Copyright Statement

The digital copy of this thesis is protected by the Copyright Act 1994 (New Zealand).

This thesis may be consulted by you, provided you comply with the provisions of the Act and the following conditions of use:

- Any use you make of these documents or images must be for research or private study purposes only, and you may not make them available to any other person.
- Authors control the copyright of their thesis. You will recognise the author's right to be identified as the author of this thesis, and due acknowledgement will be made to the author where appropriate.
- You will obtain the author's permission before publishing any material from their thesis.

To request permissions please use the Feedback form on our webpage.
http://researchspace.auckland.ac.nz/feedback

General copyright and disclaimer

In addition to the above conditions, authors give their consent for the digital copy of their work to be used subject to the conditions specified on the Library Thesis Consent Form and Deposit Licence.
Corneal microstructure in diabetes mellitus and its association with peripheral neuropathy and cardiac autonomic neuropathy

Stuti Misra

Abstract

The overall focus of this research project was the ocular and systemic neurological complications of diabetes mellitus (DM) in man, and how they interrelate, with a view to being better able to understand and predict their development for earlier therapeutic intervention and prognostic accuracy. Therefore, a series of inter-related studies in this thesis investigated complications of DM involving the cornea and ocular surface, and their association with peripheral neuropathy and cardiac autonomic neuropathy.

Established nerve biopsy techniques with electron microscopy, and ex vivo confocal microscopy of skin punch biopsies allow direct examination of long nerve fibre damage and repair in DM. However, both are invasive procedures and may induce persistent pain at the biopsy site, cold intolerance and sensory deficits. However, laser-scanning in vivo corneal confocal microscopy (IVCM), as demonstrated in this thesis, enables non-invasive imaging of living human corneal nerves, potentially providing an alternative means of grading and monitoring nerve damage in DM.

The human corneal sub-basal nerve plexus architecture in normal and in those with DM was evaluated. The normal and diabetic corneal sub-basal nerve plexus exhibits a clockwise ‘whorl’ orientation that may have implications for post-surgical nerve regeneration. Other ocular parameters including corneal sensitivity, hysteresis, central thickness and topography along with the pre-ocular tear film metrics were compared between eyes of normal participants and participants with DM. Significant changes in the corneal sub-basal nerve density, corneal sensitivity threshold, and pre-ocular tear film in those with DM indicates a compromised ocular surface that may result in diabetic keratopathy.
Having established differences in the sub-basal nerve plexus between DM and normal corneas, the effect of the most common treatment for diabetic retinopathy, pan retinal photocoagulation (PRP), on corneal nerves was explored. The corneal nerve changes after PRP were established to be related primarily to DM and appeared independent of the laser treatment.

The relationship between ocular parameters including corneal sub-basal nerve plexus density, corneal sensitivity, diabetic retinopathy, peripheral neuropathy and cardiac autonomic neuropathy were evaluated. The correlation of corneal sub-basal nerve density with corneal sensitivity and total neuropathy score, a measure of peripheral neuropathy, confirms that reduced sub-basal nerve density reflects peripheral neuropathy in DM. This enables assessment of nerve damage without the need for painful and invasive skin biopsies and suggests a potential surrogate role for corneal IVCM in the diagnosis and assessment of peripheral diabetic neuropathy and potentially the monitoring of novel treatments.

The nexus of studies that constitute this research thesis investigated the crucial importance of early assessment of the cornea to determine the neuropathic effect of DM on the eye and, by association, upon the extremities of the body. The integration of IVCM corneal evaluation in screening programs, in addition to retinal screening, could aid in monitoring disease progression in DM at an early stage thus enabling better prognosis and more timely interventions.
For mum and dad
Acknowledgements

A somewhat surreal feeling strikes me as I contemplate the many people who accompanied me in this major journey of a lifetime. The completion of this thesis would not have been possible without the selfless contributions of so many. A page or two is insufficient to thank everyone and I apologise to those I may have omitted but to whom I am ever grateful.

First and foremost, I am grateful to my supervisors, Dr. Jennifer Craig, Professor Charles McGhee and Associate Professor Dipika Patel, who guided me throughout the process of inception, development and execution of this project.

Dr. Jennifer Craig has been very friendly and approachable, even contacting her on weekends was never a problem. I have learnt the significance and the art of attention to scientific detail under her excellent supervision. She was always happy to discuss any work related or personal issue. I am extremely thankful to her for supporting me through complexities and difficulties of this project.

Professor Charles McGhee has been one of the pillars of this work. In spite of being a busy clinician-scientist and a supervisor to eight other students, he always gave one-on-one guidance and constant encouragement and inspired me to strive for perfection. Not only has he been a great mentor professionally but also personally. I am grateful to him for funding me in the last few months to get me to the finish line and further my research career.

Associate Professor Dipika Patel is the most efficient person I’ve ever known. She has always given prompt and detailed feedback on documents of any length. Dipika has vast knowledge of the subject and always made time to share her expertise in a friendly
supportive manner, despite her busy schedule. I am grateful to her for teaching me the importance of working to tight deadlines.

Additionally, I truly appreciate Associate Professor Geoffrey Braatvedt for recruiting participants and constantly providing expert scientific and clinical input to this research. Dr. Dean Kilfoyle and Dr. Kevin Ellyett have also been extremely supportive advisors of this project.

I wish to sincerely thank Associate Professor Trevor Sherwin and Professor Colin Green who kept me motivated through tough times. Thanks to Ilva Rupenthal, James McKelvie, Carol Greene and Joanna Black for friendly and humorous support at University and conferences overseas. At this juncture, I must also mention my friends at the post graduate students’ association (PGSA) with whom I share some of the most memorable moments of my doctoral student life.

I must acknowledge all the research funding bodies for the project and travel grants that supported me. Without their monetary help this project would not have existed. Save Sight Society NZ Incorporated, New Zealand Association of Optometrists (NZAO), New Zealand Society for the Study of Diabetes (NZSSD), Sir John Logan Campbell travel award, FMHS-PGSA travel grant and University of Auckland doctoral scholarship have provided invaluable support to this project.

Finally, I truly value the support of my family who constantly encouraged me to achieve my best professional endeavours, especially my loving Mum, Dr. Pramila Misra and Dad, Rajiva Misra. A special thanks to my friends in New Zealand (Amita, Smitha, Prem and Harshad) and also in USA and India who kept in touch, despite different time zones and busy schedules. Lastly, only the sheer patience, understanding and support of my husband, Adelbert, gave me strength to complete this mammoth task.
# Table of Contents

Abstract...............................................................................................................................................ii

Acknowledgements.............................................................................................................................v

Chapter 1: Normal ocular surface anatomy and physiology

1. Introduction ........................................................................................................................................ 2

1.1. The pre-ocular tear film................................................................................................................... 2

1.1.1. The structure ............................................................................................................................. 2

1.1.2. The layers of tear film ............................................................................................................... 3

1.1.3. The pre-ocular tear film in diabetes mellitus .............................................................................. 5

1.2. Cornea ........................................................................................................................................... 5

1.2.1. Corneal epithelium .................................................................................................................... 7

1.2.2. Bowman’s layer ......................................................................................................................... 10

1.2.3. Stroma .................................................................................................................................... 10

1.2.4. Descemet’s membrane ............................................................................................................. 12

1.2.5. Endothelium ............................................................................................................................ 12

1.3. Limbus......................................................................................................................................... 13

1.4. Conclusion ................................................................................................................................... 15

1.5. References ................................................................................................................................... 16

2. Introduction ....................................................................................................................................... 24

2.1. Classification ................................................................................................................................. 25

2.2. Type 1 diabetes mellitus .............................................................................................................. 25

2.3. Type 2 diabetes mellitus .............................................................................................................. 26

2.4. Gestational diabetes mellitus ....................................................................................................... 27
9. Introduction ................................................................................................................... 153

9.1. Methods.................................................................................................................. 154

9.2. Results ................................................................................................................... 155

9.3. Discussion .............................................................................................................. 159

9.4. References............................................................................................................. 162

10. Introduction ................................................................................................................. 165

10.1. The two-dimensional architecture of the corneal sub-basal nerve plexus in diabetes mellitus .......................................................................................................................... 166

10.2. Corneal assessment using in vivo confocal microscopy in diabetes mellitus ......... 166

10.3. The cornea in patients with and without diabetes mellitus......................... 167

10.4. Evaluation of relationships between corneal nerve density, corneal sensitivity, retinopathy, cardiovascular autonomic neuropathy and peripheral neuropathy .............. 168

10.5. The effect of panretinal photocoagulation on the corneal sub basal nerve plexus in diabetes mellitus .......................................................................................................................... 169

10.6. The pre-ocular tear film in patients with and without DM ......................... 170

10.7. Final conclusion ................................................................................................. 170

11. Appendix ..................................................................................................................... 173

11.1. Related publications ............................................................................................. 173

11.2. Conference and seminar presentations ............................................................... 173

11.3. Alcohol consumption questionnaire ................................................................... 176
# List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>External ocular structures</td>
<td>2</td>
</tr>
<tr>
<td>1.2</td>
<td>The layers of normal tear film</td>
<td>4</td>
</tr>
<tr>
<td>1.3</td>
<td>Slit-lamp biomicroscopy images of the cornea</td>
<td>6</td>
</tr>
<tr>
<td>1.4</td>
<td>The layers of cornea stained with Masson’s trichrome</td>
<td>7</td>
</tr>
<tr>
<td>1.5</td>
<td>Limbal palisade morphology</td>
<td>14</td>
</tr>
<tr>
<td>2.1</td>
<td>Herpes simplex viral infection</td>
<td>36</td>
</tr>
<tr>
<td>2.2</td>
<td>Digital images using non-mydriatic retinal camera</td>
<td>40</td>
</tr>
<tr>
<td>3.1</td>
<td>Slit lamp biomicroscope</td>
<td>51</td>
</tr>
<tr>
<td>3.2</td>
<td>McMonnies Dry Eye Questionnaire</td>
<td>53</td>
</tr>
<tr>
<td>3.3</td>
<td>Patterns of lipid layer</td>
<td>55</td>
</tr>
<tr>
<td>3.4</td>
<td>A Keeler Tearscope Plus™</td>
<td>56</td>
</tr>
<tr>
<td>3.5</td>
<td>Phenol Red Thread test</td>
<td>57</td>
</tr>
<tr>
<td>3.6 a, b</td>
<td>The Non-Contact Corneal Aesthesiometer (NCCA)</td>
<td>58</td>
</tr>
<tr>
<td>3.7</td>
<td>Oculus Pentacam</td>
<td>59</td>
</tr>
<tr>
<td>3.8</td>
<td>Typical Oculus Pentacam report</td>
<td>59</td>
</tr>
<tr>
<td>3.9</td>
<td>Ocular Response Analyzer (ORA)</td>
<td>61</td>
</tr>
<tr>
<td>3.10</td>
<td>Diagrammatic representation of the ocular response analyser technique</td>
<td>62</td>
</tr>
<tr>
<td>3.11</td>
<td>HRT II RCM-IVCM</td>
<td>63</td>
</tr>
<tr>
<td>3.12</td>
<td>Layers of the cornea as imaged by HRTII RCM-LCSM</td>
<td>64</td>
</tr>
<tr>
<td>3.13</td>
<td>Example of nerve density analysis</td>
<td>65</td>
</tr>
<tr>
<td>3.14</td>
<td>Confoscan 4 SSCM</td>
<td>66</td>
</tr>
<tr>
<td>3.15</td>
<td>DR grading as photographed by a non-mydriatic retinal camera</td>
<td>68</td>
</tr>
<tr>
<td>3.16</td>
<td>Neuropathy symptom questionnaire</td>
<td>70</td>
</tr>
<tr>
<td>3.17</td>
<td>(a) Nerve conduction study in progress (b) Biothesiometry- quantitative sensory testing (QST)</td>
<td>70</td>
</tr>
</tbody>
</table>
Figure 3.18: Heart rate recording ................................................................. 71
Figure 3.19: Autonomic nerve analysis ....................................................... 72
Figure 4.1: IVCM montage of the sub basal nerve plexus ............................. 86
Figure 4.2: IVCM montage of the sub-basal nerve plexus whorl .................... 86
Figure 4.3: Electronic tracings of the IVCM images of the corneal sub-basal nerve plexus ................................................................. 87
Figure 4.4. Bland Altman plot ..................................................................... 88
Figure 4.5. Bland Altman plot ..................................................................... 88
Figure 4.6: IVCM montage of the sub basal nerve plexus ............................. 89
Figure 4.7: Representative ‘whorl’ region IVCM montage .............................. 90
Figure 5.1: Representative in vivo confocal images of HRT-II corneal module showing the sub-basal nerve plexus ................................................................. 101
Figure 5.2: Representative in vivo confocal images of the basal epithelium .......... 102
Figure 5.3: Typical in vivo confocal images of the corneal endothelium .......... 103
Figure 7.1: Scatter plot of Pearson correlation analysis of corneal sensitivity vs corneal sub-basal nerve density ................................................................. 127
Figure 8.1: Representative laser scanning IVCM images ............................... 142
Figure 8.2: Scatter plot graph showing a weak positive correlation between corneal sensitivity and biothesiometry ................................................................. 143
List of Tables

Table 3.1: Lipid layer thickness estimates ................................................................. 54
Table 5.1: Participant characteristics for corneal sub-basal nerve density analysis .... 101
Table 5.2: Participant characteristics for corneal epithelial cell density analysis ........ 102
Table 5.3: Participant characteristics for endothelial cell analysis ............................ 103
Table 5.4: Comparison of corneal cell densities between DM and control corneas ... 104
Table 6.1: Number of subjects and patient characteristics ........................................ 115
Table 6.2: Comparison of corneal parameters between controls and subjects with DM. ... 116
Table 7.1: Demographics of those with DM .............................................................. 126
Table 7.2: Pearson correlation analysis .................................................................... 128
Table 7.3: Pearson correlation analysis .................................................................... 129
Table 7.4: Pearson correlation analysis .................................................................... 130
Table 8.1: Patient demographics ............................................................................. 142
Table 8.2: Comparison of parameters between DM and controls. ............................ 144
Table 8.3: Correlations of parameters in patients with DM ...................................... 145
Table 9.1: DM and control group sample sizes and characteristics ......................... 156
Table 9.2: Comparison of symptoms and tear characteristics ................................. 156
Table 9.3: Pearson and Spearman correlation analysis ............................................. 158
Chapter 1
Normal ocular surface anatomy and physiology
1. Introduction

The ocular surface includes the tear film, the cornea, the conjunctiva and the transitional limbal zone. These regions serve as the structural support and provide a conduit for fluids and nutrients. The function of the ocular surface is also highly dependent upon the conformation and normal function of the eyelids. This chapter will review the anatomy and physiology of the pre-ocular tear film, cornea and limbus.

![External ocular structures as imaged by Slit-lamp biomicroscopy under diffuse illumination](image)

**Figure 1.1:** External ocular structures as imaged by Slit-lamp biomicroscopy under diffuse illumination

### 1.1. The pre-ocular tear film

#### 1.1.1. The structure

The pre-ocular tear film is a vital component of the optical system. There are four main functions of tear film. The first is to provide a smooth refracting surface by forming an air-tear film interface to enhance the quality of vision. Any irregularity can lead to symptoms of visual fatigue and photophobia. Secondly, tears keep the eye moist and lubricated to maintain
comfort. Any dysfunction in the interaction between the tear film and the eyelids can result in increased epithelial desquamation and the induction of pathological apoptosis. The third function of the pre-ocular tear film is to deliver nutrients to the cornea, since the cornea is avascular and entirely dependent on oxygen that diffuses from the tear film (and the aqueous humour). Lastly, being exposed to environment, the ocular surface is more vulnerable to infections. The pre-ocular tear film provides anti-bacterial defence against environmental and infectious insults.2

1.1.2. The layers of tear film

1.1.2.1. The lipid layer

The superficial lipid layer is a thin oily layer which is secreted by the meibomian glands that are embedded in the upper and lower tarsal plates. The glands of Moll and Zeis secrete additional lipid onto the lid margin. The lipid layer is responsible for retarding tear evaporation and preventing tear spillage onto the eyelid.3 This bilayer consists of both polar and non-polar lipids. Polar lipids, including phospholipids, ceramides and cerebrosides, form the lipid-aqueous interface,4 while non-polar lipids such as wax esters and cholesterol are located on the lipid-air interface. A substantial increase in tear evaporation is observed in the absence of lipid layer.3
1.1.2.2. The aqueous layer

The middle aqueous layer comprises about 90% of the total tear film thickness. It contains dissolved oxygen, electrolytes and a number of proteins that help lower the surface tension to facilitate ocular surface wetting and maintain epithelial integrity. Osmotic regulation and antimicrobial control (containing IgA and lysozyme amongst other components) are other functions of the aqueous layer. The aqueous layer is secreted primarily by the lacrimal gland, but also by the accessory lacrimal glands of Krause and Wolfring. The main lacrimal gland is responsible for reflex tear secretion that can be of peripheral or central sensory origin, caused by irritative stimuli or central nervous system disorders.

1.1.2.3. The mucin layer

The inner mucin layer is derived from the goblet cells of conjunctiva and the crypts of Henle in the fornices. The mucin layer allows the tear film to spread uniformly over the corneal and conjunctival surfaces, thereby providing protection against the shear force of blinking and
maintaining tear film viscosity at the same time. Additionally, the mucin layer may aid in the healing process and smooth micro-irregularities on the corneal surface.

1.1.3. The pre-ocular tear film in diabetes mellitus

Several clinical manifestations of diabetes mellitus impact upon the tear film and ocular surface. Abnormalities include compromised tear secretion, decreased corneal sensitivity, corneal epitheliopathy, recurrent erosions, persistent epithelial defects, delayed wound healing and higher risk of infections. Most of these present in the form of dry eye symptoms such as grittiness or burning sensation. The correlation between duration and severity of diabetes and its effect on the pre-ocular tear film are still to be fully established.

1.2. Cornea

The cornea is an avascular and highly specialised tissue in the anterior part of the eye. It is approximately 550µm in thickness centrally (range 490-620 µm) and pachymetry increases gradually towards the periphery. The cornea occupies almost one-sixth of the surface area of the globe in total. The anterior cornea is convex and aspheric, with a slightly greater horizontal than vertical diameter, with a mean diameter of 11.7mm. The average radius of curvature is 7.8mm, which, based on the corneal refractive index of 1.376, is equivalent to a dioptic value which approximates 44.00D. All the corneal layers are uniformly arranged to make light refraction and transmittance possible, an important corneal function essential for sight. The cornea is innervated by sensory fibres from the ophthalmic division of the trigeminal nerve and is the most sensitive part of the body; about five times more sensitive than the skin.
As previously noted, being avascular, the cornea requires a supply of essential nutrients via the fluids adjacent to its surfaces that is, oxygen, glucose, amino acids, etc., through the tear film, aqueous humour and limbal capillaries. When the lids are closed, the corneal oxygenation reduces and the lid vasculature provides minimal amount of oxygen via the tear film. The concentration of oxygen in the palpebral conjunctiva is almost one-third with that in air. Any significant disturbance in the tear film or aqueous humour can lead to corneal hypoxia and associated corneal oedema. In diabetes mellitus, hyperglycaemia causes excessive glycosylation of proteins, possibly due accumulation of advanced glycation end products (AGEs). Chapter 2 will further discuss the effect of diabetes mellitus on the cornea.

The cornea comprises of five layers, namely from anterior to posterior: epithelium, Bowman’s layer, the stroma, Descemet’s layer and the endothelium.
1.2.1. Corneal epithelium

The most anterior corneal epithelial layer comprises multi layered non-keratinized stratified squamous superficial cells, elongated cytoplasmic processes called wing cells and cuboidal-cylindrical shaped basal cells. The corneal epithelium is regular in thickness, ranging between 43 and 63 µm, over the entire tissue. Its smooth arrangement plays a major role in optical refraction. Another important function of the corneal epithelium is to protect the eye against microbial ingress and provide a barrier to fluid loss. The corneal epithelium consists of two to three layers of superficial epithelium, two to three layers of wing cell layer and a single layer of basal epithelium.
1.2.1.1. Superficial epithelium

The superficial epithelium consists of two to three layers of stratified flattened squamous cells that are bound by tight-junctional complexes to limit the entry of tears and external material, including fluorescein dye, into the intercellular spaces. The self renewing, non-keratinized, superficial layer has a turnover rate of five to seven days. The superficial layer contains microvilli and microplicae that secrete glycocalyx facilitating adhesion and promoting stability of the tear film.

1.2.1.2. Wing cell layer

These flattened cells, sandwiched between the superficial epithelium and the basal cell layer, are referred as wing cells. This transitional layer is approximately two to three cells deep and has tight, intercellular junctions with an average cell number of 5070 cell/mm². Wing cells are rich in keratin upon which the corneal epithelial cells are dependent for differentiation.

1.2.1.3. Basal Epithelium

A layer of basal epithelial cells sits on the 0.05µm thick basement membrane through a series of hemidesmosomes by anchoring fibrils of type VII collagen. This system helps the epithelium to firmly adhere to Bowman’s layer which is particularly important since the exposed corneal epithelium needs to resist abrasion to maintain function. Electron microscopy has revealed two zones within the basement membrane; a pale layer anteriorly known as the lamina lucida and an electron-dense layer posteriorly called the lamina densa.
The basement membrane also contains extra cellular matrix which is instrumental in maintaining the regularly arranged stratified corneal epithelium.\textsuperscript{34, 39} It is therefore also vital for corneal wound healing. In the case of acute epithelial injury, fibronectin is produced within the basement membrane, whereas, in normal conditions, type IV collagen and laminin provide stability to the corneal epithelium.\textsuperscript{34, 40}

Only the basal cells are capable of epithelial mitotic activity; superficial cells and wing cells do not possess this quality. Furthermore, these basal cells move centripetally at a rate of 26 $\mu$m / day to maintain epithelial equilibrium.\textsuperscript{41} The total epithelial turnover occurs in approximately 7 to 14 days when the daughter cells are sloughed off into the tear film.\textsuperscript{2, 42}

The epithelial basement membrane is believed to be compromised in the case of diabetic keratopathy as evidenced by abnormal light scattering.\textsuperscript{43} Differential regulation of the proliferation and migration of basal cells during wound healing may be due to alterations in corneal epithelial cell function.\textsuperscript{44}

Underneath and parallel to basal epithelium, nerve bundles run to form corneal sub-basal nerve plexus.\textsuperscript{45} These bundles are derived from the ophthalmic division of the trigeminal nerve and run through stroma before ending between Bowmans layer and basal epithelium.\textsuperscript{46, 47} These sub-basal nerve endings are from myelinated A-\(\delta\) and unmyelinated C-nerve fibres.\textsuperscript{48} In diabetic peripheral neuropathy, early damage to myelinated A-\(\delta\) and unmyelinated C-nerve fibres leads to hyperesthesia, paraesthesia, and loss of pain and temperature sensation.\textsuperscript{49} The involvement of corneal sub-basal nerve endings in diabetes mellitus will be explored in Chapter 5, 7 and 8.
1.2.2. Bowman’s layer

The acellular layer situated directly beneath corneal basal epithelium, known as Bowman’s layer, does not regenerate post injury. The thickness of this ranges between 10-20µm and consists of randomly, but closely arranged, collagen fibrils and proteoglycans in an amorphous matrix.\textsuperscript{50, 51} The collagen fibrils are mainly type I and III in this layer.\textsuperscript{52} Anteriorly, Bowman’s layer abuts the lamina densa of the basement membrane of the epithelium\textsuperscript{53} and posteriorly the layer is contiguous with anterior stromal collagen fibrils.\textsuperscript{54}

1.2.3. Stroma

The middle connective tissue layer of the human cornea, known as the stroma, is approximately 500µm thick, or around 90% of the total corneal thickness.\textsuperscript{55} The stroma stands out in its organisation from the rest of the collagenous tissues of the body because of its regularly arranged fibres and extracellular matrix which afford it transparency.\textsuperscript{21} The parallel arrangement of collagen fibrils, densely packed in layers or lamellae, allow clear passage of light through the cornea.\textsuperscript{56}

Collagen, keratocytes, extra-cellular matrix and stromal nerves together form the corneal stroma. The stroma depends on the keratocytes to create and maintain the extra cellular matrix environment.\textsuperscript{57} They also play a crucial role in stromal homeostasis by synthesising collagen molecules and glycoaminoglycans.\textsuperscript{58} The anterior stroma is home to the largest number of keratocytes in the cornea; the average density being 33,050 cell/mm\textsuperscript{3} when imaged by \textit{in vivo} confocal microscopy.\textsuperscript{59} An earlier study by the same group showed a higher keratocytes density of 47,100 cell/mm\textsuperscript{3} within the anterior stroma.\textsuperscript{60} However, there is a general trend of reduction in keratocyte density from the anterior to mid stroma, followed by a slight increase in density in the posterior stroma.\textsuperscript{61, 62} The keratocyte density in mid
stroma ranges from 11,610 to 19,578 cell/mm³ in comparison with 14,100 to 27,900 cell/mm³ in posterior stroma.\textsuperscript{59, 61, 63}

