL-6 assay*

The ELISA kit used in the preliminary study (Quantikine® HS Human IL-6, R&D Systems Inc., Abingdon, UK) was selected to analyse the tear fluid samples for IL-6. After sample preparation, the IL-6 assay was conducted according to the product insert. A brief overview of the procedure is described below.

The supplied reagents and Standards were prepared, according to the manufacturer guidelines. A serial dilution of the supplied Standard was performed. 100µl assay diluent was added to each well, followed by 100µl Standard or sample to the relevant wells according to the layout of the 96-well plate. When all Standards and samples were loaded, the plate was left to incubate at room temperature for two hours on a horizontal orbital microplate shaker set at 500rpm.

The plate was washed six times with wash buffer (supplied) in a plate autowasher immediately after incubation. After the last wash, the plates were inverted and blotted against dry paper towels to remove excess wash buffer. 200μl supplied IL-6 conjugate was added to each well and the plates were covered and left to incubate for two hours at room temperature on the orbital plate shaker. After the second incubation, the plate was washed six times in the autowasher using wash buffer and blotted to remove excess wash buffer. 50μl supplied substrate was added to each well, covered and left to incubate for 60 minutes at room temperature. Following incubation 50μl amplifier solution was added to each well and left to incubate at room temperature for a further 30 minutes. Finally, 50μl Stop solution was added to each well.

The optical density (OD) of each well was determined immediately after the addition of the Stop solution using a plate reader (FLUOstar Omega, BMG Labtech Ltd., UK) set to 490nm and 650nm. A standard curve plotting the OD against the concentration of the IL-6 standard to a four parameter logistic curve fit was generated using plate reading software (MARS Data Analysis Software, BMG Labtech Ltd., UK).

This procedure is summarised in Figure 5.10.

1. Samples prepared & serial dilution of supplied Standard performed
↓
2. 100µl assay diluent added to each well
↓
3. 100µl standard/sample added to each well
↓
4. Plate incubated at room temperature for 2 hours on orbital shaker at 500rpm
↓
5. Plate washed 6 times with wash buffer, inverted and blotted against dry paper towels.
↓
6. 200µl conjugate added to each well
↓
7. Plate incubated at room temperature for 2 hours on orbital shaker
↓
8. Wash plate (see step 5)
↓
9. 50µl substrate added to each well and incubated for 60 minutes at room temperature
↓
10. 50µl amplified solution added to each well and incubated for 30 minutes
↓
11. 50µl Stop solution added
↓
12. Plate read using microplate reader

Figure 5.10 Summary of IL-6 ELISA procedure using the R&D Systems Quantikine® HS Human IL-6

5.6.1.6   Statistical analysis

Statistical analysis was performed using SPSS 18.0 (www.ibm.com/SPSS_Statistics). Data were tested for normality using a Shapiro-Wilk test. The data was not normally distributed ($p<0.0001$) but as the data is continuous, parametric statistics were still applied as the sample of results are representative of a normal population (Bland and Altman, 2009).

Cytokine concentrations were examined for carry-over and treatment-period interaction using independent samples t-tests and no carry-over effects were detected. Independent samples t-tests were conducted for a comparison of ELI and ELO samples after one day and seven days of intervention. Two-sided p-values<0.05 were considered statistically significant.

5.6.2 Results

The r² values from the standard curves were 1 and 0.999 for both IL-8 and IL-6 plates respectively. The intra-assay CV of each assay plate is summarised in Table 5.10 and was based upon replicates of the calibrator standards and any samples run in duplicate. All plates had a CV less than 8% which is generally accepted as good precision and low variability (Crowther, 2001).

Table 5.10 Intra-assay CV for IL-8 and IL-6 cytokine assays. Two IL-8 plates were used, the average intra-assay CV of the two plates is specified.

Cytokine	Intra-assay CV (%)
IL-8	3.34%
IL-6	4.03%

5.6.2.1 Cytokine IL-8

Tear samples collected for 24 subjects across all six visits (baseline, 1 day and 7 day post-ELI, washout, 1 and 7 day post-ELO) were analysed and compared.

Appendix IX shows the IL-8 assay results for all subjects. One subject was unable to supply sufficient volumes of tear fluid for analysis across all six visits. Eight subjects had missing data from one or more visits due to insufficient tear fluid volumes after storage. Table 5.11 shows the data in 15 subjects of which a complete data set was collected.

Table 5.11 IL-8 concentrations (pg/ml) according to subject and visit. All samples, unless shaded in grey, were diluted by 25x. Cells shaded were samples diluted by 30x and values have been corrected by a factor of 1.2 to reflect the additional dilution

Subject	Pre-ELO	1 Day ELO	7 Days ELO	Pre-ELI	1 Day ELI	7 Days ELI
2	321.8	251.5	388.4	440.4	451.5	885.6
10	181.6	144.8	353.7	240.4	295.8	225.7
3	240.4	163.3	100.6	146.7	111.7	93.1
11	148.6	122.8	176.1	96.8	119.1	102.4
6	130.2	133.9	179.8	207.4	281.1	166.9
12	130.2	214.7	185.3	159.6	292.1	137.5
14	158.5	204.5	337.6	642.1	177.2	153.7
19	209.0	148.9	343.6	226.6	144.1	177.2
20	411.0	209.0	222.2	98.5	124.4	167.9
15	258.9	188.7	191.0	244.0	208.9	280.1
18	906.5	651.6	552.0	286.3	375.5	241.8
23	211.2	286.3	228.8	278.0	202.2	267.4
21	3577.6	1152.4	1128.0	2184.3	1267.4	731.5
5	629.0	201.8	358.8	329.1	108.0	89.3
17	202.2	177.2	172.5	181.8	103.8	191.0

IL-8 cytokine concentrations across all six visits were detected for 15 out of 23 subjects. The mean IL-8 concentration of these 90 samples was 338.8±461.4pg/ml. Table 5.12 below summarises the mean concentration of IL-8 in tear fluid samples after each eyeliner intervention. This data is also illustrated in Figure 5.11. There was no statistically significant difference between baseline values before each eyeliner intervention (p=0.352, independent samples t-test).

Table 5.12 Mean IL-8 concentration (pg/ml) for data where cytokine detection was possible across all 6 visits (n=15). Statistical analysis conducted using independent samples t-tests.

IL-8 (n=15)	ELO	ELI	ELO minus ELI	P-value
Baseline	511.8±874.7	374.3±514.8	137.4	*0.220*
Δ 1 day	281.8±272.9	284.2±291.9	-2.4	*0.943*
p-value (baseline vs. 1 day)	*0.174*	*0.203*		
Baseline	511.8±874.7	374.3±514.8	137.4	*0.220*
Δ 7 days	320.1±250.4	260.8±231.8	59.4	*0.282*
p-value (baseline vs. 7 day)	*0.267*	*0.300*		

This data shows a decline in IL-8 concentration after 1 day of eyeliner application, followed by a small increase in IL-8 concentration after seven days of ELO, and a small continued decline after seven days of ELI. One-way repeated measures ANOVAs were conducted and showed no significant difference between short- or long-term changes in cytokines in either method of eyeliner application (ELO p=0.475; ELI p=0.672). Furthermore, the results from a one way repeated measures ANOVA showed no statically significant difference in IL-8 concentration at of the six visits (p=0.697).
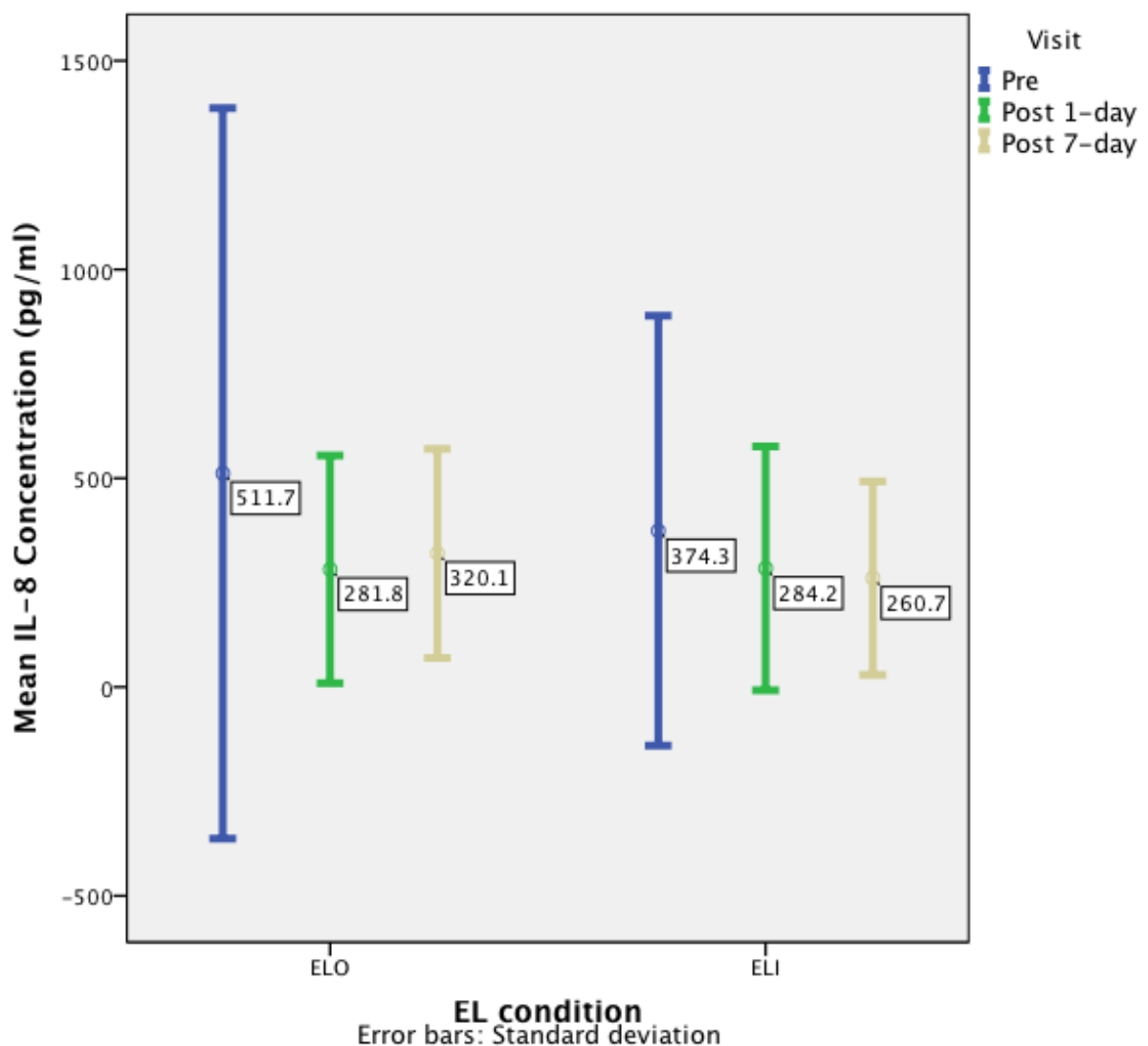
Figure 5.11 Mean plot of IL-8 concentration at baseline, 1 day and 7 day in ELO and ELI application (n=15)

5.6.2.2 Cytokine IL-6

Due to tear fluid volumes, there is no data for all six visits for any subjects in the IL-6 assay. IL-6 was detectable in eleven subjects at any point of the study. Table 5.13 shows the results of all samples detectable for IL-6. One subject (#21) exhibited cytokine concentrations over five times greater than the mean IL-6 concentration.

Table 5.13 Detectable IL-6 concentrations (pg/ml), according to subject and visit. "-" indicate an inadequate tear volume sample for analysis thus no value was obtained. All samples were diluted 25x

Subject	Pre-ELO	1 day ELO	7 days ELO	Pre-ELI	1 day ELI	7 days ELI
2	2.3	3.9	3.0	2.3	-	-
23	10.1	12.5	-	-	9.5	4.9
5	-	-	4.3	-	-	-
20	-	-	13.5	15.9	9.7	24.9
18	-	15.5	11.4	11.4	-	-
11	-	-	-	7.3	4.8	-
4	-	-	8.8	26.2	-	-
12	-	-	-	-	5.0	-
6	-	17.1	-	8.9	13.1	-
10	-	-	-	-	-	12.3
21	-	724.4	-	-	-	35.9

Table 5.14 below shows the number of detectable samples, mean and standard deviations of IL-6 concentrations for each visit. The mean IL-6 concentration for 27 detectable samples was 37.7±137.5pg/ml. Due to the small number of samples per visit, statistical analysis to compare between eyeliner conditions was not appropriate.

Table 5.14 Number of detectable samples and mean and SD of IL-6 concentrations (pg/ml) for each visit.

	Pre-ELO	1 day ELO	7 days ELO	Pre-ELI	1 day ELI	7 days ELI
n	2	5	5	6	5	4
Mean IL-6 concentration	6.2	154.7	8.2	12.0	8.4	14.0
SD	5.6	318.5	4.5	8.3	3.5	10.1

Figure 5.12 shows the concentrations of detectable IL-6 samples according to each visit.

