Cellular changes at the lid margin

by

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Author's declaration

This thesis consists of material all of which I authored or co-authored: see Statement of Contributions included in the thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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

Statement of Contributions

I would like to acknowledge the names of my co-authors who contributed to this thesis:

- Dr. Lyndon Jones

- Dr. Lakshman Subbaraman

- Dr. Kevin van Doorn

Abstract

Purpose

The hypothesis underlying this thesis is that CL wear, lid wiper epitheliopathy (LWE), and symptoms of dryness and discomfort may be manifest as cellular changes of the lid marginal epithelium, as a result of mechanical action (e.g. friction). The purpose of this thesis was to elucidate the histology of the lid margin epithelium in relation to CL wear, with a focus on ocular discomfort and dryness. The specific aims of each chapter are outlined below:

- Chapter 1: to review the relevant literature and to introduce the reader to the topic area;
- Chapter 2: to define the rationale and objectives of this thesis;
- Chapter 3: to optimize a method of collecting, staining and imaging cells from the lid margin using impression cytology (IC);
- Chapter 4: to assess the utility of the IC method developed in chapter 4, towards characterizing the epithelial cell morphology of the upper lid margin in symptomatic and asymptomatic soft lens (SCL) wearers and non-lens wearers with distinct levels of LWE;
- Chapter 5: to assess the lid margins of symptomatic and asymptomatic SCL wearers;
- Chapter 6: to assess the lid margins of rigid gas permeable (RGP) and non-CL wearers;
- Chapter 7: to cross-compare findings from chapters 5 and 6, and to determine differences between the upper and lower lid margins.
- Chapter 8: to conclude the findings and knowledge gained following the above projects, and to point out potential future work directions.

Methods

- Chapter 3: Upon anesthesia (proparacaine hydrochloride, 0.5%), the upper lids of 5 subjects (n=10) were everted and IC was conducted using various membranes (mixed cellulose esters, hydrophilic PTFE, polyethersulfone). Several fixatives (100% methanol, 95% ethanol), cytological stains (Papanicolaou (hematoxylin Gill No.1, OG-6, EA-65), Periodic Acid-Schiff (PAS) and Alcian Blue (AB)) and soak times (1, 3, 5 minutes) were tested. Varying concentrations of fluorescent dyes (Calcein AM, Ethidium homodimer-1, Annexin V) were tested and imaged using confocal laser scanning microscopy (CLSM);

- Chapter 4: Fifteen participants were enrolled in three study groups: 5 asymptomatic non-lens wearers with low LWE (average grade of 1.0 or lower in both eyes); 5 adapted, asymptomatic SCL wearers with low LWE; 5 adapted, SCL wearers with high LWE (average grade of 2.0 or higher). Participants completed subjective comfort ratings and LWE was assessed using the Korb Protocol B. IC samples were taken from the upper lid margin using Millicell Cell Culture Inserts and cellular features and sample cellularity evaluated after histochemical and immuno-cytochemical staining as described in the previous chapter;

- Chapter 5: Forty adapted SCL wearers were enrolled and equally distributed in two study groups based on self-reported CL-related comfort levels. Comfort was assessed using the Young scheme, the Ocular Surface Disease Index (OSDI), the Contact Lens Dryness Evaluation Questionnaire (CLDEQ-8) and diurnal 0-100 scales for comfort and dryness. LWE was assessed using lissamine green (LG) and IC performed on the upper and lower lid margins as in the previous chapters. The lid wiper (LW) and muco-cutaneous junction (MCJ) cellular areas were defined and dimensioned using a custom programmed software and ImageJ;

- Chapter 6: Eighteen RGP wearers and 19 non-lens wearers (nCL) were enrolled in two study groups. Comfort, LWE and IC were assessed as in the previous chapter;

- Chapter 7: Study groups analyzed in chapters 5 and 6 were cross-compared (n=77) with regards to clinical signs, comfort scores, LWE and lid margin morphology at both lid margins and width measurements for the LW and MCJ areas. Upper and lower lid margins were also compared.

Results

- Chapter 3: IC delivered optimal results using the hydrophilic PTFE membrane. Fixing in 95% ethanol for >20 minutes, then staining in 500μl each of AB, hematoxylin Gill No.1, OG-6 and EA-65 for 3 minutes revealed the presence of goblet cells, mucins, cell nuclei and various degrees of pre- and para-keratinization. Calcein AM (4μM) and Ethidium (4μM) were combined to successfully show cell esterase activity and compromised cell membranes. Up to 200 microscopy digital images were captured for each sample and stitched into a high-resolution, large scale image of the entire IC span;

- Chapter 4: Three distinct cellular morphologies were identified, spanning between the tarsal/marginal conjunctiva, through the LW conjunctiva, to the MCJ at the Marx line. Epithelial cell morphology did not vary with LWE grade or lens wear. Sample cellularity may or may not be altered by lens wear, LWE and/or symptoms. No association was found between LWE and ocular discomfort;

- Chapter 5: Average (±SD) upper and lower LWE grades were identical in both groups (0.8 ± 0.7) and did not correlate with any subjective comfort score or other study variable. The average width in the upper LW (415 ± 131 μm) and MCJ (114 ± 43), and lower LW (187 ± 120) and MCJ (90 ± 41) was measured (n=139). Wider LW and MCJ areas correlated with higher LWE grades ($p<0.05$, r=0.61 to 0.86);

- Chapter 6: RGP wearers reported overall similar or better comfort than nCL wearers ($p>0.05$). Average LWE grades (±SD) were significantly different, for both upper (RGP: 1.66 ± 0.97; nCL: 0.44 ± 0.75; $p=0.0002$) and lower (RGP: 1.48 ± 0.94; nCL: 0.39 ± 0.49; $p=0.0001$) lid margins. The average width of the upper (RGP: 666 ± 219 μm; nCL: 265 ± 64; $p<0.0001$) and lower LW areas (RGP: 518 ± 211; nCL: 224 ± 101; $p<0.0001$) was significantly higher in RGP wearers, and correlated well with the LWE grade ($p<0.01$, r=0.78 to 0.89);

- Chapter 7: The average (±SD) LWE grade of SCL wearers (0.8 ± 0.8) was greater than in nCL (0.4 ± 0.7, $p=0.0125$) and lower than in RGP wearers (1.6 ± 0.9, $p=0.0015$). No significant difference was found between the upper and lower LWE grades in any of the four groups. Longer average CL wear times and older age were correlated with higher LWE grades (Spearman r range: 0.27 to 0.31, $p<0.05$) and better comfort scores (Spearman r range: 0.25 to 0.44, $p<0.05$). The width of the upper LW of SCL wearers (415 ± 132 μm) was greater than in nCL (266 ± 64, $p=0.0003$) and narrower than in RGP wearers (667 ± 219, $p=0.0004$). The

width of the lower LW of SCL wearers (187 ± 120) was up to 2.8 times smaller than in RGP wearers (519 ± 212, $p<0.0001$), but similar to nCL (225 ± 102, $p=0.072$). The upper LW was significantly wider than the lower LW in all participants ($p<0.05$), except for RGP wearers.

Conclusions

A protocol for collecting, staining, imaging and analyzing cells from the lid marginal epithelium was developed and showed appropriate sensitivity for identifying distinct cellular morphology and varying degrees of keratinization. We presented the first account to show a correlation between LWE grade and widths of the LW and MCJ areas after histological inspection. By identifying enlarged areas of keratinization in the LW of LWE versus non-LWE subjects, we provide evidence to support the frictional etiology of LWE and possibly also the Marx line. This is the first study to show that SCL lens wear is associated with enlarged LW areas in the upper and lower lid margins, providing strong evidence that the mechanical interaction with a CL may alter the cyto-morphology of the lid margin epithelium. The effect of RGP lenses is similar and significantly more pronounced. Regardless of CL wear, the LW at the upper lid margin is wider than the lower one, upholding the frictional role of the LW during habitual blinking.
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Acknowledgements

Completing a PhD is often thought of being a solitary task. That's because pressing a single button for hours on end, in a dark laboratory, at 3am on a Saturday is no fun. Neither is trying to dig yourself out from underneath a crushing pile of papers, books, and journals, nor staring at an open MS Word document immodestly titled "Thesis", followed by endless white space. *Alone*.

And when it's suddenly all over, those solitary moments seem benign, few and far in between. You realize that for the most part of this strange journey, you were not alone. Instead, you were surrounded by numerous individuals caring for you, tolerating you, guiding you and paving your way to the end.

And if you didn't lose your mind in the lab or the library, it's only because you weren't *actually* alone.

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Dedication

To my wife, Madalina. We did this together.

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List of Abbreviations

AB	Alcian Blue
AKA	Also known as
aSCL	Asymptomatic soft contact lens wearer
BAK	Benzalkonium Chloride
C-cells	Cells of the conjunctiva (tarsal)
CL	Contact lens
CLD	Contact lens dryness
CLDEQ	Contact lens dry eye questionnaire
CLSM	Confocal laser scanning microscope
CRF	Case report form
DED	Dry eye disease
DEWS	Dry eye workshop
DNA	Deoxyribonucleic acid
DW	Daily wear
ELM	Eyelid margin
EthD	Ethidium homodimer
EW	Extended wear
FOI	Feature of interest
GC	Goblet cells
HEMA	Hydroxyethylmethacrylate
IC	Impression cytology
IQR	Inter-quartile range
L-cells	Cells of the lid wiper area
LG	Lissamine green
LW	Lid wiper
LWE	Lid wiper epitheliopathy
M-cells	Cells of the muco-cutaneous junction or Marx' line
MCJ	Muco-cutaneous junction
MG	Meibomian gland
MGD	Meibomian gland disfunction
NaFL	Sodium fluorescein
NLW	Non-lens wearer
OSDI	Ocular Surface Disease Index
PAS	Periodic Acid-Schiff
PMMA	Polymethylmethacrylate
PTFE	Polytetrafluoroethylene
RB	Rose Bengal
RGP	Rigid gas permeable contact lens
ROI	Region of interest
SCL	Soft contact lens
SD	Standard deviation
sSCL	Symptomatic soft contact lens wearer
TFOS	Tear Film and Ocular Society

Chapter 1
Literature review

The eyelid margin (ELM) is the anatomical structure that glides over the ocular surface or a contact lens (CL) during blinking. Its primary function is to spread the layers of the tear film, moisturizing the ocular surface and thus ensuring its integrity and functionality. Only a 1-2 mm narrow portion of the lid margin is supposed to touch or wipe over the eye during habitual blinking, and has therefore been termed "the lid wiper" (LW). Given that the upper eyelid executes over 10,000 blinks per day, traveling 10-12 mm vertically and gliding over the eye or a CL, a frictional force is expected at the interface between the LW region on the eyelid surface and the surface it rubs against (the ocular surface or CL surface). Evidence suggests that increased friction in this region may be related to ocular discomfort and dryness. With these symptoms standing at the forefront of CL research, primarily due to such symptoms being the most common cause of dissatisfaction among wearers and the number one reason for CL-dropout, the LW region has become an area of increased focus in ocular research over the past two decades.

In this chapter, we shall review the literature on the ELM, beginning by outlining the methods of investigating this region, followed by its anatomical and histological descriptions. Finally, we shall investigate its relationship to CL wear and CL-related discomfort.

1.1 Methods of investigating the eyelid margin

1.1.1 Clinical methods (in vivo)

1.1.1.1 Eversion

The lid margins are routinely assessed during an ocular examination, typically using a slit-lamp bio-microscope. To expose the upper lid margin, participants are asked to look down, while the investigator pulls on the lashes with their fingers and places a downwards pointing cotton-tip swab in the suprapalpebral sulcus, thus creating a wedge for the lid to flip over the tarsal plate, exposing the upper palpebral conjunctiva and lid margin (Figure 1). To prevent the eyelid from "flipping back", the investigator singlehandedly presses the eyelashes against the participant's eyebrows while the ocular inspection is underway. The lower lid margin is exposed by gently pulling down on the outer lid, just inferior to the lashes, which reveals the inferior palpebral conjunctiva and lid margin.

Figure 1: Everted upper eyelid showing staining of the lid margin with lissamine green

1.1.1.2 Vital stains

Traditionally, sodium fluorescein is used as the main vital dye to inspect corneal staining, while rose Bengal (RB) was used to detect damage to the conjunctiva [1]. Both dyes exhibit different staining properties and mechanisms [2]. At the lid margin, RB was first used by Marx in 1924, to describe "a most curious effect on the posterior eyelid edge" [3], which shall be elaborated on in section 1.2.2. In 1973, lissamine green (LG) was introduced for ocular surface staining and was reported to have identical properties to RB [4]. Ever since, it has mostly replaced RB, which has a dose-dependent toxicity and causes stinging. LG stains dead or degenerate, but not healthy, cells [5]. All three dyes have been used alone or in combination to characterize the ELM [6–12].

1.1.1.3 Confocal microscopy

Confocal laser scanning microscopy (CLSM) is an optical imaging technique used to increase the resolution and contrast of a sample, by employing an array of pinholes for limiting out-of-focus light in image formation. Using this technique coupled with laser light, two-dimensional images can be obtained at different depths beneath the surface of a sample; by "sectioning" a sample in this manner, a three-dimensional image of a specific feature can be created. In recent years, CLSM has been used for imaging anterior as well as posterior segment structures of the eye, with Knop et al. being the first to use in vivo CLSM to describe the lid margin. They imaged and dimensioned Meibomian gland openings, goblet cells (GC) and features of epithelial cells at depths of up to 85 μm underneath the surface [13]. Two other accounts used CLSM to report the presence of inflammatory cells at the lid margin following CL wear [14], and an increase in Langerhans cells in subjects with CL-induced dry eye [15].

1.1.2 Histological methods (ex vivo)

To investigate the histology or cytology of tissue or cell samples, specimens are first fixed in solvents such as ethanol or methanol [16], before being processed with various stains to highlight specific features. Early reports more commonly employed periodic acid-Schiff (PAS) to stain goblet cells and their secretions, together with hematoxylin as a counterstain to stain epithelial cells [17,18]. The latter was subsequently complemented and refined by Papanicolau stains (e.g. GILL-1, EA-65, OG-6 etc.) to better interpret epithelial changes such as squamous metaplasia and keratinization degrees [19]. The PAS is also occasionally substituted by Alcian Blue, for a better chromatic contrast to other pink/red dyes [20].

1.1.2.1 Cadaver excisions

Historical descriptions of the lid margin date back to 1877 [21], with few others scantly describing the lid margin and the methodology used to obtain tissue [22,23]. The first account to accurately illustrate the excision of lid marginal biopsies from cadavers was published in 2011 by Knop et al., providing the most accurate description of the lid margin conformation available to date [13]. They excised the complete conjunctival sac from 14 eyes, 1-2 mm distal (exterior) to the outer lid border along the entire lid margin, in both upper and lower eyelids. This way, they were able to obtain the whole posterior lamella and conjunctiva of both eyelids in one piece, while the upper and lower lids remained connected at the nasal canthus. After sectioning the tissue into 5-mm wide strips from the lid margin towards the bulbar side and fixing and embedding these in paraffin blocks, serial sections of 5-10 μm were cut using a rotary microtome. Samples were stained with hematoxylin and eosin, and Masson-Goldner's trichrome stain, and imaged using a standard bright-field microscope [13] (Figure 2).
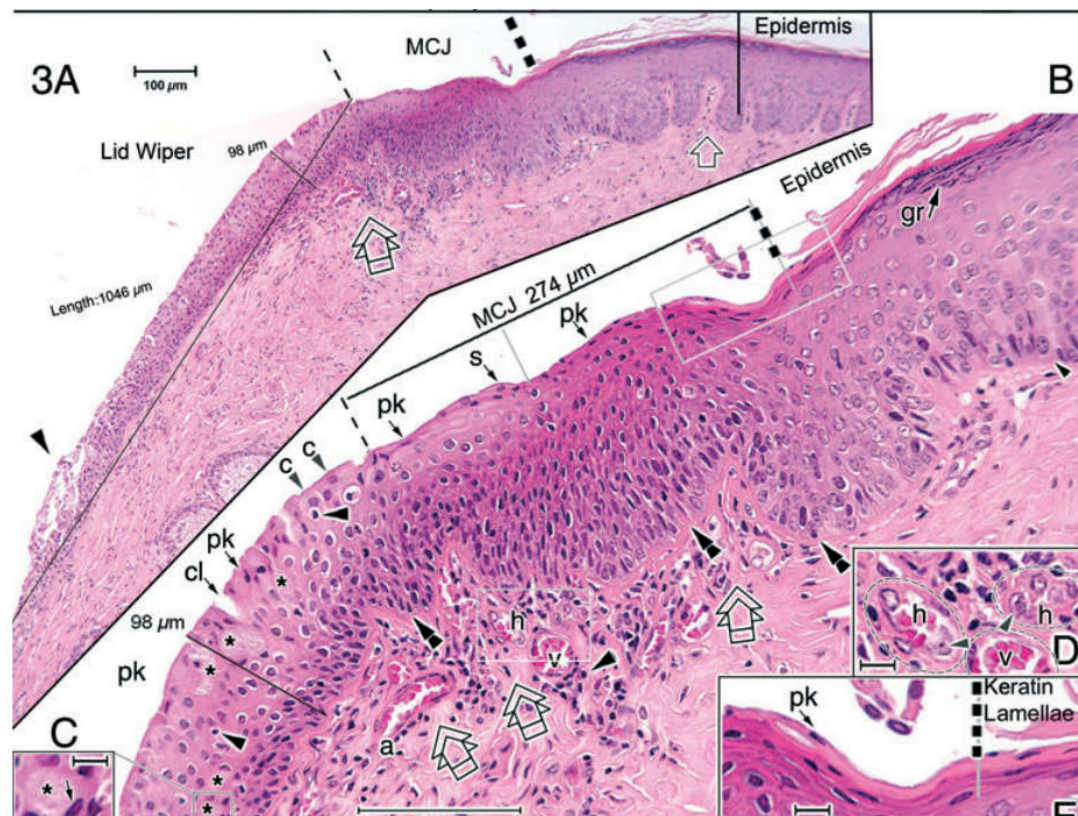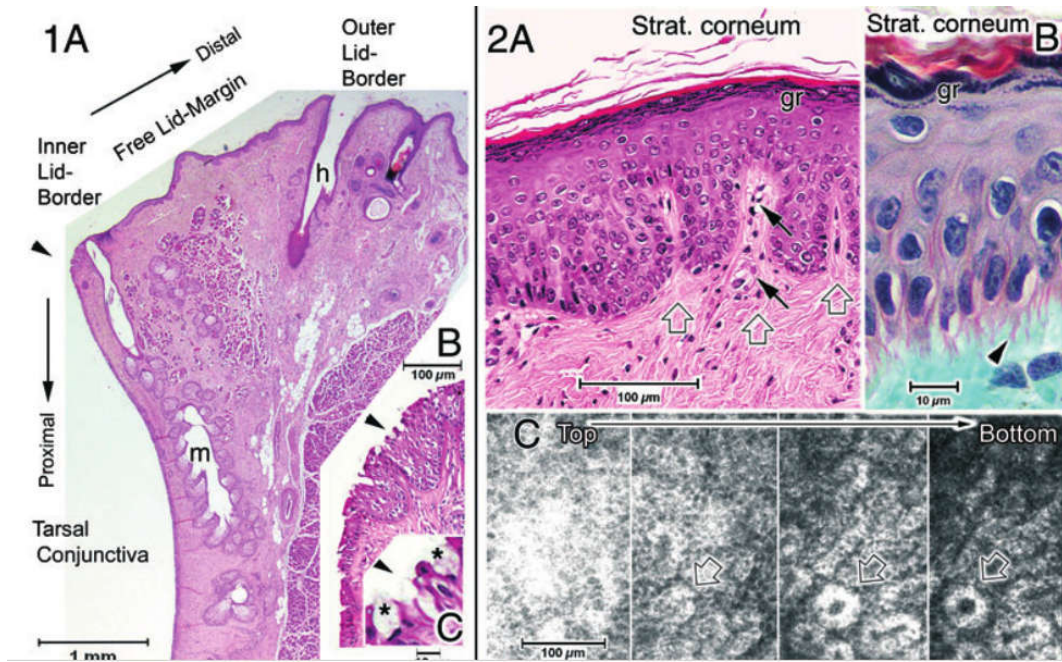
Figure 2: Histological description of the lid margin [13]

SECTION 1: Overview of a section through the center of an upper human eyelid margin. The epidermis extends over the roundish outer lid border onto the free lid margin. A ciliary hair follicle (h) and a Meibomian gland (m) are seen. The inner lid border (arrowhead at the crest) has a zone of increased epithelial thickness (as seen in higher magnification, B), which forms an elevation apposed to the globe. This zone is only 0.3 mm wide here and contains goblet cells (C, asterisks). H&E. Scale bar: 1 mm (A); 100 µm (B); 10 µm (C). SECTION 2: Epidermal rete pegs line narrow dermal papillae (open arrows) that contain vessels (dark arrows). A distinct granular layer (gr in A, B) is covered by the stratum corneum. The denser basal cells have extensive basal processes into which epithelial filaments (arrowhead in B) terminate. RLSM z-scan through the epidermis shows a bright hyper-reflective meshwork of cell borders at the epithelial surface. Papillae have bright rings of basal epithelial cells with a dark core; the same papilla is marked by an open arrow in the sub-figures of (C). (A) H&E, bar = 100 µm; (B) MG stain, bar = 10 µm; (C) RLSM, bar = 100 µm). SECTION 3: Higher enlargements of the inner lid border of an upper lid in a mid-temporal position (A–E). The narrow dermal papillae (open arrow in A) become irregular and stop. The epidermal cornified and granular (gr in B) layers stop abruptly (thick interrupted line in A, B, E). The MCJ forms a large epithelial peg lined by pointed papillae (double open arrows in A, B). The MCJ surface (here 274 µm wide) first has a zone of continuous pk cells for 150 µm (B, grey line), followed by a zone of discontinuous pk interspersed among ordinary squamous (s) cells. Small dense roundish basal cells continue underneath the initial part of the lid wiper. (B) At the start of the lid wiper (narrow interrupted line in A, B) a conjunctival epithelial structure with cuboidal (c in B) surface cells occurs. It reaches a maximal thickness here of 98 µm soon after its start. It gradually thins down, forms a slope, and extends for here about a 1000-µm width (A) until it transforms into that of the sub-tarsal fold; a preparation artefact is seen (A, arrowhead). The lid wiper is composed mainly of cuboidal cells, some columnar cells, and contains goblet cells (asterisks in B). Some interspersed pk cells of flat to columnar shape occur at the surface (B). Goblet cells with faint staining of granular content or a reticular meshwork and a flat basal nucleus are also located in the depth of the epithelium (asterisk and arrow on nucleus in enlarged detail, (C). A few intraepithelial lymphocytes (arrowheads in B) are seen, Occasional smaller clefts (cl in B) occur between epithelial cells. An increased number of lymphocytes (B, arrowhead) and vessels, including high endothelial venules (h) with brighter, roundish endothelial nuclei (arrowheads), ordinary venules (v), and arterioles (a) underneath the MCJ is better seen in higher magnification (D, vessels are encircled by dotted lines). In another magnification (E), pk cells are clearly identified. H&E stain. Scale bar: 100 µm (A, B); 10 µm (C–E).

1.1.2.2 Impression cytology

Impression cytology (IC) is a quick and simple, non-invasive way of collecting superficial cells from the ocular surface by application of a membrane; upon removal, cells adhere to the membrane, allowing for subsequent cyto-chemical processing [20]. It has been used for over 40 years on the bulbar, tarsal or limbal conjunctiva and cornea as an effective tool for assessing conditions such as Sjögren's

syndrome or squamous metaplasia [17,18]. The most common membrane materials are paper or cellulose based, although a few exceptions such as plastic discs, glass slides, nitrocellulose or polyether sulfone filters exist [20]. More recently, Biopore membranes such as the Millicell-CM (Figure 3) have been preferentially used for immunohistochemistry due to their good transparency when immersed in liquids. The application is sometimes preceded by the instillation of a drop of anesthetic (such as 0.5% proparacaine or 0.5% proxymetacaine hydrochloride) to alleviate the slightly uncomfortable sensation during membrane removal [24]. The impact of anesthetics on the morphology and functionality of cells is debated [18,20,25], since preservatives in some solutions (e.g. proparacaine hydrochloride, AKA Alcaine) are composed of Benzalkonium Chloride (BAK), a chemical known for its biocidal properties, used in in vitro studies to effectively kill off cultured cells.
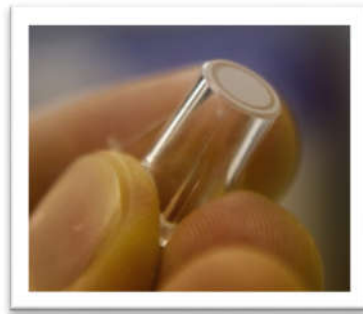
Figure 3: Biopore Millicell-CM cell culture insert used for IC of the ELM.

The membrane is made from hydrophilic PTFE (Teflon).

Only two accounts report the use of IC on the ELM. Doughty [9] collected cells along the lower Marx line of 10 healthy male adults using Millicell-CM filters and reported the occurrence of "three to eight lines of squamous-appearing cells", after fixing and staining cells with Giemsa [26]. Cell images were projected at a final magnification of 1000x and an overlay was generated; using a digitizer pad, cells and nucleus areas were dimensioned and the nucleus-to-cell (NC) ratio was calculated [27,28] (Figure 4).
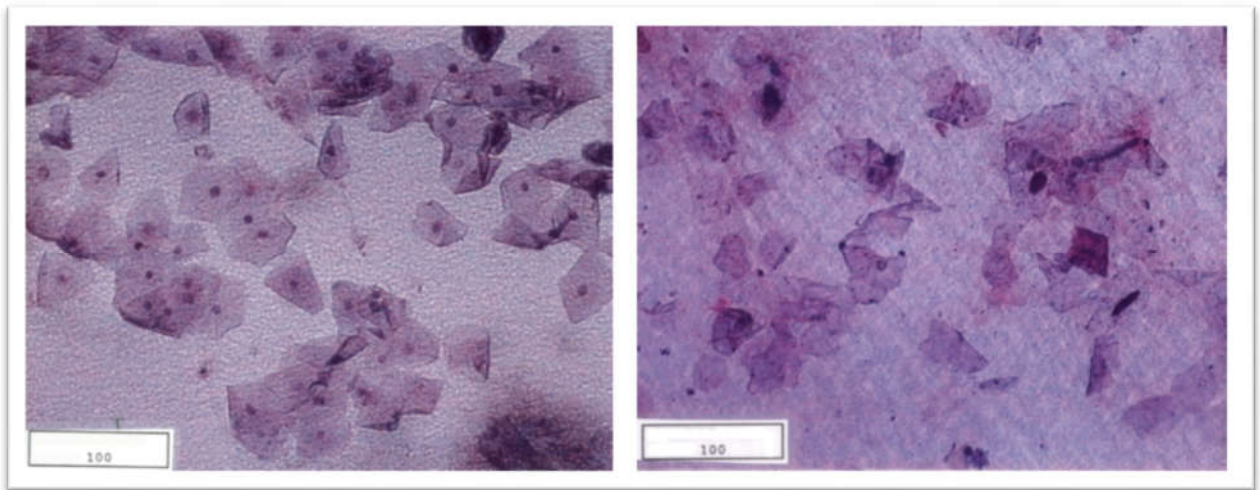
Figure 4: Impression cytology of the lid wiper area [9]

Example of squamous cells showing more prominent nuclei collected by impression cytology from the marginal zone to Biopore (Millicell) filter. The specimen was air-dried, glutaraldehye-fixed, Giemsa-stained. The amorphous staining at the bottom right of the image is due to the meibomian gland oils, while the palpebral conjunctiva is beyond the top edge of the image (left). Example of squamous cells showing weakly staining nuclei collected by impression cytology from the marginal zone to Biopore (Millicell) filter. The specimen was air-dried, glutaraldehye-fixed, Giemsa-stained (right). Length of rectangular box (scale bar) = 100 mm.

Jalbert et al. applied Biopore membranes on the everted upper eyelid of 40 healthy CL and non-CL wearers, and described the cellular appearance and NC-ratio following histological staining with PAS and hematoxylin, as well as immunocytochemical staining with anti-human primary antibodies (mouse filaggrin, rabbit TGase1 and mouse cytokeratin $1/10$) to show the expression of keratinization [24]. Samples were imaged using bright-field, fluorescence and CLSM (Figure 5).

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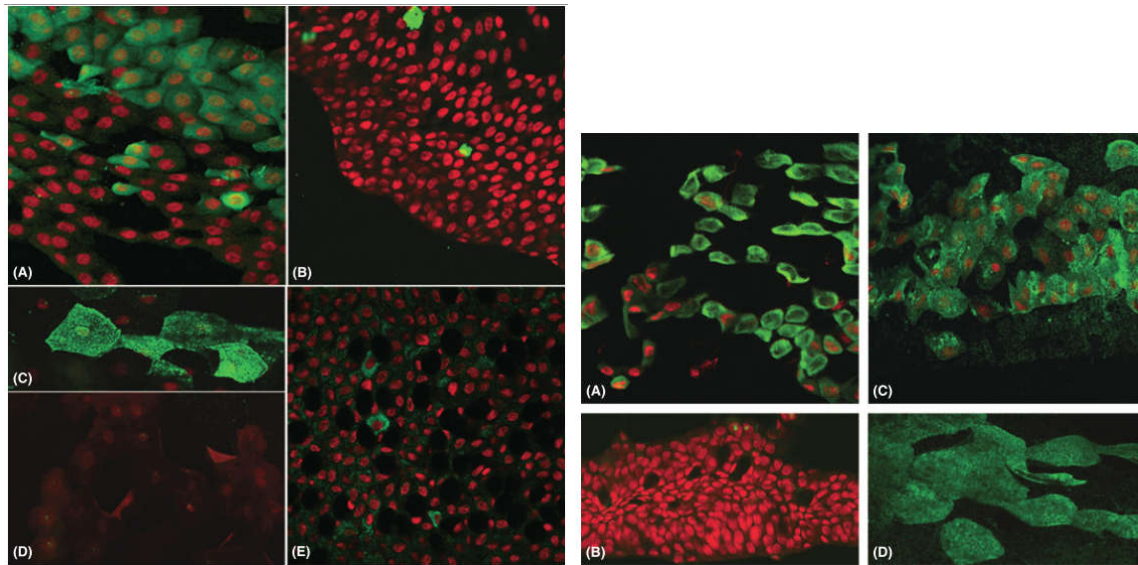
Figure 5: Immunocytochemical staining of lid marginal cells, imaged using fluorescence microscopy [24]

Left panes: representative images of filaggrin immunostaining in the mucocutaneous junction (A), with minimal immunostaining in the marginal epithelium (lower field shown in A, B) and bulbar conjunctiva epithelium (E). Squamous epithelium of the lid margin showed patchy areas of filaggrin immunostaining; note the flattened morphology and small / absent nuclei (C). Immunostaining is not apparent in the Ig negative control (D). Right panes: Representative images showing lid margin epithelium immunostaining for cytokeratin 1 / 10 (A, B) and TGase1 (C, D). The squamous epithelium is cytokeratin 1 / 10 positive (A); however, the epithelium proximal to this shows no immunostaining; goblet cells are visible within this region (B). The mucocutaneous junction zone (C) and squamous epithelium (D) dis- played TGase1 immunoreactivity.

1.2 Anatomy and histology of the eyelid margin

1.2.1 General

Although Sattler observed a "thickened epithelium" at the ELM almost 150 years ago [21], Parsons is credited with being the first to describe the 'sharp' inner lid border that lies in close contact with the globe, and to postulate its contribution to the distribution of tears [23]. The common belief at the time, which to a certain extent persists to this day, was that other palpebral conjunctival structures, such as the tarsal conjunctiva, may be in contact with the ocular surface during blinking. But in 1965, Ehlers suggested that the ELM may be the only structure touching the eye, by acting as a "wind-screen wiper" during the spread of tears [22]. Only a few years later, Kessing confirmed that only a narrow region at the ELM is in contact with the eye during blinking; the area between the cornea and the tarsal

conjunctiva soon became known as the "Kessing space" [29]. Its exact dimensions have not been confirmed, but it is likely less than 250 µm deep and potentially filled with a thick mucus based layer [30]. This gel-like substance is produced by Goblet cells and is believed to be essential for lubrication during blinking (Figure 6).
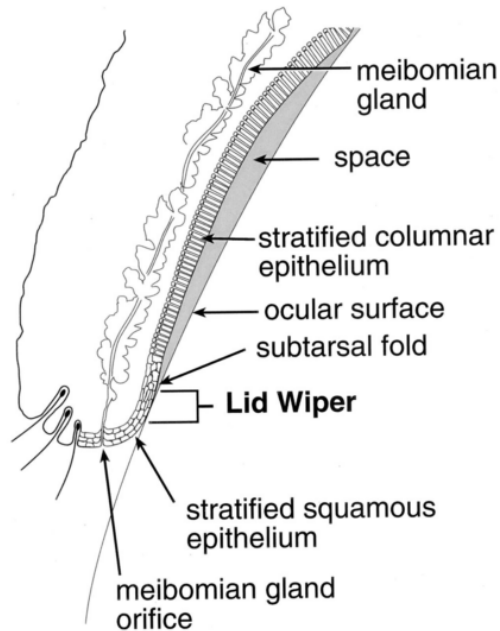
Figure 6: Aspects of the palpebral conjunctiva in the upper eyelid. The "space" refers to the Kessing space [6]

The current understanding is that the upper and lower ELMs broadly consist of three areas, spanning between the lacrimal puncta and the lateral canthus horizontally, and vertically between the outer skin and the eyelashes distally and the tarsal conjunctiva/sulcus proximally. The latter vertical layout encompasses the Meibomian gland openings, the muco-cutaneous junction or the Marx line, and the lid wiper conjunctiva (Figure 7), with a total reported width ranging between 0.3 and 0.7 mm [13,31,32]. In 2010, Knop et al. published a review titled "The lid margin is an underestimated structure for preservation of ocular surface health and development of dry eye disease" [33], which was followed by a series of detailed anatomical and cytological descriptions of the ELM [13,34,35].

While the tarsal conjunctiva is assumed not be in direct contact with the globe during blinking (representing the palpebral aspect of the Kessing space), two fundamental structures are differentiated at the lid margin in the context of friction during blinking: the MCJ and the LW.
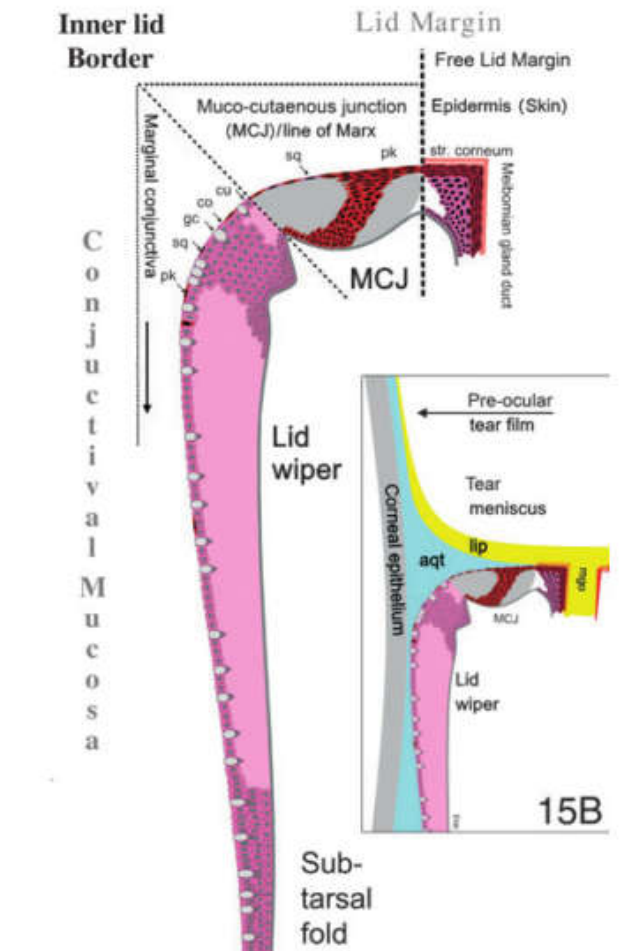
Figure 7: Diagram of section of the ELM [13]

1.2.2 The Marx line and muco-cutaneous junction (MCJ)

The junction between the keratinized epithelium and the palpebral conjunctiva of the upper and lower ELMs was first described by Marx in 1924, who coined the eponymous line after observing its staining with RB and other dyes [3].

Until recently, as evidenced by its sparse occurrence in the literature, interest in the Marx line was minimal [36–39] partly because the nature, precise location, functional implication and morphological basis of the observed Marx line were unclear. Some descriptions regarded the whole epithelial thickening as the MCJ [40,41] (this was later confirmed to be the LW [6,13,42]) and others defined the MCJ as only a narrow division line between the cornified epidermis and the conjunctiva [33]. As evidenced by recent histological work of Knop et al. [13], cells in this region stained very intensely

with the red acidic fuchsine stain (or Masson-Goldners trichrome stain), which Marx had also used. It was concluded that this narrow zone of para-keratinized cells at the surface of the MCJ represents the histological equivalent of the vital staining line of Marx [33].

Several in vitro and in vivo techniques have since been employed to dimension the MCJ and accounts on its vertical width range between 0.09 mm [32,43] and 0.5 mm [9,13].

Knop proposes a bi-zonal composition of the MCJ [13]. Analyzing biopsies, the authors found the cornified and granular epidermis of the skin of the eyelid to stop abruptly, just behind the posterior margin of the Meibomian openings. They defined this point as the beginning of the MCJ, with a total width of 150 to 350μm. This tissue structure then becomes a continuous surface layer of para-keratinized cells for the first 150 to 200 μm (the continuous para-keratinized zone), followed by discontinuous para-keratinized cells interspersed with ordinary squamous cells for the remaining 100 to 150 μm (the squamous transition zone). Knop thus defined the MCJ as a "transitional zone of stratified epithelium populated at the surface by adjacent zones of continuous and discontinuous para-keratinized and ordinary squamous cells" [13]. Doughty similarly concluded that "cells along the line of Marx are moderate-sized squamous cells with nuclei smaller than in normal bulbar conjunctival cells, at times even pyknotic (shrunken) and/or anucleate" [9]. In comparison, Jalbert et al. found that epithelial cells at the line of Marx displayed para-keratinized features, with dense cytoplasm but more regular cell size, shape and nuclei [24]. This description alludes to a more non-squamous nature of cells in this region.

To this day, the etiology and functional significance of this vital staining line have remained a subject of speculation. Marx noted that this line: (1) has a relation to the outer margin of the tear meniscus; (2) may be caused by the interaction of the tears with the epithelium; (3) may serve to guide the tears along the lid margin to the lacrimal punctum [3]. Bron et al. hypothesized that the Marx line may be the result of evaporative water loss from the tear meniscus and subsequent hyperosmolar stress to this region [44]. Ehlers suggested it might be caused by friction during blinking [22], while Norn observed that it represented the bottom of the tear meniscus, which argues against a direct contact with the globe [45]. Others have also considered this line to be the natural site of contact between the ELM and the ocular surface [31,46]. However, Knop considers this to be unlikely, arguing that the line of Marx is too far outside, i.e. distal, on the posterior lid border to touch the globe. Further, they insist that this zone is too narrow and appears to be too rigid to prevent destruction of the sensitive bulbar epithelia, given the frequent physiological eye blinks [33].

Progressing further proximally, towards the tarsal sulcus, the MCJ transitions into an epithelium with a conjunctival structure, composed of roundish cells of less density. This epithelial cushion forms the lid wiper (LW).

1.2.3 The lid wiper

Parsons first advanced the idea of a wiping surface at the ELM, which is "covered with stratified epithelium […] and is in closest apposition with the globe, and mutual pressure of the two may perhaps be the cause of the flattening of the superficial cells", thus suggesting a squamous nature of the epithelium in this area [23]. Ehlers also supported this view [22], as surfaces exposed to friction are commonly composed of squamous epithelium (e.g. cornea, oral epithelium, esophagus [47]). Yet, these descriptions predated those of the MCJ, leaving room for interpretation as to the exact location of these cells.

In 2011, Knop et al. described the LW as consisting of a "stratified epithelium with a conjunctival structure of cuboidal cells, some para-keratinized cells and goblet cells" [13]. Jalbert et al. suggests a squamous view of LW histology that agrees more with historical descriptions [22,23], describing "flat and polygonal shaped epithelial cells, with dense (keratinized) cytoplasm and small or no nuclei" [24]. Efron et al. have signaled the ambiguity of Jalbert's descriptions, since cells were reportedly collected "around Meibomian gland openings", as well as the inconsistent labeling of some of their figures. Moreover, the authors also raise the question whether the undisturbed three-dimensional epithelial morphology (i.e. squamous, cuboidal or columnar) of cells can be determined, in light of the pressure-based application of the IC membrane [48].

The dimensions of the LW region are unclear, with Ehlers stating that the squamous epithelium extends away from the lid margin on to the conjunctival side of the lid "...for some distance, until, rather abruptly, it continues in a single- or multi-layered, almost cubical epithelium with goblet cells" [22], and Knop reporting a width of the LW of "0.3–1.5 mm or more" [13]. It is noted that although the LW shows a typical conjunctival structure, the change of the epithelial surface morphology at its beginning is not sharply defined, bearing the characteristics of a transition zone [13]. For example, Goblet cells, located in both superficial and deeper layers of the LW epithelium, tend to increase in numbers towards the tarsal conjunctiva [34], highlighting the importance of localized lubrication during blinking. Recent evidence emerged to suggest that decreased lubrication in this region may cause a clinical observation called lid wiper epitheliopathy (LWE).

1.3 Lid wiper epitheliopathy

LWE is a clinical condition proposed and introduced by Korb et al. in 2002, after observing that the ELMs of symptomatic SCL wearers stained more intensely than in asymptomatic SCL wearers [6]. The staining was measured after the instillation of fluorescein and RB and graded based on the horizontal length and the sagittal width of the staining on a scale from 0 to 3 (Table 1). This grading (and the corresponding descriptors) does not feature an accepted threshold above which LWE would be considered a disease, partly because LWE is not considered a disease per se. It should also be noted that grade 0 LWE ("none") is typically represented by the natural occurrence of the Marx line, described earlier.

SCL-associated LWE was originally proposed to most likely result from an altered tear film [49] between the CL and the LW. In a subsequent multicenter study, Korb et al. excluded CL wearers and showed that LWE occurs when symptoms of dry eye are experienced, even in the absence of routine clinical dry eye findings [42]. After replacing RB with LG [50], Korb et al. suggested that the prevalence of LWE was six times greater, and more severe grades of LWE are 16 times more likely in cases of dry eye, compared to controls [51]. These results have been mirrored by others, showing an increased incidence of LWE in patients with dry eye symptoms [52,53]. As of 2016, close to 50 publications on LWE were published in papers or conference abstracts, denoting increased interest in this topic [48], with multiple staining patterns [8] and grading schemes and techniques [7,54–59] being advanced by others.

Table 1: LWE grading scheme introduced by Korb et al.

Grading:			
Horizontal length of staining	Grade	Sagittal width of staining	Grade
<2 mm	0	<25% of the width of wiper	0
2-4 mm	1	25% - <50% width of wiper	1
5-9 mm	2	50% - <75% width of wiper	2
>10 mm	3	≥ 75% of the width of wiper	3

Classification:

	Fluorescein Grade (FI)	Lissamine Green Grade (LG)
Horizontal	_____	_____
Sagittal	_____	_____
Average	_____	_____
Final [(FI + LG)/2]	_____	
Classification	_____	

Subjects will be classified according to their final LWE grade for each eye.

Final LWE Grade	
0	None
0.25 – 1.00	Mild
1.25 – 2.00	Moderate
2.25 – 3.00	Severe

1.3.1 LWE and CL wear

To date, the number of publications which confirm the proposed relationship between SCL-associated symptoms and increased LWE [60–65] is roughly equal to those who did not find such an association [11,66–68]. The latter group also includes a meta-analysis conducted by Efron et al., involving 587 subjects across multiple studies, in which no significant relationship was found between the grade of LWE and various CL-related comfort metrics [48]. The authors also note that curiously, and most likely coincidentally, all studies failing to demonstrate a difference in LWE between symptomatic and asymptomatic CL wearers occurred *after* the studies which did. However, this inconclusiveness may be caused by small sample sizes and the limited statistical power of some studies, as well as the inappropriate sensitivity of techniques to detect fine differences between LWE and/or

comfort and dryness. And yet, these results are relevant not just in the context of diagnosis, but more so with respect to the pathogenesis and etiology of this condition [48].

1.3.2 Etiology of LWE

While the etiology of LWE (with CL) is believed to be mainly linked to frictional aspects [6,42,69–72], numerous other theories were put forward, ranging from changes in the aqueous [73] or mucin [65] components of the tear film or its osmolarity [74–76], to bio-tribological aspects of blinking [77,78], abnormal blinking activity [79], saccadic eye movements [48], inflammation [80], eyelid pressure and elasticity [32,81] or even psychological factors [82].