The stroma is composed primarily of three types of proteins; collagens, proteoglycans and glycoproteins.\textsuperscript{64} Collagen is the most abundant protein in the human body.\textsuperscript{65} It is present in various corneal structures including the lamellae of the stroma. Largely, collagen types I, III and V constitute the normal cornea whereas type XII plays an important role in the morphogenesis of corneal scar tissue.\textsuperscript{56} Collagen types VI and XIV have also been found in trace amounts in the cornea.\textsuperscript{56, 66}

The precise parallel and regular arrangement of collagen fibrils is captured by transmission electron microscopy, however, X-ray diffraction arguably provides more accurate data, highlighting the diameter of collagen fibrils to be 31nm in youth, progressing to around 34 nm with increasing age.\textsuperscript{67} The spacing between the fibrils varies from the centre of the cornea (57nm) to the periphery (60nm) and increases further at the site of the limbus.\textsuperscript{67} Using X-ray diffraction, Maurice was able to confirm the fibrillar packing arrangement (and associated destructive interference) required for corneal transparency.\textsuperscript{68}

The stromal collagen fibrils are surrounded by proteoglycans that are responsible for regulating hydration and maintaining the stroma’s structural properties.\textsuperscript{69} Proteoglycans include a protein core to which one or more glycosaminoglycan (GAG) chains are attached. Proteoglycans consist of keratin sulphate (about 65%), chondroitin-4 sulphate and dermatan sulphate. Some studies also suggest the involvement of proteoglycans in maintaining both the diameter and separation of collagen fibrils.\textsuperscript{70}

The corneal stroma tends to swell when the corneal endothelial pump fails and this leads to irregular spacing between the collagen fibres. As a result of inter-fibril irregularity, there is scattering of incident light, and consequently the cornea appears hazy.\textsuperscript{71}
1.2.4. Descemet’s membrane

Descemet’s layer is an acellular layer, with a thickness of approximately 3µm at birth and reaching 20µm as a normal ageing process.\textsuperscript{55, 72} It lies between the stroma and the endothelial layer of the cornea.\textsuperscript{73} It acts as the basement membrane of the corneal endothelium and is composed largely of collagen type IV. Electron microscopy has revealed that this membrane is divided into two distinct parts. The anterior portion contains vertically arranged collagen fibrils that have a hexagonal arrangement when sectioned horizontally.\textsuperscript{74} Conversely, the posterior part has a granular appearance. Unlike Bowman’s layer, Descemet’s membrane has the capability of regenerating post-injury.\textsuperscript{73}

1.2.5. Endothelium

The endothelium is a confluent monolayer of 400-500,000\textsuperscript{75} non-replicating cells which forms a honeycomb mosaic that by regulating hydration, is partly responsible for maintaining corneal clarity.\textsuperscript{69, 76} The cells are flattened and tightly adherent to one another, covering the entire posterior corneal surface in an approximately 6µm thick monolayer that fuses with the cells of trabecular meshwork at the periphery.\textsuperscript{77, 78} The endothelial cell density at birth ranges from 3400 cells/mm\textsuperscript{2} to 7500 cells/mm\textsuperscript{2} and gradually reduces with age.\textsuperscript{80-82} Using specular microscopy, laser scanning \textit{in vivo} confocal microscopy and slit scanning microscopy, various studies have shown normal cell density in an adult ranging from approximately 1953 to 3125 cells/mm\textsuperscript{2}.\textsuperscript{75, 63-87} The endothelium secretes the thick basal lamina described as Descemet’s membrane, bound together by modified desmosomes.

Classically, an endothelial cell has a round nucleus which is 7µm in diameter and the cell surface is covered by several posteriorly projected microvilli and marginal folds.\textsuperscript{71, 75} In addition to the nucleus, the presence of cytoplasm-rich organelles, abundant mitochondria,
widespread endoplasmic reticulum, free ribosomes and Golgi apparatus confirms the active metabolic role of these cells.\textsuperscript{16}

Endothelial morphology and cell density are known to alter from childhood to later stages of life. The endothelial cell density in the normal cornea deteriorates at a rate of approximately 0.6\% per year.\textsuperscript{69}

The endothelial cell layer is also responsible for fluid equilibrium within the stroma to maintain stable corneal osmolarity levels.\textsuperscript{88} The ionic "pump" proteins, Na\(^+\) and K\(^+\) ATPase, located in the baso-lateral plasma membrane of endothelial cells counterbalance the inward fluid flow by promoting transfer of excess fluid from the stroma back into the aqueous humour.\textsuperscript{89} The selective barrier allows ion flux only sufficient to satisfy the osmotic gradient. This process acts to step down the osmotic gradient from hypotonic to hypertonic equilibrium.\textsuperscript{75} Any breach in the membrane bound Na\(^+\) and K-ATPase sites of the fluid pump leads to immediate stromal swelling.\textsuperscript{90}

Trauma, inflammation and inherited endothelial disorders lead to a decreased endothelial cell count and a deviation of cell shape from their original hexagonal shape.\textsuperscript{91-95} Endothelial cell counts may reduce following intra ocular surgery, such as phacoemulsification of cataract.\textsuperscript{71, 96}

\subsection*{1.3. Limbus}

The zone between the cornea and sclera is known as the limbus and is divided into two anatomical components: the corneal limbus and the scleral limbus. In total, the limbus is approximately 1.5mm - 2.0mm wide.\textsuperscript{17} The limbal region also includes Schlemm's canal, the trabecular meshwork, loose connective tissue and on the ocular surface the non-keratinized
epithelium that connects the corneal and conjunctival epithelia.\textsuperscript{97} The location of the corneal epithelial stem cells at the corneo-scleral limbus makes it a region of interest for clinicians and researchers alike.\textsuperscript{98} Palisades of Vogt are abundant in the lower limbus; although they are also found in the superior limbus. It is these fibrovascular palisade-like structures that provide a conducive microenvironment, or niche, for limbal stem cells.\textsuperscript{99} Protection from ultra-violet radiation to stem cells is provided by melanin pigment which, naturally varies with skin colour.\textsuperscript{100} These self-renewing cells are important in maintaining corneal epithelial integrity.

Figure 1.5: Limbal palisade morphology as imaged by laser scanning in vivo confocal microscopy. (a) Absent palisade ridges (b) limbal palisade in a non-pigmented subject (c) hyper-reflective cells observed in palisade basal cells in patients with moderate pigmentation and (d) hyper-reflective cells within the rete pegs in patients with marked pigmentation.\textsuperscript{98}
1.4. Conclusion

The ocular surface including pre-ocular tear film, cornea and corneo-scleral limbus, is a complex unit that is crucial in protecting the eye from primary infections and providing the optimum refractive system for the eye. As described in the current chapter, this system is affected by many local as well as certain systemic disorders, including diabetes mellitus. The next chapter will discuss how diabetes mellitus can affect the ocular surface along with other complications of this disease.
1.5. References


55. Doughty MJ, Martin Herranz R, Corrales R. Comparative anatomy and physiology of the cornea and conjunctiva. *Ocular Surface: Anatomy and Physiology, Disorders and Therapeutic Care* 2012;32.


89. Joyce NC. Cell Cycle Control and Replication in Corneal Endothelium. *Cornea and External Eye Disease* 2010;69-86.


Chapter 2
Diabetes mellitus: definition, classification and complications
Chapter 2

2. Introduction

Diabetes mellitus (DM) is defined as a metabolic disorder characterised by chronic hyperglycaemia with disturbance of carbohydrate, fat and protein metabolism. In this condition, the pancreas either does not produce enough insulin or the body is unable to utilise it efficiently, or both. Thirst, polyuria and weight loss are classic symptoms of diabetes mellitus. These symptoms are often subtle initially, which can result in delayed diagnosis of DM, but the disease lasts a lifetime once diagnosed. Eventually, DM can lead to blindness, cardiovascular disease, nephropathy, and neuropathy with risk of foot ulcers and amputation.

In 1980, DM was estimated to affect 153 million people worldwide and this figure had doubled to 347 million by 2008 according to a study by Danei et al. Interestingly, the estimated figures reported by the World Health Organisation (WHO) Global Burden of Disease Study were somewhat lower. They estimated the global prevalence of DM as 220 million in 2010 and predicted a rise to 366 million by the year 2030. Regardless of the precise global figure (220-347 million), the prevalence of diabetes is inevitably rising due to the ageing population and trends in lifestyle choices. Substantial measures need to be taken to detect and manage the disease and better equip already over-burdened health care systems to tackle this worldwide problem.

This chapter will describe the classification, diagnostic criteria, and complications of DM, most specifically those relating to the eye.
2.1. Classification

An attempt to provide an international classification of DM was made by the National Diabetes Data Group (NDDG) and published in 1979. One year later an alternative classification was published by the Expert Committee on Diabetes in conjunction with the WHO. However, WHO consultation further amended the classification in 1985 to align more closely with NDDG. The two major categories were named IDDM (Insulin Dependent Diabetes Mellitus) or type 1 diabetes mellitus and NIDDM (non-Insulin Dependent Diabetes Mellitus) or type 2 diabetes mellitus. More recently (2003) it has been suggested that the terms IDDM and NIDDM should be abolished due to classification on the basis of treatment and not aetiology. Over the past 25 years, a number of amendments have been suggested and accepted by various health organisations.

The characteristics of the components of the widely accepted aetiological classification are described here. In this thesis, mainly type 1 DM and secondarily, type 2 DM (within Chapter 8 only) were investigated.

2.2. Type 1 diabetes mellitus

The type 1 form of DM constitutes approximately 5 to 10% of the total diabetic population and occurs due to pancreatic islet β-cell destruction that eventually causes ketoacidosis, coma and death if left untreated. This form is characterised by the presence of islet cells, glutamic acid decarboxylase (GAD), IA-2, IA-2β, and other insulin antibodies that reflect the autoimmune process that leads to β-cell destruction. The degree of β-cell destruction varies in these individuals; some patients present with ketoacidosis as the first manifestation of the disease, whereas, others retain residual β-cell function for several years. However, this latter group ultimately becomes dependent on insulin for survival. During later stages of the
disease, due to low or undetectable levels of plasma C-peptide, there is virtually no insulin secretion.¹

Type 1 DM is believed to be a multistep autoimmune disease. A widely accepted hypothesis was put forward in 1986 that is still considered a benchmark.¹³ It stated that some individuals are genetically prone to the disease. At a certain stage, they face environmental triggers that initiate islet autoimmunity leading to decay in β-cell mass, development of autoantibodies, hyperglycaemia and ultimately loss of C-peptide.¹¹

Type 1 DM develops in association with certain hereditary factors including Human Leukocyte Antigen (HLA) alleles and environmental factors, for instance a viral infection. Unfortunately, to date, environmental factors are poorly understood in this form of diabetes. Obesity is not always present but is not necessarily incompatible with the diagnosis.¹ The presence of other autoimmune disorders is not uncommon, for example, Grave’s disease, Hashimoto’s thyroiditis, vitiligo, Addison’s disease, myasthenia gravis, autoimmune hepatitis and coeliac sprue.¹² Notably type 1 can occur at any age, even in the ninth decade of life and not necessarily in the younger age group as previously understood.¹²,¹⁴

Some patients suffering from type 1 DM are autoantibody negative, but insulin dependent, with no known aetiology; they belong to the ‘idiopathic’ type 1 category.¹⁴ These patients suffer from periodic ketoacidosis and exhibit varying degrees of insulin dependency. This idiopathic form is more common in persons of African and Asian origin.⁸

2.3. Type 2 diabetes mellitus

The most common type of DM, previously referred to as non-insulin dependent diabetes mellitus (NIDDM) or adult-onset diabetes, is now known as type 2 DM. It accounts for about
90-95% of the total diabetic population. Those affected show a combination of insulin resistance and relative insulin deficiency. The exact aetiology of this form of diabetes is not known, although it is understood that there is no autoimmune destruction of β-cells. Insulin treatment is not required by patients during the initial stages of the disease and, indeed, throughout their lifetime in many cases.

Several genetic factors are involved in type 2 DM that are augmented by lifestyle, such as obesity and lack of exercise; and environmental factors. Although not all patients fall into the clinically obese category, obesity can cause some degree of insulin resistance by itself. Type 2 diabetes often remains undiagnosed for a number of years due to its asymptomatic nature and slow progression. The onset of type 2 DM is believed to occur, on average, 7 years prior to confirmation of a clinical diagnosis, by which time the presence of micro and macro vascular complications is not uncommon.

Insulin secretion is reduced in these patients and is insufficient to compensate for insulin resistance. However, weight reduction and pharmacological treatment serve to improve insulin resistance. Individuals with a previous history of gestational DM, a lack of physical activity, older age, a genetic predisposition, and certain ethnic groups, are most prone to this form of diabetes. The genetics of type 2 DM are complicated and still not clearly defined.

### 2.4. Gestational diabetes mellitus

Gestational diabetes mellitus (GDM) is one of the common complications that occur during pregnancy. GDM refers to glucose intolerance that is exacerbated during pregnancy, particularly during the 3rd trimester. GDM contributes to almost 90% of all pregnancy complications. Once diagnosed with higher than normal glucose levels, the development of
insulin resistance progresses at a similar rate to that in an individual with type 2 diabetes.\textsuperscript{16} Some ethnic groups have a higher than average incidence of GDM, for example, Hispanic, Asian, African, and Pacific Island populations.\textsuperscript{8}

The prevalence of GDM is on a constant rise as obesity among women at reproductive age increases. However, higher levels of physical activity before or during early stages of pregnancy reduces the risk of developing GDM.\textsuperscript{18} Women diagnosed with this form of DM have an increased risk of perinatal morbidity and are predisposed to develop type 2 DM in later stages of their lives. Furthermore, the children of these women are more likely to suffer from diabetes in childhood or early adulthood. The body mass index (BMI) of these children is more likely to be affected along with adiposity during late adolescence / early adulthood.\textsuperscript{19}

### 2.5. Other forms of diabetes mellitus

There are several less frequently encountered forms of DM. Genetic defects of the β-cell are inherited in an autosomal dominant pattern and were described in the early literature as maturity onset diabetes of the young (MODY).\textsuperscript{12} Certain genetically determined anomalies of insulin action can also lead to diabetes. However, the mutations of insulin receptors are not identical and result in variable metabolic changes ranging from hyper-insulinaemia to severe diabetes.\textsuperscript{1}

An excess amount of hormones including growth hormone, cortisol, glucagon, epinephrine; iatrogenic factors and some genetic syndromes including Down and Turner syndromes can induce additional forms of DM.\textsuperscript{12}
2.6. Diagnostic criteria of diabetes mellitus

Hyperglycaemia is a consequence of diabetic metabolic disturbance and not the cause. Since the underlying molecular pathophysiology of the disease is not clear, the measurement of plasma glucose and/or HbA1c (glycated haemoglobin) remain major diagnostic criteria. Efforts were made in 1979 to standardise the original diagnostic criteria involving glucose concentrations in the plasma, with the aim of identifying the high-risk population. These criteria were amended eighteen years later to specifically target individuals at greater risk of diabetic complications. The regular review of the rationale for retaining the diagnostic criteria is crucial. The current cut-off point with respect to these criteria reflects the risk for microvascular complications including retinopathy and nephropathy.

Today, the most commonly used tests for diagnosing DM are glycated haemoglobin (HbA1c) level, casual blood glucose measurement and the oral glucose tolerance test (OGTT). Patients are advised to fast overnight prior to blood collection for the OGTT, which is conducted 2 hours after drinking at least 75 g of dissolved glucose. Although this is one of the most routinely performed tests in pregnancy, the post-load test has been reported to be expensive and poorly reproducible.

The HbA1c test, which provides a measurement of glycated proteins, is widely accepted as a routine monitoring examination for patients with DM. It is defined as the haemoglobin (Hb) with glucose attached to the N-terminal of one, or possibly more, β chains. Levels of glycated proteins other than Hb differ and their quantification as a clinical tool lacks efficacy as their concentrations have, as yet, failed to correlate significantly with the chronic complications of diabetes. The combination of the HbA1c result and fasting plasma glucose level is currently considered to deliver a definitive diagnosis of diabetes.
Chapter 2

The 2003 report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus described four major diagnostic criteria; (1) the glycated haemoglobin (HbA1c) should be ≥ 6.5% (48 mmol/mol); or (2) the presence of diabetic symptoms in addition to a casual plasma glucose concentration ≥11.1 mmol/l), where casual refers to any time of the day without regard to the time since last meal, and common diabetic symptoms include polyuria, polydipsia and unexplained weight loss; or (3) The fasting plasma glucose should be ≥7.0 mmol/l where fasting means no caloric intake for at least 8 hours; or (4) Two hour post-load value of ≥11.1 mmol/l in an OGTT.8,20

Although the presence of islet cell autoantibodies is not routinely used in the diagnosis of type 1 DM, a standard islet cell autoantibody test can help classify the disease. Performance of this test is recommended for the relatives of affected individuals due to DM’s familial association.20

In cases of GDM, abnormal glucose tolerance is diagnosed when the plasma glucose values are over 7.0 mmol/l after fasting; ≥10.0 mmol/l after an hour and ≥8.5 mmol/l after 2 hours.12

2.7. Complications

Increasing duration of diabetes further compounds the impact of the complications, both macro- and microvascular. Common complications include neuropathy, nephropathy, keratopathy and retinopathy, and small vessel vasculopathy.3,26 The principle aim of this thesis was to attempt to correlate neuropathy, keratopathy and retinopathy, with a view to being able to predict other features by performing in vivo confocal microscopy imaging of the corneal nerves. This section details the systemic complications that were investigated in the current study, and the major ocular complications.
Chapter 2

2.7.1. Cardiovascular autonomic neuropathy

Cardiovascular autonomic function controls the heart rate, blood pressure and myocardial contractility and hence is crucial for proper functioning of the cardiovascular system. Cardiovascular autonomic neuropathy (CAN) is considered to be a severe complication of DM due to its prognostic consequences. The presence of CAN increases mortality rate almost ten-fold in comparison to diabetic individuals without CAN. The mortality rate rises in those with DM because of arteriovenous shunting and sudomotor dysfunction which increases their susceptibility to foot ulceration and amputations.

To avoid or delay reaching the final stages of the disease, early diagnosis and good glycaemic control are essential. One of the earlier signs of CAN is resting tachycardia caused by parasympathetic damage, followed by abnormal circadian rhythm. Previous studies have reported a reduction in circadian heart rate variability in type 1 diabetics as a consequence of impaired parasympathetic innervation. The most common measurement to characterise autonomic dysfunction is heart rate variability (HRV) assessment. HRV is discussed in more detail in Chapter 3.

2.7.2. Peripheral neuropathy

Peripheral neuropathy is considered to be one of the most common long-term complications of DM and affects around 50% of diabetic patients. Diabetic neuropathy is recognised to be heterogenous and, as such, can be divided into mononeuropathies, polyneuropathies, plexopathies and radiculopathies. An increased influx of glucose occurs through the polyol pathway with the increased enzymatic activity of aldose reductase, which causes an elevation of intracellular sorbitol concentrations and fructose levels in peripheral nerves. The prevalence of peripheral neuropathy increases with age, with duration of DM and with
poor glycaemic control. The symptoms of neuropathy in type 2 DM typically occur after around 10 years of the disease, reflecting the silent nature of this form of DM. On the contrary, severe diabetic neuropathy can develop after only a few months of a type 1 DM diagnosis. Primary clinical features include small nerve fibre dysfunction caused by impairment of the process that supports nerve fibre regeneration in diabetes. Neuropathy of the lower limbs presents itself with a variable degree of pain and sensory loss associated with proximal muscle weakness and atrophy. Common patient complaints are of numbness or a tingling sensation, or pain in the lower limbs that worsens at night. The weakness of the quadriceps and iliopsoas muscles causes difficulty climbing stairs and is compounded by a decreased patellar reflex. The symptoms progress over weeks or months in most cases before stabilising. Neurological changes tend to be asymmetrical and unilateral.

A recent study demonstrated that subclinical sensory diabetic neuropathy affects both large and small fibres in type 1 DM. Several diagnostic techniques are used to assess individuals with diabetic neuropathy. Monofilaments are widely used for touch sensitivity for initial lower limb evaluation, and this is conducted along with a symptom questionnaire such as the Michigan Neuropathy Screening Instrument. A more comprehensive tool includes a combination of clinical assessment, a symptoms questionnaire, vibration perception threshold and a nerve conduction test as explained by Cornblath, and discussed in Chapter 3.

2.7.3. Ocular

Arguably, the most investigated ocular complication of DM is diabetic retinopathy reported to be the leading cause of blindness amongst the working age group in the developed world. In recent times, the effects of DM on the individual ocular tissues in diabetes mellitus has become clearer, most specifically those on the tear film, cornea, iris and lens.
2.7.3.1. LIDS AND LASHES

It is generally accepted that diabetics are more susceptible to infections. Subjects with DM are prone to get the most common lid disease, blepharitis.\textsuperscript{39, 40} Recurrent styes, resulting from infected sebaceous glands, have also been noted as one of the initial signs of diabetes.\textsuperscript{40, 41} Xanthelasma is a further potential consequence of DM.\textsuperscript{41}

2.7.3.2. ORBIT

A few reports of orbital cellulitis in patients with diabetes have been described in literature. One such example, involved an undiagnosed case of type 2 DM, which presented with a bacterial form of orbital cellulitis.\textsuperscript{42} In an independent case, a rare opportunistic fungal infection, rhinocerebral mucormycosis, was reported in a patient with type 1 DM.\textsuperscript{43} Predictably, uncontrolled diabetes is considered to be one of the predisposing factors for rhino-orbito-cerebral mucormycosis.\textsuperscript{44}

2.7.3.3. CONJUNCTIVA

The effects of diabetes on the conjunctiva tend to surface at later stages.\textsuperscript{39} The most common risk in the diabetic conjunctiva is development of an acute bacterial infection.\textsuperscript{45} Patients with a history of an extended duration of diabetes often present with microvascular abnormalities including micro-vessel dilation\textsuperscript{46, 47} and increased tortuosity and leakage of the conjunctival capillaries.\textsuperscript{48} Interestingly, these changes in the capillaries mirror vessel changes observed in the retina.\textsuperscript{48} Several instances of microaneurysms in the bulbar conjunctiva have also been reported.\textsuperscript{49} However, there is need for further detailed studies to more fully explain the effects of this chronic disease on the conjunctiva.
Those with DM have a higher prevalence of tear film abnormalities, causing dry eye. One of the major contributing factors to the development of dry eye in DM is the decreased neural reflexes causing reduced corneal sensitivity (discussed later in this chapter) and leading to an abnormal ocular surface. Patients typically complain of regular dry eye symptoms including burning and foreign body sensation. In more severe cases, apparent tolerance to dryness and epitheliopathy can occur as a result of decreased corneal sensitivity associated with the development of diabetic neurotrophic keratopathy. In terms of the tear fluid itself, the higher glucose concentration in tears in DM, arising from conjunctival vessel leakage, alters the wound healing capability of the cornea and damages the microvascular supply to the lacrimal gland decreasing lacrimation.

Tear film stability, as measured by the tear break up time, has been found to be reduced in diabetes. This decline correlates with peripheral neuropathy and with poorly controlled disease. Decreased goblet cell density is believed to contribute to the decrease in tear film stability in DM. As described in Chapter 1, the goblet cells are the main source of the tear film mucins that function to protect the cornea and promote a stable tear film. In addition to its effect on tear film stability, peripheral neuropathy is also believed to disrupt lacrimal gland function resulting in diminished basal tear production in patients with DM. Assessment of lacrimal gland function, with the Schirmer test, has shown lower tear production rates in diabetics than in normal individuals. It has also been suggested that retinopathy and pan retinal photocoagulation may further increase the likelihood of dry eye in diabetic individuals.

Hyperglycemia and oxidative stress can lead to the formation of advanced glycation end-products (AGEs) that accumulate and modify the structure of the ocular surface protein.
Additionally, the cellular response to stress in the eye causes increased expression of nuclear factor kappa-B resulting in widespread tissue damage and dysfunction\textsuperscript{51}, including diabetic keratopathy.\textsuperscript{58} Inflammatory alterations have been observed in the lacrimal gland of diabetic rats, as a result of increased levels of AGEs and nuclear factor kappa-B.\textsuperscript{40, 59}

The effects of diabetes mellitus on the tear film in the patient cohort studied are reported in Chapter 9.