Figure 5.12 Scatter plot illustrating the spread of IL-6 data for all detectable sample according to eyeliner condition

5.6.3   Discussion

The results demonstrate that variations in tear cytokine expression may occur with eyeliner use. The observed variations differed according to the method and duration of eyeliner application. Additionally, trends of cytokine variations were different for IL-8 and IL-6.

IL-8 and IL-6 are have been successfully detected in human tear fluid using ELISA consistently across the literature. This was one of the reasons for choosing these two cytokines for investigation in this study. The practical difficulties of detecting these cytokines using this platform have typically involved the large sample volumes required to run assays, hence the need for pooling samples (Nakamura *et al.*, 1998; Thakur and Willcox, 2000; Kallinikos *et al.*, 2006). In this study, the Quantikine® IL-6 and IL-8 assays required 100µl and 50µl per well, respectively. However, during the storage of the tear fluid samples, sample loss due to sublimation was unexpectedly encountered. This occurrence has been documented in long-term storage of small sample volumes of plasma, where between 4-17% volume loss due to sublimation can be encountered (Craft *et al.*, 1993; Gislefoss, 2010), even when capped tubes are used (Holland *et al.*, 2003). Consequently:

- Pooling RE and LE of tear fluid samples became a necessity
- Difficulties with cytokine detection occurred in some subjects who could only deliver a small quantity of tear fluid
- Unable to run samples for IL-8 in duplicates
- Less samples were tested for IL-6 than at first forecasted
- Statistical analysis was not possible on the IL-6 data due to the small number of results yielded therefore a lacking in statistical power

An estimation of the percentage of sublimation could have been calculated by freezing known quantities of the IL-8 and IL-6 standards at the same time as freezing each tear fluid sample and observing the quantity of standard that could be recovered after long term storage. However for future studies involving small volumes of samples, tear fluid could be diluted prior to storage.

The values for detectable IL-8 and IL-6 samples in this study were lower than the mean, but within the range of values of cytokine concentrations of healthy, normal subjects described

in the literature and summarised in Table 5.8. The pattern of IL-8 concentration in tear fluid at each eyeliner intervention followed a decrease from baseline after 1 day of use and an increase after 7 days of use. These changes were more pronounced in the ELO application compared with the ELI. Furthermore, these findings are similar to those in the optimisation study (section 5.5.6.3 and 5.5.6.4) where a reduction in IL-8 and -6 occurred after 7 days of ELI application. These findings confound the hypothesis that the ELI would be the intervention which would be more likely to induce an ocular surface inflammatory response. It was hypothesised that the migration of pencil eyeliner, which has been shown to increase in ELI application in Chapter 3, would cause low levels of inflammation which, if not evident by Day 1, would manifest by Day 7 of eyeliner usage. The chemical formulation of the pencil eyeliner was hypothesised to be a substance foreign to the ocular surface, thus causing the immune system to respond. Debbasch *et al.* (2005) alluded to the study of IL-8 as a measure of eye tolerance to cosmetic formulations for human *in vivo* testing. In this study, it may be the case that seven days of eyeliner application may not be long enough to observe a significant alteration in cytokine expression.

IL-8 is one chemokine whereby detection in tear fluid is sensitive to reflex tearing (Nakamura *et al.*, 1998; Sonoda *et al.*, 2006; Zakaria *et al.*, 2012). While care was taken to avoid stimulating reflex tearing during tear fluid collection, Appendix VI shows the subjective comments at each visit during the eyeliner interventions. There were several reports of reflex tearing and some volunteers reported re-applying the product after several hours as they found the eyeliner had "disappeared". Reflex tearing may result in a reduction in IL-8 concentrations during Day 1 of each eyeliner intervention where volunteers are "adapting" to the application process. To better understand the role of reflex tearing, further studies could utilise the examination of tear fluid for a protein arising from the lacrimal gland, such sIgA. The concentration of sIgA in tear fluid has consistently been shown to be reduced in reflex tears (Fullard and Snyder, 1990; Fullard and Tucker, 1991; Sitaramamma, Shivaji and Rao, 1998) therefore assessing the concentration of sIgA in collected tear samples could be a useful way in taking tear reflexing into account.

While the investigation of cytokine expression is being developed for clinical diagnostic procedures and therapies, interestingly, the diurnal variation and repeatability of cytokine detection is poorly understood. Uchino *et al.* (2006) found very little variability of mean IL-8

concentration in tear fluid between 09:00 to 24:00 however a separate study indicated almost 25% reduction from 09:00 to 12:00 using the same multiplex bead assays (Uchino *et al.*, 2006a; Uchino *et al.*, 2006b). Huang *et al.* (2012) found good intraclass correlations (ICC) in IL-6 and IL-8 concentration when two visits seven days apart were compared using multiplex bead arrays. No data is found for diurnal variation in cytokine concentration using ELISA however it may be assumed the trend is followed as there is evidence of good correlations between multiplex bead assays and ELISA (Elshal and McCoy, 2006). Thus the diurnal and between visit fluctuations of IL-8 are unlikely to be an influence in this study. One variable which was difficult to control was the duration of the one day exposure to pencil eyeliner which ranged between 4-8 hours of first day application.

The difficulty of collecting large tear fluid volumes without stimulating reflex tearing has resulted in under-sampling, in addition to sample loss, for IL-6 analysis. Future studies could employ the "flush" or "washout" technique of tear fluid collection as an alternative method to basal tear collection. Markoulli *et al.* (2011) first validated this technique where 60µl saline was instilled into the inferior fornix and fluid sample immediately collected. The results indicated the total profile of proteins detected in the tear fluid was comparable to basal tear collection. Zakaria *et al.* (2012) found the concentration of IL-6 using this technique was significantly reduced compared to basal tear collection. Most recently, Guyette *et al.* (2013) found statistically significant correlations in cytokines with flush and basal tear collection techniques, although found a subtle difference between aqueous deficiency and normals. While the concentration of IL-6 may be slightly lower than basal tear fluid collection, the quantity of fluid collected is significantly greater, enabling a greater chance of IL-6 detection, which is present in tear fluid in much smaller amounts compared with IL-8. However, a major disadvantage of implementing the flush technique in this study would be the risk of washing pencil eyeliner into the collected tear fluid samples. It is unknown how the clarity of samples affects ELISA outcomes, and methods of "purifying" the tear fluid samples may be required.

The results from this study had one outlier which demonstrated elevated IL-6 and -8 concentrations greater than 2 times the standard deviation. The reason for this subject to have such elevated cytokine concentrations is unestablished however data in the literature presents vast ranges of cytokine concentrations amongst healthy controls. The volunteer

selection procedure for this study ensured that all subjects had no history of previous or active ocular surface pathology and no history systemic pathology. There is some evidence that respiratory tract viruses, particularly the influenza virus, demonstrate ocular surface tropism, without initially manifesting as an ocular surface disease (Olofsson *et al.*, 2005; Belser *et al.*, 2012; Belser, Rota and Tumpey, 2013). It may be the case that this subject with markedly increased IL-6 and -8 cytokines in tear fluid may have been inoculated with an influenza virus which had not manifest on the ocular surface or respiratory tract.

The results from this study indicate that the use of pencil eyeliner, in conjunction with eye cosmetic remover, does not appear to induce subclinical inflammation over the period of the study. While the number of samples analysed is small, these findings reflect the nature of the safety of these products approved by the EU. However, the reduction of reported ocular comfort and increase in OSDI scores highlighted in Chapter 4 and Appendix VI indicate other processes may be occurring with eyeliner application.

This study has shown variations of inflammatory cytokines occur with the use of cosmetic pencils and eye cosmetic removers. The reduction of IL-8 after short-term (1 day) use of eyeliner pencil may be due to reflex tearing during the adaption of using new cosmetic products. The recovery of IL-8 concentration to nearly baseline levels suggests long-term subclinical changes are not present with pencil eyeliner use. Thus cosmetic products do not appear to induce a cascade of inflammatory events in healthy subjects.

6   Conclusions and Future Work

6.1   Overall conclusions

The primary aim of this thesis was to establish the impact of eye cosmetic usage on ocular comfort, investigate the migration of cosmetic products that are applied externally around the eyes, determine the clinical effects of eye cosmetics usage on the ocular surface and tear film and to explore any subclinical, biochemical effects resulting from eye cosmetics application. This thesis had the following hypotheses:

- Eye cosmetic usage negatively impacts ocular comfort, affecting both contact lens wearers (CLW) and non-contact lens wearers (NCLW), with CLW being affected the greatest. The application of certain types of eye cosmetics close to the ocular surface, such as eyeliner, will cause the largest amount of discomfort.

- When applied externally around the eyes, pencil eyeliner readily contaminates the tear film, particularly when applied with closer proximity to the ocular surface

- Pencil eyeliner applied behind the lash line will most likely induce clinically detectable changes affecting tear quality and stability as it readily migrates into the tear film, compared to when it is applied along peri-ocular skin. These clinical changes will be more obvious with longer duration of eyeliner usage.

- The application of pencil eyeliner will induce inflammation of the ocular surface, at subclinical levels. The amount of inflammation will be more obvious with the application of pencil eyeliner behind the lash line and with longer duration of eyeliner usage.

These hypotheses were tested with a series of studies, documented from Chapter 2 to Chapter 5. Chapter 2 examined the results of an online survey investigating cosmetic usage in a UK population. This was undertaken to evaluate the impact of potential issues which may be uncovered by subsequent experiments in this thesis. The results from this survey confirmed the extensive use and popularity of eye cosmetic usage in a UK population. Additionally, the results showed eye cosmetic usage habits in CLW and NCLW did not differ.

Ocular comfort was measured using two scales in the survey: perceived ocular comfort (an ordinal scale from 0 to 10) and the OSDI questionnaire. Amongst eye cosmetic users, perceived ocular comfort was significantly reduced when cosmetics were used. While an increase in OSDI scores, a measure of dry eye symptom severity, was found amongst cosmetic users, this was not statistically significant. Additionally, CLW who chose to not use eye cosmetics had greater OSDI scores (i.e. worse dry eye symptoms) than CLW who applied eye cosmetics. This is thought to be due to self-selection: CLW who already experience discomfort with relation to CL wear may choose to avoid eye cosmetics in fear of further exacerbating dry eye symptoms. The frequency of eye cosmetics usage did not appear to have an impact upon OSDI scores. The use of eyeliner was of particular interest in this thesis, as this product, out of all eye cosmetic products, is commonly applied with the closest proximity to the ocular surface. The results from the survey showed the use of eyeliner significantly increased dry eye symptoms, particularly when NCLW used the product regularly (defined as usage ≥ three times a week). Lastly, the online survey examined the opinions of cosmetic users. The results indicated that cosmetic users with negative perceptions of how eye cosmetics may affect ocular health and wellbeing experienced the greatest amount of ocular discomfort when using eye cosmetics.

Anecdotal reports from eye care practitioners often note the observation of eye cosmetic products contaminating the tear film and contact lenses of patients. Chapter 3 described an experiment observing and quantifying the migration of pencil eyeliner applied a) to the peri-ocular skin and b) behind the lash line as proof of concept. While the migration of eyeliner following application to the peri-ocular skin did occur, the migration of eyeliner was comparatively slower and the contamination was less compared to when the product was applied behind the lash line. This finding led to a final clinical study which was considered in two separate parts (Chapter 4 and Chapter 5).

The final study in this thesis involved a randomised crossover trial of 24 subjects. Each subject used cosmetic pencil eyeliner for duration of one week in each eyeliner application method: a) applied to the peri-ocular skin (ELO) and b) behind the lash line (ELI). Each subject was assessed for clinical signs of inflammation and ocular surface stress (redness, ocular surface staining), ocular comfort and tear film parameters (quality and stability). Subjects were assessed following one day and seven consecutive days of eyeliner usage to

examine short- and long-term changes, respectively. It was hypothesised that the usage of eyeliner, particularly in ELI application, would induce mild clinical signs of inflammation which would be most evident after long-term usage. These signs were not expected to be marked as the cosmetic eyeliner pencil had to conform to standards set by the European Commission for cosmetic product safety. The outcomes from the clinical observations formed the basis of Chapter 4. The results showed that clinical signs of inflammation and ocular surface stress were not detectable after one or seven consecutive days of eyeliner usage in either application method. However, repeated usage of ELI increased tear lipid layer thickness, although no clinically significant changes in tear stability were observed. Repeated usage of ELI also resulted in decreased ocular comfort and increased dry eye symptoms, measured with the OSDI questionnaire. Although no clinically significant changes were detected, it was hypothesised the reports of decreased ocular comfort arose from subclinical measures of inflammation which would be apparent in the expression of cytokines present in tear fluid.