Given the vast diversity of proposed theories and the interaction of factors during CL wear, it has been suggested that LWE may actually be a multi-factorial condition, taking on many different forms [48]. This is noteworthy, particularly in the context of dry eye disease (defined below), which is a multi-factorial condition itself. As such, Efron et al. propose the use of the term "lid wiper epitheliopathies", or even refraining from the use of the term "epitheliopathy" altogether, and referring to "lid wiper staining" instead. It is noted that more fundamental research studies, such as the work by Jalbert et al. [24], may be more compelling than clinical trials towards understanding LWE [48]. Curiously, there is no knowledge on the cytology of the lid wiper region, in LWE versus non-LWE subjects.

1.4 Contact lens-related discomfort

1.4.1 Definitions

The scientific community has dedicated considerable efforts towards developing a better understanding of CL-related discomfort (CLD) as well as dry eye disease (DED), both of which are among the leading causes of CL-related dissatisfaction and drop-out [82–84]. CLD and DED appear to be associated, although the direct mechanisms are not clear [85]. Similar pathophysiological changes that occur in dry eye can be observed in CL wearers, and conversely CL wear can be a precipitating factor in DED [86,87]. As a result, a number of large-scale, expert-led workshops (the Tear Film and Ocular Society's (TFOS) Workshops on Dry Eye (DEWS & DEWS II)) have been recently conducted in topic-specific subcommittees, and published in the form of freely available reports [88,89]. One of the goals hereby was to define CLD and DED. The currently accepted definitions are:

"CLD is a condition characterized by episodic or persistent adverse ocular sensations related to lens wear, either with or without visual disturbance, resulting from reduced compatibility between the contact lens and the ocular environment, which can lead to decreased wearing time and discontinuation of contact lens wear [88]".

The newly developed TFOS DEWS II definition states:

"DED is a multifactorial disease of the ocular surface characterized by a loss of homeostasis of the tear film, and accompanied by ocular symptoms, in which tear film instability and hyperosmolarity, ocular surface inflammation and damage, and neurosensory abnormalities play etiological roles" [89].

Numerous factors may be related to CLD and the proposed classification is certainly not exhaustive (Figure 8).
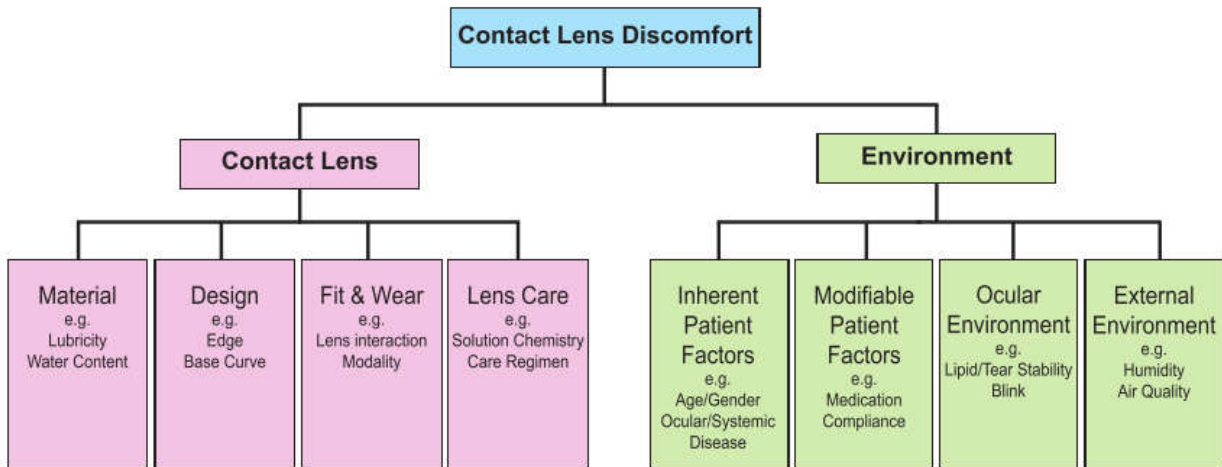
Figure 8: Classification of CLD. Examples of each subcategory are provided, but not intended to list all potentially related factors within each subcategory. [85]

1.4.2 Diagnosis

Optometric exams rely on a number of clinical tests, many of which may be helpful in the diagnosis of CLD. The assessment of the pre-lens tear film [90,91], Meibomian glands [92], bulbar and limbal hyperemia [93], and corneal and conjunctival staining [94,95] have been shown to be related to CLD, but there does not appear to exist a single common sign that is present in all patients experiencing CLD [96]. The lack of association between clinical signs and symptoms is frequently reported [97,98] and it

is considered that investigating symptoms in SCL wearers is likely to have more diagnostic value than conducting clinical tests [96].

As such, CLD is primarily reported according to symptomatology as opposed to the observation of signs. The frequency and intensity with which these symptoms are reported can be assessed with the use of questionnaires. While most questionnaires were developed to assess dry eye symptoms in non–lens wearers [99–101], only more recently were instruments specifically developed to assess symptoms in CL wearers. The Contact Lens Dry Eye Questionnaire (CLDEQ) [102,103] and the recently revised version (CLDEQ-8) [104] have been shown to be effective tools. At the same time, the long-standing absence of a single validated questionnaire for measuring discomfort has hampered cross-comparisons between studies [105].

1.4.3 Contact lens properties

1.4.3.1 Material

   CLs can be broadly differentiated by their material composition into soft (SCL) and rigid materials. The latter also include now obsolete poly-methyl methacrylate (PMMA) materials, as well as more modern rigid gas permeable (RGP) compositions. Approximately 90% of the world's CL wearers wear soft lenses [105]. Among many factors, the commercial success of SCL materials is largely due to their superior initial comfort, compared to rigid materials, but this considerable difference may only be true in the short term. There is little evidence that long-term comfort is substantially different between SCL and RGP wear, past the initial adaptation phase [106,107]. In fact, experienced RGP wearers reported better comfort than all other wearers in one study, suggesting that long-term RGP lens wear may ultimately be the ''most comfortable'' option [107]. Additional lens-specific features may be more relevant with regard to CLD.

1.4.3.2 Friction and lubricity

   The coefficient of friction is the ratio of the frictional force between two contacting surfaces in relative motion to the normal force between those surfaces. "Lubrication" is defined as any means capable of controlling friction and wear of interacting surfaces in relative motion. Materials with low friction are considered well lubricated, or having good lubricity [105]. Multiple in vitro measurements of friction on CLs have been conducted and considerable support is emerging for the role of friction in CL wear and comfort [71,72,108–113]. A range of technologies have been used, but none has yet shown

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to be representative of the in vivo environment, and in vivo measurements of friction at the ELM remain unfeasible to this day. Nevertheless, it has been pointed out that low friction should not be considered in isolation, and that other CL characteristics may determine comfort [48].

1.4.3.3 Lens Edge Profile

Various lens edge designs have been proposed, with modern molded designs generally tapering to a thinner edge than lathe-cut and older molded designs (**Figure 9**). Generally, thicker, rounded edge shapes tend to exhibit poorer comfort than sharper edges [114,115]. Considerably less comfortable than SCL designs at first [107,116,117], the discomfort in RGP lenses appears to stem from the interaction between the lens edge and the ELM, particularly the upper lid margin, as evidenced by the various strategies adopted here to minimize the discomfort [118]. Several studies have shown that the interaction of the lens edge with the eyelid is the most important factor in determining comfort in rigid lens wear [119–121]. It is worth noting that the LW zone has the highest neural sensitivity of all conjunctival and lid regions, and is similar in this respect to the central cornea [122,123].
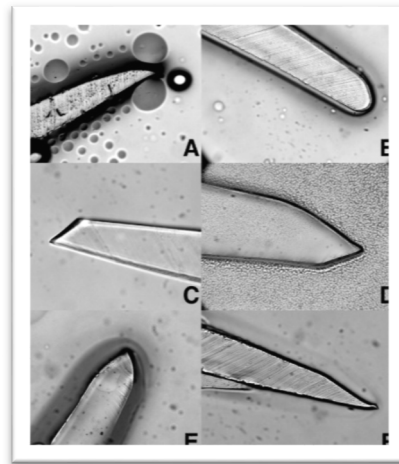
Figure 9: Edge profiles of common soft CLs

(A: Johnson & Johnson Acuvue Oasys, B: Bausch & Lomb PureVision, C: CIBAVision Dailies, D: CooperVision Clariti monthly, E: CooperVision Biofinity, F: Johnson & Johnson 1-Day Acuvue Moist). Images taken under 40x magnification after freezing the CLs and sectioning using a cryo-microtome.

1.4.3.4 Lens Movement

Excessive lens movement is typically associated with discomfort, presumably caused by the repeated motion of the lens over the cornea. However, an alternative explanation might be irritation through

excessive interaction between the lens and the lids, but this has not been quantified [124]. A single study so far has found a correlation between lens movement and comfort, with less mobile lenses being rated more comfortable [125].

1.4.4 Morphological Changes associated with CL-wear

CL wear has a range of effects on ocular tissue, ranging from cytological changes in the conjunctiva, to topographical changes of the cornea [126–128] and structural transformations in the Meibomian glands [92,129,130]. Daily wear (DW) of SCLs has a limited impact on the corneal epithelium [131,132], but extended wear (EW) modes are associated with an increase in cell size and area [133–135]. During DW of RGP lenses, cells increase in size by 10% to 30% [136]. Conjunctival squamous metaplasia, which is the flattening of epithelial cells and increase in cell diameter with loss of goblet cells [137], is known to occur in the conjunctiva around the limbus of most SCL wearers [137,138]. These and other changes [139,140] are believed to occur as a result of mechanical friction on the epithelial cell surface, and may be reversed by cessation of lens wear [137]. Further evidence suggests that CL wear produces different effects on the upper and lower eyelids. While RGP wear appears to cause MG dropout in the upper eyelids, SCLs are associated with the shortening of glands in the lower lids [92]. Although some studies support a potential causative link between cytological alterations in CL wear and CLD [138,141], to date, no direct correlation between any of these morphological changes with CLD has been reported.

Chapter 2
Rationale and objectives

Contact lenses have spawned a multi-billion-dollar industry [142], with an estimated 140 million wearers worldwide, as of 2013 [88,143]. However, since their commercialization over 50 years ago and despite substantial innovations such as silicone hydrogel materials and daily disposable lenses, CLs have been unable to become a truly successful and viable visual aid for everyone. In the United States, nearly three million, or 10% of lens users discontinue CL wear every year [144]. The most commonly cited reasons are symptoms of discomfort and dryness [84,102,103,145–147]. Dryness is *the* most common symptom with SCL wear, with prevalence estimates ranging from 50% to 94%, depending on the test population [102,103,148–150]. And yet, despite substantial research efforts over the past decades, the etiology of CL-related dryness and discomfort remain speculative.

Inserting a "foreign body" such as a CL onto the surface of the eye disrupts the tear film [151]. Additionally, both conventional and silicone hydrogel CL materials absorb and adsorb tear film components [152], and cause denaturation of tear lysozyme [153] and degradation of tear film lipids [154]. Over 1500 individual tear film components (many with unknown functions) have been identified, and their interactions with the complex and diverse chemistry of CL materials and surface treatments certainly warrants further investigation.

More recently, the mechanics of the blink and the interaction of eyelid margins with the ocular (or CL) surface have become the focus of comfort-related research. A 1-2 mm narrow region recently termed the "lid wiper" [6] is presumed to travel over 400 m over the surface of the eye or a CL each day, given an average of 10,000 blinks that humans execute daily [155]. The supposed increased friction in this area during habitual blinking is typically alleviated by the tear film, yet disturbances to the normal tear film, whether physiological, pathological or by wearing a CL, may affect the comfort we perceive. Specifically, the coefficient of friction (which varies with different lens materials) appears to be related to the perceived comfort during CL wear. While the implications of friction in CL wear were recognized as early as 1936 by Feinbloom [69], the coefficients of friction of contact lenses were not reported until 1995 [70]. In 2002, Korb et al. published their seminal paper on LWE, an increased vital staining of the lid margin in dry eye subjects versus normals [6]. This occurrence is likely of frictional origin, caused by inadequate lubrication during habitual blinking. In combination with a number of accounts showing correlations between friction and comfort [71,72,108–111], the discovery of LWE has

catalyzed further research into the potential role of CL surface friction and the lid wiper in explaining discomfort and dryness symptoms associated with CL wear [78,108,156–158]. As of 2016, a total of 47 conference abstracts and publications were recorded on the topic of LWE [48].

While its utility as a clinical tool and the ability to reliably predict dry eye are still being debated, a significant gap exists between the observed vital staining and its alleged clinical meaning: *what is the significance of this staining at a cellular level and how is the lid margin of lid wiper epitheliopathic subjects different than normals?* Or, in the words of Efron et al. from their comprehensive review on LWE [48], "the question as to the histology of the lid wiper needs to be resolved". Specifically, it is unknown whether the vital staining with LG (i.e. the status of cells being "dead" or "degenerate") equates with an altered cytology of epithelial cells (in comparison to normals (i.e. non-LWE subjects), and whether additional, more subtle features of the state and morphology of these cells can be assessed using histological observations and measurements.

So far, only a handful of accounts exist on the lid-margin histology, but they are difficult to reconcile, for a number of reasons. Historical reports of Parsons [23] and Ehlers [22], as well as a more recent paper by Jalbert et al. [24], suffer from a lack of clarity as to the precise anatomical locations to which histological descriptions pertain. Knop et al. [13] undoubtedly provides the most accurate description of the lid margin to date, but studied excised tissue from cold stored cadavers with an average age of 77 years, which is significantly higher than the age of typical CL wearers, with a worldwide average of around 30 years [159].

This raises the question of the relevance of the observations of Knop [13]. While Jalbert's report is the only one to include CL wearers and non-wearers in their analysis, it is not possible to determine from their work the extent to which the observed cell morphology relates to contact lens wear or symptoms. . In light of the mechanical or frictional considerations outlined above,

> *The hypothesis that underlies this thesis is that CL wear, LWE, and symptoms of dryness and discomfort may be manifest as cellular changes of the lid marginal epithelium, as a result of mechanical action (e.g. friction).*

Part of the reason why ex vivo accounts of the cytology of living subjects are largely missing is the difficulty of obtaining cells from this narrow region. Impression cytology, a technique routinely used

for collecting superficial cells from the bulbar conjunctiva or cornea has been employed only a number of times on the lid margin, but with limited success, with authors reporting "scarce collections" and obtaining only sub-par images of the cells in this region [9,24].

Therefore, the purpose of this thesis was to develop and optimize a method to collect and analyze cells from the lid margin, by which to elucidate their histology in relation to CL wear, hopefully shedding more light on the mechanics of ocular discomfort and dryness.

In chapter 3, we begin by identifying the ideal combination of membranes, cytochemical stains, imaging and analysis techniques to obtain cellular samples from the eyelid margins of humans. By carefully optimizing every step of the procedure, from the application angle of the membrane to the lid margin, to digitally enhancing the quality of the obtained images, we aimed to maximize the quantity and quality of cellular information obtained from this region.

The above method was then employed in a pilot study in chapter 4, to assess its practicality in differentiating the lid marginal cyto-morphology of lens wearers and non-wearers (n=15) with varying degrees of LWE and subjective ocular discomfort.

In chapter 5, the sample size was expanded (n=40) and the manifestations of CL-related discomfort at the upper and lower eyelid margins were compared between two groups of symptomatic and asymptomatic SCL wearers. Subjective comfort was assessed using a battery of questionnaires, LWE was evaluated using lissamine green and the width of the lid wiper and muco-cutaneous junction cellular regions were determined using a custom-programmed software used in conjunction with the obtained images of IC collections.

Using the same methodology employed above, chapter 6 explored the effects of lens wear in and of itself at the lid margin, by comparing twenty RGP wearers to twenty non-lens wearers.

Finally, in chapter 7, a cross-comparison between chapters 5 and 6 was conducted, to illuminate the effects of SCL wear on the eyelid margins in contrast to non-lens wearers, as well as to compare SCL wear to RGP lens wear. Lastly, the upper and lower lid margins of all participants were compared in terms of LWE and cyto-morphology.

Chapter 3

Method optimization to collect, stain and image cells from lid margin

Parts of this chapter are published as follows:

Muntz, A., van Doorn, K., Subbaraman, L. N., Jones, L. W. (2016). Impression cytology of the lid wiper area. JoVE (Journal of Visualized Experiments), (114), e54261-e54261

Muntz, A., van Doorn, K., Subbaraman, L. N., Jones, L. W. (2015) Impression cytology of the lid wiper area. Invest. Ophthalmol. Vis. Sci.;56(7):4432.

3.1 Overview

PURPOSE: Few reports on the cellular anatomy of the lid wiper (LW) area exist and only one makes use of cytological methods. Impression cytology (IC) is typically performed on bulbar and tarsal conjunctiva and thus requires optimization for use on the LW. The purpose of this study was to optimize a method of collecting, staining and imaging cells from the LW region using IC.

METHODS: Upon anesthesia (proparacaine hydrochloride, 0.5%), the upper lids of 5 subjects (n=10) were everted and IC was conducted using various membranes (mixed cellulose esters, hydrophilic PTFE, polyethersulfone). Several fixatives (100% methanol, 95% ethanol), cytological stains (Papanicolaou (hematoxylin Gill No.1, OG-6, EA-65), Periodic Acid-Schiff (PAS) and Alcian Blue (AB)) and soak times (1, 3, 5 minutes) were tested. Varying concentrations of fluorescent dyes (Calcein AM, Ethidium homodimer-1, Annexin V) were tested and imaged using bright-field and confocal laser scanning microscopy (CLSM).

RESULTS: IC delivered optimal results when using the hydrophilic PTFE membrane. Fixing in 95% ethanol for >20 minutes then staining in 500µl each of AB, hematoxylin Gill No.1, OG-6 and EA-65 for 3 minutes revealed the presence of goblet cells, mucins, cell nuclei and various degrees of pre- and para-keratinization. Calcein AM (4µM) and Ethidium (4µM) were combined to successfully show cell esterase activity and compromised cell membranes. Up to 200 microscopy digital images were captured for each sample and stitched into a high-resolution, large scale image of the entire IC span.

CONCLUSION: We have developed a protocol consisting of an optimal selection of membrane, stains and imaging procedures and successfully showed that the sensitivity of IC is appropriate for identifying distinct cellular morphologies surrounding the LW area, as well as showing varying degrees of metabolic activity. To our knowledge, this is the first time this selection of fluorescent dyes was used to image LW IC membranes. This protocol will be effective in future studies to reveal undocumented details of the lid wiper area, such as assessing cellular particularities of contact lens wearers or patients with dry eye or lid wiper epitheliopathy.

3.2 Introduction

As outlined in section 1.4, much attention has been devoted to the study of associations between symptoms of ocular discomfort and clinical signs [53,64,97], yet little is known about clinically-relevant variations in the (sub-) cellular anatomy and physiology of the lid wiper area [9,24], especially in conjunction with contact lens (CL) wear.

Existing reports on the cellular composition of the lid margin mainly rely on excised tissue samples [13,34] conducted on cadavers; with an average age of 77, these reports may not be representative of the structure that exists in the predominantly young contact lens wearing population. With increasing age, lid margin structures such as Marx line are reported to modify shape and position [160]. *In vivo* confocal microscopy [13] has also been employed to characterize the structural, but less the functional composition of the lid margin.

Impression cytology (IC) is a quick and simple way of collecting superficial cells from the ocular surface by application and removal of a membrane. It has been used for over 40 years on the bulbar, tarsal or limbal conjunctiva and cornea [17,18] as an effective tool for assessing conditions such as Sjögren's syndrome or dry eye. Given the focus that the lid margin area has recently gained with respect to CL-related discomfort, it is somewhat surprising that there are currently just two reports on the use of IC to assess the lid margin. While Doughty [9] only focused on the lower Marx line of non-CL wearers, Jalbert and colleagues [24] examined the upper lid margin, including the lid wiper, of both CL and non-CL wearers, but could not demonstrate differences between these groups.

Images presented by Doughty only span a maximum width of ca. 10 cells and do not offer good histological color distinction, as the author describes the effect of rose Bengal on the cells prior to staining with Giemsa as persisting and appearing "quite dramatic" (Figure 10). As noted by Efron [48], IC samples imaged by Jalbert et al. are inconsistently labeled and their descriptions are somewhat ambiguous, including the precise area that analyzed cells were collected from. As such, their comparison between CL and non-CL wearers may be somewhat doubtful.
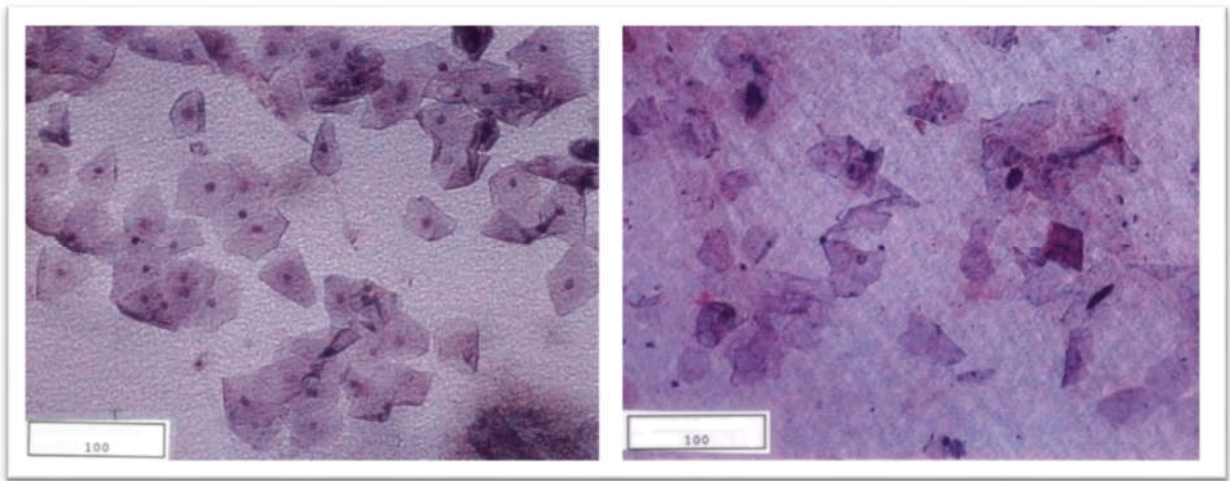
Figure 10: Impression cytology of the lid marginal zone [9]

Example of squamous cells showing more prominent nuclei collected by impression cytology from the marginal zone to Biopore (Millicell) filter. The specimen was air-dried, glutaraldehye-fixed, Giemsa-stained. The amorphous staining at the bottom right of the image is due to the meibomian gland oils, while the palpebral conjunctiva is beyond the top edge of the image (left). Example of squamous cells showing weakly staining nuclei collected by impression cytology from the marginal zone to Biopore (Millicell) filter. The specimen was air-dried, glutaraldehye-fixed, Giemsa-stained (right). Length of rectangular box (scale bar) = 100 mm.

Both authors note that samples had "modest numbers of cells but in adjacent regions across the surface of the filter, the number of adherent cells was sometimes rather lower" [9] and that "samples of marginal epithelial cells that were continuous and could be graded" could be obtained for only 67% of all subjects [24]. This may have to do with the distinct surface geometry and cellular composition of the lid margin, both of which greatly differ from the bulbar conjunctiva, which IC is typically used on. The histological stains employed in these studies (Giemsa and PAS) are favored for their ease of use, particularly towards characterizing the morphology, or dimensioning cellular samples. But, as indicated by Doughty, this comes at the cost of inferior color representation. Other stains, such as the Papanicoloau family of stains, are specifically geared towards a subtler differentiation of keratinization states. These may be better employed in studying the effects of friction at the lid margin.

It appears that a more thorough optimization of the classical bulbar/corneal IC procedure, including the selection of an adequate membrane, its correct application to the conjunctiva and a improved selection of cytochemical stains and imaging techniques, is required for the lid margin.

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3.3 Purpose

The purpose of this study was to optimize a method of collecting, staining and imaging cells from the lid margin using impression cytology, to allow for improved evaluation of the impact of friction in this region. This involved:

a) the selection of an appropriate membrane for collecting the cells from the lid margin;

b) determining the mechanics of an effective application of this membrane on the lid margin;

c) perfecting a cytochemical and immunocytochemical staining protocol to better characterize the collected cells;

d) optimizing techniques to maximize the microscopic imaging of samples from the lid margin.

The procedures developed here were employed in subsequent chapters of this thesis.

3.4 Materials

3.4.1 Subjects

Twenty healthy subjects were enrolled (n=40 eyes) and had IC performed multiple times over the course of a year, with at least eight weeks in between collections. Ethics approval and informed consent were obtained prior to study procedures, and the study was conducted in accordance with the Helsinki declaration for protection of research participants.

A standard optometric slit lamp inspection of the anterior segment was performed before every impression, to confirm eye health. The participant was comfortably seated in a reclined chair, with appropriate head support. Prior to IC, a drop of anesthetic (Alcaine, proparacaine hydrochloride ophthalmic solution, USP, 0.5%, Alcon Laboratories, Fort Worth, Texas) was dispensed in the lower conjunctival sac and the participant asked to close their eyes for a minute. Following IC, participants were administered a drop of ocular lubricant (Bion Tears, Alcon, Fort Worth, Texas) to alleviate any discomfort and underwent a slit lamp inspection to confirm ocular integrity. Participants were instructed to not wear contact lenses for the rest of the day.

3.4.2 Membranes

Three different membranes were compared for their suitability for IC of the lid margin: MF-Millipore Membrane Filters, hydrophilic mixed cellulose esters, 25 mm sheets, 0.45 μm pore size (#HAWP02500, Merck Millipore, Darmstadt, Germany), Millicell Cell Culture Inserts, hydrophilic

PTFE (Teflon), 12 mm inserts, 0.4 μm pore size, (#PICM01250, Merck Millipore, Darmstadt, Germany) and the Eyeprim, a proprietary polyethersulfone membrane (Opia Technologies, Paris, France) [161].

3.4.3 Cytochemical solutions and reagents

Several cytochemical solutions for assessing keratinization and cellular morphology were purchased from Sigma-Aldrich, St. Louis, Missouri: Periodic-Acid-Schiff Kit (PAS) (#395B), Hematoxylin Solution Gill No.1 (#GHS1128), Papanicolaou Stains OG-6 (#HT40132) and EA-65 (#HT40432), Alcian Blue, 1% in 3% acetic acid (AB) (#A3157) and Phloxine B (#P2759).

A fluorescent immunocytochemical stain for determining the viability of cells, the LIVE/DEAD Viability/Cytotoxicity Kit (#L3224) was purchased from Thermo Fisher Scientific Inc., Burlington, Canada, consisting of Ethidium Homodimer-1 (#A23204), Calcein AM (#MP03224) and the Annexin Binding Buffer (#PNN1001).

A selection of fixating solvents and rinsing agents were employed: 100% methanol (v/v), 70-100% ethanol (v/v), RiOS water, xylene, sodium bicarbonate.

3.4.4 Laboratory equipment

Membranes were handled and processed in a chemical fume hood, using nitrile gloves, autoclave-sterilized micro scissors and tweezers. Staining was performed in glass bottom well-plates (#P35G-0-14-C, MatTek Corp.), Falcon and Eppendorf tubes, pipetting using standard laboratory Eppendorf pipettes, using single-use 1 ml and 10 µl pipette tips. For microscopy, membranes were placed either on standard Corning microscope glass slides with coverslips, or in glass bottom well-plates, coverslipped and sealed using laboratory parafilm tape.

3.4.5 Imaging devices

Samples were imaged using a Zeiss bright-field microscope (Zeiss Inc. Toronto, Canada), with an AxioCam digital acquisition system, connected to a PC running AxioVision image acquisition software. This microscope was located at the facility where the IC and membrane processing were performed. A Zeiss 510 META 18 confocal laser scanning microscope (CLSM) with an inverted motorized microscope Axiovert 200M (Zeiss Inc. Toronto, Canada), connected to a PC running the Zeiss ZEN image acquisition software was used for imaging the immunocytochemically stained samples. This microscope was located at a separate facility, 15 minutes' walk away from the main

collection and processing laboratory. Microscope settings were determined during the optimization process.

3.4.6 Software

For image acquisition, the Zeiss AxioVision software was used for bright-field microscopy and the Zeiss ZEN software for confocal imaging. Uncompressed files were saved in their native format (*.zvi and *.czi respectively) and exported to *.jpeg or *.tiff files of varying compression ratios. Image processing and enhancing were performed using Image J (National Institutes of Health (NIH), Bethesda, Maryland). Adobe Photoshop Elements (Adobe Systems Inc., San Jose, California) was used for stitching panoramic images. Batch processing and file management was performed with Total Commander (Ghisler Software GmbH, Switzerland). Images were inspected using IrfanView (Irfan Skiljan, Vienna, Austria).

3.5 Protocol optimization

[This section is the equivalent to results & discussion]

Developing the IC procedure involved the selection of a suitable membrane for the lid margin, determining the mechanics of its application, the selection of cytochemical dyes and the optimization of staining and imaging protocols.

This iterative, non-linear process is difficult to present in a chronological order (i.e. the selection of an appropriate membrane relies on microscopic imaging), therefore to favor readability, these steps will be organized in more relevant sections.

3.5.1 Membrane selection

Mixed cellulose ester membranes (MF-Millipore Membrane Filters) are the most common choice for IC of the bulbar conjunctiva [17,20,25]. Samples are cut in about 10 mm small pieces from bulk material sheets, sterilized, applied and removed from the ocular surface using forceps (**Figure 11**).
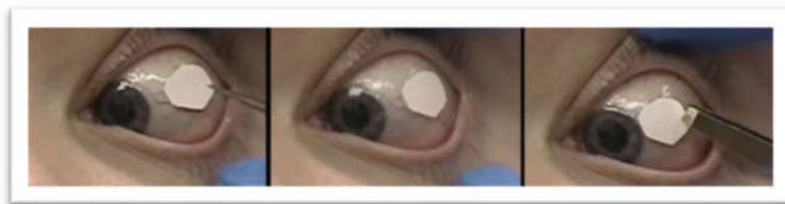
Figure 11: Impression cytology of the left eye, temporal bulbar region

While the use of forceps on the relatively flat bulbar conjunctiva is unproblematic, applying these rigid membranes to the curved and narrow lid margin was challenging. The difficulty in maintaining consistent application angle and pressure was reflected by the great variation in yielded cells, and often not collecting any cells at all. Membranes tended to break down following extensive fixation in alcohol, and could not be made transparent for confocal analysis without interfering with the staining process and were therefore eventually excluded from the optimization protocol.

Following the encountered difficulties of applying membranes using consistent pressure, a recently developed commercial device (Eyeprim, Opia Technologies, Paris), featuring a curved membrane and a piston-controlled mechanism specifically developed for the IC of the bulbar conjunctiva, was included in the study (**Figure 12**).

Figure 12: The Eyeprim device by Opia provides constant application pressure for impression cytology of the bulbar conjunctiva

Repeated attempts at adapting this device to the narrow, pronounced curvature of the lid margin did not prove successful and the device was deemed impractical for this study. The membranes were difficult to separate from their plastic holder, and could not be made transparent for confocal microscopy.

Although atypical, the use of hydrophilic Teflon cell culture inserts for IC has been documented previously [24,162]. Compared to the other tested membranes, they showed superior cellular adhesion and provided almost complete transparency when submerged in water or alcohol, making them a suitable choice for confocal microscopy. The individually packaged, sterile membrane was ideally sized (12 mm), covering a representative area of the central lid margin which is usually in touch with typical contact lens diameters of 9 to 14 mm. During the initial unmounted applications, the plastic holder was convenient for cutting out the membrane prior to IC. The flexible, thin membrane self-adhered to the lid margin (Figure 13), providing large, confluent, cell collections, but required the forceful use of forceps for its removal, which was uncomfortable for the subjects.

Figure 13: Unmounted application of the Millicell membrane to the upper lid of the right eye

This application was eventually abandoned in favor of the safer and more convenient and rapid application of the membranes while still in their holders (Figure 14).
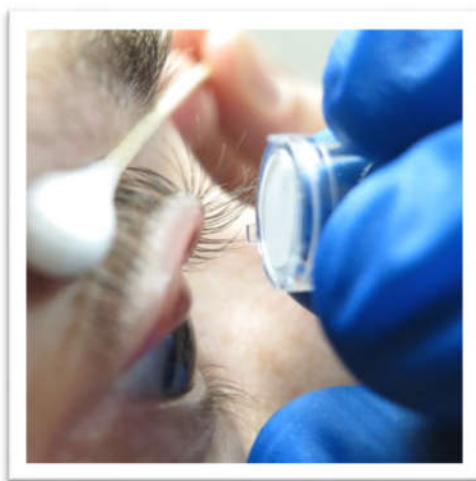
Figure 14: Impression cytology of the right eye, upper lid margin region, with the Millicell Cell Culture Insert (mounted application)

Membranes were cut out following IC and processed. Handling the separated membrane required great care due to the extremely thin and flexible membrane, which tended to fold and stick onto itself, often making it hard or impossible to separate without damaging the collection area. In these cases, the application had to be repeated, making the initial optimization steps particularly laborious and time consuming.

3.5.2 Lid eversion

To conduct IC on the lid margins, eversion of the lids is required, which is typically performed as part of a regular slit-lamp biomicroscopy exam. For the lower lid, gently pulling down on the outer lid, just inferior to the lashes, will sufficiently expose the palpebral conjunctiva for typical inspections, but a more pronounced exposure of the lid margin is required for IC. Disposable, sterile cotton swabs were used for this purpose. The participant was asked to look up and the swab placed horizontally in the infrapalpebral sulcus. While applying gentle pressure, the swab was slowly rotated towards the investigator, causing the lid to roll outwards and appropriately expose the curvature of the lid margin for IC. Avoiding the use of fingers conveniently created enough room for the membrane application (Figure 14).

For exposing the upper lid margin, the participant was asked to look down, while the investigator gently pulled on the lashes with their fingers and placed a downwards pointing swab in the suprapalpebral sulcus, thus creating a wedge for the lid to flip over the tarsal plate, exposing the upper palpebral conjunctiva and lid margin. The "flipping back" of the everted lid is typically achieved by pressing the lashes against the eyebrows using fingers, but this greatly limited the application of the IC membrane. The technique was gradually perfected to swiftly switch to the cotton swab for securing the lashes in place, which enabled a more comfortable membrane application. When handling the upper eyelid, care must be taken to only touch the lashes and avoid any contact of the lid margin. As observed by Varikooty et al. [54], this iatrogenic staining may affect the structural integrity of the epithelial cells, and produce a false positive lissamine green staining of the lid margin. This effect would likely also be observed in the cytological findings of IC.

For an optimal and safe procedure, the investigator must develop good handling skills and care to secure the open lid, avoid contact with the lid margin and leave enough room for the application of the IC membrane. Initially, an assistant investigator performed the lid eversion, since IC required the use of both hands to accurately handle membranes and forceps. With sufficient experience, an investigator was able to singlehandedly perform the eversion and membrane application.

3.5.3 Membrane application

A fundamental difference between bulbar conjunctival and lid margin IC is the surface geometry of the two surfaces. In contrast to the relatively large and flat bulbar conjunctiva, the curved, narrow shape of the latter has a great influence on the quality and consistency of cell collections, through the mechanics of the application. In this section, the location, angle, pressure and removal of the membrane placement are discussed separately.

3.5.3.1 Application location

The central, nasal and temporal regions of upper and lower lid margins were impressed. Given the relatively narrow nasal and temporal regions, placement of the membrane was challenging and yielded tightly packed cell collections which were difficult to analyze. The central lid margin was readily accessible and provided a wider area for impression, which is also most exposed to a worn contact lens. Given that subsequent chapters of this thesis were to study the impact of contact lenses on the lid margin, and in line with previous reports [24], IC was conducted here. Nevertheless, it may be worthwhile to study the cytology of the nasal and temporal areas, given the anecdotally observed

differences in LWE staining, as these distal areas often tend to stain more pronouncedly with LWE, even in cases when the central region shows little or no staining.

3.5.3.2 Application angle

Given the pronounced curvature of the lid margin, even slight variations in the application angle had a major impact on collection quality, i.e. cell number and type. The consistent, perpendicular application was particularly difficult with the rigid mixed cellulose membranes, handled with forceps. The flexible PTFE cell insert membranes were separated from the plastic holder, and conveniently followed the curvature of the lid margin, firmly adhering to its entire surface. This latter aspect was equally responsible for good collections, but also exhibited great difficulty in removing the membrane from the conjunctiva, requiring a forceful, uncomfortable removal using forceps (Figure 13). This approach was abandoned in favor of the mounted application of the membrane, using the provided plastic holder and cutting the membrane out for processing only after cell collection. Membranes were initially applied with a rolling motion, to ensure contact with the entire width of the lid margin. This technique was eventually abandoned due to inconsistent results, in favor of developing experience with a consistent, perpendicular application.

3.5.3.3 Application pressure

Cell adhesion greatly depends on the application pressure. A gentle application will result in a poor cell yield, whereas a forceful application is not only undesirable and uncomfortable, but can also expel a large amount of meibum, covering up cellular features on the membrane and rendering them hard or impossible to analyze (Figure 15).

Figure 15: IC sample of the lid margin covered by dark meibum secretion

While no means of objectively measuring the pressure were available in this study, the literature reports that a pressure of 60 g yields better results than the originally proposed 40 g or 80 g [18,20]. The optimal, moderate pressure that felt light, yet sufficient to indent and flatten the curved lid margin to yield wide, consistent collections, was identified over the course of many months of repeated applications.

3.5.3.4 Membrane removal

In line with a previous report on IC of the lid margin [24], 3-5 seconds was found to be an adequate duration for impressions. Longer applications, as recommended for bulbar IC, would be affected by either pronounced meibum secretion, or overlapping and distorted cells, likely due to the handheld, unsteady application. The thin PTFE membrane turned transparent upon adequate contact with the conjunctiva as it absorbed surface moisture. A membrane that would not turn transparent would indicate a wrong application angle, as placing the membrane on the dry, outer skin would not moisturize the membrane, whereas an impression imprint width greater than ~2 mm would indicate an inclination in the opposite direction, collecting cells from the tarsal conjunctiva. A combination of the correct location, angle, pressure and duration of the application, would be confirmed by the appearance of a 1-2 mm wide transparent area, as well as an audible, subtle "popping" sound upon removal, produced by

the tensioned membrane becoming unstuck from the moist conjunctiva. Finally, upon visually inspecting the membrane after collection, a thin, green, uninterrupted line of lissamine green stain would be visible if LWE had been assessed prior to the IC procedure.

3.5.4 Membrane processing and staining

Upon impression, PTFE and Eyeprim membranes were cut out from their holders using micro scissors, taking care to cut closely along the edges, to maximize the retained collection area. The extremely light-weight membranes required careful handling and securing using tweezers, to prevent them from being airborne due to normal fume-hood ventilation. Membranes were then fully submerged in various fixation agents (70-100% ethanol or 100% methanol), to preserve the cytologic details of samples as close as possible to the living state, in preparation for the cytochemical staining [16]. Fixation was performed for varying lengths of time, from minutes to multiple hours. Although histological samples can typically fixate for many hours or even days, it was found that fixating LW samples in methanol for more than 3 hours would negatively impact sample quality. This would dissolve any meibum deposition, which initially aided inspection of some samples, but also detached collected cells along with the meibum. Methanol was later substituted with 95% ethanol, which allowed fixation times of 20 minutes up to 3 hours. While PTFE and Eyeprim membranes responded well to prolonged fixation times, MF membranes tended to break down after 2 hours and were not analyzable.

Following fixation, membranes were hydrated by transferring them from solvent to water, in preparation for aqueous stains such as hematoxylin and Alcian blue. While most histological samples are robust to a direct transfer, this impacted LW samples by affecting stain quality and cell retention, perhaps due to the weaker adhesion of cells to the membrane. In a more gradual approach, water was slowly pipetted into the fixation agent until reaching a 75% solution, repeatedly absorbing the contents of the vial into the pipette tip. This slow, homogenous mixing of the liquids helped avert previous issues. Dehydration of samples for other stains (e.g. Papanicolaou) was achieved in the same but reverse manner.

3.5.4.1 Cytochemical staining

To better characterize cell morphology and keratinization, the ideal selection, sequence and soaking time of cytochemical stains was determined. One of the more common stains for IC of the ocular surface, the Periodic-Acid-Schiff (PAS) was initially employed. Cellular features and keratinization levels were well represented (Figure 16), but given the frictional component to be studied in subsequent

chapters, a finer distinction of keratinization levels was desired, since PAS only distinguished slightly varying hues of red, pink or brown.
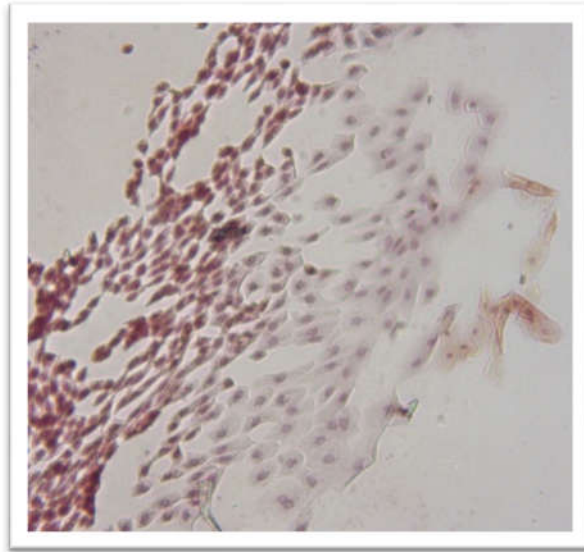
Figure 16: IC of the lid margin stained with PAS showing the transition between tarsal conjunctiva and mucocutaneous junction.

A pair of Papanicolaou stains, the OG-6 stain (indicating pre- and parakeratinization) and EA-65 stain (an indicator for early/late lifecycle cells) replaced PAS, enabling a better chromatic distinction of keratinization levels (Figure 17). While standard staining protocols for most dyes suggest that samples be soaked for 3 to 5 minutes, the EA-65 dye would overpower the other stains if used for longer than 3 minutes.

Figure 17: IC of the lid margin stained with Papanicoloau OG-6 and EA-65 dyes.

Blue indicates no/low keratinization; an increase in "redness" highlights more advanced states of keratinization.

The hematoxylin Gill-1 was used for nuclear detail representation, along with a sodium bicarbonate rinse to raise the pH and improve the nuclear detail (i.e. promotes "bluing" of the nuclei). Due to the interference with the OG-6 stain, the sodium bicarbonate rinse was eventually abandoned.

The Alcian Blue (AB) stain was introduced as an alternative to PAS to stain goblet cells and mucins, as, unlike with PAS, AB does not interfere with downstream staining. Used at the beginning of the staining protocol, AB caused an exaggerated blue coloration of the membrane (Figure 18), which decreased the color representation for other subsequent dyes. Measures to counteract this issue included diluting down the stock AB, filtration and creating an in-house AB solution from scratch; finally, the exposure time to AB was decreased from the suggested 5 minutes, to 3 minutes, which significantly reduced the coloration, yet allowed a good representation of goblet cells and their impression.
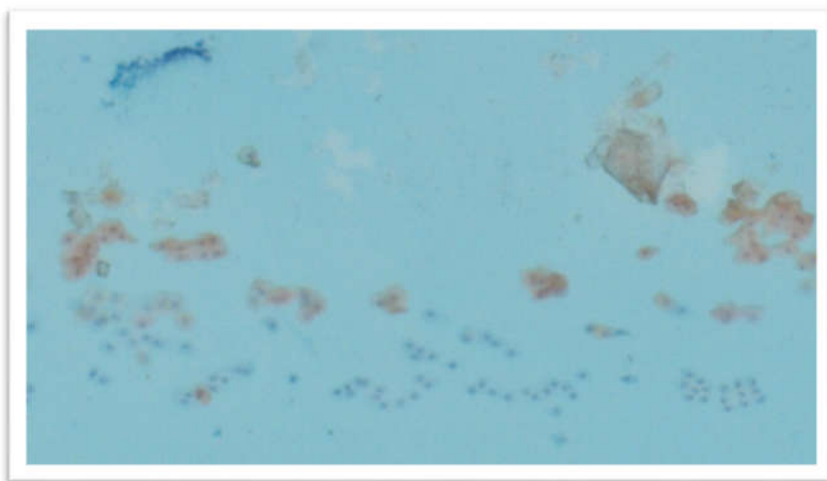
Figure 18: IC of the lid margin showing pronounced staining of the membrane with Alcian Blue

Phloxine B was introduced to color the cytoplasm and stain for orthokeratinized cells (i.e. fully keratinized cells or stratum corneum), but abandoned due to the interference with the OG-6 stain. Xylene was used for clearing membranes of the dark, opaque meibum that frequently obscured cellular features (see Figure 15), but this removal often occurred at the cost of also removing cellular features of potential interest. At this stage in the development of the protocol, it was unknown if and what meaning these meibum deposits would have. With increasing handling experience and more consistent membrane application pressure, meibum deposits became less prevalent and the xylene rinse was abandoned.

