CORNEAL NERVE STRUCTURE AND FUNCTION AS MARKERS OF DIABETIC NEUROPATHY

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Abstract

Diabetic neuropathy is a significant clinical problem that currently has no effective therapy, and in advanced cases, leads to foot ulceration and lower limb amputation. The accurate detection, characterisation and quantification of this condition are important in order to define at-risk patients, anticipate deterioration, monitor progression and assess new therapies. This thesis evaluates novel corneal methods of assessing diabetic neuropathy. Over the past several years two new non-invasive corneal markers have emerged, and in cross-sectional studies have demonstrated their ability to stratify the severity of this disease. Corneal confocal microscopy (CCM) allows quantification of corneal nerve parameters and non-contact corneal aesthesiometry (NCCA), the presumed functional correlate of corneal structure, assesses the sensitivity of the cornea. Both these techniques are quick to perform, produce little or no discomfort for the patient, and with automatic analysis paradigms developed, are suitable for clinical settings. Each has advantages and disadvantages over established techniques for assessing diabetic neuropathy. New information is presented regarding measurement bias of CCM images, and a unique sampling paradigm and associated accuracy determination method of combinations is described. A novel high-speed corneal nerve mapping procedure has been developed and application of this procedure in individuals with neuropathy has revealed regions of sub-basal nerve plexus that dictate further evaluation, as they appear to show earlier signs of damage than the central region of the cornea that has to date been examined. The discriminative capacity of corneal sensitivity measured by NCCA is revealed to have reasonable potential as a marker of diabetic neuropathy. Application of these new corneal markers for longitudinal evaluation of
diabetic neuropathy has the potential to reduce dependence on more invasive, costly, and time-consuming assessments, such as skin biopsy.
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List of Abbreviations

CCM  Corneal confocal microscopy
CNBD  Corneal nerve branch density
CNFD  Corneal nerve fibre density
CNFL  Corneal nerve fibre length
CNFT  Corneal nerve fibre tortuosity
CRF  Case report form
CS  Contrast sensitivity
JDRF  Juvenile Diabetes Research Foundation
GADAb  Glutamic acid decarboxylase antibodies
GCP  Good Clinical Practice
HREC  Human Research Ethics Committee
HRV  Heart rate variability
IGT  Impaired glucose tolerance
IHBI  Institute of Health and Biomedical Innovation
LADA  Latent Autoimmune Diabetes in Adults
LSCM  Laser-scanning confocal microscopy
NCCA  Non-contact corneal aesthesiometry
NDS  Neuropathy disability score
NET  Nerve electrophysiology testing
NHMRC  National Health and Medical Research Council
PAH  Princess Alexandra Hospital
QST  Quantitative sensory testing
QUT  Queensland University of Technology
RNFLT  Retinal nerve fibre layer thickness
RS  Retinal sensitivity
SSCM  Slit-scanning confocal microscopy
TSCM  Tandem-scanning confocal microscopy
UM  University of Manchester
List of Publications and Manuscripts

Relevant Publications During Candidature


Relevant Manuscripts Accepted For Publication


Relevant Manuscripts Submitted and Under Review By Referees

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Relevant Manuscripts Under Revision Following Referees' Reports

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A statement of acknowledgement of joint authors is presented in Appendix A. All co-authors have acknowledged the following statement:

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The authors have certified that:

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2. They take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;

3. There are no other authors of the publication according to these criteria;

4. Potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit, and

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

Signature: _________________________

Date: _________________________
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Chapter 1. Thesis Orientation

1.1 Preface

This thesis presents a series of publications focused on two ocular examination techniques; corneal confocal microscopy (CCM) and non-contact corneal aesthesiometry (NCCA). The publications arose from work performed as a cohesive series of experiments investigating the viability of these techniques as potential markers of diabetic neuropathy. A review of our current understanding of CCM and NCCA as potential markers of neuropathy is presented, as well as cross-sectional studies investigating issues relating to methodology and validation of these tests. The ability of CCM and NCCA to detect change commences with an evaluation of between- and within-observer repeatability. An exploration of a sampling paradigm is also reported. These aspects are important for ongoing investigations of corneal markers of diabetic neuropathy and have particular relevance for longitudinal trials.

Application of new corneal markers for longitudinal evaluation of diabetic neuropathy has the potential to minimise or replace more invasive and time-consuming assessments such as foot-punch biopsy. Two new corneal markers of diabetic neuropathy, corneal sensitivity and corneal nerve parameters, have been explored in this body of work providing information related to accuracy, repeatability and usefulness of novel ophthalmic tests of diabetic neuropathy. These studies may provide the benchmark for researchers on sampling of images, analysis techniques and other clinical application such as screening patients for neuropathy.

The studies presented in this thesis are associated with a five-year longitudinal observational investigation, namely the Longitudinal Assessment of Neuropathy in Diabetes using novel ophthalmic MARKers (LANDMark study). This project is funded by the National Health & Medical Research Council (NHMRC) for a cohort
with type 2 diabetes and the Juvenile Diabetes Research Foundation (JDRF) International for a type 1 cohort. The aims of the studies presented herein are closely linked to those of the LANDMark Study. The aims of the LANDMark study reflect the longitudinal nature of the study and are primarily focused on ability to detect change in corneal morphology and sensitivity over time, and the relationship with changes observed in traditional measures of neuropathy, such as nerve conduction studies and quantitative sensory testing. Other aims include evaluating CCM and NCCA as a means to detect neuropathy earlier than traditional measures. An outline of this thesis is provided below.

1.2 Thesis Outline

A review of the literature constitutes Chapter 2 and is titled “Introduction”. This is essentially a paper published in The Ocular Surface journal entitled “Corneal markers of diabetic neuropathy”. This chapter reflects on structural and functional evidence to date regarding corneal confocal microscopy and non-contact corneal aesthesiometry as potential markers of diabetic neuropathy. The last section of this chapter succinctly illustrates the hypothesis development of the subsequent chapters.

The three succeeding chapters of this thesis (Chapters 3-5) explore several factors of structural aspects of the cornea (corneal nerve assessment using corneal confocal microscopy and quantification of nerve parameters) and Chapter 6 evaluates the presumed functional correlate of corneal nerve parameters, corneal sensitivity, using non-contact corneal aesthesiometry, as a potential marker of diabetic neuropathy. Chapter 3 explores the question of repeatability of corneal measurement methods of interest and is entitled “Repeatability of measuring corneal sub-basal nerve fibre length”. This study explored inter- and intra-observer repeatability in assessing corneal nerve parameters in images of the sub-basal
nerve plexus using semi-automated analysis software, and is published in the Eye and Contact Lens journal. Good repeatability is necessary in measurement studies as it provides the ability to detect real change with some degree of confidence, if change exists. The objective was to determine the Bland-Altman repeatability and intra-class correlation coefficients for two observers measuring, on two occasions, corneal nerve fibre length and corneal nerve branch density.

Chapter 4 explores image sampling techniques in the corneal sub-basal plexus. The accuracy of corneal nerve fibre parameter measurement addresses the sampling of confocal images and is presented as a paper entitled “Optimal image sample size for estimation of corneal nerve morphology using corneal confocal microscopy”, accepted for Optometry and Vision Science. Examining a novel sampling procedure to determine accuracy of two measures of corneal nerve parameters was the aim of this study. Specifically, the objective was to determine the number of images necessary to provide an accurate estimate of corneal nerve fibre length and corneal nerve branch density – two important correlates of severity of diabetic neuropathy.

Chapter 5, encompassing a paper accepted by the Cornea journal, describes a project to develop a rapid, optimised technique of wide-field imaging of the human corneal sub-basal nerve plexus, beyond the central millimetre imaged with standard CCM. This novel technique will facilitate routine use of mapping of the sub-basal nerve plexus in clinical and research contexts.

Chapter 6, entitled “Corneal sensitivity as an ophthalmic marker of diabetic neuropathy”, investigates the diagnostic value of a corneal function test compared to traditional methods. The objective was to determine the discriminative capacity of non-contact aesthesiometry in a cohort of individuals with type 2 diabetes when
compared to a validated measure of neuropathy, the neuropathy disability score. This paper has been published in the Optometry and Vision Science journal.

Concluding remarks are presented in Chapter 7. The four studies presented here have provided necessary information regarding multiple observers analysing CCM images, as well as a novel method of assessing the number of images required for an accurate assessment of nerve fibre parameters as well as a broader area of the corneal sub-basal plexus. The discriminative capacity of non-contact corneal aesthesiometry as a marker for diabetic neuropathy has been determined.
Chapter 2. Introduction

2.1 Preface

This introductory chapter consists of a review of the literature published as a paper in The Ocular Surface journal entitled “Corneal markers of diabetic neuropathy”:


2.2 Abstract

Diabetic neuropathy is a significant clinical problem that currently has no effective therapy, and in advanced cases, leads to foot ulceration and lower limb amputation. The accurate detection, characterisation and quantification of this condition are important in order to define at-risk patients, anticipate deterioration, monitor progression, and assess new therapies. This review evaluates novel corneal methods of assessing diabetic neuropathy. Two new non-invasive corneal markers have emerged, and in cross-sectional studies have demonstrated their ability to stratify the severity of this disease. Corneal confocal microscopy allows quantification of corneal nerve parameters and non-contact corneal aesthesiometry, the functional correlate of corneal structure, assesses the sensitivity of the cornea. Both these techniques are quick to perform, produce little or no discomfort for the patient, and are suitable for clinical settings. Each has advantages and disadvantages over traditional techniques for assessing diabetic neuropathy. Application of these new corneal markers for longitudinal evaluation of diabetic neuropathy has the potential to reduce dependence on more invasive, costly, and time-consuming assessments, such as skin biopsy.
2.3 Introduction

The eye is the only organ in the human body in which nerves can be observed directly and non-invasively. Specifically, a rich nerve plexuses can be imaged at the sub-basal layer of the corneal epithelium using corneal confocal microscopy and in the retina using optical coherence topography. This paper reviews recent research into the utility of assessing the structure and function of the corneal sub-basal nerve plexus as a marker of one the most common and debilitating complications of diabetes – peripheral neuropathy.

The American Diabetes Association report mild to severe forms of nervous system damage in 60-70% of people with diabetes (American Diabetes Association 2007). This condition affects sensory, autonomic, and motor neurons of the peripheral nervous system. In advanced cases, it can lead to foot ulceration and lower limb amputation. In 2004, about 71,000 non-traumatic lower-limb amputations were performed in the US with significant attributable health care costs, (American Diabetes Association 2007) and the vast majority of these would have been due to late complications of diabetic neuropathy. The accurate detection, characterisation and quantification of this condition are important in order to define at-risk patients, anticipate deterioration, monitor progression and assess new therapies (Boulton 2007).

The definition of ‘confirmed diabetic sensorimotor polyneuropathy (DSPN)’ agreed by a recent international consensus group is “the presence of an abnormality of nerve conduction and a symptom or symptoms or a sign or signs of neuropathy” (Tesfaye, Boulton et al. 2010). Common symptoms, usually in the feet or legs, include tingling, numbness, extreme sensitivity to touch, prickling, burning and pain, and these symptoms are usually worse at night.
Diabetic neuropathy may be the result of poor control of blood glucose levels. High levels of glucose in the blood (hyperglycemia) disrupts metabolism of nerves due to reduced blood flow. This then causes accumulation of toxins which damage nerve structure and function (Veves and Malik 2007). Late sequelae of diabetic neuropathy include foot ulceration and ultimately, in some cases, lower extremity amputation. Foot ulceration is a common problem in people with neuropathy, with an annual incidence in excess of 7% compared with an incidence of less than 1% in those without neuropathy (Abbott, Vileikyte et al. 1998). Early detection or prediction of foot ulceration is vital since foot amputations are preceded by foot ulceration in 80% of cases (Abbott, Vileikyte et al. 1998).

Painful diabetic neuropathy affects approximately 30% of all diabetic patients (Shaw and Zimmet 1999; Davies, Brophy et al. 2006) and of those, 80% report the pain to be moderate or severe (Davies, Brophy et al. 2006). It has a significant impact on quality of life and on health care costs (Veves and Malik 2007). Ollendorf and co-workers (Ollendorf, Kotsanos et al. 1998), using a model based upon the incidence and cost of lower extremity amputations in diabetes, predicted potential savings of US $2 to $3 million over three years if the incidence of foot ulceration was reduced through education programs, multidisciplinary clinics and financial support for therapeutic footwear. In their hypothetical model, the economic benefit was between US$ 2 900 to $ 4 442 per person with a history of foot ulcer. Ramsey and co-workers (Ramsey, Newton et al. 1999) estimated the attributable costs for a middle-aged diabetic male patient to be US $28,000 two years after a new foot ulcer.

Risk factors associated with the development of neuropathy in diabetes include increased age, height and body mass index, duration of diabetes, hypertension, smoking, poor glycemic control, and abnormal lipid profile and albumin level (Partanen, Niskanen et al. 1995; Adler, Boyko et al. 1997; Forrest, Maser et al.
1997; Dyck, Davies et al. 1999; van de Poll-Franse, Valk et al. 2002; Tesfaye, Chaturvedi et al. 2005). While providing valuable information relating to factors associated with diabetic neuropathy, these studies shed no light on the pathological changes taking place in small nerve fibres, or the degree to which changes in symptoms and signs correlate with the rate of nerve degeneration.

Recently, two non-invasive corneal markers of diabetic neuropathy have emerged, and in cross-sectional studies have demonstrated their ability to stratify the severity of this disease (Malik, Kallinikos et al. 2003; Quattrini, Kallinikos et al. 2005; Tavakoli, Kallinikos et al. 2007; Tavakoli, Quattrini et al. 2010). Corneal confocal microscopy (CCM) allows quantification of nerve structure in the cornea (Oliveira-Soto and Efron 2001), whilst non-contact corneal aesthesiometry (NCCA) assesses a presumed correlate measure of function - corneal sensitivity (Murphy, Patel et al. 1996). The purpose of this review is to summarise our current understanding of these tests as potential markers of diabetic neuropathy. The application of these corneal tests compared to traditional methods will be described.

2.4 Traditional tests of diabetic neuropathy

Conventional techniques, such as nerve conduction studies and quantitative sensory testing, along with an assessment of neurological disability, offer a relatively robust means of defining neuropathic severity (Boulton, Malik et al. 2004). However, these procedures have potential shortcomings when they are employed to define therapeutic efficacy in clinical intervention trials (Mojaddidi, Quattrini et al. 2005). These shortcomings relate to an inability to target the fibre types demonstrating regeneration and repair.

To assess diabetic neuropathy, the American Diabetes Association recommends one measure from each of the following categories: clinical symptoms, clinical examination, electrodiagnostic studies, quantitative sensory testing (QST) and
autonomic function testing (American Diabetes Association 1988). Although this recommendation reflects current practice, the authors of this report acknowledge the limitations of this method of diagnosis. Tesfaye et al (Tesfaye, Chaturvedi et al. 2005) recommend a slightly less invasive protocol i.e. without the electrophysiological assessment. These traditional methods of assessing diabetic neuropathy are outlined below.

A. Quantitative sensory testing

There are several means of assessing sensory function in a quantitative fashion. These range from simple 5-minute clinical assessments to more complex investigations of sensory perceptions and thresholds (heat, cold and vibration) using sophisticated computer-assisted devices.

A relatively new, validated quantitative measure of neuropathy is the neuropathy disability score (NDS) (Young, Boulton et al. 1993; Abbott, Carrington et al. 2002). The NDS is based upon subjective responses to sharp/blunt stimuli, vibration and temperature as well as the presence or absence of the Achilles tendon reflex to indicate the degree of neuropathy. These responses are scored on a 0-10 scale, where 0 indicates no neuropathy and 10 denotes severe neuropathy.

The CASE IV (WR Medical Electronics Co, MN, USA) and Medoc Quantitative Sensory Analyser (Medoc Advanced Medical Systems, Ramat-Yishai, Israel) are two currently marketed devices for undertaking QST. Both devices operate by applying a stimulus device to the foot and assessing sensory thresholds for heat, cold and vibration. Patients are required to indicate when they first become aware of sensory stimuli and/or when the stimuli become uncomfortable or painful.

Quantitative sensory testing is currently accepted as an endpoint for trials in diabetic neuropathy; however, studies have shown no relationship between QST and unmyelinated fibre pathology (Veves, Malik et al. 1991; Malik, Tesfaye et al. 2005).
B. Electrophysiology

Nerve conduction velocity can measure the degree of damage in larger nerve fibres in a reliable and objective fashion. A probe electrically stimulates a nerve fibre, and an electrode measures the speed of impulse transmission along the axon. Slow transmission rates and impulse blockage purportedly indicate damage to the myelin sheath, while a reduction in the strength of impulses is a sign of axonal degeneration. The disadvantages of this test are that it is uncomfortable for the patient and must be carried out by a trained individual and performed in triplicate to reliably assess neuropathy (Skljarevski and Malik 2007).

Whilst nerve conduction studies and QST are useful and well validated measures of progression for established diabetic neuropathy, their utility in early small fibre neuropathy is limited because they primarily measure larger myelinated nerve fibre function (Boulton, Malik et al. 2004). In an interventional study, there was no relationship between the improvement of peroneal motor nerve conduction velocity and the myelinated fibre density and regenerative activity was confined almost exclusively to the small myelinated fibres, which are not assessed by conventional electrophysiology (Greene, Arezzo et al. 1999).

C. Nerve and skin biopsy

A direct examination of thinly myelinated and unmyelinated nerve fibre damage and repair is possible using sural nerve biopsy with electron microscopy (Malik, Veves et al. 2001; Malik, Tesfaye et al. 2005) and the newly refined skin-punch biopsy (Smith, Howard et al. 2005). A 3 mm skin punch is removed from the dorsum of the foot under local anaesthesia. The biopsy is cryoprotected, fixed and processed to reveal the intra-epidermal nerve fibre morphology. The number of nerves per dermal-epidermal junction is recorded (in units of number of nerves per millimetre). Small fibre abnormalities can be assessed by intra-epidermal nerve fibre density, and
longitudinal changes have been observed in patients after lifestyle intervention (Smith, Russell et al. 2006). Diabetic patients with minimal neuropathy (normal electrophysiology and quantitative sensory tests) can still show significant unmyelinated fibre degeneration (Malik, Tesfaye et al. 2005). The disadvantage of these biopsy techniques is that both sural nerve and skin-punch procedures are invasive. Furthermore, if employed to assess therapeutic efficacy, repeat assessments are required, which have to be at a different site from the original biopsy.

D. Magnetic resonance imaging

Magnetic resonance imaging (MRI) can assist in quantification of nerve structure and function. A strong magnetic field is used to produce a three-dimensional picture or a two-dimensional "slice" of the scanned area. MRI has been used to show a significant reduction in cross-sectional area of the spine in early diabetic neuropathy in the cervical and thoracic regions compared to healthy controls (Selvarajah, Wilkinson et al. 2006). However, this technique is expensive and not suitable for routine clinical use and the method has not been evaluated in prospective studies.

E. Monofilament test

The 10-gram nylon monofilament test is a rapid, reproducible and inexpensive method for testing diabetic neuropathy and is widely used as a predictor of ulceration risk of the foot (Coppini, Young et al. 1998). The test is abnormal if the patient cannot sense the touch of the monofilament when it is pressed against the foot with just enough pressure to bend the filament. However, this test has been shown to be less sensitive than NDS for predicting foot ulcers (Miranda-Palma, Sosenko et al. 2005).
F. Neuropad™

The commercially available Neuropad™ (Trigocare International GmbH, Wiehl, Germany) has recently been proposed as a simple test to diagnose peripheral neuropathy. An adhesive pad containing cobalt salts is attached to the plantar aspect of the foot and changes colour from blue to pink within 10 minutes if cholinergic sympathetic function is normal. An abnormal Neuropad response (blue or patchy) is associated with sympathetic dysfunction and clinical neuropathy.

The Neuropad test has been correlated with clinical assessments of neuropathy by Quattrini and co-workers who demonstrated that Neuropad results significantly correlated with NDS, Neuropathy Symptom Score (NSS), QST, deep-breathing heart rate variability (see below) and intra-epidermal nerve fibre density determined by skin-punch biopsy (Quattrini, Jeziorska et al. 2008). In the same study, the sensitivity of an abnormal Neuropad response in detecting clinical neuropathy (NDS ≥ 5) was shown to be 85% (negative predictive value 71%) and the specificity was 45% (positive predictive value 69%). These findings suggest that Neuropad can be used to assess nerve function; however, its use in assessing longitudinal changes in patients with diabetes is unknown.

G. Heart rate variability

Autonomic neuropathy, a form of peripheral neuropathy, can be assessed using tests of heart rate variability, i.e. the beat-to-beat alterations in heart rate, during deep breathing in people with diabetes (Risk, Bril et al. 2001). With the patient lying down, resting heart rate is measured after a short rest period of 5-10 minutes. Patients are then asked to breathe in deeply for 5 seconds and then breathe out deeply for 5 seconds for 8 consecutive respiratory cycles while cardiac electrical activity is recorded using an electrocardiogram. The expiration/inspiration (E/I) ratio is one variable that can be calculated as an indicator of autonomic neuropathy. This
ratio is the average of the quotient between the longest R-R intervals (time duration between two consecutive R waves of the electrocardiogram) during expiration and shortest R-R intervals during inspiration. Values equal to or higher than one are typically accepted as normal (Agelink, Malessa et al. 2001).

### 2.5 Corneal Innervation

The human cornea is richly innervated primarily by sensory nerve fibres originating from the ophthalmic division of the trigeminal nerve. Sympathetic innervations, arising from the superior cervical ganglion, however are scare (Toivanen, Tervo et al. 1987). It is unclear if the human cornea receives parasympathetic innervations (Muller, Marfurt et al. 2003). Nerve bundles enter the cornea in the limbal region in a radial fashion and travel parallel to the corneal surface. Bundles of nerves enter the peripheral cornea in a radial fashion and lose their myelin sheath approximately 1 mm from the corneal limbus (this is essential in order to maintain corneal transparency) and subdivide into smaller branches. The nerve branches travel from the periphery to the centre below the anterior third of the stroma to accommodate the lamella nature of the stromal collagen (Muller et al 2001), and divide into several smaller branches. The nerves in the stromal layers take a 90 degree turn, proceed toward the corneal surface and penetrate Bowman’s membrane and continue parallel to the corneal surface between Bowman’s membrane and the basal epithelial cell layer (Muller, Pels et al. 1996). The penetration points in Bowman’s membrane are primarily in the periphery, but do occur in the centre but to a lesser degree (Muller, Pels et al. 1996, Marfurt et al 2010). Epithelial leashes, a mixture of straight and beaded nerve fibres, extend between the basal cells. The diameter of individual nerve fibres in the sub-basal plexus varies between 0·05 and 2·5 μm and most are in the range of 0·1–0·5 μm, consistent with A-delta and C fibres (Muller, Pels et al. 1996). In vitro evaluation of human cadaver eyes has greatly expanded our understanding of the corneal sub-basal nerve complex (Muller, Pels et al. 1996;
Muller, Vrensen et al. 1997; Al-Aqaba, Alomar et al. 2010; Al-Aqaba, Fares et al. 2010; He, Bazan et al. 2010; Marfurt, Cox et al. 2010).

A. In-vitro assessment of corneal nerve structure

Muller and co-workers (Muller, Pels et al. 1996; Muller, Vrensen et al. 1997) have extensively described the architecture of human corneal nerves using fresh donor corneas with transmission electron microscopy and light microscopy. Density of the corneal nerves was found to be similar in the central and para-central region of the cornea; however, density is reduced in the periphery (Muller, Vrensen et al. 1997; He, Bazan et al. 2010). Muller and co-workers determined the majority of fibres were C fibres and ranged from 0.4 to 0.7 \( \mu \)m in diameter (Muller, Vrensen et al. 1997). Marfurt et al (Marfurt, Cox et al. 2010) used a novel immunohistochemical whole-mount process to stain and examine donor corneas. They estimated, on average, that over 200 stromal nerves penetrate Bowman’s membrane to supply the central 10 mm of corneal epithelium, and they reported a mean nerve fibre density of 46 ± 5 mm/mm².

Using a three-dimensional mapping technique, He and co-workers (He, Bazan et al. 2010) also reported that nerves penetrated Bowman’s membrane primarily in the periphery. Furthermore, they reported that there was no difference in nerve density between males and females, and that a reduction of nerve fibre density was associated with ageing. A limitation of studying corneal morphology ex vivo is that nerve fibres degenerate or disappear after 13.5 hours post-mortem (Muller, Vrensen et al. 1997), although it appears a greater network of nerve fibres is appreciated when examined using fluorescent immunohistological techniques (He, Bazan et al. 2010). Several researchers have noted the whorl-like assembly of the nerves in the sub-basal nerve plexus both histologically (Al-Aqaba, Fares et al. 2010; He, Bazan et al. 2010; Marfurt, Cox et al. 2010), and using in vivo corneal confocal microscopy.
(Patel and McGhee 2005), as shown in Figure 2-1. The whorl-like assembly of nerves appears to converge more frequently in the clockwise direction (Patel and McGhee 2005; He, Bazan et al. 2010), however, counter-clockwise convergence has been noted (He, Bazan et al. 2010).

![Figure 2-1. Whorl-like assembly of the sub-basal nerve plexus in the inferior cornea, in this case converging in a clockwise pattern. (Image: courtesy of Dr Katie Edwards).](image)

**B. In vivo assessment of corneal nerve structure**

Quantification of nerve parameters of the central cornea in healthy individuals using *in vivo* corneal confocal microscopy (CCM) has been performed successfully by many researchers over the past 10 years using several types of microscopes, primarily tandem, slit-scanning and laser-scanning devices. *In vivo* corneal confocal microscopy has advanced our understanding of corneal nerve ultrastructure. The sub-basal nerve fibre bundles, surrounded by a Schwann cell sheath (Muller, Pels et al. 1996), are easily resolved (Figure 2-2), however epithelial nerves can occasionally be observed. Thick, stromal nerves are easily observed (Zhivov, Stachs et al. 2006).
The principle of the confocal microscope is that a single point of tissue is illuminated and simultaneously imaged by a camera in the same plane. This produces an image with a very high resolution, but a very narrow field of view. The narrow field of view is overcome as the microscope creates a useable field of view by instantaneously illuminating a small region of the cornea with thousands of tiny spots of light each second, with each spot of light being synchronously imaged. The spot images are reconstructed to create a usable field of view offering high resolution and magnification. A similar result can be achieved using a scanning slit beam of light (Efron 2007). Because the cornea is transparent, white light, or more recently laser light, can be used to image tissue in vivo at the cellular level and at a high resolution.

Tandem-scanning confocal microscopes (TSCM) and, more commonly, slit-scanning confocal imaging microscopes (SSCM) have been used extensively to examine the cornea in vivo. The fact that corneal nerves could only be viewed in 81% of healthy individuals using TSCM makes it essentially unsuitable to quantify corneal nerve parameters (Patel, McLaren et al. 2002). The most recent device, however, is a laser scanning corneal confocal microscope (LSCM), the Heidelberg Retina Tomograph 3 with Rostock Corneal Module (HRT3) (Heidelberg, Germany). The primary advantage of laser scanning confocal microscopy is the ability to produce very high contrast images of thin layers from the cornea and conjunctiva.

Examination of a corneal nerve structure using the HRT3 involves using a drop of topical anaesthetic (benoxinate hydrochloride 0.4%) in the eye to be examined. The patient is instructed to fixate a target with the eye that is not being examined. The objective lens of the laser microscope is housed within a sterile disposable Perspex cap. A drop of visco-elastic gel is placed on the tip of the objective lens before the cap is mounted on top. The gel optically couples the objective lens to the Perspex cap. The surface of the sterile Perspex cap is brought gently into contact with the
cornea; this procedure is facilitated by a side-mounted CCD camera which displays a magnified, real-time image of the cap contacting the cornea.

Images are obtained using one of three possible examination modes. Section mode enables manual acquisition and storage of one or more single images. The cornea is scanned manually in x, y and z axes and image capture is effected with the aid of a foot pedal. Volume scan mode allows automatic acquisition of up to 40 images, approximately 2 μm apart, in the z-axis. Thus, a section of cornea 80 μm in depth can be scanned in this way (using the 400 μm field lens). Figure 2-2 shows images representative of the corneal layers. Sequence scan mode allows acquisition of up to 100 images at capture rates from 1-30 frames per second.

Figure 2-2. Six representative layers of the cornea capable of being imaged by the volume scan function of the HRT3 with Cornea Rostock Module. The approximate depth is shown in the figure. (Image: Nicola Pritchard).

A variety of corneal nerve parameters have been reported by researchers and a wide range of parameter outcomes have been noted (Oliveira-Soto and Efron 2001; Grupcheva, Wong et al. 2002; Patel and McGhee 2005; Midena, Cortese et al. 2009). Table 2-1 summarises the findings of various studies for seven different
nerve parameters. Corneal nerve fibre density has been reported in two ways – count of nerves per unit area and total length of nerve material per unit area.
**Table 2-1.** Corneal nerve fibre parameters assessed with use of corneal confocal microscopy in healthy individuals. Error values are ± standard deviation unless otherwise indicated.

<table>
<thead>
<tr>
<th>Author/Year</th>
<th>N eyes (special characteristics)</th>
<th>Type of confocal microscope</th>
<th>Density (count) (nerves/mm²)</th>
<th>Length (mm/mm²)</th>
<th>Branching (branches/mm²)</th>
<th>Beading (beads/mm)</th>
<th>Width (μm)</th>
<th>Tortuosity (0-4)</th>
<th>Reflectivity (0-4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rosenberg et al, 2000</td>
<td>9</td>
<td>TSCM</td>
<td>30 ± 7</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Oliveira-Soto and Efron, 2001</td>
<td>14</td>
<td>SSCM</td>
<td>117 ± 62</td>
<td>11.1 ± 4.2</td>
<td>-</td>
<td>222 ± 43</td>
<td>2.9 ± 0.2</td>
<td>1.2 ± 0.4</td>
<td>1.1 ± 0.5</td>
</tr>
<tr>
<td>Grupcheva et al, 2002</td>
<td>25 (aged 25±5) 25 (aged 70±5)</td>
<td>SSCM</td>
<td>-</td>
<td>0.6 ± 0.3</td>
<td>-</td>
<td>213 ± 123</td>
<td>0.5 ± 0.2</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Patell et al, 2002</td>
<td>20</td>
<td>TSCM</td>
<td>32 ± 10</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Malik et al, 2003</td>
<td>18</td>
<td>SSCM</td>
<td>45 ± 14</td>
<td>13.5 ± 0.3</td>
<td>79 ± 30</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Benitez del Castillo et al 2004</td>
<td>11 (&lt;60 years) 10 (≥ 60 years)</td>
<td>SSCM</td>
<td>62 ± 11 42 ± 12</td>
<td>10.3 ± 1.2</td>
<td>8.4 ± 1.2</td>
<td>-</td>
<td>1980 ± 650 1820 ± 630</td>
<td>2.1 ± 0.4</td>
<td>1.1 ± 0.5 1.5 ± 0.5</td>
</tr>
<tr>
<td>Kalilnikos et al, 2004</td>
<td>18</td>
<td>SSCM</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>25.9 ± 9.9*</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Erie et al, 2005</td>
<td>65</td>
<td>TSCM</td>
<td>28 ± 10</td>
<td>8.4 ± 2.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Patel et al and McGhee, 2005</td>
<td>3 (central) 3 (whorl)</td>
<td>LSCM</td>
<td>-</td>
<td>21.7 ± 1.4</td>
<td>25.2 ± 0.6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mocan et al, 2006</td>
<td>24</td>
<td>SSCM</td>
<td>34 ± 6</td>
<td>-</td>
<td>59 ± 12</td>
<td>3.1 ± 0.4</td>
<td>1.7 ± 0.6</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Benitez del Castillo et al, 2007</td>
<td>10 (&lt;60 years) 10 (≥ 60 years)</td>
<td>SSCM</td>
<td>62 ± 11 43 ± 12</td>
<td>10.6 ± 1.4</td>
<td>8.3 ± 1.2</td>
<td>-</td>
<td>1920 ± 610 1970 ± 500</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Li et al, 2007</td>
<td>26</td>
<td>SSCM</td>
<td>-</td>
<td>17.6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mehr et al, 2007</td>
<td>15</td>
<td>SSCM</td>
<td>42 ± 3</td>
<td>9.7 ± 0.7</td>
<td>27 ± 3</td>
<td>-</td>
<td>-</td>
<td>19.6±1.3*</td>
<td>-</td>
</tr>
<tr>
<td>Niederer et al, 2007</td>
<td>85</td>
<td>LSCM</td>
<td>-</td>
<td>20.3 ± 6.5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Quattrini et al, 2007</td>
<td>15</td>
<td>SSCM</td>
<td>43 ± 5 (se)</td>
<td>6.1 ± 1.2</td>
<td>27 ± 3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Erie et al, 2008</td>
<td>18 18</td>
<td>TSCM  SSCM</td>
<td>-</td>
<td>5.5 ± 1.9 10.7± 5.6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Niederer et al, 2008</td>
<td>52</td>
<td>LSCM</td>
<td>-</td>
<td>22.4 ± 6.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Patel et al, 2009</td>
<td>31</td>
<td>LSCM</td>
<td>-</td>
<td>25.9 ± 7.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>De Cilla et al, 2009</td>
<td>50</td>
<td>LSCM</td>
<td>18 ± 5</td>
<td>-</td>
<td>-</td>
<td>2.0 ± 0.8</td>
<td>2.6 ± 0.9</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Patel et al, 2009</td>
<td>20 (aged 26±3) 20 (aged 43±5) 20 (aged 61±7)</td>
<td>LSCM</td>
<td>-</td>
<td>10.6 ± 6.8 10.1 ± 6.8 10.6 ± 6.6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>20.8± 9.5* 23.2± 13.7* 25.7± 14.0*</td>
<td>-</td>
</tr>
<tr>
<td>Tavakoli et al, 2010</td>
<td>17</td>
<td>SSCM</td>
<td>46 ± 4</td>
<td>11.2 ± 0.9</td>
<td>25 ± 3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

se – standard error; TSCM – tandem scanning confocal microscope; SSCM – slit scanning confocal microscope; LSCM – laser scanning confocal microscope; *tortuosity coefficient
When ‘major nerve branches’ were counted in images from a SSCM (Figure 2-3 left), nerve fibre density in healthy individuals ranged from 13 nerves/mm$^2$ (Quattrini, Tavakoli et al. 2007) to 46 nerves/mm$^2$ (Tavakoli, Quattrini et al. 2010). The subjective nature of determining what constitutes a ‘major nerve’ may explain this range of outcomes. Corneal nerve fibre length, calculated from images captured using SSCM, ranged from 0.6 mm/mm$^2$ (Grupcheva, Wong et al. 2002) to close to 14 mm/mm$^2$ (Malik, Kallinikos et al. 2003). The low values of nerve fibre length obtained by Grupcheva et al (Grupcheva, Wong et al. 2002) can be attributed to the inclusion of only bright superficial nerves (Figure 2-3 left). The LSCM generates clearer, brighter images of nerves hence one would expect a greater density of nerve material reported per unit area when using this instrument (Figure 2-3 right). Indeed, nerve fibre length per unit area has been consistently reported at 20-22 mm/mm$^2$ using LSCM (Patel and McGhee 2005; Niederer, Perumal et al. 2007). Figure 2-3 shows the difference in image contrast and measurement outcomes.

Figure 2-3. A SSCM 400 µm x 300 µm image (left) and a LSCM 400 µm x 400 µm image (right). The corneal nerve fibre length for the SSCM image (IDCaseWest137BL: courtesy Dr Scott Howell) was 7.5 mm/mm$^2$ and for the LSCM image (ID32LG-3: Nicola Pritchard) was 24.5 mm/mm$^2$. The corneal nerve branch
density for the SSCM image was 33.3 branches/mm² and for the LSCM image, 62.5 branches/mm².

Authors differ in their sampling and quantification processes; for example, some authors use just one or two images per individual to calculate nerve parameters (Oliveira-Soto and Efron 2001; De Cilla, Ranno et al. 2009; Patel, Tavakoli et al. 2009), while others use between three and five ‘high-quality’ images per individual to quantify nerves (Kallinikos, Berhanu et al. 2004; Mehra, Tavakoli et al. 2007; Niederer, Perumal et al. 2007). Other parameters such as corneal nerve fibre branching, beading, width, tortuosity and reflectivity have also been reported (Oliveira-Soto and Efron 2001; Grupcheva, Wong et al. 2002; Malik, Kallinikos et al. 2003; Benítez del Castillo, Wasfy et al. 2004; Kallinikos, Berhanu et al. 2004; Benítez del Castillo, Acosta et al. 2007; Mehra, Tavakoli et al. 2007; Quattrini, Tavakoli et al. 2007; Patel, Tavakoli et al. 2009; Tavakoli, Quattrini et al. 2010).