Chapter 5 describes the biochemical investigations of tear fluid following eyeliner usage and subsequent migration to the ocular surface. In order to effectively analyse the tear fluid, optimisation of enzyme-linked immunosorbent assay (ELISA) protocols were undertaken. The optimisation was conducted to determine a) which inflammatory cytokines were detectable in tear fluid b) the dilutions of tear fluid required to obtain detectable results yet maximise the number of observations. From the results of the optimisation study, IL-6 and IL-8 were decided to be the cytokines of choice for further investigation. During the final study (in which the clinical observations were described in Chapter 5), a sample of tear fluid was collected from each subject at each visit to assess the concentrations of inflammatory cytokine concentrations. IL-8 concentrations were assessable in 15 out of 24 subjects. IL-8 concentration reduced after short-term use of eyeliner pencil in ELO and ELI application, which may have been due to reflex tearing during the adaption of using a new cosmetic product. This may be an artefact within the data due to one participant having exceedingly high IL-8 concentration at baseline. Thus pencil eyeliner, used in conjunction with eye cosmetic remover, did not appear to induce a cascade of inflammatory events in healthy subjects. Due to inadequate tear fluid collection from some subjects and sublimation of tear fluid samples which can occur during long-term fluid storage at -80°C, the detection of IL-6

was less successful and no firm conclusions can be drawn from the available data. It was hypothesised that the decrease in ocular comfort was as a result of increased inflammatory markers present in tear fluid. Since the concentration of IL-6 and IL-8 were not elevated following pencil eyeliner usage, it is anticipated that the reduction in ocular comfort is caused by other mechanisms.

In summary, this thesis found:

- Eye cosmetic usage negatively impacts ocular comfort, affecting CLW and NCLW. The application of certain types of eye cosmetics close to the ocular surface, such as eyeliner was found to cause a significant reduction in comfort.

- When applied externally around the eyes, pencil eyeliner readily contaminated the tear film. Migration was maximal when eyeliner is applied behind the lash line.

- When eyeliner was applied behind the lash line or to peri-ocular skin, the clinical signs of inflammation and ocular surface stress were minimal, even after seven days of consecutive application. Lipid layer thickness increases after repeated application of eyeliner along the MCJ however improvements in tear stability were not evident.

- Seven consecutive days of pencil eyeliner application, in conjunction with eye cosmetic remover, did not induce inflammation of the ocular surface, at subclinical levels.

6.2   General discussion

This thesis has shown contamination of the tear film occurs most readily when eyeliner is applied behind the lash line. This was determined by the observing highly pigmented glitter particles present in cosmetic pencil eyeliner. Migration of eye cosmetics applied to peri-ocular skin was also shown to occur, however at a slower rate. This study would have benefited from tear fluid analysis over the period of anterior eye observation. Analysis of tear fluid using high performance liquid chromatography (HPLC) would allow the detection of cosmetic product chemicals contaminating the tear fluid. HPLC could also be performed on tear fluid when no pigmented glitter particles are no longer visible as it is possible that some of the chemical products may still reside within the tears, on the ocular surface and in

the cul-de-sac after all particles have been cleared. Although the size of pigmented particles may cause a change in ocular surface comfort measures, residual chemicals arising from cosmetic products may also induce this change. For example, castor oil eye drops have been detected in tear fluid using HPLC up to four hours post-instillation (Maissa *et al.*, 2010). Furthermore, the observation of eyeliner pencil in Chapter 4 was conducted on Caucasian subjects. Patients of South East Asian descent often have different ocular anatomical features, including epicanthal folds, shortened supratarsal creases and lash ptosis (Malik *et al.*, 2007; Lee *et al.*, 2013). These features may have effect upon the speed of cosmetic product migration.

The usage of eye cosmetics applied close to the ocular surface has a minimal impact upon clinical observations and reported ocular comfort in a small sample of young, healthy volunteers. In this thesis, the maximal duration of seven consecutive days of eyeliner and eye cosmetic remover usage may not be long enough to observe any clinical (or immunological) changes. Increasing the duration of eyeliner usage in future work would be beneficial, particularly as 80% of respondents to the online survey reported using eye cosmetics at least 3-5 times a week. Additionally, it is anticipated that clinical observations may be greater in a cohort of older patients with dry eye signs and symptoms therefore extending the age range of patients would be beneficial in future studies. It is interesting to note that the effect of an increase in tear lipid layer thickness was observed only after repeated use of ELI, although Bonferroni corrections for multiple comparisons were not made during the statistical analysis of this study. This may have occurred due to increased manipulations of the eyelid margins with ELI application. Firstly, the daily application of ELI may have resulted in an element of eyelid margin debridement, a form a meibomian gland dysfunction therapy which has been shown to have therapeutic benefits in the management of dry eye disease (Blackie and Korb, 2013). Secondly, the repeated regular removal of ELI with make-up remover may have caused inadvertent "eyelid massaging", another form of treatment in the management of meibomian gland dysfunction (Geerling *et al.*, 2011). Therefore, the negative connotations associated with eye cosmetics usage found in the online survey could be reversed if eyeliner usage could be used as a form of dry eye therapy. What has been previously been described in the literature is the reduction of meibomian gland obstructions following regular use of castor oil eye drops (Goto *et al.*, 2002b). With

the high lipid content in eyeliner pencils, meibum lipids capping the meibomian orifices may be broken down with lipid-based eyeliner pencils, in a similar way lipids are broken down by sebum in skin (Lautenschläger, 2004).

The results from the biochemical investigations suggested that seven days of eyeliner and eye cosmetic remover usage does not induce an inflammatory response. This confounded the hypothesis that stated that an increase in inflammatory cytokines would occur, as the ocular surface would recognise the chemicals present in eyeliner formulations to be foreign and subsequently causing a reduction in ocular comfort. The small sample size for the immunological investigation makes a firm conclusion regarding this observed trend difficult – a larger sample size would make for a more robust conclusion, particularly for IL-6 investigations. If indeed no inflammatory cascade is induced with repeated eyeliner use, this further emphasises the nature of the safety of this cosmetic eyeliner pencil for sale in the UK and may further support the potential therapeutic benefits, as already discussed.

This thesis focussed primarily on cosmetic pencil eyeliner due to the nature of application. The results of the online survey indicated that not one respondent used only one type of eye cosmetic product. In some instances, the usage of several eye cosmetic products may cause an alteration in ocular comfort, rather one sole product.

6.3   Future work

Anticipated future works following this thesis include:

- Examining the presence of pencil eyeliner in tear fluid using HPLC Conducting HPLC analysis on tear fluid samples would not only reinforce the evidence of eyeliner migration from the eyelid margins, but could also aid with discerning the residency time of the waxes and lipids present in the cosmetic pencil. Correlations between the presence of lipid types in tear fluid with tear lipid layer thickness observations could be conducted to examine the source of lipid layer thickness. As previously discussed, the origin of a thicker lipid layer may arise from supplementation from the eyeliner pencil, or may arise from "debridement" of the lid margin and meibomian gland orifices.

- Observing the migration of pencil eyeliner in healthy Caucasian subjects from a wider age group would be of particular benefit as it is well recognised that pretarsal skin length and eyelid crease height increases with age (Cartwright *et al.*, 1994; van den Bosch, Leenders and Mulder, 1999; Erbagci *et al.*, 2005; Price *et al.*, 2009). For example, the influence of dermatochalasis may have an impact on how readily cosmetic products migrate into the tear film.

- Observing the migration of pencil eyeliner in subjects from different ethnicities including those of African and South East Asian descent would be of benefit due to the established anatomical differences in eyelid structure (Malik *et al.*, 2007; Price *et al.*, 2009; Lee *et al.*, 2013).

- Extending the duration of eyeliner usage up to three months to examine for clinical and immunological changes in a larger scale, to include a wider age range of patients, including those with meibomian gland dysfunction and dry eye disease. For completeness, assessment and visualisation of the meibomian glands prior and after the duration of eyeliner usage with meibography would allow visualisation of the meibomian glands and any influence the intervention may have upon their structure and function.

- By exploiting the fact that products applied around the eyelid margins migrate, pharmacological drugs could be incorporated into a product formulated in a manner similar to an eyeliner pencil, which is applied behind the lash line. This method of drug delivery would be particularly beneficial for those with limited manual dexterity. Additional research into the most appropriate excipients to maintain drug stability and mobility would be required. Furthermore, careful monitoring of the minimum and maximum contact time of the product with the ocular surface would be required as this may differ to conventional cosmetic products.

- Investigating the impact of other commonly used eye cosmetic products such as mascara and alternative formulations of eyeliner (such as liquid eyeliner) on clinical and subclinical changes of the ocular surface and tear film.

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Appendix I Eye cosmetic formulations

All formulations from COPLIA Cosmetic Frame Formulations 2000 (Guidelines realised in collaboration with The European Association of Poison Centres and Clinical Toxicologists) which provide information regarding the type of ingredients and their maximum concentration for cosmetic products available in the European market.

Table 15 Typical formulation of cake mascara. Frame formulation number 10.16 – 2000

Product	Ingredient	Example of ingredient	Maximum levels (%w/w)	Function(s) and additional properties
Mascara (cake)	Emulsifying agents, surfactants	TEA-stearate	50	Emulsifier, surfactant
	Waxes	Ozokerite Cera alba (beeswax) Carnauba wax	50	Binding, emulsion stabilising, opacifying agent, viscosity controlling Emollient, emulsifying, film-forming, viscosity controlling Absorbent, binding, film-forming, viscosity controlling
	Viscosity controlling agents	Polyethylene derivatives Stearalkonium hectorite	20	Viscosity controlling
	Aqua		20	Solvent
	Cosmetic colorants	Pearlescent agents	25	Colouring agents
	Additional ingredients	Vitamins Plant extracts	5	
	Preservatives, antimicrobials		1	

Table 16 Typical formulation of liquid mascara. Frame formulation number: 10.16 – 2000

Product	Ingredient	Example of ingredient	Maximum levels (%w/w)	Function(s) and additional properties
Liquid mascara (regular)	Oils, fat, waxes	Carnauba wax Cera alba (beeswax) Petrolatum/petroleum distillate	25	Absorbent, binding, film-forming, viscosity controlling Emollient, emulsifying, film-forming, viscosity controlling Emollient / Antifoaming, solvent
	Cosmetic colorants (including pearlescent agents)		25	Colouring agents
	Emulsifying agents	TEA-stearate	20	Emulsifier, surfactant, helps maintain pigment in solution
	Silicones, volatile silicones	Cyclomethicone	20	Emollient, humectant, solvent, viscosity controlling agent
	Humectants	Propylene glycol Glycerin	15	Humectants
	Viscosity controlling agents	Magnesium silicate Aluminium silicate Polyethylene glycol (PEG) derivatives	15	Viscosity controlling
	Film forming agents	Ammonium acrylates copolymer Cellulose derivatives Acetyl trihexyl citrate	15	Film forming agents

	Ethanol	Alcohol Alcohol denat.	11	Antifoaming, antimicrobial, astringent, masking, solvent, viscosity controlling
	Fibres	Cellulose Nylon fibres	10	Lengthens the appearance of lashes
	Isoparaffins		5	Solvent, viscosity controlling
	Emollients	Isopropyl myristate		Binding, emollient, perfuming, skin conditioning, solvent
	Additional ingredients	Vitamins Plant extracts	5	
	Preservatives Antimicrobials	"Parabens" – methyl, ethyl, propyl, butyl, isopropyl isobutylparaben	2.5	Preservative
	Parfum		02	Deodorant, masking, perfuming
	Aqua		To 100	Solvent

Table 17 Typical formulation of waterproof liquid mascara. Frame formulation number: 10.16 – 2000

Product	Ingredient	Example of ingredient	Maximum levels (%w/w)	Function(s) and additional properties
Liquid mascara (waterproof)	Volatile mineral spirits	Branched-chain isoparaffins (C11-C14)	80	Solvent, viscosity controlling
	Silicones, volatile silicones	Cyclomethicone	80	Emollient, humectant, solvent, viscosity controlling agent
	Waxes, oils and fats	Ozokerite\n\nCarnauba	30	Binding, emulsion stabilising, opacifying agent, viscosity controlling\n\nAbsorbent, binding, film-forming, viscosity controlling
	Cosmetic colorants (including pearlescent agents)		25	Colouring agents
	Emulsifying agents	Cera alba (beeswax)	20	Emollient, emulsifying, film-forming, viscosity controlling
	Resins, film forming agents	Aluminium distearate\n\nPVP/ Hexadecane copolymer	15	PVP reduces smudging
	Fillers	Talc\nKaolin\nZinc stearate	15	Improves lash thickening effect
	Viscosity controlling agents	Stearalkonium hectorite	12	Viscosity controlling, gel-forming
	Ethanol	Alcohol\n\nAlcohol denat	10	Antifoaming, antimicrobial, astringent, masking, solvent, viscosity controlling

	Aqua		10	Solvent
	Humectants	Propylene glycol	5	Humectant
	Additional ingredients	Vitamins Plant extracts Silk powder	5	
	Parfum		1	Deodorant, masking, perfuming
	Sodium borate		1	Antimicrobial pH regulator Prevents separation of emulsion
	Antioxidants		1	
	Preservatives Antimicrobials		1	