After all of the trouble-shooting outlined above, a final cytochemical staining protocol was decided upon. IC membranes were gradually hydrated by slowly adding water to the tube used for fixation, and transferred to an all-water tube for several seconds. Water was added to the tube and after a few seconds removed. Membranes were transferred to AB for 3 min, followed by 3 consecutive water rinses. Membranes were transferred to Hematoxylin # 1 stain for 3 min, followed by 3 water rinses. Membranes were dehydrated and transferred to the Papanicolaou OG-6 stain for 3 min, followed by one 95% ethanol rinse. Membranes were transferred to the Papanicolaou EA-65 stain and left for 3 min, followed by three 95% ethanol rinses, three 100% ethanol rinses and stored in a sealed tube with 100% ethanol until imaging.

This protocol provided good representation of cellular structures and a finer distinction between different keratinization grades than previously observed [9,24].

3.5.4.2 Immunocytochemical staining

While a single study reports the use of immunocytochemical dyes on impressions from the lid margin [24], to date, this is the first time that the LIVE/DEAD Kit has been employed for lid margin samples. Since the optimal stain concentration depends on the cell type, concentrations had to be adjusted. Immediately upon collection, samples were hydrated with a drop of Annexin buffer to preserve the state of the collected cells and to prepare membranes for immunocytochemical staining. Samples were then halved and half of the cells killed with BAK, the other half undergoing different staining concentrations, ranging from 0.1 to 10 µM EthD-1 and Calcein AM respectively. Concentrations were increased until sufficient green and red fluorescence was generated by calcein and ethidium respectively, as observed by fluorescence microscopy. For the final immunocytochemical staining protocol, 5 µl of EthD-1 and calcein AM each were mixed in 2.5 ml of Annexin buffer solution. IC samples were stained using a drop of this composition and imaged as promptly as possible. The green and red fluorescence of the stains indicated cell viability and compromise, respectively (Figure 19).
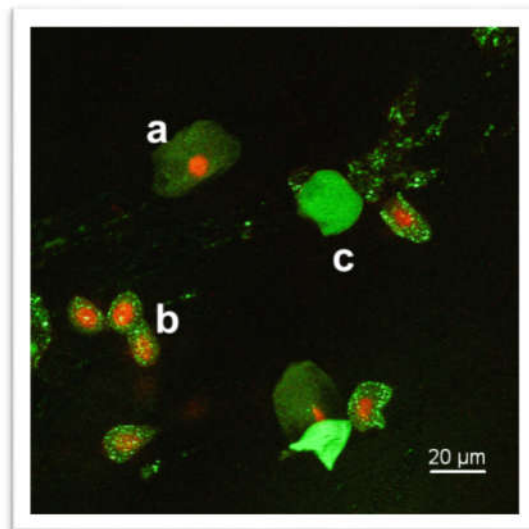
Figure 19: Confocal laser scanning microscopy showing fluorescent cells from the lid margin.

Varying morphology is visible between squamous (a) and columnar/cuboidal cells (b). Green

fluorescence of Calcein AM indicates esterase activity in the cell body (i.e., cell viability). Red

fluorescence of Ethidium reveals nucleic acids, indicating cell membrane compromise. Few cells show intense green and no red fluorescence, possibly indicative of cell membrane integrity (c).

3.5.5 Imaging

3.5.5.1 Bright-field microscopy

Histological imaging typically involves the acquisition of a good overview of the entire sample, followed by high magnification images of features or regions of interest (FOI, ROI). The desire to better evaluate the occurrence of finer details, only visible at high magnification, across the entire collection area, lead to the development of a novel imaging technique. The iterative optimization of the imaging process is described below.

Initially, samples were inspected for collection and staining quality using adequate and consistent illumination and magnification settings. A 2.5x magnification objective was used for the overview of the entire collection area, 10x and 20x objectives ensured a good balance between captured area and cellular detail, whereas the 40x and 63x objectives were used for very high magnifications, such as visualizing nucleic detail (Figure 20). The microscope illumination was adjusted using an analog potentiometer for every magnification level.
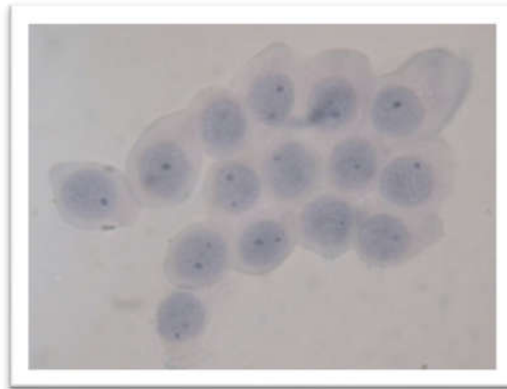
Figure 20: Lid wiper conjunctiva imaged using 63x magnification objective.

Small dots inside of nuclei indicate nucleolae.

Following satisfactory image and collection quality as judged by visual inspection, the computerized digital imaging system was activated and software parameters such as exposure, white balance, contrast

or gamma were determined for any given microscope illumination and magnification. Any automated metering was disabled, settings were saved as templates for future use and images were captured.

The novelty of this protocol consisted in the stitching of images captured using the 10x or 20x microscope objective into one single, large image file, which allowed the overview of the entire collection, as well as the ability to zoom in to high level detail (Figure 21). For the stitching software to automate the process, adjacent images were required to overlap horizontally as well as vertically to a certain extent. If the common area between two neighboring images was too small, the stitching software would not recognize the common features. On the other hand, capturing large overlapping areas between images was time consuming and would generate large numbers of files (>200 images per sample), that would considerably slow down the stitching process. A 20-30% area overlap was found to be an ideal balance for a reliable stitching process. Imaging the entire collection area was accomplished by "scanning" the membrane, acquiring images starting at the top right corner of the collection, progressing towards the bottom left corner of the collection, precisely advancing the microscope stage and constantly adjusting the focus. Accurately determining these start and end points required the precise orientation of the membrane on the glass slide, which in turn was critical for an accurate stitching process. The alignment with either X or Y axis of the microscope stage was ensured by adjusting the orientation of the membrane in a sensitive process, in which the cover slip was lifted, the membrane carefully realigned using a pipette tip, rehydrated with 100% ethanol and coverslipped again with great care, to avoid its movement. The thin layer of pure ethanol was volatile, requiring frequent rehydration of the sample. This was also necessary during the lengthy imaging process, which often lasted over an hour per sample.
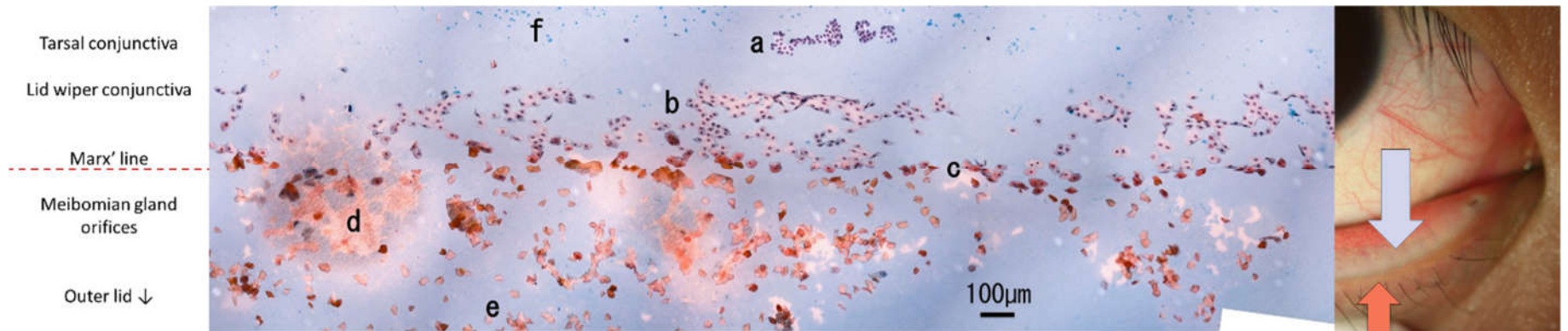
Figure 21: Panoramic image composed of >100 individual pictures of lid margin IC.

Features: (a) small columnar/cuboidal epithelial cells of the tarsal/marginal conjunctiva. Cells here exhibit blue/green/purple color indicating no keratinization; (b) cells of the lid wiper conjunctiva, transitional in morphology and stain color between regions (a) and (c); (c) large squamous cells of the muco-cutaneous junction/Marx' line. Red/orange/pink stain color indicates keratinization; (d) Meibum impression; (e) anuclear, cornified cells of the epidermis; (f) Goblet cell impression or tear film mucins.

Acquired images were stored in their original, uncompressed, lossless *.zvi format, but required conversion to more common file types for viewing or processing using external software. A series of formats were tested for this purpose. While the uncompressed *.tiff format allowed maximum detail retention, the file size of this format (usually ~30 MB/file) was unpractical for post-processing hundreds of files. The more common *.jpeg file format is small but impractical due to its heavy compression and resulting loss of detail. A custom *.jpeg file format was developed by identifying an ideal compression ratio of 5%, which balanced good detail retention and a manageable file size of 2-3 MB/image. Once exported, images were batch processed using ImageJ or IrfanView to improve color balance, contrast or exposure. This step was crucial towards improving and streamlining the subsequent stitching and analysis processes. Finally, images were stitched together into a single file, initially using a time-consuming manual procedure, followed by the programing of an automated script for Adobe Photoshop Elements (Appendix A). The resulting files were visually inspected and compared with 2.5x magnification images to ensure the accuracy and reliability of the process.

3.5.5.2 Confocal microscopy

One previous report used a confocal laser scanning microscope (CLSM) in conjunction with a keratinization-related protein [24]. In the present study, the immunocytochemical LIVE/DEAD stains were imaged using CLSM, for a more precise analysis of cellular viability and morphology. This highly sensitive laser-based imaging system, enables high-resolution three-dimensional visualization of semi-transparent biological samples by using the optical confocality principle, along with Z-stacking. The precision of the CLSM relies on the fine adjustment of a variety of imaging settings.

After setting up the microscope per the absorption and emission spectra of ethidium homodimer-1 (528/617 nm Ex/Em maxima in the presence of DNA) and calcein AM (494/517 nm Ex/Em maxima), parameters such as pinhole size, detector gain, amplifier offset and gain, laser transmission, frame size, scan speed and pixel depth have to be precisely determined for every sample. This is unproblematic for the more commonly visualized *in vitro* cultured cells, which are distinctly visible against the low background noise of the support medium or glass slide, and allow for determining and maintaining these settings for one or a series of samples. *Ex vivo* cell samples, particularly lid margin impressions, were often covered or surrounded by other cellular structures and secretions, creating artifacts which impeded the visibility of cellular features, and required the continuous optimization of imaging parameters for every sample and even different region of interest on the same sample, resulting in a significantly more time-consuming imaging process. While the powerful CLSM enabled sub-cellular

resolution imaging, superior to that of bright-filed microscopy or previous CLSM reports, scanning around relatively large samples such as IC samples which span over 10 mm, was time-consuming and unpractical. For this reason, to localize the collection area and stained regions of interest, the sample surface had to be inspected using an auxiliary Hg light source. Once the location of ROI was determined, the digital imaging system of the CLSM was activated and the three-dimensional location of ROI and the maximal staining intensity were determined more precisely using the live-time scanning preview of the ZEN software. Given the uneven surface of IC samples and the shallow depth-of-field of the confocal system, focusing on samples required great precision. Repeatedly switching between the CLSM and the Hg lamp was necessary, along with exchanging the water-immersion based microscope objectives, which caused further delays. Finally, images of single ROI were acquired, as, due to the system's high resolution and time constraints, obtaining enough images for panoramic stitching was not practical. Files were saved in the native, uncompressed *.czi file format and exported to a universally accessible, uncompressed *.tiff file format for analysis.

3.5.6 Limitations

While this protocol brings improvements to the reported IC techniques, the collection of cells from the lid margin is not without limitations. A major concern lies in the variability and repeatability of collections, as originally observed by Jalbert [24], who reported a 67% success rate in obtaining confluent patches of cells from the lid margin. The mechanics of the application (as discussed in section 1.5.3) have a major impact on the number and type of collected cells, which can only be addressed by developing investigator experience through many impressions. At this stage, it is uncertain how collection quality correlates with ocular health, since superior cell layers are forcefully removed from the conjunctiva and this may alter interpretations of viability and keratinization. While the immunocytochemical stains are intended to reflect cell viability (and esterase activity is observed in all collections), it is unclear what the red fluorescence of cell nuclei in our collections indicate, and whether this is tied in, e.g. with the 15-minute transportation delay between staining and imaging. Clinical studies with larger sample sizes are necessary to fully validate this technique and confirm these observations and theories.

Finally, developing and conducting the techniques presented here can be extremely time consuming. Optimizing the technique is a long, multi-stage, iterative process, subject to many variables which can influence collection, staining and imaging quality. While the impression itself is rapid, preparing,

staining and imaging a single sample can take up to 6 hours. The particularly tedious imaging process is severely limiting, as only a single sample can be imaged at a time.

3.6 Conclusion

A novel protocol for conducting IC on the lid margin was developed, consisting of a dedicated lid eversion technique, the selection of an adequate IC membrane and its correct application to the lid margin, two cytochemical staining protocols that allow the detailed representation of cellular features at the lid margin and the characterization of their morphology, keratinization and viability, as well as specialized microscopy and imaging protocols that enable the acquisition of detailed images of IC samples.

The PTFE cell culture inserts are convenient because they are sterilized, ideally sized and can be handled manually, without further equipment, ensuring rapid collections. An optimized cytochemical protocol was developed by replacing the commonly used but chromatically very intense periodic acid-Schiff stain with Alcian blue, which in conjunction with the subsequent Papanicolaou dyes OG-6 and EA-65, displayed a finer chromatic variation, enabling a more detailed perspective on the cellular keratinization level than that reported previously [9,24]. Overall, morphological features of collected cells coincide with previous literature reports [9,13,24,33]. The immunocytochemical staining protocol revealed concomitant cell viability and cell membrane compromise in most cells. A novel and superior microscopic imaging technique was developed, by which high magnification images of the entire collection area were acquired and stitched into a single, large, panoramic file. While slightly more time consuming, this technique is advantageous, as it provides an overview of the full membrane, as well as the ability to zoom in to nuclear detail, all in a single image, allowing for the computation of quantitative metrics such as cell count and nuclear-cytoplasmic (NC) ratio [163]. Confocal microscopy enabled a very detailed representation of cell morphology and viability.

Both the optimization of the procedures presented here, as well as their clinical application are time consuming and highly dependent on developing investigator experience and dexterity. Yet, obtaining data of the cellular structure of the lid wiper region in a larger sample of contact lens and non-lens wearers may help verify the correlation between the friction that occurs between these cells and the ocular surface or the lens, and subjective comfort. This may provide valuable knowledge and permit future clinical trials to explore the cellular particularities of contact lens wearers, subjects with lid wiper

epitheliopathy, dry eye or dryness symptoms, in contrast to asymptomatic subjects, to hopefully shed light on the topic of ocular discomfort.

Chapter 4

Impression cytology of the lid wiper region

4.1 Overview

PURPOSE: A pilot study to assess the epithelial cell morphology of the upper lid margin in symptomatic and asymptomatic soft contact lens (SCL) wearers and non-lens wearers (nCL) with low and high levels of lid wiper epitheliopathy (LWE).

METHODS: Fifteen participants were enrolled in three study groups: 5 asymptomatic nCL with low LWE; 5 adapted, asymptomatic SCL wearers with low LWE; 5 adapted, SCL wearers with high LWE. Participants completed subjective comfort ratings and LWE was assessed using the Korb Protocol B. Impression cytology (IC) of the upper lid margin was performed using Millicell Cell Culture Inserts and cellular features and sample cellularity evaluated after histochemical and immuno-cytochemical staining.

RESULTS: Three distinct cellular morphologies were identified, spanning between the tarsal/marginal conjunctiva, through the lid wiper (LW) conjunctiva, to the muco-cutaneous junction (MCJ) at the Marx line; their features coincide with recent literature reports. Epithelial cell morphology did not vary with LWE grade or lens wear; the relationship with subjective symptoms could not be studied due to the nature of the study design. Sample cellularity may or may not be altered by lens wear, LWE and/or symptoms. No association was found between LWE and ocular discomfort.

CONCLUSION: The employed IC, staining and imaging techniques are adequate for characterizing epithelial cells at the lid margin. A larger sample size and an improved study design should be employed to further explore the trends observed in this pilot study.

4.2 Introduction

In the healthy eye, the tear-film acts as a lubricant between the lid and the ocular surface or a CL, reducing friction and preventing ocular damage [22,164]. It is postulated that an altered tear film [165], decreased mucins [65] and CL surface alterations [6,49] may contribute to an increase in friction and shear forces between the lids and the cornea, bulbar conjunctiva or a CL during blinking. While the surfaces of CL materials may exhibit different coefficients of friction *in vitro* [111], evidence is emerging to suggest that this friction may play a leading role in ocular comfort during CL wear [71,72,108–111].

In their seminal paper on dry eye symptoms and CL wear, Korb et al proposed that properties of CL may be associated with clinically observable phenomena, notably LWE. This condition is observed as vital staining of the upper and lower lid margins, which may be an early sign of tissue disturbance, possibly linked to symptoms of dryness and even predicting an underlying or emerging ocular surface disease [6]. While the cause of lid margin staining is not known, LWE has been shown to occur in CL wearers [6,61,64,65] as well as non-wearers [61–63,67,68]. While some reported increased LWE levels in lens wearers compared to non-wearers [62,67], others failed to observe this relationship [61,63]. Similarly, the number of publications noting an association between higher LWE levels and ocular discomfort [6,61,63–65] is approximately equal to the ones that do not find such a link [48,62,67,68]. In the fifteen years since the initial proposal for LWE, over 45 papers and conference abstracts have been published on LWE [48]. With ongoing interest in this topic, our understanding of the topic is considered far from complete and its clinical relevance is under much debate.

As concluded in a recent review article [48], "the question as to the histology of the lid wiper needs to be resolved". Efron et al propose that more fundamental research studies (such as the work of Jalbert et al.[24]) could prove more compelling than the conduct of clinical trials towards the understanding of LWE.

So far, only a handful of publications have explored the surface cellular morphology of the lid margin. Arguably, the most comprehensive account was published by Knop et al, wherein cytological samples were excised from cold-stored cadavers and meticulously characterized [13]. But, with an average age of 77 years, the samples from their donors may not be representative of today's typical CL wearing population, who have an average age of 31 years [166].

Using IC, a technique described elsewhere [20] and optimized in the previous chapter of this thesis, Doughty precisely dimensioned and described epithelial cells and their transitional nature at the lid margin. However, they only examined cells from the lower lid margin and their 10 subjects were non-CL wearers [9].

Jalbert et al. recruited 40 CL wearers and non-wearers, assessed LWE using LG and performed IC on their upper lid margin [24]. They found no association between lens wear and LWE staining or Nelson grade of the collected cell samples. LG staining was reported in 17% of all subjects, yet the authors did not provide information on the difference between LWE and non-LWE subjects. Additionally, some of their descriptions are ambiguous and image labeling sometimes discrepant.

While the uncertainty in describing these transitional zones at the lid margin is recognized [48] and, in absence of a standardized nomenclature in this emerging field, understandably, this surely warrants further exploration. To our knowledge, the lid margin cytology of symptomatic and asymptomatic lens and non-lens wearers, with varying degrees of LWE, remains unexplored.

Using the optimized method for collecting epithelial cells from the lid margin developed in the previous chapter of this thesis, the present pilot study sought to investigate the cytology of the lid margin in symptomatic and asymptomatic CL wearers and non-wearers with low and high grades of LWE.

4.3 Materials and methods

This study was a prospective, randomized, contralateral eye (participants were wearing their habitual CL only in one eye), 3-day, non-dispensing pilot study, involving one screening and two study visits. One unmasked observer was responsible for collecting all data.

4.3.1 Subject recruitment

All clinical studies have been designed to follow the ethical principles in the Declaration of Helsinki, with the International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidelines for Good Clinical Practice (GCP), with the University of Waterloo's Guidelines for Research with Human Participants and with the Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans, 2nd Edition. Informed consent was obtained from all participants prior to enrolment. The study received approval from the Office of Research Ethics at the University of Waterloo, Waterloo, Ontario, Canada (ORE #19739). The study was advertised using the recruitment system at the Centre for Contact Lens Research at the University of Waterloo.

4.3.2 Inclusion and exclusion criteria

Participants were eligible for inclusion in the study if they: were at least 17 years of age and had full legal capacity to volunteer; had read, understood and signed an information consent letter; were willing and able to follow instructions and maintain the appointment schedule; were adapted, daily CL wearers with a minimum of 2 years of lens wearing experience, wear their lenses for at least 3 days a week, if in a CL wear group, and brought a pair of prescription spectacles to the study visit, if they were a CL wearer with a prescription greater than ± 1.50 diopters.

Participants were excluded from the study if they: were participating in any concurrent clinical or research study; had any known active[1] ocular disease and/or infection and/or allergy; were an extended lens wearer; had a systemic condition that in the opinion of the investigator may affect a study outcome variable; were using any systemic or topical medications that in the opinion of the investigator may affect a study outcome variable; this included rewetting drops (participants must refrain from using rewetting drops on the day of screening and study visit 1); had any known sensitivity to the diagnostic pharmaceuticals (Sodium Fluorescein, Lissamine Green) used in the study; were pregnant, lactating or planning a pregnancy at the time of enrolment; were aphakic, or had undergone refractive error surgery.

4.3.3 Study procedures

To determine eligibility and study group assignment, several subjective and objective clinical tests were performed at the screening visit as well as throughout the study (see below). Data were manually recorded onto case report forms (CRF).

4.3.3.1 Subjective comfort questionnaires

The subjective comfort was assessed using three different questionnaires. CL wearing participants rated their CL-related dryness based on the model by Young et al. [150]. Frequency was rated between "Never", "Rarely", "Sometimes", "Frequently" and "Constantly", and intensity assigned a value between zero ("Never have it") and five ("Very intense dryness sensation"). Based on their responses, subjects were considered symptomatic or asymptomatic (Table 2).

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[1] For the purposes of this study, active ocular disease was defined as an infection or inflammation, which requires therapeutic treatment. Mild (i.e. not considered clinically relevant) lid abnormalities (blepharitis, meibomian gland dysfunction, papillae), corneal and conjunctival staining were not considered active ocular disease. Neovascularization and corneal scars are the result of previous hypoxia, infection or inflammation and are therefore not active.

Table 2: Classification of asymptomatic and symptomatic CL wearers by dryness

Intensity of contact lens dryness		Frequency of contact lens dryness				
		Never	Rarely	Sometimes	Frequently	Constantly
Never have it	0					
Not intense at all	1		Asymptomatic			
	2				Symptomatic	
	3					
	4					
Very Intense	5					

Non-lens wearing participants evaluated their ocular dryness using the Ocular Surface Disease Index (OSDI) questionnaire [99]. A series of questions relating to different symptoms (e.g. gritty eyes, blurred vision etc.) in different environments (e.g. reading, driving etc.) were scored from zero ("None of the time") to four ("All of the time") and summed up to a total score, representing the "OSDI score".

All participants rated their overall comfort and overall dryness on a scale from zero ("poor comfort/intolerable") to 100 ("excellent comfort/cannot be felt").

4.3.3.2 Clinical techniques

Several typical optometric tests were performed on all participants. Participant demographic data and the medical/ocular history was recorded. The visual acuity was determined using a logMAR chart. Next, for CL wearers, the pre-lens non-invasive tear break-up time (PLNITBUT) and for non-lens wearers the pre-corneal non-invasive tear break-up time (NITBUT) were measured using a corneal topographer (Oculus K4, Oculus GmbH, Wetzlar, Germany). Using a slit-lamp biomicroscope, the anterior ocular surface and the fit of CLs were assessed. Corneal and conjunctival staining were assessed after applying a small amount of sodium fluorescein, by application of a sterile fluorescein strip (Fluorets, Laboratoire Chauvin, France) to the lower conjunctival lid margin.

4.3.3.2.1 Lid wiper epitheliopathy evaluation

Upper lid margin staining was evaluated at the screening visit, after two instillations of sodium fluorescein (FL) dye (two superimposed 1.0 mg strips, Fluorets® from Laboratoire Chauvin, France) and, after the completion of the bio-microscopy exam, an identically repeated application of lissamine green (LG) dye (two superimposed 1.5 mg strips from HUB Pharmaceuticals, CA). Staining of the upper lid margin region was graded according to the scheme presented in **Table 3** below [50]. Digital images of LWE were obtained using a slit-lamp mounted digital camera.

Table 3: LWE grading and classification scheme by Korb et al.

Grading:			
Horizontal length of staining	Grade	Sagittal width of staining	Grade
<2 mm	0	<25% of the width of wiper	0
2-4 mm	1	25% - <50% width of wiper	1
5-9 mm	2	50% - <75% width of wiper	2
>10 mm	3	≥ 75% of the width of wiper	3

Classification:

	Fluorescein Grade (Fl)	Lissamine Green Grade (LG)
Horizontal	_____	_____
Sagittal	_____	_____
Average	_____	_____
Final [(Fl + LG)/2]	_____	
Classification	_____	

Subjects will be classified according to their final LWE grade for each eye.

Final LWE Grade	
0	None
0.25 – 1.00	Mild
1.25 – 2.00	Moderate
2.25 – 3.00	Severe

4.3.3.3 Impression cytology

IC samples from the upper lid wiper regions were collected using the Millicell Cell Culture Inserts

(Merck Millipore, Darmstadt, Germany) as detailed in the previous chapter of this thesis. Artificial tears (Bion Tears, Alcon, Fort Worth, TX) were dispensed to alleviate any potential discomfort following the IC procedure. The membrane underwent immediate processing, while an additional investigator completed the clinical investigation.

4.3.3.4 Sample processing

Following the collection, the membrane was separated from its holder and cut in half using micro-scissors, dividing the collection in two equal parts. One half was submerged in 95% Ethanol for fixation prior to treatment with four histologic dyes; the other half was placed in a glass bottom dish for cytochemical staining assessment. Alcian Blue (AB), Hematoxylin Gill-1, Papanicoloau OG-6 and EA-65 dyes were used for histological staining.

For immunocytochemical staining, the apoptotic indicator Annexin V Alexa Fluor 647 was added to the standard staining protocol with the LIVE/DEAD Kit (Calcein AM (4μM), Ethidium (4μM) and Annexin V (5μl in 250μl buffer)). The detailed staining protocols are described in chapter 3 of this thesis.

4.3.4 Group assignment

After signing the informed consent form and being considered eligible for inclusion in the study, participants were divided up in three study groups, according to the following criteria (Table 4).

Group A: five asymptomatic non-lens wearers with no LWE

Participants who had never worn CL, had an OSDI score of 12 or lower and an average LWE grade of 1.0 or lower in both eyes were considered for inclusion in this group.

Group B: five adapted, asymptomatic soft lens wearers with no LWE

Adapted, habitual CL wearers, considered "asymptomatic" based on the Young et al. classification scheme (Table 2), reporting good end-of-day comfort (average wear time minus comfortable wear time = 2 hours or less) and an average LWE grade of 1.0 or lower in both eyes were considered for inclusion in this group.

Group C: five adapted soft lens wearers with high LWE

Adapted, habitual CL wearers, considered "symptomatic" based on the Young et al. classification scheme (Table 2), reporting low end-of-day comfort (average wear time minus comfortable wear time

= 3 hours or more) and an average LWE grade of 2.0 or higher in both eyes were considered for inclusion in this group.

NOTE: Due to difficulties encountered in recruiting participants with high LWE grades, the eligibility criteria for group C was eventually expanded to include asymptomatic participants as well.

Participants who did not meet the criteria for one of the groups described above were discontinued from the study.

Table 4: Study group criteria

Study group	A	B	C
Lens wear	Never worn	Habitual SCL wearer	Habitual SCL wearer
Classification by Young	NA	Asymptomatic	Symptomatic (later also included asymptomatic subjects)
OSDI Score	<12	NA	NA
End of day comfort, self-reported (average wear time – comfortable wear time)	NA	<2 hours	>3 hours
LWE grade average	0 to 1.0	0 to 1.0	2.0 to 3.0

4.3.5 Study visits

This study consisted of three study visits occurring on separate days. Eligibility and grouping were determined at the screening visit (visit code 0-0). On the next day, after wearing a CL in one eye only, participants came in for the collection visit (visit code 1-0), where IC was performed on the upper lid margins. Samples underwent histochemical and immunocytochemical processing and analysis as described in Chapter 3. Ocular health was checked and the participants completed the study at the study exit visit (visit code 2-0). The study visits are detailed below. Table 5 shows an overview of the procedures conducted at each study visit.

4.3.5.1 Screening visit (0-0)

Screening appointments were scheduled in the afternoon to ensure that CL wearers had worn their habitual lenses for at least eight hours before the scheduled appointment. Written informed consent was obtained and a study ID number assigned prior to any study procedures or evaluations. Eligibility and grouping were determined using a series of self-administered questionnaires and the inspection of the ocular surface using a slit lamp biomicroscope. CL wearers were instructed to only wear one of their habitual lenses in a randomly determined eye for the collection visit on the following day.

4.3.5.2 Collection visit (1-0)

Twenty-four hours after the screening visit, participants came in wearing only one CL in one eye for the collection visit. Subjective and objective measures were conducted and after removal of the CL, IC was performed on the upper lid margin of both eyes. Artificial tears (Bion Tears, Alcon, TX) were administered to alleviate any potential discomfort following the IC procedure. The visual acuity was checked and a slit-lamp biomicroscopy performed to ensure the integrity of the lid margins and ocular surface. Participants were instructed to wear their spectacles for the rest of the day and refrain from wearing CLs.

4.3.5.3 Study exit visit (2-0)

The Study exit visit took place after a minimum of one and a maximum of seven days after the collection visit. Participants came in wearing their spectacles and a series of subjective and objective measures were conducted to ensure ocular integrity.

Table 5: Overview of procedures conducted at each study visit

Procedure, measurement	Visit code		
	0-0	1-0	2-0
Information consent	✓		
History	✓		
Medical history	✓		
Visual acuity	✓	✓	✓
Pre-lens and pre-corneal non-invasive tear break-up time (PLNITBUT, NITBUT)	✓	✓	
Biomicroscopy	✓	✓	✓
Lid Wiper assessment	✓		✓
Digital images of LWE	✓		
Subjective comfort ratings (0-100)	✓	✓	✓
Lens fit	✓	✓	
IC of lid wiper region		✓	

4.3.6 Data analysis

Analysis of the histological and immunocytochemical samples involved qualitative and quantitative descriptions, by visual inspection and grading by a single investigator. Measures included stain color (keratinization), intensity and other observed patterns, as well as sample cellularity. No inferential statistics were performed on this data set.

4.4 Results

4.4.1 Demographics

Twenty-three participants were screened and enrolled into the study. Of these, 15 met the eligibility criteria and successfully completed the study. There were 10 females and 5 males with mean age of 28

years (median 27 years, ranging from 21 to 50 years). Eight participants were ineligible at the screening visit and were discontinued.

Five participants wore daily disposable CLs and 5 were monthly replacement silicone hydrogel wearers. Two subjects in Group C were symptomatic according to the Young et al. classification scheme, the other three were asymptomatic.

4.4.2 Lid wiper epitheliopathy

At the screening visit, an average LWE grade of 0 was recorded in groups A and B, while group C averaged an LWE grade of 2.1. There was no difference in LWE grade between the lens wearing and the non-lens wearing eye, in either of the study groups. Representative images for low and high LWE degrees are shown below.
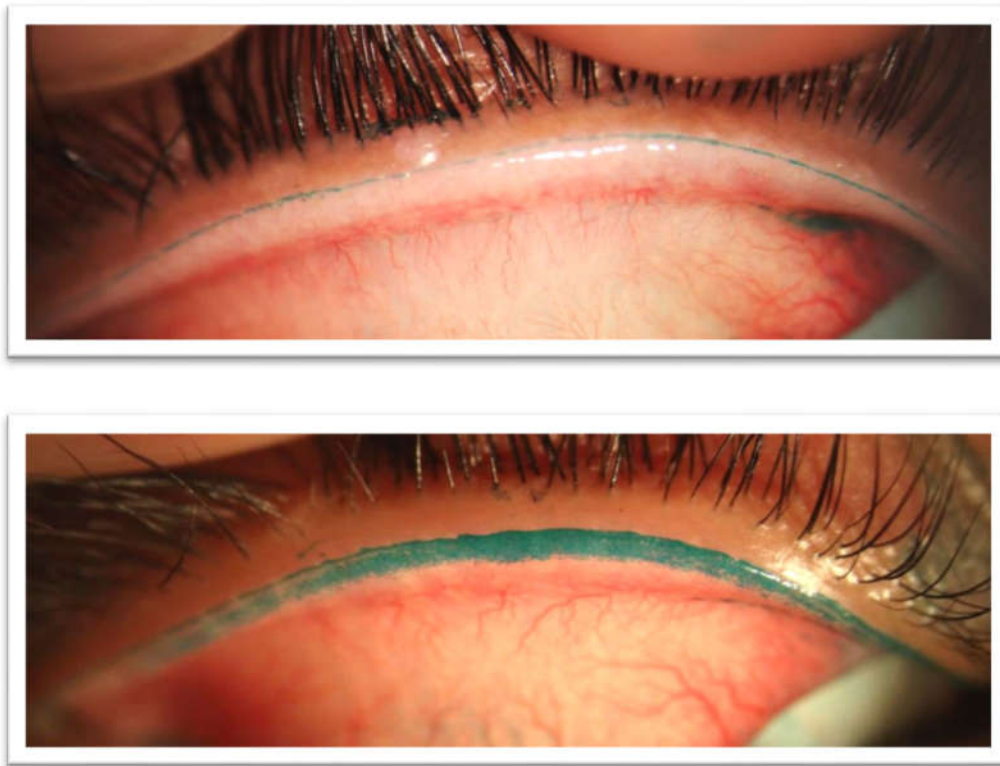
Figure 22: Examples of LWE grade 0.0 (above) and 3.0 (below) as shown by LG staining on the everted upper lid

Note that the green line staining the lid margin graded 0 for LWE represents the (anatomical) Marx line.

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4.4.3 Histological features

The AB stain detected goblet cells, haematoxylin stained cell nuclei and the Papanicolaou stains indicated more generalized changes such as keratinization. Microscopy of the samples indicated the presence of three distinct cellular morphologies progressing from the tarsal sulcus region through the lid wiper region to the muco-cutaneous junction (MCJ) at the Marx line. Despite variability in the transitional areas between these regions, some common features were observed. Marginal/tarsal conjunctival cells (for simplicity we call these "C-cells" here) have small columnar/cuboidal cell bodies, large nuclei and stain green with Papanicolaou indicating metabolic activity and absence of keratinization. Goblet cells were occasionally detected in this region.

Adjacent to this region was the "lid wiper conjunctival" band, spanning a width of approximately 6-9 epithelial cells ("L-cells"). Their large nuclei and the light blue/green stain contrast with the adjacent large, squamous cells of the MCJ and Marx' line ("M-cells"), which exhibit pyknotic nuclei and red, pink or orange staining, characteristic of reduced metabolic activity and para-keratinization. Cornified cells of the epidermis were also found in the MCJ region, surrounding the openings of Meibomian glands, often covered in meibum. The membrane representing the background stained various hues of blue, green and brown.

Cells in the marginal conjunctiva stain blue, indicating viability; through the lid wiper region, towards the Marx' line, the stain changes color to orange/pink. The L-cells present in the lid wiper conjunctival area take on different (light) hues of blue or red, depending on their keratinization state. An overview of these characteristics is provided in Figure 23, and a more detailed view of the L-cells in the lid wiper conjunctival area is presented in Figure 24.
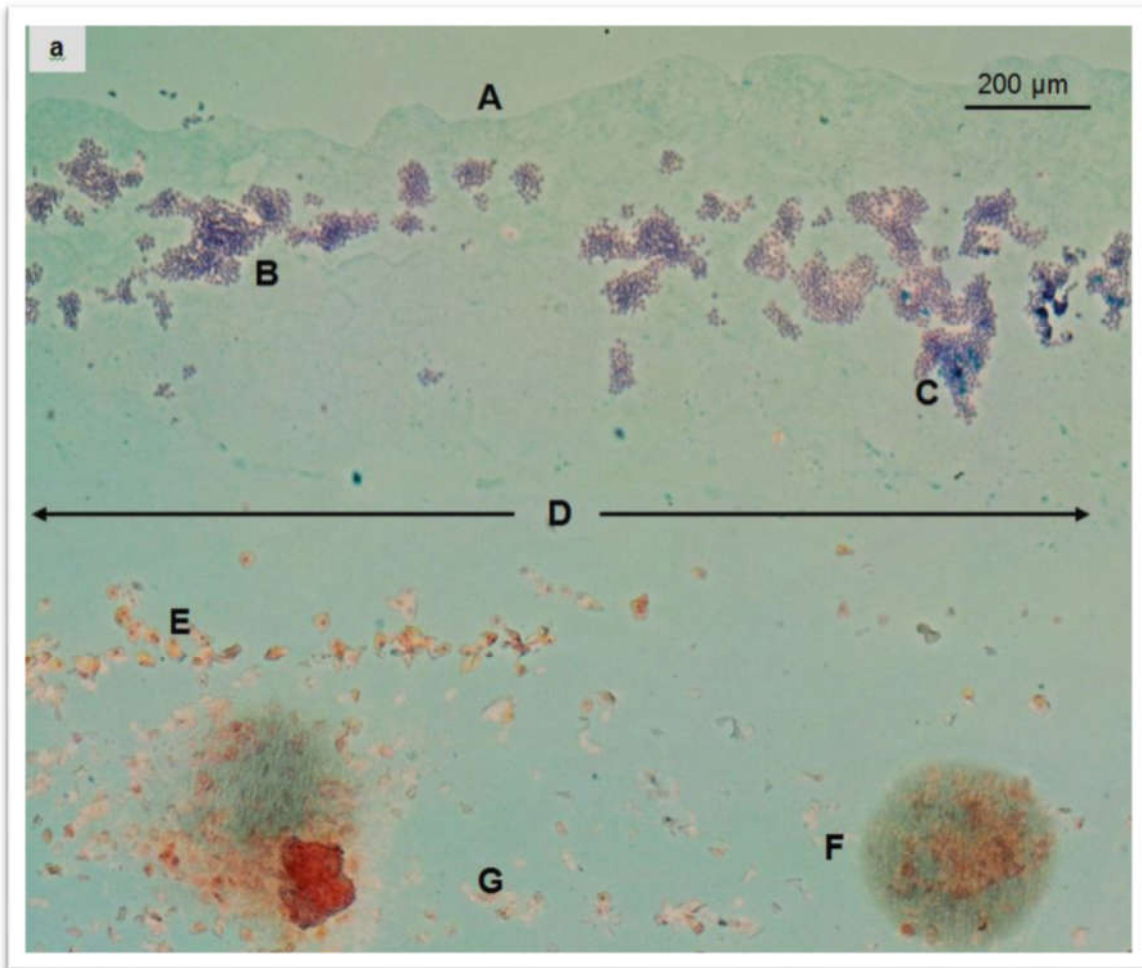
Figure 23: Typical IC collection after histological staining.

From the top of the image towards the bottom, the collection represents the transition from the tarsal conjunctiva to the Marx line and Meibomian glands. Boundary of contact area between membrane and lid indicated by tear film components, likely mucins, stained light blue by AB (A); columnar/cuboidal cells of the tarsal/marginal conjunctiva, stained mostly dark blue or violet by Hematoxylin and PAP (B); Goblet cells or their impressions, stained intense blue by AB (C), to be carefully distinguished from tear film mucins, which stain in a similar fashion; lid wiper conjunctiva between M and C-cells, with very few L-cells (D); squamous cells of the MCJ (M-cells) arranged in a linear, narrow band, reflective of the Marx line, stained pink, red or orange by Hematoxylin and PAP, with occasionally absent nuclei (E); impressions of Meibomian gland openings, mostly dark, covering other cells and structures underneath (F); anucleic, cornified cells of the epidermis (G).
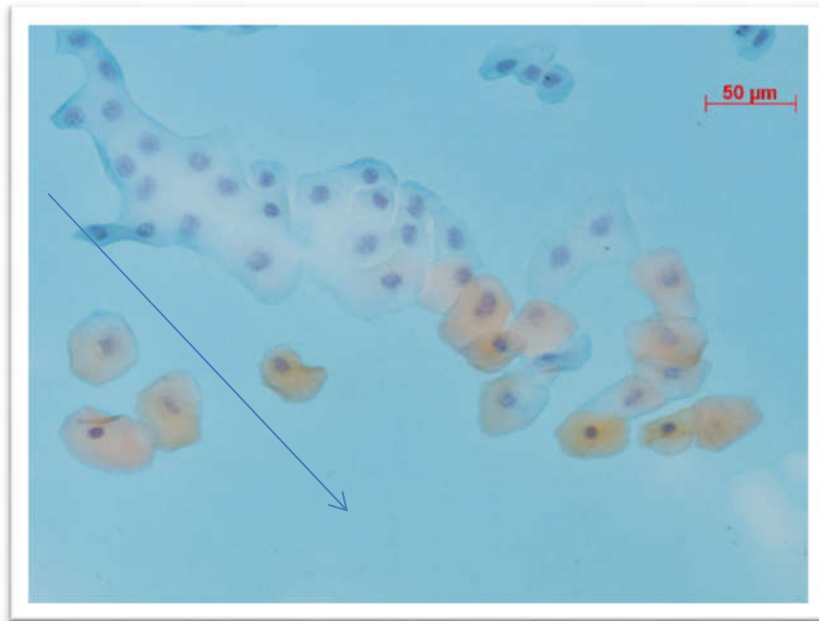
Figure 24: Detail of transition between cell types at the lid margin.

L-cells in the lid wiper region (light blue) and M-cells at the MCJ (orange). L-cells (present in the D-area of Figure 2) are usually the size of M-cells at the MCJ/Marx line and feature a light blue stain, similar to C-cells. The arrow points towards the MCJ

Overall, the changing cellular characteristics observed in most samples progressing from the tarsal conjunctiva through the lid wiper region, towards the muco-cutaneous junction at the Marx line, can be described as a decrease in cell patch size and cell number, an increase in nucleus size, a change from blue to red staining and less goblet cells towards the Marx line. These trends are depicted in Figure 25.
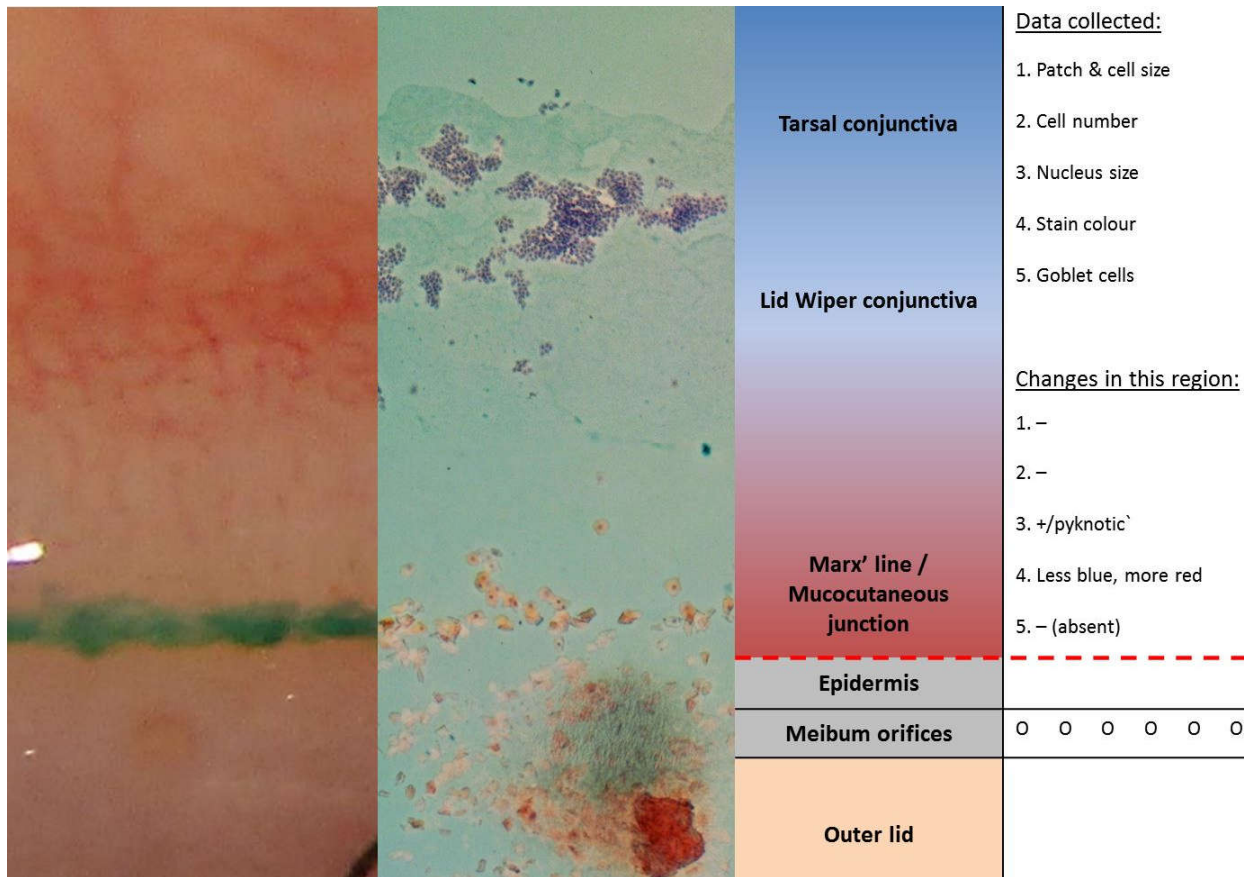
Figure 25: Schematic diagram of cellular changes across the lid wiper area.