### 2.7.3.5. CORNEA

It has been reported that patients suffering from DM are at greater risk of several corneal abnormalities including superficial punctate keratitis, recurrent corneal erosions, persistent epithelial defects, and endothelial damage.\textsuperscript{60, 61} One published study detected corneal abnormalities in 73.6\% of all the diabetic patients who were examined.\textsuperscript{62} Due to the generally decreased immunity of diabetics, those suffering from diabetes risk developing severe complications such as corneal ulcers and infections following corneal trauma.\textsuperscript{63} A higher prevalence of viral and fungal infections, including herpetic eye disease\textsuperscript{64} and fungal keratitis\textsuperscript{65} has also been reported in those with DM.
Consequences of diabetes on the corneal epithelium include superficial punctuate keratitis, microcystic oedema, full thickness breaks and the formation of abnormal epithelial basement membrane.\textsuperscript{39, 66} Recurrent epithelial defects, arising from corneal basement membrane abnormalities,\textsuperscript{40} reduce the ability of the cornea to act as a barrier against infection.\textsuperscript{50, 56, 60, 66, 67}

In a study by Tavakoli et al. (2007), a significant negative association was identified between corneal sensitivity, increasing age and the severity of neuropathy.\textsuperscript{68} Independently, other researchers have demonstrated a correlation between weakened corneal barrier function and higher HbA1c levels.\textsuperscript{67}

Rosenberg et al. (2000) correlated corneal sub-basal nerve density, as determined from \textit{in vivo} confocal microscopy (IVCM) images, with corneal sensation in a small group of 23 patients with type 1 DM.\textsuperscript{60} These early studies confirmed the relationship between corneal nerve density and corneal sensitivity\textsuperscript{60} and suggested a strong correlation between corneal nerve changes and the stage of peripheral neuropathy.\textsuperscript{69} Corneal nerve tortuosity has since been confirmed to be significantly greater in diabetics suffering from severe peripheral neuropathy.\textsuperscript{70}
In addition to changes in the sub-basal nerves, stromal nerves have also been shown to be affected in DM, with significantly reduced nerve thickness and increased tortuosity.\textsuperscript{71} Interestingly, researchers have identified marked improvements in sub-basal nerve density, nerve branch density and tortuosity in association with significant improvements in HbA1c.\textsuperscript{72} Therefore, it appears that corneal IVCM has the potential to act, not only as a surrogate marker for peripheral neuropathy but also to be used as a routine examination tool to review the progress of this systemic disease, perhaps in a manner akin to retinal photo screening.

Decreased innervation of the sub-basal nerve layer in the cornea of subjects with DM is considered to be responsible for atypical cell densities, both within the epithelium and endothelium.\textsuperscript{73} In a study of 220 patients, polymegathism and pleomorphism of the corneal endothelial cells were observed in type 2 diabetes.\textsuperscript{74} Central corneal thickness has been shown to be increased in diabetes\textsuperscript{75} and correlates with duration of the disease.\textsuperscript{76} This suggests that hyperglycemia disrupts corneal endothelial function, causing swelling. Interestingly, conflicting reports on diabetic corneal biomechanics have been published. While one study reported higher\textsuperscript{77} corneal hysteresis values in diabetes, another observed the contrary.\textsuperscript{78} In a third study no difference in corneal biomechanics between the control and diabetic group was demonstrated.\textsuperscript{79}

The current project investigated the sub-basal nerve plexus in the normal and diabetic cornea (Chapters 4 and 5), in addition to assessing corneal biomechanics (Chapter 6). Of principle interest was the identification and exploration of possible correlations between sub-basal nerve density, corneal sensitivity, retinopathy and peripheral neuropathy. These data are discussed in Chapter 7.
2.7.3.6. IRIS AND PUPIL

Iris neovascularisation or rubeosis iridis is the most common complication of the iris, in diabetes, and has the potential to involve the entire iris surface. Neovascularisation emerges as small tufts of blood vessels along the pupillary margin or in the anterior chamber and, left unattended, can progress to rubeotic glaucoma. This occurs mainly in patients with proliferative diabetic retinopathy.

Pupil size and responses are commonly affected in DM. Individuals with longstanding disease typically have miotic pupils with reduced physiological hippus and show a poor response to pharmacological mydriasis. This poor response to dilation suggests that DM has a more profound effect on sympathetic than on parasympathetic innervation.

2.7.3.7. CRYSTALLINE LENS

Changes in ocular refraction and accommodation are universally recognised to be affected in DM. Although it has been reported that the change in refractive power could be due to the contribution of the posterior cornea these changes are largely the result of changes in lenticular refraction. In hyperglycaemic conditions, the difference in the osmotic gradient between the crystalline lens and aqueous humour leads to lenticular swelling and myopia.

Cataract is another well documented cause of vision impairment in patients with DM. The Blue Mountains Eye Study highlighted that an abnormal fasting glucose results is a major risk factor for the development of cortical cataract. Increasing duration of DM can further increase the risk of developing cataract. Also, cataract in DM generally progresses at a more rapid rate than in non-diabetics, and typically affects those with DM at an earlier age.
For age-matched individuals, the incidence of cataract surgery has been shown to be significantly greater in type 1 than in type 2. Prompt management of significant cataract in diabetic patients is crucial not only for vision improvement but also to enable accurate evaluation of the retina. However, this has to be weighed against the potential for cataract extraction to promote development of, or exacerbate, diabetic macular oedema.

The incidence of posterior capsular opacification has also been shown to be higher following cataract extraction in diabetic individuals compared to controls, highlighting the importance of post-operative follow-up to perform YAG capsulotomy when required.

2.7.3.8. Retina

A landmark study, the Wisconsin Epidemiological Study of Diabetic Retinopathy (WESDR), demonstrated that almost every individual suffering from DM has a 100% risk of developing some form of diabetic retinopathy over the course of 30 years of the disease. Similarly to other complications of DM, poor glycaemic control, age and duration of DM contribute greatly towards the rate of development of retinopathy in both type 1 and type 2 DM. Over 80% of those with type 1 DM become affected, though largely clinically significant retinopathy is seen in patients with DM of more than 5 years duration. In comparison, around 20% of those with type 2 DM present with retinopathy at the time of diagnosis due to its insidious clinical onset.
Clinical retinopathy is categorised into three developmental stages, namely, mild or background, non-proliferative, and proliferative retinopathy. Early signs include retinal vasodilation due to thickening of the capillary basement membrane, microaneurysms, intraretinal haemorrhages, and hard and soft exudates. Moderate and severe non-proliferative retinopathy represent increasing levels of ischaemia, with venous beading and looping alongside microvascular abnormalities. The most advanced stage, proliferative retinopathy, shows signs of neovascularisation progressing across the surface of either the retina or the posterior vitreous face. An additional category, diabetic maculopathy, is actually the most common cause of visual impairment in DM. The clinical signs include retinal thickening, presence of hard exudates within 500 µm of the fovea and ischemia around the fovea. The blood retinal barrier is compromised at this stage and fluids, proteins and lipids begin to leak into the sensory retina. The development of DR is also influenced by age related changes in Bruch’s membrane and the vitreous. The classification used in this thesis is described in Chapter 3.
longitudinal comparison, the most common tool used to screen those with DM for retinopathy is retinal photography,\textsuperscript{104} described in detail in Chapter 3.

Pan-retinal photocoagulation (PRP) is the current standard treatment for proliferative diabetic retinopathy.\textsuperscript{105} A recent study demonstrated changes in the corneal microstructure that suggested that PRP could have unexpected, adverse effects on the cornea.\textsuperscript{106} Chapter 8 of this thesis reports investigation of the effect of PRP on the cornea by comparing corneas of those with DM, with and without treatment by PRP.
2.8. References


Chapter 3
Evaluation techniques
Chapter 3

3. Introduction

The patients recruited with diabetes mellitus (DM) underwent detailed ocular evaluation including tear film, corneal and retinal assessment in addition to an extensive battery of neurology and autonomic nerve analyses and blood analysis. The control participants underwent detailed ocular evaluation and a blood test to exclude undiagnosed DM. This chapter will discuss in detail the techniques involved in these evaluations.

3.1. Visual acuity

Visual acuity is defined as the ability to discriminate two contrast points in space. Visual acuity assessment of each eye (with the other eye occluded) was performed in all the patients with their current spectacles using a standardised logMAR chart. The same technique was used to obtain visual acuity with a pinhole on top of their spectacles to confirm whether they were wearing their optimum correction. The values were recorded in logMAR format where each letter carries a value of 0.02. The projector chart was calibrated for a distance of six metres and the same chart was used throughout the study. The room illumination was dimmed to ensure appropriate and constant contrast.

3.2. Slit lamp biomicroscopy

Examination of the external and anterior ocular structures was performed using a Topcon slit lamp biomicroscope (Topcon Medical Systems, NJ, USA) (Figure 3.1). Both direct and indirect illumination techniques were employed to observe specific structures. The slit lamp biomicroscope consists of an observation and an illumination system. These systems are coupled such that the illumination system and the microscope share a common focal plane. Direct illumination techniques used include diffuse, focal illumination (optical section, parallelepiped, conical beam) and indirect techniques included retro-
illumination, sclerotic scatter and specular reflection.\textsuperscript{7} Diffuse illumination allowed gross examination of the external ocular structures whereas focal illumination exposed both transparent and translucent structures including the corneal layers, tear debris, blood vessels and corneal scars. Retro-illumination facilitated observation of structures that scatter light and appear lighter/darker than the background, such as corneal oedema and keratic precipitates.\textsuperscript{7} Sclerotic scatter follows the principle of total internal reflection and any corneal opacities, or corneal clouding was made visible by the scattering of light which appeared as bright patches against the dark background of the iris and pupil. Lastly, specular reflection was possible by aligning the beam of light and microscope while utilising the model of equal angle of incidence and the angle of reflection.\textsuperscript{8} The corneal endothelium was observed using this monocular technique.\textsuperscript{9}

![Figure 3.1: Slit lamp biomicroscope used in this study](image)
3.3. Dry eye assessment

The dry eye assessment performed on each study participant included symptomatic evaluation with the McMonnies Dry Eye Questionnaire, lipid layer assessment by interferometry and an evaluation of aqueous deficiency with the Phenol Red Thread (PRT) test.

The McMonnies Dry Eye Questionnaire (Figure 3.2) incorporates 12 questions concerning risk factors for dry eye including age, gender, contact lens history, dry eye symptoms (with or without external stimuli), history of dry eye treatments, systemic illnesses associated with dry eye syndrome (arthritis, thyroid disease), associated dryness of mucous membrane (mouth, throat, chest, or vagina) and use of topical or systemic medication which might predispose the individual to dry eye.\(^{10, 11}\) The McMonnies questionnaire has been validated for reliability and accuracy,\(^{11, 12}\) and has been widely used in clinical research as a tool for quantifying dry eye symptoms.\(^{11-15}\)

Utilising the principle of thin film interferometry, an interferometer can provide information about the thickness of the tear film lipid layer and its fluidity, in vivo. One of the first interferometers was developed in 1989.\(^{16}\) The interferometer uses bright white light as the illumination source. The pattern of white light interference allows precise and detailed information regarding the topography and thickness of the lipid layer of tear film to be obtained from specular images.\(^{17}\) The Keeler Tearscope Plus™ (Keeler Instruments Ltd, Berkshire, UK) was used in this study for interferometry analysis.
McMonnies Dry Eye Questionnaire

Please answer the following questions by underlining the responses most appropriate to you:

Gender: female / male.

Age: less than 25 years / 25 - 45 years / more than 45 years.

Currently wearing: no contact lenses / hard contact lenses / soft contact lenses.

1. Have you ever had drops prescribed or other treatment for dry eyes?
   - Yes
   - No
   - Uncertain

2. Do you ever experience any of the following eye symptoms? (Please underline those that apply to you.)
   - Soreness
   - Scratchiness
   - Dryness
   - Grittiness
   - Burning

3. How often do your eyes have these symptoms? (underline)
   - Never
   - Sometimes
   - Often
   - Constantly

4. Are your eyes unusually sensitive to cigarette smoke, smog, air conditioning, or central heating?
   - Yes
   - No
   - Sometimes

5. Do your eyes become very red and irritated when swimming?
   - Not applicable
   - Yes
   - No
   - Sometimes

6. Are your eyes dry and irritated the day after drinking alcohol?
   - Not applicable
   - Yes
   - No
   - Sometimes

7. Do you take (please underline) antihistamine tablets or use antihistamine eye drops, diuretics (fluid tablets), sleeping tablets, tranquillisers, oral contraceptives, medication for duodenal ulcer, digestive problems, high blood pressure, antidepressants or ..............................................................................................................................? (Write in any medication you are taking that is not listed.)

8. Do you suffer from arthritis?
   - Yes
   - No
   - Uncertain

9. Do you experience dryness of the nose, mouth, throat, chest or vagina?
   - Never
   - Sometimes
   - Often
   - Constantly

10. Do you suffer from thyroid abnormality?
    - Yes
    - No
    - Uncertain

11. Are you known to sleep with your eyes partly open?
    - Yes
    - No
    - Sometimes

12. Do you have eye irritation as you wake from sleep?
    - Yes
    - No
    - Sometimes

---

For office use only:

<table>
<thead>
<tr>
<th>Age: M0F0/M1F3/M2F6</th>
<th>1: 6/0/0</th>
<th>3: 0/1/2</th>
<th>4: 4/0/2</th>
<th>5: 0/2/0</th>
<th>6: 0/4/0</th>
</tr>
</thead>
<tbody>
<tr>
<td>T: Anti-diur 2, tranq, pill / ulcer / dig / bp / antidep 1</td>
<td>2/0/0</td>
<td>5: 0/1/2</td>
<td>4: 2/0</td>
<td>11: 2/0</td>
<td>12: 2/0</td>
</tr>
</tbody>
</table>

Total score: [ ]
The normal lipid layer ranges in thickness from 20-160nm, and the varying thicknesses are represented by different tear film surface patterns which have been classified as open meshwork, closed meshwork, wave or flow, amorphous and colour fringes (Figure 3.3).\textsuperscript{18-20} The lipid layer is continually stretched and compressed between upper and lower lid margins by the blinking mechanism.\textsuperscript{21} The dynamic appearance of the tear film thus needs to be taken into consideration when grading the lipid layer pattern. Using this method and classification criteria, lipid layer thicknesses of the normal and dry eye have been determined with good repeatability.\textsuperscript{19, 20, 22}

<table>
<thead>
<tr>
<th>Lipid layer pattern</th>
<th>Thickness (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>Open meshwork</td>
<td>13-50nm~</td>
</tr>
<tr>
<td>Closed meshwork</td>
<td>30-50 nm~</td>
</tr>
<tr>
<td>Flow or wave</td>
<td>50-80 nm~</td>
</tr>
<tr>
<td>Amorphous</td>
<td>80-90 nm~</td>
</tr>
<tr>
<td>Colour fringes</td>
<td>90-180 nm~</td>
</tr>
</tbody>
</table>
A second part of the Tearscope Plus™ assessment involved measurement of non-invasive tear break-up time (NIBUT). This was the preferred technique over the clinical fluorescein break-up time test as it is recognised that the instillation of fluorescein can adversely affect tear film stability. The Keeler Tearscope grid attachment, when inserted into the Tearscope, projects a grid pattern onto the pre-corneal tear film surface (Figure 3.4). The patient, gazing straight ahead, was asked to blink twice, and then to refrain from blinking. The time until the first break or distortion within the projected grid pattern was recorded as the NIBUT. The procedure was repeated and an average of three measurements calculated. A thicker tear film lipid layer has been shown to be associated with increased tear film stability. Increasing age and gender also play a role in the stability of tear film. The tear film stability decreases with age, and women over 45 years exhibit a further deterioration in tear film stability.
The non-invasive tests described above were followed by the Phenol Red Thread (PRT) test (Figure 3.5). The PRT test provides an indication of the basal tear secretion with minimal stimulation of reflex tear production. This test involves hooking a thread, impregnated with pH sensitive phenol red dye, over the lower temporal lid margin for a test period of 15 seconds. The phenol red dye changes from a yellow to red colour in the presence of the slightly alkaline tear fluid and the colour conversion facilitated distinction of the length of cotton thread wetted by tears. Participants were advised to continue blinking normally. A PRT test result of less than 6mm is considered to be indicative of dry eye. This technique has high intra-subject repeatability. Conversely, the Schirmer test has poor repeatability and reproducibility in normal as well as those with dry eye. Hence, the use of Schirmer test was avoided in the current study.
3.4. Corneal sensitivity assessment

Central corneal sensitivity (CST) was evaluated non-invasively with the Non-Contact Corneal Aesthesiometer (NCCA, Glasgow Caledonian University, Glasgow, UK). The NCCA emits a controlled jet of air, lasting for 0.9 seconds, which is directed towards the cornea and produces a localised reduction in surface temperature (Figure 3.6). The change in temperature is detected by the corneal nerves and the minimum force of air required from the machine to induce a sensation is expressed in milli bars (mBAR). The technique has been validated against the Cochet-Bonnet aesthesiometer, and deemed accurate and repeatable. CST is known to reduce in patients with DM and has been correlated with the severity of neuropathy.
Figure 3.6 a, b: The Non-Contact Corneal Aesthesiometer (NCCA) is attached to the slit lamp biomicroscope for stability, accuracy and repeatability of positioning during assessment of corneal sensitivity.

In the current studies, the stimulus was first demonstrated to the patient at a supra-threshold pressure, before the CST was assessed using a forced-choice double-staircase technique. The CST was measured three times and the average calculated.

3.5. Corneal topography and central thickness measurement

The Oculus Pentacam Rotating Scheimpflug Tomographer (Oculus Optikgerate GmbH, Wetzlar, Germany) was used for evaluating corneal topography and thickness (Figure 3.7). The instrument combines slit illumination with blue light emitting diodes (LEDs) and a Scheimpflug camera, which rotates to analyse the anterior segment. Around 25 images of the anterior eye segment are acquired by the rotating camera over a period of approximately two seconds. The instrument automatically captures and corrects any eye movements by a static camera that detects the pupil contours and compensates for fixation drift. Both two and three dimensional models of the anterior segment of the eye are created by the instrument software. The 3-D reconstruction is based on the measured elevation data.

Room lights were switched off during the assessment and the participant was instructed to fixate on the target, with both eyes open. The machine was aligned to the pupil plane and the subject requested to blink before holding their eyes open for the duration of the
examination. The instrument automatically commenced image acquisition once correctly focussed.40

Figure 3.7: Oculus Pentacam being used to evaluate the left eye of a patient.

Figure 3.8: Typical Oculus Pentacam report combining optical, diagrammatic and numeric data.
Corneal topography can be reported in three ways; instantaneous curvature/power, axial distance/power and elevation. The corneal power estimation uses instantaneous curvature data and radius of curvature data, and an average corneal refractive index. Corneal thickness is computed by calculating point to point differences in elevation between the anterior and posterior corneal surfaces. The corneal SimK values and central corneal thickness (CCT) measurements are extrapolated from the report generated by the instrument software.\textsuperscript{38-40} The Pentacam provides precise evaluation of corneal and pachymetry maps (Figure 3.8) with high intra observer repeatability.\textsuperscript{41} The instrument provides reliable ‘K’ measurements in comparison studies with an automated keratometer and a conventional corneal topographer (TMS-4; Tomey, Erlangen, Germany).\textsuperscript{42} The inter observer intra class correlation coefficients ranged from 0.95 to 0.99 in a recent study.\textsuperscript{42} Even though several studies have reported excellent repeatable and reproducible results with Pentacam for central corneal topography,\textsuperscript{40, 43, 44} the results are not are interchangeable with other corneal topography instruments.\textsuperscript{45, 46} A number of studies have also shown agreement and repeatable results for the CCT measurement with Pentacam, Orbscan II and ultrasound pachymetry.\textsuperscript{39, 41, 43, 47}

3.6. Measurement of biomechanical properties of the cornea

Corneal hysteresis (CH) and corneal resistance factor (CRF), as measured by the Ocular Response Analyzer (ORA) (Reichert Ophthalmic Instruments, Depew, NY, USA) (Figure 3.9), are two unique biomechanical parameters of the cornea that provide additional information to clinicians in evaluating patients with keratoconus,\textsuperscript{48} following refractive surgery\textsuperscript{49} and after corneal collagen cross-linking treatment.\textsuperscript{50, 51} The instrument applies a precisely metered collimated air pulse to the cornea and under this force, the cornea flexes inwards, beyond applanation, and into a slight concavity. Within milliseconds after applanation, the cornea then move back outwards, resuming its normal shape, when the air pump shuts off in an inverse-time symmetrical system (Figure 3.10).\textsuperscript{52}
The delay between the inward and the outward applanation actions gives rise to two different intraocular pressure values. The average of the two pressure values is described as the Goldmann correlated intra ocular pressure (IOPg). However, the ORA is recognised to overestimate the IOPg value.  

The difference between the inward and outward pressure values obtained during the dynamic bi-directional applanation process, caused by viscous damping within the cornea, is termed the CH. Accounting for this viscous damping within the corneal tissues, which is created by the viscosity of glycoaminoglycans (GAGs), proteoglycans (PGs) and collagen matrix interaction, gives rise to two additional parameters; corneal compensated IOP (IOPcc) and CRF. IOPcc is an empirical measurement whereas CRF is an indicator of overall resistance of the cornea. A recent study showed good inter and intra observer reproducibility of CH, CRF and IOPcc measurements. The inter observer reproducibility of CH was 7.3 ± 8.6% for eight examiners in a different study. It has been shown that CH decreases following eye rubbing for 2 minutes, therefore during the current study care was taken to advise participants to refrain from eye rubbing to avoid unreliable results.
Figure 3.10: Diagrammatic representation of the ocular response analyser technique, where the difference between the inward and outward pressure values is named corneal hysteresis.\textsuperscript{52}

Although CH is believed to be independent of the radius of curvature of the cornea, corneal astigmatism, visual acuity and axial length,\textsuperscript{59} it has been shown to be influenced by age, IOP and CCT.\textsuperscript{60, 61} CH is also reported to be lower in patients with Fuch’s corneal dystrophy\textsuperscript{62} and glaucoma\textsuperscript{63} conditions seen predominantly in older age. DM is also believed to affect corneal biomechanics and lead to lower CH values compared to healthy controls.\textsuperscript{64} The CH and CRF alterations have been reported to depend on long term glycaemic control.\textsuperscript{65}

3.7. \textit{In vivo} confocal microscopy

Both slit-scanning \textit{in vivo} confocal microscopy (SSCM) and laser scanning \textit{in vivo} confocal microscopy (LSCM) allow non-invasive acquisition of two-dimensional corneal layers, at a cellular level.\textsuperscript{66-73} The two types of \textit{in vivo} confocal microscopy IVCM used in the current study were Confoscan 4 (NIDEK, Aichi, Japan) (SSCM) and Heidelberg Retina Tomograph (HRT) II Rostock Corneal Module (RCM) (Heidelberg Engineering GmbH, Germany) (LSCM).
In HRT II RCM, the laser beam (670nm diode laser) is focused on the cornea and periodically deflected by oscillating mirrors to allow sequential scanning of the cornea. A light sensitive detector measures the amount of reflected light. IVCM images are an optical section through the cornea at the location of the focal plane. Light scattered outside the focal plane is suppressed. The focal plane is moved manually to scan through the corneal layers. Each IVCM image is about 400 × 400 µm in size with a lateral resolution of 2 µm and optical section thickness of 4 µm.

Figure 3.11: HRT II RCM-IVCM, (a) Probe with viscous gel (above) and TomoCap in place (below) (b) IVCM in clinical use

Utmost importance was given to prepare the instrument and the subject. Corneal anaesthesia was achieved by instilling a drop of 0.4% oxybuprocaine (Chauvin Pharmaceuticals Ltd., UK) in the conjunctival sac of the patient. After sterilising the probe tip, a pea sized amount of viscous gel (Viscotears® Carbomer, Novartis Pharmaceuticals Ltd., UK) was applied to the tip, while taking care to avoid the formation of any air bubbles. A disposable TomoCap was mounted over the probe without contamination while mounting. Lubricating viscous gel was additionally applied to the contralateral eye, not under examination, to protect it from drying during the procedure. Before beginning the scan, the focus position control was reset.
The subject was positioned and asked to fix their gaze, with the contralateral eye, on a central target (black dot) on a board mounted on the wall. The cornea was scanned from epithelium to endothelium (Figure 3.12) by rotating the appropriate knob to modify the imaging plane. If the imaging was being performed for the preparation of a nerve map, the subject would then be requested to look sequentially at the blue and red coloured dots arranged in a radial, circular pattern on the same fixation board (Figure 3.11). The board was kept at a distance of two metres for each assessment. Intermittent rest breaks were offered as and when required by the participants.