Appreciation of a large, montaged proportion of the human sub-basal nerve plexus, using *in vivo* corneal confocal microscopy, has also been described (Patel and McGhee 2005; Efron 2007), and is shown in Figure 2-4 A. Patel and McGhee (2005) were the first to describe the use of CCM to capture a large number of images across the cornea, which are then stitched or mapped together to form a montage. This allows the overall pattern of the sub-basal nerve plexus to be appreciated. Using this technique the authors also demonstrated that the sub-basal nerve plexus radiates towards a whorl-like complex centred one to two millimetres below the corneal apex (Figure 2-4 B and Figure 2-4 C). Clockwise (Patel and McGhee 2005; He, Bazan et al. 2010) and counter-clockwise (He, Bazan et al. 2010) convergence of nerves has been noted in this region (Figure 2-1). Developing this technique for quantitative analysis of the nerve plexus will enhance our understanding of neuropathic nerve changes in diabetes and other neuropathic disorders.
Figure 2-4. A. Montage of about 100 images obtained with laser scanning CCM depicting the architecture of the sub-basal nerve (scale bar 400 μm) (Efron 2007) constructed using the technique of Patel and McGee (Patel and McGhee 2005). B. Blended montage of approximately 1300 images produced using an evolved technique of corneal nerve mapping developed in our laboratory (see text). C. Nerve tracing of the same image as shown in B. (Images: courtesy Nicola Pritchard and Dr Katie Edwards).
The technique devised by Patel and McGhee is tedious, requiring long sessions of image capture and many hours to form a montage of the image. We have developed a novel, faster (several hours compared to several weeks) and semi-automated technique for mapping the corneal sub-basal nerve plexus using the video capture facility of the LSCM, described in Chapter 5. This is achieved by having the participant track a moving target on a large computer screen with the contralateral eye. This procedure, which takes about 20 seconds and results in the capture of 100 contiguous images, is repeated along 13 radial meridians. The second stage of montaging is performed with Image-Pro Plus 7 (MediaCybernetics, Bethesda MD, USA) to align and blend the radial image strips together. A complete map is displayed in Figure 2-4 B and the corresponding nerve tracing in Figure 2-4 C.

2.6 Assessment of corneal sensitivity

A. Assessment of corneal nerve function

Quantification of corneal nerve function by means of measuring corneal sensitivity has also contributed to our understanding of corneal innervation. Ocular surface sensations arise from nerves derived from the anterior ciliary branches of the ophthalmic division of the trigeminal nerve (Bron, Tripathi et al. 1997). Using CCM, corneal nerve density has been measured in the centre of the cornea, and on average is approximately 45 nerve bundles per 350 µm² (Table 2-1). If the human cornea is 90 mm² (and equal regional distribution is assumed) approx 12 000 nerve bundles are present in the human sub-basal plexus; Muller et al (2003) (Muller, Marfurt et al. 2003) estimate approx 7000 nociceptors per mm². Using the in vivo corneal mapping technique, Patel and McGee have demonstrated some differences may exist in corneal fibre density in different regions of the cornea (Patel & McGee 2005).
Murphy and co-workers (Murphy, Patel et al. 1996; Murphy, Lawrenson et al. 1998; Murphy, Patel et al. 2004) have described how corneal sensitivity can be measured using a novel non-invasive method, the non-contact corneal aesthesiometer (NCCA). This instrument uses controlled pulses of air of varying pressures to stimulate the cornea and it measures the corneal sensitivity threshold to a composite stimulus consisting of air pressure, and tear film evaporation and disruption (Murphy, Patel et al. 1996; Murphy, Patel et al. 2001).

There are two frequently used methods for determining an absolute threshold to a sensory stimulus, the method of limits and the method of constant stimuli (Ehrenstein and Ehrenstein 1999). The method of limits involves presentation of stimuli below threshold then ascending to the cross-over point (i.e. when the sensation is first felt), then repeating the procedure in a descending fashion. Both ascending and descending procedures are alternated several times and the average cross-over point reveals the threshold. The method of constant stimuli involved presenting a pre-determined range of (usually 5-9) stimuli which encompasses the threshold (determined by e.g. the method of adjustment where the stimulus is adjusted to be just felt). The stimuli are presented in a quasi-random order equally often, usually not less than 20 times. The proportion of ‘detected” and “not detected” are plotted against the stimulus intensity (the psychometric function); the stimulus intensity corresponding to 50% perceived responses is the absolute threshold. A staircase procedure, based on the method of limits, starts with a descending series in one unit steps of stimuli until the observer cannot detect the stimulus; the stimulus is increased by one step until it is detected, then decreased and increased around the threshold value, usually 6-9 times. The threshold is the average of all the intensities where the observer’s response changed. A García-Pérez staircase is a forced-choice technique, usually of at least 30 reversals, with a larger step-up than step-down (and the step up is between 2/3 and 3/3 of the spread of the
psychometric function) (García-Pérez 2000). A modified approach to the Garcia-Perez staircase has been applied to measuring corneal sensitivity with the NCCA (Golebiowski, Papas et al. 2005; Pritchard, Edwards et al. 2010); the staircase begins above the corneal sensitivity threshold, a step-up of 0.2 mbars and a step-down of 0.15 mbars is applied to 4 reversals. The average of the last two reversals is recorded as the corneal sensitivity threshold.

Corneal sensation is mediated primarily by the Aδ fibres (mechanosensory) and C fibres (thermo- and chemosensory) (Muller, Pels et al. 1996). The advantage of NCCA over the traditional von Frey hair or Cochet-Bonnet aesthesiometer (using a fine nylon filament) is that a large, continuous range of stimulus intensities can be produced. In addition, the stimulus is more precise, testing is less variable than when using a filament, and there is minimal patient apprehension (Murphy, Patel et al. 1996). Carbon dioxide (CO₂) aesthesiometers are used to stimulate specific corneal receptors by continuous flow of CO₂ (or air), such that the sensitivity is measured in flow per unit time (e.g. mL/min) (Belmonte, Acosta et al. 1999). Both the NCCA and CO₂ aesthesiometers can assess the corneal sensation threshold in an accurate and repeatable manner (Murphy, Lawrenson et al. 1998; Belmonte, Acosta et al. 1999) and the NCCA has been demonstrated to be more able to measure lower stimulus thresholds than Cochet-Bonnet aesthesiometry. (Murphy, Lawrenson et al. 1998)

Human corneal sensitivity using pneumatic non-contact aesthesiometry varies between 0.35 millibars to 1.53 millibars in healthy individuals (Murphy, Patel et al. 1996; Murphy, Patel et al. 2004; Patel, Ku et al. 2008; Patel, Tavakoli et al. 2009; Tavakoli, Quattrini et al. 2010), as shown in Table 2-2. Corneal sensation thresholds are age-dependent (Murphy, Patel et al. 2004; Patel, Tavakoli et al. 2009). It appears that corneal sensitivity measured with this instrument decreases on average 0.1 millibar per year of increased age when the results of these researchers
are extrapolated. Corneal sensitivity is influenced by the psychophysical procedure used to determine the threshold. A forced-choice double staircase technique provides the most accurate measure of corneal sensitivity (Murphy, Patel et al. 1996), and more recently a modification of this technique, the Gomez-Perez staircase, has been proven to be reliable in CO₂ corneal aesthesiometers (Golebiowski, Papas et al. 2005; Situ, Simpson et al. 2007).

Table 2-2. Corneal sensitivity of healthy individuals measured using the pneumatic non-contact corneal aesthesiometer. Error values are ± standard deviation unless otherwise indicated.

<table>
<thead>
<tr>
<th>Author</th>
<th>n</th>
<th>Corneal sensitivity (mbars)</th>
<th>Participant age (years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Murphy et al, 1996</td>
<td>14</td>
<td>0.45 ± 0.20</td>
<td>29 (range 21-38)</td>
</tr>
<tr>
<td></td>
<td>69</td>
<td>0.58 ± 0.25</td>
<td>23 (range 19-29)</td>
</tr>
<tr>
<td>Murphy et al, 2004</td>
<td>30</td>
<td>0.81 ± 0.56</td>
<td>42 (range 30-59)</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>1.53 ± 0.80</td>
<td>69 (range 60-80)</td>
</tr>
<tr>
<td>Mehra et al, 2007</td>
<td>15</td>
<td>0.77 ± 0.02</td>
<td>46 ± 3</td>
</tr>
<tr>
<td>Tavakoli et al, 2007</td>
<td>18</td>
<td>0.73 ± 0.14</td>
<td>56 ± 17</td>
</tr>
<tr>
<td>Patel et al, 2009</td>
<td>31</td>
<td>0.35 ± 0.11</td>
<td>35 ± 12</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>0.44 ± 0.21</td>
<td>26 ±3 (range 20-34)</td>
</tr>
<tr>
<td>Patel et al, 2009</td>
<td>20</td>
<td>0.39 ± 0.18</td>
<td>43 ± 5 (range 35-50)</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>0.50 ± 0.29</td>
<td>61 ± 7 (range 51-65)</td>
</tr>
<tr>
<td>Tavakoli et al, 2010</td>
<td>17</td>
<td>0.72 ± 0.36 (se)</td>
<td>55 ± 5</td>
</tr>
<tr>
<td>Pritchard et al (unpublished)</td>
<td>65</td>
<td>0.60 ± 0.31</td>
<td>52 ±15 (range 15-75)</td>
</tr>
</tbody>
</table>

se – standard error  mbars – millibars
2.7 Corneal dysfunction in diabetes

In diabetes, deficiencies have been noted in all layers of the cornea. Individuals with diabetes have increased corneal thickness (Busted, Olsen et al. 1981; Schultz, Peters et al. 1983), and alterations to cellular morphology and the basement membrane (Taylor and Kimsey 1981). These features invariably affect other parameters such as reducing corneal sensitivity (Nielsen and Lund 1979; Rosenberg, Tervo et al. 2000; Dogru, Katakami et al. 2001; Murphy, Patel et al. 2004; Nuho, Subekti et al. 2004; Creuzot-Garcher, Lafontaine et al. 2005), and tear secretion (Seifart and Strempel 1994), increasing epithelial fragility (abnormal adhesion) (O’Leary and Millodot 1981), reducing cell density (Quadrado, Popper et al. 2006), as well as leading to abnormal hydration control (Herse and Hooker 1994) and an increased risk of corneal infection (Schein, Glynn et al. 1989).

Parameters of corneal nerve fibre mass, assessed using corneal confocal microscopy, are reduced or altered in diabetes (Rosenberg, Tervo et al. 2000; Morishige, Chikama et al. 2001; Malik, Kallinikos et al. 2003; Kallinikos, Berhanu et al. 2004; Hossain, Sachdev et al. 2005; Quattrini, Kallinikos et al. 2005; Chang, Carrel et al. 2006; Li, Wang et al. 2006; Mocan, Durukan et al. 2006; Quadrado, Popper et al. 2006; Tavakoli, Quattrini et al. 2006; Mehra, Tavakoli et al. 2007; Quattrini, Tavakoli et al. 2007; Tavakoli, Quattrini et al. 2010), shown in Table 2-3 and Figure 2-5.
Figure 2-5. Corneal nerve fibre length in controls and individuals with varying degrees of diabetic neuropathy (after Tavakoli et al. (Tavakoli, Quattrini et al. 2010). Error bars represent standard error of the mean.

Compared to individuals without diabetes, corneal nerve fibre quantity and branching are reduced (Rosenberg, Tervo et al. 2000; Malik, Kallinikos et al. 2003; Hossain, Sachdev et al. 2005; Quattrini, Kallinikos et al. 2005; Chang, Carrel et al. 2006; Li, Wang et al. 2006; Mocan, Durukan et al. 2006; Quadrado, Popper et al. 2006; Tavakoli, Quattrini et al. 2006; Mehra, Tavakoli et al. 2007; Quattrini, Tavakoli et al. 2007; Tavakoli, Quattrini et al. 2010) and tortuosity is increased (Kallinikos, Berhanu et al. 2004; Quattrini, Kallinikos et al. 2005; Mocan, Durukan et al. 2006; Tavakoli, Quattrini et al. 2006; De Cilla, Ranno et al. 2009; Tavakoli, Quattrini et al. 2010). Reduced reflectivity of nerves (De Cilla, Ranno et al. 2009) may be associated with alterations in light scatter at the level of Bowman’s membrane in individuals with diabetes (Morishige, Chikama et al. 2001; De Cilla, Ranno et al.
Table 2-3 shows a comparison of corneal nerve fibre parameters in individuals with and without diabetes.

The first report of CCM being successfully applied for patients with various degrees of diabetic neuropathy was by Rosenberg and co-workers (Rosenberg, Tervo et al. 2000) in 2000. Malik and co-workers (Malik, Kallinikos et al. 2003), and subsequently Hossain and co-workers (Hossain, Sachdev et al. 2005) validated this approach by demonstrating a significant inverse relationship between two corneal anatomical measures (corneal nerve fibre density and length), and neuropathic severity in diabetic patients.

Kallinikos et al (Kallinikos, Berhanu et al. 2004) and Midena et al (Midena, Cortese et al. 2009) both showed an increase in corneal nerve fibre tortuosity in diabetic patients compared to control individuals. These findings, especially in relation to nerve fibre density, have been confirmed by subsequent workers (Chang, Carrel et al. 2006; Li, Wang et al. 2006; Mocan, Durukan et al. 2006). Significant nerve pathology has been observed in diabetic patients without neuropathy (Quattrini, Tavakoli et al. 2007) which suggests CCM has a role in the early detection of this debilitating condition.

Reduced corneal sensitivity in diabetes has been well documented over the past 40 years (Schwartz 1974; Nielsen and Lund 1979) using crude measures such as the nylon filament style of aesthesiometer. Using more sensitive, non-invasive techniques, a loss of corneal sensitivity in diabetic patients has been confirmed by Murphy et al (Murphy, Patel et al. 2004). Corneal sensitivity measured with NCCA can be reduced by up to 30% in people with diabetes (Table 2-3).

Corneal functional loss is also associated with small fibre neuropathy in diabetes (Nielsen and Lund 1979; Cousen, Cackett et al. 2007; Mehra, Tavakoli et al. 2007; Quattrini, Tavakoli et al. 2007; Tavakoli, Kallinikos et al. 2007; Tavakoli, Quattrini et
al. 2010). Creuzot-Garcher et al (Creuzot-Garcher, Lafontaine et al. 2005) and Nuho et al (Nuho, Subekti et al. 2004) have demonstrated that the loss of corneal sensitivity in diabetic patients is more significant in those with neuropathy. This work was extended by Tavakoli et al, (Tavakoli, Kallinikos et al. 2007; Tavakoli, Quattrini et al. 2010) who demonstrated that corneal sensitivity, measured using NCCA, was significantly reduced in diabetic patients with varying degrees of neuropathy, compared to controls (Figure 2-6).

![Figure 2-6. Corneal sensitivity threshold measured using NCCA in controls and individuals with varying degrees of diabetic neuropathy (after Tavakoli et al (Tavakoli, Quattrini et al. 2010). Error bars represent standard error of the mean.](image)

2.8 Corneal tests as surrogate markers for neuropathy

Markers for a disease are ideally rapid to conduct, non-invasive, sensitive, specific for the disease, reproducible, affordable and easy to use in a clinical setting. Corneal nerve parameters measured using CCM have been reported as potential
surrogate markers for neuropathic damage and repair in diabetes (Malik, Kallinikos et al. 2003; Kallinikos, Berhanu et al. 2004; Tavakoli, Kallinikos et al. 2007; Lalive, Truffert et al. 2009). Corneal sensitivity, measured using NCCA, is also being investigated as a corneal marker of diabetic neuropathy (Quattrini, Tavakoli et al. 2007; Tavakoli, Kallinikos et al. 2007; Pritchard, Edwards et al. 2010). Figure 2-5 and Figure 2-6 show the mean corneal nerve fibre length and corneal sensation threshold respectively, as a function of severity of neuropathy.

CCM is non-invasive, although requiring the installation of a local anaesthetic, and can be performed in a few minutes. Direct observation of nerve pathology, until the arrival of *in vivo* CCM, required sectioning and staining of excised skin, nerve or corneal tissue. However, CCM has been demonstrated as superior to skin punch biopsy for quantifying nerve damage due to diabetes (Tavakoli, Quattrini et al. 2006). Using SSCM, sensitivity and specificity of the CCM for assessing neuropathy by corneal nerve fibre density (nerves per unit area) is 0.82 and 0.52, respectively (Tavakoli, Quattrini et al. 2010), as shown in Table 2-4. For a higher risk group, i.e. those at risk of foot ulceration, the sensitivity of nerve fibre density was 0.71 with a specificity of 0.64. Improved resolution of the corneal nerve plexus is achieved with LSCM compared to other available instruments, hence discriminative capacity of this instrument may be different to that reported using SSCM technology.
Table 2-3. Corneal nerve fibre length and corneal sensation thresholds of healthy individuals and patients with diabetes†.

<table>
<thead>
<tr>
<th>Author</th>
<th>Corneal nerve fibre length (mm/mm²)</th>
<th></th>
<th></th>
<th>Diabetes‡</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n Healthy individuals</td>
<td>n Diabetes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malik et al, 2003</td>
<td>18 13.5 ± 0.3</td>
<td>7 7.5 ± 1.1 (mod)</td>
<td>7 4.3 ± 1.5 (sev)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Quattrini et al, 2007</td>
<td>15 6.1 ± 1.2</td>
<td>18 3.9 ± 0.6 (mild)</td>
<td>15 3.6 ± 0.6 (mod)</td>
<td>11 3.7 ± 0.4 (sev)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mehra et al, 2007</td>
<td>15 9.7 ± 0.7</td>
<td>20 2.2 ± 0.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tavakoli et al, 2010</td>
<td>17 11.2 ± 0.9</td>
<td>34 8.1 ± 0.7</td>
<td>37 5.5 ± 0.5</td>
<td>16 3.0 ± 0.4</td>
<td>14 3.0 ± 0.3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

|                        | Corneal sensation threshold (mbars) |                   |               |               |               |               |               |
|------------------------|------------------------------------|-------------------|---------------|---------------|---------------|---------------|
|                        | n Healthy individuals | n Diabetes |                   |               |               |               |               |
| Murphy et al, 2004     | 69 0.6 ± 0.3 (young) | 5 0.7 ± 0.4 | 30 0.8 ± 0.6 (middle age) | 54 0.8 ± 0.4 | 20 1.5 ± 0.8 (older) | 52 1.0 ± 0.5 |               |
| Cousen et al, 2007     | 25 1.7 ± 0.3 | 25 1.9 ± 0.3 |               |               |               |               |               |
| Mehra et al, 2007      | 15 0.8 ± 0.0 | 20 1.5 ± 0.3 |               |               |               |               |               |
| Tavakoli et al, 2010   | 17 0.7 ± 0.4 (se) | 34 1.2 ± 0.1 (no) | 37 1.3 ± 0.1 (mild) | 16 1.5 ± 0.2 (mod) | 14 2.2 ± 0.5 (sev) |               |               |
| Pritchard et al, 2010  | 65 0.6 ± 0.3§ | 81 0.9 ± 0.6 |               |               |               |               |               |

† age ranges for corneal sensitivity were similar for all authors (average 55 years)
‡ No - no neuropathy, mild - mild neuropathy, mod - moderate neuropathy, sev - severe neuropathy
§ Pritchard et al, (unpublished)
se – standard error
A key advantage of CCM is that it is a simple and rapid clinical procedure that could be undertaken by a trained medical assistant. In a clinical environment the test would involve a 3-minute examination assuming paradigms involving automated image analysis are developed to provide a rapid diagnosis without the need for specialist interpretation. The instrumentation is currently expensive, but cost is likely to decrease as the instrument gains wider use in the ophthalmic community, at which time CCM could be readily translated into routine clinical practice.

The discriminative capacity of NCCA as a test for diabetic neuropathy has been examined in the UK and Australia (Table 2-4). In a group of 101 individuals with diabetes in Manchester (Tavakoli, Quattrini et al. 2010), a sensation threshold of 0.60 for a corresponding specificity of 0.61 for the presence of neuropathy (defined as NDS >3) was reported. In a Brisbane population of 81 individuals with type 2 diabetes, Pritchard and co-workers have reported a sensitivity of 0.70 (Pritchard, Edwards et al. 2010), as described in Chapter 6. Both groups show that NCCA is unable to differentiate those at risk of foot ulceration (NDS > 6). Like CCM, NCCA is quick and easy to use. This instrument is not currently commercially available, and development of computer-assisted testing of a psychophysical method is necessary for it to be applied in a clinical setting (Situ, Simpson et al. 2007). NCCA offers a large, continuous range of stimulus intensity, the stimulus is precise and there is minimal patient apprehension with its application. The NCCA can assess the corneal sensation threshold, using a composite stimulus consisting of air pressure, tear film evaporation and disruption, in an accurate and repeatable manner (Murphy, Lawrenson et al. 1998). Variability of repeated measures with NCCA was approximately 10% of the mean (Murphy, Lawrenson et al. 1998).
Table 2-4. Discriminative capacity of nerve fibre parameters and corneal sensitivity as indicators of diabetic neuropathy determined by NDS.

<table>
<thead>
<tr>
<th>Author</th>
<th>Parameter</th>
<th>n</th>
<th>Sensitivity NDS &gt; 3</th>
<th>Specificity NDS &gt; 3</th>
<th>Sensitivity NDS &gt; 6</th>
<th>Specificity NDS &gt; 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Tavakoli, Quattrini et al. 2010)</td>
<td>Corneal nerve fibre density (nerves/mm²)</td>
<td>101</td>
<td>0.82</td>
<td>0.52</td>
<td>0.71</td>
<td>0.64</td>
</tr>
<tr>
<td></td>
<td>Corneal nerve fibre length (mm/mm²)</td>
<td>101</td>
<td>0.64</td>
<td>0.79</td>
<td>0.64</td>
<td>0.71</td>
</tr>
<tr>
<td></td>
<td>Corneal sensitivity (mbars)</td>
<td>101</td>
<td>0.60</td>
<td>0.61</td>
<td>0.23</td>
<td>0.89</td>
</tr>
<tr>
<td>(Pritchard, Edwards et al. 2010)</td>
<td>Corneal sensitivity (mbars)</td>
<td>81</td>
<td>0.70</td>
<td>0.75</td>
<td>0.52‡</td>
<td>0.85‡</td>
</tr>
</tbody>
</table>

NDS – neuropathy disability score

mbar – millibars

‡NDS ≥ 6

The intra- and inter-observer repeatability of measuring corneal nerve parameters from images captured using SSCM has been reported to be acceptable; intra-class correlation coefficients ranging from 0.60 for inter-operator repeatability for tortuosity to 0.96 (which is excellent rather than acceptable) for intra-operator repeatability for nerve fibre length (Midena, Cortese et al. 2009). Automated analysis of corneal nerve parameters has been attempted (Ruggeri, Scarpa et al. 2006) such that a tracking algorithm was applied to identify nerve tissue using a grey/white detection threshold process and connections between nerve segments were created.
Repeatability may be affected by other factors such as poor reflectivity of the basement membrane in individuals with diabetes (Morishige, Chikama et al. 2001).

Markers of disease should also be able to monitor changes – either in the course of the natural history of the disease or in response to therapeutic intervention. CCM can be used to monitor long-term changes in corneal nerve morphology. Iqbal and co-workers (Iqbal, Kallinikos et al. 2005) reported that diabetic patients who maintained good health and were compliant with their therapeutic regimen showed an increase in corneal nerve fibre density after an average of 25 months, indicating reduced neuropathic severity. The increase in nerve fibre density was related to a statistically significant improvement in HbA1c. Small fibre repair has also been observed within six months of successful pancreas transplantation in patients with type 1 diabetes (Mehra, Tavakoli et al. 2007). An anecdotal report by Lalive and co-workers (Lalive, Truffert et al. 2009) noted improvement of corneal nerve fibre characteristics (reduced thickness and reduced tortuosity) in the recovery phase of a patient with anti-myelin-associated glycoprotein neuropathy.

The findings outlined above suggest that CCM can be used to assess longitudinal changes in nerve morphology, and therefore should be able to monitor progress of neuropathic severity in patients with diabetes. Observational studies are currently underway to explore the usefulness of CCM and NCCA to monitor nerve fibre parameter and corneal sensitivity parameters in people with, and those at risk of developing, diabetic neuropathy over a five-year period.

In clinical trials, when a clinical endpoint is undesirable (e.g. foot amputation), surrogate markers are sought. For corneal nerve morphology and function to be considered biomarkers for neuropathy, they must be proven to be equivalent to the existing clinical endpoint. However, regulatory authorities require interventional trials to show that the effect of the intervention on the biomarker matches that of the
clinical endpoint. It is then that the surrogate marker is considered to be valuable (Fleming and DeMets 1996). Researchers are currently examining the potential for employing CCM and NCCA in therapeutic trials, taking into account the natural history of diabetic neuropathy, so they may well be established as non-invasive surrogate endpoints for diabetic neuropathy.

2.9 Summary and Conclusions

Although in its early stages, developments of CCM and NCCA show promise as potential markers of diabetic neuropathy. Application of these tests for longitudinal evaluation of diabetic neuropathy have several advantages, primarily reducing dependence on more invasive assessments, such as foot-punch biopsy, in this prevalent and clinically significant complication of diabetes. Further research is warranted to support the cross-sectional studies demonstrating the ability of CCM and NCCA to stratify the severity of neuropathy. Longitudinal observational studies will further clarify the true efficacy of these tests in the near future.

2.10 Hypotheses for subsequent chapters

Reduction of observer, participant (subject) and instrument bias enhances precision, accuracy and reliability. These are important aspects of any diagnostic tool, can be assessed by inter- and intra-observer consistency and comparisons to gold standards (Hulley, Cummings et al. 1988) and improved by standardising testing, training operators and participants, automating procedures, repetition, masking and instrument calibration.

In vivo corneal confocal microscopy relies on examiner expertise, patient cooperation and fixation, and reduced eye movements, to achieve good quality images for measurement. Quantifying the morphology of the sub-basal nerve plexus has been achieved through manual, semi-automated and fully-automated means to date (Grupcheva, Wong et al. 2002; Malik, Kallinikos et al. 2003; Oliveira-
Soto and Efron 2003; Meijering, Jacob et al. 2004; Darwish, Brahma et al. 2007; Darwish, Brahma et al. 2007; Patel and McGhee 2007; Scarpa, Grisan et al. 2008; Tavakoli, Marshall et al. 2009; Dabbah, Graham et al. 2010; Tavakoli, Marshall et al. 2010). The repeatability of measurement of nerve fibres in the corneal sub-basal nerve plexus using CCM, however, has not been reported in the literature and observer bias has not been explored. The objective of the experiment (Chapter 3) was to determine the Bland-Altman repeatability (Bland and Altman 1986) and intra-class correlation coefficients for two observers measuring, on two occasions, corneal nerve fibre length and corneal nerve branch density.

An adjunct to understanding observer and instrument bias in measurement studies is accuracy, which is influenced by sampling procedures. To date inferences on overall corneal nerve parameters have been made from the central area of the cornea representing less than 1 mm$^2$ of CCM images with size ranging from 300 to 400 square microns (Zhivov, Stachs et al. 2006). Through the production of corneal sub-basal nerve maps using the technique of Patel and McGee (Patel and McGhee 2005) it is apparent that repeatedly sampling such a small area of the central cornea (via the alignment techniques recommended by the manufacturer), measurement error and bias may occur. The purpose of the study described in Chapter 4 was to present a technique for quantifying the number of random central corneal images required for a predetermined level of accuracy of any characteristic, and also to assess the number of images which need to be assessed for achieving an acceptable level of accuracy for the two corneal characteristics of interest in our study, namely corneal nerve fibre length and corneal nerve branch density.

The techniques used to determine an appropriate sampling paradigm for corneal nerve fibre parameters revealed a significant limitation to the process – time. The chair and follow-up time required to create a map of the corneal sub-basal nerve plexus (Patel and McGhee 2005) limits the routine use of this approach in many
patients, particularly those who are older and have health problems. The purpose of this study presented in Chapter 5 was to develop a novel, faster technique for mapping the corneal sub-basal nerve plexus and to demonstrate the additional clinical potential of this approach.

CCM shows promise as a potential surrogate marker for diabetic neuropathy. Non-invasive surrogate markers are sought in diseases where the endpoint is undesirable (Lassere, Johnson et al. 2007) e.g. foot amputation as a late sequelae of diabetic neuropathy. NCCA, a non-invasive test of corneal sensitivity, has been shown to differentiate groups with and without diabetes (Murphy, Patel et al. 2004; Tavakoli, Kallinikos et al. 2007) and can stratify individuals with diabetes by neuropathy severity (Tavakoli, Kallinikos et al. 2007), however, the sensitivity and specificity of the test has not been reported. The aim of this study presented in Chapter 6 was to explore the discriminative capacity of NCCA for predicting the outcome of the neuropathy disability score, a validated clinical method for diagnosing minimal to advanced neuropathy.
Chapter 3. Repeatability of measuring corneal sub-basal nerve fibre length

3.1 Preface

The paper presented in this chapter describes an essential component of research methodology – intra- and inter-observer repeatability. A repeatable test measure is essential for determining the effect of change and/or establishing differences between samples. Understanding repeatability is one of the main principles of conducting scientific investigations and assists in avoiding misinterpretation of data. Repeatability, in this instance, is participant (subject)-, observer- and equipment-dependent; the agreement of test results between labs, locations and apparatus is referred to as reproducibility and is not addressed in this paper. Once repeatability is established, further evaluations such as sampling paradigms (Chapter 4) and more complex examination techniques (Chapter 5) can be pursued. The repeatability of measurement of nerve fibres in the corneal sub-basal nerve plexus using CCM has not been reported in the literature, and observer concordance has not been explored. These are important aspects of any diagnostic tool, especially when subjective judgements of observers are involved. This paper is the first of two papers presented in this thesis addressing methodology-related factors of measuring corneal nerve morphology from CCM images and is published in the Eye & Contract Lens journal:

3.2 Abstract

To analyse the repeatability of measuring nerve fibre length from images of the human corneal sub-basal nerve plexus using semi-automated software, images were captured from the corneas of 50 diabetic participants with type 2 diabetes mellitus with varying severity of neuropathy, using the Heidelberg Retina Tomograph 3 with Rostock Corneal Module. Semi-automated nerve analysis software was independently used by two observers to determine nerve fibre length (NFL) from images of the sub-basal nerve plexus. This procedure was undertaken on two occasions, three days apart. The intraclass correlation coefficient values were 0.95 (95% confidence intervals 0.92 to 0.97) for between-observer and 0.95 (0.74, 1.00) for within-observer comparisons. Bland-Altman plots of the NFL values indicated a reduced spread of data with lower NFL values. The overall spread of data was less for (a) the observer who was more experienced at analysing nerve fibre images, and (b) the second measurement occasion. Semi-automated measurement of NFL in the sub-basal nerve fibre layer is repeatable to an acceptable degree. Repeatability can be enhanced by using experienced observers. It may be possible to improve repeatability when measuring this anatomical structure using fully automated image analysis software.

3.3 Introduction

Corneal confocal microscopy (CCM) is a useful tool for (a) evaluating the integrity of the cornea in conditions such as keratoconus (Efron and Hollingsworth 2008) and acanthamoeba and fungal keratitis, (Efron 2007) (b) assessing the response of the cornea to contact lens wear, (Efron 2007) and (c) monitoring corneal repair following ophthalmic surgery (Darwish, Brahma et al. 2007). The clinical utility of this technique has also been demonstrated for tracking small nerve fibre damage and repair in the cornea following certain forms of corneal refractive surgery when the
nerve fibre layer is severed (Darwish, Brahma et al. 2007) and in a range of peripheral neuropathies including diabetic neuropathy (Malik, Kallinikos et al. 2003), idiopathic small fibre neuropathy (Tavakoli, Marshall et al. 2010) and Fabry’s disease (Tavakoli, Marshall et al. 2009).

Two corneal confocal microscopes are currently available: the white light, slit scanning Confoscan 4 (NIDEK Co., Ltd., Aichi, Japan) (Efron 2007; Patel and McGhee 2007) and the laser-scanning Heidelberg Retinal Tomograph 3 with Rostock Corneal Module (HRT3; Heidelberg Engineering GmbH, Dossenheim, Germany) (Efron 2007; Patel and McGhee 2007). Although these instruments have limited capacity to undertake certain forms of automated or semi-automated assessment of corneal cell structures, neither is equipped to undertake a morphometric analysis of the corneal sub-basal nerve plexus. Measurement of this structure is required to facilitate quantitative descriptions of nerve fibre degeneration and repair in conditions such as those outlined above.

Although various authors have reported the application of manual, semi-automated and fully-automated approaches to quantifying morphological aspects of the sub-basal nerve plexus (Grupcheva, Wong et al. 2002; Malik, Kallinikos et al. 2003; Oliveira-Soto and Efron 2003; Meijering, Jacob et al. 2004; Darwish, Brahma et al. 2007; Darwish, Brahma et al. 2007; Patel and McGhee 2007; Scarpa, Grisan et al. 2008; Tavakoli, Marshall et al. 2009; Dabbah, Graham et al. 2010; Tavakoli, Marshall et al. 2010), the repeatability of measurement of nerve fibres in the corneal sub-basal nerve plexus using CCM has not been reported in the literature. Furthermore, the potential bias due to different observers has not been explored. These are important aspects of any diagnostic tool, especially when judgement of an individual observer is involved.
Meijering et al (Meijering, Jacob et al. 2004) have noted in some studies that authors have failed to detail their method of nerve fibre analysis, which limits useful comparison between studies. Difficulties in the manual measurement of sub-basal nerves (such as the time involved and measurement error) have also been discussed in the ophthalmic literature (Grupcheva, Wong et al. 2002; Oliveira-Soto and Efron 2003). In view of these concerns, and of the imperative to establish the measurement capability of corneal sub-basal nerve fibre analysis, we have undertaken a study of the repeatability of a semi-automated technique for quantifying images of corneal nerves captured using the Heidelberg CCM.

3.4 Materials and methods

A. Participants

Images used in this analysis were obtained from 50 study participants with type 2 diabetes mellitus with varying severity of neuropathy, who were recruited from the outpatient clinic of the Princess Alexandra Hospital, Brisbane, Australia. Ethical clearance was granted by the Princess Alexandra Hospital and Queensland University of Technology Research Ethics Committees. The study participant characteristics are presented in Table 3-1.
Table 3-1. Study participant characteristics.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>50</td>
</tr>
<tr>
<td>Sex F/M</td>
<td>17 / 33</td>
</tr>
<tr>
<td>Age (years)</td>
<td>60 ± 9</td>
</tr>
<tr>
<td>T1/T2</td>
<td>0 T1 / 50 T2</td>
</tr>
<tr>
<td>Duration of diabetes (years)</td>
<td>13 ± 8</td>
</tr>
<tr>
<td>Insulin use (Y/N)</td>
<td>18 / 30 *</td>
</tr>
<tr>
<td>Eye used R/L</td>
<td>41 / 9</td>
</tr>
<tr>
<td>Neuropathy disability score (0-10)</td>
<td>4.1 ± 3.2</td>
</tr>
</tbody>
</table>

* 2 did not indicate; Y yes, N no, T1 type 1, T2 type 2, R right, L left

B. Observers

The observers were two individual researchers (NR – Observer 1, KE – Observer 2) engaged in ongoing studies of corneal markers of diabetic neuropathy at the Queensland University of Technology, Australia. Both were familiar with corneal confocal microscopy, although observer 2 was more experienced with the technique from a clinical perspective.

C. Corneal confocal microscopy

All images of the sub-basal plexus were captured using the Heidelberg Retinal Tomograph (HRT3) with Rostock Corneal Module. This is a laser scanning confocal microscope using a 670 nm red wavelength diode laser source. Images produced measured 400µm x 400µm with a digital image size of 384 x 384 pixels. A large drop of a highly viscous eye gel (GenTealEyes, Novartis, North Ryde, NSW, Australia) was placed between the microscope objective and a Perspex ‘TomoCap’ that covered the objective. The eye under examination was anesthetised with a drop
of 0.4% benoxinate hydrochloride (Chauvin Pharmaceuticals, Chefaro, UK) and Viscotears (Novartis, North Ryde, NSW, Australia) was applied as a coupling agent between the TomoCap and the cornea. Participants were asked to fixate on an LED target with one eye (generally corresponding to the side of non-dominant hand) while the central cornea of the contralateral eye was examined. At least eight images of the sub-basal nerve plexus were captured.

D. Corneal sub-basal nerve plexus image analysis

Observer 1 chose one CCM image for each participant on the basis of being the first captured, in-focus, high contrast image of the sub-basal nerve plexus. All 50 images were analysed by this observer using a custom-designed semi-automated nerve analysis software package (CCMetrics (Dabbah, Graham et al. 2010); University of Manchester, UK) that involved tracing the nerves on a Wacom Graphics Tablet (Wacom Co. Ltd., Saitama, Japan.) using a grip pen.