From left to right, a magnified photograph of the lid margin with LG-stained Marx' line is shown, the corresponding IC collection (shown in Figure 5), the described regions and the overall trends in shown in the last pane.

A total average of 126 ± 199 (mean $\pm$ SD) cells were collected for every IC sample in this study (n=30). After removing the outliers (300, 500 and 1000 cells collected from one eye of one participant in group A and both eyes of a participant in group B respectively), the total average was 66 ± 46 cells per sample. All subsequently reported values exclude these outliers. Tables 6-9 depict the differences between groups, variables such as lens wear and symptoms and the total average cell counts for the study groups.

Table 6: Total average (C+L+M) cell count for every sample in each study group and standard deviations (SD)

Group	Mean	SD
A	45	13
B	64	32
C	89	67
Total	**66**	**46**
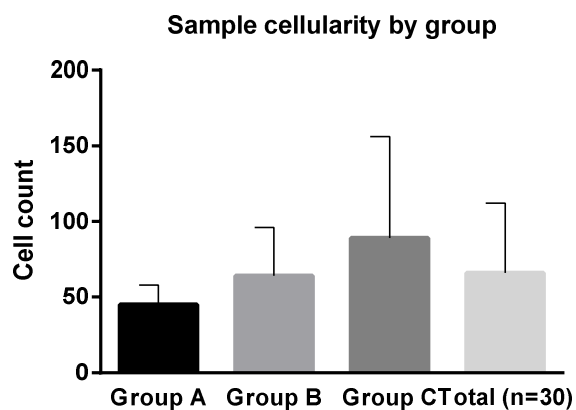
Figure 26: Total average (C+L+M) cell count between study groups.

Table 7: Average cell count for every sample and cell type in each study group (n=30).

	Cell Type					
	C		L		M	
	Mean	SD	Mean	SD	Mean	SD
Group A	24	12	6	7	18	11
Group B	12	14	19	11	36	26
Group C	35	60	22	19	33	27
Total	**25**	**38**	**15**	**14**	**29**	**23**
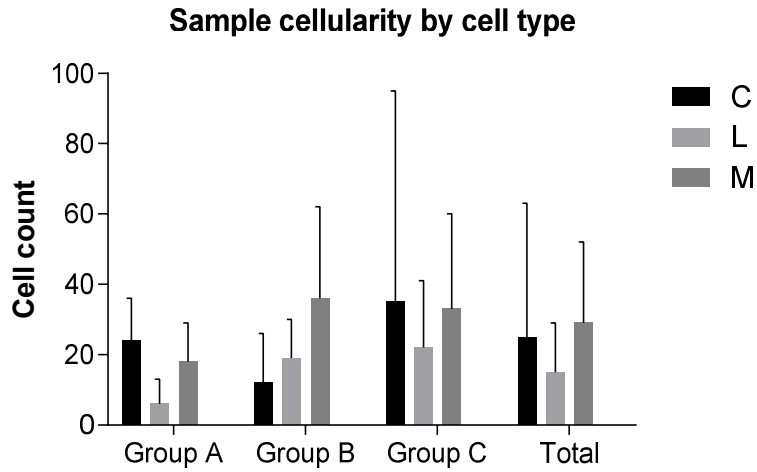
Figure 27: Average cell count differentiated by cell type in each study group.

Table 8: Average cell count for every sample and cell type differentiated by lens wear

	Cell Type					
	C		L		M	
nCL (n=10)	Mean	SD	Mean	SD	Mean	SD
Group B	15	17	19	8	46	31
Group C	45	87	22	13	41	36
Total	**30**	**64**	**21**	**10**	**44**	**32**
CL (n=10)	Mean	SD	Mean	SD	Mean	SD
Group B	9	10	18	14	25	17
Group C	25	20	21	25	24	14
Total	**17**	**18**	**20**	**19**	**25**	**14**

Sample cellularity by lens wear
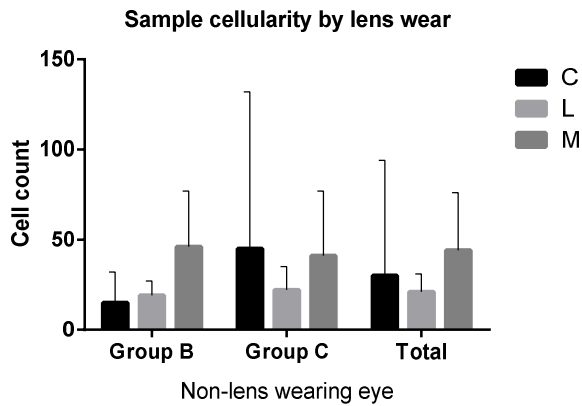
Figure 28: Average cell count in non-CL wearing eye between groups.

Sample cellularity by lens wear
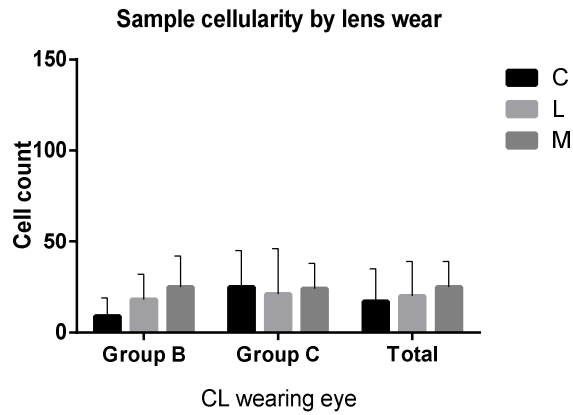
Figure 29: Average cell count in CL wearing eye between groups.

Table 9: Average sample cellularity for symptomatic and asymptomatic high LWE subjects in Group C.

	Cell Type					
	C		L		M	
Symptomatic (n=4)	Mean	SD	Mean	SD	Mean	SD
Group C	18	22	17	16	24	19
Asymptomatic (n=6)	Mean	SD	Mean	SD	Mean	SD
Group C	47	77	25	21	38	32
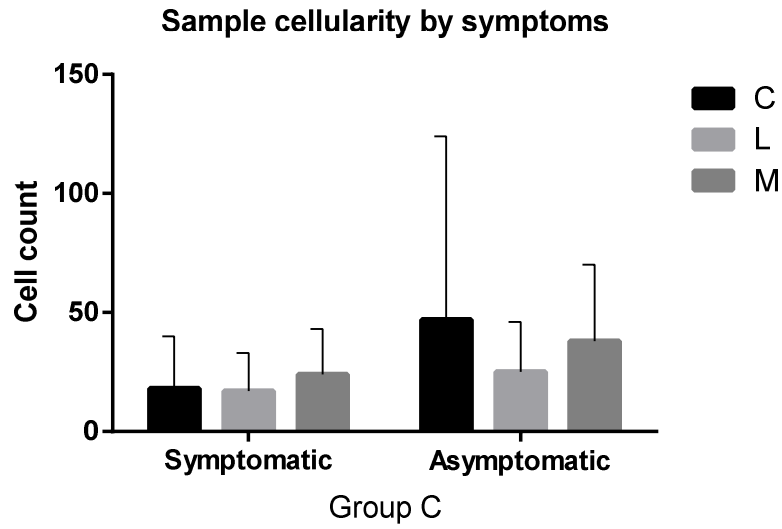
Figure 30: Average cell count differentiated in symptomatic and asymptomatic CL wearers with high LWE.

4.4.4 Immunocytochemistry and confocal microscopy

All collected and inspected cells showed red Ethidium fluorescence of the nuclei, indicating membrane penetration, and green fluorescence of Calcein, indicating esterase activity (Figure 31), suggesting that all cells were equally compromised and viable. There were differences between cells collected from low and high lid wiper epitheliopathic subjects, contact lens wearers and non-lens wearers, and symptomatic and asymptomatic participants.
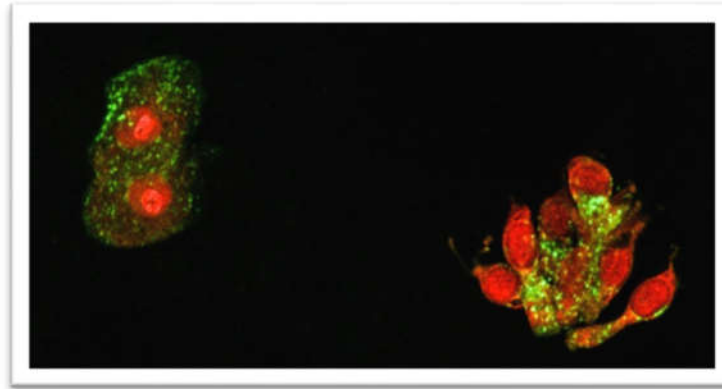
Figure 31: Two squamous cells (left) typical of the MCJ area, and columnar cells representative of the tarsal conjunctiva adjacent to the lid wiper area.

All cells have compromised cell membranes as shown by the penetration of Ethidium causing red fluorescence of the cell nucleus, yet show a degree of viability, as demonstrated by the green fluorescence of Calcein, indicating esterase activity.

Examples of images acquired with the CLSM are shown below and discussed in the following section.
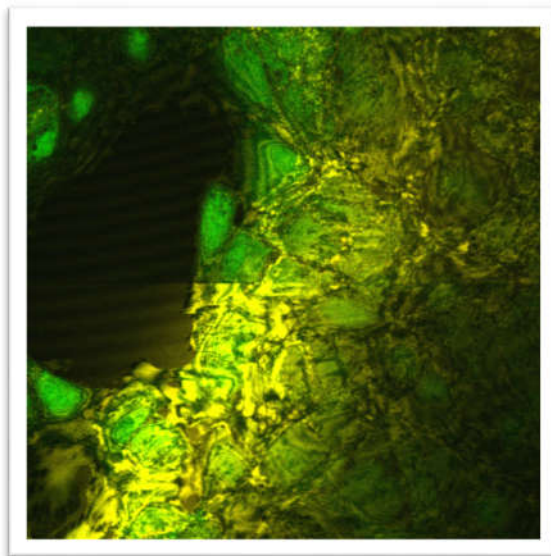
Figure 32: Interference with Annexin V dye (yellow).

This pattern was present in all samples.
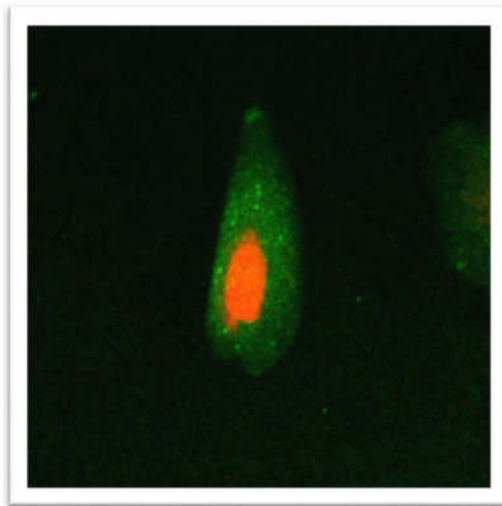
Figure 33: High resolution image of a cell in the tarsal conjunctiva.
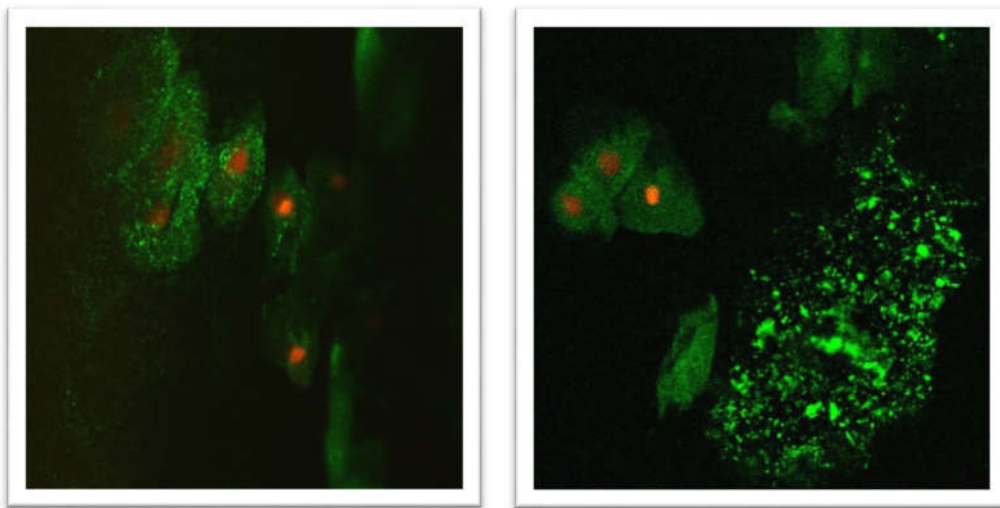
Figure 34: Images taken at different depths of field in the same region.

Cell visibility highly depends on imaging depth. The strongly fluorescing structure in the right image is unknown, and structures underneath are not visible.
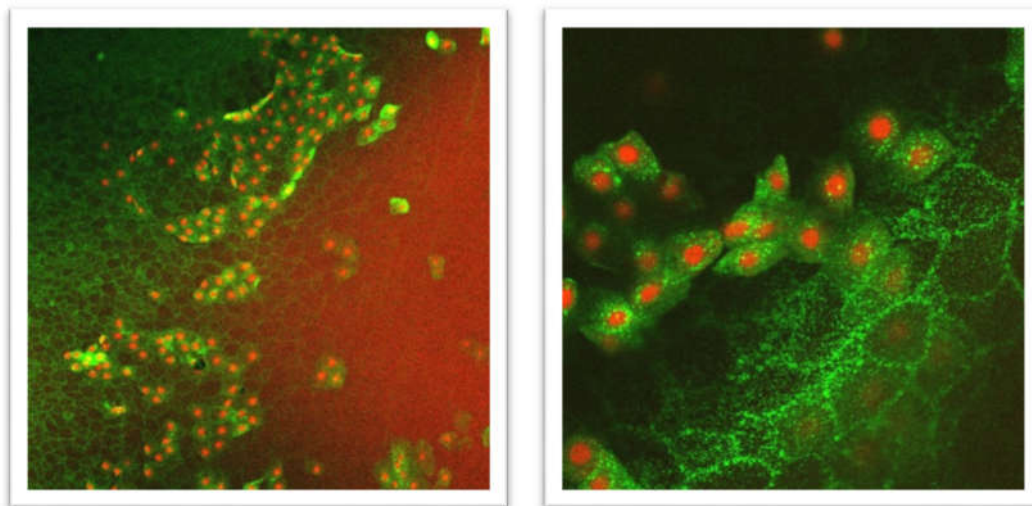
Figure 35: Web-like structure between cells and red interference from Ethidium stain (left) and close-up (right).
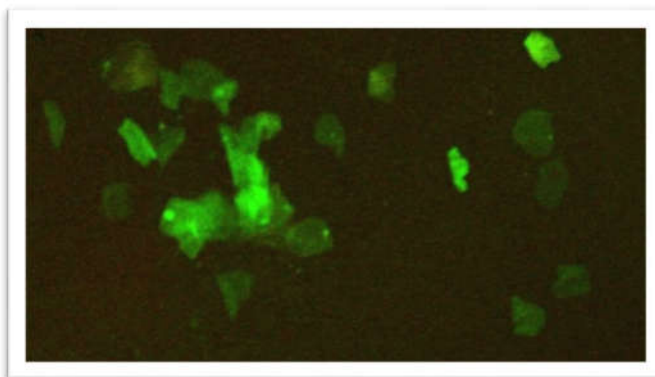
Figure 36: Unidentified, possibly intact cellular structures as shown by increased esterase activity (Calcein AM fluorescence) and absence of Ethidium fluorescence.

4.5 Discussion

After the IC collection and analysis technique was optimized in the previous chapter of this thesis, an increased sample size was required to confirm the initial observations. The objective of this pilot study was to investigate the cytological characteristics of the lid margin in symptomatic and asymptomatic contact lens wearers and non-wearers with low and high grades of LWE. For this

purpose, fifteen participants were recruited and enrolled into three study groups for which these criteria were amassed. Specifically, group C participants were required to be symptomatic and exhibit high LWE. This requirement was based on the alleged associations between LWE and discomfort [6,64,65], but also to better highlight potential cellular particularities by polarizing the small number of participants into distinct groups. As increasing numbers of participants could not be enrolled due to mismatched criteria (i.e. high LWE participants were not symptomatic and vice-versa), the progress of the study was significantly delayed. Of note, participants who in previous research studies had exhibited high degrees of LWE, showed minimal LWE at screening for this study, suggesting that LWE may not be consistent over time. Eventually, the study protocol was modified to accommodate symptomatic as well as asymptomatic subjects with high LWE in group C. Therefore, three out of five participants in group C exhibited high degrees of LWE and no or low symptoms of discomfort. While more LWE subjects stained positively with LG than with NaFL, no difference in LWE grade was recorded between the lens and the non-lens wearing eye with either dye, in either study group. No relationship between lens wear and LWE, or symptoms and LWE could be proven in this study, which is in agreement with other recent publications which also failed to demonstrate such a link [48,66,167].

Qualitatively, our observations coincide with previously published findings [9,13,24], in that there are "three main lid margin zones", showing a "transition of epithelial features across the lid margin", as pointed out by Jalbert et al [24]. Doughty notes that "cells along the Marx's line are moderate-sized squamous cells with nuclei smaller than in normal bulbar conjunctival cells or pyknotic (shrunken) or the cells may be anucleate" [9], which fully concurs with the present findings. The small number of publications on this topic suffers from a lack of standardized nomenclature, therefore the descriptions of these transitional areas and their location in relationship to each other are not consistent (Efron, 2016, p.10-11[48]). Where the literature does seem to agree, is in the reported difficulty in obtaining continuous cell samples from the lid wiper conjunctiva. Jalbert notes that only "for 67% (27∕40) of all subjects, we obtained PAS∕ haematoxylin-stained samples of marginal epithelial cells that were continuous and could be graded" [24], while Doughty reports that the samples "have modest numbers of cells but in adjacent regions across the surface of the filter, the number of adherent cells was sometimes rather lower" [9]. While this aspect is only tangentially touched on in the respective reports, the variation in cell collection quality became evident well into the progress of this study, at a point when increasing the sample size was not feasible. Notwithstanding the scarce and variable collections, no effect of lens wear, symptomology or LWE was observed in the cellular morphology. A larger sample size would be imperative for future studies investigating these effects. Jalbert concluded that

"lid margin impression cytology could be used to test the hypothesis that higher grades of lid margin lissamine green staining (>grade 2) associated with changes in epithelial morphology, including metaplasia of the marginal epithelial cells". This was not evident in the qualitative observations of cellular morphology in this study, nor in the quantitative analysis of samples. While metaplasia is determined by dimensioning individual cells and NC-ratio scoring [18], a more sensible metric was opted for at this preliminary stage of studying the lid margin in different populations. The cell count, or sample cellularity, is a common metric for assessing bulbar conjunctival IC [13,168,169]. Previously discussed collection scarcity and variability also affected the quantitative analysis of samples, reflected in the large standard deviations of cell counts. While the findings did not warrant performing inferential statistics and are therefore to be interpreted with caution, some trends are worth noting. The keratinized M-cells at the MCJ are likely easier to detach onto the IC membrane, than non- (or less) keratinized L or C-cells. This may explain why M-cell counts were generally higher. On the other hand, C-cells from the tarsal conjunctival region are more likely to attach to the membrane if the application angle is incorrect, as discussed in section 3.5.3.2. This is most likely the cause for the three outliers, but also for the fact that C-cell counts have the overall highest SD among all three cell types. Accordingly, it can be argued that L-cells, positioned in between C and M-cell regions and representing an elevated, epithelial thickening of the lid margin [13], are subject to a more consistent application angle, allowing more reliable cell counts than the other cell types. This area is of particular interest in the assessment of the lid margin, given its proposed exclusive apposition to the ocular globe or contact lens [13], as opposed to the more outwards position of the MCJ [45]. Therefore, the overall lower number of L-cells is possibly explained by its proposed "wiping" function, as being exposed to increased friction may require epithelial cells to be more resistant to mechanical forces. To this end, groups B and C record higher L-cell counts than group A, suggesting that lens wear may affect or be related to an increased cell sloughing in the lid wiper epithelium. Finally, the question remains whether the slightly higher L-cell count in group C compared to groups A and B, can be accounted to either LWE or symptoms, either of which could further increase cell sloughing. Since LWE may extend from the MCJ well into the tarsal conjunctiva, particularly for higher LWE grades [8], this may explain why group C samples showed the overall highest cell counts across all groups. This interesting observation should be further investigated in future studies using a larger sample size.

The presence of goblet cells on the palpebral conjunctiva is well documented [13,34], but unlike the bulbar or tarsal conjunctiva, goblet cells at the lid margin are located in deeper epithelial layers, making their collection by IC difficult [9]. Yet, expressions of goblet cells were visible on the membranes in

the C and L regions, less so in the M-region, as shown by the Alcian Blue stain. These must be carefully differentiated from other tear film components (allegedly mucins), as both stain in similar patterns.

Another interesting observation was karyorrhexis, a common pre-apoptotic state in which the cell nucleus undergoes fragmentation [170,171]. Some cells in the samples exhibit this pattern (Figure 37). It was observed that in samples where nucleus fragmentation was present, not all cell nuclei were always fragmented. This may reflect the actual cell status or may have been induced by the fixation agent; this is not fully understood at this moment. Furthermore, inconsistent nucleus fragmentation was observed in half of all participants of groups B and C. This behavior is particularly interesting as nuclei appear fragmented in the non-lens wearing eye in group B, but fragmented in the lens wearing eye in group C.
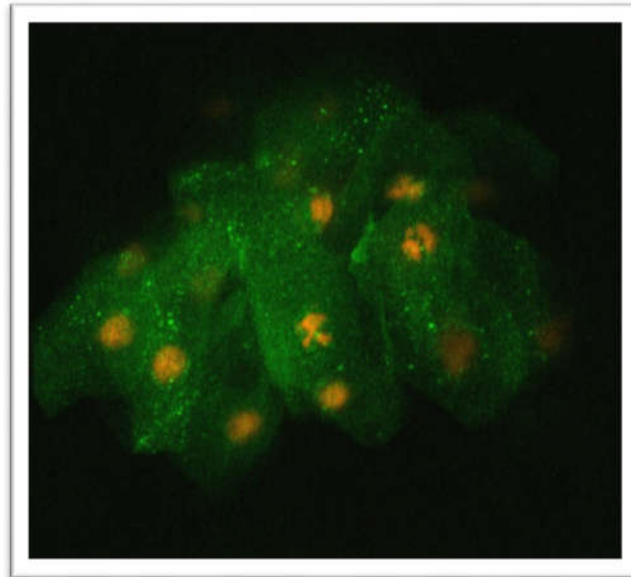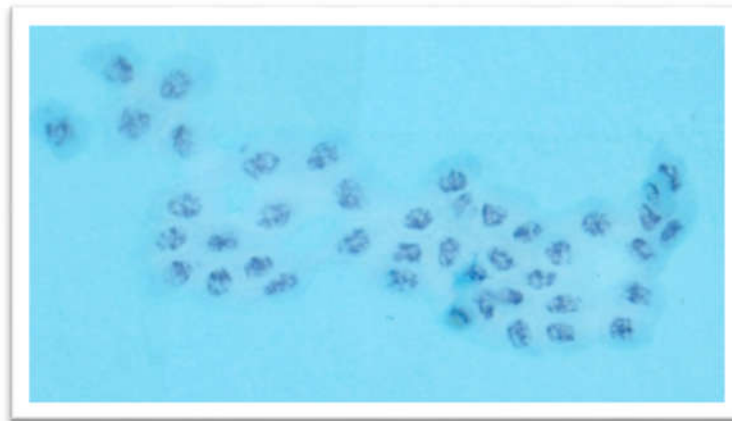
Figure 37: Karyorrhexis in L-cells as shown by histological and immunocytochemical staining.

Observations using the immunocytochemical stains and the CLSM system were similar to the optimization procedure, in that all collected cells in this study equally showed membrane compromise as well as cell viability. This remains perhaps the most relevant question, whether the results reflect the true status of cells, or rather a flaw induced by the technique itself, wherein the IC procedure would damage the cells at removal. Since this finding was consistent across all groups in this study, no conclusion can be drawn about the effect of grouping variables on the viability of cells.

The limitations of the CLSM system once again outweighed the benefits of high resolution imaging (Figure 33) and many challenges were faced with this technique. The apoptotic indicator Annexin V

added to the previous protocol, induced a significant interference pattern (Figure 32), which was present in all samples. Despite lengthy troubleshooting (including varying concentrations of the dye, ratios to the other dyes, order of staining, as well as varying imaging parameters such as the wavelengths, transmission coefficients and filter selection), we were unable to determine the cause of this effect and imaging with this dye was impossible.

As described before, tear film products collected on the membrane (presumed to be mostly meibum) and interfered with the visualization of cells (Figure 34). This interference was present in almost every sample, irrespective of study grouping variables. Additionally, several structures could not be identified, such as the web-like pattern found in many cell collections (Figure 35). No explanation could be found for this appearance. The etiology of a different recurrent structure is also unclear. Mostly found underneath patches of meibum, these cell-like formations feature a stronger green Calcein AM fluorescence than seen in all other cells, indicating high esterase activity, but show no Ethidium fluorescence, indicating an intact membrane. The morphology resembles that of L- or M-cells found in this region, which usually show nucleus fluorescence and much lower esterase activity. These structures may be cornified cells of the epidermis, with a high degree of keratinization causing the fluorescence (Figure 36).

Finally, issues of membrane and slide alignment, as well as three-dimensional localization of cellular structures, required lengthy troubleshooting and imaging times of up to three hours per sample.

4.6 Conclusion

In this chapter, the previously developed IC collection and analysis protocol was successfully used to characterize cellular structures of the lid margin. Three distinct cellular morphologies were detected, spanning between the tarsal/marginal conjunctiva, through the lid wiper conjunctiva, to the muco-cutaneous junction at the Marx' line. Cellular features between these regions are transitional in nature, broadly coinciding with previous reports. While neither lens wear nor LWE seemed to affect cellular morphology, the relationship with subjective symptoms could not be studied due to erroneous study design. The sample cellularity may or may not be altered by lens wear, LWE and/or symptoms, particularly in the lid wiper conjunctiva. Future work ought to probe these trends, imperatively using a larger sample size.

While an improvement in IC collection technique was noticed from optimization work in the previous chapter, sample consistency is still problematic albeit unclear whether this reflects poor technique or actual study design variables.

Cellular viability and membrane compromise were equally found in all cells across all study groups. It remains unclear whether this stems from the true status of cells, or whether it was induced by the IC collection technique itself. The high-resolution imaging capabilities of the CLSM were outweighed by its drawbacks; at this point, this technology may be superfluous and would perhaps be best employed at a later stage in the study of the lid margin morphology, for characterizing single cells or cell patches, rather than membrane-wide quantification.

High LWE and ocular discomfort were not correlated in this pilot study, contrary to previous suggestions. Pairing up these variables as inclusion criteria impeded the progress of the study and should be omitted in future studies. Rather than LWE, cytological assessments should consider subjective symptoms as a grouping variable, as to date, LWE is equivocal with respect to its predictability of dry eye and the present findings suggest a rather indiscriminate nature of LWE.

Chapter 5

Manifestations of subjective discomfort at the lid margin of symptomatic and asymptomatic soft contact lens wearers

5.1 Overview

PURPOSE: The purpose of this study was to assess the lid margin epithelium of symptomatic (sSCL) and asymptomatic (aSCL) soft lens wearers.

METHODS: Forty adapted SCL wearers were enrolled and equally distributed in two study groups based on self-reported contact lens (CL)-related comfort levels. Comfort was assessed using the Young scheme, Ocular Surface Disease Index (OSDI), Contact Lens Dryness Experience Questionnaire (CLDEQ-8) and diurnal 0-100 scales for comfort and dryness. Lid wiper epitheliopathy (LWE) was assessed using lissamine green (LG) and impression cytology (IC) performed on the upper and lower lid margins using Millicell cell culture inserts. Samples were stained with Alcian Blue, Hematoxylin and Papanicoloau dyes, and the lid wiper (LW) and muco-cutaneous junction (MCJ) cellular areas defined and dimensioned using ImageJ.

RESULTS: The average upper and lower LWE grades were similar in both groups (0.8 ± 0.7) and did not correlate with any subjective comfort score or other study variable. The width of lid marginal areas was not significantly different between symptomatic and asymptomatic SCL wearers (n=139). The average width (±SD) measured 415±131 µm at the upper LW, 114±43 for the MCJ, and 187±120 at the lower LW and 90±41 for the MCJ. Some of these measures were found to correlate with LWE ($p<0.05$, r=0.61 to 0.86).

CONCLUSION: We present the first account to show a correlation between LWE grade and widths of the LW and MCJ areas after histological inspection, and provide evidence to support the frictional etiology of LWE and of the Marx line.

5.2 Introduction

The human upper eyelid executes around 10,000 blinks every day [155] , traveling 10-12 mm per blink, with just a 1-2 mm narrow conjunctival region apposing the eye. This so-called "lid wiper" (LW) [6] is therefore exposed to 400 m of daily travel. It is the role of the tear film to alleviate the supposed increased friction in this area during habitual blinking. Any disturbance to the normal tear film, whether physiological, pathological or by wearing a CL, may affect the comfort we perceive. Recent evidence suggests that the lid margin may reflect or predict dry eye conditions [6,53,62,172].

The lid wiper and MCJ are two different structures

The lid margin was originally described as early as the 20th century, and while its wiping function was proposed in 1904 [23], it wasn't until much later that two separate structures were differentiated. Marx was the first to describe the MCJ in 1924 and eponymously termed it Marx line, identifying it as an elevated epithelial structure which stains with various vital dyes, and is different from the epidermis of the eyelid or the tarsal conjunctiva [3,35]. While initial descriptions believed that it is this sharp inner border of the lid that glides over the eye [22,23], it was only through Korb's introduction of LWE [6] and Knop's detailed histological descriptions of the lid margin [13], that the lid wiper was established as a posterior, or proximal, structure to the MCJ (Figure 38). The consensus appears to be now that it is the LW which is wiping over the eye, and the Marx line is not. Yet, to this date we have limited knowledge of the location, size and role/relevance of the MCJ and the lid wiper.
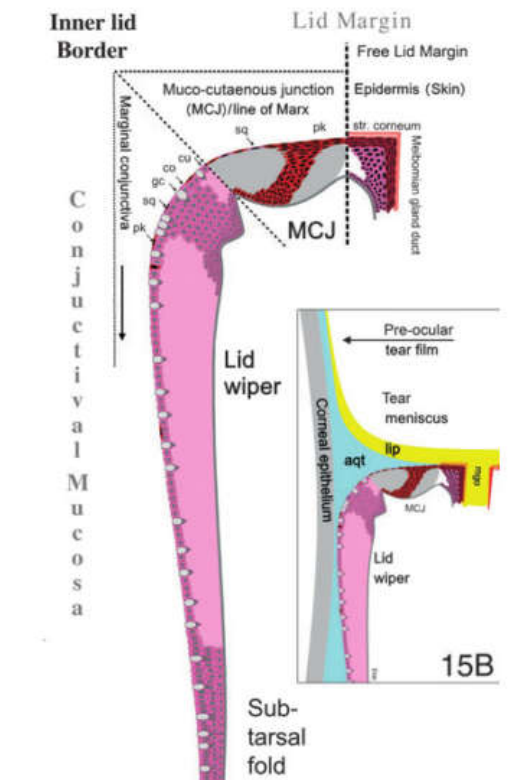
Figure 38: The lid margin, as described by Knop et al. [13]

The cellular morphology of the lid margin

Clinically, the lid margin is routinely assessed using vital dyes, as detailed in section 1.1.1. The MCJ/Marx line region stains with vital dyes in nearly all individuals, while an extended staining of the LW has been proposed as an "epitheliopathy", and an indicator or predictor for dry eye [6]. The extent of this staining is visually estimated and computed into a score known as the "Korb grade", which ranges from mild to severe [6,50,51]. Interestingly, there are no cytological reports that mirror this staining. Simply put, we do not actually know what we are staining. Using IC, Jalbert collected and described lid margin cells, but did not find any difference between CL wearers and non-wearers, yet suggested that IC "may be used to investigate LWE" [24]. Others [9] have employed IC, in vivo confocal microscopy, cadaver excisions [13] and pressure sensitive paper [31] to investigate the lid wiper, and these reports largely agree on the size of the MCJ. Measures range from 0.09 ± 0.02 mm [31] to "0.3 mm wide but when stained with LG, up to 0.5 mm wide" [9] or "three to eight lines of squamous appearing cells" [9], with other accounts falling within this range [13,34,43].

One of the earliest descriptions of the lid wiper notes that "the squamous epithelium extends away from the lid margin on to the conjunctival side of the lid *for some distance*, until, rather abruptly, it continues in a single- or multi-layered, almost cubical epithelium with goblet cells" [22]. Knop observes that "The start of the lid wiper was defined by the occurrence of cells with a cuboidal shape at the epithelial surface" and that "Interspersed para-keratinized cells of different shapes (squamous to columnar) continued in decreasing number from the MCJ over the surface of the lid wiper onto the tarsal conjunctiva", but does not clearly identify the end of the lid wiper area, only noting that it "gradually decreased over a distance of about 0.3–1.5 mm or more", adding that "the lid wiper tended to be wider, i.e. longer, in more nasal and temporal positions along the lid margin compared to the center of the eyelid" [13]. Using pressure sensitive paper adhering to a rigid CL and that was removed after 10 seconds of wear, Shaw [31] measured a width of 0.6 ± 0.16 mm of an area presumed to correspond to the LW. However, in their comprehensive review of LWE [48], Efron and colleagues notes that it is difficult to reconcile these accounts for a number of reasons, mostly due to the transitional character of cells in this region and the resulting lack of clarity regarding the precise anatomical locations to which histological descriptions pertain.

Insufficient information

While there seems to be a consensus that the LW is exclusive in its wiping role, there is no clear answer as to where it ends. Further, despite increased interest in LWE and its potential to reveal or predict dry eye, its clinical measurement is limited to visual estimates, with no accounts to compare the vital staining observed in vivo, with cytological analyses.

The small number of reports on the cytology of the LW may not be representative of the typical CL-wearing population, or sufficiently reliable. Knop analyzed tissue samples excised from ten cold-stored cadavers with an average age of 77 [13], but the average age of CL wearers is less than half of that [159] and the position of the Marx line is known to change after the age of 50 [173]. Jalbert conducted IC on 40 CL wearers and non-wearers, but did not accurately describe the origin of their collections, stating that cells were collected "around meibomian gland openings" [24]. Moreover, as Efron pointed out, some of their images are incorrectly labelled [48].

In the previous chapter, IC samples were visually inspected and provided some insight into the lid margin morphology. With an increased sample size, which would counterbalance previously discussed issues of repeatability and consistency of cell collections, a more precise analysis of IC collections would be possible with the aid of computerized algorithms. Software such as ImageJ offers powerful

dimensioning tools, which can be re-programmed and streamlined to automate measurements for specific applications.

With CL-related discomfort being such a highly prevalent phenomenon [145], we feel it is imperative to further explore the lid margin and the proposed LWE as promising new avenues towards a better understanding of ocular discomfort. Therefore, the aim of this study was to assess whether different degrees of subjective ocular (dis-)comfort in CL wearers are associated with cyto-morphological changes at the lid margin.

5.3 Materials and methods

Forty habitual CL wearers were enrolled in this prospective, non-dispensing study and differentiated by self-reported CL-related discomfort into two study groups. LWE was graded using LG and IC conducted on the upper and lower lid margins. IC samples were processed and evaluated using methods described in the previous chapters.

5.3.1 Subject recruitment

All clinical studies have been designed to follow the ethical principles in the Declaration of Helsinki, with the ICH guidelines for Good Clinical Practice (GCP), with the University of Waterloo's Guidelines for Research with Human Participants and with the Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans, 2nd Edition. Informed consent was obtained from all participants prior to enrolment. The study received approval from the Office of Research Ethics at the University of Waterloo, Waterloo, Ontario, Canada (ORE #20958). The study was advertised using the recruitment system at the Centre for Contact Lens Research at the University of Waterloo.

5.3.2 Inclusion and exclusion criteria

Participants were subject to the same inclusion and exclusion criteria outlined in section 4.3.2.

5.3.3 Study procedures

Subjective and objective clinical tests were performed at the study visits by a single investigator. Data was manually recorded onto CRFs.

5.3.3.1 Subjective comfort questionnaires

All participants completed four different questionnaires to assess their CL-related dryness and discomfort symptoms. The symptomatic/asymptomatic classification scheme by Young et al. [150], the

Ocular Surface Disease Index (OSDI) questionnaire [99] and the 0-100 scale for diurnal changes in comfort and dryness have all been described in section 4.3.3.1. Additionally, participants rated their CL-related discomfort using the Contact Lens Dryness Evaluation Questionnaire (CLDEQ-8), a shortened version of the complete CLDEQ [104,174,175], which assesses the frequency and intensity of symptoms such as eye discomfort, dryness, blurry vision. These are rated between "Never (0)" and "Constantly (5)", and "Not at all intense (0)" and "Very intense" (5), and compared using the sum of scores (0-37).

5.3.3.2 Clinical techniques

Subjects underwent optometric tests as described in section 4.3.3.2. LWE of the upper and lower lid margins was evaluated after two instillations of lissamine green dye (two superimposed 1.5 mg strips, wetted with a drop of saline solution, HUB Pharmaceuticals, CA), one minute apart, and graded according to the Korb scale [50].

5.3.3.3 Impression cytology

IC samples from the upper and lower lid margin of both eyes were collected using Millicell Cell Culture Inserts (Merck Millipore, Darmstadt, Germany) as detailed in the previous chapter of this thesis, under section 4.3.3.3. Artificial tears (Bion Tears, Alcon, Fort Worth, TX) were dispensed to alleviate any potential discomfort following the procedure.

5.3.3.4 Sample processing

Following collection, all four cell culture inserts were promptly submerged in 95% Ethanol and fixated for approximately 30 minutes. Membranes remained attached to the plastic insert throughout the staining procedure. The detailed histological staining protocol using Alcian Blue (AB), Hematoxylin Gill-1, Papanicoloau OG-6 and EA-65 (all Sigma-Aldrich, St. Louis, Missouri), as well as the panoramic imaging and stitching method, are described in section 4.3.3.4.

5.3.4 Group assignment

After signing the informed consent form and being considered eligible for inclusion in the study, participants were allocated to one of the two study groups based on their responses to the classification and polarization scheme described by Young et al. Asymptomatic CL wearers were enrolled in group aSCL and symptomatic participants were included in group sSCL (Table 10).

Table 10: Classification of asymptomatic and symptomatic CL wearers by dryness

Intensity of contact lens dryness		Frequency of contact lens dryness				
		Never	Rarely	Sometimes	Frequently	Constantly
Never have it	0					
Not intense at all	1					
	2	**Asymptomatic (Group aSCL)**		Marginal (DO NOT ENROL)		
	3			**Symptomatic (Group sSCL)**		
	4					
Very Intense	5					

Participants who reported marginal discomfort or did not meet the criteria for one of the groups described above were discontinued from the study.

5.3.5 Study visits

Eligibility and study grouping were determined at the screening visit, and LWE measured and IC performed on the upper and lower lid margins at the study collection visit. The two study visits were consecutive, totaling 1.5 hours and only one participant was enrolled per day. Samples underwent histochemical processing and analysis thereafter. Data were recorded on CRFs.

5.3.6 Analysis

5.3.6.1 IC sample collection quality grading

To overcome the varying quality of IC samples discussed in previous chapters and reported in the literature [24], a grading system was developed, to triage analyzable samples. Collection quality was visually assessed and graded by a single investigator. Sample cellularity, cell diversity and any other features that would aid or impede the analysis of the cells were considered, based on which, samples were graded from 1 – 4 (Table 11). One week later, assessment and grading were repeated, with masking of the initial marks. An error rate of 5% and lower was established as a threshold for repeatability between grading sessions. Examples of collection quality grades are shown in Figure 39.

Table 11: Criteria for grading sample collection quality

Grade	Sample characteristics						Outcome
	Sample cellularity	Cell type diversity	Cell grouping	Cell area delimitation	Stain color differentiation	Obstructing features	
1	No or few cells	One type	Single, scattered				Considered unanalyzable
2	Few cells (dozens)	1-2 types	Occasionally grouped or in continuous patches,	Not always distinguishable	Weak	Meibum occasionally covering cells	Considered unanalyzable
3	Lots of cells (hundreds)	2-3 types	Individual clusters of adjacent cells	Mostly distinguishable	Good	Mostly free from obstructing features	**analyzable**
4	Lots of cells (>hundreds)	3 types	Continuous collections featuring multiple/large clusters of adjacent cells	Well defined	Good	No obstructing features	**analyzable**
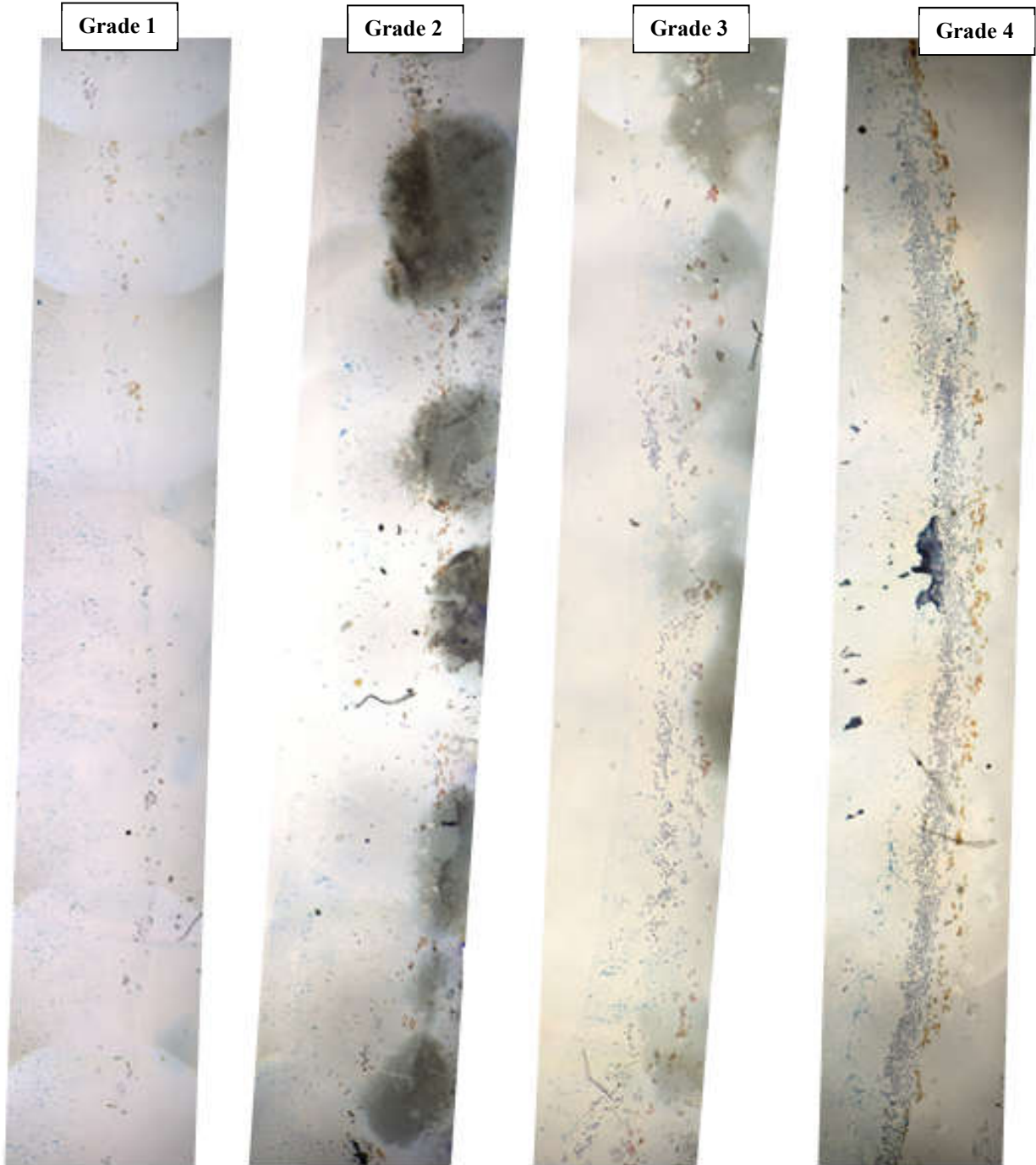
Figure 39: Examples of collections graded 1-4, according to criteria described in Table 11

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5.3.6.2 Sample dimensioning

For samples graded 3 and 4, the average width of the L- and M-cell areas was measured. In this context, and as shown in Figure 40 and Figure 44, the width of a cellular area is defined as the vertical distance between two cell types (or features, as described below) on the IC sample. Measurements were computed using ImageJ (National Institutes of Health (NIH), Bethesda, Maryland). Three lines were manually traced onto each image: the first line (red line in Figure 41) indicated the direction of the collection, i.e. the line to which the measured distance was perpendicular to; for the lid wiper width, the second line travelled along the proximal margin of the MCJ, as indicated by red/orange keratinized squamous cells (yellow line in Figure 41), and the third line delimited the visible transition of L-cells to C-cells of the tarsal conjunctiva, i.e. from large, squamous cells with small nuclei, to small cell bodies with large nuclei (Figure 42), as well as the occurrence of goblet cell (GC) impressions and expressions (Figure 43). The boundaries of the MCJ were delimited by the occurrence of keratinised cells with nuclei (green and yellow lines in Figure 41). The directional line (red line in Figure 41) was a straight line, while the two delimiting lines (green and yellow lines in Figure 41) were segmented lines, to accurately fit the curved shape of the cell collection and account for variation and irregularities. For discontinuous cell collections, delimiting lines were interpolated according to the surrounding morphology. The average width of the LW and MCJ was computed as the difference in orthogonal (x,y) coordinates between approximately 1000 points on the two delimiting lines, using a custom plug-in programmed for this purpose (see Appendix A for complete source code). Distances were recorded in pixels and converted to micrometers after calibration of the imaging system. The Zeiss AxioVert microscope, the AxioCam ICc5 camera and the AxioVision (Zeiss Inc. Toronto, Canada) image acquisition software produced scales of 0.6714 and 2.593 µm/pixel for the 10x and 2.5x objectives respectively.
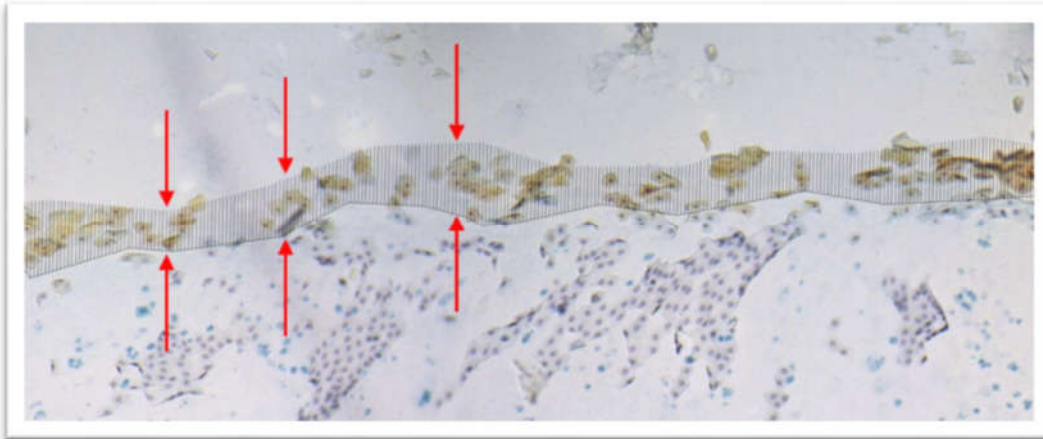
Figure 40: Width of MCJ (arrows), delimited by occurrence of M cells.