Table 18 Typical formulation of pencil eyeliner. Frame formulation number 10.14 -2000

Product	Ingredient	Example of ingredient	Maximum levels (%w/w)	Function(s) and additional properties
Eye pencil	Waxes, oils and fats	Ozokerite Carnauba Hydrogenated vegetable oil	70	Binding, emulsion stabilising, opacifying agent, viscosity controlling Absorbent, binding, film-forming, viscosity controlling Emollient, skin-conditioning
	Cosmetic colorants, colour additives	Pearlescent agents	50	Colouring agents
	Silicones, volatiles silicones		50	Emollient, humectant, solvent, viscosity controlling agent
	Fillers	Talc	30	Bulking
	Polymers, resins	Nylon	20	Bulking, viscosity controlling agent
	Surfactants	Non-ionics – polysorbate 60, PEG-6 sortbitan stearate	5	Surfactants
	UV filters		5	
	Additional ingredients	Vitamins Plant extracts	5	
	Preservatives, antimicrobials, antioxidants		1	
	Parfum		0.3	Deodorant, masking, perfuming

Table 19 Typical formulation of liquid and cream eyeliners. Frame formulation number 10.15 -2000

Product	Ingredient	Example of ingredient	Maximum levels (%w/w)	Function(s) and additional properties
Eyeliner (liquid and cream)	Cosmetic colorants, colour additives		25	Colouring agents
	Film forming agents	Acrylic resin Ammonium acrylates copolymer	25	Film forming agents
	Waxes, emollients	Silicone Fatty alcohols	25	Emollients, binding, viscosity controlling
	Humectants	Propylene glycol Glycerin	20	Humectant
	Emulsifying agents	Polysorbates PEG oleate TEA-oleate	10	Emulsifying agents
	Viscosity controlling agents	Carbomer Magnesium/aluminium silicate Cellulose	10	Viscosity controlling agents
	Ethanol and/or isopropanol	Alcohol Alcohol denat. Isopropyl alcohol	10	Antifoaming, antimicrobial, astringent, masking, solvent, viscosity controlling
	Oils	Mineral oil	5	Emollient, skin protecting, solvent
	Additional ingredients	Vitamins Plant extracts	5	
	Preservatives antimicrobials		1	

	Parfum		1	Deodorant, masking, perfuming
	Aqua		To 100	Solvent

Table 20 Typical formulation of cake eyeliner. Frame formulation number 10.15 -2000

Product	Ingredient	Example of ingredient	Maximum levels (%w/w)	Function(s) and additional properties
Eyeliner (cake)	Fillers	Talc	70	Bulking
	Cosmetic colorants, colour additives		50	Colouring agents
	Emulsifying agents, binding agents	Fatty esters	10	Emulsifying and binding
	Additional ingredients	Vitamins Plant extracts	5	
	Parfum		1	Deodorant, masking, perfuming
	Preservatives, antimicrobials		1	

Table 21 Typical formulation of powder eye shadow. Frame formulation number 10.12 – 2000

Product	Ingredient	Example of ingredient	Maximum levels (%w/w)	Function(s) and additional properties
Eye shadow (Powder)	Cosmetic colorants Colour additives	Pearlescent agents	80	Colouring agents
	Oils, binding agents	Cera alba Lanolin Silicone	20	Emollient, emulsifying, film-forming, viscosity controlling Emollient, emulsifying, skin conditioning, surfactant
	Binders	Zinc or magnesium stearates Zinc or magnesium laurates	10	Prevents flaking or dusting
	Additional ingredients	Humectants Vitamins UV filters	5	
	Parfum		1	Deodorant, masking, perfuming
	Preservatives, antimicrobials		1	
	Fillers	Talc Silica Starch Nylon powder Polyethylene powder	To 100	Bulking Absorbent, anticaking, bulking, opacifying Absorbent, bulking, opacifying

Table 22 Typical formulation of stick eye shadow. Frame formulation number 10.12 – 2000 taken COLIPA Cosmetic Frame Formulations 2000

Product	Ingredient	Example of ingredient	Maximum levels (%w/w)	Function(s) and additional properties
Eye shadow (Stick)	Waxes Oils Fats Emollients	Cera alba Paraffinum liquidum Lanolin Oleyl alcohol	70	Emollient, emulsifying, film-forming, viscosity controlling Emollient, skin-protecting, solvent Emollient, emulsifying, skin conditioning, surfactant Emollient, emulsifying, opacifying, viscosity controlling
	Cosmetic colorants, pigments, colour additives		50	Colouring agents
	Additional ingredients	Vitamins Plant extracts	5	
	Parfum		1	Deodorant, masking, perfuming
	Preservatives, antimicrobials, antioxidants		1	

Table 23 Typical formulation of cream eye shadow. Frame formulation number: 10.13 – 2000 taken COLIPA Cosmetic Frame Formulations 2000

Product	Ingredient	Example of ingredient	Maximum levels (%w/w)	Function(s) and additional properties
Eye shadow (Cream)	Waxes, oils	Petrolatum Candelilla cera	70	Emollient Emollient, film forming
	Cosmetic colorants, colour additives	Pearlescent agents	50	Colouring agents
	Fillers	Talc	20	Bulking
	Emulsifying agents	PEG stearate Polysorbates	10	Emulsifying agents
	Silicones	Dimethicone	10	Antifoaming, emollient, skin conditioning, skin protecting
	Viscosity controlling agents	Carbomer	5	Emulsion stabilising, gel forming, viscosity controlling
	Additional ingredients	Vitamins Plant extracts	5	
	UV filters		2	
	Parfum		1	Deodorant, masking, perfuming
	Preservatives, antimicrobials		1	
	Antioxidants		1	
	Aqua		To 100	Solvent

Table 24 Typical formulation of anhydrous eye shadow. Frame formulation number 10.13 -2000 taken

COLIPA Cosmetic Frame Formulations 2000

Product	Ingredient	Example of ingredient	Maximum levels (%w/w)	Functions(s) and additional properties
Eye shadow (anhydrous – cream or stick)	Oils Volatile mineral spirits	Paraffinum liquidum Branched chain isoparaffin (C11-C14)	70	Emollient, skin-protecting, solvent Solvent, viscosity controlling
	Silicones and volatile silicones	Cyclomethicone	70	Emollient, humectant, solvent, viscosity controlling agent
	Cosmetic colorants, colour additives	Pearlescent agents	35	Colouring agents
	Fillers	Talc	35	Bulking
	Waxes, oils and fats	Cera alba Lanolin	25	Emollient, emulsifying, film-forming, viscosity controlling Emollient, emulsifying, skin conditioning, surfactant
	Viscosity controlling agents	Aluminium stearate	15	Anticaking, cosmetic colorant, emulsion stabilising, viscosity controlling
	Additional ingredients	Vitamins Plant extracts	5	
	Antioxidants		1	
	UV filters		1	
	Preservatives, antimicrobials		0.1	

Appendix II Common cosmetic ingredients known to cause allergic dermatitis

From: Special considerations in eye cosmetics. *Clin Dermatol* 19: 424-430. (Draelos, 2001)

Agent	Ingredient
Preservatives	*Parabens*
	Phenyl mercuric acetate
	Imidazolidinyl urea
	Quaternium-15
	Potassium sorbate
Antioxidants	*Butylated hydroxyanisole*
	Butylated hydroxytoluene
	Di-tert-butyl-hydroquinone
Resins	*Colophony*
Pearlescent additives	*Bismuth oxychloride*
Emollients	*Lanolin*
	Propylene glycol
Fragrances	
Pigment contaminants	*Nickel*

Appendix III Statistical analysis of OSDI scores with relation to frequency of eye cosmetic usage amongst contact lens wearers and non-contact lens wearers
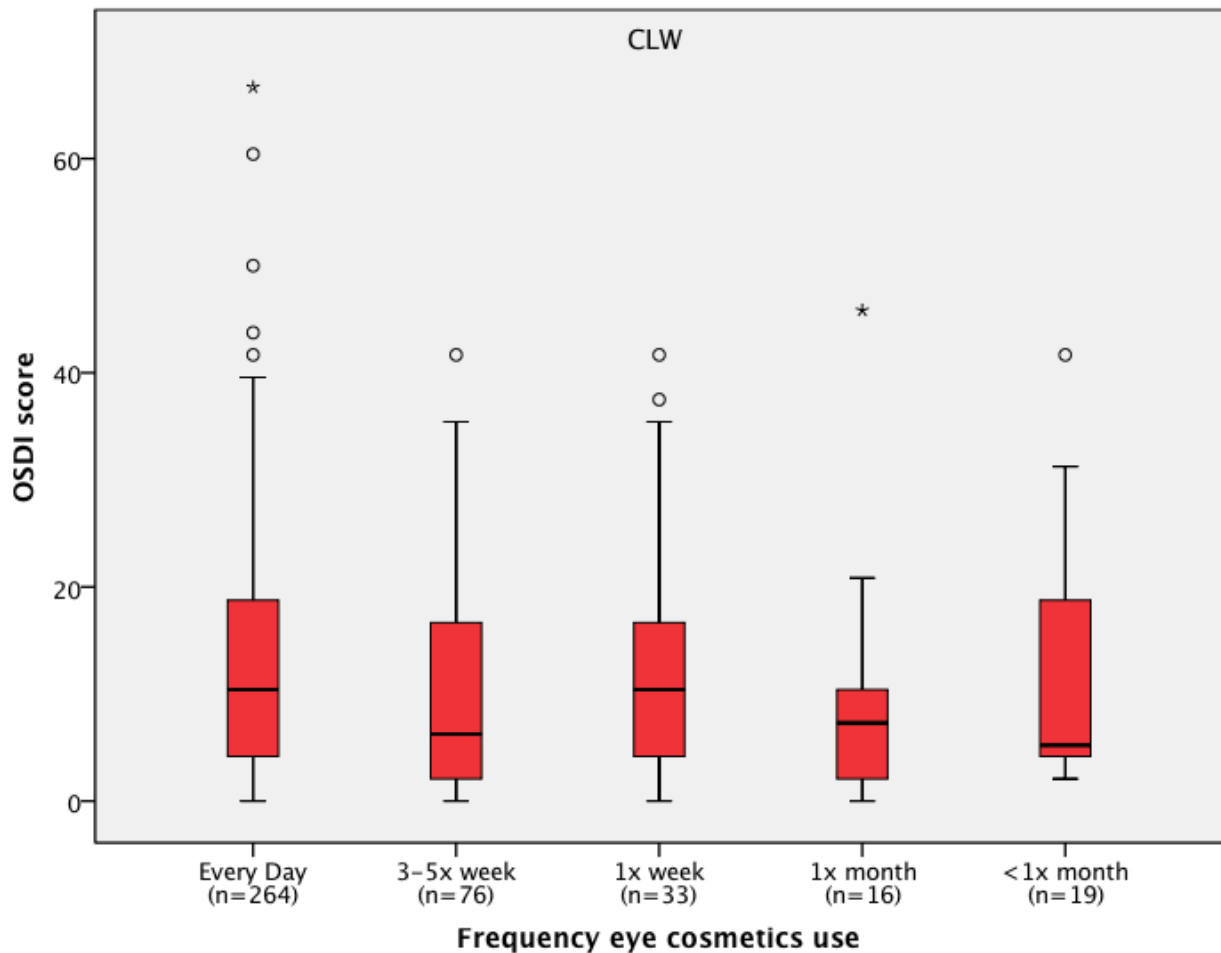
Boxplot showing OSDI scores with frequency of eye cosmetic usage amongst CLW. There was no significant difference between median OSDI scores with frequency of eye cosmetic usage (Kruskal-Wallis test, p=0.229)
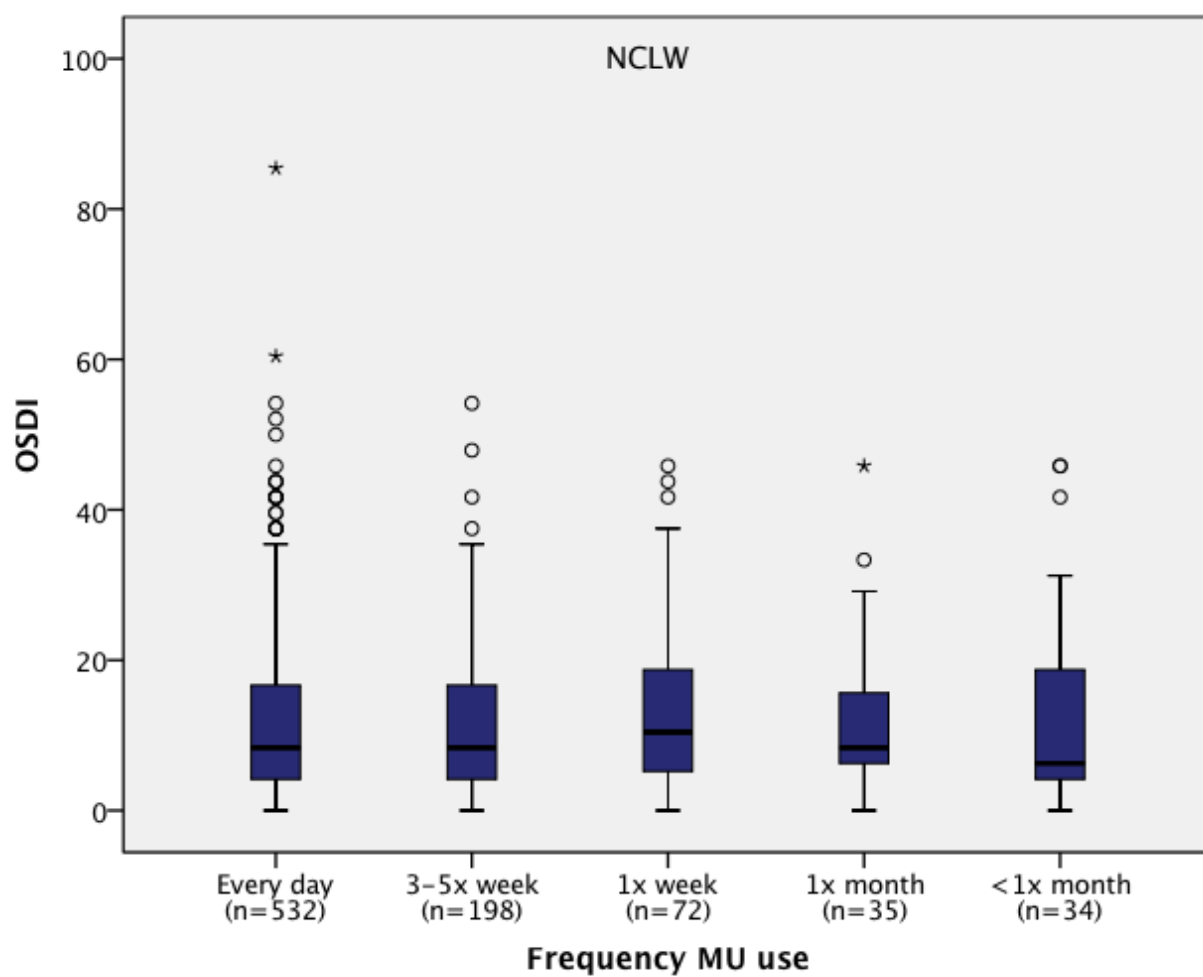
Boxplot showing OSDI scores with frequency of eye cosmetic usage amongst NCLW. There was no significant difference between median OSDI scores with frequency of eye cosmetic usage (Kruskal-Wallis test, p=0.625)