Numerous characteristics of the sub-basal nerve plexus can be quantified. For this experiment, we assessed ‘nerve fibre length’ (NFL) because this parameter is considered to be the better test for diagnosing patients with diabetic neuropathy (compared with the parameters ‘nerve fibre density’ and ‘nerve branch density’) based on receiver operator characteristic analysis (Tavakoli, Quattrini et al. 2010). Nerve branch density was also examined as both parameters could be measured simultaneously. We classified a nerve fibre in an image as a continuous white line exhibiting a clearly defined path against the grey amorphous background. All nerve fibres and branch offshoots visible in the frame were measured. Any short white line, dot or mark that was not attached to another nerve fibre (and could thus be a keratocyte, Langerhans cell or image artefact) was not considered as a nerve element and therefore was not included in the measurement. Any bifurcation noted in a continuous white line described above was counted as a branch. Nerve fibre
length (NFL) was expressed as the total length of all nerves and branches in the image (mm) per image frame of known dimensions (mm²); thus, the units of NFL were mm/mm². Nerve branch density (NBD) was expressed as the total number of branches per unit area in branches/mm².

After a period of three days, all 50 images were analysed again by the same observer. This process was then repeated by observer 2 using the 50 images originally selected by observer 1. Thus, both observers assessed the same set of 50 images on two occasions separated by at least three days.

The CCMetrics algorithm converts the traced nerve tissue and branch density points identified from the 400 µm x 400 µm (384 pixel x 384 pixel) image to mm/mm² via a user-determined scaling factor, in this instance 0.00104167. A screen snapshot is shown in Figure 3-1.

Figure 3-1. Screen snapshot of CCMetrics. The scaling factor is applied by the observer in mm/pixel.  (Image: Nicola Pritchard)
E. Statistical analysis

Estimates of NFL and NBD were determined for both ‘individual’ (i.e. comparison of the measurements of individual images by both observers between successive analysis occasions) and ‘observer’ (comparison of the measurements of individual images between observers across both analysis occasions) and repeatability was assessed using the intraclass correlation coefficient (ICC). This analysis was undertaken using the ‘Psychometric’, ‘ICC.lme’ and ‘ICC.CI’ functions of the ‘Applied Psychometric Theory’ statistical package (Fletcher 2006). The correlation between the two ‘occasions’ was also estimated using the statistical package ‘R’ (R Development Core Team 2011).

Bland-Altman plots (Bland and Altman 1986) were generated to facilitate an appreciation of the extent of between-observer and between-occasion discrepancies, and the relation between these discrepancies and the overall magnitude of NFL and NBD. Log transformation was applied to corneal nerve fibre length when an association between the difference and the mean was found.

3.5 Results

The ICC values were 0.95 (95% confidence intervals 0.92 to 0.97) for individual and 0.95 (0.74, 1.00) for observer. The estimated correlation between the two occasions was 0.96 (0.95, 0.98). Table 3-2 shows the ICC and 95% CI for both corneal nerve fibre length and branch density.

Bland-Altman plots of the NFL values obtained on the two measurement occasions by observers 1 and 2 are shown in Figure 3-2 A and Figure 3-2 B, respectively. For observer 1 (Figure 3-2 A), a reduced spread of data is associated with lower NFL values. The upward slope of the line in Figure 3-2 A (from left to right) indicates that, for higher mean NFL values, a higher value was assigned to NFL on the first measurement occasion than the second, however, the mean was not significantly
associated with the difference (P=0.06). The mean was significantly associated with the difference for observer 2, occasion 1 and occasion 2 (P<0.001) and Figure 3-2 B to D show similar trends. The overall spread of data was less for observer 2 (standard deviation [sd] = 1.22) than for observer 1 (sd = 2.26).

Table 3-2. Intraclass correlation coefficients (ICC) and 95% confidence intervals (shown in brackets) for nerve fibre length and nerve branch density.

<table>
<thead>
<tr>
<th></th>
<th>CNFL ICC (95% CI)</th>
<th>CNBD ICC (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Occasion 1 vs Occasion 2</td>
<td>Occasion 1 vs Occasion 2</td>
</tr>
<tr>
<td>Observer 1</td>
<td>0.96 (0.92-0.97)</td>
<td>0.9 (0.84-0.94)</td>
</tr>
<tr>
<td>Observer 2</td>
<td>0.97 (0.95-0.98)</td>
<td>0.94 (0.89-0.96)</td>
</tr>
<tr>
<td>Observer 1 vs Observer 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Occasion 1</td>
<td>0.93 (0.88-0.96)</td>
<td>0.89 (0.81-0.93)</td>
</tr>
<tr>
<td>Occasion 2</td>
<td>0.94 (0.89-0.96)</td>
<td>0.86 (0.77-0.92)</td>
</tr>
</tbody>
</table>
Figure 3-2. Bland-Altman repeatability of NFL difference versus NFL mean for n=50 representing (A) observer 1, (B) observer 2, (C) occasion 1, and (D) occasion 2. On each graph, the solid line represents the linear regression and the dotted lines are the 95% limits of agreement.
Bland-Altman plots of the NFL values obtained by the two observers on occasions 1 and 2 are shown in Figure 3-2 C and D, respectively. On occasion 1 (Figure 3-2 C), a reduced spread of data is associated with lower NFL values. The upward slope of the line in Figure 3-2 C (from left to right) indicates that, for higher mean NFL values, observer 2 assigned a higher NFL value compared to observer 1. Similar trends were observed for occasion 2 (Figure 3-2 D). The overall spread of data was less on occasion 2 (sd = 1.69) than on occasion 1 (sd = 2.33).

Figure 3-3. Sample image of the sub-basal nerve plexus (A) without any tracings, (B) showing the tracing performed by observer 1, and (C) showing the tracing performed by observer 2. The green dots indicate designated nerve branches. (Images: courtesy Dr Katie Edwards)

Figure 3-3 A shows an image of the sub-basal nerve plexus captured with the CCM that yielded discrepant results between the two observers. The nerve tracing performed by observers 1 and 2 are shown in Figure 3-3 B and Figure 3-3 C, respectively. The green dots indicate designated nerve branches.
Figure 3-4. Bland-Altman repeatability of NBD difference versus NBD mean for n=50 representing (A) observer 1, (B) observer 2, (C) occasion 1, and (D) occasion 2.
2. On each graph, the solid line represents the linear regression and the dotted lines are the 95% limits of agreement.

Table 3-3. Descriptive (mean ± standard deviation) and comparative statistics (t-test) for corneal nerve fibre length (mm/mm²) and corneal nerve branch density (branches/mm²) for two observers at two occasions.

<table>
<thead>
<tr>
<th></th>
<th>Occasion 1</th>
<th>Occasion 2</th>
<th>Mean difference</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNFL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Observer 1</td>
<td>22.2 ± 8.2</td>
<td>21.6 ± 7.6</td>
<td>-0.6 ± 2.3</td>
<td>0.07</td>
</tr>
<tr>
<td>Observer 2</td>
<td>18.0 ± 6.7</td>
<td>16.8 ± 5.9</td>
<td>1.2 ± 1.5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Mean difference</td>
<td>4.2 ± 2.8</td>
<td>4.7 ± 2.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P value</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CNBD</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Observer 1</td>
<td>103.63 ± 76.84</td>
<td>98 ± 73.06</td>
<td>5.63 ± 32.74</td>
<td>0.230</td>
</tr>
<tr>
<td>Observer 2</td>
<td>73 ± 59.34</td>
<td>70.63 ± 50.62</td>
<td>2.38 ± 19.88</td>
<td>0.402</td>
</tr>
<tr>
<td>Mean difference</td>
<td>30.63 ± 32.69</td>
<td>27.38 ± 33.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P value</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 3-4. Mean difference, upper and lower limits (mm/mm²) calculated via log transformation applied to corneal nerve fibre length for two observers at two occasions.

<table>
<thead>
<tr>
<th>CNFL</th>
<th>Mean difference</th>
<th>Upper Limit</th>
<th>Lower Limit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Observer 1</td>
<td>0.10</td>
<td>1.25</td>
<td>0.84</td>
</tr>
<tr>
<td>Observer 2</td>
<td>0.03</td>
<td>1.22</td>
<td>0.93</td>
</tr>
<tr>
<td>Occasion 1</td>
<td>0.09</td>
<td>1.60</td>
<td>0.95</td>
</tr>
<tr>
<td>Occasion 2</td>
<td>0.11</td>
<td>1.55</td>
<td>1.05</td>
</tr>
</tbody>
</table>

Figure 3-5. Log transformation using Bland-Altman repeatability of NFL difference versus NFL mean for n=50 representing (A) observer 1, (B) observer 2, (C) occasion 1, and (D) occasion 2. On each graph, the solid line represents the linear regression and the dotted lines are the 95% limits of agreement.
3.6 Discussion

The results of this experiment indicate high between-observer and within-observer repeatability in the measurement of NFL and NBD of the human corneal sub-basal nerve plexus.

The reduced variance in data observed with lower mean NFL and NBD values in Figure 3-2 and Figure 3-4, respectively, is to be expected, as a lower number of nerve fibres and branches decrease the possibility of inconsistent scoring and measurement error. Thus, for example, a repeated estimate of NFL in an image containing no nerve fibres, or one nerve fibre, is likely to be in greater agreement than for a repeated estimate of NFL in an image containing 20 nerve fibres. The reason why both observers tended to assign a higher NFL value on the first occasion than the second occasion is unclear, but may indicate a shift in criteria towards a more conservative approach when identifying nerve fibres on the second occasion, and although unlikely given the short duration of each measurement, some degree of fatigue with the task. This phenomenon was less apparent with the measurement of branch density, which may indicate a nerve branch is a less ambiguous feature than a nerve.

Between-observer differences in scores given on the two separate occasions may be explained by the different skill levels of the two observers. Observer 1 was a visiting researcher to our laboratory who had no prior experience in assessing images of the corneal sub-basal layer prior to this experiment. Observer 2, on the other hand, was an experienced post-doctoral researcher who had been performing confocal microscopy and assessing images of the corneal sub-basal layer for 12 months prior to this experiment. The lower variation of the data points pertaining to the measurements of observer 2 (Figure 3-2 B, Figure 3-4 B) is consistent with the higher level of pertinent experience of this observer compared with that of observer...
1 (Figure 3-2 A, Figure 3-4 A). Figure 3-3 illustrates a large discrepancy in estimating NFL and NBD, as evidence by the greater number white lines and green dots in the confocal image (Figure 3-3A) deemed to be true nerves and branches by observer 2 (Figure 3-3C) compared to observer 1 (Figure 3-3B). A within- or between-observer bias suggests that longitudinal data may not reveal differences where differences exist over time or reveal real differences between populations or time-points. The greatest bias was observed between observers, therefore, until automated analysis is available, training observers will continue to be an important process, as well as large sample sizes.

Within-observer differences in the spread of data are possibly explained by a ‘learning effect’ or an alteration in the criteria used by the observer. By the time measurements were undertaken on the second occasion, both observers were more experienced, having already undertaken this exercise previously (i.e. the ‘first’ measurement occasion), as well as undertaking further image analysis tasks during the interim period. It is possible that, on the second measurement occasion, both observers adopted a more purposeful and informed approach to the assessment task; hence the narrower 95% confidence intervals on the second measurement occasion. This effect could be greater in respect of the inexperienced observer (observer 1), who might have become more consistent; thus, the discrepancy in the scores recorded by observer 1 relative to observer 2 would be smaller on the second occasion.

Less variability at lower NFL and NBD values is noted in the present study, however, fewer data points at the lower and higher ends of the plot does not necessarily mean variance truly changes with amount of nerve fibre tissue. The fact that there are only 3 or 4 data points makes it more of an artefact than a real difference in variance.
Log transformation is recommended when an association between the difference and the mean is observed (Bland and Altman 1986). When log transformation was applied to the four scenarios, the maximum within-observer difference was 25% above and 16% below; the maximum between-observer difference was 45% above and 5% below (Table 3-4 and Figure 3-5). This between-observer difference was reflected in the SDs of 1.22 mm/mm² for observer 2 and 2.26 mm/mm² for observer 2. However, with 49 degrees of freedom, these are not considered to be significantly different – as a rule of thumb, a difference would be indicated if a 3-fold difference was observed (http://www.ats.ucla.edu/stat).

This work documents reasonable repeatability in the semi-automated assessment of images of the human sub-basal nerve plexus. The growing interest in assessing this nerve plexus in relation to a variety of ophthalmic applications (Darwish, Brahma et al. 2007; Darwish, Brahma et al. 2007; Efron 2007; Efron and Hollingsworth 2008) and peripheral neuropathic disease (Malik, Kallinikos et al. 2003; Tavakoli, Marshall et al. 2009; Tavakoli, Marshall et al. 2010) highlights the need for accurate and repeatable quantification of nerve fibre morphology, especially if this measure is to be used in longitudinal studies or indeed to assess the benefits of interventions.

In conclusion, semi-automated measurement of the sub-basal nerve fibre layer— in particular measurement of NFL and NDB — is shown to be repeatable to an acceptable degree. Using more experienced or trained observers can enhance repeatability. Fully automated systems for analysing such images, such as those being developed by Scarpa and co-workers. (Scarpa, Grisan et al. 2008) and Dabbah et al. (Dabbah, Graham et al. 2010), hold promise of virtually eliminating remnant errors.
3.7 Subsequent studies of repeatability

The findings of the above study are supported by a recent study by Hertz and co-workers (Hertz, Bril et al. 2011) who found corneal nerve fibre length to be the only parameter with consistently good reproducibility. The authors used one image each from right and left eyes selected for contrast and clarity from two sets of 40 images per eye sampled via the ‘volume’ scan mode of the identical instrument at two occasions. Images captured at two different times will be less similar than two images captured at one session and measured by an observer at two different times. The difference in study design and sampling method explains the slightly lower intra-observer intraclass correlation coefficient of 0.72 and inter-observer intraclass correlation coefficient of 0.73, compared with the present study.
Chapter 4. Optimal image sample size for estimation of corneal nerve morphology using corneal confocal microscopy

4.1 Preface

The number of images used for determination of corneal nerve morphology was investigated in this study. Inferences made for corneal nerve parameters to date are for the whole or ‘central’ corneal area, yet each image represents only approximately 0.2% of the average corneal surface. Capturing and assessing a small number of images may result in misleading inferences and potential bias, as the small sample estimates may not accurately represent the broader picture. Measurement of such biological parameters needs to be a balance between accuracy, and what is logistically possible and feasible, especially in a clinical setting. A novel statistical method for determining the minimum number of images that represents corneal nerve morphology is presented in the paper, accepted for publication in the journal of Optometry and Vision Science:


4.2 Introduction

Corneal confocal microscopy (CCM), developed in 1968 by Petran and co-workers (Petran, Hadravsky et al. 1968), offers researchers and clinicians the opportunity to directly and non-invasively examine small nerve fibres in the living human cornea at a magnification of approximately 700X (Efron 2007). The optical principle of CCM is
that field of view is sacrificed for resolution but is regained by scanning, which allows essentially transparent tissue to be viewed with good contrast and high lateral resolution. Quantification of corneal nerve parameters is widely documented in both healthy individuals (Masters and Bohnke 2001; Jalbert, Stapleton et al. 2003; Patel and McGhee 2005; Stachs, Zhivov et al. 2007), and in those with a range of neurological and corneal disorders and compromise (Malik, Kallinikos et al. 2003; Weed, McGhee et al. 2005; Midena, Brugin et al. 2006; Darwish, Brahma et al. 2007; Patel, Tavakoli et al. 2009; Tavakoli, Quattrini et al. 2010).

Corneal confocal microscopy allows the capture of images of tissue, both on the horizontal or x-y plane, and at different depths of the cornea, the z plane. In studies conducted to date, arbitrary numbers of images have been used for analysis of nerve parameters under the implicit assumption that these are a representative sample of the central nerve plexus. Small numbers of images (typically between one and three (Pritchard, Edwards et al. 2011) have been assessed by researchers, as the process is extremely laborious and typically done manually by a trained observer. Inferences made for corneal nerve parameters are for the whole corneal area, yet each image represents only approximately 0.2% of the average corneal surface. Therefore, capturing and assessing a small and hence possibly non-representative number of images may result in misleading inferences and potential bias. Hence, the number of images used for determination of corneal nerve morphology needs to be a balance between accuracy of these parameters, and what is logistically possible and feasible, especially in a clinical setting. In the absence of automated software which could analyse most of the corneal area efficiently, an appropriately-targeted effort for image analysis becomes crucial, especially in a research environment where large sample sizes and/or longitudinal studies are required. Given the laborious nature of corneal confocal image analysis,
it is important to assess how a small sample of the central corneal region may accurately represent a larger area in terms of nerve fibre parameters.

Measurement of corneal nerve parameters from single images was shown to be relatively repeatable in Chapter 3 (intraclass correlation coefficient of 0.95 within-and between-observer) (Efron, Edwards et al. 2010). However, to our knowledge, no study has been conducted to determine the number of images required for the outcome parameters to have a desired, predetermined level of accuracy within range of an assumed “true” value (as the true value at this time is unknown). The purpose of this paper is two-fold: (i) to present a technique for quantifying the number of random central corneal images required for a predetermined level of accuracy of any characteristic, and (ii) to assess the number of images which need to be assessed for achieving an acceptable level of accuracy for the two corneal characteristics of interest in our study, namely corneal nerve fibre length and corneal nerve branch density.

4.3 Materials and methods

A. Image capture

Corneal confocal microscopy using the Heidelberg Retinal Tomograph III with Cornea Rostock Module (Heidelberg Engineering GmbH, Germany) is ideal for imaging the central corneal layers. In our study, the central cornea was defined as the point of contact between the disposable Perspex cap with a flat anterior surface (Tomocap™) and the cornea when the participant was fixating centrally, typically using the contralateral eye. A minimum of 20 images of the central cornea were captured on each of 20 individuals so that approximately 2.5 mm² of the central cornea was imaged (Figure 4-1). These images were montaged using a technique called corneal mapping, with an example of a resulting image shown in Figure 4-1 as described by Patel and McGhee (Patel and McGhee 2005) and Efron (Efron
2007). Imaging the central cornea in this way determines the reference, or most accurate measure, for determination of the corneal nerve parameters examined.

![Image](image.png)

Figure 4-1. Example of a montage of approximately 40 images captured from the central region of the cornea for one participant. The red squares represent the random sample of 16 images selected which overlap not more than 20%. (Image: courtesy Dr Katie Edwards)

Twenty individuals with type 2 diabetes mellitus were enrolled for this study and written, informed consent was obtained. These individuals were part of a longitudinal, observational trial, investigating new ophthalmic markers of diabetic neuropathy. Only individuals without history of ocular trauma or surgery, ocular disease or systemic disease affecting the cornea were included. Ethical clearance was granted by the Princess Alexandra Hospital in Brisbane and the Queensland University of Technology Research Ethics Committees. After explaining the procedure to the participant, typically one or two drops of local anaesthetic (benoxinate hydrochloride 0.4%, Chauvin, France) were applied to the cornea. The
best 16 out of 20 images from the centre of the cornea that did not overlap by more than 20% were deemed to adequately represent the central part of the cornea covering approximately 3% of the total corneal area (Figure 4-1). Sixteen images produce a 4 x 4 image area that was (a) clinically simple to achieve and therefore, (b) anecdotally a region likely to coincide with the region of the cornea imaged based on the manual positioning of the instrument via the instrument-generated corneal reflex. The 20 individuals had neuropathy categorized at four different stages: no neuropathy, mild, moderate and severe with five individuals in each group. Selection bias was minimised by using data from the first 20 individuals enrolled with the appropriate number of images available until five per group were identified.

Table 4-1. Study participant characteristics.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>20</td>
</tr>
<tr>
<td>Sex F/M</td>
<td>6 /14</td>
</tr>
<tr>
<td>Age (years)#</td>
<td>60 ± 10</td>
</tr>
<tr>
<td>T1/T2</td>
<td>0 T1 / 20 T2</td>
</tr>
<tr>
<td>Duration of diabetes (years)#</td>
<td>12 ± 9</td>
</tr>
<tr>
<td>Insulin use (Y/N)</td>
<td>10 / 8 *</td>
</tr>
<tr>
<td>Eye used R/L</td>
<td>15 Right / 5 Left</td>
</tr>
<tr>
<td>Neuropathy disability score (0-10)#</td>
<td>5.2 ± 3.7</td>
</tr>
</tbody>
</table>

* 2 did not indicate; #average ± SD; T1 type 1, T2 type 2, M male, F female, Y yes, N no, R right, L left

B. Corneal nerve image analysis

Corneal nerve fibre length, defined as the total length of nerves per unit area (mm/mm²), and corneal nerve branch density, defined as the total number of nerve
branches per unit area (branches/mm$^2$), were quantified for each image. The observer traced the nerve fibres using a digital pen and tablet (Wacom, Cintiq, USA) for each image and the nerve parameters were calculated using an algorithm written in Matlab (CCMetrics (Dabbah, Graham et al. 2010); University of Manchester, Manchester, United Kingdom). Each image was assessed twice, however, after showing strong within-observer and within-participant repeatability (ICC= 0.95 for both, widest 95% CI 0.74-1.00 in Chapter 3 (Efron, Edwards et al. 2010), only the first measure of one observer was used. Each image took approximately 5 minutes to calculate the desired parameters. These parameters were chosen as they are currently being explored as surrogate markers of peripheral nerve damage, particularly in diabetes, and require little or no multilevel judgements by the observer during the quantification process e.g. the presence or absence nerve tissue is a dichotomous judgment, however, some authors attempt to differentiate major nerve branches from minor prior to defining it’s dimensions, i.e. a multilevel judgement.

Despite several corneal nerve fibre parameters being reported by other authors quantifying corneal morphology or neuropathic disorders, only two parameters were selected for these analyses. All parameters are highly correlated with each other, the parameters of corneal nerve fibre length and corneal nerve branch densities have shown to have greater predictive value in diabetic neuropathy (Tavakoli, Quattrini et al. 2010; Hertz, Bril et al. 2011) and both require less subjective input from the observer (Pritchard, Edwards et al. 2011). For these reasons these two parameters have been selected for evaluation in this experiment.

**C. Statistical Analysis**

To determine the minimum number of images required to provide an accurate representation of the central cornea, a benchmark or ‘true value’ against which a parameter estimated from a lower number of images can be compared, has to be
established. The most appropriate benchmark (‘gold standard’ value) in this case was deemed to be the mean nerve fibre length and nerve branch density of the maximum number of different high quality images available per participant, i.e. 16 images.

This benchmark value, the average of 16 central images, was compared to a number of estimates of the mean corneal nerve fibre length and branch density from a number of images up to the maximum number of images available, i.e. from two to 16 images. If the images to be used for these estimates are sampled at random, there will be random noise added to the comparisons with the gold standard and, potentially, bias. A rigorous sampling procedure was employed to determine the minimum number of images (k) out of a total of 16 which would provide the minimal sampling variation and bias.

The sampling technique applied here is based on the creation of appropriate combinations (as defined in a mathematical sense) of the existing images. A combination is an unordered collection of k elements taken from a total of n elements (k ≤ n). Thus, if we have n elements of which k will be selected disregarding the order of selection, we seek combinations (C) of n elements taken k at a time. In the case of combinations, selecting items “1”, “2” and “3” in this order is the same as selecting them in any other order (i.e.. 2, 3, 1, and 3, 2, 1 are not distinct selections). Combinations are unlike permutations where order matters and for which 3, 2, 1 would be considered distinct from 2, 3, 1. The formula for estimating the total number of combinations of n elements taken k at a time is:

\[
\binom{n}{k} = \frac{n!}{(n-k)! \cdot k!}
\]

(where “!” signifies factorial). For example in this study, if we seek the combinations of 16 images (n) taken 2 (k) at a time (i.e. pairs) we have a total of:
optimal image sample size for estimation of corneal nerve morphology using corneal confocal microscopy

\[ \binom{16}{2} = \frac{16!}{(16-2)!2!} = 120 \]

distinct combinations. Thus, if we take for each participant two images at a time from a total of 16 images, and we estimate all unique means, we would have 120 mean values. The total unique combinations for each participant’s 16 images are shown in Table 4-2. This technique is similar to the permutation tests and bootstrap methods (Good 2000; Hesterberg, Moore et al. 2009). These techniques take repeated samples with replacement from one sample, whereas in this case, we estimated all possible combinations, sampling without replacement.

Table 4-2. Number of combinations of k images able to be sampled from 16 images taken k at a time

<table>
<thead>
<tr>
<th>Number of Images (k)</th>
<th>Number of Combinations</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16</td>
</tr>
<tr>
<td>2</td>
<td>120</td>
</tr>
<tr>
<td>3</td>
<td>560</td>
</tr>
<tr>
<td>4</td>
<td>1820</td>
</tr>
<tr>
<td>5</td>
<td>4368</td>
</tr>
<tr>
<td>6</td>
<td>8008</td>
</tr>
<tr>
<td>7</td>
<td>11440</td>
</tr>
<tr>
<td>8</td>
<td>12870</td>
</tr>
<tr>
<td>9</td>
<td>11440</td>
</tr>
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</tbody>
</table>
To allow appropriate visual and computational comparisons between and within participants, each mean was converted to a relative mean i.e. the ratio of the calculated mean for each combination with the benchmark mean of 16 images. Since a large number of means for each combination level is estimated, the estimated means (and consequently of the mean ratios) for each combination level are expected to be approximately normally distributed in accordance with the central limit theorem. For estimating a required confidence interval (CI) around the mean, the classic formula $CI = \mu \pm t \times SD$ was used, where $\mu$ is the mean ratio, $t$ is the appropriate quantile from a $t$ distribution and $SD$ is the estimated standard deviation of the mean ratio. For example, for the 95% CI intervals around the mean, the formula $CI = \mu \pm 1.96 \times SD$ would be used, assuming that we have a large sample (> 120). If the number of observations are not “large” enough, the appropriate number with which to multiply the standard deviation is estimated based on the quantiles of a $t$-distribution instead of the above value of 1.96. In the current analysis, the actual quantiles from a $t$-distribution were used, based on the number of observations, rather than the theoretical ones. However, the reader is reminded that due to the large number of combinations for each level, the outcome will be similar to the theoretical calculations, as described below.

The above “theoretical” approach for estimating intervals will be asymptotically accurate, (when the sample size ($k$) is large) as it is based on the central limit theorem. However, for small $k$ the approximation could be inaccurate. Therefore, an alternative “empirical” interval was also estimated. The empirical confidence intervals were calculated from the values of the estimated means for each combination. This interval contains the actual, estimated proportion of values around the mean ratio, e.g. 95% of the actual values estimated around the mean rather than an estimated theoretical method. If the underlying assumption of theoretical calculations is correct, i.e. that the values form a normal distribution, the
two methods (theoretical and empirical) should give similar answers. The theoretical has the limitation that it assumes a symmetrical, theoretical distribution which may or may not be the case in each problem, whereas the empirical calculations could be skewed toward the specific data sample and thus not able to be generalised.

Because the above individuals had different levels of neuropathy as explained previously, the first issue to be addressed is if the observations from different neuropathy groups could be analysed together. Although the above procedure has been devised so as to account for differences in the means of corneal parameters, potential systematic differences in variation of different neuropathy levels could have implications for analysing the observations from different neuropathy groups in one analysis. To determine if severity of neuropathy had an effect on the variation observed, outcomes were graphically compared for four groups based on the severity of neuropathy.

All calculations were performed and figures were constructed in R (Version 2.11.1, R Development Core Team, http://www.r-project.org/). The 95, 90, 85 and 80% intervals were calculated for each number of combinations and plotted to determine the minimum number of images for an acceptable degree of accuracy, both with the theoretical and the empirical approach.

4.4 Results

Corneal nerve branch density and corneal nerve length mean ratio (relative to one) for all image combinations for each participant are shown in B Figure 4-2. It is apparent that the greater number of images used for the estimation of the mean ratio, the less variation around the mean ratio. Differing amounts of variation around the mean ratio are observed among participants. Branch density (Figure 4-3 A) and fibre length (Figure 4-3 B) were plotted for different severities of neuropathy. Qualitatively, the dimension of the spread of points for both parameters
was similar in all groups of neuropathy and therefore these groups could be analysed together, regardless of neuropathy status. The distribution of the mean ratio of all individuals combined is shown in Figure 4-4 for corneal nerve branch density and corneal nerve fibre length. Variation around the mean is greater for corneal nerve branch density than corneal nerve fibre length. Furthermore, corneal nerve fibre length has a more symmetrical distribution than corneal nerve branch density when observed qualitatively.

Table 4-3. Descriptive statistics for corneal nerve branch density (CNBD) and corneal nerve fibre length (CNFL).

<table>
<thead>
<tr>
<th>Degree of neuropathy</th>
<th>CNBD (#/mm²)</th>
<th>CNFL (mm/mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total N=20</td>
<td>None n=5</td>
</tr>
<tr>
<td>CNBD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(#/mm²)</td>
<td>61.3 ± 29.3</td>
<td>89.2 ± 26.9</td>
</tr>
<tr>
<td>CNFL</td>
<td>16.1 ± 4.2</td>
<td>19.8 ± 4.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANOVA between neuropathy, P=)</td>
<td>0.085</td>
<td>0.028</td>
</tr>
</tbody>
</table>
Figure 4-2. Individual participant scatterplots (numbered 1 to 20) of (mean ratio) for (A) corneal nerve length and (B) corneal nerve branch density calculated from every combination of the corneal nerve images, The number of images used to calculate the mean ratio is plotted on the x-axis. The mean ratio, centred around 1, is the calculated mean for the combination relative to the true mean for that participant, ie. of all 16 images.
Figure 4-3. Mean ratio for the different groups of neuropathy for corneal nerve branch density (A) and corneal nerve fibre length (B).
Figure 4-4. Mean ratio for (A) corneal nerve branch density and (B) corneal nerve length for all 20 participants.
Confidence intervals for four quantiles from 80% to 95% are shown for corneal nerve branch density (Figure 4-6 A) and corneal nerve fibre length (Figure 4-6 B). Since the results of the theoretical and the empirical approach were very similar only the ones for the theoretical approach are given since they are likely to be more generalisable and independent of the sample. As the number of images assessed increases from two to 16 the intervals become narrower. The same results in a tabular form are provided in Tables 4-4 and 4-5 for corneal nerve branch density and corneal nerve fibre length respectively. From these, the degree of certainty of being within a specific amount of the mean can be determined. For example, calculating the mean corneal nerve branch density using five randomly selected images (Figure 4-5 A) from the central cornea would provide a mean that would be maximum 29% different of the true mean 80% of the time, or 44% different of the true mean 95% of the time. Using eight randomly chosen images, however, increases the likelihood of a better proximity to the true mean; 95% of the time the mean corneal nerve branch density will be within 30% of the true mean. Similarly for corneal nerve fibre length, a sampling of five images would ensure the calculated mean was 13% within the true mean 80% of the times sampled. If eight images were used an equivalent accuracy would be achieved 95% of the times sampled for corneal fibre length.

A

Figure 4-5. Average corneal nerve branch density (CNDB) (A) and corneal nerve fibre length (CNFL) (B) plotted against the number of images used to calculate the mean.
Figure 4-6. Confidence intervals (80%, 85%, 90% and 95%) of mean ratio for corneal nerve branch density (A) and corneal nerve fibre length (B).
A 95% interval of 0.70 to 1.30 for corneal branch density is obtained through a selection of eight images per person. This means that the estimated mean will be no more than 30% higher or 30% lower than the true mean 95% of the time. The equivalent interval for corneal nerve fibre length is 0.86 to 1.14, i.e. 95% of the time the estimated mean will be no more than 14% higher or lower than the true mean. For varying degrees of accuracy, at the cost of workload, Figure 4-5 and Table 4-3 indicate the number of images required.
Table 4-4. Confidence intervals calculated by the theoretical approach for different number of images measured relative to the mean of 16 images for corneal nerve branch density.

<table>
<thead>
<tr>
<th>No of images</th>
<th>95%</th>
<th>90%</th>
<th>85%</th>
<th>80%</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>1.79 -0.21</td>
<td>1.67-0.33</td>
<td>1.58-0.42</td>
<td>1.52-0.48</td>
</tr>
<tr>
<td>3</td>
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<td>1.46-0.54</td>
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</tr>
<tr>
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</tr>
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<td>1.33-0.67</td>
<td>1.29-0.71</td>
</tr>
<tr>
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<td>1.32-0.68</td>
<td>1.28-0.72</td>
<td>1.25-0.75</td>
</tr>
<tr>
<td>7</td>
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<td>1.29-0.71</td>
<td>1.25-0.75</td>
<td>1.22-0.78</td>
</tr>
<tr>
<td>8</td>
<td>1.30-0.70</td>
<td>1.25-0.75</td>
<td>1.22-0.78</td>
<td>1.20-0.80</td>
</tr>
<tr>
<td>9</td>
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<td>1.22-0.78</td>
<td>1.19-0.81</td>
<td>1.17-0.83</td>
</tr>
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<td>1.13-0.87</td>
</tr>
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<td>1.11-0.89</td>
</tr>
<tr>
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<td>1.12-0.88</td>
<td>1.11-0.89</td>
<td>1.09-0.91</td>
</tr>
<tr>
<td>14</td>
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<td>1.10-0.90</td>
<td>1.08-0.92</td>
<td>1.07-0.93</td>
</tr>
<tr>
<td>15</td>
<td>1.08-0.92</td>
<td>1.07-0.93</td>
<td>1.06-0.94</td>
<td>1.05-0.95</td>
</tr>
</tbody>
</table>
Table 4-5. Confidence intervals calculated by the theoretical approach for different number of images measured relative to the mean of 16 images for corneal nerve fibre length.

<table>
<thead>
<tr>
<th>No of images</th>
<th>95%</th>
<th>90%</th>
<th>85%</th>
<th>80%</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>1.35-0.65</td>
<td>1.30-0.70</td>
<td>1.26-0.74</td>
<td>1.23-0.77</td>
</tr>
<tr>
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<td>1.15-0.85</td>
</tr>
<tr>
<td>5</td>
<td>1.20-0.80</td>
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<td>1.14-0.86</td>
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</tr>
<tr>
<td>6</td>
<td>1.17-0.83</td>
<td>1.14-0.86</td>
<td>1.13-0.87</td>
<td>1.11-0.89</td>
</tr>
<tr>
<td>7</td>
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<td>1.13-0.87</td>
<td>1.11-0.89</td>
<td>1.10-0.90</td>
</tr>
<tr>
<td>8</td>
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<td>1.10-0.90</td>
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</tr>
<tr>
<td>9</td>
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<td>1.09-0.91</td>
<td>1.08-0.92</td>
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<tr>
<td>10</td>
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<td>1.08-0.92</td>
<td>1.07-0.93</td>
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<tr>
<td>11</td>
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<td>1.08-0.92</td>
<td>1.07-0.93</td>
<td>1.06-0.94</td>
</tr>
<tr>
<td>12</td>
<td>1.08-0.92</td>
<td>1.06-0.94</td>
<td>1.06-0.94</td>
<td>1.05-0.95</td>
</tr>
<tr>
<td>13</td>
<td>1.06-0.94</td>
<td>1.05-0.95</td>
<td>1.05-0.95</td>
<td>1.04-0.96</td>
</tr>
<tr>
<td>14</td>
<td>1.05-0.95</td>
<td>1.04-0.96</td>
<td>1.04-0.96</td>
<td>1.03-0.97</td>
</tr>
<tr>
<td>15</td>
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<td>1.03-0.97</td>
<td>1.03-0.97</td>
<td>1.02-0.98</td>
</tr>
</tbody>
</table>
4.5 Discussion

The purpose of this study was to present a technique for quantifying the number of random central corneal nerve plexus images required for a predetermined level of accuracy. A further objective was to assess the number of images which need to be assessed for achieving an acceptable level of accuracy for the two characteristics of interest in our study, namely corneal nerve branch density and corneal nerve fibre length. For a chosen level of confidence, the accuracy of the two corneal nerve parameters was calculated, and the number of images required for this level of accuracy was determined. A balance between desired accuracy and logistical issues such as time to capture and analyse can be determined.

The required level of accuracy depends on several factors and often is a balance between it and the expense of labour and time (e.g. person-hours) involved to analyse additional samples. Each image takes approximately 5 minutes to analyse, hence to obtain a true mean for one individual, approximately 80 minutes. Figure 4-7 shows the distance from the true mean relative to the time saved by analysing fewer images. In the current study, a decision was made by the authors that eight images per person would give a sufficiently accurate mean of corneal nerve branch density, the parameter with the greatest variation. The authors propose the selection of eight images, which provides a 95% confidence interval of 0.70 - 1.30 for branch density, and achieves a balance between accuracy and workload for corneal nerve parameters. With this number of images sampled, the estimated mean will be no more than 30% different from the true mean 95% of the time and represents a 50% time saving. This same number of images analysed for nerve fibre length affords a narrower difference of approximately 14% from the true mean, hence giving us increased degree of confidence. An estimated mean of nerve branch density that was close to 30% different from the true mean would not be
considered reasonable, so it unlikely that branch density will be used as a ‘stand-alone’ surrogate for neuropathy.