The outer lid and the Meibomian glands are above the line, the lid wiper (blue cells) and tarsal conjunctiva are below. Cropped section, represents ca. 20% of span of full IC collection.
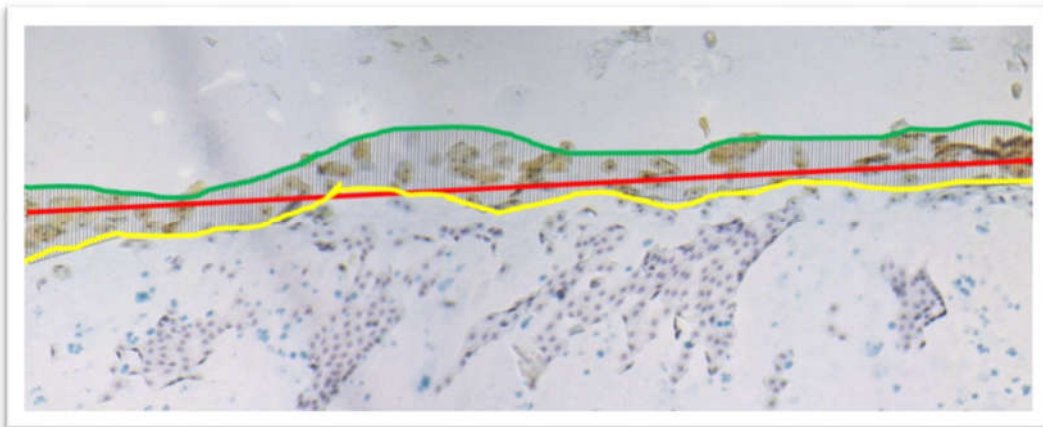
Figure 41: Directional (red), distal (green) and proximal delimiting lines (yellow) used in ImageJ to compute width of cellular areas.
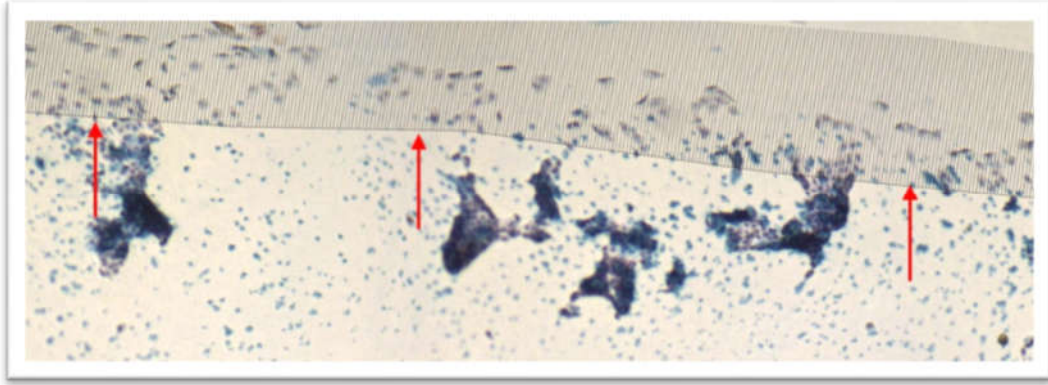
Figure 42: End of lid wiper area (arrows) as indicated by a decrease in cell size as well as GC presence and their expressions.
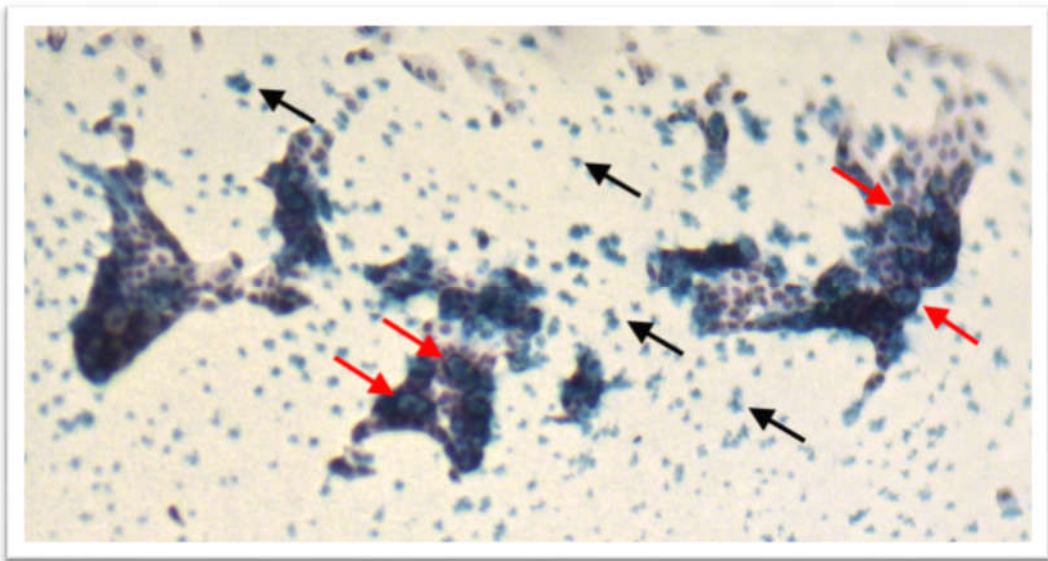
Figure 43: GC impressions (red arrows) and GC expressions on the IC membrane (black arrows)
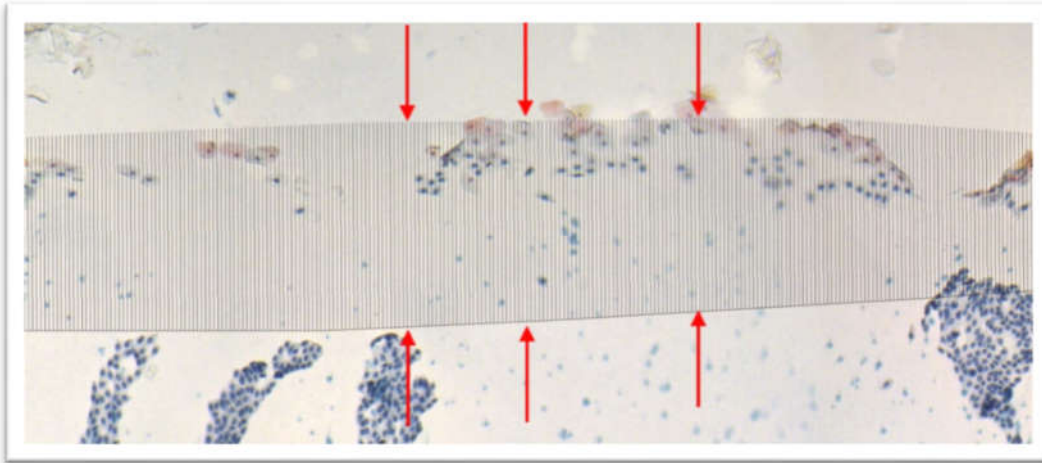
Figure 44: Width of lid wiper (arrows). Upper boundary marked by keratinized M cells, lower boundary by the occurrence of large patches of small co/cu C cells, and GC expressions.

At this point, it is worth noting the dimensional terminology of the two staining types (vital and histological). In both cases, the "width" refers to the vertical extent of staining, also termed "sagittal" in Korb's LWE grading scale (**Table 3**), while "length" describes its horizontal dimension. Note that, in the case of histological staining, the most commonly used descriptor in the present thesis is "width", as indicated by the red arrows in Figure 45.
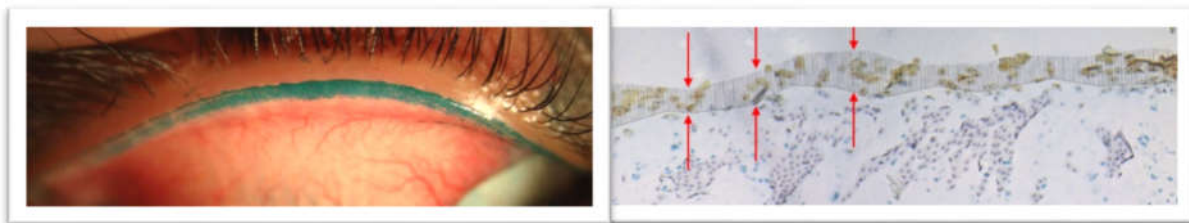
Figure 45: Vital and histological staining of lid margin cells.

Red arrows indicate width of cellular area; this dimension is the equivalent of the sagittal width of the vital staining, as defined by Korb et al.

5.3.6.3 Data analysis and plotting

Statistical analysis and graphs were created using GraphPad Prism (GraphPad Software, Inc., San Diego, California). For boxplot graphs, the bottom and top of the box represent the first and third

quartiles, and the band inside the box signifies the second quartile (the median). The ends of the whiskers indicate the lowest datum still within 1.5 IQR of the lower quartile, and the highest datum still within 1.5 IQR of the upper quartile. Dots outside the whiskers stand for outliers identified using the Tukey method. Asterisks denote statistically significant differences between the two groups comprised within the square brackets.
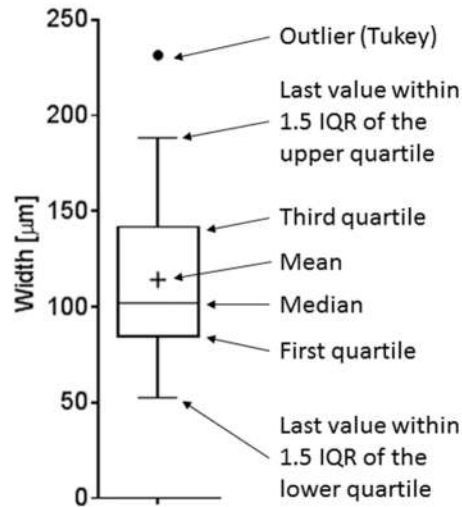
Figure 46: Elements of a boxplot graph

Differences between groups and study parameters were determined using the unpaired t-test or the Mann-Whitney test, and correlations between study parameters and variables were tested using Pearson and Spearman coefficients. A significance level (p) of α=0.05 was assumed for every statistical test unless otherwise noted.

5.4 Results

5.4.1 Demographics

Forty participants were screened and completed the study between November 2015 and December 2016. In group aSCL, 20 participants (75% females, average age ± SD of 39 ± 16 years, ranging between 18 and 67 years) and in group sSCL, 20 participants (80% females, average age ± SD of 29 ± 12 years, ranging between 18 and 64 years) were enrolled. Differences between the group mean ages were statistically significant (p= 0.0264). Distribution by age and by comfort are displayed in Figure 47 and Figure 48.

89
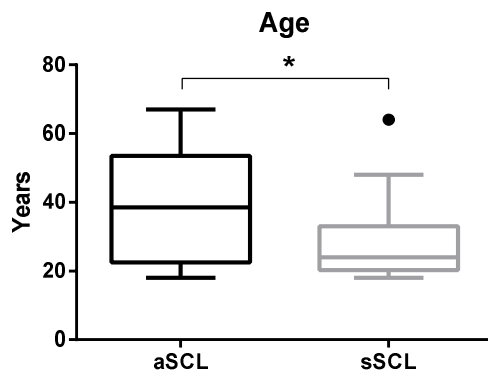
Figure 47: Distribution of participant age by study group
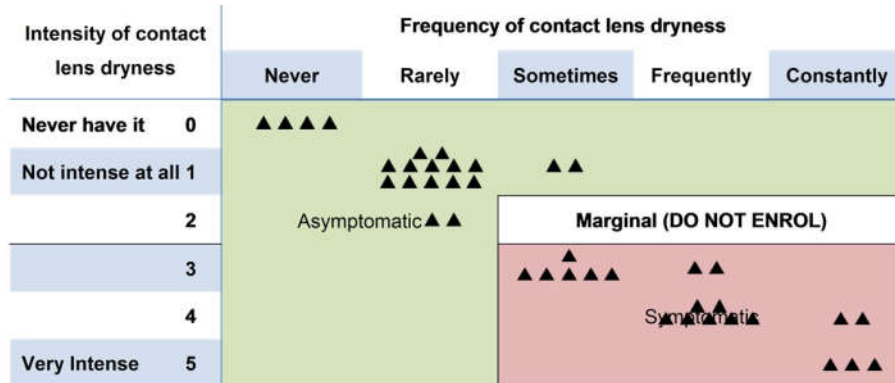
Figure 48: Distribution of participants in the two study groups by CL-related dryness. Each symbol represents one participant.

Participants in group aSCL reported an average daily wear time (mean ± SD) of 13.1 ± 2.4 hours per day, out of which 12.1 ± 2.5 hours were reported as being comfortable. Lenses were typically worn for 6.1 ± 1.2 days per week, with an average lens wear experience of 17.8 ± 11.9 years. Participants in group sSCL reported an average daily wear time (mean ± SD) of 10.4 ± 3.3 hours per day, out of which 5.8 ± 3.4 hours were reported as being comfortable. Lenses were typically worn for 5.2 ± 1.2 days per week, with an average lens wear experience of 9.0 ± 7.0 years (Figure 49). These values were all significantly different between the two groups (Table 12).

Table 12: Average self-reported wear times (±SD). Bold values indicate statistical significance

	Group aSCL	Group sSCL	aSCL vs. sSCL (Mann Whitney U)
Average daily wear [hours]	13.1 ± 2.4	10.4 ± 3.3	**p=0.0108**
Comfortable daily wear [hours]	12.1 ± 2.5	5.8 ± 3.4	**p< 0.0001**
Average weekly wear [days]	6.1 ± 1.2	5.2 ± 1.2	**p=0.0210**
Wear experience [years]	17.8 ± 11.9	9.0 ± 7.0	**p=0.0067**
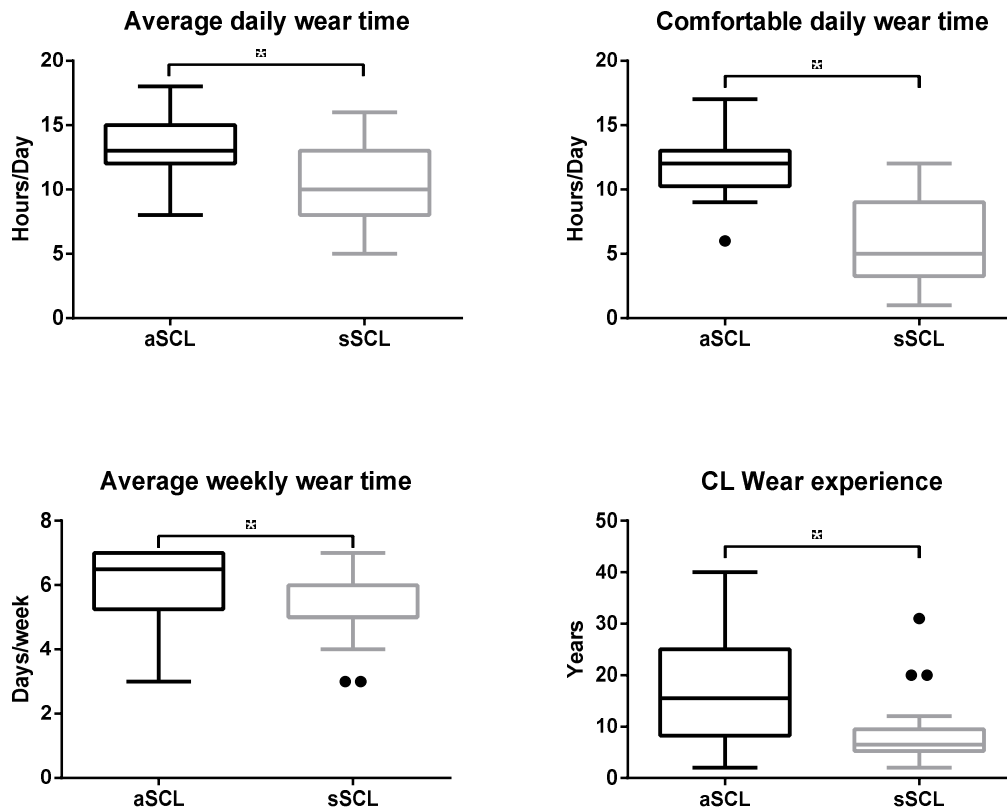
Figure 49: CL wear experience and wear habits between study groups.

Thirty percent of all participants in each group reported allergies, half of these having some ocular manifestation, such as in seasonal allergies, none of these being reported as currently active episodes during the study or in the weeks leading up to the study visit. Four participants in group sSCL reported the daily use of lubricating drops. Among both groups, twenty percent of participants were daily

disposable CL wearers, the others wore monthly replacement CLs. Seventy-five percent of all worn CLs were silicone-hydrogel lenses, and the others were hydrogel materials.

5.4.2 Subjective comfort scores

5.4.2.1 Ocular Surface Disease Index (OSDI)

Average (± SD) OSDI scores of 8 ± 7 and 24 ± 13 were recorded in group aSCL and sSCL respectively. These were statistically significantly different (p< 0.0001, unpaired t-test).
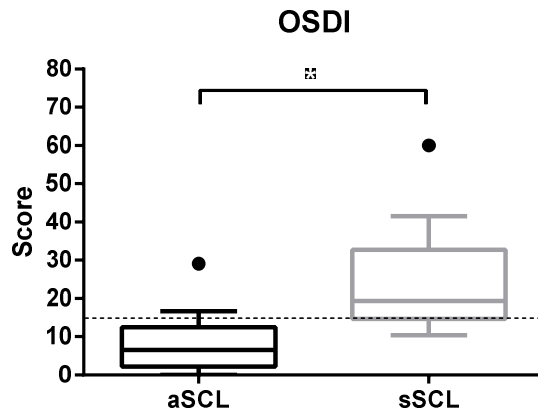
Figure 50: Distribution of OSDI scores between study groups.

Dashed line indicates threshold value (15) above which patients are considered symptomatic.

5.4.2.2 Contact Lens Dry Eye Questionnaire (CLDEQ-8)

An average CLDEQ-8 score (± SD) of 6.25 ± 2.7 was recorded in Group aSCL, while Group sSCL averaged at 19.7 ± 4.6. The difference was statistically significant (p<0.0001, Mann Whitney U test).
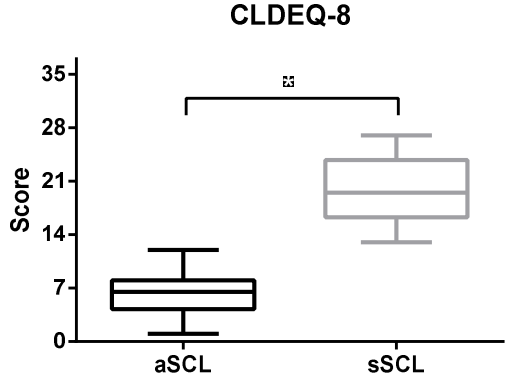
CLDEQ-8

Figure 51: Distribution of CLDEQ-8 scores between study groups. Higher values indicate inferior comfort.

The distributions of responses are displayed below.

CLDEQ-8 responses for Group A

CLDEQ-8 responses for Group B
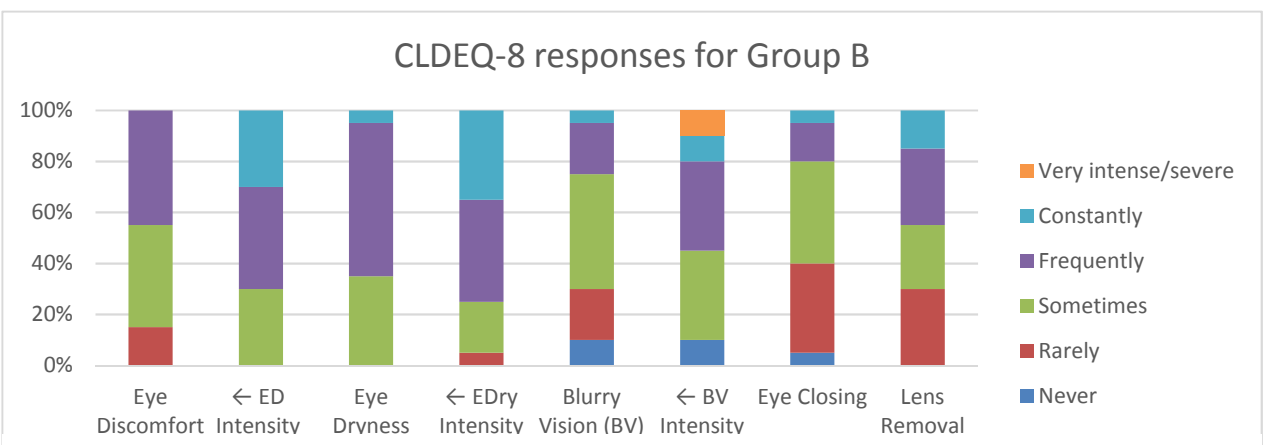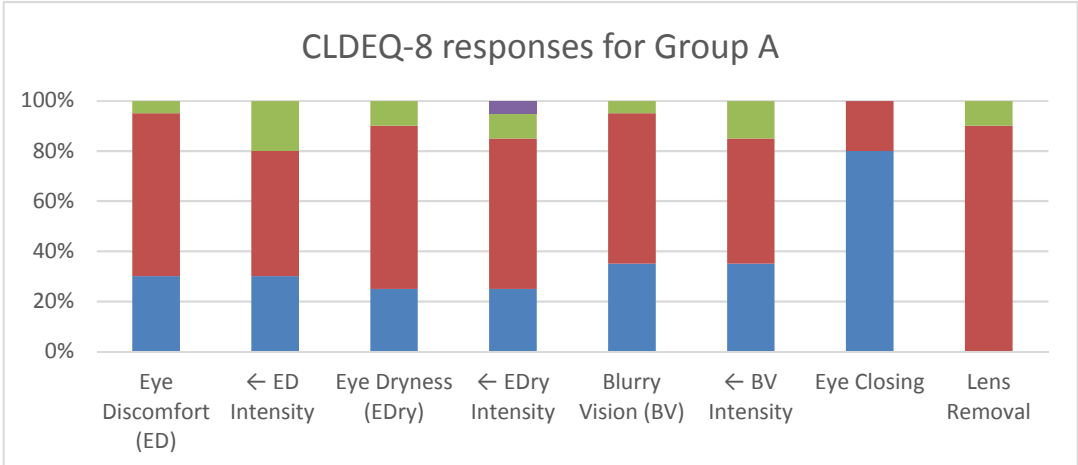
Figure 52: Distributions of CLDEQ-8 responses in the two study groups

5.4.2.3 Diurnal comfort and dryness

The average scores for diurnal comfort and dryness are summarized in Table 13. Both comfort and dryness scores were significantly different between groups aSCL and sSCL at every time point (p<0.001, Mann Whitney U test).

Table 13: Average (±SD) diurnal scores for comfort and dryness. Bold values indicate statistical significance

		aSCL	sSCL	aSCL vs. sSCL (Mann Whitney U)
Comfort	Morning	97 ± 7	87 ± 13	**p=0.0063**
	Noon	98 ± 3	73 ± 15	**p< 0.0001**
	Evening	91 ± 6	51 ± 21	**p< 0.0001**
Dryness	Morning	98 ± 5	86 ± 16	**p=0.0054**
	Noon	97 ± 5	71 ± 15	**p< 0.0001**
	Evening	92 ± 6	48 ± 21	**p< 0.0001**
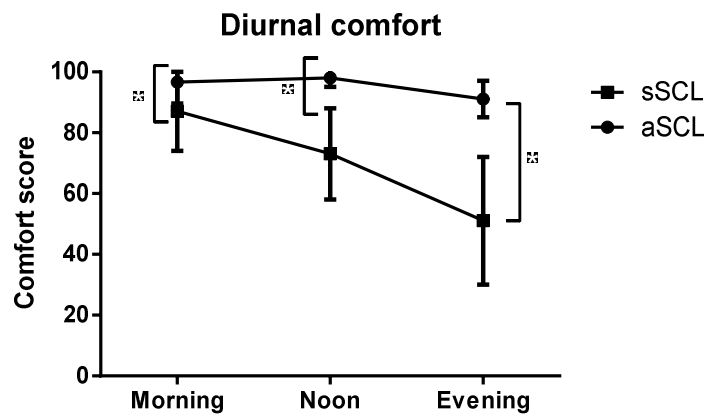
Figure 53: Average diurnal scores for comfort, error bars indicate SD. Higher values indicate inferior comfort.
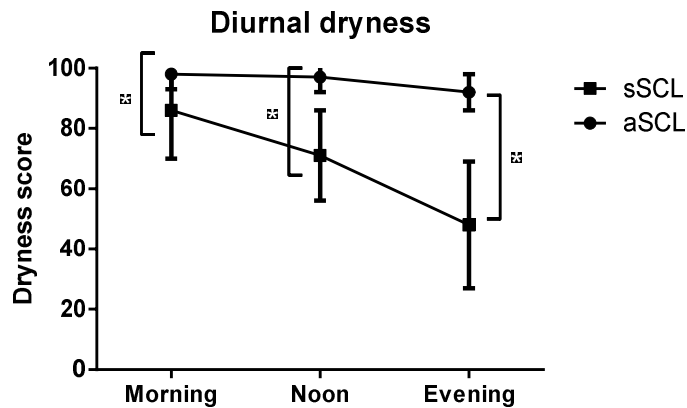
Figure 54: Average diurnal scores for dryness, error bars indicate SD.

5.4.3 Lid wiper epitheliopathy

The average LWE grades are summarized in Figure 55 and their distribution is shown in Figure 56. There were no significant differences between the groups (Table 14) and the grades were well correlated bilaterally (Table 15).

Table 14: Average (±SD) LWE grade (OU) observed with LG.

LWE	Group aSCL	Group sSCL	aSCL vs. sSCL (Mann Whitney U)
Upper	0.8 ± 0.8	0.8 ± 0.7	p>0.05
Lower	0.8 ± 0.6	1.0 ± 0.7	p>0.05
Upper vs. lower (Spearman correlation)	r=0.7 **p<0.05**	r=0.7 **p<0.05**	
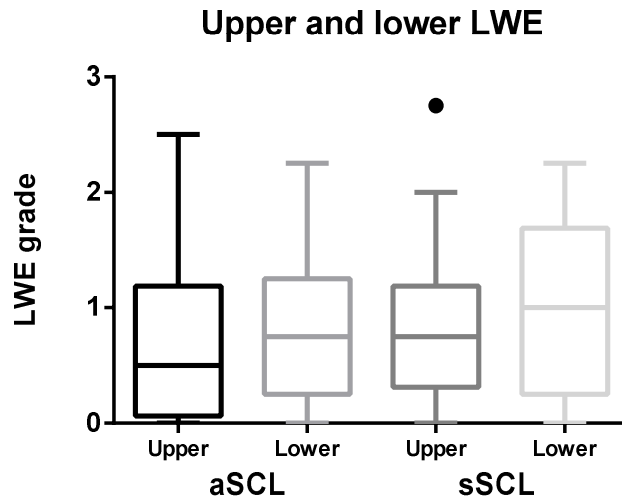
Figure 55: Upper and lower LWE grade in the two study groups.
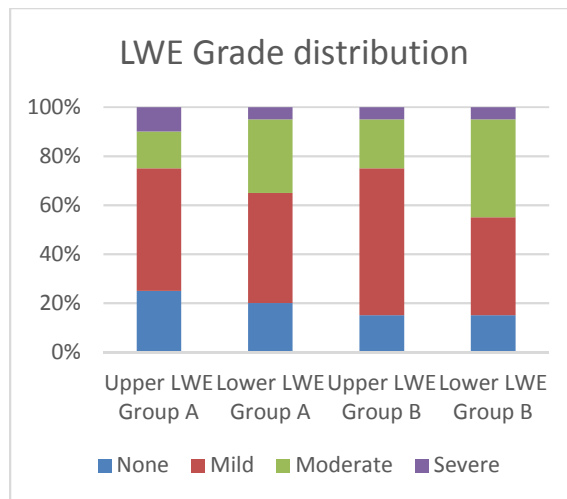
Figure 56: Distribution of LWE grades.

Table 15: Correlation of LWE grades between the left and right eye (Spearman correlation).

Group	Upper LWE	Lower LWE
aSCL	**r=0.8**	**r=0.66**
	p<0.0001	p=0.0016
sSCL	**r=0.66**	**r=0.88**
	p=0.0014	p<0.0001

LWE grade correlated with other study variables in only two instances: the upper LWE grade (OU) in Group sSCL was correlated with the average daily wear time (hrs/day) (r=0.56, p=0.01, n=20, Spearman correlation), and the lower LWE grade (OU) in Group sSCL was correlated with the average weekly wear time (days/week) (r=0.49, p=0.02, n=20, Spearman correlation). No other significant correlations were identified between LWE grade and study variables.

5.4.4 Histological analysis

5.4.4.1 Collection quality grading

The accuracy/repeatability of the double grading of samples in group aSCL and sSCL was 97.5% and 96.5% respectively. A total of 92 samples (57.5 ± 8.5 % of total of 160 samples) were graded either 3 or 4 and were considered analyzable.
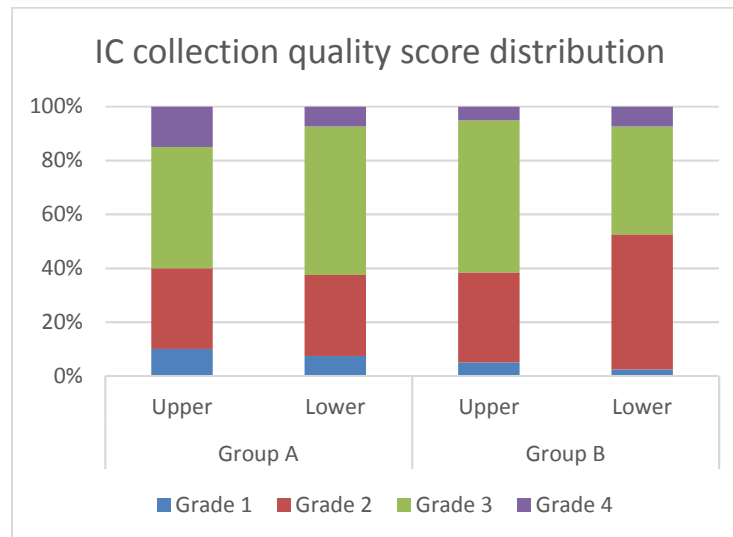
Figure 57: Distribution of collection quality grading of IC samples

5.4.4.2 Dimensional analysis

The width of the lid wiper and MCJ areas was measured in 139 instances. Each measurement was computed from up to 1000 single width measurements per sample and reported as an average value and SD. While analysis and comparison of widths employ the average values, SDs were not included in the analysis. These ranged between 11.9% and 31.5% of each respective mean width value, as shown in Figure 58.

Lid marginal width measurements
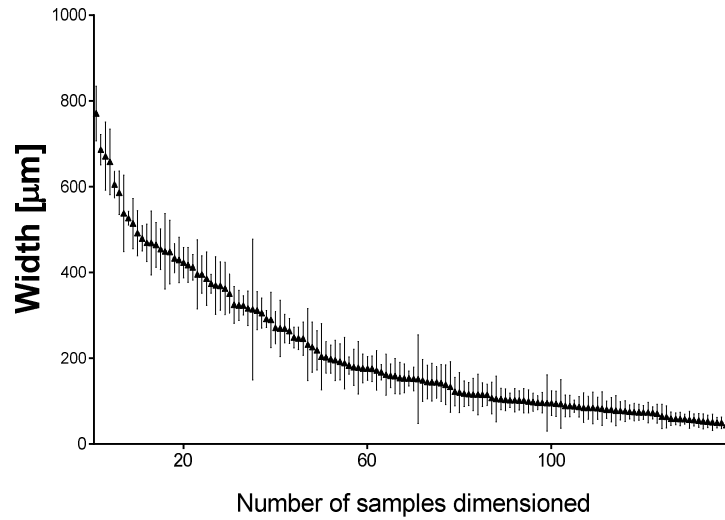
Figure 58: Average width of pooled L and M cell areas across study population (sorted). Error bars indicate SDs (n=139).

The average width of lid wiper and MCJ areas is depicted in Figure 59. Bilateral comparisons revealed no statistically significant difference (Mann Whitney U test), therefore only the right eye value was considered for further comparisons. The value from the left eye was used in 17 instances (out of 139), where the right eye value was not available.
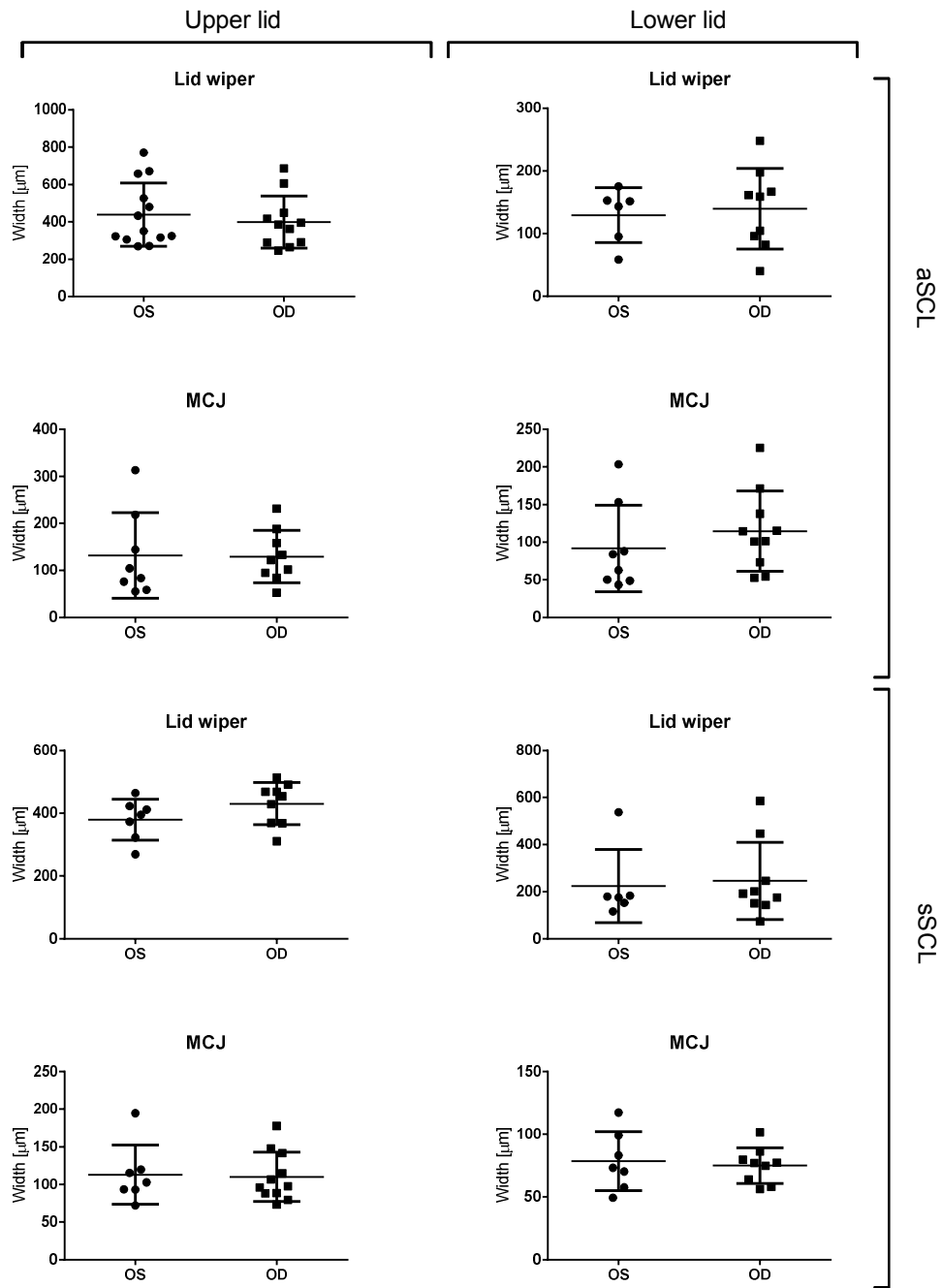
Figure 59: Widths of the lid wiper and MCJ areas.

Each point represents a separate sample; middle line represents mean value. Bilateral differences were not statistically significant (Mann Whitney U test).

Widths of the lid wiper (Figure 60) or MCJ (Figure 61) were not significantly different between groups (Table 16). No statistically significant correlation was found per sample between the two measures.
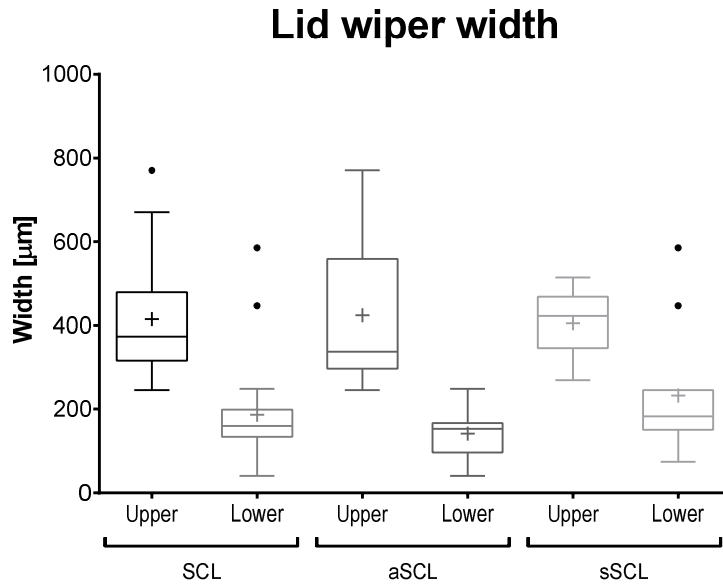
Lid wiper width

Figure 60: Width of the upper and lower lid wiper area.

MCJ width
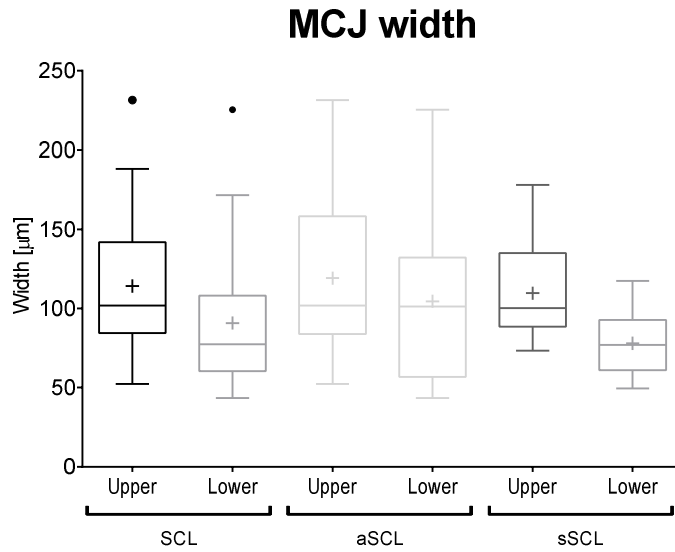
Figure 61: Width of the upper and lower MCJ area.

Table 16: Widths of the lid wiper and MCJ in μm.

Study group	Upper lid margin						Lower lid margin					
	L			M			L			M		
	Mean	**SD**	**n**	**Mean**	**SD**	**n**	**Mean**	**SD**	**n**	**Mean**	**SD**	**n**
aSCL	424.4	171.0	14	119.0	55.3	11	141.9	57.7	11	104.5	54.0	12
sSCL	404.8	75.5	13	109.6	31.2	12	232.1	150.1	11	78.0	19.4	13
SCL	415.0	131.7	27	114.1	43.6	23	187.0	120.2	22	90.7	41.3	25
aSCL vs. sSCL (Mann Whitney U)	p=0.84			p=0.96			p=0.13			p=0.37		

5.4.4.3 Correlation with study variables

The width of the lid wiper and MCJ areas was verified for correlation with all other measured study variables (described in section 5.3.3). A total of 668 correlation pairs were tested and the statistically significant results are summarized in Table 17.

Table 17: Summary of statistically significant correlations between cell area width and all other recorded study parameters (of total of 668 pairs). The asterisk indicates ipsilateral correlations.

