In Vivo Assessment of Inflammatory Cells in Contact Lens Wearers

Yahya Ahmed Alzahrani

BSc (Optom), MSc (Optom)

Supervisors:

Professor Nathan Efron

Dr. Nicola Pritchard

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Institute of Health and Biomedical Innovation,

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Queensland University of Technology,

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Abstract

Langerhans cells are antigen presenting cells that can be observed in ocular tissues using laser scanning confocal microscopy. It is hypothesised that contact lens wear upregulates Langerhans cells, especially in people with dry eye symptoms. This thesis set out to determine the impact of contact lens wear on the density of Langerhans cells in the cornea, conjunctiva and lid wiper of contact lens wearers with and without dry eye.

A series of preliminary studies was conducted to validate and refine the experimental methodology. The first study explored the repeatability of measurements of presumed Langerhans cell density in the ocular surface in a cohort of healthy people, and found that such measurements were repeatable with a high degree of reliability. The number of images required for optimal sampling of Langerhans cells in the central cornea and bulbar conjunctiva was determined. Extraneous factors that may impact upon the immunological response of the ocular surface, such as mechanical effects (eye rubbing) and eye closure were explored; Langerhans cell density increased in both of these conditions.

A longitudinal study over a 24-week period was conducted on two groups of contact lens wearers. One group reported contact lens-induced dry eye and the other group had no symptoms or signs of dry eye. Participants who did not wear contact lenses were recruited as controls. Contact lens wear induced an immediate two-fold increase in the number of Langerhans cells in the corneal centre, nasal bulbar

conjunctiva and lid wiper, with the response being significantly greater in those with contact lens-induced-dry eye. These changes are thought to reflect an up-regulated ocular immune response.

This thesis establishes a protocol for Langerhans cell assessment, which appears to be a sensitive marker of the inflammatory status of the anterior eye during contact lens wear.

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Statement of Original Authorship

The work contained in this thesis has not been previously submitted to meet requirements for an award at this or any other higher education institution. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made.

QUT Verified Signature

Signature:

Date: 01-03-2016

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Chapter 1: Introduction

Preface

Contact lens discomfort is one of the major untreated problems in ophthalmic science. Up to half of all contact lens wearers complain of discomfort-related contact lens wear (Nichols et al. 2013). Contact lens–related discomfort is characterised by episodic or persistent adverse ocular sensations secondary to contact lens wear. It is introduced as a consequence of reduced compatibility between the ocular surface and contact lens (Nichols et al. 2013). It considered the main reason for contact lens discontinuation (Nichols et al. 2013).The majority of contact lens wearers use different descriptions to explain symptoms of discomfort, but the overwhelming majority categorise this discomfort as *dry eye* (Nichols et al. 2013).

The term *dry eye* was first introduced in 1950 by Andrew De Roetth (De Roetth 1950). It is one of the most common clinically established eye diseases around the world, and one of the most frequently encountered ocular morbidities (Gayton 2009). It is defined as 'a multifactorial disease of the tears and ocular surface that results in symptoms of discomfort, visual disturbance, and tears 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).

Dry eye may be associated with the recruitment of inflammatory cells (Langerhans cells) in the ocular surface. For example, aqueous tear-deficient dry eye, (either Sjögren's syndrome, or non-Sjögren's syndrome), was found to have increased the

number of Langerhans cells in the ocular surface, typically, in the cornea (Lin et al. 2010, Villani et al. 2013).

In 1910, Engelmann was the first to identify Langerhans cells in the cornea (cited by (Machetta et al. 2014). They were described as being similar to those in the skin, the so-named Langerhans cells (Langerhans 1868, Jolles 2002). As noted earlier, Langerhans cells are antigen presenting cells that are derived from hematopoietic bone-marrow progenitor cells (Banchereau et al. 1998). In the cornea, dendritic cells were reported to have membrane-associated Ia antigens, which led to ophthalmologic interest in Langerhans cells; these cells were presumed to be Langerhans cells (Sacks et al. 1986). However, it had long been thought that the normal corneal centre lacked Langerhans cells (Gillette et al. 1982, Suzuki et al. 2000). Thus, the real nature of these cells became a matter of controversy, while the distribution of these cells in the cornea had long been a challenge for ophthalmic clinicians and researchers.

There are no standard protocols for measuring dry eye. However, there are a number of tools that can be used to evaluate dry eye, either with or without a contact lens in place such as phenol red thread (Kurihashi et al. 1976), non-invasive break- up time test (cited by (Fullard et al. 1990)), Schirmer's test (Hamano et al. 1983). Dry eye can be predicted through recruitment of dry eye questionnaires such as McMonnies questionnaire (McMonnies 1986), Dry Eye Questionnaire-5 (Chalmers et al. 2010), Contact Lens Dry Eye Questionnaire-8, (Chalmers et al. 2012), and Dry Eye Questionnaire (Begley et al. 2002).

Evaluating the presence and distribution of Langerhans cells in the ocular surface in dry eye can be conducted by means of laser scanning confocal microscopy. It is a unique system that enables visualising the anterior ocular tissues *in vivo* at the cellular level (Machetta et al. 2014). To date, there are no studies into the inflammatory response of the ocular surface to contact lens wear in dry eye. The purpose of this thesis was to improve understanding of Langerhans cells in the ocular surface, typically in the corneal centre and bulbar conjunctiva as well as in the lid wiper, *in vivo* among contact lens wearers, as well as to report on the inflammatory response to contact lens wear in dry eye. It was hypothesised that contact lens wear will increase Langerhans cell density in the cornea, conjunctiva and, lid wiper.

The following paragraphs comprise an overview of the effects of contact lens wear on the eye. Specifically, it identifies the following areas of interest: Langerhans cells and the ocular surface, quantification of presumed Langerhans cell density in the cornea, conjunctiva and lid wiper in both non-contact lens and contact lens wearers.

1.1 Langerhans Cells and the Ocular Surface

A number of researchers (Banchereau et al. 1998, Steinman et al. 1999, Steinman et al. 2003, Ohl et al. 2004, Adams et al. 2005), since Paul Langerhans first described the presence of dendritic cells in the skin late in the nineteenth century (Langerhans 1868, Jolles 2002), showed that Langerhans cells migrate to draining lymph nodes in the steady state through the lymphatic vessels of the skin and stabilise in the T-cell

area of the draining lymph nodes. During inflammation, Langerhans cell migration rates increase. Thus, these cells are important since they release pro-inflammatory cytokines that are essential to stimulate the immune responses against the host (Langerhans 1868, Jolles 2002).

The ocular surface is a unique surface in the body; it is frequently exposed to environmental factors, such as toxic, antigen, and microbiological factors. These factors may induce negative effects on the integrity and function of the eye. Therefore, a defence system, that can provide a protective agent for the ocular surface, is essential to ensure good ocular function and integrity. Langerhans cells are a key component of the ocular surface defence system (Langerhans 1868, Jolles 2002). Subsequently these cells have been called different names and ascribed different functions, including peculiar branched wiry bodies (Billingham et al. 1950); polymorph elements (Scharenberg 1955), basal layer branched cells (Whitear 1960), Langerhans cells (Langerhans 1868, Jolles 2002) and, polygonal cells (Sugiura 1969).

The ocular surface contains two basic structures: the conjunctiva and the cornea. Studies revealed that the central cornea was devoid of Langerhans cells, whereas the peripheral cornea does contain Langerhans cells (Gillette et al. 1982, Suzuki et al. 2000). However, this study conflicts with many studies that showed that Langerhans cells can be observed in both the centre and peripheral part of the cornea (Asbell et al. 1987, Hamrah et al. 2002, Rosenberg et al. 2002, Novak et al. 2003, Patel et al. 2005, Yamagami et al. 2005, Yamagami et al. 2005, Zhivov et al. 2005).

Studies have shown that dendritic cells can be found in the iris, ciliary body and choroid (Williamson et al. 1989, Streilein et al. 1990, McMenamin et al. 1992, Streilein et al. 1992, McMenamin et al. 1994, McMahon et al. 2006). In the iris and ciliary body, immunological cells were observed to be spread throughout the stroma and able to migrate out during inflammation (Camelo et al. 2003). An associated study reported that extracellular spaces communicate with lymphatic channels assisting antigens to be transported to the anterior chamber of the eye (Camelo et al. 2003, Camelo et al. 2004).

According to Bergstresser et al. (1980), it was observed that the corneal stroma contains leukocytes and dendritic cells, which are decreased gradually from the peripheral part of the cornea to the centre. In a steady-state and under inflammatory conditions of the eye, dendritic cell migration is controlled by cytokines (which are important for the interaction between cells in the immune response) and chemokines (they are peptide activators of G protein-coupled receptors regulate inflammatory cell recruitment).

Interestingly, most studies (Rosenberg et al. 2000, Rosenberg et al. 2002, Patel et al. 2005, Zhivov et al. 2005, Zhivov et al. 2007, Resch et al. 2008, Efron et al. 2009, Guthoff et al. 2009, Lin et al. 2010, Le et al. 2011, Tavakoli et al. 2011, Marsovszky et al. 2012) on confocal microscopy of the ocular surface have referred to dendritic cells observed on the ocular surface as Langerhans cells. There is evidence in the literature to suggest that there is a direct correlation between *in vivo* corneal confocal microscopy and immunohistochemistry observations of dendritic cells found in the

corneal epithelium. This correlation was not established for corneal stromal dendritic cells (Mayer et al. 2012). The expression of Langerhans cell-specific cell surface markers by dendritic cells in the corneal or limbal epithelium has also been reported (Chen et al. 2007, Mayer et al. 2007).

Therefore, this formation of cells is termed in the current study as 'presumed Langerhans cells' for the following reasons. Firstly, the classification system of Zhivov et al. (2005) and Zhivov et al. (2007) has been adopted to identify corneal Langerhans cells. Secondly, the majority of the ophthalmic literature has termed these dendritic cells as Langerhans cells, so the term Langerhans cells appears to be the more accepted term for these cells (Rosenberg et al. 2000, Patel et al. 2005, Zhivov et al. 2005, Mastropasqua et al. 2006, Zhivov et al. 2007, Sindt et al. 2012, Resch et al. 2015). Thirdly, the term used here will align with the contemporary ophthalmic literature (Lin et al. 2010, Tavakoli et al. 2011, Marsovszky et al. 2012, Sindt et al. 2012, Villani et al. 2013, Machetta et al. 2014, Resch et al. 2015).

1. 2 Quantification of Presumed Langerhans Cell Density in the Cornea

The cornea is a transparent avascular connective tissue that acts as the primary infectious and structural barrier of the eye. In cooperation with the tear film, the cornea induces a typical anterior refractive surface for the eye (DelMonte et al. 2011). The human cornea is composed of three cellular layers (epithelium, stroma, and endothelium) and two interfaces (Bowman's and Descemet's membranes) and Dua's layer (Dua et al. 2013). These layers are discussed below.

Epithelium: The corneal epithelium protects the corneal layers from the outside environment. The epithelium is composed of about five to six rows of stratified squamous epithelial cells (Pedler 1962), consisting of three cell layers; the superficial cells (40 to 50 µm in diameter) - (Efron et al. 2001), the wing cells (40 to 45 µm in diameter) -(Hollingsworth et al. 2001), and the basal cells (10 µm in diameter)- (Efron 2007). These cells remain for seven to ten days before undergoing involution, apoptosis (programmed cell death) and desquamation (Hanna et al. 1961). The corneal epithelium creates flat polygonal cells in an average of two to three layers, which helps the tear film mucinous layer and the cell membrane to be adhered (DelMonte et al. 2011). It also contains transient amplifying cells (cells capable of multiple cellular divisions) and basal cells. The epithelial cells are created as a result of differentiation of limbal stem cells that migrate to the corneal centre. When a corneal epithelial cell is disrupted, the whole cell is damaged which causes an epithelial layer defect (Hanna et al. 1961).

The corneal epithelium has a symbiotic association with tear film. Conjunctival goblet cells produce the mucinous layer of the tear film which react with the corneal epithelial cells. This interaction allows the spreading of the tear film during the blinking process (Gipson et al. 1992).

Based on confocal microscopic measurements, superficial and basal epithelial cell densities vary from 759 ± 162 to 1213 ± 370 cells/mm², and 3601 ± 408 to 8996 ± 1532 cells/mm², respectively (Mustonen et al. 1998, Vanathi et al. 2003, Popper et al. 2004, Eckard et al. 2006), and range in size from 546 ± 151 to 913 ±

326 µm², and 65 ± 13 to 192 ± 20 µm², respectively (Mustonen et al. 1998, Mutalib 1999, Harrison et al. 2003).

Subbasal nerve plexus: These nerves are positioned between the basal epithelium and the Bowman's layer, and contains straight and beaded fibres, which have been described as axonal efferent and sensory terminals (reviewed in (Muller et al. 2003)). The nerve densities in the sub-basal nerve plexus were estimated by Oliveira-Soto et al. (2001), and Patel (2006) to be 11,101 ± 4290 µm/mm², and 14,713 ± 6056 µm/mm² respectively. Some studies reported bright corpuscular or specular elements at the level of the corneal sub-basal nerve plexus, which are considered to be presumed Langerhans cells (Auran et al. 1994, Zhivov et al. 2005, Mastropasqua et al. 2006). These cells are 12 to 15 µm in diameter and can be found in different forms: cells without dendrites, cells with small dendritic processes forming a local network, cells forming a 'wire net-like structure' through long interdigitating dendrites and Y and X shaped dendritic forms with a small central cell body - Figure 1 - (Hazle et al. 1999, Zhivov et al. 2005, Efron et al. 2010). Banchereau and Steinman (1998) proposed that Langerhans cells have two phenotypes: immature Langerhans cells (which are suggested to be responsible for capturing antigens), and mature Langerhans cells (which have the ability to sensitise naïve T-cells via major histocompatibility complex and the secretion of interleukin-12, as well as co-stimulatory molecules) - (Banchereau et al. 1998). Thus, they represent an integral part of the immune system (Zhivov et al. 2006).

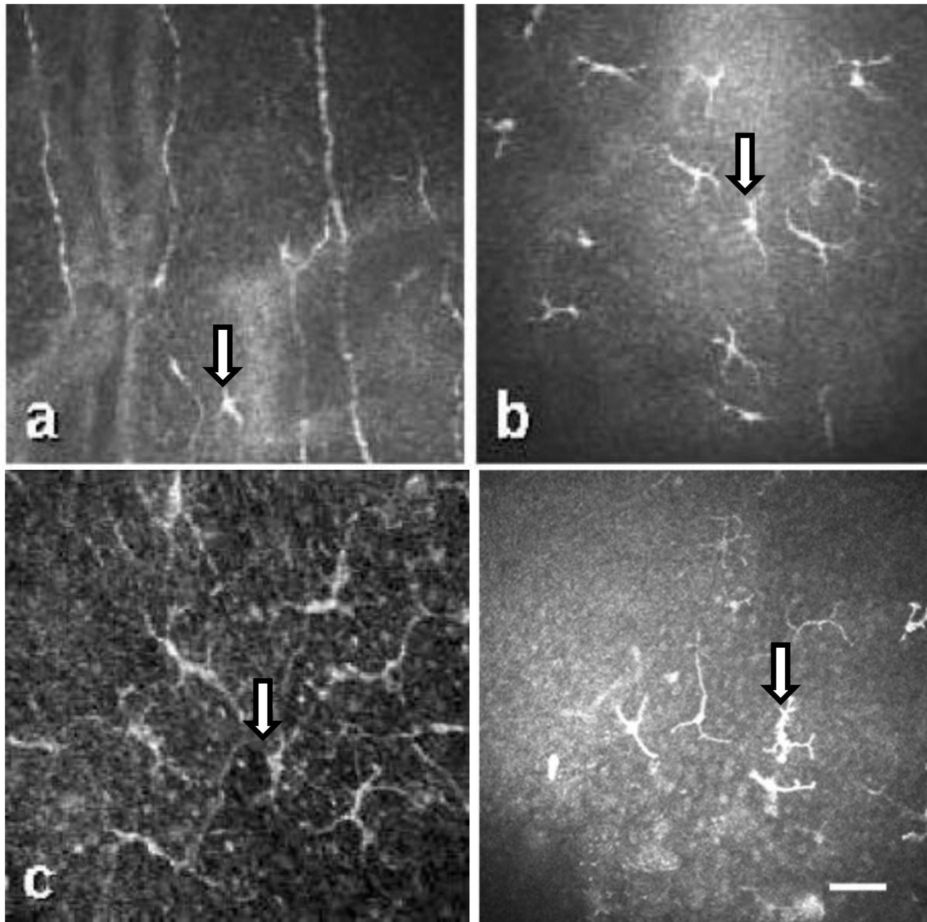


Figure 1. *In vivo* confocal microscopic images showing different appearances of presumed Langerhans cells in the human cornea. The white arrows indicate (a) cells without dendrites, (b) cells with small dendritic processes forming a local network, (c) cells forming "wire net-like structure" through long interdigitating dendrites and (d) Y and X shaped dendritic forms with a small central cell body; image size is 400× 400 µm (Zhivov et al. 2007, Efron et al. 2010).

Bowman's layer: It lies anterior to the corneal stroma. It is argued that Bowman's layer is not a membrane but rather the acellular condensate of the most anterior part of the corneal stroma (DelMonte et al. 2011). It is condensed layer of collagen; 15

μm thick assisting the cornea in maintaining its shape. Bowman's layer can be regenerated in case of disruption (Kayes et al. 1960, Worthen 1972)

Stroma: It is essential in the transparency and strength of the cornea, occupying 80 to 85% of the corneal (Jakus 1960, Worthen 1972). The stroma contains 200 to 250 different lamellae of collagen fibrils (to maintain corneal transparency), ground substance, keratocytes (which produces the collagen and proteoglycans) and nerve fibres. Keratocytes are located inside the anterior stroma and they are the main cell type of the corneal stroma. They are responsible for maintaining the extracellular matrix environment and are capable of synthesising collagen molecules and glycosaminoglycans that help to maintain stromal homeostasis (DelMonte et al. 2011). Corneal stroma is divided into the anterior and posterior zones. (DelMonte et al. 2011).

***Descemet's membrane*:** It is comprised of an amorphous ultrastructural texture; it is observed between the posterior stroma and the endothelium, with a thickness of 10 μm (Dohlman et al. 1955, Jakus 1960). Collagen is the main component of the extracellular matrix which has many types including type I, type II and type IV. Type IV collagen molecule represents the main constitutions of Descemet's membrane (Konomi et al. 1984). At the eight week stag in utero Descemet's membrane is secreted by endothelial cells. At adulthood, the anterior one-third of Descemet's membrane has showed to have a hexagonal lattice (Hogan et al. 1971, Tamura et al. 1991)

Endothelium: It is found as a single cell layer of hexagonal cells, at 10 µm in thickness. The endothelial cell thickness undergoes many changes over time, before stabilisation in adulthood. It maintains the corneal stroma in a relatively dehydrated state (78 % water content), and contains several hemides-mosomes that support adhesion to Descemet's membrane (Leuenberger 1972, Kreutziger 1976, Hirsch et al. 1977)

Dua et al. (2013) characterised a novel pre-Descemet's layer in the human cornea, subsequently termed Dua's layer. However, Bergmanson (2014) and Mckee et al. (2014) argued that Dua's layer does not exist in the cornea.

Recently, numerous studies (Asbell et al. 1987, Hamrah et al. 2002, Novak et al. 2003, Yamagami et al. 2005) have shown different precursors and maturation stages of presumed Langerhans cells in the normal cornea. Patel and McGhee (2005), and Rosenberg et al. (2002) observed hyper-reflective dendritic-like structures at the level of the basal epithelium and Bowman's layer in the central cornea. They had a mean density of 34 ± 3 cells/mm². *Ex vivo* histological research on the mouse model revealed that presumed Langerhans cells were located throughout the entire corneal epithelium (Hamrah et al. 2002). The *ex vivo* studies (Gillette et al. 1982, Catry et al. 1991, Hamrah et al. 2003) showed that the central cornea contains a number of presumed Langerhans cells. Also presumed Langerhans cell density was seen as higher in the central cornea of healthy infants (40 ± 13 cells/mm²) compared to adults (8 ± 6 cells/mm²) - (Chandler et al. 1985).

Similarly, Gillette et al. (1982) investigating ocular surface antigens using *adenosine triphosphatase* staining and immunofluorescence for human leukocyte antigen detection; illustrated that presumed Langerhans cells were highly represented in the human corneal periphery (75 to 150 cells/mm²) compared to the centre, where no cells where observed. They also, observed that presumed Langerhans cells resided in the peripheral corneas of guinea pigs (15 to 20 cells/mm²) and mice (50 to 100 cells/mm²) - (Gillette et al. 1982). A quantitative study of the *adenosine triphosphatase*-stained epithelial sheets revealed that the peripheral third of the human cornea showed a presumed Langerhans cell density of 15 to 20 cells/mm² (Chandler et al. 1985).

In vivo confocal microscopy has opened up a new and promising method by which to investigate presumed Langerhans cells in the corneal layers at the cellular level (Auran et al. 1995, Patel et al. 2005, Zhivov et al. 2005). Auran et al. (1995), investigating human corneas, by scanning slit confocal microscopy, observed bright objects that appeared to be aligned along the basal epithelial nerve. It was presumed that these objects were presumed Langerhans cells, confirming the same finding as that of Rosenberg et al. (2000). However, the later study by Zhivov et al. (2005) that used *in vivo* laser scanning confocal microscopy, found presumed Langerhans cell recruitment in the corneal epithelium of healthy volunteers. The distribution was 98 ± 8 cells/mm² (ranging from 0 to 208 cells/mm²) in the periphery of the cornea, and 34 ± 3 cells/mm² (ranging from 0 to 64 cells/mm²) in the centre, regardless of the participant's age. There was no significant correlation identified between presumed Langerhans cell density in the centre and in the periphery of the cornea. Presumed Langerhans cell density in males and females in the centre of the healthy cornea was

35 ± 4 cells/mm² and 33 ± 5 cells/mm², respectively, and 95 ± 11 cells/mm² and 101 ± 12 cells/mm² in the periphery respectively. Importantly, as highlighted by Zhivov et al. (2005) the corneal presumed Langerhans cells were found as bright particles 15 µm in diameter, located at a depth of 35 to 60 µm. These results were similar to those of earlier studies (Schimmelpfennig 1982, Rosenberg et al. 2000, Rosenberg et al. 2002). The peri-central cornea observation revealed a presumed Langerhans cell density of 25 to 50 cells/mm² which was similar to the result obtained by Gillette et al. (1982) -Tables 1 and 2.

Given the above explanations of presumed Langerhans cells in the normal cornea, it is expected that presumed Langerhans cells in the cornea may be influenced by various types of corneal manipulations, such as corneal suturing, grafting, cauterisation and latex bead injection (Suzuki et al. 2000). Many studies (Gillette et al. 1982, Asbell et al. 1987, Lewkowicz-Moss et al. 1987) have shown that presumed Langerhans cell density increases with cornea infections, such as herpes simplex virus keratitis, and *Pseudomonas* infection. A study conducted by Resch et al. (2005) on nine injured eyes, using confocal microscopy, determined that the presence of a foreign body increased presumed Langerhans cell density in the cornea epithelium from 35 ± 21 cells/mm² in healthy eyes to 68 ± 24 cells/mm² in the affected eyes (p= 0.012) - (Resch et al. 2008). Corneal presumed Langerhans cell density was three times higher in patients with rheumatoid arthritis than in the controls. Presumed Langerhans cell densities in the central and peripheral cornea presented were 68 ± 71 cells/mm² and 126 ± 104 cells/mm² in rheumatoid arthritis respectively, compared to 23 ± 33 and 69 ± 33 cells/mm² in healthy individuals respectively (Marsovszky et al. 2012). The potential increase in presumed Langerhans cell density for patients with

rheumatoid arthritis might be due to a decrease in tear production (Marsovszky et al. 2012). Systemic diseases play a vital role in terms of the increase in corneal presumed Langerhans cell density, such as diabetes. In 2005, Popper et al. found that patients with mild and moderate retinopathy had a high number of presumed Langerhans cells: 2 ± 1 cells/mm² and 3 ± 1 cells/mm², respectively, compared to 1 ± 1 cells/mm² in the control participants ($p < 0.023$) - (Popper et al. 2005). A recent study demonstrated a significantly higher presumed Langerhans cell density in the corneas of diabetic individuals (17 ± 1 cells/mm²) compared to healthy people (6 ± 1 cells/mm², $p = 0.001$) - (Tavakoli et al. 2011). However, no significant correlation was found between presumed Langerhans cell density and the duration of the diabetes, sex, corneal sensitivity, or corneal nerve morphology. Interestingly, it appears that corneal diseases lead to the migration of presumed Langerhans cells to the cornea, while conjunctival inflammation can influence presumed Langerhans cell densities in the cornea (Suzuki et al. 2000). Tables 1 and 2 summarise the outcomes of presumed Langerhans cell density assessment, in the cornea, based on previous studies.

Table 1. Summary of the results of quantitative presumed Langerhans cells assessment in the healthy human corneas with different techniques.

Authors & Year	PLCD/ Central (cells/mm²)	PLCD/ Peripheral (cells/mm²)	Tool
Gillette et al. (1982)	0	75-150	ATPase-staining
Chandler et al. (1985)	7 ± 6 39 ± 13	142 ± 25 (Adults) 165 ± 60 (Infants)	ATPase-staining
Rosenberg et al. (2000)	34 ± 3	None	LSCM
Patel & McGhee (2005)	34 ± 3	None	TSCM
Mastropasqua et al.(2006)	24 ± 11	None	LSCM
Zhivov et al. (2005)	34 ± 3	98 ± 8	LSCM
Resch et al. (2008)	35 ± 22	None	LSCM
Guthoff et al. (2009)	59 ± 46	102 ± 27	LSCM
Lin et al. (2010)	34 ± 6	90.7 ± 8	LSCM
Tavakoli et al. (2011)	6 ± 2	None	Slit scanning CM
Marsovszky et al. (2012)	23 ± 34	69 ± 33	LSCM
Sindt et al. (2012)	29 ± 23	None	LSCM
Villani et al. (2013)	None	53 ± 34	LSCM
Machetta et al. (2014)	15	44	LSCM
Resch et al. (2015)	21 ± • 21	78 ± 40	LSCM

Abbreviations: None, no data; PLCD, presumed Langerhans cell density; LSCM, laser scanning confocal microscopy; CM, confocal microscopy; ATPase, *adenosine triphosphatase*

Table 2. Summary of the results of quantitative presumed Langerhans cells assessment in corneas of healthy animals.

Authors (Year)	PLCD Central (cell/mm²)	PLCD Peripheral (cell/mm²)	Tool	Study Models
Bergstresser et al. (1980)	0 ± 0	None	ATPase staining	Guinea pigs, hamsters & mice
Rowden (1980)	0 ± 0	None	ATPase staining	Guinea pigs& mice
Gillette et al.(1982)	None None None	15 – 20 25-50 50 - 100	ATPase staining	Guinea pigs Rats Mice
Rubsamen et al. (1984)	0 ± 0	None	ATPase staining & Immunofluore-scence (IF) assay	Mice
Lewkowicz-Moss et al. (1987)	9	None	ATPase-staining	Mice
van Klink et al. (1993)	≈ 10	None	ADPase staining	Chinese hamsters
Sankaridurg et al. (2000)	0 ± 0	13 ± 6	ATPase-staining	Guinea pigs

Abbreviations: None, no data ; PLCD, presumed Langerhans cell density; ATPase, *adenosine triphosphatase*.

1.3 Quantification of Presumed Langerhans Cell Density in the Conjunctiva

The conjunctiva is a highly vascular, immunologically tolerant tissue that covers the inner surface of the eyelids and the eye globe. It plays a role in maintaining a moist and hydrophilic ocular surface through the secretion of mucus. It consists of two components the epithelium, and the stroma. It contains significant numbers of immune cells (Forrester et al. 2010).

The conjunctiva is usually described in terms of three parts (palpebral conjunctiva; bulbar conjunctiva; and conjunctival fornix), according to the area of the anterior eye, where it is found, as discussed below:

The palpebral conjunctiva**:** The palpebral conjunctiva is a thin and vascular tissue covering the inner surfaces of the eyelid and comprises two major layers of tissue: the epithelial layer, and the stroma. The conjunctival epithelium consists of goblet cells, and non-epithelial cells, such as Langerhans cells (Efron et al. 2009).

The bulbar conjunctiva**:** The bulbar conjunctiva is thin, and transparent, and covers the outer surface of the globe. It is composed of two layers: epithelial (approximately 5-7 µm thick), and stroma. Based on light microscope observations, the bulbar conjunctiva contains Langerhans cells (Lawrenson 2002).

The conjunctival fornix: **C**onjunctival fornix is the zone between the bulbar and the palpebral conjunctiva. It allows the eyeball to move from side to side and up and down.

Böck et al. (1971) were the first to identify inflammatory cells in the conjunctiva of the normal guinea pig. A decade later, another study by Rodrigues et al. (1981) in the normal conjunctiva of selected species which evaluated presumed Langerhans cells. Based on *adenosine triphosphatase* staining, this identification revealed that the human conjunctiva has 250 to 300 cells/mm². Also presumed Langerhans cells were found in the conjunctiva of the Lewis rat, guinea pigs, and in mice. As observed in the previous study, presumed Langerhans cells, were identical to those explored by Bodaghi et al. (1997). They identified presumed Langerhans cells in the normal limbal conjunctiva with a density of 272 ± 37 cells/mm². Other studies of the conjunctiva reported the observation of presumed Langerhans cells in the conjunctiva (Steuhl et al. 1995, Suzuki et al. 2000, Kobayashi et al. 2005, Efron et al. 2009, Efron et al. 2009)- Table 3. Recently, however, Villani et al. (2013) were unable to detect presumed Langerhans cells in the bulbar conjunctiva

Table 3. Assessment of presumed Langerhans cell density in the healthy conjunctiva

Authors (Year)	Zone	PLCD (cells/mm²)	Tool
Steuhl et al. (1995)	Lateral superior	1	Langerhans-specific anti-CD1a antibody
Steuhl et al. (1995)	Bulbar conjunctiva	4.7	Langerhans-specific anti-CD1a antibody
Rodrigues et al. (1981); Sacks et al. (1986)	Conjunctiva	2.5-300	ATPase staining
Bodaghi et al. (1997)	Limbal conjunctiva	272 ± 37	Conjunctival biopsy CD1a antigen
Efron et al. (2009)	Conjunctiva	23 ± 25	LSCM
Le et al. (2011)	Conjunctiva	42 ± 9	LSCM

Abbreviations: PLCD, presumed Langerhans cell density; LSCM, laser scanning confocal microscopy.

Based on the anti-CD1a antibody, a quantitative analysis of presumed Langerhans cell density in excised human conjunctival biopsy specimens revealed that presumed Langerhans cell density in the superior temporal zone of the bulbar conjunctiva to be 1 cell/mm² (Steuhl et al. 1995). This contrasted with the results of Efron et al. (2009) who investigated presumed Langerhans cell density using laser scanning confocal microscopy and found a presumed Langerhans cell density of 23 ± 25 cells/mm² in

healthy bulbar conjunctiva. The later study found no significant difference in presumed Langerhans cell density between the nasal (32 ± 38 cells/mm²), temporal (43 ± 42 cells/mm²), superior (no cells where observed), and inferior (17 ± 49 cells/mm²) regions of the bulbar conjunctiva ($p = 0.082$) - (Efron et al. 2009). In contrast, Sacks et al. (1986) noticed significant regional variations in presumed Langerhans cell density in different zones of the conjunctiva. These disparate findings could be due to the lower sensitivity of the novel laser scanning confocal microscopy used by Efron et al. (2009) compared to the monoclonal antibodies employed by Sacks et al. (1986). The more sensitive method, allowed the researchers to more easily examine presumed Langerhans cell density. Interestingly, Steuhl et al. (1995) showed a significant reduction in presumed Langerhans cell density with age in the central inferior palpebral. However, one recent study by Wei et al. (2011) reported no alterations in presumed Langerhans cell density in the palpebral conjunctiva with age. These discrepancies may be due to the differences in the selection criteria of the participants.

Over the past decade, studies have revealed a significant amount about presumed Langerhans cells and have raised many questions about their role in disease. For example, McArdle et al. (1986) and Chen et al. (1989) reported an increase in presumed Langerhans cell density in a specific diseased conjunctivas, namely in Bowen disease tumours, squamous cell carcinomas, basal cell carcinomas, and in non-neoplastic epithelium adjacent to these neoplasms. Vernal conjunctivitis was identified as causing an increase in presumed Langerhans cell density in the conjunctiva (Takeuchi et al. 1983). Another study observed high presumed Langerhans cell density in patients with atopic dermatitis and ocular complications.

These findings illustrate that presumed Langerhans cells play a vital role in the immunopathology of the diseased conjunctiva (Yoshida et al. 1997). A recent study revealed that vernal keratoconjunctivitis increases presumed Langerhans cell density in both bulbar and tarsal conjunctiva (365 ± 216 and 232 ± 145 cells/mm² respectively) compared to the normal conjunctiva (42 ± 8 and 0 ± 0 cells/mm² respectively) - (Le et al. 2011). Bielory (2000) and Tabbara (2003) confirmed that the ocular allergic inflammatory response involved presumed Langerhans cells. Recently, presumed Langerhans cell recruitment in the ocular surface is associated with *contact lens wear* (Zhivov et al. 2007, Sindt et al. 2012).

1.4 Quantification of Presumed Langerhans Cell Density in the Ocular Surface of Contact Lens Wearers.

Contact lens wear has become a common solution for refractive error correction because of the significant advancements in contact lens designs and materials. Over the past few decades, these advancements have led to more affordable and safer contact lenses, with more convenient wearing modalities. Nevertheless, a contact lens still represents a foreign body in the eye. Thus, it may have physiological and mechanical impacts on the eye. This means that fitting a lens on the eye presents a direct interaction with the ocular surface; it creates anatomical and physiological changes on the globe. Practically, a soft contact lens covers the whole cornea, the limbus, and extends about two millimetres onto the bulbar conjunctiva to induce optimum vision and comfort. The soft lens can be temporarily displaced further onto the bulbar conjunctiva, up to about 2 to 4 mm from the limbus, a result of eye movement and blinking. Consequently, a potential effect on the ocular surface

structure and function can be induced. Excess staining, deep arcuate band staining with fluorescein staining (Lakkis et al. 1996), hyperemia, chemosis (Guillon et al. 2005) and an alteration of goblet cell density (Knop et al. 1992, Connor et al. 1994, Çakmak et al. 2003, Lievens et al. 2003) are reported complications caused by the compromise of the bulbar conjunctiva. The following paragraphs discuss the effect of contact lens wear on presumed Langerhans cell density in the conjunctiva and cornea, as well as the lid wiper.

1.4.1 Impact of Contact Lens Wear on Presumed Langerhans Cell Density in the Cornea.

The distribution of presumed Langerhans cells in the cornea has not been given more attention because it was investigated in only two studies (Zhivov et al. 2007, Sindt et al. 2012). It has been argued that the central cornea lacks presumed Langerhans cells; however, *ex vivo* studies have shown that the central cornea has presumed Langerhans cells (Catry et al. 1991, Hamrah et al. 2003). Maturation of presumed Langerhans cells and their migration to the cornea has become the focus of a number of studies. It was assumed that presumed Langerhans cells in the cornea were influenced by a variety of stimuli, such as irritation. One of the main sources of ocular irritation was assumed to be *contact lens wear*. As shown in Table 4, several studies have evaluated the effects of contact lens wear on presumed Langerhans cell density in the cornea. One study assessed extended wear of contact lenses (a period of two weeks) in the eyes of rabbits (Hazle et al. 1999). The eyes were enucleated when the lenses were removed; the findings showed that this type of lens encouraged presumed Langerhans cells to migrate into the cornea within two weeks of wear. In

addition, contact lens removal after a period of two weeks did not reduce the number of presumed Langerhans cells in the centre of the cornea (Hazle et al. 1999). Sankaridurg et al. (2000) fitted hydrogel contact lenses into the eyes of a guinea pig to investigate the possibility of contact lenses inducing the migration of presumed Langerhans cells into the central cornea. They evaluated the density of presumed Langerhans cells at the conjunctiva, and the peripheral and central corneal epithelia (the baseline for presumed Langerhans cell densities were 126 ± 16, 13 ± 6 and 0 ± 0 cells /mm² respectively). The number of cells in the peripheral cornea, central cornea and limbal conjunctival found in the eyes of the guinea pigs were 25 ± 6 ,7 ± 3 and 135 ± 24 cells/mm², respectively, one week after the lens fitting.