Figure 4-7. Percent from the true mean as a function of time saving by analysing reduced number of images relative to 16 images. It is assumed that each image takes 5 minutes to analyse, hence 16 images, 80 minutes. Time saving (%) is calculated as the reduction in time by analysing less images relative to 80 minutes.

To date investigators differ in their sampling and quantification processes. If just one or two images per individual to calculate nerve parameters are used (Oliveira-Soto and Efron 2001; De Cilla, Ranno et al. 2009; Patel, Tavakoli et al. 2009) this study has demonstrated that the estimate for corneal nerve branch density should be within 79% of the true mean 95% of the time, or 52% of the mean 80% of the time. Similarly three or five images per individual used to quantify nerves (Kallinikos, Berhanu et al. 2004; Mehra, Tavakoli et al. 2007; Niederer, Perumal et al. 2007) could be up to 62% from the true mean. The decision on the number used was typically determined by the authors, subjectively, taking into account the limitations of the method of capture and instrument used. For example, using the Tomey
instrument, z-axis scans are performed, then the best few images available of the nerve fibre plexus is utilised. With the Heidelberg instrument, many individual sections at the level of Bowman’s membrane can be easily captured, such that in a 5-minute examination, 100 images of analysis quality could easily be captured.

Parameters that have a subjective nature to the measurement technique will inevitably have a wider confidence interval than those determined in an objective way. Corneal nerve branch density, where an observer must identify and select each branch in the nerve path is likely to have wider confidence intervals than corneal nerve fibre length, where few, clearly defined nerve fibres will account for most of the fibre length mean and less clearly defined fibres (and thus more subjective ones) will account for a relatively low proportion of the mean. In our experience less clearly defined branches will account for a relatively greater proportion of the overall mean such that branch density mean will demonstrate a greater degree of noise. As automated algorithms are developed for analysis of these corneal nerve images (Dabbah, Graham et al. 2010), the subjective nature of measurements will be diluted and the measurements will become faster, more objective and thus will lead to the estimation of narrower CIs (better precision). Increased accuracy, however, is yet to be determined. Such programmes are under development by several groups undertaking corneal confocal microscopy. However, currently these procedures have to be performed manually.

A range of disciplines utilise statistical approaches for assessing the number of samples that are required to achieve required accuracy (Good 2000; Hesterberg, Moore et al. 2009). The main difference in these methods with the one employed in this paper is that they (i) either do not have repeated measurements per individual or (ii) they do not fully utilise the repeated measurements available in each individual or sample. In our approach, by using all possible combinations of images for each individual we utilise all available information, since every possible sample is
generated and analysed. Furthermore, we examined the results both using the theoretical distribution based on the t-distribution and the empirical based on the actual quartiles of the distribution produced. Other methods found in the literature do not use such a rigorous approach (Ruffolo and Shakoor 2009).

One basic assumption of the methods used in Ruffolo and Shakoor (Ruffolo and Shakoor 2009) is that the exact population mean and standard deviation are known. It could be argued that hundreds of individuals without diabetes or any corneal or neurological abnormalities be used to determine the true mean. The analyses presented here were performed on 20 participants with type 2 diabetes with 16 images per participant, non-overlapping by more than 25%.

It is conventionally acceptable to use a 95% confidence interval, when estimating parameters from a dataset. In this case, as it can be seen in Figure 4-6 A and Figure 4-6 B, the intervals are narrower for fibre length than they are for branch density. Thus, visually assessing the appropriate number of images based on the desired accuracy for branch density would lead to a greater accuracy than the required one for fibre length. Since this is not an undesired outcome and it would simplify logistical procedures if both were assessed on the same number of images, the selection of number of images to be assessed was based on branch density.

Several other corneal nerve parameters are yet to be investigated such as corneal nerve beading (Grupcheva, Wong et al. 2002; Midena, Cortese et al. 2009), corneal nerve tortuosity (Kallinikos, Berhanu et al. 2004) and nerve fibre width (Grupcheva, Wong et al. 2002; Darwish, Brahma et al. 2007). In this study two highly correlated corneal nerve parameters were calculated from all combinations of two to 16 images for each individual, and a range of confidence intervals determined. Applying this statistical approach to corneal nerve fibre tortuosity, corneal nerve fibre width and corneal nerve beading will reveal the number of images required for a desired level
of confidence and accuracy for each of these variables. Conducting this work in a healthy control population with further our understanding of corneal nerve parameter measurement, and allow comparisons in other neurological disease processes.

4.6 Conclusions

This study is the first to examine the effect of sampling of corneal nerve images from the centre of the cornea to calculate corneal nerve fibre parameters. Sample combination analysis can be used to determine the sampling required for corneal nerve parameters where a true and accurate parameter mean can be agreed. The findings of this paper have implications for researchers investigating corneal nerve parameters and the methodology has applications in other biological sampling studies.
Chapter 5. Wide-field assessment of the sub-basal corneal nerve plexus using a novel mapping technique

5.1 Preface

The previous chapters focused on quantitative aspects of central corneal nerve morphology. This chapter describes the development of a technique aimed at assessing the extensive branching pattern of nerves across a larger proportion of the cornea. Adjacent and other regions of the sub-basal nerve plexus may shed valuable information regarding degeneration of nerves and most studies to date have focused only on quantification of the central corneal region. This paper, accepted by Cornea journal, is as follows:


5.2 Abstract

Purpose: To develop a rapid, optimised technique of wide-field imaging of the human corneal sub-basal nerve plexus.

Methods: A dynamic fixation target was developed and, when coupled with semi-automated tiling software, a rapid method of capturing and montaging multiple corneal confocal microscopy (CCM) images was created. To illustrate the utility of this technique, wide-field maps of the sub-basal nerve plexus were produced in two participants with diabetes; one with and one without neuropathy.
Results: The technique produced montages of the central 3mm of the sub-basal corneal nerve plexus. The maps appear to show a general reduction in the number of nerve fibres and branches in the diabetic participant with neuropathy compared to the individual without neuropathy.

Conclusion: This novel technique will allow more routine and widespread use of sub basal nerve plexus mapping in clinical and research situations. The significant reduction in the time to image the corneal sub-basal nerve plexus should expedite studies of larger groups of diabetic patients and those with other conditions affecting nerve fibres. The inferior whorl and surrounding areas may show the greatest loss of nerve fibres in individuals with diabetic neuropathy, but this should be further investigated in a larger cohort.

5.3 Introduction

Imaging of the in vivo human corneal sub-basal nerve plexus using corneal confocal microscopy (CCM) has become a useful tool in the investigation of both ophthalmic (Moilanen, Vesaluoma et al. 2003; Oliveira-Soto and Efron 2003; Calvillo, McLaren et al. 2004; Patel and McGhee 2006; Darwish, Brahma et al. 2007; Darwish, Brahma et al. 2007) and systemic conditions (Tavakoli, Marshall et al. 2009). In particular, this approach has been used as a novel non-invasive technique for the measurement of diabetic neuropathy (Rosenberg, Tervo et al. 2000; Malik, Kallinikos et al. 2003; Kallinikos, Berhanu et al. 2004; Tavakoli, Quattrini et al. 2010). Most studies to date have focused on the central cornea for assessment of nerve morphology. Chapter 3 and Chapter 4 address two key aspects of nerve parameter measurement – repeatability of measurement and sampling for accuracy. However, the corneal nerves form an extensive branching pattern across the entire cornea, converging in the inferior-central cornea in a whorl like pattern (Patel and McGhee 2005; Patel and McGhee 2008; He, Bazan et al. 2010). There is significant
within-corneal regional variation in this structure (Patel and McGhee 2005), and appreciable structural changes can be observed over a period of as little as six weeks (Patel and McGhee 2008). Therefore, an assessment of the complete corneal nerve plexus may be of considerable value especially if it could define earlier evidence of degeneration or indeed regeneration of corneal nerves (Darwish, Brahma et al. 2007; Mehra, Tavakoli et al. 2007).

Patel and McGhee were the first to image the overall architecture of in vivo sub-basal nerves, and demonstrated this in three healthy volunteers using CCM (Patel and McGhee 2005). They captured images using a fixation grid and used manual mapping software to provide a confluent montage of the central 5-6 mm of the nerve plexus. However, this technique involved image capture “chair time” of 50 minutes and several hours of additional time to manually montage up to 700 black and white images (Patel and McGhee 2005). The extended chair time required to capture enough images to create a map limits the routine use of this approach in many patients, particularly those who are older and have health problems.

We describe a novel, faster technique for mapping the corneal sub-basal nerve plexus and demonstrate the additional clinical potential of this approach in two diabetic patients — one with and one without neuropathy.

5.4 Research Design and Methods

A. Imaging Technique

Laser scanning in vivo CCM was performed on the right cornea of participants using the Heidelberg Retinal Tomograph (HRT3) with Rostock Corneal Module (Heidelberg Engineering GmbH, Heidelberg, Germany). The dimension of images produced using this instrument is 400 µm x 400 µm. The image capture setting of the CCM was set to “sequence”, with a frame rate of five frames per second which gives videos of 20 seconds in length and captures 100 frames.
The cornea of each participant was anesthetised with a drop of 0.4% benoxinate hydrochloride (oxybuprocaine hydrochloride; Minims). The fixation target was placed at a distance of 1.5m from the front of the cornea of the participant, and was a screen-based interactive moving target, made with Flash (Adobe CS4) software (Figure 5-1). The target comprised of 13 target “loops” that each originated and terminated at point A, taking 20 seconds to complete a loop, with the participant tracking the target for the full 20 seconds. The participants were asked to fixate on point A with their contralateral eye, which was the left eye.

Figure 5-1. Sequence of scans to acquire images of the whole corneal plexus. (every second scan is represented by a dotted line to differentiate neighbouring scans). (Image: courtesy Kevin Gosschalk)
The CCM was moved forward until gentle contact was established between the front of the applanation cap and the cornea. The CCM was moved up and down until the inferior whorl or vortex of the nerve plexus of the right eye was in view. The first target loop and video sequence were commenced simultaneously. The starting point for all 13 videos was when point A was fixated and the inferior whorl was in view at the same time, providing a common starting point. As the eye tracked the loop, the CCM captured a video of 20 seconds duration that tracked a two-dimensional strip of the entire corneal nerve plexus. This was repeated until all 13 target loops had been tracked and imaged to produce 13 video sequences with a common starting point, each containing 100 frames that covered a different section of the nerve plexus. Image capture took approximately 15-20 minutes per participant.

Each video was then converted into single image frames and montaged or ‘tiled’ together. This was done in two stages using two different software applications. First, to give an appreciation of the completeness of the map, Image Pro Plus 7.0 software was used to tile the 13 videos of 100 images using an automated function. This took approximately 10 minutes. The full nerve map was then montaged together using Photoshop (Adobe CS4). This process results in some inaccuracies in montaging, but it allowed sufficient mapping of the nerve plexus such that missing sections caused by errors in target fixation as well as loss of contact of the cornea and CCM applanation cap could be identified and repeated immediately, taking approximately 1-2 minutes per loop.

The second stage of montaging was with Photoshop, using the Photomerge panoramic functionality to align and blend the single images together. The purpose of this step was to create the final refined version of the corneal nerve map. Each video was aligned separately and all the strips were pieced together to form a complete map of the nerve plexus (Figure 5-2). A black on white version of the
nerve plexus was traced manually using an interactive digital tablet and pen (Wacom Cintiq 12WX) (Figure 5-3), taking approximately 2 hours per map.

B. Participants

Two diabetic participants consented to wide-field mapping of their corneal nerve plexus using CCM at the Anterior Eye Laboratory, Institute of Health and Biomedical Innovation, Queensland University of Technology. Participants had no history of eye disease, or of systemic disease that may have affected the cornea, and had no other cause of neuropathy apart from diabetes.

Both participants underwent a complete medical examination, as well as an assessment of glycaemic control and lipid profiles. Neurophysiological (peroneal, tibial and sural nerve conduction velocity and amplitude) and neurological (Neuropathy Disability Score (NDS)) examinations - which includes an assessment of vibration perception, pin-prick perception and temperature perception, as well as the presence or absence of ankle reflexes - were undertaken (Table 5-1).

To determine if a masked, trained observer could differentiate nerve plexus deficiency from the black on white maps, 12 maps from individuals with varying duration of diabetes were graded. Observers graded both the central and whorl region of each nerve plexus on a 0-10 scale where 0 indicated no deficiency and 10 indicated marked deficiency. An unpaired t-test was used to compare the scores of those with and without neuropathy and p<0.05 was considered significant.
Table 5-1. Clinical characteristics of participants and results of neurological testing (normal ranges are indicated in parenthesis in left column).

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Participant ID 206MR</th>
<th>Participant ID 120AF</th>
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</thead>
<tbody>
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<td>Age (years)</td>
<td>64</td>
<td>50</td>
</tr>
<tr>
<td>Gender</td>
<td>Female</td>
<td>Male</td>
</tr>
<tr>
<td>Duration of diabetes (years)</td>
<td>6</td>
<td>39</td>
</tr>
<tr>
<td>HbA1c (%) (4-6)</td>
<td>10.7</td>
<td>7.7</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L) (3.9-5.5)</td>
<td>4.2</td>
<td>6.1</td>
</tr>
<tr>
<td>Triglycerides (mmol/L) (0.6-2.0)</td>
<td>0.8</td>
<td>3.9</td>
</tr>
<tr>
<td>HDL Cholesterol (mmol/L) (0.9-1.5)</td>
<td>1.8</td>
<td>0.8</td>
</tr>
<tr>
<td>LDL Cholesterol (mmol/L) (0.0-4.0)</td>
<td>2.0</td>
<td>3.5</td>
</tr>
<tr>
<td>eGFR (mL/min) (&gt;59)</td>
<td>&gt;90</td>
<td>87</td>
</tr>
<tr>
<td>Urine albumin/creatinine ratio (0.0-2.6)</td>
<td>&lt;0.5</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>Neuropathy Disability Score (0-2)</td>
<td>2/10</td>
<td>7/10</td>
</tr>
<tr>
<td>Peroneal NCV (m/s) (≥43 if &lt; 54 years; &gt;37 if ≥ 54 years)</td>
<td>41.5</td>
<td>35.1</td>
</tr>
<tr>
<td>Tibial NCV (m/s) (&gt;42)</td>
<td>44.0</td>
<td>39.7</td>
</tr>
</tbody>
</table>

HbA1c glycosylated haemoglobin; HDL high density lipid, LDL low density lipid, NCV nerve conduction velocity, eGFR estimated glomerular filtration rate.

The study was approved by the Queensland University of Technology Human Research Ethics Committee and was conducted in accordance with the tenets of the Declaration of Helsinki.

5.5 Results

Figure 5-2 and Figure 5-3 show a 2-dimensional representation of the central 3 mm of the sub-basal nerve plexus of the two individuals with diabetes - one with
neuropathy (left) and one without neuropathy (right). Both participants had 2-3 ‘loops’ missing that were identified and then repeated after the first montaging stage.

Figure 5-2 shows the raw montaged map from each patient. These maps demonstrate a complex network of branching and anastomosing corneal nerves in the central 3 mm of the cornea which appear as a series of fine white lines (long thin arrow) converging on the inferior whorl (short arrow). Also evident in both these images are artifacts (long thick arrow) caused by contact and pressure of the Tomocap against the cornea during the examination. These artifacts appear as broad linear structures, some with apparent branching throughout the field, which should not be interpreted as large nerve fibres. Figure 5-3 shows the black on white traced montage of nerve fibres from each participant.

Figure 5-2. Montage and corneal map of the whole corneal nerve plexus from a type 1 diabetic participant with (a) and without (b) neuropathy. Corneal nerves - long thin arrow; inferior whorl - short arrow; image artifacts - long thick arrow. (Images: courtesy Dr Katie Edwards)
Figure 5-3. Traced maps of the montage of whole corneal nerve plexus from type 1 diabetic participant with (a) and without (b) neuropathy. Further examples of montage and traced maps are shown in Appendix G. (Images: courtesy Dr Katie Edwards)

To define the changes in the corneal plexus morphology related to diabetic neuropathy, a type 1 diabetic participant without neuropathy was compared to a type 1 participant with severe neuropathy with an NDS of 7. The individual with severe neuropathy had a marked reduction in both peroneal and tibial nerve conduction velocities and amplitudes and an unattainable sural nerve response (Table 5-1). The participant with neuropathy was older and had a longer duration of diabetes with a past history of retinopathy, though there was no evidence of nephropathy (Table 5-1). Interestingly, although the current HbA1c was better in the participant with neuropathy, the lipid profile was much worse as evidenced by a higher total cholesterol, LDL and triglycerides with a lower HDL.
Comparison of the two maps shows an overall reduction in the number of nerve fibres in the diabetic patient with neuropathy. The density of the entire nerve plexus is reduced and branching is markedly reduced. Overall there appears to be fewer nerve fibres in the inferior and temporal sectors and the inferior whorl appears to be affected more severely.

Twelve maps from individuals with varying degree of duration of diabetes (0-52 years) were graded by a trained observer with regard to nerve plexus deficiency in both the central region and the whorl (Figure 5-4). The mean grade of the nerve plexus deficiency in the central and whorl regions of the neuropathy group was higher than that of the no neuropathy group, but not statistically so. Table 5-2 shows the participant characteristics of the sample used for this pilot study and Figure 5-4 shows the maps graded.

Table 5-2. Clinical characteristics of participants and results of nerve plexus grade and neurological testing (normal ranges are indicated in parenthesis in left column).

<table>
<thead>
<tr>
<th></th>
<th>No neuropathy</th>
<th>Neuropathy*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>43 ± 3</td>
<td>59 ± 5</td>
</tr>
<tr>
<td>Gender</td>
<td>F5 M2</td>
<td>F1 M4</td>
</tr>
<tr>
<td>Duration of diabetes (years)</td>
<td>10 ± 10</td>
<td>35 ± 17</td>
</tr>
<tr>
<td>HbA1c (%) (4-6)</td>
<td>6.7 ± 1.2</td>
<td>8.2 ±1.6</td>
</tr>
<tr>
<td>Abnormal peroneal NCV</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Abnormal sural NCV</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>Central nerve plexus deficiency (0-10)</td>
<td>3.4 ± 2.9</td>
<td>5.0 ± 2.5$</td>
</tr>
<tr>
<td>Whorl nerve plexus deficiency (0-10)</td>
<td>3.7 ± 2.5</td>
<td>4.5 ± 2.2$</td>
</tr>
</tbody>
</table>

Neuropathy by San Antonio criteria. HbA1c glycosylated heamoglobin; NCV nerve conduction velocity $ P>0.05$ unpaired t-test.
Abnormal peroneal and sural NCV are compared to lab normal ranges are shown in Table 5-3.

Table 5-3. Nerve conduction cut-off values to classify participants as ‘normal’ or ‘abnormal’ in terms of their electrophysiological response.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Cut-off (m/s)(^a,b)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Age &lt; 54</td>
</tr>
<tr>
<td>Peroneal NCV ankle to fh</td>
<td>&lt; 45 m/s</td>
</tr>
<tr>
<td>Peroneal NCV fh to pf</td>
<td>&lt; 45 m/s</td>
</tr>
<tr>
<td>Sural NCV</td>
<td>&lt; 40 m/s</td>
</tr>
<tr>
<td>Tibial NCV</td>
<td>&lt; 43 m/s</td>
</tr>
</tbody>
</table>

\(^a\) < 10\(^{th}\) percentile for healthy individuals without neuropathy at Brisbane site.

\(^b\) Nerve conduction (NC) is considered abnormal if either peroneal or sural nerve conduction velocity (NCV) is below age-reference cut-off values.

\(^c\) If sural not present, NCV is considered abnormal if tibial NCV is below 43 m/s for any age.
Figure 5-4. Maps of the corneal nerve plexus from participants with type 1 diabetes with and without neuropathy. (Images: courtesy Dr Katie Edwards and Nicola Pritchard)
5.6 Discussion

The original nerve mapping technique developed by Patel and McGhee (Patel and McGhee 2005) was a significant advance in imaging the human corneal nerve plexus. However, we found it challenging to replicate their technique in our laboratory as we found the chair time needed for image capture was too long for many participants and the image montaging was prohibitively resource-intensive. Another difficulty was an inability to accurately determine the area of cornea being assessed and indeed Patel and McGhee (Patel and McGhee 2009) acknowledge this issue and attribute it to disconjugate eye movements, microsaccades, involuntary participant movements and difficulty aligning the center of the grid with the corneal apex. This makes montaging images particularly troublesome given the uniformity of the black and white images.

We have addressed some of these issues using a dynamic target and video instead of single frame image capture. Additionally, the inferior whorl was used as a unique landmark to initiate and terminate the video capture sequence, making montaging substantially easier. The use of faster, automated software has also lead to montaging being performed almost in real time to enable identification of missing sections and immediate reimaging.

One disadvantage of the technique is the artifact induced in the nerve images due to corneal compression. Anecdotally, even an extremely experienced clinician cannot avoid these artifacts using the dynamic target and capture method. Heidelberg did introduce a non-contact version of the objective, however, in our experience, is unacceptable for imaging the corneal nerve plexus.

Zhivov et al (Zhivov, Blum et al. 2010) recently described a technique to generate maps of the \textit{in vivo} human sub basal nerve plexus in real time. However, this was using online, specialised software. As well, significant gaps were present in the
published maps. The technique we have developed uses commercially available equipment and software and hence, could be replicated easily. Additionally, the novel use of a dynamic fixation target with pre-determined target areas gives more accurate and complete coverage of the areas of interest, making the method less operator-dependent, which has previously been acknowledged as a limitation of mapping techniques (Zhivov, Blum et al. 2010).

This is the first time that wide-field maps of the corneal nerve plexus have been generated using in vivo CCM in patients with diabetic neuropathy. We confirm previous studies which have shown a reduction in corneal nerve fibre and branch density in the central cornea (Quattrini, Tavakoli et al. 2007; Tavakoli, Quattrini et al. 2010). In addition, the nerve maps we have generated provide insights into changes in a large proportion of the corneal nerve plexus, with significant loss of nerve fibres, particularly, in the inferior cornea. It appears that the inferior whorl and surrounding areas may show the greatest loss of nerve fibres and should therefore be targeted to increase the sensitivity of CCM as a measure of diabetic neuropathy. It is also possible a broader region of the cornea must be examined to fully appreciate nerve loss and make a sound diagnosis regarding the neuropathy status of an individual.

This technique is representing just one third of the entire corneal area. The suggested approach, however, needs to be investigated in a larger cohort with further quantitative assessment of the maps. Furthermore, as neuropathic changes do not affect all nerves equally (Llewelyn, Gilbey et al. 1991; Herrmann, Griffin et al. 1999), in vitro studies may be able to elucidate the best approach and corneal location to apply CCM as a marker of diabetic neuropathy.

The overall significant reduction in the time taken to image the whole corneal nerve plexus (2 hours vs. 2 weeks) should expedite studies of larger groups of patients and control participants. It offers several advantages over the current technique of only imaging the central cornea. This is particularly relevant in assessing nerve
damage and subsequent repair localised to a particular part of the cornea following refractive surgery (Darwish, Brahma et al. 2007; Darwish, Brahma et al. 2007), where an assessment of the entire plexus may provide additional insights into nerve regeneration. Furthermore, if the inferior cornea does indeed have a greater predilection for nerve damage, as indicated in our preliminary analysis, then perhaps imaging this area may enable detection of earlier nerve damage in diabetic (Malik, Kallinikos et al. 2003) and other neuropathies (Tavakoli, Marshall et al. 2009; Tavakoli, Marshall et al. 2010).

The pilot study conducted on 12 individuals with and without neuropathy revealed the potential of assessing the central and whorl regions of the cornea for evaluation of nerve plexus deficit. Although no statistical difference was observed on this small sample, the mapping of the corneal nerve plexus appears to have value and is worthy of investigating in greater numbers of participants with additional quantitative assessments.

In conclusion, we describe a novel, faster technique for mapping the corneal sub-basal nerve plexus and demonstrate the additional clinical potential of this approach. The significant reduction in the time to image the corneal sub-basal nerve plexus should expedite studies of large groups of individuals with conditions affecting the nerve fibres. The inferior whorl and surrounding areas may show the greatest loss of nerve fibres in individuals with diabetic neuropathy, but this should be further investigated in a larger cohort.
Chapter 6. Corneal sensitivity as an ophthalmic marker of diabetic neuropathy

6.1 Preface

The earlier chapters explore aspects of observation and analysis of the anatomical features of the corneal sub-basal nerve plexus. Repeatable and accurate measurement of clinical parameters provides vital information necessary when pursuing alternative applications for a procedure, in these instances corneal nerves imaged with CCM as a marker of diabetic neuropathy. A presumed correlate of corneal nerve anatomy is corneal sensitivity. As demonstrated by work in the earlier chapters, the cornea is a highly innervated tissue. Damage to the nerves supplying the cornea has been shown to result in reduction in corneal sensation (Chang-Ling, Vannas et al. 1990). A reduction in corneal sensitivity has been noted with increasing severity of damage to nerves due to diabetes (Malik, Kallinikos et al. 2003), therefore, the established technique of non-contact corneal aesthesiometry (NCCA) has the potential to serve as an important, non-invasive, sensitive measure of nerve fibre damage and repair in human diabetic neuropathy along side CCM. This chapter reflects a study published in Optometry and Vision Science, the journal of the American Academy of Optometry:


6.2 Abstract

Purpose. The objective of this study was to explore the discriminative capacity of non-contact corneal aesthesiometry (NCCA) when compared with the neuropathy
disability score (NDS) score - a validated, standard method of diagnosing clinically significant diabetic neuropathy.

Methods. Eighty-one participants with no history of ocular disease, trauma or surgery, and no history of systemic disease which may affect the cornea, were enrolled. Participants were ineligible if there was history of neuropathy due to non-diabetic causes or current diabetic foot ulcer or infection. Corneal sensation threshold was measured on the hand-dominant side eye at a distance of 10 mm from the centre of the cornea using a stimulus duration of 0.9 seconds. The NDS was measured producing a score ranging from 0–10. To determine the optimal cut-off point of corneal sensation threshold that identified the likely presence of neuropathy (diagnosed by NDS), the Youden index and ‘closest-to-(0,1)’ criteria were used.

Results. The receiver operator characteristic curve for NCCA for the presence of neuropathy (NDS ≥ 3) had an area under the curve of 0.73 (AUC null hypothesis = 0.5, P=0.001) and for the presence of moderate neuropathy (NDS ≥ 6), 0.71 (AUC null hypothesis = 0.5, P=0.003). Using the Youden index, for an NDS ≥ 3 the sensitivity of NCCA was 70% and specificity was 75%, and a corneal sensation threshold of 0.66 mbars or higher indicated the presence of neuropathy. When NDS ≥ 6 (indicating risk of foot ulceration) was applied, the sensitivity was 52% with a specificity of 85%.

Conclusions. NCCA is a sensitive test for the diagnosis of minimal and more advanced diabetic neuropathy and may serve as a useful surrogate marker for diabetic and perhaps other neuropathies.

6.3 Introduction

Diabetic neuropathy is a significant and prevalent complication of diabetes that has no effective treatment once established, and can ultimately result in foot ulceration
Corneal sensitivity as an ophthalmic marker of diabetic neuropathy

and lower extremity amputation (Abbott, Vileikyte et al. 1998). Current preventative therapies are primarily aimed at maintaining near normal glycaemia control, as poor control has been shown to be a significant risk factor in the severity of the disease (Dyck, Davies et al. 1999). While a number of disease-modifying agents are currently under investigation their efficacy is as yet unproven. Diabetic patients with minimal neuropathy (normal electrophysiology and quantitative sensory tests) show significant unmyelinated fibre (Malik, Tesfaye et al. 2005) and intraepidermal nerve fibre degeneration (Quattrini, Tavakoli et al. 2007; Umapathi, Tan et al. 2007; Loseth, Stalberg et al. 2008). However, these current methods of assessing nerve morphology, such as sural nerve or foot-punch biopsy are invasive and painful (Malik, Veves et al. 2001).

The cornea derives its innervation from the ophthalmic division of the trigeminal nerve and contains primarily Aδ and unmyelinated C fibres. The same class of fibres assessed both histologically and functionally in the foot are impaired in diabetic neuropathy (Loseth, Stalberg et al. 2008). Using corneal confocal microscopy (CCM), Malik and co-workers were the first to describe the significant association between corneal nerve fibre damage and neuropathic severity in diabetic patients (Malik, Kallinikos et al. 2003). Since 2003, investigations have further explored this relationship (Kallinikos, Berhanu et al. 2004; Hossain, Sachdev et al. 2005; Iqbal, Kallinikos et al. 2005; Quattrini, Kallinikos et al. 2005; Quattrini, Tavakoli et al. 2007; Tavakoli, Kallinikos et al. 2007; Tavakoli, Kallinikos et al. 2007; Lalive, Truffert et al. 2009; Tavakoli, Marshall et al. 2009; Tavakoli, Quattrini et al. 2010) and quantification techniques (Dabbah, Graham et al. 2010; Efron, Edwards et al. 2010), described in Chapters 3 and 4. Malik and co-workers (Malik, Kallinikos et al. 2003) also demonstrated a reduction in corneal sensation with increasing neuropathic severity (Tavakoli, Kallinikos et al. 2007). Therefore, the relatively new technique of non-contact corneal aesthesiometry (NCCA) has the potential to serve as an important, non-invasive,
sensitive measure of nerve fibre damage and repair in human diabetic neuropathy (Tavakoli, Kallinikos et al. 2007). A key advantage of NCCA is that it is a simple and rapid clinical procedure that could be undertaken by a trained ophthalmic or medical assistant.

Murphy and co-workers (Murphy, Patel et al. 1996; Murphy, Lawrenson et al. 1998; Murphy, Patel et al. 2004) have described how corneal sensation threshold can be measured using the NCCA. This instrument uses controlled pulses of air of variable pressure to stimulate the cornea and thus measures the corneal nerve threshold to a composite stimulus consisting of air pressure, tear film evaporation and disruption (Murphy, Patel et al. 1996; Murphy, Patel et al. 2001). Differentiation of mechanical and chemical stimuli have further been investigated using a CO₂ aesthesiometer (Belmonte, Acosta et al. 1999).

The advantages of NCCA over the traditional and more conventional von Frey hair or Cochet-Bonnet aesthesiometer (using a fine nylon filament) are that a large, continuous range of stimulus intensity can be produced, the stimulus is more precise, testing is less variable than when using a filament, and there is minimal patient apprehension (Murphy, Patel et al. 1996). The NCCA can assess the corneal sensation threshold in an accurate and repeatable manner (Murphy, Lawrenson et al. 1998). Murphy et al (Murphy, Lawrenson et al. 1998) have also determined that NCCA allows measurement of lower stimulus thresholds than Cochet-Bonnet aesthesiometry.

The potential costs of diabetic neuropathy could be minimised if NCCA proves effective at diagnosing and monitoring the progression of diabetic neuropathy as people at risk can be targeted early for improved glycemic control. The aim of this study was to explore the discriminative capacity of NCCA for predicting the outcome
of the neuropathy disability score (NDS) - a validated clinical method for diagnosing minimal to advanced neuropathy.

6.4 Methods

A. Participants

Eighty-one patients with type 2 diabetes were recruited from the Department of Diabetes and Endocrinology at Princess Alexandra Hospital in Brisbane, Australia, and were enrolled after obtaining written informed consent. All diagnoses were made by endocrinology consultants from this department and patients with type 1 diabetes, gestational diabetes or impaired glucose metabolism were not included. Patients were excluded if they had a history of ocular trauma or surgery, ocular disease or systemic disease affecting the cornea; systemic disease (e.g., malignant disease, large fibre neuropathy, congestive heart failure, major psychosis); history of neuropathy of non-diabetic cause, current diabetic foot ulcer or infection or participation in any interventional research trial. Participant characteristics are shown in Table 6-1. Ethical clearance was granted by the Princess Alexandra Hospital and Queensland University of Technology Research Ethics Committees and the study was conducted in accordance with the principles of the Declaration of Helsinki.

B. Corneal sensitivity assessment

An NCCA was designed and constructed for the Institute of Health and Biomedical Innovation's (IHBI) Anterior Eye Lab (Figure 6-1) based on the original work of Murphy and co-workers (Murphy, Patel et al. 1996).
The instrument was employed to measure corneal sensitivity using a modified Garcia-Perez staircase technique (Golebiowski, Papas et al. 2005) to determine the corneal sensation threshold. NCCA was selected as the preferred method for assessing corneal sensitivity due to its repeatability and precision advantages over the Cochet-Bonnet test (Murphy, Patel et al. 1996; Murphy, Lawrenson et al. 1998; Tavakoli, Kallinikos et al. 2007). The test was explained to the participant, and the eye on the side of hand dominance was measured using a stimulus duration of 0.9 seconds in a quiet room without distractions. The participant was instructed to fixate on a target, and the 0.5 mm nozzle tip distance was adjusted such that it was 10 mm from the centre of the cornea (measured intermittently throughout testing using a horizontal rule). The participant was provided with training prior to conducting the test and a stimulus well above expected threshold (6 mbar) was presented to demonstrate the sensation of the stimulus. The participant was asked to blink immediately prior to the stimulus presentation to avoid lid artefacts and improve attention. Pressure adjustment was made using a mechanical valve on the NCCA. A response was required for each presentation, but if the participant was unsure or felt the sensation on the eyelids or eyelashes, the stimulus was recorded as “not
felt”. If the stimulus was not felt the pressure was increased by 0.20 mbar, and if felt, decreased by 0.15 mbar, and a change from “felt” to “not felt” constituted a reversal point. An average of the last two of four reversals was taken as the recorded threshold measure. The minimum or maximum pressures produced by the NCCA were recorded if, after retraining, the participant still indicated feeling the minimal pressure of 0.3 mbar or not feeling the maximal pressure of 17 mbar (respectively). If the participant responded “felt” to at least two out of three false positive (presentation of no, or sham stimulus) after retraining, they were considered unreliable and excluded from analysis. The NCCA user manual included examples of the staircase procedure and the standard operating procedure, including calibration procedure, are shown in Appendix F. No participants’ data needed to be removed because of unreliability in the study.
Table 6-1. Study participant characteristics.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>Female 29&lt;br&gt;Male 52</td>
</tr>
<tr>
<td>Age (years mean ± SD, range)</td>
<td>60 ± 8 (37 - 71)</td>
</tr>
<tr>
<td>Duration of diabetes (years)</td>
<td>13 ± 9 (1 – 42)</td>
</tr>
<tr>
<td>Insulin dependence</td>
<td>Yes 31 (38%); No 47 (58%); Not recorded 3 (4%)</td>
</tr>
<tr>
<td>Corneal sensitivity (mbars, mean ± SD, range)</td>
<td>0.91 ± 0.63 (0.25 – 4.00) by NCCA</td>
</tr>
<tr>
<td>Eye used for testing</td>
<td>Right 66 / Left 15</td>
</tr>
<tr>
<td>Average neuropathy level (NDS units, mean ± SD, range)</td>
<td>4 ± 3 (0 - 10) by NDS</td>
</tr>
<tr>
<td>Presence of neuropathy (Young, Boulton et al. 1993) (NDS ≥ 3)</td>
<td>Present 53 (65%); Absent 28 (35%)</td>
</tr>
<tr>
<td>Risk of foot ulceration (Abbott, Carrington et al. 2002) (NDS ≥ 6)</td>
<td>Present 27 (33%); Absent 54 (67%)</td>
</tr>
</tbody>
</table>
C. Neuropathy assessment

The neuropathy disability score (NDS) is based on a clinical scoring system obtained by establishing vibration perception using a 128Hz tuning fork; pin-prick perception using Neurotip™ (Owen Mumford Ltd, Oxford, UK) on the plantar aspect of the big toe; temperature perception using warm and cold metal rods on the central plantar surface (arch) of both feet and the presence or absence of ankle reflexes using a tendon hammer. Both feet were examined such that each abnormal response afforded a 1-point increment in score, producing a score ranging from 0–10, whereby NDS of 2 or less indicates ‘no neuropathy (Young, Boulton et al. 1993).

All participants underwent a detailed clinical history and examination to rule out any other cause of neuropathy. NDS was carried out in a masked fashion to avoid inadvertent experimenter bias; NDS and NCCA examinations were conducted by different investigators and the NCCA investigator was masked with respect to NDS status of the participant. The coefficient of variation with NCCA has been reported to be 5.6% (Tavakoli, Marshall et al. 2010).