Lid	Cell area	Study group	Parameter	p	r	n	Correlation
Lower	L	aSCL	Weekly average wear days [days/week]	**0.02**	0.67	11	Spearman
Lower	L	aSCL	Limbal hyperemia*	**0.04**	0.69	9	Spearman
Lower	M	aSCL	OSDI score	**0.03**	-0.61	12	Spearman
Lower	M	aSCL	Wear modality [daily, monthly]	**0.0001**	0.04	12	Spearman
Lower	L	SCL	LWE*	**0.001**	0.67	19	Spearman
Lower	L	SCL	Palpebral papillae*	**0.04**	0.46	19	Spearman
Lower	M	SCL	Age [years]	**0.04**	0.43	25	Pearson
Lower	M	SCL	Years of wear [years]	**0.01**	0.5	25	Pearson
Lower	M	SCL	OSDI score	**0.03**	-0.43	25	Spearman
Lower	L	sSCL	LWE*	**0.01**	0.77	10	Spearman
Lower	M	sSCL	Age [years]	**0.03**	0.6	13	Pearson
Lower	M	sSCL	Gender	**0.0001**	-0.39	13	Spearman
Lower	M	sSCL	Wear modality [daily, monthly]	**0.0001**	-0.53	13	Spearman
Lower	M	sSCL	Bulbar hyperemia*	**0.01**	-0.8	7	Spearman
Upper	L	aSCL	Weekly average wear days [days/week]	**0.01**	0.65	14	Spearman
Upper	M	aSCL	Lens type [Hy, SiHy]	**0.03**	0.67	11	Spearman
Upper	L	aSCL	LWE*	**0.03**	0.61	13	Spearman
Upper	M	aSCL	Palpebral hyperemia*	**0.02**	-0.73	8	Spearman
Upper	L	SCL	Weekly average wear days [days/week]	**0.03**	0.41	27	Spearman
Upper	M	sSCL	Gender	**0.0001**	-0.3	12	Spearman
Upper	M	sSCL	LWE*	**0.02**	0.86	7	Spearman

Examples of IC collections from high LWE grade participants are shown in Figure 62.
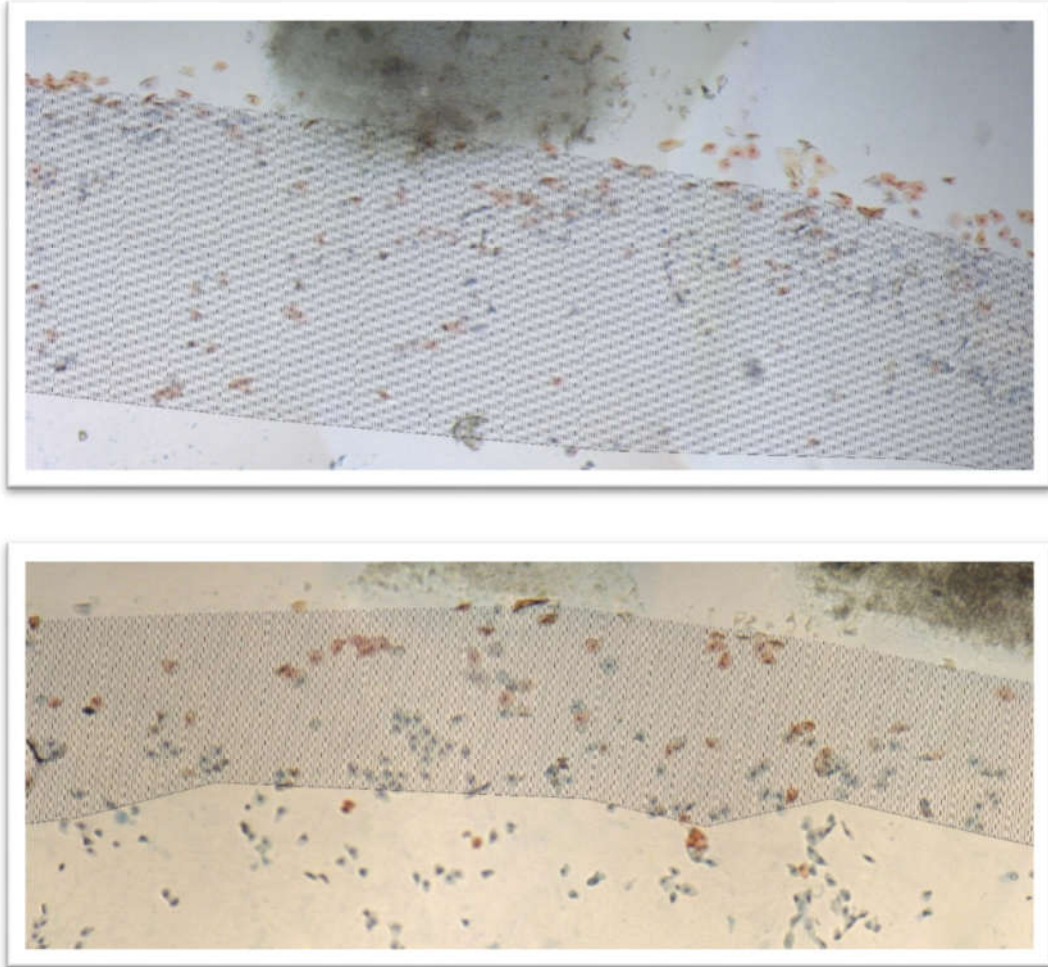
Figure 62: Two cropped IC collections from high LWE participants.

Keratinized (red/orange) cells extend away from the MCJ, interspaced in the lid wiper area. Shaded area shows measured width of lid wiper.

5.5 Discussion

5.5.1 Improved study design and methodology

The IC collection, staining, imaging and analysis techniques were developed and optimized in chapter 3, and tested in a pilot study described in chapter 4. This study expands, refines and enriches the knowledge gained on the epithelium at the lid margin. While both upper and lower lid margins were investigated, their comparison is reserved for the final chapter of this thesis.

A compelling improvement from the previous chapter was rethinking and loosening up the recruitment criteria, by focusing participant enrolment on symptomology, rather than LWE, and thus enabling a larger sample size. LWE was assessed only with LG (and not LG + NaFl), since LWE studies in recent years have mostly abandoned the use of NaFl for this purpose [48]. Study visits were not scheduled on separate days, but rather consecutive and on the same day, allowing a more efficient work-flow. Still, due to the high volume of laboratory and time-consuming image acquisition work, no more than one participant could be enrolled per day.

The histochemical processing was performed without separating the membrane from the plastic holder, which was convenient, efficient and allowed a consistent staining duration for all four samples stained simultaneously. This improvement not only ensured stain color consistency between samples, but also an increased cell collection area (edge-to-edge), as membranes remained in the holder, minimizing the need for handling with tweezers and the potential loss or damage of cells in the preparation process.

Given the larger number of histochemical images and their previously discussed variation in quality, a novel grading system was developed, which provided reliable, repeatable results and aided a more efficient method of dimensioning cellular areas, discussed below. Sample grading and dimensioning was performed using masking, ensuring an unbiased analysis. With increasing investigator experience, more consistent applications of the membrane to the lid margin were possible, presumably reducing previously discussed effects of application angle, pressure etc. on collection quality. As collection quality varied nevertheless, discontinuous cell collections may be less likely related to investigator error, and perhaps resemble individual variation and differences in structural features of the epithelium. Furthermore, we observed that the application pressure itself may express Meibum, which appears to hinder the adherence of cells to the membrane. While Jalbert reports that 67% of all samples were analyzable [24], less than half of all samples in this study were analyzed. This discrepancy may stem from the fact that Jalbert analyzed much smaller samples, whereas our panoramic stitching technique allowed for much greater collection areas, which in turn diminished the likelihood of obtaining continuous collections.

5.5.2 Participant demographics and clinical findings

The distribution of participants according to Young's CL-related dryness classification scheme was uniform (Figure 48), while the exclusion of marginal subjects ensured a clear polarization between symptoms in the two groups. After enrolment, all three subjective comfort questionnaires mirrored this division, upholding the reliability of this polarization system as a quick and efficient tool to triage study

participants by symptomology. The OSDI scores for group aSCL and sSCL were significantly different, and respectively well below and above the critical threshold of 15, shown to separate normal from dry eye type responses [99]. Statistically significant differences were recorded between CLDEQ-8 scores, and for diurnal 0-100 scores for comfort and dryness at all time-points. The differences in CL wear experience and wear habits further underpinned this trend. In group sSCL, only half of the daily CL wear time was considered comfortable, while group aSCL subjects reported comfortable wear until the last hour before lens removal. This may also be reflected in the significantly lower average age of group sSCL participants (p=0.04, Mann Whitney U test) compared to that of group aSCL, and their much shorter wear experience, almost half of that found in group aSCL. An explanation for this may be that nearly half of symptomatic lens wearers are known to eventually drop out of CL wear [145], and are likely doing so at an earlier age, which would result in a shorter wear experience (than asymptomatic wearers). Ultimately, this has affected our efforts of age-matching the groups. Nevertheless, the average participant age in both groups is close to that of the average CL wearer [159], while opening up recruitment to the general population of a middle-sized urban area (as opposed to limiting to university students), allowed for a diverse and more relevant sample that resembled the typical CL wearing population.

Sample size and the average age were improved compared to those of Knop's cytological report on the lid margin (ten cold stored cadavers, average age of 77 years) [13], and more similar to that of Jalbert's paper, which reports an identical sample size and a lower participant age (26 years) [24]. In contrast to this study, Jalbert does not distinguish symptomology, but compares CL-wear versus neophytes, mentioning that subjects were healthy and free from ocular disease. Jalbert also graded upper LWE stained with LG, and their distributions are similar to ours for grades 2 and 3, but differ for grades 0 and 1. This is less likely related to symptomology, as distributions in groups aSCL and sSCL are identical in this study, and rather resembles a common issue with LWE grading, wherein the Marx' line is not always clearly distinguished from grade 1 LWE [6,48,81]. Similar to the results of the previous chapter, no significant difference in LWE grade was found between symptomatic and asymptomatic CL wearers, which is more in line with other recent publications [48,66,167] and fails to demonstrate the initially proposed relationship between dry eye symptoms and high LWE grades [6,35,42]. This is further supported by significantly different comfort of the study groups according to four separate comfort questionnaires, yet the LWE grade remained unchanged between the groups. Furthermore, LWE did not correlate with any other study variable or clinical finding, with the exception of a weak but significant correlation with the average wear time in group sSCL.

All study variables and clinical findings were tested for correlation with the width of the LW and MCJ areas (discussed below), resulting in 668 correlation pairs. Of these, 24 (less than 4%) were significant. Given the large number of pairs, many of the significant results may be spurious, which is also highlighted in the relatively low r-values of under 0.5. From these results, it appears that clinical signs or patient history do not correlate with the width of cellular zones at the lid margin. A single exception, perhaps a most exciting one, is that, more than any other study parameter, LWE correlates in four separate instances with the width of the lid wiper and the MCJ, showing some of the largest correlation coefficients (r-values of up to 0.86). Although not statistically significant, LWE correlated with L- and M-cell widths in six more instances, with p-values between 0.06 and 0.08, and r-values between 0.6 and 0.8. This further reinforces the consistency and reliability of the analysis method and follows up on Jalbert's suggestion that IC could help investigate LWE [24].

5.5.3 Cytological findings

Before delving into the discussion of the width of the lid wiper and MCJ areas, it is essential to revisit their definitions, since, as shown in the introduction, these appear to be somewhat equivocal in the literature. In addition, the increased sample size from the previous chapter helped refine some of our initial observations. In contrast to Knop's trans-sectional excisions accurately detailing the deeper layers of the lid margin, our wide samples offer a much better view of the surface of the epithelium; it is this uppermost surface that is subject to friction through the immediate contact with the cornea or a CL during blinking.

As pointed out in the methods section, the end of the lid wiper was defined as the line where large squamous cells transition to columnar/cuboidal cells. According to Parsons [23], it is the pressure between the lid wiper and the ocular surface which may be the cause of flattening of the superficial cells, convention suggesting that surfaces exposed to a certain degree of mechanical friction are typically composed of squamous epithelium, such as the cornea, oral epithelium, or esophagus [47]. The expectation would therefore be that the lid wiper – as a surface that would experience extensive frictional forces because of blinking – would largely be comprised of squamous epithelium and that as soon as pressure and/or friction would "abruptly" cease, cells would transition to a different morphology, identified as C-cells in the previous chapter. In dimensioning over 100 IC samples and as shown in Figure 59, this width rarely exceeded 800 μm, and typically averaged ca. 400 μm, which is less than reported by Knop et al. [13]. Additionally, our observation is that most often this change in cell size coincides with the occurrence of GC and their expressions (Figure 43), presumably as a result

of membrane application pressure. From an anatomical and physiological perspective, GC at the surface of the tarsal conjunctiva or Kessing space would move to the deeper layers in the LW [34], being substituted by cells designed to withstand mechanical action at the lid wiper. This further supports the theory that contact with the surface of the eye only occurs here. The presence of (para-) keratinized epithelial cells in the LW of high LWE participants (Figure 62), suggests that friction – or some form of mechanical action – is increased here, reinforcing the mechanical etiology of LWE, presumably as a result of an altered tear film or the presence of a foreign body, such as a CL. According to the Stribeck curve of classical tribology, three different regimes are recognized in the relationship between friction, load, speed and lubricious fluid properties, ranging from the hydrodynamic regime, in which a full lubricant film is present between surfaces in opposition and motion, through the mixed regime allowing occasional contact between solid surfaces, to the boundary lubrication dominated by the close contact of solid surfaces (high friction) (Figure 63) [176].
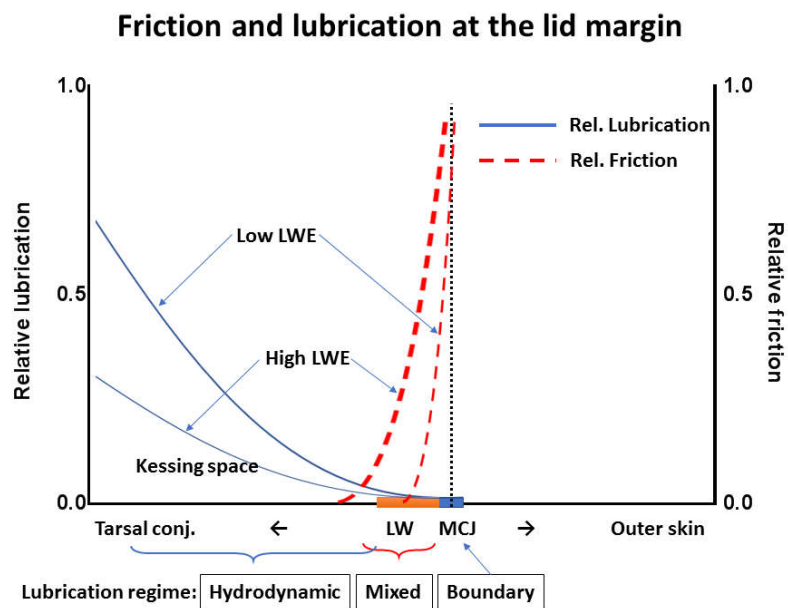
Figure 63: Proposed relationship between friction and lubrication at the lid margin during the blink.

As observed in chapter 4, as we approach the MCJ, cell size increases, GC impressions and expressions disappear altogether and keratinization increases, abruptly culminating at the MCJ. Assuming that the proposed relationship between friction and LWE is true – and the correlation between keratinization and LWE discussed above certainly supports that – then that would suggest that the MCJ, representing

a maximum in cell size and keratinization, actually makes <u>most</u> contact with the ocular surface, supposedly for a very brief moment during the blink, implying that boundary lubrication occurs here. This would refute Knop's stance on the MCJ not being part of the lid wiper [13,33], and rather align with earlier descriptions by Shaw [31], Doughty [46] and even Ehlers [22], who suggest that the Marx line *does* come in contact with the eye. Our findings suggest that both LWE and the Marx line may have a common, frictional etiology. While the microscopic dynamics of the lid margin during blinking are not fully understood, it is conceivable that the (upper) blinking motion may exert sufficient friction at the lid margin to elastically retract the Marx line just enough, such that it would briefly touch the ocular surface and return to its "resting position" after the completion of the blink (Figure 64). Far from being a static structure, the Marx line is known to travel distally with age and even alter its location and morphology depending on friction, in patients with Meibomian Gland Dysfunction (MGD) [173]. If the upper lid travels 10-12 mm during a blink, assuming that the proximal border of the MCJ (i.e. the beginning of the lid wiper) is in contact with the ocular surface and its width is about 0.3 mm, it would mean that its displacement would measure 0.3 mm/10 mm, or 30 μm/mm. Cells in this region have diameters of 30-50 μm [9], meaning that the MCJ would displace at a rate of <1 cell/mm during the blink. Such a minute mobility during blinking should not be excluded. Yet surprisingly, the MCJ/Marx line are conventionally described as static structures, whether in tissue excisions, but especially clinically, and for the lower lid in particular, as the Marx line is visible without everting the lid, in the open eye position. It may be worthwhile remembering that these biological structures should be considered in motion, as part of a dynamic system. Instead, we should consider the possibility that the eversion act itself may be "invasive", as the applied pressure could exacerbate the dimensions and misrepresent the location of these minuscule features.
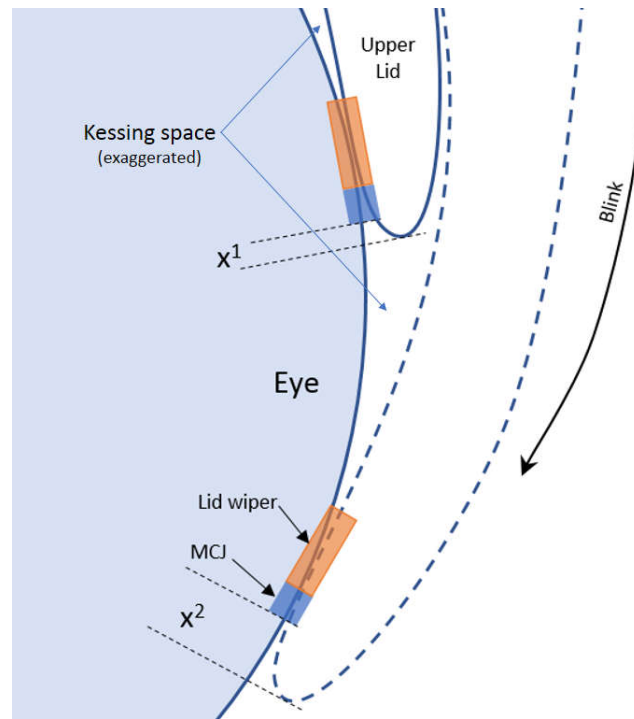
Figure 64: Proposed dislocation ($x_1 - x_2$) of lid wiper and MCJ during the blink, resulting in a brief contact between MCJ and the ocular surface.

At the same time, it is well established that the lower lid margin does not travel nearly as much as the upper lid during habitual blinking, and so the blink-related frictional etiology (of LWE and the Marx line) may not sufficiently explain these phenomena. It has been suggested that other mechanisms, for instance horizontal micro-movements of the eye during the inter-blink phase, fixation saccades or micro-tremor may additionally contribute to LWE-causing friction [48]. These differences between the upper and lower lid margins shall be further explored in the final chapter of this thesis.

5.5.4 Dimensional analysis

The width of the MCJ and the lid wiper were bilaterally comparable, suggesting a reliable collection and analysis technique. Yet, it is still unclear whether the fact that only around half of all collections were analyzable is attributed to individual subject variation or the previously discussed application variables. Despite this, the measured lid wiper and MCJ widths were almost identical in the two study groups, suggesting that CL-related discomfort is not reflected in the epithelial cyto-morphology of the lid margin. This complements our observations of LWE staining indiscriminately of comfort, yet does

not fully absolve the role of the lid margin in the relationship with discomfort, as for instance, the neuroanatomy of the lid margin still remains largely unexplored [48].

Considering individual, anatomical variations, as well as methodological differences outlined above, our findings broadly align with previous reports, perhaps with the exception of the lid wiper width, which tends to be somewhat smaller than Knop's description [13]. They note that the LW can be up to 1.5mm wide or more, mentioning differences between locations along the lid margin, with nasal/temporal locations having much wider lid wipers, compared to the central lid margin. Although our observations were limited to the central lid margin, individual variations were noticed, as indicated in Figure 60. While mathematically these are considered outliers, they have been treated as natural variation and included in the analysis, as this work is still largely exploratory in nature. The positive correlations of these values with high LWE grades further support the view that they might not be outliers, but, in light of the earlier discussed frictional aspects, may underpin the proposed etiology of LWE. Nevertheless, this hypothesis should be carefully considered, given the relatively small number of high LWE participants (Figure 65, Figure 66). Future studies with larger sample sizes and perhaps even stronger comfort polarization schemes may be able to further investigate this observation.
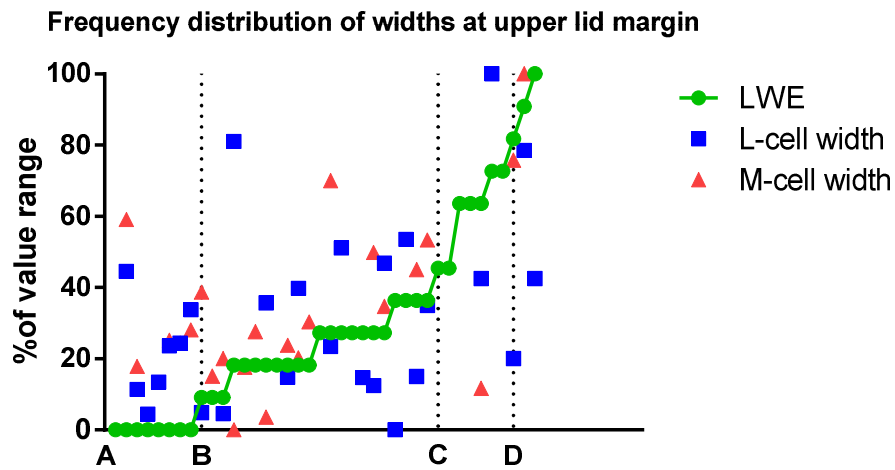
Figure 65: Frequency distribution of LWE grade, width of LW and width of MCJ at the upper lid margin.

Values have been normalized and are expressed as percentages of the total range of each metric. Each metric's maximum (100%) is: LWE=Grade 2.75; L-cell width=770 μm; M-cell width=231 μm. The x-axis represents grouping bins with reference to the LWE grade: A – B = None; B – C = Mild; C – D = Moderate; D+ = Severe.
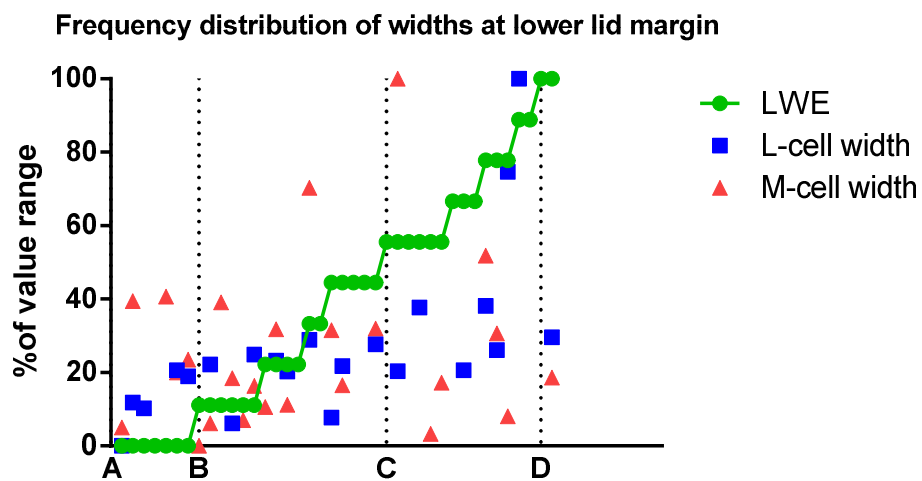

Figure 66: Frequency distribution of LWE grade, width of LW and width of MCJ at the lower lid margin.

Values have been normalized and are expressed as percentages of the total range of each metric. Each metric's maximum (100%) is: LWE=Grade 2.25; L-cell width=585 μm; M-cell width=225 μm. The x-axis represents grouping bins with reference to the LWE grade: A – B = None; B – C = Mild; C – D = Moderate; D+ = Severe.

5.6 Conclusion

This is the first account of a cytological description and comparison of the lid margin conjunctiva between symptomatic and asymptomatic CL wearers. Featuring a larger sample size, a representative demographic and improved measurement criteria from the previous chapter, this study offered a more detailed and concise description of the lid wiper and MCJ surface dimensions, than available to date. These measurements are an essential complement and improvement to the detailed, classical histological excisions, as well as a first account on the cytological perspective of LWE stained with LG, showing a correlation between high LWE grades, and enlarged LW/MCJ, and increased para-keratinization in these areas. We also propose that the Marx line does make contact with the ocular surface or a CL during blinking, representing a frictional, and keratinization maximum in the transitional morphology of the lid margin. Comfort (or the lack thereof) does not appear to be reflected in the width of cellular areas of the lid margin, both symptomatic and asymptomatic SCL wearers exhibiting comparable LW and MCJ widths, as measured by histology. Future studies may consider employing a stronger polarization scheme, by recruiting severe dry eye patients, or people with Sjogrens syndrome, and comparing them with asymptomatic normals.

We have followed up on Jalbert's suggestion that LWE may be investigated using IC and confirmed this in our findings. At the same time, it was not possible to determine from their work whether cell morphology relates to CL wear in and of itself. Since discomfort remains a central issue for CL wearers and researchers alike, this relationship shall be further investigated in the upcoming chapter.

This chapter was limited to the separate comparison of the upper and lower lid margins, yet there were noticeable differences observed between the upper and lower lid margin widths. Several publications have suggested that the lower lid margin is subject to different mechanisms and interactions than the upper lid margin, therefore this relationship shall be investigated in the final chapter of this thesis.

Chapter 6

Manifestations of lens wear at the lid margin in rigid and non-contact lens wearers

6.1 Overview

PURPOSE: The purpose of this study was to assess the lid margin epithelium of rigid gas permeable (RGP) contact lens (CL) wearers and non-lens wearers (nCL).

METHODS: Eighteen RGP wearers and 19 nCL were enrolled in two study groups. Comfort was assessed using the Ocular Surface Disease Index (OSDI), the short Contact Lens Dryness Experience Questionnaire (CLDEQ-8) and diurnal 0-100 scales for comfort and dryness. Lid wiper epitheliopathy (LWE) was assessed using lissamine green (LG) and impression cytology (IC) performed on the upper and lower lid margins using Millicell cell culture inserts. Samples were stained with Alcian Blue, Hematoxylin and Papanicoloau dyes, and the lid wiper (LW) and muco-cutaneous junction (MCJ) cellular areas dimensioned using ImageJ.

RESULTS: RGP wearers reported overall similar or better comfort than nCL ($p>0.05$). Average LWE grades ($\pm$SD) were significantly different, for both upper (RGP: 1.66$\pm$0.97; nCL: 0.44$\pm$0.75; p=0.0002) and lower (RGP: 1.48$\pm$0.94; nCL: 0.39$\pm$0.49; p=0.0001) lid margins. The average width of the upper (RGP: 666$\pm$219 μm; nCL:265$\pm$64; p<0.0001) and lower LW areas (RGP: 518$\pm$211; nCL: 224$\pm$101; p<0.0001) was significantly higher in RGP wearers, and correlated well with the LWE grade (p<0.01, r=0.78 to 0.89).

CONCLUSION: RGP lens wearers experience higher LWE grades but similar or better comfort than nCL. This is the first study to show that rigid lens wear is associated with up to three times wider LW areas in the upper and lower lid margins than those who do not wear lenses, providing strong evidence that mechanical interactions with a CL may alter the cyto-morphology of the lid margin epithelium.

6.2 Introduction

In the previous chapter, CL-related discomfort did not appear to relate to changes in cells of the lid margin, as both vital staining (LWE) and cellular morphology did not differ between symptomatic and asymptomatic lens wearers. However, we could show that there may be an association between higher LWE grades and the width of cellular areas and/or cellular keratinization, presumably as a result of increased friction at the lid margin. While the idea that the presence of a CL may alter friction on-eye was proposed as early as 1936 and again in 1965 [22,69], it has only recently become the subject of increased attention [71,111], after Nairn and Jiang [70] measured coefficients of friction *in vitro* on CL in 1995 and Korb et al. introduced the idea of LWE [6]. Yet to this day, measuring friction at the eyelid margin remains impractical.

Using a diverse range of methodologies of measuring friction on CL, a number of recent accounts have showed that comfort with CL is closely tied to friction [71,72,108–111]. With the primary hypothesis for the etiology of LWE being increased friction, these findings have spurred further research into the role of the LW for CLD. Yet, Efron et al. recommend erring on the side of caution and not assuming a causal relationship. Instead, they advanced the idea that other common factors, such as lens modulus and edge design, may determine or contribute to comfortable CL wear [48].

The introduction of soft, flexible poly-hydroxyethyl methacrylate (HEMA) materials markedly improved comfort from the original poly-methyl methacrylate (PMMA) lens designs. The larger size, reduced on-eye mobility and, particularly, the decreased stiffness of SCL are thought to be among the causes for superior wearer comfort. To this end, the edge design of modern silicone hydrogel lenses has received a great deal of attention, with various geometries existing today (Figure 67). Hereby, the assumption is that the lens edge profile may be a decisive factor in avoiding the common "foreign body sensation" experienced by CL wearers, since the lid margin needs to surmount this lip over 10,000 times per day during the process of blinking.
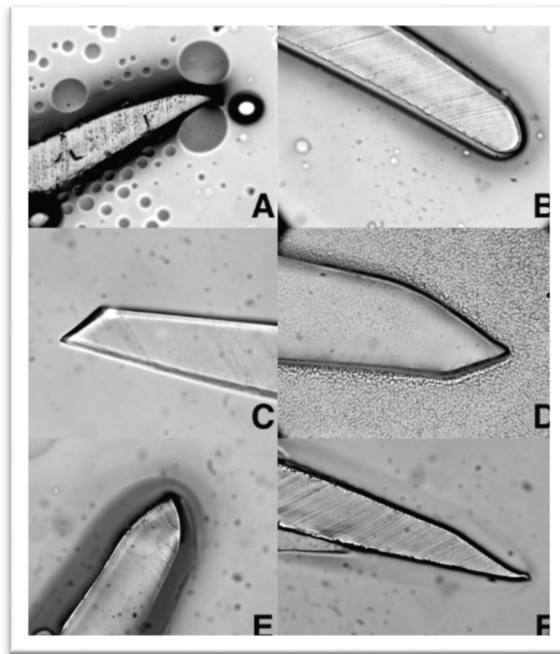
Figure 67: Edge profiles of common soft CLs

(A: Johnson & Johnson Acuvue Oasys, B: Bausch & Lomb PureVision, C: CIBAVision Dailies, D: CooperVision Clariti monthly, E: CooperVision Biofinity, F: Johnson & Johnson 1-Day Acuvue Moist). Images taken under 40x magnification after freezing the CLs and sectioning using a cryo-microtome.

In RGP lenses, edge design and its interaction with the eyelid margin was confirmed to play a major role in CL comfort and success [177]. These lenses are markedly thicker, smaller and up to 1000 times stiffer than SCLs [178–180], conceivably effecting a greater mechanical action between ocular tissues and the CL during the blink. These same features, which RGP lenses are generally praised for as ideal choices for bio-physiological reasons, offering superior tear exchange, oxygen permeability and a lower risk of infectious events [180], may have also lead to their decline in popularity over the past decades. Rigid lenses are commonly associated with reduced initial comfort, particularly in contrast to modern soft daily disposable materials. While market penetration rates of RGPs still vary greatly around the globe (compare a world average of 7% of all CL fits representing RGP CL, with 50% in Germany [166]), their popularity has dwindled. Today, the number of RGPs being fit has been overtaken by modern soft materials, which is in no small part, thanks to their unparalleled initial comfort.

Contact lens related discomfort

At the same time, discomfort remains the leading cause for ceasing CL wear, whether for rigid (58% of drop-outs), or soft lenses (40% of cases) [181]. The mechanism by which CL-related discomfort occurs remains unknown, but it appears that lens wear leads to corneal sensitivity loss and a reduction in Meibomian gland function [92]. While the mechanism for corneal sensitivity loss with different materials is debated [92,182], there are numerous studies demonstrating a reduction in corneal sensitivity with PMMA, RGP [182–185] and even conventional hydrogel [182,186] CLs, although this effect appears to have subsided with recent material developments [187].

There is comparatively little information available on the effects of CL wear on the lid margin. A decrease in lid margin and tarsal conjunctival sensitivity in response to PMMA, RGP, and low oxygen transmissibility SCL was noted as early as 1968, but it is unclear which specific lid marginal region(s) were assessed in those studies [123,187], as the MCJ and LW had not been clearly differentiated at the time. To date, a single account of assessing LWE in rigid lens wearers exists [188]. Featuring a large sample size of over 500 participants, upper and lower LWE was measured in Japanese soft, rigid and non-lens wearers. Rigid lens wearers were shown to exhibit significantly more and higher grades of LWE compared to both other groups, and a higher prevalence of LWE was noted in younger patients, which they attributed to the higher eyelid tension at younger ages, as opposed to increased laxity in older age [189]. Unfortunately, the authors do not report subjective comfort scores and the racial distribution of patients is omitted. Given the different eyelid anatomy and probable eyelid pressure and tension in Asian populations, this may have been a valuable discussion with regards to LWE. Finally, Shiraishi does not elaborate on the findings in rigid lens wearers, limiting their results and discussion to only reporting the values of prevalence and severity in the upper and lower lid margins.

Therefore, investigating LWE as well as the cellular morphology in rigid lens wearers may be of great value, particularly in contrast with a well-matched non-lens wearing control group, to better understand CL-induced changes at the lid margin.

6.3 Materials and methods

Eighteen habitual RGP CL wearers and 19 non-lens wearers were enrolled in this prospective, non-dispensing study. LWE was graded using LG and IC conducted on the upper and lower lid margins. IC samples were processed and evaluated using methods described in the previous chapters.

6.3.1 Subject recruitment

All clinical studies followed the ethical principles in the Declaration of Helsinki and complied with the ICH guidelines for Good Clinical Practice (GCP), with the University of Waterloo's Guidelines for Research with Human Participants and with the Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans, 2nd Edition. Informed consent was obtained from all participants prior to enrolment. The study received approval from the Office of Research Ethics at the University of Waterloo, Waterloo, Ontario, Canada (ORE #20958). The study was advertised using the recruitment system at the Centre for Contact Lens Research at the University of Waterloo.

6.3.2 Inclusion and exclusion criteria

Participants were subject to the same inclusion and exclusion criteria outlined in chapter 5.

6.3.3 Study procedures

All study procedures employed in this study, including clinical techniques, impression cytology and IC sample processing have been described in section 4.3.3.

6.3.3.1 Subjective comfort questionnaires

All participants completed the OSDI questionnaire and the 0-100 scales for diurnal changes in comfort and dryness as described in section 4.3.3.1. Participants in group RGP also responded to the CLDEQ-8 questionnaire.

6.3.4 Group assignment

After signing the informed consent form and being considered eligible for inclusion in the study, CL wearing participants were allocated to group RGP and non-lens wearers to group nCL (Table 18). Ineligible participants were discontinued from the study.

Table 18: Overview of study groups comprised in the analysis

Study group code	Description
RGP	Rigid gas permeable lens wearers
nCL	Non-lens wearers

6.3.5 Study visits

Eligibility and study grouping were determined at the screening visit, and LWE measured and IC performed on the upper and lower lid margins at the study collection visit. The two study visits were consecutive, totaling 1.5 hours and only one participant was enrolled per day. Samples underwent histochemical processing and analysis thereafter. Data were recorded on CRFs.

6.3.6 Analysis

IC sample quality grading and analysis are described in section 5.3.6.

6.3.6.1 Data analysis and plotting

Statistical analysis and graphs were created using GraphPad Prism (GraphPad Software, Inc., San Diego, California). For boxplot graphs (Figure 68), the bottom and top of the box represent the first and third quartiles, and the band inside the box signifies the second quartile (the median). The ends of the whiskers indicate the lowest datum still within 1.5 IQR of the lower quartile, and the highest datum still within 1.5 IQR of the upper quartile. Dots outside the whiskers stand for outliers identified using the Tukey method. Asterisks denote statistically significant differences between the two groups comprised within the square brackets.
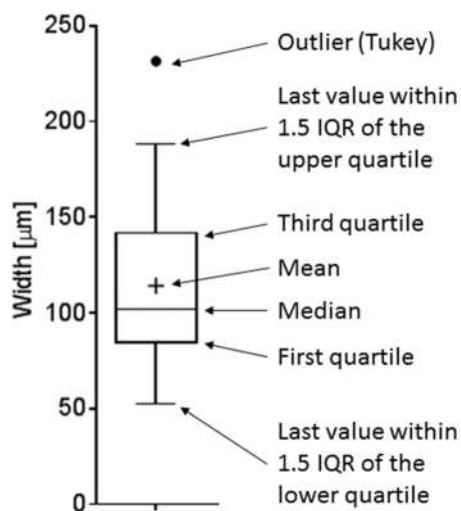
Figure 68: Elements of a boxplot graph

Differences between groups and study parameters were determined using the unpaired t-test or the Mann-Whitney test, and correlations between study parameters and variables were tested using Pearson and Spearman coefficients. A significance level (p) of $\alpha=0.05$ was assumed for every statistical test unless otherwise noted.

6.4 Results

6.4.1 Demographics

Thirty-seven participants were screened and completed the study between November 2015 and December 2016. In group RGP, 18 participants (89% females, average age ± SD of 42 ± 21 years, ranging between 18 and 72 years) and in group nCL, 19 participants (85% females, average age ± SD of 41 ± 20 years, ranging between 20 and 75 years) were enrolled (Figure 69).
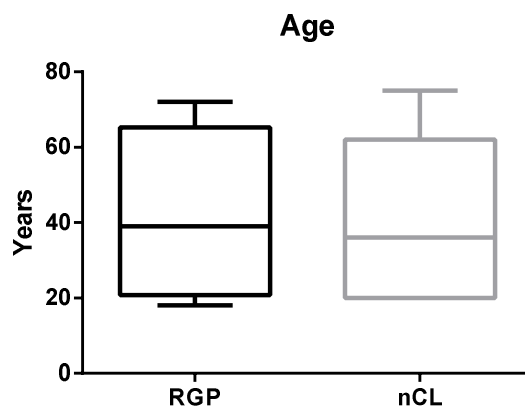
Figure 69: Distribution of participant age by study group

Participants in group RGP reported an average daily wear time (mean ± SD) of 12.7 ± 2.7 hours per day, out of which 11.6 ± 3.1 hours were reported as being comfortable. Lenses were typically worn for 6.3 ± 0.9 days per week, with an average lens wear experience of 24.3 ± 18 years.

Thirty-three percent of participants in group RGP and 15 percent of participants in group nCL reported allergies, none of these reporting any currently active episodes of ocular allergies. Six participants in group RGP and one in group nCL reported the daily use of lubricating drops.

6.4.2 Subjective comfort scores

6.4.2.1 Ocular Surface Disease Index (OSDI)

Average (± SD) OSDI scores of 8.3 ± 5.1 and 14 ± 15 were recorded in group RGP and nCL respectively (Figure 70). These were not statistically significantly different.
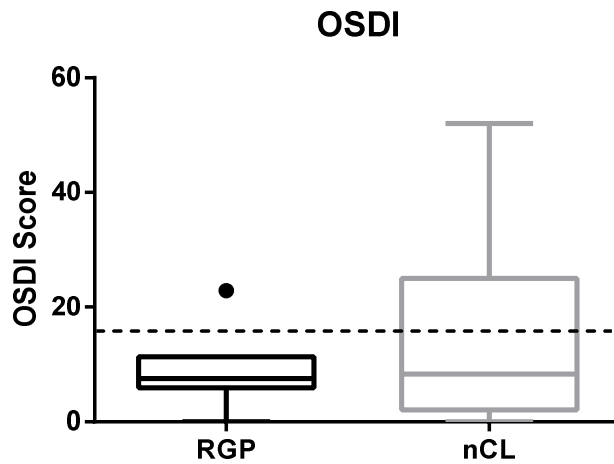
OSDI

Figure 70: Distribution of OSDI scores between study groups.

Dashed line indicates threshold value (15) above which patients are considered symptomatic.

6.4.2.2 Contact Lens Dry Eye Questionnaire (CLDEQ-8)

An average CLDEQ-8 score (± SD) of 9.5 ± 5.2 was recorded in Group RGP. The distributions of responses are displayed in Figure 71.
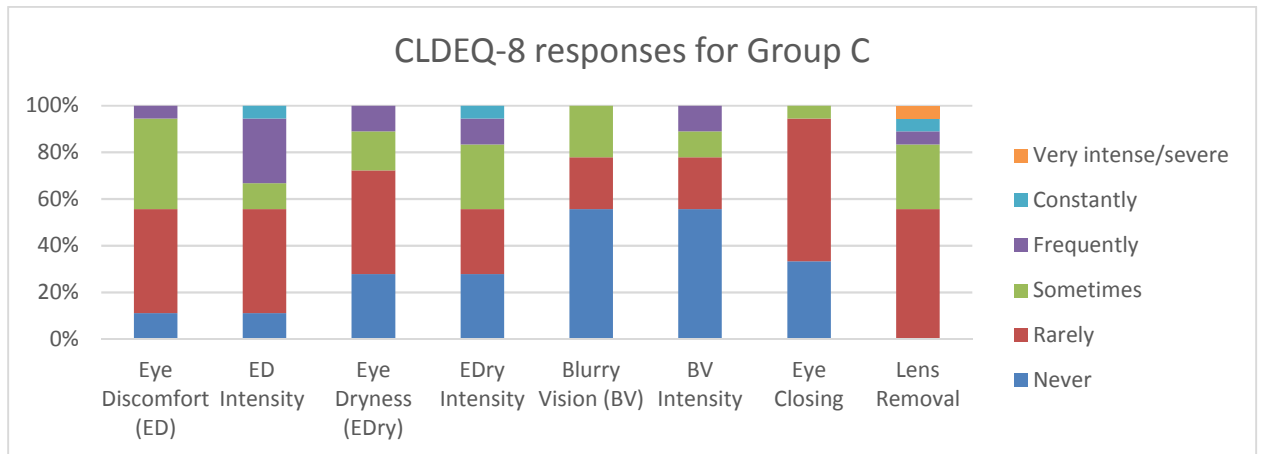
Figure 71: Distributions of CLDEQ-8 responses in the two study groups

6.4.2.3 Diurnal comfort and dryness

The average scores for diurnal comfort and dryness are summarized in Table 19 and displayed in Figure 72 and Figure 73.

Table 19: Average (±SD) diurnal scores for comfort and dryness. Bold values indicate statistical significance.

		Group RGP	Group nCL	RGP vs. nCL (Mann Whitney U)
Comfort	**Morning**	94 ± 10	81 ± 18	**p=0.0272**
	Noon	89 ± 20	91 ± 13	p = 0.64
	Evening	81 ± 19	81 ± 25	p = 0.63
Dryness	**Morning**	95 ± 7	81 ± 26	p = 0.12
	Noon	88 ± 19	82 ± 22	p = 0.67
	Evening	83 ± 20	81 ± 25	p = 0.61
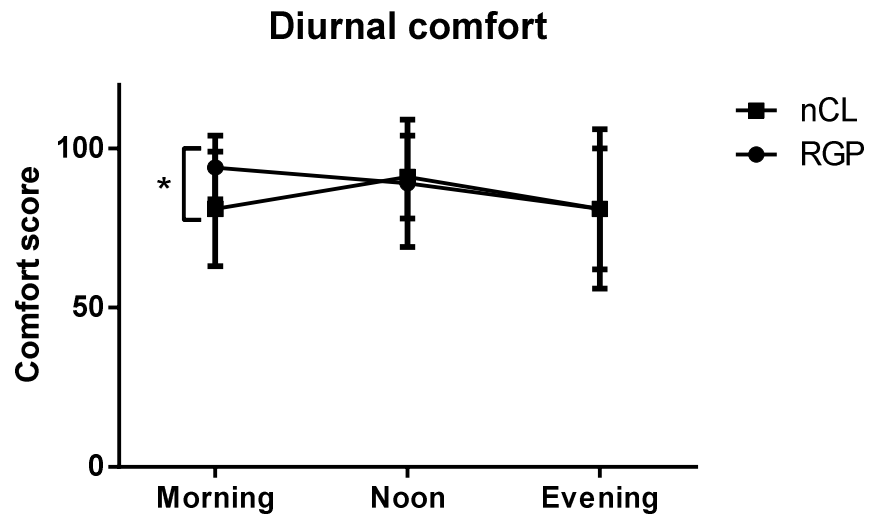
Figure 72: Average diurnal scores for comfort, error bars indicate SD.
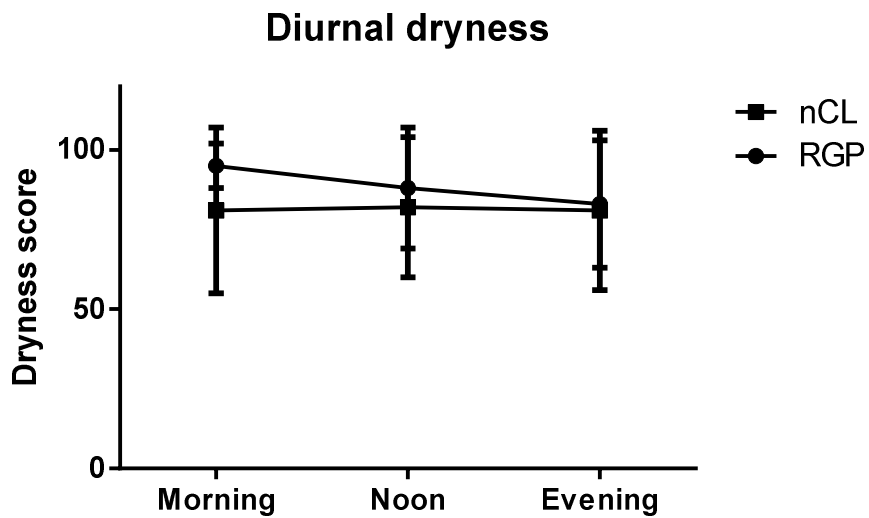
Figure 73: Average diurnal scores for dryness, error bars indicate SD.

6.4.3 Lid wiper epitheliopathy

The average LWE grades are summarized in Figure 74 and their distribution is shown in Figure 75. Differences between the groups were significant (Table 20) and the grades were well correlated bilaterally Table 21.

Table 20: Average (±SD) LWE grade (OU). Bold values indicate statistical significance.