Zhivov et al. (2007) investigated the density of presumed Langerhans cells in the cornea and found that the cells were present in the centre (34 ± 3 cells/mm²) and periphery (98 ± 8 cells/mm²) of the healthy corneas. Further, presumed Langerhans cell density in contact lens wearers was reported to be two-fold greater (78 ± 25 cells/mm² centrally, and 210 ± 24 cells/mm² peripherally) than in healthy participants, implying chronic irritation of the eye. Interestingly, the study illustrated that presumed Langerhans cell density in the central cornea was lower in long-term contact lens wearers compared to those who had worn contact lenses for less than ten years. Recently, Sindt et al. (2012) established that the density of presumed Langerhans cells was significantly higher in the central corneas of lens wearers (64 ± 71 cell/mm²) than in those of a control group (29 ± 23 cell/mm²) - Table 4. Presumed Langerhans cell density in the corneal centre was affected by the lens materials and the lens care solutions. For example, a study by Sindt et al. (2012) examined the effect of a traditional polymer hydrogel lens (n =12) on presumed

Langerhans cell population in the cornea compared with silicone hydrogel lenses (n =41).Traditional lenses in this study included Biomedics® (American Hydron), Acuvue® types (Johnson and Johnson), Proclear® (Cooper Vision), Frequency® 55 (Cooper Vision), Freshlook® Daily (Ciba Vision), Dailies (Ciba Vision), and Soflens® 38 (Bausch and Lomb). Silicone hydrogel lenses, on the other hand, included O2 Optix (Ciba Vision), Biofinity (Cooper‑Vision), PureVision (Bausch and Lomb), and Acuvue Advance and Oasys (Johnson and Johnson). A higher population of presumed Langerhans cells was found in the cornea of silicone hydrogel lens wearers (47 ± 44 vs 69 ± 77 cells/mm², respectively, $p = 0.212$) - Table 4.

Further studies (van Klink et al. 1993, Su et al. 2006, Szliter et al. 2006) observed that presumed Langerhans cells were found in the corneas when eyes are fitted with contact lenses. Occasionally, the central cornea contains only immature presumed Langerhans cells (cells lacking dendrites, and being mobile in nature), while the peripheral part of the cornea contains immature and mature (cells bearing dendrites) phenotypes (Hamrah et al. 2002, Yamagami et al. 2005).

Table 4. Summary of outcomes of quantitative presumed Langerhans cell densities in contact lens wearers and non-lens wearers.

Tissue	Authors (Year)	Study Models	Technique used	Lens type	PLCD (control) (cells/mm²)	PLCD CL wearers (cells/mm²)	Effect of CL wear on PLCD
Cornea	van Klink et al. (1993)	Animal (Chinese hamster)	[1]ADPase stain	Parasite-laden	*Central* ≈ 10	*Central* ≈ 60	↑ Density
Cornea	Hazle et al. (1999)	Animal (Rabbits)	Zeiss Axiophot microscope	EW	*Central* 0	*Central* 80 ± 23	↑ Density
Cornea	Sankaridurg et al. (2000)	Animal (Guinea pigs)	ADPase stain+ Olympus light microscope at 5X magnification	Hydrogel	*Peripheral* 13 ± 6 *Central* 0	*Peripheral* 25 ± 6 *Central* 7 ± 3	↑ Density

Abbreviations: CR, Case report; PT, Pilot study; PLCD, presumed Langerhans cell density; EW, extended wear ; CM, confocal microscopy ;

[1] Adenosine diphosphatase (ADPase) staining is a convenient and sensitive method for detecting the existence of Langerhans cells in skin or cornea

Cornea& Conjunctiva	Szliter et al. (2006)	Animal (Rats)	Zeiss Axiophot microscope with Axiocam digital imagery at 20X magnification	Silicone hydrogel (EW)	No data available	No data available	No effect (conj.) ↑Density (cornea)
Cornea	Su et al. (2006)	Human(CR)	CM (NIDEK)	Soft daily wear	*Central* 0 *Peripheral* 0	No data available	↑ Density
Cornea	Zhivov et al. (2007)	Human	HRT/RCM	Hard & soft	*Central* 34 ± 3 *Peripheral* 98 ± 8	*Central* 78 ± 25 *Peripheral* 210 ± 24	↑ Density
Cornea	Sindt et al. (2012)	Human*(PT)	HRT/RCM	Soft lenses	*Central* 29 ± 23	*Central* 64 ± 71	↑ Density

Whether acute or chronic, the clinical complications of contact lens wear are discussed comprehensively in the literature. Contact lens wear can introduce disruption of tear film physiology (Holly 1981), devastating tear film reformation (Holly 1981), and increase tear film evaporation (Tomlinson et al. 1982). These lead to further complications such as dry eye (Baudouin et al. 1999, Tsubota et al. 1999, Brignole et al. 2000), and long-term ocular treatment as a result of formation of some contact lens complications such as severe corneal oedema (Efron et al. 1988).

Contact lenses, as superficial foreign bodies, may cause subclinical alterations to the ocular surface. It has been argued that daily or extended contact lens wear, especially soft lenses, causes subclinical inflammation of the conjunctival epithelium (Baudouin et al. 1999, Tsubota et al. 1999, Brignole et al. 2000) and decreases in goblet cell density and mucin production (Pisella et al. 2000, Pisella et al. 2001).

1.4.2 Impact of Contact Lens Wear on Presumed Langerhans Cell Density in the Conjunctiva.

A recent qualitative study by (Efron et al. 2010) aimed at examining the response of the bulbar conjunctiva to contact lenses using laser scanning confocal microscopy, observed presumed Langerhans cells in the conjunctiva of contact lens wearers. No significant difference in presumed Langerhans cell density between contact lens wearers (n= 11) (17 ± 17 cells/mm²) and healthy controls (n=11) (23 ± 25 cells/mm², $p = 0.545$) . However, the small sample size may have influenced the overall results, and it is highly likely that a larger sample size is necessary for greater statistical

power. To date, no other study has illustrated the effect of contact lens wear on presumed Langerhans cell density in the human conjunctiva.

Presumed Langerhans cell density in contact lens wearers in other parts of ocular surface, such as the lid wiper, may be affected by lens wear. Therefore, the next section will provide an overview about the lid wiper.

1.5 The Lid Wiper

The lid wiper is that portion of the upper eyelid that wipes along the anterior surface of contact lens or the ocular surface (Korb et al. 2002). It has a distinctly different conjunctival structure, composing of cuboidal, conjunctival, and para-keratinized cells, together with interspersed goblet cells, creating multiple layers of up to 15 layers. The lid wiper epithelium height varies from 100 µm in its initial portion (the crest of the inner lid border) to about 0.3 - 1.5 mm in the tarsal conjunctiva area, becoming wider in the nasal and temporal portions (Knop et al. 2011) - Figure 2.

Increase presumed Langerhans cell density in the ocular surface may related with contact lens-induced dry eye. Therefore, the next section will provide an overview about dry eye.

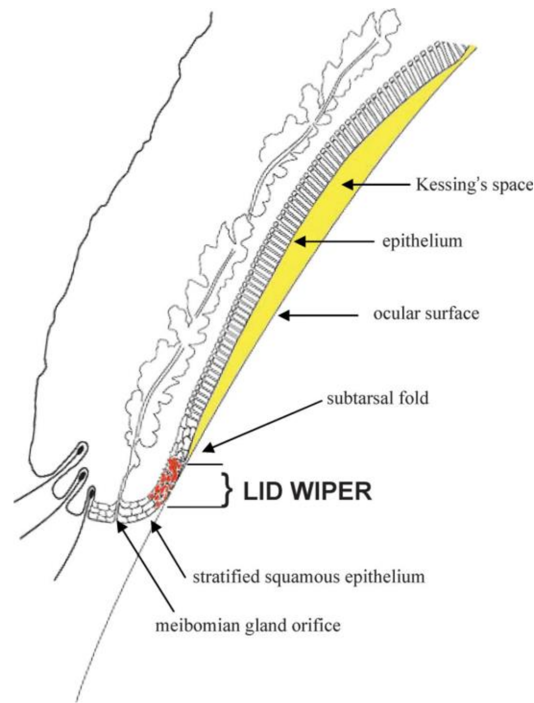


Figure 2. The lid wiper area (Korb et al. 2010).

1.6 Dry Eye

Dry eye may associate with the recruitment of presumed Langerhans cells in the cornea and conjunctiva. For example, aqueous tear–deficient dry eye, (either Sjögren's syndrome, or non-Sjögren's syndrome), was found to have a negative impact on presumed Langerhans cell density in the cornea. The number of presumed Langerhans cells in the cornea of the Sjögren's syndrome individuals increased from 34 ± 5 cells/mm² in the centre and 90 ± 8 cells/mm² in the periphery in normal corneas, to 89 ± 10 in the centre and 106 ± 10 cells/mm² in the periphery. The presumed Langerhans cell density of the Sjögren's syndrome group increased at both the centre and periphery of the cornea to 127 ± 23 and 157 ± 29 cells/mm² respectively, compared to the controls (Lin et al. 2010). In 2013, Villani et al. observed increased numbers of presumed Langerhans cells in patients with Sjögren's

syndrome (169 ± 48 cells/mm²) and meibomian gland disease (82 ± 38 cells/mm²) compared to controls (53 ± 34 cells/mm²) - (Villani et al. 2013).

In 2009, Waduthantri et al. conducted a study on 54,052 patients, to illustrate the cost and patterns of expenditure of dry eye treatment in Singapore. They found that the cost of dry eye treatment from one pharmacy only was more than US $1 million (Waduthantri et al. 2012). Another study showed that dry eye has a negative impact on quality of life (Waduthantri et al. 2012, Lemp et al. 2007). Also, dry eye is strongly related to anxiety and depression. A recent study reported that dry eye participants suffer from slower reading rate and reading difficulties (Ridder et al. 2014). However, dry eye symptoms are not affected by socioeconomic and demographic factors (Waduthantri et al. 2012). This emphasises the importance of finding a successful solution to the problem of dry eye.

In the normal eye, many ocular tissues, such as the lacrimal glands, cornea, conjunctiva, meibomian glands, and eyelids help regulate the tear film. These tissues are connected to each other via the sensory and motor nerves; they are known as the lacrimal functional unit. The lacrimal functional unit is responsible for maintaining the transparency of the cornea, the quality of the retinal image, and tear film integrity (Stern et al. 1998). In dry eye, the lacrimal functional unit is disturbed; this leads to a sensory, motor nerve and/or glandular damage or disease, in which many alterations to the operations of the lacrimal functional unit occur. These alterations include decreased tear secretion, altered tear composition and disturbed tear clearance (Stern et al. 1998, Lemp et al. 2007). Dry eye may lead to lid congruity alteration, low blink

rate, and meibomian gland dysfunction (Lemp et al. 2007). Extrinsic dry eye causative factors include low humidity environments and high ambient temperatures in which the ocular surface tear evaporation increases. Nakamori et al. (1997) and Wolkoff et al. (2006) have illustrated that work environments may lead to dry eye through long-term computer use alongside low blink rates. Contact lens wear plays a pivotal role in dry eye development because one-half to three-quarters of dry eye symptoms are secondary to contact lens wear. Dry eye symptoms were also the main reason for discontinuation of contact lens wear (Doughty et al. 1997, Pritchard et al. 1999, Nichols et al. 2005, Richdale et al. 2007).

In summary, dry eye is a challenging disease. It may occur due to internal factors (such as ocular inflammation) or due to external factors (such as low environmental humidity, high ambient temperatures or contact lens wear). Importantly, dry eye presents as different severities, whilst its signs and symptoms do not always correlate with each other (Nichols et al. 2004).

1.6.1 Prevalence and Characteristics of Dry Eye

The human tear film thickness is approximately 3 μm (King-Smith et al. 2000). The thickness depends on many factors, including sex, age, room temperature, and humidity (King-Smith et al. 2000). It contains three structures: an outer lipid layer, an intermediate aqueous phase, and an inner mucus layer.

There is also a variety of descriptions of the function of the tear film. For example, one description refers to four functions:

- Providing a smooth optical surface for normal vision;
- The maintenance of epithelial cell health;
- The maintenance of ocular surface comfort; and
- Protection from infectious insults (Pflugfelder et al. 2004).

Efron (2012) suggested two additional functions of the tear film:

- Supply the cornea with the necessary materials, such as oxygen, amino acids, glucose and vitamins; and
- Removal of waste, such as carbon dioxide and lactate (Efron 2012).

It would appear that any alteration to these functions may lead to irritation, inflammation, visual disturbance or infection (Pflugfelder et al. 2004).

The prevalence of dry eye in the general population have not been identified or reported precisely– Table 5. Studies showed that Asian eyes revealed a higher risk of dry eye disease than Caucasian (Albietz et al. 2004, Albietz et al. 2005).

Table 5. Prevalence of dry eye among people in different countries

Author(s)	Country	Participant number	Dry eye prevalence (%)
Moss et al. (2000)	US (Beaver Dam	3722	14.4
Lee et al. (2002)	Indonesia (Sumatra)	1058(age >20 years)	27.5%
Schaumberg et al. (2003)	United States	39,876	7.8% (among women aged 50 years and above)
Chia et al. (2003)	Sydney	1075	15.3%
Lin et al. (2003)	Taiwan	1361(age >64)	33.7%
Sahai et al. (2005)	India	500 (age >20 years)	18.4%
Rege et al. (2013).	India	4750	15.4%
Onwubiko et al. (2014).	Nigeria	402	19.2%
Vehof et al. (2014)	UK	3824 females	9.6%

Because of the selective bias in these hospital- based samples, the calculated prevalence rates are likely to be higher than the population-based studies. The level of these percentages makes dry eye a developing public health issue as it is one of the most common conditions seen in eye care clinics- Tables 5 and 6.

Table 6. The dry eye frequency among contact lens wearers.

Authors (Year)	No. of Participants	Dry eye (%)
Brennan et al. (1989)	104	75%
Doughty et al. (1997)	13517	50%
Begley et al. (2001)	1054	78%
Nichols et al. (2002)	367	45%
Guillon et al. (2005)	502 (soft CL wearers)	43%
Nichols et al. (2006)	360	55%
Jansen et al. (2010)	15 (soft CL wearers)	86%
Giannoni et al. (2012)	457	40%
Onwubiko et al. (2014)	402	19%

*CL, contact lens.

The discrepancies in the results could be explained by the differences of the ethnicity and age of the participants as well as the variety of definitions for dry eye. Further, there are no standardised and uniform diagnostic criteria for dry eye, and this will have contributed in part to the discrepancies in the prevalence reported. Another reason for the differences could be that some studies were population-based while others were hospital-based. This should drive researchers and clinicians to figure out a uniform, internationally-recognized diagnostic protocol for dry eye testing. Cut-off values for dry eye test are still devoid of an appropriate and uniform definition. In addition, environmental influences such as ambient temperature, aridity, wind, and irritants, are not taken into the consideration during dry eye testing (Savini et al. 2008). More attention should also be paid to test repeatability, sensitivity and specificity, as well as to the clinical circumstances. Dry eye is usually a symptomatic

disorder that varies from mild, through moderate to severe (Lee et al. 2002) and it can present as burning, stinging, grittiness, a foreign body sensation, tearing, ocular fatigue, and dryness (Lemp et al. 2007).

1.6.2 Techniques for Measuring Dry Eye

This section describes four dry eye tests: phenol red thread, non-invasive break- up time test, fluorescein staining, and dry eye questionnaires.

Phenol Red Thread Test

Schirmer's test has many drawbacks, such as low reproducibility, specificity, and sensitivity, lack of a definite site of paper placement in the conjunctival sac, as well as the potential of injury to the conjunctiva and cornea. It also has an uneven absorption of tears by the paper strip and discomfort is frequently reported (Cho et al. 1993), hence the phenol red thread test was developed. The idea, first introduced by Kurihashi et al in 1975 (cited by (Kurihashi et al. 1976)), used a thread to measure tear secretion. This idea was modified by Hamano et al. (1983) who used a cotton thread, impregnated with phenol red (a pH-sensitive indicator), to measure tear secretion. The wet portion of the thread changed from yellow to red when wetted with tears. The thread is inserted in the lower conjunctiva for 15 seconds; dry eye is suspected when less than 10 mm of thread was wetted.

The test was initially conceived as a test for tear secretion but later it was used to measure tear volume and/or the residual tears located on the lower conjunctival sac (Sakamoto et al. 1993, Cho et al. 1996). However, Tomlinson et al. (2001) argued

that the phenol red thread test is actually a test to measure the uptake of a small amount of fluid residing in the eye, which stimulates a low degree of reflex tearing rather than measuring the tear volume or residual tears on the conjunctiva. The phenol red thread test may be minimally uncomfortable, and thus, produce low reflex tear secretion compared with other invasive tests (Kurihashi et al. 1976, Sakamoto et al. 1993, Yokoi et al. 2000, Tomlinson et al. 2001).

According to a number of authors (Cho 1993, Cho et al. 1996, Nakaishi et al. 1999), the eyes should be kept closed during the phenol red thread test. In later studies (Hamano et al. 1990, Sakamoto et al. 1993, Little et al. 1994, Kwong et al. 1998, Cho et al. 2003, Miller et al. 2004, Glasson et al. 2006), researchers recommended that patients should be asked to keep their eyes open during the test and to blink naturally. Recent studies (Doughty et al. 2007, Bitton et al. 2013) assessing the outcomes from using the phenol red thread test with the eyes being kept open, versus the closed eye protocol, found no difference between the two protocols. While many researchers prefer to perform the test with the eyes closed, the majority administer the test with the eyes open. Nevertheless, it would be useful for this aspect of the protocol to be more clearly articulated in the reports related to the use of the protocol.

Non-Invasive Break-Up Time Test

The non-invasive break-up time test, first introduced by Mengher and colleagues in 1983 (cited by (Fullard et al. 1990)), is where an illuminated rectangular grid pattern is placed onto the surface of the cornea and observed through a Xeroscope . It was defined as ‘the time taken in seconds between the last complete blink and the

appearance of the first random disturbance of a grid'. The HIR-CAL grid system was also used to measure a non-invasive tear break-up time, based on a modified Bausch and Lomb keratometer. It mainly measures the pre-corneal tear film (Hirji et al. 1989). In 1993, Loveridge introduced a new kertoscope target with Loveridge grid. It is a thick sheet of perspex fitted into a hand-held Klein Kertoscope (Loveridge 1993).

These tests eliminate the effect of fluorescein instillation on the tear film, and thus is potentially more reliable and reproducible. However, the light source may lead the tear film to evaporate during periods of dry eye measurement, which then produces an artificial reduction of the tear break-up time (reviewed in (Lemp et al. 2007)).

The normal range of the non-invasive break-up time test is greater (40 to 60 seconds) than the normal range of the fluorescein break-up time test (Tonge et al. 1991) . The test uses the Toposcope, the Keratometer, the Tearscope or the Xeroscope. A reading of less than 9 seconds is considered to indicate dry eye (Morris 2006).

Fluorescein Staining

Fluorescein was first applied to the human cornea in 1970 (Norn 1970). Fluorescein staining is considered one of the most reliable methods for dry eye diagnosis as it can penetrate the interrupted part of the corneal epithelium. It stains the corneal cells without intrinsic cellular toxicity (Bron et al. 2003, Foulks 2003, Turner et al. 2005, Behrens et al. 2006). Fluorescein can be instilled either by fluorescein strips or by

1% or 2% sterile dose. It is recommended that the strip should be placed onto the lower palpebral conjunctiva (not onto the cornea) - (Efron 2012). To better recognise the staining, a yellow barrier filter (such as Wratten # 12 yellow) is recommended to enhance the visualisation of the staining over the conjunctiva, even for mild dry eye (Koh et al. 2003). Nevertheless, Savini et al. (2006) argued that corneal staining is not necessarily a sign of dry eye. It is argued this measurement tool is not sensitive and can only diagnose dry eye in approximately 10% of the total dry eye cases (Schiffman et al. 2000, Lin et al. 2004, Nichols et al. 2004). Fluorescein staining is unable to present direct proof of dry eye as it is not a sensitive and specific diagnostic dry eye tool (Savini et al. 2006, Yoon et al. 2011).The ideal volume of applied fluorescein is 2 to 5 μL (Zeev et al. 2014). Zeev et al. (2014) postulated that fluorescein may cause ocular surface toxicity.

Corneal staining among individuals with dry eye can be estimated using the Efron system (Efron 1999). It indicates five levels of severity of staining: normal (no staining), trace, mild, moderate, and severe. This system evaluates contact lens complications through corneal staining, conjunctival redness and papillary conjunctivitis (Efron 1999, Efron 2012). The Efron system is one of many that can be used to grade the severity of the dry eye and quantify anterior eye changes.

2.8.6 Dry Eye Questionnaires

Subjective responses in dry eye are of equal relevance as they assess the patient experience. There are more than fourteen dry eye-related questionnaires, for

example, the McMonnies questionnaire (McMonnies 1986), the Dry Eye Questionnaire (DEQ)-(Begley et al. 2003), the Contact Lens Dry Eye Questionnaire-8 (CLDEQ-8) (Chalmers et al. 2012) and the McCarty Symptom Questionnaire (McCarty et al. 1998) -Table 7.

Table 7. The standard dry eye diagnostic tools

Dry Eye Diagnostic Tool	Description	Purpose	Test Problems
Phenol Red Thread (mm/15 sec) *(PRT)*	Non-invasive tear test. The thread is 75 mm long. The last 3mm is placed on the lower conjunctiva for 15 sec. The red colour change of the thread is measured end to end in millimetres.	Tear volume.	Not clear whether the test is for tear volume or for tear production (Sakamoto et al. 1993, Cho et al. 1994, Cho et al. 1996, Tomlinson et al. 2001).
Schirmer's *I test (mm/15 sec)*	The rounded wick end is inserted into the lower fornix for 5 minutes. The wet area is measured in millimetres.	Aqueous tear deficiency.	Low dry eye detecting sensitivity and it takes a long time. (Hamano et al. 1983, Lucca et al. 1990)
Fluorescein Break-Up Time (BUT or FBUT)	BUT is the time between the last complete blink and the first appearance of a dry spot, using fluorescein dye.	Tear Film Stability.	The results may be affected by an extra amount of fluorescein. It is not reliably reflect disease (cited by (Fullard et al. 1990))

Non-Invasive Break-Up Time test (sec)(NIBUT)	NIBUT is the time measurement, in seconds, between the last complete blink and the first break in the tear film. The test uses the Toposcope (cited by (Fullard et al. 1990)), the Keratometer (Madden et al. 1994, Kojima et al. 2010), the Tearscope (cited by (Fullard et al. 1990)) or the Xeroscope (Pflugfelder et al. 1998).	Stability of the tear film.	Proper patient participation is critical.
Grading Staining techniques	-Fluorescein installation. - Rose Bengal staining. - Lissamine Green staining - Efron Grading Scales for corneal staining (Efron 1997, Efron 1999).	Estimates damage in dry eye.	Fluorescein installation provides stronger staining than RB. -Intra- observer agreement (Lemp 1995).
Tear Film Osmolarity	A lab-on-a-chip test that requires 50 nL sample of tears (Tomlinson et al. 2006).	Highly useful in dry eye diagnosis.	It is high cost.

		(Tomlinson et al. 2006)	
Optical coherence tomography	Reproducible, objective, and non-invasive instrument (Nguyen et al. 2012, Altan-Yaycioglu et al. 2013).	Measures the tear lake. Measure tear film thickness	Expensive. Time-consuming.
Interferometer	In vivo, non-invasive instrument, compares images for uniformity and colour (Doane 1989).	Measures lipid layer of the tear	High cost.. Time-consuming.
Biomarkers	They have different techniques to analyse the tear film (TearScan MicroAssay System (Fujishima et al. 1996) Sjö (Shen et al. 2012) Inflam-maDry Detector (Sambursky et al. 2013) & EyePrim (Lee et al. 2013))	Measure tear protein patterns	Expensive
McMonnies questionnaire	Self-administered questionnaire; consisting of 12 questions.	Focus on risk factors for dry eye disease	It is unable to detect the severity of dry eye (McMonnies 1986).

Dry Eye Questionnaire	Self-administered dry eye questionnaire; consisting of 23 questions.	Measure frequency. Intensity in the morning and late in the day. Degree of bother. (Begley et al. 2003)	Not suitable for contact lens wearers. Long list of questions. Symptoms are not important to determine the disease. (Begley et al. 2001, Begley et al. 2002)
Dry Eye Questionnaire-5	Self-administered DE questionnaires; consisting of only 5 questions.	Reports absence and presence of dry eye. Sjögren syndrome.	Not for contact lens wearers. (Chalmers et al. 2010)
Contact Lens Dry Eye Questionnaire	36 questions specific to symptoms of CL-related dry eye.	Focuses on ocular surface symptoms	Does not presume risk factors for DE syndrome.(Begley et al. 1994, Begley et al. 2000, Begley et al. 2001).
Contact Lens Dry Eye Questionnaire-8	A short form of the CLDEQ questionnaire; consisting of 8 questions.	Investigate frequency. Late day intensity of dryness. Discomfort.	It is unable to detect the severity of dry eye. (Chalmers et al. 2012)

Abbreviations: DE; dry eye; CL, contact lens.

Dry eye diagnosis is still a challenging task as the definitions of dry eye cover many aspects. Also, there are no particular universal protocols to estimate tear film deficiency, despite the availability of more than twenty dry eye examinations, and more than 100 published works about dry eye diagnosis.

1.7 Corneal Confocal Microscopy

Corneal confocal microscopy is a unique system that enables visualising the anterior ocular tissues *in vivo* - Figure 3 and Table 8. This device introduced by Stave et al. (2002) is currently used to investigate different parts of the eye, such as the cornea, limbus, tear film and conjunctiva (Stave et al. 2002, Zhivov et al. 2006, Guthoff et al. 2009). The laser scanning confocal microscope, uses a high intensity light source and a set of galvanometer scanning mirrors to assist the laser beam to be scanned over the back of the microscope (Webb et al. 1980, Webb et al. 1987, Masters et al. 1990). There are many clinical applications for the scanning confocal microscope, including the diagnoses of corneal dystrophy, keratitis, ocular surface disorders, meibomian gland diseases, corneal nerves, uveitis and glaucoma (Kymionis et al. 2013).

In summary, presumed Langerhans cell density in the ocular surface was discussed in a significant number of studies both in healthy and diseased conditions, using different investigative methods. However, the effects of contact lens wear on presumed Langerhans cell density in the cornea have been examined in very few publications, which were cross-sectional studies and have not discussed the effect of contact lens wear on the cells over time. The impact of contact lens wear on presumed Langerhans cell density in the conjunctiva was discussed in one study, which had a small sample size and was not evaluated the cells over

time. To date, no study has investigated Langerhans cell density in the lid wiper. Moreover, no study investigated the effect of contact lens wear on presumed Langerhans cell density in the cornea, conjunctiva and lid wiper in contact lens-induced dry eye. Therefore, the aim of the current studies were to address these issues.

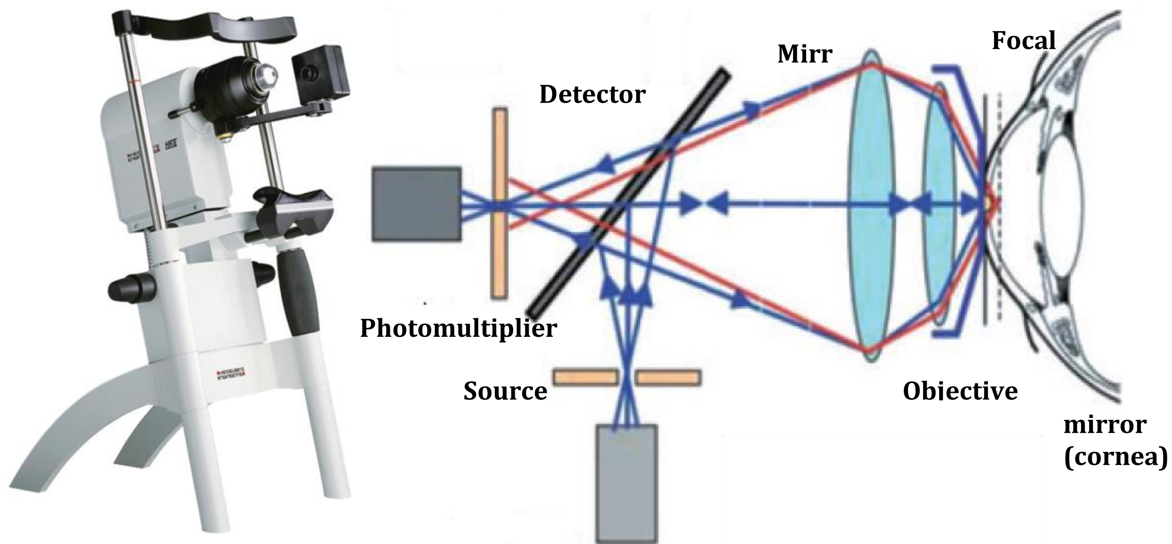


Figure 3. Laser-scanning confocal microscope: the Heidelberg Retina Tomograph 3 with Rostock Corneal Module (Courtesy of Heidelberg Engineering, GmBH, Dossenheim, Germany, (Guthoff et al. 2009)

Table 8. Specification of corneal confocal microscopy.

Component	HRTIII **(Efron et al. 2009, Efron et al. 2009, Efron et al. 2010)**
Scanning wavelength	670nm
Light source	Diode laser
The objective lens	60X water immersion
Working distance	-----
Image frame size	400 × 400 µm
Numerical aperture	0.9
Transverse resolution	2 µm
Optical section thickness	4 µm

In vivo confocal microscopy has obvious advantages compared with other techniques, not least being a more sensitive technique to document the ocular surface steady state compared with other techniques such as impression or brush cytology (Kojima et al. 2010, Alhatem et al. 2012). This advantage encourages researchers and practitioners to use corneal confocal microscopy to investigate ocular surface cells such as goblet cells and conjunctival epithelial cells (Kojima et al. 2010, Alhatem et al. 2012). Confocal microscopy is able to evaluate ocular surface cells at the cellular levels and simultaneously provide clear images without creating any significant effect on the target area (Villani et al. 2014). This technique is able to analyse thin, avascular, and multilayered tissue. It is suitable for investigating alterations in epithelial cells, meibomian glans, and corneal nerves (Villani et al. 2013). Corneal confocal microscopy enables the differential diagnosis of different pathogens, such as corneal

verticillata, keratitis, and conjunctival scar, compared with slit lamp biomicroscopy (Eckard et al. 2006, Messmer 2008). The technique is capable of investigating epithelial dendritic cells in the limbus and in the human cornea as compared with slit-lamp biomicroscopy and impression cytology (Nubile et al. 2009). However, confocal microscopy is unable to distinguish differentiated cells, because it induces only morphological details. This is mean that the identification of cell phenotypes requires *ex vivo* histology (Nubile et al. 2009). The finding of confocal microscopy needs interpretation by practitioners as this finding is based on cell reflectivity and light-scattering phenomenon (Nubile et al. 2009). Confocal microscopy has a limited field of view when scanning a tissue (Hillenaar et al. 2012). It is an expensive technique compared to slit-lamp biomicroscopy (Hillenaar et al. 2012). It requires extensive knowledge of ocular surface morphology to identify any alterations in the ocular surface structure (Niederer et al. 2010, Hillenaar et al. 2012). It needs calibration, and an experienced operator, which makes it hard to use compared to slit-lamp biomicroscopy (Hillenaar et al. 2011). Optical coherence tomography induces a larger field view, higher depth penetration and a shorter examination time compared with corneal confocal microscopy (Hillenaar et al. 2012).

Chapter 2: Overall Methodology

2.1 Participants

Pre-study investigations

Before undertaking the longitudinal study, repeatability of measuring presumed Langerhans cell density in the bulbar conjunctiva was conducted on 15 healthy participants (described in Chapter 3). This investigation was followed by further evaluation aimed at determining the number of images needed for an accurate measurement of the presumed Langerhans cell density in the cornea and conjunctiva. Ten healthy participants were recruited for this investigation (described in Chapter 3). All participants met the eligibility criteria described

Three primary studies addressed the research aims, presented in this thesis, with a total of 106 participants screened for eligibility. Eighty-three (83) non-contact lens wearers (47 females and 36 males) were eligible to participate. This cohort, with an average age of 30 ± 8 (mean ± SD) years (range, 18-50 years old) were enrolled. The distribution of the participants for the three studies is described below:

- Eighty-three (83) non-contact lens wearers participated in the study entitled “Effect of contact lens wear on presumed Langerhans cell recruitment in the cornea in dry eye and non-dry eye contact lens wearers” presented in Chapter 4.
- The same 83 participants participated in the study entitled “Effect of contact lens wear on presumed Langerhans cell recruitment in the conjunctiva in dry eye and non-dry eye contact lens wearers (Chapter 5).

- A subset of 66 participants participated in the study "Effect of contact lens wear on presumed Langerhans cell recruitment in the lid wiper in dry eye and non-dry eye contact lens wearers" described in Chapter 6.

The three additional studies were conducted with different population samples to investigate the effect of short-term contact lens wear, eye rubbing, and eye closure on presumed Langerhans cell recruitment to the central cornea. A total of 63 individuals were recruited for the aforementioned studies (Sections 3.3, 3.4 and 3.5, respectively). These studies used a contralateral design were comparisons were made between right and left eyes; therefore 126 eyes were evaluated.

Study Participants

To explore the effect of contact lens wear on corneal and conjunctival presumed Langerhans cells, participants were assigned to one of two groups after one week of contact lens wear. Thirty-five participants of 60 (58%) were assigned to the group with no contact lens induced-dry eye and 25 participants (42%) were assigned to the contact lens induced-dry eye group. Subsequent examinations were conducted after four weeks, and twenty-four weeks from the baseline visit. The remaining 23 age-balanced non-lens wearers were monitored over the same time course, and served as the control group.

In the study to explore the effects of contact lens wear on the lid wiper, participants were also assigned to the dry eye and non-dye eye groups. Of the 46 participants evaluated in this study, 29 participants (63%) had no contact lens induced-dry eye and 17 participants (37%) had contact lens induced-dry eye, and completed six months of lens wear. The remaining 20 age-balanced non-lens wearers were monitored over the same time period, and served as the control group.

A detailed explanation of the study was provided to the participants throughout the study, and written informed consent was obtained before the study began. Ethics approval for this study was obtained from the Queensland University of Technology Human Research Ethics Committee (Approval number 1300000117). The study was conducted in accordance with the principles of the Declaration of Helsinki.

To recruit potential participants, emails were sent to the Queensland University of Technology staff and students seeking their interest in participation. Invitations were sent to potential participants via twitter, the Queensland University of Technology classifieds, the Saudi Arabian Students Association in Brisbane, and Facebook, as well as by visiting with undergraduate students in the Queensland University of Technology International College.

The refractive correction of the contact lens required by each participant was determined by a subjective refraction. The participants were trained in the use of disposable contact lenses by the examiner, and they were provided with a leaflet and video recording of contact lens insertion, removal and care.

General Participant Inclusion and Exclusion Criteria

The inclusion and exclusion criteria were the same for all studies; however the individuals assigned to the contact lens wearing group had three additional exclusion criteria, as listed below. The inclusion and exclusion criteria are factors that are related to eye health and/or the success of contact lens wear.

Inclusion Criteria:

- Healthy volunteers providing written informed consent,
- No history of contact lens wear for six months prior to the first examination day,
- Age ranging from 18 to 50 years.

Exclusion Criteria:

- Recent history of ocular inflammation,
- Any history of ocular trauma or surgery,
- Current or long-term topical ocular medication with the exception of non-preservative artificial tear supplements,
- Systemic disease that may affect the cornea or conjunctiva,
- Blood pressure instability,
- Diabetes,
- Dry eye,
- Pregnancy or breastfeeding,
- Using oral contraceptives.

Additional exclusion criteria for the contact lens wearing group

- Astigmatism of more than -1.50 D,

- Myopia more than -7.00 DS,
- Hyperopia more than +2.00 DS.

These additional criteria were to ensure that the participant would have clear vision with the correction and have a good chance of success with contact lens wear.

2.2 Design of the Primary Studies

The design was a prospective, case-controlled study evaluating presumed Langerhans cell density in contact lens wearers. The control group and contact lens group (fitted with hydrogel contact lenses) made four visits to the Vision Testing room at IHBI. The baseline examination was followed by appointments at one week, 4 weeks, and 24 weeks. Figure 4 describes the number of participants who attended the screening, and follow-up visits. Presumed Langerhans cell density in the lid wiper was evaluated at the 24 week visit only.

Since the study aimed to understand components of the immunologic status of the eye during contact lens wear, the study duration needed to ensure there the confounding effect of adaptation to contact lens wear was considered. As adaptation to spherical contact lens wear typically takes up to one week, the six month evaluation should account for the effect of adaptation.

Slit-lamp biomicroscopy was performed, before and after confocal microscopy, to verify the integrity of the ocular surface.