D. Statistical method

To investigate the discriminative capacity of NCCA the sensitivity and specificity (Altman and Bland 1994) of the test were determined against the diagnosis of different grades of neuropathy defined using the NDS. Sensitivity is defined as the proportion of true positives and specificity as the proportion of true negatives that are correctly identified by the test. The receiver operating characteristic (ROC) curve is a plot of sensitivity versus false positive rate (1 – specificity), and can be used to evaluate the effectiveness of a new test compared to a validated test for disease status and thus can used to compare the performance of two or more tests (Altman and Bland 1994; Kaivanto 2008).
The area under the ROC curve, a plot of sensitivity versus false positive rate (1 – specificity), represents an overall performance of the test – a measure of diagnostic accuracy (Zweig and Campbell 1993). In this study ROC curves were derived using commercially available software (SPSS Inc, Chicago, Illinois, USA) from values of corneal sensation threshold to determine the level of sensation which best agrees with determination of neuropathy using NDS, an established clinical measure which has been used to stratify patients with differing severity of neuropathy (Young, Boulton et al. 1993). Confidence intervals for sensitivity and specificity were also calculated. Two different diagnostic levels of NDS were used to define different grades of neuropathy to allow the assessment of the ability of NCCA to detect minimal neuropathy (NDS ≥ 3) (Young, Boulton et al. 1993) and more advanced neuropathy (NDS ≥ 6) associated with a risk of foot ulceration (Abbott, Carrington et al. 2002). Statistical significance was defined as p value of less than or equal to 0.05.

To determine the optimal cut-off point of corneal sensation threshold that identifies the presence of neuropathy (diagnosed by NDS), two methods were used: the ‘closest-to-(0,1)’ criterion and the Youden index (Perkins and Schisterman 2006; Kaivanto 2008). The closest-to-(0,1) point is determined using a formula for the radius of a circle with centre at (0,1), that is the top left corner of the plot, which is a tangent to the ROC curve and found by solving the equation of the circle:

\[ r^2 = x^2 + y^2 - 1 \]

for sensitivity (y) and 1-specificity (x) which minimise the radius (r) of the circle. The Youden index measures how far away from the diagonal each point on the ROC curve is located. It is estimated as:

\[ (\text{sensitivity} + \text{specificity} - 1) \]
for each cut-off point; the maximum value is the point of interest (Perkins and Schisterman 2006). The Youden index is currently advocated as the preferred method, as it indicates a cut-point as far away from the diagonal (i.e. which represents a test with random diagnosis) as possible (Perkins and Schisterman 2006; Kaivanto 2008).

To further assess the usefulness of the test, positive predictive values (PPV) and negative predictive values (NPV) (Altman and Bland 1994) were calculated using a proposed NCCA cut-off for neuropathy. The positive predictive value is the proportion of participants with positive test results (i.e., corneal sensation threshold $\geq$ cut-off in mbar) who are correctly diagnosed with neuropathy. The negative predictive value is the proportion of participants with negative test results (i.e., corneal sensation threshold $< \text{cut-off in mbar}$) who are correctly diagnosed without neuropathy.

Regression analysis was performed to determine if corneal sensation threshold was associated with corneal nerve parameters in the same individuals.

6.5 Results

The ROC curves show the discriminative capacity of NCCA as a marker for neuropathy and risk of foot ulceration in a group of people with type 2 diabetes (Figure 6-2). If NCCA were a perfect test, one which correctly identifies 100% of the population with or without neuropathy, the ROC curve would have an area under the curve of 1.0. The ROC curve for NCCA as a diagnostic marker for neuropathy (NDS $\geq 3$) had an area under the curve (AUC) of 0.73 ($P=0.001$) and 0.71 when NDS $\geq 6$, that is, when used as a marker for risk of ulceration ($P=0.003$, Table 6-2).
Figure 6-2. Receiver operator characteristic (ROC) curves illustrating the diagnostic performance of the NCCA against NDS ≥ 3 (solid line) and NDS ≥ 6 (dashed line) in 81 participants with type 2 diabetes.

From the ROC curve data the Youden indices and closest-to-(0,1) cut-points were calculated to find the optimal threshold of corneal sensation threshold at which neuropathy would be diagnosed. Using the Youden index, the sensitivity of NCCA was 70% (specificity 75%) when NDS ≥ 3. An identical optimal threshold was calculated using the closest-to-(0,1) method. When an NDS ≥ 6 (a cut-off indicating
risk of foot ulceration (Abbott, Carrington et al. 2002)) was applied the sensitivity was 52% with a specificity of 85%. The (0,1) method indicated a lower threshold cut-off for NCCA, hence higher sensitivity and lower specificity (63% and 69% respectively).

Table 6-2. Area under the curve (AUC), P values and NCCA cut-offs using Youden and closest-to-(0,1) methods for NDS ≥ 3 and NDS ≥ 6.

<table>
<thead>
<tr>
<th>Neuropathy</th>
<th>AUC</th>
<th>P value</th>
<th>Youden Cut-off</th>
<th>Closest-to-(0,1) Cut-off</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Index (mbar)</td>
<td>Criteria (mbar)</td>
</tr>
<tr>
<td>NDS ≥ 3</td>
<td>0.731</td>
<td>0.001</td>
<td>0.45 0.66</td>
<td>0.15 0.66</td>
</tr>
<tr>
<td>NDS ≥ 6</td>
<td>0.707</td>
<td>0.003</td>
<td>0.37 1.12</td>
<td>0.24 0.83</td>
</tr>
</tbody>
</table>

NDS neuropathy disability score
Table 6-3 shows the number of participant correctly and incorrectly diagnosed by NCCA using the Youden cut-off of 0.66 mbars. The positive predictive value for NCCA (i.e., the proportion of participants with abnormal corneal sensation threshold who are correctly diagnosed with neuropathy) was 84% for NDS $\geq 3$ and 64% for NDS $\geq 6$. The negative predictive value of NCCA (i.e., the proportion of participants with normal corneal sensation threshold who were correctly diagnosed without neuropathy) was 57% for NDS $\geq 3$ and 78% for NDS $\geq 6$. 
Table 6-3. Frequency of abnormal and normal diagnoses with NCCA using the Youden cut-offs and the gold standards of a) NDS ≥ 3 (presence of neuropathy) and b) NDS ≥ 6 (risk of foot ulceration). Sensitivity and specificity, positive predictive values (PPV) and negative predictive values (NPV) are indicated for each criteria used.

<table>
<thead>
<tr>
<th></th>
<th>Neuropathy (NDS ≥ 3)</th>
<th>Risk of foot ulceration (NDS ≥ 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Present (+)</td>
<td>Absent (-)</td>
</tr>
<tr>
<td>a. NCC ≥ 0.66</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abnormal (+)</td>
<td>37</td>
<td>7</td>
</tr>
<tr>
<td>Normal (-)</td>
<td>16</td>
<td>21</td>
</tr>
<tr>
<td>Total</td>
<td>53</td>
<td>28</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>70%</td>
<td></td>
</tr>
<tr>
<td>Specificity</td>
<td>75%</td>
<td></td>
</tr>
<tr>
<td>b. NCC ≥ 1.12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Abnormal (+)</td>
<td>14</td>
<td>8</td>
</tr>
<tr>
<td>Normal (-)</td>
<td>13</td>
<td>46</td>
</tr>
<tr>
<td>Total</td>
<td>27</td>
<td>54</td>
</tr>
<tr>
<td>Sensitivity</td>
<td>52%</td>
<td></td>
</tr>
<tr>
<td>Specificity</td>
<td>85%</td>
<td></td>
</tr>
</tbody>
</table>

NDS neuropathy disability score
There was negligible association between corneal sensation threshold and corneal nerve parameters, shown in Table 6-4, however, the two corneal parameters are highly correlated (Pearson r = 0.93, P<0.001).

Table 6-4. Correlation coefficient (Pearson r) estimating association between corneal sensation threshold and corneal nerve fibre length and corneal nerve branch density for n=63 observations. Corneal nerve fibre length and corneal branch density were assessed using semi-automated software (CCMetrics, University of Manchester) applied to images captured using the Heidelberg Retina Tomograph with Cornea Rostock Module.

<table>
<thead>
<tr>
<th>Corneal sensation threshold (mbars)</th>
<th>Corneal nerve branch density (#/mm²)</th>
<th>Corneal nerve fibre length (mm/mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.00</td>
<td>0.17 (p=0.09)</td>
<td>0.12 (p=0.169)</td>
</tr>
<tr>
<td>Corneal nerve branch density (#/mm²)</td>
<td>1.00</td>
<td>0.93 (p=&lt;0.001)</td>
</tr>
<tr>
<td>Corneal nerve fibre length (mm/mm²)</td>
<td></td>
<td>1.00</td>
</tr>
</tbody>
</table>

6.6 Discussion

This study has demonstrated that NCCA has sufficient discriminative capacity to be considered a useful test of diabetic neuropathy. The clinical usefulness of NCCA to identify a systemic disease depends on its ability to show high sensitivity (a low false negative rate) and high specificity (low false positive rate). A sensitivity of 70% and specificity of 75% was established for the IHBI NCCA. This means that in patients with neuropathy, seven out of 10 would be correctly identified using NCCA and of those without neuropathy, one in four of people would be misidentified with
neuropathy (specificity 75%, Table 6-3). Of those patients without risk of foot ulceration, NCCA would correctly diagnose 85% (specificity). Of those diagnosed without risk of foot ulceration using NCCA, approximately one in five would be misdiagnosed (NVP 78%). A corneal sensation threshold measured using NCCA of 0.66 mbars or higher indicates the presence of neuropathy in an individual with type 2 diabetes, and if not currently under care, requires medical investigation.

Early detection is important because effective intervention must be aimed at a stage when there is a capacity for the nerve to repair, specifically in the sub-clinical or early phase of identifiable nerve damage. Impaired glucose tolerance (IGT)-related neuropathy may represent the earliest stage of diabetic neuropathy, since several groups have demonstrated that up to 40% of individuals with idiopathic neuropathy have IGT compared to less than 15% in the age-matched general population (Malik, Tesfaye et al. 2005). Therefore, NCCA may well be a suitable screening device for detecting nerve damage even in patients with IGT. However, the disadvantages of low specificity also need to be considered; it may cause the patient needless anxiety, inconvenience in having to undergo further diagnostic tests and may result in considerable personal and health service resources.

NDS has been shown to be clinically useful in detecting neuropathy and in particular to help predict those at risk of foot ulceration (Abbott, Carrington et al. 2002). Quantitative sensory testing (QST) and electrophysiology are more sophisticated and are considered to be more sensitive for diagnosing and staging diabetic neuropathy; however, their utility in detecting early neuropathy where small fibres are damaged is limited, because QST and electrophysiology tests primarily measure large myelinated nerve fibre function. Several recent studies show significant small fibre abnormalities in diabetic patients with normal electrophysiology and QST (Quattrini, Tavakoli et al. 2007; Umapathi, Tan et al. 2007; Loseth, Stalberg et al. 2008). Furthermore, Malik and co-workers (Malik, Tesfaye et al. 2005) have shown
there is no apparent relationship between quantitative sensory testing and unmyelinated fibre pathology.

The wide range of definitions of neuropathy used when investigating the discriminative capacity of new measures of this condition makes comparison with this study difficult. In a systematic review conducted by Dros et al., (Dros, Wewerinke et al. 2009) sensitivity of the 10-gram monofilament test ranged from 41% to 93% in three studies, with differences attributed to site of application of the test, definitions of threshold and populations examined. The sensitivity of NCCA reported in the present study is situated approximately midway in this range, and as the authors indicate, these tests, like NCCA, need clear guidelines on use and an additional test (or tests) for diagnostic accuracy. NeuroQuick, a novel screening device for assessment of small nerve fibre function using cold threshold perception, had a reported area under the ROC curve similar to NCCA (0.76 vs. 0.73, respectively) (Ziegler, Siekierka-Kleiser et al. 2005), however, the definition of neuropathy was quite different. In the present study neuropathy was defined as at least two abnormal responses to a range of motor and sensory tests, including nerve conduction. The Neuropad test measures sweat gland function and is an indicator of autonomic neuropathy (Quattrini, Jeziorska et al. 2008). This test is reported to have a better sensitivity than NCCA (85%) when the NDS cut-off for neuropathy was ≥ 5 (Quattrini, Jeziorska et al. 2008), which suggests that the Neuropad test is superior to NCCA for identifying individuals with risk of foot ulceration. Results from the present study indicate that using NCCA to identify individuals at risk of foot ulceration is not worthwhile with a sensitivity value close to that of chance i.e. 52%. Only half the individuals with the risk of foot ulceration would be correctly identified. Combining this test with another test of neuropathy may reveal it be a useful adjunct. It is possible that NCCA has greater predictive than concurrent validity.
A limitation of the present study is the relatively small sample size of this cohort. This prevented applying a method where a completely unbiased estimate of sensitivity and specificity can be determined. One method of reducing bias is using holdout validation, which involves randomly selecting a subset of observations from the initial sample to form a validation (or testing) set. The remaining observations are used as the prediction (or training) data. However, both samples must be from the same population. Conducting ROC analysis firstly on a prediction sample of, for example 2/3 of the sample then on a holdout sample (e.g. the remaining 1/3) has the advantage of an unbiased estimated of sensitivity and specificity. If the test’s initial performance was determined on only a specific population group (e.g. older individuals with type 2 diabetes), and is subsequently applied to another population (e.g. younger type 1 individuals), the validation results from the holdout sample may differ greatly from the predictive performance. It has been suggested that validation samples contain at least 100 events (e.g. cases of neuropathy) and 100 non-events (e.g. cases with no neuropathy) (Vergouwe, Steyerberg et al. 2005; Toll, Janssen et al. 2008) and of different but related participants compared to the prediction cohort (Altman and Royston 2000). The degree to which the participant groups are related may include patients suspected of the same disease or at risk of the same event (Altman and Royston 2000). NCCA will be applied to additional populations in the future, which may well serve as suitable validation populations.

The lack of association between corneal nerve anatomy and function suggests the nerves observed with in vivo corneal confocal microscopy do not reflect the number and or integrity of the nerve endings eliciting the sensation threshold. Corneal nerve morphology has been shown to be associated with corneal sensation when measured by relatively crude devices such as Cochet-Bonnet aesthesiometer (Rosenberg et al 2000) and in populations with severe levels of corneal hypoesthesia (Patel, Ku et al. 2009; Hamrah, Cruzat et al. 2010). When more
sensitive aesthesiometry is applied, no association between corneal morphology and corneal sensation was observed in Sjogren’s syndrome sufferers, suggesting morphological alterations and inflammatory factors may influence sensation more than presence of nerve fibres (Tuisku, Konttinen et al. 2008).

Developing objective tools or markers for nerve damage is vital to future development and assessment of new therapies for human diabetic neuropathy. Although simple clinical sensory tests or measures of patient symptoms allow clinicians to infer the level of nerve damage, (Meijer, van Sonderen et al. 2000) CCM can directly identify anatomical loss of small nerve fibre integrity, and has recently been shown to be a sensitive ophthalmic marker of diabetic neuropathy (Tavakoli, Quattrini et al. 2010). This study confirms that its putatively associated functional response (loss of corneal sensation as measured by NCCA) can similarly be employed as a marker of neuropathy.

To improve human health, new discoveries or existing technologies applied to different problems must be translated into practical applications. NCCA has been shown to be a relatively sensitive test for the diagnosis of diabetic neuropathy and may well serve as a useful adjunct surrogate marker for diabetic and other neuropathies. Further longitudinal studies are required to determine its true application in investigation of the small fibre neuropathies.

NCCA and other screening tools that show promise as markers of diabetic neuropathy have the scope to enhance the role eye care professionals play in managing complications of diabetes. Eye care practitioners already play a significant role in the diagnosis of a number of systemic disease consequent to the large number of associated ocular manifestations (Nagpal, Goldberg et al. 1977; Cockburn, Gutteridge et al. 1994; Atkin 1996; Jain and Gottlob 2001). It is possible that the first signs of conditions such as diabetes, connective tissue disease (Atkin
1996), sickle haemoglobinopathies (Nagpal, Goldberg et al. 1977), gastrointestinal disorders (Jain and Gottlob 2001) as well as heart disease, migraine, hypertension, and carcinoma (Cockburn, Gutteridge et al. 1994) can present at a routine eye examination. Diagnosing systemic disease through a comprehensive eye examination can lead to improved treatment outcomes and the possibility of lowered overall health care costs through early detection.
Chapter 7. Summary and Conclusion

7.1 Work Embodied in this Thesis

This body of work examines aspects of corneal nerve anatomy and function in the context of employing two novel corneal tests as prospective markers of diabetic neuropathy. Identifying new markers of a disease are important when the current methods of evaluation are invasive, the outcome of the disease is undesirable, early intervention is productive, and to take advantage of therapeutic improvements. The intention of this thesis was threefold – firstly to summarise our current understanding of two new potential corneal markers of diabetic neuropathy; secondly, to explore specific aspects of measurement characteristics of these potential tools, and thirdly to investigate the clinical efficacy of one such tool, corneal sensation threshold, as a marker of diabetic neuropathy - a highly prevalent and debilitating complication of diabetes. This thesis, comprising five publications, forms a growing body of evidence which adds to the current knowledge of functional and anatomical corneal parameters and their suitability as potential markers of a diabetic neuropathy. This thesis provides new information regarding measurement bias of CCM images, describes a unique sampling paradigm and associated accuracy determination using the method of combinations. A novel high-speed corneal nerve mapping procedure has been developed. Application of this procedure has revealed regions of sub-basal nerve plexus that dictate further evaluation as they appear to show earlier signs of damage than the currently assessed central 1 mm² region of the cornea. Finally, this thesis presents well-timed information of the discriminative capacity of corneal sensitivity measured by NCCA as it is explored as a potential marker of diabetic neuropathy.

Automation of analysis of CCM images to obtain corneal nerve morphology data will alleviate or eliminate several of the limitations of manual and semi-automated
systems (Dabbah, Graham et al. 2010). Until these tools are widely available, intra- and inter-observer repeatability remain important aspects of a diagnostic tool (Hulley, Cummings et al. 1988). The objective of the repeatability experiment presented in Chapter 3 was to determine the Bland-Altman repeatability (Bland and Altman 1986) and intra-class correlation coefficients for two observers measuring, on two occasions, corneal nerve fibre length and corneal nerve branch density. Corneal nerve fibre length and branch density assessed by corneal confocal microscopy (CCM) were quantitatively proven to be parameters that can be measured in a repeatable fashion. Therefore, assessing anatomical aspects of corneal nerves has been shown to be a repeatable process in the central cornea when two trained observers use semi-automated analysis. One important finding of this study that can be applied to ongoing studies is that there may be significant inter-observer variability. Use of a single observer for nerve fibre measurement when using semi-automated systems may be appropriate, or, if more than one observer is undertaking such work, steps to reduce inter-observer variability by training and/or statistically normalising the data could be applied.

Achieving the most repeatable system of image analysis has reduced value if the image sampling technique prevents accurate representation of the parameter of interest. Appraisal of accuracy, influenced by sampling methodology, is an important adjunct to understanding observer and instrument measurement bias. The two next studies, presented in Chapter 4 and 5, address a limitation of CCM studies to date with the intention of measuring corneal nerve morphology. Inferences on central corneal nerve parameters have been made from the central area of the cornea representing less than 1 mm$^2$ based on CCM image sizes ranging from 300 to 400 square microns (Zhivov, Stachs et al. 2006). Through the assembly of corneal sub-basal nerve maps using the technique described by Patel and McGee (Patel and McGhee 2005) it has become apparent that repeatedly sampling such a small area
of the central cornea (via the alignment techniques recommended by the manufacturer) may induce measurement error and bias. A technique for quantifying the number of random central corneal images required for a predetermined level of accuracy of any characteristic was determined. The number of images necessary for achieving an acceptable level of accuracy for corneal nerve fibre length and corneal nerve branch density was determined using combination sampling of up to 16 images. A thoughtful sampling of more than five central (8 is suggested), although not completely overlapping, corneal nerve images from CCM provides a reasonable level of accuracy for corneal nerve morphology measures. This sampling method in the central cornea allows measurement of fibre length and branch density with predictable accuracy.

Evaluation of central corneal nerve morphology can be improved using an appropriate sampling paradigm and semi-automated measurement techniques using one or more observers. Development of an improved technique to qualitatively, and eventually quantitatively, assess broader areas of the cornea means that evaluation of the sub-basal nerve plexus moves towards application in a clinical setting. The time required to assess individuals and manage the data to create a map of the corneal sub-basal nerve plexus (Patel and McGhee 2005) limits the routine use of this approach in many patients, particularly those who are older and have health problems. The purpose of the wide-field mapping study, presented in Chapter 5, was to develop a novel, faster technique for a broader appreciation of the corneal sub-basal nerve plexus and to establish the additional clinical potential of this approach. A dynamic fixation target followed by the participant with high-rate video capture of CCM images plus a stitching algorithm in ImageProPlus allowed appreciation of the sub-basal nerve plexus after minutes rather than weeks of data handling. Appreciation of the a broader area of the plexus in individuals with neuropathy suggests the whorl region 1 to 2 mm inferior to the corneal apex may be
a region requiring closer examination in terms of early nerve damage. This novel technique using CCM to map the sub-basal nerve plexus will provide a time-efficient method of assessing the overall architecture of the corneal nerves.

Just as CCM has shown promise as a potential surrogate marker for diabetic neuropathy, NCCA, a non-invasive test of corneal sensitivity, has also been shown to differentiate groups with and without diabetes (Murphy, Patel et al. 2004; Tavakoli, Kallinikos et al. 2007). Furthermore, NCCA appears to be able to stratify individuals with diabetes by neuropathy severity (Tavakoli, Kallinikos et al. 2007). Since the sensitivity and specificity of the test had not been reported, the aim of study presented in Chapter 6 was to explore the discriminative capacity of NCCA for predicting the outcome of the neuropathy disability score, a validated clinical method for diagnosing minimal to advanced neuropathy. Corneal sensation threshold measured by non-contact corneal aesthesiometry (NCCA) was shown to have fair clinical utility in differentiating individuals with and without diabetic neuropathy. The sensitivity and specificity of the test is probably inadequate as a stand-alone diagnostic tool, however the predictive validly of the test when combined with other tests may reveal a useful clinical tool.

Non-invasive surrogate markers are sought in diseases where the endpoint is undesirable (Lassere, Johnson et al. 2007), e.g. foot amputation as late sequelae of diabetic neuropathy. The clinical utility of corneal sensation threshold, measured using NCCA, as a marker of diabetic neuropathy has been shown to be reasonable, yet further evaluation of the test is probably necessary. Accurate and unbiased measurement of cornea nerve morphology imaged by CCM as another marker of diabetic neuropathy has also been demonstrated. These works adds to the current knowledge regarding application of these functional and anatomical corneal parameters and their suitability as potential markers of a diabetic neuropathy.
Summary and Conclusion

There are several practical implications of this work including establishing a benchmark for corneal nerve parameter analysis in terms of repeatability and sampling paradigms. These analyses form the foundation stages for further exploration of the full nerve plexus using novel methods such as wide-field mapping. Anatomical and functional measures of the cornea in health and disease provide clinicians and researchers with necessary benchmarks for assessing the tissue and monitoring change. The knowledge gained by the studies presented will provide a point of reference for longitudinal assessments.

7.2 Future Directions

The studies represented in this thesis are cross-sectional in nature. The longitudinal observational study following the natural history of diabetic neuropathy (LANDMark Study), primarily focused on the ability to detect change in corneal morphology and sensitivity over time, will continue to assess corneal nerve structure and function in terms of their applicability as surrogate measures of neuropathy. In the long run they may serve as potentially useful non-invasive surrogate endpoints in therapeutic trials for diabetic neuropathy. Other analyses will include assessment of risk factors for loss (or gain) of structure and function. Regional analysis of the corneal sub-basal nerve plexus will be further investigated using static and dynamic means. The corneal whorl, the region of the cornea just inferior to the corneal apex representing the distal portion of the corneal nerves, will be explored qualitatively and quantitatively and investment in new mapping techniques will further our understanding of the anterior eye. Developing models where combinations of tests are used to improve predictive validity is also work for the future.

A validation ‘field trial’ could be conducted with CCMs and NCCAs deployed in hospitals around Australia and the performance and utility of this approach evaluated. A cost-benefit investigation of CCM and NCCA compared to traditional
measures of neuropathy may be necessary. Techniques for automated measurement of nerve parameters need to be accelerated, validated, tested, and deployed in clinical settings. Once established as valid and repeatable tests for neuropathy, CCM and NCCA may be used to evaluate the effectiveness of therapies that may be developed in the future.


Bibliography


Bibliography


Bibliography


Appendices

Appendix A: Author Permissions
Appendix B: Journal Version of Relevant Publications
Appendix C: Other Publications During and Prior to Candidature
Appendix D: Ethics Clearances
Appendix E: Case Report Forms
Appendix F: Standard Operating Procedures
Appendix G: Additional Nerve Maps
Statement of Contribution of Co-Authors for Thesis by Published Paper

The authors listed below have certified that:

1. they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
2. they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
3. there are no other authors of the publication according to these criteria;
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<td>Wrote the manuscript</td>
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<td>Nathan Efron*</td>
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<td>Kevin Gosschalk</td>
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</table>

Principal Supervisor Confirmation

I have sighted email or other correspondence from all co-authors confirming their certifying authorship.

Name: Professor Nathan Efron  Signature:  Date: 27/6/11
Statement of Contribution of Co-Authors for Thesis by Published Paper

The authors listed below have certified* that:

1. they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
2. they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
3. there are no other authors of the publication according to these criteria;
4. potential conflicts of interest have been disclosed to (a) granting bodies, (b) the editor or publisher of journals or other publications, and (c) the head of the responsible academic unit, and
5. they agree to the use of the publication in the student’s thesis and its publication on the Australasian Digital Thesis database consistent with any limitations set by publisher requirements.

Publication:


Note: this study was part of the broader LANDMark study, a Longitudinal study Assessing Neuropathy in Diabetes using novel ophthalmic Markers.

<table>
<thead>
<tr>
<th>Contributor</th>
<th>Statement of contribution*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicola Pritchard</td>
<td>Wrote the manuscript, research design, data analysis, LANDMark research design and SOP development, responsible for all ethics clearances for LANDMark, supervised the study</td>
</tr>
<tr>
<td>Signature:</td>
<td></td>
</tr>
<tr>
<td>Date: 10/8/10</td>
<td></td>
</tr>
<tr>
<td>Katie Edwards*</td>
<td>LANDMark research design and SOP development, data collection, contribution to the manuscript</td>
</tr>
<tr>
<td>Dimitrios Vagenas*</td>
<td>Aided with data analysis, contribution to the manuscript</td>
</tr>
<tr>
<td>Ayda M Shahidi*</td>
<td>Data collection, contribution to the manuscript</td>
</tr>
<tr>
<td>Geoff P Sampson*</td>
<td>Aided in data analysis and interpretation of results, contribution to the manuscript</td>
</tr>
<tr>
<td>Anthony W Russell*</td>
<td>LANDMark research design, instrumental in funding of LANDMark, facilitated recruitment of participants, contribution to the manuscript</td>
</tr>
<tr>
<td>Rayaz A Malik*</td>
<td>LANDMark research design, instrumental in funding of LANDMark contribution to the manuscript</td>
</tr>
<tr>
<td>Nathan Efron*</td>
<td>LANDMark research design, awarded funding of LANDMark, supervised the LANDMark project, contribution to the manuscript</td>
</tr>
</tbody>
</table>

Principal Supervisor Confirmation
I have sighted email or other correspondence from all co-authors confirming their certifying authorship.

Name: Professor Nathan Efron  Signature:  Date: 10/8/10
Corneal Markers of Diabetic Neuropathy

NICOLA PRITCHARD, BAppSc(Optom),1 KATIE EDWARDS, PhD,1
AYDA M. SHAHIDI, BOptom,1 GEOFF P. SAMPSON, PhD,1
ANTHONY W. RUSSELL, MD, PhD,2,3 RAYAZ A. MALIK, MD, PhD,4 and
NATHAN EFRON, PhD, DSc1

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http://dx.doi.org/10.1016/S1542-0124(11)70006-4

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Repeatability of Measuring Corneal Subbasal Nerve Fiber Length in Individuals With Type 2 Diabetes


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http://dx.doi.org/10.1097/ICL.0b013e3181eea915
Corneal Sensitivity as an Ophthalmic Marker of Diabetic Neuropathy

Nicola Pritchard*, Katie Edwards†, Dimitrios Vagenas‡, Ayda M. Shahidi‡, Geoff P. Sampson†, Anthony W. Russell§, Rayaz A. Malik‖, and Nathan Efron*
Other Publications

Other Publications During Candidature


7. Efron N, Al-Dossari M, Pritchard N. Confocal microscopy of the bulbar conjunctiva in contact lens wear. Cornea 2009; 21(9); 43-52.


Other Manuscripts Accepted For Publication

nil
Other Manuscripts Submitted and Under Review By Referees


Other Manuscripts Under Revision Following Referees' Reports

nil

Peer-Reviewed Publications Prior to Candidature


10. Fonn D; MacDonald KE; Richter D; Pritchard N. The ocular response to extended wear of a high DK silicone hydrogel contact lens. Clin Exp Optom 2002:85 (3);176-82.

11. du Toit R; Pritchard N; Heffeman S; Simpson T; Fonn D. A comparison of three different scales for rating contact lens handling. Optom Vis Sci 2002:79 (5);313-20.


Book Chapter

Dear Dr Efron

Re: HREC/08/QPAH/58
Ophthalmic Markers Of Diabetic Neuropathy.

On the 1st June 2010 the Metro South Health Service District Human Research Ethics Committee, reviewed the following amendment for the above study and approval was granted:-

- PCIF version 6 dated 4th May 2010

It should be noted that all requirements of the original approval still apply.

If you have any queries, please do not hesitate to contact the Metro South Health Service District Human Research Ethics Committee, Executive Support Officer on (07) 3176 7672.

Best wishes for the progress of the study.

Yours sincerely

Dr Jennifer Fleming
Chair
Metro South Health Service District
Human Research Ethics Committee
Centres for Health Research
Princess Alexandra Hospital

Cc: Nicola Pritchard - Trial Coordinator

Queensland Health

Enquiries to: Metro South Health Service District
Human Research Ethics Committee
Telephone: 07 3176 7672
TTY: 07 3176 7737
Facsimile: 07 3176 7667
Email: PAH_Ethics_Research@health.qld.gov.au
Our Ref: HREC/08/QPAH/58
Date: 1st June 2010
Dear Professor Efron

Re: Longitudinal Assessment of Neuropathy in Diabetes using Novel Ophthalmic Markers Ref No. 1483C

I write to advise that the Mater Health Services Human Research Ethics Committee considers the above study to meet the requirements of the National Statement on Ethical Conduct in Human Research (2007) and has granted ethical approval for your research proposal. Please accept our very best wishes for the success of this study. In all future correspondence with the Committee please quote the Mater reference number.

Documents reviewed and approved include:

- Correspondence received 5th and 15th March 2010 in response to Committee questions
- Completed Mater Cover Sheet
- Addendum to NEAF
- Financial Costing Summary
- Parent/Guardian Information Sheet and Consent Form (including Revocation of Consent Form, Revocation of Consent to Biobank Form, Future Contact Consent Form) dated 5th March 2010
- Participant Information Sheet and Consent Form – Adults (including Revocation of Consent Form, Revocation of Consent to Biobank Form, Future Contact Consent Form) Version 2 dated 5th March 2010
- Participant Information Sheet and Assent Form – Minors Version 2 dated 5th March 2010
- LANDMark Participation Questionnaire, revised March 2010
- LANDMark Study Case Report Form
- LANDMark Study Protocol Version 4 dated December 2009
- HREC approval letters from QUT and PAH
- Biosketch – Professor Nathan Efron
- Multi-Institutional Agreement between Mater Health Services, Queensland University of Technology, The University of Queensland and University of Manchester
- NEAF Version 2.0

This approval is valid until 16.03.13. Please note the following conditions of approval.

- Any departure from the protocol detailed in your proposal must be reported immediately to the Committee.
When you propose a change to an approved protocol, which you consider to be minor, you are required to submit a written request for approval to the Chairperson, through the Secretary. Such requests will be considered on a case by case basis and interim approval may be granted subject to ratification at the next meeting of the Committee.

Where substantial changes to any approved protocol are proposed, you are required to submit a full, new proposal for consideration by the Human Research Ethics Committee.

You are required to advise the Research Ethics Coordinator immediately of any complaints made, or expressions of concern raised, in relation to the study, or if any serious or unexpected adverse events occur.

Under the NHMRC National Statement on Ethical Conduct in Research Involving Humans, research ethics committees are responsible for monitoring approved research to ensure continued compliance with ethical standards, and to determine the method of monitoring appropriate to each project. You are required to provide written reports on the progress of the approved project annually, the first report being due on 16.03.11 and finally on completion of the project. (The Progress Report is located at http://www.mater.org.au/Home/Research/Human-Research-Ethics-Committee.aspx or can be accessed through the Mater Intranet, Applications, Research Register then under the project name or alternately can be emailed to you). Please inform the Committee of publications, presentations at Conferences, education and quality improvement outcomes from this study. The Committee may also choose to conduct an interim audit of your research.

Please be aware that all study procedures including follow up of participants and data analysis should be completed within the approval time frame or an extension should be requested.

Please contact the Executive Director in the participating hospital/hospitals prior to commencing of the study. To access medical records, for the purpose of this study, please provide a copy of this approval letter to the Corporate Health Information Manager. I would also be grateful if you could confirm the date of commencement. (All correspondence should be directed to the Mater Research Ethics Coordinator.)

Yours sincerely

Dr Helen Liley
Chairperson
Mater Health Services Human Research Ethics Committee

Research Ethics Coordinator Room 235 Aubigny Place Ph: 07 3163 1585 Fax: 07 3163 1571 Email: research.ethics@mater.org.au
Dear Prof Nathan Efron

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

Within this Approval Certificate are:

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

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

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

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

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

### Project Details

Category of Approval: Administrative Review

Approved From: 28/05/2008

Approved Until: 28/05/2013 (subject to annual reports)

Approval Number: 0800000167

Project Title: Ophthalmic markers of diabetic neuropathy

Experiment Summary: Employ novel non-invasive ophthalmic markers of peripheral nerve dysfunction to investigate peripheral nerve morphology and function in Type 1 and 2 diabetic patients with and without neuropathy.

### Investigator Details

Chief Investigator: Prof Nathan Efron
Other Staff/Students:

<table>
<thead>
<tr>
<th>Investigator Name</th>
<th>Type</th>
<th>Role</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dr Tony Russell</td>
<td>External</td>
<td>Associate Investigator</td>
</tr>
<tr>
<td>Ms Nicola Pritchard</td>
<td>Internal</td>
<td>Associate Investigator</td>
</tr>
<tr>
<td>Prof John Prins</td>
<td>External</td>
<td>Associate Investigator</td>
</tr>
<tr>
<td>Ms Katie P Edwards</td>
<td>Student</td>
<td>Student</td>
</tr>
<tr>
<td>Dr Robert Henderson</td>
<td>External</td>
<td>Associate Investigator</td>
</tr>
<tr>
<td>Ms Garima Tyagi</td>
<td>Internal</td>
<td>Research Assistant</td>
</tr>
<tr>
<td>Ms Ophelia Ho</td>
<td>External</td>
<td>Research Team Member</td>
</tr>
<tr>
<td>Ms Kelly Bennett</td>
<td>External</td>
<td>Research Team Member</td>
</tr>
<tr>
<td>Mr Andrew Knuckey</td>
<td>Internal</td>
<td>Research Team Member</td>
</tr>
<tr>
<td>Ms Jay Lee</td>
<td>Internal</td>
<td>Research Assistant</td>
</tr>
<tr>
<td>Dr Andrew Cotterill</td>
<td>External</td>
<td>Associate Investigator</td>
</tr>
<tr>
<td>Ms Kath Macintosh</td>
<td>External</td>
<td>Associate Investigator</td>
</tr>
<tr>
<td>Ms Sangeetha Srinivasan</td>
<td>Student</td>
<td>Ethics- Student- Course- Doctoral</td>
</tr>
<tr>
<td>Ms Colleen Wooten</td>
<td>Internal</td>
<td>Research Team Member</td>
</tr>
<tr>
<td>Dr Dimitrios Vagenas</td>
<td>Internal</td>
<td>Associate Investigator</td>
</tr>
<tr>
<td>Ms Anne Warne</td>
<td>Internal</td>
<td>Associate Investigator</td>
</tr>
<tr>
<td>Dr Geoff Sampson</td>
<td>Internal</td>
<td>Associate Investigator</td>
</tr>
</tbody>
</table>

Participant Details

Participants:
Approximately 220

Location/s of the Work:
Anterior Eye Laboratory, IHBI QUT; Centre for Diabetes and Endocrinology, Princess Alexandra Hospital; Cardiovascular and Endocrine Sciences, Manchester Royal Infirmary

Conditions of Approval

Specific Conditions of Approval:
No special conditions placed on approval by the UHREC. Standard conditions apply.