Appendix IV List of ingredients in Avon Glimmerstick Liqui-glide (Graphite)

Ingredients:

Aqua, butylene glycol, sodium stearate, glycerin, propylene glycol, VP/VA copolymer, phenoxyethanol, isododecane, nylon-12, aloe barbadensis leaf juice, PPG-5-CETETH-20, allantoin, hydroxyethylcellulose, ethylhexyglycerin, tocopheryl acetate, distearimonium hectorite, propylene carbonate, cucumis sativus extract [+/- CI 77491, CI 77492, CI 77499, CI 77891, Mica]

Appendix V Period interaction and carry-over effects using paired t-tests for Chapter 4 Clinical Outcomes

Table showing p-values from paired t-tests examining period interaction and carry-over effects; since all p-values>0.05, no effects were detected.

	Period effect	Carry-over effect
Bulbar redness temporal	0.38	0.21
Bulbar redness nasal	0.90	0.16
NITBUT	0.34	1.00
LLT	0.93	0.57
Conjunctival staining temporal	0.33	0.33
Conjuctival staining nasal	0.65	0.40

Appendix VI Volunteer comments from each visit of the clinical study (relevant to Chapter 5 & 6)

Subject	1 day ELO	7 day ELO	1 day ELI	7 day ELI
2	-	-	Noticed ↑ lacrimation after application and product had faded by 4 hours post-application	Noticed product faded very quickly
23	Found it more difficult to apply compared with ELI	-	Easy to apply, good staying power, lasted for at least 5 hours	Happy with eyeliner application but noticed Simple Eye Make-up remover stings and makes eyes feel dry.
5	Smudged easily after using facial moisturiser	-	Aware some eyeliner has worn off over the day (after 8 hours wear)	-
13	Difficult to apply	Moves off eyes very quickly	Noticed faded a lot by 4 hours	Aware eyeliner moving "outwards" (i.e. spilling over onto peri-ocular skin over course of the day
20	-	Aware very little eyeliner left by the end of the day – more eyeliner stayed on with ELI	Lots of eyeliner collected in the inner canthus	Noticed ↑ bulbar redness from 5 days use onwards, build-up of eyeliner in inner canthus.

Subject	1 day ELO	7 day ELO	1 day ELI	7 day ELI
22	More comfortable applying ELO than ELI	Eyeliner moves off around eyes readily	Stung for a few seconds after application	-
18	-	-	Noticed majority of eyeliner had disappeared 1.5 hours after application	Noticed little eyeliner remaining by end of the day
14	-	-	-	Eyes feel more sore than normal
17	Difficult to apply	-	-	-
7	-	Aware eyeliner moved spread out around skin by end of the day	Noticed eyeliner faded as day went on	Noticed eyeliner faded by end of the day
8	-	Eyes felt more dry on 1 day	Noticed eyeliner faded quickly, topped up application 3 hours after first application	Eyes felt more watery over last 7 days of ELI application
21	Stayed on well all day	-	Smudges away very quickly	-
15	Feels some eyeliner has smudged off	-	Noticed eyeliner fades away very quickly – reapplied 2 hours after first application	Noticed eyeliner fading away very quickly – moves outwards onto the skin

Subject	1 day ELO	7 day ELO	1 day ELI	7 day ELI
11	-	-	Faded away after 1.5 hours of initial application – reapplied	Feels eyeliner "runs" out of eyes quite a lot – frequent wiping/patting of eyes to remove excess
1	Aware some eyeliner has smudged off	-	Fades away after 3 hours	-
19	-	-	Felt majority of eyeliner had faded 3 hours after application	Very aware rapid fading of eyeliner by 3 hours of wear
4	Some fading after 1.5 hours of initial application	-	-	-
12	-	-	Some reflex tearing after initial application, eyes felt ok after reflex tearing	Eyes felt more tired & heavy over course of the 7 days
6	-	Feels eyeliner fades off quickly by early afternoon	-	Very aware of eyes, ↑ blinking by end of day and ↑ dryness sensation
16	-	-	-	-

Subject	1 day ELO	7 day ELO	1 day ELI	7 day ELI
24	-	Happy with eyeliner but eyes felt a bit itchy after using the eye make-up remover	Feels product stays on base of lashes	-
9	Some fading of eyeliner after 6 hours of use	-	RE felt a bit itchy after a few hours of wear but no soreness	Aware eyeliner fades away quite quickly
10	-	Eyes feel less irritated compared to ELI	Slightly gritty sensation after application	Eyes feel gritty after application of eyeliner and notices ↑ lacrimation. Eyeliner fades quickly
3	-	Occasionally itchy eyes over the past 7 days	-	Feels eye look a little more red than normal and eyes occasionally itchy

Appendix VII Summary of studies using ELISA to measure inflammatory mediators in tear fluid samples

(CRT=corneal refractive therapy; SiH=Silicone Hydrogel contact lenses; RGP=Rigid Gas Permeable contact lenses; MGD=Meibomian Gland Dysfunction)

Study	Inflammatory mediators examined + min detectable unit	Concentration of inflammatory mediator	Kit used	Tear fluid sample volume collected	Other
Gonzalez-Perez *et al.* (2012)	IL-6 IL-8 EGF MMP-9 (No minimum detectable unit given)	IL-6 – 2pg/ml (control), 3pg/ml (SiH), 5pg/ml (CRT) IL-8 – 602pg/ml (control), 660pg/ml (SiH), 953pg/ml (CRT) EGF – 698pg/ml (control), 1537pg/ml (SiH), 2349pg/ml (CRT) MMP-9 – 39ng/ml (control), 45ng/ml (SiH), 74ng/ml (CRT)	Commercially available quantitative sandwich ELISA (Quantikine; R&D systems Europe, Abingdon)	At least 15µl per subject	Centrifuged at 13,000 5 mins Samples diluted to 1:20, 1:80, 1:100 for cytokines, EGF and MMP-9 respectively with sample calibrator diluents – final results were corrected according to dilution factor
Poyraz *et al.* (2012)	IL-6 – 0.9pg/ml Il-8 – 2.0pg/ml	IL-6 – 7-11pg/ml (baseline) IL-8 – 107- 176pg/ml (baseline)	ELISA kits BenderMed BMS213 and BenderMed BMS204, Vienna, Austria	Not specified	Centrifuged at 4,000 rpm 10 min Diluted to a volume of 200 µL in 1:4 and 1:20 ratios with sample diluent

Study	Inflammatory mediators examined + min detectable unit	Concentration of inflammatory mediator	Kit used	Tear fluid sample volume collected	Other
Lema *et al.* (2009)	IL-6 TNF-α MMP-9 (No minimum detectable unit given)	IL-6 - 2.2pg/ml TNF-α - 1.8pg/ml MMP-9 - 6.1ng/ml	Quantitative sandwich ELISA kits (Quantikine, R&D systems, Minneapolis, MN) MMP-9 measured with ELISA from Biotrack, Amersham Pharmacia Biotech, Buckinghamshire, UK	10µl of tear sample in micropipettes	Samples were diluted 1:20 with sample solvent to a final volume of 300µl. Final results were multiplied by the dilution factor (×20).
Acera *et al.* (2008)	IL-1β IL-6 MMP-9 (No min detectable unit given)	IL-1 – 50pg/ml (controls) IL-6 – 8pg/ml (controls) MMP-9 – 24ng/ml (controls)	Double-sandwich ELISAs for human IL-1β (17 kDa), IL-6 (28 kDa) and MMP-9 (92 kDa) with commercial kits (Calbiochem, Darmstadt, Germany)	Not specified – used absorbent PVA sponges but 10µl tear sample per patient used in assays	
Yoon *et al.* (2007)	Il-6 – 0.7pg/ml TNF-α – 0.5pg/ml	Il-6 – 4pg/ml (controls), 19pg/ml (dry eye), 25pg/ml (Sjőgrens) TNF-α – <0.5pg/ml (controls), 4pg/ml (dry eye + Sjőgrens)	Commercial ELISA kits – Quantikine; R&D Systems, Minneapolis, MN	30µl	Samples diluted to 100µl (IL-6) and 200µl (TNF-α) with PBS

Study	Inflammatory mediators examined + min detectable unit	Concentration of inflammatory mediator	Kit used	Tear fluid sample volume collected	Other
Fodor *et al.* (2006)	IL-6 <0.1pg/ml (0.16-10pg/ml) IL-8 <0.1pg/ml (0.39-25pg/ml)	IL-6 – 110pg/ml (controls) IL-8 – 572pg/ml (controls)	Commercially available human ultrasensitive, solid phase ELISA kits (Bio Source International Inc., Nivells, Belgium).	No information given – tear collection for 2 minutes	Samples diluted with 100μl tris-buffered saline Samples not centrifuged
Kallinikos *et al.* (2006)	EGF – 0.7pg/ml HGF – 40pg/ml IL-8 – 10pg/ml	EGF – 579pg/ml (control), 1401pg/ml (RGP), 1383pg/ml(SiH) HGF – not detected IL-8 – 414pg/ml (control), 593pg/ml (RGP), 476pg/ml (SiH)	Commercially available ELISA – R&D Systems, Europe Ltd	No information – but samples from both eyes per subject pooled	Centrifuged at 13,000rpm 2 mins Small sample volumes so each sample diluted 1:25
Leonardi *et al.* (2006)	IL-4 – 5-40pg/ml IL-13 – 25-1000pg/ml IFN-γ – 25-1000pg/ml (Minimum and maximum detectable unit)	IL-4 – not detected in normals IL-13 – not detected in normals IFN-γ - 29±35pg/ml	Measured in triplicate with sandwich ELISAs (Biotrak; Amersham, UK)	Collection with capillary micropipette – no volume given but 2μl were removed for cytology	Centrifuged at 1500rpm for 10 mins. Samples diluted to give duplicate readings within the standard curve – final cytokine concentrations calculated as means of duplicate assays and adjusted for dilution factor