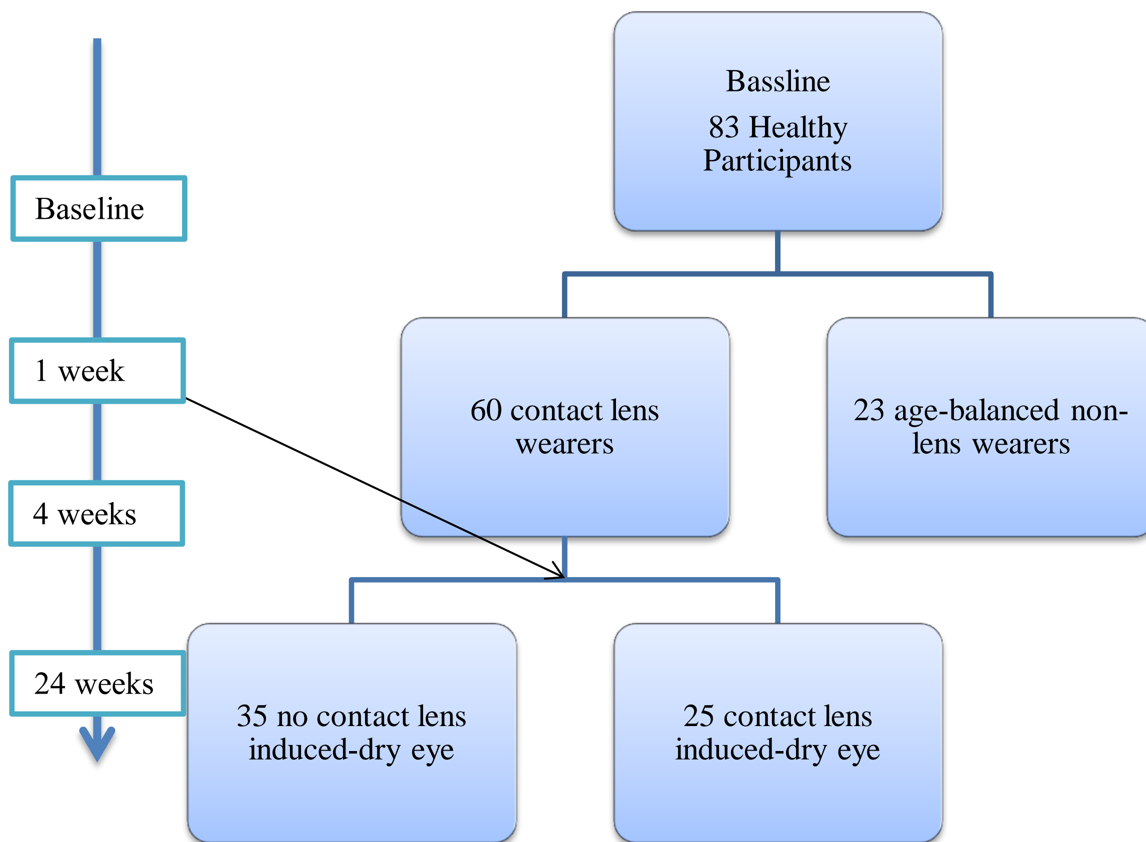


Figure 4. Number of participants enrolled into the study and subsequent group assignment.

The group with and without contact lens induced dry eye were assigned at the week visit.

The studies were conducted alongside a study undertaken by a fellow PhD student, Luisa Holguin, at Institute of Health and Biomedical Innovation, Queensland University of Technology. These concurrent studies utilised the same participants, and applied a similar methodology however addressed different aims and tissue assessments. The duties were distributed between candidates to assist with masking and labour distribution.

2.3 Dry Eye Evaluation

At the baseline examination of this investigation, four different dry eye diagnostic tools were chosen to ensure that dry eye was diagnosed using a comprehensive battery of validated subject questionnaires and objective tests.

The Dry Eye Questionnaire-5 (DEQ-5)-(Begley et al. 2003), the non-invasive break-up time test, the phenol red thread test and ocular surface staining. In the contact lens-wearing group, the Contact Lens Dry Eye Questionnaire-8 (CLDEQ-8) was also used.

Dry Eye Questionnaire-5

The Dry Eye Questionnaire-5 is a self-administrated questionnaire consisting of five questions. Through the questionnaire, dry eye symptoms were investigated, including: frequency; intensity in the morning (AM intensity); intensity late in the day (PM intensity); and degree of discomfort bother (Begley et al. 2003). A Likert-type scale was used, ranging

from ‘I don’t have dry eye’, being represented by the number 0, to ‘extremely severe’, being represented by the number 5. If a participant presented with a total score of 0 to 6 (out of 22), the individual was considered to have normal, healthy eyes without dry eye. However, a participant was considered to have dry eye, if the achieved score was more 6 (out of 22) (Appendix 4.1).

Non-Invasive Break-Up Time Test

Time taken for the tears to break up can be measured by observing the integrity mires of the keratometer. The participant was seated carefully with the chin on the chin rest of the keratometer and the forehead on the headrest. The keratometer was then adjusted and focused on the eye to be examined . The participant was asked to blink once and then stop blinking. A stopwatch was started after the last complete blink. At the first sign of any distortion of the image of the keratometer mires (three identical circles), the stopwatch was stopped, and the time recorded. If the participant blinked between measurements, the test was halted, and then repeated after several more blinks. The time interval between the last blink and the first sign of mire distortion was noted in seconds. The right and left eyes were assessed and the average of the three readings per eye was taken as the mean value. A reading of less than 9 seconds was considered to indicate dry eye (Morris 2006).

Phenol Red Thread Test

The phenol red thread test can be used to assess tear volume and utilises a yellow cotton thread impregnated with phenol red (Pcot-test, Tianjin Jingming New Technology Development Ltd., Tianjin Hi-Tech Industrial Park, China) placed in the lower conjunctival sac. In this study the thread was placed in the conjunctival sac on the temporal side of the eye for 20 seconds; a wet length of 10 mm or less was considered to indicate dry eye (Hamano et al. 1983).

Ocular Surface Staining

To observe ocular surface integrity, a drop of saline was installed on a fluorescein-impregnated strip which was then touched gently on the lower bulbar conjunctiva. The blue light on the slit-lamp biomicroscope and a yellow filter were used to evaluate the corneal epithelial disruption as an indicator ocular dryness (Bron et al. 2003). Corneal staining was graded from 0 to 4 (normal to severe staining) using the Efron Grading Scales (Efron 2012) as shown in Figure 5. Moderate or severe fluorescein staining was considered to indicate dry eye (Efron et al. 2001).

Figure 5. Efron grading scales for corneal staining (Efron 2012).

Contact Lens Dry Eye Questionnaire-8 (CLDEQ-8)

The Contact Lens Dry Eye Questionnaire-8 was developed to examine the distribution of dry eye symptoms among contact lens wearers, using a self-administered survey (Chalmers et al. 2012). This questionnaire consists of eight questions, and results in scores ranging from of 0 to 37, in which dry eye is represented by a score of more than 17 out of 37 (Appendix 4.2).

Dry eye examinations took place at each visit. During the baseline examinations, the participants underwent the four dry eye examinations unrelated to contact lens wear described above: Dry Eye Questionnaire-5, non-invasive break-up time test, phenol red thread test, and ocular surface staining. A participant who passed the Dry Eye Questionnaire-5, and one of the other dry eye examinations (non-invasive break-up time test, phenol red thread or ocular surface staining), was considered eligible for inclusion in the study. One week, four weeks and 24 weeks from the baseline examinations, the control participants underwent four dry eye examinations: Dry Eye Questionnaire-5, non-invasive break-up time test; phenol red thread test, and ocular surface staining. The Contact Lens Dry Eye Questionnaire-8 was used to assess the contact lens-wearing group, to facilitate classification into two subgroups: contact

lens-induced dry eye; and no contact lens-induced dry eye. The non-invasive break-up time test, the phenol red thread test, and the ocular surface staining test were conducted after four, and 24 weeks from the baseline examination.

2.4 Corneal Confocal Microscopy

Presumed Langerhans cell density was examined from images captured using a Heidelberg laser scanning confocal microscopy (HRT3) in combination with the Rostock Corneal Module. The Heidelberg laser scanning confocal microscopy utilizes a 60X objective lens with a numerical aperture of 0.9. The resultant image from this instrument has dimension of 400 × 400 µm and provides a transverse resolution of 2 µm and about 4 µm of an optical section thickness (Efron et al. 2010). Further, a 670 nm red wavelength helium-neon diode laser is used as the illumination source. As a class 1 laser system, it does not pose any ocular safety hazard. A new disposable Perspex applanating cap (Heidelberg Engineering GmbH Tiergartenstra βe 1569121 Heidelberg) was used for each participant. Before fitting the TomoCap to the Rostock Corneal Module, it was filled with a GenTeal Gel (Novartis Pharmaceuticals Australia Pty Limited, North Ryde, NSW, Australia). The gel facilitates an optical coupling of the Rostock Corneal Module objective lens with the back surface of the TomoCap. Before touching the eye, an anaesthetic drop (0.4% oxybuprocaine hydrochloride (Chauvin Pharmaceuticals Ltd, UK)) was applied to the participant eyes to control the ocular sensation. When capturing the images, the participant was advised to look at the fixation target for the corneal centre examination and at the opposite side, to the area of measurement for the nasal bulbar conjunctival examination. This procedure is supported using a side-mounted CCD camera that allows visualisation of a magnified and real-time image on the computer screen. The applanating lens was moved in small increments in the vertical and

horizontal axes, while the focal plane of the device was gradually moved into the sub-corneal and conjunctival tissue with the aim of capturing different groups of presumed Langerhans cells.

The number of presumed Langerhans cells was counted using the in-built counting tool of the instrument. From each (400 × 400 μm) image the cell density was determined as the number of cells per square millimetre. A mean cell density was determined from five images from the cornea and six images from the conjunctiva.

It has been demonstrated that presumed Langerhans cells are located at the level of lower intermediate epithelial cells, basal epithelial cells, Bowman's membrane, and subepithelial nerve plexus of the cornea (Zhivov et al. 2007). They have also been located within the epithelium and the stroma of the conjunctiva (Efron et al. 2009). Therefore, this study focused on the areas in which presumed Langerhans cell were previously reported.

2.5 Contact Lenses

The 'Biomedics® 1 day Extra' soft contact lens was used for the study -Figure 6. The hydrogel material is oculficlon D material with 55% water content. The lens does not provide high oxygen performance compared to the latest generation of silicone hydrogel lens. For example, the oxygen permeability (Dk) and oxygen transmissibility (Dk/t) of the hydrogel lens Biomedics® 1 day Extra at -3.00 D is 45 barrer or 10^{-11} (cm^3 O_2 cm)/(cm^3 sec mmHg) and 27 x 10^{-9} (cm/s)(mlO_2/ml x mm Hg), respectively. On the other hand, oxygen permeability of silicone hydrogel lenses such as Focus Night & Day, O_2 Optix, Acuvue Oasys, PureVision and Acuvue Advance are 140, 10, 103, 91 and 60 barrer respectively

(Efron et al. 2007). Being a hydrogel lens, it provided a greater physiological challenge to the anterior ocular structures. The refractive correction of the contact lens was determined by subjective refraction. The participants were trained in the use of disposable contact lenses by the examiner. They were provided with a leaflet and video recording of contact lens insertion, removal, and care.

Figure 6. The 'Biomedics® 1 day Extra' soft contact lens

2.6 Statistical Analysis

To examine repeatability, Bland-Altman (Bland et al. 1986) analyses were performed to determine the agreement between two different methods or techniques.

The Bland-Altman plot presents the difference between the two methods plotted against the average of the two methods of measure, with an indication of the mean difference between two methods of measurement and 95% limits of agreement (LoA) between the methods (Bland et al. 1986).

SigmaPlot version 12.5 (Systat Software, Inc. SigmaPlot for Window) was used to generate the Bland-Altman plot.

In the current study, an unpaired *t*-test was used to compare continuous outcome variables between the two different groups (i.e dry eye vs non-dry eye) of unequal sample size.

Analysis of variance (ANOVA), with post-hoc tests, was used for the comparison of three or more groups of data (i.e contact lens induced-dry eye vs. contact lens induced-dry eye vs non-contact lens wearers). A repeated measure is robust to normality violations (Fitzmaurice et al. 2012).

Because the linear mixed model includes missing values in its analysis, it was used in the current studies to determine the significance of any differences between and within the groups. A *p*-value of less than 0.05 was considered significant.

A natural log transformation was also explored for rendering the data more normally distributed. However, this has the drawback of altering the original data and an interpretation would now be only valid for the log transformed data and not the original. Given the robustness of liner mixed model to departures from normality, it was decided to use the raw data and present these results (Fitzmaurice et al. 2012).

Chapter 3: Factors Influencing Presumed Langerhans Cell Density in the Ocular Surface

3.1 Repeatability of Measuring Presumed Langerhans Cell Density in the Bulbar Conjunctiva

Preface

In the beginning of this thesis, this section investigates a fundamental component of the research methodology for the current thesis, which is intra-observer repeatability. It is essential to evaluate the effect of change or differences between studies. Exploring repeatability is a main principal of conducting a scientific research as it assesses to avoid any misinterpretation of data. There have been no prior reports of the repeatability of measuring of presumed Langerhans cells in the cornea and conjunctiva.

3.1.1 Introduction

A major ophthalmic innovation this century is the corneal confocal microscope, which is an essential tool in investigating the integrity of ocular tissues, such as the cornea, (in various conditions including keratoconus (McCarty et al. 1998), and keratitis (Efron 2007)), conjunctiva (Messmer et al. 2006, Efron et al. 2009) and limbus (Miri et al. 2012). The microscope also aids the assessment of the responses of the cornea and conjunctiva to contact lens wear (Efron 2007).

3.1.2 Research Question and Hypothesis

A. Research Problem

The repeatability of measuring cell densities in the ocular tissues is unknown and there have been no previous published reports of the repeatability of measuring presumed Langerhans cell density in the bulbar conjunctiva.

The current study was carried out to identify the degree of repeatability when measuring presumed Langerhans cell measurements, using *in vivo* confocal microscopy in conjunctival tissue.

B. Aim

The aim of the current study was to determine the repeatability of measuring presumed Langerhans cells in the ocular surface .

C. Research Questions

Is *in vivo* measurement of presumed Langerhans cell recruitment in the ocular surface repeatable?

D. Hypothesis

It is hypothesised that *in vivo* measurement of presumed Langerhans cell recruitment in the ocular surface is repeatable when undertaken by one observer under identical testing

conditions 24 hours apart. This is because temporary superficial ocular surface defects were observed after conducting confocal microscopy examination, as a result of the direct touch of TomoCap with the examined tissue. This defect recovered a few hours after the examinations.

3.1.3 Methods

A. Participants

Fifteen (15) healthy participants (4 females and 11 males; aged 31 ± 9 (mean ±SD) years, range 21 to 57 years) from the Queensland University of Technology staff and students, participated in this study after fully understanding the concept and the possible consequences of the study. Written informed consent was obtained from all participants before their inclusion in the study. A slit-lamp examination of the anterior ocular surface was performed before commencing the study.

Individuals with history of corneal surgery or trauma, diabetes, blood pressure instability, current or long-term topical ocular medication, history of contact lens wear, who were pregnant and/or breastfeeding, or who were using oral contraceptives were excluded.

B. Corneal confocal microscopy

Confocal microscopy was performed using the technique described in Chapter 2, section 2.4. Laser scanning confocal microscopy, was conducted on one eye on two separate occasions. This occasions were separated by at least 24 hours, with more attention being given to the measurements in the morning period (7:30 am to 12:00 pm) to avoid the potential confound of diurnal variations in cell number or appearance.

C. Statistical analysis

The intra-class correlation coefficient was used to compare the Langerhans cell density from the first test with the second test. The Bland-Altman plots shows the mean difference between the two methods of measurement and the 95% limits of agreement between the methods (Bland et al. 1986). The significance of any differences between the test and the retest was evaluated using a paired *t*-test, and a p-value of less than 0.05 was considered significant.

3.1.4 Results

Participant characteristics are shown in Table 9. The study revealed no significant difference between the presumed Langerhans cell measurements for the test and the retest (pair *t*-test; p = 0.466). The mean difference between the test and retest was 0.53 cells/mm², or 6% of the mean, while the intra-class correlation coefficient was 0.950, with a 95% limit of agreement (average difference ± 1.96 standard deviation of the difference) of -5.85 to 4.78. The Bland-Altman plot visually illustrates the repeatability of the technique shown in Figure 7.

Table 9. Characteristics of study participants.

Participants Details	Figures	Range
Age (mean ± SD)	31 ± 9 years	21 to 57 years
Sex (male/female)	4 females/ 11 males	--------
*PLCD(cells/mm²) (mean ± SD)	Test= 9 ± 8 Re-test= 8 ± 8	0 to 27 0 to 25

*PLCD, presumed Langerhans cell density.

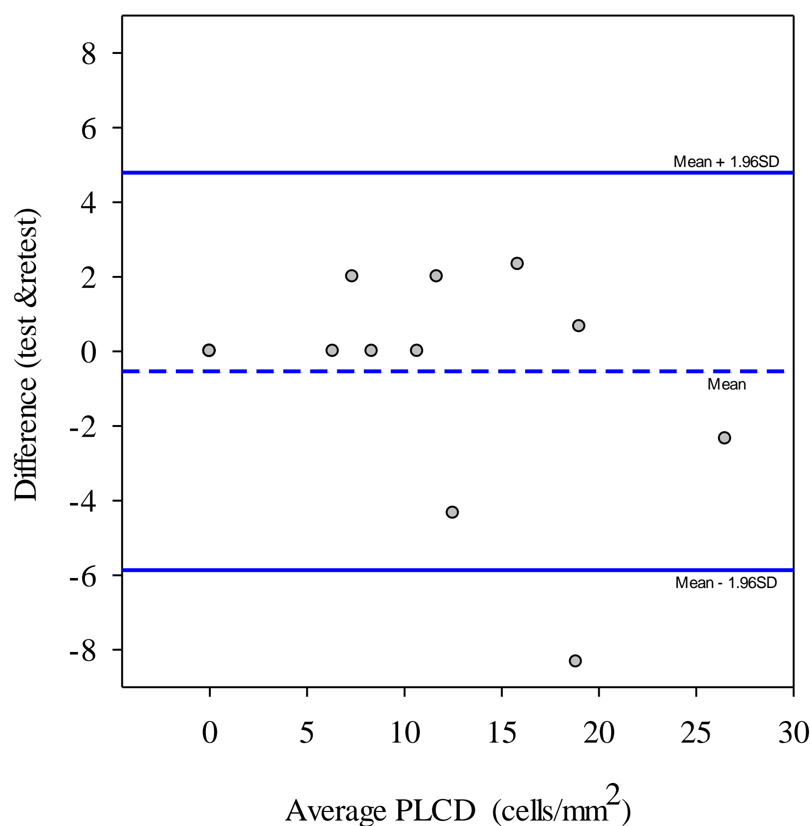


Figure 7. Bland-Altman plot of the differences in presumed Langerhans cell density between the first and second visit against the mean of the two visits of the 15 participants in the study. The solid line represents the 95% limits of agreement and the dotted line represents the mean difference between test and retest. PLCD, presumed Langerhans cell density.

3.1.5 Discussion

The purpose of this study was to investigate the repeatability of measuring presumed Langerhans cell density in the bulbar conjunctiva. To date, few publications have examined the variability in the measurements of the ocular tissue using laser scanning confocal microscopy. One study has been identified investigating repeatability using corneal confocal microscopy in the cornea. Makrynioti et al. (2010) examined the central, mid-peripheral, and limbal cornea of eight healthy individuals, and found that confocal microscopy is repeatable when evaluating corneal layers at various locations. The current study is the first to evaluate changes in presumed Langerhans cell density in the conjunctiva, using laser scanning confocal microscopy in healthy participants.

The results of the study indicate good within-observer repeatability in the measurement of presumed Langerhans cell density in the human bulbar conjunctiva (the intra-class correlation coefficient was 0.950). Intrasessional repeatability was 9 ± 9 cells/mm^2 in the first visit and 9 ± 8 cells/mm^2 in the second visit, with a range of 0 to 28 cells/mm^2 and 0 to 25 cells/mm^2, respectively. The study showed no significant difference in presumed Langerhans cell density in the human bulbar conjunctiva between the test and retest measurements, which indicated that the measurement can be applied.

3.1.6 Conclusion

The current study demonstrated a good intra-sessional repeatability for presumed Langerhans cells in the human conjunctiva such that any differences observed between test (contact lens) and control (no contact lens) or over time was not due to the technique. Corneal confocal microscopy is capable of assessing presumed Langerhans cell density in the ocular surface.

This information are important in evaluating the immune response in healthy and diseased eye conditions using corneal confocal microscopy. The between-observer repeatability is yet to be explored.

3.1.7 Subsequent Study

The above study illustrated an important finding in terms of repeatability of measuring presumed Langerhans cells in the ocular surface. It illustrated that measuring presumed Langerhans cells in the bulbar conjunctiva is repeatable. The study also showed that corneal confocal microscopy is able to evaluate presumed Langerhans cells in the ocular surface without any significant effects on presumed Langerhans cell recruitment. The number of analysed images captured by confocal microscopy, in order to actually evaluate presumed Langerhans cells, has not been explored. The number of selected images should be a scientifically based-selection. Therefore, the following section of this chapter will carefully explore optimal image sampling for determination of presumed Langerhans cell population in the central of the cornea and bulbar conjunctiva. The findings of the study will help to avoid misinterpretation of presumed Langerhans cells in the ocular surface as a result of arbitrary selection of analysed images.

3.2 Optimal Image Sampling for Determination of Presumed Langerhans Cell Density in the Central of the Cornea and Bulbar Conjunctiva

3.2.1 Introduction

Images of a tissue using confocal microscopy in the horizontal (X-Y) plane, and in the Z plane, at different ocular tissue depths. In studies published to date, *arbitrary numbers* of images have been used for the analysis of cell variables under the implicit assumption that these are a representative sample of the inflammatory cells on the ocular surface. A few images, typically between two and eight, have been assessed by a number of researchers (Zhivov et al. 2005, Mastropasqua et al. 2006, Mayer et al. 2007, Zhivov et al. 2007, Efron et al. 2009, Le et al. 2011, Tavakoli et al. 2011, Villani et al. 2011, Wei et al. 2011, Marsovszky et al. 2012, Sindt et al. 2012, Villani et al. 2013). However, these images were chosen without evidence-based studies, and represent only approximately 0.2% of the average corneal and conjunctival surface. Thus, capturing and analysing a small number of images may lead to the misinterpretation of the results and a possible bias. In clinical practice in particular, a balance between accuracy and logistic feasibility is essential in the investigation of such a biological parameter. Given the complicated corneal and conjunctival physiology, it is critical to find a scientific method that identifies the minimum number of images that would represent the whole corneal or conjunctival tissue.

3.2.2 Method

This was a prospective cross-sectional study. Ten participants were enrolled in this study; written informed consent was obtained from all participants before their inclusion in the study. A slit-lamp examination of the anterior ocular surface was performed before commencing the study. Participants had no history of ocular trauma, surgery, ocular disease or systemic disease affecting presumed Langerhans cells in the ocular surface. The mean ± SD age for both experiments was 30 ± 4 years with a range of 25 to 38 years. After the participants' inclusion in the study, a local anaesthetic (benoxinate hydrochloride 0.4%, Chauvin, France) was applied to the eye. The images were captured using a laser scanning confocal microscope (see Chapter 2; section 2.4). The microscope is suitable for imaging the central corneal and conjunctival layers as it is capable of generating good quality, high-contrast images. A minimum of 100 images from the central cornea and 100 images from the bulbar conjunctiva were captured, so that significant area of the assessable tissue was examined. Of these, three, four, five etc, up to ten clear frames from the high-quality digital images, with an overlap of no more than 20%, were randomly selected for the analyses. In the study, the corneal centre was defined as the point of contact between the corneal surface and the disposable Perspex cap. The participants were advised to look at the fixation target, typically using the contralateral eye. To examine the conjunctiva, participants were asked to fixate a target so the Perspex cap made adequate contact with the tissue under examination. The number of images used (from three to ten) was plotted against the standard deviation to assess the optimal images necessary to observe a stable level of variability.

3.2.3 Results

Figures 8 and 9 show the number of images against the standard deviation of each image. The figures show that standard deviation decreases initially then plateaus as the number of images increases. At around five or more frames from the cornea centre and approximately six or more frames from the bulbar conjunctiva, the averages of the standard deviation become stable, illustrating that the five frames from the central region of the cornea and the six frames from the bulbar conjunctiva are the minimum frames need to achieve valid data.

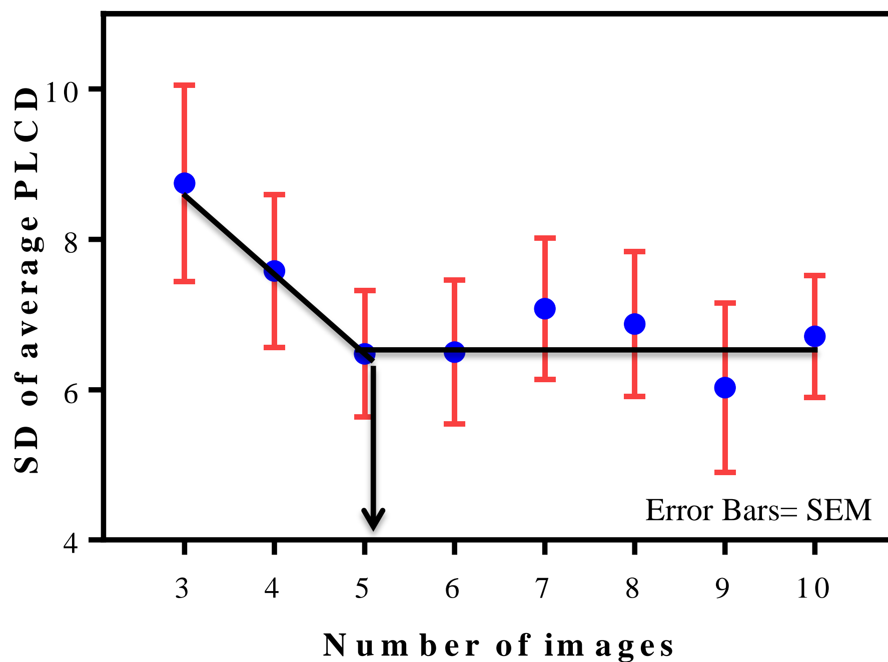


Figure 8. Number of images versus standard deviation of the mean values of presumed Langerhans cell density (PLCD) in the corneal centre, measured by corneal confocal microscopy of ten normal control participants. Since taking the mean PLCD from greater than five images does not appreciably improve the standard deviation, this number of images was assessed for determining PLCD of the central cornea throughout the thesis.

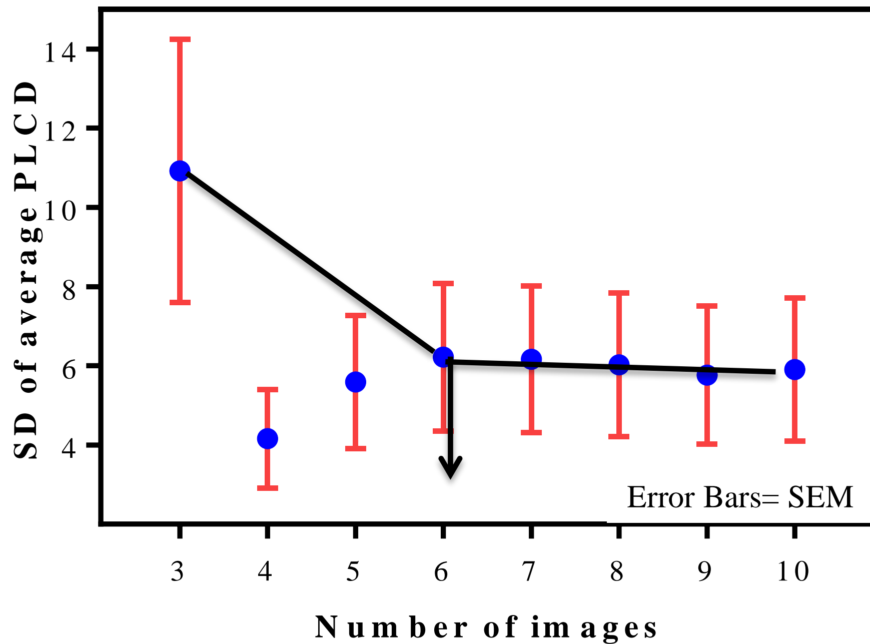


Figure 9. Number of images versus standard deviation of the mean values of presumed Langerhans cell density (PLCD) in the nasal bulbar conjunctiva, measured by corneal confocal microscopy ten normal control participants. Since taking the mean PLCD from greater than six images does not appreciably improve the standard deviation, this number of images was assessed for determining PLCD of the nasal bulbar conjunctiva throughout the thesis.

3.2.4 Discussion

The purpose of current study was to identify the number of images from the conjunctiva and cornea, using laser scanning confocal microscopy, to achieve an accurate and repeatable presumed Langerhans cell density count. The number of images required to achieve the goals were determined, with the level of accuracy depending on factors that include image quality and participant cooperation. Each image takes approximately three minutes to analyse. In the

current study, the analysis of determination of the number of images was started with three frames as this number was the minimum number taken to calculate presumed Langerhans cell density in the ocular surface as shown in the previous studies (Efron et al. 2009, Le et al. 2011, Villani et al. 2011, Villani et al. 2013).

This study presents what is believed to be the first *in vivo* evaluation of the optimum number of images required to analyse presumed Langerhans cells in the cornea and conjunctiva. The previous investigators (Efron et al. 2009, Le et al. 2011, Villani et al. 2011, Villani et al. 2013) - captured at least five good-quality frames; the average of the three clearest images were analysed. As the number of the analysed images was much smaller than the number of images used in the main study, there was the potential that different results for the investigated cells would be found.

The number of images used to represent presumed Langerhans cells in the cornea and conjunctiva vary between studies. For example, in their studies, Efron et al. (2010) used three images, Zhivov et al. (2007) and Sindt et al. (2012) used four images, while Marsovszky et al. (2012) used five images to represent presumed Langerhans cells either in the cornea and conjunctiva. The aforementioned studies selected different numbers of image frames to represent presumed Langerhans cells in the cornea and conjunctiva. This variation in sample size may be the main reason of the differing study results.

3.2.5 Conclusion

This study showed that that the minimum number of images required to properly evaluate presumed Langerhans cells is five frames for the central cornea and six frames for the bulbar conjunctiva. The future studies apply this finding to the sampling procedures.

3.2.6 Subsequent Study

A number of analysed images that evaluated presumed Langerhans cell recruitment in the ocular surface, using corneal confocal microscopy, shows an obvious variation most likely due to arbitrary selection of the analysed images, which may cause a significant difference in the final outcomes. The above study illustrated that at least five images from the corneal centre and six images from the nasal bulbar conjunctiva should be evaluated when investigating presumed Langerhans cells in the ocular surface.

The association between contact lens wear and presumed Langerhans cell population has been evaluated in the previous literatures, but both were cross-sectional studies (Zhivov et al. 2007, Sindt et al. 2012). These studies showed that presumed Langerhans cell recruitment increased significantly after many years of lens wear. It is obvious that the time course of this upregulation in the cell density is unknown. Therefore, following experiment will explore the short-term effect of contact lens wear on presumed Langerhans cell density in corneal centre.

3.3 Short-Term Time Course of Presumed Langerhans Cell Recruitment into the Cornea during Contact Lens Wear.

This section discusses the effect of short-term contact lens wear on the number of presumed Langerhans cells in the centre of the cornea.

3.3.1 Introduction

It may be important to understand the short-term time course to interpret the longer-term impact of changes in presumed Langerhans cells in the ocular surface. This might lead to further understanding of the sub-clinical inflammatory response in the ocular surface.

One of the main components of the ocular surface defence system is the presence of Langerhans cells (Gillette et al. 1982). These Langerhans cells provide a protective cover for the ocular surface (conjunctiva and cornea) to ensure good ocular function and integrity. The cells have been described in the published literature since the earliest microscopic examinations of the ocular surface (Segawa 1964). Nevertheless, only a few studies have evaluated their density in the ocular surface, and those studies have typically focused on the cornea. The research evaluated Langerhans cells through cross-sectional studies which provide little information about the cells over either long or short term periods.

3.3.2 Research Question and Hypothesis

Research Problem and Aim

The effect of contact lens wear on presumed Langerhans cell density in the ocular surface has been addressed in other studies (Zhivov et al. 2007, Sindt et al. 2012). However, the short-term effect of contact lens wear on presumed Langerhans cell density in the ocular surface, over an eight-hour time course, has previously not been investigated. Therefore, the aim of the study was to illustrate the impact of contact lens wear on presumed Langerhans cell density in the central cornea over an eight hour period of wear, using *in vivo* confocal microscopy.

Research Question

What is the impact of contact lens wear on presumed Langerhans cell recruitment in the central cornea in a period of eight-hour time course?

Hypothesis

For the current study, it is hypothesised that presumed Langerhans cell density may rapidly increase during the first few hours of lens wear as a result of an immunological reaction of the eye, and reach a significant number by the end of the eight hours.

3.3.3 Methods

This was a short-term, case-controlled prospective study, whereby one eye served as the test eye and the contralateral eye served as the control. The ten healthy participants, students and staff from the Queensland University of Technology (aged 30 ± 5 (mean ±SD) years, range, 23 to 39 years) were informed fully, at the outset, about the concept and possible consequences of the study. Written informed consent was obtained from all participants before their inclusion in the study. The study procedures were performed in accordance with the principles of the Declaration of Helsinki and the Queensland University of Technology Human Research Ethics Committee provided ethics approval (Ref 1300000117) (see Appendix 1). The right and left eyes of the ten participants were included for the examination. Individuals with a history of corneal surgery or trauma, diabetes, blood pressure instability, current or long-term topical ocular medication, a history of contact lens wearing, being pregnant or breastfeeding, taking oral contraceptives, or with symptoms of dry eye, were excluded. A slit-lamp examination of the anterior ocular surface was performed before the study commenced.

After conducting the baseline measurements of both eyes, using corneal confocal microscopy, a low power soft hydrogel lens (Biomedics® 1 day Extra- CooperVision) was fitted into one eye and the fellow eye served as the control. Corneal confocal microscopy (explained in detail in Chapter 2; section 2.4) was conducted again for both eyes after two hours of the baseline measurements. The measurements were repeated every two hours for a period of eight hours. At each 2-hour time point, a new contact lens of the same power (either -0.25 or +0.50 dioptres) was inserted into the test eye to avoid lens infection. For each eye, five high-quality digital images (out of 100), not overlapping by more than 20% were randomly selected for

analyses To ensure that a similar corneal location was re-measured at the each visit, the participants were advised to look at the red reflex of the instrument; the reflex was aligned at approximately the centre of the pupil. A slit-lamp biomicroscopy examination was performed after each 2-hour time point to verify that the integrity of the ocular surface had not been compromised during the study.

In this study hydrogel lenses were used because they give impart greater physiological stress on the cornea compared to silicone hydrogel lenses as a result of their lower oxygen transmissibility, despite both hydrogel and silicone hydrogel being safe and effective for daily wear. To maximise the effect, the hydrogel lens was chosen to attempt to provide a physiological stress for the cornea, within the normal accepted clinical guidelines.

A post-hoc power analysis conducted in respect of the size effect observed at the two hours-time point revealed that, for a 1-sided test and $\alpha = 0.05$, 80% power was achieved with the sample size of 10 participants per group.

Statistical methods

The difference in the presumed Langerhans cell density between experimental and control eyes caused by short-term contact lens wear was evaluated using a linear mixed model analysis (forcing control eye and time two hours as the reference value). A supplementary mixed model analysis was undertaken to analyse differences in presumed Langerhans cell density values between the two-hour subsequent time points. A *p*-value of less than 0.05 was

considered statistically significant. The data were analysed using IBM SPSS Statistics for Windows, Version 21.0 IBM Corp. Armonk, NY, USA.

3.3.4 Results

At baseline no statistically significant differences in presumed Langerhans cell density measurements was observed between the experimental and the control eyes – Figure 10. The baseline mean of presumed Langerhans cell density for the experimental eyes, prior to contact lens wear, was 18 ± 19 cells/mm^2 (range from 6 to 69 cells/mm^2), while the mean for the control eyes 20 ± 19 cells/mm^2 (range from 6 to 68 cells/mm^2)-(p=0.262). Presumed Langerhans cells density significantly increased after two hours of lens wear in the test eye to 36 ± 32 cells/mm^2 (baseline vs 2 hours $p < 0.001$), and decreased gradually over the next six-hour period to eight hours where presumed Langerhans cells density was 30 ± 31 cells/mm^2, being still greater than the baseline (p = 0.045). Tables 10 and 11 show the mean and p-values of presumed Langerhans cell density over the eight-hour time course. Figure 10 shows that the interaction between the group (eye) and time was significant ($F_{(4,80)}$= 3.099, p = 0.020); for example, the contact lens wearing eyes and the control eyes behave differently over time (p = 0.020). When accounting for the repeated measures nature of the experiment in a linear mixed model, the contact lens wearing eyes at 2-hours and 8-hours had a greater presumed Langerhans cells density than at the baseline ($p < 0.001$, and p = 0.045, respectively). At 2 hours, the contact lens wearing eyes had a significantly higher presumed Langerhans cells density than the control eyes (p = 0.001).

Table 10. The average density of presumed Langerhans cell density in experimental and control eyes of 10 healthy volunteers based on the second visit as a reference.

Time / hours	Experimental eyes (mean ± SD)- (cells/mm^2)	P-value	Control eyes (mean ± SD)- (cells/mm^2)	P-value
Baseline	18 ± 19	p = 0.001	20 ±19	p = 0.262
2 hours	36 ± 32	-----------	20 ± 20	-----------
4 hours	33 ± 32	p = 0.397	20 ± 19	p = 0.106
6 hours	32 ± 30	p = 0.351	20 ± 19	p = 0.799
8 hours	30 ± 31	p = 0.173	19 ± 20	p = 0.140

Mixed model analysis results (forcing control eye and time 2 hours as the reference value).

Table 11. The average density of presumed Langerhans cell density in experimental and control eyes of 10 healthy volunteers.