Figure 3.12: Layers of the cornea as imaged by HRTII RCM-LCSM (a) Epithelium (b) Sub-basal nerve plexus (c) Anterior stroma (d) Mid stroma (e) Post stroma (f) Endothelium

Using the HRT II RCM, approximately 500 images from each subject were required for preparation of the sub-basal nerve map. A large number of images were acquired to minimise the risk of having gaps in the final montage map. Images were overlapped and
manually computer-stitched to form a contiguous montage using Macromedia Freehand 10 (Macromedia Inc, San Francisco, CA, USA).

The sub-basal nerve density was evaluated in individual images, by tracing visible nerves with an electronic pen (Wacom Technology Group, Vancouver, BC, Canada) (Figure 3.13) and measuring the length of the traced nerves with a digital calliper tool (analySIS 3.1, Soft Imaging System, Münster, Germany). The IVCM images of the epithelial cell layer from the HRTII-RCM were used to determine epithelial cell density. Three high quality, clear and focused representative images from each subject were processed using ImageJ software to calculate the epithelial cell density. A central image area of 200 µm² was used for the calculation.

Figure 3.13: Example of nerve density analysis using an electronic pen

SSCM images of the endothelial cell layer were acquired by Confoscan 4 (NIDEK, Aichi, Japan) for semi-automated measurement of endothelial cell density. The slit is illuminated by a halogen lamp (“white” non-coherent light unlike the 670nm source in the HRTII RCM), allowing longer examination times due to low light intensity and the scanning and de-scanning is done by a double-sided mirror. The detector is a charge-coupled device (CCD)
camera. Viscous gel (Viscotears® Carbomer, Novartis Pharmaceuticals Ltd., UK) acts as a medium that optically couples the tip of the microscope objective to the cornea.

Figure 3.14: Confoscan 4 SSCM, (a) In clinical use (b) Objective lens probe with lubricating gel

The coefficient of variation in cell shape and coefficient of variation in cell area were semi-automatically calculated with the NAVIS (Nidek Advanced Vision Information System) endothelial analysis software to quantify pleomorphism and polymegathism of the endothelial cells. Mean endothelial cell densities were calculated for each eye in this current study. Only the central 200 µm by 200 µm area of each image was used for analysis purposes.

In vivo confocal microscopy has become the standard tool for assessing the living cornea at a cellular level in health, disease and post-ocular surgery. A reduction in keratocytes and endothelial density has been reported with increasing age, when the cornea is imaged by LSCM. However, there are conflicting findings regarding the influence of increasing age on the corneal sub-basal nerve density, with the different types of IVCM. More recently, IVCM has been used to detect and monitor the progress of diabetes mellitus and its complications (discussed in Chapter 2). The instrument shows excellent
repeatability and reproducibility in evaluation of corneal sub-basal nerve density, in both controls and in DM.\textsuperscript{82, 90, 91}

3.8. Retinal Imaging

Digital images of the central and peripheral retina were captured using a non-mydriatic retinal camera (Non-Mydriatic Retinal Camera DR-DGi, Canon Inc, USA) for the purpose of diabetic retinopathy (DR) grading. In order to image a large area of the retina the subject was initially asked to focus straight ahead, followed by primary gaze positions. At least two focused images of the central and two of the peripheral retina were captured. Blurred images were discarded. For the purpose of this study DR was graded by an independent medical retina specialist. The standard criteria from the Early Treatment Diabetic Retinopathy Study (ETDRS) report are accepted worldwide, hence utilised in this thesis.\textsuperscript{92} DR was divided into non-proliferative DR, mild proliferative diabetic retinopathy (mild PDR), moderate PDR, severe PDR (Figure 3.15). Diabetic maculopathy was also taken into account during grading.
Figure 3.15: DR grading as photographed by a non-mydriatic retinal camera, (a) no active DR (b) mild DR and (c) moderate DR
3.9. Peripheral neuropathy assessment

The Total Neuropathy Score (TNS) scoring system provides a simple, inexpensive and rapid, yet sensitive, measure of peripheral neuropathy. This score is derived from a combination of the symptomatic neuropathy score (Figure 3.16), clinical assessment by an experienced neurologist, biothesiometry-quantitative sensory testing (QST) or vibration perception threshold (VPT) (Figure 3.17) and nerve conduction study (NCS). The questionnaire concentrates on grading the severity of the sensory, motor or autonomic symptoms, with the exception of non-distal symptoms. The clinical assessment further involves establishing the presence and severity of distal length-dependent neuropathy that is predictive of diabetic neuropathy. Biothesiometry-QST has been described as being as crucial to neuropathy assessment as visual acuity measurement is to an eye examination. More sensitive than the monofilament test, an alternative test in common use, the QST is acknowledged to be an important non-invasive and practical screening tool to detect peripheral neuropathy.

The final component of the TNS is from a more sophisticated test, the NCS, which determines myelinated nerve function by preferentially assessing the fastest conducting subset of the alpha motor axon population. Polyneuropathy does not affect nerves uniformly during the early stages, and reduced sensitivity of conduction velocity can indicate partially affected nerves. Reduction in nerve conduction velocity is associated with the presence of other microvascular diabetes complications including DR. The use of all these entities, jointly as the TNS, provides a reliable and sensitive scoring system.
Chapter 3

**Questionnaire**

Please answer the following questions about your symptoms. If your symptoms fluctuate in severity please score them at their WORST in the last 6 weeks.

Please score the RIGHT side unless your symptoms are much worse on the left.

**Which side have you scored? RIGHT / LEFT**

1. How much numbness (loss of feeling), tingling (*gone to sleep* feeling), or burning pain do you experience on a regular basis? (Circle one)
   a. None
   b. In your fingers and/or toes only
   c. Finger and/or toes and extending up to wrist or ankle
   d. Symptoms extending from fingers/toes up to knee or elbow
   e. Symptoms above the knees or elbows

2. How much muscle weakness (decreased strength) do you experience in your arms or legs? (Circle one)
   a. None
   b. Causes slight difficulty
   c. Causes moderate difficulty but you can still manage most activities yourself
   d. You require some help or assistance for some routine activities
   e. You have some muscles with paralysis (complete loss of movement)

3. Have you experienced **ON MORE THAN ONE OCCASION** in the past few months the following symptoms? (Circle all that apply)
   a. Urinary incontinence (involuntary leakage of urine)
   b. Diarrhoea that wakes you from sleep and is not due to food poisoning
   c. Unusual sweating soon after a meal (within 30 minutes)
   d. Vomiting soon after a meal (within 30 minutes)
   e. Palpitations or severe dizziness soon after standing up

---

**Figure 3.16: Neuropathy symptom questionnaire**

**Figure 3.17: (a) Nerve conduction study in progress (b) Biothesiometry- quantitative sensory testing (QST)**
3.10. Autonomic nerve analysis

Cardiorespiratory reflexes are compromised in DM and it is possible to identify parasympathetic impairment of cardiac innervation in some patients prior to the advent of clinical signs of autonomic neuropathy.\textsuperscript{102} The common clinical autonomic function measurements involve an end-organ response to a physiological provocation.

![Heart rate recording during assessment of autonomic function](image)

**Figure 3.18:** Heart rate recording during assessment of autonomic function

The measurement of heart rate (Figure 3.18) variability (HRV) in different physiological states is the most common index of cardiac parasympathetic function, and was chosen as the appropriate metric for investigation and comparison with corneal nerve function in the current study.\textsuperscript{103} According to validated protocols, the variation between beats or HRV was measured in four stages, namely, resting breathing, deep breathing, Valsalva and orthostatic manoeuvre (Figure 3.19).\textsuperscript{104} The subjects were asked lie down for the resting breathing manoeuvre. For Valsalva manoeuvre, subjects were given a mouth piece to blow air as long as possible. Subjects were asked to ask stand quickly and remain standing for two minutes for orthostatic manoeuvre. Finally, subjects were asked to sit down and breathe deeply for the last ‘deep breathing’ manoeuvre. In addition, the key measures expiration/inspiration ratio and tachycardia ratio were obtained from the established ‘deep breathing’ manoeuvre.\textsuperscript{104} The subject was requested to refrain from caffeine and alcohol consumption.
for 48 hours to avoid adversely affecting test results and all measurements were performed by a respiratory physiologist.

Figure 3.19: Autonomic nerve analysis during (a) resting breathing (b) Valsalva manoeuvre (c) orthostatic manoeuvre, and (d) deep breathing. Photographs were obtained with kind permission of the subject.
3.11. References

Chapter 3


42. Modis L, Jr., Szalai E, Kolozsvari B, Nemeth G, Vajas A, Berta A. Keratometry evaluations with the Pentacam high resolution in comparison with the automated keratometry and conventional corneal topography. *Cornea* 2012;31:36-41.


Chapter 4
The two-dimensional architecture of the corneal sub-basal nerve plexus in diabetes mellitus
Chapter 4

4. Introduction

As described in Chapter 1, corneal nerve microstructure has, until recent years, been relatively poorly understood. Nerve fibre bundles enter the cornea multi-directionally at the limbus through the mid-stroma and form a radial nerve network. The nerves traverse towards the anterior stroma and perforate Bowman’s layer perpendicularly. The perforation sites are predominantly located in the mid-peripheral cornea and, after passing through Bowman’s layer, the nerves terminate into bulb-like thickenings from which multiple sub-basal nerves arise. Müller et al., using light and electron microscopy *ex vivo*, elegantly revealed a dense regular meshwork of sub-basal nerves with equal density throughout the central and mid-central region.

Chapter 3 discusses in detail the *in vivo* confocal microscopy (IVCM) technique that allows real time microscopic examination of the living human cornea at the cellular level. Using this technology, Patel and McGhee elucidated the two dimensional architecture of the corneal sub-basal nerve plexus in the living human eye, demonstrating a radiating pattern of nerve fibre bundles converging towards an area approximately 1-2 mm inferior to the corneal apex, in a whorl-like pattern. In published data of ten human eyes, the sub-basal nerve plexus, observed with IVCM, consistently exhibited a clockwise whorl. The dynamic nature of the normal sub-basal nerve plexus has also recently been reported, demonstrating continuous centripetal movement.

Although this configuration has subsequently been confirmed by immunohistochemical analyses, the whorl pattern is not always observed in *ex-vivo* human corneas. A recent *ex-vivo* study of seven human corneas observed a clockwise orientation in four and anti-clockwise in three. Interestingly, immunohistochemical studies on 12 Fisher-344 Sprague-
Dawley rats and 20 Pax6+-/ mice revealed a mixture of clockwise and anti-clockwise, but predominantly clockwise, orientations.

Despite the obvious utility of these IVCM techniques to increase our understanding of corneal innervation in health and disease, to date, only sub-basal nerve maps of unpaired eyes have been described in the literature. Additionally, there are only two published sub-basal nerve maps from the corneas of patients with diabetes mellitus (DM). The current chapter investigates the two-dimensional architecture of the corneal sub-basal nerve plexus in paired healthy eyes and in those with DM.

4.1. Materials & Methods

Six healthy subjects and five with DM were recruited for the study. No personal or family history of eye disease and no history of contact lens wear, ocular trauma/surgery, or systemic diseases that may affect the cornea were reported amongst the subjects. Informed, written consent was obtained from all subjects after explanation of the nature and possible consequences of the study. Both eyes of the healthy subjects and the right eye of those with DM were examined by slit-lamp biomicroscopy and were confirmed to have clinically intact epithelium.

Laser scanning in vivo confocal microscopy using the Heidelberg Retina Tomograph II Rostock Corneal Module (RCM) (Heidelberg Engineering GmBH, Germany) was performed on all subjects, each eye being examined on separate days within a time span of two weeks. The examination technique was used as described in Chapter 3. Intermittent rest breaks were provided as and when requested by the participant. Minimal corneal staining with fluorescein was observed in all participants, on post-IVCM slit-lamp examination. The method described is similar to that described by Patel and McGhee, although the
acquisition time was shorter due to a smaller region of mapping, and the total number of images acquired was larger to optimise completeness of the final maps.

Each captured image measured 400 µm × 400 µm. For each subject, Macromedia Freehand 10 (Macromedia Inc, San Francisco, CA, USA) enabled manual arrangement of images to form a contiguous montage. Blurred and duplicate images were discarded. Each montage took approximately 10 hours to compile. Subsequent sub-basal nerve density was assessed with the aid of a calliper tool (analySIS 3.1, Soft Imaging System, Münster, Germany). All nerves in an area of 700 µm × 700 µm within the whorl region were traced manually with an electronic pen (Wacom Technology Group, Vancouver, BC, Canada) to enable quantification of sub-basal nerve density. This measurement area was selected as the largest area, centred on the whorl, that could be consistently measured on every montage without encountering gaps. On a separate occasion, the images were traced again by the same, and an independent, experienced, examiner to determine inter and intra observer repeatability. Nerve density was computed by the analySIS software and the numerical data stored as a Microsoft Excel file.

To estimate the location of the whorl, the central corneal location was determined by use of a CCD camera attachment that enabled live imaging of the cornea from the temporal aspect during examination. The corneal apex was identified and used as the ‘central image’. This distance between this “central” image and the centre of the whorl on the resulting montage was measured using Adobe Photoshop CS4 (Adobe systems Incorporated, San Jose, CA, USA).

Data were imported to SPSS v15, (IBM, IL, USA). Statistical analysis included determination of the intra-observer and inter-observer limits of agreement, according to Bland and Altman. A t-test enabled comparison of sub-basal nerve density measurements between the two
eyes of each individual. Reported data are specified as mean ± standard deviation, and p<0.05 was considered statistically significant.

4.2. Results

4.2.1. Sub-basal nerve maps of healthy subjects

All 12 eyes of the 6 healthy participants were examined. Participants comprised 3 males and 3 females, with a mean age ± standard deviation of 30.5 ± 5.8 years.

A mean of 887 ± 50 consecutive images of the sub-basal nerve plexus were captured for each eye and mapped across a mean linear distance of 3.7 ± 0.5mm vertically and 4.4 ± 0.8mm horizontally. The sub-basal nerves were observed to be more vertically orientated in the central cornea, converging in the infero-central area to form an anastomosing network, or “whorl” in both eyes of all subjects. Figure 4.1 displays a single sub-basal nerve plexus montage. Figure 4.2 demonstrates the whorl region in the right and left eyes of the same subject. The sub-basal nerve plexus whorl assumed a clockwise orientation in both eyes of all subjects (Figure 4.3). The mean distance between the central cornea and the centre of the whorl was 1.70 ± 0.33 mm for right eyes and 1.78 ± 0.31 mm for left eyes.
Figure 4.1: IVCM montage of the sub basal nerve plexus of the right eye of a 27 year old male. The montage was prepared from 584 individual images.

Figure 4.2: IVCM montage of the sub-basal nerve plexus whorl of the right (a) and the left (b) eyes of a 36 year old female.

The nerve density in a 0.49mm² area of the whorl region was 39.17 ± 4.95 mm/mm² and 41.36 ± 4.19 mm/mm² in right and left eyes respectively. There was no statistically significant
difference in nerve density between the right and left eyes ($p=0.61$). Repeated measurement of nerve density by the same examiner and by an independent observer, for the right eyes, gave results of $39.13 \pm 3.79$ mm/mm$^2$ and $40.38 \pm 4.43$ mm/mm$^2$, respectively.

Figure 4.3: Electronic tracings of the IVCM images of the corneal sub-basal nerve plexus whorl, with background data removed, from paired corneas for all six subjects, A-F. The overall appearance is of a clockwise whorl in each pair of right and left corneas. The gender and age of each of the subjects were as follows: A, male aged 33 years, B, male aged 27 years, C, female aged 26 years, D, female aged 25 years, E, male aged 35 years and F, female aged 36 years. Each image is $800 \mu m \times 800 \mu m$ in dimension.

Bland and Altman analysis showed good intra-observer repeatability (Figure 4.4), with 95% of measurements falling within $1.12$ mm/mm$^2$ of each other. Bland and Altman analysis of inter-observer repeatability showed that 95% of measurements fell within $5.05$ mm/mm$^2$ of each other (Figure 4.5).
Figure 4.4. Bland Altman plot of the agreement between sub-basal nerve density measurements made by a single observer on two separate occasions.

Figure 4.5. Bland Altman plot of the agreement between the sub-basal nerve density measurements of two observers.
4.2.2. Sub-basal nerve maps of subjects with DM

IVCM was performed on 5 eyes of 5 male DM subjects with the intention to prepare corneal sub-basal nerve map montages. The mean duration of DM was 15.8±8.5 years. Only one map could be prepared with a linear distance of 4.1 mm vertically and 3.0 mm horizontally (Figure 4.6). Only ‘whorl’ region maps could be montaged for the remaining 4 eyes. The apparent reduction of sub basal nerve density in those with DM created significant problems in the preparation of contiguous montages. Figure 4.7 demonstrates a clockwise ‘whorl’ orientation of the right eye of a subject.

Figure 4.6: IVCM montage of the sub basal nerve plexus of the right eye of a 39 year old male with 18 year history of DM. The montage was prepared from 397 individual images.
4.3. Discussion

The data of 17 human eyes presented in this chapter strongly suggest that an infero-central sub-basal nerve plexus whorl is the norm in the living human cornea and that an overall clockwise pattern prevails in both right and left eyes. As noted, a similar pattern has been shown in all other IVCM sub-basal corneal nerve plexus maps in healthy individuals published to date.\textsuperscript{7, 8, 16, 19} Interestingly, the whorl pattern is believed to be lost in diseases such as diabetes mellitus\textsuperscript{20} and keratoconus.\textsuperscript{14} In the current study, the sub-basal nerve maps in those with DM demonstrated a clockwise orientation, albeit with significant gaps in sub-basal nerves.

The reason for this consistent clockwise orientation is not clear, but several theories may contribute to the explanation of the whorl development in the sub-basal corneal nerve plexus. Human histological studies by Schimmelpfennig et al\textsuperscript{3} have shown that epithelial nerve branches are orientated perpendicular to the corneal surface. This suggests that if...
there is centripetal epithelial slide, corneal epithelial cells and sub-basal nerves may migrate centripetally in tandem. This theory is supported by the observation of a similar whorl pattern in cases of hurricane epitheliopathy,\textsuperscript{21} and by the whorl pattern of migration of corneal epithelial cells observed in mice near the central cornea.\textsuperscript{22} It has been suggested that whorl orientation may be influenced by chemotropic guidance from limbal capillary vessels and population pressure from the limbus.\textsuperscript{22, 23} The location of the whorl in the infero-central corneal region has also been proposed to be influenced by shearing forces exerted by the eyelids on blinking.\textsuperscript{7}

Recently, Edwards et al\textsuperscript{17} employed a novel technique for imaging and montaging the sub-basal nerve plexus both in normals and in DM. Their technique involved image capture using a dynamic target and video rather than a static target and multiple single-frame images. However, many smaller nerve branches are not detectable and only the main nerve trunks are visible.\textsuperscript{24} Additionally, the compression produces Kobayashi-structures or K-structures to appear and obscure some of the nerve architecture.\textsuperscript{24} In a similar manner to that described by Edwards et al,\textsuperscript{17} the ‘whorl’ region was used throughout this thesis as the main reference point during the preparation of confluent montages. The apparently low sub-basal nerve density in DM and the presence of fragmented nerves led to difficulties in producing confluent maps in these cases. The sub-basal nerve density in DM will be discussed in Chapter 5 and its correlation with peripheral neuropathy, in Chapter 7.

The results of this study indicated high intra-observer repeatability in sub-basal nerve density quantification, consistent with that reported by Efron et al.\textsuperscript{25} Inter-observer repeatability reported here is also comparable to that reported by Efron et al.\textsuperscript{25} The sub-basal nerve density of 39.17 ± 4.95 mm/mm\textsuperscript{2} (of right eyes) over the whorl region in the current study is higher than the previously reported sub-basal nerve density of 25.2 ± 6.1 mm/mm\textsuperscript{2}.\textsuperscript{7} However, the area selected for analysis in the current study, focussing specifically on the whorl region was 0.49mm\textsuperscript{2} in comparison to the previously analysed area of 0.64 mm\textsuperscript{2}. As
nerve density appears to decrease with increasing distance from the whorl, measuring nerve density over a larger area might explain the difference in values obtained in the two studies. Interestingly, a recent immunohistochemical analysis of human ex vivo corneas reported a relatively similar central sub-basal nerve density of 45.94 mm/mm². The latter result may reflect the higher resolution and consequently the ability to image smaller diameter nerve fibre bundles with this technique compared with IVCM. Although this study has attempted to estimate the location of the whorl relative to the central cornea, it is stressed that this is an estimate because determination of the exact point location of the corneal apex is not sufficiently accurate in IVCM imaging.

Measurements such as the sub-basal nerve diameter and beading frequency were not assessed in this study as laser scanning IVCM optimises image quality by continuously varying the illumination intensity. For measurements of the diameter of thin highly reflective structures (such as sub-basal nerves), or the beading frequency, all images would need to be acquired using a fixed illumination intensity to be comparable, since illumination intensity affects the apparent thickness of corneal nerves, particularly as they approach the limit of resolution.

Enantiomorphism or non-superimposable mirror-image symmetry of the limbs is well recognised and is responsible for bimanual and multi-joint movements. Enantiomorphism of corneal topographic patterns and collagen organisation is also well documented in the literature and it might be reasonably anticipated that enantiomorphism would be similarly observed in the configuration of the sub-basal nerve plexus in paired eyes. However, the sub-basal nerve plexus in both the right and left eyes of all the individuals examined in the current study exhibited whorl patterns which consistently displayed a clockwise orientation, consistent with the non-paired right and left eyes described in the literature previously. The comparable nerve plexus configuration in the right and left eyes therefore suggests that the mirror imaging in corneal topographic patterns is not obeyed with respect to the configuration.
Chapter 4

of the corneal sub-basal nerve plexus. This has significant implications when considering post-surgical nerve healing, particularly following LASIK. On the basis of these data, flap position is likely to have a different impact on nerve regeneration in the two eyes.

This study extends our knowledge of the architecture of normal corneal innervation, and reports a comparison of the sub-basal corneal nerve plexus configuration in paired human eyes. There was no statistically significant difference in nerve density between the eyes, but it was surprising to note that, unlike other aspects of the cornea that exhibit mirror image symmetry, the sub-basal nerve plexus whorl exhibited a clockwise orientation in both right and left eyes, in healthy eyes and in those with DM.
Chapter 4

4.4. References


94
Chapter 5
Corneal assessment using *in vivo* confocal microscopy in diabetes mellitus
Chapter 5

5. Introduction

As discussed in Chapter 2, diabetes mellitus (DM) is associated with a range of ocular complications including retinopathy, cataract, refractive error and keratopathy. Diabetic keratopathy is characterised by superficial punctate epitheliopathy, increased epithelial fragility, recurrent corneal erosions, reduced corneal sensitivity, altered epithelial and endothelial morphological appearance and barrier function, and increased susceptibility to corneal ulceration and oedema.

The role of the epithelium in physiological shedding of the cells for continuous renewal and the role of the endothelium, for maintaining corneal transparency by regulating corneal hydration, are explained in Chapter 1.

The corneal epithelium in patients with type 2 DM has been shown, in some studies, to have a decreased mean cell density compared to the healthy cornea, however, in other studies it has been shown to have an increased mean cell density compared to normal. The disruption in corneal epithelial metabolism can also decrease the corneal sub-basal nerve plexus density in DM patients.

Changes in epithelial and sub-epithelial layers can be imaged using in vivo confocal microscopy (IVCM). IVCM has been regarded, in recent years, as a rapid, non-invasive clinical technique that has scope for detecting corneal nerve damage in patients with DM. In addition to changes in the corneal epithelium and sub-basal nerve plexus, previous studies have consistently reported that the endothelial cell density is reduced in patients with DM when compared to healthy participants. Increased endothelial pleomorphism and polymegathism are also reported features in patients with DM. Nonetheless, similar endothelial morphological changes have been observed in the normal aging cornea.
The changes in diabetic keratopathy are thought to be due to epithelial basement membrane abnormalities which occur throughout the body. However, only a few studies have actually quantified corneal epithelial and endothelial cell densities in patients with type 1 DM and healthy corneas. As discussed in Chapter 2, DM also affects the corneal sub-basal nerve plexus in terms of nerve density and structure. This chapter quantitatively analyses corneal epithelial, sub-basal nerve plexus and endothelial cell layers imaged by in vivo confocal microscopy in patients with type 1 DM compared to healthy controls.