Study	Inflammatory mediators examined + min detectable unit	Concentration of inflammatory mediator	Kit used	Tear fluid sample volume collected	Other
Lema and Duran, (2005)	IL-4 IL-6 IL-10 TNF-α MMP-9 (No min detectable unit given)	IL-4 – 6.1pg/ml IL-6 – 2.2pg/ml IL-10 – 1.7pg/ml TNF-α – 1.8pg/ml MMP-9 – 6.1ng/ml (66.5pg/ml in keratoconus px) (Normal results shown only)	Quantitative sandwich ELISA kits (Quantikine, R&D systems, Minneapolis, MN)	15µl using micropipettes	Samples diluted 1:20 with sample diluents (reagent provided by ELISA kit) to final volume of 300µl
Solomon et al. (2001)	IL-1α Mature IL-1β Pre-cursor IL-1β IL-1Ra (No min detectable unit given)	IL-1α – 43pg/ml (normals), 254pg/ml (MGD), 443pg/ml (Sjőgrens) Mature IL-1β – 30pg/ml (normals), 188pg/ml (MGD), 81pg/ml (Sjőgrens) Pre-cursor IL-1β – 380pg/ml (normals), 1pg/ml (MGD), 0.4pg/ml (Sjőgrens) IL-1Ra – 295ng/ml (normals), 940ng/ml (MGD), 2396ng/ml (Sjőgrens)	R&D systems, Minneapolis, MN	Uncertain – collection using polyester rods	Centrifuged rods at 12,000rpm for 5 mins to extract tears. For reflex study in controls, tears were pooled. For dry eye study, tears not pooled. All samples were diluted with supplied ELISA buffer to 100-200µl

Study	Inflammatory mediators examined + min detectable unit	Concentration of inflammatory mediator	Kit used	Tear fluid sample volume collected	Other
Thakur and Willcox, (2000)	IL-1β – 50pg/ml IL-6 – 17.5pg/ml IL-8 – 120pg/ml	IL-1β – not detected IL-6 – 94pg/ml IL-8 – 24ng/ml	R&D Systems, Minneapolis, MN	At least 20µl	Samples diluted 1:25. For each assay, 4µl diluted to 100µl (see paper for more detailed methodology)
Uchio et al. (2000)	IFNγ – 4pg/mL, IL-2 – 5pg/mL, IL-4 – 65fg/mL IL-5 – 4pg/mL	IFNγ – 92pg/ml (normals) IL-2 – 37pg/ml (normals) IL-4 – 60fg/ml (normals) IL-5 – 40pg/ml (normals)	Two-epitope 'sandwich' ELISA kit (CytoscreenTM human IFNγ, IL-2, IL-4 and IL-5 ELISA kits; BioSource International, Camarillo, CA, USA)	40µl	The tears were diluted 10-fold
Thakur and Willcox, (1998)	IL-1β – 50pg/ml IL-6 – 17.5pg/ml IL-8 – 120pg/ml	IL-1β – no absolute value given IL-6 – 20pg/ml IL-8 – no absolute value given	Antibody sandwich ELISA R&D Systems, Minneapolis, MN	No information given	Samples diluted 1:25 PBS containing 1% BSA (see paper for more detailed methodology)
Tishler et al. (1998)	IL-6 – 0.095pg/ml	IL-6 – 42pg/ml (normals), 89pg/ml (Sjőgrens)	Quantitative ELISA kit (Quantikine High Sensitivity, R&D Systems Inc., Minneapolis, MN)	5µl	No indication of any dilution

Study	Inflammatory mediators examined + min detectable unit	Concentration of inflammatory mediator	Kit used	Tear fluid sample volume collected	Other
Nakamura *et al.* (1998)	IL-1α IL-1β IL-6 IL-8 (No min detectable unit given)	IL-1α – 11pg/ml (basal), 9pg/ml (reflex) IL-1β – 13pg/ml (basal), ND (reflex) IL-6 – 226pg/ml (basal), 12pg/ml (reflex) IL-8 – 731pg/ml (basal), 277pg/ml (reflex)	R&D systems, Minneapolis, MN	Basal mean = 9µl (range 2-20µl) Reflex mean = 115µl (range 80-200µl)	200µl required for IL-1α and IL-1β assay 100µl required IL-6 and IL-8 assay Basal tears pooled from 11-25 eyes Reflex tears pooled from 1-2 eyes No diluted tears were used in the assays; samples not centrifuged

Appendix VIII Reports of IL-8 and IL-6 detection in the literature using ELISA and multiplex bead analysis

Reports of IL-8 in the literature: The mean IL-8 concentration in normals using ELISA is 956±1396pg/ml (range 5-4600pg/ml)

	Method of detection	Mean concentration in Normals/Controls	Mean concentration in other conditions	Correlations with symptoms/clinical signs?
Gonzalez-Perez *et al.* (2012)	ELISA	602pg/ml	660pg/ml (EW-SiH) 953pg/ml (CRT)	
Poyraz *et al.* (2012)	ELISA	Not investigated	107pg/ml (SiH) 176pg/ml (Conventional hydrogel)	
Fodor *et al.* (2006)	ELISA	572pg/ml	Penetrating keratoplasty 220pg/ml Corneal foreign body 203pg/ml Cataract operation 171pg/ml Bacterial conjunctivitis 661pg/ml	
Kallinikos *et al.* (2006)	ELISA	414pg/ml	476pg/ml (EW-SiH) 593pg/ml (EW-RGP)	
Berry and Jeffreys (2001)	ELISA	<5pg/ml	Varied depending on presentation of chemical injury	IL-6 increased with chemical injury

	Method of detection	Mean concentration in Normals/Controls	Mean concentration in other conditions	Correlations with symptoms/clinical signs?
Thakur and Willcox (2000)	ELISA	Not investigated	After 3 hours: 24ng/ml neophyte 31ng/ml Non-adapted CLW 122ng/ml Adapted CLW After 8 hours: 148ng/ml neophyte 70ng/ml NACLW 229ng/ml ACLW	
Thakur and Willcox (1998)	ELISA	No value given	CLARE range 65-420ng/ml CLPU range 0.9-3.4pg/ml	CLARE tears showed a significant correlation with severity rating and IL-8 concentration
Nakamura *et al.* (1997)	ELISA	731pg/ml (basal) 227pg/ml (reflex)	-	
Huang *et al.* (2012)	Multiplex bead analysis	4.60ng/ml (4600pg/ml)	Mild DES 3.31ng/ml Mod DES 5.38ng/ml Severe DES 9.73 ng/ml	IL-1Ra and IL-8 shown to be correlate best with corneal staining
Hagan *et al.* (2013)	Multiplex bead analysis	991.8pg/ml (range 246-9078.5pg/ml) 457.7pg/ml without outlier	DES 1156pg/ml	
Guyette *et al.* (2013)	Multiplex bead analysis	194pg/ml (non-stimulated) 125pg/ml (washout method)	403pg/ml (aqueous deficient, non-stimulated) 245pg/ml (aqueous deficient, washout method)	

	Method of detection	Mean concentration in Normals/Controls	Mean concentration in other conditions	Correlations with symptoms/clinical signs?
Balasubramanian *et al.* (2012)	Multiplex bead analysis	1168FIU/mg (Fluorescent intensity unit to total protein ratio)	2893FIU/mg (keratoconus) 1308FIU/mg (collagen cross linking)	
Zakaria *et al.* (2012)	Multiplex bead analysis	No specified concentration	No specified concentrations but significantly high IL-8 in px with neovascularised corneas	
Carreno *et al.* (2010)	Multiplex bead analysis	323pg/ml		
Enriquez-de-Salamanca *et al.* (2010)	Multiplex bead analysis	Unable to read from graph	Unable to read from graph (evap DES)	IL-8/CXCL8 inversely correlated with Schirmer, lysozyme and TBUT IL-8 shown to have an association with pain scores
Lam *et al.* (2009)	Multiplex bead analysis	176pg/ml	1510pg/ml (dysfunctional tear syndrome - DTS) 1303pg/ml (DTS with MGD) 1657pg/ml (DTS without MGD)	Significant positive correlation with fluorescein staining and conjunctival lissamine green staining but not with symptoms No significant inverse correlation with symptom severity scores

	Method of detection	Mean concentration in Normals/Controls	Mean concentration in other conditions	Correlations with symptoms/clinical signs?
Massingale *et al.* (2009)	Multiplex bead analysis	16791pg/ml	48509pg/ml (DES)	R^2 value with OSDI = 0.98
Malvitte *et al.* (2007)	Multiplex bead analysis	356pg/ml	4650pg/ml (glaucoma treatment)	
Uchino *et al.* (2006)	Multiplex bead analysis	1084pg/ml		
Uchino *et al.* (2006)	Multiplex bead analysis	283pg/ml (baseline)		
Sonoda *et al.* (2005)	Multiplex bead analysis	~1000pg/ml (basal) ~550pg/ml (reflex)		

Reports of IL-6 detection in the literature. The mean IL-6 concentration in normal subjects using ELISA is 45±76pg/ml (range 2-226pg/ml).

	Method of detection	Mean concentration in Normals/Controls	Mean concentration in other conditions	Correlations with symptoms/clinical signs?
Gonzalez-Perez *et al.* (2012)	ELISA	2pg/ml	3pg/ml (EW-SiH) 5pg/ml (CRT)	
Lema *et al.* (2009)	ELISA	2.2pg/ml	5.5pg/ml keratoconus 5.7pg/ml (subclinical keratoconus)	
Acera *et al.* (2008)	ELISA	8pg/ml	51.78pg/ml (conjunctivochalasis) 16.53pg/ml (blepharitis) 16.52pg/ml (dry eye) 33.11pg/ml (allergic eye disease)	
Yoon *et al.* (2007)	ELISA	4pg/ml (controls)	19pg/ml (dry eye) 25pg/ml (Sjőgrens)	
Fodor *et al.* (2006)	ELISA	110pg/ml	Penetrating keratoplasty 170pg/ml Corneal foreign body 109pg/ml Cataract operation 189pg/ml Bacterial conjunctivitis 366pg/ml	
Lema *et al.* (2005)	ELISA	2.2pg/ml	6.7pg/ml (keratoconus)	
Thakur and Willcox (2000)	ELISA		After 3 hours: 94pg/ml neophyte	

	Method of detection	Mean concentration in Normals/Controls	Mean concentration in other conditions	Correlations with symptoms/clinical signs?
			65pg/ml Non-adapted CLW 175pg/ml Adapted CLW After 8 hours: 147pg/ml neophyte 60pg/ml NACLW 218pg/ml ACLW	
Thakur and Willcox (1998)	ELISA		CLARE range 19-320pg/ml CLPU range 46-125pg/ml	
Tishler *et al.* (1998)	ELISA	42.1pg/ml	88.6pg/ml (Sjőgrens)	
Nakamura *et al.* (1997)	ELISA	226pg/ml (basal) 12pg/ml (reflex)	-	
Guyette *et al.* (2013)	Multiplex bead analysis	43pg/ml (non-stimulated) 37pg/ml (washout method)	57pg/ml (ADDE, non-stimulated) 48pg/ml (ADDE, washout method)	
Zhang *et al.* (2013)	Impression cytology + immunohistochemical staining	(Mean optical densities values only, not expressed as concentration)		IL-6 expression positively correlates with OSDI scores and TBUT but not Schirmer I
Balasubramanian *et al.* (2012)	Multiplex bead analysis	66.7FIU/mg (Fluorescent intensity unit to total protein ratio)	313.6FIU/mg (keratoconus) 109.7FIU/mg (collagen cross linking)	
Zakaria *et al.* (2012)	Multiplex bead analysis	No specified concentration	No specified concentrations but significantly high IL-6 in patients with neovascularised corneas	
Carreno *et al.* (2010)	Multiplex bead analysis	130pg/ml		

	Method of detection	Mean concentration in Normals/Controls	Mean concentration in other conditions	Correlations with symptoms/clinical signs?
Enriquez-de-Salamanca *et al.* (2010)	Multiplex bead analysis	Unable to read from graph	Unable to read from graph (evaporative DES)	IL-6 inversely correlated with Schirmer, lysozyme and TBUT. IL-6 positively correlated with rose bengal and fluorescein staining IL-6 shown to have an association with pain scores and sandy/gritty sensations
Lam *et al.* (2009)	Multiplex bead analysis	26.5pg/ml	238pg/ml (dysfunctional tear syndrome - DTS) 289pg/ml (DTS with MGD) 210pg/ml (DTS without MGD)	Significant positive correlation with fluorescein staining and conjunctival lissamine green staining Significant inverse correlation with symptom severity scores
Massingale *et al.* (2009)	Multiplex bead analysis	623pg/ml	1626pg/ml (DES)	R^2 value with OSDI = 0.99
Malvitte *et al.* (2007)	Multiplex bead analysis (Bioplex)	1000pg/ml	~3000pg/ml (glaucoma treatment)	
Uchino *et al.* (2006)	Multiplex bead analysis	29pg/ml		
Uchino *et al.* (2006)	Multiplex bead analysis	32pg/ml		
Sonoda *et al.* (2005)	Multiplex bead analysis	~175pg/ml (basal) ~150pg/ml (reflex)		

	Method of detection	Mean concentration in Normals/Controls	Mean concentration in other conditions	Correlations with symptoms/clinical signs?
Schultz and Kunert (2000)	Polyacrylamide gel electrophoresis (PAGE) and immunoblot analysis	No IL-6 detected in non-CLW	43.8pg/5µl (CLW)	3 CLW told to stop wearing CLs for 1 week, IL-6 not detected after 1 week cessation. IL-6 returned to original levels within 24hours of CLW

Appendix IX Full set of results for IL-8 assay

IL-8 concentrations (pg/ml) according to subject and visit. "-" indicate an inadequate tear volume sample for analysis thus no value was obtained. All samples were diluted by 25x unless highlighted which indicate 30x dilution.