Time / hours	Experimental eyes (mean ± SD)- (cells/mm^2)	Control eyes (mean ± SD)- (cells/mm^2)	P-value Experimental vs control eyes
Baseline	18 ± 19	20 ± 19	0.262
2 hours	36 ± 32	20 ± 20	0.004
4 hours	33 ± 32	20 ± 19	0.024
6 hours	32 ± 30	20 ± 19	0.020
8 hours	30 ± 31	19 ± 20	0.042

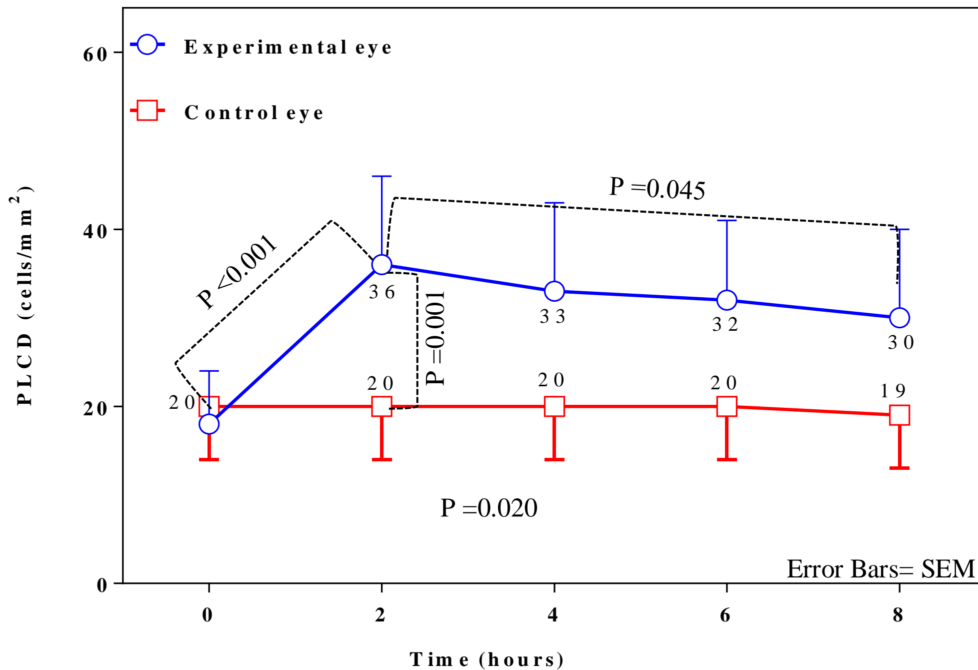


Figure 10. The impact of contact lens wear on presumed Langerhans cell recruitment in the central cornea in a period of eight-hour time course. PLCD, presumed Langerhans cell density; SEM, standard error of mean.

3.3.5 Discussion

Laser scanning confocal microscopy is an emerging, non-invasive technology that is useful for *in vivo* assessment of the histopathology of several eye diseases. This tool enables the study of the surface structure of the eye at the cellular level, under both healthy and disease conditions. As noted by Rolando et al. (1994), examiners are able to repeat the tests on a particular tissue without producing any alteration. In the current study, the measurements were repeated four times without any eye alteration being observed in the examined tissue.

The current study is important as it has shown that presumed Langerhans cells can be seen in the central normal cornea; this finding is in agreement with previous studies (Asbell et al. 1987, Hamrah et al. 2002, Novak et al. 2003, Yamagami et al. 2005, Zhivov et al. 2007, Resch et al. 2015), but contradicts the earlier claims of Gillette et al. (1982) and Suzuki et al. (2000), that the normal cornea lacks presumed Langerhans cells.

The current study reported for the first time, the short-term recruitment of presumed Langerhans cells in the corneal centre of contact lens wearers *in vivo*. After examining the effect of contact lens wear on presumed Langerhans cell density in the corneal centre over an eight hour period using laser scanning confocal microscopy, the study found that presumed Langerhans cells behaved differently, over time, in the experimental eyes compared to the control eyes ($p = 0.020$). The greatest cell recruitment was present after two hours of lens wear. The results illustrate that contact lens wear has a rapid effect on presumed Langerhans cell density in the short-term, typically in the first two hours of contact lens wear ($p < 0.001$). Interestingly, the impact of lens wear on the cells continued even after eight hours of lens wear ($p = 0.045$), implying chronic irritation of the eye secondary to lens wear.

A number of studies (Mustonen et al. 1998, Wakamatsu et al. 2010, Villani et al. 2013) reported the presence of dendritic cells (presumed Langerhans cells) on the ocular surface as explaining the presence of inflammation. However, the current study shows presumed Langerhans cell recruitment in both the experimental eyes (increased after lens wear) and the control eyes (no changes in the cell density before and after lens wear) in the corneal centre of each participant.

3.3.6 Conclusion

This study provides important new data regarding presumed Langerhans cells in the cornea of contact lens wearers over a short period of time. The study demonstrated that presumed Langerhans cells increased rapidly, approximately two-fold, during the first eight hours of lens wear. These cells mediate immune and inflammatory responses in the cornea of contact lens wearers. These findings provide a useful foundation for further investigations, particularly in the subsequent days of lens wear

3.3.7 Subsequent Study

The above observations represent a paradigm shift in understanding the ocular response to contact lens wear. It shows that the apparent adaptive response following the initial upregulation of presumed Langerhans cells reported in the aforementioned study would suggest that end-of-day discomfort is not of inflammatory origin, and may instead be, for example, a purely mechanical phenomenon. Importantly, mechanical and/or biological inflammatory stimuli, such as eye rubbing and/or eye closure, may induce significant impacts on the cell recruitment. To confirm this, this thesis evaluated the effect of eye rubbing and eye closure on presumed Langerhans cell density in the cornea. The following two studies will investigate and discuss these factors in more detail.

3.4 Impact of Eye Rubbing on Short-Term Presumed Langerhans Cell Recruitment.

This section discusses the effect of eye rubbing, as a mechanical inflammatory stimulus, on the number of presumed Langerhans cells in the centre of the cornea.

3.4.1 Introduction

Eye rubbing is a physiological response to uncomfortable eyes caused by factors such as fatigue and exposure to dust or allergens. Eye rubbing may be reported before or after sleep, as well as during contact lens wearing. The parts of the body used most for eye rubbing are the finger pads, the knuckles, or the palms of the hand. Eye-rubbing averages only a few seconds for most individuals (McMonnies et al. 2003). However, the frequency of eye rubbing may increase significantly under a number of ocular conditions, including dry eye (Pflugfelder et al. 2002), keratoconus (McMonnies et al. 2003), eye allergy, conjunctivitis - (Senaratne et al. 2005), trichiasis and blepharitis (Sihota et al. 2011). Eye rubbing involving the eyelids may be reported for some skin conditions, such as eczema and atopic dermatitis (De Benedetto et al. 2009).

When the frequency, intensity and duration of eye-rubbing episodes, over a particular period of time has increased, eye rubbing is considered as an abnormal condition (Balasubramanian et al. 2013). The duration of eye rubbing varies among individuals with affected eyes (from less than 15 seconds to 180 seconds) compared to individuals with normal eyes (usually less than 5 seconds) - (McMonnies et al. 2003). Therefore, differentiation between normal and

affected eyes can be made through the average duration of eye rubbing (Balasubramanian et al. 2013).

Eye rubbing influences the corneal properties. Using rabbits, Greiner et al. (1997) investigated the effect of 5 minutes of eye rubbing on ocular surface tissues at zero-, four-, eight-,and twelve-hour intervals. The authors found that eye rubbing caused corneal epithelial thinning and conjunctival cell swelling. In their study Liu et al. (2011) assessed the impact of eye rubbing on the corneal thickness and intraocular pressure between allergic and control eyes. Their participants rubbed one eye for 20 seconds every two minutes, during two episodes. The study found that eye rubbing caused no significant decrease neither in corneal thickness nor intraocular pressure. Another study by Prakasam et al. (2012), on ten healthy participants sought to understand the effect of eye rubbing on the total corneal, epithelial and Bowman's membrane thickness; they found no significant effects of eye rubbing on the corneal layers thicknesses. However, their sample size (n=10), alongside the short time period of eye rubbing (30 seconds) may have precluded them from observing significant effects.

Mansour et al. (2002) investigated the impact of eye rubbing on corneal topography in twenty-nine healthy participants; they found that eye rubbing caused a distortion of corneal topography. The correlation between eye rubbing, and keratocyte densities and interleukin-8 was examined by Kallinikos et al. (2004). They found a significant reduction in the keratocyte densities, as well as a significant increase of interleukin-8 in the rubbed eyes compared to the unrubbed eyes. The study by McMonnies et al. (2010) identified a displacement of the corneal epithelial wing cells from the rubbed area towards the corneal periphery. They demonstrated

that the corneal epithelial intercellular water can be displaced from the rubbed area towards the corneal periphery.

The finding by Elder (1993) showed that eye rubbing does not only lead to alterations in corneal properties, it can lead to eye diseases such as keratoconus (Copeman 1965, Karseras et al. 1976, Rabinowitz 1998, Bawazeer et al. 2000, McMonnies et al. 2003, Ioannidis et al. 2005). Rubbing may also lead to the rupture of the cell membrane and loss of cytoplasm (Mansour et al. 2002), or it may lead to a temporary refractive error, such as astigmatism of -0.50 to -0.75 D (Mansour et al. 2002), as well as to the development of a transient visual blur (Ladage et al. 2001). The blur may result from corneal moulding, disruption of the tear film, increased intra-ocular pressure, vitreomacular traction, and/or altered macular perfusion (Mansour et al. 2002). Over the long term, eye rubbing may expose the post–LASIK cornea to ectasia (Rabinowitz 1998). It may also cause cone formation, or rupture of Descemet's membrane in keratoconus (McMonnies 2007, McMonnies 2009).

A study conducted on 53 healthy participants aged 15 to 50 years found that eye rubbing can cause a significant change in corneal parameters, such as a decrease in the corneal resistance factor (Oltulu et al. 2014).

Eye rubbing may lead to a raised corneal temperature, epithelial thinning, increased intraocular pressure and/or changes to keratocytes (McMonnies 2007, McMonnies 2009, McMonnies et al. 2010). Oedema may also be developed in a closed eye condition as a result of overnight hypoxia leading to an increase in eye rubbing after waking up (Greiner et al.

1997). There is also an increase in eye rubbing associated with physical tiredness (Greiner et al. 1997, McMonnies 2009). Table 12 summaries the effect of eye rubbing on the ocular tissue.

Table 12. Effect of eye rubbing on the ocular tissues.

Author (Year)	Study aim	Participant's no.	ER duration	Results
Greiner et al. (1997)	Effect of ER on ocular surface tissue (Animal model)	Rabbits	At 0, 4, 8 &12 intervals, after 5 min	Alteration in ocular surfaces tissue.
Raizman et al. (2000)	Effect of ER on signs & symptoms of allergic conjunctivitis	30 (60 eyes)	15 times (after 5, 15, 30, & 60 min)	Transient increase in ocular itching, chemosis, & hyperemia.
Mansour et al. (2002)	Effect of ER on corneal topography	20	5 min	Transient visual blur & corneal topography's transient distortion.
Kallinikos et al. (2004)	Effect of ER on the cornea in both the absence and presence of a CL	20 (40 eyes)	For 10 sec/min for 30 min	Significant reduction in *KD in the rubbed eyes compared with the control eyes.
Kalogeropoulos et al. (2009)	Effects ER on basal epithelial and epithelial thickness.	10 (20 eyes)	For 20 sec/min for 30 min	ER had no effect on corneal epithelial thickness and basal cells.

Liu et al. (2011)	Effect of ER on corneal thickness & IOP	40 (80 eyes)	2 episodes, each lasting 20 sec, with a 2 min break between episodes.	No effect.
Prakasam et al. (2012)	Effect of ER on the cornea thickness	10 (20 eyes)	30 sec	No effect.
Balasubramanian et al. (2013)	Effect of ER on MMP-13, IL-6 and TNF- α in tears	17	60 sec	Increased the level of tear MMP-13, IL-6. and TNF- α.

Abbreviations: min, minute; sec, second; IOP, intraocular pressure; MMP, matrix metalloproteinase; IL, interleukin; TNF- α, tumour necrosis factor; KD, keratocyte density.

3.4.2 Research Question and Hypothesis

Research Problem

The ocular responses to eye rubbing (and possible causal links) may include epithelial thinning, increased intraocular pressure, changes to ocular surface tissues, changes to corneal topography, such as transient distortion and reduction in keratocyte density, and higher concentrations of inflammatory mediators in the pre-corneal tear fluid (Greiner et al. 1997, Raizman et al. 2000, Mansour et al. 2002, Kallinikos et al. 2004, McMonnies 2009, Balasubramanian et al. 2013). These findings are summarised in Table 12. However, no studies have directly investigated the effects of eye rubbing on presumed Langerhans cells in ocular tissues.

Aim

Eye rubbing influences corneal tissues and causes a significant impact on its integrity and function including its immunological response. Therefore, the aim of the current study was to employ *in vivo* laser scanning confocal microscopy, to investigate the effect of eye rubbing on presumed Langerhans cell density in the corneal centre.

3.4.2.3 Research Question

What is the impact of eye rubbing (as a mechanical inflammatory stimulus) on short-term presumed Langerhans cell recruitment?

3.4.2.4 Hypothesis

It is hypothesised that presumed Langerhans cell density in the central cornea will be increased as a result of the mechanical processes induced by eye rubbing.

3.4.3 Methods

This was a case-controlled, prospective study whereby one eye served as the test eye and the contralateral eye served as the control. The analysis of images conducted by confocal microscopy from experimental eyes and control eyes was masked. Thirty healthy participants (aged 32 ± 6 (mean ± SD) years, (range 23 to 48 years)) from the Queensland University of Technology staff and student cohort, and Brisbane city residents, participated in this study. They did so after fully understanding the concept and possible consequences of the study. Written informed consent was obtained from all participants before inclusion in the study. The study procedures were performed in accordance with the University Human Research Ethics Committee at the Queensland University of Technology (Ethics approval number 1300000117), as well as in accord with the principles of the Declaration of Helsinki. The right and left eyes of all 30 participants were included for examination. Individuals were excluded if they had a history of corneal surgery or trauma, diabetes, blood pressure

instability, current or long-term topical ocular medication, history of contact lens wearing, or were pregnant or breastfeeding, using oral contraceptives, or with a symptom of dry eye. A slit-lamp examination of the anterior ocular surface was performed before commencing the study.

In order to determine the sample size, a pilot study of ten healthy participants was conducted. The number of participants recruited for the study was determined using power analysis (G*Power 3.1.) Table 13

Table 13. Sample size calculation using G*Power 3.1.

t tests - Means: Difference between two dependent means (matched pairs)

Analysis:	A priori: Compute required sample size		
Input:	Tail(s)	=	Two
	Effect size dz	=	0.547022
	α err prob	=	0.05
	Power (1-β err prob)	=	0.8
Output:	Noncentrality parameter δ	=	2.945804
	Critical t	=	2.048407
	Df	=	28
	Total sample size	=	29
	Actual power	=	0.811513

Eye rubbing

In the current study, gentle eye rubbing in a circular pattern over the corneal centre was performed over the closed eye using the index finger parallel to eyebrow. The examiner advised the participants to rub their eyes as they usually would if their eyes were itchy and to keep rubbing the eye in the same manner for each session of the test. The rubbed eye was randomly chosen by the participants and the fellow eye

served as the control. To ensure that the corneal centre was being rubbed, the participants were advised to keep a steady and primary gaze fixation. Typically, they were advised to look at a target straight ahead, with the fellow eye opened. Before performing the eye rubbing task, the laser scanning microscopy was performed on the cornea of both eyes. The procedure was explained in Chapter 2; section 2.4. Next the eye rubbing task was undertaken, then both eyes underwent laser scanning confocal microscopy.

After conducting the baseline measurements, the participants were instructed to rub one eye for ten seconds every minute, for a total period of half an hour. They were advised to keep both eyes open after stopping their eye rubbing based on the approach of Kallinikos et al. (2004). Immediately after 30 minutes of eye rubbing, laser scanning confocal microscopy, was performed on both eyes. For each eye, five high-quality digital images (out of 100), not overlapping by more than 20% were randomly selected for analyses. To ensure that a similar corneal location was re-measured at the second session; the participants were advised to look at the red reflex of the instrument and the reflex was aligned at approximately the centre of the pupil. A slit-lamp examination was performed before and after confocal microscopy to verify that the integrity of the ocular surface was unaltered.

Statistical methods

The significance of any differences of eye rubbing effect between the experimental eyes and the controls was evaluated using a repeated-measures analysis of variance

(ANOVA). As there are no missing values in the data, a repeated-measures analysis of variance is robust to normality violations (Fitzmaurice et al. 2012). A repeated measures multivariate analysis of variance (using Hotelling's Trace) was performed for the groups. A *p*-value of less than 0.05 was considered significant. The data were analysed using IBM SPSS Statistics for Windows, Version 21.0 IBM Corp. Armonk, NY, USA.

3.4.4 Results

There were no significant differences between presumed Langerhans cell density measurements obtained from the experimental eyes and the control eyes at baseline (n = 30; p = 0.132). The baseline mean of presumed Langerhans cell density for the right eyes was 26 ± 19 cells/mm^2 (range from 5 to 76 cells/mm^2) and the left eyes was 26 ± 19 cells/mm^2 (range from 5 to 76 cells/mm^2). Table 14 shows the data obtained from the controls and the experimental eyes, indicating that the minimum value achieved was at the baseline, while the maximum value was for the right eye, post-rubbing. After 30 minutes of eye rubbing, there was a significant difference in presumed Langerhans cell density between the rubbed eyes and the controls (31 ± 21 cells/mm^2 and 27 ± 20 cells/mm^2, respectively) - ($F_{(0.16,9.46)}$= 57.00, p =0.003). Figures 11 shows that, in the same time period, when the cell densities went up in the rubbed eyes, the control eyes maintained the same pre-rubbing density. However, the experimental eyes showed an increase in presumed Langerhans cell density after eye rubbing – see Figures 11 and 12.

Table 14. Presumed Langerhans cell density (PLCD) in the cornea at baselines and after eye rubbing (ER) in the experimental and control eyes.

Time	Experimental eyes -right (ER)	Control eyes -left (no ER)	P-value
Before ER PLCD (cells/mm^2) (range)	26 ± 19 (5-76)	26 ± 19 (5-76)	p=0.682
After ER PLCD (cells/mm^2) (range)	31 ± 21 (7-81)	27 ± 20 (4-75)	p=0.004

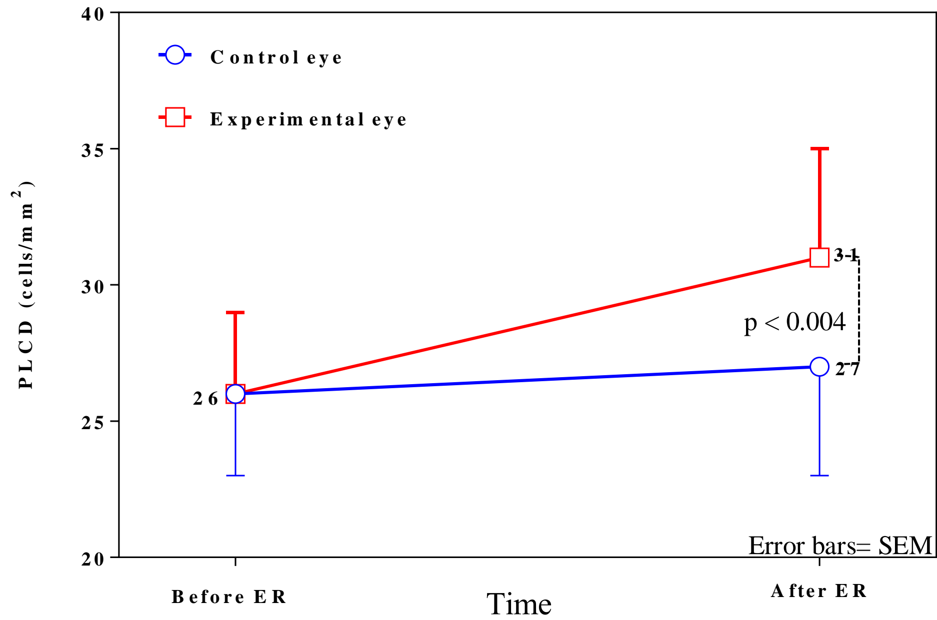


Figure 11. Shows the effect of eye rubbing (ER) on presumed Langerhans cell density (PLCD) in the human cornea of 30 healthy participants. SEM, standard error of mean.

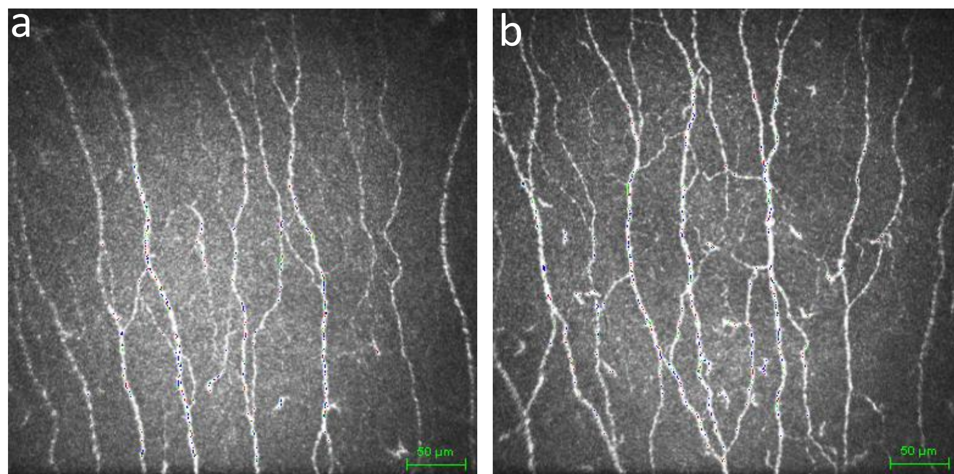


Figure 12. *In vivo* confocal microscopy images (before and after 30 min of eye rubbing) of presumed Langerhans cells in the level of sub-basal nerve plexus of the corneal centre (a) unrubbed eye at a depth of 63 µm (b) rubbed eye at a depth of 63 µm. The images captured from the right eye of 37- year-old male. *Bar* represents 50 µm.

3.4.5 Discussion

To the best of the authors knowledge, no study has investigated the impact of eye rubbing on the inflammatory response of the human cornea, despite the significant reports associating eye rubbing with changes in eye tissues or/and cells. The current study investigated the impact of eye rubbing (as a mechanical inflammatory stimulus) on short-term presumed Langerhans cell recruitment. No effect of eye rubbing was observed in presumed Langerhans cell density in the corneal centre between the experimental and control eyes pre-eye rubbing ($p = 0.682$). A significant increase in presumed Langerhans cell density in the corneal centre was reported after eye rubbing in the experimental eyes compared with the controls ($p = 0.004$). This result indicates that eye rubbing produces some changes in the corneal condition, which can lead to a sub-clinical inflammatory response.

The effect of eye rubbing on the ocular tissues, especially the cornea, has been discussed in a few studies – see Table 12. Different rubbing period were used in these studies, which may have led to different results. However, neither the current nor previous studies measured the amount of force created by the finger during the process of eye rubbing. Nevertheless, the current study advised participants to rub their eyes the way they usually would if their eyes were itchy. This method was different from that used in the previous studies (Kalogeropoulos et al. 2009, McMonnies et al. 2010, Liu et al. 2011, Prakasam et al. 2012). During the process of eye rubbing, the participants might not be able to replicate the same rubbing force at each episode. Therefore, the participants may have found it difficult to apply similar

rubbing patterns at each episode, which might have impacted upon the overall outcome of the study. Nonetheless, they were supervised during the study and were encouraged to maintain a similar pattern each time.

There is a possible association between increase presumed Langerhans cell density and eye rubbing, as well as their development of some eye diseases (Table 12). For example, the results of various studies suggest that keratoconus development is associated with eye rubbing (Lindsay et al. 2000, Jafri et al. 2004, McMonnies 2007, Weed et al. 2007, Efron et al. 2008).

3.4.6 Conclusion

Using laser scanning confocal microscopy, an evaluation of the effect of eye rubbing on presumed Langerhans cell density in the centre of the cornea, after 30 minutes of eye rubbing, shows an increase in the presumed Langerhans cell density. Eye rubbing appears to upregulate the immune status of the cornea, which may help explain the physiological mechanisms underpinning previous reports of the ocular response to eye rubbing (Pflugfelder et al. 2002, McMonnies et al. 2003, Senaratne et al. 2005, Sihota et al. 2011). The recovery period would be of interest to examine in future studies.

3.5 Impact of Eye Closure on Short-Term Presumed Langerhans Cell Recruitment.

This section discusses the effect of eye closure, as a biological inflammatory stimulus, on the number of presumed Langerhans cells in the centre of the cornea

3.5.1 Introduction

The ability of the cornea to withstand the physiological stresses of the closed-lid environment has been of crucial interest to practitioners and researchers for many years. Previous studies (Hill et al. 1965, Freeman et al. 1973) have shown that the cornea experiences a number of adverse environmental influences when the eyelid is closed. For example, the eye experiences an increase in temperature leading to an increase in the metabolic activity of the epithelial cells which then cause a greater oxygen demand (Hill et al. 1965, Freeman et al. 1973). Eye closure also leads to a decrease in the level of the oxygen available to the cornea at approximately one-third of that available under open eye conditions (Efron et al. 1979). The closed eye causes a reduction in the tear evaporation, which plays a role in maintaining corneal deturgescence. Thus closed eye conditions lead to corneal swelling (Terry et al. 1978). Moreover, a tear film acidic shift has been reported during prolonged lid closure (Carney et al. 1976), possibly by changing the corneal demand for oxygen (Carney et al. 1980).

Only a few studies have examined the effect of eye closure on ocular tissues; with most referring to contact lens wearers - Table 15. A study investigated the effect of

short-term closed eye conditions on the cornea in contact lens wearers. They showed that three hours of eye closure causes corneal swelling (O'Neal et al. 1984). Another study showed that contact lens wear leads to an inflammatory response in the cornea in the closed eye (Conners et al. 1995). Overnight sleep showed an increase in corneal swelling of about four percent, while closed eye conditions lasting for an hour or more were found to introduce changes in the corneal properties, probably because of the evaporation of water from the tear (Mandell et al. 1965, Mertz 1980).

Temperature and oxygen tension were found to be the most effective factors on corneal thickness. Maurice (1978) found that eye closure leads to an increase in the corneal temperature of approximately 4° C when compared with that of the open eye. In 2003, investigation the effect of eye closure on the thickness of the tear film at post-lens during contact lens wear conducted by Nichols and King-Smith. They reported that the post-lens tear film was affected by eye closure in contact lens wearers. Table 15 summarises the results of previous studies that illustrate the effect of eye closure on the cornea. However, all the studies were conducted on contact lens wearers only. In contrast, the current study focused on the effect of eye closure on presumed Langerhans cell density in the human cornea of non-contact lens wearers.

Table 15. Summary of the results of the effect of eye closure on the cornea during contact lens wear.

Author (Year)	Study aim	Participant's no.	EC duration	Results
Efron et al. (1979)	Effect of CL wear on corneal oxygen uptake rate in the closed-eye.	8	5 minutes	Eye closure reduced corneal oxygen uptake.
Efron (1981)	EOP beneath a lens of known oxygen transmissibility in closed-eye condition.	8	5 minutes	Marginal increase in EOP beneath the contact lens.
O'Neal et al. (1984)	The effect of EC on the corneas of CL wearers.	14	3 hours	Corneal swelling was increased
Conners et al. (1995)	Effect of CL wear on inflammatory response and	Animal model (Rabbit)	9 days of lens wear	Increased in inflammatory response in the anterior surface.

	corneal thickness in the closed-eye.			
Nichols et al. (2003)	The effect of EC on the thickness of the tear film during CL wear.	10	30 minutes	Tear film thickness was decreased.

Abbreviations: EC, eye closure; CL, contact lens; EOP, equivalent oxygen percentage.

3.5.2 Research Question and Hypothesis

Research Problem

Eye closure is a natural concept which a person experiences every day, either during the blinking process or sleep. Neither short nor long-term effects of eye closure have been given much attention by researchers, except in a very few studies related to contact lens wearers (Maurice 1978, Conners et al. 1995, Nichols et al. 2003). However, much remains unknown about the effect of eye closure on ocular tissues. The closed eye environment is often considered as a state of sub-clinical inflammation characterised by increase inflammatory response in the tear film and corneal swelling (Sack et al. 1992, Tan et al. 1993). There is a possibility that this might be reflected by an increase in presumed Langerhans cell density in the ocular surface when the eye is closed.

Aim

No previous study has investigated the impact of eye closure on presumed Langerhans cell density. Therefore, the aim of the current study was to employ *in vivo* laser scanning confocal microscopy, to investigate the impact of eye closure (as a biologic inflammatory stimulus) on short-term presumed Langerhans cell density recruitment.

Research Question

What is the impact of eye closure (as a biologic inflammatory stimulus) on short-term presumed Langerhans cell recruitment?

Hypothesis

It is hypothesised that presumed Langerhans cell density in the central cornea of the closed eye will be increased after sleep.

3.5.3 Methods

This was a case-controlled, prospective study. A total of 46 healthy volunteers were enrolled in the current study. The analysis of images conducted by confocal microscopy from experimental eyes and control eyes was masked. Thirty healthy participants aged 31 ± 6 (mean ± SD) years, range 20 to 48 year-old) were enrolled in the experimental group. The remaining sixteen healthy participants aged 32 ± 4 (mean ±SD) years, range 26 - 38 year-old) were monitored over the same time course, and served as the control group. The participants, recruited from the Queensland University of Technology staff and student cohort, as well as Brisbane city residents, participated after fully understanding the concept and possible consequences of the study. Written informed consent was obtained from all participants before their inclusion in the study. The study procedures were performed

in accordance with the University Human Research Ethics Committee at Queensland University of Technology (Ethics approval number 1300000117) and the principles of the Declaration of Helsinki. Individuals were excluded if they had a history of corneal surgery or trauma, diabetes, blood pressure instability, current or long-term topical ocular medication, contact lens wearing, being pregnant or breastfeeding, taking oral contraceptives, or with a symptom of dry eye

In order to determine the sample size, a pilot study of 10 healthy participants was conducted. The number of participants recruited for the study was calculated using power analysis (G*Power 3.1.)-Table 16.

Table 16. Sample size calculation using G*Power 3.1.

t tests - Means: Difference between two dependent means (matched pairs)

Analysis:	A priori: Compute required sample size	
Input:	Tail(s)	Two
	Effect size dz	0.419633
	α err prob	0.05
	Power (1-β err prob)	0.8
Output:	Noncentrality parameter δ	2.87686
	Critical t	2.012896
	Df	46
	Total sample size	47
	Actual power	0.804102

Eye Closure

The participants were advised to cover one eye as soon as they got up from sleep. Each participant was provided with an eye patch (Nexcare Opticlude Orthoptic Eye Patch 20 Junior Patches) and cotton pads to be placed underneath the eye patch over the

closed upper eyelid to ensure that the eye was not opened under the eye cover. An earlier study by Ehlers et al. (1997), conducted on 61 healthy volunteers, found that the normal variability of daily sleep ranged from between six and eight hours per day. The participants in the current study were advised to sleep for at least six hours prior the visit.

The baseline images were taken the day before the experiment. The next morning, the examiner ensured that the eye was still patched properly and questioned the participants about any occurrence of eye opening during the night, before removing the patch. From these self-reports it was verified that all participants were able to keep the patch in place and keep the test eye closed during the previous night until arrival at the appointment. Laser scanning confocal microscopy on the corneal centre was repeated on the patched eye as soon as the participant arrived. The procedure of examining the corneal centre using laser scanning confocal microscopy is explained in Chapter 2, section 2.4. In terms of the control participants, the baseline measurements were conducted in the morning time (9:00 am -12:00 pm) on one eye. The measurements were repeated after 6 to 8 hours following the baseline measurements. During the process, the participants were advised not to sleep during the time between the two visits, even for short time, with the expectation that activities such that normal blinking pattern occurred in controls.

For each eye, five high-quality digital images (out of 100), not overlapping by more than 20% were randomly selected for analyses. To ensure that a similar corneal location was re-measured at the second session, the participants were advised to look at the red reflex of the instrument and the reflex was aligned at approximately the

centre of the pupil. Slit-lamp biomicroscopy was performed before and after the confocal microscopy to verify that the integrity of the ocular surface remained unaltered.

Statistical Methods

The significance of any differences of eye closure effect between the experimental group and the controls was evaluated using a repeated-measures analysis of variance (ANOVA). As there are no missing values in the data, a repeated-measures analysis of variance is robust to normality violations (Fitzmaurice et al. 2012). A repeated measures multivariate analysis of variance (using Hotelling's Trace) was performed for the groups. A *p*-value of less than 0.05 was considered significant. The data were analysed using IBM SPSS Statistics for Windows, Version 21.0 IBM Corp. Armonk, NY, USA.

3.5.4 Results

There were no significant differences between presumed Langerhans cell density measurements obtained from the eyes of the experimental and the control groups at the baseline (n = 30 vs 16, respectively; p =0.841). The baseline mean of presumed Langerhans cell density for the experimental group was 27 ± 19 cells/mm^2 (range from 5 to 76 cells/mm^2) and the control group of 26 ± 17 cells/mm^2 (range from 7 to 62 cells/mm^2). Post-eye-closure, showed that there was a significant difference in presumed Langerhans cell density in the experimental group compared to their

baseline data (30 ± 18 cells/mm^2, $F_{(0.11,4.91)}$= 4.911, p = 0.032), while control group was remain stable over time (25 ± 18 cells/mm^2, p = 0.117). The analysis of images conducted by confocal microscopy from experimental eyes and control eyes was masked. Figure 13 shows that presumed Langerhans cell density in the both groups was almost similar at the baseline; indicating that there was no significant effect of eye closure on presumed Langerhans cell density in the experimental and control groups before eye closure. However, the cell population was increased significantly after eye closure within the experimental group. The difference in presumed Langerhans cell density between experimental and control groups, post and pre-closure, is shown in Table 17 and Figure 14.

Table 17. Presumed Langerhans cell density at baseline and after eye closure in the experimental and control groups.

Time	Experimental eyes- right (EC)	Control eyes- Left (no EC)
Before EC PLCD (range)	27 ± 19 cells/mm^2 (5-76)	26 ± 17 cells/mm^2 (7-62)
After EC PLCD (range)	30 ± 18 cells/mm^2 (12-75)	25 ± 18 cells/mm^2 (5-64)
P-Value	p = 0.032	p = 0.117

EC, eye closure; PLCD, presumed Langerhans cells density; SD, standard deviation.

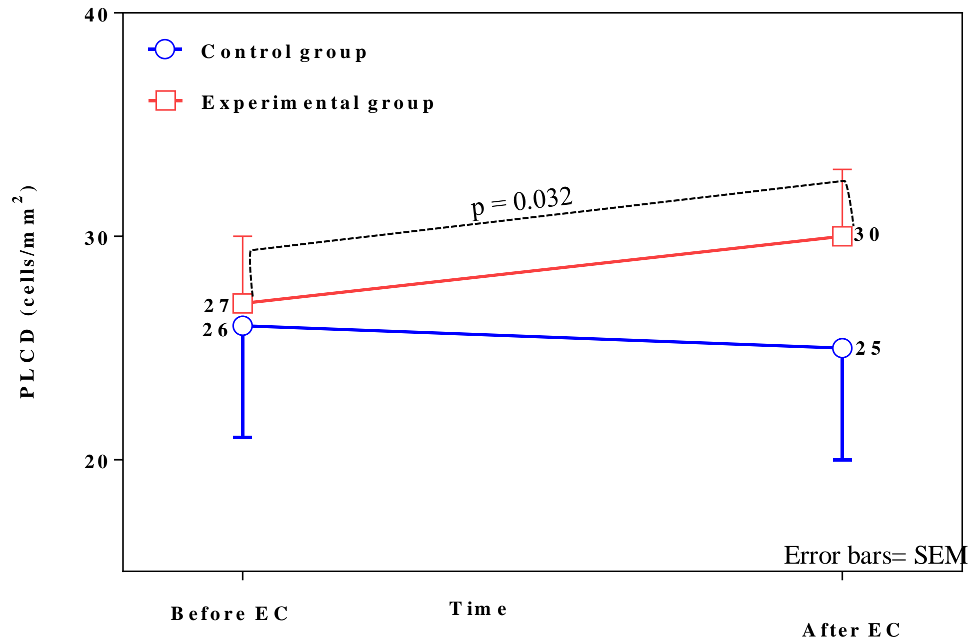


Figure 13. The effect of eye closure (EC) on presumed Langerhans cell density (PLCD) in the human cornea of 30 participants who covered their eyes vs 16 controls. SEM, standard error of mean.