Standard Conditions of Approval:
The University's standard conditions of approval require the research team to:

1. Conduct the project in accordance with University policy, NHMRC / AVCC guidelines and regulations, and the provisions of any relevant State / Territory or Commonwealth regulations or legislation;

2. Respond to the requests and instructions of the University Human Research Ethics Committee (UHREC);

3. Advise the Research Ethics Coordinator immediately if any complaints are made, or expressions of concern are raised, in relation to the project;

4. Suspend or modify the project if the risks to participants are found to be disproportionate to the benefits, and immediately advise the Research Ethics Coordinator of this action;

5. Stop any involvement of any participant if continuation of the research may be harmful to that person, and immediately advise the Research Ethics Coordinator of this action;

6. Advise the Research Ethics Coordinator of any unforeseen development or events that might affect the continued ethical acceptability of the project;
7. Report on the progress of the approved project at least annually, or at intervals determined by the Committee;

8. (Where the research is publicly or privately funded) publish the results of the project is such a way to permit scrutiny and contribute to public knowledge; and

9. Ensure that the results of the research are made available to the participants.

Modifying your Ethical Clearance:
Requests for variations must be made via submission of a Request for Variation to Existing Clearance Form (http://www.research.qut.edu.au/ethics/forms/hum/var/var.jsp) to the Research Ethics Coordinator. Minor changes will be assessed on a case by case basis.

It generally takes 7-14 days to process and notify the Chief Investigator of the outcome of a request for a variation.

Major changes, depending upon the nature of your request, may require submission of a new application.

Audits:
All active ethical clearances are subject to random audit by the UHREC, which will include the review of the signed consent forms for participants, whether any modifications / variations to the project have been approved, and the data storage arrangements.
LANDMARK BioBank
Participant Information and Consent Form

for a joint project by Princess Alexandra Hospital
and Queensland University of Technology

Principal Researcher: Prof Nathan Efron
Associates: Prof Andrew Boulton, Prof Rayaz Malik, Prof John Prins, Dr Anthony Russell, Ms Nicola Pritchard, Dr Katie Edwards

1 Queensland University of Technology, 2 University of Manchester, 3 Princess Alexandra Hospital.

1. Introduction
As a participant in the study entitled “Ophthalmic Markers of Diabetic Neuropathy (LANDMARK)” you are also invited to donate blood and skin tissue (Manchester site only) to the LANDMARK BioBank or tissue bank. The purpose of this tissue bank is collecting and storing fresh or archived tissue blood for future research purposes. Your involvement entails: donation of blood and skin tissue (Manchester site only) which can be stored in the tissue bank and allowing access to your relevant medical records.

This Participant Information and Consent Form tells you about the BioBank research project. It explains the procedures involved. Knowing what is involved will help you decide if you want to take part in the BioBank project.

Please read this information carefully. Ask questions about anything that you don’t understand or want to know more about. Before deciding whether or not to take part, you might want to talk about it with a relative, friend or healthcare worker.

Participation in this research is voluntary. If you don’t wish to take part, you don’t have to. You will receive the best possible care whether you take part or not.

If you decide you want to take part in the research project, you will be asked to sign the consent section. By signing it you are telling us that you:

• understand what you have read;
• consent to take part in the research project;
• consent to participate in the research processes that are described;
• consent to the use of your personal and health information as described

You will be given a copy of the Participant Information Sheet and Consent Form to keep.

If you are the parent or guardian of a child or young person, as the ‘person responsible’ for the patient, you are invited to consider the patient’s participation in the research project. Both the child/young person and the ‘person responsible’ must consent to participation in the study.

2. What is the LANDMARK BioBank?
The LANDMARK BioBank is a non-profit organisation that collects and stores tissue and blood and other biological samples for future research purposes. These samples will be labelled with a unique code and when a researcher wishes to study certain aspects of diabetes, he/she can apply to the BioBank for samples of tissue, blood or other biological samples. The researcher will not know who donated the samples (i.e. the samples are ‘de-identified’). These samples can only be given to researchers with your permission and the BioBank will require written evidence of ethical approval before researchers are allowed access to samples.
Associated with the BioBank is the collection of the clinical study data acquired during the LANDMARK study and other relevant medical history and clinical information of people who have donated tissue, blood and/or other biological samples. This information is stored in a secure clinical database.

It is entirely up to you to decide how much or how little to contribute to the BioBank. The tissue and clinical data will be stored indefinitely in the BioBank to maximise the potential for contribution to future diabetes research. You may access information stored about you by the BioBank, by contacting the Project Manager, Ms Nicola Pritchard, or the Chief Investigator, Professor Nathan Efron.

3. What is the purpose of sample collection?

The purpose of this BioBank is a resource to diabetes researchers working with and developing new techniques to look at genes and their protein products in cells which may eventually be developed into better diagnostic tools and treatment options for diabetic neuropathy. This project involves the collection of some blood samples and skin tissue (Manchester site only) from you. The small amount of tissue and/or blood samples that we will bank will be used in a variety of ways. The most important of these will be directed to improvements in the diagnosis and treatment of diabetes, including diabetic neuropathy.

4. How are the blood and/or tissue samples collected?

We would also like to obtain an additional blood sample (20-25 mls, or 2-3 tubes) from you. After collection, the samples will undergo initial processing, and then the components will be stored frozen to allow future research to be undertaken into blood sample features linked to diabetes. You can donate a blood sample to the BioBank, even if you are not donating tissue. If you consent to having a blood sample collected it will be done at a time that is convenient to you; for example, rather than having blood collected at your study visit, it can be arranged for the blood samples to be collected at another time.

Manchester only: You are about to have a biopsy to remove tissue to do some tests. Your surgeon will remove some tissue as part of the study. Generally not all the tissue is needed for assessing the nerves by examination under the microscope. It is this ‘left over’ tissue that we invite you to donate to the BioBank. The amount stored will be approximately 1.5mm in diameter.

5. Are there any risks involved?

As stated in the information provided for the primary LANDMARK study, having a blood sample taken may cause some discomfort or bruising. Sometimes, the blood vessel may swell, or blood may clot in the blood vessel, or the spot from which tissue is taken could become inflamed. Rarely, there could be a minor infection or bleeding. If this happens, it can be easily treated.

Manchester site only

The skin biopsy may produce some discomfort; however, local anaesthetic will be used to numb the area prior to the biopsy. You will be left with a small scar which will fade over a period of 6 months and will be barely visible at 1 year.

We would also like to collect other relevant information about you and store it on a secure computer database, so that we can link your tissue samples to the data collected in the study and other ongoing information about your health (e.g. Hb A1c levels). This information will be obtained from health care providers, for example medical practitioners, consultants and hospitals and will be stored in the secure database. This information will remain strictly confidential and protected by a password. Only authorised BioBank staff will be able to access this information. All information will be dealt with and stored in accordance with State and Federal Legislation and National Health & Medical Research Council National Statement 2007. Your information will not be used other than for the purpose of this protocol. The BioBank may be asked to supply proof of your consent.

6. What are the benefits involved?
There will be no direct benefit to you from donating your tissue, blood and/or other biological samples but the information obtained from this project is likely to be of benefit to people with diabetes and diabetic neuropathy in the future.

7. What if I change my mind?

Your participation in this project is voluntary and the choice to donate your tissue, blood and/or other biological samples is entirely up to you. No matter what you decide, it will not affect your care in any way. You are under no obligation to donate blood/tissue or other biological samples. If you change your mind at any time, please notify the Project Manager, Ms Nicola Pritchard, or the Chief Investigator Professor Nathan Efron. Samples and information (data) already sent out of the BioBank may not be able to be recovered.

8. Will I find out about the results of the research using my tissue?

You will receive the results of your study participation if you would like them, but you will not receive results from the research done with your tissue. This is because research can take many years and uses samples from a large number of people and so will not affect your care right now.

10. Who will use my tissue?

Your tissue sample may be used by a team of researchers such as those associated with the JDRFI or other researchers based in Australia, the UK or anywhere in the world. Only researchers who have institutional research ethics committee approvals are able to apply for or obtain samples from the BioBank. The LANDMARK BioBank Management Committee, which includes a consumer representative, meets and discusses all requests for material.

11. Who can I contact if I have more questions?

We encourage you to call us with any concerns or questions you may have. At IHBI please contact the Project Manager Ms Nicola Pritchard (07) 3138 6414 or the Chief Investigator Professor Nathan Efron on 07 3138 6401.

12. What else do I need to know?

The BioBank is committed to making sure that information about you is kept safe and in strict confidence. Personal information that can identify you, such as your name or date of birth, is removed and replaced with a unique BioBank number code. Only authorised BioBank staff will be able to link this code with your information. Future researchers are only supplied with re-identifiable samples and data. This ensures that nothing that can identify you or your family will ever be sent to other researchers, or appear on any public or published reports.

Is this research project approved?

The ethical aspects of this research project have been approved by the Human Research Ethics Committee of the Princess Alexandra Hospital and Queensland University of Technology. This project will be carried out according to the National Statement on Ethical Conduct in Research Involving Humans (March 2007) produced by the National Health and Medical Research Council of Australia. This statement has been developed to protect the interests of people who agree to participate in human research studies.

Should you wish to discuss the study with someone not directly involved, in particular in relation to matters concerning policies, information about the conduct of the study or your rights as a participant, or should you wish to make an independent complaint, you can contact the Ethics Manager, Princess Alexandra Hospital Human Research Ethics Committee, Ph: (07) 3240 58 56, Email: PAH_Ethics_Research@health.qld.gov.au; or the QUT Research Ethics Officer, Queensland University of Technology Human Research Ethics Committee, Ph: (07) 3138 2340, E-mail: ethicscontact@qut.edu.au.
15. Consent

a) I, ______________________________________ (please print your full name) consent to the collection and storage of samples of my tissue, fresh or archived, blood and/or other biological samples, in the LANDMARK Bio Bank. I also agree to complete a short questionnaire.

b) I have read and understood the LBB Participant Information Sheet. This sheet describes the purpose of the tissue bank and what my involvement entails if I take part. The research objectives have been fully explained to me and I have been given the opportunity to ask questions. I have been given the opportunity to have a member of my family or a friend present while the study was explained to me.

c) I give permission for the research team to access my medical records from the appropriate state public or private health care facility that I have attended for health care concerning my diabetes diagnosis and/or treatment, for example medical practitioners, consultants and hospitals. I give my permission for the release of information concerning my disease and treatment from any of the above.

d) I understand that all information will be treated in the strictest confidence and used for research purposes only.

e) I understand that I will not be personally identified in any reports from this or associated projects and that only de-identified data will be given to other researchers following written ethical approval. I am informed that any further research, using this tissue or established primary cell lines, will only be done following the full approval granted by the Human Research Ethics Committee to which that Research Team must report.

f) I understand that the tissue, blood and/or other biological samples (including their constituents and anything derived from it) will be stored indefinitely in the LANDMARK BioBank or other approved storage facility and may be used for future biochemical and genetic studies of diabetes or diabetic neuropathy.

g) While I understand that the purpose of this research project is to improve the quality of medical care, it has also been explained that my involvement may not be of any benefit to me.

h) I understand that while my involvement in the study is voluntary it will not affect my relationship with my medical advisers in their management of my health. I also understand that I am free to withdraw from the project at any stage without my future treatment being affected. I may do this by contacting the Project Manager (or local Coordinator) and obtain a “withdrawal of consent” form.

i) I give permission for my tissue, blood and/or other biological samples (including their constituents and anything derived from it), to be used in any way that LANDMARK investigators deems most beneficial and assign and waive all claims to patents, commercial returns, property or any material or products which may form part of or arise from these studies.

j) I have been told that this project has been approved by the Queensland University of Technology, Princess Alexandra Hospital, and University of Manchester Human Research Ethics Committees.

k) I understand that this research will comply with the National Health and Medical Research Council’s Statement on Ethical Conduct 2007 and relevant State and Federal Legislation.

l) I understand that I will be given a signed copy of this document to keep.

Participant’s name (printed) _______________________
Signature ______________________________________ Date ____________________

Declaration by parent, guardian or person responsible (where appropriate): I agree for my child/young person or the person named above who I am responsible for to participate in this
research and I believe that they have understood the explanation of the study, its procedures and
risks.

Name of parent/guardian to participant’s (printed) _______________________________
Signature ______________________________________ Date __________________

Name of witness to participant’s signature (printed) ______________________________
Signature ______________________________________ Date __________________

Declaration by researcher*: I have given a verbal explanation of the research project, its procedures
and risks and I believe that the participant has understood that explanation.

Researcher’s name (printed)  _______________________________________________
Signature ______________________________________ Date __________________

* A senior member of the research team must provide the explanation and provision of information
concerning the research project.

Note: All parties signing the consent section must date their own signature.

16. Who else can I contact?

Who you may need to contact will depend on the nature of your query; therefore, please note the
following:

If you want any further information concerning this project or if you have any medical problems
which may be related to your involvement in the project (for example, any side effects), you can
contact Dr Anthony Russell Ph: 07 3240 5914 or the following people:

Nicola Pritchard
Ph: 07 3138 6414
Email: n.pritchard@qut.edu.au

Prof. Nathan Efron
Ph: 07 3138 6401
Email: n.efron@qut.edu.au

Katie Edwards
Ph: 07 3138 6154
Email: katie.edwards@qut.edu.au

If you feel emergency medical care is required, then go to the nearest hospital Emergency
Department.

For complaints:

If you have any complaints about any aspect of the project, the way it is being conducted or any
questions about being a research participant in general, then you may contact:

Ethics Manager
Ph: (07) 3240 5856
Email: PAH_Ethics_Research@health.qld.gov.au

QUT Research Ethics Officer
Ph: (07) 3138 2340
E-mail: ethicscontact@qut.edu.au

Researcher Ethics Officers/Managers are not connected with the research project and can facilitate
a resolution to your concern in an impartial manner.
Future Contact Consent

I *give permission / do not give permission to the LANDMARK research team to contact me in the future regarding further research projects (*delete whichever is not applicable)

I *wish / do not wish to be notified if research using my tissue reveals information which has significant implications for me or my family. I understand that if I do wish to be notified about any information which has significant implications for me or my family, in accordance with the National Statement on Ethical Conduct in Research Involving Humans, this will be done with appropriate counselling and support provided by experienced health professionals. (*delete whichever is not applicable)

In the event of my death or I am un-contactable I wish / do not wish my family to be notified if research using my tissue reveals information with significant implications for my family. I understand that if I do wish my family to be notified about any information which has significant implications for them, in accordance with the National Statement on Ethical Conduct in Research Involving Humans, this will be done with appropriate counselling and support provided by experienced health professionals. (*delete whichever is not applicable)

If family is to be notified please contact the following person/s if I am not contactable for any reason:

Name of 1st nominee if I cannot be contacted _______________________________________
Relationship to me: ___________________________________________________________
Address ________________________________________________________________
_______________________________________________________________________
Phone _________________________________________________________________
Name of 2nd nominee if I cannot be contacted __________________________________
Relationship to me: _______________________________________________________
Address ________________________________________________________________
_______________________________________________________________________
Phone _________________________________________________________________
Signature: _______________________________ Date ________________.
Witness name _______________________________ Date ________________
Witness Signature _______________________________ Date ________________

I confirm that to the best of my knowledge, the participant has understood the information provided to him, the implications of this information and that the participant will be provided with a copy of this document.

Investigator full name ____________________________________________
Investigator signature ____________________________ Date: ________________
Participant Information and Consent Form

for a joint project by Princess Alexandra Hospital

and Queensland University of Technology

Project Title (official): Ophthalmic Markers of Diabetic Neuropathy

Project Title (simplified): Examining the eyes to diagnose nerve problems in patients with diabetes.

Principal Researcher: Prof Nathan Efron

Associates: Prof Andrew Boulton, Prof Rayaz Malik, Prof John Prins, Dr Anthony Russell, Nicola Pritchard, Katie Edwards, AProf Andrew Cotterill

1 Queensland University of Technology, 2 University of Manchester, 3 Princess Alexandra Hospital, 4 Mater Children’s Hospital

1. Introduction

You (or your child or the person you are responsible for) are invited to take part in this research project. This is because you (or your child or the person you are responsible for) are in the age range of 14-75 years and either have a history of diabetes, or have no history of disease that might affect the nerves of the eye or the body. People who have had eye injury or surgery, other eye diseases (e.g. glaucoma), other general health diseases which may affect the front ‘clear window’ of the eye, known as the cornea (e.g. keratoconus) or body (e.g. carcinoma, leukemia), large fibre neuropathy (damage to the large nerve fibres), congestive heart failure (weakening of the heart’s pumping ability), major mental health problems, HIV-AIDS or diabetic foot ulcer or infection, or those participating in any other research trial will not be eligible.

The research project is aiming to investigate relationship between the nerves of the eye and a condition which involves the peripheral nerves of the body in people with and without diabetes. We hope to determine if some of the measures of the nerves in the eye and the sensitivity of the eye are reduced in people with peripheral nerve damage due to diabetes.

This Participant Information and Consent Form tells you (and your child or the person you are responsible for) about the research project. It explains the procedures involved. Knowing what is involved will help you (or your child or the person you are responsible for) decide if you (or they) want to take part in the research.

Please read this information carefully. Ask questions about anything that you (or your child or the person you are responsible for) don’t understand or want to know more about. Before deciding whether or not to take part, you (or they) might want to talk about it with a relative, friend or healthcare worker.

Participation in this research is voluntary. If you (or your child or the person you are responsible for) don’t wish to take part, you (or they) don’t have to. You (or your child or the person you are responsible for) will receive the best possible care whether you (or they) take part or not.
If you (or your child or the person you are responsible for) decide you (or they) want to take part in the research project, you (or they) will be asked to sign the consent section. By signing it you (or they) are telling us that you (or they):

- understand what you (or they) have read;
- consent to take part in the research project;
- consent to participate in the research processes that are described;
- consent to the use of your (or their) personal and health information as described.

You (or your child or the person you are responsible for) will be given a copy of this Participant Information and Consent Form to keep.

If you are the parent or guardian of a child or young person, as the ‘person responsible’ for the patient, you are invited to consider the patient’s participation in this research project. Both the child/young person and the ‘person responsible’ must consent to participation in the study. If you (or they) decide to take part and later change your mind, you (or they) are free to withdraw from the project at any stage for any reason (stated or unstated) without comment or penalty.

2. What is the purpose of this research project?

This research project focuses on patients with different types of diabetes. As you (or your child or the person you are responsible for) may know, diabetes is associated with high sugar levels in the blood due to the body not producing enough insulin to convert this sugar into energy. We think there might be some differences in the nerves of the eyes of people who have different types of diabetes and we can measure this by using new, simple methods that measure the actual nerves and nerve function. These are the eye tests: corneal confocal microscopy (CCM; high magnification microscope) can be used to look at the nerves in the front of the eye; and corneal non-contact aesthesiometry (NCCA) is used to measure the sensitivity of the front of the eye; ocular coherence tomography (OCT) is used to assess the nerves and tissues at the back of the eye and flicker perimetry (FP) measures how well you can see dim lights (both these techniques are described in Section 3). The measures of nerves and nerve function made by these techniques are thought to be related to diabetic neuropathy, the damage of nerves in the peripheral limbs associated in some patients with diabetes. In the research project we aim to investigate the following:

- Changes in corneal (front of eye) nerve counts and corneal sensitivity over time.
- Changes in retinal (back of eye) nerve layer thickness and sensitivity to light over time.
- The relationship between the progression of nerve damage with the results of other traditional nerve tests such as electrophysiology, measuring electrical signals from the body), measuring how easily you can detect vibration and temperature sensitivity and assessment of level of pain and discomfort in people with different types of diabetes.
- The ability of these eye tests to detect nerve damage earlier than traditional means.
- Identify risk factors associated with changes in nerves and nerve function in people with different types of diabetes; these may include age, height, weight, duration of diabetes, blood pressure, smoking, and poor blood-sugar control.

Understanding these aspects of the nerves may provide healthcare professionals with a quick, simple, cost-effective and repeatable means to identify patients at risk, anticipate and monitor deterioration, and assess new treatments.
Diabetic nerve damage is a significant clinical problem that currently has no effective treatment, and in advanced cases, it is a major cause of ill-health and death worldwide. If left unmanaged, diabetic nerve damage can lead to foot ulceration and ultimately, in some cases, foot amputation. It is therefore important to have the capacity to detect this condition early, monitor its progression and assess the benefits of any treatments.

The results of this study will develop a better understanding of small fibre peripheral nerves in the arms and legs in patients suffering from diabetic nerve damage, and will determine the extent to which these changes are associated with the clinical signs and symptoms of the condition. The significance of this study is that it will reveal the potential for these eye tests to serve as sensitive, rapid, repeatable, ‘patient-friendly’ eye tests for the detection, diagnosis and monitoring of the progression of diabetic nerve damage. This information will provide a sound basis for the design of trials of treatments for diabetic nerve damage. Data will also be generated which will reveal the importance (or otherwise) of blood sugar control and other metabolic abnormalities and lifestyle factors which may impact on the progression of nerve damage in diabetic patients.

A total of 298 participants will take part in this study at the Institute of Health and Biomedical Innovation (IHBI) at QUT in Brisbane and a further 202 at the University of Manchester in the United Kingdom.

Five groups of people will be recruited in Brisbane:

Group 1: Patients with Type 1 diabetes and without nerve damage
Group 2: Patients with Type 1 diabetes with nerve damage
Group 3: Patients with latent autoimmune diabetes in adults (LADA; similar to Type 1 diabetes but occurring later in life) with nerve damage
Group 4: Patients with Type 2 diabetes with and without nerve damage
Group 5: Non-diabetic participants without nerve damage.

Some of the results of this research will be used by the researchers Ayda Moavenshahidi and Nicola Pritchard to obtain Doctor of Philosophy degrees.

This research is a collaborative project between researchers at QUT, Princess Alexandra Hospital (PAH) and University of Manchester (UM). It has been initiated by the investigators Professors Nathan Efron (QUT), Rayaz Malik, Andrew Boulton (UM), and John Prins (PAH); Dr Anthony Russell (PAH) and optometrists Nicola Pritchard and Dr Katie Edwards (QUT).

This research has been funded in part by the Juvenile Diabetes Research Foundation International and Australia’s National Health & Medical Research Council and the George Weaber Foundation (to support Ms Moavenshahidi).

3. What does participation in this research project involve?

Your participation (or that of your child or the person you are responsible for) will involve asking you (or they) to reveal eye and past medical problems, and undergo an examination of the front part of the eye using a high powered microscope, read letters on an eye chart, and have the pressure of the eyes measured. We will ask you (or your child or the person you are responsible for) to complete a questionnaire about pain in your (or their) lower limbs, and undergo simple tests of your (or their) sensation of pain/ouch, vibration and temperature. The tests are quick and involve use of a pointed tip, a tuning fork and warm and cool metal rods to test these three sensations. The presence of absence of the reflexes in your knees and ankles using a small hammer will be tested. Your (or their) height, weight and blood pressure will also be measured and a picture will be taken of the back of the eye.
Another high powered microscope, known as a corneal confocal microscope (CCM) will be used to examine the number of nerves at the front part of the eye, the cornea. A drop of anaesthetic is applied to numb the front of the eye and you (or they) will be asked to sit at an instrument and look at a target while several images are captured. Initially the drop may sting for 1 or 2 seconds. Because the drop numbs the eye it is possible to scratch the eye without noticing it. Therefore please do not rub the eyes for at least 45 minutes after the drop has been placed in the eye.

Another test of your (or their) ability to feel different sensations will be done using an instrument that can measure when you (or they) just notice sensations of cool, warm and vibration on the foot. For example, for the coolness test you (or they) may feel like “a pulse of cooling” has touched the foot. It is important that before these tests no sedatives, tranquillisers, opiates, or stimulants have been taken in the preceding 12 hours, and not more than one hot drink has been consumed prior to the test.

Another test that can reveal alterations to the nerves is a test of heart rate variability. A measure of heart rate variability will also be conducted to show how the heart responds to deep breathing and to changes in blood pressure and posture.

Corneal non-contact aesthesiometry (NCCA) will be conducted to measure the sensitivity of the cornea. The smallest noticeable air pressure is determined by directing gentle, almost imperceptible puffs of air to the eye, and you (or they) indicate whether the air on the eye can be felt or not. We will also take a small sample of tears (50 µl) to examine the proteins; this involves holding a tiny glass tube near the eye for a few seconds. A swab of the conjunctiva (the white part of the eye) will be taken to investigate how diabetes affects the normal microbiota (microscopic living organisms) of the eye.

The speed the nerves conduct messages will also be tested as a measure of nerve damage. Nerve conduction velocity will be measured by putting sensors on the ankle, wrist and elbow. The limb will be kept warm with a heat lamp if necessary. A small electrical current will be applied to the sensor which may feel like a tingling sensation and it may be uncomfortable for you (or them). You (or they) should feel no discomfort once the test is finished.

Ocular coherence tomography (OCT) involves having a drop inserted into one eye to dilate the pupil. Then you (or they) will be asked to fixate a target while seated at the instrument, and at least two OCT images are captured. A photograph of the back of the eye will also be taken using a specialised digital camera. Due to the increased size of the pupil, your (or their) sensitivity to glare may be increased for 4 to 6 hours, so you (or they) may wish to wear dark glasses when outside and/or have someone drive or escort you (or them) home.

Flicker perimetry (FP) involves viewing a light stimulus of varying intensity, and sometimes flickering, which appears in different parts of the visual field. You (or your child or the person you are responsible for) will be required to click a button if you (or they) see the light while looking at a central spot.

At the end of the study procedures the eye will be examined again; follow-up appointments will be made if the investigator believes it is in your (or their) best interests. This study will be carried out at IHBI at QUT.

We expect the visit will be approximately 3 to 5 hours at IBHI at QUT, Kelvin Grove at a time suitable to you. You (or they) will not be paid for participation in this research, but will be provided transport to and from QUT (e.g. parking / vouchers for petrol or cab vouchers will be provided up to approximately $40) and will receive light refreshments during the visit (approximate value $10).

4. What will happen to my test samples?
You (or your child or the person you are responsible for) will be asked to provide consent for the collection of your (or their) blood (approximately 30-35ml, or 3-4 tubes) and urine (approximately 10ml) during the research project. From these samples the levels of protein, glucose, lipid and a test for antibodies for glutamic acid decarboxylase (GADAb) and antibodies to islet cells (ICAAb) will be determined and recorded. This will help investigators decide which group to assign you (or them) to. All samples will be individually identifiable at the time of collection, analysis and report; a re-identifiable code will be assigned your (or their) blood results. All blood and urine samples will be assessed through a contracted pathology service and samples are usually destroyed 7 days after collection. Separate consent will be obtained regarding storage of blood samples. Unused tear and swab samples will be destroyed typically within 7 days of collection.

5. What are the possible benefits?

There will be no direct benefit to you (or your child or the person you are responsible for) from your (or their) participation in this research. However, it may benefit the many people who have problems with diabetic neuropathy, because with these instruments and techniques we are able to look at the tissues of the eye under very high magnification. Also these new technologies may reveal features that have not, to date, been discovered but which might serve as sensitive, rapid and useful techniques for the detection, quantification and monitoring of the progression of nerve disease in patients with diabetes as well as other diseases where the nerves of the body are affected. Some people find the opportunity to learn and be a part of something new an interesting experience.

We can provide you (or your child or the person you are responsible for) with state-of-the-art images of your (or their) eye if you (or they) would like them.

6. What are the possible risks?

The risks associated with participation in this study are minimal, and similar to routine diabetic and primary eye care. Minimal scratching the front surface of the eye can occur with corneal confocal microscopy, similar to that which might occur if you (or they) rub the eyes too hard; however, in our experience it is like that noted with normal daily wear of contact wearers. This type of abrasion heals quickly, without intervention, typically within 12 hours.

Having a blood taken may cause some discomfort or bruising. Sometimes, the blood vessel may swell, or blood may clot in the blood vessel, or the spot from which tissue is taken could become inflamed. Rarely, there could be a minor infection or bleeding. If this happens, it can be easily treated.

Nerve conduction tests involve applying a small electrical current to the limb which may feel like a tingling sensation; this may be uncomfortable for you (or them). You (or they) should feel no discomfort once the test is finished.

If you (or your child or the person you are responsible for) become upset or distressed as a result of your (or their) participation in the research, the researcher is able to arrange for counselling or other appropriate support. Any counselling or support will be provided by staff who are not members of the research team. In addition, you (or they) may prefer to suspend or end participation in the research if distress occurs without comment or penalty.

There may be additional risks that the researchers do not expect or do not know about. Tell a member of the research team immediately about any new or unusual symptoms that you (or they) get.

7. What if new information arises during this research project?

During the research project, new information about the risks and benefits of the project may become known to the researchers. If this occurs, you (or your child or the person you are responsible for)
8. Can I have other treatments during this research project?

It is important to tell your (or their) doctor and the research staff about any treatments or medications you (or they) may be taking, including over-the-counter medications, vitamins or herbal remedies, acupuncture or other alternative treatments. You should also tell your (or their) doctor and the researchers about any changes to these during participation in the research.

9. Are there alternatives to participation?

Since this study does not involve any treatments, you (or your child or the person you are responsible for) will receive the best possible care whether you (or they) take part or not. Participation in the study does not replace full eye or medical care. You (or they) may also request that your (or their) general practitioner be informed of participation in the study.

10. Do I have to take part in this research project?

Participation in any research project is voluntary. If you (or they) do not wish to take part you (or they) don’t have to. If you (or they) decide to take part and later change your mind, you (or they) are free to withdraw from the project at any stage for any reason (stated or unstated) without comment or penalty.

The decision whether to take part or not to take part, or to take part and then withdraw, will not affect your (or their) routine treatment, your relationship with those treating you (or them), nor your (or their) relationship with Princess Alexandra Hospital or Queensland University of Technology.

11. What do I need to do if I decide to withdraw from this research project?

If you (or your child or the person you are responsible for) decide to withdraw, please notify a member of the research team before you (or they) withdraw.

If you (or they) decide to leave the project, the researchers would like to keep the personal and health information about you (or them) and your (or their) blood results that have been collected. This is to help them make sure that the results of the research can be measured properly. If you (or they) do not want them to do this, you (or they) must tell them before joining the research project.

12. Could this research project be stopped unexpectedly?

There are no foreseeable reasons why this research project would be terminated before completion. In the unlikely event this did occur, you (or they) will be informed in writing and asked to attend a final study visit.

13. How will I be informed of the results of this research project?

The research team will provide regular newsletters on the progress of the study. You (or your child or the person you are responsible for) will also receive a copy of any publications that are generated as a result of this study. We expect this research project to be completed in approximately 5 years and a full summary of the results will be provided to you (or them) then. Results from the tests we perform will be sent, with your (or their) permission, directly to your (or their) medical practitioners.

We would like to receive your feedback about your participation and may ask you (or the person you are responsible for) to complete a questionnaire after the visit. These responses will be matched to your visit dates by a team member who has no interaction with you.
means that the results of these questionnaires will be anonymous to the staff you meet at your visits.

14. What else do I need to know?

Any information obtained in connection with this research project that can identify you (or your child or the person you are responsible for) will remain confidential and will only be used for the purpose of this research project. It will only be disclosed with your (or their) permission, except as required by law. Information about you (or them) may be obtained from your (or their) health records held at PAH (where applicable) for the purposes of this research e.g. additional blood results related to your (or their) PAH clinic visit. If you attend another clinic we will seek your (or the person you’re responsible for) permission to obtain your (or their) blood results from your (their) doctor.

Data is stored on paper records in locked filing cabinets at QUT, and the data in electronic form (i.e. entered into a computer) is only available to the research team members and is kept secure by using password-protected limited-access environment to protect your privacy. Data is stored during the project in an identifiable format i.e. with your name attached. In any publication and/or presentation, information will be provided in such a way that you (or they) cannot be identified, except with your (and/or their) permission. This will be done by only using the code number assigned to you (or them) for the purpose of this study; this will provide anonymity.

At completion of the project your (or their) data will be decoded, or de-identified, such that it will not be possible to determine which data belong to which participant. Data for this project will be kept for a minimum of 15 years or 5 years after the last publication, and de-identified data may be shared with national and international data registries. Paper files will be shredded.

Information about your (or their) participation in this research project may be recorded in your (or their) health records.

How can I access my information?

In accordance with relevant Australian privacy and other relevant laws, you have the right to access the information collected and stored by the researchers about you (or your child or the person you are responsible for). You also have the right to request that any information, with which you disagree, be corrected. Please contact one of the researchers named at the end of this document if you (or they) would like to access your (or their) information.

What happens if I am injured as a result of participating in this research project?

If you (or they) suffer an injury as a result of participating in this research project, hospital care and treatment will be provided by the public health service at no extra cost to you (or them) if you (or they) elect to be treated as a public patient.

Is this research project approved?

The ethical aspects of this research project have been approved by the Human Research Ethics Committee of the Princess Alexandra Hospital and Queensland University of Technology.

This project will be carried out according to the National Statement on Ethical Conduct in Human Research (2007) produced by the National Health and Medical Research Council of Australia. This statement has been developed to protect the interests of people who agree to participate in human research studies.
15. Consent

I have read, or have had read to me in a language that I understand, this document and I understand the purposes, procedures and risks of this research project as described within it.

I give permission for my doctors, other health professionals, hospitals or laboratories outside this hospital to release information to Queensland University of Technology concerning my health and treatment that is needed for this project. I understand that such information will remain confidential.

I consent to the use of blood samples taken from me for use in this specific research project only, as described in Section 4 of this document.

I have had an opportunity to ask questions and I am satisfied with the answers I have received.

I freely agree to participate in this research project as described.

I understand that I will be given a signed copy of this document to keep.

Participant’s name (printed) ______________________________________________
Signature __________________________________________ Date ______________

Declaration by parent, guardian or person responsible (where appropriate): I agree for my child/young person or the person named above who I am responsible for to participate in this research and I believe that they have understood the explanation of the study, its procedures and risks.

Name of parent/guardian to participant’s (printed) ___________________________
Signature __________________________________________ Date ______________

Name of witness to participant’s signature (printed) ___________________________
Signature __________________________________________ Date ______________

Declaration by researcher*: I have given a verbal explanation of the research project, its procedures and risks and I believe that the participant has understood that explanation.

Researcher’s name (printed) ______________________________________________
Signature __________________________________________ Date ______________

* A senior member of the research team must provide the explanation and provision of information concerning the research project.

Note: All parties signing the consent section must date their own signature.
16. Who can I contact?

Who you (or your child or the person you are responsible for) may need to contact will depend on the nature of your (or their) query; therefore, please note the following:

For further information or appointments:

Landmark Study Email: landmark@qut.edu.au or Katie Edwards, Ph: 07 3138 6154, Email: katie.edwards@qut.edu.au.

If you (or they) have any medical problems which may be related to your (or their) involvement in the project (for example, any side effects), you can contact Dr Anthony Russell Ph: 07 3240 5914 If you (or they) want any further information concerning this project you can contact the following people:

- Katie Edwards
  - Ph: 07 3138 6154
  - Email: katie.edwards@qut.edu.au

- Nicola Pritchard
  - Ph: 07 3138 6414
  - Email: n.pritchard@qut.edu.au

- Prof. Nathan Efron
  - Ph: 07 3138 6401
  - Email: n.efron@qut.edu.au

If you (or they) feel emergency medical care is required, then go to the nearest hospital Emergency Department.