Subject	Pre-ELO	1 day ELO	7 days ELO	Pre-ELI	1 day ELI	7 days ELI
1	163.27	-	-	133.87	-	151.80
2	321.75	251.54	388.45	440.37	451.50	885.59
3	200.33	163.27	100.57	146.74	111.71	93.098
4	529.30	749.84	325.45	410.70	203.68	-
5	6289.0	201.84	358.79	329.15	108.00	89.35
6	130.18	133.87	179.79	207.35	281.06	166.94
7	1526.60	-	861.088	1163.67	-	-
8	-	166.94	-	-	277.37	-
9	178.01	111.33	-	174.29	200.33	126.50
10	181.63	120.69	294.74	200.33	295.84	225.75
11	148.58	122.81	176.12	96.84	119.12	102.43
12	130.18	214.71	185.30	159.60	292.15	137.55
13	-	-	-	191.52	155.92	196.31
14	158.49	204.49	337.60	535.11	177.21	153.73
15	258.88	188.68	190.96	243.98	208.94	280.06
16	-	-	363.71	-	-	-
17	202.22	177.20	172.52	181.81	103.82	190.96
18	906.49	651.62	551.97	286.34	375.45	241.82
19	208.95	148.93	286.35	226.61	144.10	177.21
20	411.03	208.95	222.23	98.54	124.36	167.91
21	3577.585	1152.417	1127.965	2184.279	1267.38	731.494
22	-	-	-	698.942	-	-
23	211.174	286.344	228.795	277.972	202.2215	267.448

Appendix X Publication: Eye Cosmetics Usage and Associated Ocular Comfort (2012)

Appendix X

Eye cosmetic usage and associated ocular comfort

Alison Ng, Katharine Evans, Rachel North and Christine Purslow

School of Optometry and Vision Sciences, Cardiff University, Cardiff, UK

Abstract

Purpose: Eye cosmetics usage is commonplace and whilst some products such as eyeliner are applied with close proximity to the ocular surface, there is little knowledge of the short- and long-term ocular effects of eye cosmetic formulations. This study aimed to investigate the use of eye cosmetics and identify any relationships between ocular comfort and cosmetic usage.

Methods: Results were collated from an online survey comprising 23 questions that recorded demographics, Ocular Surface Disease Index (OSDI) score, extent and range of eye cosmetic use and perceived comfort differences with and without eye cosmetics.

Results: The 1360 female respondents (median age 25, interquartile range 20–34 years) completed the survey; 83% reported using eye cosmetics regularly ($\geq$ 3 times per week) with mascara being most commonly used. Fifty three per cent used at least three different eye cosmetics products regularly. OSDI scores of cosmetics users were similar to non-users ($p = 0.083$), but perceived comfort was greater when cosmetics were not used ($p < 0.001$). In occasional cosmetics users (use of products < 3 times per week), 65% reported a reduction in comfort when cosmetics were used. Median OSDI scores suggested a trend towards reduced comfort amongst eyeliner users ($p = 0.07$) although frequency and type of cosmetic products used did not appear to influence OSDI scores.

Conclusions: This study shows the use of multiple eye cosmetics is extensive and associated with the perception of ocular discomfort. With such widespread use of these products, more research is required to assess the effect on the ocular surface and tear film, which may be underestimated.

Introduction

Eye cosmetics are part of everyday life around many parts of the world. Women and men judge eye cosmetic use as a factor in facial attractiveness[1] and the psychosocial, and even economic impact of cosmetics use is well documented.[2] The cosmetics market is certainly large, with UK sales ranking fourth in Europe, exceeding €8.5 billion.[3] Indeed, Mintel have reported a 38% increase in eye cosmetic sales since 2004.[4]

Lead toxicity and changes in conjunctival and periocular pigmentation are documented complications arising from the use of kohl, commonly used in Indian and Middle Eastern cultures,[5–8] but there is little published literature that

reviews the long term side effects of Western eye cosmetic use. All cosmetic products manufactured for sale in Europe must undergo rigorous safety assessments to comply with the European Cosmetics directive (76/768/EEC) to ensure that the product does not cause harm to human health. There are several reported cases where the use of Western formulations of mascara and eyeliner have caused increased conjunctival pigmentation, ranging from diffuse pigmentation of the tarsal conjunctiva and conjunctival fornices to discrete, punctate deposits.[9–11] However these publications are dated in light of modern cosmetic product formulations. More recently, there have been case studies reporting the accumulation of cosmetic products within the lacrimal system and on the ocular surface which have resembled

melanomas.[12–14] However, the reported and published incidence of these unusual circumstances are rare compared with the incidence of allergic contact dermatitis (ACD) around the eyelids caused by cosmetics use, which is approximately 4%.[15] Preservatives and fragrances added to products are the primary causative agents of cosmetic ACD and irritant contact dermatitis.[16,17] If cosmetic products induce a dermatological reaction, a localised response involving redness, swelling, small vesicles/blisters and sweating may be present which may generate a range of symptoms including tingling, burning, tightness, itchiness or pain.[18–20]

Although regulated eye cosmetic products should cause no harm or morbidity to the ocular surface, mild undesirable effects may go undetected or unreported – presumably many consumers simply choose to omit the use of incompatible products from their daily regime.[17] There is no published evidence about any association between ocular comfort and use of cosmetics. This is of particular interest because of the known association between ocular comfort and quality of life. Dry eye patients have higher scores relating to anxiety and depression than those without the condition[21] and even in those who have good vision, dry eye symptoms have a significantly negative impact on the quality of life.[22] Although the influence of external factors such as the regular use of eye cosmetics upon dry eye symptoms have been suggested to cause symptoms of dryness and discomfort,[23,24] these findings are not yet well established. The mechanisms of eye irritation is unproven but potentially arises from several factors: particles and pigments from cosmetic products may cause foreign body sensations and may reduce tear film stability, the presence of preservatives and fragrances may induce toxic and allergic responses[25] and may also play a role in altering tear film pH and osmolarity.

The way in which eye cosmetics are applied in the Western world is largely dictated by fashion trends and personal preference. Of particular interest is the use of kohls and eyeliners. Traditional kohl is applied within the mucocutaneous junction (*Figure 1a*) in many Eastern cultures as it was once believed that this reduced the effects of glare in sunny climates.[26] Alternatively, eyeliner is also commonly applied outside the eyelash line, directly onto periorbital skin (*Figure 1b*).

There is evidence of the migration of cosmetic products into the tear film when applied to periorbital skin,[27] resulting in contamination of the tear film. Cosmetics such as eyeliner can be applied with even closer proximity to the ocular surface when used along the lid margin and over the meibomian glands (*Figure 1a*). Meibomian gland dysfunction (MGD) is a major cause of dry eye disease.[28] However, to date, there are no published data reporting the changes in ocular comfort when such eye cosmetics are used within the mucocutaneous junction.

This paper reports the results from a UK survey that aimed to investigate the use of eye cosmetics and identify any relationships between perceived ocular comfort and cosmetic usage. The use of eyeliner was also explored as it is hypothesised that the position of application of these products may result in differences in reported levels of ocular comfort due to migration of products around the eyelid margin.

Methods

An online survey was designed using the Bristol Online Survey software tool (http://www.survey.bris.ac.uk) and hosted on the Cardiff University network. An initial focus group of eye care professionals and lay persons reviewed initial survey designs before the final survey of 22 questions was established, which are summarised in *Table 1*. The initial 10 questions collected subject demographics, followed by questions to elicit information about the type and frequency of eye cosmetic use, and to calculate an Ocular Surface Disease Index (OSDI) score.[29] The OSDI questionnaire scores dry eye symptomology from 0 to 100, where higher scores indicate increasing severity of symptoms. It has been suggested that the cut-off score between normals and dry eye subjects is 15.[29] Perceived ocular comfort with and without eye cosmetic use was also recorded using a simple scale of 0–10, where 10 indicated maximal comfort.
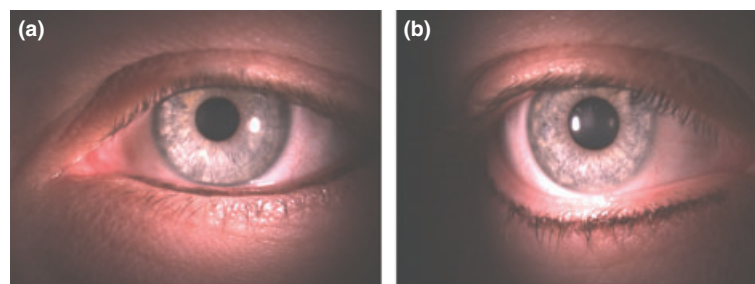
Figure 1. Eyeliner applied within the lash line (a) and outside the lash line (b).

Table 1. Summary of questions analysed in the cosmetics survey

Questions 1–10	Collection of demographic data (age, gender, previous history of allergies and eye sensitivity)
Questions 11–13	OSDI questionnaire
Questions 14–20	Use of eye cosmetics, type and frequency of cosmetic use
Questions 21–22	Perceived ocular comfort with and without eye cosmetic use

Data were collected between February and April 2011. All staff and students at Cardiff University were emailed with the online link to the survey inviting participation. All responses were anonymous and data were securely stored electronically.

Data analysis

Data from the survey were downloaded into a Microsoft Excel worksheet (www.microsoft.com/Office) and statistical analysis of data was performed using Microsoft Excel 2007 and SPSS 16.0 for Windows (www.ibm.com/SPSS_Statistics).

Data were tested for normality using a Kolmogorov–Smirnov test. Since the data were not normally distributed, non-parametric statistics were applied to the data, and two-sided p values< 0.05 were considered significant for all statistical tests. Data are presented as median values with interquartile (IQ) ranges.

Results

The survey was completed by 1462 respondents. Although 7% ($n = 102$) of responses were from males, these were excluded from data analysis to avoid bias in the results,

leaving 1360 female responses for analysis. The median age of female respondents was 25 (IQ range 20–34) years.
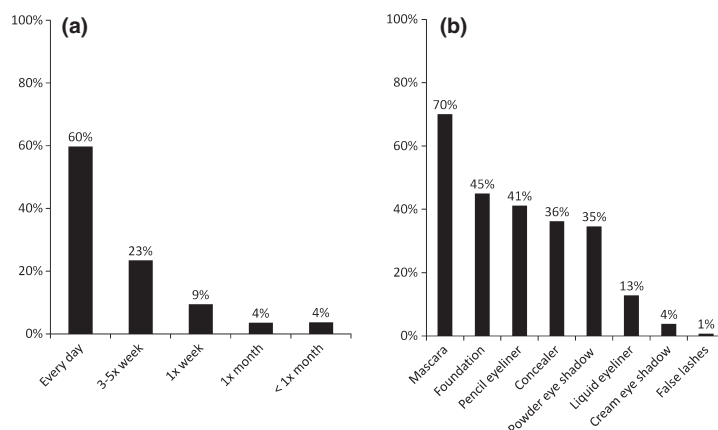
Use of cosmetic products

About 89% ($n = 1206$) of respondents reported using eye cosmetics and 83% ($n = 1006$) reported using eye cosmetics at least three times per week (*Figure 2a*). The median age of eye cosmetics users was 24 (IQ range 20–33) years. The median age of non-users ($n = 154$) was 25 (IQ range 21–37) years which was statistically different to the make-up users (Mann–Whitney *U* test, $p = 0.031$).

'Regular use' of a cosmetic product was defined as use of the product three or more times a week. The most regularly used (≥ 3 times per week) cosmetic products applied around the eyes were mascara (70%), foundation (45%) and pencil eyeliner (41%) (*Figure 2b*). The use of a single cosmetic product was relatively rare in these regular makeup users (12%, $n = 143$); 53% ($n = 638$) used at least three different products regularly.

Ocular comfort

The median OSDI score for the cohort was 10.4 (IQ range 4.2–16. 7). OSDI scores ranged between 0 and 85.4, with 30% ($n = 409$) of respondents scoring > 15. Although eye cosmetic users indicated greater dry eye symptoms with higher OSDI values compared to non-users (10.4, IQ range 4.2–16.7 vs 8.3, IQ range 2.1–15.1 respectively), this difference was not statistically significant (Mann–Whitney *U* test, $p = 0.083$, *Figure 3*).