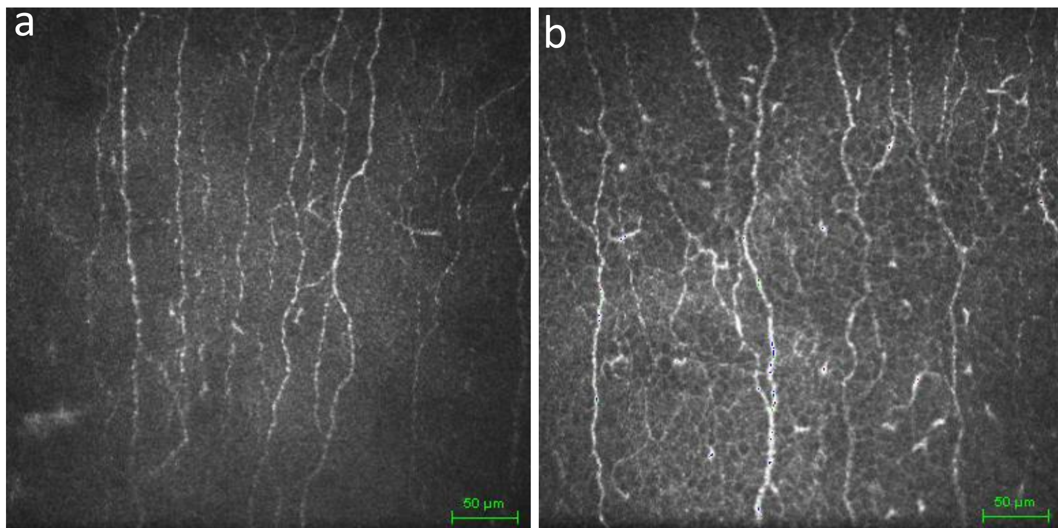


Figure 14. *In vivo* confocal microscopy images (before and after eye closure) of presumed Langerhans cells in the level of sub-basal nerve plexus of the corneal centre

(a) Before eye closure at a depth of 60 µm,

(b) After eye closure at a depth of 60 µm. The images were captured from a 33-year-old male. It shows higher density of presumed Langerhans cells in the participant eye after eye closure than before closure. *Bar* represents 50 µm.

3.5.5 Discussion

The result indicates that eye closure produces significant changes in the presumed Langerhans cell density. The effect of eye closure on the ocular tissues, especially the cornea, has been discussed by a few researchers, as outlined below; the these studies focused on the association between contact lens wear and eye closure. Efron et al. (1979) illustrated that contact lens wear in the closed eye leads to corneal oxygen deficiency, and decreases the equivalent oxygen percentage beneath the contact lens (Efron et al. 1981). Also overnight wear increases corneal swelling (O'Neal et al. 1984), increases corneal thickness and appearance of eye inflammation (Conners et al. 1995), and decreases tear film thickness (Nichols et al. 2003). No previous study investigated the effect of eye closure on presumed Langerhans cell density in the human cornea. Therefore, the current study was first to illustrate that eye closure causes an initial heightened sub-clinical inflammatory response in the cornea.

3.5.6 Conclusion

The current study provides interesting new data regarding presumed Langerhans cells in the corneal centre after overnight eye closure. Eye closure was shown to induce a short-term increase in the number of presumed Langerhans cells in the corneal centre. The effects of overnight lens wear on the presumed Langerhans cell needs to be determined. Further investigations of the effect of eye closure on presumed Langerhans cells after longer period of eye closure are needed. The recovery period would be of clinical interest and it is recommend to examine this issue in future studies.

Overall Summary

The aforementioned studies, and their highly novel and dramatic findings, represent a watershed contribution to the contact lens literature. It demonstrates that it is possible to monitor, in real time, the sub-clinical inflammatory status of the eye non-invasively. The above results have the potential to fundamentally alter thinking about the sub-clinical response to lens wear as well as the thinking about mechanical and biological inflammatory stimuli that influence the ocular surface, and are therefore of profound importance. The results may help to unlock the stalemate in respect of the understanding of the reasons behind of end-of-day discomfort with contact lens wear. There is no doubt that the above studies, particularly, the short-term effect of contact lens wear on presumed Langerhans cell recruitment, leaves open the question of what is happening after wearing contact lenses for long time. Answering this question, the

following three chapters (4, 5, and 6) will longitudinally evaluate the effect of contact lens wear on presumed Langerhans cell population in three areas of the ocular surface: the cornea, the conjunctiva and the lid wiper.

Chapter 4: Effect of Contact Lens Wear on Presumed Langerhans Cells Recruitment in the Cornea in Dry Eye and Non-Dry eye Contact Lens Wearers

Preface

The effect of contact lens wear on presumed Langerhans cell density has previously been evaluated in two studies (Zhivov et al. 2007, Sindt et al. 2012), and both were cross-sectional studies. These studies demonstrated that presumed Langerhans cell density increased significantly after many years of lens wear but have not actually explained the time course of this upregulation. It is still unknown when this upregulation occurs; for instance, after a week, after a month or even after a year. The previous chapter, particularly section 3.3, has demonstrated that there is an initial upregulation of presumed Langerhans cell in the first two hours in the corneal centre of contact lens wearers, but this study has not explored this effect longitudinally. It also investigated this effect on the corneal centre only. The following studies will longitudinally investigate the effects of contact lens wear on presumed Langerhans cell recruitment in the corneal centre (Chapter 4) and nasal bulbar conjunctiva (Chapter 5) in a larger cohort. It will also explore this upregulation in contact lens-induced dry eye.

4.1 Introduction

The ocular surface is frequently exposed to toxic, antigenic, and microbiological insults. One of the main components of the ocular surface is the cornea, which acts as the primary infectious and structural barrier of the eye. Together with the tear film, the cornea forms the anterior refractive surface for the eye (DelMonte et al. 2011). The posterior surface of a contact lens covers the cornea and overlaps onto the conjunctiva whereas the posterior surface of rigid contact lenses covers a significant proportion of the cornea. The anterior surface of a contact lens rests against the superior palpebral conjunctiva during eye closure. Dry eye, which affects the corneas of contact lens wearers, is the main reason for the discontinuation of contact lens wear (Doughty et al. 1997, Pritchard et al. 1999, Nichols et al. 2005, Richdale et al. 2007). It affects approximately thirty million people over the world. Women represent the greater proportion of dry eye (reviewed in (Phadatare et al. 2015). Dry eye is characterised by tear production deficiency and/or poor quality of the tear film, leading to tear film instability and increased tear evaporation. It leads to an interpalpebral ocular surface damage, causing symptoms of discomfort (reviewed in (Phadatare et al. 2015). Dry eye is more common among middle-aged and older adults as a result of longer contact lens usage, effects of systemic drugs, refractive surgeries and autoimmune diseases (Schein et al. 1997, Moss et al. 2000, Pflugfelder 2008). Dry eye may be associated with environmental factors such as dry weather, air pollution, chemical burns, computer work, and television watching (Miljanovic et al. 2007, Tong et al. 2010, Pouyeh et al. 2012). Dry eye is a challenging disease, which requires more studies. For patients and practitioners, and although many

studies have addressed prevalence and contributing factors, more studies are needed to understand the inflammatory aspects of dry eye.

Several studies have reported inflammatory cells in the normal corneas. The distribution and density of these cells may be influenced by external factors such as contact lens wear. The density of the cells was found to increase to approximately twice the normal density after contact lens wear (van Klink et al. 1993, Hazle et al. 1999, Sankaridurg et al. 2000, Szliter et al. 2006, Zhivov et al. 2007, Sindt et al. 2012). The number of inflammatory cells, typically presumed Langerhans cells may increase in contact lens-induced dry eye than no contact lens-induced dry eye and non-contact lens wearers.

4.2 Research Question and Hypothesis

4.2.1 Research Problem

In 2005, there were approximately 140 million contact lens wearers worldwide (Morgan et al. 2012). Contact lens wear discontinuation is still the major dilemma that affects successful contact lens wear, with wearers reporting dryness and discomfort as the main reasons for discontinuation (Doughty et al. 1997, Young et al. 2002, Chalmers et al. 2006, Richdale et al. 2007). To date, the effects of contact lens wear on presumed Langerhans cell density in the cornea have been examined in very few publications, which were cross-sectional studies and have not discussed the

effect of contact lens wear on the cells over time. No previous study has examined the effect of contact lens wear on presumed Langerhans cell density in dry eye.

4.2.2 Aim

The aim of the current study was to improve our understanding of presumed Langerhans cells in the cornea *in vivo* among contact lens wearers, as well as to report on the inflammatory response to contact lens wear in dry eye.

4.2.3 Research Questions

The key research questions for the present study are as follows:

1) What is the effect of contact lens wear on presumed Langerhans cell populations in the cornea?
2) What is the effect of contact lens wear on presumed Langerhans cell density in the corneas in people with contact lens induced dry eye?

4.2.4 Hypotheses

1) Corneal presumed Langerhans cell population will be higher among contact lens wearers than in non-lens wearing control participants.

2) Corneal presumed Langerhans cell density will be higher in individuals with contact lens -induced dry eye than contact lens wearers without dry eye.

3) Higher levels of presumed Langerhans cell populations in the corneal centre are associated with dry eye severity among contact lens wearers.

4)

5) Corneal presumed Langerhans cell density increases over time after introduction to contact lens wear and reaches a steady state as the eye adjusts to contact lens wear.

4.3 Methods

4.3.1 Participants

Eighty-three non- contact lens wearers (47 females and 36 males) aged 30 ± 8 (mean ± SD) years, (range 18-50 years) from the Queensland University of Technology staff and students and Brisbane city residents participated in this study. They did so after fully understanding the concept and possible consequences of the study. Written informed consent was obtained from all participants before their inclusion in the study. Study procedures were performed in accordance with the guidelines of the University Human Research Ethics Committee at the Queensland University of Technology (Ethics approval number 1300000117), and the principles of the Declaration of Helsinki. The inclusion and exclusion criteria described in Chapter 2 were applied to these 83 participants .

A continuous response outcome variable is divided from independent control and experimental groups. In this pilot study, the response within each participant group was normally distributed, with a standard deviation of 8. If the true difference in the experimental and control means is 9.5, then it would be necessary to study 12 experimental participants, 12 subgroup participants and 12 control participants in order to reject the null hypothesis that the population means of the experimental and control groups are equal with a probability (power) of 0.8. The Type I error probability associated with the test of this null hypothesis is 0.05. Longitudinal studies often have a high drop-out rate; if the study have a 20% drop-out rate, the total sample size at the end of the study was 45 participants- Table 18.

Table 18. Sample size calculation using G*Power 3.1

F tests - ANOVA: Repeated measures, between factors

Analysis:	A priori: Compute required sample size	
Input:	Effect size f	= 0.477
α err prob	=	0.05
Power (1-β err prob)		= 0.80
Number of groups		= 2
Number of measurements		= 4
Corr among rep measures		= 0.5
Output:	Noncentrality parameter λ	= 8.7371136
Critical F	=	4.3009495
Numerator df		= 1.0000000
Denominator df		= 22.0000000
Total sample size		= 24
Actual power		= 0.8064208

4.3.2 Study Design

The design is a prospective, case- controlled study. Eighty-three non-contact lens wearers were enrolled. Sixty participants were fitted with daily disposable hydrogel lenses and examined after one week, four weeks and 24 weeks. The remaining

twenty-three age-balanced non-lens wearers were monitored over the same time course, and served as the control group (refer to Chapter 2). At the one-week visit, contact lens wearers were divided into two subgroups according to their responses to the Contact Lens Dry Eye Questionnaire-8 (CLDEQ-8). This resulted in 25 individuals with contact lens-induced dry eye and 35 without contact lens-induced dry eye.

Dry eye examinations were performed at each visit (refer to Chapter 2, section 2.3).

The eligible participants underwent a subjective refraction. When the subjective refraction showed that the participants had a refractive error, he/she was encouraged to participate in the contact lens group. Then, the participants were trained in the use of disposable contact lenses by the examiner. The training included one-on-one instruction, a leaflet and a video recording about contact lens insertion; removal, and care.

4.3.3 Corneal Confocal Microscopy

Presumed Langerhans cell density in the corneal centre was examined using a Heidelberg laser scanning confocal microscope in combination with the Rostock Corneal Module (refer to Chapter 2, section 2.4). Corneal confocal microscopy was conducted on one eye only. When capturing the images, the participants were advised to look at the fixation target. The corneal epithelial layer, Bowman's membrane, and sub-epithelial nerve plexus of the cornea were scanned by using the device "section" mode to obtain images of a single area of the cornea at a certain depth. To ensure that a similar corneal location was re-measured at the follow-up

visits, the red reflex of the instrument was aligned at approximately the centre of the pupil - Figure 15. The study time on confocal microscopy was between 5 to 7 minutes per participant. Five high-quality digital images (out of 100), not overlapping by more than 20% were randomly selected for analyses. The number of presumed Langerhans cells was counted manually in each visual field (384 × 384 pixels) per section using the instruments in-built counting tool, and a grade system with a 50- μm grid width and given as cells per square millimetre.

4.3.4 Statistical Analysis

A linear mixed model was performed to examine changes over time in presumed Langerhans cell density and whether the changes were different in contact lens-induced dry eye compared to no contact lens-induced dry eye and controls. Although the data set displayed relatively high kurtosis, a linear mixed model was deemed most suitable for this longitudinal data set, bearing in mind that these models are robust to normality violations (Fitzmaurice et al. 2012). Repeated measures multivariate analysis of variance (using Pillai's Trace and Wilks' Lambda) were performed for the groups and include the factors age and sex. The data were analysed using IBM SPSS Statistics for Windows, Version 21.0 IBM Corp. Armonk, NY, USA. A *p*-value of less than 0.05 was considered statistically significant.

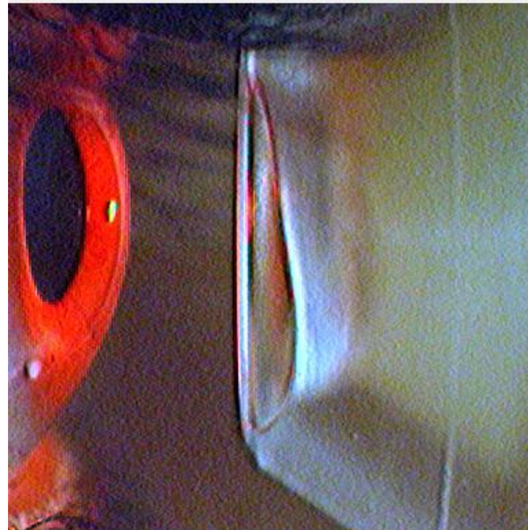


Figure 15. Definition of the corneal centre using light red reflex of confocal microscopy.

4.4 Results

Summary details of the contact lens wearers and control participants examined in this study are shown in Table 19. The current study screened 106 participants and excluded approximately 23 participants of them, because of pregnancy (n= 1), ocular allergy (n= 2), trauma (n =2), surgery (n= 1), systemic disease (n= 1), dry eye (n= 5), medication use (n= 4), a corneal scar (n=1), and contact lens fitting difficulty (n= 6). After recruitment, 16 participants withdrew from the study, mainly because of contact lens-induced dry eye (n= 8), lost contact (n= 2), or not being interested in participating in the study (n=6). All recruited participants were included in the results below.

The cohort was sex balanced (46% males) - ($\chi^2 = 0.373$, $p = 0.541$). There were no significant differences between the three groups in age (contact lens-induced dry eye, no contact lens-induced dry eye and non-contact lens wearers) - ($F = 0.788$, $p = 0.469$). The data had relatively high kurtosis but linear mixed models are robust to normality violations (Fitzmaurice et al. 2012). Presumed Langerhans cells are located in the corneal epithelial layer, Bowman's membrane and sub-epithelial nerve plexus of the cornea at a depth of 15 to 70 µm using corneal confocal microscopy - Figure 16.

Table 19. Characteristics of study participants

Characteristic	CL-NDE	CL-DE	Controls
Sex (F/ M)	25/10	18/7	5/18
Age (year)	30 ± 8	30 ± 8	31 ± 9
Duration of lens wear (hour/day)	9 ± 3	9 ± 3	n/a
Contact lens power (D) R/L	-1.70 ± 1.98/ -1.63 ± 1.79	-1.75 ±1.99/ -1.64 ± 1.80	n/a

*CL-NDE, no contact lens –induced dry eye; CL-DE, contact lens –induced dry eye

All data are mean ± SD.

No changes in presumed Langerhans cell density in the cornea were observed over time in the control group ($F = 1.337$, $p = 0.272$) throughout the 24 week observation period. Presumed Langerhans cell density was found to be 24 ± 15 cells/mm^2 (mean

± SD) in contact lens-induced dry eye and 26 ± 18 cells/mm^2 in non-contact lens wearers at baseline ($p = 0.720$). The difference between contact lens-induced dry eye and non-contact lens wearers became significant after one week of lens wear (55 ± 35 vs 27 ± 19 cells/mm^2, $p < 0.001$)- Figure 17. Four weeks after contact lens wear, presumed Langerhans cell recruitment remained significantly different from baseline measurement (41 ± 26 vs 27 ± 19 cells/mm^2, $p = 0.004$). No significant difference between individuals with contact lens-induced dry eye and non-contact lens wearers was reported at the 24-week visit (35 ± 25 vs 28 ± 18 cells/mm^2, $p = 0.681$) - Table 21.

Presumed Langerhans cell density was found to be 24 ± 18 cells/mm^2 (mean ± SD) in no contact lens-induced dry eye and 26 ± 18 cells/mm^2 in non-contact lens wearers at baseline ($p = 0.770$). The cell density was significantly increased in no contact lens-induced dry eye compared to non-contact lens wearers, after one week of lens wear (43 ± 25 vs 27 ± 19 cells/mm^2, $p = 0.010$) before recovering at the four week (35 ± 24 vs 27 ± 19 cells/mm^2, $p = 0.190$) and 24- week visits (29 ± 16 vs 28 ± 18 cells/mm^2, $p = 0.865$) - Table 20. There was a significant difference was observed in contact lens-induced dry eye compared to no contact lens-induced dry eye at one-week visit ($p= 0.041$). However, no significant difference was identified between these two groups at 4 ($p= 0.062$) and 24-week ($p= 0.068$) visits. Figures 19 and 20 show *in vivo* confocal microscopy images of different layers of the cornea in contact lens wearers and non-lens wearers.

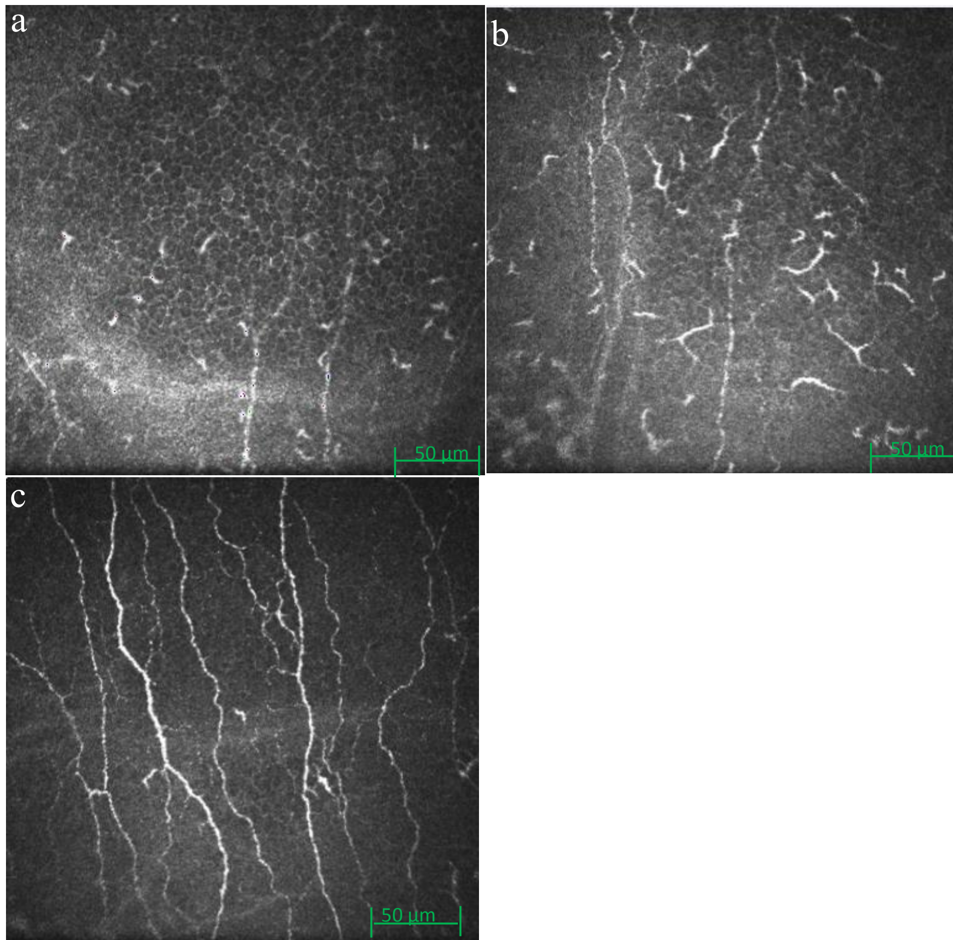


Figure 16. *In vivo* corneal confocal microscopy images of presumed Langerhans cells at different depths of the corneal layers,

a) At the level of the epithelial layer (and at a depth of 55 µm) in the central cornea of a 28 year-old male contact lens wearer,

b) At the level of Bowman's membrane and the sub-basal nerve plexus (at depth of 55 µm) at the corneal centre of a 32 year-old male contact lens wearer,

c) At the level of sub-basal nerve plexus (and at a depth of 62 µm) at the central cornea of a 32 year-old male non-contact lens wearer. Bar represents 50 µm.

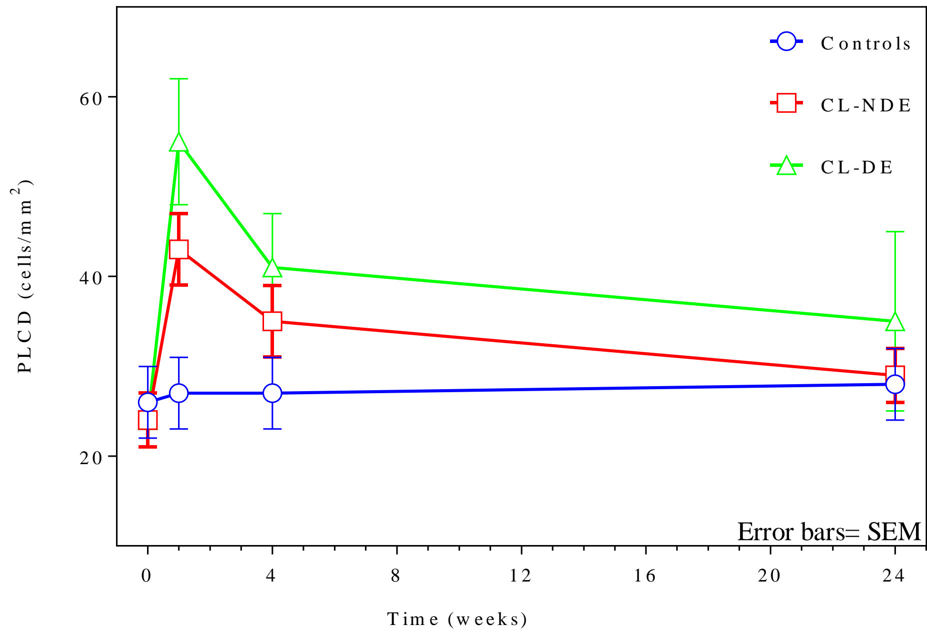


Figure 17. The presumed Langerhans cell density observed in the cornea over a 24 week period in the three groups. CL-NDE, no contact lens-induced dry eye, CL-DE; contact lens-induced dry eye. P-values between groups and the points is shown in Table 20. SEM, standard error of mean.

Table 20. P values of comparisons addressed in this study. The presumed Langerhans cell density (PLCD) in the control group baseline is compared to each of the subsequent visits, and the control group (CG) is compared to the contact lens induced dry eye group (CL-DE) and the contact lens wearers without dry eye (CL-NDE). A *p*-value of less than 0.05 was considered statistically significant.

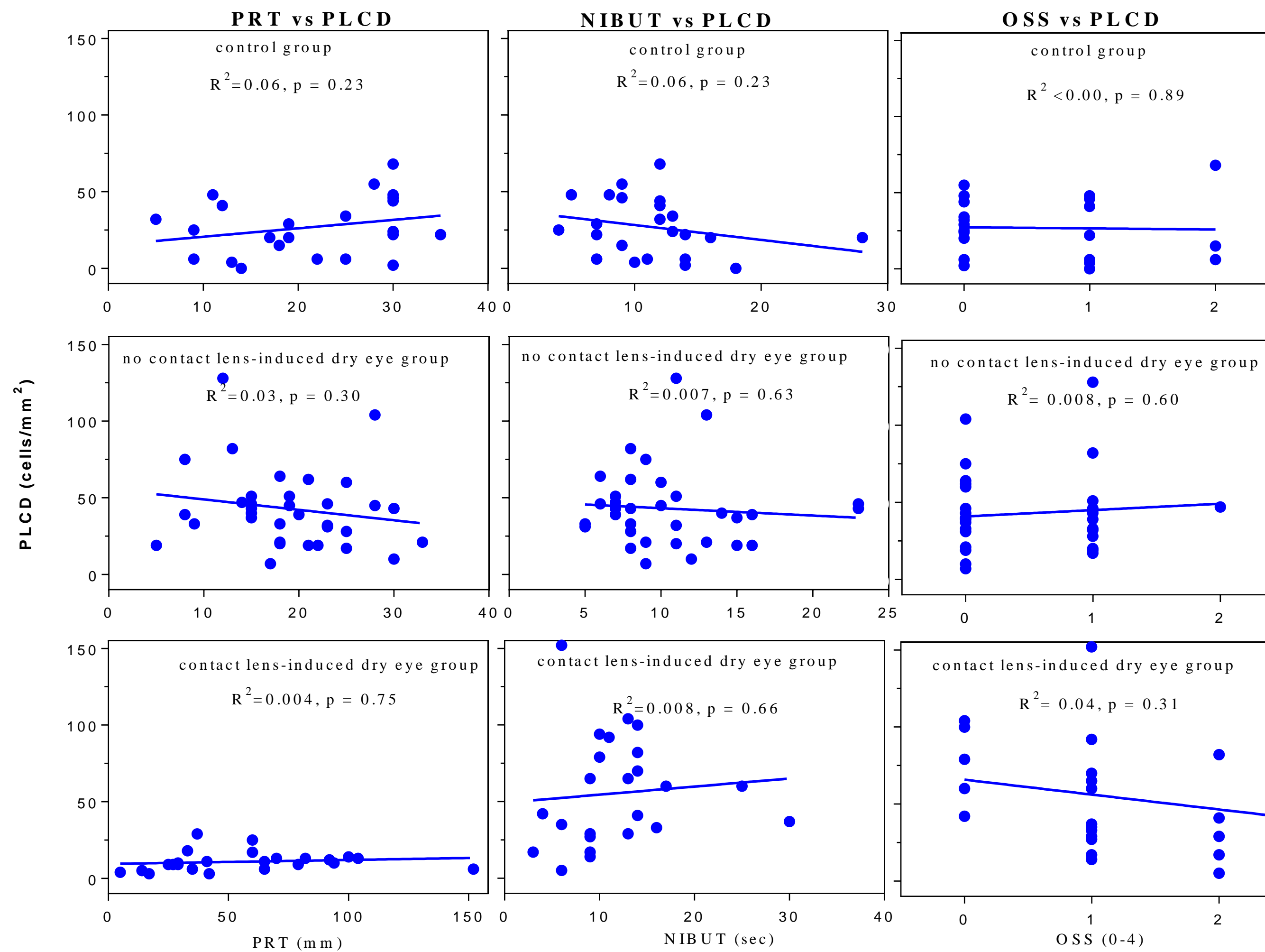
	0W	1W	4W	24W
CG baseline vs 1,4 & 24		0.681	0.865	0.852
CG vs CL-DE	0.720	**< 0.001**	**0.004**	0.068
CG vs CL-NDE	0.770	**0.010**	0.190	0.865
CL-DE vs CL-NDE	0.927	**0.041**	0.062	0.068

There were significant multivariate effects for time (V = 0.782, F = 33.983, p < 0.001) and an interaction between group and time (V = 0.735, F = 5.618, p < 0 .001). However, there were no significant multivariate effects for group (V = 0.088, F (= 1.434, p = 0.227), sex (V = 0.024, F = 0.752, p = 0.476), age group (V = 0.035, F= 1.121, p = 0.332), interaction between sex and time (V = 0.146, F = 1.629, p = 0.156), or interaction between age group and time (V = 0.069, F = 0.706, p = 0.646) when participants were grouped by age: ≤ 30 year-old, and >30 year-old.

Univariate, within-group analyses showed no significant interaction between time and sex for corneal presumed Langerhans cell density (F = 1.061, p = 0.376). The

analyses also showed no significant interaction between the time and age group for corneal presumed Langerhans cell density ($F = 1.316$, $p = 0.272$).

The current study used five types of dry eye diagnostic tools depicted in Table 21 where the severity/grade of the test is plotted against presumed Langerhans cell density. There was no significant correlation between presumed Langerhans cell density in the corneal centre and the severity/grade of the dry eye diagnostic tools used in the study - Figure 18.


PRT vs PLCD
NIBUT vs PLCD
OSS vs PLCD
control group
R2=0.06, p = 0.23
R2=0.06, p = 0.23
R2 <0.00, p = 0.89
no contact lens-induced dry eye group
R2=0.03, p = 0.30
R2=0.007, p = 0.63
R2= 0.008, p = 0.60
contact lens-induced dry eye group
R2=0.004, p = 0.75
R2=0.008, p = 0.66
R2= 0.04, p = 0.31
PLCD (cells/mm2)
PRT (mm)
NIBUT (sec)
OSS (0-4)

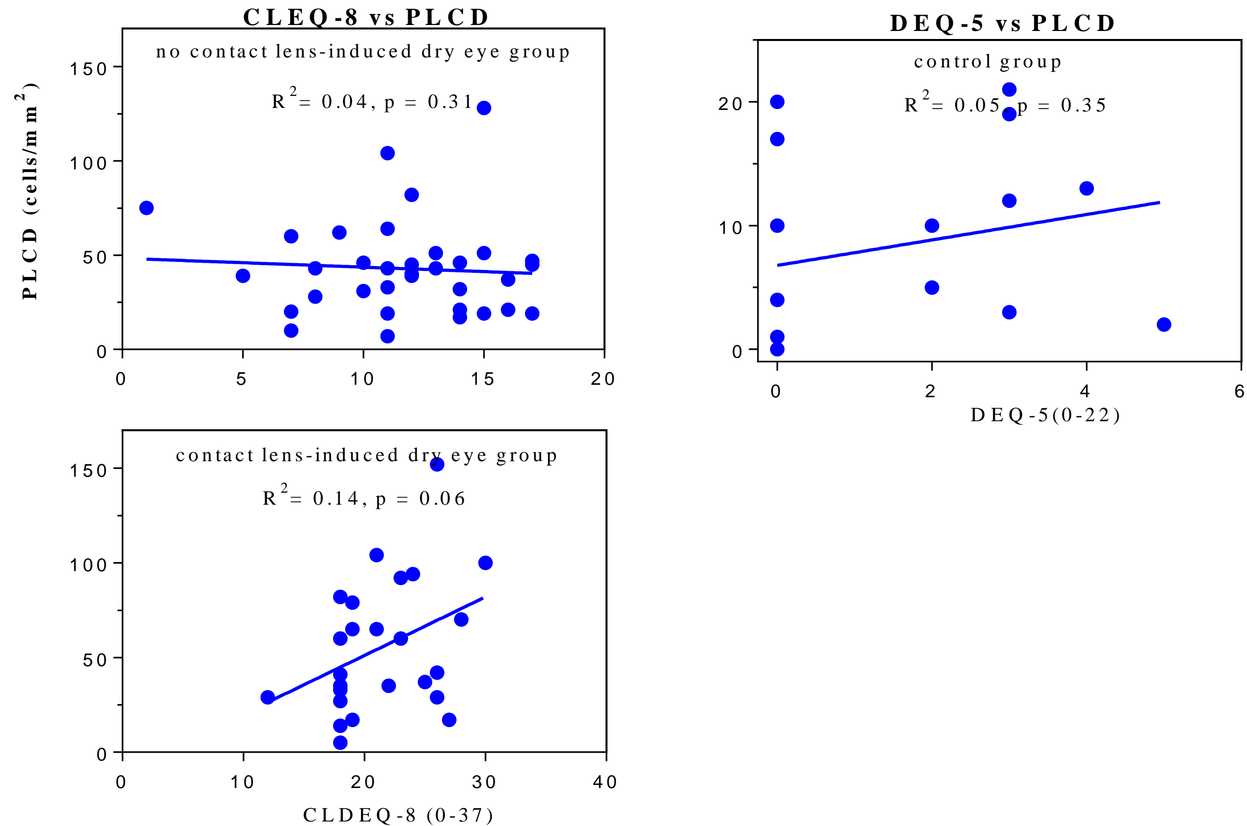


Figure 18. The association between presumed Langerhans cell density (PLCD) in the corneal center and the severity/grade of the dry eye diagnostic tools (phenol red thread (PRT), non-invasive break up time test (NIBUT), ocular surface staining (OSS), contact lens dry eye questionnaire-8 (CLDEQ-8) and dry eye questionnaire-5 (DEQ-5)). The square of the correlation coefficient (R^2) shows the outcome of linear regression. The data represent the correlation between the cell density and the tools at 1-week visit only.

Table 21 Summary of the dry eye diagnostic tool results over the 24 week period.

		control				CL-NDE				CL-DE			
Time (weeks) & Dry eye test		BL	1w	4W	24W	BL	1W	4W	24W	BL	1W	4W	24W
DEQ-5	mean ± SD	2 ± 2	1 ± 2	2 ± 3	2 ± 2	4 ± 2	n/a	n/a	n/a	4 ± 2	n/a	n/a	n/a
	min- max	0-6	0-5	0-13	0-6	0-6							
NIBUT	mean ± SD	13 ± 2	11 ± 1	11 ± 1	10 ± 2	12 ± 2	11 ± 1	11 ± 2	12 ± 2	13 ± 3	12 ± 2	13 ± 2	10 ± 2
	min- max	4-25	4-28	4-20	3-19	5-30	5-23	4-30	5-22	6-28	3-30	3-30	3-27
PRT	mean ± SD	23 ± 3	21 ± 3	20 ± 2	19 ± 4	20 ± 3	19 ± 3	18 ± 4	13 ± 4	18± 5	15 ± 3	16 ± 3	9 ± 3
	min- max	8-38	5-35	9-30	7-30	6-40	5-30	8-30	4-40	3-30	3-30	3-30	3-19
OSS	mean ± SD	1 ± 0	1 ± 0	1 ± 0	0 ± 0	0 ± 0	0 ± 0	1 ± 0	1 ± 0	0 ± 0	1 ± 0	1 ± 0	1 ± 0
	min- max	0-2	0-2	0-2	0-2	0-2	0-2	0-2	0-2	0-2	0-3	0-2	0-3
CLDEQ-8	mean ± SD	n/a	n/a	n/a	n/a	n/a	11 ± 4	12 ± 6	11 ± 6	n/a	21 ± 4	18 ± 5	19 ± 9
	min- max						1-17	1-28	1-29	n/a	12-30	9-28	5-36

BL, baseline; w, week; DEQ, dry eye questionnaire-5; NIBUT, non-invasive break-up time test; PRT, phenol red thread test; OSS ocular surface staining; CLDEQ-8, contact lens dry eye questionnaire-8.

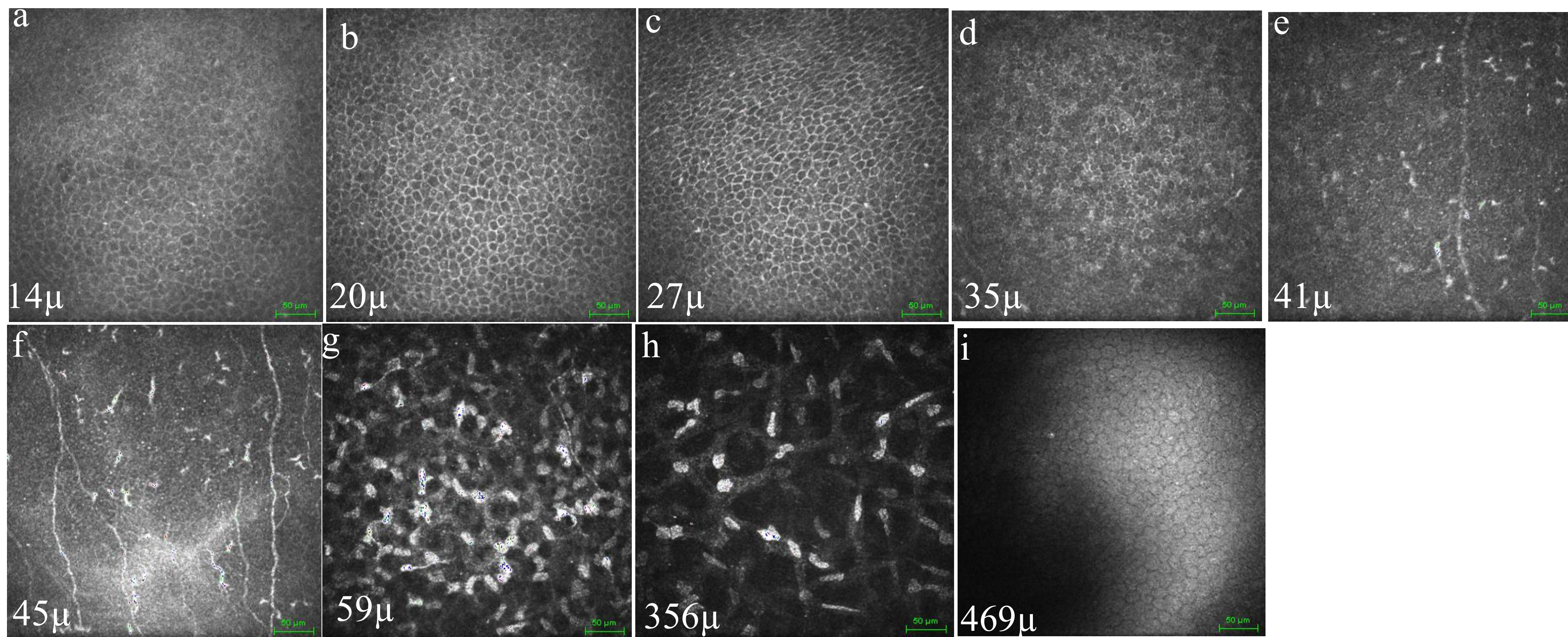


Figure 19. *In vivo* confocal microscopy images of different layers of the cornea. The images were captured from the central cornea of a 38 year-old male contact lens wearer.(a,b,c,d,e) epithelial layer, (f) sub-basal nerve plexus , (g,h) stroma, (i) endothelial layer. Bar represents 50 µm.