For complaints:

If you (or they) have any complaints about any aspect of the project, the way it is being conducted or any questions about being a research participant in general, then you (or they) may contact:

- Ethics Manager
  - Princess Alexandra Hospital Human Research Ethics Committee
  - Ph: (07) 3240 5856
  - Email: PAH_Ethics_Research@health.qld.gov.au

- QUT Research Ethics Officer
  - Queensland University of Technology Human Research Ethics Committee
  - Ph: (07) 3138 2340
  - Email: ethicscontact@qut.edu.au

Researcher Ethics Officers/Managers are not connected with the research project and can facilitate a resolution to your (or their) concern in an impartial manner.
<table>
<thead>
<tr>
<th>Distiller ID:</th>
<th>Participant Initials:</th>
<th>Visit:</th>
<th>Baseline</th>
<th>Date:</th>
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<tbody>
<tr>
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</table>

**Participant Contact Information**

Name (First, Middle, Last):  
DOB: / /  
Postal address:  
Phone 1:  
Phone 2:  
Email address:  
Preferred method of contact:  
Home  
Mobile  
Email  
Other (specify)  
Additional contacts  
Person 1:  
Person 2:  
in case we lose touch:  
Phone:  
Phone:  

**Update to contact details**

Date:  
Name (First, Middle, Last):  
DOB: / /  
Postal address:  
Phone 1:  
Phone 2:  
Email address:  
Preferred method of contact:  
Home  
Mobile  
Email  
Other (specify)  
Additional contacts  
Person 1:  
Person 2:  
in case we lose touch:  
Phone:  
Phone:  

Date:  
Name (First, Middle, Last):  
DOB: / /  
Postal address:  
Phone 1:  
Phone 2:  
Email address:  
Preferred method of contact:  
Home  
Mobile  
Email  
Other (specify)  
Additional contacts  
Person 1:  
Person 2:  
in case we lose touch:  
Phone:  
Phone:  

Date:  
Name (First, Middle, Last):  
DOB: / /  
Postal address:  
Phone 1:  
Phone 2:  
Email address:  
Preferred method of contact:  
Home  
Mobile  
Email  
Other (specify)  
Additional contacts  
Person 1:  
Person 2:  
in case we lose touch:  
Phone:  
Phone:  

V:\MOP and CRF\CRFs\CRFs_masked.xlsx Contact Details Page 1 of 1
## MEDICAL

<table>
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<tr>
<th>Distiller ID:</th>
<th>Participant Initials:</th>
<th>Visit: Baseline</th>
<th>Date:</th>
</tr>
</thead>
</table>

| Investigator: **Medical Investigator responsible for consent form and allocating Distiller ID** |
|--------------|------------------------------------------------------------------|

<table>
<thead>
<tr>
<th>Consent</th>
<th>Original Maintained</th>
<th>Duplicate Provided</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Participant's preferred name</th>
<th>Age</th>
<th>Gender:</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Male</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Female</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Check:</th>
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<tbody>
<tr>
<td>Hospitality arranged</td>
</tr>
<tr>
<td>No coffee/tea/stimulants</td>
</tr>
<tr>
<td>Inbound cab receipt</td>
</tr>
<tr>
<td>Breakfast</td>
</tr>
<tr>
<td>Only took diabetes medication today</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Ethnicity:</th>
</tr>
</thead>
<tbody>
<tr>
<td>* Please complete based on ethnic background/phenotype, not country of birth</td>
</tr>
<tr>
<td>European (eg. European Australian, English, German, Spanish)</td>
</tr>
<tr>
<td>South East Asian (eg. Chinese, Japanese, Korean, Indonesian, Thai, Malaysian)</td>
</tr>
<tr>
<td>Asian (eg. Indian, Pakistani, Bangladeshi, Sri Lankan)</td>
</tr>
<tr>
<td>Middle eastern (eg. Iranian, Iraqi, Lebanese, Syrian)</td>
</tr>
<tr>
<td>Australian Aboriginal or Torres Strait Islander</td>
</tr>
<tr>
<td>Other; please specify;</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Hand dominance</th>
</tr>
</thead>
<tbody>
<tr>
<td>What hand do you write with?</td>
</tr>
<tr>
<td>Right</td>
</tr>
<tr>
<td>Left</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Medical History</th>
<th>General medical history</th>
</tr>
</thead>
<tbody>
<tr>
<td>Do you have any allergies? If yes, please describe</td>
<td>Yes</td>
</tr>
<tr>
<td>** *********** If allergy to any anaesthetic and/or medical dye, please notify ophthalmic observer ***********</td>
<td></td>
</tr>
<tr>
<td>Has a doctor ever told you that you have hypertension?</td>
<td>Yes</td>
</tr>
<tr>
<td>If yes, what is the current status of your hypertension?</td>
<td></td>
</tr>
<tr>
<td>Treated with medication and controlled</td>
<td></td>
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<tr>
<td>Treated with medication, but not controlled</td>
<td></td>
</tr>
<tr>
<td>Treated with lifestyle (diet and exercise) and controlled</td>
<td></td>
</tr>
<tr>
<td>Treated with lifestyle (diet and exercise), but not controlled</td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Medical History cont.</th>
<th>Medications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Please attach medications and medical practitioner contact details lists, including dosage and frequency if possible;</td>
<td></td>
</tr>
<tr>
<td>Include over-the-counter (including vitamin supplements/painkillers)</td>
<td></td>
</tr>
<tr>
<td>Include meds prescribed by an alternate therapist (eg. homeopath, naturopath, herbalist)</td>
<td></td>
</tr>
</tbody>
</table>
**Diabetes History**

In what year, and how old were you when you were first diagnosed with diabetes?

<table>
<thead>
<tr>
<th>Year:</th>
<th>Age:</th>
</tr>
</thead>
</table>

Do you use insulin?  
Yes  No

If yes;

- When did you start using insulin?  
- How many months between diabetes diagnosis and insulin use?  
  (please be as accurate as possible)

<table>
<thead>
<tr>
<th>Dosage</th>
<th>Months</th>
</tr>
</thead>
</table>

Dosage
(see medications list; please record name, daily dosage and injections per day)

In the past, has a doctor ever told you you have any of the following, directly related to your diabetes?

<table>
<thead>
<tr>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
</table>

Nephropathy (damage to the kidneys)
Retinopathy (damage to blood vessels in the back of the eye)
Neuropathy (damage to the nerves)
Stroke
Heart disease (if yes, please describe)

**Smoking**

Have you ever been a smoker?

Yes (smoked more than 100 cigarettes in total in your lifetime)
No (either never smoked, or smoked less than 100 cigarettes in total in your lifetime)-go to next section

Do you currently smoke?

Yes  No; years since quitting  

If you are a current or former smoker;

How many years in total have you been/ were you a smoker?  

On average, how many cigarettes do/did you smoke per day?  

**Alcohol**

Do you drink alcohol

Yes  No; go to next section

Yes, but not currently. If so;  

i) did you stop following medical advice?  

ii) how many years ago did you stop?  

If you are a current or former drinker;

On average, in the past year (or if a former drinker, approx), how many days per month did you drink?  

On each of these occasions, how many drinks would you have?  

**LANDMark Study Case Report Form**

**MEDICAL**

Attach Short Form McGill Pain Questionnaire - please staple to MEDICAL CRF

### Diabetic Neuropathy Symptom Score

The questions should be answered ‘yes’ (positive: 1 point) if a symptom occurred one or more times a week during the last 2 weeks or ‘no’ (negative: zero points) if it did not. Questions should be answered no if there is another explanation for the symptom (eg. injury)

<table>
<thead>
<tr>
<th>Question</th>
<th>Yes (1)</th>
<th>No (0)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Do you suffer from unsteadiness in walking? (ie. need for visual control, increase in the dark, walk like a drunk man, lack of contact with floor)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. Do you have a burning, aching pain or tenderness at your legs or feet? (ie. occurring at rest or at night, not related to exercise, exclude claudicatio intermittens)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. Do you have prickling sensations at your legs and feet? (ie. occurring at rest or at night, distal&gt;proximal, stocking glove distribution)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. Do you have places of numbness on your legs or feet? (ie. distal&gt;proximal, stocking glove distribution)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Total score**  [4/4]

### Pregnancy (ask women <50yo)

Is it likely you are pregnant?  
- Yes  
- No  
- N/A

### Blood Pressure

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Systolic</th>
<th>Diastolic</th>
</tr>
</thead>
<tbody>
<tr>
<td>BP (Sitting)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BP (Finger (sitting))</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BP (Toe (lying down))</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SpO2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Height, Weight & Waist Circumference

- **Height**  
  1 m (record to nearest 0.001m)

- **Weight**  
  1 kg (record to nearest 0.1kg)

- **Waist**  
  1 cm  
  2 cm  
  Average: cm

*If >1 cm between 2 waist measures, repeat*
**Quantitative Sensory Testing**

<table>
<thead>
<tr>
<th></th>
<th>Foot tested:</th>
<th>Right</th>
<th>Left</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensation first if odd ID#</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vibration first if even ID#</td>
<td>Initial foot temp:</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Sensation always precedes pain

<table>
<thead>
<tr>
<th></th>
<th>Room temp:</th>
<th>Humidity:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temp (°C)</td>
<td>Correctly identified</td>
<td></td>
</tr>
<tr>
<td>Cold sensation</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Warm sensation</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Cold induced pain</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Warm induced pain</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

**Averages**

<table>
<thead>
<tr>
<th></th>
<th>Cold sensation</th>
<th>Warm sensation</th>
<th>Cold induced pain</th>
<th>Warm induced pain</th>
<th>Vibration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial temp</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Compared to normalised values

- Cold sensation: Normal, Abnormal
- Warm sensation: Normal, Abnormal
- Vibration: Normal, Abnormal

**APPLY NEUROPAD & start timer**

**Heart Rate Variability**

<table>
<thead>
<tr>
<th></th>
<th>File name:</th>
<th>Comments:</th>
</tr>
</thead>
<tbody>
<tr>
<td>80 secs DB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 min rest</td>
<td></td>
<td></td>
</tr>
<tr>
<td>80 secs DB</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

E/I Ratio: _____________________

**Neuropad (10 mins)**

<table>
<thead>
<tr>
<th></th>
<th>Foot tested:</th>
<th>Right</th>
<th>Left</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pink (normal)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patchy</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blue (abnormal)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Approx % pink: __________ %

Automated analysis % pink: __________ %

**Monofilament**

<table>
<thead>
<tr>
<th></th>
<th>Foot tested:</th>
<th>Right</th>
<th>Left</th>
</tr>
</thead>
<tbody>
<tr>
<td>Points felt:</td>
<td>3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Risk of foot ulceration: Yes, No

V:\MOP and CRF\CRFs\CRFs_masked.xlsxCRFs_masked.xlsxMedical-Baseline Page 4 of 6
**Neuropathy Deficiency Score**

Record ‘✓’ for correct or ‘✗’ for incorrect response then record ‘0’ for normal or ‘1’ for abnormal (≥2/3 correct responses = normal)

<table>
<thead>
<tr>
<th>Pain Sensation</th>
<th>R1</th>
<th>R2</th>
<th>R3</th>
<th>Score (/1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>L1</td>
</tr>
<tr>
<td>Vibration Sensation</td>
<td>R1</td>
<td>R2</td>
<td>R3</td>
<td>Score (/1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>L1</td>
</tr>
<tr>
<td>Temperature Sensation</td>
<td>R1</td>
<td>R2</td>
<td>R3</td>
<td>Score (/1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>L1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Achilles Tendon Reflex</th>
<th>R</th>
<th>Present</th>
<th>Present w/ reinforcement</th>
<th>Absent</th>
<th>Score (/2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>L</td>
<td>Present</td>
<td>Present w/ reinforcement</td>
<td>Absent</td>
<td>(Total) NDS</td>
</tr>
</tbody>
</table>

**Final score**

None (0-2)  Mld (3-5)  Signif (6-8)  Severe (9-10)

**Blood Pressure**

<table>
<thead>
<tr>
<th>Supine</th>
<th>Standing (after 1min)</th>
<th>Standing (after 3min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BP</td>
<td>Systolic</td>
<td>Diastolic</td>
</tr>
<tr>
<td>Time</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 min</td>
<td>Systolic: (Supine-Standing)</td>
<td>Drop &gt; 20?</td>
</tr>
<tr>
<td></td>
<td>Diastolic: (Supine-Standing)</td>
<td>Drop &gt; 10?</td>
</tr>
<tr>
<td>3 min</td>
<td>Systolic: (Supine-Standing)</td>
<td>Drop &gt; 20?</td>
</tr>
<tr>
<td></td>
<td>Diastolic: (Supine-Standing)</td>
<td>Drop &gt; 10?</td>
</tr>
</tbody>
</table>

**Blood/urine collection at this visit?**

<table>
<thead>
<tr>
<th>Yes</th>
<th>No</th>
<th>Type 2</th>
</tr>
</thead>
</table>

**If no, referral to collection centre?**

<table>
<thead>
<tr>
<th>Centre:</th>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
</table>

**Biobank sample logged?**

<table>
<thead>
<tr>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
</table>

If no, why?
<table>
<thead>
<tr>
<th>Eligibility Checklist</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Aged 14 to 75 years</td>
<td></td>
</tr>
<tr>
<td>Signed written informed consent</td>
<td></td>
</tr>
<tr>
<td>Type 1 diabetes, Type 2 diabetes or LADA (or absence of diabetes for the control group)</td>
<td></td>
</tr>
<tr>
<td>Be willing to participate and comply with the experimental protocol</td>
<td></td>
</tr>
<tr>
<td>No history of systemic disease (e.g. malignant disease, congestive heart failure NYHA Grade III or IV, major psychosis (i.e. schizophrenia or bipolar), certain autoimmune diseases - hypothyroidism, coeliac)</td>
<td></td>
</tr>
<tr>
<td>No history of neuropathy due to non-diabetic cause (e.g. alcoholism, amyloidosis, autoimmune disorders, chronic kidney failure, connective tissue disease, infectious disease (e.g., Lyme disease, HIV/AIDS, hepatitis B, leprosy), liver failure, radiculopathy, vitamin deficiencies (e.g. pernicious anaemia, B12 deficiency)</td>
<td></td>
</tr>
<tr>
<td>No presence of severe diabetic neuropathy indicated by NDS &gt; 8</td>
<td></td>
</tr>
<tr>
<td>(NB. NDS &gt; 8 is acceptable in the Type 2 diabetic group; Group 4).</td>
<td></td>
</tr>
<tr>
<td>No current or active diabetic foot ulcer or infection</td>
<td></td>
</tr>
<tr>
<td>Not participating in any other interventional (e.g. drug) research trial.</td>
<td></td>
</tr>
</tbody>
</table>

Protocol deviation: Yes
If yes, explanation:

Place Contact Details & Medical CRFs in masking folder

Medical Investigator Signature

CRF completeness reviewed by:

Data entry completed by:
## MEDICAL

<table>
<thead>
<tr>
<th>Distiller ID:</th>
<th>Participant Initials:</th>
<th>Visit:</th>
<th>Date:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1YR</td>
<td>2YR</td>
</tr>
<tr>
<td>Investigator:</td>
<td></td>
<td>3YR</td>
<td>4YR</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Participant’s preferred name</th>
<th>Age</th>
<th>Gender:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Male</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Female</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Check:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hospitality arranged</td>
</tr>
<tr>
<td>Inbound cab receipt</td>
</tr>
<tr>
<td>Only took diabetes medication today</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Hand dominance</th>
</tr>
</thead>
<tbody>
<tr>
<td>What hand do you write with?</td>
</tr>
<tr>
<td>Right</td>
</tr>
</tbody>
</table>

### Medical History

- **General medical history, particularly since last visit**
- **Do you have any allergies? If yes, please describe**
  - Yes | No
- **If allergy to any anaesthetic and/or medical dye, please notify ophthalmic observer**
- **Has a doctor told you in the last 12 months that you have hypertension?**
  - Yes | No
  - If yes, what is the current status of your hypertension?
  - Treated with medication and controlled
  - Treated with medication, but not controlled
  - Treated with lifestyle (diet and exercise) and controlled
  - Treated with lifestyle (diet and exercise), but not controlled
  - Untreated

#### Medications

- **Please attach medications and medical practitioner contact details lists, including dosage and frequency if possible;**
  - Include over-the-counter (including vitamin supplements/painkillers)
  - Include meds prescribed by an alternate therapist (e.g., homeopath, naturopath, herbalist)

### Diabetes History Update

- **In the past 12 months, has a doctor ever told you you have any of the following, directly related to your diabetes?**
  - Yes | No
  - Nephropathy (damage to the kidneys)
  - Retinopathy (damage to blood vessels in the back of the eye)
  - Neuropathy (damage to the nerves)
  - Stroke
  - Heart disease (if yes, please describe)

#### Smoking

- **On average, how many cigarettes do/did you smoke per day? _____/day**
- **Comments:**

#### Alcohol

- **On average, in the past year, how many days per month did you drink? _____ days**
- **On each of these occasions, how many drinks would you have? _____ drinks**

**Attach Short Form McGill Pain Questionnaire - staple to MEDICAL CRF**
### MEDICAL

#### Diabetic Neuropathy Symptom Score

The questions should be answered 'yes' (positive: 1 point) if a symptom occurred one or more times a week during the last 2 weeks or 'no' (negative: zero points) if it did not. Questions should be answered no if there is another explanation for the symptom (eg. injury).

<table>
<thead>
<tr>
<th>Question</th>
<th>Yes (1)</th>
<th>No (0)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Do you suffer from unsteadiness in walking? (ie need for visual control, increase in the dark, walk like a drunk man, lack of contact with floor)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. Do you have a burning, aching pain or tenderness at your legs or feet? (ie. occurring at rest or at night, not related to exercise, exclude claudicatio intermittens)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. Do you have pricking sensations at your legs and feet? (ie occurring at rest or at night, distal&gt;proximal, stocking glove distribution)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. Do you have places of numbness on your legs or feet? (ie. distal&gt;proximal, stocking glove distribution)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Total score /4

#### Pregnancy (ask women <50yo)

Is it likely you are pregnant?  
- Yes  
- No  
- N/A

#### Blood Pressure

<table>
<thead>
<tr>
<th>BP</th>
<th>Sitting</th>
<th>SpO2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic</td>
<td></td>
<td>Finger (sitting)</td>
</tr>
<tr>
<td>Diastolic</td>
<td></td>
<td>Toe (lying down)</td>
</tr>
</tbody>
</table>

#### Height, Weight & Waist Circumference

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Unit</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Height</td>
<td>m</td>
<td></td>
</tr>
<tr>
<td>Weight</td>
<td>kg</td>
<td></td>
</tr>
<tr>
<td>Waist</td>
<td>cm</td>
<td></td>
</tr>
</tbody>
</table>

*if >1cm between 2 waist measures, repeat*
## Quantitative Sensory Testing

<table>
<thead>
<tr>
<th></th>
<th>Cold Sensation</th>
<th>Warm Sensation</th>
<th>Cold Induced Pain</th>
<th>Warm Induced Pain</th>
<th>Vibration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temp (°C)</td>
<td>Correctly identified</td>
<td>Temp (°C)</td>
<td>Correctly identified</td>
<td>Temp (°C)</td>
<td>Correctly identified</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Averages</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Compared to normalised values:
- Cold sensation: Normal, Abnormal
- Warm sensation: Normal, Abnormal
- Vibration: Normal, Abnormal

## Heart Rate Variability

- 80 secs DB
- 5 min rest
- 80 secs DB

E/I Ratio:

## Neuropad (10 mins)

- Pink (normal)
- Patchy
- Blue (abnormal)

Approx % pink: %

Automated analysis % pink: %

## Monofilament

Points felt: 3

Risk of foot ulceration: Yes, No
**Neuropathy Deficiency Score**

Record ‘✓’ for correct or ‘✗’ for incorrect response then record ‘0’ for normal or ‘1’ for abnormal (≥2/3 correct responses = normal)

<table>
<thead>
<tr>
<th>Pain Sensation</th>
<th>R1</th>
<th>R2</th>
<th>R3</th>
<th>Score (/1)</th>
<th>L1</th>
<th>L2</th>
<th>L3</th>
<th>Score (/1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vibration Sensation</td>
<td>R1</td>
<td>R2</td>
<td>R3</td>
<td>Score (/1)</td>
<td>L1</td>
<td>L2</td>
<td>L3</td>
<td>Score (/1)</td>
</tr>
<tr>
<td>Temperature Sensation</td>
<td>R1</td>
<td>R2</td>
<td>R3</td>
<td>Score (/1)</td>
<td>L1</td>
<td>L2</td>
<td>L3</td>
<td>Score (/1)</td>
</tr>
<tr>
<td>Achilles Tendon Reflex</td>
<td>R</td>
<td>Present</td>
<td>Present w/ reinforcement</td>
<td>Absent</td>
<td>Score (/2)</td>
<td>L</td>
<td>Present</td>
<td>Present w/ reinforcement</td>
</tr>
</tbody>
</table>

(Total) NDS

**Final score**

None (0-2)  Mild (3-5)  Significant (6-8)  Severe (9-10)

**Comments**

**Blood Pressure**

<table>
<thead>
<tr>
<th>BP</th>
<th>Systolic</th>
<th>Diastolic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time</td>
<td>Postural hypotension?</td>
<td>Yes</td>
</tr>
<tr>
<td>1 min</td>
<td>Systolic: (Supine-Standing)</td>
<td>Drop &gt; 20?</td>
</tr>
<tr>
<td>3 min</td>
<td>Diastolic: (Supine-Standing)</td>
<td>Drop &gt; 10?</td>
</tr>
</tbody>
</table>

**Blood/urine collection at this visit?**

Yes  No  Type 2

If no, referral to collection centre?

Yes  No

Biobank sample logged?

Yes  No

Centre: ___________

If no, why? ___________

**Protocol deviation:**

Yes  No

If yes, explanation:

---

**Place Contact Details & Medical CRFs in masking folder**

**Medical Investigator Signature**

__________

**Initials**

__________

**CRF completeness reviewed by:**

__________

**Data entry completed by:**

__________
# LANDMark Study Case Report Form

## OPHTHALMIC

<table>
<thead>
<tr>
<th>Distiller ID:</th>
<th>Participant Initials:</th>
<th>Visit:</th>
<th>Date:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Investigator:</td>
<td>Participant's preferred name:</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Hand dominance

What hand do you write with?
- [ ] Right
- [ ] Left

### Ophthalmic History

**General ophthalmic history**

<table>
<thead>
<tr>
<th>Question</th>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>History of corneal surgery?</td>
<td>[ ]</td>
<td>[ ]</td>
</tr>
<tr>
<td>History of cataract surgery?</td>
<td>[ ]</td>
<td>[ ]</td>
</tr>
<tr>
<td>History of other ocular surgery?</td>
<td>[ ]</td>
<td>[ ]</td>
</tr>
<tr>
<td>History of corneal trauma?</td>
<td>[ ]</td>
<td>[ ]</td>
</tr>
<tr>
<td>History of other ocular trauma?</td>
<td>[ ]</td>
<td>[ ]</td>
</tr>
</tbody>
</table>

If yes to any, please describe, including year of event:

Have you ever had an allergic reaction to eye drops?
- [ ] Yes
- [ ] No

If yes, please describe:

Are you a previous contact lens wearer?
- [ ] Yes
- [ ] No

Are you a current contact lens wearer?
- [ ] Yes
- [ ] No

If yes, are your lenses soft or hard?
- [ ] Soft
- [ ] Hard

How long have you been a CL wearer for? years

How frequently do you wear your lenses? _hrs/day_ days/wk

Ocular history excludes from study?
- [ ] Yes
- [ ] No

### VA (logMAR)

<table>
<thead>
<tr>
<th>OD</th>
<th>OS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unaided</td>
<td>PH:</td>
</tr>
<tr>
<td>Spectacle correction</td>
<td>OD</td>
</tr>
<tr>
<td>Over-correction</td>
<td>OS</td>
</tr>
</tbody>
</table>

### Spectacle Rx (current)

<table>
<thead>
<tr>
<th>OD</th>
<th>OS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Add</td>
<td></td>
</tr>
<tr>
<td>Notes</td>
<td></td>
</tr>
</tbody>
</table>

### Slit Lamp Examination

<table>
<thead>
<tr>
<th>OD</th>
<th>OS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Observations</td>
<td>Observations</td>
</tr>
</tbody>
</table>

Corneal compromise?
- [ ] Yes
- [ ] No
**OPHTHALMIC**

**Non-Contact Corneal Aesthesiometry**

(Record in millibars)

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Felt?</th>
<th>Stimulus</th>
<th>Felt?</th>
<th>Stimulus</th>
<th>Felt?</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Test eye only required

Final threshold:

* to 4 reversals (threshold is average of last 2)

reversal = yes -> no (start count AFTER first 5 presentations)

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Tear Collection**

Able to collect tears?

Yes  No

**PUPIL DILATION - INSERT MYDRIATIC DROPS NOW (obtain consent)**

0.5% Tropicamide  1.0% Tropicamide

**Perimetry**

FDT 1st if odd ID#  Eye tested:  OD  OS

Medmont W/W 1st if even ID#

*Test eye only required

Medmont W/W Perimetry (L’mark static)  Matrix FDT Perimetry (N-30-F)

Test 1

Test 2

performed once  performed twice

**Comments**

Good fixation by your observation?

Yes  No
**OPHTHALMIC**

**Fundus Image(s)**
*3 fields montage at time of data collection
*test eye only required

<table>
<thead>
<tr>
<th></th>
<th>OD</th>
<th>OS</th>
</tr>
</thead>
<tbody>
<tr>
<td>ETDRS Score</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Comments

**Ocular Coherence Tomography (OCT)**
*test eye only required

<table>
<thead>
<tr>
<th></th>
<th>OD</th>
<th>OS</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNFL 3.45</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ONH</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3D DISK</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MM6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Comments

**Corneal Confocal Microscopy**
*test eye only required

Eye tested:

<table>
<thead>
<tr>
<th></th>
<th>OD</th>
<th>OS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Filenames:</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Comments

**Slitlamp Examination**
Corneal epithelium
NaFl
*test eye only required

Observations
<table>
<thead>
<tr>
<th>OPHTHALMIC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IOP</strong></td>
</tr>
<tr>
<td><em>test eye only required</em></td>
</tr>
<tr>
<td>Eye tested:</td>
</tr>
<tr>
<td>Pressure:</td>
</tr>
<tr>
<td>Instrument:</td>
</tr>
<tr>
<td>Time:</td>
</tr>
<tr>
<td>Comments:</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Eligibility</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<tr>
<td></td>
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<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Corneal only</td>
</tr>
</tbody>
</table>

| Protocol deviation: | Yes | No |
| If yes, explanation: | | |

| Is any immediate follow-up for this participant required? | Yes | No |
| If yes, please explain: | | |

| Suitable for mapping? | Yes | No |
| Comments: | | |

| Suitable for/agreeable to contact for additional experiments? | Yes | No |

| Ophthalmic Investigator is responsible for 24hr follow up |

| Report by: | Optom | Admin |

| Ophthalmic Investigator Signature | | |
**OPHTHALMIC**

<table>
<thead>
<tr>
<th>Distiller ID:</th>
<th>Participant Initials:</th>
<th>Visit:</th>
<th>Date:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1YR</td>
<td>2YR</td>
</tr>
</tbody>
</table>

Investigator: Participant's preferred name:

**Hand dominance**

What hand do you write with? Right Left

**Ophthalmic History**

Recent ophthalmic history, particularly since last visit

In the past 12 months:
- History of corneal surgery?
- History of cataract surgery?
- History of other ocular surgery?
- History of corneal trauma?
- History of other ocular trauma?

If yes to any, please describe, including year of event:

Have you ever had an allergic reaction to eye drops? Yes No

If yes, please describe

Are you a previous contact lens wearer? Yes No

Are you a current contact lens wearer? Yes No

If yes, are your lenses soft or hard?

How long have you been a CL wearer for? years

How frequently do you wear your lenses? hrs/day days/wk

**VA (logMAR)**

<table>
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<td>OS</td>
</tr>
<tr>
<td>Specify:</td>
<td></td>
</tr>
</tbody>
</table>

**Spectacle Rx (current)**

<table>
<thead>
<tr>
<th>OD</th>
<th>OS</th>
</tr>
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<tbody>
<tr>
<td>Add</td>
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</tr>
<tr>
<td>Notes</td>
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</tr>
</tbody>
</table>

**Slit Lamp Examination**

<table>
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<tr>
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<tbody>
<tr>
<td>Observations</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Corneal compromise?</th>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>OS</th>
<th></th>
</tr>
</thead>
</table>

| Observations |

<table>
<thead>
<tr>
<th>Corneal compromise?</th>
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<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
# LANDMark Study Case Report Form

## OPHTHALMIC

### Non-Contact Corneal Aesthesiometry

*record in millibars*

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Felt?</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>

*test eye only required

Eye tested: [ ] OD  [ ] OS

---

<table>
<thead>
<tr>
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<tbody>
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<td></td>
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<th>Felt?</th>
</tr>
</thead>
<tbody>
<tr>
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<td></td>
</tr>
</tbody>
</table>

*test eye only required

Final threshold: [ ]

---

### Tear Collection

Able to collect tears? [ ] Yes  [ ] No

---

### PUPIL DILATION - INSERT MYDRIATIC DROPS NOW (obtain consent)

- 0.5% Tropicamide
- 1.0% Tropicamide

---

### Perimetry

<table>
<thead>
<tr>
<th>Test 1</th>
<th>Test 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>performed once</td>
<td>performed twice</td>
</tr>
</tbody>
</table>

Eye tested: [ ] OD  [ ] OS

---

### Fundus Image(s)

*3 fields montage at time of data collection

<table>
<thead>
<tr>
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<th>OS</th>
</tr>
</thead>
</table>

---

**Comments**

Good fixation by your observation? [ ] Yes  [ ] No

---

**ETDRS Score**

<table>
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<th>OS</th>
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</table>

---

**Comments**
OPHTHALMIC

Ocular Coherence Tomography (OCT)
*test eye only required

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<td></td>
<td></td>
</tr>
<tr>
<td>MM6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Comments

Corneal Confocal Microscopy
*test eye only required

Eye tested: [ ] OD [ ] OS

Filenames:

Comments

Slitlamp Examination
Corneal epithelium

NaFl

OD [ ] OS [ ]

Observations

IOP
*test eye only required

Eye tested: [ ] OD [ ] OS

Pressure:

Instrument: [ ]

Time: [ ]

Protocol deviation: Yes [ ] No [ ]

If yes, explanation:

<table>
<thead>
<tr>
<th><strong>OPHTHALMIC</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Is any immediate follow-up for this participant required?</strong></td>
</tr>
<tr>
<td>[ ] Yes</td>
</tr>
<tr>
<td>If yes, please explain: <strong>Comments:</strong></td>
</tr>
<tr>
<td>[ ] Yes</td>
</tr>
<tr>
<td><strong>Suitable for mapping?</strong></td>
</tr>
<tr>
<td>[ ] Yes</td>
</tr>
<tr>
<td><strong>Suitable for/agreeable to contact for additional experiments?</strong></td>
</tr>
<tr>
<td>[ ] Yes</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Ophthalmic Investigator is responsible for 24hr follow up</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Health care provider report by:</strong></td>
</tr>
<tr>
<td>Optom</td>
</tr>
</tbody>
</table>

**Ophthalmic Investigator Signature**
Please describe any pain you have today, or most days, that is not caused by obvious or specific cause e.g. knee replacement surgery pain. Choose the two most significant if you have more than two. Please leave the shaded cells blank.

Pain Location & Descriptors

Write location of pain

Indicate pain location on torso

Please circle the level of pain for each of the descriptors below

<table>
<thead>
<tr>
<th>Pain 1</th>
<th>Pain 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Throbbing</td>
<td>Throbbing</td>
</tr>
<tr>
<td>Shooting</td>
<td>Shooting</td>
</tr>
<tr>
<td>Stabbing</td>
<td>Stabbing</td>
</tr>
<tr>
<td>Sharp</td>
<td>Sharp</td>
</tr>
<tr>
<td>Cramping</td>
<td>Cramping</td>
</tr>
<tr>
<td>Gnawing</td>
<td>Gnawing</td>
</tr>
<tr>
<td>Hot-Burning</td>
<td>Hot-Burning</td>
</tr>
<tr>
<td>Aching</td>
<td>Aching</td>
</tr>
<tr>
<td>Heavy</td>
<td>Heavy</td>
</tr>
<tr>
<td>Tender</td>
<td>Tender</td>
</tr>
<tr>
<td>Splitting</td>
<td>Splitting</td>
</tr>
<tr>
<td>Tiring-Exhauting</td>
<td>Tiring-Exhauting</td>
</tr>
<tr>
<td>Sickness</td>
<td>Sickness</td>
</tr>
<tr>
<td>Fearful</td>
<td>Fearful</td>
</tr>
<tr>
<td>Punishing-Cruel</td>
<td>Punishing-Cruel</td>
</tr>
</tbody>
</table>

Total

Continued over page…
**Short Form McGill Pain Questionnaire**

<table>
<thead>
<tr>
<th>Visual Analogue Scale</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Please place a vertical line on the line below that best represents the pain you have on a from no pain to worst possible pain.</td>
<td></td>
</tr>
<tr>
<td><strong>NO PAIN</strong></td>
<td><strong>WORST</strong></td>
</tr>
<tr>
<td><strong>POSSIBLE</strong></td>
<td><strong>PAIN</strong></td>
</tr>
<tr>
<td>Length 0-10mm</td>
<td>Length 0-10mm</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Pain Index</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Please circle one word from the index below that best describes your pain.</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>No Pain</td>
</tr>
<tr>
<td>1</td>
<td>Mild</td>
</tr>
<tr>
<td>2</td>
<td>Discomforting</td>
</tr>
<tr>
<td>3</td>
<td>Distressing</td>
</tr>
<tr>
<td>4</td>
<td>Horrible</td>
</tr>
<tr>
<td>5</td>
<td>Excruciating</td>
</tr>
<tr>
<td>Score</td>
<td>Score</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Comments</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Please write any comments you have below:</td>
<td></td>
</tr>
</tbody>
</table>

*Thanks for completing the questionnaire.*

Investigator signature:
The LANDMark Participation Questionnaire

Our research team is investigating attitudes to participation in the LANDMark Study. We would like to find out if there is anything we can do to improve the experience of people who volunteer for our research studies.

Your participation in this questionnaire is voluntary and if you agree to complete the questionnaire on this occasion, you can choose not to be involved with this part of the project following any future visits. We will match up the answers in this questionnaire against your decision to remain or not to remain in the LANDMark study in future years. However the feedback provided by you will be analysed by a staff member who has no interaction with you when you attend for research visits. The answers from this questionnaire will at all times be kept anonymous from research staff that you meet and who are familiar with you personally.

This survey will take approximately 10 minutes to complete. Please try to answer each question in a way that reflects your true attitudes, even if these are critical, as all responses are beneficial to the study.

The following questions concern your thoughts around participating in the LANDMark Study.

Please indicate to what extent you agree or disagree with these statements.

<table>
<thead>
<tr>
<th></th>
<th>Strongly disagree</th>
<th>Mostly disagree</th>
<th>Neither agree or disagree</th>
<th>Mostly agree</th>
<th>Strongly agree</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. I thought the research staff were kind and friendly.</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
</tr>
<tr>
<td>2. I believe that the researchers in this project are driven by a desire to help people like me.</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
</tr>
<tr>
<td>3. I don’t think that my individual contribution will make much of a difference.</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
</tr>
<tr>
<td>4. The environment at ihbi struck me as relaxed and comfortable.</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
</tr>
<tr>
<td>5. It feels purposeful to participate in this study.</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
</tr>
<tr>
<td>6. I feel that the staff members have treated me with respect.</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
</tr>
<tr>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
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<tr>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>7.</td>
<td>The researchers only seemed interested in my results and not in me as a person.</td>
<td>Strongly disagree</td>
<td>Mostly disagree</td>
<td>Neither agree or disagree</td>
<td>Mostly agree</td>
</tr>
<tr>
<td></td>
<td></td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
</tr>
<tr>
<td>8.</td>
<td>I wish the appointments could have been more flexible to suit my needs.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
</tr>
<tr>
<td>9.</td>
<td>I’m curious to find out what the results from this study will be.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
</tr>
<tr>
<td>10.</td>
<td>It would be interesting to receive more feedback regarding my results.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
</tr>
<tr>
<td>11.</td>
<td>I believe that I may, through the involvement in this study, contribute to the advancement of medical knowledge.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
</tr>
<tr>
<td>12.</td>
<td>I plan to continue participating in this study.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
</tr>
<tr>
<td>13.</td>
<td>I trust that my results will be kept confidential and that information about me will not be misused.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>○</td>
<td>○</td>
<td>○</td>
<td>○</td>
</tr>
<tr>
<td>14.</td>
<td>The transport arrangements and the refreshments provided were a reasonable compensation for my participation.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>15.</td>
<td>I feel that that my participation is appreciated.</td>
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<td>16.</td>
<td>It is hard for me to find the time to participate in this study.</td>
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<td>17.</td>
<td>The refreshments I was offered were reasonable considering the duration and time of day of my visit.</td>
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<td>18.</td>
<td>I appreciated receiving the participant’s gold medal.</td>
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<td>19.</td>
<td>Participating in this study has been inconvenient for me.</td>
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20. The information and instructions given to me regarding my participation were clear and informative.  

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<tr>
<th>Strongly disagree</th>
<th>Mostly disagree</th>
<th>Neither agree or disagree</th>
<th>Mostly agree</th>
<th>Strongly agree</th>
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If you feel that your participation has been inconvenient, please specify. If you have any comments related to any of the questions, please indicate these here also.

________________________________________________________________________
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This section concerns your experience of the testing itself.

The testing consisted of a medical part and an ophthalmic part. Please indicate how you felt about the testing by circling one number for each question.

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21. How did you feel about the medical tests (e.g., measuring your weight, height, temperature and vibration threshold).  

22. How did you feel about the ophthalmic tests (any tests that were done on your eyes).

23. How did you feel about the nerve conduction tests (the tests that were done on your legs).

More questions on the next page.
Finally, we would like to ask you a few questions regarding your preferences for your involvement in this study. These questions are designed to give us a general idea of specific interests amongst participants. As with all answers in this survey, the responses below will be linked to your identity but only by a team member not directly involved with you.

Please answer the following statements by ticking the Yes or No box

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<th>Yes</th>
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<td>24. I would like to receive updates about the LANDMark Study every six months.</td>
<td>□</td>
<td>□</td>
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<td>25. I would like to participate in an annual information session about the study.</td>
<td>□</td>
<td>□</td>
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<td>26. I would be willing to complete confidential online surveys related to the LANDMark Study. The surveys may concern things like symptoms of dry eyes, etc.</td>
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<td>27. I would be able to volunteer more than one or two appointments (up to 4 hours each) per year.</td>
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Please write some suggestions of how we may further improve your participation in the LANDMark Study.