LWE	Group RGP	Group nCL	RGP vs. nCL (Mann Whitney U)
Upper	1.66 ± 0.97	0.44 ± 0.75	**p=0.0002**
Lower	1.48 ± 0.94	0.39 ± 0.49	**p=0.0001**
Upper vs. lower (Spearman correlation)	r=0.65 **p=0.0038**	r=0.67 **p=0.0018**	
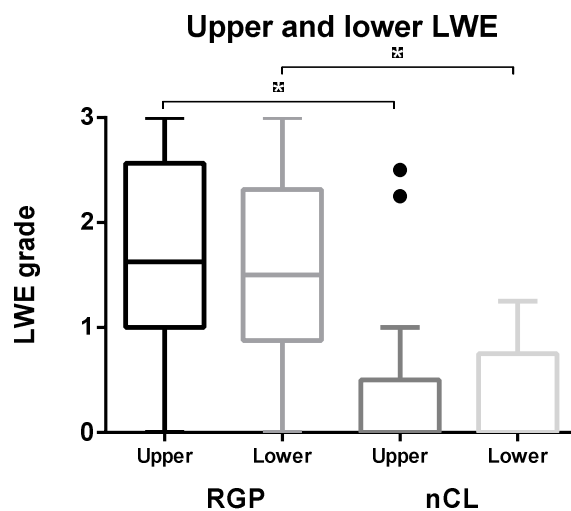
Figure 74: Upper and lower LWE grade in the two study groups.

The prevalence of LWE grades in group RGP was 11% for "none", 22% for "mild", 33% for "moderate" and 33% for "severe", in both upper and lower lid margins. In group nCL, 58% and 52% had no LWE in the upper and lower lid margins respectively, while 31% of all cases were "mild" grades, with "moderate" and "severe" grades combined totaling less than 20% in this group.
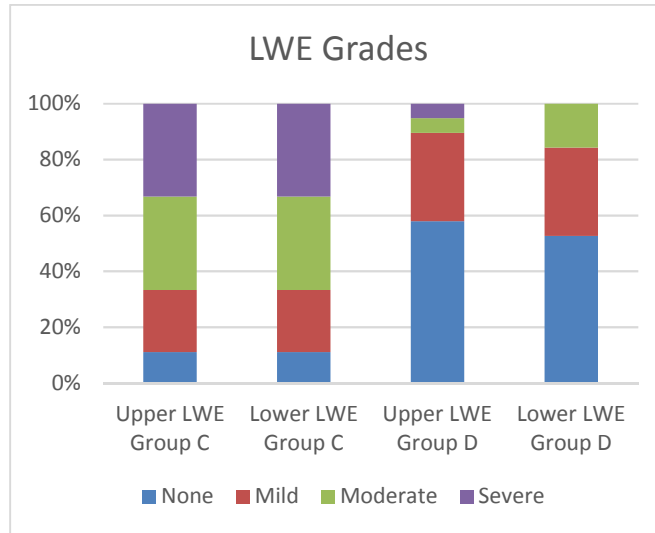
Figure 75: Distribution of LWE grades.

Table 21: Correlation of LWE grades between the left and right eye (Spearman correlation).

Group	Upper LWE	Lower LWE
RGP	r=0.78	r=0.85
	p=0.0001	**p<0.0001**
nCL	r=0.94	r=0.91
	p<0.0001	**p<0.0001**

The LWE grade correlated with other study variables solely in group RGP, and only in the lower lid. The statistically significant correlations are depicted in Figure 76.
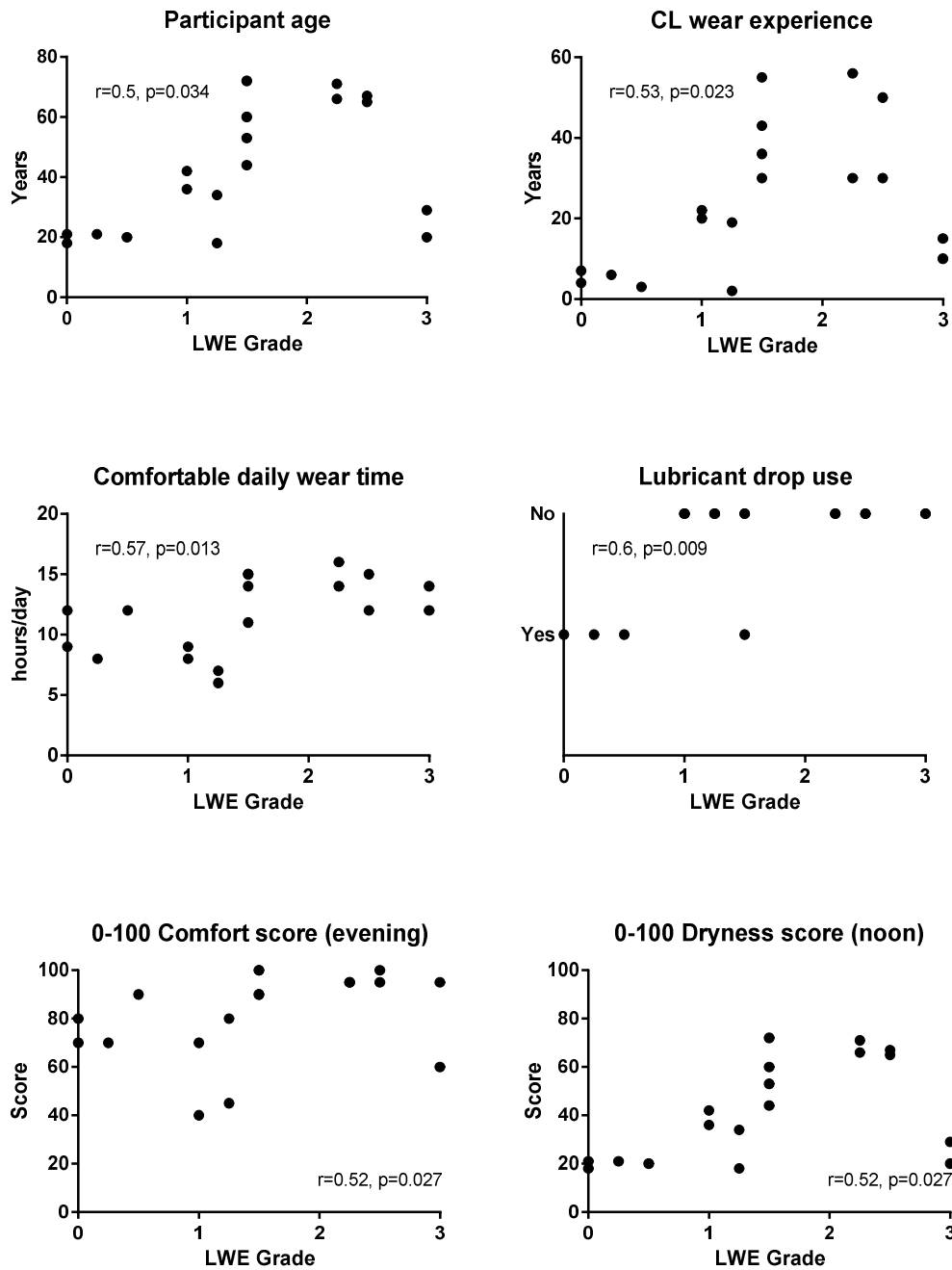
Figure 76: Statistically significant correlations between LWE grade and study variables. Spearman r and the p-value are given in each case.

Figure 77: LWE stained with LG in the upper lid of RGP wearers in group RGP. Staining is limited to the central part of the lid margin, corresponding to the area touched by the CL.

6.4.4 Histological analysis

6.4.4.1 Collection quality grading

The accuracy/repeatability of the double grading of samples in group RGP and nCL was 96.5% and 98% respectively. A total of 77 samples (52.3% of total of 147 samples) were graded either 3 or 4 and were considered analyzable (Figure 78).
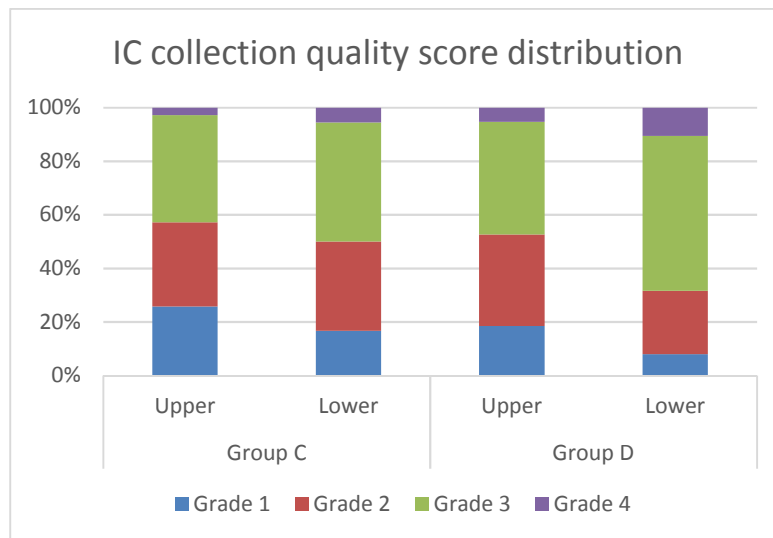
Figure 78: Distribution of collection quality grading of IC samples

6.4.4.2 Dimensional analysis

The lid wiper and MCJ areas were measured in 103 instances. Each measurement was computed from up to 1000 single width measurements per sample and reported as an average value and SD. While analysis and comparison of widths employ the average values, SDs were not included in the analysis. These averaged at 28.2 ± 13.2 % of each respective mean width value, as shown in Figure 79.
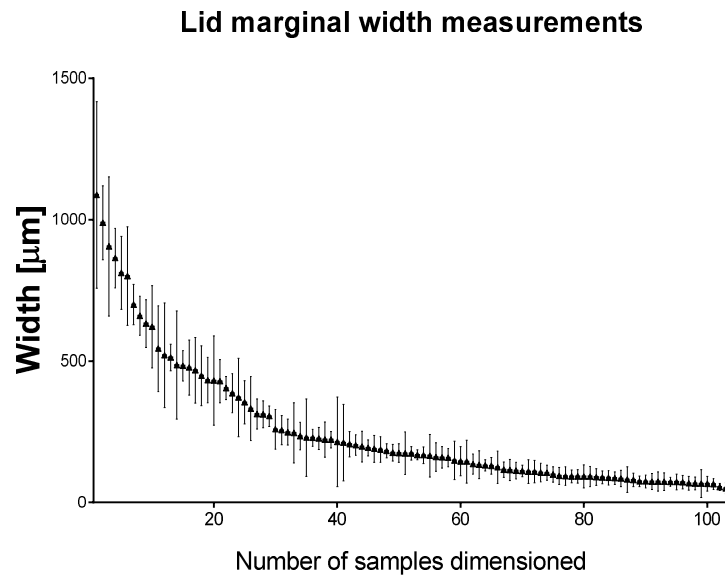
Figure 79: Average width of pooled L and M cell areas across study population (sorted). Error bars indicate SDs (n=103).

The average width of lid wiper and MCJ areas is depicted in Figure 80 and Figure 81, the values and differences between the groups are summarized in Table 22.
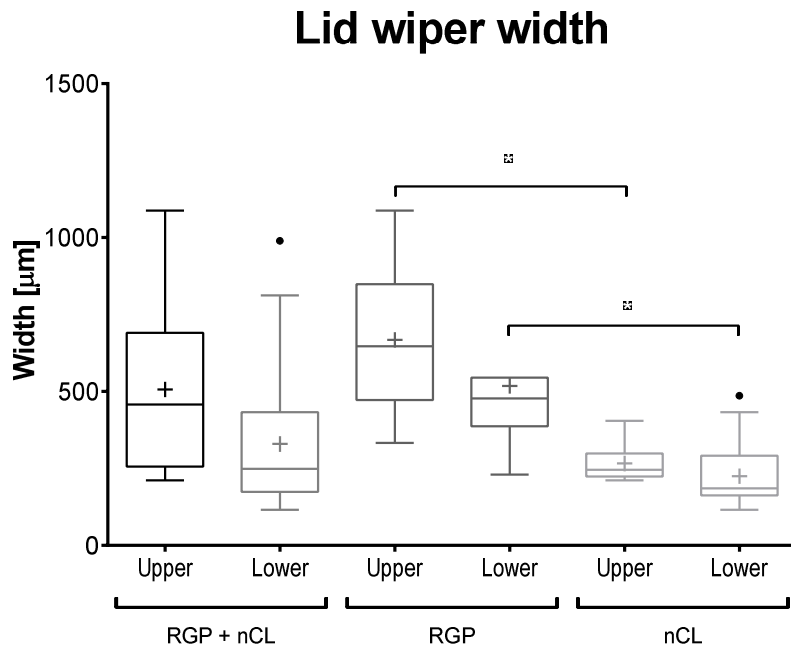
Lid wiper width

Figure 80: Width of the upper and lower lid wiper area.

MCJ width
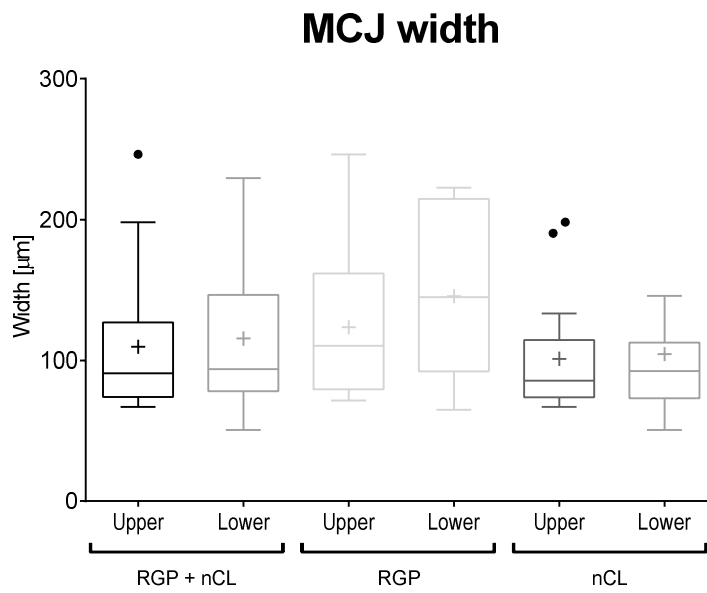
Figure 81: Width of the upper and lower MCJ area.

Table 22: Widths of the lid wiper and MCJ in μm.

Study group	Upper lid margin						Lower lid margin					
	L			M			L			M		
	Mean	SD	n	Mean	SD	n	Mean	SD	n	Mean	SD	n
RGP	666.9	219.1	12	123.5	54.37	10	518.5	211.8	11	145.7	58.14	7
nCL	265.8	64.35	8	101.2	40.98	16	224.9	101.9	20	104.4	45.96	19
RGP+nCL	506.5	264.5	20	109.8	46.85	26	329.1	204.7	31	115.5	51.78	26
RGP vs. nCL (Mann Whitney U)	p<0.0001			p=0.2844			p<0.0001			p=0.1353		

6.4.4.3 Correlation with study variables

The width of the lid wiper and MCJ areas was verified for correlation with all other measured study variables (age, gender, lens wear habits and experience, allergies, drop use, corneal and conjunctival staining, bulbar hyperemia, LWE, OSDI, CLDEQ-8 and diurnal scores for dryness and discomfort). Statistically significant results are summarized in Table 23.

Table 23: Summary of statistically significant correlations between cell area width and all other recorded study parameters.

Lid	Cell area	Study group	Parameter	p	r	n	Correlation
Lower	L	RGP	LWE	0.016	0.78	9	Spearman
Upper	L	RGP	Years of wear [years]	0.03	0.73	9	Spearman
Upper	L	RGP	Gender	0.0001	-0.72	9	Spearman
Lower	L	RGP & nCL	LWE	0.0001	0.89	21	Spearman
Upper	L	RGP & nCL	OSDI score	0.006	0.66	16	Spearman
Upper	L	RGP & nCL	LWE	0.0001	0.87	14	Spearman
Lower	L	nCL	LWE	0.004	0.78	12	Spearman

The relationships between LWE and lid margin area widths are shown in Figure 82.
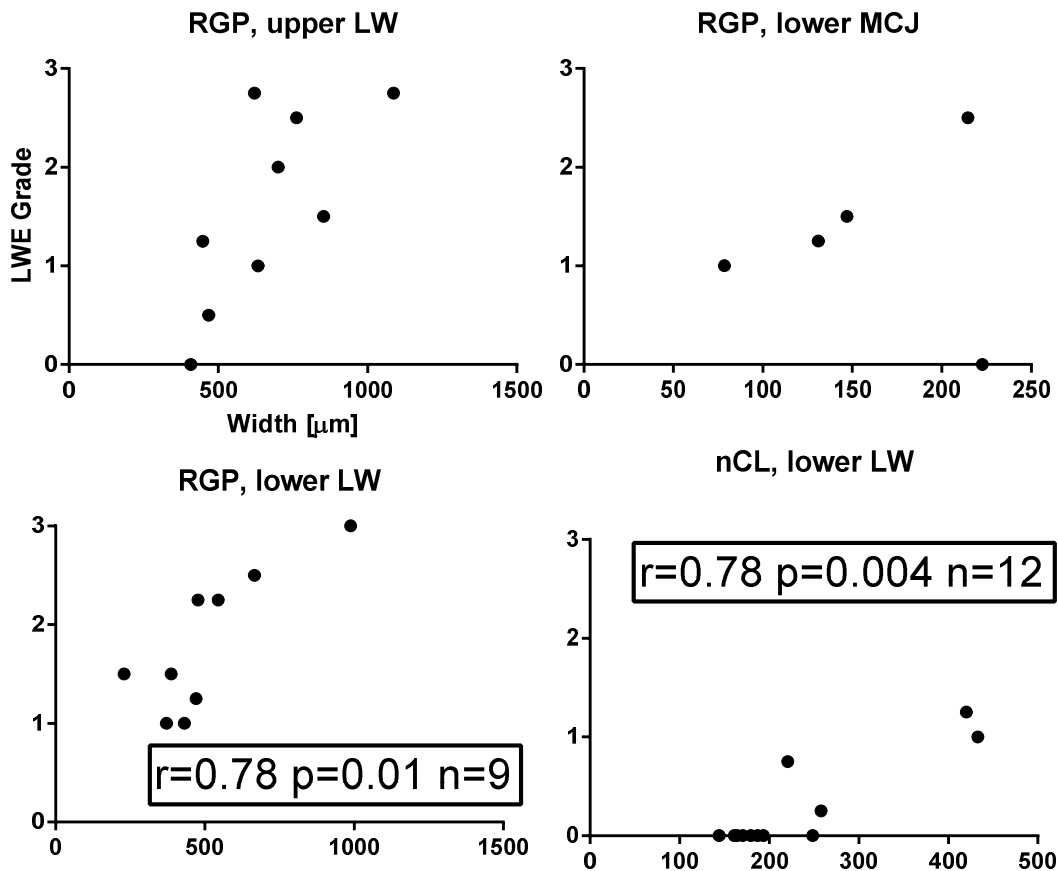
Figure 82: Correlations between LWE grade and the width of the LW and MCJ areas. Statistically significant instances are denoted by Spearman r and p-values.

6.5 Discussion

6.5.1 Demographics and clinical findings

In the previous chapter, differences in subjective comfort of CL wearers did not appear to materialize in cyto-morphological changes at the lid margin, nor were these reflected in the severity of LWE. If the lid margin does play a role in the perception of discomfort, this may extend far beyond changes in its most superficial epithelial layer, and multiple avenues have been and are yet to be explored, as outlined in the introduction. However, our results so far could not address the question whether lens wear in and of itself is associated with the observed changes. Analogous to polarizing study participants by

discomfort in the previous chapter, and presuming that CLD is indeed a function of mechanical interactions (encompassing frictional forces, tension, boundary interaction etc.), it may be worthwhile demarcating the most contrasting forms of CL wear – non- vs. rigid lens wear – to highlight any underlying differences.

Yet, given the decreasing popularity of these lenses outside of atypical cornea geometries and pathological cases, recruiting healthy RGP wearers was no easy feat and eventually had to be aborted, leading to the discrepant group sizes. The near perfect age-matching was superior to that of the previous chapter.

Although statistically not significant, RGP lens wearers reported similar or better comfort compared to non-lens wearers, as shown by the OSDI score and the diurnal variation of comfort and dryness (Figure 70, Figure 72, Figure 73). While perhaps surprising at first, this finding resonates with the cited effects of corneal and lid margin sensitivity loss, which occur within minutes of rigid lens wear, and exacerbate after many years of wear [182]. This mechanism may also translate in the adaptation which occurs in terms of comfort with wearers of rigid CL [190]. As inferred by Efron et al., if the lid wiper is indeed a primary source of discomfort during rigid lens wear, then some form of adaptation may be taking place in the lid wiper to enable this effect. Investigation of such adaptive mechanisms in both rigid and soft lenses would provide useful insights into the pathophysiology of LWE [48].

Akin to the data of Shiraishi et al., the only other study to investigate LWE in rigid lens wearers, our staining grades were both comparable in terms of prevalence and severity, and were significantly higher in RGP than in non-lens wearers [188]. LWE was consistent bilaterally, as well as between the upper and lower lids, in both groups, although Shiraishi does note that the prevalence and grade of lower lid LWE were significantly higher than those in the upper lid. Our images of the LG-stained upper lid (Figure 77) strongly indicate a mechanical etiology of LWE, as the horizontal length of the staining closely matches the diameter of typical RGP lenses, ranging between 10 and 12 mm. This pattern was only observed in the upper lid, which, unlike to SCL wear, typically "holds" the rigid lenses in position. The lower lid is in less contact with the lens, supposedly only during the blink, when the lower margin of the lens meets the lower lid upon eye closure. Given the edge and stiffness of RGP lenses versus SCL materials as well as the repeated interaction with the lid margin during the blink, it is somewhat surprising that this pattern was only observed at the upper lid margin. Even more curiously, correlations with LWE and other clinical signs were instead limited to the lower lid of RGP wearers (Figure 76). While Shiraishi detected an inverse relationship between both upper and lower LWE grades and

participant age, in the present study lid margin staining increased with age, and correspondingly with CL wear experience. Further, supposedly arbitrary and incongruous correlations were detected between LWE and lubricant drop use, comfortable daily wear time, as well as diurnal comfort and dryness scores. The absence of any other association between LWE and comfort further supports findings of the previous chapters, suggesting a missing link in the proposed concordance of symptomology and LWE severity. While participants enrolled in this study did not manifest severe forms of dry eye (e.g.: Sjogren's Syndrome), it may be worthwhile assessing these associations in future studies.

6.5.2 Cytological findings

Similar to the previous chapter, just over half of all collected samples were qualitatively acceptable to be considered for analysis. Slightly lower numbers may be due to the fact that non-lens wearers in group nCL were not as experienced as CL wearers to have their eyes handled, therefore the IC procedure was more difficult to conduct at times, which may have led to occasional variations in the consistency of the membrane application. Nevertheless, the SDs of the width measurements were in the same range as before, denoting a consistent collection and analysis technique. The combination of smaller group sizes and slightly inferior collection quality from the last chapter, lead to poor bilateral availability of analyzable samples, and did not warrant unilateral comparisons as before. Instead, the average value was computed between the left and right (where applicable) for comparisons and correlations with other study variables. Given previously discussed aspects of consistency, these results may nevertheless be considered reliable.

There are two central findings of this study, one of which reinforces observations from the previous chapter, namely that higher LWE grades are associated with enlarged LW regions and increased keratinization at the lid margin. The other original discovery indicates that (rigid) CL wear appears to be linked to significant morphological changes in the lid margin epithelium. Specifically, the LW region in rigid lens wearers was close to three times wider than in non-lens wearers, suggesting that mechanical interactions between the lens and the lid margin leads to cellular changes in the lid wiper. Cells here were proximally larger (i.e. squamous) for a longer distance, before transitioning to small columnar / cuboidal cells of the tarsal conjunctiva. The occurrence of GC and their impressions were also shifted proximally. It is unclear whether the reason for these changes are attributed to the edge of the CL, its increased modulus, its small size and increased mobility on eye, the increased frictional forces between the upper lid and the lens, the increased tension of the upper lid across the lens, or a combination of the above. In their paper on blepharoptosis in RGP wearers, Thean et al. advanced another potential

explanation for eyelid margin changes associated with rigid lens wear. Hereby, the majority of contact lens wearers presenting with blepharoptosis gave a history of prolonged use of hard contact lenses. One explanation for this would be the mechanisms of removal of hard contact lenses. This involves pulling the lids laterally at the lateral canthus followed by a harsh blink, which over years can lead to levator aponeurosis dehiscence [191]. While typically only performed once a day for removing the lenses, this pronounced pressure at the lid margin may contribute to cyto-morphological changes as well. In this context, the positive correlation between the increased LW width and the years of lens wear experience is particularly noteworthy.

Differences in width were observed in the upper and lower lid wiper regions between the groups, but not in the MCJ regions, supporting the theory that it is the lid wiper that is in contact with the lens for most of time. While not significantly different, the width of the MCJ region in the lens wearing group spanned a much wider interval than in non-lens wearers (Figure 61). Notwithstanding the observed and defined transitional features between MCJ and LW, the exact boundary can occasionally be ambiguous, given the interspersed presence of para-keratinized cell in the lid wiper region. Instead, the exterior boundaries of these areas are usually very clearly defined: distally, the M cells are delimited by the abrupt disappearance of cell nuclei in ortho-keratinized cells of the outer skin, as well as the visible expressions of Meibomian glands; proximally, the inner-most boundary of the L cells feature distinctly smaller cells and GC impressions and expressions. Therefore, it may be worthwhile comparing the sum of L and M cell widths, which may be a more representative measure for the total mechanical interaction at the lid margin, considering the frictional theories put forward in the previous chapter. This comparison will be undertaken in the following chapter of this thesis.

Finally, further supporting observations in the previous chapter, the width of both lid marginal areas correlated well with the LWE grade, particularly at the lower lid margin of both rigid and non-lens wearers. While only statistically significant in two out of four instances, and bearing relatively small sample sizes, this relationship shown in Figure 82 certainly underscores the suggested link between LWE and cellular changes at the lid margin, namely that higher levels of LWE are associated with enlarged LW areas, showing an increased occurrence of keratinized epithelial cells.

6.6 Conclusion

Both LWE grade and the width of the lid wiper of rigid CL wearers were significantly greater than those of non-lens wearers. This suggests that a mechanical component innate to (rigid) CLs may be

responsible for this structural and morphological change at the lid margin. Future work could investigate these effects in scleral lens wearers, to isolate the effect of the lens edge interaction with the lid margin.

In this proof-of-concept type of study, these findings are limited to the rather obsolete rigid CLs and it would be invaluable to illuminate these relationships in SCL wearers. This comparison shall be tackled in the following chapter of this thesis.

Chapter 7

Comparisons between the upper and lower lid margins of soft, rigid and non-contact lens wearers

7.1 Overview

PURPOSE: The purpose of this study was a cross-comparison of the results from the previous two chapters of the present thesis. The lid margin epithelium of soft (SCL), rigid (RGP) and non-lens wearers (nCL), as well as the upper vs. lower lid margins were compared.

METHODS: Four distinct groups of subjects (asymptomatic and symptomatic SCL, RGP & nCL) comprising 77 participants were compared. Cross-comparisons between all study groups included clinical signs, comfort scores, lid wiper epitheliopathy (LWE) and histo-chemically stained impression cytology (IC) samples taken from both lid margins and width measurements for the lid wiper (LW) and muco-cutaneous junction (MCJ) areas. Upper and lower lid margins were also compared.

RESULTS: The average (±SD) LWE grade of SCL wearers (0.8 ± 0.8) was greater than in nCL (0.4 ± 0.7, p=0.0125) and lower than in RGP wearers (1.6 ± 0.9, p=0.0015). No significant difference was found between the upper and lower LWE grades in any of the four groups. Longer average CL wear times and older age were correlated with higher LWE grades (Spearman r range: 0.27 to 0.31, p<0.05) and better comfort scores (Spearman r range: 0.25 to 0.44, p<0.05). The width of the upper LW of SCL wearers (415 ± 132 μm) was greater than in nCL (266 ± 64, p=0.0003) and narrower than in RGP wearers (667 ± 219, p=0.0004). The width of the lower LW of SCL wearers (187 ± 120) was up to 2.8 times smaller than in RGP wearers (519 ± 212, p<0.0001), but similar to nCL (225 ± 102, p=0.072). The upper LW was significantly wider than the lower LW in all participants (p<0.05), except for RGP wearers.

CONCLUSION: SCL wearers exhibit more LWE than nCL, and less than RGP wearers, in both upper and lower lid margins. CL wear may be associated with an enlarged LW area: while RGPs affect both upper and lower lid margins, the impact of SCLs is largely limited to the upper lid margin. Regardless of CL wear, the LW at the upper lid margin is wider than the lower one, upholding the frictional role of the LW during habitual blinking.

7.2 Introduction

In chapter 5 of this thesis, symptomatic and asymptomatic SCL wearers showed similar LWE grades and lid marginal cytology; discomfort appeared not to be related to the eyelid margin cellular changes. Consequently, in chapter 6, we investigated the effect of lens wear itself on the lid margin, by polarizing RGP wearers and non-lens wearers. A sizable difference in both LWE and lid margin cytology was noted, suggesting that lens wear does affect the eyelid margin. But since SCL are far more popular than RGP CLs, it would be invaluable to review differences between SCL and non-lens wearers, as well as SCL and RGP wearers, to learn whether different lens types substantiate changes at the lid margin.

As a potential indicator for dry eye and discomfort, LWE studies are typically focused on populations differentiated by symptomology, whether CL-wearing or not. Only a handful of reports compared the prevalence and grade of LWE between lens wearers and non-wearers. Varsani and Wong found similar staining patterns irrespective of lens wear [61], while another study found that 25% of a presenting population to an eye clinic, including CL and non-wearers, had LWE [55]. Similarly, Alghamdi et al. examined the lid wiper in current, previous and non-CL wearers and found no difference in the severity of LWE between the three groups [192]. On the other hand, Best et al. fitted silicone hydrogel lenses to neophytes and found that after 6 months of lens wear, lid wiper grades had increased a full step over baseline levels [66]. As Efron et al. pointed out in their comprehensive review on LWE, there are roughly equal numbers of papers that have found LWE to be greater in contact lens wearers as those that have not [48]. In these cases, "lens wearers" typically only encompasses SCL wearers, yet little is known about LWE in RGP wearers. A single account so far reports the difference in LWE between SCL and RGP wearers, noting a significantly higher prevalence and mean grade of the latter in the upper lid margin, but similar values at the lower lid margin [188].

These equivocal findings between the upper and lower lid margins are a further topic of debate in LWE research. Beyond prevalence and severity, it is the etiology of lower LWE itself that is disputed, and there are reasons to believe that it is not identical to upper LWE. The different motion of the upper and lower lid margins during a blink have been known since 1980, thanks to the advent of high frame-rate video recordings [193]. Using only 64 frames/sec, "four times the normal silent film rate", the authors showed that the upper eyelid has a large vertical movement while the lower lid has a shorter horizontal nasal-ward movement. In 2015, using an advanced high-speed camera, Yamamoto et al. recorded the movements of the eyelids and displacement of the eyes during spontaneous blinking and concluded that higher pressure from the eyelid may be one of the causes for the development of lower LWE [81]. In

their paper, Shiraishi reported a significantly higher prevalence and severity of lower LWE than that of upper LWE, and suggested that this may be due to the small repeated lateral lower lid excursions. They concluded that the examination of the lower lid margin would be preferable to that of the upper lid in studies of LWE [188]. In contrast, Berry et al. found that upper LWE scores of symptomatic SCL wearers were significantly greater than those of asymptomatic wearers, and that this association was limited to the upper LWE [65]. Although friction-related damage appears to be the causal mechanism for upper LWE, tear hyperosmolarity may also be of relevance [48]. A positive correlation was observed by Golebiowski et al. between upper LWE and tear osmolarity measured in the inferior meniscus [76]. As a consequence of the reduced travel in the lower versus the upper lid margin, McMonnies argues that there is less opportunity in the lower lid wiper for friction-related damage, and any epitheliopathy observed here is more likely due to hyperosmotic insult [74]. This contradicts the suggestions of Shiraishi et al. [188] and is inconsistent with the observations of Stahl et al. [75], who could not prove a relationship between tear or lens osmolality, comfort and LWE at the upper lid margin.

The ambiguities of the histology of the lid margin have already been outlined in the previous chapters. These discrepancies are only exacerbated by the fact that the few cytological reports inconsistently report on either the upper [24] or the lower [9] lid margin, with only a single account investigating both [13].

For these reasons, and because the upper and lower lid margins were viewed separately in previous chapters, this chapter will dedicate a closer look at the differences between upper and lower lid margins, both in terms of vital staining (LWE), as well as cytological findings. Furthermore, this chapter will review differences between SCL and RGP wearers, and SCL and non-lens wearers, in a cross-comparison of the findings from chapters 5 and 6.

7.3 Materials and methods

This chapter comprises comparisons between chapters 5 and 6. All materials and methods have been described in the respective sections. Table 24 reiterates the study groups analyzed and compared in this chapter.

Table 24: Overview of study groups comprised in the analysis

Study group code	Description	Analyzed in
aSCL	Asymptomatic soft lens wearers	Chapter 5
sSCL	Symptomatic soft lens wearers	
RGP	Rigid gas permeable lens wearers	Chapter 6
nCL	Non-lens wearers	

7.4 Results

7.4.1 Demographics

A total of seventy-seven participants were included in the present analysis, with an average age of 37.6 ± 17.8 years across all groups (Figure 83). The average age of SCL wearers (SCL) was not significantly different than that of RGP (p=0.27) or non-lens wearers (p=0.23).
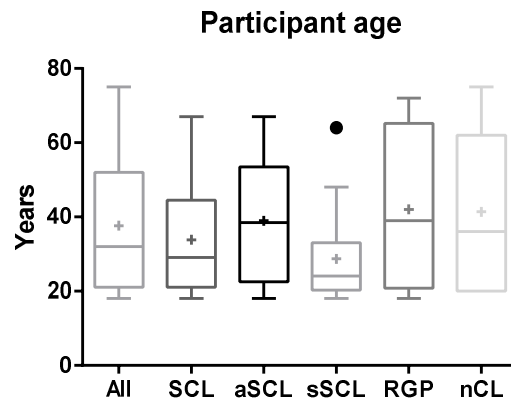
Figure 83: Distribution of participant age by study group

SCL wearers wore their lenses for an average of 11.78 ± 3.1 hours/day for 5.7 ± 1.2 days/week, while RGP wearers reported a daily average of 12.72 ± 2.7 hours/day, for 6.3 ± 0.9 days/week. These differences were not statistically different (p=0.27; p=0.057). RGP wearers reported significantly more comfortable wear hours per day (12 ± 3.1 hours/day), compared to SCL wearers (9.0 ± 4.4 hours/day) (p=0.02). Overall, SCL wearers had worn their lenses for 13.4 ± 10.6 years, while RGP wearers had an experience of 24.3 ± 18 years; this difference was statistically significant (p=0.04). These trends are depicted in Figure 84.
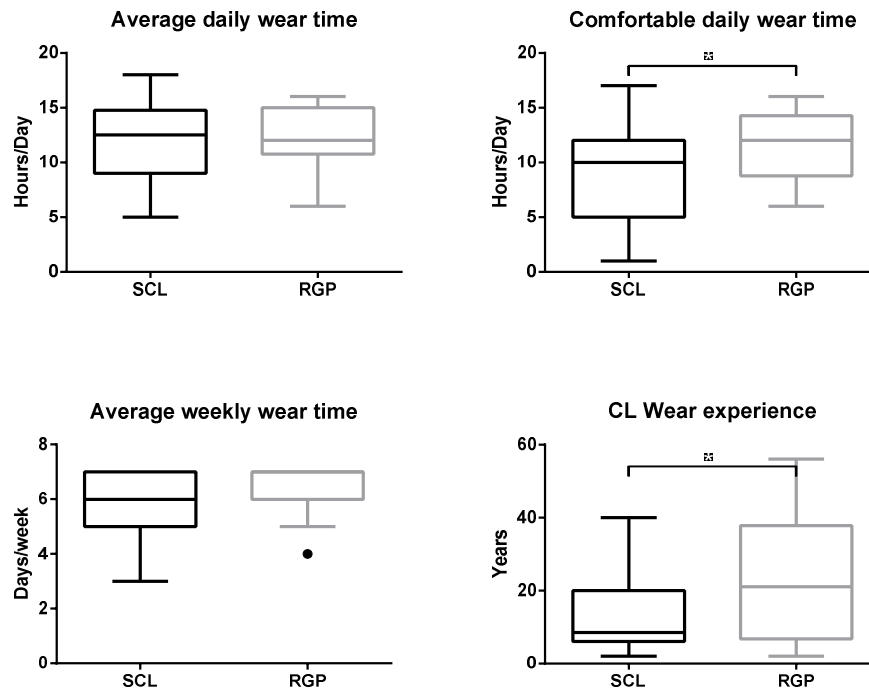
Figure 84: Self-reported CL wear habits and experience

7.4.2 Subjective comfort scores

SCL wearers scored a significantly higher OSDI score (16 ± 13) than RGP lens wearers (8.3 ± 5.1; p=0.018). The CLDEQ-8 score was not significantly different between the groups (SCL: 13 ± 7.8; RGP: 9.6 ± 5.3; p=0.17) (Figure 85).
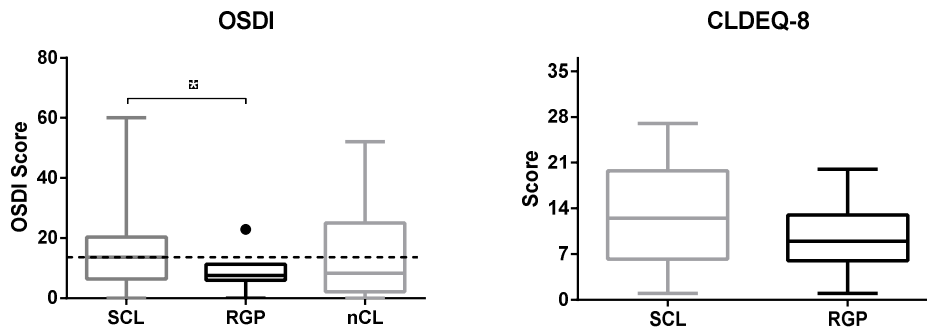
Figure 85: Distribution of OSDI and CLDEQ-8 scores between study groups.
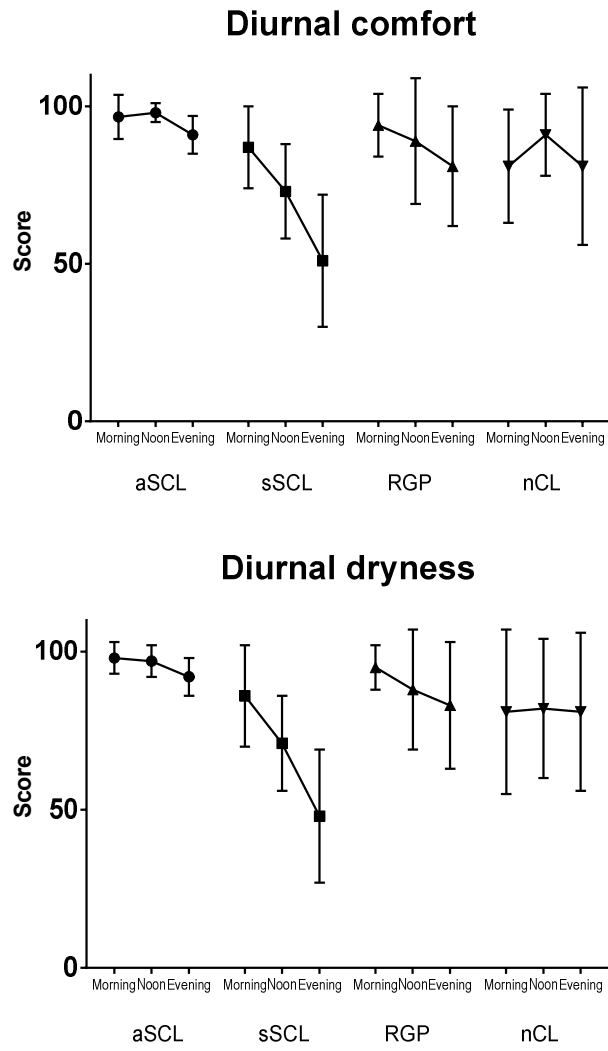
Figure 86: Average diurnal scores for comfort and dryness across groups.

OSDI, CLDEQ-8 and the diurnal scores for comfort and dryness across all groups combined were tested for correlations against all other measured study variables (age, gender, lens wear habits and experience, allergies, drop use, corneal and conjunctival staining, bulbar hyperemia, LWE). The statistically significant correlations are summarized in Table 25 and Figure 87. These were limited to the self-reported CL wear experience and the age of participants. Overall, younger participants and more novice CL wearers, reported worse comfort than older ones.

Table 25: Summary of statistically significant correlations between comfort data and clinical data (Spearman correlations).

Comfort scale	Study variable	p	r	n
CLDEQ-8	Age	**0.017**	-0.31	58
CLDEQ-8	Years of lens wear	**0.0058**	-0.36	58
OSDI	Years of lens wear	**0.027**	-0.29	58
Diurnal comfort morning	Years of lens wear	**0.0001**	0.44	58
Diurnal comfort noon	Age	**0.002**	0.25	77
Diurnal comfort noon	Years of lens wear	**0.003**	0.38	58
Diurnal comfort evening	Years of lens wear	**0.001**	0.41	58
Diurnal comfort evening	Age	**0.046**	0.23	77
Diurnal dryness morning	Years of lens wear	**0.0064**	0.35	58
Diurnal dryness noon	Years of lens wear	**0.0022**	0.39	58
Diurnal dryness evening	Years of lens wear	**0.0019**	0.40	58
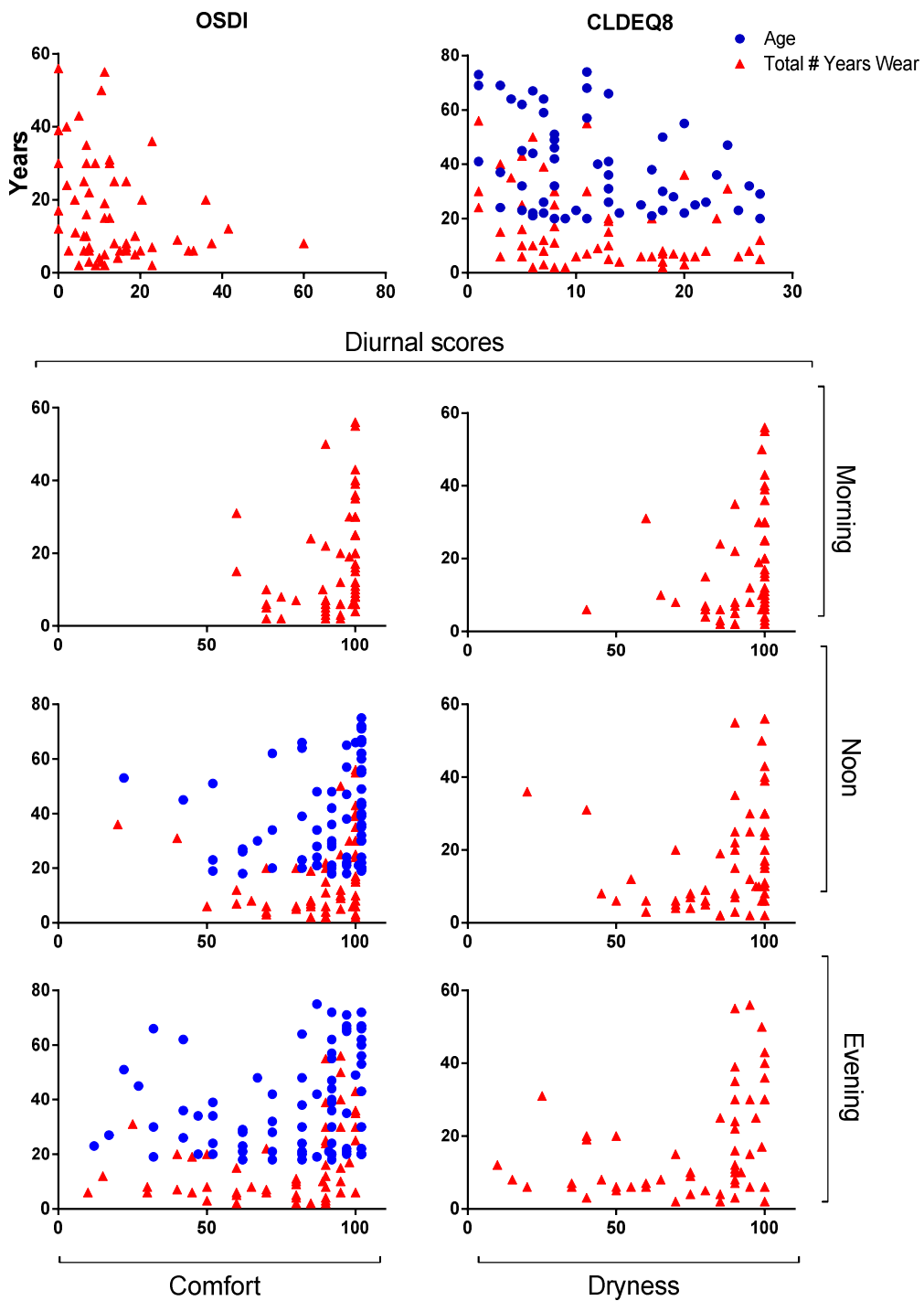
Figure 87: Statistically significant correlations between comfort and participant age (blue dots) and total lens wear experience (red triangles).

7.4.3 Lid wiper epitheliopathy

7.4.3.1 Differences between groups

SCL wearers had significantly less LWE than RGP wearers at both upper (p=0.0015) and lower (p=0.0238) lid margins. SCL wearers also exhibited higher LWE grades than nCL at the upper (p=0.0125) and lower (p=0.0055) lid margins. Figure 88 depicts these differences, while average LWE grades are summarized in Table 26.
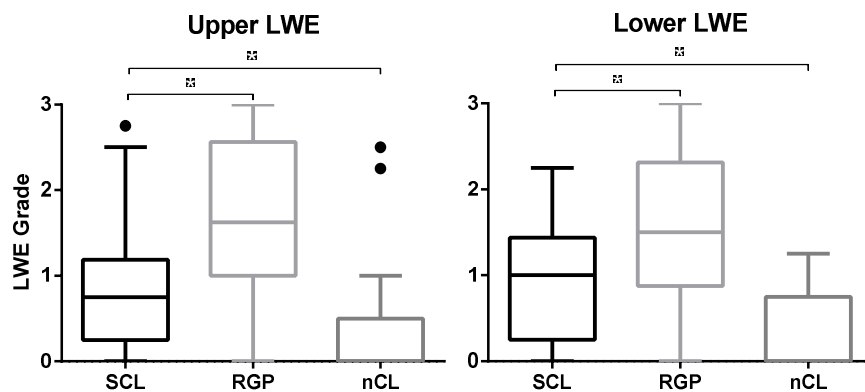
Figure 88: Differences between LWE grades of SCL and RGP lens wearers, and SCL and non-lens wearers, at the upper and lower lid margins.

7.4.3.2 Differences between lid margins

Differences between the upper and lower lid margins were not statistically significant in any of the study groups (Table 26).

Table 26: Average upper and lower LWE grades in each study group.

	aSCL	sSCL	RGP	nCL
Upper LWE	0.8 ± 0.8	0.8 ± 0.7	1.6 ± 0.9	0.4 ± 0.7
Lower LWE	0.8 ± 0.6	1.0 ± 0.7	1.4 ± 0.9	0.3 ± 0.4
Upper vs. lower LWE (Mann Whitney U)	p=0.81	p=0.51	p=0.58	p=0.75

7.4.3.3 Correlations with LWE

Upper and lower LWE grades across all groups were tested for correlations with all other measured study variables (age, gender, lens wear habits and experience, allergies, drop use, corneal and conjunctival staining, bulbar hyperemia, LWE, OSDI, CLDEQ-8 and diurnal scores for dryness and discomfort). Two modest correlations were found between the average daily CL wear time and the upper LWE grade (r=0.31, p=0.019, n=58), and the weekly average CL wear time and the lower LWE grade in all participants (r=0.27, p=0.04, n=58). These relationships are shown in Figure 89.
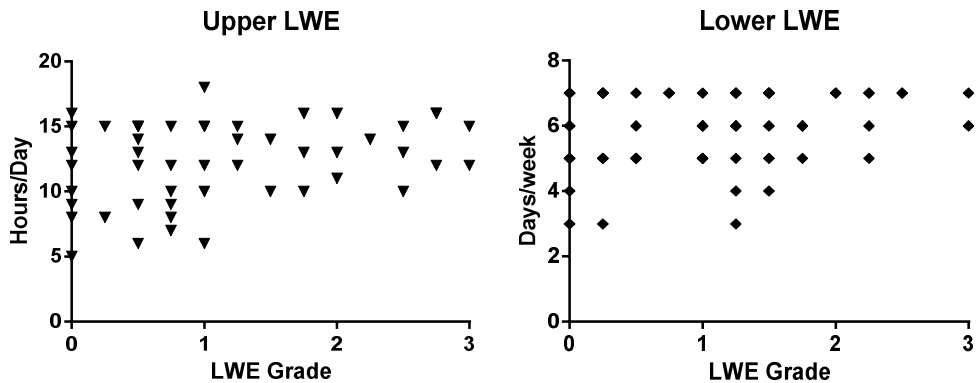
Figure 89: Summary of statistically significant correlations between LWE grade and the average daily/weekly CL wear time in hours (Spearman correlation)

7.4.4 Histological analysis

The widths of the LW, MCJ, and the combined (LW+MCJ) cellular areas at the upper and lower lid margins were compared between study groups in this section. Table 27 summarizes the average width values, which are compared by study group and by lid margin in the following two sections.

Table 27: Average width of lid marginal areas across study groups (in μm).

Study group	Cell area	Lid margin	Mean	SD	n
SCL	LW+MCJ	Upper	528	126	19
		Lower	266	96	18
	MCJ	Upper	114	44	23
		Lower	91	41	25
	LW	Upper	415	132	27
		Lower	187	120	22
aSCL	LW+MCJ	Upper	549	171	9
		Lower	261	100	9
	MCJ	Upper	119	55	11
		Lower	104	54	12
	LW	Upper	424	171	14
		Lower	142	58	11
sSCL	LW+MCJ	Upper	509	73	10
		Lower	243	50	7
	MCJ	Upper	110	31	12
		Lower	78	19	13
	LW	Upper	405	76	13
		Lower	232	150	11
RGP	LW+MCJ	Upper	674	168	7
		Lower	557	77	3
	MCJ	Upper	124	54	10
		Lower	146	58	7
	LW	Upper	667	219	12
		Lower	519	212	11
nCL	LW+MCJ	Upper	342	70	6
		Lower	285	88	15
	MCJ	Upper	101	41	16
		Lower	104	46	19
	LW	Upper	266	64	8
		Lower	225	102	20

7.4.4.1 Differences between groups

Given that lid marginal widths were comparable between symptomatic and asymptomatic SCL wearers (section 5.4.4.2), for the purpose of comparison with RGP and non-lens wearers, SCL wearers were combined into a single group (SCL). The upper LW was significantly wider in SCL wearers versus

nCL and significantly narrower than in RGP wearers. The MCJ was similar across groups (Table 28). The difference between SCL and RGP wearers at the lower LW was even greater, but not significantly different between SCL and nCL. The MCJ was somewhat wider in RGP wearers versus SCL (Table 29). These results are depicted in Figure 90.

Table 28: Average widths of the upper LW, MCJ, and the combined (LW+MCJ) cellular areas in μm, for SCL (SCL), RGP (RGP) and non-lens wearers (nCL).

Study group	Upper lid margin								
	LW+MCJ			MCJ			LW		
	Mean	SD	n	Mean	SD	n	Mean	SD	n
SCL	528	126	19	114	44	23	415	132	27
RGP	674	168	7	124	54	10	667	219	12
nCL	342	70	6	101	41	16	266	64	8
Mann Whitney U test (p-values)									
SCL vs. RGP	**0.0829**			0.7702			**0.0004**		
SCL vs. nCL	**0.0007**			0.236			**0.0003**		

Table 29: Average widths of the lower LW, MCJ, and the combined (LW+MCJ) cellular areas in μm, for SCL (SCL), RGP (RGP) and non-lens wearers (nCL).

Study group	Lower lid margin								
	LW+MCJ			MCJ			LW		
	Mean	SD	n	Mean	SD	n	Mean	SD	n
SCL	266	96	18	91	41	25	187	120	22
RGP	557	77	3	146	58	7	519	212	11
nCL	285	88	15	104	46	19	225	102	20
Mann Whitney U test (p-values)									
SCL vs. RGP	**0.003**			**0.0175**			**< 0.0001**		
SCL vs. nCL	0.46			0.2775			0.0753		
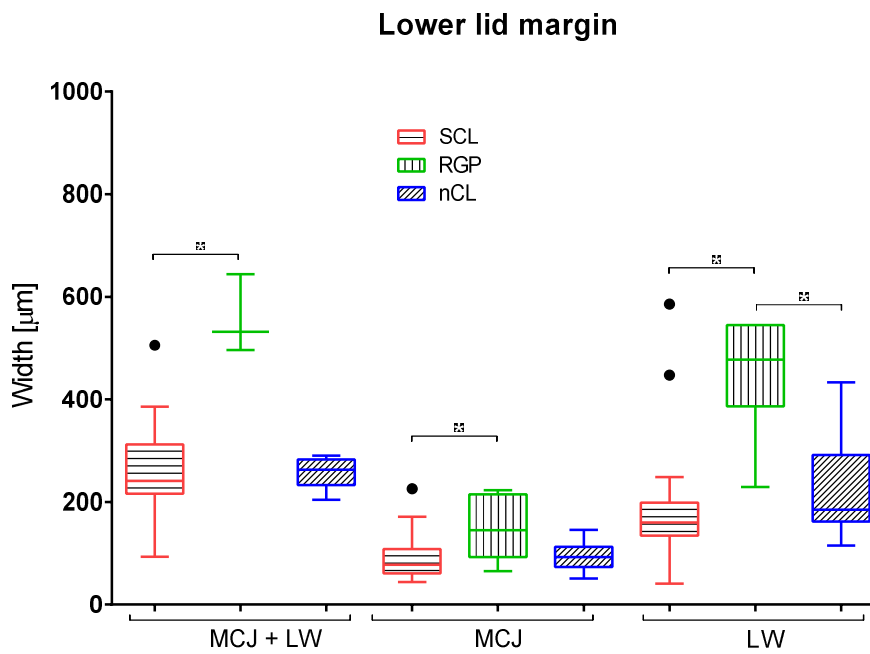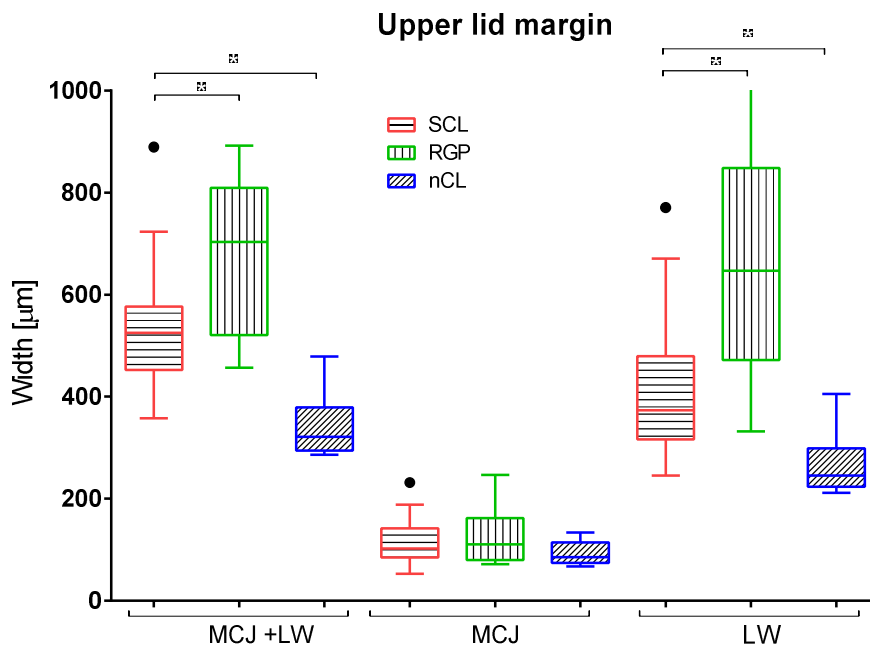
Figure 90: Widths of the upper and lower LW, MCJ, and the combined (LW+MCJ) cellular areas between soft (SCL), rigid (RGP) and non-lens wearers (nCL).

7.4.4.2 Differences between lid margins

The LW and the combined (MCJ+LW) widths were significantly greater at the upper lid margin compared to the lower lid in all study groups except for RGP wearers. No relevant differences were found between the upper and lower MCJ areas. These trends are depicted in Figure 91 and Table 30.
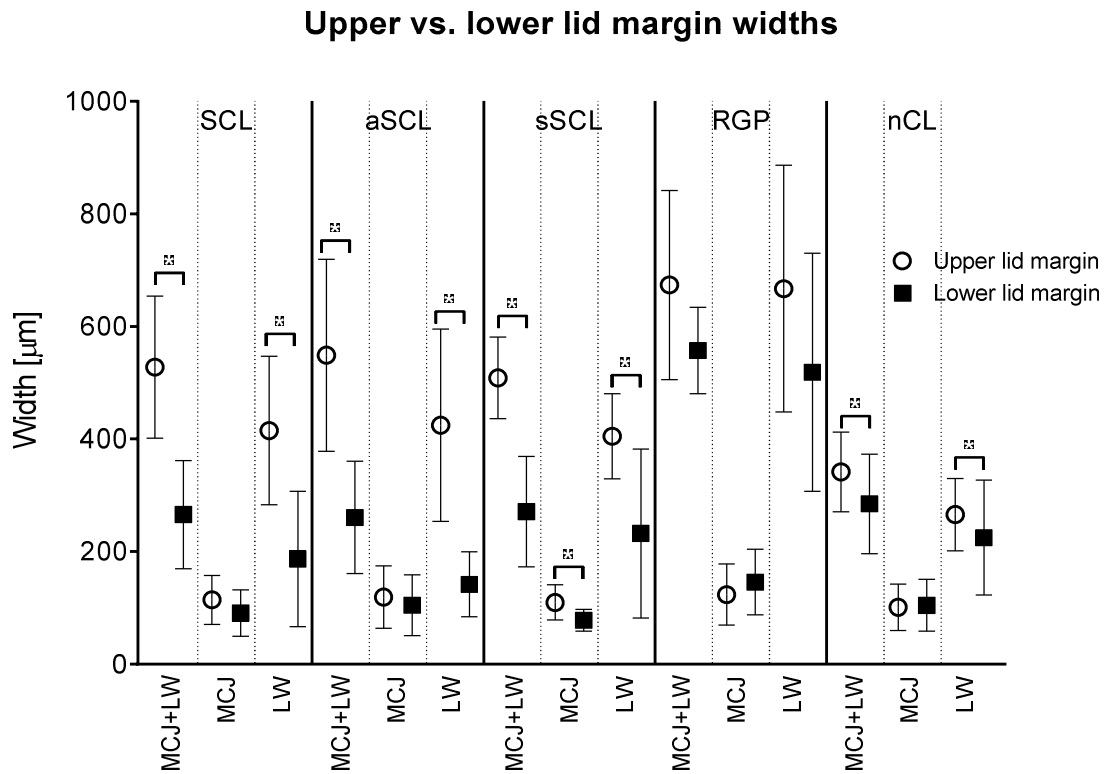
Figure 91: Comparison between the upper (circle) and lower (square) LW, MCJ, and the combined (LW+MCJ) cellular area widths.

Table 30: p-values for comparison between upper and lower lid margin areas within each group (Mann Whitney U). Average values are shown in Table 27.

|	Upper vs. lower lid margin areas		
Study group	MCJ+LW	MCJ	LW
SCL	**< 0.0001**	0.0262	**< 0.0001**
aSCL	**< 0.0001**	0.5181	**< 0.0001**
sSCL	**0.0001**	**0.0055**	**0.0025**
RGP	0.3333	0.4705	0.1027
nCL	**0.0084**	0.822	**0.0428**

7.5 Discussion

This chapter represents a relevant extension of the work conducted and presented in chapters 5 and 6, comparing findings in the SCL groups with those of RGP and non-lens wearers.

7.5.1 Demographics & Clinical findings

What may initially seem surprising, is that RGP wearers reported overall superior comfort to SCL and even nCL. This may partly be explained by the loss of ocular surface sensitivity known to occur with rigid lens wear [190], although the precise location (cornea, conjunctiva, lid margin etc.) of this phenomenon has not been determined. Another proposed mechanism is the psychological adaptation to discomfort known to occur in RGP wearers [82], who may have lower or more realistic expectations towards lens wear, perceiving discomfort less intensely than SCL wearers. While few study variables and parameters were found to correlate with comfort data in the previous chapters, an interesting trend was observed when assessing comfort across the entire study population. Specifically, comfort appeared to correlate with either participant age or the number of years of lens wear experience. Although weakly correlated, these trends were significant across several comfort measurement methods, including OSDI, CLDEQ-8 and diurnal comfort and dryness questionnaires (Figure 87). We observed that older participants rated better comfort than younger ones; this was especially visible in the total number of years of lens wear. As suggested earlier, this may be related to increasing desensitization occurring with lens wear, known to exacerbate over time, but may also, or as a result of this phenomenon, suggest that younger wearers tend to be warier of discomfort and rate it worse than their older counterparts. Another explanation may lie in the fact that participants who experienced discomfort at an early age would have dropped out early, while asymptomatic lens wearers will have continued to wear lenses into their later years. Interestingly, this trend is opposite to the proposed

relationship with LWE, which is said to increase with decreasing comfort [6]. Yet, in our studies, participants with longer CL wear experience reported less discomfort. At the same time, longer average wear times were correlated with higher LWE grades, hinting at a frictional etiology of LWE, as lubrication is assumed to worsen with extended periods of lens wear, as discussed in previous chapters. Still, counter to both Pult's and Shiraishi's findings [53,188], who reported higher LWE grades in older and younger participants respectively, LWE did not correlate with age in our studies. Further, LWE in SCL wearers was significantly higher than in non-lens wearers and significantly lower than RGP wearers, which is akin to Shiraishi's results [188]. They found LWE to be more prevalent and more severe at the lower lid margin, while in our studies both upper and lower lid margins were consistent. Our findings suggest that lens wear may be associated with higher degrees of LWE, and that this relationship is more pronounced with rigid materials, be it for their increased modulus, edge design, increased movement or different frictional coefficient, as discussed in the previous chapter. Yet at the same time, LWE did not correlate with any comfort measures across the entire study population, suggesting that, while friction may indeed be the central mechanism of discomfort, LWE is not necessarily an appropriate clinical tool for its investigation.

7.5.2 Cytological findings

The cross-comparison of lid marginal areas between study groups further underpinned the fact that lens wear does affect conjunctival morphology and structure. This was seen most predominantly at the upper lid margin, where the lid wiper area was wider in SCL wearers, compared to non-lens wearers. At the same time, and mirroring the LWE results described above, both upper and lower lid wiper areas of SCL wearers were significantly narrower than those of RGP wearers, suggesting that the mechanics in rigid lens wear have a more pronounced impact on the lid margins. SCL wear does not appear to affect the lower lid margin when compared to non-lens wearers. RGP lenses instead, possibly due to their greater movement and repeated interaction with the lower lid margin during blinking, appear to be associated with changes across the entire lower lid margin, as lid wiper, the MCJ and their combined width were significantly wider than in SCL wearers.

The upper lid margin was significantly wider than the lower one across all groups, except for RGP wearers. While the stark differences between the upper and lower lid margins in groups aSCL and sSCL may indicate that this difference could be induced by lens wear, this trend is also present in the non-lens wear group, albeit to a smaller extent. This may suggest that the upper lid margin is fundamentally anatomically different, which may be a result of the different blinking patterns between the upper and

lower lids. At the same time, this difference was not consistent with LWE, as upper and lower grades were similar within every study group, counter to the findings of Shiriashi et al. [188].

This discrepancy, along with previously discussed inconsistencies in LWE staining patterns within our studies as well as in the literature, continues to leave the question regarding the etiology of LWE open. Perhaps it is worth noting that most clinical tools and measures used in this study and others, including comfort questionnaires and even the IC sample dimensioning method developed in the present work, are either validated or objective and/or automated. Meanwhile, Efron points out that there is an equal number of studies that were and were not able to prove the proposed function of LWE [48]. Instead, the LWE grading system has often been the subject of debate in the literature [48], as well as anecdotally among clinicians, due to its insufficient repeatability between investigators and between studies. Hereby, the confusion between the omnipresent staining of the Marx' line and mild to moderate grades of LWE seem to be the central controversy [194]. More severe forms of LWE also suffer from the subjectivity of visually assessing the horizontal and sagittal extents of staining, which, coupled with the generous grading categories proposed (millimeters instead of micrometers, particularly for the sagittal extent of staining), along with the slightest investigator bias, may easily lead to the observed incongruities between studies. In this sense, and given the technological advancements of today's photography, image processing and analysis capabilities, it may seem almost trivial to develop and adopt a simple software, similar to the work of Varikooty [58] and Kunnen [59] (or to the method developed in the present study for dimensioning IC samples), to make use of the existent slit-lamp-mounted imaging equipment and deliver real-time, automated metrics of LWE, without the need of extensive post-processing and manual tracing, but geared more towards an efficient clinical application.

7.6 Conclusion

This chapter provided a cross-comparison between results obtained in chapters 5 and 6 of this thesis. Findings pertaining to SCL wear were compared against RGP and non-lens wear, and the upper lid margin contrasted against the lower. Additionally, we examined the total study population included in the previous two chapters.

There appears to be a link between the age and/or the number of years of lens wear experience and subjective comfort ratings. Younger participants tended to report worse comfort than older ones, as expressed by OSDI, CLDEQ-8 and diurnal scores for dryness and discomfort. This finding may or may not result from the proposed mechanisms of corneal and/or lid marginal desensitization, as well as from

a psychological adaptation to CL-related discomfort, particularly as some of the oldest study participants were RGP wearers.

SCL wearers exhibit significantly more LWE than non-lens wearers, and significantly less LWE than RGP lens wearers. The LWE grade was similar at both upper and lower lid margins, in every study group. We observed a weak but statistically significant correlation between LWE grade and CL wear habits: participants who reported longer average wear times displayed higher LWE grades.

Finally, and most prominently, we provide a novel insight into the lid marginal cytology of CL wearers, showing that SCL wear has a far lesser impact on the lid wiper conjunctiva and the MCJ compared to RGP wear, particularly at the lower lid margin. At the upper lid margin, the lid wiper of SCL wearers was narrower than that of RGP wearers, but also significantly larger than that of non-lens wearers, indicating that SCL wear may be associated with morphological changes at the lid margin. Overall, the width of the upper lid wiper area was significantly greater than that of the lower lid margin across all study groups, except for RGP wearers. Because this phenomenon was apparent in non-lens wearers as well, this may suggest that the upper lid margin experiences a greater frictional force during habitual blinking than the lower one, hinting at a fundamental anatomical difference in the LW structure of humans.

Chapter 8

Conclusions and future research directions

The work presented in this thesis encompasses the development and optimization of a technique for collecting, processing, imaging and analyzing epithelial cells from the eyelid margin, and applying it towards better understanding the lid margin epithelial cyto-morphology in symptomatic and asymptomatic soft, rigid and non-CL wearers. Beyond the impact of CL wear and discomfort on the eyelid margin, these results have contributed to improving and refining our understanding of the human eyelid morphology.

In this final chapter, a unifying look at the work conducted during the course of this thesis will critically highlight the value of the presented results and will hopefully spur new research avenues.

The main results, findings and achievements of this thesis are summarized in

Table 31.

8.1 On methodology

A substantial portion of the work described in this thesis involved developing and continuously adapting and improving the IC collection technique for lid margin cells, the selection of cyto-chemical stains, as well as the imaging and analysis methods. By developing investigator experience and dexterity, qualitative histological samples were obtained, which enabled a more detailed perspective on the cellular features than reported previously [9,24]. The microscopic imaging and panoramic stitching technique described in Chapter 3 was adequately complemented by the subsequently method of dimensioning LW and MCJ areas developed in Chapter 5. This provided a first account on the size of these anatomical features on a representative population, as opposed to the cadaver excisions previously reported.

Although confocal microscopy offered a very detailed representation of cell morphology, the employed selection of dyes may not be appropriate to determine cellular viability, as it was unclear whether the ambivalent nature of results represented the real state of cells, or whether that was induced by the IC collection technique itself. Another drawback was that samples required immediate processing and imaging, which were typically lengthy in nature, due to the intricacies of the imaging system and the required optimization of parameters. This was a logistical inconvenience in this study, but future studies employing this technique may circumvent them by investigating the possibility of storing samples for subsequent (batch) processing. The high-resolution imaging capabilities of the CLSM would perhaps be best employed at a later stage in the study of the lid margin morphology, for characterizing single cells or cell patches from clearly determined lid marginal regions, rather than using it for membrane-wide quantification.

Given the pronounced curvature of the lid margin, the quality of IC collections will always be highly dependent on the application angle of the membrane to the lid marginal surface. While superior handling experience by the investigator is irreplaceable, we believe that a maximum yield in cellularity and IC collection quality may have been reached using the presented technique. With a sufficient sample size, this variability can be circumvented, yet we propose a variation of this technique, which may considerably increase the sample cellularity. By cropping out 3-4 mm wide rectangular strips of the Millipore membrane and applying these to the lid margin, significantly higher IC collection quality samples can be obtained, as the thin membrane will closely follow the curvature of the lid margin, and

quite firmly adhere to the conjunctiva. This procedure requires nearly surgical precision, as it cannot be conducted without the use of forceps. The removal of the membrane should occur very promptly, within 2 or 3 seconds after application, and would necessarily require leaving an edge of the membrane to remain unattached to the conjunctiva, in order to facilitate its maneuvering. This technique has been initially tested out in the optimization phase of our work, albeit with the full membrane (Figure 92), which adhered to the tarsal conjunctiva and involved a forceful and unpleasant removal. Yet this technique provided some of the highest quality cell collections, alleviating issues of membrane application angle and pressure. We highly recommend the use of this technique to be adapted by a skillful investigator who wishes to pursue similar work in the future. In this context, it may be worthwhile considering the use of ocular surface anesthetics, carefully considering the effects of preservatives and other ingredients on cellular morphology discussed earlier. While proparacaine hydrochloride (Alcaine) has been employed during the method development stage of this work, it was deliberately abandoned during later studies (Chapters 4-7), as IC demonstrated minimal to no discomfort when performed at the ELM, and because of the biocidal properties of its preservative (BAK).

However, more so than LWE, IC of the ELM is a new area of study, undoubtedly requiring further validation studies. Unlike bulbar or corneal IC, where appropriate measures and gold standards for collection quality have been established, the repeatability of the method presented in this work solely relies on investigator experience acquired through numerous applications, and by visually inspecting and evaluating the samples.

Figure 92: Unmounted application of the Millipore membrane to the lid margin

8.2 On the impact of CL wear on the lid margins

By successfully employing the above IC collection and analysis techniques in a pilot study (Chapter 4), we were able to obtain a rapid insight into the cellular structures of the lid margin, which broadly coincided with previous reports in the literature, providing a more detailed and concise description of the LW and MCJ surface morphology and dimensions than available to date, complementing the detailed, classical histological excisions.

By expanding the sample size and refining the methodology in chapters 5 and 6, we provided a first account to show that CL wear *does* alter the lid marginal morphology. Specifically, lens wear appears to cause an increase in the LW and/or MCJ width, particularly in the upper lid margin. These morphological changes are considerable with rigid materials, potentially relating to lens modulus, lens edge design, increased on-eye movement or different coefficients of friction. Likewise, the lid wiper region of SCL wearers was significantly larger than that of non-lens wearers, indicating that even SCL wear is associated with morphological changes in this area. Future work should seek to polarize different SCL types, materials or lens edge designs, to narrow down the causative factors leading to the observed changes. For instance, the impact of scleral lenses on the lid margin may be worth exploring, as these lenses feature an identical modulus to typical RGP lenses, while their lens edges do not contact the lid margin during habitual blinking.

The significantly larger width of the upper vs. the lower LW in all participants suggests that the upper lid margin experiences a greater frictional force during habitual blinking than the lower one. The fact that this finding also pertained to non-lens wearers, may be hinting at a fundamental anatomical difference in the LW structure of humans.

8.3 On lid wiper epitheliopathy

Pursuing Jalbert's suggestion that LWE may be investigated using IC, we offered the first account of a cytological description and comparison of the lid margin conjunctiva of symptomatic and asymptomatic SCL, RGP and non-lens wearers.

We observed that high LWE grades are correlated with enlarged LW and/or MCJ areas and increased para-keratinization in these areas, indicating a frictional – or at least mechanical – etiology of LWE. Subjects with high LWE exhibited wider lid marginal areas, which were more densely populated by large, keratinized cells, compared to non-LWE subjects. Regardless of the cause of this manifestation (induced by the presence, or design, of a CL, an altered tear film and/or other factors), epithelial keratinization indicates a physiological response to exterior stimuli causing distress to the exposed tissue, resembling the typical protective mechanism encountered in various other bodily epithilia (buccal, esophageal etc.). Our observations also suggest that the Marx line *does* contact the ocular surface (or a CL surface) during blinking, representing a frictional and keratinization maximum in the transitional morphology of the lid margin.

SCL wearers exhibited significantly more LWE than non-lens wearers, and significantly less LWE than RGP lens wearers, with comparable LWE grades between the upper and lower lid margins in all 4 study groups. There was no association between LWE and subjective comfort, in neither of the four questionnaires used, nor was comfort (or the lack thereof) reflected in the cellular morphology of the lid margin. This was in spite of positive correlations between longer average wear times and higher LWE grades, again indicating inadequate lubrication towards the end of the day. Future studies may consider employing a stronger polarization scheme, by recruiting severe dry eye patients, or people with Sjogrens syndrome, and comparing them with asymptomatic normals. Our work failed to confirm Korb et al.'s hypothesis regarding the association between LWE and dryness and discomfort. Nevertheless, as Efron stated, LWE is still in its infancy, and work in this area should continue, particularly as our results – albeit exploratory – seem to endorse the frictional origin of this phenomenon.

A significant step forward in refining the process of LWE grading, would be the development of an easy to use, automated LWE grading software, to be efficiently employed during routine assessments. The insights gained while dimensioning the lid marginal areas in these studies, have lead us to believe that the proposed metrics for dimensioning LWE grades may be too generous for the minute changes seen in the cyto-morphology of the LW and MCJ areas (millimeters vs. micrometers). Utilizing such a

technology in future LWE studies may aid the repeatability of this measure and help alleviate the reported inconclusiveness of many LWE studies to date, including the ones presented in this thesis.

Table 31: Thesis summary

Main achievements		
We have developed an enhanced impression cytology method for the lid margin.		
We deliver a first account on the cellular width of the lid margin of a CL-representative population.		
Main findings		
CL- wear		• Is associated with an increase in the width of the lid wiper region, particularly at the upper lid margin. • Rigid lenses have the greatest impact on the lid margin cytology, and even soft CL wear is manifest at the lid margin, in comparison to non-lens wear.
LWE		• Higher grades are associated with enlarged lid wiper regions, characterized by many large, squamous cells, denoting increased keratinization, compared to non (or low) LWE. • Etiology may be frictional • Is greater in RGP vs. SCL wearers, and greater in SCL vs. nCL wearers. Grades are comparable between the upper and the lower lid margins. • Is not directly associated with comfort.
Comfort		• Symptoms are not manifest in the lid marginal cytology. • RGP wearers had better/equal comfort than non-lens wearers or even asymptomatic SCL wearers

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Figure 2

Figure 4, Figure 10

Figure 5

Figure 6

Figure 7, Figure 38

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```
import ij.*;
import ij.plugin.*;
import ij.process.*;
import ij.gui.*;
import ij.measure.Calibration;
import java.awt.event.*;
import java.util.EventListener;
import java.awt.Rectangle;
import java.awt.geom.*;
import java.awt.*;
import ij.measure.ResultsTable;
import javax.swing.Timer;


/**
      This plugin implements the KeyListener interface and listens
      for key events generated by the current image.
*/
public class Distance_Between_Polylines implements PlugIn, KeyListener,
ActionListener {

      private static ResultsTable results = new ResultsTable();
      ControlWindow window;
      ImagePlus img;
      int action;
      int x0, y0;
      int num_points;
      double x_step, y_step;
      double dx, dy;
      PolygonRoi polyline1, polyline2;


      public void run(String args) {

            this.img = WindowManager.getCurrentImage();
            if ( this.img == null ){
                  IJ.noImage();
                  return;
            }
            ImageWindow win = this.img.getWindow();
            ImageCanvas canvas = win.getCanvas();
            EventListener[] listeners =
canvas.getListeners(KeyListener.class);  // kan bruke getKeyListeners
            for ( int i=0; i < listeners.length; ++i ){
```

```java
                        if ( listeners[i].getClass() == this.getClass() ){
                                IJ.error("Distance_Between_Polylines already
running for this window");
                                return;
                        }
                }

                canvas.addKeyListener(this);
                window = new ControlWindow("Plugin Message Window", "Please
draw a direction line and press [Enter]", this);

                this.action = 0;
                doNextAction();
        }

        // Methods for handling key presses
        public void keyPressed(KeyEvent e) {
                doNextAction();
        }

        public void keyReleased(KeyEvent e) {}
        public void keyTyped(KeyEvent e) {}

        // Methods for managing the window:
        public void actionPerformed( ActionEvent e ) {

                window.setVisible( false );
                window.dispose();
                this.img.getWindow().getCanvas().removeKeyListener(this);
        }

        void terminatePlugin() {

                Timer timeout = new Timer(2000, this);
                timeout.setRepeats(false);
                timeout.start();   // execute actionPerformed() in 2 seconds
        }

        // The important methods:
        void doNextAction(){

                switch (action) {
                case 0:
                        window.setMessage("Please draw a direction line and
press [Enter]");
                        break;
                case 1:
                        if ( ! readRefLine() ){
                                return;
                        }
                        window.setMessage("Draw the first line and press
[Enter]");
                        break;
```

```
        case 2:
            if ( ! readFirstLine() ){
                return;
            }
            window.setMessage("Draw the second line and press
[Enter]");
            break;
        case 3:
            if ( ! readSecondLine() ){
                return;
            }

            if ( window.isShowing() ){  // another small hack
                doCalculations();
            }

            terminatePlugin();  // also removes the KeyListener

            break;
        default:
            window.setMessage("Another time in action switch (reset
counter?)");
            break;
        }

        ++this.action;
    }

    boolean readRefLine(){

        Roi roi = this.img.getRoi();

        if ( roi != null && roi.getType() == Roi.POLYLINE ){
            PolygonRoi polyline = (PolygonRoi) roi;
            if ( polyline.getNCoordinates() != 2 ){
                IJ.error("The direction line must have only two
points");
                return false;
            } else {
                int[] x_coords = polyline.getXCoordinates();
                int[] y_coords = polyline.getYCoordinates();
                Rectangle offset = polyline.getBounds();
                roi = (Roi) new Line(x_coords[0]+offset.x,
y_coords[0]+offset.y,
                                     x_coords[1]+offset.x,
y_coords[1]+offset.y);
            }
        } else if ( roi == null || roi.getType() != Roi.LINE ){
            IJ.error("The direction line must be a line");
            return false;
        }

        Line line = (Line) roi;
```

```java
            this.dx = line.x2 - line.x1;
            this.dy = line.y2 - line.y1;

            double length = Math.sqrt( dx*dx + dy*dy );

            this.num_points = (int) Math.floor( length ) + 1;

            this.x_step = dx / (num_points - 1);
            this.y_step = dy / (num_points - 1);

            this.x0 = line.x1;
            this.y0 = line.y1;

            return true;
        }

    boolean readFirstLine(){

            Roi roi = this.img.getRoi();
            if ( roi != null && roi.getType() == Roi.LINE ){

                    Line line = (Line) roi;
                    int[] x_coords = { line.x1, line.x2 };
                    int[] y_coords = { line.y1, line.y2 };
                    roi = (Roi) new PolygonRoi( x_coords, y_coords, 2,
Roi.POLYLINE );

            } else if ( roi == null || !(roi.getType() == Roi.POLYLINE ||
roi.getType() == Roi.FREELINE) ){

                    IJ.error("This plugin only work with polylines");
                    return false;
            }

            this.polyline1 = (PolygonRoi) roi;

            return true;
        }

    boolean readSecondLine(){

            Roi roi = this.img.getRoi();
            if ( roi != null && roi.getType() == Roi.LINE ){

                    Line line = (Line) roi;
                    int[] x_coords = { line.x1, line.x2 };
                    int[] y_coords = { line.y1, line.y2 };
                    roi = (Roi) new PolygonRoi( x_coords, y_coords, 2,
Roi.POLYLINE );

            } else if ( roi == null || !(roi.getType() == Roi.POLYLINE ||
roi.getType() == Roi.FREELINE) ){
```

```java
                IJ.error("This plugin only work with polylines");
                return false;
        }

        this.polyline2 = (PolygonRoi) roi;

        return true;
    }

    double scaleX( double x ) {

        Calibration calib = this.img.getCalibration();
        return (x-calib.xOrigin)*calib.pixelWidth;
    }

    double scaleY( double y ) {

        Calibration calib = this.img.getCalibration();
        return (y-calib.yOrigin)*calib.pixelHeight;
    }

    int sign( double num ) {

        return (num < 0) ? -1 : 1;
    }

    void doCalculations(){

        double x, y;
        double l1_x, l1_y;
        double l2_x, l2_y;
        double x_diff, y_diff;
        double[] distances   = new double[num_points];
        int    num_distances = 0;
        double avg_distance  = 0;
        double dist_variance = 0;

/*
// debugging:
IJ.write("refline: " + x0 + "," + y0 + " -> " + (x0+dx) + "," + (y0+dy));
IJ.write("");
int[] x_coords, y_coords;
Rectangle offset;
IJ.write("polyline1: " + polyline1.getNCoordinates() + " coordinates");
x_coords = polyline1.getXCoordinates();
y_coords = polyline1.getYCoordinates();
offset = polyline1.getBounds();
for ( int i = 0; i < polyline1.getNCoordinates(); ++i ){
      IJ.write("polyline1["+i+"] = " + (x_coords[i]+offset.x) + "," +
(y_coords[i]+offset.y));
}
IJ.write("");
```

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```
IJ.write("polyline2: " + polyline2.getNCoordinates() + " coordinates");
x_coords = polyline2.getXCoordinates();
y_coords = polyline2.getYCoordinates();
offset = polyline2.getBounds();
for ( int i = 0; i < polyline2.getNCoordinates(); ++i ){
      IJ.write("polyline2["+i+"] = " + (x_coords[i]+offset.x) + "," +
(y_coords[i]+offset.y));
}
IJ.write("");
// end debugging
*/

            for ( int point = 0; point < num_points; ++point ){
                // the actual point on the reference/direction line
                x = scaleX(this.x0 + point*this.x_step);
                y = scaleY(this.y0 + point*this.y_step);

                Point2D.Double p1 = getOrthogonalPoint( x, y, polyline1
);
                l1_x = p1.getX();
                l1_y = p1.getY();
                if ( l1_x == 0 && l1_y == 0 ){
                     // et lite hack...
                     continue;
                }

                Point2D.Double p2 = getOrthogonalPoint( x, y, polyline2
);
                l2_x = p2.getX();
                l2_y = p2.getY();
                if ( l2_x == 0 && l2_y == 0 ){
                     // et lite hack her ogs�...
                     continue;
                }

                // calculate the distance between the lines
                x_diff = l2_x - l1_x;
                y_diff = l2_y - l1_y;

                // store the distance in an array
                distances[num_distances] = Math.sqrt( x_diff*x_diff +
y_diff*y_diff );
                ++num_distances;

                if ( point % 10 == 0 ) {
                     Calibration calib = this.img.getCalibration();
                     img.getProcessor().drawLine(
                          (int)(l1_x/calib.pixelWidth  +
calib.xOrigin),
                          (int)(l1_y/calib.pixelHeight +
calib.yOrigin),
                          (int)(l2_x/calib.pixelWidth  +
calib.xOrigin),
```

```
                                    (int)(l2_y/calib.pixelHeight +
calib.yOrigin));
                        img.updateAndRepaintWindow();
                }
            }

            // calculate average distance
            for ( int i = 0; i < num_distances; ++i ) {
                    avg_distance += distances[i] / num_distances;
            }
            window.setMessage("Average distance: " + avg_distance);

            // calculate standard deviation (variance first)
            for ( int i = 0; i < num_distances; ++i ) {
                    dist_variance += (distances[i] - avg_distance) *
(distances[i] - avg_distance) / (num_distances - 1);
            }

            results.incrementCounter();
            results.addLabel("Filename", this.img.getTitle());
            results.addValue("Avg distance", avg_distance);
            results.addValue("Std deviation", Math.sqrt(dist_variance));
            results.show("Average distances");
        }

    public Point2D.Double getOrthogonalPoint( double x, double y,
PolygonRoi polyline ) {

            // first find the approximately orthogonal point

            int[] x_coords = polyline.getXCoordinates();
            int[] y_coords = polyline.getYCoordinates();
            Rectangle offset = polyline.getBounds();

            int i, lo_i, hi_i;
            double val, lo_val, hi_val;
            lo_i = 0;
            hi_i = polyline.getNCoordinates() - 1;
            lo_val = this.dx*(x-scaleX(x_coords[lo_i]+offset.x))
                    + this.dy*(y-scaleY(y_coords[lo_i]+offset.y));
            hi_val = this.dx*(x-scaleX(x_coords[hi_i]+offset.x))
                    + this.dy*(y-scaleY(y_coords[hi_i]+offset.y));

            if ( sign(lo_val) == sign(hi_val) ){
                    return new Point2D.Double();    // 0,0 indikerer feil :-
}
            }

            for ( int diff = (hi_i-lo_i)/2; diff >= 1; diff = (hi_i-
lo_i)/2 ){
                    i = lo_i + diff;
                    val = this.dx*(x-scaleX(x_coords[i]+offset.x))
                        + this.dy*(y-scaleY(y_coords[i]+offset.y));
```

```
                    if ( sign(val) == sign(lo_val) ){
                          lo_i = i;
                          lo_val = val;
                    } else {
                          hi_i = i;
                          hi_val = val;
                    }
             }

// debuggin:
if ( hi_i - lo_i != 1 ){
      IJ.error("hi_i - lo_i != 1");
}

             // then find the exact orthogonal point
             double lo_x = scaleX(x_coords[lo_i]+offset.x);
             double lo_y = scaleY(y_coords[lo_i]+offset.y);
             double hi_x = scaleX(x_coords[hi_i]+offset.x);
             double hi_y = scaleY(y_coords[hi_i]+offset.y);

             double a1; // slope for the polyline between lo_x,lo_y and
hi_x,hi_y
             if ( lo_x == hi_x ){
                    // vertical line
                    a1 = 1e14; // use a really large number
             } else {
                    a1 = ( hi_y - lo_y )/( hi_x - lo_x );
             }
// debugging:
if ( Math.abs(a1) > 1e14 ){
      IJ.error("a1 is a really big number: "+a1);
}


             double a2; // slope for the orthogonal to the direction line
at x,y
             if ( this.dy != 0 ){
                    a2 = -( this.dx/this.dy ); // the orthogonal line has
coordinates (x,y)+a2*(-dy,dx)
             } else {
                    // direction line horizontal, and thus the orthogonal is
vertical
                    //a2 = 1e14; // use a really large number
                    return new Point2D.Double( x, lo_y + a1*(x-lo_x) );
             }
// debugging:
if ( Math.abs(a2) > 1e14 ){
      IJ.error("a2 is a really big number: "+a2);
}

             if ( a1 == a2 ){  // do I need some fuzziness here?
                    // the line segment between lo_x,lo_y and hi_x,hi_y is
```

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```
                          // perpendicular to the direction line. Just use the
middle point.
                          return new Point2D.Double( (hi_x+lo_x)/2, (hi_y+lo_y)/2
);
               } else {
                          double xn = ( (y-a2*x) - (lo_y-a1*lo_x) )/(a1-a2);
                          return new Point2D.Double( xn, y+a2*(xn-x) );
               }

       }

}

class ControlWindow extends Dialog {
       Label label;

       public ControlWindow(String title, String message, ActionListener
listener) {
               super( IJ.getInstance(), title, false );

               setLayout( new BorderLayout() );
               if ( message==null ){
                      message = "";
               }

               Panel center = new Panel();
               center.setLayout( new FlowLayout( FlowLayout.CENTER, 15, 15 )
);
               add( "Center", center );

               this.label = new Label();
               center.add( this.label );
               setMessage( message );

               Button button = new Button( "  End plugin  " );
               button.addActionListener( listener );
               Panel panel = new Panel();
               panel.setLayout( new FlowLayout() );
               panel.add( button );
               add( "South", panel );

               if ( ij.IJ.isMacintosh() ){
//                    setResizable( false );
               }

               pack();
               placeUpperRight( this );
               show();
       }

       public void setMessage( String new_message ) {

               label.setText( new_message );
```

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```
            if ( label.getMinimumSize().getWidth() >
label.getSize().getWidth() ){
                    // how do I make the label and window larger?
                    this.validate();
            }
        }

        static void placeUpperRight(Window win) {
            Dimension screen =
Toolkit.getDefaultToolkit().getScreenSize();
            Dimension window = win.getSize();

            if (window.width==0){
                    return;
            }

            win.setLocation( screen.width-window.width, 0 );
        }

}
```