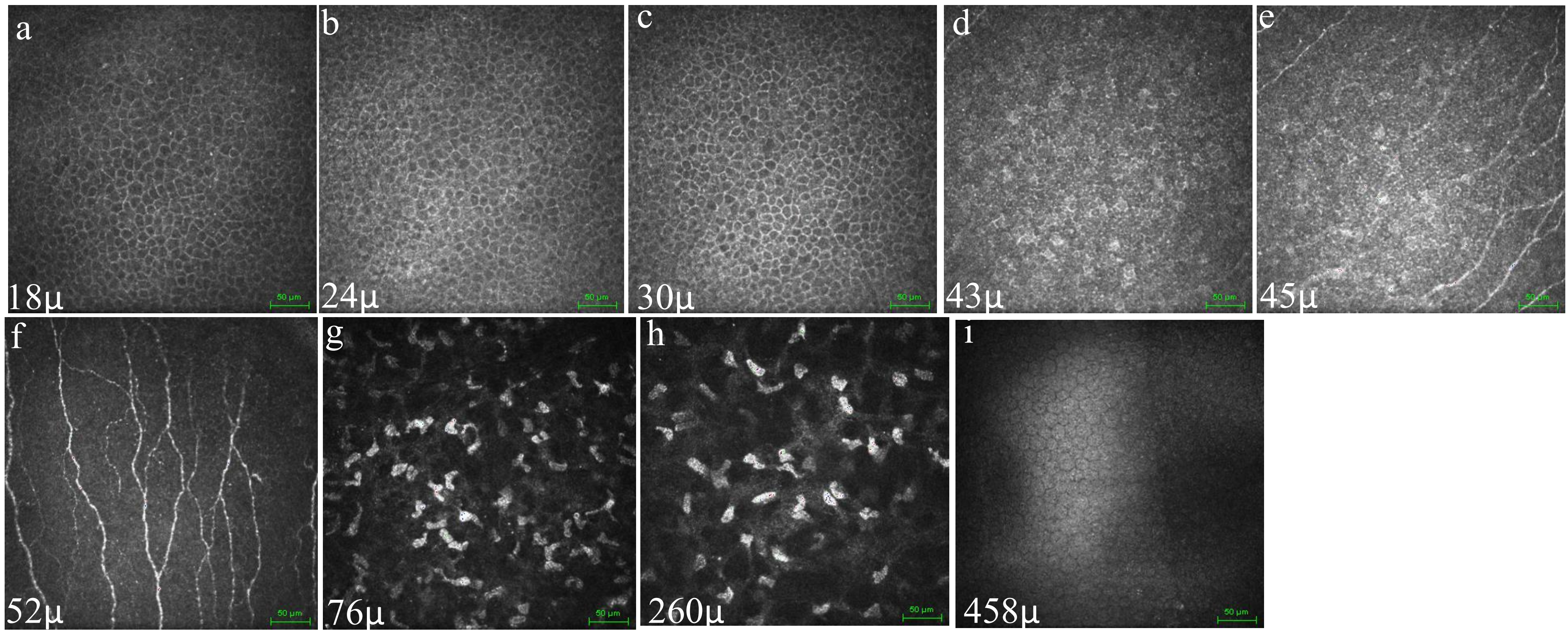


Figure 20. *In vivo* confocal microscopy images of different layers of the cornea. The images were captured from the central cornea of a 34 year-old male non- contact lens wearer. (a,b,c,d,e) epithelial layer, (f) sub-basal nerve plexus , (g,h) stroma, (i) endothelial layer. Bar represents 50 µm.

4.5 Discussion

Corneal presumed Langerhans cell population were higher among contact lens wearers than non-lens wearing control participants. The largest number of cells was observed at one-week post-wear, followed by a tendency to plateaux, suggesting adaptation. The adaptation process could be attributed to a diminished physical impact of the lens over time, as the tear film and anterior ocular structures adopt a new steady-state level of homeostasis.

The immune system (including presumed Langerhans cells) of the eye plays a role in protecting the integrity and function of the ocular surface (Zhivov et al. 2005). Presumed Langerhans cells have the ability to migrate via tissues and are able to capture, process and present antigens. These cells have the capability to stimulate and activate T cell responses (Liu et al. 2001). In non-inflamed conditions, presumed Langerhans cells are the only cells that are able to the expresses the major histocompatibility complex class II in the corneal epithelium (Klareskog et al. 1979). They help the cornea to respond to insults more rapidly and completely, and they improve the responsiveness of immune system (Asbell et al. 1987, Williamson et al. 1987, Jager 1992). It was reported that CCR7 plays a vital role in dendritic cell migration in steady-state and inflammatory conditions, as it allows dendritic cells to enter lymph nodes (Ohl et al. 2004). CCR7 is up-regulated in the iris in endotoxin-induced inflammation and is introduced in antigen-presenting cells of the cornea as a secondary to injuries (Jin et al. 2007).

Presumed Langerhans cell migration into the cornea of a contact lens wearer was supported by the study of Hazle et al. (1999) who found a return of the cell density to baseline values after fourteen days of lens wear. This process of recovery could explain that the cornea is adapting to lens wear.

This study provides an *in vivo* evaluation of presumed Langerhans cells in the central cornea of contact lens and non-contact lens wearers. The results show changes in presumed Langerhans cells in the central cornea of contact lens -induced dry eye and no contact lens-induced dry eye over a 24- week period. The sixty contact lens wearers in this study were fitted with soft hydrogel lenses only. Thus, the results in this study relate to only this type of soft contact lens. It is possible that other types of contact lenses, such as rigid or silicone hydrogel contact lenses, may induce different behaviours of the presumed Langerhans cells. This study used hydrogel lens as it gives more physiological stress, as a result of their lower oxygen transmissibility, on the cornea and the authors aimed to stress the cornea as much as possible within the normal accepted clinical guidelines. As a result of this inferior oxygen performance, hydrogel lenses might be associated with decreased comfort and greater levels of physiological alteration to anterior ocular structures compared with silicone hydrogel lenses. These factors may in turn lead to up-regulation of the immune response of the eye.

The current study confirmed that presumed Langerhans cells can be found in the normal cornea. The results of this study are consistent with the laser scanning

confocal microscopy studies conducted by other authors on the healthy corneas (Rosenberg et al. 2000, Popper et al. 2005, Zhivov et al. 2005, Mastropasqua et al. 2006, Resch et al. 2008, Guthoff et al. 2009, Lin et al. 2010, Marsovszky et al. 2012, Sindt et al. 2012, Villani et al. 2013, Machetta et al. 2014). Upon maturation, presumed Langerhans cells develop different phenotypes, mainly dendritic-like structure forms, and different cell functions (Teunissen et al. 1990, Banchereau et al. 1998). Further investigations about characterisation of presumed Langerhans cells ex vivo in enucleated, diseased and healthy eyes may be needed to understand the maturation and migration of the cells. This is because the characterisation of these cells in healthy conditions may not necessary apply to diseased eyes.

The current study illustrated that contact lens wear produces higher presumed Langerhans cells recruitment in the corneal centre of contact lens wearers compared to non-contact lens wearers, which is in agreement with previous *in vivo* corneal confocal microscopy studies on the corneas of contact lens wearers(Zhivov et al. 2007, Sindt et al. 2012).

The present study indicated that there was no significant impact of age on presumed Langerhans cells in the central cornea, which is in agreement with Sindt et al. (2012) and (Machetta et al. 2014), but was incongruent with the findings of Zhivov et al. (2007). Table 22 compares the results from this study to those of previous studies. In the previous studies, (Asbell et al. 1987, Auran et al. 1995, Dekaris et al. 1999, Hazle et al. 1999, Hamrah et al. 2002, Yamagami et al. 2005, Yamagami et al. 2005,

Zhivov et al. 2005, Mastropasqua et al. 2006, Sindt et al. 2012, Machetta et al. 2014) presumed Langerhans cells in the central cornea of non-contact lens wearers was found to range from 24 to 34 cells/mm^2. In the present study, the cell density was reported to be 26 ± 18 cells/mm^2 (mean ± SD), which was in the expected range. The difference in presumed Langerhans cell density between studies could be due to any one of the following:

1- Discrepancies in the inclusion and exclusion criteria between the studies. The current study had a strict inclusion criterion for healthy voulanteers.
2- The number of images used to represent the cell density was varied. The present study used six and five randomly selected images from the cornea and conjunctiva, respectively, which may have contributed to improving the standard deviation.
3- The study protocols differed among the studies.
4- The majority of the studies showed large variations in the number of cells within the control groups, which makes the sample size important in the evaluation of the total density of the cells. The current study recruited participants, based on a calculation of the required sample size to demonstrate an effect.

One week after contact lens wear, presumed Langerhans cells in the corneal centre increased from 26 ± 18 cells/ mm^2 to 55 ± 35 cells/ mm^2 in contact lens-induced dry eye ($p < 0.001$) and 43 ± 25 cells/ mm^2 in no contact lens dry eye ($p = 0.010$). The results, which show that contact lens wear increased the number of presumed Langerhans cells two-fold in the central cornea, is in agreement with the studies by

Sankaridurg et al. (2000), Zhivov et al. (2007), Sindt et al. (2012), and (Machetta et al. 2014). At the four-week visit, presumed Langerhans cell density in contact lens-induced dry eye vs no contact lens-induced dry eye was significantly different (55 ± 35 vs 35 ± 24 cells/mm^2, $p < 0.001$). However, presumed Langerhans cell density was not significantly different after 24 weeks of lens wear. This study also evaluates the cells in contact lens-induced dry eye and in no contact lens induced-dry eye.

Corneal presumed Langerhans cell density was found to be higher in individuals with contact lens -induced dry eye than contact lens wearers without dry eye. Presumed Langerhans cell density increased rapidly after one week of lens wear in the central corneas of contact lens-induced dry eye ($p < 0.001$) and no contact lens induced- dry eye ($p = 0.010$). Higher levels of presumed Langerhans cell populations were not found to be associated with dry eye severity among contact lens wearers (Figure 18).

The current study confirmed that presumed Langerhans cells are present in the centre of healthy corneas. The cells are found at the basal epithelium, sub-basal nerve plexus, and Bowman's layer. The studies of Rosenberg et al. (2000), Patel et al. (2005), Popper et al. (2005), Resch et al. (2008), Guthoff et al. (2009), Lin et al. (2010),Tavakoli et al. (2011), Marsovszky et al. (2012), Sindt et al. (2012) and (Machetta et al. 2014) are congruent with the current finding.

Migration of presumed Langerhans cells to the cornea is a controversial issue. The traditional model of migration of the cells from and into the epidermis is not appropriate to be applied to the cornea as the cornea is an avascular tissue (Dekaris et al. 1999). Some researchers have argued that the cells migrate to the central cornea from the underlying area of the cornea using sub-epithelial nerve plexus to move inside the cornea (Thoft et al. 1983, Auran et al. 1995, Ladage et al. 2003). Cell immigration to the corneal centre in contact lens wearers, possibly indicates that the cornea is responding to contact lens wear as a foreign body through the recruitment of more immune cells (Hazle et al. 1999, Zhivov et al. 2007). Increased presumed Langerhans cells in the cornea of contact lens wearers may also be occurring in response to hypoxia, cytokine-mediated, and mechanical effects (Kallinikos et al. 2004).

Hamrah et al. (2003) argued that presumed Langerhans cells in the corneal centre transformed to mature phenotypes through formation of dendritic-like processes. Mature phenotypes are able to secrete interleukin-12, and represent an integral part of the immune system (Banchereau et al. 1998, Hamrah et al. 2003). The exact molecular mechanisms that regulate the maturation of presumed Langerhans cells or those that maintain the high levels of presumed Langerhans cells, in the cornea in an immature phenotype, are still unknown. Promoting immune-inflammatory responses on the ocular surface appears to be controlled by centripetal migration of presumed Langerhans cells (Dekaris et al. 1999, Randolph et al. 2008).

Increased presumed Langerhans cell density in the ocular tissue either in disease conditions or contact lens wearers does not affect the functioning of the eye and therefore does not lead to further problems. This is because the continued elevation of presumed Langerhans cells places the ocular surface in a ' response-ready mode'. Thus, the ocular tissues can react rapidly to challenges such as toxic, traumatic, viral and/or microbial insults (Efron 2012).

Dry eye is one of most common ophthalmic conditions worldwide (Lemp et al. 2007). Untreated dry eye may increase the risk of ocular infection. Unfortunately, there are no standardised and uniform diagnostic criteria for dry eye. There exists vastly different signs and symptoms of dry eye, and the aetiology of dry eye can be difficult to diagnose clinically. In this study, dry eye participants had shown at least one symptom and one sign of dry eye to be so diagnosed.

Table 22. The impact of age and sex on presumed Langerhans cell density (PLCD) in the corneal centre reported in the current study compared to previous studies. The table divided the participants into two groups: less than 30 and more than 30 year-old. Dividing participants into these two groups was because of that mean ages of the current study was 30 years and median was 28 years. Also, Zhivov et al. (2007)found that PLCD was higher in males less than 30 year-old and in females older than 30 years. SD, standard deviation; SEM, standard error of mean

Current study, n=60			Sindt et al. (2012) n= 53		Zhivov et al. (2007) n=55	
	n	PLCD (cells/mm^2) mean ± SD	n	PLCD (cells/mm^2) mean ± SD	n	PLCD (cells/mm^2) mean ± SEM
Lens wearer age						
>30	36	48 ± 31	23	48 ± 38	19	130 ± 60
30-50	24	49 ± 29	22	41 ± 6	29	40 ± 25
Lens wearer sex						
Females	43	18 ± 19	6	69 ± 72	16	52 ± 36
Males	17	15 ± 11	47	39 ± 71	39	90 ± 31

4.6 Conclusion

The more pronounced increase in presumed Langerhans cell density in the corneal centre observed at the one-week visit, suggests that contact lens wear causes an initial heightened sub-clinical inflammatory response in the cornea, which subsequently subsides but remains elevated compared to non-lens wearers indicating a degree of adaptation. Contact lens–induced dry eye causes higher recruitment of presumed Langerhans cells in the cornea than no contact lens–induced dry eye. This inward cell migration may serve as marker of the inflammatory status of the ocular tissues in dry eye. This increase in presumed Langerhans cell density renders the ocular tissues ready to react to external insult.

4.7 Overall Summary of the Result of Chapters 3 and 4

This section addresses the relative effects of short-term contact lens wear, eye rubbing, eye closure and the effect of contact lens-induced dry eye on presumed Langerhans cell density in the corneal centre. The aim of the comparison was to understand which factors elicited a greater recruitment of presumed Langerhans cell density into the cornea.

The presumed Langerhans cell density was compared between the 10 participants enrolled in the short-term effect of lens wear study (section 3.3). There were 30 participants in the eye rubbing study (section 3.4), 30 participants in the eye closure study (section 3.5), 25 contact lens-induced dry eye participants, and 35 no contact lens-induced dry eye participants (Chapter 4).

Analysis of variance (ANOVA) was used to determine the overall effect of activity on presumed Langerhans cell density and post-hoc testing (with Tukey HSD) was applied to determine the significance of differences between individual groups. The data were analysed using IBM SPSS Statistics for Windows, Version 21.0 IBM Corp. Armonk, NY, USA. A *p*-value of less than 0.05 was considered significant.

The presumed Langerhans cell recruitment differs by the stimulus applied ($p < 0.001$). In the samples observed, eye rubbing had the lowest number of presumed

Langerhans cell density (0.4 ± 2 cells/mm^2) and contact lens-induced dry eye the greatest (14±16 cells/mm^2). Significant differences were noted (using Tukey HSD post-hoc test) between the short-term contact lens wear and the eye rubbing studies ($p= 0.016$), between the eye rubbing and long-term contact lens-induced dry eye studies ($p < 0.001$), and between the long-term studies of contact lens-induced dry eye and no contact lens-induced dry eye ($p =0.030$) studies (24 weeks of lens wear). The effect of contact lens-induced dry eye presented a higher level of recruitment of presumed Langerhans cells in the corneal centre than did the other stimuli - Figure 21.

The corneas under the experimental conditions (eye rubbing and eye closure) underwent different effective stimuli (mechanical inflammatory and biologic inflammatory stimuli, respectively) and shorter corneal effect (30 minutes of eye rubbing and up to 8 hours of eye closure, compared to 24 weeks of contact lens wear in the other group). The discrepancies in the type of stimuli and time might have impacted on the overall outcomes from these two studies. Further, the study compared the eight hour contact lens wear effect on corneal presumed Langerhans cell recruitment, with 24 week contact lens wear. Despite the similarity of contact lens wearing time between contact lens-induced dry eye and no contact lens-induced dry eye (24 weeks of lens wear), a higher corneal presumed Langerhans cell population was found in contact lens-induced dry eye than in no contact lens-induced dry eye.

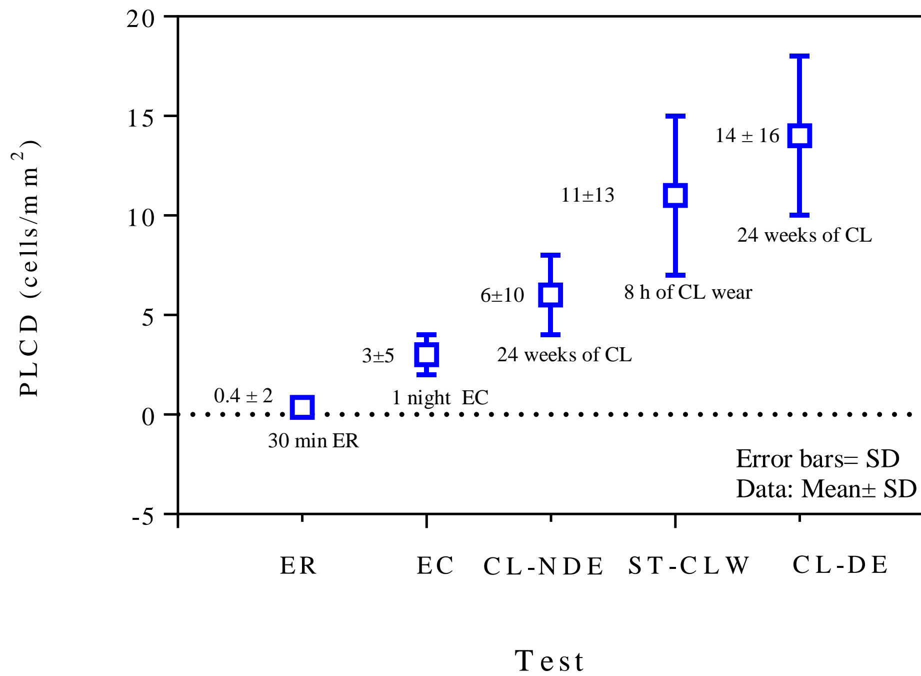


Figure 21. The effects of eye rubbing (ER), eye closure (EC), no contact lens -induced dry eye (CL-NDE), short term contact lens wear (ST-CLW) and contact lens -induced dry eye (CL-DE), on presumed Langerhans cell recruitment in the corneal center. SD, standard deviation.

Chapter 5: Effect of Contact Lens Wear on Presumed Langerhans Cells Recruitment in the Conjunctiva in Dry Eye and Non-Dry eye Contact Lens Wearers

Preface

The aforementioned chapter (Chapter 4) has investigated the effect of contact lens wear on presumed Langerhans cell recruitment in the corneal centre in dry eye and non-dry eye contact lens wearers. The study was conducted on a large cohort (n= 83) and it observed them over a six month period. The study showed that contact lens-induced dry eye is associated with an initial upregulation of presumed Langerhans cells in the cornea, which suggests an inflammatory basis for this condition during the initial phases of contact lens wear.

It has now become an obvious that there is an upregulation in presumed Langerhans cell in the cornea centre in contact lens wearers both in short (over 8 hours) and long-time (over 6 months) lens wear. However, the existence of this upregulation in the other parts of the ocular surface needs to be evaluated as well. The current chapter will concentrate on the evaluation of the effect of contact lens wear on presumed Langerhans cell density in the conjunctiva through recruiting the same sample as previous chapter (Chapter 4) over the same time course (six months).

5.1 Introduction

The conjunctiva is a highly immunologically sensitive tissue that contains a significant number of inflammatory cells (Stapleton et al. 2006). It acts as an immunological mediator and plays a role in maintaining a moist and hydrophilic ocular surface through the secretion of mucus (Becquet et al. 1996, Yang et al. 2000, Efron et al. 2010). Several studies have investigated presumed Langerhans cell density in the normal, healthy conjunctiva, both in humans and in animals (Böck et al. 1971, Rodrigues et al. 1981, Steuhl et al. 1995, Suzuki et al. 2000, Kobayashi et al. 2005, Zhivov et al. 2005, Zhivov et al. 2007, Efron et al. 2009, Efron et al. 2009). Possibly due to methodological issues, only one study failed to detect presumed Langerhans cells in the normal conjunctiva (Villani et al. 2013).

The individuals affected by dry eye has reached up to 30 million people worldwide, with most of the sufferers being women (reviewed in (Phadatare et al. 2015)).

5.2 Research Question and Hypothesis

5.2.1 Research Problem

There are approximately 140 million contact lens wearers worldwide (Morgan et al. 2012). Contact lens wear can induce or exacerbate dry eye. Dry eye among contact lens wearers often leads to discontinuation of lens wear (Doughty et al. 1997,

Pritchard et al. 1999, Nichols et al. 2005, Richdale et al. 2007). Contact lens -induced dry eye may lead to an increase in the density of presumed Langerhans cells in the conjunctiva. The effect of contact lens wear on presumed Langerhans cells in in the conjunctiva, in dry eye has received little attention in the literature. Only one study has examined the effect of contact lens wear on presumed Langerhans cells in the conjunctiva, which had a small sample size (Efron et al. 2010). This study was cross-sectional study and have not evaluated the cells over time.

5.2.2 Aim

The aim of the current study was to improve our understanding of presumed Langerhans cells in the conjunctiva *in vivo* among contact lens wearers, as well as to report on the inflammatory response to contact lens wear in dry eye.

5.2.3 Research Questions

The key research questions for the present study are as follows:

1) What is the effect of contact lens wear on presumed Langerhans cell populations in the conjunctiva?
2) What is the effect of contact lens wear on presumed Langerhans cell density in the conjunctiva in dry eye associated with contact lens wear?

5.2.4 Hypotheses

1) Conjunctival presumed Langerhans cell population will be higher among contact lens wearers than in non-lens wearing control participants.

2) Conjunctival presumed Langerhans cell density will be higher in individuals with contact lens -induced dry eye than contact lens wearers without dry eye

3) Higher levels of presumed Langerhans cell populations in the conjunctiva are associated with dry eye severity among contact lens wearers.

4) Conjunctival presumed Langerhans cell density increases over time after introduction to contact lens wear and reaches a steady state as the eye adjusts to contact lens wear.

5. 3 Methods

5.3.1 Participants

The recruited participants were identical to the population sample of the earlier study (Chapter 4). The same inclusion and exclusion criteria of the study were applied (refer to Chapter 2; section 2.1).

5.3.2 Study Design

The study design into the effect of contact lens wear on the presumed Langerhans cell recruitment in the conjunctiva of dry eye and non-dry eye contact lens wearers was discussed in detail in Chapter 2, section 2.2.

Dry eye examinations took place at each visit (refer to Chapter 2, section 2.3).

5.3.3 Corneal Confocal Microscopy

Presumed Langerhans cell density in the nasal bulbar conjunctiva was examined using a Heidelberg laser scanning confocal microscopy in combination with the Rostock Corneal Module. Corneal confocal microscopy was conducted on one eye only. To measure the nasal part of the conjunctiva, the surface of the TomoCap was positioned on the nasal bulbar conjunctiva, such that the centre of the applanating surface was about 2 to 4 mm from the limbus - Figure 22- (refer to Chapter 2 section 2.4).

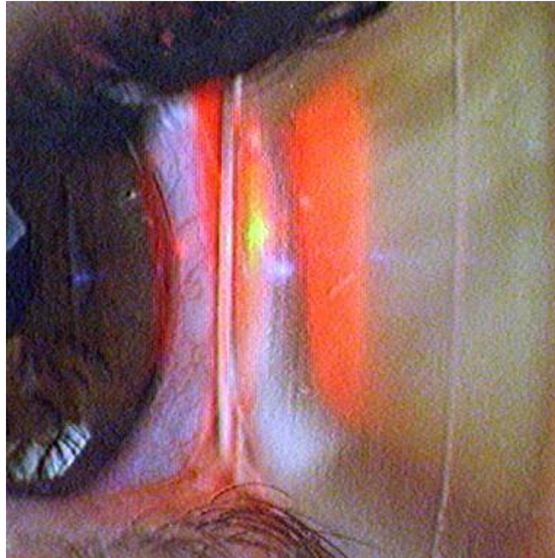


Figure 22. Image of the nasal bulbar conjunctiva applanated by the Tomocap while conducting using confocal microscopy.

5.3.4 Statistical Analysis

The statistical analysis is described in Chapter 4, section 4.3.4.

5.4 Results

Summary details of the contact lens wearers and control participants examined in this study are shown in Table 19 in Chapter 4. The cohort was sex-balanced (46% males) and no significant differences existed between the samples - ($\chi^2 = 0.373$, p = 0.541). There was no significant difference in age among the control, contact lens –induced dry eye and no contact lens –induced dry eye groups (F= 0.788, p = 0.469). Presumed Langerhans cells are located at any depths and layers of the conjunctiva

and appear in different forms when imaged using corneal confocal microscopy - Figure 22.

Over the 24 week observation period no changes were identified in presumed Langerhans cell density in the nasal bulbar conjunctiva in the control group ($F = 0.126$, $p = 0.944$).

Non-contact lens wearers at baseline had an average of 7 ± 7 cells/mm^2 similar to 7 ± 5 cells/mm^2 (mean ± SD) in the contact lens-induced dry eye group ($p = 0.857$). After one week of lens wear the cell population was significantly increased in contact lens-induced dry eye (17 ± 13 vs 7 ± 7 cells/mm^2, $p = 0.003$) - Figure 24. Four weeks after contact lens wear, presumed Langerhans cell populations in contact lens-induced dry eye was similar to baseline values (14 ± 11 vs 7 ± 7 cells/mm^2, respectively, $p = 0.094$). At the 24-week visit presumed Langerhans cell density was also similar to the baseline level (10 ± 8 vs 8 ± 7 cells/mm^2, respectively, $p = 0.292$).

At baseline presumed Langerhans cell density was similar to those with and without contact lens –induced dry eye (8 ± 9 cells/mm^2 (mean ± SD) vs 7 ± 7 cells/mm^2, $p = 0.886$). After one week of lens wear, the cell density was significantly different to the baseline levels in no contact lens-induced dry eye (17 ± 20 vs 7 ± 7 cells/mm^2, $p < 0.001$), then similar to baseline at the four-week visit (12 ± 12 vs 7 ± 7 cells/mm^2, $p = 0.173$) and 24-week visit (10 ± 11 vs 8 ± 7 cells/mm^2, $p = 0.516$) - Table 23.

Presumed Langerhans cell density was similar between contact lens-induced dry eye and no contact lens-induced dry eye at all-time points - Table 24.

Table 23. Presumed Langerhans cells behaviour in the nasal bulbar conjunctiva over the 24 week period. The result presented by their p values. A p-value of less than 0.05 was considered significant.

	0W	1W	4W	24W
CG vs CG at 1,4 &24		0.898	0.997	0.983
CG vs CL-DE	0.857	**0.003**	0.094	0.292
CG vs CL-NDE	0.886	**0.001**	0.173	0.516
CL-DE vs CL-NDE	0.739	0.952	0.656	0.623

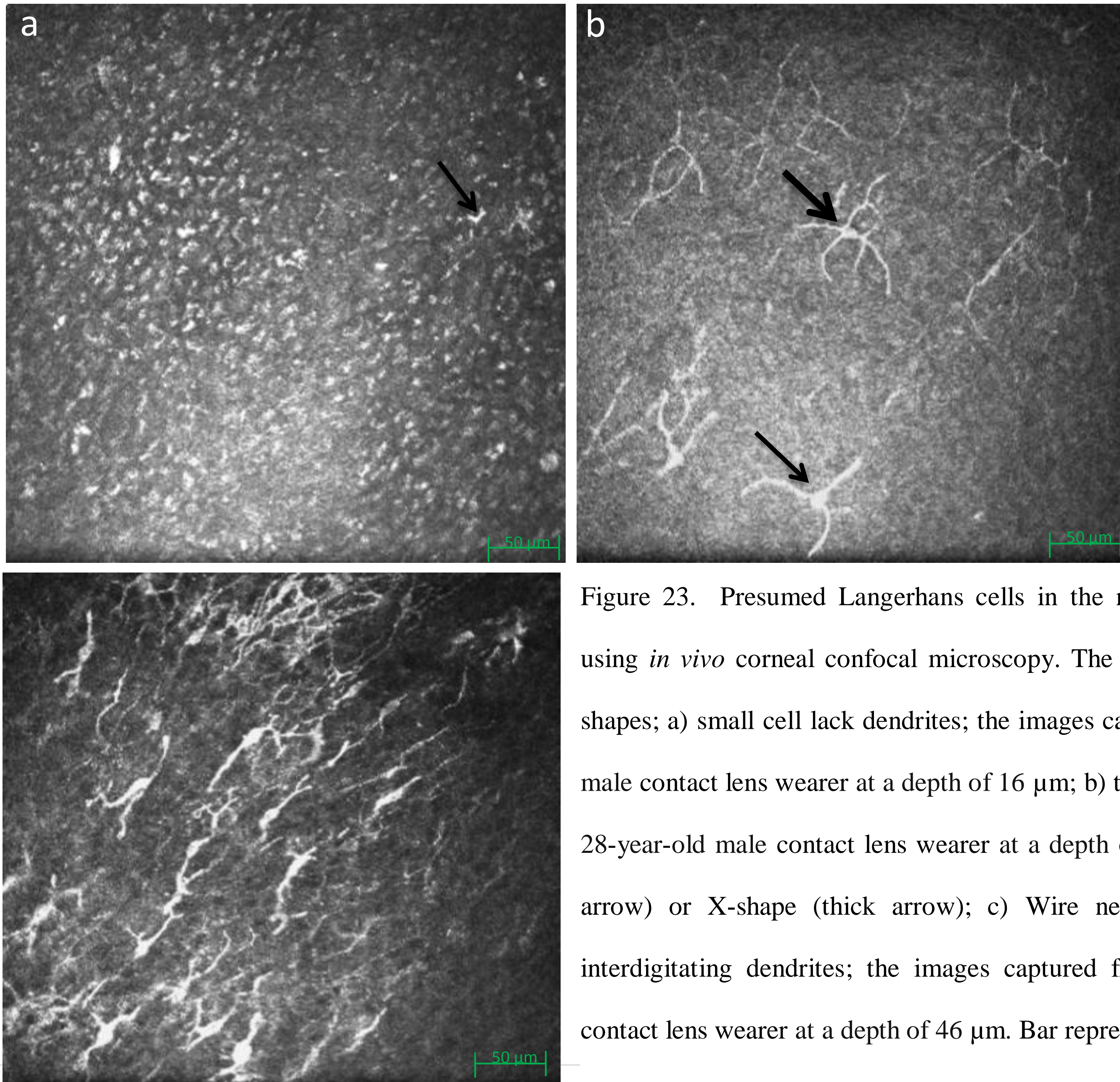


Figure 23. Presumed Langerhans cells in the nasal bulbar conjunctiva using *in vivo* corneal confocal microscopy. The cells appear in different shapes; a) small cell lack dendrites; the images captured from 22-year-old male contact lens wearer at a depth of 16 µm; b) the images captured from 28-year-old male contact lens wearer at a depth of 20 µm; Y-shape (thin arrow) or X-shape (thick arrow); c) Wire netting pattern with long interdigitating dendrites; the images captured from 25-year-old female contact lens wearer at a depth of 46 µm. Bar represents 50 µm.

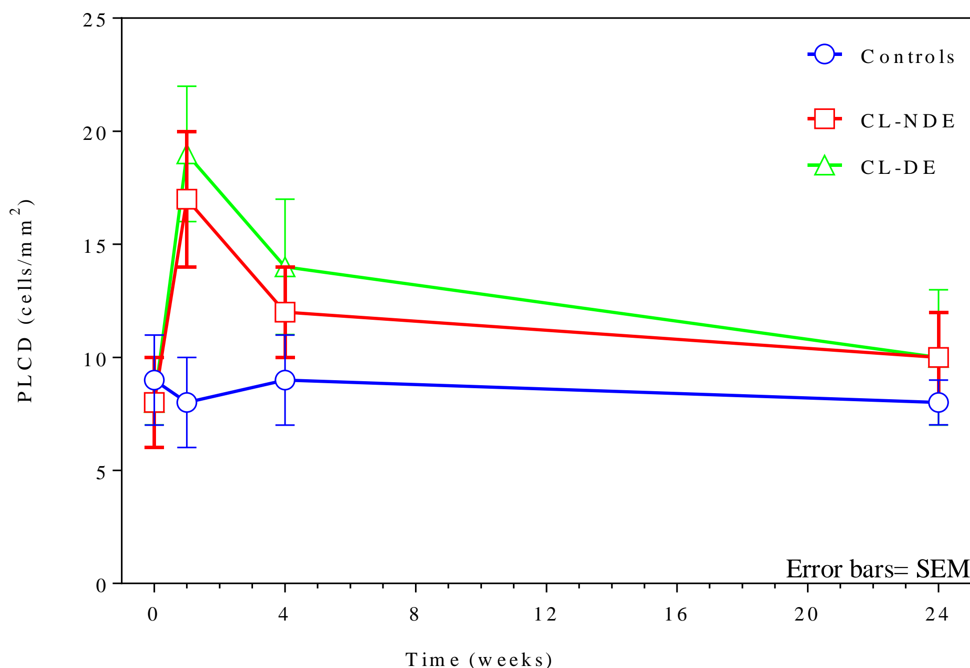
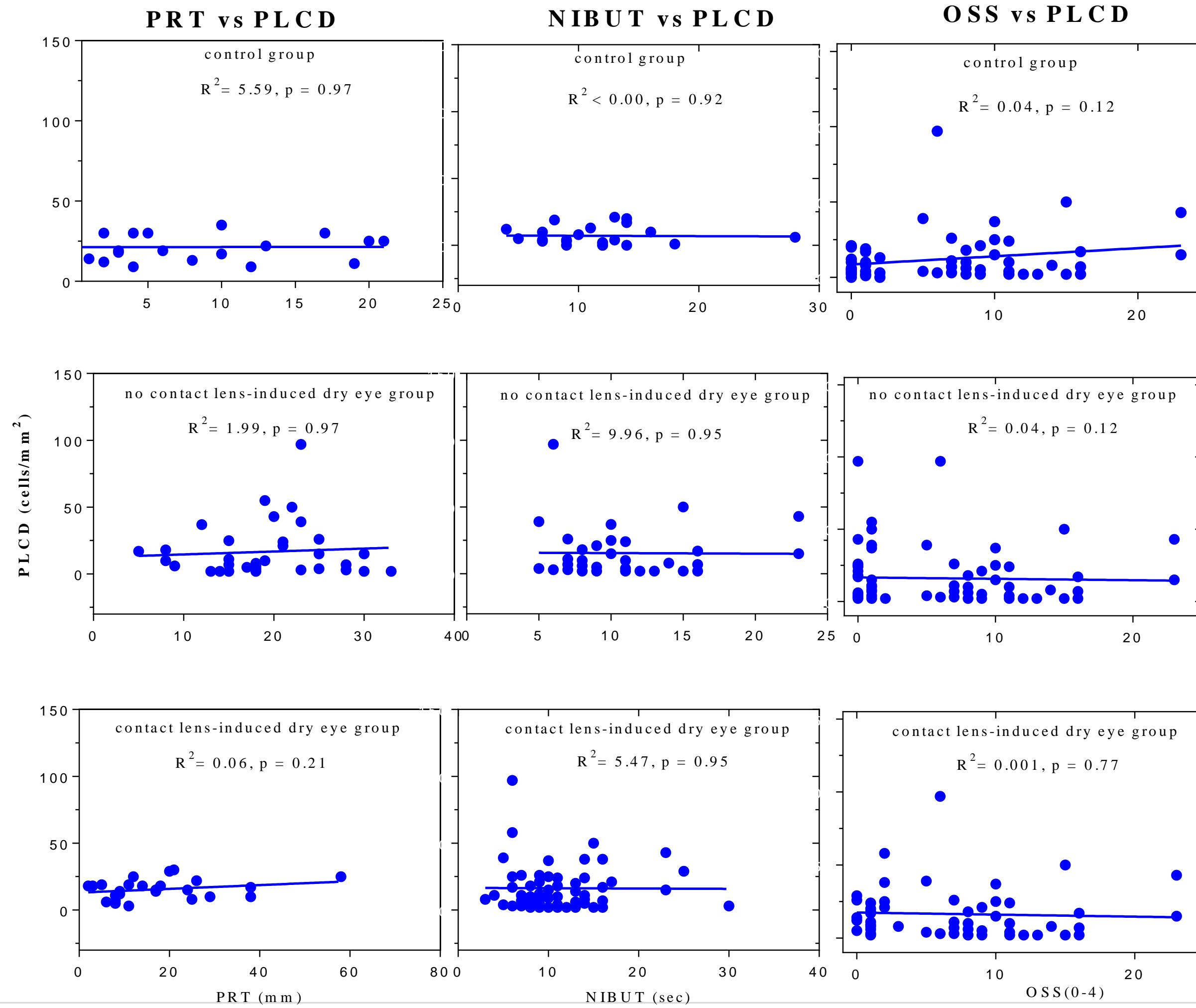


Figure 24. Presumed Langerhans cell density in the nasal bulbar conjunctiva over a 24 week period in the three groups: CL-NDE, no contact lens-induced dry eye, CL-DE; contact lens-induced dry eye. SEM, standard error of mean.