____________________________________________________________________________________
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Thank you for taking the time to fill out this questionnaire.
# LANDMark Study Case Report Form

## Study Exit

<table>
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<tr>
<th>Distiller ID:</th>
<th>Participant Initials:</th>
<th>Date:</th>
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**Investigator:**

Reason for study exit:

- **Completed study**

Discontinued due to:

- **Adverse effect**
- **Lost-to-follow-up**
- **Signed consent - not eligible**
- **Other**

Please indicate reason

**Comment:**

- 
- 
- 

Signature:
Appendix F – Standard Operating Procedures
Standard Operating Procedures

for
Corneal structural and functional markers of diabetic neuropathy
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1 Neuropathy Disability Score (NDS)

1.1 Equipment
- 2 Neurotips™ (Owen Mumford Ltd., Oxford, UK) loaded in Neuropens
- 128 Hz tuning fork
- Metal rods (length 120 mm x diameter 6 mm)
- 2 beakers
- Hot and cold water
- Tendon hammer

1.2 Testing Conditions
- The participant should either be seated comfortably with their feet on a foot rest such that their legs are horizontal, or lying down on a bed for the first 3 tests, then in the appropriate position for the reflex testing.
- The room should be quiet, with normal illumination.
- NDS protocols involve testing both feet of participants. However, in the event that a participant has undergone an amputation, or has another medical condition that precludes bilateral testing, one foot can be tested and the score doubled.

1.3 Pain Sensation
- Press the sharp end of the Neurotip™ onto the forearm to induce a painful stimulus then repeat with the blunt end, to ensure that the participant can tell the difference between the sharp and blunt stimuli.
- Ask the participant to close their eyes and apply the sharp and blunt ends of the Neurotip™, in turn, on the pulp of the great toes (R and L). Apply the first stimulus (e.g. sharp) whilst saying “1”, then immediately follow with the second stimulus (blunt) whilst saying “2”. Instruct the participant to tell you whether they think the painful stimulus occurred during time period “1” or period “2”.
- Do this procedure 3 times on each toe, randomly changing the order of the stimuli.
- Record 0 for normal or 1 for abnormal (≥2/3 correct responses = normal).

1.4 Vibration Sensation
- Vibrate the 128Hz tuning fork by striking the two prongs together against your palm. For non-vibration, strike the tuning fork flat with prongs apart against the palm. Ensure the participant can differentiate vibration and non-vibration by holding the tuning fork against the participant’s wrist so that the circular base rests lightly against the skin.
- Ask the participant to close their eyes and hold the circular base of the vibrating and non-vibrating tuning fork, in turn, against the end of the great toes (R and L). Apply the first stimulus (vibrating) whilst saying “1”, then immediately follow with the second stimulus (non-vibrating) whilst saying “2”. Instruct the participant to tell you whether the vibration occurred during time period “1” or period “2”.
- Do this procedure 3 times on each toe, randomly changing the order of the stimuli.
- Record 0 for normal or 1 for abnormal (≥2/3 correct responses = normal).

### 1.5 Temperature Sensation

- Fill two containers (beakers), one with hot water and the other with cold water. Place one of the metal rods in cold water and the other in hot water and leave for about 30 seconds. Ensure the participant can tell the difference between warm and cold sensation on their forearm.

- Ask the participant to close their eyes and press the rods in turn against the foot dorsum. Apply the first stimulus (warm) whilst saying “1”, then immediately follow with the second stimulus (cold) whilst saying “2”. Instruct the participant to say whether the warm sensation occurred during time period “1” or period “2”.

- Do this procedure 3 times on each foot dorsum, randomly changing the order of the stimuli.

- Record a 0 for normal or 1 for abnormal (≥2/3 correct responses = normal).

### 1.6 Achilles Tendon Reflex

- Instruct the participant to sit with legs horizontal, and bent so that the knee faces outward from the body. Alternatively, the participant should kneel on a chair.

- Hold the plantar surface of the foot with your left hand so that the Achilles tendon is under moderate tension and the tendon hammer is left to fall under its own weight on to the Achilles tendon with your right hand. Look for a reflex movement in the foot and a contraction of the gastrocnemius muscle. If absent, ask the participant to pull their hands together in the reinforcement position (join hands and pull against them as hard as possible, and clench teeth if necessary) just prior to hammer strike. Record 0 for normal, 1 for present with reinforcement, or 2 for absent reflex.

- Finally, record the total NDS ( __ /10) and designation (see below).

### 1.7 NDS Score

- NDS = 0-2 no neuropathy
- NDS = 3-5 mild neuropathy
- NDS = 6-8 significant neuropathy
- NDS = 9,10 severe neuropathy
2 Ocular Screening Procedures

2.1 Visual Acuity
- Measure monocular acuity OD and OS. Record as Snellen acuity.
- If acuity on targeted test eye is worse than 6/9.5, check acuity with pinhole. If improvement, refract if necessary and record best achieved acuity. If best achieved acuity remains worse than 6/9.5, consider other eye for test. If neither eye achieves 6/9.5, participant may be ineligible for part or all of the study, depending on the cause of the reduced acuity.
- In Distiller the field is ‘text’ so VA can be recorded with additional letters correct e.g. 6/9.5+2.

2.2 Slit Lamp Examination
- Position participant comfortably in chair, and position them in the chin and forehead rest.
- White light examination: Examine entire cornea, iris and lens at a high magnification. Examine conjunctiva and lids. Note the presence of any adverse findings, and indicate on diagram. Perform and record for OU.
- Where no adverse findings or staining are observed, mark this on the record as “NAD”.

2.3 Intraocular Pressure (IOP)
- IOP measurement on test eye will be made using the iCare-Tonometer, or equivalent. No anesthetic is required with the iCare Tonometer.
- Loading a new disposable probe: Remove the top of a new disposable probe container. Place the opened container against the collar. Turn the tonometer and probe up-side down at the same time so that the probe container is upright and the probe slides into the collar. Check that the probe has dropped into the collar before removing the container.
- Turning the device on & magnetising the probe: Raise the tonometer to the vertical position, taking care not to let the probe fall out. Press the measurement button. The probe will be magnetised by moving back and forward for a short time. The probe should now not fall out. The tonometer is ready for measurement when 00 appears in the display.
- Adjusting the forehead support: The forehead rest can be adjusted by turning the adjustment wheel such that the tip of the probe should be 4-8mm from the central cornea.
- Participant position: the participant should be seated on a stool, with their eye at the height of your hand in measurement position. Ask the participant to fixate on a target/object in the distance (a few meters at least), at eye level.
- Measurement: Bring the tonometer to rest on the participant's forehead, such that the probe is 4-8mm from their cornea. If necessary, adjust the distance by turning the forehead-support adjustment wheel. The central groove should be in a horizontal position. Take a measurement by lightly pressing the measurement button (press carefully to avoid shaking the tonometer). The tip of the probe should hit the central cornea. Make 6 consecutive measurements. After each successful measurement, there is a short beep. Two beeps indicate an erroneous measurement.
• Measurement display: After the 6 measurements, the IOP is shown on the display after the letter P. Record IOP. When the P is blinking, it means the standard deviation of the measurements is greater than normal;
  \[ P_- = \text{SD slightly bigger, but OK.} \]
  \[ P_-- = \text{SD greater than normal. Repeat if IOP >19mmHg} \]
  \[ P^{-} = \text{SD is great. Repeat measurements.} \]

• Turning the tonometer off: Press either of the selector buttons until the display shows End. Press the measurement button for 2 seconds. The display will then show ByE and turn off. The probe is ejected, and should be placed back in it's container in the sharps bin.

• If average reading with iCare-Tonometer is >21mmHg, repeat IOP measurement using an applanation tonometer at the completion of the entire ophthalmic protocol (topical anaesthetic is required).

• If applanation reading \(<=21\text{mmHg}, \text{retinal and corneal data is admissible. If applanation reading}>21\) (consider participant as ocular hypertensive, thus), exclude retinal and field data from analyses and refer participant to an appropriate health care provider.

• The data of the ocular hypertensive/glaucoma suspects may be analyzed separately.

2.4 Verto meter

• If participant has glasses, measure the prescription using the vertometer.

• Note that an estimate of refractive error is only required for the purpose of calculating a compensation lens for visual field assessment.

3 Non-Contact Corneal Aesthesiometry

3.1 Equipment

• A non-contact corneal aesthesiometer (NCCA) was designed and constructed for the IHBI Anterior Eye Lab by Kimble Dunster and Lincoln Hudson based on the original work of Murphy et al 1996. The instrument is intended to measure corneal sensitivity using a staircase threshold sequence.

3.2 Testing Conditions

• Make sure the room is quiet during the tests. It is extremely important that the participant is not distracted during the tests by noises (e.g. conversation, telephone ringing etc, rustling papers, people entering the room, walking round etc.).

• The room temperature should be in the range of 18-22°C, and the NCCA should not be placed under or next to any fans, air conditioning outlets, or any places where drafts may occur. The individual conducting the test should be careful not to cause any air movement around the participant’s face by their own movement.
3.3 Instrument Set-Up

- First turn the power on. The unit goes through a brief self test as a quick check of the system. Once the reference light is on (green LED), this indicates that the test is over.
- Turn the air cylinder tap on. There should now be a continuous stream of air emitted from the reference nozzle. (note; always ensure that the reference nozzle and test nozzles are the same (check by colour), or the displayed pressure will be different to that coming from the test nozzle)
- If you need to set a new upper limit (no need to do this regularly);
  - Turn the stimulus pressure knob (front panel) fully anti-clockwise.
  - Turn the larger knob on the back SLOWLY to set the maximum pressure you think you will need during the test. (having a higher limit will still allow you to go down to almost 0.00 millibars (mB), but will make adjustments in the lower range more difficult than a lower set upper limit). If you wish, you can lock the back knob into position by fastening the nut (not essential) (note: the rear panel pressure may require adjustment when output nozzle size is changed).
  - Be cautious when setting the pressure at the higher end of the range. The sensor in the instrument is able to sense and display up to approx 17 mB. However, the pressure is able to go vastly higher than 17 mB, but will continue to display just 17.

3.4 Threshold Determination

- Explain the procedure to the participant.
- Test the eye on the side of hand dominance. If that eye is ineligible (ie history of surgery, trauma), then use the other eye, but ensure that all other ophthalmic tests are performed on that eye also.
- Select the stimulus time using the push buttons to increment and decrement the seconds and tenths-of-seconds quantities. This should be 0.9s for the LANDMark protocol.
- Pull the instrument fully away from the participant. The head of the participant is placed in the head and chin rest, and the overall height of the instrument table is adjusted for comfort. Instruct the participant to look straight ahead and gaze at the fixation target.
- Move the instrument forward until the cornea is about 10 mm from, and aligned with, the nozzle tip.
- Lock slit lamp in place.
- Pressure adjustment is via a mechanical valve on the front panel. Give the participant a ‘test’ stimulus (above threshold, approx 6 millibars) so that they are aware of what the stimulus will feel like. Ask the participant to blink immediately before giving stimulus. With the participant in place, count “blink, 2, 1” , with the stimulus given on ‘1’.
- Determine the corneal sensitivity threshold using the forced choice, staircase psychophysical technique.
- Ask the participant to verbally respond, if a stimulus was felt or not.
  - If unsure, they should be instructed to respond “no”
  - Ask the participant to respond only to corneal and not to lid/lash sensation if possible
3.5 Modified Staircase Technique

- This protocol is employing a modified Garcia-Perez Staircase technique (Golebiowski et al, 2005) to determine corneal sensitivity threshold
- Participants are presented with a stimulus and asked if they felt it.
- Participants are required to give an answer for each presentation, but if unsure, should be instructed to answer no (not felt).
- Sham presentations should be included to monitor participant responses.
- An initial stimulus above threshold (6 mB) should be presented to demonstrate the sensation.
- Participants will generally respond "yes" to the demonstration 6 mbars stimulus. If so, step size is halved (3 mbars, 1.5 mbars...) in subsequent trials until first "no".
- From then, use the following criterion steps
  - If "no" (not felt), increase pressure by 0.20 mbars
  - If "yes" (felt), decrease pressure by 0.15 mbars
- A change from "yes" to "no" constitutes a reversal point
- Staircases are terminated after 4 reversals (at the criterion step size), and the average of the last 2 reversals is taken as the recorded threshold measure

3.6 “Floor” technique

- The NCCA has a stimulus pressure floor of around 0.30 mbar so it is not possible to continue testing beyond this if a participant is consistently responding "yes" at this level. If a participant responds with 2 consecutive "yes" responses at 0.30 mbar (i.e. test floor level for the instrument), the following procedure should be applied:
  - Re-check alignment, test distance and set-up, and re-train participant regarding expected sensation (including cornea v lids/lashes)
  - AFTER this, if there are 3 further consecutive "yes" responses at 0.30 (floor) at any stage in the staircase, take the following action:
    - Turn off medical air supply without alerting the participant
    - Press Stimulus Trigger once, which will release available pressure and the stimulus pressure reading should drop to zero or negative. The participant should still be aligned in slit lamp head/chin rest at this stage
    - Retest the participant by pressing stimulus trigger again (valve switching noise will still occur but there will be no air release - the participant will think there is a stimulus from the noise). This will constitute a false positive catch-trial
    - If the participant gives two out of three "yes" responses to this, conclude they are unreliable (>66% false positives) and retrain them before re-testing. If the participant does the same thing again, data should be excluded from analyses.
    - If the participant is more reliable after re-training, subsequent data is admissible
    - If the participant gives at least 2 "no" responses to 3 false positive catch trials (FP < 34%), assume they are reliable (i.e. have demonstrated they can tell air from no air), discontinue trial and allocate an admissible "floor" level threshold of 0.30 (as they will never theoretically get 8 reversals to finish the staircase if their true threshold is <0.30)
• The NCCA also is unable to measure pressures above 17 mbar. Therefore, participants responding ‘no’ to pressures of 17 mbar should be assigned a threshold of 17 mbar.

3.7 Instrument Care

• Once finished, the gas should be turned off first, then the power.
• To clean the instrument, alcohol wipes can be used.

3.8 NCCA Calibration

The manometer should be set up as above.
Every 6 months record the plot of the stimulus pressure on the NCCA display in mbars vs. the manometer reading in mbars on the attached graph. The plot should be signed by the individual conducting the calibration and filed with LANDMark documentation. Discrepancies should be brought to the attention of investigators at both sites.
Calibration graph April 2009.
4 Corneal Confocal Microscopy

4.1 Equipment

- The Heidelberg Retina Tomograph 3 (HRT3) with Rostock Cornea Module (Heidelberg Engineering, Germany) is a laser-scanning confocal microscope (LSCM) which operates by scanning a laser beam spot of less than 1 µm in diameter sequentially over each point of the examined area. In order to scan the image, the laser beam spot must be deflected in two perpendicular directions. This is achieved using two scanning mirrors: a resonant scanner deflects the beam horizontally to produce a scan line and a galvanometric scanner deflects this scan line vertically, to produce a scan field. Descanning of reflected light is performed by the same two scanning mirrors. The reflected light is deflected to a detector which is an avalanche photo diode (a point-like detector). The signal of the photo diode is digitized to form the image.

- This instrument has a field of view of 400 X 400 µm when used with a 63X objective lens that has a numerical aperture of 0.9. It uses a 670 nm red wavelength Helium-Neon diode laser as its illumination source. This is a class 1 laser system and therefore does not pose any ocular safety hazard; however, the manufacturer recommends a maximum period of exposure of 45 minutes in a single examination period. A section of about only 4 to 10 µm thick is observed at any one time.

4.2 Testing Conditions

- There are no specific conditions required, however, this should be the last test performed in the sequence, due to the occasional, temporary and minor (<1hr) blurring of vision and corneal epithelial changes. It is essential that this test be performed prior to corneal aesthesiometry, due to the requirement of corneal anaesthetization during confocal microscopy.

- Images should only be taken on the side of hand dominance.

4.3 Examination Procedure

- The participant should be familiarized with the instrument and procedures.

- Place a large drop of GenTeal Gel (carbomer 940, Ciba Vision Ophthalmics) onto the tip of the lens, avoiding air bubbles in the drop, then place the Tomocap™ over the objective lens. Disinfect the front of the Tomocap™ with an alcohol wipe (isopropyl alcohol 70% v/v), or use a new sterile Tomocap™.

- Click on the ‘Heidelberg Eye Explorer’ icon.

- Flip the forehead rest over toward the participant, and pull the chin rest out as far as possible. Adjust the LED fixation target for central fixation. Position the camera so that the optical axis of the camera runs perpendicular to the optical axis of the laser scanning camera. Remove the cap from the camera lens.

- Either create a new participant record or select an existing participant and create a new examination (both icons are red people)

- If creating a new participant record, enter; Last=Landmark, First=participant’s study code, and enter DOB and sex.

- In examination data, enter; Device type=HRT-Cornea, Operator=your initials, Study=Landmark
On the cornea module settings, the field lens should be FOV 400um (default)

The laser is now on. To turn the laser off at any time, press the green (I) icon.

Focus the objective lens onto the front surface of the Tomocap™ (bright white field), and hit **Reset**. The cap is approx 500um thick, so a similar white field is observed at the back surface of the cap, after passing though a stellate-like field ie. the gel. As such, to check that it is the front surface, the back surface is approx -500um back.

Set the depth to 40um.

Anaesthetize the eye with one drop of benoxinate hydrochloride 0.4% (oxybuprocaine hydrochloride, minim). Apply GenTeal Gel to both eyes to avoid blinking (use a new tube for each participant).

**Target set up**

A target should be set up at a distance of 1m from the participant’s eye.

The target should have 2 points on it, separated vertically by 14cm, with point “C”, for central fixation and point “I” for fixation allowing imaging of the inferior whorl.

Pull the instrument fully away from the participant. The head of the participant is placed in the head and chin rest, and the overall height of the instrument table is adjusted for comfort. Instruct the participant to look straight ahead and gaze at the red fixation light.

Move the instrument forward until the cornea is about 15mm from the Tomocap.

Using the chinrest adjustment, and the position adjustment knobs (inner=up/down, outer=left/right), align the instrument with the central cornea while the participant looks directly at the red fixation light. The laser beam should fall in the centre of the pupil. Ask the participant to blink, then keep their eyes open.

Slowly advance the instrument onto the cornea using the advancing knob. As you move closer to the cornea, you will see the Tomocap enter the image on screen and advance toward the cornea.

As the Tomocap makes contact with the cornea, the laser beam reflex should appear to be positioned immediately between the anterior pole of the cornea and the front surface of the Tomocap. Stop advancing.

On the screen to the left, an image of the tear film should now be present. As you focus forward, cell layers should appear. At approx 40-55um, the nerve plexus should appear.
• Capture images of nerves according the Image collection protocols.
• Move the instrument away from the cornea. Ask the participant to blink several times. Repeat this process until there are at least 15 images that are suitable for analysis. Each of these 15 images should not be overlapping another image by more than approximately 20%.

4.4 Image Collection Protocols

Protocol 1: Inferior whorl
• Instruct the participant to look at point I.
• Move the instrument forward until the cornea is about 15mm from the Tomocap.
• Using the chinrest adjustment, and the position adjustment knobs (inner=up/down, outer=left/right), align the instrument so that the red reflex is aligned horizontally in the middle of the cornea and vertically with approximately the bottom margin of the pupil.
• Slowly advance the instrument towards the cornea using the advancing knob. As you move closer to the cornea, you will see the Tomocap enter the image on screen.
• Ask the participant to blink, then keep their eyes open.
• Ensure that the vertical alignment looks correct (at the bottom of the (undilated) pupil margin)
• As the Tomocap makes contact with the cornea, cell layers should appear on the screen. Adjust focus until the nerve plexus appears.
• Move vertically and horizontally until the inferior whorl appears.
• The direction of the nerves should give you an indication as to where you need to move to image the whorl;

![Diagram of nerve fibers](image-url)
- Capture approx 9 images of nerves. Images should not overlap by more than 20% and be suitable for analysis.
- The 9 images should be taken as shown, with one image centred right on the centre of the whorl and another 8 surrounding;

- Move the confocal off the participant’s cornea.

**Protocol 2: Central cornea**

- Instruct the participant to look at point I.
- Move the instrument forward until the cornea is about 15mm from the Tomocap.
- Using the chinrest adjustment, and the position adjustment knobs (inner=up/down, outer=left/right), align the instrument so that the red reflex is aligned horizontally in the middle of the cornea and vertically with approximately the centre of the pupil.
- Slowly advance the instrument towards the cornea using the advancing knob. As you move closer to the cornea, you will see the Tomocap enter the image on screen.
- Ask the participant to blink, then keep their eyes open.
• As the Tomocap makes contact with the cornea, cell layers should appear on the screen. Adjust focus until the nerve plexus appears.
• Nerves should run in an approximately vertical orientation.
• Capture a number of nerves images.
• Move the instrument away from the cornea. Ask the participant to blink several times. Repeat this process until there are at least 8 images that are suitable for analysis. Each of these images should not be overlapping another image by more than approximately 20% and be suitable for analysis.

Protocol 3:

• Align the central cornea with the red reflex

5 Adverse Events

It is a requirement of the regulatory and granting bodies that all serious and unexpected adverse events (SAEs) be reported by investigators to the HRECs.
In the rare instance that an adverse event should occur, the Investigator will complete the HREC applicable adverse event form to document the condition. The Investigator will report the adverse event to the local HREC and to the study coordinator within 5 working days of discovery of the event.

The Investigator will use his/her clinical judgment as to whether or not the participant reporting with an adverse event should continue in the study.

According to NHMRC National Statement on Ethical Conduct in Human Research 2007, the definition of a serious adverse event (SAE) is any untoward medical occurrence that:

- results in death;
- is life-threatening;
- requires in-participant hospitalisation or prolongation of existing hospitalisation;
- results in persistent or significant disability/incapacity;
- is a congenital anomaly/birth defect; or
- is a medically important event or reaction.

SAEs include all of the above events if they occur during the research project. The PAH HREC SAE form will be printed on Queensland Health letterhead. The Principal Investigator (or most appropriate investigator) at each site will sign the form before submission to the HREC to indicate they have viewed the SAE and considered, in his/her expert opinion, whether the event is related or not related to the study. SAEs at the PAH shall be reported to the Ethics Secretariat as soon as practicable using the On-site SAE Form (1 original + 15 copies) from the following:

6 Case Report Form Management

The purpose of this SOP is to ensure the project maintains correct, legible and complete data in the case report forms (CRF).

These procedures are applicable to all research team members involved in the collection, notation and recording of data. Ink shall be used.

RESPONSIBILITIES

All research team members who gather information in any form from the participant are responsible for the correct collection of that information.

PROCEDURES

- Use black or blue ball point pen; do not use pencil.
- The CRF indicates inclusion/exclusion criteria met.
- All data specified in the protocol to be collected and if not, the field is to be marked with a value of “none” or in the case inability/omission, “not done”.
- All data is to be entered correctly, legibly and completely. If there is an error in transcription, the investigator is to draw a line through the mistake, write the correct response either beside or above the corrected error then date and initial the change.
- Errors are not to be overwritten, scratched out or have liquid paper applied in an attempt to correct.
- All participant file pages are to have participant ID and initials on them. These include such documents as the participants medication lists whether typed, handwritten or photocopied, the pain questionnaire, doctors or pharmacy letters and the taxi receipt.
- Please ensure all these forms as well as the PICF and consent are also present in the participant’s file.
- Where a data field is found missing, the relevant investigator is alerted immediately so as to rectify the omission.
- When all fields are complete, the CRF is to be scanned to pdf in the appropriate directory (eg VisLandmark/Data/FG85/CRF’s_Baseline_120509).
- A CRF reviewer is to co-initial the bottom of each page checked.
- The paper documents to be filed in a secure filing cabinet.
- Participant is phoned 24 hours after visit by an investigator and this is noted in their file.
7 Side to Test

The hand dominant side is tests unless otherwise indicated for medical, ophthalmic and nerve conduction tests.

If an alternate side is indicated, this is shall be noted on the case report form.

For the tests designated Medical there are several instances when the hand-dominant side is contraindicated e.g. amputated toes, active ulcer in which case all Medical lab tests shall be done on the non-dominant hand side. In the instance of NDS, the tests are performed on one side only and the score is doubled.

For ophthalmic tests there are several instances when the hand-dominant side is contraindicated e.g central corneal scar.

If the test side is contraindicated at subsequent visits, this is noted as a protocol deviation, e.g. using the contralateral eye for CCM due to recent cataract surgery, potential eye inflammation or scar.
The IHBI Non-Contact Corneal Aesthesiometer

(after Murphy 1996)

User Guide

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Introduction
This non-contact corneal aesthesiometer (NCCA) was designed and constructed for the IHBI Anterior Eye Lab by Kimble Dunster and Lincoln Hudson based on the original work of Murphy et al 1996. The instrument is intended to measure corneal sensitivity using a stair case threshold sequence. Murphy and co-workers (1998) found a stimulus of 0.9s and bore size of either 0.5mm or 1.0mm to provide the most reliable measures of sensitivity. In 2004, the same group found that healthy non-diseased eyes had a corneal threshold sensitivity of between 0.1 and 1.0 millibars and 0.1 to 2.4 millibars in diabetics and elderly participants. The stimulus area on the cornea was found to be approx 0.196 mm² using the 1.0mm diameter bore (Murphy et al, 1998). Tavakoli and co-workers in 2007 found, using the same instrument in their lab in Manchester, a range of 0.5 to 4.11 millibars for diabetic and control participants.

Operation

• For the instruments made in Australian for the UK, UK IEC power leads will be required and UK style medical air connectors will need to be added to the hoses.

• The units must only be used with medical grade air; oxygen must not be used.

• The units must be switched on when setting pressure
• The maximum pressure that may be displayed is \(~17\text{mbar}\). If pressures above that are set, the display will only indicate \(~17\text{mbar}\).

When changing nozzles, follow this procedure:

• Fit reference and stimulus nozzles – both must be the same size
• Set the rear regulator to minimum by turning anti-clockwise
• Set the front flow control to maximum by turning fully anti-clockwise
• Turn the unit on and wait until it boots up
• SLOWLY increase the pressure using the rear regulator until the maximum pressure to be used in the test is reached. Continue to about 3mbar above that pressure.
• Lock the rear regulator
• Adjust the pressure with the front regulator during the test.
• If there is insufficient range using the front control, SMALL adjustments of the rear regulator may be required.

The pressure of the air is monitored using an electronic pressure sensor which displays its reading digitally in millibars (mbars) above atmospheric pressure. The air outflow, and thus pressure, of the reserve is regulated using a manually controlled valve. Two electronically controlled two-way switch valves direct the flow of the air either to an exhaust jet or, when a stimulus is to be applied to the eye, via the stimulus jet.

The stimulus jets are made out of brass and are 35mm in length and 8mm in external diameter. There are 3 different internal diameters, 0.5 mm (blue), 1.25 mm (white) and 2.0 mm (red) (Figure 5) through which passes the air stimulus.

Using Schlieren interferometry, Murphy and co-workers demonstrated the air flow at 1cm distance had a turbulent fringe diameter of 3 mm, however, the fringe having a lower pressure intensity than the central core of the flow stimulated an area of 0.8 mm\(^2\) (Murphy, Patel et al. 1996).

The time duration of the stimulus can be varied by electronic control of the switch valves to deliver an air pulse of any desired duration from 0.1 to 9 seconds. The various components of the device are connected using nylon tubing, of 4 mm diameter with a 2.5 mm central bore. The valve, pressure sensor and its display, and the connecting tubing are all placed within a self-contained polycarbonate sealed box shown in Figures 1 to 3.
Figure 1: Front of instrument

Figure 2: Back of instrument

Figure 3: Front of instrument with nozzle and external push button attached

The nozzle, air connector and cylinder arrangement are shown in Figures 4 to 6.
The stimulus jet is positioned close to the eye by means of a slit-lamp attachment, which allows accurate alignment of the jet. This air jet mounting attachment is made up of a vertical metal bar, which is placed in the hole found at the top of the slit-lamp’s rotational pivot. The testing distance of 1 cm is set by using a clear, plastic centimetre ruler attached to the side of the plastic mount, and extending towards the eye, similar to the scale on an exophthalmometer.

**Electronic operation:**

*Power on self test (POST)*

When power is turned on, the unit goes through a brief self test to be used by the operator as a quick check of the system.
Figure 7: The reference and stimulus output valves and LEDs.

The sequence is as follows:

1) The two panel LEDs (one red one green) and the pressure display backlight will flash twice in unison

2) The reference valve and led will turn on for one second then off

3) The stimulus valve and led will turn on for one second then off

4) A delay of 2 seconds before going into normal operational mode

Operational mode
At rest, the reference valve will be on, directing air flow through the reference outlet.

Select the stimulus time using the push buttons to increment and decrement the seconds and tenths-of-seconds quantities.

Figure 8: The stimulus time gauge.
Depressing the external push button briefly will turn the reference valve off and the stimulus valve on, directing air flow through the stimulus outlet for the selected time. At the end of the time period, both valves will switch over.

**Figure 9:** Pressure release button.

If a time of zero is selected, the valves will switch in response to the external push button. That is, the air flow will be directed to the stimulus outlet for as long as the button is held depressed.

**Sleep mode**

After 15 minutes of inactivity, the unit goes into sleep mode. Both valves and the pressure display backlight are turned off. To wake from sleep mode, briefly press the external push button.

**Display**

The pressure display is set to show the pressure before the output in millibars.

**Figure 10:** Pressure stimulus display.
**Power**

Power to the unit is 24 VDC supplied by a hard-wired external supply connected to the 240VAC mains via a standard IEC power lead.

**Serial output**

The board has the provision for serial RS232 communications. A level converter is required for connection to a PC. Connection is via a header on the board. Currently the port is set to 9600 baud, 8 data bits, 1 stop bit, no parity. With DIP switch 4 off, the unit will output the calculated pressure and the state variable once every second. With DIP switch 4 on, more information is sent each second. Reboot the unit to see the column headings that describe what this extra information is. Both data streams use comma separation making it suitable for input to spreadsheets. On reboot, the unit will also output the software version and the serial number of the board.

**Pressure system:**

**Pressure adjustment**

Pressure adjustment is via a mechanical valve on the front panel. A more coarse adjustment is available on the rear panel. The rear panel pressure may require adjustment when output nozzle size is changed. The same size nozzle must be connected to the reference and stimulus output.

![Figure 11: Pressure stimulus adjustment knob.](image)

Consideration must be given to the fact that the pressure does not necessarily equate to a particular flow through the nozzle. For example, changing the nozzle from a narrow to a wide one, keeping the pressure constant, will result in an increase in flow. Here, pressure is constant but flow changed. The converse example is keeping the nozzle the same and increasing the pressure. This will result in an increased flow, with increased pressure, but probably not a proportional increase.

**Nozzle diameters**

Three nozzle diameters have been constructed with internal diameters of 0.5mm, 1.25mm and 2.0mm, banded with blue, white and red respectively.
Clinical Use

The stimulus jet is positioned close to the eye by means of a slit-lamp attachment, which allows accurate alignment of the jet. This air jet mounting attachment is made up of a vertical metal bar, which is placed in the hole found at the top of the slit-lamp’s rotational pivot. It is 17 cm tall, from the bottom of the base plate to the air jet, which is mounted in a holder. The bar is also set back 2.5 cm from the pin which fits into the hole. The testing distance of 1 cm is set by using a clear, plastic centimetre ruler attached to the side of the plastic mount, and extending towards the eye, similar to the scale on an exophthalmometer. Alignment of the air jet with the centre of the cornea is carried out visually. The sensation felt by the subject is described in a variety of ways, the most common being as a ‘cold’ sensation. It is also described as feeling like a breeze on the eye, or as a pressure type sensation; however, it is not an unpleasant sensation. Unsurprisingly, subjects report the stimulus as being quite difficult to describe, especially when close to threshold.

The subject is asked to place his or her head against the slit-lamp headrest and look straight ahead, at a distant fixation target, using the non-test eye. The slit-lamp was then adjusted until the end of the air jet was aligned with the centre of the cornea, 1 cm away. The duration of the air pulse was set at 0.9 seconds, and once the subject was comfortable, a stimulus was released at a predetermined air pressure.

There are several well-documented methods for the evaluation of the threshold in psychophysical tests (Goldstein, 1989). Two popular experimental techniques for the estimation of reliability and the threshold level are: (1) the method of constant stimuli; and (2) the method of limits.

A modified approach to the Garcia-Perez staircase has been applied to measuring corneal sensitivity with the NCCA (Golebiowski, Papas et al. 2005; Pritchard, Edwards et al. 2010); the staircase begins above the corneal sensitivity threshold, a step-up of 0.2 mbars and a step-down of 0.15 mbars is applied to 4 reversals. The average of the last two reversals is recorded as the corneal sensitivity threshold. Three examples of the staircase procedure are shown in the following figure.
LANDMark Study Case Report Form

**OPHTHALMIC**

Non-Contact Corneal Aesthesiometry
(record in millibars)

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Felt?</th>
</tr>
</thead>
<tbody>
<tr>
<td>&quot;test eye only required&quot;</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>✓</td>
</tr>
<tr>
<td>5</td>
<td>✓</td>
</tr>
<tr>
<td>1.5</td>
<td>✓</td>
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</tr>
<tr>
<td>0.35</td>
<td>✓</td>
</tr>
<tr>
<td>0.30</td>
<td>x</td>
</tr>
<tr>
<td>0.20</td>
<td>✓</td>
</tr>
<tr>
<td>0.10</td>
<td>✓</td>
</tr>
<tr>
<td>0.05</td>
<td>✓</td>
</tr>
<tr>
<td>0.00</td>
<td>✓</td>
</tr>
</tbody>
</table>

Eye tested: [ ] OD [ ] OS

Stimulus Felt?

Final threshold: [unreliable → failed 4\frac{1}{5} false positives.]

* to 4 reversals (threshold is average of last 2)
reversal = yes -> no (start count AFTER first 5 presentations)

Non-Contact Corneal Aesthesiometry
(record in millibars)

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Felt?</th>
</tr>
</thead>
<tbody>
<tr>
<td>&quot;test eye only required&quot;</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>✓</td>
</tr>
<tr>
<td>5</td>
<td>✓</td>
</tr>
<tr>
<td>1.5</td>
<td>✓</td>
</tr>
<tr>
<td>0.75</td>
<td>x</td>
</tr>
<tr>
<td>1.00</td>
<td>✓</td>
</tr>
<tr>
<td>0.95</td>
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<tr>
<td>0.70</td>
<td>x</td>
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<tr>
<td>0.05</td>
<td>x</td>
</tr>
<tr>
<td>0.00</td>
<td>✓</td>
</tr>
</tbody>
</table>

Eye tested: [ ] OD [ ] OS

Stimulus Felt?

Final threshold: 1.275
Average of reversals 3+4.

* to 4 reversals
reversal = yes -> no but do not include first change
Safety and Contacts

Inside the unit there is a blow-off valve that has been set to release excess pressure in the event of a failure in the internal regulator. The setting of this valve should not require further adjustment. Medical gas cylinder heads should be serviced at the interval recommended by the manufacturer. The unit must not be used with gases other than medical air. Oxygen must not be used at any time. It is further recommended that internal pneumatic components are replaced every five years.

Diagnostic mode (Intended for use by service personnel)

Set DIP switch 1 to enter diagnostic mode.

DIP switch 2 turns the reference solenoid valve and led on and off.

DIP switch 3 turns the stimulus solenoid valve and led on and off.

The external push button turns the display backlight off and on.

Contacts:

Kimble Dunster k.dunster@qut.edu.au, 0412 122 690

Lincoln Hudson l.hudson@qut.edu.au, 3138

There are no formal arrangements with any person or group at QUT or elsewhere regarding ongoing maintenance and service and may be provided on a fee-for-service basis.

References

Goldstein, 1989

Murphy et al 1998

Tavakoli et al 2007
Appendix G – Additional Nerve Maps
## Additional Nerve Maps

### Table 1. Study participant characteristics

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Participant ID 119DH</th>
<th>Participant ID 121MJ</th>
<th>Participant ID 123MH</th>
<th>Participant ID 129JP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td>M</td>
<td>M</td>
<td>M</td>
<td>M</td>
</tr>
<tr>
<td>Age (yrs)</td>
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<td>54</td>
<td>57</td>
<td>33</td>
</tr>
<tr>
<td>T1/T2</td>
<td>T1</td>
<td>T1</td>
<td>T1</td>
<td>T1</td>
</tr>
<tr>
<td>Duration of diabetes (yrs)</td>
<td>22</td>
<td>45</td>
<td>52</td>
<td>2</td>
</tr>
<tr>
<td>Insulin dependence</td>
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<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Eye used R/L</td>
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<td>Right</td>
<td>Right</td>
<td>Right</td>
</tr>
<tr>
<td>VA test eye</td>
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<td>6/12-</td>
<td>6/9.5</td>
<td>6/6</td>
</tr>
<tr>
<td>Neuropathy disability score (0-10)</td>
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<td>1</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>