5.1. Methods

Fifty three patients with type 1 DM were recruited by a collaborating endocrinology physician and forty control subjects were recruited by the principal investigator. Patients with previous ocular surgery or trauma, corneal disease, systemic disease (other than DM) with the potential to affect the cornea, or current or significant previous contact lens wear (of ≥ 3 months duration), were excluded from the study. HbA1c levels of all the control participants were determined to confirm the absence of diabetes. Informed consent was obtained from all participants as discussed in earlier chapters. IVCM was performed, using two different in vivo confocal microscopes, to acquire images of the central corneal basal epithelium, and endothelium.

The HRTII-Rostock corneal module (RCM) was used to capture IVCM images of the basal epithelial cell layer and sub-basal nerve plexus from the right cornea of each participant. The left eye was examined in three patients due to history of ocular surgery or trauma in the right eye. IVCM images of the endothelial cell layer of the same eye were acquired using the Confoscan 4 (NIDEK). Both IVCM techniques are described in detail in Chapter 3. For each patient, three of the clearest images from each of the epithelium, sub-basal nerves, and endothelium were randomly selected for analysis and were allocated unique codes using
online research randomizer software. Sub-basal nerve (SBN) density (nerve length/mm²) was analysed using computer software (analySIS 3.1; Soft Imaging System, Münster, Germany). The mean epithelial cell density was analysed using ImageJ software. Only the central 200 µm by 200 µm area of each image was used for epithelial and endothelial measurements. Endothelial cell density and the percentage of coefficient of variation (COV) in cell shape and coefficient of variation in cell area were calculated using NAVIS (Nidek Advanced Vision Information System) software. More than 33% COV in cell shape was considered polymorphic and over 54.1% COV in cell area was considered pleomorphic. An area of 0.04 mm² was assessed for endothelial cell analysis in all the patients.

The Shapiro-Wilk test was performed to confirm normality of the data distributions for both patients with DM and control subjects. The Student t test was employed to assess the difference between the diabetic and healthy control corneal data. A p-value of <0.05 was considered significant. All the techniques are described in Chapter 3. The sample size calculation prior to data collection suggested a minimum patient recruitment of 50 and 31 controls for 80% power and 95% confidence. Using sub-basal nerve density the published literature, the power calculation for the study has allowed enough number to detect differences between control and diabetic group where 25% change is expected in the later group.

5.2. Results

Fifty three patients with type 1 DM (26 male and 27 female), with a mean age of 48.6 ± 11.8 years, were identified. The patients had been diagnosed with DM for a mean duration of 25.8 ± 11.4 years (range 4 to 57 years). Forty controls (17 male and 23 female), mean age, 44.3 ± 14.7 years, with no previous ophthalmic history or contact lens wear, were also assessed for comparison. The control and the DM group were reasonably age (p=0.12) and gender
matched. Table 5.1 and Table 5.2 show the demographics of the participants from whom HRT-II RCM confocal images were acquired. Patients for whom focused and clear epithelial images could not be obtained, were excluded from the analysis.

Typical IVCM images of the sub-basal nerve plexus in a normal and a diabetic cornea are shown in Figure 5.1. and Figure 5.2 shows representative IVCM images of the basal epithelium from a control cornea and from a patient with DM. Table 5.2 describes the demographics of the subset of participants in whom analysis of the endothelium was performed. Unfortunately, IVCM images of the endothelium could be captured for only 25 control and 19 DM subjects due to unanticipated instrument malfunction during this aspect of the study.

Patients with DM had a significantly lower sub-basal nerve density compared to healthy controls (46.8% lower, p<0.0001) (Table 5.4). The number of cells per unit area in the epithelial and endothelial layers and the relative differences between controls and patients with DM are shown in Table 5.4. There was no significant difference in epithelial or endothelial cell densities between aged matched DM and control subjects. The endothelial layer showed signs of polymegathism and pleomorphism in both DM and control subjects.
Table 5.1: Participant characteristics for corneal sub-basal nerve density analysis

<table>
<thead>
<tr>
<th>Participant characteristics for sub-basal nerve density analysis</th>
<th>Controls</th>
<th>DM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subjects</td>
<td>40</td>
<td>53</td>
</tr>
<tr>
<td>M:F ratio</td>
<td>17:23</td>
<td>26:27</td>
</tr>
<tr>
<td>Age (years)</td>
<td>44.3 ± 14.7</td>
<td>48.6 ± 11.8</td>
</tr>
<tr>
<td>Age Range (years)</td>
<td>22-73</td>
<td>19-70</td>
</tr>
<tr>
<td>Diabetes duration (mean ± SD) (years)</td>
<td>NA</td>
<td>25.8 ± 11.4</td>
</tr>
</tbody>
</table>

Figure 5.1: Representative in vivo confocal images of HRT-II corneal module showing the sub-basal nerve plexus of (a) a 30 year old male control subject and (b) a 28 year old male with 18 year history of DM. The scale bar denotes 100 µm
Table 5.2: Participant characteristics for corneal epithelial cell density analysis

<table>
<thead>
<tr>
<th>Participant characteristics for epithelial cell analysis</th>
<th>Controls</th>
<th>DM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subjects</td>
<td>38</td>
<td>48</td>
</tr>
<tr>
<td>M:F ratio</td>
<td>16:22</td>
<td>23:25</td>
</tr>
<tr>
<td>Age (years)</td>
<td>45.1 ± 14.6</td>
<td>47.9 ± 11.8</td>
</tr>
<tr>
<td>Age Range (years)</td>
<td>22-73</td>
<td>19-69</td>
</tr>
<tr>
<td>Diabetes duration</td>
<td>NA</td>
<td>25.3 ± 11.7</td>
</tr>
<tr>
<td>(mean ± SD) (years)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 5.2: Representative in vivo confocal images of the basal epithelium of (a) a 57 year old male control subject and (b) a 48 year old female with 25 year history of DM. The scale bar denotes 100 µm
Table 5.3: Participant characteristics for endothelial cell analysis

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>DM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subjects</td>
<td>25</td>
<td>19</td>
</tr>
<tr>
<td>M:F ratio</td>
<td>9:16</td>
<td>9:10</td>
</tr>
<tr>
<td>Age (years)</td>
<td>52.3 ± 21.5</td>
<td>48.8 ± 10.5</td>
</tr>
<tr>
<td>Age Range (years)</td>
<td>22-73</td>
<td>33-70</td>
</tr>
<tr>
<td>DM duration (mean ± SD) (years)</td>
<td>NA</td>
<td>28.3 ± 5.52</td>
</tr>
</tbody>
</table>

Figure 5.3: Typical in vivo confocal images of the corneal endothelium captured by Confoscan 4 from (a) a 22 year old female control subject and (b) a 25 year old female with 15 year history of DM. The scale bar denotes 100 µm
Table 5.4: Comparison of corneal cell densities between DM and control corneas.

<table>
<thead>
<tr>
<th>Layer</th>
<th>Controls [Mean ± SD]</th>
<th>DM [Mean ± SD]</th>
<th>Relative (%)</th>
<th>( P^* )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal Epithelial density</td>
<td>5115 ± 664</td>
<td>5406 ± 774</td>
<td>+5.70</td>
<td>0.069</td>
</tr>
<tr>
<td>(cells/mm²)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endothelial density</td>
<td>2286 ± 454</td>
<td>2328 ± 223</td>
<td>+1.80</td>
<td>0.715</td>
</tr>
<tr>
<td>(cells/mm²)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endothelial Polymegathism</td>
<td>39.5</td>
<td>45.9</td>
<td></td>
<td>0.287</td>
</tr>
<tr>
<td>(%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Endothelial Pleomorphism</td>
<td>52.5</td>
<td>52.4</td>
<td></td>
<td>0.949</td>
</tr>
<tr>
<td>(%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sub-basal nerve density</td>
<td>21.17 ± 4.2</td>
<td>11.04 ± 3.8</td>
<td>-46.8</td>
<td>&lt;0.001**</td>
</tr>
<tr>
<td>(mm/mm²)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
5.3. Discussion

Maintenance of normal corneal epithelial cell density depends on the healthy regulation and balance between cell proliferation, differentiation, migration and death. The vulnerability of the diabetic cornea to infection and erosions is widely recognised confirming definite corneal abnormality in this disease. An ex vivo study by Tsubota et al\textsuperscript{23} has demonstrated altered maturation of epithelial cells in the corneas of DM patients. Another study revealed focal epithelial degeneration and accumulation of glycogen granules.\textsuperscript{24} However, currently, there is no general consensus amongst researchers regarding epithelial cell density in DM.\textsuperscript{3, 6}

Abnormal epithelial basement membrane has been observed in the nervous system, skin, kidney, retina and cornea in patients with DM.\textsuperscript{25-28} The corneal epithelial basement membrane becomes irregularly thickened and multi-laminated.\textsuperscript{29, 30} This leads to subclinical scattering of light in the cornea visible by IVCM but not detectable on routine clinical examination.\textsuperscript{31} Increased light scattering in the cornea has also been reported in patients with retinal vascular hyper-permeability.\textsuperscript{32} The accumulation of advanced glycation end products on the epithelial basement membrane also plays a pivotal role in the epithelial disorders in patients with DM.\textsuperscript{33}

In this study, the epithelial and endothelial cell densities for control participants were slightly lower than values reported in previous studies.\textsuperscript{3, 34} Interestingly, the cell density in the basal epithelium was relatively higher (5.70\%) in patients with DM, however, compared to controls this was not statistically significant. This observation supports the results of a study carried out by Frueh et al,\textsuperscript{6} however, it contradicts the data reported by Quadrado et al.,\textsuperscript{3} which showed a significantly lower epithelial cell density in patients with type 2 DM. These authors concluded that the lower epithelial cell density may be a result of basal cell depletion caused by reduced corneal innervation.\textsuperscript{3}
Chapter 5

Under typical physiological conditions, the corneal sub-basal nerve plexus is responsible for a healthy epithelial surface by maintaining corneal sensitivity, normal epithelial metabolism and by liberating neuropeptides and growth factors. The corneal nerves are known to be affected by DM. These changes include reduction in sub-basal nerve density, decreased nerve branching and increased nerve tortuosity, the latter possibly indicating nerve regeneration in diabetic keratopathy. The current study showed a significantly lower sub-basal nerve density in patients with DM compared to eyes of age matched control subjects. Sub-basal nerve branching and tortuosity were not considered or analysed in the present study due to the previously established lack of reproducibility of these measurements in patients with DM.

A study by Rehany et al inferred that the corneal endothelial damage in DM occurs secondary to continuous hyperglycaemic glycosylation of collagen, although the literature to date has failed to demonstrate consistent differences in cell densities between those with and without hyperglycaemia. In the current study the endothelial cell densities in DM patients were similar to those reported in previous studies, while the cell densities of the control subjects appeared to be slightly lower than those in the published literature. Overall, however, the 1.8 % greater mean endothelial cell density of the DM corneas compared to control subjects in the current study was not statistically significant. The differences between the current data and previous studies may be related to variable duration of DM and glycaemic control, local population characteristics, or methodology of IVCM data acquisition and analysis.

Diabetes can lead to changes in corneal endothelial cells not only in terms of cell density but also morphology, that is, polymegathism and pleomorphism. There were no significant differences in corneal endothelial morphology between normal control and DM patients in this study. Some studies have reported these changes prior to diabetic retinopathy at early stages of the disease. An early ex vivo study did suggest significant morphological
differences in type 1, type 2 DM patients and control subjects. However, an appropriately age-matched, controlled, clinical study is of utmost importance in assessing corneal endothelium as changes in endothelial morphology are evident simply with increasing age.

In conclusion, the current study identified no significant differences in corneal epithelial or endothelial cell densities, nor variation in endothelial morphology, when data from subjects with DM were compared to data from age-matched control subjects. However, a significantly lower corneal sub-basal nerve plexus density was identified in DM eyes - an important observation which strongly supports data reported in a number of previous studies. Chapter 7 will discuss how the accurate measurement of such decreased sub-basal nerve density is pivotal if IVCM is to increasingly become a useful biomarker for the assessment of peripheral neuropathy in type 1 DM.
5.4. References


Chapter 6
The cornea in health and in diabetes mellitus
6. Introduction

As highlighted in Chapter 2, diabetes mellitus (DM) can lead to numerous complications throughout the body. With regard to ocular complications, the changes in the posterior segment are generally more easily detectable than those of the anterior segment. Even when the ocular surface appears smooth on slit lamp examination in patients with DM, altered corneal sensitivity, biomechanical changes within the cornea and underlying ultrastructural abnormalities may exist.\textsuperscript{1-4}

A controlled pulse of air to the cornea stimulates many receptive fields due to extensive branching of the corneal nerve fibres.\textsuperscript{5, 6} The non-contact corneal aesthesiometer, or NCCA, uses an air pulse to stimulate the corneal nerves and the change in temperature is perceived by the subject.\textsuperscript{7} The reduction in sub-basal nerve density in those with DM, as elucidated in Chapter 5, could lead to decreased corneal sensitivity.\textsuperscript{8}

The advent of another non-contact instrument, the Ocular Response Analyzer (ORA), has made non-invasive assessment of the biomechanical properties of the cornea possible. Biomechanical properties include corneal hysteresis (CH) and corneal resistance, as described by the corneal resistance factor (CRF). CH indicates corneal viscous dampening and CRF is a correction property, related to corneal elasticity, that helps account for the effect of central corneal thickness (CCT) on intra-ocular pressure (IOP) measurement, as described in Chapter 3.\textsuperscript{9} The normal corneal thickness depends largely on intact corneal endothelial pump function and it is postulated to be thicker in DM due to corneal endothelial dysfunction and increased corneal hydration.\textsuperscript{10, 11} Topographic and refractive changes in the cornea and the crystalline lens are other consequences of unstable plasma glucose concentration in patients with DM.\textsuperscript{12} Examination of corneal topography and corneal
thickness are therefore useful clinical techniques for analysing the cornea in patients with DM.

The current chapter evaluates differences in corneal sensitivity threshold, corneal biomechanical properties, corneal thickness and topography in individuals with and without DM.

6.1. Methods

Posters were distributed within the University of Auckland, Grafton campus and forty-five individuals volunteered as control participants for the study. Respondents with any history of contact lens wear, ocular surgery or trauma, or neuropathy unrelated to DM were excluded. Fifty three patients with a history of type 1 DM provided written informed consent to participate after being identified as potentially eligible by a specialist endocrinology (diabetes) physician. All ocular assessments were performed on the right eye only unless the participant reported a history of unilateral surgery or trauma to the right eye, in which case the left eye was examined. A detailed medical history was obtained regarding ethnicity, duration of DM, and medication use. Additionally, a standard hospital questionnaire regarding smoking and alcohol consumption was completed by each subject. Smoking was recorded in pack years which was calculated by multiplying the number of packs of cigarettes smoked per day with the number of years smoked. The alcohol questionnaire included two questions on frequency and one on quantity of alcohol consumed by an individual (Appendix 11.3). Scores for each response were summed to obtain the final alcohol score.

Central corneal sensitivity threshold was evaluated by NCCA (Glasgow Caledonian University, UK). CH, CRF and IOP were assessed using ORA (Reichert Ophthalmic
Instruments, Depew, NY, USA). IOP was also measured by Goldmann tonometry as the clinically accepted gold-standard. The CCT measurement and corneal topographical maps were obtained using the Oculus Pentacam Rotating Scheimpflug tomographer (Oculus Optikgerate DmbH, Wetzlar, Germany). Digital images of the central and peripheral retina were captured with a non-mydriatic retinal camera (Non-Mydriatic Retinal Camera DR-DGi, Canon Inc, USA) for diabetic retinopathy (DR) grading, which was assessed by an independent medical retina specialist. HbA1c levels were evaluated from blood testing. All the assessments were performed as described in Chapter 3.

Statistical analysis was performed using IBM SPSS v19.0 (Chicago, IL, USA). A one-way ANOVA was carried out to establish differences between subjects with or without DM. A p-value of <0.05 was considered significant. A minimum sample size of 50 was determined to detect a correlation between different variables within the same group of subjects with DM, with 80% power and 95% confidence on the basis of previously reported data.

6.2. Results

Patient characteristics including gender, age, ethnicity, body mass index, duration of diabetes, smoking history (pack years), alcohol history score, HbA1c levels and presence of diabetic retinopathy are stated in table 6.1.

The cut off for HbA1c levels in control subjects was 5.5% and, as a consequence, 5 subjects were excluded from the study after the initial blood test. The ethnicity of control subjects included 47.5% European, 22.5% Indian, and 20% Asian (excluding Indian and 4 others (European/Japanese, European/Latino, Israeli, and Brazilian). The duration of DM ranged from 4 to 57 years.
Chapter 6

Table 6.1: Number of subjects and patient characteristics including, gender, age (years), BMI (kg/m²), smoking history (pack years), alcohol history score, ethnicity, duration of DM (years) and diabetic retinopathy (DR) grading.

<table>
<thead>
<tr>
<th></th>
<th>DM</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subjects (n)</td>
<td>53</td>
<td>40</td>
</tr>
<tr>
<td>M:F ratio</td>
<td>26:27</td>
<td>17:23</td>
</tr>
<tr>
<td>Age (years)</td>
<td>48.6 ± 11.8</td>
<td>44.3 ± 14.7</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.9 ± 4.6</td>
<td>25.0 ± 3.8</td>
</tr>
<tr>
<td>Smoking history (pack years)</td>
<td>4.2 ± 10.3</td>
<td>1.7 ± 1.0</td>
</tr>
<tr>
<td>Alcohol score</td>
<td>3.8 ± 2.3</td>
<td>2.1 ± 0.2</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>7.7 ± 1.0</td>
<td>5.3 ± 0.2</td>
</tr>
<tr>
<td>Ethnicity (n)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>European</td>
<td>51</td>
<td>19</td>
</tr>
<tr>
<td>Indian</td>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td>Asian (excluding Indian)</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>Māori</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Others</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Mean DM Duration (years)</td>
<td>25.8 ± 11.4</td>
<td></td>
</tr>
<tr>
<td>&lt;10</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>10-20</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>21-31</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>&gt;31</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>No DR</td>
<td>32 (60.3%)</td>
<td></td>
</tr>
<tr>
<td>Mild DR</td>
<td>11 (20.7%)</td>
<td></td>
</tr>
<tr>
<td>Moderate DR</td>
<td>10 (18.8%)</td>
<td></td>
</tr>
<tr>
<td>Severe DR</td>
<td>0 (0%)</td>
<td></td>
</tr>
</tbody>
</table>

Subjects with DM had significantly lower corneal sensitivity compared to controls (p<0.0001) (Table 6.2). However, there were no significant differences between the two groups of subjects in terms of CH, CRF, CCT, IOP and simulated keratometry (SIM-K) (Table 6.2).
Table 6.2: Comparison of corneal parameters between controls and subjects with DM. The mean values of corneal sensitivity threshold (mBAR), corneal hysteresis (mmHg), corneal resistance factor (mmHg), Minimum K and maximum K (Dioptres) and central corneal thickness (µm) in subjects with and without DM.

<table>
<thead>
<tr>
<th></th>
<th>DM (Mean ± SD)</th>
<th>Controls (Mean ± SD)</th>
<th>ANOVA (p values)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corneal sensitivity</td>
<td>1.3 ± 1.3</td>
<td>0.2 ± 1.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>threshold (mBAR)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corneal hysteresis</td>
<td>11.1 ± 1.7</td>
<td>10.7 ± 1.7</td>
<td>0.20</td>
</tr>
<tr>
<td>(mmHg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corneal resistance</td>
<td>10.9 ± 1.6</td>
<td>10.4 ± 2.0</td>
<td>0.18</td>
</tr>
<tr>
<td>Factor (mmHg)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IOPg-ORA (mmHg)</td>
<td>14.7 ± 4.4</td>
<td>15.2 ± 2.9</td>
<td>0.50</td>
</tr>
<tr>
<td>IOP-applanation (mmHg)</td>
<td>13.8 ± 4.4</td>
<td>13.8 ± 2.9</td>
<td>0.50</td>
</tr>
<tr>
<td>SIM-keratometry</td>
<td>42.9 ± 1.5</td>
<td>42.8 ± 1.4</td>
<td>0.67</td>
</tr>
<tr>
<td>flat axis (Dioptres)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SIM-keratometry</td>
<td>43.5 ± 1.4</td>
<td>43.5 ± 1.6</td>
<td>0.90</td>
</tr>
<tr>
<td>steep axis (Dioptres)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average Keratometry</td>
<td>43.2 ± 1.4</td>
<td>43.3 ± 1.4</td>
<td>0.79</td>
</tr>
<tr>
<td>(Dioptres)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Central corneal</td>
<td>555.8 ± 32.2</td>
<td>554.3 ± 32.4</td>
<td>0.83</td>
</tr>
<tr>
<td>thickness (µm)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

6.3. Discussion

DM affects the cornea’s morphological, physiological, metabolic and clinical state. Although decreased corneal sensitivity in DM is well documented,\(^8\), \(^{13}\), \(^{14}\) researchers have thus far failed to agree on corneal biomechanical and central corneal thickness changes in DM.\(^9\), \(^{10}\), \(^{15-17}\)

The current study showed significantly lower corneal sensitivity in participants with DM compared to controls. An increase in corneal sensitivity threshold and abnormal neural regulation in the diabetic cornea is acknowledged to result in delayed epithelial wound
healing and recurrent corneal erosions.\textsuperscript{18} Corneal sensitivity gradually diminishes with increasing age in patients with or without DM.\textsuperscript{13}

Although Patel et al\textsuperscript{19} demonstrated differences in corneal sensitivity in various age groups they were unable to establish a direct correlation between reduction in sensitivity and increasing age. Another study found no difference in corneal sensitivity until the fifth decade of life but showed a sharp reduction subsequently.\textsuperscript{20} Irrespective of age, patients with DM show decreased corneal sensitivity when measured with contact or non-contact aesthesiometry suggesting the potential for corneal sensitivity to become an ophthalmic marker for diabetic polyneuropathy.\textsuperscript{14, 21} The significant difference in corneal sensitivity thresholds in patients with DM and control subjects confirms the importance of this parameter in the ophthalmic assessment of patients with DM.

Central corneal thickness is an anatomical property of the cornea whereas CH and CRF are biomechanical parameters. It has been shown that hyperglycemia has a strong influence on corneal biomechanical properties due to induced stromal collagen crosslinking through glycosylation and lysyl oxidase enzymatic activity.\textsuperscript{22} Additionally, several studies have reported greater CCT in patients with DM compared to control subjects.\textsuperscript{11, 23, 24}

One study inferred that the increased corneal thickness and collagen crosslinking in DM results in increased corneal stiffness and elevated damping effect (or increased CH) in patients with DM.\textsuperscript{25} Interestingly, collagen crosslinking with riboflavin is used as a mode of treatment in keratoconus with an intention of stiffening the cornea.\textsuperscript{26} Some studies have shown greater CH, CRF and CCT in those with DM compared to healthy eyes suggesting increased corneal stiffness and the authors proposed this as a diabetic protective phenomenon.\textsuperscript{4, 15} In contrast, Sahin \textit{et al.}, showed significantly lower CH values in patients with DM, which was attributed to the alteration in collagen cross linking.\textsuperscript{9} Other corneal
disorders such as Fuchs’ dystrophy and keratoconus are also associated with low CH values.\textsuperscript{27}

No statistically significant differences were observed in terms of CH, CRF and CCT between the corneas of the control group and the patients with DM in the current study. This may be related to the degree of repeatability and limitations of ORA measurements. Indeed, a previous study of 49 normal subjects observed a moderate level of variability in CH measurements with a repeatability coefficient value of 2.6 and coefficients of variation of 12.3 %.\textsuperscript{28} Another study reported inadequate coefficient of variation value of 17.5\% for CH, 18\% for CRF and 8.9\% for IOP.\textsuperscript{29}

Patients with a longer duration of DM and varying plasma glucose concentrations are more likely to get transient refractive changes.\textsuperscript{30-32} Historically, these refractive changes are believed to be largely due to increased lenticular thickness along with changes in the lens morphology, thereby resulting in a myopic shift.\textsuperscript{33, 34} However, the relative hyperopic shift typically occurs as a result of the reversal of the thickened lens changes and induced myopia.\textsuperscript{30, 35, 36} The alterations in refractive index associated with water influx within the lens also contribute to the transient hyperopic shift.\textsuperscript{37, 38} A positive correlation of these refractive changes to HbA1c levels has been reported.\textsuperscript{12, 39} Previous studies have suggested but not confirmed changes in corneal curvature.\textsuperscript{12, 37, 40} The present cross-sectional study did not show any statistically significant differences in corneal curvature between normal subjects and patients with DM. However, a longitudinal evaluation may have highlighted refractive changes in the patients.