There was no significant interaction between time and sex for conjunctival presumed Langerhans cell density ($F = 1.473$, $p = 0.234$). The analyses also showed no significant interaction between time and age group for conjunctival presumed Langerhans cell density ($F = 0.303$, $p = 0.661$), when participants were grouped by age: ≤ 30 year-old, and >30 year-old.

The current study used five dry eye diagnostic tools are reported in Table 21 in Chapter 4. The severity/grade of the test is plotted against conjunctival presumed Langerhans cell density. There was no significant correlation between presumed

Langerhans cell density in the nasal bulbar conjunctiva and the severity/grade of the dry eye diagnostic tools, used in the study - Figure 25.



Chapter 5: Effect of contact lens wear on presumed Langerhans cells recruitment in the conjunctiva in dry eye and non-dry eye contact lens wearers.

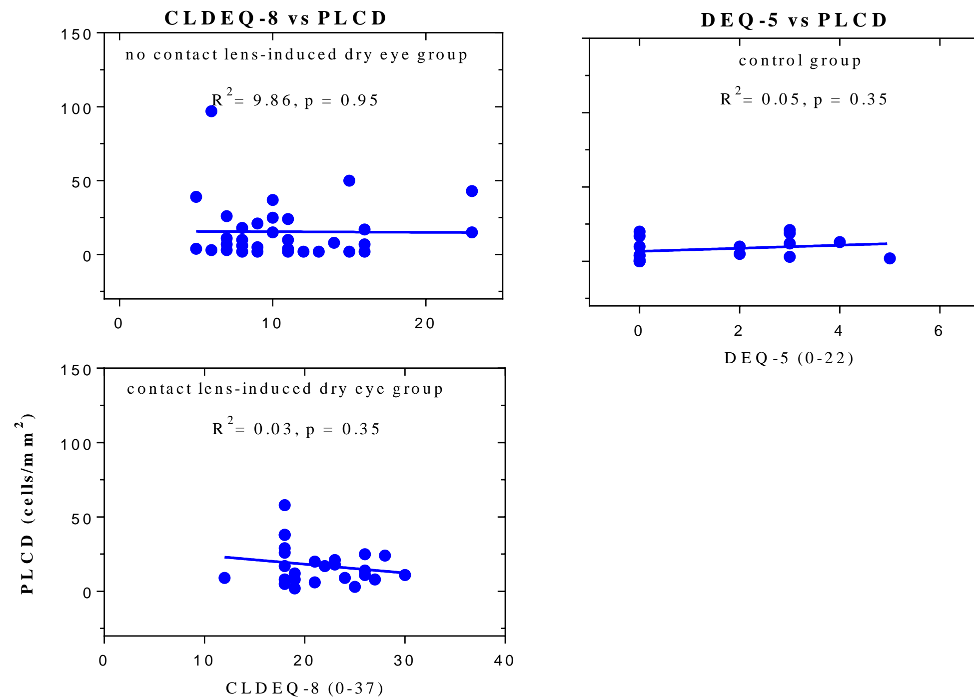


Figure 25. Correlation between presumed Langerhans cell density (PLCD) in the nasal bulbar conjunctiva and dry eye diagnostic tools used in the current study (phenol red thread (PRT), non-invasive tear break up time test (NIBUT), ocular surface staining (OSS), contact lens dry eye questionnaire-8 (CLDEQ-8) and dry eye questionnaire-5 (DEQ-5)). The data present the correlation between PLCD and the tools at the 1-week visit only.

5.5 Discussion

Conjunctival presumed Langerhans cell population were higher among contact lens wearers than non-lens wearing control participants and increased over time after introduction to contact lens wear. The largest number of cells was observed after one-week of lens wear, followed by a tendency to plateaux, suggesting adaptation. Higher levels of presumed Langerhans cell populations in the conjunctiva were not found to be associated with dry eye severity among contact lens wearers -Figure 25.

The conjunctiva is a vascular, immunologically tolerant, flat tissue that covers the inner surface of the eyelids and the eye globe. It plays a role in maintaining a moist and hydrophilic ocular surface through the secretion of mucus. It consists of two components: the epithelium and the stroma (Bron et al. 1985, Forrester et al. 2010). The conjunctival stroma contains different types of cells such as fibroblasts, leukocytes, and presumed Langerhans cells (Hogan et al. 1971).

This study has discussed the changes in presumed Langerhans cell population in the nasal bulbar conjunctiva of contact lens wearers. The sixty contact lens wearers were fitted with soft hydrogel lenses. Therefore, the changes in the presumed Langerhans cells were attributed to the soft lenses only. The results may change with the use of the other types of contact lenses.

In the current study, presumed Langerhans cells were reported in the healthy conjunctiva, using laser scanning confocal microscopy. This finding is in agreement with the studies of Efron et al. (2010) and Le et al. (2011). At the baseline measurement of the current study, presumed Langerhans cell density in the nasal bulbar conjunctiva of non-contact lens wearers was found to be 7 ± 7 cells/mm^2. This finding was lower than the findings of Efron et al. (2010) - (23 ± 25 cells/mm^2) and Le et al. (2011) – (42.1 ± 8.8 cells/mm^2). The fact that the cells were presented in a low density in the healthy participants could be explained by the strict inclusion of healthy voulanteers and the large sample size of the present study. These results are not in agreement with those of Villani et al. (2013) findings, who reported no presumed Langerhans cells in the conjunctiva.

The contact lens wearing group in this study revealed an approximately two-fold increase in the presumed Langerhans cell density one week after lens wearing. This finding contrasts with the study of Efron et al. (2010), who reported no significant difference in the cell recruitment between contact lens wearers and non-lens wearers. This result might have arisen because Efron et al. recruited a small number of participants in their study.

The increase in presumed Langerhans cell density in the nasal bulbar conjunctiva of contact lens wearers could be best explained by that fact that contact lens wear leads to disruption of the ocular homoeostasis (Dart et al. 1991, Stapleton et al. 1993, Ladage 2004). Changes in conjunctival Langerhans cell density may also be

attributed to hypoxia, cytokine-mediated, and mechanical effects on the anterior ocular surface (Kallinikos et al. 2004).

Presumed Langerhans cell density was reported at various layers of the conjunctiva. The cells are often hyper-reflective, and appear as small cells lacking dendrites, Y-shapes, X-shapes or/ and wire netting patterns with long interdigitating dendrites (Zhivov et al. 2005, Efron et al. 2010). The current study reported these shapes as well -Figure 23. The cells are easily identified because of their large size and their dendritic shape.

5.6 Conclusion

Contact lens wear increases presumed Langerhans cell recruitment two-fold in the nasal bulbar conjunctiva. The increment observed at the one-week visit, suggests that contact lens wear causes an initial heightened sub-clinical inflammatory response in the conjunctiva. There is a higher presumed Langerhans cell population presenting in the conjunctivas of contact lens-induced dry eyes compared to no contact lens-induced dry eyes. Dry eye induces upregulation of presumed Langerhans cells and may lead to a modulation and maturation of the cells. This upregulation contributes to the inward migration of presumed Langerhans cells toward the cornea. Release pro-inflammatory cytokines and/or phenotypes created by chemokines may contribute to the formation of mature presumed Langerhans cell phenotypes. Corneal confocal microcopy is capable of assessing the immune response to contact lens wear

by assessing presumed Langerhans cells in the conjunctiva. Further studies are required to understand the role of presumed Langerhans cells in the cornea of healthy and dry eye contact lens-wearing individuals.

5.7 Subsequent Study

The above observation noted an increase in conjunctival presumed Langerhans cell recruitment after one week of lens wear but no difference after one month of wear. During the blinking process, the area of the eyelid, known as the lid wiper, wipes over the ocular surface and causes a rubbing effect on the ocular surface (Korb et al. 2002). In the case of contact lens wear, the lid wiper interacts with the anterior surface of contact lenses (Korb et al. 2002). This interaction between the lid wiper and anterior surface of a contact lens may cause sub-clinical inflammatory response in the lid wiper, especially in contact lens-induced dry eye. The next chapter will recruit the same participants participated in the study in Chapters 4 and 5 and evaluate the lid wiper in contact lens-induced dry eye and no contact lens- induced dry eye after six months of lens wear.

Chapter 6: Effect of Contact Lens Wear on Presumed Langerhans Cells Recruitment in the Lid Wiper in Dry Eye and Non-Dry eye Contact Lens Wearers

Preface

The aforementioned studies (Chapters 4 and 5) showed that contact lens -induced dry eye is associated with an upregulation of presumed Langerhans cells in the corneal centre and nasal bulbar conjunctiva. In the case of contact lens-induced dry eye, the lid wiper travels across the ocular or lens surface during blinking and there might be a mechanical or frictional effect on the lid wiper. This effect may lead to an upregulation of presumed Langerhans cells in the lid wiper. The following section will investigate the effect of contact lens wear on presumed Langerhans cell density in the lid wiper in contact lens-induced dry eye and no contact lens-induced dry eye after six months of lens wear.

6.1 Introduction

The lid wiper is the area of marginal conjunctiva of the upper and lower portion of the eyelid (Knop et al. 2012). The lid wiper wipes over the ocular surface during the blinking process and, so, is named the lid wiper (Korb et al. 2002, Korb et al. 2005, Korb et al. 2010). A normal lid wiper is 0.4 to 0.6 mm in width and 100 µm height, in its initial portion, and decreases gradually towards the tarsal conjunctiva. The lid wiper tends to be narrower at the centre of the eyelid compared to the temporal side - Figure 2.

The lid wiper epithelium is thicker than the tarsal conjunctival epithelium. It contains approximately 8 to 15 cell layers, and is up to 100 mm thick. However, its thickness varies among individuals (Korb et al. 2010, Knop et al. 2012). In 1904, Parsons demonstrated for the first time that the inner part of lid border interacts with the ocular surface (cited by (Korb et al. 2005)).

The lid wiper is essential for maintaining ocular surface integrity through distribution of the tear film. Therefore, participants with dry eye are more likely to report symptoms of lid wiper epitheliopathy compared to healthy, non-dry eye individuals (Korb et al. 2002, Korb et al. 2005). Since reporting the alterations of the inner lid border (lid wiper epitheliopathy), the lid wiper became of increased interest to many researchers such as Korb et al. (2010) and Shiraishi et al. (2014) particularly with respect to contact lens wear.

A study conducted by Matsumoto et al. (2009) examined inflammatory cell density in patients with obstructive meibomian gland disease, using *in vivo* corneal confocal microscopy. They found that the cells were observed in the eyelid in controls (882 ± 301 cells/mm²) and in the experimental group (1216 ± 328 cells/mm²). They demonstrated that patients with obstructive meibomian gland disease showed 10 to 13 times higher cell densities than healthy controls. However, both control and experimental groups had eyelid treatment for 12 weeks before undergoing ocular examinations. Another study conducted by Ibrahim et al. (2010) investigated the density of inflammatory cells in the eyelid, using *in vivo* corneal confocal microscopy. It was found that the cells were present in the eyelid in controls (56± 32 cells/mm²), and in patients with meibomian gland dysfunction (1026 ± 537 cells/mm²). However, both control and experimental groups had Sjögren's syndrome, ocular surface surgery or a procedure that affected in the integrity of the ocular surface. No previous study investigated the lid wiper region in contact lens wearers, and healthy controls, using *in vivo* corneal confocal microscopy.

The lid wiper plays an essential role in ocular comfort through the interaction between the tear film and the ocular surface. Experiencing dry eye after contact lens wear may also be associated with lid wiper defects (Korb et al. 2002). Dryness is usually reported as a result of insufficient tear film thickness to separate the lid wiper and the ocular surface (Korb et al. 2005). Lid wiper defects may lead to inflammation of the ocular surface (Korb et al. 2010). In contact lens-induced dry eye, the lid wiper region has a greater degree of interaction with the contact lens surface (instead of the tear film), causing a trauma to the lid wiper. However, this disorder not only affects dry eye contact lens wearers, but it influences non-contact

lens wearers too (Korb et al. 2002, Korb et al. 2010). Indeed, contact lens wear is a major factor that may lead to instability of the tear film. Lens wear may also lead to an increase in presumed Langerhans cell populations in the ocular surface, typically in the cornea. This finding should encourage to do more research into the effect of lens wear on the other parts of the ocular surface, such as the lid wiper. Investigating presumed Langerhans cell recruitments in the lid wiper will lead to further understanding of the immune response of the ocular surface to contact lens wear and the pathophysiology of the lid wiper. Therefore, an investigation is necessary into the effect of contact lens wear on the ocular cells, such as inflammatory cells.

6.2 Research Question and Hypothesis

6.2.1 Research Problem

In a dry eye, the lid wiper wipes the ocular surface during blinking leading to possibly mechanical trauma of lid border and epithelial cells. This interaction is automatic and frequent as the normal blink rate ranges from three to fifteen times per minute, in an attempt to keep lubricated (Monster et al. 1977, Carney et al. 1982). In contact lens wearers this process causes the lid wiper to interact with the surface of the contact lens, which leads to dry eye symptoms, even if dry eye signs are absent (Korb et al. 2002). This interaction may also affect lid wiper integrity, leading to an increase in inflammatory cell density in this portion. No previous studies have examined the effect of contact lens wear on inflammatory cell recruitment (presumed Langerhans cells) in the lid wiper in non- contact lens and contact lens wearers.

6.2.2 Aim

The aim of the present study was to improve our understanding of the behaviour of presumed Langerhans cells *in vivo* in the lid wiper of contact lens wearers, and also provide an indication of the inflammatory response to contact lens wear which results in dry eye.

6.2.3 Research Questions

The key research questions for the present study are as follows:

1) What is the effect of contact lens wear on presumed Langerhans cell populations in the lid wiper?
2) What is the effect of contact lens wear on presumed Langerhans cell density in the lid wiper or people with contact lens induced dry eye?

6.2.4 Hypotheses

1) Lid wiper presumed Langerhans cell population will be higher among contact lens wearers than in non-lens wearing control participants.
2) Lid wiper presumed Langerhans cell density will be higher in individuals with contact lens-induced dry eye than contact lens wearers without dry eye.

6.3 Methods

6.3.1 Participants

The current study used the same participant sample as the study reported in Chapter . Only those participants who completed the 24 week visit were recruited for this study. Sixty-six non-contact lens wearers (37 females and 29 males) aged 30 ± 8 (mean ± SD) years, (range 18-50 years) were enrolled in the study. Written informed consent was obtained from all participants before their inclusion in the study. The study procedures were performed in accordance with the University Human Research Ethics Committee at the Queensland University of Technology (Ethics approval number 1300000117), and the principles of the Declaration of Helsinki. The inclusion and exclusion criteria of the project were applied (refer to Chapter 2)

6.3.2 Study Design

The design was a cross-sectional study, which observed participants after six months of lens wear, who were compared to individuals who had not worn contact lenses. Sixty-six participants completed the six-months from the baseline visit. Seventeen participants with contact lens-induced dry eye and twenty-nine with no contact lens-induced dry eye, were in the study groups. Twenty age-matched non-lens wearers served as the control group. A full explanation of dry eye examination tools and contact lens used in this study is addressed in Chapter 2; sections 2.3 and 2.5.

6.3.3 Corneal Confocal Microscopy

In vivo corneal confocal microscopy was used in this study (refer Chapter 2; section 2.4).

After six months, an assessment of the lid wiper was undertaken. To reduce the ocular sensation, one drop of anaesthetic (0.4% Oxybuprocaine hydrochloride, Chauvin Pharmaceuticals Ltd, UK) was applied to the eyes. Disposable cotton buds were used to evert the upper eyelid and prevent direct contact with the finger. The length of the lid wiper extends from superior punctum to the lateral canthus (Korb et al. 2005). To keep everted eyelid in position, participants were instructed to the keep looking down at all times. The examination was performed expeditiously as keeping the upper eyelid everted for long time is uncomfortable and may reduce tears secretion. The applanating lens was moved slightly in a vertical and a horizontal movement, while the focal plane was gradually moved into the lid wiper with the aim of capturing different groups of presumed Langerhans cells - Figure 26.

No previous study has determined the actual location of presumed Langerhans cells in the lid wiper. Therefore, images were captured at different depths of the lid wiper tissue. Six high-quality digital images (out of 100), not overlapping by more than 20% were randomly selected for analyses. All the examinations were performed on the same eye that was investigated earlier, and reported in Chapters 4 and 5. After

conducting the test, the eye was returned to its normal position and artificial tear drops were applied to the ocular surface to reduce discomfort

Figure 26. Image of lid wiper region applanated by the Tomocap while using confocal microscopy.

6.3.4 Statistical Analysis

Analysis of variance (ANOVA) was used to determine the overall effect of activity on presumed Langerhans cell population and post-hoc testing (with Tukey HSD) was applied to determine the significance of differences between individual groups. The data were analysed using IBM SPSS Statistics for Windows, Version 21.0 IBM Corp. Armonk, NY, USA. A *p*-value of less than 0.05 was considered to be statistically significant.

6.4 Results

The demographic data of the three study groups (20 non-lens wearers, 17 contact lens -induced dry eye and 29 no contact lens-induced dry eye) is shown in Table 24. There were no significant differences in age among the three groups ($F = 0.587$, $p = 0.559$). There were significant differences in presumed Langerhans cell density in the lid wiper among the three groups ($p < 0.001$). The cell density was significantly greater in the lid wiper of contact lens-induced dry eye compared to the control group (17 ± 10 vs 8 ± 4 cells/mm^2; $p < 0.001$), but similar in no contact lens-induced dry eye compared to the control group (10 ± 5 cells/mm^2 vs 8 ± 4 cells/mm$^{2;}$ $p = 0.489$). The presumed Langerhans cell density was significantly higher in contact lens-induced dry eye than no contact lens-induced dry eye (17 ± 10 vs 10 ± 5 cells/mm^2; $p = 0.002$). Figure 27 illustrates the differences in presumed Langerhans cells among the three groups. Presumed Langerhans cells were located at a depth of 1 to 154 µm, and were seen in immature and/or mature phenotypes – Figure 28.

Table 24. Characteristics of study participants.

Characteristic	CL-NDE	CL-DE	Controls
Sex (F/ M)	20/9	12/5	5/15
Age (year)	30 ± 9	29 ± 9	30 ± 9
Duration of lens (hour/day)	9 ± 3	9 ± 3	n/a
Contact lens power (D)-R/L	-1.70 ± 1.98/ -1.63 ± 1.79	-1.75 ±1.99/ -1.64 ± 1.80	n/a

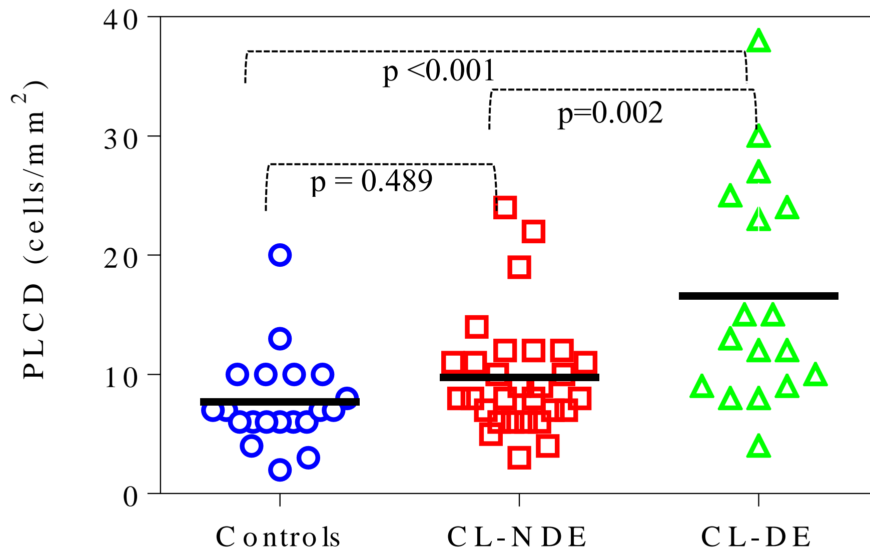


Figure 27. Presumed Langerhans cell density in the lid wiper after 24 weeks of lens wear. CL-NDE, no contact lens-induced dry eye, CL-DE; contact lens-induced dry eye . A *p*-value of less than 0.05 was considered significant. Data present mean ± SD.

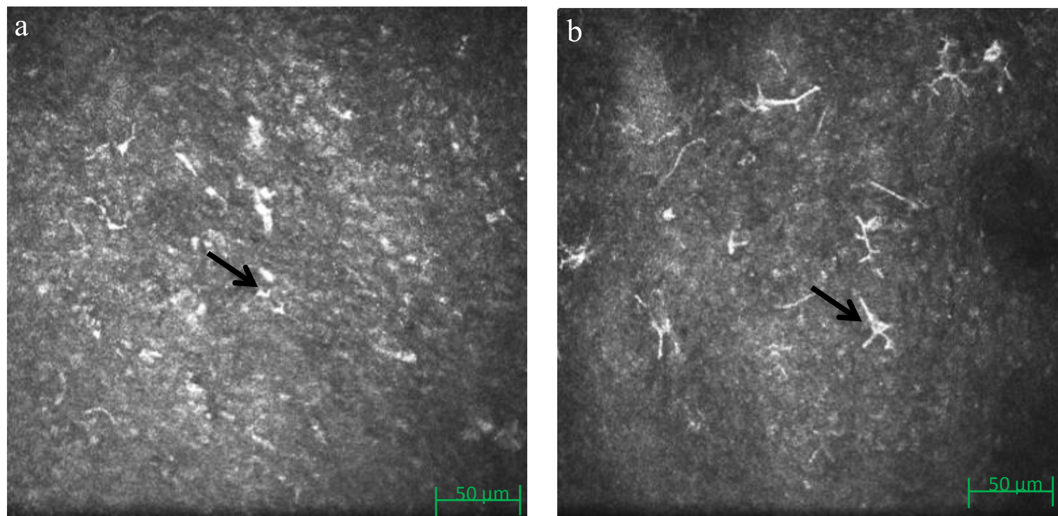


Figure 28. Presumed Langerhans cells in the lid wiper region using *in vivo* corneal confocal microscopy. The cells appear as different phenotypes; a) small cell lacking dendrites (black arrow); b) cells with long dendrites (black arrow). The images were captured from a 28-year-old male contact lens wearer at a depth of: a) 23 µm; b) 43 µm. Bar represents 50 µm.

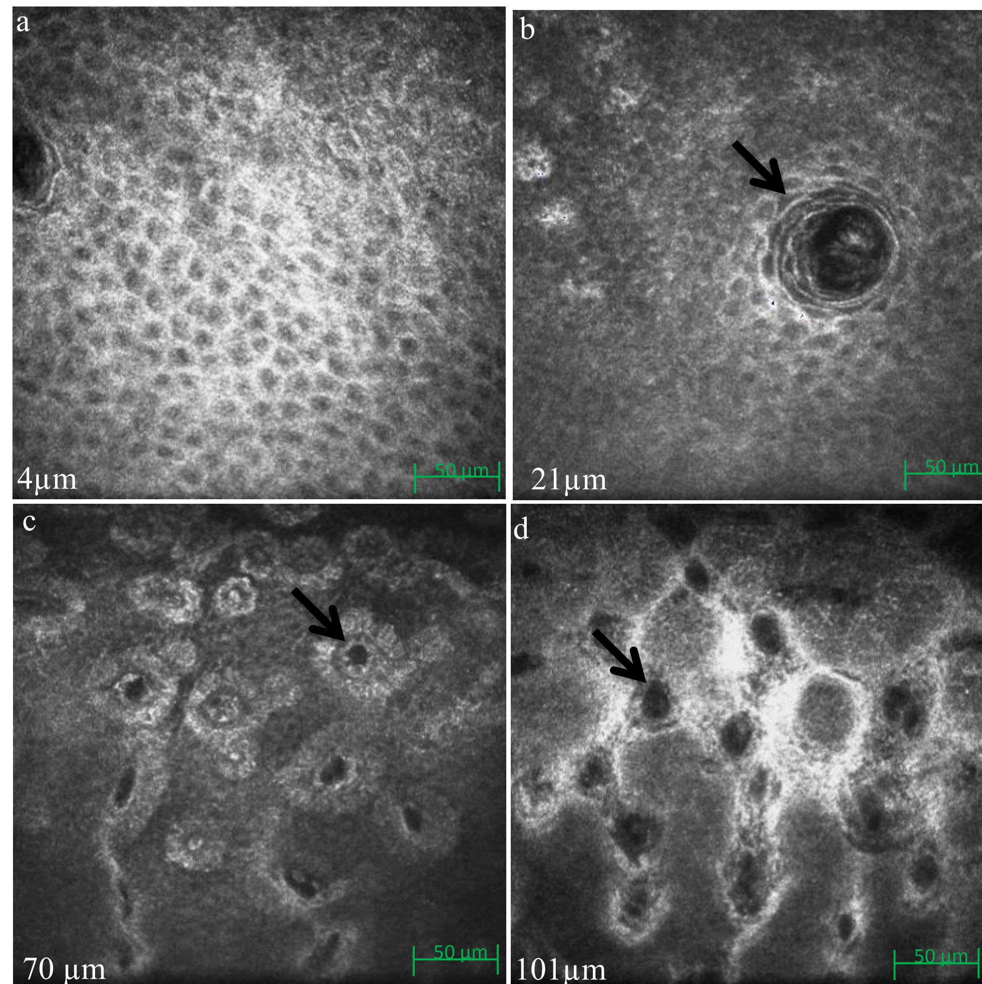


Figure 29. *In vivo* corneal confocal microscopy images of presumed Langerhans cells in the lid wiper: a) surface of lid wiper - the image was from a 22-year-old male non- contact lens wearer at a depth of 4 µm; b) Meibomian orifice (thick arrow) - on the area beside the lid wiper (the image was from a 32-year-old female contact lens wearer at a depth of 21 µm); c, d) Presumed goblet cells appear large in size, with a roundish to elongated shape, faint staining, and dense basal indented nuclei (thick arrows). They appeared dark possibly because they had released their contents onto the ocular surface - the image was from a 24-year-old male contact lens wearer at depths of 70 µm and 101 µm. µm, respectively. Bar represents 50 µm.

6.5 Discussion

The aim of the present study was to determine the effect of contact lens wear on presumed Langerhans cell populations in the lid wiper and investigate wearers with contact lens induced dry eye. This study has demonstrated, for the first time, that contact lens wear increases presumed Langerhans cell populations in the lid wiper of contact lens wearers *in vivo*. It is also illustrated that contact lens-induced dry eye increases the number of presumed Langerhans cells two-fold in the lid wiper compared to non-lens wearers ($p < 0.001$). This increment in presumed Langerhans cell recruitment could be explained by that fact that the lid wiper cells are exposed to mechanical trauma through the interaction of the lid wiper surafce with the contact lens suraface during the movement of the lid wiper on a contact lens-wearing eye (Korb et al. 2002, Korb et al. 2005, Korb et al. 2010). It was shown that the shear stress between the lid wiper and a contact lens suraface is greater than that between the lid wiper and the conjunctiva and cornea (Korb et al. 2005, Korb et al. 2010, Shiraishi et al. 2014). This increased frictional stress could lead to higher presumed Langerhans cell recruitment in the lid wiper. It could also damage the lid wiper causing and/or exacerbating dry eye. Dry eye is characterised by insufficient tear film thickness to separate the lid wiper and ocular surface. This deficiency may lead to lid wiper trauma as it as it comes into greater contact with the ocular surface during blinking. The effect is expected to be higher in contact lens wearers than non-lens wearers, as the lid wiper is negatively impacted by its interaction with the harder contact lens surface (Korb et al. 2002, Korb et al. 2005). This conclusion makes investigating immune responses of the lid wiper to the effects of contact lens wear important to understand, with specific regard to the recruitment of inflammatory cells (presumed Langerhans cells).

The lid wiper has a rubbing effect on the ocular surface through blinking process, which helps to spread the tears over the ocular surface or the surface of a contact lens on the eye (Ehlers et al. 1997, Knop et al. 2012). The lid wiper epithelium consists of large cuboidal and goblet cells, which together from eight to fifteen cell layers (Knop et al. 2011). In the case of insufficient tear production or contact lens-induced dry eye, excessive mechanical friction on the ocular surface may result. This effect is likely to lead to a trauma of the lid wiper region and, perhaps, lid wiper epitheliopathy (Mathers et al. 1992, Korb et al. 2002, Korb et al. 2005, Korb et al. 2008). This study aimed to understand the effect of contact lens wear on presumed Langerhans cell populations in the lid wiper region in participants with contact lens-induced dry eye, no contact lens-induced dry eye, and non-lens wearers. In the present study, presumed Langerhans cells were reported to be present in both contact lens and non-contact lens wearers. Contact lens-induced dry eye presents the highest density of the cells. This agrees with the results of Knop et al. (2011), in which dry eye sufferers present a six-fold greater frequency of lid wiper epitheliopathy than healthy controls. The present study found no difference in the cell recruitment between no contact lens-induced dry eye sufferers and non-contact lens wearers without dry eye ($p > 0.05$).

An increase in presumed Langerhans cell populations in the lid wiper presumably occurs as a result of more friction in the contact lens-induced dry eye group than in the healthy volunteer group. Therefore, further investigations are needed about the impact of the contact lens wear on the lid wiper goblet cell density.

Despite introducing a variety of dry eye evaluation tools, predicating contact lens-induced dry eye before lens fitting remains difficult. Bron (2001) and Nichols et al. (2004) had unsuccessful attempts in predicting the onset of dry eye in patients before wearing a contact lens. Pult et al. (2009) have described a Contact-Lens-Predictive-Test, which is able to predict dry eye in new contact lens wearers. This test may help eye practitioners to predict any potential increase in presumed Langerhans cells on the ocular surface or on the lid wiper before lens fitting.

Interestingly, the study of the effect of contact lens wear on presumed Langerhans cell recruitment in the cornea and conjunctiva showed that the cell recruitment recovered after 6 months of lens wear. However, the cells in the lid wiper region recovered in no contact lens-induced dry eye, and remained elevated in contact lens-induced dry, even after long-time lens wear. This could be attributed to the ocular surface cells underneath contact lens being protected by tears. The process of protection produces a lower effect from contact lens wear on the ocular surface cells than the effect on the lid wiper cells. Further, the lid wiper is a different eye tissue which behaves differently in response to contact lens wear when compared to the ocular surface response. For example, presumed Langerhans cells in the lid wiper may be modulated by blood supply compared to the cornea, in which there is no vascular modulation.

Presumed goblet cells were observed in the lid wiper epithelium *in vivo* - Figure 29. This finding was in agreement with the findings of Argüeso et al. (2001) and Knop et al. (2011). The significance of deep goblet cells has not yet been understood (Knop et al. 2012). These

cells can be observed individually (or in a cluster) in the epithelial layer of the lid wiper. They are characterised by their large size (20 to 25 µm in length and 10 to 15 µm width), are roundish to elongated in shape, show faint staining and a dense basal indented nuclei (Knop et al. 2012)- Figure 29. Presence of goblet cells may indicate need for more lubrication in the lid wiper region through an internal lubrication system (Knop et al. 2012). Based on histological findings, goblet cells have been observed in different locations of the lid wiper epithelium (at the surface and deep in the epithelium) in a thickness of 80 µm. These characteristics are distinct to those of conjunctival goblet cells. The cells play a vital role in reducing any potential friction and/or trauma in the interaction between the lid wiper and the ocular surface (Pult et al. 2009).

The structures observed in the cornea, conjunctiva and lid wiper, using cornea confocal microscopy, were presumed Langerhans cells. These cells could represent another form of immune cell such as macrophages or mast cells. However, macrophages are mainly observed in the corneal stroma and are found in a low densities (Hamrah et al. 2003). Mast cells may participate in immune reactions through the release of an array of biologically active media (Da Silva et al. 2014). Mast cells populate connective tissues surrounding blood vessels and mucus glands (Galli et al. 2005). A significant number of studies have found that migration of mast cells to different locations both in inflammatory and non-inflammatory conditions are directed by cytokines, chemokines and adhesion molecules (reviewed in (Collington et al. 2011). However, the mechanism of mast cell recruitment in inflammatory conditions has not been fully explained (Da Silva et al. 2014). Ocular structures are supplied with mast cells which can be found in the conjunctiva and limbus. They are best seen in histologic sections (Smith 1961, Smith et al. 1970, Larkin et al. 2010, Klintworth 2012).

Hyper-reflective, irregular, ovoid, multilobate, comma-shaped and non-dendritic objects have been observed in the conjunctiva. These cells were presumed to be conjunctival polymorphonuclear cells (Leukocytes)-(Messmer et al. 2006, Wakamatsu et al. 2009, Wakamatsu et al. 2010, Villani et al. 2011, Villani et al. 2013). Leukocyte cells can be seen in the cornea in immune-mediated diseases such as corneal graft rejection and herpes stromal keratitis. Leukocyte cells are the most abundant granulocytes in white blood cells which are responsible for healing of corneal epithelial wound (Gan et al. 1999, Li et al. 2006, Mastropasqua et al. 2006).

Polymorphonuclear cells are able to release of granular products and cytokines (Bourghardt Peebo et al. 2007). Studies found a high population of polymorphonuclear cells in the tears of dry eye, possibly an indication of severe damage of the ocular surface (Augustin et al. 1995, Lin et al. 2010).

Despite the similarity in the aforementioned cells with presumed Langerhans cells, *in vivo* corneal confocal microscopy can differentiate between dendritic cells and the other cells by virtue of their morphology and size. Development of new technology that can provide higher image resolutions than the current techniques will enable further exploration and understanding of different immunological cells in the eye.

6.6 Conclusion

Contact lens wearers with dry eyes have approximately two time the number of presumed Langerhans cells in the lid wiper compared to non-dry eye patients and non-lens wearers. The increment suggests that contact lens wear causes a sub-clinical inflammatory response in the lid wiper region. Further studies on the effect of contact lens wear on the lid wiper cells in short-term lens wear are required.

Chapter 7 Discussion and Conclusions

This thesis presents a series of studies using laser scanning confocal microscopy to investigate a particular cell type present in the ocular surface. The studies focused on assessing presumed Langerhans cell density in the cornea centre, nasal bulbar conjunctiva, and lid wiper in three groups of people: individuals with contact lens induced-dry eye; those wearing contact lenses without contact lens induced-dry eye, and non-contact lens wearers.

Of the 60 participants who were recruited into the contact lens group of this thesis, 25 (42%) of them developed contact lens induced-dry eye after one week. The results showed a heightened sub-clinical inflammatory response in the cornea and conjunctiva of those with contact lens-induced dry eye compared with those without contact lens-induced dry eye and non-lens wearers.

After six months of lens wear, presumed Langerhans cells in the lid wiper were evaluated. The study showed an increase in the cell density in the lid wiper in contact lens-induced dry eye compared with those without this condition. The results showed that contact lens-induced dry eye has associations with presumed Langerhans cell upregulation in the lid wiper.

These studies were characterised by their careful selection of the participants in respect of those with and without contact lens-induced dry eye as well as strict selection of participants in the control group. The operator assessing presumed Langerhans cells from the clinical status of the participants was masked in order to minimise any potential of bias. The study used the same lens type and the same length of time which helped to exclude any possible

confounding variables. Differences in presumed Langerhans cell between the three groups were sufficiently powered.

On the other hand, the study presents a limitation related to the gender imbalance of contact lens-induced dry eye and no contact lens-induced dry eye versus the control group. The imbalance may have confounded comparisons between those groups if significant gender differences exist in the parameters assessed in this work. The control group was recruited separately, rather than having been randomly assigned from a single combined recruitment cohort of potential study participants, which is considered as another limitation of these studies. Effect of contact lens wear on presumed Langerhans cell recruitment was limited to six months; thus the effect of longer time of lens wear is not explored.

Given that five participants dropped out of the study that investigated the effect of lens wear on presumed Langerhans cell recruitment as a result of lens discomfort from contact lens-induced dry eye versus one participants from the no contact lens-induced dry eye and none from the control group, the results may underestimate to full extent of presumed Langerhans cell upregulation in the lid wiper in contact lens-induced dry eye. The study also did not measure lid wiper epitheliopathy using staining techniques as a part of the lid wiper-related experiment. This decision was made in order to avoid any potential impacts of the staining on inflammatory response. However, the demonstration of a possible association between lid wiper epitheliopathy and increased presumed Langerhans cell population was precluded. Nonetheless, this association could to be investigated in future studies.

The limitation of laser scanning confocal miscopy for unambiguously identifying the observed bright features in corneal confocal microscopy images as presumed Langerhans

cells is another weakness, Notwithstanding the histochemical studies discussed in this thesis that strongly suggest that these dendritic cells are Langerhans cells (Chen et al. 2007, Mayer et al. 2007, Romani et al. 2012). The identification of the bright, dendritic features observed in corneal confocal microscopy images as presumed Langerhans cells may present another limitation. Immature forms of presumed Langerhans cells, which have short or absent dendrites, could be misidentified for other inflammatory cells types known to reside in ocular surface tissues, such a monocytes and polymorphs. Even if these bright features are some other cell type involved in the immunologic cascade of events, my conclusions are essentially unaltered because my observations support the overriding principle of inflammatory cell upregulation in contact lens-induced dry eye. Misidentification of tissue features as Langerhans cells would constitute a random error that would manifest without bias across all groups.

The thesis used one type of contact lens for the contact lens group, which limited data variability. This also precluded investigation into the effect of variety of surface characteristics (e.g. lubricity and wettability) that may have influenced comfort and thus upregulated presumed Langerhans cells.

7.1 Works Embodied in This Thesis

The works embodied in this thesis comprised many studies. The results of these studies add to the current knowledge regarding the relationship between contact lens wear and the one aspect of the immune system of the eye, namely presumed Langerhans cell behaviour in the cornea and conjunctiva. Before conducting the primary studies, a study was conducted to

identify the degree of repeatability in the assessment of presumed Langerhans cell recruitment. The results demonstrated that the measurement of presumed Langerhans cell recruitment on the ocular surface, using *in vivo* corneal confocal microscopy, is repeatable such that any differences observed between test (contact lens) and control (no contact lens) or over time was not due to the technique. No previous study had determined the minimum number of images that accurately represent the presumed Langerhans cell density in the cornea and conjunctiva. Therefore, a second study was conducted and showed that six and five randomly selected images from the cornea and conjunctiva, respectively, were sufficient to accurately define Langerhans cell density in the ocular surface.

The other important studies illustrated that factors, such as eye closure (i.e. more than six hours) and eye rubbing, can influence presumed Langerhans cells in the corneal centre, as these factors lead to a physiological stress on the cornea. This stress influences the cornea properties and lead to an increase in presumed Langerhans cell density. These factors appear to upregulate the immune status of the cornea, which may help explain the physiological mechanisms underpinning previous reports of the ocular response to eye rubbing and connect them to the clinical practice.

The primary studies provided evidence that contact lens wear increases the number of presumed Langerhans cells in the corneal centre and nasal bulbar conjunctiva. This increase in the cell density is greater in contact lens-induced dry eye, indicating a heightened sub-clinical inflammatory response in the cornea, conjunctiva and lid wiper. Cell populations in the cornea and conjunctiva appear to recover over time as the eye adjusts to a contact lens wear; however, cell numbers remain elevated in the lid wiper region in those with contact

lens dry eye. Dry eye results from insufficient tear film thickness, which makes the tear film unable to adequately separate the lid wiper and anterior surface of a contact lens. This deficiency in separating the two surfaces leads to a lid wiper trauma through continuous interaction of the lid wiper, and the anterior surface of the contact lens, during the blinking process.

The outcomes from the primary studies encouraged the authors of this work to conduct another study to understand the effect of eight hours of contact lens wear on the presumed Langerhans cell recruitment to the corneal centre. This study generated novel information regarding the effect of a one-day (typically eight hours) lens wear on presumed Langerhans cell recruitment to the corneal centre. This study showed that the cell recruitment rapidly increases after a few hours of lens wear and gradually recovers in the following hours, but not back to baseline levels.

These outcomes support the notion that contact lens wear induces physiological stress upon the ocular surface (Kallinikos et al. 2004). The results also show that both the cornea and the conjunctiva respond to a contact lens as a foreign body (Su et al. 2006, Zhivov et al. 2007, Sindt et al. 2012).

7.2 Recommendations and Future Directions of this Research

The studies discussed in this thesis were longitudinal, focused on the effect of contact lens wear on the ocular surface , typically the corneal centre, nasal bulbar conjunctiva, and the lid

wiper. The information derived from these studies will inform eye researchers and practitioners that contact lens wear causes a constant sub-clinical eye inflammation. Such information will encourage researchers to undertake more research into the behaviour of inflammatory cells and mediators during contact lens wear. Future research should include an examination of the impact of a variety of lens types, materials, modalities, lens care solutions, and lens replacement frequencies on presumed Langerhans cell recruitment, as an indicator of the inflammatory status induced by these various factors. Research should be undertaken into the influence of sleep with contact lenses, or eye rubbing during lens wear on inflammatory cells.

The outcomes of these studies, will help researchers, and clinicians to further understand the inflammatory response to contact lens wear. The outcome may also help them understand the physiological basis of a range of contact complications, such as staining of the cornea and conjunctiva, contact lens induced-dry eye and papillary conjunctivitis (Efron 2012). Assessment of presumed Langerhans cell density may also assist the contact lens industry to determine optimal lens design characteristics. Further, this approach could be used as a potential marker of the sub-clinical inflammatory status of the eye, which could be used by contact lens industry to assess the ocular response to different lens designs and solution formulations. Further laboratory experiments are essential to provide immunohistological evidence, supporting that these formation of cells are Langerhans cells.

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Appendices

Appendix 1: Ethics Approval Certificate

University Human Research Ethics Committee

HUMAN ETHICS APPROVAL CERTIFICATE

NHMRC Registered Committee Number EC00171

Date of Issue: 22/5/13 (supersedes all previously issued certificates)

Dear Miss Luisa Holguin Colorado

A UHREC should clearly communicate its decisions about a research proposal to the researcher and the final decision to approve or reject a proposal should be communicated to the researcher in writing. This Approval Certificate serves as your written notice that the proposal has met the requirements of the *National Statement on Research involving Human Participation* and has been approved on that basis. You are therefore authorised to commence activities as outlined in your proposal application, subject to any specific and standard conditions detailed in this document.

Within this Approval Certificate are:

* Project Details
* Participant Details
* Conditions of Approval (Specific and Standard)

Researchers should report to the UHREC, via the Research Ethics Coordinator, events that might affect continued ethical acceptability of the project, including, but not limited to:

(a) serious or unexpected adverse effects on participants; and
(b) proposed significant changes in the conduct, the participant profile or the risks of the proposed research.

Further information regarding your ongoing obligations regarding human based research can be found via the Research Ethics website http://www.research.qut.edu.au/ethics/ or by contacting the Research Ethics Coordinator on 07 3138 2091 or ethicscontact@qut.edu.au

If any details within this Approval Certificate are incorrect please advise the Research Ethics Unit within 10 days of receipt of this certificate.

Project Details

Category of Approval:	Human non-HREC		
Approved From:	20/05/2013	**Approved Until:**	20/05/2018 (subject to annual reports)
Approval Number:	1300000117		
Project Title:	Impact of contact lens wear on conjunctival goblet and langerhans cells		
Experiment Summary:	Examine the characteristics of these cells in the cornea the front clear window of the eye) and conjunctiva (the glistening clear tissue seen covering the white of the eye) and to identify any potential consequences of contact lens wear.		

Investigator Details

Chief Investigator: Miss Luisa Holguin Colorado

Other Staff/Students:

Investigator Name	**Type**	**Role**
Prof Nathan Efron	Internal	Supervisor
Dr Nicola Pritchard	Internal	Supervisor
Mr Yahya Ahmed M Alzahrani	Student	Ethics- Student- Course- Doctoral

RM Report No. E801 Version 4

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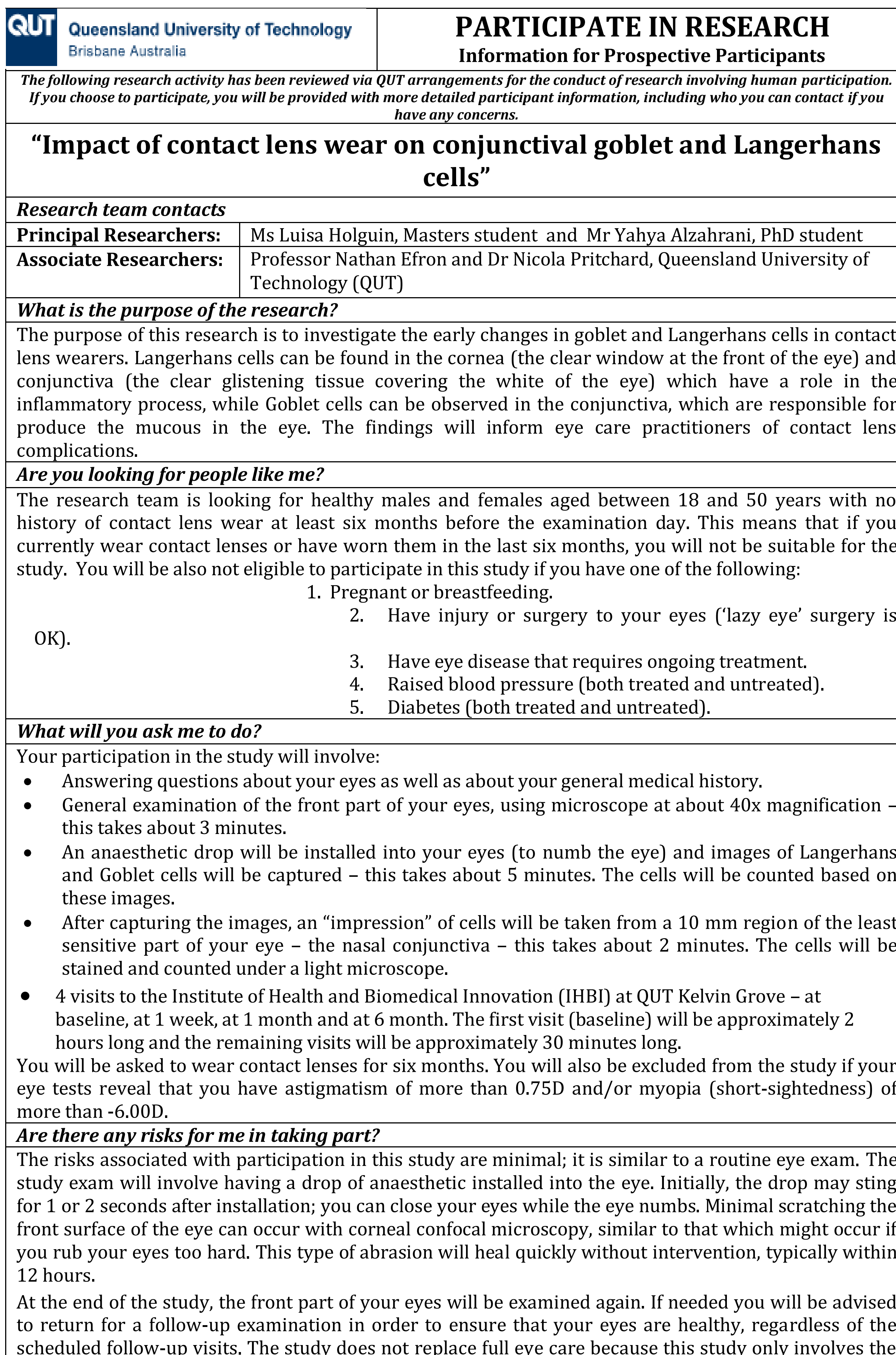
Appendix 1: Ethics approval certificate

Appendix 2: Participant Information and Consent Form



QUT Queensland University of Technology
Brisbane Australia

PARTICIPATE IN RESEARCH

Information for Prospective Participants

The following research activity has been reviewed via QUT arrangements for the conduct of research involving human participation. If you choose to participate, you will be provided with more detailed participant information, including who you can contact if you have any concerns.

"Impact of contact lens wear on conjunctival goblet and Langerhans cells"

Research team contacts

Principal Researchers:	Ms Luisa Holguin, Masters student and Mr Yahya Alzahrani, PhD student
Associate Researchers:	Professor Nathan Efron and Dr Nicola Pritchard, Queensland University of Technology (QUT)

What is the purpose of the research?

The purpose of this research is to investigate the early changes in goblet and Langerhans cells in contact lens wearers. Langerhans cells can be found in the cornea (the clear window at the front of the eye) and conjunctiva (the clear glistening tissue covering the white of the eye) which have a role in the inflammatory process, while Goblet cells can be observed in the conjunctiva, which are responsible for produce the mucous in the eye. The findings will inform eye care practitioners of contact lens complications.

Are you looking for people like me?

The research team is looking for healthy males and females aged between 18 and 50 years with no history of contact lens wear at least six months before the examination day. This means that if you currently wear contact lenses or have worn them in the last six months, you will not be suitable for the study. You will be also not eligible to participate in this study if you have one of the following:

1. Pregnant or breastfeeding.
2. Have injury or surgery to your eyes ('lazy eye' surgery is OK).
3. Have eye disease that requires ongoing treatment.
4. Raised blood pressure (both treated and untreated).
5. Diabetes (both treated and untreated).

What will you ask me to do?

Your participation in the study will involve:

- Answering questions about your eyes as well as about your general medical history.
- General examination of the front part of your eyes, using microscope at about 40x magnification – this takes about 3 minutes.
- An anaesthetic drop will be installed into your eyes (to numb the eye) and images of Langerhans and Goblet cells will be captured – this takes about 5 minutes. The cells will be counted based on these images.
- After capturing the images, an "impression" of cells will be taken from a 10 mm region of the least sensitive part of your eye – the nasal conjunctiva – this takes about 2 minutes. The cells will be stained and counted under a light microscope.
- 4 visits to the Institute of Health and Biomedical Innovation (IHBI) at QUT Kelvin Grove – at baseline, at 1 week, at 1 month and at 6 month. The first visit (baseline) will be approximately 2 hours long and the remaining visits will be approximately 30 minutes long.

You will be asked to wear contact lenses for six months. You will also be excluded from the study if your eye tests reveal that you have astigmatism of more than 0.75D and/or myopia (short-sightedness) of more than -6.00D.

Are there any risks for me in taking part?

The risks associated with participation in this study are minimal; it is similar to a routine eye exam. The study exam will involve having a drop of anaesthetic installed into the eye. Initially, the drop may sting for 1 or 2 seconds after installation; you can close your eyes while the eye numbs. Minimal scratching the front surface of the eye can occur with corneal confocal microscopy, similar to that which might occur if you rub your eyes too hard. This type of abrasion will heal quickly without intervention, typically within 12 hours.

At the end of the study, the front part of your eyes will be examined again. If needed you will be advised to return for a follow-up examination in order to ensure that your eyes are healthy, regardless of the scheduled follow-up visits. The study does not replace full eye care because this study only involves the

front part of the eye. It should be noted that if you have agreed to participate in the study, you can discontinue at any time during the project without comment or penalty.	
Are there any benefits for me in taking part?	
If you are suitable for contact lenses, you will be fitted with disposable soft contact lenses (one day replacement). These lenses will be provided for **free throughout the duration of the study (for 6 months).** You will be required to return unused lenses at the end of the study and if you discontinue the study. A prescription for the lenses will be provided to you by the examiner.. The knowledge gained will benefit people who wear contact lenses.	
Will I be compensated for my time?	
We would very much appreciate your participation in this research. ***To compensate you for your contribution, the research team will provide you with***d free soft contact lenses .	
I am interested – what should I do next?	
If you would like to participate in this study, please contact the following research team members: **Ms Luisa Holguin** **0731386404** luisafernanda.holguincolorado@student.qut.edu.au **Mr Yahya Alzahrani** **0421 808 117** y.alzahrani@student.qut.edu.au ***You will be provided with further information to ensure that your decision and consent to participate is fully informed***.	
Thank You!	**QUT Ethics Approval Number: 1300000117**

Queensland University of Technology
Brisbane Australia

CONSENT FORM FOR QUT RESEARCH PROJECT

Impact of contact lens wear on conjunctival goblet and Langerhans cells

QUT Ethics Approval Number 1300000117

RESEARCH TEAM CONTACTS

Ms Luisa Holguin

3138 6404

luisafernanda.holguincolorado@student.qut.edu.au

Mr Yahya Alzahrani

0421 808 117

yahyaahmedm.alzahrani@student.qut.edu.au

Prof Nathan Efron

3138 6401

n.efron@qut.edu.au

Dr Nicola Pritchard

3138 6414

n.pritchard@qut.edu.au

STATEMENT OF CONSENT

By signing below, you are indicating that you:

- Have read and understood the information document regarding this project.
- Have had any questions answered to your satisfaction.
- Understand that if you have any additional questions you can contact the research team.
- Understand that you are free to withdraw at any time, without comment or penalty.
- Understand that you can contact the Research Ethics Unit on 3138 5123 or email ethicscontact@qut.edu.au if you have concerns about the ethical conduct of the project.
- Agree to participate in the project.

Name ....

Signature ....

Date ....

- ***Please return this sheet to the investigator. You will be given a copy of the document to keep***

PARTICIPANT INFORMATION FOR QUT RESEARCH PROJECT
Short-term time course of cell recruitment into the cornea during contact lens wear **- "Impact of contact lens wear on conjunctival goblet and Langerhans cells".** **(QUT Ethics Approval Number 1300000117)**

RESEARCH TEAM

Principal Researchers:	Mr Yahya Alzahrani, PhD student
Associate Researchers:	Professor Nathan Efron and Dr Nicola Pritchard, Queensland University of Technology (QUT)

DESCRIPTION

This project is being undertaken as part of PhD research for Yahya Alzahrani, and is an optional addition to the project entitled "Impact of contact lens wear on conjunctival goblet and Langerhans cells" (QUT Ethics Approval Number 1300000117).

I wish to invite you to participate in this study, which will be conducted either after 2months from the baseline examination or after finishing the abovementioned study. The aim of this study is to investigate the effect of short time contact lens wear (one day) on Langerhans cell density in the central cornea (the front clear part of the eye). I will fit you with a very low prescription (-0.50D) soft CLs in one eye (such that it will not adversely affect their vision) and the eye that we don't fit with the lens will serve as a comparison to the eye that we do fit. After lens insertion, you will be assessed every two hours for eight hours.

A confocal microscope will be used to take pictures of the eye to investigate the changes in the number of Langerhans cells in the cornea as a result of lens use over one day period.

You are invited to participate in this project because you are eligible for the main study as a participant in the control group (the group not wearing contact lenses).

PARTICIPATION

Your participation will involve, over one day, five additional visits to the Anterior Eye Lab at IHBI, each visit of approx 15 minutes duration:

- An examination of the front part of your eyes using microscope at about 40x magnification – this takes about 3 minutes;
- Fitting you with a very low prescription (-0.50D) soft CLs in one eye only.
- Sitting an instrument, having a drop of anaesthetic placed in both eyes (to numb the eye) and having images captured of the superficial layers of cells of the eye – this takes about 5 minutes. This test will be performed every two hours for eight hours.
- You will be asked not to rub your eyes for at least 40 minutes after the tests because the drop numbs the eye, and it is possible for you to damage the front layer of cells of your eye without noticing it.

- You will be able to leave the building as required during the day, returning for the 2-hourly examinations.

Your participation in this project is entirely voluntary. If you do agree to participate you can withdraw from the project without comment or penalty. If you withdraw, on request any identifiable information already obtained from you will be destroyed. Your decision to participate or not participate will in no way impact upon your current or future relationship with QUT (for example your grades) as well as will not impact on your participation in the main study.

EXPECTED BENEFITS

You will be reimbursed for travel expenses and out-of-pocket expenses with a Coles-Myer voucher of $40 at the end of the study. The knowledge gained from the study may benefit people who wear contact lenses in the future. Some people find participating in studies an interesting experience.

RISKS

There is no risk beyond that involved in a regular eye examination. During and at the end of the study the front of your eyes will be examined again. If the investigator believes it is in your best interests, you may be asked to return for a follow-up examination. You will involve having a drop of anaesthetic applied to the eye. Initially the drop may sting for 1 or 2 seconds. You can close your eyes while the eye numbs.

PRIVACY AND CONFIDENTIALITY

All comments and responses will be treated confidentially, and if presented or published, you will be anonymous. Only the principal researcher and the supervisors will have access to your records. Your data may be used in the future for subsequent analysis or investigation related to eye and visual improvement.

CONSENT TO PARTICIPATE

We would like to ask you to sign a written consent form (enclosed) to confirm your agreement to participate.

QUESTIONS / FURTHER INFORMATION ABOUT THE PROJECT

If have any questions or require any further information please contact one of the research team members below.

Mr Yahya Alzahrani– Principal Researcher
School of Optometry and Vision Science and IHBI
3138 0455 y.alzahrani@student.qut.edu.au

Dr Nicola Pritchard – Supervisor
School of Optometry and Vision Science and IHBI
3138 6414 n.pritchard@qut.edu.au

CONCERNS / COMPLAINTS REGARDING THE CONDUCT OF THE PROJECT

QUT is committed to research integrity and the ethical conduct of research projects. However, if you do have any concerns or complaints about the ethical conduct of the project you may contact the QUT Research Ethics Unit on 3138 5123 or email ethicscontact@qut.edu.au. The QUT Research Ethics Unit is not connected with the research project and can facilitate a resolution to your concern in an impartial manner.

Thank you for helping with this research project.

Please keep this sheet for your information.

Queensland University of Technology Brisbane

CONSENT FORM FOR QUT RESEARCH PROJECT

Short-term time course of cell recruitment into the cornea during contact lens wear

QUT Ethics Approval Number 1300000117

RESEARCH TEAM CONTACTS

Mr Yahya Alzahrani

0421 808 117

y.alzahrani@student.qut.edu.au

Prof Nathan Efron

3138 6401

n.efron@qut.edu.au

Dr Nicola Pritchard

3138 6414

n.pritchard@qut.edu.au

STATEMENT OF CONSENT

By signing below, you are indicating that you:

- Have read and understood the information document regarding this project.
- Have had any questions answered to your satisfaction.
- Understand that if you have any additional questions you can contact the research team.
- Understand that you are free to withdraw at any time, without comment or penalty.
- Understand that you can contact the Research Ethics Unit on 3138 5123 or email ethicscontact@qut.edu.au if you have concerns about the ethical conduct of the project.
- Agree to participate in the project.

Name ....

Signature ....

Date ....

Please return this sheet to the investigator.

You will be given a copy of the document to keep.

PARTICIPANT INFORMATION FOR QUT RESEARCH PROJECT

Impact of eye rubbing (as a mechanical inflammatory stimulus) on short-term cell recruitment

-"Impact of contact lens wear on conjunctival goblet and Langerhans cells".

(QUT Ethics Approval Number 1300000117)

RESEARCH TEAM

Principal Researcher:	Mr Yahya Alzahrani, PhD student
Associate Researchers:	Professor Nathan Efron and Dr Nicola Pritchard, Queensland University of Technology (QUT)

DESCRIPTION

This project is being undertaken as part of PhD research for Yahya Alzahrani, and is an optional addition to the project entitled "Impact of contact lens wear on conjunctival goblet and Langerhans cells" (QUT Ethics Approval Number 1300000117).

I wish to invite you to participate in this study, which will be conducted either after 2months from the baseline examination or after finishing the abovementioned study. The aim of this study is to investigate the effect of eye rubbing on Langerhans cell density in the central cornea (the front clear part of the eye). You will be instructed to create mechanical stimulation of one eye (you can choose which eye) through gentle rubbing through the lid of the closed eye. The eye that you don't rub will serve as a comparison to the eye that you do rub. You will be advised to rub the same eye for 10 seconds, every 1 minute, for a total period of 30 minutes. The eye will be kept open except when eye rubbing is being performed.

A confocal microscope will be used to take pictures of the eye to investigate the changes in the number of Langerhans cells in the cornea as a result of eye rubbing.

You are invited to participate in this project because you are eligible for the main study as a participant in the control group (the group not wearing contact lenses).

PARTICIPATION

Your participation will involve one additional visit to the Anterior Eye Lab at IHBI, of approx 45 minutes duration:

- An examination of the front part of your eyes using microscope at about 40x magnification – this takes about 3 minutes;
- You rubbing one eye for 10 seconds, every 1 minute, for a total period of 30 minutes.
- Sitting an instrument, having a drop of anaesthetic placed in both eyes (to numb the eye) and having images captured of the superficial layers of cells of the eyes – this takes about 5 minutes.

Your participation in this project is entirely voluntary. If you do agree to participate you can withdraw from the project without comment or penalty. If you withdraw, on request any identifiable information already obtained from you will be destroyed. Your decision to participate or not participate will in no way impact upon your current or future relationship with QUT (for example your grades) as well as will not impact on your participation in the main study.

EXPECTED BENEFITS

You will be reimbursed for travel expenses and out-of-pocket expenses with a Coles-Myer voucher of $10 at the end of the study. The knowledge gained from the study may benefit people who wear contact lenses in the future. Some people find participating in studies an interesting experience.

RISKS

There is no risk beyond that involved in a regular eye examination. During and at the end of the study the front of your eyes will be examined again. If the investigator believes it is in your best interests, you may be asked to return for a follow-up examination. You will involve having a drop of anaesthetic applied to the eye. Initially the drop may sting for 1 or 2 seconds. You can close your eyes while the eye numbs.

PRIVACY AND CONFIDENTIALITY

All comments and responses will be treated confidentially, and if presented or published, you will be anonymous. Only the principal researcher and the supervisors will have access to your records. Your data may be used in the future for subsequent analysis or investigation related to eye and visual improvement.

CONSENT TO PARTICIPATE

We would like to ask you to sign a written consent form (enclosed) to confirm your agreement to participate.

QUESTIONS / FURTHER INFORMATION ABOUT THE PROJECT

If have any questions or require any further information please contact one of the research team members below.

Mr Yahya Alzahrani– Principal Researcher
School of Optometry and Vision Science and IHBI
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Thank you for helping with this research project.

Please keep this sheet for your information.

QUT Queensland University of T
Brisbane Australia

CONSENT FORM FOR QUT RESEARCH PROJECT

Impact of eye rubbing (as a mechanical inflammatory stimulus) on short-term cell recruitment

QUT Ethics Approval Number 1300000117

RESEARCH TEAM CONTACTS

Mr Yahya Alzahrani

0421 808 117

y.alzahrani@student.qut.edu.au

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3138 6401

n.efron@qut.edu.au

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- Agree to participate in the project.

Name ....

Signature ....

Date ....

Please return this sheet to the investigator. You will be given a copy of the document to keep.

PARTICIPANT INFORMATION FOR QUT RESEARCH PROJECT
Impact of eye closure (as a biologic inflammatory stimulus) on short-term cell recruitment **"Impact of contact lens wear on conjunctival goblet and Langerhans cells".** **(QUT Ethics Approval Number 1300000117)**

RESEARCH TEAM

Principal Researcher:	Mr Yahya Alzahrani, PhD student
Associate Researchers:	Professor Nathan Efron and Dr Nicola Pritchard, Queensland University of Technology (QUT)

DESCRIPTION

This project is being undertaken as part of PhD research for Yahya Alzahrani, and is an optional addition to the project entitled "Impact of contact lens wear on conjunctival goblet and Langerhans cells" (QUT Ethics Approval Number 1300000117).

I wish to invite you to participate in this study, which will be conducted either after 2months from the baseline examination or after finishing the abovementioned study. The purpose of this study is to illustrate the effect of eye closure on Langerhans cell density in the central cornea (the front clear part of the eye). You will be advised to cover one eye, as soon as you get up from sleep, using a given eye patch, and keep the other eye open. The eye that you don't close will serve as a comparison to the eye that you do close.

A confocal microscope will be used to take pictures of the eye to investigate the changes in the number of Langerhans cells in the cornea as a result of eye closure.

You are invited to participate in this project because you are eligible for the main study as a participant in the control group (the group not wearing contact lenses).

PARTICIPATION

Your participation will involve one additional visit to the Anterior Eye Lab at IHBI, of approx 15 minutes duration:

- An examination of the front part of your eyes using microscope at about 40x magnification – this takes about 3 minutes;
- Advising you to cover one eye, as soon as you get up from sleep, using a given eye patch.
- After getting to the clinic, the patch will be removed.
- Sitting an instrument, having a drop of anaesthetic placed in both eyes (to numb the eye) and having images captured of the superficial layers of cells of both eyes – this takes about 5 minutes.

Your participation in this project is entirely voluntary. If you do agree to participate you can withdraw from the project without comment or penalty. If you withdraw, on request any identifiable information already obtained from you will be destroyed. Your decision to participate or not participate will in no way impact upon your current or future relationship with QUT (for example your grades) as well as will not impact on your participation in the main study.

EXPECTED BENEFITS

You will be reimbursed for travel expenses and out-of-pocket expenses with a Coles-Myer voucher of $20 at the end of the study. The knowledge gained from the study may benefit people who wear contact lenses in the future. Some people find participating in studies an interesting experience.

RISKS

There is no risk beyond that involved in a regular eye examination. During and at the end of the study the front of your eyes will be examined again. If the investigator believes it is in your best interests, you may be asked to return for a follow-up examination. You will involve having a drop of anaesthetic applied to the eye. Initially the drop may sting for 1 or 2 seconds. You can close your eyes while the eye numbs.

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CONSENT TO PARTICIPATE

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QUESTIONS / FURTHER INFORMATION ABOUT THE PROJECT

If have any questions or require any further information please contact one of the research team members below.

Mr Yahya Alzahrani– Principal Researcher

School of Optometry and Vision Science and IHBI

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Please keep this sheet for your information.

Queensland University of Technology
Brisbane Australia

CONSENT FORM FOR QUT RESEARCH PROJECT

Impact of eye closure (as a biologic inflammatory stimulus) on short-term cell recruitment

QUT Ethics Approval Number 1300000117

RESEARCH TEAM CONTACTS

Mr Yahya Alzahrani

0421 808 117

y.alzahrani@student.qut.edu.au

Prof Nathan Efron

3138 6401

n.efron@qut.edu.au

Dr Nicola Pritchard

3138 6414

n.pritchard@qut.edu.au

STATEMENT OF CONSENT

By signing below, you are indicating that you:

- Have read and understood the information document regarding this project.
- Have had any questions answered to your satisfaction.
- Understand that if you have any additional questions you can contact the research team.
- Understand that you are free to withdraw at any time, without comment or penalty.
- Understand that you can contact the Research Ethics Unit on 3138 5123 or email ethicscontact@qut.edu.au if you have concerns about the ethical conduct of the project.
- Agree to participate in the project.

Name ....

Signature ....

Date ....

Please return this sheet to the investigator. You will be given a copy of the document to keep

Appendix 3: Case Reports

Contact Lens Case Report Form

Name: **Baseline** Date:

Refraction:

	Sph	**Cyl**	**Axis**	**Spherical Equivalent**
OD				
OS				

K-reading:

OD		**OS**	
K1:		**K1:**	
K2:		**K2:**	

Initial Lens Parameters:

	BC	**Dia**	**Power**
OD			
OS			

	OD	**OS**
Coverage:		
Movement:		
Centration:		

VA OD:

OS:

Over-refraction:

OD:

OS:

Final Lens Parameters:

	BC	**Dia**	**Power**
OD			
OS			

Comment:

Contact Lens Follow-up Form

Name: **Visit:** **Date:**

VA OD:

OS:

Over-refraction:

OD:

OS:

Slit-lamp biomicroscopy: *(Lens on)*

	OD	OS
Coverage:		
Movement:		
Centration:		

Slit-lamp biomicroscopy: *(Lens off)*

Limbal vasculature:

Lid evaluation:

Cornea evaluation:

Injection:

Microcysts:

Striae:

Polymegethism:

Limbal engorgement:

Tarsal conjunctiva (follicles and papillae):

Corneal staining:

Final Lens Parameters:

	BC	Dia	Power
OD			
OS			

Comment:

Appendix 4: Dry Eye Questionnaires

4.1 Dry Eye Questionnaires (DEQ-5)

1- Questions about EYE DISCOMFORT:

a. During a typical day in the past month, **how often** did your eyes feel discomfort?

0 Never
1 Rarely
2 Sometimes
3 Frequently
4 Constantly

b. When your eyes felt discomfort, **how intense was this feeling of discomfort** at the end of the day, within two hours of going to bed?

Never have it	Not at All Intense				Very Intense
0	1	2	3	4	5

2- Questions about **EYE DRYNESS**:

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

0 Never
1 Rarely
2 Sometimes
3 Frequently
4 Constantly

b. When your eyes feel dry, **how intense was this feeling of dryness** at the end of the day, within two hours of going to bed?

Never have it	Not at All Intense				Very Intense
0	1	2	3	4	5

3- Questions about **WATERY EYE:**

During a typical day in the past month, **how often** did your eyes look or feel excessively watery?

0 Never
1 Rarely
2 Sometimes
3 Frequently
4 Constantly

Score: 1a +1b +2a+3 = Total

Source: Chalmers RL, Begley CG, Caffery B. Validation of the 5-Item Dry Eye Questionnaire (DEQ-5): Discrimination across self-assessed severity and aqueous tear. deficient dry eye diagnoses. Cont Lens Anterior Eye. 2010 Apr;33(2):55-60.

4.2 Contact Lens Dry Eye

Questionnaire-8 (CLDEQ-8)

1-Questions about **EYE DISCOMFORT:**
a-During a typical day in the last past 2 weeks, **how often** did your eyes feel discomfort while wearing your contact lenses?
1 Never
2 Rarely
3 Sometimes
4 Frequently
5 Constantly

When your eyes felt discomfort with your contact lenses**, how intense was this feeling of discomfort...**
b. At the end of your wearing time?
Never Not at All Very
have it intense intense
0 1 2 3 4 5

2. Questions about **EYE DRYNESS**:
A. During a typical day in the past 2 weeks, **how often** did your eye feel dry?
1 Never
2 Rarely
3 Sometimes
4 Frequently
5 Constantly

When your eyes feel dry, **how intense was this feeling of dryness...**
b. At the end of your wearing time?
Never Not at All Very
have it intense intense
0 1 2 3 4 5

Source: *Chalmers RL, Begley CG, Moody K, Hickson-Curran SB. contact lens dry eye questionnaire-8 (CLDEQ-8) and opinion of contact lens performance. Optom Vis Sci. 2012 Oct;89(10):1435-42*

3. Question about **CHANGEABLE, BLURRY VISION**:
a. During a typical day in the past 2 weeks, **how often** did your vision change between clear and blurry or foggy while wearing your contact lenses?
1 Never
2 Rarely
3 Sometimes
4 Frequently
5 Constantly

When your vision was blurry, **how noticeable was the changeable, blurry, or foggy vision....**
b. At the end of your wearing time?
Never Not at All Very
have it intense intense
0 1 2 3 4 5

4. Question about **CLOSING YOUR EYES:**
During a typical day in the past 2 weeks, **how often did your eyes bother you so much that you wanted to close them?**
1 Never
2 Rarely
3 Sometimes
4 Frequently
5 Constantly

5. Questions about **REMOVING YOUR LENSES:**
How often during the past 2 weeks, did your eyes *bother you so much* while wearing your contact lenses that you felt as if you needed to stop whatever you were doing and **take out your contact lenses?**
1 Never
2 Less than once a week
3 Weekly
4 Several times a week
5 Daily
6 Several times a day

Appendix 5: Conference Presentations

1- Alzahrani Y, Holguin L, Pritchard N, Efron N. In vivo assessment of inflammatory cells in contact lens wearers. American Academy of Optometry Conference, Denver, USA, 2014.

2- Alzahrani Y, Holguin L, Pritchard N, Efron N. In vivo assessment of inflammatory cells in the cornea and conjunctiva of contact lens wearers. Institute of Health and Biomedical Innovation (IHBI) Inspires Postgraduate Conference, Gold Coast, Queensland, Australia, 2014.

3- Alzahrani Y, Holguin L, Pritchard N, Efron N. In vivo assessment of inflammatory cells in contact lens wearers. International Cornea & Contact Lens Congress, Gold Coast, Queensland, Australia, 2015.

4- Alzahrani Y, Holguin L, Pritchard N, Efron N. In vivo assessment of inflammatory cells in contact lens wearers. International Society for Contact Lens Research Conference, Budapest, Hungary, 2015

Appendix 6: Achievements

- Awarded a 2014 Cornea and Contact Lens Society of Australia research award;

- Submission to the Scientific Program Committee of American Academy of Optometry selected as a candidate for recognition as the Best Presentation

- Awarded 2015 student travel award by the International Society for Contact Lens Research to present a paper of the forthcoming biennial meeting of that Society in Budapest, Hungary, in August 2015.