The data set of makeup users was further explored for differences in OSDI scores according to regularity of makeup use, as previously defined. The OSDI scores of regular users of eye cosmetics were similar to those who used eye

Figure 2. Frequency of eye cosmetic use ($n = 1206$) (a). Percentage of respondents who use each type of cosmetic product ≥ 3 times a week ($n = 1206$) (b).
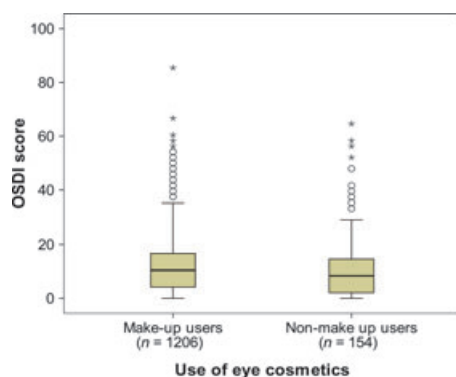
Figure 3. Comparison of OSDI scores in make-up users and non-users. Circles represent values 1.5 times greater than the IQ range; asterisks represent values 3 times greater than the IQ range.

cosmetics less than three times a week (10.4, IQ range 4.2–16.7, vs 10.4, IQ range 4.2–18.8 respectively) and there was no significant difference in median OSDI scores with increasing frequency of make-up use (Mann–Whitney *U* test, $p = 0.47$).

Analysis of the OSDI score of users was conducted for each cosmetic product comparing regular or light use, using the Mann–Whitney *U* test, summarised in *Table* 2.

The number of respondents who were regular users of false lashes was comparatively small, so no statistical comparison was performed. There was no statistical difference in mean OSDI scores between regular and light users of any other product.

The survey was designed to explore eyeliner use according to both product type and position and this data was analysed accordingly (*Figure 4*). Median OSDI scores indicated reduced comfort in the eyeliner-using group (10.4, IQ range 4.2–18.8) compared to the non-using group (8.3, IQ range 2.1–16.7), but this was not statistically significant (Mann–Whitney *U* test, $p = 0.07$). Furthermore, OSDI scores were similar irrespective of the position of eyeliner application (within the lash line vs outside the lash line, $p = 0.25$, Mann–Whitney *U* test) and the type of eyeliner

applied (pencil vs liquid within the lash line, $p = 0.40$; pencil vs liquid outside the lash line, $p = 0.53$).

Ocular comfort with and without eye cosmetics

Eye cosmetic users were asked to rate on an ordinal scale the perceived comfort of their eyes on days that eye cosmetics are applied and not applied, where 0 = uncomfortable and 10 = very comfortable. The median perceived comfort score was significantly greater without make-up compared to when make-up was used (*Figure 5*), with scores of 9 (IQ range 8–10) and 8 (IQ range 7–9) respectively (Wilcoxon Signed Rank test, $p < 0.001$).

These differences in perceived comfort scores were further analysed according to regular and light eye cosmetics use, to determine whether increased use was associated with reduced comfort. Among light cosmetics users, 65% reported a reduction in perceived comfort when cosmetics were used and 35% reported no difference. Among regular cosmetics users, the division between comfort differences was more equal: 49% reported a reduction, and 51% reported no difference in perceived comfort when cosmetics were used. A Chi-squared test for independence showed a significant association between changes in perceived comfort and the regularity of make-up use ($p < 0.01$).

Discussion

The product choice of the respondents in this survey is similar to reported trends in the US market which indicated 65% and 62% of women are regular users of mascara and eyeliner respectively.[4] The large number of cosmetics-using female respondents reflects the rise in popularity of eye cosmetics use, where sales in this sector have increased by 38% since 2004.[4]

In this study, significant differences in OSDI scores were not established between eye cosmetics users and non-users, although a trend towards higher scores for eye cosmetics users was evident. The relatively small sample size of non-users (11%) may not have been sufficient to detect

Table 2. Comparison of OSDI scores in regular and light use of eye cosmetic products

	Concealer	Foundation	Mascara	Powder eye shadow	Cream eye shadow	Eyeliner	False lashes
Regular users (≥ 3 times a week)							
n	437	543	845	418	46	571	9
Median OSDI	10.4	10.4	10.4	10.4	10.4	10.4	8.3
IQ range	4.2–18.8	4.2–18.8	4.2–16.7	4.2–18.8	6.3–19.8	4.2–18.8	2.0–25.0
Light users (< 3 × times a week)							
n	297	286	315	599	418	487	321
Median OSDI	10.4	10.4	10.4	10.4	10.4	8.3	10.4
IQ range	4.2–17.7	5.7–18.8	4.2–18.8	4.2–16.7	4.2–18.8	4.2–16.7	4.2–16.7
p value	0.82	0.21	0.53	0.62	0.74	0.21	–
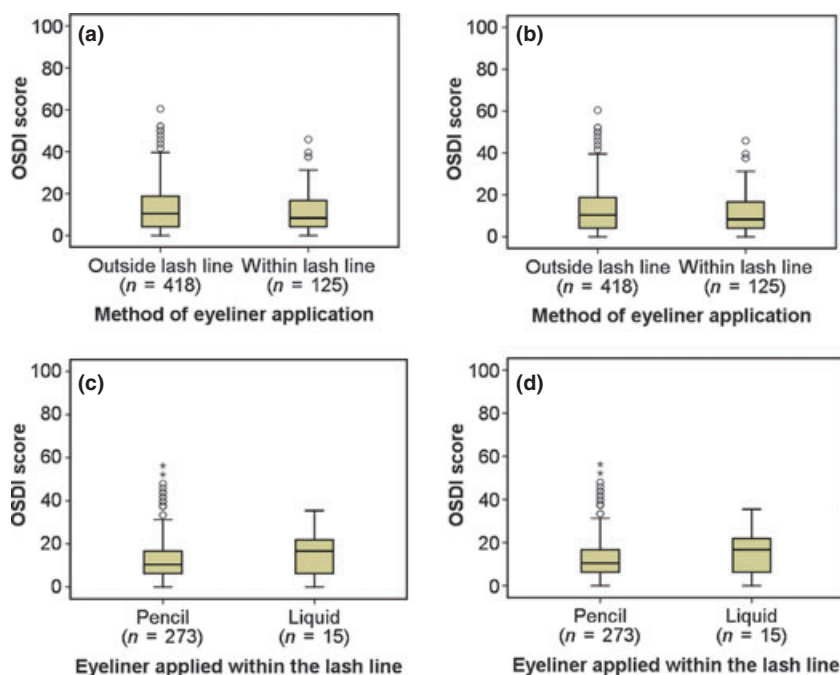
Figure 4. OSDI scores in four different eyeliner conditions comparing: eyeliner use with non-use (a); application of eyeliner outside and within the lash line (b); pencil with liquid eyeliner when applied within the lash line (c); pencil with liquid eyeliner when applied outside the lash line (d). (b–d) The respondents who use the products in the specified way specifically (i.e. respondents who used eyeliner in both conditions in each chart were excluded).
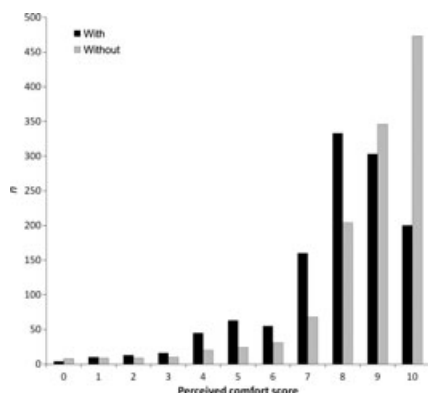
statistically significant differences. The motivation to complete the survey is likely to have been greater in make-up users due to increased relevance and existing interest, reflected in the large number of female respondents. This finding may also reflect the fact that the OSDI is based upon symptoms of dry eye rather than irritation. Although the OSDI questionnaire has been shown to be a valid and reliable tool with good sensitivity for assessing and monitoring dry eye severity and intervention,[29] the OSDI scale

Figure 5. Perceived comfort scores when make-up is used compared to when it is not used (*n* = 1206).

may not be the most appropriate measure of ocular comfort for this study. Furthermore, the respondents to this survey were predominately healthy, with only 30% of female respondents having an OSDI score greater than 15, which is the recommended cut-off for symptomatic dry eye diagnosis using the OSDI questionnaire.[29]

A separate study has shown that 17% of normal subjects reported symptoms of 'soreness, scratchiness, dryness or burning' after using make-up or make-up remover.[24] In this study, perceived ocular comfort amongst cosmetics users was found to be significantly improved when eye cosmetics were not used. While 65% of light cosmetics users reported a reduction in perceived comfort, this was the case in only 49% of regular users. Self-selection is likely here: light cosmetic users may be choosing to limit their frequency of make-up use due to experiences in discomfort and the converse may be true for regular cosmetics users.

Alternatively, regular make-up users may be more tolerant to changes in perceived comfort due to a variety of other influential factors (e.g. habits and psychological factors relating to cosmetics use).

The potential origins of ocular discomfort following eye cosmetic use are multi-factorial. While every effort is made to formulate products which are safe for application to periocular skin, there will be inadvertent migration of these products onto the ocular surface.

The migration of cosmetic products applied around the eyes may occur as a result of manual transfer. This will generate an immediate response of discomfort. Direct experimental instillation of 30 mg of neat mascara and powder eye shadow into the eyes of human subjects has been shown to cause the greatest subjective discomfort amongst a battery of cosmetic products.[30] However the gradual migration of ointments applied around the eyes onto the ocular surface has been ascribed to the action of the muscles of Riolan. The fine vertically-oriented muscles lie directly beneath eyelid skin allows vertical movement of lid skin, facilitating the gradual migration of ointments over the lid margin and onto the ocular surface.[31] Once cosmetic products are in the tear film, changes in pH and tear osmolarity may occur which may lead to reduced tear film stability, resulting in further discomfort. However, the likelihood of cosmetic product migration in this manner is likely to be product-dependent. With an increasing trend of formulating 'long-wear' and 'waterproof' cosmetic products, the improved residency on skin and lashes may alter or even minimise patterns of migration compared to products which may be powder-based.

The addition of preservatives, surfactants and emulsifiers to cosmetic products may cause irritation to the ocular surface,[25] in a similar way it may irritate periocular skin. Preservatives commonly used in cosmetic products include[32]: parabens, imidazolydinil urea, diazolydinil urea, formaldehyde, benzalkonium chloride and 2-bromo-2-nitropropane-1,3-diol, all of which are cosmetics ingredients which responsible are for eyelid allergic contact dermatitis.[18,33] Pigments and particles suspended in coloured eye cosmetic products, varying in particle size, may provoke foreign body sensations when in contact with the ocular surface. Certainly, in the formulation of ophthalmic pharmaceutical preparations, it has been recommended that particle sizes are no larger than 10 μm to minimise eye irritation.[34] Dermatologists recommend changing products or application techniques for patients with sensitive skin and eyes. The use of unpigmented (clear) mascara and restricting application to the tips of the lashes can cause less irritation for some patients.[18] Cosmetic manufacturers may formulate hypoallergenic products which contain less sensitising ingredients, further reducing the potential for irritation which may be ideal for sensitive patients. While alternatives to daily colour cosmetics application exist (eyelash tinting, permanent make-up), these invasive procedures carry their own risks[35,36] and should only be carried out by suitably trained professionals.[36]

In this study, no trends were identified with frequency of eye cosmetics use or the type of products used in relation to OSDI scores. As previously discussed, the use of the OSDI questionnaire to detect changes in irritation for this cohort may not be suitable. However the confounding influence of multiple product usage is unknown. The results indicated that the majority of cosmetics users applied two or more products as part of their weekly routine; only a small number of users chose to apply one product only.

Eyeliner use was analysed according to formulation (pencil or liquid) and the position of eyeliner application (within or outside the lash line) to explore the hypothesis that product placement closer to the lid margin may contaminate the tear film resulting in tear film changes and subsequent discomfort. MGD remains a leading cause of evaporative dry eye and obscuring these gland openings with cosmetic products might be factorial in dysfunction. A host of associated ophthalmic risk factors for the condition have been summarised by the International Workshop on Meibomian Gland Dysfunction,[28] but there are no published data which explore the effects of applying eye cosmetics close to, or along the meibomian glands. While the acute effects of eyeliner application due to tear film contamination might be tear film instability, long term effects of eyeliner application on meibomian gland morphology or meibum lipid profiles are unknown. Conversely the application, and particularly removal, of eye cosmetics inevitably involves digital manipulation of the lids which also may encourage expression of meibum into the tear film. Increased meibum in the tear film will increase tear film lipid layer thickness which, in turn, can retard tear film evaporation. Indeed digital expression of meibomian glands is a recognised therapy for MGD[37] which has been shown to significantly reduce tear evaporation rates.[38]

In summary, the results of this study indicate widespread regular use of multiple eye cosmetics but did not indicate significant differences in comfort with respect to formulation and position of eyeliner application. This may be due to the limited sensitivity of the OSDI for this predominately young healthy cohort who may only experience short-term changes in ocular comfort from cosmetic use. It is clear that with such widespread use of these products more research is required to assess the effect on the ocular surface and tear film, which may be underestimated.

Conclusion

This study shows the use of eye cosmetics is extensive and associated with the perception of ocular discomfort.

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