Chapter 7 will examine the association of ocular parameters with peripheral neuropathy in patients with DM.
6.4. References

Chapter 6


Chapter 7
Evaluation of relationships between corneal nerve density, corneal sensitivity, retinopathy, cardiovascular autonomic neuropathy and peripheral neuropathy in type 1 diabetes mellitus
7. Introduction

The heterogeneous nature of diabetic neuropathy is discussed in detail in Chapter 2. Diabetic neuropathy can affect any system in the body leading to increased morbidity, especially if there is cardiovascular autonomic neuropathy (CAN).\textsuperscript{1} CAN causes increased heart rate variability and is associated with poorer disease prognosis and an increased risk of mortality.\textsuperscript{2} Early diagnosis of diabetic peripheral neuropathy is essential to reduce the risk of foot ulceration and premature death.\textsuperscript{3}

A number of tests are used in evaluating peripheral neuropathy. Typically, electrophysiological methods, such as nerve conduction testing are utilised to detect nerve dysfunction objectively and together with subjective neuropathic symptoms and signs that detect presence and severity of distal length-dependent neuropathy, are combined to form a total neuropathy score.\textsuperscript{4, 5} Nerve conduction study (NCS) enables large fibre dysfunction to be detected at secondary stages of diabetic neuropathy, and can identify crucial small fibre damage during the early stages of diabetic neuropathy.\textsuperscript{1} Identification of an anomaly in the terminal small nerve fibres is otherwise difficult by regular objective neuropathy tests.\textsuperscript{6} The gold standard assessment of neuropathy is a skin punch biopsy but this enables only examination of morphology, and not function.\textsuperscript{7} Although the skin punch biopsy offers detailed assessment of nerve damage in distal symmetric polyneuropathy and small fibre sensory polyneuropathy, its invasive nature limits its adoption as a standard screening tool.\textsuperscript{7} Conversely, \textit{in vivo} corneal confocal microscopy (IVCM) allows non-invasive visualisation of small fibre corneal nerve microstructure, as explained in Chapters 3, 4 and 5. It should be noted that distal symmetric polyneuropathy affects long nerves whereas small nerve fibre sensory polyneuropathy involves autonomic and somatic C fibres.\textsuperscript{8, 9}
A recognised relationship exists between glucose metabolism and diabetic retinopathy, such that individuals who experience increased blood glucose levels are at an increased risk of developing retinopathy in comparison to individuals with normal glucose metabolism. However, the relationship between blood glucose metabolism and neuropathy is not yet clearly understood.

This Chapter seeks to examine the relationship between corneal nerve microstructure, retinopathy, cardiovascular autonomic neuropathy and peripheral neuropathy in subjects with type 1 DM.

### 7.1. Methods

Patients with a history of type 1 DM (n=207) were identified by a collaborating physician. Fifty-three, of those, fulfilled the criteria and provided written, informed consent to participate in this study. None of the patients had a history of contact lens wear, ocular surgery or trauma in the eye to be examined, or neuropathy unrelated to DM. Consistent with the methodology in previous chapters, ocular assessments were performed on the right eye only, with the exception of individuals with a history of right eye surgery or trauma, in which case the left eye was examined. A detailed medical history was obtained regarding duration of DM, medication use, smoking habits and alcohol consumption (as described in Chapter 6).

Laser scanning IVCM was performed using the Heidelberg Retina Tomograph II, corneal module (Heidelberg Engineering GmbH, Germany) as described in Chapter 3. A central corneal sensitivity threshold was evaluated by non-contact aesthesiometry or NCCA (Glasgow Caledonian University, UK). All patients underwent a neuropathy assessment. An overall neuropathy score was obtained from a combination of the symptomatic neuropathy
score, clinical neuropathy assessment by a collaborating neurologist, biothesiometry (quantitative sensory testing) and NCS.\textsuperscript{4} Autonomic nerve analysis was performed by a collaborating respiratory physiologist. The expiration/inspiration ratio during deep breathing (ANS-EI), valsalva ratio and tachycardia ratio were noted as key observations. Digital images of the central and peripheral retina were captured using a non-mydriatic retinal camera (Non-Mydriatic Retinal Camera DR-DGi, Canon Inc, USA) for diabetic retinopathy (DR) grading. DR was graded by an independent, fellowship-trained, medical retina specialist. All the assessments were performed as described in Chapter 3.

Appropriate statistical analyses were discussed with an experienced bio-statistician. The Shapiro-Wilk test was performed to confirm normality of the data distributions for those with DM. On the basis of previously reported data, a power calculation for this study (Statistical solutions, LLC) determined a minimum sample size of 50 was required to detect a correlation between different variables within the same group of subjects with DM, with 80% power and 95% confidence. A Pearson correlation (2-tailed) analysis was applied to determine the correlation between variables. A p-value <0.05 was considered significant.

### 7.2. Results

Patient demographics including gender, mean age, body mass index (BMI), duration of diabetes, smoking history (pack years), alcohol history score, HbA1c level and severity of diabetic retinopathy are documented in Table 7.1. Following the methodology of comparative studies, mean data of neuropathy assessments and key variables of autonomic nerve analysis are also stated in Table 7.1.

Pearson correlation analyses of the four components of total neuropathy score (NCS-Total), patient demographics including BMI, DM duration, smoking and alcohol score with corneal
sub-basal nerve (SBN) density and corneal sensitivity (CST) are tabulated (Table 7.2). Body mass index (BMI) did not correlate with the neuropathy scores or ocular assessments. Longer disease duration showed moderate correlation with total neuropathy score \((r=0.33, p=0.01)\), clinical neuropathy assessment score \((r=0.27, p=0.04)\) and nerve conduction test \((r=0.34, p=0.01)\). Elevated HbA1c blood values were associated with higher smoking score \((r=0.29, p=0.03)\), total neuropathy scores \((r=0.30, p=0.02)\) and neuropathy clinical assessment \((r=0.38, p=0.004)\). Higher smoking scores were associated with elevated total neuropathy score \((r=0.47, p<0.001)\).

Table 7.1: Demographics of those with DM, diabetic retinopathy grade, neuropathy score and autonomic nerve analyses (expiration/inspiration ratio during deep breathing, valsalva, and tachycardia ratios).

<table>
<thead>
<tr>
<th>Demographics</th>
<th>Subjects (n)</th>
<th>53</th>
</tr>
</thead>
<tbody>
<tr>
<td>M:F ratio</td>
<td>26:27</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>48.6 ± 11.8</td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.9 ± 4.6</td>
<td></td>
</tr>
<tr>
<td>Smoking history (pack years)</td>
<td>4.2 ± 10.3</td>
<td></td>
</tr>
<tr>
<td>Alcohol score</td>
<td>3.8 ± 2.3</td>
<td></td>
</tr>
<tr>
<td>HbA1c</td>
<td>61.3 ± 12.0</td>
<td></td>
</tr>
<tr>
<td>Mean DM Duration (years)</td>
<td>25.8 ± 11.3</td>
<td></td>
</tr>
<tr>
<td>&lt;10</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>10-20</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>21-30</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>&gt;30</td>
<td>16</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Diabetic Retinopathy grade</th>
<th>Subjects (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No active DR</td>
<td>32 (60.3%)</td>
</tr>
<tr>
<td>Mild DR</td>
<td>11 (20.7%)</td>
</tr>
<tr>
<td>Moderate DR</td>
<td>10 (18.8%)</td>
</tr>
<tr>
<td>Severe DR</td>
<td>0 (0%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Neuropathy score</th>
<th>Subjects (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCS</td>
<td>5.3 ± 5.1</td>
</tr>
<tr>
<td>Symptoms</td>
<td>0.5 ± 0.9</td>
</tr>
<tr>
<td>Clinical</td>
<td>1.1 ± 1.7</td>
</tr>
<tr>
<td>Biothesiometry</td>
<td>0.8 ± 1.4</td>
</tr>
<tr>
<td>NCS</td>
<td>2.7± 2.8</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Autonomic nerve analysis</th>
<th>Subjects (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANS-EI</td>
<td>-0.4 ± 1.3</td>
</tr>
<tr>
<td>ValsalvaRatio</td>
<td>0.2 ± 1.6</td>
</tr>
<tr>
<td>TachycardiaRatio</td>
<td>0.2 ± 0.6</td>
</tr>
</tbody>
</table>
The correlation between CST and corneal SBN density was statistically significant ($r=-0.36$, $p=0.008$). The clinical component of NCS also showed positive correlation with corneal sensitivity ($r=0.31$, $p=0.02$). The corneal sub-basal nerve density showed significant correlation with total neuropathy score ($r=-0.33$, $p=0.01$), neuropathy symptoms ($r=-0.34$, $p=0.01$) and nerve conduction test ($r=-0.34$, $p=0.01$) (Table 7.2).

Table 7.3 illustrates the autonomic nerve analysis (ANS); ANS-EI, ANS-valsalva ratio, ANS-tachycardia ratio; compared with corneal sensitivity, sub-basal nerve density and body mass index, DM duration and HbA1c. The components of autonomic nerve analysis are explained in detail in Chapter 3. ANS-EI ($r=-0.29$, $p=0.03$) and tachycardia ratio ($r=0.42$, $p=0.002$) showed positive correlation with HbA1c in this study. BMI ($r=0.38$, $p=0.005$) and duration of DM ($r=0.29$, $p=0.03$) showed correlation with tachycardia ratio. Moreover, corneal sensitivity was found to be correlated with ANS-EI ($r=-0.36$, $p=0.008$).
Table 7.2: Pearson correlation analysis between body mass index (BMI), DM duration (years), smoking (pack years), alcohol score, HbA1c, Corneal sensitivity or CST (mBAR), sub-basal nerve (SBN) densities (mm/mm²) and total neuropathy score (NCS-total) which has four components: neuropathy symptoms (NCS-symptoms), neuropathy clinical assessment score (NCS-clinical), NCS-QST (quantitative sensory testing in Volts) and NCS-test (nerve conduction test). The statistically significant values are highlighted in orange. Significant correlations within the different components of NCS are not highlighted.

<table>
<thead>
<tr>
<th></th>
<th>BMI R</th>
<th>DM Duration R</th>
<th>Smoking score R</th>
<th>Alcohol score R</th>
<th>HbA1c R</th>
<th>CST R</th>
<th>NCS-symptoms R</th>
<th>NCS-clinical R</th>
<th>NCS-QST R</th>
<th>NCS-test R</th>
<th>SBN Density R</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI</td>
<td>1.02</td>
<td>0.67</td>
<td>0.19</td>
<td>0.22</td>
<td>-0.14</td>
<td>0.09</td>
<td>-0.18</td>
<td>-0.01</td>
<td>-0.05</td>
<td>0.25</td>
<td>0.26</td>
</tr>
<tr>
<td>DM Duration</td>
<td>0.02</td>
<td>0.19</td>
<td>0.11</td>
<td>0.32</td>
<td>0.54</td>
<td>0.20</td>
<td>0.95</td>
<td>0.73</td>
<td>0.07</td>
<td>0.07</td>
<td>0.06</td>
</tr>
<tr>
<td>Smoking score</td>
<td>0.15</td>
<td>0.29</td>
<td>0.02</td>
<td>0.18</td>
<td>0.33</td>
<td>0.23</td>
<td>0.27</td>
<td>0.05</td>
<td>0.35</td>
<td>-0.25</td>
<td>-0.19</td>
</tr>
<tr>
<td>Alcohol score</td>
<td>0.03</td>
<td>0.88</td>
<td>0.29</td>
<td>0.98</td>
<td>0.00</td>
<td>0.04</td>
<td>0.00</td>
<td>0.17</td>
<td>0.01</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>HbA1c</td>
<td>1.02</td>
<td>0.77</td>
<td>0.63</td>
<td>0.57</td>
<td>-0.08</td>
<td>-0.04</td>
<td>0.79</td>
<td>0.33</td>
<td>0.63</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>CST</td>
<td>1.24</td>
<td>0.08</td>
<td>0.20</td>
<td>0.31</td>
<td>0.02</td>
<td>0.17</td>
<td>-0.36</td>
<td>0.87</td>
<td>0.23</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>NCS-total</td>
<td>1.50</td>
<td></td>
<td></td>
<td></td>
<td>0.56</td>
<td>0.56</td>
<td>0.85</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td></td>
</tr>
<tr>
<td>NCS-symptoms</td>
<td>0.31</td>
<td></td>
<td></td>
<td></td>
<td>0.33</td>
<td>0.33</td>
<td>0.33</td>
<td>0.02</td>
<td>0.01</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>NCS-clinical</td>
<td>0.48</td>
<td></td>
<td></td>
<td></td>
<td>0.48</td>
<td>0.48</td>
<td>0.48</td>
<td>0.48</td>
<td>0.48</td>
<td>0.48</td>
<td></td>
</tr>
<tr>
<td>NCS-QST</td>
<td>0.17</td>
<td></td>
<td></td>
<td></td>
<td>0.17</td>
<td>0.17</td>
<td>0.17</td>
<td>0.17</td>
<td>0.17</td>
<td>0.17</td>
<td></td>
</tr>
<tr>
<td>NCS-test</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td>-0.34</td>
<td>-0.34</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>SBN Density</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Correlation is significant at the 0.05 level (2-tailed).
** Correlation is significant at the 0.01 level (2-tailed).
Table 7.3: Pearson correlation analysis between body mass index (BMI), DM duration (years), HbA1c, corneal sensitivity or CST (mBAR), sub-basal nerve (SBN) densities (mm/mm²) and three variable of autonomic nerve analysis including ANS-EI, ANS-Valsalva ratio, ANS-tachycardia ratio. The statistically significant values are highlighted in orange. Significant correlations within the different components of ANS are not highlighted.

<table>
<thead>
<tr>
<th></th>
<th>BMI</th>
<th>DM Duration</th>
<th>HbA1c</th>
<th>CST</th>
<th>SBN</th>
<th>ANS-EI</th>
<th>ANS-Valsalva ratio</th>
<th>ANS-Tachycardia ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI</td>
<td>R 1</td>
<td>0.02</td>
<td>0.22</td>
<td>-0.14</td>
<td>0.26</td>
<td>-0.08</td>
<td>-0.21</td>
<td>.38**</td>
</tr>
<tr>
<td></td>
<td>P 0.87</td>
<td>0.11</td>
<td>0.32</td>
<td>0.06</td>
<td>0.54</td>
<td>0.14</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>DM Duration</td>
<td>R 1</td>
<td>0.02</td>
<td>0.18</td>
<td>-0.25</td>
<td>-0.04</td>
<td>-0.18</td>
<td>.29*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>P 0.87</td>
<td>0.20</td>
<td>0.07</td>
<td>0.77</td>
<td>0.20</td>
<td>0.03</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HbA1c</td>
<td>R 1</td>
<td>0.13</td>
<td>-0.07</td>
<td>-0.29</td>
<td>-0.20</td>
<td>.42**</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>P 0.37</td>
<td>0.65</td>
<td>0.03</td>
<td>0.15</td>
<td>0.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CST</td>
<td>R 1</td>
<td>-0.36</td>
<td>-0.36</td>
<td>-0.21</td>
<td>-0.21</td>
<td>0.22</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>P 0.01</td>
<td>0.01</td>
<td>0.14</td>
<td>0.01</td>
<td>0.01</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SBN</td>
<td>R 1</td>
<td>0.06</td>
<td>0.06</td>
<td>-0.11</td>
<td>-0.11</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>P 0.65</td>
<td>0.66</td>
<td>0.42</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANS-EI</td>
<td>R 1</td>
<td>0.05</td>
<td>0.73</td>
<td>-0.16</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>P 0.73</td>
<td>0.26</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANS-Valsalva Ratio</td>
<td>R 1</td>
<td>-0.64**</td>
<td></td>
<td>0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>P 0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANS-Tachycardia Ratio</td>
<td>R 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>P 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).
Table 7.4: Pearson correlation analysis between diabetic retinopathy grading (DR grade), smoking (pack years), alcohol score, HbA1c, Corneal sensitivity or CST (mBAR), neuropathy clinical assessment score (NCS-clinical), ANS-EI and sub-basal nerve (SBN) densities (mm/mm²). Only statistically significant components are shown in the table from total neuropathy score variables and ANS components.

The results of Pearson correlation analysis of diabetic retinopathy (DR) grading and other ocular and neuropathy components are detailed in table 7.4. HbA1c levels were strongly correlated with diabetic retinopathy grading ($r=0.46$, $p=0.001$), as were NCS-clinical ($r=-0.32$, $p=0.02$) and ANS-EI ($r=-0.40$, $p=0.003$) scores.
7.3. Discussion

Currently, nerve electrophysiology and quantitative sensory tests are used as a gold standard for diagnosing and quantifying diabetic peripheral neuropathy. However, the ability of these tests to detect small nerve fibre damage is limited. Sural nerve and skin punch biopsies are used for accurate analysis of small nerve fibre injury. In randomised controlled trials, subjective assessment including health survey or mood states or pain scores are utilised. Clinical correlation of diabetic peripheral neuropathy with invasive test, such as skin punch biopsy, is not practical for the purpose of routine screening and as objective measure in randomised controlled trials to test the efficacy of various treatment strategies.

Peripheral neuropathy causes early damage to Aδ and unmyelinated C-class small nerve fibres leading to hyperesthesia, paraesthesia, and loss of pain and temperature. Perhaps, small corneal nerve fibres are also affected at this early stage. These early changes are identified using IVCM, as described previously, which allows detection of small nerve fibres non-invasively in a clinical setting. In this study, 53 subjects with type 1 DM underwent an extensive series of ocular, electrophysiological and neuropathy assessments.

The sub-basal nerve density was markedly reduced in patients with diabetes mellitus, as previously discussed in Chapter 5. Corneal sensitivity exhibited a significant relationship with corneal sub-basal nerve density in this study \((r=0.36\) and \(p=0.008\)). Such a progressive decrease in corneal sensitivity in diabetic patients is not surprising. Due to this reduction, corneal sensitivity is regarded as a valuable ophthalmic marker.

Importantly, the inverse correlation of corneal sub-basal nerve density with total neuropathy score emphasises the parallel involvement of the trigeminal (cranial) nerve in the context of diabetic peripheral neuropathy \((r=-0.33, p=0.01)\). Interestingly, an independent study of 25
patients with a history of idiopathic small fibre neuropathy also showed significant corneal sub-basal nerve density damage. Corneal and intra-epidermal nerve length measurements are reported to be significantly lower in patients with painful neuropathy compared to those with painless neuropathy. IVCM not only detects the involvement of corneal microstructure but may also reflect improvement in corneal nerve morphology with improvement in risk factors of diabetic neuropathy, including better glycaemic control. Semi-automated analysis of IVCM images has previously shown repeatable results in those with DM which should make such clinical analyses easier in the future.

Although a study has shown that corneal sub-basal nerve density reduces with worsening diabetic retinopathy, the current study did not identify any significant correlation between retinopathy grading and sub-basal nerve density, despite the presence of peripheral neuropathy. The findings of the present research are supported by a recent publication by Zhivov et al, where authors found sub-basal nerve density impairment in those with and without any retinopathy, irrespective of peripheral neuropathy. A microvascular manifestation of DR as opposed to the both neuropathic and microvascular contribution in peripheral neuropathy could be a possible explanation.

The current study confirmed the previously established relationships between elevated HbA1c, diabetic retinopathy, higher total neuropathy score and aberrant cardiovascular autonomic control, corroborating the importance of glycaemic control. A longer smoking history correlates with peripheral small fibre neuropathy, CAN, retinopathy and poses a challenge in the maintenance of satisfactory HbA1c values due to metabolic alterations and endothelial dysfunction in type 1 DM. Evidence shows that patients who have smoked more than 30 pack years are at three-fold risk of acquiring neuropathy. The present study also showed significant correlation between high smoking score and total neuropathy score as well as poor glycaemic control.
Any abnormality in heart rate variability (HRV) is regarded as an early sign of CAN. Few studies have reported dysfunctional HRV in DM patients with CAN using the instantaneous cardiac rate-meter.\textsuperscript{35, 36} Patients who failed glucose testing are reported to suffer from some degree of neural dysfunction with small fibre neuropathy and CAN.\textsuperscript{37} We believe this is the first study to explore relationships between CAN, total neuropathy score, retinopathy and corneal sub-basal nerve damage. CAN with reduced heart rate variability and parasympathetic loss is typically observed within 2 years of diagnosis of type 1 DM.\textsuperscript{38} The strong correlation between total neuropathy score and autonomic nerve analysis was not surprising ($r=-0.33$, $p=0.01$). However, the strong inverse correlation of corneal sensitivity with autonomic nerve analysis is a novel finding ($r=-0.36$, $p=0.008$). DR has been shown to be associated with cardiac autonomic dysfunction, similar to the current study ($r=-0.40$, $p=0.003$).\textsuperscript{29, 39, 40} DR affects autonomic function through oxidative stress leading to endothelial dysfunction. This endothelial anomaly is linked to abnormal heart rate variability and cardiac autonomic dysfunction.\textsuperscript{41}

The ocular complications, peripheral neuropathy and CAN in DM occur concurrently. The common electrophysiological tests detect changes only at secondary stages of the disease while nerve punch biopsies are invasive in nature and merely provide morphological evidence. Hence it might be possible that one or other, or both of the ocular parameters, corneal sub basal nerve density and corneal sensitivity, could act as a non-invasive biomarker for these neuropathies as a prognostic tool in monitoring disease progression and also in randomised controlled trials of new treatment modalities, for example naltrexone hydrochloride treatment for diabetic keratopathy. In conclusion, the integration of corneal IVCM and corneal sensitivity assessment into regular retinal screening programs could assist in identifying patients who are most vulnerable to developing complications, predict deterioration relating to peripheral neuropathy or CAN, and assess the efficacy of novel treatments for ocular and peripheral complications of DM.
7.4. References


Chapter 8
The effect of pan-retinal photocoagulation on the sub-basal nerve plexus in diabetes mellitus
8. Introduction

Chapter 2 discussed the prevalence and various complications (including ocular) of diabetes mellitus (DM). Diabetic retinopathy (DR) is one of the leading causes of blindness worldwide. Important therapeutic measures in the management of DR include optimising glycaemic, lipid and hypertensive control. However, in cases of proliferative or severe pre-proliferative DR, laser pan-retinal photocoagulation (PRP) is indicated with the aim of preventing progression of DR and vision loss. Although “sight-saving” and effective in the treatment of advanced diabetic retinal disease PRP is fundamentally associated with extensive tissue destruction and underlying anatomical changes.

Diabetic keratopathy can be associated with significant ocular complications and decreased corneal sensitivity has been reported following PRP in diabetes. This has been attributed to direct thermal injury to ciliary nerves running between the choroid and sclera. However, only one study has directly investigated the effects of PRP on corneal nerve microstructure. The aim of this chapter is to investigate the effect of PRP on the sub-basal nerve plexus in patients with DM, and to evaluate the relationship between corneal sub-basal nerve density, corneal sensitivity and peripheral neuropathy.

8.1. Subjects and Methods

The study was conducted with ethical approval granted by the Regional Ethics Committee (NTX/09/12/122) and adhered to the tenets of the Declaration of Helsinki. Inclusion criteria were: ≥10 year history of DM (type 1 or 2) or the presence of any DR. Patients with a history of ocular surgery or trauma, contact lens wear, or a history of corneal disease or systemic disease with the potential to affect the cornea (other than DM) were excluded. Patients were categorised into two groups according to their treatment history; a PRP group with a history
of PRP at least 6 months ago, and a non-PRP (N-PRP) group with no history of PRP. Patients with recent PRP (<6 months ago) were excluded from the study.

A detailed medical history was obtained from all patients regarding duration of DM, other medical problems or diabetic complications, medications, and smoking and alcohol history. A validated questionnaire, the Michigan Neuropathy Screening Instrument (MNSI), as described in Chapter 3, was administered to calculate the severity score of neuropathic symptoms. HbA1c (glycated haemoglobin) levels, from within the preceding 3 months, were also recorded. Although fasting glucose remains a useful diagnostic criterion for DM, it only provides an instantaneous, single point, indication of glucose control. In contrast HbA1c gives an indication of the degree of control over the previous 3 months and is considered to be a major diagnostic criterion of DM.

Central corneal sensitivity threshold (CST) was measured using a non-contact corneal aesthesiometer (NCCA). Vibration perception threshold (VPT) of the feet was measured using a biothesiometer (Bio-Medical Instrument Co. Newbury, Ohio, USA). The examination was performed at the medial malleolus, and at the plantar and dorsal aspects of the distal inter-phalangeal (DIP) joint of the great toe of the foot ipsilateral to the examined eye.

Digital images of the central and peripheral retina were captured using a non-mydriatic retinal camera (Non-Mydriatic Retinal Camera DR-DGi, Canon Inc, USA) for DR grading. All retinal images were graded by a fellowship-trained medical retina specialist.

Laser scanning in vivo confocal microscopy was performed on all subjects using the Heidelberg Retina Tomograph II Rostock Corneal Module (RCM) (Heidelberg Engineering GmBH, Germany). Sub-basal nerve (SBN) density was analysed using computer software.
Chapter 8

(analySIS 3.1; Soft Imaging System, Münster, Germany). All of these examination techniques were conducted as described in chapter 3.

SPSS 19.0 for Windows (Chicago, IL) was used for statistical analysis. An online statistical tool (Statistical solutions, LLC) calculated a minimum sample size of 10 in each group with 95% confidence interval. Independent samples t-test compared the patient characteristics of the PRP and non-PRP groups, and, one-way ANOVA was performed to compare ocular and peripheral neuropathy features between the two groups. Bivariate Pearson correlation (2-tailed) was used to determine the correlations between a number of different factors. A p-value of <0.05 was considered significant. A correlation with a correlation coefficient (Pearson’s r) of <±0.3 was considered weak; ±0.3 to ±0.6 was considered moderate; and >±0.6 was considered strong.

8.2. Results

Thirty eight eyes of 38 participants with DM were enrolled for this study, of which 19 eyes had previously undergone PRP. Fifteen left eyes and 23 right eyes were assessed. There was no significant difference in the mean age, body mass index (BMI), current severity of retinopathy, smoking and alcohol history between the PRP and non-PRP groups (Table 8.1). The mean duration of DM and HbA1c values were not significantly different between the two groups (Table 8.2).

Figure 8.1 shows three typical IVCM images of the central cornea showing sub-basal nerves in a healthy eye and 2 diabetic eyes (one previously treated with PRP). The mean SBN densities were 12.27 ± 4.28 mm/mm² in the PRP group and 12.75 ± 3.59 mm/mm² in the non-PRP group. There were no significant differences between the PRP and the non-PRP
groups in terms of SBN density ($p=0.71$), CST ($p=0.84$), MNSI score ($p=0.19$), and biothesiometry ($p=0.77$) (Table 8.2).

![Image](74x565 to 509x718)

Figure 8.1: Representative laser scanning IVCM images of (a) the central cornea showing sub-basal nerves in a healthy eye, (b) Visibly reduced sub-basal nerve density in a subject with diabetes mellitus without a history of PRP; (c) Sub-basal nerves in a subject with diabetic retinopathy treated with PRP. Scale bar =100µm

Table 8.1: Patient demographics including male: female ratio, age, body mass index (BMI), smoking history (pack years), alcohol score, ethnicity, duration of diabetes mellitus (years) and type of diabetic retinopathy (subject percentage included) in subjects with retinopathy treated by pan-retinal photocoagulation (PRP) and untreated retinopathy (Non-PRP) group.

<table>
<thead>
<tr>
<th>Groups</th>
<th>PRP</th>
<th>non-PRP</th>
<th>t-test (p values)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subjects (n)</td>
<td>19</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>M:F ratio</td>
<td>13:06</td>
<td>14:05</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>57.0 ± 7.9</td>
<td>59.5 ± 14.2</td>
<td>0.50</td>
</tr>
<tr>
<td>BMI ($\text{kg/m}^2$)</td>
<td>29.6 ± 4.0</td>
<td>29.2 ± 5.1</td>
<td>0.78</td>
</tr>
<tr>
<td>Smoking history (pack years)</td>
<td>5.8 ± 12.1</td>
<td>4.4 ± 9.75</td>
<td>0.70</td>
</tr>
<tr>
<td>Alcohol score</td>
<td>2.20</td>
<td>2.3</td>
<td>0.75</td>
</tr>
<tr>
<td>Type 1 : Type 2 Mean DM Duration (years)</td>
<td>1:18</td>
<td>1:18</td>
<td></td>
</tr>
<tr>
<td>&lt;10</td>
<td>18.0 ± 10.4</td>
<td>13.6 ± 7.2</td>
<td></td>
</tr>
<tr>
<td>10-20</td>
<td>2</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>&gt;20</td>
<td>11</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>Mean DM Duration (years) No active DR</td>
<td>6</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>&lt;10</td>
<td>0 (0%)</td>
<td>1 (5.3%)</td>
<td></td>
</tr>
<tr>
<td>10-20</td>
<td>0 (0%)</td>
<td>10 (52.6%)</td>
<td></td>
</tr>
<tr>
<td>&gt;20</td>
<td>3 (15.8%)</td>
<td>2 (10.5%)</td>
<td></td>
</tr>
<tr>
<td>Mean DM Duration (years) Mild NPDR</td>
<td>4 (21.1%)</td>
<td>5 (26.3%)</td>
<td></td>
</tr>
<tr>
<td>&lt;10</td>
<td>12 (63.2%)</td>
<td>1 (5.3%)</td>
<td></td>
</tr>
<tr>
<td>10-20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;20</td>
<td>3 (15.8%)</td>
<td>2 (10.5%)</td>
<td></td>
</tr>
<tr>
<td>Mean DM Duration (years) Moderate NPDR</td>
<td>4 (21.1%)</td>
<td>5 (26.3%)</td>
<td></td>
</tr>
<tr>
<td>&lt;10</td>
<td>12 (63.2%)</td>
<td>1 (5.3%)</td>
<td></td>
</tr>
<tr>
<td>10-20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;20</td>
<td>3 (15.8%)</td>
<td>2 (10.5%)</td>
<td></td>
</tr>
<tr>
<td>Mean DM Duration (years) Severe NPDR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10-20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;20</td>
<td>3 (15.8%)</td>
<td>2 (10.5%)</td>
<td></td>
</tr>
<tr>
<td>Mean DM Duration (years) PDR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10-20</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;20</td>
<td>3 (15.8%)</td>
<td>2 (10.5%)</td>
<td></td>
</tr>
</tbody>
</table>
The variables from both the groups included in the correlation analysis were SBN density, HbA1c, CST and biothesiometry (medial malleolus, great toe plantar and great toe dorsal) and MNSI score (Table 8.3).

HbA1c showed no significant correlations with nerve density, corneal sensitivity, biothesiometry or neuropathy symptoms score. Sub-basal nerve density showed a moderate correlation with CST (Pearson’s correlation $r=0.30$, $p=0.06$), and corneal sensitivity was modestly correlated with biothesiometry (medial malleolus) ($r=0.26$, $p=0.11$) (Figure 8.2).

All three biothesiometry measurements were strongly correlated with each other (Table 8.3). Symptomatic MNSI scores were also significantly correlated with the biothesiometry measurements ($r=0.83$, $p<0.001$).

![Corneal sensitivity vs biothesiometry](image)

Figure 8.2: Scatter plot graph showing a weak positive correlation between corneal sensitivity and biothesiometry of medial malleolus with $r=0.26$ and $p=0.11$. 

Table 8.2: Comparison of parameters between DM and controls. The sub-basal nerve density, HbA1c, corneal sensitivity, Michigan neuropathy symptomatic score and biothesiometry for medial malleolus (MM), great toe plantar (GTP) and great toe dorsal (GTD) of patients with (n=19) and without (n=19) a history of pan-retinal photocoagulation (PRP) are tabulated.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>PRP (Mean ± SD)</th>
<th>Non-PRP (Mean ± SD)</th>
<th>ANOVA (p values)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sub-basal nerve density (mm/mm²)</td>
<td>12.27±4.28</td>
<td>12.75±3.59</td>
<td>0.71</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>9.12±1.89</td>
<td>8.56±1.64</td>
<td>0.33</td>
</tr>
<tr>
<td>Corneal sensitivity (mBar)</td>
<td>2.32±2.23</td>
<td>2.46±1.76</td>
<td>0.84</td>
</tr>
<tr>
<td>MNSI Score</td>
<td>3.00±2.60</td>
<td>2.10±1.37</td>
<td>0.19</td>
</tr>
<tr>
<td>Biothesiometry - MM (V)</td>
<td>27.15±10.04</td>
<td>26.67±8.89</td>
<td>0.77</td>
</tr>
<tr>
<td>Biothesiometry - GTP (V)</td>
<td>20.21±10.50</td>
<td>20.94±8.62</td>
<td>0.81</td>
</tr>
<tr>
<td>Biothesiometry - GTD (V)</td>
<td>19.630±10.11</td>
<td>20.63±8.59</td>
<td>0.71</td>
</tr>
</tbody>
</table>
Table 8.3: Correlations of parameters in patients with DM including sub-basal nerve density (mm/mm²), HbA1c (%), corneal sensitivity (mBAR), Michigan neuropathy symptomatic score and Biothesiometry (Volts) for medial malleolus (MM), great toe plantar (GTP) and great toe dorsal (GTD)

<table>
<thead>
<tr>
<th></th>
<th>SBN density (µm/mm²)</th>
<th>HbA1c (%)</th>
<th>CST (mBar)</th>
<th>VPT - MM (V)</th>
<th>VPT - GTP (V)</th>
<th>VPT - GTD (V)</th>
<th>MNSI Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBN density (µm/mm²)</td>
<td>R</td>
<td>1</td>
<td>0.098</td>
<td>0.065</td>
<td>0.907</td>
<td>0.929</td>
<td>0.763</td>
</tr>
<tr>
<td>P</td>
<td></td>
<td>0.557</td>
<td>0.028</td>
<td>0.016</td>
<td>0.015</td>
<td>-0.051</td>
<td>0.028</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>R</td>
<td>1</td>
<td>0.084</td>
<td>-0.154</td>
<td>-0.094</td>
<td>-0.041</td>
<td>-0.043</td>
</tr>
<tr>
<td>P</td>
<td></td>
<td>0.617</td>
<td>0.356</td>
<td>0.574</td>
<td>0.807</td>
<td>0.797</td>
<td>0.797</td>
</tr>
<tr>
<td>CST (mBar)</td>
<td>R</td>
<td>1</td>
<td>0.263</td>
<td>0.155</td>
<td>0.285</td>
<td>0.148</td>
<td>0.148</td>
</tr>
<tr>
<td>P</td>
<td></td>
<td>0.11</td>
<td>0.153</td>
<td>0.082</td>
<td>0.374</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VPT - MM (V)</td>
<td>R</td>
<td>1</td>
<td>.811**</td>
<td>.753**</td>
<td>0.24</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P</td>
<td></td>
<td></td>
<td>0</td>
<td>0</td>
<td>0.147</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VPT - GTP (V)</td>
<td>R</td>
<td>1</td>
<td>.836</td>
<td>.528</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P</td>
<td></td>
<td></td>
<td>0</td>
<td>0</td>
<td>0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VPT - GTD (V)</td>
<td>R</td>
<td>1</td>
<td>.586</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

* Correlation is significant at the 0.05 level (2-tailed).
** Correlation is significant at the 0.01 level (2-tailed).

8.3. Discussion

The corneal sub-basal nerve plexus lies between the basal epithelium and Bowman’s layer.\(^{17}\) The sub-basal nerve density in diabetic corneas has been widely reported to be reduced, particularly in patients with associated diabetic retinopathy and neuropathy.\(^{18-20}\)
Chapters 4 to 6 have already highlighted the alterations in architecture and density of the subbasal nerve plexus and corneal sensitivity in those with DM. The concurrent changes in corneal sensitivity, retinopathy, peripheral neuropathy and cardiac autonomic neuropathy with reduced corneal subbasal nerve plexus density were outlined in Chapter 7. This study set out to determine if PRP in advanced retinopathy is associated with potentially confounding alterations in the subbasal nerve plexus, when considering the relationship with diabetic peripheral neuropathy.

Messmer et al., reported mean sub-basal nerve density of 16.1 mm²/mm² in patients with a mean of 14.8 year history of type 2 DM as compared to 12.2 and 12.7mm/mm² in PRP and non-PRP patients in the current study, using the same IVCM.21

Importantly, a recent global study showed that the patients with a less than 10 year history of DM had only a 21.0% prevalence of any degree of DR as compared to 54.2% in patients with a 10-20 year history of DM.22 Additionally, only 1.2% of the former group developed proliferative retinopathy compared to 9.0%, possibly requiring treatment, in the latter group.22

The normal sub basal nerve density has been reported to be between 21.6 and 45.9mm/mm² as measured by in vivo and ex vivo studies.23-25 The reduction in nerve density is associated with a loss of corneal sensitivity in these eyes.26 The corneal sensitivity threshold in the normal population is noted to be significantly lower than that found in the diabetic subjects of the current study, at 0.38mBAR.15 A number of previous studies have investigated the corneal nerve changes in DM patients compared to healthy controls21, 27-29 but there has been little exploration of the possible effects of PRP on corneal nerves.8

Interestingly, De Cilla et al (2009) reported lower sub-basal nerve density in patients with proliferative diabetic retinopathy (PDR) treated by pan-retinal photocoagulation when compared to untreated PDR.8 However, it is important to note that the extent of diabetic
retinopathy *per se* has been shown to correlate with reduced corneal sensitivity irrespective of the treatment.\(^{30}\) The presence of basement membrane abnormalities in the corneal epithelium and retinal blood vessels in DM has been put forward as a possible explanation for this correlation.\(^{31}\), \(^{32}\) A recent study highlighted differences in corneal sensitivity between the diabetic participants and control subjects, but identified no differences in corneal sensitivity threshold between PRP treated and PRP untreated diabetic participants.\(^{33}\)

The current chapter has shown that the PRP and non-PRP groups were not significantly different, not only with respect to nerve density, but also corneal sensitivity, biothesiometry at the medial malleolus and subjective neuropathy score. Thus pan-retinal photocoagulation did not appear to have any adverse effect on the corneal SBNP density or corneal sensitivity in this study when compared to comparable, non-PRP treated, diabetic eyes. Therefore, these data suggest the reduction in corneal sub-basal nerve density observed in the PRP group appear to be attributable to the effect of diabetes itself rather than laser treatment.

Reflectivity and tortuosity are two other parameters that have been used to quantify IVCM sub-basal nerves in previous studies. Some studies have simply used a subjective grading system for sub-basal nerve tortuosity.\(^{8},^{20}\) More complex objective mathematical methods have also been used to define a tortuosity coefficient in diabetes mellitus using a measure of nerve fibre curvature with the observer selecting nerve branches for calculation.\(^{27},^{34},^{35}\).

Unfortunately, as noted in earlier chapters these subjective and objective measurements of nerve tortuosity are not directly comparable. Furthermore, in the absence of a repeatable method for analysing tortuosity\(^ {35}\) this technique was deemed unsuitable for evaluation of subjects in this study. Indeed, in the context of reflectivity, the laser scanning in vivo confocal microscope used in the current study automatically adjusts the illuminating brightness to maximise image quality, Whilst generally producing higher quality images than “white” light IVCM this automatic adjustment leads to inconsistent reflectivity of highly reflective
structures, such as corneal sub-basal nerves. For these reasons, corneal nerve density was chosen as the most reliable parameter for comparison between the groups in this study.

Previous studies have demonstrated that sub-basal nerve density and corneal sensitivity correlate significantly with the severity of diabetic peripheral neuropathy. Biothesiometry (to measure VPT) is a non-invasive and practical technique that can be easily performed in a clinical setting and is recognised as a reliable predictor of diabetic peripheral neuropathy. Therefore, significant correlations between sub basal nerve density and corneal sensitivity; between nerve density and biothesiometry (VPT); and between MNSI score and biothesiometry might be anticipated in this study. Indeed, both of the correlation pairs: CST and VPT, MNSI score and VPT, showed a positive correlation with each other. The correlation between MNSI score and VPT suggests that severe peripheral neuropathy is associated with increased symptoms and vice versa. Therefore, in agreement with other studies, these data suggest that non-invasive clinical tools such as corneal aesthesiometry and the MNSI questionnaire have the potential to rapidly predict peripheral neuropathy severity in lower limbs. Other studies have also shown positive correlation of peripheral neuropathy with corneal sensitivity and the MNSI questionnaire.

In conclusion, pan retinal photocoagulation treatment does not have any significant effect on corneal sub-basal nerve density or corneal sensitivity in patients with diabetes mellitus compared to subjects with diabetes who have not undergone PRP. Therefore, this observation provides key additional information, in relation to the possibility of reliably using IVCM of the corneal subbasal nerve plexus as an indicator of peripheral neuropathy in DM where the subject has undergone PRP.
8.4. References


Chapter 9

The pre-ocular tear film in patients with and without diabetes mellitus
9. Introduction

Various ocular manifestations of diabetes mellitus (DM) are explained in Chapter 2. Whilst diabetic cataract and diabetic retinopathy feature extensively in the literature, only a fraction of the literature has been dedicated to the corneal or ocular surface complications of DM. Historically, patients with DM complain of dry eye symptoms and experience epithelial fragility, punctate keratopathy, persistent epithelial defects, and decreased corneal sensitivity.1-4 The compromised innervation to the cornea and microvasculature changes in the retina in DM have been discussed in Chapters 5 to 8. The close dynamics of the cornea and the pre-ocular tear film suggests that affected cornea in DM would result in impaired tear film. The tear film in DM is primarily characterised by impairment in tear quantity and quality.5, 6

The tear film has been reported to be unstable in those with DM and the longer duration of DM could prompt damage to the microvasculature and innervation of the lacrimal gland causing impaired lacrimation.3, 5, 7 However, it is still unclear if DM complications in the extremities of the body are reflected on the tear film. Few studies have investigated the association of these ocular surface changes to peripheral neuropathy.5, 8, 9 Some studies have suggested anomalous innervation of the lacrimal gland in those with diabetic sensory neuropathy.3, 5

This chapter compares tear film parameters in patients with DM and control subjects. Correlations of these tear film metrics with age, DM duration, diabetic retinopathy and peripheral neuropathy are also described.
Chapter 9

9.1. Methods

Fifty-three patients, with a history of type 1 diabetes mellitus, were identified by a specialist endocrinology physician and invited to participate, took part in this study. Through advertisements distributed within the University of Auckland, Grafton campus 45 volunteers were recruited to participate in the study as controls. Exclusion criteria included a ≥ 3 month history of contact lens wear, any ocular surgery, trauma, disease or a diagnosis of neuropathy unrelated to DM. Additionally, a standard hospital questionnaire concerning smoking and alcohol usage were completed by the subjects. HbA1c was obtained for all the subjects with a cut-off for exclusion as a control subject of 5.5 %.

A detailed dry eye history was obtained from each of the subjects in addition to a medical and general history. The validated McMonnies dry eye questionnaire was completed by all subjects. Gross ocular examination was performed by slit-lamp biomicroscopy (Topcon Medical Systems, NJ, USA). Tear film interferometry was performed with the Keeler Tearscope Plus™ (Keeler Ltd, Berkshire, UK), enabling estimation of the thickness of the tear film lipid layer and also measurement of the tear film stability as the non-invasive tear break up time (NIBUT). The phenol red thread test (PRTT) (Zone-Quick™, FCI Ophthalmics, Pembroke, MA, USA) provided an index of tear secretion with minimal stimulus to reflex tearing. All ocular assessments were performed on one eye only; generally the right eye, with the exception of subjects reporting unilateral surgery or trauma to the right, in which case the left eye was examined.

Correlation analysis was performed between the results of the tear film assessment techniques and corneal sub-basal nerve density (from HRT IVCM), corneal sensitivity (NCCA), diabetic retinopathy (graded by a medical retina specialist) and total neuropathy score (derived by neurological assessment).
On the basis of previously reported data, a power calculation determined a minimum sample size of 50 to detect a correlation between different variables within the same group of subjects with DM, with 80% power and 95% confidence. Statistical analysis was accomplished using IBM SPSS v19.0 (Chicago, IL, USA). A logarithmic transformation of the positively skewed raw NIBUT values normalised the data and enabled use of parametric statistical tests. A one-way ANOVA for parametric and Friedmann for non-parametric variables were carried out to establish differences in test results between subjects with and without DM. Pearson and Spearman correlation (2-tailed) analysis was performed for parametric and non-parametric variables, respectively. A p-value of <0.05 was considered statistically significant.

9.2. Results

Five of the volunteer control subjects were excluded on the basis of an HbA1c level higher than 5.5%. Table 9.1 describes the primary patient characteristics including gender, age, ethnicity and duration of diabetes. The patients with DM were European with the exception of one Asian and one Māori participant. Although the majority (48%) of the control subjects were also of European descent (n=19), the volunteers were more varied in ethnicity, and included participants reporting Asian, Indian, Japanese, Latino, Israeli, and Brazilian backgrounds.

The mean age of those with DM was 48 ± 11 years compared to 44 ± 14 years in the control group. Table 9.2 illustrates comparative data between control subjects and patients with DM in terms of their dry eye symptomatology and clinical features. There was no statistically significant difference in dry eye symptom scores between the DM and control groups (p=0.33). Interferometry results, however, were statistically significantly different between the two groups, with both lipid layer thickness (p=0.02) and NIBUT (p<0.0001) observed to be
reduced in the DM group. PRTT results were also found to be statistically significantly different between the groups with and without DM (p=0.01).

Table 9.1: DM and control group sample sizes and characteristics including gender, age, ethnicity, smoking and alcohol history, and duration of DM.

<table>
<thead>
<tr>
<th></th>
<th>DM</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subjects (n)</td>
<td>53</td>
<td>40</td>
</tr>
<tr>
<td>M:F ratio</td>
<td>26:27</td>
<td>17:23</td>
</tr>
<tr>
<td>Age (years)</td>
<td>48.6 ± 11.8</td>
<td>44.3 ± 14.7</td>
</tr>
<tr>
<td>Smoking history (pack years)</td>
<td>4.2 ± 10.3</td>
<td>1.7 ± 1.0</td>
</tr>
<tr>
<td>Alcohol score</td>
<td>3.8 ± 2.3</td>
<td>2.1 ± 0.2</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>7.7 ± 1.0</td>
<td>5.3 ± 0.2</td>
</tr>
<tr>
<td>Ethnicity</td>
<td></td>
<td></td>
</tr>
<tr>
<td>European</td>
<td>51</td>
<td>19</td>
</tr>
<tr>
<td>Indian</td>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td>Asian (excluding Indian)</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>Māori</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Others</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Mean DM Duration (years)</td>
<td>25.8 ± 11.4</td>
<td></td>
</tr>
<tr>
<td>&lt;10</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>10-20</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>21-31</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>&gt;31</td>
<td>16</td>
<td></td>
</tr>
</tbody>
</table>

Table 9.2: Comparison of symptoms and tear characteristics for control and DM groups (mean/median), together with the significance of their differences (ANOVA/Friedmann). Non-invasive tear break up time (NIBUT) values are extrapolated from logarithmically transformed data. PRTT refers to the measurements of the phenol red thread test.

<table>
<thead>
<tr>
<th></th>
<th>DM</th>
<th>Controls</th>
<th>ANOVA (p values)</th>
</tr>
</thead>
<tbody>
<tr>
<td>McMinnies questionnaire (Mean ± SD)</td>
<td>8.8 ± 6.7</td>
<td>7.6 ± 4.6</td>
<td>0.33</td>
</tr>
<tr>
<td>Lipid layer thickness (median)</td>
<td>2</td>
<td>3</td>
<td>0.02</td>
</tr>
<tr>
<td>NIBUT (Mean ± SD)</td>
<td>6.0 ± 1.9</td>
<td>8.2 ± 2.5</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>PRTT (Mean ± SD)</td>
<td>13.7 ± 4.7</td>
<td>16.3 ± 4.9</td>
<td>0.01</td>
</tr>
</tbody>
</table>
A number of correlations between ophthalmic and non-ophthalmic parameters were discussed in Chapter 7 but the association between the tear film and other parameters are primarily discussed in this chapter. Pearson and Spearman correlation analysis (for parametric and non-parametric parameters respectively) between meibomian gland function, tear film stability, tear volume, corneal sensitivity and sub-basal nerve density (SBN), diabetic retinopathy grading and total neuropathy score are shown in Table 9.3.

Dry eye symptoms correlated positively with age (r=0.33, p=0.015) whereas tear film stability was inversely related (r=-0.28, p=0.046). The duration of DM also negatively correlated with age (r=-0.511, p<0.010). The thickness of the lipid layer of the tear film was positively correlated with NIBUT (r=0.56, p<0.001) and basal tear secretion quantity (r=0.38, p<0.01).

The tear film stability was noted to have a weak positive association with sub basal nerve density (r=0.28, p=0.04). NIBUT showed an inverse relationship with total neuropathy score (r=-0.29, p=0.03). Corneal sensitivity did not correlate statistically with any of the tear film parameters.
Table 9.3: Pearson and Spearman correlation analysis between age, DM duration, smoking score, alcohol score, McMonnies questionnaire scores (DEQ), phenol red thread test (PRTT) (mm), tear film interferometry including lipid layer patter (grading:-Lipid thickness), stability (NIBUT), corneal sensitivity (CST) (mBAR), sub-basal nerve density (SBN) (mm/mm²), diabetic retinopathy grading (DR grade) and total neuropathy score (NCS total).

<table>
<thead>
<tr>
<th></th>
<th>Age</th>
<th>DM Duration</th>
<th>Smoking Score</th>
<th>Alcohol Score</th>
<th>DEQ</th>
<th>Lipid Thickness</th>
<th>NIBUT</th>
<th>PRTT</th>
<th>CST</th>
<th>SBN</th>
<th>DR grade</th>
<th>NCS total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age R</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td>0.51**</td>
<td>0.00</td>
<td>0.73</td>
<td>0.22</td>
<td></td>
<td>-0.17</td>
<td>0.02</td>
<td>0.05</td>
</tr>
<tr>
<td>P</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-0.05</td>
<td>-0.28*</td>
<td>0.20</td>
</tr>
<tr>
<td>DM Duration R</td>
<td></td>
<td>1.00</td>
<td>-0.17</td>
<td>0.21</td>
<td>-0.09</td>
<td>-0.29*</td>
<td>0.21</td>
<td>0.18</td>
<td>-0.25</td>
<td>0.00</td>
<td>0.27</td>
<td>0.04</td>
</tr>
<tr>
<td>P</td>
<td></td>
<td></td>
<td></td>
<td>0.29</td>
<td>0.13</td>
<td>0.52</td>
<td>0.03</td>
<td>0.14</td>
<td></td>
<td>0.20</td>
<td>0.07</td>
<td>0.79</td>
</tr>
<tr>
<td>Smoking Score R</td>
<td>1.00</td>
<td>-0.17</td>
<td>0.21</td>
<td>-0.26</td>
<td>-0.26</td>
<td>-0.26</td>
<td>0.00</td>
<td>-0.19</td>
<td>-0.19</td>
<td>0.27</td>
<td>0.47**</td>
<td>47**</td>
</tr>
<tr>
<td>P</td>
<td></td>
<td></td>
<td></td>
<td>0.88</td>
<td>0.92</td>
<td>0.06</td>
<td>0.07</td>
<td>0.06</td>
<td></td>
<td>0.98</td>
<td>0.17</td>
<td>0.05</td>
</tr>
<tr>
<td>Alcohol Score R</td>
<td>1.00</td>
<td>-0.03</td>
<td>-0.07</td>
<td>0.26</td>
<td>-0.03</td>
<td>0.26</td>
<td>0.27</td>
<td>0.06</td>
<td></td>
<td>0.13</td>
<td>-0.08</td>
<td></td>
</tr>
<tr>
<td>P</td>
<td></td>
<td></td>
<td></td>
<td>0.11</td>
<td>0.05</td>
<td>0.06</td>
<td>0.85</td>
<td>0.63</td>
<td></td>
<td>0.07</td>
<td>0.34</td>
<td>0.57</td>
</tr>
<tr>
<td>DEQ R</td>
<td></td>
<td></td>
<td></td>
<td>-0.25</td>
<td>0.01</td>
<td>0.14</td>
<td>-0.17</td>
<td></td>
<td>-0.17</td>
<td>-0.04</td>
<td>-0.05</td>
<td></td>
</tr>
<tr>
<td>P</td>
<td></td>
<td></td>
<td></td>
<td>0.66</td>
<td>0.07</td>
<td>0.93</td>
<td>0.33</td>
<td></td>
<td></td>
<td>0.22</td>
<td>0.75</td>
<td>0.73</td>
</tr>
<tr>
<td>Lipid thickness R</td>
<td>1.00</td>
<td>-0.10</td>
<td>-0.10</td>
<td>0.28</td>
<td>-0.10</td>
<td>-0.03</td>
<td>-0.29*</td>
<td></td>
<td></td>
<td>-0.03</td>
<td>-0.29*</td>
<td></td>
</tr>
<tr>
<td>P</td>
<td></td>
<td></td>
<td></td>
<td>0.28</td>
<td>0.46</td>
<td>0.04</td>
<td>0.82</td>
<td></td>
<td></td>
<td>0.04</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>NIBUT R</td>
<td>1.00</td>
<td></td>
<td>-0.10</td>
<td>0.13</td>
<td>-0.16</td>
<td>-0.16</td>
<td>0.05</td>
<td></td>
<td>0.01</td>
<td>0.17</td>
<td>0.24</td>
<td></td>
</tr>
<tr>
<td>P</td>
<td></td>
<td></td>
<td></td>
<td>0.37</td>
<td>0.71</td>
<td>0.26</td>
<td>0.96</td>
<td></td>
<td></td>
<td>0.37</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PRTT R</td>
<td></td>
<td></td>
<td>-0.10</td>
<td>0.55</td>
<td>-0.29*</td>
<td>-0.29*</td>
<td>0.01</td>
<td></td>
<td>0.01</td>
<td>0.17</td>
<td>0.24</td>
<td></td>
</tr>
<tr>
<td>P</td>
<td></td>
<td></td>
<td></td>
<td>0.36**</td>
<td>0.01</td>
<td>0.23</td>
<td>0.08</td>
<td></td>
<td></td>
<td>0.23</td>
<td>0.08</td>
<td></td>
</tr>
<tr>
<td>CST R</td>
<td></td>
<td></td>
<td></td>
<td>0.13</td>
<td>0.05</td>
<td>0.05</td>
<td>0.11</td>
<td></td>
<td>0.17</td>
<td>0.17</td>
<td>0.24</td>
<td></td>
</tr>
<tr>
<td>P</td>
<td></td>
<td></td>
<td></td>
<td>0.05</td>
<td>0.13</td>
<td>0.23</td>
<td>0.46</td>
<td></td>
<td></td>
<td>0.46</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>SBN R</td>
<td></td>
<td></td>
<td></td>
<td>-0.33*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.25</td>
<td></td>
</tr>
<tr>
<td>P</td>
<td></td>
<td></td>
<td></td>
<td>0.11</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.11</td>
<td>0.07</td>
<td></td>
</tr>
<tr>
<td>DR grade R</td>
<td></td>
<td></td>
<td></td>
<td>0.25</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.25</td>
<td>1.00</td>
</tr>
<tr>
<td>P</td>
<td></td>
<td></td>
<td></td>
<td>0.07</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.00</td>
</tr>
</tbody>
</table>

** Correlation is significant at the 0.01 level (2-tailed).
* Correlation is significant at the 0.05 level (2-tailed).
9.3. Discussion

The current study assessed the state of the tear film in patients with DM and control subjects. The thickness of the lipid layer (as estimated from the pattern grading), NIBUT and tear secretion (including minimal reflex and basal tear secretion)\(^{11}\) were significantly reduced in patients with DM confirming the increased risk to the ocular surface in this disease. All of the tear film parameters were tested for correlation with age, DM duration, corneal sensitivity, sub basal nerve density, diabetic retinopathy grading and total neuropathy score. Various studies have argued the effect of increasing age on dry eye symptoms.\(^{12-15}\) Yet, the effect of age on the tear film and dry eye symptoms is not clear. In the present study, age matched subjects with and without DM had similar dry eye symptom scores. The current study also showed marked instability of the tear film in patients with DM compared to control subjects.\(^{5}\) Although age and DM duration were associated with decreased tear film stability, stage of retinopathy was not found to be a factor, contrary to the observations of Saito et al.\(^{16-18}\). The tear film stability including lipid layer thickness has been shown to be influenced by gender, with older women tending to have thinner and contaminated lipid layers.\(^{19}\) A decrease in androgens in post-menopausal women regulates meibomian gland function, supporting female gender as a risk factor in dry eye disease.\(^{20}\) Several studies have shown a reduction in tear film stability and basal tear secretion in patients with DM.\(^{5,17,18,21}\) However, Goebbles et al reported no difference in the tear film stability between age-matched individuals with or without DM.\(^{22}\)

All the three layers of the tear film could be affected in DM. The meibomian and lacrimal glands are responsible for the secretion of lipid and aqueous layer of the pre-ocular tear film. Only two studies have reported compromised tear lipid layer in those with DM.\(^{6,23}\) The lipid layer of the tear film was also observed to be altered in the current study. The elevated expression of advanced glycation end-products in the lacrimal gland is indicative of their
involved in signalling and inflammatory changes in DM.\textsuperscript{7} The reduction in goblet cell numbers as a result of compromised mucin production also contribute to the tear film instability in DM.\textsuperscript{5,24}

Inoue \textit{et al} inferred that reduction in tear break up time may lead to occurrence of superficial punctate keratitis (SPK) in those with DM.\textsuperscript{6} Functional abnormalities of corneal innervation may also contribute to the incidence of SPK in DM.\textsuperscript{25} The observation of a correlation between tear stability (NIBUT) and corneal sub-basal nerve density was not surprising. Chapter 7 discussed the association of corneal sub-basal nerve density and peripheral neuropathy. Interestingly, a significant correlation between NIBUT and total neuropathy score (a measure of peripheral neuropathy) was demonstrated in the current study.

The reduced basal tear production suggests that lacrimal gland function is affected, alongside diabetic peripheral neuropathy, as seen in this study where a statistically significant difference between the diabetic and non-diabetic groups was observed.\textsuperscript{26} This could be caused by dysfunction of the ocular surface secretory glandular functional unit resulting from peripheral neuropathy, in addition to, disease of the sensory afferent nerves innervating the ocular surface and the autonomic (efferent) nerves innervating the tear-secreting glands.\textsuperscript{26-28}

An earlier study noted a difference between the two groups in total and reflex tear secretion but did not find any change in basal tear secretion as measured by Schirmer test with anaesthesia.\textsuperscript{17} Previously, diminished lacrimation when measured with Schirmer’s test and the cotton thread test has also been reported.\textsuperscript{6} Although, altered tear secretion in DM has been reported to be associated with peripheral neuropathy and poor metabolic control; the current study did not show any such relationship.\textsuperscript{5} In recent years, naltrexone hydrochloride, an opioid antagonist, has shown signs of improvement in tear production and corneal sensitivity in diabetic rats.\textsuperscript{29} The authors suggested that the naltrexone hydrochloride
restores tear production and corneal sensitivity by interfering with opioid-opioid receptor actions.\textsuperscript{29}

In summary, the current study confirms the threat to the state of the ocular surface health in those with DM as compared to control subjects. The reduction in tear production in patients with DM and the association between tear film stability and sub-basal nerve density and diabetic peripheral polyneuropathy suggests that peripheral neuropathy may affect lacrimal gland function.\textsuperscript{29} Furthermore, the promising results of naltrexone hydrochloride treatment\textsuperscript{30, 31} in diabetic rats may pave the way for randomised controlled trials in patients with DM using standard tests to assess the ocular surface.
9.4. References

Chapter 10
Conclusions
10. Introduction

This thesis comprises an extensive series of inter-related clinical studies that investigated corneal microstructure, using laser scanning in vivo confocal microscopy, especially in regard to the subbasal nerve plexus, in subjects with and without diabetes mellitus (DM).

It is important to first understand and delineate the status of the normal cornea before studying the diseased cornea. Therefore, the study in Chapter 4 explored the inter-ocular comparisons of corneal sub-basal nerve montage maps in normal healthy subjects in addition to unilateral corneal sub-basal nerve maps in those with DM. Laser scanning in vivo confocal microscopy was also used to investigate the corneal microstructure in subjects with and without DM, as detailed in Chapter 5.

In addition, other corneal parameters including corneal sensitivity, topography, hysteresis and central corneal thickness were compared in Chapter 6. All of the ocular parameters assessed were evaluated for correlation with diabetic peripheral neuropathy and autonomic neuropathy. Chapter 7 elucidated and discussed these associations.

Chapter 8 studied the effect of a common treatment for diabetic retinopathy, pan retinal photocoagulation (PRP), on corneal nerve microstructure. Finally, the effect of DM on the pre-ocular tear film was studied as described in Chapter 9.

The final conclusions that can be drawn from the studies contained in each of these Chapters are highlighted in the sections hereafter.
10.1. The two-dimensional architecture of the corneal sub-basal nerve plexus in diabetes mellitus

Chapter 4 investigated the two-dimensional architecture of the corneal sub basal nerve plexus in paired healthy eyes. The sub-basal nerve architecture of unilateral eyes of those with DM was also examined. It was observed that:

1) Based on 12 eyes, the normal corneal sub-basal nerve architecture in living human eyes, displayed an infero-central whorl configuration with a clockwise orientation in both right and left eyes. The lack of enantiomorphism might have a significant impact on post-surgical nerve healing process, especially subsequent to LASIK surgery.

2) The sub-basal nerve maps in those with DM similarly demonstrated a clockwise orientation, albeit with an obvious reduction in the density of sub-basal nerves.

3) High intra and inter-observer repeatability in the measurement of sub basal nerve density confirms excellent reliability of the analytical technique reported in these studies (and where employed elsewhere in this thesis).

10.2. Corneal assessment using in vivo confocal microscopy in diabetes mellitus

Laser scanning in vivo confocal microscopy (IVCM) of the cornea was performed to enable quantitative analysis of the corneal epithelium, sub-basal nerve plexus and endothelium. The main conclusions of Chapter 5 were:
1) Conclusive evidence of a significantly lower corneal sub basal nerve density in DM compared to subjects without DM.

2) There was no difference between the two groups (with and without DM) with regard to epithelial and endothelial cell density, or in respect to endothelial polymegathism and pleomorphism, indicating that the disturbed corneal innervation precedes, or is independent of, any abnormality in the corneal epithelium or endothelium in DM.

10.3. The cornea in patients with and without diabetes mellitus

Corneal sensitivity, topography, hysteresis, resistance factor and intraocular pressure were assessed in those with DM and compared to age matched normal subjects in Chapter 6. It was observed that:

1) The corneal sensitivity threshold was increased in patients with DM compared to those without. This difference in corneal sensitivity is highly relevant as it might predispose to delayed epithelial wound healing and promote the development of recurrent corneal erosions in those with DM.

2) There was no statistically significant difference in central corneal thickness, keratometry, hysteresis and resistance factor and intraocular pressure in DM compared with the normal eye. These inter-related corneal variables are not affected in DM at the stage where corneal sensitivity is compromised.
Chapter 10

10.4. Evaluation of relationships between corneal nerve density, corneal sensitivity, retinopathy, cardiovascular autonomic neuropathy and peripheral neuropathy

The associations between the corneal sub-basal nerve density, corneal sensitivity, retinopathy grading, cardiovascular autonomic neuropathy and peripheral neuropathy were evaluated in Chapter 7. The conclusions of the chapter were that:

1) A reduction in corneal sub-basal nerve density is associated with decreased corneal sensitivity in patients with DM. The small corneal nerve fibres are affected at an early stage of DM exhibiting an increase in corneal sensitivity threshold.

2) The corneal sub-basal nerve density was inversely associated with the degree of peripheral neuropathy. This implies a comparable involvement of the cranial (trigeminal) nerve and the long nerves involved in diabetic peripheral neuropathy. Sub basal nerve density could potentially become a biomarker for peripheral neuropathy.

3) The decreased corneal sub basal nerve density and diabetic retinopathy in the posterior segment appear to develop independently in those with DM.

4) Poor glycaemic control is responsible for the worsening of diabetic retinopathy, peripheral neuropathy and autonomic cardiovascular neuropathy.

5) Being related, diabetic peripheral neuropathy and cardiac autonomic neuropathy probably originate simultaneously in DM. The neural dysfunction was observed to occur along with small fibre neuropathy and variation in heart rate.
6) A significant relationship between high smoking score and peripheral neuropathy was observed in the current study. This association suggests that patients who smoke are at higher risk of developing neuropathy.

7) Reduced corneal sensitivity and diabetic retinopathy are independently associated with cardiac autonomic dysfunction. Hence, the assessment of these two ocular variables could be helpful in predicting cardiac autonomic neuropathy, which is associated with an increased risk of mortality.

10.5. The effect of panretinal photocoagulation on the corneal sub basal nerve plexus in diabetes mellitus

The aim of this study was to investigate the effect of pan-retinal photocoagulation (PRP), a treatment for diabetic retinopathy, on the corneal sub-basal nerve plexus and corneal sensitivity in patients with type 2 DM. Additionally, associations between corneal sub-basal nerve density, corneal sensitivity and peripheral neuropathy were evaluated in Chapter 8. The results showed that:

1) PRP treatment had no impact on corneal sub basal nerve density, corneal sensitivity, biothesiometry and subjective neuropathy score in DM. Hence, PRP does not appear to cause any adverse effect on the cornea. Thus, any effects observed in the cornea are due to the DM disease process and are not related to retinal laser treatment.
2) A significant relationship was observed between sub basal nerve density and corneal sensitivity in type 2 DM, similar to that identified in type 1 DM earlier in the thesis.

3) Biothesiometry was correlated with sub basal nerve density and symptomatic neuropathy score suggesting an association between peripheral neuropathy and corneal sub-basal nerve density in type 2 DM, similar to that observed in type 1.

10.6. The pre-ocular tear film in patients with and without DM

Chapter 9 explored the effect of DM on the quantity and stability of the pre-ocular tear film. Further relationships between pre-ocular tear film metrics, diabetic retinopathy grading and peripheral neuropathy were studied. The results showed that:

1) The thickness of the lipid layer, tear film stability, and tear secretion were significantly reduced in those with DM compared to those without DM. Such alterations of the pre-ocular tear film may contribute to an already compromised ocular surface in DM.

2) The parallel relationships between pre-ocular tear film stability, corneal sub basal nerve density, and peripheral neuropathy is suggestive of concurrent alteration in lacrimal gland and trigeminal nerve function in tandem with development of diabetic peripheral neuropathy.

10.7. Final conclusion

The inter-related studies in this thesis highlight the effect of DM on the ocular surface including the cornea and the pre-ocular tear film. Alterations in the corneal nerve architecture
and reduced sub basal nerve density indicate nerve degeneration. The decreased sub basal nerve density plays an important role in decreased corneal sensitivity. However, these changes in the sub-basal nerve plexus are not compounded by laser treatment for diabetic retinopathy (PRP). Diabetes related changes in the pre-ocular tear film may have a detrimental effect on the ocular surface.

Diabetes mellitus-related corneal abnormalities, including reduced sub basal nerve density and altered corneal sensitivity, occur together with peripheral, and cardiac autonomic, neuropathy. Both of these ocular parameters therefore have the potential to be used as a non-invasive biomarker for diabetic peripheral, and cardiac autonomic, neuropathy allowing earlier prediction of these DM complications. Automated sub basal nerve density analysis would assist in obtaining prompt IVCM results. Therefore, corneal IVCM and corneal sensitivity could compliment regular diabetic retinal screening programs in identifying “at risk” patients with DM who are more prone to complications. Importantly, these non-invasive ocular assessments, instead of invasive skin punch biopsies, could also test the efficacy of novel treatments for ocular as well as peripheral complications of DM in randomised controlled trials.

The relatively limited availability of laser scanning in vivo confocal microscopy and appropriately skilled clinicians/technicians at the present time remains a (temporary) barrier to the widespread introduction into the clinical environment as a routine investigation. None-the-less it is entirely possible, with refinements in IVCM technology, particularly image handling and reconstruction, that IVCM with become an integral tool in the investigation and management of diabetes mellitus in the next five to ten years.
Appendix
11. Appendix

11.1. Related publications

Misra S, Ahn HN, Craig JP, Pradhan M, Patel DV, McGhee CNJ
Effect of Pan-Retinal Photocoagulation on Corneal Sensation and the Corneal Sub-Basal Nerve Plexus in Diabetes Mellitus
Investigative Ophthalmology and Visual Science 2013; Jul 2; 54(7): 4485-90

Misra S, Craig JP, McGhee CNJ, Patel DV
Inter-ocular comparison by in vivo confocal microscopy of the two-dimensional architecture of the normal human corneal sub-basal nerve plexus
Cornea 2012 Dec; 31(12):1376-80

11.2. Conference and seminar presentations

Misra S
Diabetes and corneal innervation; 9th International Symposium of Ophthalmology (ISO), 9th to 11th November, 2013, Gunagzhou, China
(Invited lecture)

Misra S, Patel DV, McGhee CNJ, Pradhan M, Braatvedt GB, Kilfoyle D, Craig JP
Peripheral neuropathy and pre-ocular tear film dysfunction in diabetes mellitus; American Academy of Optometry, 23rd to 26th October, 2013, Seattle, USA
(Selected for ‘Press conference’ and ‘Hot topics symposium’)

Misra S
Imaging the cornea in diabetes mellitus; 28th Asia-Pacific Academy of Ophthalmology Congress, 17th to 20th January, 2013, Hyderabad, India
(Invited lecture)
Misra S, Patel DV, Braatvedt GB, Kilfoyle D, Craig JP, McGhee CNJ
Correlation of Corneal Nerve Microstructure and Function with Peripheral Neuropathy in Diabetes Mellitus using In Vivo Confocal Microscopy; 28th Asia-Pacific Academy of Ophthalmology Congress, 17th to 20th January, 2013, Hyderabad, India

Misra S, Patel DV, Braatvedt GB, Kilfoyle D, Craig JP, McGhee CNJ
In vivo corneal confocal microscopy: an ophthalmic marker for peripheral neuropathy in diabetes mellitus; FMHS Doctoral Showcase, 20th November, 2012, Auckland, NZ (prize winning presentation)

Misra S, Patel DV, Braatvedt GB, Kilfoyle D, Craig JP, McGhee CNJ
In vivo corneal confocal microscopy: an ophthalmic marker for peripheral neuropathy in diabetes mellitus; The Royal Australian and New Zealand College of Ophthalmologists (RANZCO); 24th to 28th November, 2012, Melbourne, Australia

Misra S
In vivo corneal confocal microscopy: an ophthalmic marker for peripheral neuropathy in diabetes mellitus; New Zealand Association of Optometrists Annual conference; 26th to 28th October, 2012, Taupo, NZ (Invited lecture)

Misra S, Patel DV, Braatvedt GB, Kilfoyle D, Craig JP, McGhee CNJ
Correlation of Corneal Nerve Microstructure and Function with Peripheral Neuropathy in Diabetes Mellitus using In Vivo Confocal Microscopy; The Association for Research in Vision and Ophthalmology (ARVO), 6th to 10th May, 2012, Fort Lauderdale, FL, USA.

Misra S, Patel DV, Braatvedt GB, Kilfoyle D, Craig JP, McGhee CNJ
In vivo corneal confocal microscopy: an ophthalmic marker for peripheral neuropathy in diabetes mellitus; New Zealand society for the study of Diabetes Annual scientific meeting; 2nd to 4th May, 2012, Auckland, NZ (prize winning presentation)

Misra S
Corneal microstructural changes in diabetes; 27th Asia-Pacific Academy of Ophthalmology Congress (APAO), 13th to 16th April, 2012, Busan, Korea (Invited lecture)
Misra S, Ahn HN, Craig JP, Pradhan M, Patel DV, McGhee CNJ.
The effect of pan-retinal photocoagulation on the corneal sub-basal nerve plexus in patients with diabetes mellitus; 27th Asia-Pacific Academy of Ophthalmology Congress (APAO), 13th to 16th April, 2012, Busan, Korea

Misra S, Patel DV, Craig JP, McGhee CNJ
The architecture of the corneal sub-basal nerve plexus in patients with type 1 diabetes Mellitus; Joint Congress of Society of Ophthalmology (SOE) and American Academy of Ophthalmology (AAO), 4th to 7th June, 2011, Geneva, Switzerland

Misra S, Patel DV, Craig JP, McGhee CNJ
Corneal sub-basal nerve plexus architecture in the diabetic cornea; 26th Asia-Pacific Academy of Ophthalmology Congress 20 to 24th March, 2011, Sydney, Australia

Misra S
Diabetes and corneal innervation; 25th Asia-Pacific Academy of Ophthalmology Congress, 16-20th September, 2010, Beijing, China
(Invited lecture)

Misra S, Craig JP, McGhee CNJ, Patel DV
Inter-ocular comparison of corneal nerve plexus configuration;
Royal Australian and New Zealand College of Ophthalmology, 11th to 14th May, 2010, Wellington, NZ

Misra S, Craig JP, McGhee CNJ, Patel DV
Configuration of the corneal sub-basal nerve plexus whorl; World Cornea Congress VI, 2nd to 5th April, 2010, Boston, MA, USA

Misra S, Craig JP, McGhee CNJ, Patel DV
Comparison of corneal sub-basal nerve plexus configuration between paired eyes
Calvin Ring Seminar, 25th March, 2010, Auckland, NZ
11.3. Alcohol consumption questionnaire

<table>
<thead>
<tr>
<th>SOCIAL HISTORY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Occupation:</td>
</tr>
<tr>
<td>Tobacco:</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Alcohol:</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

Provide Brief Intervention if:

MEN: score > 5  WOMEN: score > 4  = TOTAL SCORE

Other substance use: