LONGITUDINAL ASSESSMENT OF CORNEAL SUBBASAL NERVE MORPHOLOGY AS A POTENTIAL MEASURE OF DIABETIC PERIPHERAL NEUROPATHY

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Submitted in fulfilment of the requirements for the degree of
Doctor of Philosophy

School of Optometry and Vision Science

Institute of Health and Biomedical Innovation

Faculty of Health

Queensland University of Technology

2015

KEYWORDS

Corneal confocal microscopy

Diabetic peripheral neuropathy

Subbasal nerve morphology

Natural history

ABSTRACT

It is estimated that currently more than 382 million people are living with diabetes worldwide. Up to half of the people with diabetes are also affected by diabetic neuropathy as the most common complication of diabetes. Diabetic peripheral neuropathy (DPN) which begins with symptoms such as burning pain, tingling and numbness of the lower extremities, is the main factor predisposing diabetic patients to ulceration and subsequently to amputation. Other than glycaemic control and pain management, there is no effective therapy to prevent the DPN or halt its progression. Proper detection and management of DPN can improve quality of life and prevent morbidity and mortality of these patients. Limitations of conventional measures of neuropathy prompted the search for simple, rapid and non-invasive markers for screening, diagnosis and follow-up of DPN.

While quantification of the corneal subbasal nerve plexus (SNP) using corneal confocal microscopy (CCM) has been shown to be a promising marker for detection and stratification of DPN in several studies over the past decade, no data is available concerning the natural course of changes to this nerve plexus in health or diabetes. Furthermore, there is uncertainty as to whether age influences the SNP.

Prior to utilizing the SNP morphometric parameters in the longitudinal context to investigate these research gaps, two important studies were conducted relating to methodological development. The first experiment provided information in respect to employment of an automated algorithm for quantification of corneal nerve morphology and the suitability of using this technique in diabetic individuals without and with neuropathy compared with manual and semi-automated methods. In the second experiment, the repeatability of CCM in combination with automated analysis has been examined in a cohort of diabetic and healthy individuals, in which corneal nerve fibre length was found to be the most repeatable and reliable SNP morphometric parameter. Having addressed these two main methodological issues, application of corneal confocal microscopy combined with automated

image analysis in a cohort of healthy participants without diabetes or neuropathy revealed the effect of age on central corneal nerve morphology as well as the stability of this nerve plexus over three years using longitudinal data. Finally, the natural history of three main SNP structural parameters in a cohort of diabetic individuals with and without DPN was assessed and the longitudinal relationship between these parameters and established measures of neuropathy was determined. Corneal nerve fibre density decreased over time in DPN group compared to controls. Moreover, the SNP parameters found to be associated with some neuropathy measures. Overall, this study demonstrated stability of corneal nerve morphology in the healthy state and dynamic small fibre damage at the SNP associated with DPN, thus providing justification for ongoing efforts to establish corneal nerve morphology as an appropriate adjunct to conventional measures of DPN.

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LIST OF ABBREVIATIONS

CCM: Corneal confocal microscopy

CI: Confidence interval

CNBD: Corneal nerve branch density

CNFD: Corneal nerve fibre density

CNFL: Corneal nerve fibre length

CoR: Coefficient of repeatability

DNSS: Diabetic neuropathy symptom score

DPN: Diabetic peripheral neuropathy

DPN+ve: Diabetic participant with peripheral neuropathy

DPN-ve: Diabetic participant without peripheral neuropathy

EDB: Extensor digitorum brevis

FH: Fossa head

HbA_{1c}: Glycosylated haemoglobin

HDL: High density lipoprotein

ICC: Intraclass correlation coefficient

IENFD: Intraepidermal nerve fibre density

IGT: Impaired glucose tolerance

IOP: Intra ocular pressure

LDL: Low density lipoprotein

LoA: Limits of agreement

LSCM: Laser-scanning confocal microscopy

NCS: Nerve conduction studies

NDS: Neuropathy disability score

QST: Quantitative sensory tests

SD: Standard deviation

SEM: Standard error of mean

SNP: Subbasal nerve plexus

SSCM: Slit-scanning confocal microscopy

T1DM: Type 1 diabetes mellitus

VPT: Vibration perception threshold

STATEMENT OF ORIGINAL AUTHORSHIP

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

QUT Verified Signature

Signature:

Date: June 1, 2015

LIST OF PUBLICATIONS AND MANUSCRIPT DURING CANDIDATURE

Relevant publications during Candidature

1. Pritchard, N., Edwards, K., Dehghani, C., Fadavi, H., Jeziorska, M., Marshall, A., Petropoulos, I. N., Ponirakis, G., Russell, A. W., Sampson, G. P., Shahidi, A. M., Srinivasan, S., Tavakoli, M., Vagenas, D., Malik, R. A., Efron, N. (2014). Longitudinal assessment of neuropathy in type 1 diabetes using novel ophthalmic markers (LANDMark): study design and baseline characteristics. *Diabetes Research and Clinical Practice, 104*(2), 248-256.
2. Dehghani, C., Pritchard, N., Edwards, K., Russell, A. W., Malik, R. A., & Efron, N. (2014). Fully automated, semiautomated, and manual morphometric analysis of corneal subbasal nerve plexus in individuals with and without diabetes. *Cornea, 33*(7), 696-702.
3. Dehghani, C., Pritchard, N., Edwards, K., Vagenas, D., Russell, A. W., Malik, R. A., & Efron, N. (2014). Morphometric stability of the corneal subbasal nerve plexus in healthy individuals: a 3-year longitudinal study using corneal confocal microscopy. *Investigative Ophthalmology and Vision Science, 55*(5), 3195-3199.
4. Dehghani, C., Pritchard, N., Edwards, K., Vagenas, D., Russell, A. W., Malik, R. A., & Efron, N. Natural history of corneal nerve morphology in mild neuropathy associated with type 1 diabetes: development of a potential measure of diabetic peripheral neuropathy. *Investigative Ophthalmology and Vision Science*, 55(12), 7982-7990.

Relevant manuscript accepted for publication

Nil

ACKNOWLEDGMENT OF JOINT AUTHORS AND VERIFICATION OF PERMISSIONS

A statement of acknowledgement of co-authors is presented in Appendix 1. All co-authors have acknowledged the following statement:

"Statement of Contribution of Co-Authors for Thesis by Published Paper"

The authors 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 QUT ePrints database consistent with any limitations set by publisher requirements.

ACKNOWLEDGMENTS

It would not have been possible to make this work without the encouragement, help and support of the kind people around me, to only some of whom it is possible to give particular mention here.

I owe my deepest gratitude to my supervisory team Professor Nathan Efron, Dr. Nicola Pritchard and Dr. Katie Edwards for their aspiring guidance, continuous encouragement and support during this work. Their mentorship was paramount in providing confidence and a well-rounded experience consistent with my long-term career goals.

It gives me great pleasure in acknowledging the support and help of the LANDMark team at IHBI. I am also deeply grateful to the LANDMark study participants for giving up their time for this study.

I express my warmest gratitude to Queensland University of Technology (QUT) for providing me the QUTPRA scholarship which made this journey possible.

I would like to thank Dr. Dimitrios Vagenas for his invaluable statistical support. Advice and comments given by Professor Rayaz Malik and Associate Professor Anthony Russell has been a great help in enhancing the quality of papers embedded in this thesis.

Finally, and most importantly, I would like to express appreciation to my beloved wife Zohreh, whose love, kindness, patience and support enabled me to accomplish this work.

CHAPTER 1. INTRODUCTION

1.1 Foreword

In this chapter, diabetic peripheral neuropathy (DPN) as one of the most common complications of diabetes is briefly introduced. The significance of this body of work is then reviewed, followed by the aims and the research questions. An outline of the design is presented and the structure of the thesis is outlined. This chapter concludes with the candidate's contribution towards this research project and to the LANDMark study.

1.2 Background

Diabetes is one of the most common diseases, with an estimate of 382 million people living with this complex condition worldwide (International Diabetes Federation, 2013). This disease can lead to serious complications including nephropathy, retinopathy and neuropathy. Diabetes can cause a wide variety of nervous system problems among which DPN is one of the most important complications (Chin & Rubin, 2010). DPN is the most commonly encountered form of neuropathies and imposes significant public health burdens. Up to half of patients with diabetes have neuropathy (Boulton et al., 2004b) which leads to numbness, tingling, pain or weakness and typically begin in lower extremities and may progress proximally. Lack of awareness of foot injury may lead to foot ulcers which in advanced stages can result in lower limb amputation (Tesfaye, 2007).

While poor glycaemic control is considered as the main risk factor for developing DPN, several studies have shown that other risk factors such as duration of diabetes, hypertension, hyperlipidaemia, obesity and smoking are involved as well (Tesfaye & Selvarajah, 2012). Studies investigating the natural history of neuropathy in diabetes mostly show a gradual worsening of DPN over time, despite differences between studies in the tests for neuropathy assessment (see section 2.2.1 Natural history of DPN). Although various aetiologies have been suggested, the precise pathogenesis responsible for loss and damage of nerve fibre underlying clinical DPN

remains controversial and may involve direct metabolic compromise and microvascular ischemia (Tesfaye & Selvarajah, 2012).

Other than glycaemic control, there is no effective therapy to prevent DPN (Callaghan et al., 2012b); however there are modifiable risk factors that have an important role in developing and worsening of DPN (Tesfaye et al., 2005). Accurate detection and management of complications can improve quality of life and prevent morbidity and mortality of these patients. Furthermore, lack of an early biomarker for nerve changes in diabetic neuropathy is one of the most important impediments of pharmacologic intervention in clinical research (Malik, 2014a; Ziegler & Luff, 2002).

The development of a simple, non-invasive method for screening, diagnosis and follow-up of DPN has been explored because conventional techniques for assessment of diabetic neuropathy including nerve and skin biopsy, quantitative sensory tests (QST) and nerve conduction studies (NCS) are invasive, uncomfortable, expensive, unable to detect small fibre damage and repair or require highly specialised medical expertise and equipment (Skljarevski & Malik, 2007). Therefore, the establishment of an appropriate surrogate marker for DPN which can identify patients at risk and prompt more intense intervention including improved glycaemic, blood pressure and lipid control is crucial. Furthermore, a sensitive surrogate marker would significantly pave the way for development of effective disease-modifying therapeutics. As the most innervated tissue in the body (Müller et al., 1997) and being directly assessable to inspection using light, the cornea became a natural target for this purpose.

1.3 Significance of the study

Structural analysis of the corneal subbasal nerve plexus (SNP) using confocal microscopy (CCM) has been introduced as useful measure for assessing DPN. Several studies have suggested that this potential corneal measure can be used to monitor, non-invasively and cost-effectively, the progression of DPN and the effects of any clinical/therapeutic interventions (Ahmed et al., 2012; Hertz et al., 2011; Malik et al., 2003; Mehra et al., 2007;

Midena et al., 2006; Pritchard et al., 2011; Rosenberg et al., 2000; Tavakoli et al., 2010b). Hence, by identifying abnormalities in corneal nerve morphology, CCM might help clinicians to detect neuropathy more easily, and by repeat examination assess the benefits of interventions, such as improved glycaemic control and treating conventional vascular risk factors. Given the promising role of corneal nerve structure in screening and assessment of DPN, in the current literature there is no general agreement in regards to the effect of age on the SNP. Additionally, to date, no data exists on longitudinal changes in corneal morphology, either in the healthy eye or in neurological dysfunction. Moreover, a longitudinal study was required to support the cross-sectional studies that demonstrated the capability of CCM for detection and evaluation of DPN.

This study focuses on the temporal changes of the corneal nerve microstructure and neuropathy measures in healthy individuals and participants with type 1 diabetes. The reason for including this type of diabetic participants (and not participants with type 2 diabetes) is that the underlying mechanisms in these two main types of diabetes are different and the pathogenesis of nerve damages may be differently influenced by metabolic factors in type 1 and type 2 diabetic patients (Kasalova et al., 2006). Furthermore, evidence exists that these patients are prone to develop neuropathic changes sooner than type 2 diabetic patients (Kamiya et al., 2005; Kasalova, et al., 2006).

1.4 Aims of the study

To date various methods have been developed and introduced to quantify corneal nerve parameters from CCM images. The first purpose of this research project was to assess the extent to which a newly developed fully automated method of SNP morphometric analysis agrees with two manual and semiautomated methods, which was essential particularly for this longitudinal study with repeated measurements over time where multiple images per participants needed to be analysed. The second aim was to explore the age-dependent alterations and the natural history of SNP morphology in healthy state. This would provide more information about the

dynamic changes of this nerve plexus which has applications not only in respect to its utility in assessing DPN, but also in various ocular and systemic conditions. The third aim of this study was to investigate longitudinal changes in corneal nerve morphology in type 1 diabetic participants with and without neuropathy. Additionally, the relationship of this non-invasive corneal measure with conventional measures of neuropathy was examined and important risk factors associated with small nerve fibre damage has been addressed.

1.5 Research questions

From the main aims of this study, the following main research questions were derived:

1. How does the fully automated method of SNP analysis agree with manual and semiautomated techniques in terms of SNP parameter quantification and detection capability in a cohort of healthy controls and diabetic individuals with and without neuropathy?
2. Is SNP influenced by age and what is the behaviour of this nerve plexus over time in healthy state?
3. What is the natural history of SNP in diabetic individuals? Is this different in diabetic people with and without neuropathy vs. healthy individuals?
4. Is there any relationship between longitudinal changes in corneal nerve structure and conventional measures of neuropathy?

1.6 Hypotheses

The following specific hypotheses were tested in this study:

1. Automated quantification of corneal nerve parameters provides comparable neuropathy detection ability to manual and semiautomated methods.
2. CCM is able to detect the age-dependent alterations of SNP morphology in healthy individuals.

3. Temporal changes of the SNP are different in diabetic participants with and without DPN.
4. Changes in corneal nerve structure relates to traditional measure of DPN.

1.7 Structure of the thesis

This thesis is presented for the PhD by publication and comprised of a series of cross-sectional and longitudinal studies to address the research questions. The overall structure of this thesis takes the form of eight chapters, including this introductory chapter. In Chapter 2, the literature relevant to the study is reviewed. This chapter begins with an introduction to diabetes and DPN, followed by a detailed review of corneal nerve morphology as potential measure of DPN. The third chapter presents the findings of the comparison study of the three quantification methods for SNP morphometric analysis, and has been published in the journal *Cornea*. Chapter 4 examines intraobserver repeatability and interobserver reproducibility of the SNP parameters quantification and serves as a linkage between chapters 3 and 5. The longitudinal nature of the study necessitated conducting interobserver and intraobserver repeatability study concerning corneal nerve morphology, which is important when measurements are repeated over time to detect real changes with some confidence levels, if there is any change.

Chapter 5 presents the baseline characteristics of the participants in this longitudinal study. Data presented here is a part of data used to form a paper entitled “Longitudinal assessment of neuropathy in type 1 diabetes using novel ophthalmic markers (LANDMark): Study design and baseline characteristics” which has been published in the *Diabetes Research and Clinical Practice* journal. Chapter 6 explores the longitudinal assessment of corneal nerve structure over time and also highlights the effect of age on SNP morphology in healthy individuals. This is essentially basis of a paper which was published in the journal *Investigative Ophthalmology and Visual Science*. Chapter 7 examines the natural history of corneal nerve parameters in diabetic individuals with and without neuropathy and also explores the longitudinal relationship between potential corneal measures and the

traditional measures of DPN. This chapter encompasses a paper that has been published in the journal *Investigative Ophthalmology and Visual Science*. Finally, the last chapter (Chapter 8) gives a brief summary with a reflection on how effectively the research aims have been addressed.

A list of references cited in this work is presented following Chapter 8 in APA style. Appendices included in this thesis are Acknowledgement of Joint Authors and Verification of Permissions, Human Ethics Approval Certificate and Participant Information and Consent Form.

1.8 Candidate's contribution to this research project and to the LANDMark study

The studies included in this PhD thesis are associated with the existing database of the LANDMark study (Longitudinal Assessment of Neuropathy in Diabetes using novel ophthalmic MARKers) (Pritchard et al., 2014) which is a two-site four-year longitudinal observational study. The LANDMark study is a broad area of research that employed corneal confocal microscopy (CCM), non-contact corneal aesthesiometry, optical coherence tomography and visual field perimetry to investigate peripheral nerve morphology and function in individuals with type 1 and type 2 diabetes as well as control participants. The Brisbane site has completed the four-year longitudinal study in July 2014; however, the Manchester site still is in progress and is scheduled to finish this year.

This PhD project focused specifically on the utility of CCM for investigating longitudinal changes of corneal nerve morphology and comparison with conventional tests of neuropathy in healthy controls and type 1 diabetic individuals with and without DPN. Participant enrolment of the two study groups (controls and type 1 diabetes) began in late 2009 in LANDMark study and recruitment continued until November 2011. The candidate joined the LANDMark team and commenced his PhD in February 2012. By adhering to his defined role and understanding the end goals of the LANDMark project, the candidate pursued his specific research aims and actively helped the

team to accomplish its goals. As a member of the LANDMark research team, the candidate's contribution involved:

- Conducting ophthalmic examination for about 250 2-hour participant-visits during duration of the PhD candidature from May 2012 to July 2015 (Table 1-1)
- Data collection and recording observation on Case Report Forms
- Data management including exporting and uploading participants' data to the integrated data base
- Preparation of documentation for health care practitioners (when required) and 24-hour follow up phone calls after their visits
- Providing ophthalmic exit reports for LANDMark participants

The specific contribution of the candidate in relation to his PhD project:

- Conducting intra- and interobserver repeatability study of subbasal nerve parameters using CCM for 16 and 11 healthy participants and individuals with diabetes, respectively
- Analysing 400 CCM images using each of the three techniques of manual, semi-automated and fully-automated (i.e. 1200 images analysed in total)
- Automated analysis of CCM images for all control and type 1 participants from baseline to final visits (approximately 960 case visits)
- Analysing, plotting and presenting the results related to this PhD project

Table 1-1 Numbers of participant-visits conducted by the candidate

Year (LANDMark visit)	2012 (Year 2-3)	2013 (Year 3-4)	2014 (Year 4)	Total
Diabetes	51	74	30	155
Controls	25	43	26	94
Total	76	117	56	**249**

CHAPTER 2. LITERATURE REVIEW

2.1 Foreword

This chapter provides a detailed overview of diabetic peripheral neuropathy (DPN), as one of the most important and prevalent complications of diabetes. Then, an overview of corneal anatomy and physiology and in particular corneal innervation is presented, followed by the effects of diabetes on the cornea. A review of current application of corneal confocal microscopy for *in vivo* assessment of subbasal nerve plexus with particular focus on the utility of this technique in relation to the assessment of DPN will be presented, subsequently. Finally, a summary of the literature review and the implications for this study are presented.

2.2 Diabetic peripheral neuropathy

Diabetes is a complex and chronic metabolic disorder characterized by hyperglycemia resulting from impaired glucose metabolism of the body. This condition occurs due to either deficiency in insulin secretion, insulin resistance or both. The most common types of diabetes are type 1 and type 2 diabetes. Type 1 diabetes is an autoimmune response where destruction of the insulin-producing β-cells of the pancreatic islets occurs, while type 2 diabetes results from both impaired insulin secretion and resistance to insulin action (Holt & Hanley, 2011). Type 1 diabetes can present at any age but this condition mainly occurs in children and young adults, while type 2 diabetes which is the most common form of diabetes, has been regarded a disease of middle-aged or elderly people (Meeking, 2011).

Polyneuropathy or peripheral neuropathy is characterised by nerve abnormalities that are predominantly distal and symmetric with beginning in the lower extremities, which may gradually ascend (Chin & Rubin, 2010) (Figure 2-1). Diabetic distal symmetric polyneuropathy or diabetic peripheral neuropathy (DPN) is a chronic, symmetrical length-dependent neuropathic syndrome and the most common subtype of neuropathies (Chin & Rubin, 2010; Dyck et al., 1993; Tesfaye, 2007).

Development of nerve loss in DPN usually follows a “stocking and glove” distribution. This is because the most distal part of nerves which are furthest from the nucleus in the dorsal root ganglion or anterior horn cell are affected first, although the pathophysiology underlying this phenomenon is not understood (Chin & Rubin, 2010; Ziegler et al., 2014a).

DPN develops following long term hyperglycaemia, associated metabolic disturbances and cardiovascular risk factors (Tesfaye et al., 2010). The prevalence of DPN has been reported to be between 7.1% and 54% depending on study design, definition of DPN and type of diabetes, with the prevalence slightly higher amongst patients with type 2 diabetes (Dyck et al., 1991; Harris et al., 1993; Tapp et al., 2003; Walters et al., 1992).

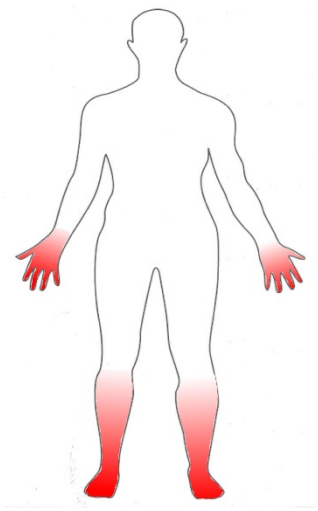

Figure 2-1 Pattern of nerve damage in diabetic peripheral neuropathy

In its early stages, symptoms of DPN which appear due to predominantly involvement of small nerve fibres of Aδ and C types, start with burning feet, tingling and numb toes while the finding of neurologic examination and nerve conduction studies may be normal (Tavee & Zhou, 2009). Clinically, the process of DPN deterioration begins with decreased vibration sensation in the toes accompanied by a reduction or loss of ankle reflexes and may progress to more severe symptoms such as pain and loss of temperature and vibration sensation (Chin & Rubin, 2010). In some patients, foot ulcerations and even amputation are the late sequelae of DPN. Up to 15% of diabetic patients develop foot ulcers (Boulton et al., 2004a). Developing an

ulcer is accompanied with an increased risk of wound progression that may finally lead to amputation (Clayton Jr & Elasy, 2009).

Therefore detecting diabetic patients with neuropathy from those without neuropathy is crucial. Evidence of reduced incidence of lower limb amputation, following a 1-hour education session for “high-risk” patients, plus a significantly lower incidence of new foot problems for patients as a result of an intensive education program (Barth et al., 1991; Malone et al., 1989) support this notion. These studies demonstrated that a simple education program significantly reduced the incidence of ulcer or foot and limb amputation in diabetic patients with neuropathy.

2.2.1 Natural history of DPN

It has been argued that the natural history of DPN has not been well understood, mainly due to scarcity of well-conducted prospective studies and inadequate knowledge of DPN pathogenesis (Tesfaye, 2007). In a 4-year follow-up study of 39 patients with DPN, Boulton et al. (1983) found a significant fall in median nerve motor conduction velocities that reflects continuing deterioration in nerve function. The Diabetes Control and Complications Trial (Leiter et al., 1995) provides valuable information regarding the development and progression of neuropathy in type 1 diabetes. This study was primarily designed as a therapeutic survey in which two group of patients were followed, one treated conventionally (control treatment) and another treated intensively. After 6.5 years of follow-up, the prevalence of clinical neuropathy increased from 8% at baseline to 22%. They also reported a very high (50%) prevalence of abnormal nerve conduction at study closeout in this group. Partanen et al. (1995) reported that the prevalence of neuropathy increased from 8.3% at baseline to 16.7% after 5 years and 41.9% after 10 years in their cohort. In a 7-year longitudinal study of almost 200 patients from the Rochester Diabetic Neuropathy Study cohort using a composite score of examinations and tests, Dyck et al. (1997) demonstrated that the average diabetic patient in their study worsened by 0.34 points per year (slope), whereas patients with diabetic polyneuropathy worsened by 0.85 points per year. They also suggested that longitudinal assessment of

diabetic neuropathy would need to be conducted for a period of at least 3 years to achieve a clinically meaningful effect.

A 12-year prospective study of DPN by Coppini et al. (2001), using vibration perception thresholds (VPT), showed that approximately 20% of diabetic patients with a normal age-corrected VPT at baseline developed an abnormal VPT over this period. A prospective study from The Epidemiology of Diabetes Complications reported that 15% of their type 1 diabetes subjects who were free of DPN at the baseline developed DPN during the first 6 years of follow-up (Forrest et al., 1997). Adler et al. (1997) reported that 20% of their participants without neuropathy at baseline developed neuropathy after an average period of 2.6 years. Similarly, van de Poll-Franse et al. (2002) in a 4-year longitudinal assessment of DPN in type 2 diabetes found that 21.3% of diabetic patients without DPN at baseline, developed significant neuropathy. A prospective follow-up (mean follow-up, 4.7 years) of 231 people with type 2 diabetes and without DPN at baseline revealed an incidence rate of 6.1 per 100 person-year (Sands et al., 1997). In the placebo arm of a study, Brown et al. (2004) found a significant worsening of DPN using nerve conduction studies (NCS) and quantitative sensory tests (QST) over 12 months in a mild to moderate affected population. The European Diabetes (EURODIAB) Prospective Complication Study (Tesfaye, et al., 2005) assessed risk factors for the development of distal symmetric neuropathy in 1172 patients with type 1 diabetes and reported that over a mean of 7.3 years of follow up, approximately one quarter of type 1 diabetic patients developed DPN.

Although the differences in study design do not allow for precise comparison between studies, the above review clearly shows a significant gradual worsening of DPN in diabetic patients over time. However, more recent studies have documented lower deterioration and more stability of neuropathy measures compared to the older reports. The NATHAN 1 trial reported that NCS and QST results did not deteriorate in the placebo-treated group over 4 years (Ziegler et al., 2011). No significant changes to symptom scores, QST and NCS has also been found in a 3-year study of diabetic patients (Gibbons et al., 2013). Overall, there seems to be some evidence of

lower rates of DPN development and worsening and hence changing the natural history of DPN which to some extent may be explained by improvements in the patient care and management of diabetes compared to previous decades.

2.2.2 Pathogenesis and risk factors of DPN

The mechanisms leading to nerve degeneration in diabetic individuals are not completely understood and although several theories have been proposed, the overall mechanism is probably multifactorial and complex (Chin & Rubin, 2010). The two main hypotheses are explained in the following sections.

Metabolic Hypothesis: Development, progression and severity of DPN is related to hyperglycaemia. The significant association between glycaemic control and DPN has been found in several studies (Dyck et al., 1999; Tesfaye et al., 1996). The damaging effect of hyperglycaemia is further confirmed by the occurrence of neuropathy associated with impaired glucose tolerance (IGT). It has been shown that the neuropathy associated with IGT is milder than the neuropathy associated with newly diagnosed diabetes (Sumner et al., 2003).

Despite the strong association between hyperglycaemia and DPN, the exact mechanism is not completely clear. One of the proposed mechanisms is accumulation of polyols (particularly sorbitol) in nerves. The aldose reductase pathway is activated by intracellular hyperglycaemia, resulting in increased sorbitol formation. Accumulation of sorbitol and fructose leads to reduced nerve myo-inositol, decreased sodium-potassium ATPase activity, alteration in protein kinase C subunits and slowed nerve conduction velocities (Chin & Rubin, 2010; Clark & Lee, 1995). Whilst the animal model experiments revealed consistent association between increased polyol pathway flux and decreased nerve conduction velocity, human studies are not consistent and most of clinical trials with aldose reductase inhibitors have failed (Li et al., 2013; Malik & Veves, 2007).

Hyperglycaemia also leads to advanced glycation end-products (AGE) formation (Clark & Lee, 1995). Formation and accumulation of AGE is another important factor for peripheral nerve damage by directly affecting structural and functional proteins or indirectly activating receptors for AGEs (RAGE) (Wada & Yagihashi, 2005). The formation of AGE can be restricted by inhibitors and the interaction of AGE-RAGE may be hindered by recombinant RAGE (Malik & Veves, 2007). Animal studies demonstrated that development and progression of microvascular complications might be preventable by inhibition of AGE production (Tahrani et al., 2010).

Oxidative stress is known as one of the most important mechanisms in the pathogenesis of DPN in animal studies, but to a lesser extent in human neuropathy (Malik & Veves, 2007). Both chronic and acute hyperglycaemia cause oxidative stress in the peripheral nerve system that can promote the development of DPN (Vincent et al., 2004).

Vascular Hypothesis: It has been hypothesised that microvascular disease can result in nerve ischemia. Sural nerve biopsies revealed defects in endoneurial vessels and reduced oxygen tension in diabetic patients with DPN, compared with diabetic patients without DPN. Tesfaye et al. (1993) used sural nerve photography (3 cm of sural nerve is exposed at the ankle using an operating microscope) and fluorescein angiography and found microvascular abnormalities in epineurial arteries and veins as well as arteriosclerosis on the nerve surface and impaired blood flow in subjects with chronic DPN. Blood vessel thickening, reduplication of basal lamina and occlusion with platelet aggregates are further evidences of vascular hypothesis in DPN (Chin & Rubin, 2010).

A considerable amount of literature has been published on risk factors for DPN. DPN increases with both age (from 5% in the 20-29 year age group to 44.2 % in the 70-79 year age group) and duration of diabetes (Young et al., 1993). Using multiple regression modelling, Adler et al. (1997) reported that age at enrolment ($P < 0.0001$, OR = 1.05), duration of diabetes ($P = 0.003$, OR = 1.03) and HbA_{1c} ($P = 0.03$, OR = 1.06) were significant risk factors for DPN. A longitudinal study of risk factors for DPN severity showed that age,

diabetes duration, HbA_{1c}, height and body mass index ($P < 0.05$) were the most important risk factors during follow up (van de Poll-Franse, et al., 2002). Significant correlations have been found between the presence of DPN with age ($P < 0.05$), duration of diabetes ($P < 0.001$), quality of metabolic control ($P < 0.001$), height ($P < 0.01$), the presence of background or proliferative diabetic retinopathy ($P < 0.01$), cigarette smoking ($P < 0.001$), high-density lipoprotein cholesterol ($P < 0.001$) and the presence of cardiovascular disease ($P < 0.05$) (Tesfaye, et al., 1996). Several subsequent studies confirmed the aforementioned risk factors as significant risk factors for DPN (Booya et al., 2005; Dyck, et al., 1999; Forrest, et al., 1997; Gomez-Viera et al., 2001; Manes et al., 2002; Morkrid et al., 2010; Tesfaye, et al., 2005; Wiggin et al., 2009; Ziegler et al., 2008).

In summary, neural structural alterations as a result of diabetes occur through several biochemical pathways, comprising interactions between glycaemic control, duration of diabetes and other cardiovascular risk factors. Although metabolic and vascular factors are the main aetiology for developing DPN, recent studies have shown that these mechanisms are likely to interact and are involved at all stages of DPN (Cameron et al., 2001; Tesfaye & Selvarajah, 2012).

2.2.3 Social and economic burden of DPN

DPN significantly reduces the quality of life of patients and also is a substantial burden both for society and health insurance (Happich et al., 2008). Damage to Aδ and unmyelinated C-class nerve fibres is responsible for most of the symptoms and signs experienced by diabetic patients with peripheral neuropathy, which include: burning or stabbing pain, hyperaesthesia, paraesthesia, and loss of pain and temperature sensation (Boulton, et al., 2004b; Tavee & Zhou, 2009). Symptoms are usually worse at night and often affect patient's sleep (Tavee & Zhou, 2009). When DPN is associated with neuropathic pain, it potentially affects both mental and physical components of quality of life (Van Acker et al., 2009). The associated sensory (e.g. loss of sensation and numbness) and motor

symptoms (e.g. weakness) initially affect the foot and toe and can ascend proximally.

Up to 15% of diabetic patients develop foot ulcers and 80% of amputations are preceded by foot ulceration (Boulton, et al., 2004a; Boulton et al., 2005; Frykberg et al., 2006). Although development of foot ulcers are multifactorial, the main cause is unperceived trauma due to reduced pain sensation (Frykberg, et al., 2006).

A longitudinal study by Abbott et al. in a large population of diabetic patients demonstrated that the foot ulceration is more common in diabetic neuropathy, with the annual incidence rising from less than 1% in those without neuropathy to 7.2% in patients with established DPN (Abbott et al., 1998). Ramsey et al. (1999) investigated 8905 patients with type 1 and type 2 diabetes and found a cumulative incidence of 5.8% of developing foot ulcer over three years observation and reported that the attributable cost for a 40 to 65 year-old male with a new ulcer was nearly $28,000 during the two years after diagnosis. The risk of foot amputation has been reported to be 23 fold in patients with diabetes compared to people without diabetes (Holman et al., 2012). Strategies that reduce amputation risk by earlier detection may potentially save $2 to $3 million of medical costs over three years in the United States (Ollendorf et al., 1998). The total annual cost of managing DPN in the United States and United Kingdom has been reported to be $16.8 billion and $1.2 billion, respectively (Gordois et al., 2003).

It is clear that from a public health perspective, DPN leads to extensive reduction of the quality of life and imposes considerable economic burdens; therefore, appropriate diagnosis and early detection of DPN can provide several benefits for both diabetic patients as well as society.

2.2.4 Diagnostic tests for DPN

A broad range of tests are commonly used to screen for, diagnose and to monitor the progression of DPN. The majority of these tests assess functional loss due to disease; however, direct structural observation of nerve tissue

itself is also possible. An outline of the most common methods is presented here.

Signs and symptoms: Neuropathy disability score (NDS) is a quantitative measure of neuropathy which includes pain sensation, vibration sensation, temperature sensation and Achilles tendon reflex of both feet and is recorded from 0 to 10 (Abbott, et al., 1998; Young, et al., 1993). Diabetic neuropathy symptom score (DNSS) is a validated and fast measure of neuropathic symptoms for clinical practice (Meijer et al., 2002) which includes four questions and it is completed by the patient. The total score is recorded from 0 to 4. Although assessment of signs and symptoms are among the most commonly used test for DPN, a recent study (Dyck et al., 2010) has found that they are associated with poor diagnostic reproducibility.

Quantitative sensory tests (QST): QST provide valuable quantitative information on sensory function in polyneuropathies, particularly in DPN (Perkins & Bril, 2003). They offer a relatively robust mean of defining neuropathy severity (Boulton, et al., 2004b) and have shown adequate proficiency (Dyck et al., 2014). However, these tests require the cooperation and concentration of the examinee and they may also be affected by anthropometric variables (Boulton, et al., 2004b; Skljarevski & Malik, 2007) which are the main disadvantages of this method. Furthermore, given that the QST of thermal and pain sensation has been proposed to assess small fibre damage and dysfunction (Arezzo, 1999), a subsequent study has shown the lack of relationship between QST and small myelinated or unmyelinated fibre pathology identified using nerve biopsy technique (Malik et al., 2001).

Nerve conduction studies (NCS): NCS stimulate a nerve at one point along its course and measure the signal at another point. Whilst NCS have been reported to be reliable and objective tests for assessment of large nerve function (Dyck, et al., 1997; Husstedt et al., 1997; Kohara et al., 2000), their reproducibility have been shown to be limited (Litchy et al., 2014). Moreover, they need trained individuals and studies in subjects with impaired glucose tolerance (IGT) and diabetes demonstrated that earliest nerve fibre damage

occurs in small fibres and NCS may not be sensitive enough to detect early functional changes (Malik et al., 2011; Skljarevski & Malik, 2007).

Nerve and skin biopsy: Nerve biopsy is an invasive and highly specialized procedure that allows the direct examination of myelinated and unmyelinated nerve fibre damage and repair using light or electron microscopy (Malik, et al., 2011). Compared to nerve biopsy, skin biopsy is a less invasive technique. Skin biopsy is an accepted means to assess small fibre nerves and to allow morphometric analysis of epidermal and dermal nerves (Lauria et al., 2009) (Figure 2-2). Intraepidermal nerve fibre density (IENFD) is used as a morphometric parameter and is expressed as the number of nerves per length of nerve section (nerve/mm). Both techniques are demanding procedures requiring expertise and laboratory for processing and quantifying. They are also not appropriate tests for longitudinal assessment as biopsies need to be taken at different sites for the purpose of re-assessment.

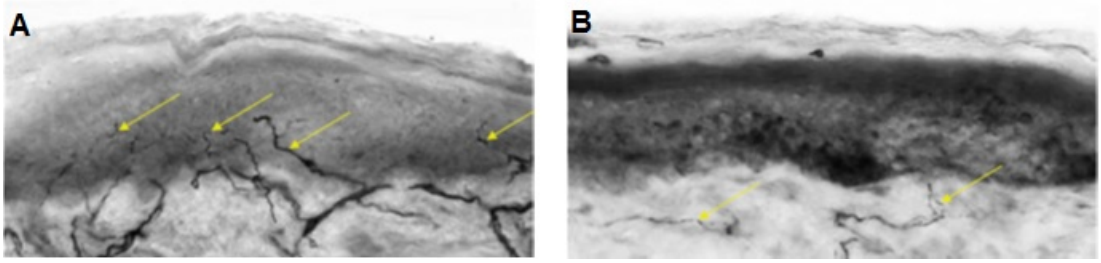

Figure 2-2 Skin biopsy of normal intraepidermal nerve fibre (IENF) (arrow) in a healthy control participant (A) and absence of IENFs with only dermal nerve fibres (arrow) in a diabetic patient with severe neuropathy (B). Figure reprinted with permission from Copyright Clearance Center. Malik, R.A., et al. Small fibre neuropathy: role in the diagnosis of diabetic sensorimotor polyneuropathy. *Diabetes/Metabolism Research and Reviews.* 2011. 27(7): 678-684.

Filament test: The 10-g monofilament examination is a simple, practical and accurate means for DPN screening; however this is not a quantitative test and there are some limitations in its specificity for DPN onset (Perkins et al., 2010).

Neuropad®: Neuropad is a relatively new adhesive visual indicator test which measures sweat production in the feet. This test has been shown to have high sensitivity and negative predictive value but low-moderate specificity and positive predictive value for the diagnosis of DPN (Papanas et al., 2013).

2.2.5 Treatment of DPN

Several studies have been performed using pharmacologic agents on the basis of pathogenetic mechanisms including aldose reductase inhibitors, AGE inhibitors, vasodilators and nerve growth factors; however, to date no effective therapy has been approved for treatment of peripheral neuropathy in diabetes (Li, et al., 2013). In the first instance, glycaemic control and considering cardiovascular risk factors are the main focus of management (Tesfaye & Selvarajah, 2012). In diabetic patients with painful DPN, pharmacological management with antidepressants, anticonvulsants, and opioids is recommended, but these drugs are often limited by unfavourable side-effects (McGreevy & Williams, 2012).

While accurate diagnosis and estimation of changes are essential to test potential therapies for DPN, lack of a reliable and sensitive clinical marker has been one of the most important impediments in clinical trials (Malik, 2014a; Ziegler & Luff, 2002). As outlined above, the inherent and associated shortcomings of the conventional measures of neuropathy indicate that a simpler, more practical and sensitive measure of neuropathy, that can be used to monitor changes over time, has to be explored.

In the past decade, corneal nerve morphology at subbasal nerve plexus (SNP) has been the centre of attention as a potential marker of DPN. In fact, the anatomical location and transparency of the cornea make this tissue ideally suited for direct observation of nerve structure pathology using *in vivo* corneal confocal microscopy (CCM). The next section deals with the SNP structure as a promising sensitive and reiterative measure of neuropathy in more detail.

2.3 Morphology of corneal subbasal neve plexus as a potential measure of DPN

2.3.1 Anatomy and physiology of the cornea

The cornea is a transparent and avascular connective tissue of the front of the eye and, in combination with the precorneal tear film, plays an important role via providing a proper anterior refractive surface and protects the eye against infection and structural damage to the deeper components of the eye (DelMonte & Kim, 2011; Farjo et al., 2008). Histologically, the human cornea consists of five basic layers, three cellular layers (epithelium, stroma, and endothelium) and two acellular interfaces (Bowman and Descemet membranes) (Figure 2-3). Corneal thickness is approximately 0.5 mm at the centre and this thickness increases gradually to the periphery.

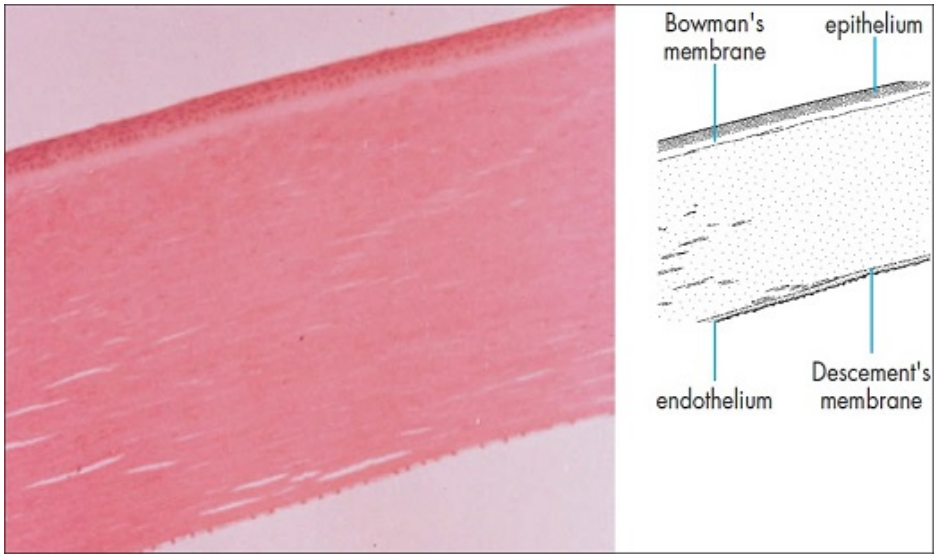

Figure 2-3 Histological cross section of the cornea. Figure reprinted with permission from Elsevier. Farjo, A. A., et al. (2008). Corneal Anatomy, Physiology and Wound Healing. In M. Yanoff & J. S. Duker (Eds.), *Ophthalmology.* 203-207.

Epithelium: In addition to its contribution as the main barrier to penetration of microorganisms and certain noxious substances, the epithelium-tear film provides approximately two third of the total refractive power of the eye. The epithelium is 4–6 cell layers thick (40–50 μm), the most superficial corneal

cells consist of 2 to 3 layers of polygonal cells, then 2-3 layers of supra-basal or wing cells and the basal layer forms a single cell layer (Farjo, et al., 2008).

Bowman layer: This is not a real membrane but rather a condensed layer of collagen. This layer is approximately 15 µm thick, protects the stroma and maintains corneal shape (Riordan-Eva, 2002).

Stroma: The stroma is the thickest layer of the cornea (80% to 85% of total corneal thickness). It is composed of densely packed, highly ordered collagen fibres (Lamella) (Farjo, et al., 2008). This highly arranged network results in corneal transparency and reduced light scattering. Collagen molecules are generated by keratocytes which are the main cell type in the corneal stroma.

Descemet's membrane: A thin acellular layer with approximate 10 µm thickness has an amorphous ultra-structural texture and represents the basement membrane of the endothelium.

Endothelium: It is a single layer of flat hexagonal (honeycomb-like) cells with a thickness of 4 µm in adulthood. This layer is responsible for maintaining the corneal stroma in a relatively deturgescent state (DelMonte & Kim, 2011).

Dua et al. (2013) reported the discovery of new acellular layer at the most posterior lamellae of the stroma with 10 µm thickness; however, there has been some debate in the literature concerning the existence of this layer (McKee et al., 2014).

2.3.2 Corneal innervation

The *ex vivo* anatomy of the corneal nerves has been studied in detail by light and electron microscopy and in combination with immunohistochemical techniques (Al-Aqaba et al., 2010; He et al., 2010; Marfurt et al., 2010; Müller et al., 2003; Müller, et al., 1997). The human cornea is the most densely innervated surface tissue of the body (606 terminals/mm^2 in the suprabasal layers of the central corneal epithelium) (Marfurt, et al., 2010). Corneal nerve fibres are mainly sensory and derived from the nasociliary branch of the

ophthalmic division of the trigeminal nerve (Müller, et al., 2003). Corneal autonomic nerve fibres consists of sympathetic fibres which are derived from the superior cervical ganglion and parasympathetic fibres that originate from the ciliary ganglion (Al-Aqaba, et al., 2010).

In the periphery, bundles of nerves enter the cornea in the middle third of the stroma and run forward anteriorly in a radial fashion to the centre. These nerves lose their perineurium and myelin within approximately 1 mm of the corneal limbus to maintain cornea transparent (Marfurt, et al., 2010; Müller, et al., 2003). In the interface between Bowman's layer and anterior stroma, the subepithelial nerve plexus is formed by the stromal nerves. After penetrating Bowman's layer, nerves continue parallel to the corneal surface between Bowman's layer and the basal epithelial cell layer (Müller, et al., 2003) and form the subbasal nerve plexus (SNP) which provides innervations to the subbasal layer of the epithelium and eventually ends within superficial epithelial layers of the cornea (Figure 2-4). The subbasal nerve plexus includes a spiral-like assemblage of long, curvilinear subbasal nerve fibres which forms a whorl-like arrangement located about 2.5 mm infronasal to the corneal apex (Marfurt, et al., 2010).

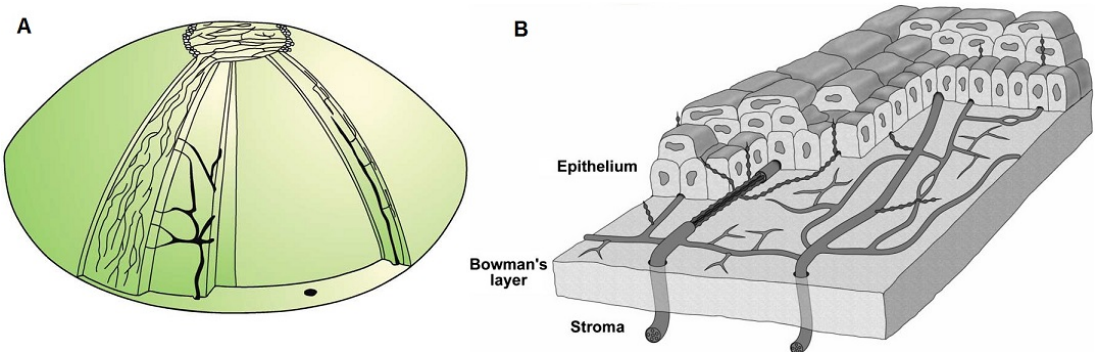

Figure 2-4 (A) Diagram of human cornea nerves in stroma and subbasal plexus. (B) 3-D representation of the corneal subbasal nerve plexus. Figures reprinted with permission from Copyright Clearance Center. (A) Müller, L.J., et al., Corneal nerves: structure, contents and function. *Experimental Eye Research*, 2003. 76(5): 521-542, and (B) Erie, J.C., et al., The effect of age on the corneal subbasal nerve plexus. *Cornea*, 2005. 24(6): 705-709.

Two main types of human corneal nerves are unmyelinated C fibres which are small diameter (2-4 μm) beaded nerves and respond to thermal and chemical stimuli and Aδ fibres that are large diameter (6 μm) straight nerves and respond primarily to mechanical stimuli (Müller, et al., 1997). Sensation of pain in human cornea results from mechanical, thermal and chemical stimulation of the cornea (Al-Aqaba, et al., 2010). Although the corneal innervation provides sensation, it has a significant role in the integrity of the ocular surface. The corneal nerve fibres also have an important influence on the corneal trophism (nourishment of the tissue) and contribute to the maintenance of a healthy corneal surface (Marfurt, et al., 2010).

2.3.3 Cornea and diabetes

Retina and cornea are two main ocular tissues that are profoundly impacted from hyperglycemia. Diabetic keratopathy, or the corneal complications of diabetes, occurs in up to 70% of diabetic patients (Lutty, 2013). Various corneal changes associated with diabetes have been reported, ranging from cellular dysfunction to failure to repair the damaged structures and functions. Gekka et al. (2004) found impairment in corneal epithelial barrier function in diabetic patients and reported that diabetic patients with higher HbA_{1c} levels were more disposed to impaired barrier function in the corneal epithelium. Lee et al. (2006) studied 200 patients with diabetes and showed that diabetic subjects had thicker corneas, lower cell density and hexagonality, and more irregular cell size. Similarly Inoue et al. (2002) reported impaired endothelial cell structure.

Increase in central corneal thickness has been reported in several studies which has been thought to be due to insufficient endothelial cell function, resulting in corneal oedema (Goldich et al., 2009; Lee, et al., 2006; McNamara et al., 1998; Rosenberg, et al., 2000); in contrast, some investigations demonstrated no difference in central corneal thickness between diabetes and control subjects (Hager et al., 2009; Inoue, et al., 2002; Wiemer et al., 2007). Diabetes has a significant effect on corneal hydration control (McNamara, et al., 1998) and can also affect corneal

biomedical parameters including increased corneal hysteresis and corneal resistance factor (Goldich, et al., 2009; Hager, et al., 2009).

Corneal nerve structure is altered by diabetes (He & Bazan, 2012; Mocan et al., 2006) and may also lead to diabetic keratopathy which is difficult to manage clinically (Bikbova et al., 2012). These patients have epithelial basement membrane and integrin alterations and impairment of epithelial wound healing (Chen et al., 2009; Ljubimov et al., 1998). Due to the structural and functional abnormalities in the diabetic cornea, these patients are theoretically at a higher risk for development of more complications such as recurrent corneal erosions, superficial punctuate keratitis, delayed wound healing and re-epithelialization, decreased sensitivity and susceptibility to injury and ulceration (Bikbova, et al., 2012; Lutty, 2013; Wiemer, et al., 2007).

2.3.4 Corneal confocal microscopy

In vivo corneal confocal microscopy (CCM) is a quick and non-invasive technique which enables reiterative microstructural imaging and evaluation of the human cornea at high resolution in health and disease. Until recently it was largely used as a tool for research laboratories, but now is considered as a powerful diagnostic tool for a variety of ocular and neurological conditions.

The principle of CCM (Figure 2-5) is that a beam of light (e.g. Laser) passes through a light source and focused by an objective lens into a small volume of a tissue (e.g. cornea) (Guthoff et al., 2009). The objective lens collects a mixture of emitted as well as reflected light from the illuminated point and projects this mixture to a conjugate spot in an “image” plane where the pinhole aperture is positioned. Light mixture is separated by a beam splitter and then reflected into the detection apparatus. The pinhole aperture blocks light from out-of-focus areas of the specimen and only the light from the focal plane passes through the pinhole to the detector where the light signal is transformed to electrical signal. Obstruction of the light that is not coming from the focal point results in sharper images (Guthoff, et al., 2009). The resultant image is an image with very high resolution but very narrow field of view. The small field of view in confocal microscopy imaging is overcome by

scanning multiple points or slits to create the image (Inoué, 2006; Jalbert et al., 2003).

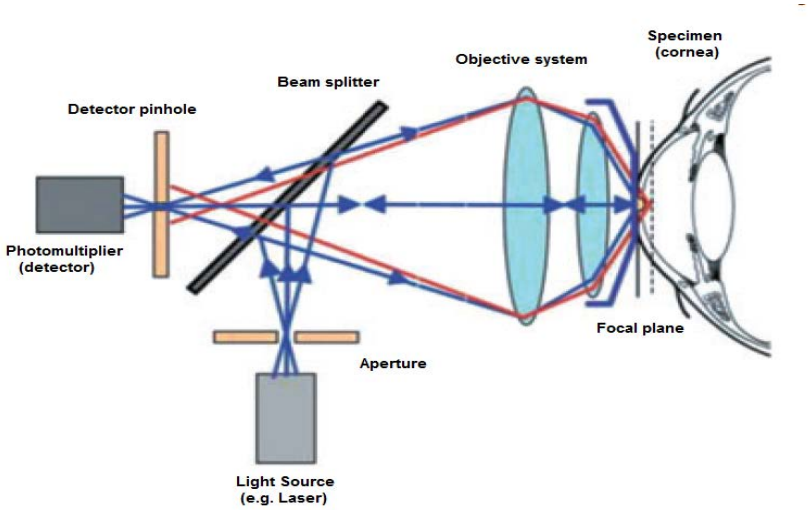

Figure 2-5 Schematic principle of the corneal confocal microscopy. Figure reprinted with permission from Copyright Clearance Center. Guthoff, R.F. et al., In vivo confocal microscopy, an inner vision of the cornea - a major review. *Clinical and Experimental Ophthalmology*, 2009. 37(1):100-117.

There are currently two CCM instruments on the market; the Nidek ConfoScan 4 which is a white-light slit scanning (SSCM) instrument, and the Heidelberg Retina Tomograph with Rostock Corneal Module which is a laser scanning (LSCM) instrument. The LSCM became available in 2004 and is able to produce images with higher contrast from different layers of the cornea, in particular the SNP.

2.3.4.1 CCM and assessment of cornea in ocular conditions and diseases

As a transparent and anteriorly located tissue of the eye, the cornea has been studied extensively at cellular level in normal status as well as in several ocular diseases using *in vivo* CCM. Rapid and non-invasive image acquisition from different corneal layers and structures helps both clinicians and researchers to extract important information in respect to changes caused by various ocular conditions and diseases. This technique has been used both qualitatively and quantitatively to characterize conditions such as

dry eye, ocular allergies and glaucoma (Benítez-del-Castillo et al., 2007; Labbé et al., 2012; Villani et al., 2013b; Zhang et al., 2011), corneal ectasia and dystrophies (Efron & Hollingsworth, 2008; Patel et al., 2009a), infectious keratitis and corneal ulcers (Hamrah et al., 2012; Labbé et al., 2009), the effect of contact lens wear (Zhivov et al., 2007), orthokeratology lens wear (Lum et al., 2012) and corneal cross-linking (Kaya et al., 2011), and the assessment of nerve regeneration after penetrating keratoplasty and different forms of corneal refractive surgery (Darwish et al., 2007a; Darwish et al., 2007b; Erie et al., 2005b).

2.3.4.2 Assessment of subbasal nerve plexus in healthy people using CCM and the effect of age

Using electron microscopy, Muller et al. performed a unique qualitative morphological analysis of corneal nerve architecture and concluded that human corneal nerves degenerate within 13.5 h after death, which results in a number of difficulties for accurate structural analysis of human corneal nerves (Müller, et al., 1997). *In vivo* CCM has addressed the problem of disappearing subbasal nerve plexus post-mortem. This rich nerve plexus has been studied extensively over the past decade. CCM observations are in agreement with histological studies (Oliveira-Soto & Efron, 2001); nerve fibres perforate Bowman's layer and eventually form a dense neural plexus just beneath the basal epithelial cell layer and appear as bright, well defined linear structures connected with anastomoses (Figure 2-6A) and organized in a vortex pattern (Figure 2-6B) in the inferior nasal quadrant of the cornea (Guthoff, et al., 2009; Patel & McGhee, 2005).

Several corneal nerve parameters have been used by researchers such as nerve fibre length, density, branching and tortousity (Grupcheva et al., 2002; Malik, et al., 2003; Oliveira-Soto & Efron, 2001; Patel & McGhee, 2005; Rosenberg, et al., 2000). Although there is no universally accepted consensus regarding the definition of corneal nerve parameters, the three main SNP parameters studied by CCM include corneal nerve fibre density (CNFD; the total number of major nerves per mm^2), branch density (CNBD; the number of branches emanating from major nerves per mm^2) and fibre

length (CNFL; total length of all nerves and branches in units of mm/mm^2) (Malik, et al., 2003; Papanas & Ziegler, 2013). However, CNFL appears to be the most standardized, generally accepted and frequently reported morphometric parameter of the SNP. Findings of several studies for reported SNP parameters from the centre of cornea in healthy corneas are presented in Table 2-1.

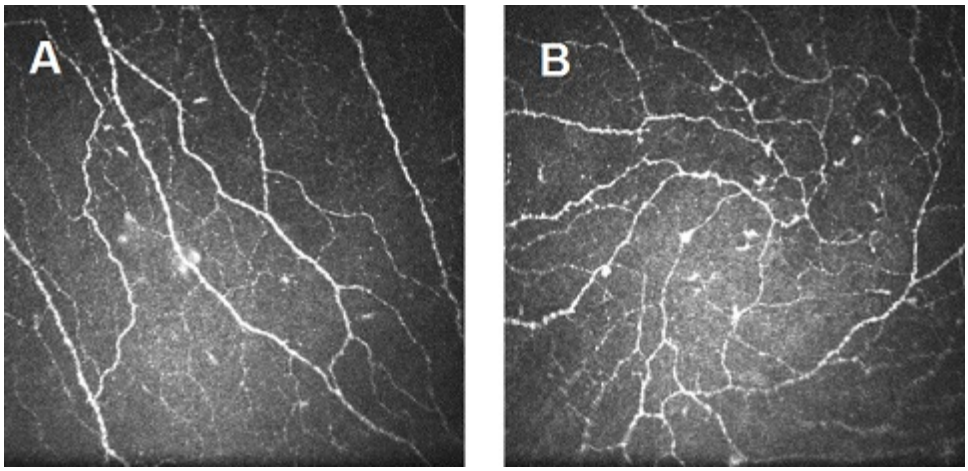

Figure 2-6 Laser-scanning confocal microscopy images of subbasal nerve plexus from centre (A) and whorl pattern (B). Each image is 400 x 400 µm.

As shown in Table 2-1, the reported SNP parameters differ significantly among studies. For example, CNFL in healthy corneas have been reported to range from 0.6 to 13.5 mm/mm^2 for SSCM and from 10.1 to 27.9 mm/mm^2 with LSCM. The disparity is likely to be related to differences in methodology such as number and quality of selected images, study participants or quantification technique. Another important difference is in the definition of the SNP parameters. For instance, some investigators have only quantified nerve branches longer than 50 mm when measuring the total length of nerves.The differences between SNP parameters reported in studies using SSCM and LSCM modalities might be because of contrast, brightness, depth of field and instrument sensitivity for detecting subbasal nerve plexus. Whilst the images acquired using LSCM have a relatively uniform contrast and brightness, images captured using SSCM are brightest along vertical strip and become darker laterally, which may potentially affect the visibility of nerve fibres at the edge of the image (Patel & McGhee, 2005).

Table 2-1 Quantification of subbasal nerve parameters in healthy individuals. Values are presented as mean ± SD, unless otherwise stated.

Author (Year)	N	Age (year)	Type of CCM	Number of images	Quantification technique	CNFD fibres/mm^2	CNBD branches/mm^2	CNFL mm/mm^2
Grupcheva et al. (2002)	25	25 ± 5	SSCM	1-3	Automated	N/A	N/A	632.3 ± 287.6*
	25	70 ± 5						582.4 ± 327.1*
Malik et al. (2003)	18	58 ± 12	SSCM	3-5	Manual	44.5 ± 14.1	78.9 ± 30.4	13.5 ± 0.3
Benitez-del-Castillo et al.	10	30 ± 6	SSCM	Various	Manual	N/A	61.9 ± 10.9	10.6 ± 1.4
(2007)	10	65 ± 3					43.0 ± 12.1	8.3 ± 1.2
Niederer et al. (2007)	85	38 ± 16	LSCM	3	Manual	N/A	N/A	20.3 ± 6.5
Quattrini et al. (2007)	15	55 ± 5	SSCM	3-5	Manual	43.2 ± 5.1	27.4 ± 3.3	6.1 ± 1.2
Erie et al. (2008)	18	38 ± 10	SSCM	2-4	Semi-automated	N/A	N/A	10.7 ± 5.6
Niederer et al. (2008)	52	26 ± 7	LSCM	3	Manual	N/A	N/A	22.4 ± 6.0
Patel et al. (2009)	31	35 ± 12	LSCM	2	Manual	N/A	N/A	25.9 ± 7.0
Patel et al. (2009b)	20	26 ± 3	LSCM	2	Manual	N/A	N/A	10.6 ± 6.8
	20	44 ± 5						10.1 ± 6.8
	20	61 ± 7						10.6 ± 6.6
Tavakoli et al. (2010b)	17	55 ± 5	SSCM	3-5	Manual	45.6 ± 4.5	25.4 ± 3.0	11.2 ± 0.9
Tavakoli et al. (2011b)	18	57 ± 3	SSCM	3-5	Manual	46.0 ± 3.8	35.6 ± 6.7	13.5 ± 0.8

Author (Year)	N	Age (year)	Type of CCM	Number of images	Quantification technique	CNFD fibres/mm^2	CNBD branches/mm^2	CNFL mm/mm^2
Hertz et al. (2011)	20	41 ± 17	LSCM	1	Manual	31.9 ± 9.4	37.2 ± 17.7	16.1 ± 4.1
Wu et al. (2012)	64	39 ± 18	LSCM	1	Manual	45.0 ± 12.0	37.0 ± 15.0	18.0 ± 4.0
Hume et al. (2012)	23	40 ± 15	LSCM	6	Manual	30.9 ± 5.8	75.3 ± 19.4	19.9 ± 3.5
Zhivov et al. (2013)	20	66 ± 13	LSCM	1	Manual	N/A	141.9 ± 85.7	20.0 ± 6.7
Tavakoli et al. (2013)	10	47 ± 3	LSCM	5	Manual	35.8 ± 1.5	100.9 ± 13.1	27.9 ± 1.3
Petropoulos. (2013b)	19	23± 1	LSCM	5	Manual	38.3 ± 3.9	58.1 ± 23.0	27.6 ± 4.0
Sivaskandarajah et al. (2013)	64	38 ± 16	LSCM	2	Manual	45.3 ± 12.0	39.7 ± 16.9	18.8 ± 4.5
Parissi et al. (2013)	106	50 (15-88)†	LSCM	4	Automated	N/A	N/A	18.6 ± 4.8
Pritchard et al. (2014)	154	46 ± 15	LSCM	3-8	Manual	N/A	83.5 ± 45.8	23.2 ± 6.3
Petropoulos et al. (2014)	55	52 ± 11	LSCM	6	Automated	30.0 ± 6.9	50.4 ± 24.7	21.2 ± 3.5

CCM, corneal confocal microscopy; SSCM, Slit-scanning confocal microscope; LSCM, Laser-scanning confocal microscope; CNFD, corneal nerve fibre density; CNBD, corneal nerve branch density; CNFL, corneal nerve fibre length; N/A, not available.
*µm/mm^2, † mean (range)

The effect of age on the SNP structure: In the current literature, there is no good agreement among previous studies concerning the age-dependent alteration of subbasal nerve plexus using *ex vivo* and *in vivo* techniques. While He et al. (2010) in an *ex vivo* study of 22 donor corneas aged from 19 to 80 years reported that subbasal nerve fibre density reduced with age, Marfurt et al. (2010) using an immunohistochemical staining technique found no significant correlation between CNFL and age in corneas of six donors aged 19 to 78 years.

Such a disagreement exists among studies using *in vivo* CCM as well. Grupcheva et al. (Table 2-1) found a significant difference in CNFL between the two age groups of healthy corneas (Grupcheva, et al., 2002), whereas a study by Erie et al. found no correlation between age and nerve fibre length in 65 individuals (aged 15-79 yeas) with healthy cornea ($r = 0.21$, $P = 0.09$) (Erie et al., 2005a). In a subsequent study, Niederer et al. (2007) reported a 0.9% per year reduction in subbasal nerve fibre density in their participants aged 18-87. In another CCM study of 60 healthy human participants, the authors reported no significant difference in mean total nerve density between their three age groups (group 1: aged < 35 years, group 2: aged 35–50 years, and group 3: aged > 50 years) (Patel, et al., 2009b). However, in a more recent study of 106 healthy participants, Parissi et al. (2013) observed a mean decline in CNFL of 0.25% to 0.30% per year.

Studies outlined above clearly illustrate the inconsistency in the literature in regard to the relationship between age and the SNP morphometric change. The design employed in previous studies reporting the effect of age on the SNP morphology has been cross-sectional, which does not necessarily mean the real age effect, because measurements are taken on subjects with different ages and the differences are attributed to the effect of age. The discrepancies between studies reporting the possible relationship between age and SNP structural parameters warrants conducting a longitudinal study in a healthy population by examining the same participants over a period of time in order to examine the real effect of age.

2.3.5 Utility of corneal nerve morphology for DPN assessment

In the process of DPN development small nerve fibres, which constitute 70-90% of peripheral nerves, are the first to be damaged (Malik, et al., 2011). Cornea which is the most innervated tissue in the body and mainly consists of sensory small nerve fibres (unmyelinated C fibres and Aδ fibres) (Al-Aqaba, et al., 2010; Müller, et al., 2003) is not an exception. Studies have also shown that the mechanisms leading to nerve degeneration at cornea such as polyol pathway and formation of advanced glycation end-products (Jacot et al., 1998; Kaji et al., 2000; Stitt, 2001) are similar to those involved in DPN.

CCM as a technique for quantitative assessment of the SNP morphology has developed during the past decade and has led to an improved understanding of nerve damage in diabetes. Using CCM, there is a large number of published studies demonstrating deficits of the SNP structural parameters in presence of diabetes (De Cilla et al., 2009; Messmer et al., 2010; Midena, et al., 2006; Mocan, et al., 2006; Nitoda et al., 2012; Zhivov, et al., 2013; Ziegler et al., 2014b).

The first study of CCM in DPN was reported by Rosenberg et al. (2000). These authors described a significant nerve fibre bundle decrease in patients with DPN compared to those without DPN ($P < 0.05$). Since then, CCM has increasingly been employed to examine the morphology of SNP in relation to DPN. The findings of various cross-sectional studies for pathology of the three most important and frequently reported SNP parameters in respect to DPN are summarized in Table 2-2. As can be seen from Table 2-2, the SNP damage is not only more pronounced in individuals with DPN, it is also associated with DPN severity. The SNP parameters also have shown moderate to high sensitivity and specificity for diagnosis of DPN.

The usefulness of CCM in DPN assessment is not limited to its diagnostic and stratification ability. It has been shown that this instrument is able to detect early corneal nerve repair after simultaneous pancreas and kidney transplantation in type 1 diabetes. While there was no significant

improvement in neurologic deficit, QST, electrophysiology, IENFD and corneal sensitivity, significant improvements occurred in CNFD ($P < 0.05$), CNBD ($P < 0.01$), and CNFL ($P < 0.05$) 12 months after successful transplantation (Tavakoli, et al., 2013). Another study by Tavakoli et al. (2011b) revealed that improvement in risk factors for DPN can result in morphological repair of the corneal nerves. In this observational study, after 24 months follow up, CNFD and CNBD increased significantly with improvement in glycaemic control and cardiovascular risk factors associated with diabetic neuropathy. They also reported that the improvement in CNFD correlated significantly with the improvement in HbA_{1c} ($r = -0.51$, $P = 0.008$).

Table 2-2 Cross-sectional studies that investigated the diagnostic ability of the corneal subbasal nerve parameters in respect to diabetic peripheral neuropathy (DPN)

Author (year)	Type of CCM	N (DM/C)	Main Outcomes
Rosenberg et al. (2000)	TSCM	44 (23/9)	A significant decrease in the nerve fibre bundles in patients with severe DPN vs. without DPN A significant decrease in the nerve fibre bundles in patients with mild to moderate neuropathy vs. without DPN
Malik et al. (2003)	SSCM	36 (18/18)	CNFD and CNFL were significantly reduced in moderate and severe neuropathy groups vs. controls CNBD was significantly reduced in mild, moderate and severe neuropathy groups vs. controls CNFD, CNBD and CNFL showed a tendency for greater reduction with increasing DPN severity
Midena et al. (2006)	SSCM	69 (42/27)	A significant decrease in the number of nerve fibres and branching pattern in diabetic patients vs. controls with a statistical trend suggesting progression of the corneal neuropathy with DPN
Quattrini et al. (2007)	SSCM	69 (54/15)	Significantly lower CNFD in mild, moderate and severe DPN vs. controls Significantly lower CNBD in mild, moderate and severe DPN as well as in diabetic participants without neuropathy vs. controls Both CNFD and CNBD showed significant reduction with increasing DPN severity Significant correlations between CNFD and IENFD (r = 0.39), between CNBD and IENBD (r = 0.41) and between CNFD and CST (r = - 0.40)
Tavakoli et al. (2010b)	SSCM	118 (101/17)	CNFD, CNBD and CNFL decreased significantly with increasing neuropathy severity CNFD, CNBD and CNFL found to be correlated with NDS (r; - 0.48, -0.51 and -0.58, respectively) CNFD of < 27.8/mm^2 showed sensitivity of 82% and specificity of 52% for diagnosis of DPN
Tavakoli et al. (2011a)	SSCM	154 (128/26)	CNFD, CNBD and CNFL significantly decreased with increasing severity of DPN CNFD, CNBD and CNFL showed significant correlations with NDS (r; -0.34, -0.31 and -0.43, respectively)
Nitoda et al. (2012)	LSCM	43 (25/18)	Moderate correlations between CNFD and CNFL with clinical and neurological tests of DPN (r, from -0.36 to -0.58)
Hertz et al. (2011)	LSCM	46 (26/20)	CNFD, CNBD and CNFL showed significant incremental decrease with increasing DPN severity

Author (year)	Type of CCM	N (DM/C)	Main Outcomes
Edwards et al. (2012b)	LSCM	292 (231/61)	CNBD and CNFL were significantly reduced in diabetic participants with DPN vs. controls CNFL was significantly reduced in diabetic participants without DPN vs. controls Modest correlations of CNBD and CNFL with NDS, cold and warm sensation thresholds, vibration perception threshold and peroneal conduction velocity (r = 0.15 to 0.25) CNFL was correlated to HbA_{1c} (r = -0.24) and duration of diabetes (r = -0.20)
Ahmed et al. (2012)	LSCM	153 (89/64)	CNFD, CNBD and CNFL were significantly lower across controls, diabetic participants without and with neuropathy CNFL (with AUC of 0.88) best discriminated participants with DPN from controls compared with CNFD and CNBD
Zhivov et al. (2013)	LSCM	38 (18*/20)	Significantly lower CNFD, CNBD and CNFL in DPN group vs. controls
Sivaskandarajah et al. (2013)	LSCM	160 (96/64)	Significantly lower CNFD, CNBD and CNFL in DPN group vs. controls and diabetic participants without DPN Modest correlation between CNFD, CNBD and CNFL and cold detection threshold (r; 0.32, 0.37 and 0.37, respectively)
Pritchard et al. (2014)	LSCM	408 (242/154)	Significantly lower CNFL in diabetic participants with neuropathy vs. without neuropathy group and controls Significantly lower CNFL in without neuropathy group vs. controls
Petropoulos et al. (2014)	LSCM	241 (186/55)	A significant reduction in manual and automated CNFD, CNBD and CNFL with increasing neuropathic severity Manually quantified CNFD and automated quantification of CNFL yielded highest AUC and sensitivity/specificity to rule out DPN

CCM, corneal confocal microscopy; DM/C, diabetes/controls; TSCM, tandem-scanning confocal microscope; SSCM, Slit-scanning confocal microscope; LSCM, Laser-scanning confocal microscope; CNFD, corneal nerve fibre density; CNBD, corneal nerve branch density; CNFL, corneal nerve fibre length; IENFD, intra-epidermal nerve fibre density; CST, cold sensation threshold; NDS, neuropathy disability score; AUC; area under curve; DPN, diabetic peripheral neuropathy.
*diabetic participants with DPN

IENF assessment using skin biopsy is an invasive and objective technique for evaluation of small nerve fibre loss and likely presents the gold-standard method (England et al., 2009). As an alternative, small nerves in the subbasal nerve plexus of the cornea have been proposed to be examined directly and more importantly non-invasively by CCM. Quattrini et al. quantified small nerve fibre pathological changes using the technique of IENF (skin punch biopsy) and CCM in 54 diabetic patients stratified for neuropathy and found that whereas both techniques accurately reflect the severity of neuropathy, CCM has a superior ability to detect earlier stages of nerve pathology compared with IENF (Quattrini, et al., 2007).

While alteration to several SNP parameters have been reported, compared with other parameters, CNFL has been proposed to be the optimal and most reliable parameter to detect nerve injury in diabetes, as demonstrated by advantages in repeatability, reproducibility and concurrent validity (Ahmed, et al., 2012; Efron et al., 2010; Hertz, et al., 2011). Furthermore, CNFL appears to be a sufficiently sensitive measure of nerve pathology and reassuringly, age and the use of contact lenses do not confound assessment of CNFL for the screening of neuropathies such as DPN (Oliveira-Soto & Efron, 2003; Wu, et al., 2012). In a more recent study by Petropoulos et al. (2013b), CNFD and CNFL were found to be the most repeatable parameters, where CNFD was superior to CNFL for intra- and inter-observer repeatability measurements. This finding is in contrast to the higher reliability of CNFL as reported by Hertz et al. (2011).

In vivo wide-field assessment of the SNP has revealed that the density and distribution of SNP nerves is different in the central and peripheral regions of human cornea either in healthy state (Patel & McGhee, 2005) or in diabetic patients with and without neuropathy (Edwards et al., 2012a). This is more evident when central cornea is compared to the whorl area (Figure 2-6). Patel and McGhee (2005) reported significantly higher CNFL in the whorl region (25.3 ± 0.6 mm/mm^2) compared with the central cornea (21.7 ± 1.4 mm/mm^2). Qualitative assessment of two generated maps has also provided some evidence of more pronounced nerve damage in the whorl region of a

diabetic patient with neuropathy compared to a diabetic patient without neuropathy (Edwards, et al., 2012a).

Although employing this novel technique of SNP assessment would offer valuable insights into identifying alterations of the SNP microstructures overtime, the image capturing and montaging are time consuming, labour and resource intensive. Because of the convenience and ease of imaging from the central cornea, which significantly reduces the chair time – an advantage for the current study with large number of participants – this region has been selected to be assessed in in the majority of previous studies.

The association between established measures of DPN and corneal nerve parameters has also been explored. Tavakoli et al. (2010b) found moderate correlations between NDS and the three main SNP parameters (CNFD r = -0.48, CNBD r = -0.51, and CNFL r = -0.58). Very modest correlations of CNBD and CNFL with NDS, cold and warm sensation thresholds, vibration perception threshold and peroneal conduction velocity (r = 0.15 to 0.25) were also reported in a subsequent study of 231 diabetic individuals with predominantly mild or no neuropathy (Edwards, et al., 2012b). Modest associations between CNFD, CNBD and CNFL and cold detection threshold (r; 0.32, 0.37 and 0.37, respectively) were also reported in a most recent study of corneal nerves and conventional small nerve fibre tests in type 1 diabetic participants (Sivaskandarajah, et al., 2013).

The review presented in previous sections clearly demonstrates the clinical relevance of corneal innervation to development of peripheral neuropathy in diabetes. The corneal sensory nerves, which consist of small nerve fibres of Aδ and C types, originate from ophthalmic division of the trigeminal nerve. These two types of nerve fibres are the earliest that undergo damage in DPN. Additionally, animal studies have shown the impairment of corneal nerve structure and function in diabetic rats (Davidson et al., 2014; Jacot, et al., 1998). Subclinical abnormalities of trigeminal and facial nerve involvement in diabetes (Urban et al., 1998) and corneal neuropathic ulcer

associated with diabetes (Schultz et al., 1983) are further evidences of involvement of the corneal nerve tissue in diabetes.

As outlined above, given the potential role of corneal nerve structure in assessment of DPN, to our knowledge, no study has been conducted concerning the natural course of the SNP structure over time in diabetic patients. Additionally, despite the fact that several cross-sectional studies have shown the existence of relationship between the SNP parameters and conventional examination methods of neuropathy, it is not clear how the longitudinal changes in the SNP parameters relate to the established measures of DPN in diabetic individuals over time. This is important because if the SNP morphology is to be considered as an adjunct to those of traditional measures, there should be comparable changes to some established measures. Otherwise the possibility remains that these measures might not be related and therefore can affect the usefulness of the SNP morphology as potential measure of DPN.

2.4 Summary of knowledge gaps and objectives of this research program

The feasibility of assessing SNP morphology via CCM and the promising role of this modality as an indicator of corneal nerve damage or repair and the potential for assessment of peripheral neuropathies, in particular DPN, has led to an increase in the scope of this approach.

The large and growing body of literature showing a relationship between quantitative analysis of SNP parameters and various ocular and systemic pathologic conditions highlights the importance of understanding the natural morphometric characteristics of the SNP over time. Besides, the uncertainty and true extent of age effect on the SNP morphology required a longitudinal study examining the same participants over a period of time which enables us to explore the true age effect in a healthy population. Therefore, the first main question in this study sought to determine the age-dependent alterations and longitudinal course of SNP structure in healthy individuals.

As reviewed above, several studies have attributed the pronounced corneal nerve pathology in diabetes to diabetic peripheral neuropathy. With reference to the lack of previous investigation concerning the natural history of corneal nerves in diabetes, the second main question explored the natural history of the SNP parameters in diabetes individuals without and with neuropathy and attempted to fill this research gap. Furthermore, the longitudinal relationship between changes in corneal nerve structure and established measures of neuropathy in individuals with diabetes was addressed.

Application of CCM in studies with large numbers of participants where multiple images from each participant need to be analysed as well as in longitudinal studies such as the present study with repeated measurements over time, necessitated employment of a fully automated analytical system to overcome shortcomings which are associated with manual and semi-automated techniques. Thus, the third research question dealt with the association, agreement and detection capability of manual, semi-automated and fully automated techniques of SNP morphometric quantification.

Since the main theme of this project was the natural history of SNP structure in participants with diabetes and healthy controls with annual repeated measurement of the SNP parameter, the fourth research question sought to examine the intra- and interobserver repeatability of these parameters in control and diabetes participants. Hence, prior to undertaking the investigation of the above mentioned first and second research questions, two studies were conducted addressing the third and fourth research questions which were related to the methodological development. Consequently, the orientation of the rest of this thesis will be as following:

- Chapter 3, where the association, agreement and detection capability of the three segmentation techniques of manual, semi-automated and fully-automated has been examined.
- Chapter 4, where the results of an intra- and interobserver study of the SNP parameters were presented.

- Chapter 5, the general methodology and the baseline characteristics of the participants included in this longitudinal study have been delineated.
- Chapter 6, the age-dependent alterations and longitudinal course of SNP structure in healthy individuals over three years have been addressed.
- Chapter 7, natural history of SNP morphology in a cohort of diabetic individuals without and with neuropathy has been investigated.
- Chapter 8, a summary of the finding and directions for possible future research have been presented.

CHAPTER 3. COMPARISON OF MANUAL, SEMI-AUTOMATED AND FULLY-AUTOMATED QUANTIFICATION OF THE SUBBASAL NERVE PLEXUS

3.1 Foreword

In line with the main aim of this PhD project to find out the natural course of corneal nerve morphology in a cohort of type 1 diabetic individuals without and with neuropathy and control participants, the paper presented in this chapter describes an important element of research methodology – comparison of a newly developed segmentation algorithm with semi-automated and manual methods. A fully-automated image analysis system which allows objective subbasal nerve quantification is essential for eliminating disadvantages that are associated with semi-automated and manual approaches. If this technique is to be employed, it must be able to detect the differences between groups and also show high association with those of manual and semi-automated methods. Once the diagnostic ability and the association of fully-automated segmentation are established, further evaluations such as intra- and interobserver repeatability study of the nerve parameters (next chapter) could be tracked. These are also of high importance for this longitudinal project and will be explained in the next chapter. The paper that is presented in this chapter has been published in the journal *Cornea*:

Dehghani C, Pritchard N, Edwards K, Russell AW, Malik RA, Efron N. Fully automated, semiautomated, and manual morphometric analysis of corneal subbasal nerve plexus in individuals with and without diabetes. Cornea 2014; 33:696-702.

3.2 Abstract

Purpose: To determine the association, agreement and detection capability of a fully-automated, semi-automated and manual method of corneal nerve fibre length (CNFL) quantification of the human corneal sub-basal nerve plexus (SNP).

Methods: Thirty-three participants with diabetes and 17 healthy controls underwent laser scanning corneal confocal microscopy. Eight central images of the SNP were selected for each participant and analysed using a manual (CCMetrics), semi-automated (NeuronJ) and fully-automated (ACCMetrics) software to quantify CNFL. The repeated-measures ANOVA analysis was used to examine the differences between the three methods. To explore the association and agreement between methods, the correlation coefficients, intraclass correlation coefficient (ICC) and Bland-Altman tests were applied.

Results: For the entire cohort, mean CNFL values quantified by CCMetrics, NeuronJ and ACCMetrics were 17.4 ± 4.3, 16.0 ± 3.9 and 16.5 ± 3.6 mm/mm^2, respectively ($P < 0.01$). CNFL quantified using CCMetrics was significantly higher than those obtained by NeuronJ and ACCMetrics ($P < 0.05$). The three methods were highly correlated (correlation coefficients from 0.87 to 0.98, $P < 0.01$). The ICC values were 0.87 for ACCMetrics vs. NeuronJ and 0.86 for ACCMetrics vs. CCMetrics. Bland-Altman plots of the CNFL values showed good agreement between the manual, semi-automated and fully-automated analysis. A small underestimation of CNFL was observed using ACCMetrics with increasing amount of nerve tissue. All three methods were able to detect CNFL depletion in diabetic participants ($P < 0.05$) and in those with peripheral neuropathy as defined by Toronto criteria compared to healthy controls ($P < 0.05$).

Conclusion: Automated quantification of CNFL provides comparable neuropathy detection ability to manual and semi-automated methods. Because of its speed, objectivity and consistency, fully-automated analysis of CNFL might be an advantage in studies of diabetic neuropathy.

3.3 Introduction

The human cornea is one of the most richly innervated surface tissues in the body (Müller, et al., 2003). The corneal sub-basal nerve plexus (SNP) is located between Bowman's layer and the corneal basal epithelium. The SNP originates from sub-Bowman's nerves penetrating Bowman's layer perpendicularly, branching into one or more subbasal nerves which run

parallel to the ocular surface (Al-Aqaba, et al., 2010; Marfurt, et al., 2010). At this interface, corneal nerves anastomose extensively with each other to form a dense and homogenous nerve plexus and eventually terminate within the superficial epithelial layers of the cornea (Marfurt, et al., 2010).

The SNP has been studied extensively *in vivo* using corneal confocal microscopy (CCM). Quantification of this nerve plexus appears to be a promising non-invasive and sensitive marker for detection and stratification of diabetic peripheral neuropathy (DPN) (Edwards, et al., 2012b; Malik, et al., 2003; Quattrini, et al., 2007; Tavakoli, et al., 2010b), a prevalent and debilitating complication of diabetes (Callaghan et al., 2012a). As such, valid and reliable quantification of the structural status of the SNP is crucial to optimize detection, monitor progression and assess possible intervention and treatment strategies in clinical disorders affecting peripheral nerves, especially DPN.

Numerous morphologic parameters of the SNP have been reported, such as nerve fibre beading, length, branching and tortuosity (Grupcheva, et al., 2002; Malik, et al., 2003; Oliveira-Soto & Efron, 2001). Compared with other parameters, corneal nerve fibre length (CNFL) has been suggested to be the optimal and most reliable parameter to detect nerve injury in diabetes, as demonstrated by advantages in repeatability, reproducibility and concurrent validity (Ahmed, et al., 2012; Efron, et al., 2010; Hertz, et al., 2011). CNFL appears to be a sufficiently sensitive measure of nerve impairment and reassuringly, age and the use of contact lenses do not confound assessment of CNFL for the screening of neuropathies such as DPN (Wu, et al., 2012).

Currently, quantification of SNP parameters from images obtained via *in vivo* CCM is mostly based on manual and semi-automated techniques (Ahmed, et al., 2012; Hertz, et al., 2011; Labbé, et al., 2012; Petropoulos, et al., 2013b; Wu, et al., 2012). These procedures include manual tracing of nerves and then calculation of the nerve fibre parameters with a segmentation algorithm method written in Matlab (Dabbah et al., 2009) or Java (Meijering, 2010), which are tedious, time-consuming and subjective, require experience and are prone to variability between and within observers (Dabbah et al., 2011;

Efron, et al., 2010; Petropoulos, et al., 2013b; Scarpa et al., 2008). Fully-automated analytical techniques have been developed to obviate the limitations of manual analysis and to extend the diagnostic value of this technique to clinical practice (Dabbah, et al., 2011; Ferreira et al., 2012; Holmes et al., 2010; Parissi, et al., 2013; Scarpa, et al., 2008). Furthermore, application of CCM in large cohort studies where multiple images from each participant need to be analysed - perhaps by team of assessors and repeated over time in longitudinal investigations - necessitates development of a fully-automated system to overcome these limitations.

The purpose of this study was to compare two methods of manual and semi-automated analysis – CCMetrics (Dabbah, et al., 2009) and NeuronJ (Meijering, 2010) - with an automated analysis system (ACCMetrics) (Dabbah, et al., 2011), for analysing SNP images obtained by *in vivo* CCM in healthy controls and individuals with diabetes. The capability of these techniques to detect reduced CNFL in individuals with DPN was also investigated.

3.4 Methods

3.4.1 Study participants

Data were accessed from a random subset of 50 participants from a total cohort of 314 participants at the Brisbane site of the ongoing LANDMark (Longitudinal Assessment of Neuropathy in Diabetes using novel ophthalmic Markers) study (Pritchard, et al., 2014). Specifically, in this retrospective, cross-sectional study data were acquired from the “year three” examinations of these participants, and included 17 healthy controls and 33 individuals with diabetes who were stratified into those with (N = 13) and without (N = 20) neuropathy. The first 20 and 40 participants’ IDs in the LANDMark database were initially selected for controls and diabetic groups, respectively. Then participants who did not have the “year three” examination were excluded.

Exclusion criteria were: a history of ocular surgery, trauma or disease; or systemic disease (apart from diabetes), which might have affected the cornea. Four participants (2 controls and 2 with diabetes), who were current

soft contact lens wearers, were asked to refrain from lens wear on the day of their examination. Diabetes was the only known cause of the presence of peripheral neuropathy.

Ethical approval was obtained from the Princess Alexandra and Mater Hospital and Queensland University of Technology research ethics committees. The study was conducted in accordance with the principles of the Declaration of Helsinki.

3.4.2 Corneal confocal microscopy

Laser-scanning CCM was conducted using the Heidelberg Retinal Tomograph (HRT3) with Rostock Corneal Module (Heidelberg Engineering GmbH, Heidelberg, Germany). This device generates 2-dimensional images, consisting of 384 X 384 pixels, covering an area of 400 X 400 µm when used with a X63 objective lens. The cornea of the dominant-hand side of the participant was anesthetized with 1 drop of 0.4% benoxinate hydrochloride (oxybuprocaine hydrochloride; Bausch & Lomb, NSW, Australia). Participants were instructed to fixate on a near target with the contralateral eye. The CCM was advanced forward, and gentle contact was established between the front of the applanation cap and the cornea; this procedure was facilitated by a side-mounted CCD camera that allowed the examiner to ensure that the central region of cornea was being examined. Using the manual focusing and section mode, multiple images of the SNP were captured from the central cornea of each participant. All captured images were saved digitally, and then the first 8 images of the SNP of each participant displaying in-focus nerves and not overlapping more than 20% among selected images (Vagenas et al., 2012) were chosen for analysis.

3.4.3 Neuropathy assessment

All participants underwent detailed assessment of neuropathy including nerve electrophysiology (peroneal motor nerve amplitude and conduction velocity), neuropathy disability score (NDS) (Young, et al., 1993) and diabetic neuropathy symptoms score (DNSS) (Meijer, et al., 2002). The Toronto

criteria (Tesfaye, et al., 2010) were used to determine the presence of neuropathy; specifically, individuals were considered to have neuropathy if they had abnormal nerve conduction (compared with age-matched controls in the LANDMark study) and a sign (NDS score ≥ 3 of 10) or symptom (DNSS ≥ 1 of 4) of neuropathy.

3.4.4 Morphometric analysis of SNP images

Eight images were analysed from each of the 50 participants using each of the three techniques described below (i.e. 1,200 images analysed in total) by one investigator (C.D), who was masked with respect to diabetes/neuropathy status of the participants. The average CNFL of eight images was calculated to determine the CNFL measure of each participant.

CCMetrics is a custom-designed manual nerve analysis software package developed at the University of Manchester (Manchester, United Kingdom) (Dabbah, et al., 2009). All clearly visible nerves were traced with a manual drawing module (Figure 3-1). The software converts manual tracings of the SNP to measures of corneal nerve fibre length (CNFL), corneal nerve density and corneal nerve fibre tortuosity. However, for purpose of this study, only the results of CNFL, which is defined as total length of all nerve fibres in the CCM image (in units of mm/mm^2), were considered.

NeuronJ is an semi-automated nerve tracing software package (Meijering, 2010) which is a plug-in module for ImageJ, a free Java-based image analysis software. Nerve tracing is initiated by locating the beginning of the nerve of interest and the tracing algorithm subsequently computes and shows the ‘optimal’ path (Figure 3-1). In some areas with low contrast nerves, the program fails to find the correct path. In such a case there is an option to switch to manual tracing mode; however, this option was not used here. CNFL was calculated by tracing all the nerve fibres and nerve branches in the image. This length was then divided by the area of the field-of-view provided by the CCM to derive the value of CNFL in units of mm/mm^2.

ACCMetrics is a fully-automated software package (Dabbah, et al., 2011) also developed at the University of Manchester (Manchester, United Kingdom), which allows automatic nerve detection (Figure 3-2). The software is optimised for 384 X 384 pixels CCM images with the field of view of 400 X 400 µm. “Multiple image analysis” mode was used to analyse the images of the SNP.

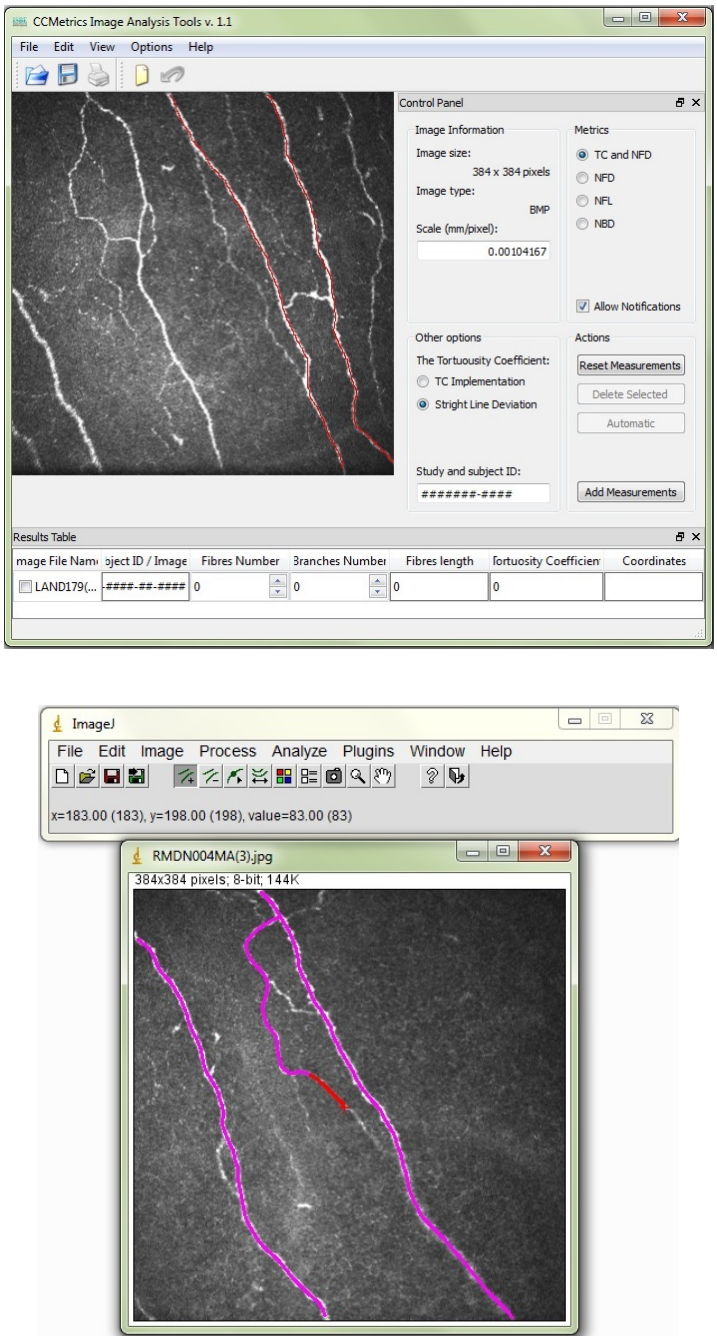

Figure 3-1 Screen snapshots of manual (CCMetrics) (top) and semi-automated (NeuronJ) (bottom) methods of corneal nerve quantification

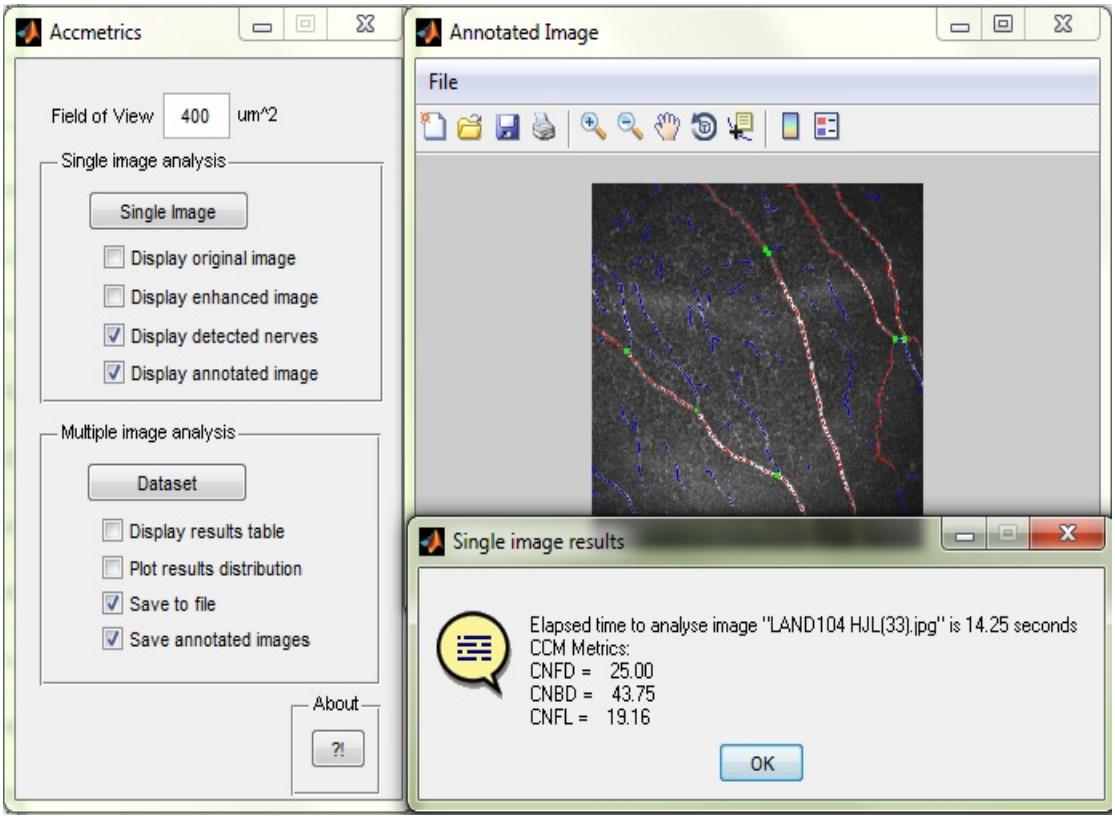

Figure 3-2 Fully-automated (ACCMetrics) analysis of corneal subbasal nerve parameters

Time analysis

The average time taken per frame for manual identification and/or tracing of nerves and software analysis was determined with a digital timing device. This procedure was conducted for all participants using each technique, all performed by the same operator (C.D.).

Interobserver variability of CNFL quantification

To determine interobserver variability in quantification of CNFL, one image from each of 15 randomly selected participants was selected from our data set. Quantification of CNFL was performed on all of these images by a second observer using each of the three techniques described above.

3.4.5 Statistical analysis

IBM SPSS (version 21.0) was used to analyse the results. All data are presented as the mean ± standard deviation (SD). Normality of the data was assessed by Shapiro-Wilk test and appropriate statistical techniques were employed. Differences between methods were examined by using repeated-measures analysis of variance (ANOVA) and Bonferroni correction. The Pearson correlation and intraclass correlation coefficients (ICC) were applied to explore the relationship between the three methods of CNFL quantification. Correlation coefficient was used to test if the measurements by a pair of methods are related. ICC, which measures the average correlation, was used to assess reliability and consistency between two methods.

Bland-Altman plots (Bland & Altman, 1986) were generated to facilitate an appreciation of the extent of between-method differences and the relation between these differences and the overall magnitude of CNFL. This statistical approach is a robust way for comparing two methods of clinical measurements and comprised of a graph of the difference between two methods against the average of the two methods as well as calculating 95% limits of agreements (Bland & Altman, 1995). We would expect 95% of differences between the pair of measurements to lie between upper and lower limits of agreement. The independent samples t-test, one-way ANOVA and Scheffe's post-hoc test were also used to establish differences between groups. *P*-values of < 0.05 were considered significant for all statistical tests. Interobserver variability of CNFL was determined using the paired-samples t-test and ICC.

The sample size determination was undertaken based on previous studies in which similar methodology (e.g. stratification, CNFL definition and analysis) were used (Ahmed, et al., 2012; Edwards, et al., 2012b). The effect size was determined using available CNFL data in different groups. Analysis using G*Power 3 software (Faul et al., 2007) showed that a total of 36 participants (12 participants per three groups), under the assumption of a type 1 error (α level) of 0.05 and 90% power, were required to discriminate the difference

among groups. Enrolment continued, until the smallest group (diabetes with DPN) contained 13 subjects, resulting in a total of 50 participants.

3.5 Results

The clinical characteristics of the 50 participants are shown in Table 3-1. Age was not significantly different between diabetic individuals without DPN (DPN-ve), with DPN (DPN+ve) and control group ($P = 0.56$). The DPN-ve and DPN-ve groups had significantly higher HbA_{1c} ($P < 0.001$) and lower total cholesterol ($P < 0.01$) compared to controls. The DPN+ve group found to have higher duration of diabetes compared with DPN-ve group.

For the entire cohort, the mean CNFL quantified by CCMetrics, NeuronJ, and ACCMetrics were 17.4 ± 4.3 mm/mm^2, 16.0 ± 3.9 mm/mm^2, and 16.5 ± 3.6 mm/mm^2, respectively (repeated-measures ANOVA, $P < 0.01$). The CNFL determined using CCMetrics was found to be significantly higher than that determined using ACCMetrics and NeuronJ (mean differences 0.9 and 1.4 mm/mm^2, respectively, $P < 0.05$). Mean CNFL values did not differ between those obtained using ACCMetrics versus NeuronJ (mean difference 0.5 mm/mm^2, $P = 0.07$).

The three methods were highly correlated (correlation coefficients 0.87–0.97, $P < 0.01$) with the strongest correlation between CCMetrics and NeuronJ ($r = 0.97$, $P < 0.001$). The calculated ICC values were 0.87 (95% confidence intervals: 0.77–0.92) for ACCMetrics versus NeuronJ and 0.86 (0.77–0.92) for ACCMetrics versus CCMetrics. Table 3-2 summarizes the results of comparison between methods in the entire cohort, control, and diabetic groups.

Table 3-1 Clinical demographic results in study participants. Data are mean ± SD unless otherwise indicated

Characteristics	C (n = 17)	DPN-ve (n = 20)	DPN+ve (n = 13)	*P*-value	Significant Differences from pairwise comparisons
Age (years)	58.8 ± 10.1	61.3 ± 11.2	62.8 ± 8.4	0.56[†]	NS
HbA_{1c} (%)	5.5 ± 0.3	7.9 ± 0.9	8.0 ± 1.2	< 0.001[‡]	C vs. DPN-ve and DPN+ve, P < 0.001
Duration of diabetes (years)	-	18.8 ± 10.2	28.2 ± 16.9	0.004[§]	DPN-ve vs. DPN+ve, P = 0.004
Total cholesterol (mmol/L)	5.9 ± 1.2	4.3 ± 1.1	4.3 ± 1.2	< 0.001[†]	C vs. DPN-ve and DPN+ve, P < 0.01
BMI	26.7 ± 3.7	28.5 ± 5.3	31.4 ± 5.3	0.03[†]	C vs. DPN+ve, P < 0.03
Systolic blood pressure (mmHg)	117.0 ± 16.5	123.2 ± 13.3	123.1 ± 9.7	0.33[†]	NS
Diastolic blood pressure (mmHg)	73.7 ± 9.4	73.4 ± 7.4	70.5 ± 5.2	0.47[†]	NS

[†]One way ANOVA t-test; [‡]Kruskal Wallis test; [§]Independent samples t-test.

C, controls; DPN-ve, diabetic individuals without DPN; DPN+ve, diabetic individuals with DPN; NS, no significant difference

Table 3-2 Comparison of corneal nerve fibre length (CNFL) as obtained using ACCMetrics (CNFL-ACCMetrics), CCMetrics (CNFL-CCMetrics) and NeuronJ (CNFL-NeuronJ) in total cohort (N = 50), controls (N= 17) and diabetic (N = 33) participants.

			CNFL-ACCMetrics			CNFL-NeuronJ		
			Total	Control group	Diabetic group	Total	Control group	Diabetic group
CNFL-CCMetrics	Total	Mean CNFL difference (mm/mm^2)	0.88*	-	-	1.40**	-	-
		Pearson correlation	0.87**	-	-	0.97**	-	-
		ICC (95% CI)	0.86** (0.77-0.92)	-	-	0.97** (0.94-0.98)	-	-
	Control group	Mean CNFL difference (mm/mm^2)	-	1.84**	-	-	1.56**	-
		Pearson correlation	-	0.79**	-	-	0.98**	-
		ICC (95% CI)	-	0.77** (0.47-0.91)	-	-	0.97** (0.92-0.99)	-
	Diabetic group	Mean CNFL difference (mm/mm^2)	-	-	0.39	-	-	1.31**
		Pearson correlation	-	-	0.88**	-	-	0.96**
		ICC (95% CI)	-	-	0.88** (0.77-0.94)	-	-	0.96** (0.91-0.98)
CNFL-ACCMetrics	Total	Mean CNFL difference (mm/mm^2)	-	-	-	0.51	-	-
		Pearson correlation	-	-	-	0.87**	-	-
		ICC (95% CI)	-	-	-	0.87** (0.77-0.92)	-	-
	Control group	Mean CNFL difference (mm/mm^2)	-	-	-	-	-0.28	-
		Pearson correlation	-	-	-	-	0.81**	-
		ICC (95% CI)	-	-	-	-	0.80** (0.54-0.92)	-
	Diabetic group	Mean CNFL difference (mm/mm^2)	-	-	-	-	-	0.92*
		Pearson correlation	-	-	-	-	-	0.87**
		ICC (95% CI)	-	-	-	-	-	0.87** (0.75-0.93)

* P-value < 0.05; ** P-value < 0.01; ICC, intra-class correlation coefficient; CI, confidence interval.

Bland–Altman plots comparing the automated technique with the semiautomated and manual procedures are shown in Figure 3-3. For the comparison of ACCMetrics versus NeuronJ (Figure 3-3A), the downward slope of the regression line indicates that, for higher mean CNFL values, a lower value was assigned to CNFL as obtained using ACCMetrics (R^2 = 0.01, P = 0.42). A similar downward trend was observed for the comparison of ACCMetrics versus CCMetrics (Figure 3-3B). However, only for the latter comparison, there was a weakly significant relationship between the difference in the CNFL and mean CNFL (R^2 = 0.09, P = 0.03). For the comparison of CCMetrics and NeuronJ (Figure 3-3C), the upward slope indicates that, for higher mean CNFL values, a higher value was assigned to CNFL as obtained by CCMetrics, and there was a modest relationship between the difference in the CNFL and mean CNFL (R^2 = 0.15, P < 0.01).

The average time to obtain a value of CNFL per image for each technique in this study was 96 ± 25 seconds for CCMetrics, 64 ± 20 seconds for NeuronJ, and 13 ± 2 seconds for ACCMetrics (repeated-measures ANOVA, P < 0.001). All 3 pairwise comparisons were significantly different (Bonferroni, P < 0.001).

Interobserver repeatability of CNFL quantification was assessed for each of the three techniques. The mean difference in CNFL between the two observers and ICC values were as follows: NeuronJ—0.62 mm/mm^2 (paired t test, P = 0.16) and 0.95 (P < 0.01); CCMetrics—0.75 mm/mm^2 (P = 0.11) and 0.97 (P < 0.01). CNFL values quantified by ACCMetrics were identical for both observers.

Three methods revealed reduced CNFL in diabetic individuals compared with controls (independent t test, P < 0.05). Using the NDS, 4 diabetic participants had mild (NDS: 3–5), 3 had moderate (NDS: 6–8), and 1 had severe (NDS: 8–10) neuropathy. Of the 33 participants with diabetes, 13 (39%) met the Toronto criteria for the presence of neuropathy.

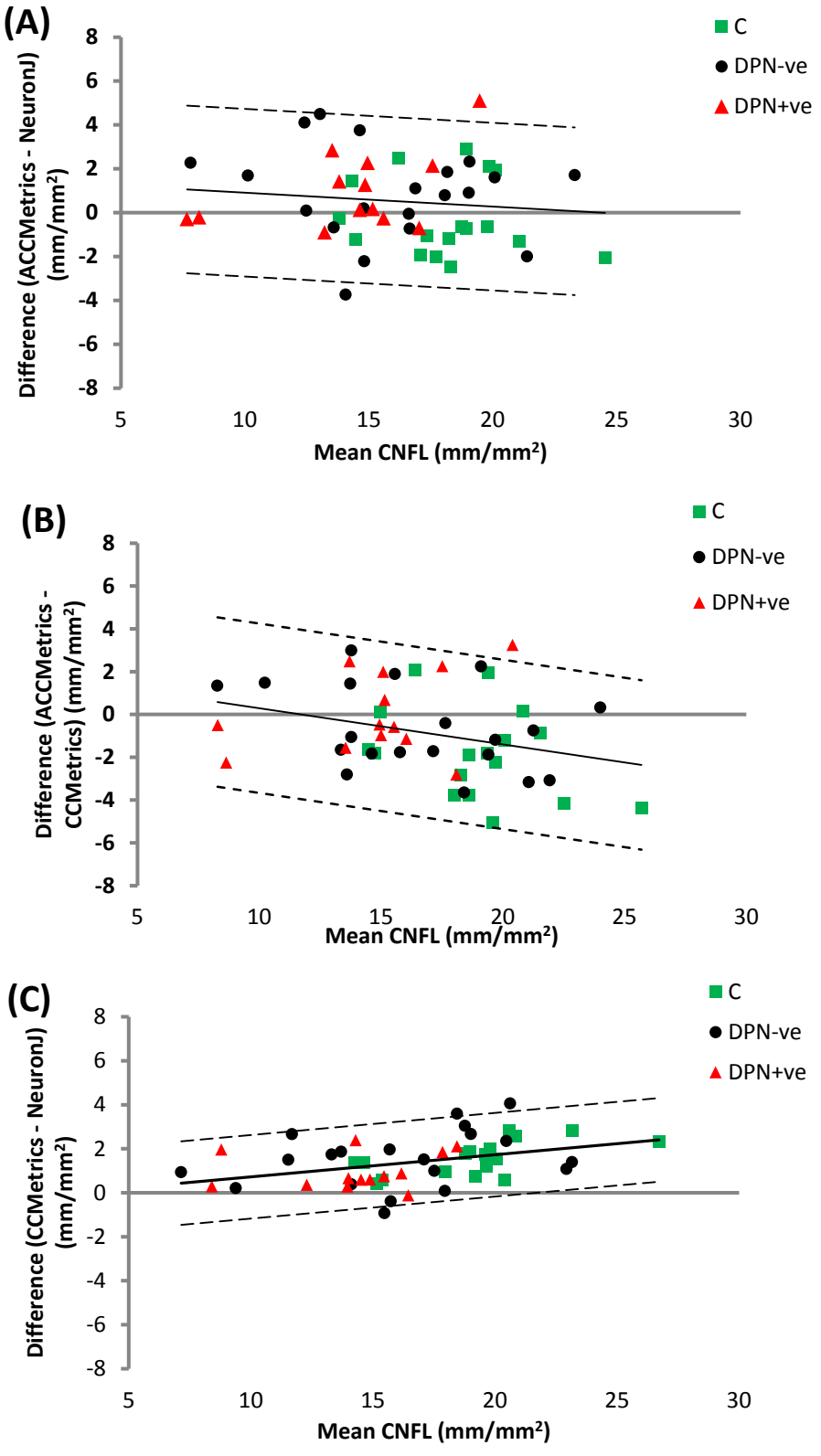

Figure 3-3 Relationship between differences in CNFL vs. mean CNFL for ACCMetrics vs. NeuronJ (A), ACCMetrics vs. CCMetrics (B) and CCMetrics vs. NeuronJ (C). On each graph, the solid line indicates the linear regression and the dashed lines indicate the 95% limits of agreement. C, controls; DPN-ve, individuals without DPN; DPN+ve, individuals with DPN.

Table 3-3 Corneal nerve fibre length (CNFL) values in healthy controls (C) and diabetic individuals without (DPN-ve) and with (DPN+ve) neuropathy.

CNFL parameter	C (n = 17)	DPN-ve (n = 20)	DPN+ve (n = 13)	P-value	Scheffe Pairwise comparison
ACCMetrics (mm/mm^2)	18.1 ± 2.7	16.3 ± 3.8	14.8 ± 3.8	0.043	C vs. DPN-ve, P = 0.307 C vs. DPN+ve, P = 0.045 DPN-ve vs. DPN+ve, P = 0.481
NeuronJ (mm/mm^2)	18.3 ± 2.9	15.4 ± 4.1	13.8 ± 3.0	0.002	C vs. DPN-ve, P = 0.046 C vs. DPN+ve, P = 0.004 DPN-ve vs. DPN+ve, P = 0.423
CCMetrics (mm/mm^2)	19.9 ± 3.4	17.0 ± 4.5	14.8 ± 3.1	0.002	C vs. DPN-ve, P = 0.074 C vs. DPN+ve, P = 0.003 DPN-ve vs. DPN+ve, P = 0.281

Values are presented as mean ± SD

Table 3-3 summarizes quantified CNFL values pertaining to the three methods of morphometric analysis, stratified according to the neuropathy status. There was a significant difference between groups for all measures ($P < 0.05$). CNFL values were significantly lower for individuals with neuropathy compared with controls for all 3 methods of morphometric analysis ($P < 0.05$). CNFL reduction as estimated with NeuronJ was marginally significant ($P = 0.046$) in individuals with diabetes without neuropathy compared with controls.

3.6 Discussion

The increasing interest in assessing morphological parameters of the SNP in relation to peripheral neuropathies highlights the need for a reliable, quick, highly repeatable, and reproducible method of analysis, particularly when these parameters are to be assessed in longitudinal studies, perhaps by multiple operators, or to examine the benefits of possible interventions. To overcome shortcomings associated with manual tracing and quantification of the SNP parameters, several research groups have developed fully

automated nerve fiber analysis software (Dabbah, et al., 2011; Ferreira, et al., 2012; Scarpa, et al., 2008; Scarpa et al., 2011). This study assessed the reliability of a manual (CCMetrics), semiautomated (NeuronJ), and fully automated software (ACCMetrics) analysis system for CNFL quantification in a diverse cohort of healthy individuals and participants with diabetes.

Age was well matched between individuals with diabetes and healthy controls (P = 0.31). Lower level of total cholesterol was observed in participants with diabetes compared to controls, as 25/33 (75%) participants with diabetes were receiving lipid lowering therapy with statins vs. none of the 17 controls. CCMetrics, the technique requiring the most observer input, yielded higher CNFL values compared with NeuronJ and ACCMetrics. The lower CNFL values obtained using ACCMetrics compared with CCMetrics was not unexpected, because a human observer is able to detect a higher number of nerves (particularly low-contrast nerves) than the automatic algorithm used in ACCMetrics. Furthermore, during the development of the algorithm for nerve detection, the threshold for detection was deliberately increased to minimize the recognition of background artefacts. Indeed, underestimation of this SNP parameter by fully automated segmentation seems to be consistent with previous studies (Dabbah, et al., 2011; Ferreira, et al., 2012; Scarpa, et al., 2008). The difference between CNFL values obtained using NeuronJ and ACCMetrics was neither clinically nor statistically significant. This may be ascribed to the fact that the “manual tracing” mode of NeuronJ was not used in this study and all tracings were performed using the “optimal” path detection mode.

The Bland-Altman plots (Figure 3-3) and ICC values (Table 3-2) confirm excellent agreement between both semi-automated and manual methods vs. fully-automated segmentation. The three methods were also strongly correlated. The correlation between each of the semi-automated and manual methods vs. the fully-automated analysis were identical (r = 0.87, $P < 0.01$), but slightly lower than that obtained by Scarpa et al. (Scarpa, et al., 2008) and Dabbah et al. (Dabbah, et al., 2011). Scarpa et al. (2008) analysed 90 images of the SNP from 76 normal and 14 abnormal subjects and reported a

correlation coefficient of 0.94. When they applied their automatic procedure to an independent source with 80 images from normal participants, the correlation coefficient between the automatic and manual method reduced to 0.89.

As noted above, an underestimation of CNFL determined using ACCMetrics compared to CCMetrics was observed with increasing amount of nerve tissue. The difference between the correlation coefficients in our study (r = 0.87) and those of Dabbah et al. (r = 0.95) in which 68 participants (20 controls and 48 diabetic participant) were investigated, can be attributed partly to the lower number of individuals with moderate and severe neuropathy (NDS ≥ 6) in the present study (8%) compared with their study (19%).

In a recent study of 106 healthy individuals, Parissi et al. (2013) reported a mean CNFL difference of 0.07 mm/mm^2 and a linear association of CNFL with slope of 0.91 between NeuronJ and automatic methods. In our study, however, the mean CNFL difference was 0.5 mm/mm^2 and the linear association slope was 0.81. The difference in results may be due to the differences between two studies in respect to the population size and composition and the number of selected images for each participant (mean 4.3 images in their study vs. 8 images in our study).

Perfect interobserver agreement in CNFL quantification when using ACCMetrics, is not surprising given that this is a fully-automated technique that requires no manual input from the observer. The high interobserver repeatability for NeuronJ and CCMetrics reported here is consistent with previous studies (Efron, et al., 2010; Hertz, et al., 2011; Petropoulos, et al., 2013b). Manual, semiautomated, and fully automated methods of CNFL quantification were able to differentiate individuals with neuropathy from controls. Fully automated nerve analysis was about 7× and 4× faster than manual and semiautomated morphometric analysis methods, respectively. These findings highlight the advantages of the fully automated versus manual and semiautomated methods of CNFL analysis, particularly for large cohort

trials and longitudinal studies that require analysis of large numbers of images.

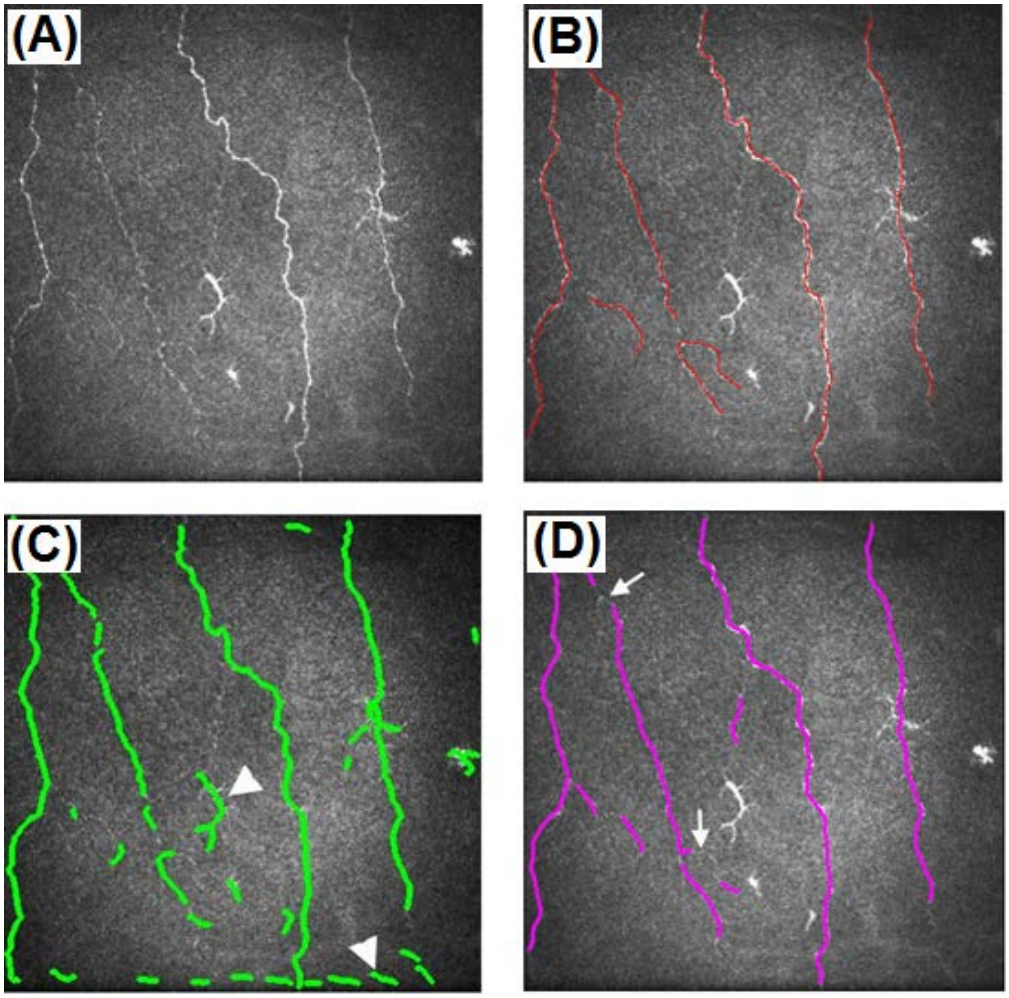

Figure 3-4 Examples of subbasal nerve length estimation. The original 400 X 400 µm image of a participant with diabetes and peripheral neuropathy (A). Analysis of the original image is shown for CCMetrics (CNFL = 11.8 mm/mm^2) (B), ACCMetrics (11.64 mm/mm^2) (C), and NeuronJ (10.0 mm/mm^2) (D). Cells and artefacts that were erroneously identified as nerve fibres are indicated with arrowheads. Low-contrast and faint nerves, which could not be identified, are indicated with arrows. All images are 400 X 400µm.

Despite the ease of fully-automated CNFL analysis, both false negative and false positive errors were evident upon close visual inspection of the processed images. Common nerve tracing errors made by the automatic

nerve-tracking algorithm included: (a) failure to detect nerves which are thin, out of focus or faint, and (b) erroneous recognition of other structures as nerve segments, dendritic cells and other artefacts (Figure 3-4). Although these shortcomings can be improved by performing manual post-analysis editing of images that have undergone initial automated segmentation, the marginal overall advantage of such a process may be offset by the introduction of inadvertent operator bias and consequent reduction of repeatability (Holmes, et al., 2010; Scarpa, et al., 2008), and significant, resource intensive and time-consuming manual input. It should also be noted that the underestimation of CNFL when using ACCMetrics, compared with CCMetrics, may limit the capacity of this software program to detect changes in CNFL in early diabetic neuropathy.

In conclusion, we have demonstrated that fully automated analysis can compute CNFL values, which are in close agreement with systems that use manual and semiautomated segmentation. These three techniques are also capable of differentiating those with and without DPN. Because of its speed, objectivity, and consistency, fully automated analysis of CNFL might be advantageous in studies of diabetic neuropathy.

3.7 Subsequent validity study of fully-automated image analysis algorithm

The findings we presented in this study in relation to the reliability of ACCMetrics for CNFL quantification are supported by a recent study by Petropoulos et al. (2014) who reported a high correlation between manual (CCMetrics) and fully-automated (ACCMetrics) quantification of CNFL ($r = 0.89$) in 186 participants with diabetes and 55 controls. Similar to this study, they found significantly reduced CNFL in participants with DPN compared with controls using both manual and fully-automated techniques while there was a slight underestimation of CNFL as obtained using ACCMetrics compared to CCMetrics. Although our study did not compare the three techniques for other SNP parameters than CNFL, Petropoulos and co-workers in their recent study also validated automated CNFD and CNBD against those obtained manually.

CHAPTER 4. INTRA- AND INTEROBSERVER REPEATABILITY OF CORNEAL NERVE PARAMETERS

4.1 Foreword

Addressing the issues of intra- and interobserver repeatability of the measurement procedures are critical in longitudinal studies where the differences in the values are to be monitored over time. Therefore, this was an essential part of the research methodology to allow application of corneal confocal microscopy (CCM) with automated image analysis for this longitudinal study. Although previous studies have evaluated repeatability and reproducibility of measurement of the SNP parameters in diabetic and healthy individuals, they either focused on the image analysis level and/or they used manual quantification method (Efron, et al., 2010; Hertz, et al., 2011; Petropoulos, et al., 2013b). However, this study was designed to assess the intra- and interobserver repeatability of the subbasal nerve parameters obtained using corneal confocal microscopy (CCM) while images were analysed by employing fully-automated quantification method.

4.2 Abstract

Purpose: To assess intra- and interobserver repeatability of the SNP parameters measurement.

Methods: For the purpose of interobserver repeatability, sixteen participants (six controls and 10 with diabetes) underwent CCM examination twice by the same observer. For another group of 11 participants (five controls and six with diabetes), a second observer then repeated the CCM examination. Eight selected central corneal images were then analysed using a fully-automated technique.

Results: There were no significant differences between mean SNP parameters of two sessions for intra- and interobserver assessment. Moderate to high intraclass correlation coefficients were found for all three SNP parameters (0.81-0.94, $P < 0.01$). The coefficients of repeatability for

intra- and interobserver assessments were: CNFD, 8% and 9.8%; CNBD, 20.1 % and 22.9% and CNFL 3% and 3.6%, respectively.

Conclusion: Among the three SNP parameters, CNFL is the most repeatable and reliable parameter and gives good observer-independent results. Assessment of SNP morphology can be used in this longitudinal study to evaluate possible changes over time.

4.3 Intraobserver test-retest repeatability of the SNP parameters

4.3.1 Methods

To assess the consistency of measurement of SNP parameters from one time to another, test-retest was conducted by performing the CCM procedure followed by automated image analysis for 16 participants on the same day of examination and each participant was tested twice, at least 30 minutes apart. Participants were enrolled from the ongoing LANDMark study (Pritchard, et al., 2014) and informed consents were obtained from all of them. Prior to the examination, participants underwent slit-lamp biomicroscopy examination to ensure the absence of any corneal compromise. The methodology of CCM examination was identical to the methods explained in the previous chapter (see section 3.4.2 Corneal confocal microscopy, page 45).

Evaluation of intraobserver repeatability was assessed by intraclass correlation coefficient (ICC), coefficient of repeatability (CoR) and Bland-Altman method (Bland & Altman, 1986). The CoR was calculated as 1.96 times the standard deviation of between the two measurements. A two-way random effects ICC was used for consistency of individual measurements. A CoR ≤ 20% was considered good and 20% to 50%, acceptable. IBM SPSS Statistics version 21 was used for all statistical analyses.

4.3.2 Results

Characteristics of the participants and the outcomes of SNP parameters are presented in Table 4-1. Paired t-test revealed no significant differences between test-retest measurements for CNFD, CNBD and CNFL (P = 0.59, P = 0.88 and P = 0.94, respectively). The results of ICC, limits of agreement

(LoA) and coefficient of repeatability (CoR) are shown in Table 4-2. Among the three SNP parameters, CNFL showed the highest ICC and the lowest CoR.

Table 4-1 Characteristics of participants in intraobserver repeatability study. Values are mean ± SD or count for categorical variables.

Parameter		Range
Age (years)	53 ± 18	17-77
Sex (Male/Female)	7/9	-
Group (Control/Diabetes)	6/10	-
CNFD (no/mm^2)		
test	20.2 ± 7.0	1.6 – 32.8
retest	19.7 ± 5.8	8.6 – 30.5
CNBD (no/mm^2)		
test	29.6 ± 18.8	1.0 – 82.8
retest	29.2 ± 16.6	4.7 – 74.2
CNFL (mm/mm^2)		
test	16.4 ± 3.0	8.5 – 21.0
retest	16.4 ± 3.2	10.6 – 22.1

CNFD, corneal nerve fibre density; CNBD, corneal nerve branch density; CNFL, corneal nerve fibre length

Table 4-2 Summary of mean difference, ICC, LoA and CoR for intraobserver repeatability study

	Mean difference	ICC	95% CI		LoA		CoR
	(test - retest)		Lower	Upper	Lower	Upper	
CNFD (no/mm^2)	0.56	0.81	0.53	0.93	-7.30	8.40	8%
CNBD (no/mm^2)	0.40	0.84	0.60	0.97	-19.50	20.20	20.1%
CNFL (mm/mm^2)	-0.02	0.90	0.75	0.97	-2.68	2.63	3%

CNFD, corneal nerve fibre density; CNBD, corneal nerve branch density; CNFL, corneal nerve fibre length; ICC, intraclass correlation coefficient; CI, confidence interval; LoA, limits of agreement; CoR, coefficient of repeatability

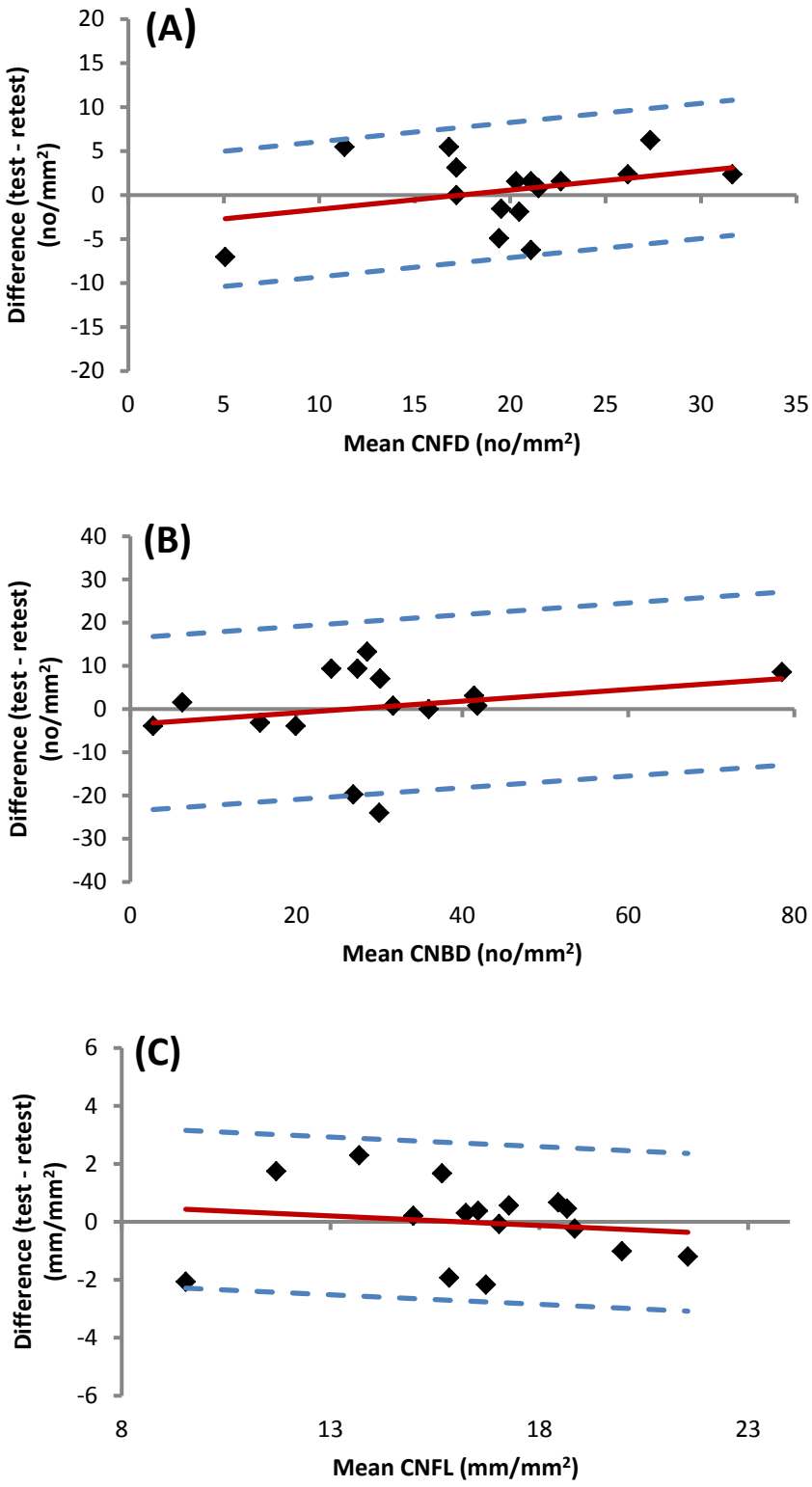

Figure 4-1 Bland-Altman plots of the relationship between differences in subbasal nerve parameters (A) CNFD, (B) CNBD and (C) CNFL vs. their mean for intraobserver test-retest study. On each graph, the solid line (red) indicates the linear regression and the dashed lines (blue) indicate the 95% limits of agreement.

Bland-Altman plots for test-retest difference and the mean of the test-retest were generated for CNFD, CNBD and CNFL and are illustrated in Figure 4-1. There was no significant relationship between mean CNFD (R^2 = 0.11, P = 0.21), CNBD (R^2 = 0.05, P = 0.40) and CNFL (R^2 = 0.02, P = 0.59) vs. their respective test-retest difference.

4.4 Interobserver repeatability of the SNP parameters

4.4.1 Methods

The same procedure was conducted to examine interobserver repeatability for 11 participants, five healthy and six with diabetes. Each participant underwent CCM examinations twice by two experienced observers on the same day of examination. Eight images per examination were collected by the observer and analysed using fully-automated algorithm.

Assessment of intraobserver repeatability was carried out by estimating ICC and CoR. A two-way random effects ICC was used to examine the consistency of measurements between two observers. A CoR ≤ 20% was considered good and 20% to 50%, acceptable. Bland-Altman plots (Bland & Altman, 1986) were also generated to depict the limits of agreement between two observers for measurement of the SNP parameters.

4.4.2 Results

Characteristics and estimates of SNP parameters of the recruited participants for assessment of interobserver repeatability are presented in Table 4-3. The mean difference between observer 2 and observer 1 was: -1.70 nerve/mm^2 for CNFD, -4.65 nerve/mm^2 for CNBD and -0.72 mm/mm^2 for CNFL. However, the differences between mean CNFD, CNBD and CNFL measured by two observers were not statistically significant (paired t-test, P = 0.29, P = 0.22 and P = 0.21, respectively).

A summary of estimated ICC, LoA and CoR are shown in Table 4-4. Similar to intraobserver study, CNFL showed the highest ICC and lowest CoR. The Bland-Altman plots of agreement between two observers for the SNP parameters are shown in Figure 4-2. No significant association was found

between mean CNFD (R^2 = 0.14, P = 0.25), CNBD (R^2 = 0.16, P = 0.21) and CNFL (R^2 = 0.01, P = 0.91) vs. their respective interobserver difference.

Table 4-3 Characteristics of the participants in interobserver repeatability study. Values are mean ± SD or count for categorical variables.

Parameter		Range
Age (years)	51 ± 11	30-65
Sex (Male/Female)	6/5	-
Group (Control/Diabetes)	5/6	-
CNFD (no/mm^2)		
observer 1	20.3 ± 10.6	7.0 – 39.8
observer 2	18.6 ± 8.7	4.7 – 32.0
CNBD (no/mm^2)		
observer 1	35.5 ± 32.7	2.3 – 100.8
observer 2	30.9 ± 28.0	2.8 – 85.9
CNFL (mm/mm^2)		
observer 1	16.9 ± 5.1	9.0 – 26.1
observer 2	16.2 ± 5.1	9.2 – 24.4

CNFD, corneal nerve fibre density; CNBD, corneal nerve branch density; CNFL, corneal nerve fibre length

Table 4-4 Summary of mean difference, ICC, LoA and CoR for interobserver repeatability study

	Mean difference (observer 2 – observer 1)	ICC	95% CI		LoA		CoR
			Lower	Upper	Lower	Upper	
CNFD (no/ mm^2)	-1.70	0.87	0.58	0.96	-11.54	8.14	9.8%
CNBD (no/ mm^2)	-4.65	0.93	0.75	0.98	-27.60	18.29	22.9%
CNFL (mm/ mm^2)	-0.72	0.94	0.78	0.98	-4.33	2.86	3.6%

CNFD, corneal nerve fibre density; CNBD, corneal nerve branch density; CNFL, corneal nerve fibre length; ICC, intraclass correlation coefficient; CI, confidence interval; LoA, limits of agreement; CoR, coefficient of repeatability

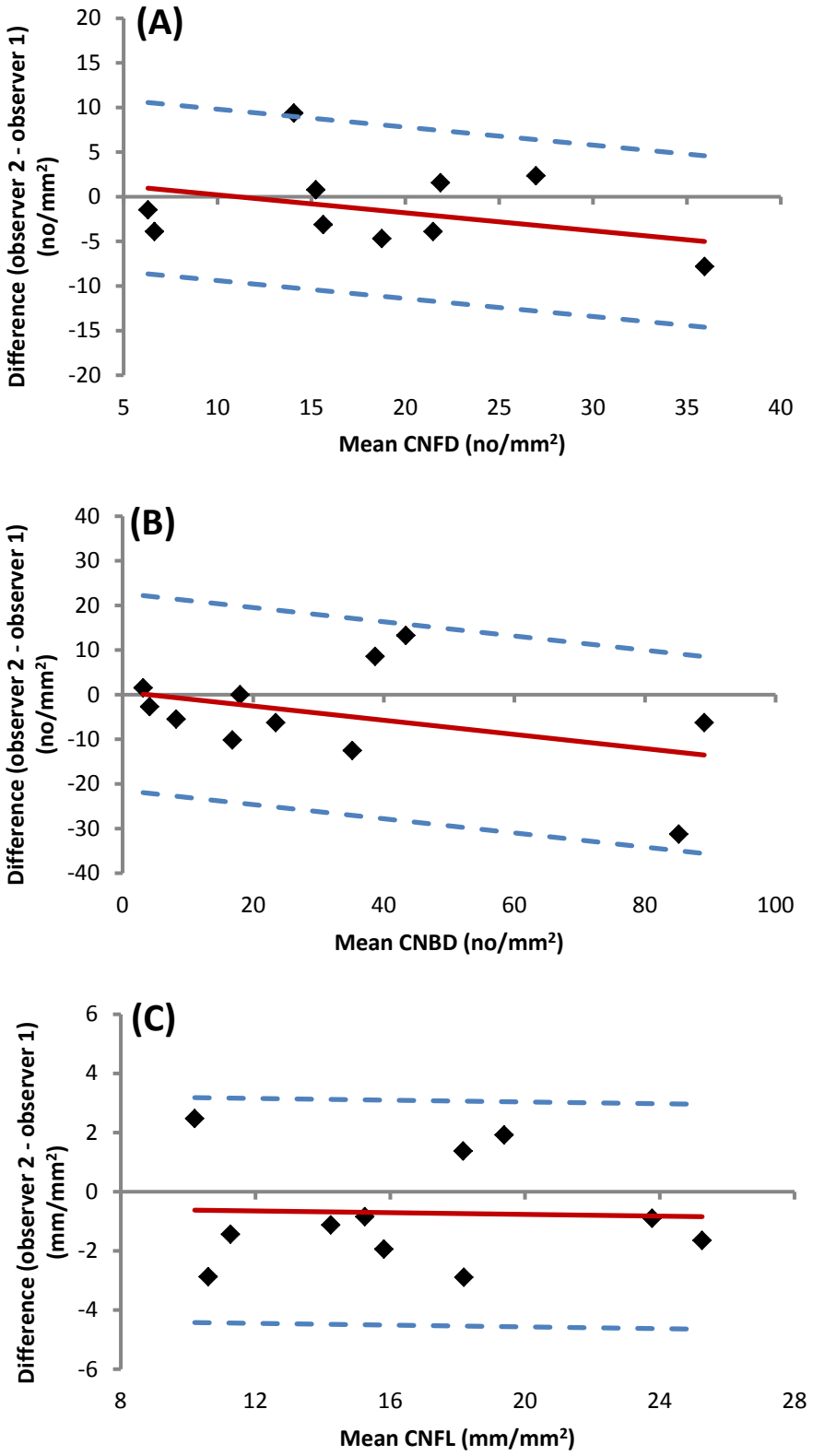

Figure 4-2 Bland-Altman plots of the relationship between differences in subbasal nerve parameters (A) CNFD, (B) CNBD and (C) CNFL vs. their mean for interobserver study. On each graph, the solid line (red) indicates the linear regression and the dashed lines (blue) indicate the 95% limits of agreement.

4.5 Discussion

In this study, the repeatability of SNP parameters within one observer (intraobserver) and between two observers (interobserver) was examined. Amongst the three evaluated SNP parameters, CNFL achieved the highest criteria for intraobserver repeatability with an ICC of 0.90, which demonstrates a very good reliability between test and retest, as well as an estimated CoR of 3% which also indicates a high repeatability of this parameter. CNFD also demonstrated a moderate ICC (0.81) and low CoR (8%). Although CNBD also showed a moderate ICC (0.84), the calculated CoR was only in the acceptable level.

Petropoulos et al. (2013b) performed an intraobserver study for a cohort of 19 healthy individuals on two separate occasions seven days apart. Similar to the current study, they found no significant difference between two sessions for CNFD, CNBD and CNFL and they reported the highest consistency for CNFL and CNFD. However, compared to our study they found lower ICC values (CNFD, 0.81 vs. 0.74; CNBD, 0.84 vs. 0.61 and CNFL, 0.90 vs. 0.70) and higher CoR (CNFD, 8% vs 17%; CNBD, 20.1% vs. 64%; and CNFL, 3% vs. 19%). These differences can be attributed to the time interval between sessions (30 minutes in our study vs. one week in their study), the image selection criteria (e.g. 8 images from the hand dominant side vs. 10 images from both eyes) and the analysis method (automated vs. manual technique).

Regarding the interobserver study, although the mean differences of the three SNP parameters were larger compared with the test-retest intraobserver study; the estimated mean values did not differ between two observers. Estimated ICCs were also moderate to high between two measurements, indicating good reliability between observers. CNFL and CNFD again showed the highest repeatability while CNBD achieved an acceptable CoR. Comparable to our findings, Ishibashi et al. (2012) reported good interobserver reproducibility for CNFD and CNFL in 14 healthy participants using coefficient of variation, while CNBD only received a poor reproducibility.

Although they performed the reproducibility study by re-examining the selected CCM images using manual quantification method, our results are in general agreement with the conclusion of Hertz et al. (2011); CNFL has the superior reliability compared to the other SNP parameters.

The mean difference of the SNP parameters in interobserver assessment implies that the observer 1 captured images with higher nerve density; however these differences, in particular for CNFL and CNFD, are minor to consider in clinical practice because they are practically insignificant and negligible. Furthermore, they showed good agreement and high ICCs between the two observers.

Variations in measurement of the SNP parameters may occur because CCM captures images from an area of 400 X 400µm, therefore difficulties in locating such a small are in the central cornea at second measurement may result in these small variations. Other sources of variability might be attributable to different focusing, participant cooperation and controlling eye movements during image acquisition. Our findings suggest that if the CCM examinations are conducted in the same centre, similar good results would be possible when done by another observer, assuming that the same methodology (e.g. image capturing and analysis) is employed. It should be noted that if CCM is to eventually be adopted widespread, it is worth repeating this study using inexperienced observers.

In conclusion, these findings indicate that measurement of the SNP parameters, in particular CNFL, using CCM and automated image analysis is highly repeatable within and between observers, which allows their application for longitudinal studies, provided that CCM examinations are done using similar methodology.

Considering the results of the study presented in the previous chapter (Chapter 3) and the outcomes of the present study, we chose to employ CCM in combination with automated algorithm to test the main hypotheses which are presented in next chapters.

CHAPTER 5. OVERALL METHODOLOGY AND BASELINE CHARACTERISTICS OF THE PARTICIPANTS

5.1 Foreword

This chapter describes the overall methodology related to this longitudinal research project. Specific methodology related to each experiment has been presented in respective chapters.

5.2 Participants

As stated in section 1.8 (page 6), this PhD project was associated with the ongoing LANDMark study. Participants were enrolled as a part of this study (Brisbane site) conducted at Anterior Eye Lab, Queensland University of Technology (QUT). Ethical clearances were granted by QUT, Princess Alexandra Hospital and Mater Hospital research ethics committees (Appendix 1).

Participants were recruited from the Centre for Diabetes and Endocrinology at Princess Alexandra Hospital and Mater Hospitals and the general population in Brisbane. Prior to their enrolment, written informed consent was obtained from all participants (Appendix 2), consistent with the Declaration of Helsinki. In LANDMark study, the following inclusion and exclusion criteria were applied at enrolment:

Inclusion criteria:

- Aged 14 to 75 years old
- Signed written informed consent
- Type 1 or type 2 diabetes, or no diabetes for control group
- Being willing to participate and comply with the experimental protocol

Exclusion criteria:

- History of corneal trauma and surgery
- History of ocular or systemic disease which may affect the cornea

- Concurrent ocular disease, infection or inflammation
- History of systemic disease (e.g. malignant disease, congestive heart failure, major psychosis, certain auto immune diseases)
- History of neuropathy due to non-diabetic cause
- Current or active diabetic foot ulcer or infection
- Participating in any other interventional research trial

The following exclusion criteria applied to control group

- Diabetes
- GADAb positive
- Presence of neuropathy

Exclusion criterion specific to this research project: Further to the overall LANDMark exclusion criteria, in this study participants with type 2 were excluded. Additionally, participants with type 1 diabetes with moderate and severe neuropathy (neuropathy disability score [NDS] ≥ 6) were also excluded in the longitudinal aspect of this research program. Figure 5-1shows the number and procedure of participant enrolment in this study.

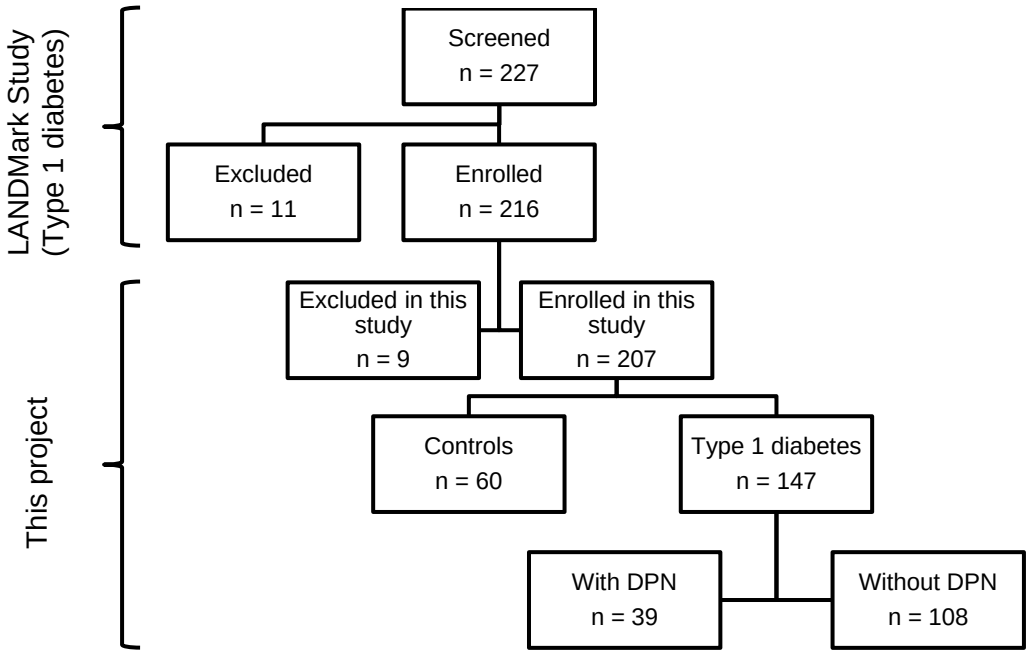

Figure 5-1 Flow chart diagram of study participants at baseline visit

5.3 Definition of neuropathy

The definition of neuropathy has been derived from the “Toronto criteria” (Tesfaye, et al., 2010) that rely on the presence of abnormal electrophysiological finding, based on age-matched controls at the site, in addition to clinical signs and/or symptoms, which was defined as one or more of the followings: (i) neuropathy disability score (NDS) ≥ 3 of 10 (Young, et al., 1993) or (ii) diabetic neuropathy symptom score (DNSS) ≥ 1 of 4 (Meijer, et al., 2002). This definition has been used throughout this thesis. The cut-off values that were applied for abnormal nerve conduction in this study are presented in Table 5-1. These cut-offs are based on age-matched control individuals at the Brisbane site.

Table 5-1 Abnormal nerve conduction criteria in this study

Parameter	Cut-off	
	Age < 54 years	Age ≥ 54 years
Peroneal CV ankle to fibula head*	< 45 m/s	< 42 m/s
Sural CV *	< 40 m/s	< 38 m/s
Tibial CV *#	< 43 m/s	

CV, conduction velocity

a. *Less than 10th percentile for healthy individuals without neuropathy

b. Nerve conduction is considered abnormal if (either) peroneal or sural CV is below age-referenced cut-off values.

c. #If sural not present, nerve conduction is considered abnormal for Toronto neuropathy if tibial CV is below 43 m/s for any age.

5.4 Assessment of neuropathy

A summary of the methods applied for neuropathy assessment are presented below.

5.4.1 Neuropathy signs and symptoms

NDS: NDS is a quantitative measure of neuropathy and was carried out using 2 Neurotips (Owen Mumford Ltd., Oxford, UK) loaded in Neuropens, 128 Hz

tuning fork, Metal rods, 2 beakers, hot and cold water and tendon hammer. This test included pain sensation, vibration sensation, temperature sensation and Achilles tendon reflex of both feet (Figure 5-2) and each abnormal response resulted in 1-point increase in score. Sharp and blunt ends of the Neurotip were applied on the pulp of the great toes in random order and the participant was asked to tell whether they think the painful stimulus occurred during sharp or blunt stimulus.

To examine the vibration sensation, the circular base of the vibrating and non-vibrating tuning fork was held against the end of the great toes in turn and the participant was instructed to tell whether the vibration occurred during time 1 or time 2. To test the temperature sensation, two beakers were filled with hot and cold water and one of the metal rods was placed in the hot water and the other in the cold water for 30 seconds. The rods were pressed in turn against the foot dorsum and the participant was asked to say whether the warm sensation occurred during time 1 or time 2.

All the above procedures were repeated 3 times and the response was considered normal if correct responses were ≥ 2/3. Achilles tendon reflex was tested with a hammer strike while participant was sitting with legs horizontal, and bent so that the knee faced outward from the body. Alternatively, the participant was asked to kneel on a chair. Finally, the NDS score was recorded from 0 to 10 (Abbott, et al., 1998; Young, et al., 1993). In this study, the DPN severity was classified using NDS score and only participants with NDS ≤ 5 were included.

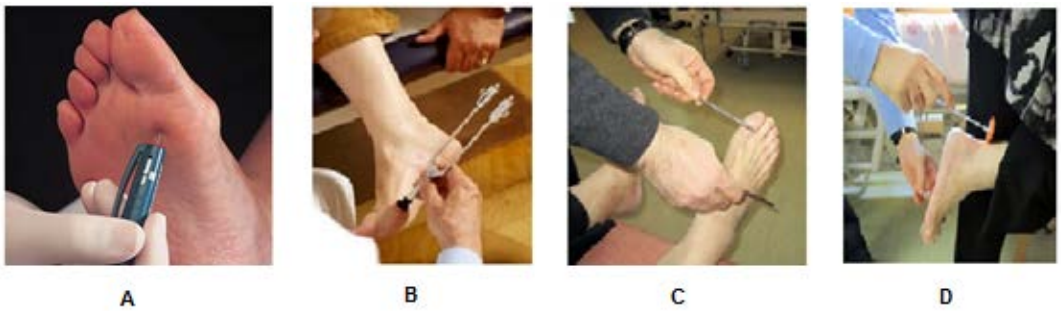

Figure 5-2 Pain sensation (A), vibration sensation (B), temperature sensation (C) and Achilles tendon reflex (D)

DNSS Questionnaire: This questionnaire (Table 5-2) is a validated and fast measure of neuropathic symptom for clinical practice (Meijer, et al., 2002) which includes 4 questions and it is completed by the participant. For each participant the total score was recorded from 0-4, based on the positive answers.

5.4.2 Quantitative sensory tests (QST)

Quantitative thermal and vibration assessment were carried out with the Medoc TSA-II NeuroSensory Analyzer and the VSA-3000 Vibratory Sensory Analyzer (Medoc Advanced Medical Systems, Ramat-Yishai, Israel) for threshold determination. Vibration perception was measured on the plantar surface of the big toe and thermal (warm and cold) sensation was assessed on the dorsal surface of the foot on the hand dominant side.

Table 5-2 Diabetic neuropathy symptom score questionnaire

1. Are you suffering of unsteadiness in walking? (i.e. need for visual control, increase in the dark, walk like a drunk man, lack of contact with floor) □ Yes (1) □ No (0) 2. Do you have a burning, aching pain or tenderness at your legs or feet? (i.e. occurring at rest or at night, not related to exercise, exclude claudicatio intermittens) □ Yes (1) □ No (0) 3. Do you have prickling sensations at your legs and feet? (i.e. occurring at rest or at night, distal>proximal, stocking glove distribution) □ Yes (1) □ No (0) 4. Do you have places of numbness on your legs or feet? (i.e. distal>proximal, stocking glove distribution) □ Yes (1) □ No (0) **Total Score _____/4**

5.4.3 Nerve conduction studies (NCS)

The Nihon Kohden Neuropack S1 (Nihon Kohden Corporation, Tokyo, Japan) was used for nerve conduction studies. The limb temperature was

maintained above 31^{o}C. Peroneal motor nerve conduction velocity (ankle to fibula head), amplitude (ankle to extensor digitorum brevis) and F wave latency were determined on the hand dominant side of the participants.

5.5 Ophthalmic procedures

All ophthalmic procedures were conducted following the medical procedures. Typically the eye on the hand-dominant side was examined unless otherwise indicated.

5.5.1 Screening procedures

This procedure began with measurement of visual acuity using Bailey-Lovie chart. Slit lamp examination of the cornea and anterior segment was performed for presence of any corneal compromise or finding that may affect the study results. Intraocular pressure measurement of the test eye was performed using iCare tonometer (Tiolat Oy, Helsinki, Finland).

5.5.2 Corneal confocal microscopy

The Heidelberg Retina Tomograph 3 (HRT3) in combination with Rostock Corneal Module (Heidelberg Engineering, Germany) was utilized to acquire multiple images of the corneal subbasal nerve plexus (SNP). This instrument is a laser-scanning confocal microscope (LSCM) and has a field of view of 400 X 400 µm when used with a 63X objective lens.

A large drop of high-viscous eye gel (GenTealEyes; Novartis, North Ryde, NSW, Australia) was placed between the microscope objective and the Perspex “TomoCap” that covered the objective. The gel optically couples the objective lens to the Perspex cap. The cornea of the dominant-hand side of the participant was anaesthetised with one drop of 0.4% benoxinate hydrochloride (oxybuprocaine hydrochloride; Bausch & Lomb, NSW, Australia). The head of the participant was placed in the head and chin rest, and the overall height of the instrument table was adjusted for comfort. The participant was instructed to fixate on a near target with the contralateral eye. The CCM was advanced forward until the laser beam fell in the centre of the

pupil (Figure 5-3). The instrument was slowly moved onto the cornea until gentle contact was established between the front of the applanation cap and the cornea; this procedure was facilitated by a side-mounted CCD camera that displays a magnified, real-time image of the cap.

Using the manual focusing, the SNP usually appeared at approximately 50-60 µm. For each participant multiple images from SNP of the central cornea were obtained using “section mode” which enables manual acquisition of a single image at a time with the aid of a foot pedal. The acquired images were saved digitally.

5.5.3 Image sampling and analysis

Investigators have used arbitrary numbers of images for analysis of the SNP morphology in the majority of previous studies. For the purpose of this research program, we followed the established protocol by Vagenas et al. (2012). This protocol offers optimized sampling paradigm for the central cornea and involves selection of a prescribed number of centrally-located images with minimum overlap that enhance the consistency of the procedure. Therefore, eight SNP images displaying in focus nerves and not overlapping by more than 20% were chosen for each participant at each visit. Selected images were then analysed using ACCMetrics (Figure 5-4), which has been explained in more detail and also compared with manual and semi-automated methods in Chapter 3. ACCMetrics as objective and fast method of corneal nerve segmentation showed good agreement with manual and semi-automated techniques, and its capability to detect depletion of the subbasal corneal nerves in individuals with DPN was comparable to those techniques.

The three quantified SNP morphometric parameters acquired using ACCMetrics include corneal nerve fibre density (CNFD; the total number of major nerves per mm^2), branch density (CNBD; the number of branches emanating from major nerves per mm^2) and fibre length (CNFL; total length of all nerves and branches in units of mm/mm^2).

Figure 5-3 Corneal light reflex at the centre of pupil

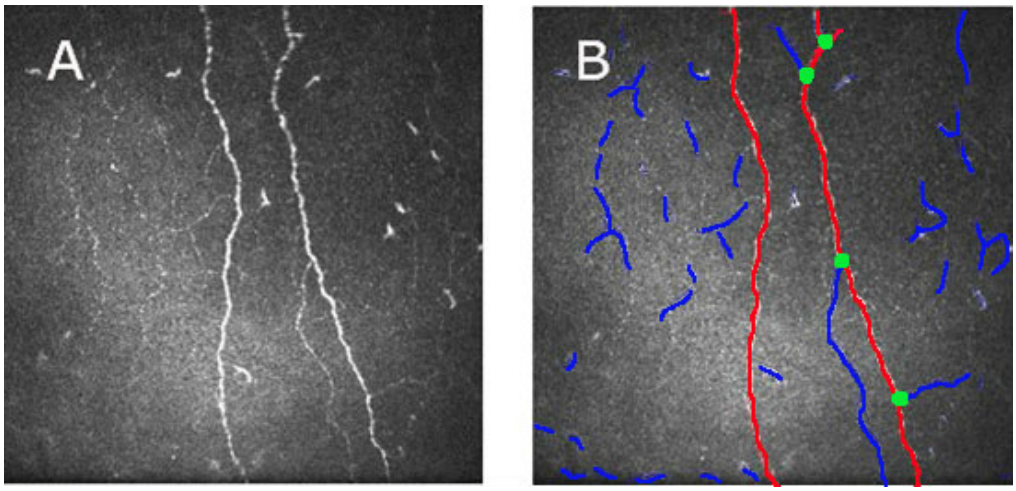

Figure 5-4 ACCMetrics analysis of corneal nerve parameters. Original 400 X 400 µm image of subbasal nerve plexus of a type 1 participant with neuropathy (A). Annotation of the same image by automatic analysis with CNFD = 12.5 nerve/mm^2, CNBD = 25.05 nerve/mm^2, and CNFL = 12.5 mm/mm^2 (B). Note that CNFD and CNBD are the number of major nerves (red lines) per mm^2 and the number of branches emanating from major nerves (green dots) per mm^2, respectively. CNFL is the total length of all nerves and branches (all blue and red lines) and expressed as mm/mm^2.

5.6 General health and metabolic measures

Information related to history of general health were collected including questions of medical history, age, duration of diabetes (self-reported or based on health care practitioner reports), and alcohol and tobacco

consumption. The following relevant health measures and blood biochemistry parameters were obtained at each annual visit:

Blood pressure: Blood pressure (systolic and diastolic) was measured using the WelchAllyn automatic digital sphygmomanometer (WelchAllyn Inc, NY, USA).

Body measurements: Weight and height were measured and body mass index (BMI) was calculated.

HbA_{1c} and lipid profiles: Visits also included an assessment of glycaemic control (HbA_{1c}), total cholesterol, low density lipoprotein cholesterol (LDL), high density lipoprotein cholesterol (HDL) and triglycerides which were assayed by a local certified pathology laboratory (Sullivan Nicolaides Pathology, Queensland, Australia).

5.7 Data management and analysis

Generated data from each participant were recorded on case report forms and then along with data from other sources such as pathology laboratory transferred to a central database. The data reported here were obtained from the database and all analyses were performed using IBM SPSS version 21.

5.8 Sequence of tests and main outcome variables

Medical and neuropathy assessments were often conducted at the beginning of the annual visits, followed by ophthalmic procedures. NCS usually were performed at the end of study visit. A summary of tests conducted and outcome variables recorded for participants at annual visits are shown in Table 5-3

5.9 Length of this longitudinal study

Lack of a previously conducted longitudinal study in respect to human corneal nerve morphology made it difficult to design a study with an appropriate length in order to allow clinically and pathologically significant changes to be observed. It has been suggested that longitudinal

assessments of diabetic neuropathy need to be conducted for a period of at least three years to achieve a meaningful and clinically significant change in QST results (Dyck, et al., 1997).

Substantial deterioration of clinical neurological examination has been shown in a previous longitudinal study with follow up duration of 2-4 years (van de Poll-Franse, et al., 2002). Significant worsening of DPN using NCS and QST over 12 months (Brown, et al., 2004) have also been reported. Considering these findings and previous studies of the relationship between traditional tests of neuropathy and corneal nerve morphology, a 4-year follow up with a baseline visit was chosen.

Table 5-3 Procedures and outcome parameters in this study

	Procedure	Outcome parameter	Type of variable
Health and metabolic	Health measures	Blood pressure, weight, height and body mass index	Continuous
	Metabolic information	HbA_{1c}, total cholesterol, low density lipoprotein cholesterol, high density lipoprotein and triglycerides	Continuous
Neuropathy measures	Quantitative sensory tests	Warm sensation threshold	Continuous
		Cold sensation threshold	Continuous
		Vibration threshold	Continuous
	Diabetic neuropathy symptoms score	DNSS	Categorical
	Neuropathy disability score	NDS	Continuous
	Nerve conduction studies	Peroneal nerve conduction velocity, F latency and amplitude	Continuous
Ophthalmic	Corneal confocal microscopy	Corneal nerve fibre density	Continuous
		Corneal nerve branch density	Continuous
		Corneal nerve fibre length	Continuous

5.10 Sample size calculation

Since the main hypothesis in this current study was the greater progression rate of SNP pathology in the neuropathy group compared to the control participants, the required sample size was calculated considering this hypothesis. The principle outcome measures for this hypothesis relate to change in CNFL and CNFD as the most reliable and repeatable parameters over a four year period. In the absence of pre-existing longitudinal data, the available baseline data of the LANDMark participants (type 1 diabetes and controls) were analysed to determine the sample size, because the mean and SD of the these parameters pertaining to the three groups of interest (control, type1 without and with neuropathy) could be obtained. The G*Power 3 software (Faul, et al., 2007) was used to calculate the effect size given the means and SD of three groups. Our desired power and significant level were set at 0.90 and 0.05, respectively and a priori analysis (sample size N is computed as a function of power level 1-β, significance level α, and the effect size) was applied, which resulted in an effect size of 0.3. Considering the required four subsequent visits, a total sample size of 100 was estimated. To compensate a 20% drop-out during study period, the total sample size was increased to 120 (40 participants in each group). Therefore, recruitment from LANDMark continued until the smallest group (diabetes with DPN) contained 40 participants resulting in a total of 207 participants.

5.11 Baseline characteristics of the participants included in this study

This section describes the recruited three groups and reports the baseline characteristics of the participants including demography, health and metabolic measures, neuropathy assessment and SNP parameters. Allocation of individuals to one of the three groups and exclusion of ineligible individuals was undertaken by details of which were outlined in previous sections (5.2 Participants and 5.3 Definition of neuropathy).

Normality of the data was examined using the Kolmogorov-Smirnov test and the appropriate tests were applied for analysis. Statistical measures including analysis of variance (ANOVA) with Scheffe post hoc test, t-test and other

nonparametric statistical tests were employed according to the characteristics of specific data elements. It should be noted that this section only gives an overview of participants at baseline who were stratified to three groups – namely controls, diabetes without DPN (DPN-ve) and diabetes with DPN (DPN+ve) - across a range of measures and more details are included in respective chapters (Chapters 6 – 7).

Table 5-4 shows the clinical characteristics and demographic data of participants at baseline based on neuropathy status. The mean age of the cohort was 48.4 ± 15.2 and 47% of the cohort were males. Although there was no significant difference between the mean age of participants with diabetes and controls (47.3 ± 15.4 vs. 51.0 ± 14.7, respectively, P = 0.11), DPN+ve group was found significantly older than controls and DPN-ve group. No sex difference was found among the three groups. DPN+ve group had longer duration of diabetes (29.6 ± 14.8) compared with DPN-ve group (16.3 ± 12.7, P < 0.001).

In regards to health and metabolic measures, there was no significant difference among groups for height, weight, BMI, diastolic blood pressure, HDL and triglycerides. As expected, mean HbA_{1c} of DPN-ve (7.9 ± 1.3) and DPN+ve (8.2 ± 1.6) groups were higher than controls (5.4 ± 0.3), however, it did not differ between DPN-ve and DPN+ve groups. Systolic blood pressure was significantly higher in DPN+ve group than DPN-ve group and controls. Mean total cholesterol and LDL were lower in both DPN-ve and DPN+ve groups compared with controls.

All the SNP parameters and established measures of neuropathy were significantly different between groups (ANOVA, P < 0.05). The results of pairwise comparisons between groups are presented in Table 5-4.

Table 5-4 Demographics and clinical characteristics of the participants at the baseline. Results are expressed as mean ± SD or counts for categorical variable.

	DPN status at baseline				
Characteristics	Controls	DPN-ve	DPN+ve	P-value (ANOVA)	Group difference (Scheffe post hoc)
Demographics					
Age (years)	51.0 ± 14.7	43.9 ± 15.7	56.5 ± 9.6	< 0.001[#]	DPN+ve vs. Controls , DPN-ve[†] DPN-ve vs. controls[†]
Sex (male/female)	26/34	49/59	22/17	0.402**	-
Diabetes duration (years)	0	16.3 ± 12.7	29.6 ± 14.8	< 0.001[§]	DPN+ve vs. DPN-ve[§]
Health and metabolic measures					
Height (cm)	170.4 ± 8.7	170.1 ± 9.8	171.2 ± 8.2	0.832*	-
Weight (kg)	76.1 ± 16.4	76.6 ± 14.7	80.2 ± 14.4	0.361*	-
BMI (kg/m^2)	26.1 ± 5.2	26.4 ± 4.3	27.4 ± 4.8	0.405*	-
Systolic BP (mmHg)	116.1 ± 13.6	117.8 ± 13.8	129.4 ± 20.0	< 0.001[‡]	DPN+ve vs. DPN-ve, Controls[§]
Diastolic BP (mmHg)	72.8 ± 7.0	72.1 ± 7.9	74.2 ± 10.3	0.392*	-
HbA_{1c} (%)	5.4 ± 0.3	7.9 ± 1.3	8.2 ± 1.6	< 0.001[‡]	Controls vs. DPN-ve, DPN+ve[§]
Total cholesterol (mmol/L)	5.4 ± 1.2	4.7 ± 0.9	4.9 ± 1.1	< 0.001[#]	Controls vs. DPN-ve, DPN+ve[†]
HDL (mmol/L)	1.5 ± 0.4	1.5 ± 0.4	1.6 ± 0.4	0.324	-
LDL (mmol/L)	3.5 ± 1.1	2.7 ± 0.7	2.7 ± 0.9	< 0.001[‡]	Controls vs. DPN-ve, DPN+ve[§]
Triglycerides (mmol/L)	1.1 ± 0.6	1.0 ± 0.6	1.1 ± 0.6	0.717	-
Corneal nerve parameters					
CNFD (number/mm^2)	22.3 ± 8.0	18.3 ± 7.1	16.3 ± 8.3	< 0.001*	Controls vs. DPN-ve, DPN+ve[†]
CNBD (number/mm^2)	35.1 ± 23.8	24.2 ± 17.4	23.7 ± 20.9	0.003[‡]	Controls vs. DPN-ve, DPN+ve[§]
CNFL (mm/mm^2)	18.1 ± 3.7	16.0 ± 3.8	15.0 ± 4.3	< 0.001*	Controls vs. DPN-ve, DPN+ve[†]

	DPN status at baseline				
Characteristics	Controls	DPN-ve	DPN+ve	P-value (ANOVA)	Group difference (Scheffe post hoc)
Quantitative Sensory Tests					
Cold sensation threshold (°C)	28.4 ± 2.8	27.4 ± 5.1	23.4 ± 7.2	< 0.001‡	Controls vs. DPN+ve§ DPN-ve vs. DPN+ve§
Warm sensation threshold (°C)	38.0 ± 4.1	37.4 ± 3.8	41.6 ± 3.7	< 0.001‡	Controls vs. DPN+ve§ DPN-ve vs. DPN+ve§
Vibration threshold (Hz)	7.0 ± 8.1	8.7 ± 10.3	25.7 ± 22.2	< 0.001‡	Controls vs. DPN+ve§ DPN-ve vs. DPN+ve§
Nerve Conduction Studies					
Peroneal F latency (ms)	49.6 ± 5.2	51.5 ± 4.9	55.7 ± 5.0	< 0.001*	Controls vs. DPN+ve† DPN-ve. vs DPN+ve†
Peroneal nerve amplitude (mV)	4.7 ± 2.3	5.2 ± 2.7	2.7 ± 1.8	< 0.001‡	Controls vs. DPN+ve§ DPN-ve vs. DPN+ve§
Peroneal nerve conduction velocity (m/s)	49.0 ± 5.5	46.7 ± 5.0	39.6 ± 5.9	< 0.001*	Controls vs. DPN-ve, DPN+ve† DPN-ve vs. DPN+ve†
Neuropathy disability score (0–10)	0.4 ± 0.9	0.6 ± 0.9	2.2 ± 1.5	< 0.001‡	Controls vs. DPN+ve§ DPN-ve vs. DPN+ve§
Diabetic neuropathy symptom score (0–4)	0.1 ± 0.3	0.2 ± 0.5	1.1 ± 1.0	< 0.001‡	Controls vs. DPN+ve§ DPN-ve vs. DPN+ve§

DPN-ve, diabetic participant without neuropathy; DPN+ve, diabetic participant with neuropathy

* One way ANOVA, **Chi-Square, †Scheffe post hoc, ‡Kruskal Wallis, §Mann-Whitney, #Welch ANOVA

5.12 Discussion

The baseline findings of the enrolled participants were presented in this section. Overall, a relatively large cohort of 207 participants enrolled in this study, including 147 type 1 individuals without and with neuropathy and 60 healthy controls, without peripheral neuropathy and/or diabetes. It should be noted that baseline characteristics are briefly discussed here and a more in-depth and specific discussion is presented in respective chapters (Chapters 6-7).

The cohort was sex-balanced (47% males) and age was well-matched between diabetes and control groups. DPN+ve group was found to be older than DPN-ve and control groups. DPN+ve group also had higher HbA_{1c}, longer duration of diabetes and higher systolic BP compared with those without DPN. These factors are among the most important risk factors for development and progression of neuropathy in patients with diabetes (see section 2.2.2 Pathogenesis and risk factors of DPN). While total cholesterol and LDL level were not different between DPN+ve and DPN-ve groups, lower level of these parameters were observed in comparison with controls. This can be attributed to the fact that 35% of diabetic participants were receiving lipid-lowering medications at baseline visit.

Using Toronto criteria and NDS, of 147 diabetic participants, 39 had mild DPN at baseline visit. The proportion of DPN+ve group to the diabetic group (27%) was suitable for assessing the natural history of SNP morphology in this group and to compare with DPN-ve and control groups. QST, NCS, NDS and DNSS were able to differentiate DPN+ve group from controls and DPN-ve group. These findings were not surprising, because symptoms, signs and NCS constitute the basis on which diabetic neuropathy was diagnosed in this study. The baseline findings showed that corneal nerve parameters obtained from CCM were able to differentiate type 1 diabetic participants without and with mild neuropathy from controls. These outcomes are in agreement with previous studies that examined the SNP morphology in relation to DPN

(Table 2-1) and confirm depletion of the SNP structural parameters in presence of DPN.

We employed an up-to-date definition for DPN which consists of NCS and signs and/or symptoms of neuropathy. Furthermore, a battery of established measures (symptoms, deficits, QST and NCS) were measured which would enable us to compare SNP structural parameters against them in terms of longitudinal changes over time. Given the lack of data in the literature regarding the natural history of corneal nerve morphology, this longitudinal study will provide insights to the usefulness of SNP morphology as a potential measure of neuropathy.

In conclusion, the data acquired at baseline indicate that the study has recruited an appropriate cohort to address the main objectives. For example the baseline glycaemic control (HbA_{1c} 8.1%) and total cholesterol (4.7 mmol/l) in the diabetic cohort of this study are comparable with previous longitudinal diabetic studies (Epidemiology of Diabetes Interventions and Complications (EDIC) Research Group, 1999; Lorbeer et al., 2011). Additionally, employment of CCM in combination with automated analysis provides an accurate and reliable method to estimate small nerve fibre damage at SNP level in diabetic individuals without and with neuropathy.

5.13 Directions for subsequent experiments

Application of CCM in combination with a fully-automated algorithm was found to be advantageous in reducing dependence of labour-intensive methods, while providing comparable results to manual and semi-automated techniques. We also found that this technique was repeatable within and between observers. Having addressed these important methodological aspects, the experiments presented in next chapters (Chapter 6-7) were conducted to address the main objective of this project.

CHAPTER 6. AGE EFFECT AND LONGITUDINAL ASSESSMENT OF SUBBASAL NERVE STRUCTURE IN HEALTHY STATE

6.1 Foreword

Using a longitudinal approach, the effect of age on three main subbasal nerve structural parameters obtained using corneal confocal microscopy was investigated in this study. These morphometric parameters include corneal nerve fibre density (CNFD), branch density (CNBD) and fibre length (CNFL). Furthermore, the longitudinal behaviour of these parameters over three years were also examined by fitting linear mixed models which are robust statistical methods for repeated measures analysis. This chapter, which addressed the second research question as defined in section 1.5 of Chapter 1, essentially forms the basis of a paper published in *Investigative Ophthalmology and Vision Science* journal as follows:

Dehghani C, Pritchard N, Edwards K, Vagenas D, Russel AW, Malik AR and Efron N. Morphometric stability of the corneal subbasal nerve plexus in healthy individuals: a 3-year longitudinal study using corneal confocal microscopy. *Invest Ophthalmology and Vision Science* 2014; 55:3195-3199.

6.2 Abstract

Purpose: To examine the age-dependent alterations and the longitudinal course of subbasal nerve plexus (SNP) morphology in healthy individuals.

Methods: Laser-scanning corneal confocal microscopy, ocular screening, and health and metabolic assessment were performed on 60 healthy participants at baseline and at 12-month intervals for 3 years. At each annual visit, eight central corneal images of the SNP were selected and analysed using a fully-automated analysis system to quantify CNFD, CNBD and CNFL. Linear mixed model approaches were fitted to examine the relationship between age and these parameters, and their longitudinal changes over three years.

Results: At baseline, mean age was 51.0 ± 14.7 years. The cohort was sex-balanced (P = 0.30). Age (P = 0.27), CNFD (P = 0.48), CNBD (P = 0.95) and CNFL (P = 0.98) did not differ between sexes. A total of 52 participants completed the 36-month visit and 49 participants completed all visits. No significant effect of age was found for CNFD ($F_{[1, 87]}$ = 0.72, P = 0.40) and CNBD ($F_{[1, 42]}$ = 0.53, P = 0.47). However, age had a significant effect on CNFL ($F_{[1, 33]}$ = 4.77, P = 0.04) with a linear decrease of 0.05 mm/mm^2 per one year increase in age. None of the three parameters showed significant changes over the 36-month period (CNFD, $F_{[1, 168]}$ = 2.32, P = 0.13; CNBD, $F_{[1, 30]}$ = 2.05, P = 0.16 and CNFL, $F_{[1, 3]}$ = 0.38, P = 0.58).

Conclusions: Corneal nerve parameters showed a stable course over a 36-month period in healthy individuals, although there was a slight linear reduction in CNFL with age. The findings of this study have implications for understanding the time-course of the effect of pathology and surgical or therapeutic interventions on the morphology of the SNP and serves to confirm the suitability of corneal nerve structure as a screening/monitoring marker for peripheral neuropathies.

6.3 Introduction

In vivo corneal confocal microscopy (CCM) is a rapid, non-invasive and reiterative technique which enables microstructural evaluation of the human cornea at high resolution. The anatomical location and transparency of the cornea make this tissue structure ideally suited for confocal microscopic assessment (Lagali et al., 2013). Image acquisition using CCM from different corneal layers and structures helps both clinicians and researchers to extract important information in respect to alterations induced by various ocular and systemic conditions.

The subbasal nerve plexus (SNP), which is a dense array of nerves located between the corneal basal epithelium and Bowman's layer, is the main corneal nerve structure studied *in vivo* using CCM as a result of distinct morphologic attributes such as length of the nerve bundles and their parallel arrangement in relation to the ocular surface. Structural analysis of the SNP

has been used to evaluate ocular conditions such as dry eye, ocular allergy and glaucoma (Benítez-del-Castillo, et al., 2007; Labbé, et al., 2012; Villani, et al., 2013b; Zhang, et al., 2011), corneal ectasia and dystrophies (Efron & Hollingsworth, 2008; Patel, et al., 2009a), the effect of contact lens wear (Efron et al., 2002; Hollingsworth & Efron, 2004) and assessment of nerve regeneration after penetrating keratoplasty (Darwish, et al., 2007a), and different forms of refractive surgery (Darwish, et al., 2007b; Erie, et al., 2005b). The CCM has also been deployed to assess small nerve fibre pathology induced by several systemic conditions including diabetes (Edwards, et al., 2012b; Tavakoli, et al., 2010b), Fabry disease (Tavakoli et al., 2009), idiopathic neuropathy (Tavakoli et al., 2010a) and chemotherapy (Ferrari et al., 2013).

Given the utility of SNP evaluation in screening, detection and monitoring of a wide range of systemic and corneal neuropathies, it is important to understand how aging might affect this nerve plexus. However, there is inconsistency in the literature with respect to the relationship between age and neural morphometric change in the SNP using *ex vivo* and *in vivo* techniques. While a number of studies have reported no significant change in the subbasal nerve morphology with age (Erie, et al., 2005a; Marfurt, et al., 2010; Patel, et al., 2009b), others have reported a decrease in nerve density with age (He, et al., 2010; Niederer, et al., 2007; Parissi, et al., 2013) and there is also uncertainty as to the age at which SNP structural loss become significant. Furthermore, to our knowledge no data are available concerning the dynamic morphologic changes of corneal nerves in health or disease over time.

The two primary objectives of this study were to investigate: (1) the relationship between age and the three main morphometric parameters of the SNP obtained from CCM (CNFD, CNBD and CNFL); and (2) longitudinal changes of these measures over three years in healthy human corneas.

6.4 Methods

6.4.1 Study participants

Following approval from the research ethics committee of Queensland University of Technology (Queensland, Australia) and obtaining written informed consent, 60 healthy participants were enrolled. Participants were recruited from the community in Brisbane, Australia, as a part of 4-year LANDMark study (Pritchard, et al., 2014). Exclusion criteria were: history of corneal surgery, trauma or disease, glaucoma, evidence of corneal compromise, ocular and systemic diseases (e.g. diabetes) that might have adversely affected the cornea and history of neuropathy. These criteria were reassessed at each annual visit.

All participants underwent assessment of visual acuity, slit lamp biomicroscopy and tonometry and all corneas were confirmed to be within clinical norms. Four participants were current soft contact lens wearers and were asked to refrain from contact lens wear on the day of examinations. Contact lens wearers were not excluded from the present study, because previous investigations of the impact of contact lens wear on morphologic changes in subbasal nerves using CCM have failed to demonstrate any impact (Oliveira-Soto & Efron, 2003; Patel et al., 2002; Wu, et al., 2012). All participants were observed at baseline and the examinations continued at 12-month intervals over three years for a total of four visits. The study was conducted in accordance with the tenets of the Declaration of Helsinki.

6.4.2 Corneal confocal microscopy and image analysis

At each visit, all participants underwent corneal confocal microscopy examination approximately at corneal apex using the Heidelberg Retina Tomograph 3 with Rostock Corneal Module (Heidelberg Engineering GmbH, Dossenheim, Germany). One eye (on the side of hand dominance) was selected and anaesthetized with a drop of 0.4% benoxinate hydrochloride (oxybuprocaine hydrochloride; Bausch & Lomb, NSW, Australia).

Eight central corneal images per participants, displaying in-focus nerves and not overlapping more than 20% (Vagenas, et al., 2012), were selected by inspection and analysed using a fully-automated analytical system (Dabbah, et al., 2011) to quantify CNFD (the total number of major nerves per mm^2), CNBD (the number of branches emanating from major nerves per mm^2) and CNFL (total length of all nerves and branches in units of mm/mm^2).

6.4.3 Blood biochemistry and health parameters

At each visit, blood biochemistry measures (HbA_{1c} and lipid profile) were assayed by a local certified pathology laboratory, and clinical measures (height, weight and blood pressure) were assessed by a research nurse.

6.4.4 Statistical analysis

Statistical analysis of the data was performed using SPSS (version 21). Normal distribution of the data was determined with the Kolmogorov-Smirnov test. Quantitative variables are expressed by the mean ± standard deviation (SD) unless otherwise indicated. For the analysis of the categorical variables, the χ^2 test was applied. The independent samples t-test was used to compare age, CNFD, CNBD and CNFL between sexes. Bivariate correlation was used, as appropriate, for assessment of association of the SNP parameters with alcohol consumption and absolute changes in those parameters with HbA_{1c}. One-way and Welch ANOVA were used to compare the SNP parameters among age groups at baseline visit. Differences in characteristics from baseline to year-3 visit were assessed using paired sample t-test (for normally distributed data) and nonparametric Wilcoxon test (for not-normally distributed data).

To analyse longitudinal data using the linear mixed model (LMM) procedure in the SPSS statistical software, the horizontal data format were converted to vertical structure; thus, there were four rows per participant corresponding to the four measurements collected over time on each participant. The relationship between age and the SNP parameters and the changes of these parameters over 3-year period were examined by fitting two linear mixed

models with restricted maximum likelihood estimation. The first model (LMM1) contained the SNP parameters, age at each annual visit and sex. The SNP parameters were individually inserted as dependent variable. Age (time-varying predictor variable) and sex (time-invariant variable) were specified as covariate and factor, respectively. Age, sex and the sex*age interaction were specified as fixed effects and Type III method of sums of squares was used.

The assessment of linear change of the SNP parameters over time (36 months) was carried out by fitting the second model (LMM2) in which these parameters were specified as dependent variable and time which was a variable capturing the order of observation, was defined as repeated variable. CNFL and sex were considered as dependent variable and factor, respectively. Time and age at enrolment were assigned as covariates.

6.5 Results

The demographic and clinical data of participants at baseline and 36-month visits are given in Table 6-1. A total of 60 participants completed the baseline visit and 52 completed the 36-month visit. Five participants discontinued during the study period due to poor health (n = 2), to loss to follow up (n = 2) and personal decision (n = 1). Two participants also missed the year-3 visit. The baseline cohort included 26 males and 34 females (χ^2 = 1.07, P = 0.30). Mean age was 51.0 ± 14.7 years. Age (males: 53.4 ± 13.8 years, females: 49.2 ± 15.3 years, P = 0.27), CNFD (males: 23.1 ± 7.9 no/mm^2, females: 21.6 ± 8.2 no/mm^2, P = 0.48), CNBD (males: 35.4 ± 26.6 no/mm^2, females: 35.0 ± 21.8 no/mm^2, P = 0.95) and CNFL (males: 18.1 ± 3.5 mm/mm^2, females: 18.1 ± 3.9 mm/mm^2, P = 0.98) did not differ between sexes.

Four participants (7%) reported to be current smokers with an average 19 cigarettes per day. CNFD, CNBD and CNFL were not significantly different between current smokers and non-smokers (P = 0.61, P = 0.27 and P = 0.20, respectively). Forty-nine participants (82%) reported current alcohol use with an average 5 units/week. No significant correlation was found between alcohol consumption (units/week) and the SNP parameters at baseline visit

(CNFD, $r_s = 0.08$, P = 0.57; CNBD, $r_s = 0.04$, P = 0.75 and CNFL, $r_s = -0.03$, P = 0.85). Nine participants were taking antidepressant medications during study period. No association was observed between using antidepressant drugs and mean SNP parameters at annual visits (independent samples t-test, P > 0.50).

Participants were divided into three age groups: group 1 aged < 45 years (*n = 19*), group 2 aged 45 - 59 years (*n = 24*) and group 3 aged ≥ 60 years (*n = 17,* Table 6-2). There was not a significant effect of age groups on CNFD (one-way ANOVA, P = 0.86), CNBD (one-way ANOVA, P = 0.65) and CNFL (Welch ANOVA, P = 0.60).

Table 6-1 Clinical demographic, metabolic and ocular screening measures of study participants at baseline and 36-month visits.

Parameter	Baseline	36 months	P-value (paired t-test)
Age (years)	51.0 ± 14.7	-	-
Sex (male/female)	26/34	24/28	-
HbA_{1c} (%)	5.4 ± 0.3	5.3 ± 0.4	<0.01
Total cholesterol (mmol/L)	5.5 ± 1.2	5.5 ± 1.2	0.84
HDL (mmol/L)	1.5 ± 0.4	1.5 ± 0.4	0.16
LDL (mmol/L)	3.5 ± 1.1	3.4 ± 1.0	0.12
Triglycerides (mmol/L)	1.1 ± 0.6	1.1 ± 0.5	0.26
Systolic blood pressure (mmHg)	116.1 ± 13.6	116.1 ± 14.0	0.95
Diastolic blood pressure (mmHg)	72.8 ± 7.0	72.3 ± 8.4	0.50
Height (cm)	170.3 ± 8.6	170.2 ± 8.8	0.70
Weight (kg)	76.3 ± 15.3	75.8 ± 13.8	0.49
BMI (kg/m^2)	26.1 ± 5.2	26.2 ± 4.8	0.68
Visual acuity (LogMAR)	0.04 ± 0.08	0.03 ± 0.08	0.17*
Intra-ocular pressure (mmHg)	13.2 ± 2.8	13.2 ± 3.1	0.98

Values shown are mean ± SD, or counts for categorical variables.

*Wilcoxon test

Table 6-2 Age and SNP parameters at baseline in three age groups.

Age groups	N	CNFD, no/mm^2 (mean ± SD)*	CNBD, no/mm^2 (mean ± SD)†	CNFL, mm/mm^2 (mean ± SD)‡	Age, years, (mean ± SD)§
Group 1: <45 years	19	23.1 ± 6.5	33.3 ± 16.3	18.7 ± 2.4	33.4 ± 8.7
Group 2: 45-59 years	24	21.8 ± 7.9	33.3 ± 22.0	18.0 ± 3.4	53.3 ± 4.5
Group 3: ≥ 60 years	27	22.0 ± 10.0	39.7 ± 32.6	17.6 ± 5.1	67.5 ± 3.6
Total group	60	22.3 ± 8.0	35.1 ± 23.8	18.1 ± 3.7	51.9 ± 14.7

*, † No significant difference among groups (one-way ANOVA, P = 0.86 and P = 0.65, respectively)

‡ No significant difference among groups (Welch ANOVA, P = 0.64)

§ Significant difference among groups (Welch ANOVA, P < 0.001)

CNFD, corneal nerve fibre density; CNBD, corneal nerve branch density; CNFL, corneal nerve fibre length

Apart from a clinically insignificant decline in HbA_{1c} (0.1 %, P < 0.01), over 36 months, there were no significant changes to health, metabolic or ocular screening measures (Table 6-1). There also was no significant correlation between absolute changes in SNP parameters and HbA_{1c} from baseline to the 36-month visit (Pearson, CNFD, r = -0.10, P = 0.50; CNBD, r = -0.09, P = 0.54 and CNFL, r = 0.05, P = 0.75).

The LMM1 was deployed to determine the association of age and SNP parameters. Using backward elimination procedure, fixed effects of sex*age interaction and sex were sequentially removed because their effects were not statistically significant. While no significant effect of age was found for CNFD ($F_{[1, 87]}$ = 0.72, P = 0.40) and CNBD ($F_{[1, 42]}$ = 0.53, P = 0.47), the Type III tests of fixed effects revealed that there was a significant influence of age on CNFL ($F_{[1, 33]}$ = 4.77, P = 0.04). Estimates of fixed effects and covariance parameters for CNFL are presented in Table 6-3.

Table 6-3 Estimates of fixed effects and covariance parameters from linear mixed model 1 in which the relationship of age and corneal nerve fibre length was statistically significant.

Parameter		Estimate	Std. Error	P-value	95% CI
Estimates of fixed effects*					
Intercept		20.89	0.89	< 0.00	19.00 - 22.81
Age		-0.05	0.02	0.04	-0.09 - -0.01
Estimates of covariance parameters*					
Residual		3.91	0.44	<0.001	3.15 – 4.87
Intercept + age	$UN_{(1,1)}$	9.88	5.65	0.08	3.15 – 30.37
	$UN_{(2,1)}$	-0.33	0.17	0.05	-0.65 - 0.00
	$UN_{(2,2)}$	0.01	0.005	0.02	0.01 - 0.03

CI: confidence interval; UN: unstructured variance-covariance matrix for random effects

* Dependent variable: corneal nerve fibre length

The natural history of the three SNP parameters over the 36-month observation period is depicted graphically in Figure 6-1. The LMM2 revealed that the linear effect of time, sex, age at enrolment and time*sex interaction were not statistically significant for any of the three SNP parameters (Table 6-4). To eliminate further the potential confounding neurogenesis effect of antidepressant drugs (Castrén & Hen, 2013) on the analysis of data relating to the longitudinal course of CNFL in healthy participants, LMM2 was repeated excluding participants who were receiving antidepressant therapy during study period. The results were similar to the total cohort with no significant effect of time for the three SNP parameters (CNFD, P = 0.23; CNBD, P = 0.15 and CNFL, P = 0.57).

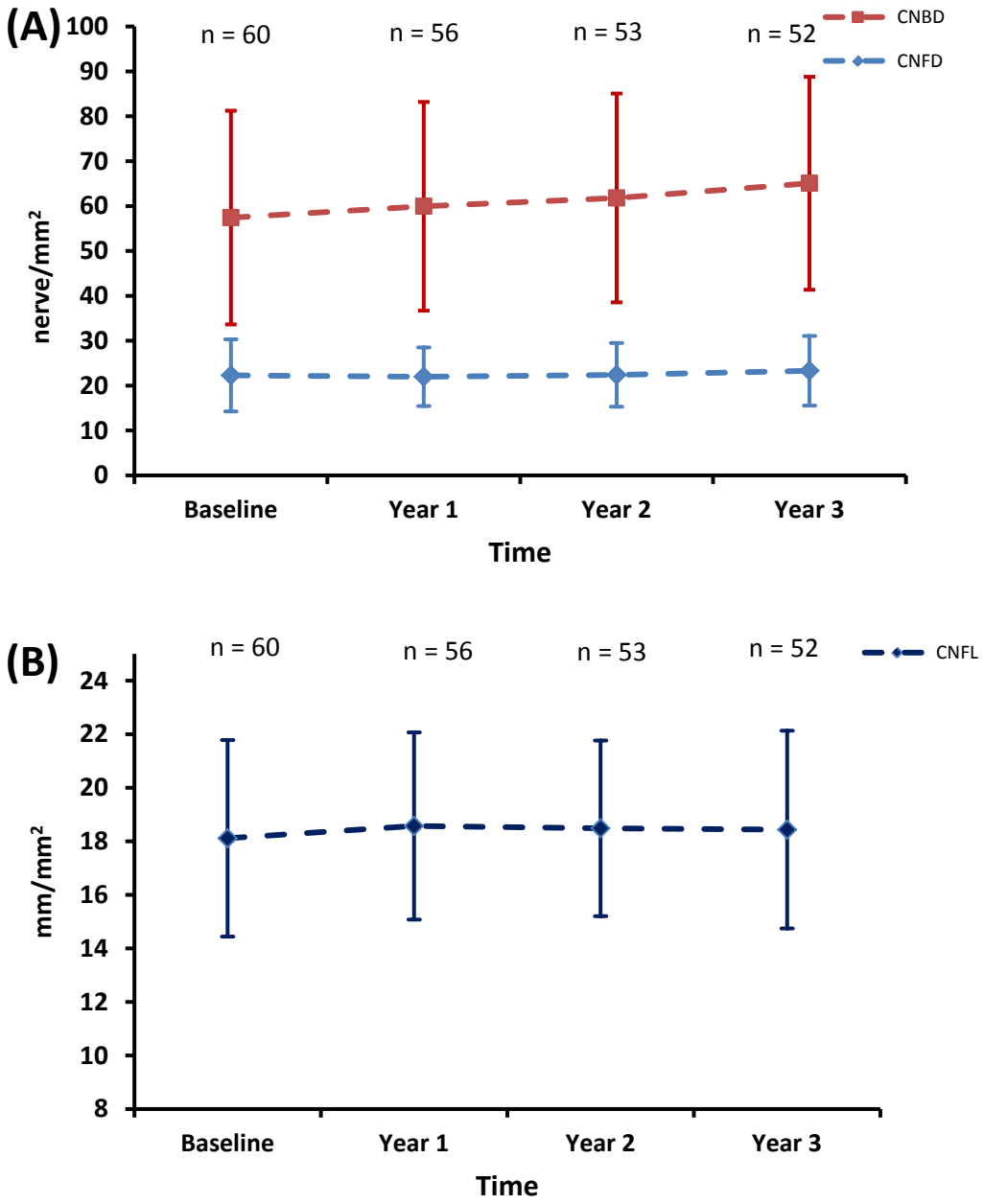

Figure 6-1 Quantification of corneal nerve fibre density and branch density (A) and corneal nerve fibre length (B) in healthy participants over 36 months. The three SNP parameters quantified in this study did not change over three years follow up (linear mixed model analysis, CNFD, P = 0.13; CNBD, P = 0.16 and CNFL, P = 0.58). Error bars represent mean ± SD.

Table 6-4 Estimates of fixed effects for the linear relationship of time and subbasal nerve parameters in linear mixed model 2.

Dependent variable: corneal nerve fibre density				
Parameter	Estimate	Std. Error	P-value	95% CI
Intercept	23.37	2.7	< 0.00	17.98 - 28.75
Time	0.19	0.47	0.69	-0.73 - 1.11
Age at enrolment	-0.02	0.05	0.49	-4.18 – 2.03
Sex				
Male*time	0.71	0.72	0.32	-0.70 – 2.12
Female*time	0†	0	-	-
Dependent variable: corneal nerve branch density				
Intercept	25.20	9.00	< 0.01	7.34 – 43.05
Time	2.12	1.53	0.18	-1.00 – 5.24
Age at enrolment	0.24	0.17	0.16	-0.10 – 0.57
Sex				
Male*time	-0.91	2.32	0.70	-5.7 – 3.84
Female*time	0†	0	-	-
Dependent variable: corneal nerve fibre length				
Intercept	19.26	1.35	< 0.00	16.56 – 21.96
Time	0.01	0.22	0.98	-0.68 - 0.67
Age at enrolment	-0.01	0.03	0.63	-0.06 – 0.04
Sex				
Male*time	0.22	0.34	0.56	-0.81 – 1.25
Female*time	0†	0	-	-
†This parameter is set at zero because it is the reference level of sex.				

6.6 Discussion

The feasibility of assessing corneal nerve morphology via CCM and the promising role of these structural parameters as an indicator of corneal nerve recovery following surgical and pharmacological intervention, and the potential for screening for peripheral neuropathies, has led to an increase in the scope of this approach. An increasing number of studies showing a relationship between quantitative analysis of the SNP parameters and various ocular and systemic pathologic conditions or surgical-induced

changes, highlights the importance of understanding the natural morphometric characteristics of the SNP over time.

In this longitudinal prospective study, participants were followed over 36 months with repeated monitoring of ocular, health and the SNP measures. At baseline, our cohort was sex balanced (45% male) and age was not significantly different between sexes. The sex of participants was also shown to have no influence on the SNP parameters. While the variability from the mean of SNP parameters increased with age (Table 6-2), mean CNFD, CNBD and CNFL between the groups was not significantly different. This finding is consistent with those of Patel et al. (2009b) who found no significant differences in mean CNFL between three age groups in a cohort of 60 healthy participants. On the other hand, Grupcheva et al. (2002) reported a significant difference in mean CNFL between two age groups (25 ± 5 years vs. 70 ± 5 years) of 50 participants.

Using laser-scanning CCM (LSCM), a great diversity has been reported in quantification of the SNP parameters in healthy individuals (Table 2-1). The mean central corneal nerve fibre length in the current study was 18.0 ± 3.6 mm/mm^2 which is similar to that reported by Wu et al. (2012) (18.0 ± 4.0 mm/mm^2), but slightly lower than the findings of Niederer et al. (2007) (20.3 ± 6.5 mm/mm^2) and Parissi et al. (2013) (18.6 ± 4.8 mm/mm^2). Differences in methodologies including number of participants, selected images, age range and method of image analysis may account for differing results.

A strength of the present study was consistency in respect to the location of corneal assessment (central), which was facilitated by an optimized sampling paradigm for the central region of the cornea that involved selection of a prescribed number of centrally-located images with minimum overlap (Vagenas, et al., 2012). As well, employment of an objective, fully-automated analysis system for image analysis facilitated reliable and objective quantification of the SNP parameters, which was important for ascertaining the natural course of these CCM measures. It has been demonstrated that fully-automated analysis of SNP parameters obtained from laser-scanning CCM images agrees very well with semi-automated and manual analysis

(Dabbah, et al., 2011; Dehghani et al., 2014; Petropoulos, et al., 2014) and yields results with a high level of reproducibility.

In the current literature, there is some discrepancy among studies as to whether corneal nerve structure changes with age. While subbasal nerve fibre density has been reported to reduce with age in an *ex vivo* study in 22 donor corneas aged from 19 to 80 years (He, et al., 2010), Marfurt et al. (2010) using an immunohistochemical staining technique, found no significant correlation between CNFL and age in corneas of six donors aged 19 - 78 years. Such a disagreement exists among studies using *in vivo* CCM as well (Erie, et al., 2005a; Niederer, et al., 2007; Parissi, et al., 2013; Patel, et al., 2009b).

The majority of studies reporting the relationship between age and corneal nerve parameters have concentrated on the total length of nerve fibres in unit of area of corneal images which is similar to the definition of CNFL adopted in this study. Hence, it is difficult to make a direct comparison of our findings in terms of CNFD and CNBD with previous reports in which these measures have not been included. Furthermore, the usual design employed in previous studies reporting the effect of age on corneal nerve morphology has been cross-sectional, in which measurements are made on participants of various ages and the detected differences are attributed to the effect of age. However, such results do not necessarily reflect real age changes. A longitudinal design with serial measurements in the same individuals over time allows true age changes for individuals to be determined. The findings of the current study (LMM1, Table 6-3) showed that while CNFD and CNBD were not affected by age, there was a significant linear decrease in the CNFL with age. The mean estimated initial status (at birth) and the linear change rate (per year) of CNFL for the total group were 20.90 mm/mm^2 and -0.05 mm/mm^2, respectively. This suggested that 1 mm/mm^2 reduction in central corneal nerve morphology would require 20 years to take place in normal participants. The cross-sectional studies of Niederer et al. (2007) and Parissi et al. (2013) reported a gradual decline in CNFL with age at a rate of 0.9%

and 0.30% per year, respectively, which exceeds the finding our longitudinal study reported here (0.05 mm/mm^2 per year).

Although marginally non-significant at $\alpha < 0.05$, the estimated covariance of the two random effects in the LMM1; that is, intercept and age ($\beta = -0.30$, $P = 0.05$) was negative (Table 6-3), which suggested individuals with high CNFL had a slower linear decrease, whereas individuals with low CNFL had a faster decrease, with age. There is also evidence of significant variance in these random effects ($\beta = 0.01$, $P = 0.02$), indicating variation among individuals in the rate of change of CNFL.

However, it is not clear why age did not have any significant effect on CNFD and CNBD which are the metrics of the major nerves and the branches emanating from them. Figure 5-4 illustrates how the three SNP parameters are quantified from CCM images. Considering the definitions of the SNP parameters and these outcomes, it can be speculated that the age-dependent alterations of the SNP mainly occur at short interconnecting links which appear to be not connected to any major nerves or branches in CCM images. The orientation of these fine, low contrast nerve fibres such as their non-parallel arrangement in relation to the ocular surface as well as the limited resolution of the current CCMs may restrict their visibility.

Apart from HbA_{1c} with a minor (0.1 %) but statistically significant difference, the average of all clinical metabolic and ocular screening measures remained stable from baseline to 36-month visit. The LMM2 (Table 6-4) showed that in this 3-year longitudinal study, the SNP parameters appeared to be stable as a function of time. The relationship of time with the change of three SNP parameters did not vary depending on sex, yielding a similar longitudinal pattern over three years for males and females. It is also worth noting that, while neuronal plasticity and regeneration can be influenced by antidepressant treatment (Baudry et al., 2011; Castrén & Hen, 2013), when our analysis was restricted to participants who were not receiving these medications, our results closely resembled those from the total cohort.

To our knowledge, no previous study has reported a longitudinal analysis of corneal nerve morphology in healthy individuals. The results presented here demonstrate, for the first time to our knowledge, stability of human corneal nerve morphology as assessed by LSCM over a 3-year period. These findings are important in demonstrating a significant, albeit weak, association between CNFL and age and the 3-year morphometric stability of the SNP structure in healthy individuals. These data provided *in vivo* evidence for stability of these structural parameters in healthy individuals and added a longitudinal perspective to consider alongside the results of cross-sectional studies demonstrating the dependence of CNFL parameter with age. The outcomes of this study may improve the ability of clinicians and researchers to understand the time-course of central corneal reinnervation following interventions such as keratorefractive surgeries and pharmacological treatment, and will assist in the interpretation of longitudinal studies using corneal nerve morphology as a screening/monitoring marker for peripheral neuropathies.

Although we found stability of the corneal nerve structure over a 36-month follow up period, this finding might not apply to the SNP changes over longer time periods. Furthermore, these findings are limited to nerve changes in the central cornea, and may not be applicable to other more peripheral regions of the human SNP. More recently, *in vivo* wide-field maps of the human SNP have been generated successfully (Edwards, et al., 2012a; Patel & McGhee, 2005) which might be useful to provide insights into changes in the entire SNP, if this procedure were to be deployed in longitudinal studies.

In conclusion, the current longitudinal *in vivo* CCM study confirms a slight reduction in CNFL as a function of age while there was no significant dynamic morphologic change in SNP morphology over 36 months. The data of this longitudinal study constitute a better understanding of SNP in living human cornea in a healthy state, which has implications in investigating the effect of corneal surgery, known transient or chronic alterations as a cause of or secondary to, local disease, or peripheral neuropathies, using corneal nerve structure as a non-invasive biomarker.

CHAPTER 7. NATURAL COURSE OF SUBBASAL NERVE STRUCTURE IN TYPE 1 DIABETES WITHOUT AND WITH MILD NEUROPATHY

7.1 Foreword

This chapter examined the natural course of subbasal nerve structural parameters in diabetic participants without and with mild neuropathy over four years and compared the trajectories to non-diabetic/non-neuropathic controls. Additionally, the longitudinal association between established measures of neuropathy with corneal nerve parameters was also assessed. In fact, this chapter addressed two main research questions defined in section 1.5 Research questions of Chapter 1. This chapter presents a study published by the *Investigative Ophthalmology and Vision Science* journal.

Dehghani C, Pritchard N, Edwards K, Vagenas D, Russel AW, Malik AR and Efron N. Natural History of Corneal Nerve Morphology in Mild Neuropathy Associated with Type 1 Diabetes: Development of a Potential Measure of Diabetic Peripheral Neuropathy. *Investigative Ophthalmology and Vision Science*, 2014;55:7982–7990.

7.2 Abstract

Purpose: To investigate longitudinal changes of subbasal nerve plexus (SNP) morphology and its relationship with conventional measures of neuropathy in individuals with diabetes.

Methods: A cohort of 147 individuals with type 1 diabetes and 60 age-balanced controls underwent detailed assessment of clinical and metabolic factors, neurologic deficits, quantitative sensory testing, nerve conduction studies and corneal confocal microscopy at baseline and four subsequent annual visits. The SNP parameters included corneal nerve fibre density (CNFD), branch density (CNBD) and fibre length (CNFL) and were quantified using a fully-automated algorithm. Linear mixed models were fitted to examine the changes in corneal nerve parameters over time.

Results: At baseline, 27% of the participants had mild diabetic neuropathy. All SNP parameters were significantly lower in the neuropathy group compared to controls (P<0.05). Overall, 89% of participants examined at baseline also completed the final visit. There was no clinically significant change to health and metabolic parameters and neuropathy measures from baseline to the final visit. Linear mixed model revealed a significant linear decline of CNFD (annual change rate, -0.9 nerve/mm^2, P = 0.01) in the neuropathy group compared to controls, which was associated with age (β = -0.06, P = 0.04) and duration of diabetes (β = -0.08, P = 0.03). In the neuropathy group, absolute changes of CNBD and CNFL showed moderate correlations with peroneal conduction velocity and cold sensation threshold, respectively (r, 0.38 and 0.40, respectively, P < 0.05). Among the important risk factors for corneal neuropathy, CNFD was found to have the highest association with HbA_{1c} (β = -0.58, P = 0.03).

Conclusion: This study demonstrates dynamic small fibre damage at the SNP, thus providing justification for our ongoing efforts to establish corneal nerve morphology as an appropriate adjunct to conventional measures of DPN.

7.3 Introduction

Diabetic neuropathy is a substantial and burdensome complication of diabetes, affecting up to 50% of these individuals (Dyck, et al., 1993). Diabetic peripheral neuropathy (DPN), which is the most common form of neuropathy, manifests as a distal, symmetric polyneuropathy that begins in the lower extremities and may progress proximally (Chin & Rubin, 2010). DPN leads to morbidity in diabetic patients in the form of painful neuropathy and foot ulceration with consequent lower limb amputation (Frykberg, et al., 2006). It accounts for reduced quality of life and imposes a significant economic burden that affects both individuals and society (Happich, et al., 2008; Van Acker, et al., 2009).

Several established tests are commonly used for screening, detection and assessment of DPN and to monitor its progression. The majority of these

tests examine neuronal function; however, direct observation of nerve structure is also possible. Neurologic symptoms and signs, quantitative sensory tests (QST) and nerve conduction studies (NCS) are the most commonly used tests for DPN (Dyck, et al., 2010). Indeed symptoms, neurological deficits and NCS constitute the basis on which diabetic neuropathy is diagnosed. QST provide quantitative measures of sensation; however, these tests require cooperation and concentration of the examinee and they may also be affected by anthropometric variables (Boulton, et al., 2004b). Whilst recent studies have shown that the proficiency of QST assessment is adequate (Dyck, et al., 2014), the reproducibility of symptoms and signs (Dyck, et al., 2010) and NCS (Litchy, et al., 2014), has been shown to be limited. Furthermore, studies in patients with impaired glucose tolerance (IGT) (Asghar et al., 2014) and recently diagnosed type 2 diabetes (Ziegler, et al., 2014b) show a marked small fibre neuropathy accompanying large fibre dysfunction.

Quantification of nerve pathology is possible through direct morphometric examinations of nerves including sural nerve biopsy (Malik et al., 2005) and skin biopsy (Lauria, et al., 2009). However, these techniques are invasive, require expertise for quantification and cannot be repeated from the same site for longitudinal studies. Accurate detection and estimation of progression are needed, especially to test putative treatments, which may alleviate the condition, and/or prevent or delay the development of sequelae. As reviewed in more detail elsewhere (Li, et al., 2013; Varkonyi et al., 2013), based on the pathogenesis of DPN, several potential therapeutic approaches have been developed targeting these mechanisms; however, apart from glucose control and pain management, currently there is no approved treatment for DPN (Callaghan, et al., 2012a; Li, et al., 2013).

Lack of a sensitive, accurate and reliable clinical endpoint has been one of the obstacles in mounting treatment trials for DPN (Malik, 2014a). Growing evidence supports a prominent association between corneal subbasal nerve plexus (SNP) morphology measured with corneal confocal microscopy (CCM) and DPN. CCM as a quick, non-invasive and reiterative technique has

a demonstrated capacity to detect early small nerve fibre damage in diabetic patients (Quattrini, et al., 2007), and diagnose (Ahmed, et al., 2012; Edwards, et al., 2012b; Malik, et al., 2003) and classify severity of DPN (Petropoulos et al., 2013a; Tavakoli, et al., 2010b). Conventional measures of neuropathy and corneal nerve parameters are also related (Edwards, et al., 2012b; Sivaskandarajah, et al., 2013; Tavakoli, et al., 2010b). Furthermore, the demonstration of early corneal nerve regeneration following simultaneous pancreas and kidney transplantation (Tavakoli, et al., 2013) and optimised glycaemic and lipid control in an observational study (Tavakoli, et al., 2011b) suggests that CCM may well fulfil some of the criteria for a surrogate end point for diabetic neuropathy.

To our knowledge, no study has been conducted to date concerning the natural course of the SNP structure over time in diabetic patients. Therefore in this study, we sought to investigate the natural history of the SNP morphology in type 1 diabetic individuals without and with mild neuropathy. Furthermore, the longitudinal relationship between changes in corneal nerve structure and established measures of neuropathy in individuals with diabetes was also addressed.

7.4 Methods

7.4.1 Study design and participants

This prospective, observational, longitudinal study was conducted following approval from Queensland University of Technology, Princess Alexandra Hospital, and Mater Hospital research ethics committees as a part of the LANDMark study (Pritchard, et al., 2014) in Brisbane, Australia. Prior to their enrolment, written informed consent was obtained from all participants and the research adhered to the tenets of the Declaration of Helsinki. Based upon the inclusion/exclusion criteria, 147 type 1 diabetic participants were recruited from Diabetes and Endocrinology Research Centre at Princess Alexandra and Mater hospitals and the general population in Brisbane. Sixty healthy participants, without peripheral neuropathy and/or diabetes were also recruited as controls. All participants were assessed at baseline and assessments continued for four annual subsequent visits (five time-points in

total and approximately 960 case visits). Participants were excluded in this study for any of the following: history of ocular trauma or surgery, ocular disease or systemic disease with potential corneal effect, and systemic disease (other than diabetes). Diabetic participants had no other known cause of neuropathy except from diabetes. Other causes of neuropathy were excluded. Diabetic participants with moderate and severe neuropathy were also excluded. All participants underwent neurologic and medical evaluation as well as ocular screening (visual acuity, slit lamp examination and intraocular pressure) and CCM, which were repeated annually.

For the definition of DPN, we followed accepted criteria (Tesfaye, et al., 2010) that rely on the presence of abnormal electrophysiological finding, based on age-matched controls at the site, in addition to clinical signs and/or symptoms, which was defined as one or more of the followings: (i) neuropathy disability score (NDS) ≥ 3 of 10 (Young, et al., 1993), or (ii) diabetic neuropathy symptom score (DNSS) ≥ 1 of 4 (Meijer, et al., 2002). The methods used during this study to assess neuropathy and health and metabolic factors have been presented in detail in Chapter 5 and will be described only briefly here.

7.4.2 Assessment of neuropathy

Neuropathy signs and symptoms: The neuropathy disability score (NDS), which is a scale of 0 to 10, was employed to assess neurological deficits. This measure included assessment of vibration, pin prick and temperature perception as well as presence or absence of ankle reflexes to both lower limbs. Diabetic neuropathy symptom score (DNSS), a scale of 0 to 4, was used to assess symptoms of neuropathy.

Quantitative sensory tests (QST): QST comprised of vibration perception, measured on the plantar surface of the big toe, and thermal (warm and cold) sensation which was assessed at the dorsal surface of the foot on the hand-dominant side.

Nerve conduction studies (NCS): Peroneal motor nerve conduction velocity (ankle to fibula head), amplitude (ankle to extensor digitorum brevis) and F wave latency were determined on the hand-dominant side of the participants.

7.4.3 General health and metabolic assessment

At each visit, all participants underwent assessment of height, weight, body mass index (BMI), blood pressure (BP), HbA_{1c} and lipid profile.

7.4.4 Corneal confocal microscopy and image analysis

CCM was carried out using Rostock Cornea Module in combination with a HRT 3 confocal microscope (Heidelberg Engineering, Heidelberg, Germany). Eight images of the SNP, showing in focus nerves and not overlapping more than 20% (Vagenas, et al., 2012), were acquired from the centre of cornea on the hand-dominant side using manual focusing and section mode. Automatic segmentation and quantification of the SNP parameters including nerve fibre density (CNFD), branch density (CNBD) and length (CNFL) was performed using ACCMetrics (Dabbah, et al., 2011), which is a fully automated analytical system. The SNP parameters for each participant were the average value obtained from the eight captured images and expressed in the unit of number/mm^2 for CNFD and CNBD, and mm/mm^2 for CNFL.

7.4.5 Intra- and inter observer repeatability of the SNP parameters

The findings of intra- and interobserver study of the SNP parameters have been explained in detail in Chapter 4. Overall, CNFL and CNFD achieved the highest values for repeatability and reproducibility, whereas CNBD showed an acceptable consistency within- and between observers.

7.4.6 Statistical analysis

Normality of the data was examined using Kolmogorov-Smirnov test and the appropriate test was applied for analysis. Data are presented as mean ± standard deviation (SD) or median and interquartile range (IQR). Four sets of analyses were conducted. First, the demographic and clinical characteristics

variables were compared between control and diabetic groups as well as between baseline and final visit. Second, using Toronto criteria, participants with diabetes were stratified as without DPN (DPN-ve) and with DPN (DPN+ve). Corneal nerve parameters and established neuropathy measures were compared between control, DPN-ve and DPN+ve. For the purpose of the two aforementioned analyses, parametric data were analysed using the independent samples t-test, paired t-test, one-way ANOVA and Scheffe post hoc test (pairwise comparison). Nonparametric data were analysed using the χ^2 test, Kruskal-Wallis test and Mann-Whitney U test.

Third, a linear mixed model was employed to examine changes over time in the SNP parameters and whether the changes were different in DPN-ve and DPN+ve compared with controls. In building a model for the data in SPSS, the following procedure was implemented. The wide format of the data was restructured to long format. The baseline values of time were set at 0, and the number of years from baseline was calculated for each time point of data collection.

Since change in the SNP parameters (i.e. CNFD, CNBD and CNFL) over time was one of the main parameters of interest in the current study, they were individually considered as response variables and time was added to the model to test the linear effect of time on the response variables. The first model contained CNFD as the response variable, group (i.e. controls, DPN-ve and DPN+ve), time and time*group interaction as primary fixed effects of interest and Type III sum of squares was selected. Group was included as a time-invariant predictor variable to explore any group differences over time.

The association between the initial CNFD parameter and the change of this parameter was estimated by calculating the covariance matrix. Here, the ‘variance components’ option was chosen and also the restricted maximum likelihood estimates for parameters was used. The process of the aforementioned model was repeated for CNBD and CNFL. Depending on whether the time*group interaction was statistically significant or not, a second set of fixed effects – namely sex, age at enrolment, duration of diabetes, HbA_{1c}, lipid profile, blood pressure, BMI, alcohol and tobacco

consumption – were included and their effects were examined. A stepwise elimination of the variables with non-statistically significant P-values was also applied.

The relation between risk factors and the changes of SNP parameters in diabetic individuals, regardless of their neuropathy status, was analysed with the latter model where all relevant risk factors were included. Control participants were excluded and group, as factor, was also removed from the model.

Finally, to explore the relationship between changes in corneal nerve parameters and functional measures of neuropathy, absolute change in all parameters was calculated (∆parameter = parameter value at final visit – parameter value at baseline). Bivariate correlations between absolute change of corneal nerve parameters and neuropathy measures were estimated using Pearson r and Spearman's rho correlation coefficients, where appropriate.

IBM SPSS 21 was used for all statistical tests and a two-tailed α=0.05 level of significance was considered for all analyses.

7.5 Results

Table 7-1 shows the clinical characteristics and demographic data of participants with diabetes and controls at baseline and final visit. Approximately 95% of the entering participants were Caucasians of European decent. There was no significant difference between the mean age of participants with diabetes and controls (P = 0.11). There were no statistically significant differences between diabetes and control groups with respect to high density lipoprotein (HDL), triglycerides, diastolic BP, BMI and number of cigarettes smoked per day (P > 0.25). Compared to controls, individuals with diabetes had a higher HbA_{1c} (P < 0.001) and systolic BP (P = 0.03) and lower total cholesterol (P < 0.001), LDL (P < 0.001) and alcohol consumption (P = 0.001).

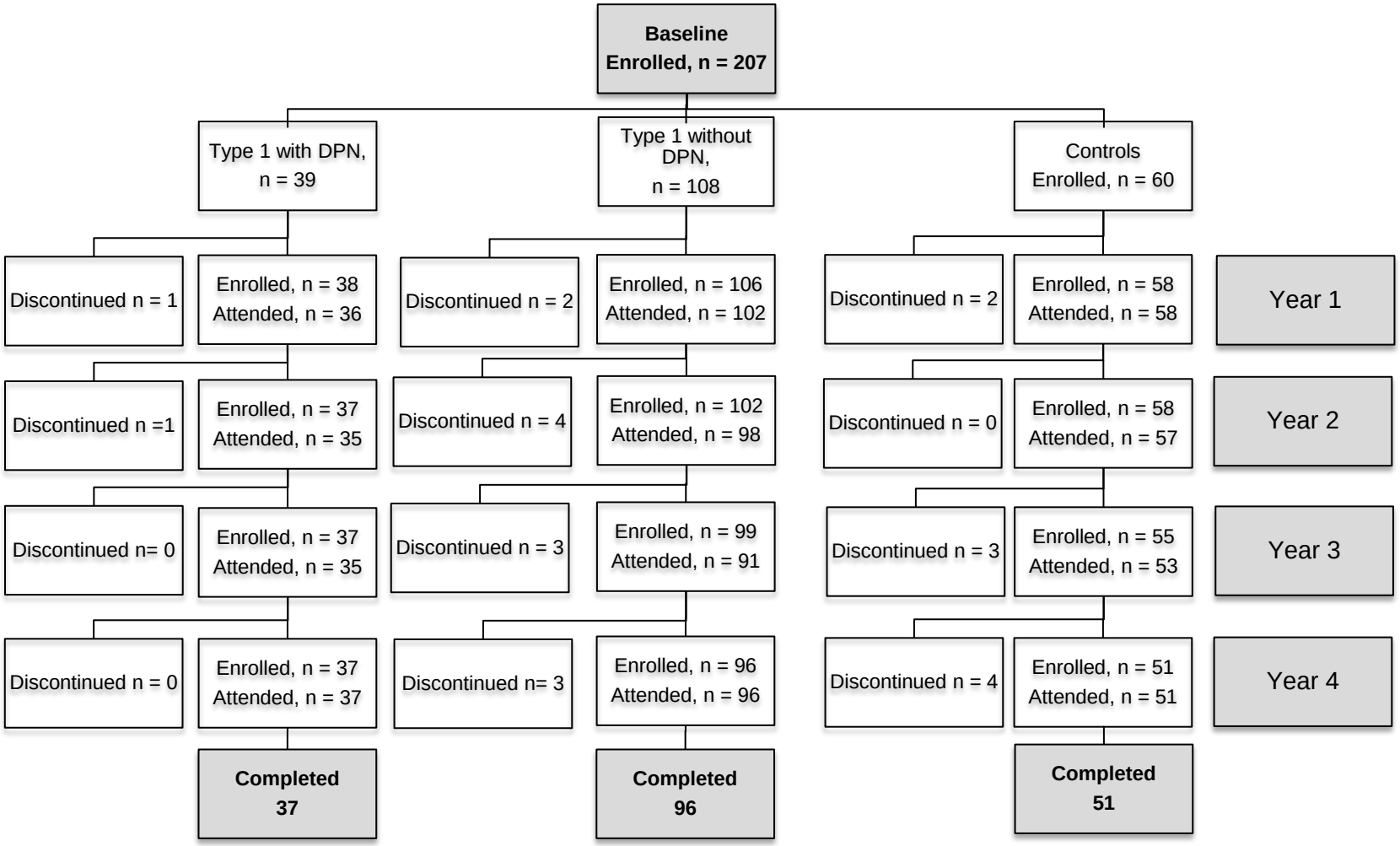

Figure 7-1 Flow chart diagram of study participants at baseline and follow-up visits

Figure 7-1 is a flow chart of study progress and shows the number of participants that enrolled, attended and discontinued at baseline and subsequent visits. Overall, 23 participants discontinued during study period. The number of participants attending annual visits is also depicted graphically in Figure 7-2. Altogether, 184 participants (89% of the baseline participants) completed the final visit. Personal decision was the main reason for withdrawal (13 participants) followed by poor health (6 participants). Four participants were also lost to follow up during the study period. The median follow up duration was 3.7 years (range, 3.4 – 4.3) for the cohort.

As can be seen from Table 7-1, at final visit HbA_{1c} showed a clinically insignificant decrease in controls (mean difference 0.2%, $P < 0.001$), while it remained the same in participants with diabetes ($P = 0.65$). Lipid profile, blood pressure, height and alcohol consumption did not differ at final visit compared to baseline visit for both diabetes and control groups ($P > 0.05$). Whilst BMI showed a statistically significant increase at the final visit in participants with diabetes ($P = 0.02$), there was no change in controls ($P = 0.42$). Both control and diabetic participants reported less smoking (number of cigarette/day) at the final visit compared to baseline ($P = 0.001$).

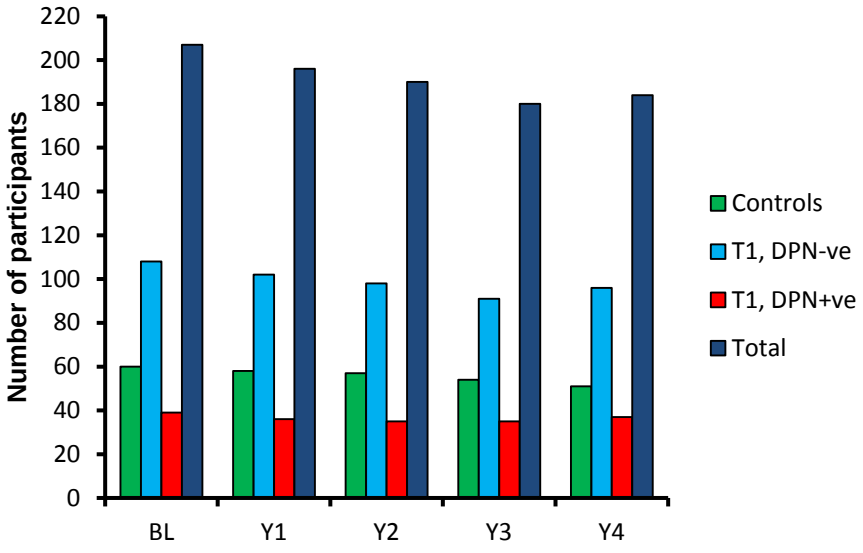

Figure 7-2 Distribution and number of participants examined at various time points. DPN-ve, diabetic participant without neuropathy; DPN+ve, diabetic participant with neuropathy.

Table 7-1 Demographic and clinical characteristics of the participants at baseline and final visit. Results are expressed as mean ± SD or counts for categorical variable.

	Baseline		Year 4 follow up		P-value		
Parameter	Control (A)	Diabetes (B)	Control (C)	Diabetes (D)	A vs. B	A vs. C	B vs D
n (male/female)	60 (26/34)	147 (71/76)	51 (22/29)	133 (67/66)	0.52*	0.98*	0.73*
Age (years)	51.0 ± 14.7	47.3 ± 15.4	57.0 ± 13.7	52.0 ± 15.3	0.11†	-	-
Duration of diabetes (years)	0	19.8 ± 14.5	0	24.0 ± 14.8	-	-	-
HbA_{1c} (%)	5.4 ± 0.3	8.1 ± 1.4	5.2 ± 0.5	8.0 ± 1.5	< 0.001‡	< 0.001§	0.65#
Total Cholesterol (mmol/L)	5.4 ± 1.2	4.7 ± 0.9	5.5 ± 1.1	4.6 ± 0.9	< 0.001†	0.83§	0.23§
HDL (mmol/L)	1.5 ± 0.4	1.6 ± 0.4	1.4 ± 0.3	1.5 ± 0.4	0.26‡	0.06§	0.78#
LDL (mmol/L)	3.5 ± 1.1	2.7 ± 0.8	3.5 ± 1.1	2.5 ± 0.7	< 0.001†	0.96§	0.07§
Triglycerides (mmol/L)	1.1 ± 0.6	1.1 ± 0.6	1.2 ± 0.5	1.1 ± 0.8	0.43‡	0.27§	0.40#
Systolic BP (mmHg)	116.1 ± 13.6	121.0 ± 16.5	117.1 ± 13.7	118.8 ± 12.1	0.03§	0.88§	0.12§
Diastolic BP (mmHg)	72.8 ± 7.0	72.7 ± 8.6	71.7 ± 8.2	71.2 ± 7.0	0.89†	0.27§	0.13§
BMI (kg/m^2)	26.1 ± 5.2	26.5 ± 4.4	26.8 ± 4.9	26.9 ± 4.7	0.46†	0.42§	0.02§
Alcohol (units/week)	5.0 ± 5.7	1.9 ± 1.8	5.2 ± 6.1	1.8 ± 1.8	0.001‡	0.78#	0.20#
Cigarettes (number/day)	6.7 ± 11.5	5.1 ± 8.0	1.3 ± 5.2	1.3 ± 5.6	0.74‡	< 0.001#	< 0.001#

*Chi square test, †Independent samples test, ‡ Mann-Whitney test, § paired samples t test, #Wilcoxon test

Comparison of the mean or median change from baseline to final visit in neuropathy measures of individuals with diabetes showed that there were no significant changes in DNSS [median 0 (0 – 0) vs. 0 (0 – 0), P = 0.56], cold sensation threshold [median 28.5 (24.8 – 28.5) vs. 28.5 (26.0 - 28.5) °C, P = 0.85], vibration threshold [median 6.8 (2.5 – 6.8) vs. 6.6 (2.9 – 6.6) Hz, P = 0.42] and peroneal F wave latency [mean 52.0 ± 5.1 vs. 52.2 ± 7.7 ms, P = 0.85]. NDS [median 1.0 (0.0 – 1.0) vs. 0.0 (0.0 – 0.0), P < 0.01], warm sensation threshold [median 37.6 (34.9 – 37.6) vs. 36.6 (34.8 - 36.6) °C, P < 0.01] and peroneal amplitude [mean 4.6 ± 2.6 vs. 5.0 ± 2.5 mV, P = 0.03] showed slight but significant improvements, whilst peroneal nerve conduction velocity [mean 45.3 ± 6.0 vs. 44.4 ± 5.8 m/s, P = 0.03] was the only measure that declined significantly from baseline to final visit.

Using Toronto criteria, in 147 individuals with type 1 diabetes, 39 (27%) were diagnosed with DPN at baseline. Table 7-2 delineates the outcomes of the SNP parameters and neuropathy assessment by DPN status. SNP parameters were significantly reduced in DPN-ve and DPN+ve groups compared to controls (P < 0.01).

All established neuropathy measures were significantly different between groups. QST, peroneal F wave latency and peroneal amplitude displayed greater deficits in DPN+ve group compared to DPN-ve and control groups (P < 0.05). Peroneal nerve conduction velocity was significantly lower in both DPN-ve and DPN+ve groups compared to controls and there also was a significant difference between DPN-ve and DPN+ve groups (P < 0.05). NDS and DNSS were significantly higher in DPN+ve group compared to control and DPN-ve groups (P < 0.001).

Figure 7-3 illustrates the 4-year time course for the SNP parameters in the cohort by neuropathy status. The results of the three created basic linear mixed model (LMM) analyses for CNFD, CNBD and CNFL can be found in Table 7-3. The Type III tests of fixed effects shows overall test of significance for the predictors included in the three basic models (LMM 1-3).

There was a significant effect of group for all three models; however the effect of time was not significant in any of them. The Type III F test for the interaction between group and time was only significant in LMM1; therefore no more models were fitted for CNBD and CNFL as response variables.

A second subset of fixed effects was included in LMM1. Upon sequential removal of non-statistically significant fixed effects and considering the lower resultant Akaike's information criteria (AIC) for comparing alternative models (Shek & Ma, 2011), a final model (LMM4) contained the fixed effects of group, time, age, duration of diabetes, HbA_{1c} and the group*time interaction was fitted. Parameter estimates and corresponding standard errors, P-values and 95% confidence intervals are given in Table 7-4.

Group and time did not show a significant effect, while the effects of age at enrolment (β = -0.06, P = 0.04) and duration of diabetes (β = -0.08, P = 0.03) were significant. LMM4 also showed a differential effect of time on the trajectory of CNFD with the slope decreasing by 0.91 nerve/mm^2 for DPN+ve individuals compared to controls (the reference level of the group).

The examination of significant risk factors for corneal neuropathy in diabetic individuals, irrespective of the baseline neuropathy status, showed that CNFD was associated with HbA_{1c} (β = -0.58, P = 0.03) and duration of diabetes (β = -0.08, P = 0.03). CNBD was found to be affected by the duration of diabetes (β = -0.21, P = 0.01) and smoking (β = -0.25, P = 0.04). No statistically significant association was found between CNFL and the included risk factors.

Table 7-2 Baseline comparison of corneal nerve parameters and neuropathy measures of the study participants according to presence and absence of neuropathy defined by Toronto criteria. Outcomes are mean ± SD.

	DPN status at baseline			
Characteristics	Controls n = 60	DPN-ve n =108	DPN+ve n =39	P Group difference
Corneal nerve parameters				
CNFD (number/mm^2)	22.3 ± 8.0	18.3 ± 7.1	16.3 ± 8.3	< 0.001* Controls vs. DPN-ve, DPN+ve†
CNBD (number/mm^2)	35.1 ± 23.8	24.2 ± 17.4	23.7 ± 20.9	0.003‡ Controls vs. DPN-ve, DPN+ve§
CNFL (mm/mm^2)	18.1 ± 3.7	16.0 ± 3.8	15.0 ± 4.3	< 0.001* Controls vs. DPN-ve, DPN+ve†
Quantitative Sensory Tests				
Cold sensation threshold (°C)	28.4 ± 2.8	27.4 ± 5.1	23.4 ± 7.2	< 0.001‡ Controls vs. DPN+ve§ DPN-ve vs. DPN+ve§
Warm sensation threshold (°C)	38.0 ± 4.1	37.4 ± 3.8	41.6 ± 3.7	< 0.001‡ Controls vs. DPN+ve§ DPN-ve vs. DPN+ve§
Vibration threshold (Hz)	7.0 ± 8.1	8.7 ± 10.3	25.7 ± 22.2	< 0.001‡ Controls vs. DPN+ve§ DPN-ve vs. DPN+ve§
Nerve Conduction Studies				
Peroneal F latency (ms)	49.6 ± 5.2	51.5 ± 4.9	55.7 ± 5.0	< 0.001* Controls vs. DPN+ve† DPN-ve. vs DPN+ve†
Peroneal nerve amplitude (mV)	4.7 ± 2.3	5.2 ± 2.7	2.7 ± 1.8	< 0.001‡ Controls vs. DPN+ve§ DPN-ve vs. DPN+ve§
Peroneal nerve conduction velocity (m/s)	49.0 ± 5.5	46.7 ± 5.0	39.6 ± 5.9	< 0.001* Controls vs. DPN-ve, DPN+ve† DPN-ve vs. DPN+ve†
Neuropathy disability score (0–10)	0.4 ± 0.9	0.6 ± 0.9	2.2 ± 1.5	< 0.001‡ Controls vs. DPN+ve§ DPN-ve vs. DPN+ve§
Diabetic neuropathy symptom score (0–4)	0.1 ± 0.3	0.2 ± 0.5	1.1 ± 1.0	< 0.001‡ Controls vs. DPN+ve§ DPN-ve vs. DPN+ve§

DPN-ve, diabetic participant without neuropathy; DPN+ve, diabetic participant with neuropathy

*One way ANOVA test, †Scheffe post hoc test, ‡Kruskal Wallis test, §Mann-Whitney test

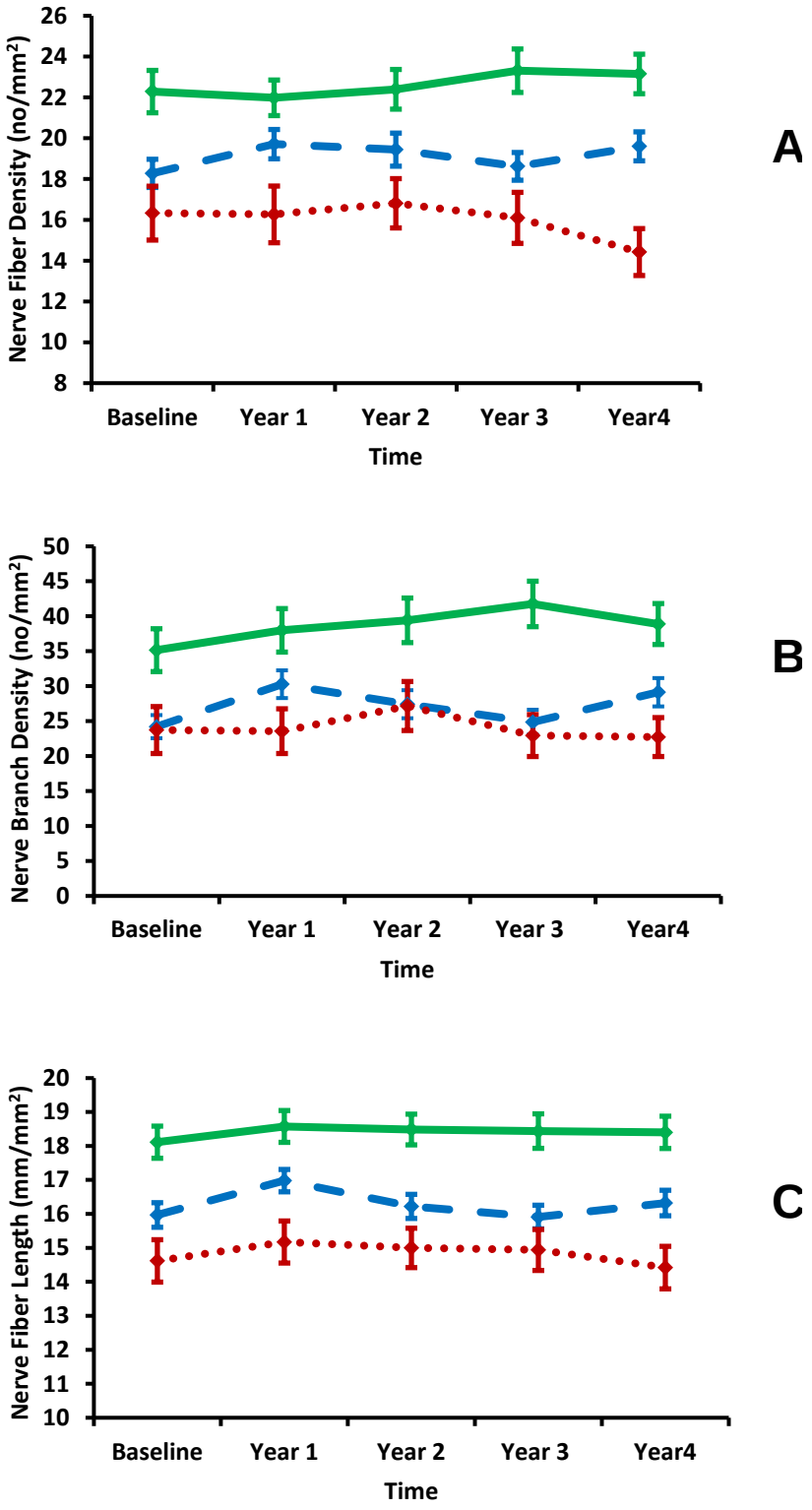

Figure 7-3 Longitudinal course of corneal nerve fibre density (A), branch density (B) and fibre length (C) over time. On each graph, the solid (green) line represents control participants, the dashed line (blue) represents diabetic participant without neuropathy and the dotted line (red) represents diabetic participant with neuropathy. Error bars indicate mean ± SEM.

Table 7-3 Results of Type III tests of fixed effects from the three initial linear mixed models analysis. Dependent variables were CNFD in linear mixed model 1 (LMM1), CNBD in LMM2, and CNFL in LMM3.

	LMM1		LMM2		LMM3	
	F	P	F	P	F	P
Intercept	1420.0	< 0.001	423.2	< 0.001	4254.4	< 0.001
Group	8.2	< 0.001	7.4	0.001	10.9	< 0.001
Time (years)	0.03	0.87	1.8	0.18	0.5	0.49
Group*Time	4.0	0.02	1.4	0.24	1.6	0.20

Table 7-4 Maximum likelihood of the fixed effect parameters for linear mixed model 4 with CNFD as the continuous response variable.

Parameter	Estimate (95% CI)	Std. Error	P-value
Intercept	27.57 (23.01-32.12)	2.32	0.00
Time	0.35 (-0.10-0.80)	0.23	0.13
Group			
DPN+ve	-1.36 (-5.17-2.45)	1.94	0.48
DPN-ve	-1.33 (-4.18-1.52)	1.45	0.36
Controls	0*	0	
Age at enrolment	-0.06 (-0.12-0.00)	0.03	0.04
Duration of Diabetes	-0.08 (-0.16 to -0.01)	0.04	0.03
HbA_{1C}	-0.41 (-0.89-0.08)	0.25	0.10
Group*Time			
DPN+ve * Time	-0.91 (-1.63 to -0.20)	0.37	0.01
DPN-ve * Time	-0.26 (-0.82-0.31)	0.30	0.37
Controls * Time	0*	0	

* This parameter is set to zero because it is the reference level of the group.

Since peroneal nerve conduction velocity was the only measure that showed a significant worsening in the diabetes group, we sought to compare the trajectories of this parameter between groups utilizing an additional mixed model (LMM5). The above-mentioned basic model was repeated with peroneal nerve conduction velocity as the response variable. There was a significant effect of time (P < 0.01) and group (P < 0.01), but the group*time

interaction was not significant (P = 0.92), indicating that the observed time effect was not different between groups (Figure 7-4).

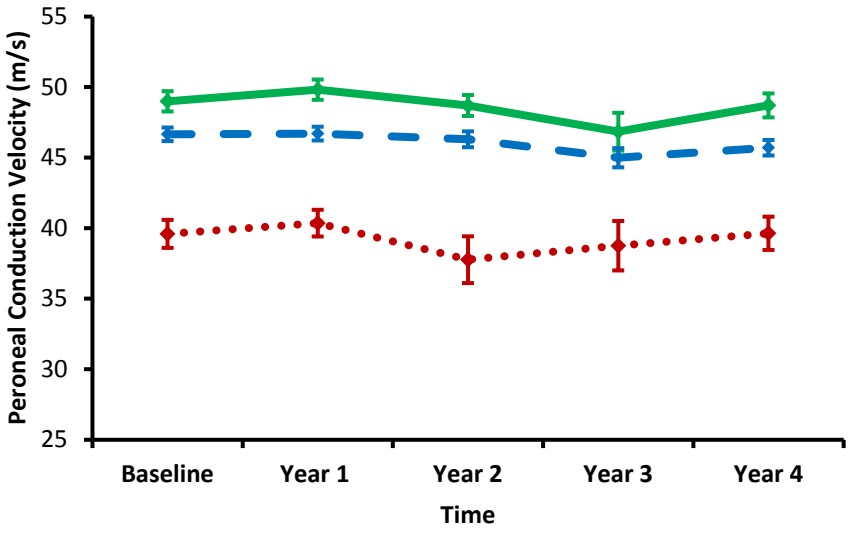

Figure 7-4 Natural course of peroneal nerve conduction velocity of the participants in this study. The solid line (green) represents control participants, the dashed line (blue) represents diabetic participant without neuropathy and the dotted line (red) represents diabetic participant with neuropathy. Error bars indicate mean ± SEM.

In the diabetic group, bivariate correlation revealed a modest association between absolute changes of CNBD and peroneal nerve conduction velocity (Pearson r = 0.23, P = 0.02) (Table 7-5, A). In the DPN+ve group, there was a significant correlation between CNBD and peroneal nerve conduction velocity (Pearson r = 0.38, P = 0.05). Absolute change in CNFL was also positively correlated with the cold sensation threshold (Pearson r = 0.40, P = 0.03) (Table 7-5, B).

Table 7-5 Correlation coefficients and estimated P-values among absolute changes (Δ) of corneal nerve parameters and established measures of neuropathy in (A) diabetic participants and (B) diabetic participants with DPN. Shaded areas indicate significant correlations (P < 0.05).

		Δ NDS	Δ DNSS	Δ CST	Δ WST	Δ VT	Δ PNL	Δ PNAm	Δ PCV
A: Diabetic participants (with and without DPN)									
Δ CNFD	Correlation coefficient	-0.02	0.01	0.00	0.12	0.09	-0.14	-0.10	0.13
	P-value	*0.81*	*0.93*	*1.00*	*0.18*	*0.34*	*0.21*	*0.27*	*0.16*
Δ CNBD	Correlation coefficient	-0.01	-0.03	0.17	0.10	0.03	-0.07	-0.01	0.23*
	P-value	*0.96*	*0.73*	*0.06*	*0.28*	*0.74*	*0.55*	*0.94*	*0.02*
Δ CNFL	Correlation coefficient	0.01	-0.06	0.06	0.08	-0.02	0.01	-0.07	0.15
	P-value	*0.97*	*0.53*	*0.50*	*0.37*	*0.85*	*0.93*	*0.46*	*0.12*
B: Diabetic participants with DPN									
Δ CNFD	Correlation coefficient	-0.08	-0.04	0.08	-0.08	0.21	-0.14	-0.10	0.24
	P-value	*0.67*	*0.84*	*0.67*	*0.67*	*0.26*	*0.58*	*0.63*	*0.24*
Δ CNBD	Correlation coefficient	-0.24	-0.13	0.33	-0.14	-0.03	-0.44	0.37	0.38*
	P-value	*0.17*	*0.46*	*0.054*	*0.42*	*0.86*	*0.08*	*0.06*	*0.048*
Δ CNFL	Correlation coefficient	-0.28	-0.23	0.40*	-0.18	-0.27	-0.29	0.36	0.24
	P-value	*0.11*	*0.18*	*0.03*	*0.30*	*0.15*	*0.26*	*0.06*	*0.23*

CNFD, corneal nerve fibre density; CNBD, corneal nerve branch density; CNFL, corneal nerve fibre length; NDS, neuropathy disability score; DNSS, diabetic neuropathy symptom score; CST, cold sensation threshold; WST, warm sensation threshold; VT, vibration threshold; PNL, peroneal nerve latency; PNAm, Peroneal nerve amplitude; PCV, peroneal nerve conduction velocity.

7.6 Discussion

In vivo assessment of the SNP morphology using CCM has emerged as a valuable clinical modality to improve our understanding of the relationship between this rich nerve plexus and various ocular and systemic conditions and diseases. As reviewed in more detail elsewhere (Villani et al., 2013a; Villani, et al., 2013b), morphometric evaluation of the SNP has been used to diagnose, assess and follow up ocular surface conditions including ocular allergy, dry eye, infectious keratitis, and glaucoma and after keratorefractive surgery and contact lens wear. Currently, considerable evidence exists that advocates the utility of CCM for assessment of small nerve fibre pathology induced by systemic and neurological conditions, in particular DPN. This study examined the longitudinal aspect of the utility of CCM to serve as an acceptable measure of DPN in clinical research and practice.

We report data from a cohort of individuals with type 1 diabetes (n = 147) and healthy controls (n = 60) collected from baseline to a median duration of 3.7 years. Although we demonstrated the stability of corneal nerve morphology in a 3-year longitudinal study in healthy individuals (Chapter 6), to our knowledge no previous study has examined the dynamic natural course of SNP microstructures in relation to DPN. With reference to the lack of previous investigation concerning the natural history of corneal nerves in diabetes, the present study is a positive response and attempts to fill this research gap.

At the baseline visit, age was matched between participants with diabetes and controls. Diabetic participants showed moderate glycaemic control and excellent control of cardiovascular risk factors including the BP and lipid profile in accordance with the current treatment guidelines (American Diabetes Association, 2014). The lower level of total cholesterol and LDL cholesterol in our diabetic patients as compared to controls is attributed to the fact that 35% were receiving lipid-lowering medications.

Comparison of the clinical parameters at baseline and final visit showed that there were no substantive and clinically significant changes to health,

metabolic and anthropometric measurements, indicating stable glucose control and desirable maintenance of cardiovascular risk factors. Although the Hawthorne effect (McCambridge et al., 2014) may have been involved, the finding of lower alcohol consumption in the diabetic patients at baseline which is maintained at follow up reflects good diabetes education. In addition, the significant reduction in tobacco consumption over time in both diabetic patients and control subjects presumably reflects overall population level education to stop smoking.

Except for peroneal nerve conduction velocity, with a statistically significant but clinically trivial decline (-0.9 m/s, P = 0.03), the remaining established neuropathy measures remained unchanged or improved slightly from baseline to the final visit. However, LMM5 showed that changes in peroneal nerve conduction velocity in DPN+ve and DPN-ve patients did not differ significantly from controls, indicating a similar effect of time for groups (Figure 7-4). The low rate of change over time in these measures can possibly be attributed to (a) the maintenance of a healthy lifestyle and compliance with medical advice among our diabetic cohort; (b) the inclusion of participants with only mild neuropathy; and (c) the relatively short duration of study. Negligible worsening or no progression of the traditional measures of DPN has also been observed in the placebo arm of a recent interventional study (Ziegler, et al., 2011) of 227 patients with predominantly type 2 diabetes, but with substantially worse glycaemic control at baseline (8.8 $\pm$ 1.9%) and a reduction of 0.67 $\pm$ 1.41% over 4 years. Our findings are further supported by a 3 year longitudinal study of 62 subjects with predominantly type 2 diabetes and good glycaemic control (HbA_{1c} 7.23 $\pm$ 1.03%), which interestingly demonstrated stability in a range of neurological examinations, symptom scores, autonomic testing, QST and nerve conduction studies with worsening only in the sural nerve amplitude and the axon-reflex vasodilation test, a measure of small fibre neuropathy (Gibbons, et al., 2013).

All three SNP parameters were significantly reduced in diabetic participants without and with neuropathy at the baseline visit. This finding is consistent with other studies, which also show a depletion of SNP tissue in diabetic

patients without and with DPN, reflecting early subclinical small fibre damage (Ahmed, et al., 2012; Petropoulos, et al., 2013a; Petropoulos, et al., 2014; Tavakoli, et al., 2010b). Based on the reported association of SNP parameters and DPN severity (Petropoulos, et al., 2013a; Tavakoli, et al., 2010b), we hypothesised that participants with diabetes and DPN would demonstrate quicker deterioration of SNP tissue than those without DPN. In order to examine this hypothesis, we built several linear mixed models. Such models afford robust methods of analysing longitudinal data with repeated measurements, in particular when the data is incomplete or unbalanced due to missing data, dropouts or differences in observation time points (Shek & Ma, 2011).

According to the three basic mixed models developed here (Table 7-3) and regardless of group, there was no significant effect of time for any of the three SNP parameters. A group*time interaction term was not significant for CNBD or CNFL (P = 0.24 and P = 0.20), indicating that the presence or absence of DPN at baseline did not appear to impact CNBD and CNFL changes over time. Mean CNBD (23.7 ± 20.9 vs. 22.7 ± 16.9, no/mm^2) and CNFL (15.0 ± 4.3 vs. 14.4 ± 4.1 mm/mm^2) declined slightly over 4 years in the neuropathy group, but to an extent that is neither clinically nor statistically significant.

However, the Type III F test for the interaction between time and group was statistically significant for CNFD (P = 0.02), suggesting that the relationship of time with CNFD change varies depending on the group. LMM4 (Table 7-4) demonstrated that whilst CNFD trajectories were not statistically different between DPN-ve and controls, the mean CNFD decreased significantly in the DPN+ve group during follow up, with a loss of approximately 1 nerve/mm^2 per year. This observed CNFD change was best predicted by participant age and duration of diabetes (both P < 0.05). One may anticipate that such a change would be influenced by glycaemic control, however, HbA_{1c} did not reach statistical significance (P = 0.10) in LMM4, where CNFD was considered as a dependent variable, possibly because of the relative stability of this factor during the study period. Although the outcome of CNFD decline

indicates dynamic structural small nerve fibre damage at the SNP, the relevance of CNFD change in the neuropathy group and the relative stability of CNBD and CNFL are not clear. Disparate changes to these three corneal nerve parameters have also been reported in diabetic individuals after improvement in risk factors for DPN (Tavakoli, et al., 2011b) and after simultaneous pancreas and kidney transplantation (Mehra, et al., 2007), suggesting a complex, dynamic and perhaps non-linear relationship between these parameters.

The baseline cross-sectional findings in the present study (Table 7-2) confirmed that all the three parameters were reduced in the neuropathy group compared to controls. The parameter that underwent the most marked reduction over time was CNFD. This suggests that branch damage (thinner branches emanating from major nerves) might represent the primary pathological change in DPN, whereas CNFD (a parameter related to the major nerve trunks) deterioration occurs later. The reduction in CNFD along with a non-significant decline of the other two parameters may also suggest degeneration of major nerve trunks with concomitant regeneration reflected by an increase in the CNBD and CNFL. Therefore, it is conceivable that loss and indeed repair of different SNP parameters may occur at different stages of the disease.

Limited studies are available documenting the link between corneal small nerve fiber change and risk factors of DPN (Edwards, et al., 2012b; Ishibashi, et al., 2012; Tavakoli, et al., 2011b). In the present study, when the data were restricted to include only diabetic individuals and upon removal of the effect of group in the linear mixed models, we found that every one-unit increase of HbA_{1c} was associated with a decrease of approximately 0.6 nerve/mm^2 in CNFD. There also was a negative effect of diabetes duration on CNFD and CNBD. Each 10-year increase of diabetes duration at baseline resulted in 0.8 and 2.0 nerve/mm^2 decline of central corneal CNFD and CNBD, respectively. CNBD was also significantly affected by smoking. Increasing one cigarette per day had a negative effect of 0.25 nerve/mm^2.

These results demonstrate the link between risk factors of DPN and morphologic parameters of corneal nerves. We have no clear explanation why HbA_{1c} has an effect on CNFD, but not CNBD and CNFL. Nevertheless, this finding is consistent with the study of Tavakoli et al. (2011b) who reported a significant correlation between changes in HbA_{1c} and CNFD ($r = -0.52$, $P < 0.01$) but not for CNBD and CNFL. In a study of 38 type 1 diabetic patients with and without neuropathy, Ishibashi et al. (2012) reported time-dependent effects of HbA_{1c} on SNP parameters. While nerve beading frequency was positively correlated to the mean HbA_{1c} levels at time of, or up to three months prior to CCM examination, no significant association was found between CNFD and CNFL with HbA_{1c} up to 6 years before CCM examination.

These findings emphasise the importance of including different SNP parameters in future studies where these parameters are to be used as measures of small nerve fibre damage and in particular repair. Additionally, in this study, only the central cornea has been investigated. Recent studies have revealed that loss of corneal neve structure in the SNP mainly occurred at the inferior whorl, which is slightly more distal than the central cornea and may therefore be expected to show more marked pathology (Davidson, et al., 2014; Edwards, et al., 2012a). Further longitudinal work assessing the inferior whorl as opposed to the central cornea may provide additional insights and ability to discriminate change in relation to DPN.

In previous cross-sectional studies, SNP parameters have been shown to correlate with functional and structural measures of neuropathy (Quattrini, et al., 2007; Sivaskandarajah, et al., 2013; Tavakoli, et al., 2010b). Quattrini et al. (2007) reported a significant correlation between CNFD versus NDS ($r = -0.30$, $P = 0.03$) and cold sensation threshold ($r = -0.40$, $P < 0.01$). In a subsequent study, moderate correlations were found between NDS and corneal nerve parameters (r; -0.48 to -0.58; $P < 0.001$) (Tavakoli, et al., 2010b). In a recent study by Sivaskandarajah et al. (2013), CNFD, CNBD and CNFL were related to cold sensation threshold (r; 0.32 to 0.37; $P \leq 0.01$). In this longitudinal study, we examined the relationship of change in

corneal nerve parameter with conventional measures of neuropathy by calculating the absolute change from baseline to final visit for participants with diabetes. We found a modest correlation between CNBD and peroneal conduction velocity (Pearson r = 0.23, P = 0.02). When the data were restricted to the DPN+ve group, this correlation increased to 0.38. Furthermore, CNFL also correlated to cold sensation threshold (r = 0.40, P = 0.03), which indicates that SNP parameters do change in a fashion comparable with some traditional measures of neuropathy.

The key strengths of this study are its longitudinal nature, inclusion of a range of traditional neuropathy measures (small and large nerve fibre dysfunction) in a relatively large number of type 1 diabetic participants, the consistency and strict adherence to technical and methodological procedures such as capturing and selection criteria of the SNP images, and employing a fully-automated image analysis algorithm, which is essential to eliminate shortcoming associated with manual and semi-automated analysis. Thus, we used a fully automated image analysis algorithm which has been validated and compared against the manual and semi-automated analysis (Dabbah, et al., 2011; Dehghani, et al., 2014; Petropoulos, et al., 2014) in individuals with diabetes.

In this study, a multimodal approach (the Toronto criteria) has been used for the case definition of DPN and comprises nerve electrophysiology and clinical signs and/or symptoms of neuropathy. Given the availability of different definitions for DPN, one may argue whether using the Toronto criteria is the appropriate approach for the utility of corneal neve morphology in diabetes. It is known from previous studies that due to high variability and poor reproducibility of signs and symptoms of neuropathy, their application in clinical research is limited if they are to be used alone (Malik, 2014b). Hence, the published consensus definitions for clinical research of DPN such as the San Antonio criteria (American Diabetes Association & American Academy of Neurology, 1988), the American Academy of Neurology, American Association of Electrodiagnostic Medicine and American Academy of Physical Medicine and Rehabilitation guidelines (England et al., 2005) and

the Toronto Diabetic Neuropathy Expert Group (Tesfaye, et al., 2010) recommended inclusion of signs and/or symptoms of neuropathy and nerve conduction studies.

The notion of including nerve electrophysiology for case definition of DPN in relation to the utility of CCM has been confirmed by Halperm et al. (2013). They studied the effect of different definitions of DPN on the validity of corneal nerve structure (CNFL) in type 1 diabetic participants and found that definitions that included electrophysiology had a better performance while including clinical criteria alone resulted in a substantially lower performance of detection capability of corneal nerve morphology.

A limitation of this study is that a majority of type 1 participants were enrolled from specialized clinics, where the glycaemic and cardiovascular factors were optimally controlled, which may not represent the typical population with type 1 diabetes. Additionally, four years of study might be insufficient to discern changes over time, particularly in the case of patients with mild neuropathy or the limited number of apparently motivated participants with well-controlled diabetes available in the neuropathy group.

In conclusion, the findings presented herein provide evidence that CCM has the potential to track the structural alterations of the small nerve fibres in DPN. Furthermore, these findings support the notion that quantification of the SNP morphology has a substantial potential to be employed as an appropriate adjunct measure to conventional measures of DPN.

CHAPTER 8. SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

In this chapter the main findings and novel contributions of the research study presented throughout this thesis are summarized. Recommendations for further research in this field are also highlighted for possible future studies.

8.1 Summary of the research project

In cross-sectional studies, morphology of corneal subbasal nerve plexus (SNP) has been suggested as a potential marker for diabetic peripheral neuropathy (DPN), which is a debilitating and prevalent complication of diabetes and currently has no effective therapy. This research project sought to examine the longitudinal aspects of the suitability of the SNP structure in the context of DPN.

Application of CCM in this longitudinal study required employment of a fully-automated quantification system of SNP parameters to reduce or eliminate the limitations that are associated with manual and semi-automated techniques. The objective of the first experiment presented in Chapter 3 was to compare a fully-automated technique (ACCMetrics) (Dabbah, et al., 2011) with manual (CCMetrics) (Dabbah, et al., 2009) and semi-automated (NeuronJ) (Meijering, 2010) methods regarding agreement, association and detection capability in a cohort of healthy participants and diabetic individuals without and with DPN using Bland-Altman method (Bland & Altman, 1986) and intraclass correlation coefficients (ICC). An important finding of this study was that the fully-automated technique could compute CNFL values which were in close agreement with manual and semi-automated methods. Furthermore, the three techniques examined in this study were able to diagnose diabetic participants with DPN from controls. Therefore, due to its speed, objectivity, and consistency, the fully-automated algorithm was chosen for analysis of CCM images in this longitudinal project.

While addressing the issues of repeatability - within and between observers - is an essential element of any scientific research, this is of more importance

when longitudinal studies with repeated measurements are conducted in order to track changes over time. The purpose of the second experiment presented in Chapter 4 was to examine the intra- and interobserver repeatability of three main SNP parameters namely – CNFD, CNBD and CNFL using statistical procedures including Bland-Altman method, ICC and coefficient of repeatability. The findings of this study showed that CNFL was the most repeatable and reliable parameters followed by CNFD, whereas CNBD achieved only an acceptable level of repeatability.

Given the cross-sectional reports of the utility of the SNP structure as a potential novel marker of DPN, there was uncertainty as to whether age influences this rich nerve plexus. To appreciate the real age effect and also to examine the time course of SNP morphology in healthy individuals, the experiment presented in Chapter 6 was conducted. An established image sampling protocol (Vagenas, et al., 2012) was implemented and all images were analysed by fully-automated algorithm to quantify CNFL. To assess the relationship between age and CNFL and the time-course of CNFL over three years, two linear mixed models were fitted using SPSS statistical software. Although the SNP morphometric parameters showed a stable course over a 3-year period in healthy individuals, there was a slight linear reduction in CNFL with age (linear decrease of 0.05 mm/mm^2 per one year increase in age). This finding clearly confirmed the age effect on SNP morphology, but not to an extent reported by some cross-sectional studies. Indeed CNFL, which is perhaps the most important structural parameter, only declined by 0.23% per year and it would take 20 years for a clinically insignificant decline of 1 mm/mm^2 to be observed in CNFL. This is an important attribute for corneal nerve structure if it is to be considered as measure of DPN.

In the last experiment, presented in Chapter 7, the longitudinal application of CCM in combination with automated image analysis was moved towards in the context of DPN assessment. The purpose of this study was to assess the natural history of SNP structural parameters in diabetic participants without and with mild neuropathy over four years and to compare their trajectories to non-diabetic/non-neuropathic controls. Additionally, the longitudinal

relationship between established measures of neuropathy with SNP parameters was also studied. While there was no clinically significant change to health and metabolic parameters and neuropathy measures in diabetic participants during study period, there was an evidence of dynamic small fibre damage at the SNP in the neuropathy group which was revealed by a significant linear decline of CNFD (decrease rate of approximately 1 nerve/mm^2 per year). The observed decline was associated with age and duration of diabetes of the participants. The findings also demonstrated that the SNP parameters did change in a fashion comparable with some traditional measures.

The findings of the studies presented in chapters 6-7 demonstrated that there was a difference between age-related and DPN-related changes in corneal nerve morphology. CNFL was the only SNP structural parameter influenced by age while CNFD and CNBD were not affected in healthy individuals. On the other hand, CNFD was the parameter that underwent the most marked decline over time in DPN+ve group. The former implies that age-dependent alterations of the SNP mainly occur at short interconnecting links while the latter indicates that major nerves are the primary target in the process of DPN. Hence, it is plausible to assume that there is a distinct difference between physiological and pathophysiological features of nerve damage associated with normal aging process versus diabetic neuropathy in the SNP. Once again, those findings highlight the importance of including the three key SNP parameters in CCM investigations.

8.2 Contribution to new knowledge

The works embodied in this thesis, which comprised of three publications and two linking chapters add to the current knowledge regarding application of *in vivo* corneal confocal microscopy and the appropriateness of the SNP morphology as potential measure of DPN. The major contributions of this project include:

- confirming the suitability of CCM combined with a fully-automated algorithm in terms of quantification of structural parameters, detection capacity and the repeatability of procedure at operator level;
- establishing the true age effect on corneal nerve morphology and the stability of this nerve plexus in healthy state, which not only confirmed the suitability of corneal nerve morphology as a potential measure for DPN, it has implication in respect to appreciation of the effect of pathology and surgical or treatment modalities on the morphology of the SNP, and
- providing evidence that CCM has the potential to track the structural alterations of the small nerve fibres in DPN, which has a major contribution to support the notion that quantification of the SNP morphology has a substantial potential to be employed as an appropriate adjunct measure to conventional measures of DPN.

8.2 Recommendations for future research

The work presented in this thesis is the first study that has employed CCM in a natural history study of SNP microstructures in relation to DPN. Although this project, in general, provides additional evidence with respect to the suitability and capacity of CCM as a small fibre structural measure of DPN, more research is required to establish this technique as a marker of DPN and to emerge as a clinical tool. This section provides some recommendations on how the outcomes of this study may enhance future research.

Accurate and reliable automated segmentation of the images obtained from CCM is still in the early stages and needs more attention. For example, while the fully-automated image analysis software reported here has been validated for the quantification of SNP parameters, the underestimation of morphometric measures compared to manual method may limit the efficacy of this technique to detect early changes in respect to DPN. Therefore, future efforts should concentrate on eliminating or alleviating such shortcomings.

These longitudinal outcomes that have been reported here are limited to nerve changes in the central cornea and may not be applicable to other more peripheral regions of the human SNP. Recent studies have shown that loss

of corneal neve structure in the SNP mainly occurred at the inferior whorl (Davidson, et al., 2014; Edwards, et al., 2012a) which may enhance the utility of CCM in relation to DPN. This area is located 1 to 2 mm inferior to the corneal apex and is slightly more distal than the central cornea. Therefore this region may be an appropriate region for future longitudinal studies to investigate more marked pathology and early nerve damage or repair.

The low rate of change in established measures of neuropathy over time, as experienced in this study, may be avoided by selecting diabetic patients with varying degrees of neuropathy severity and recruiting them in a fashion which best presents the typical population with diabetes. Additionally, considering the availability of the proper diabetic care, longer study duration is required to discern changes over time.

Nevertheless, it is hoped that the present work will serve as a basis for developing further efforts to employ CCM as a surrogate endpoint for DPN, perhaps to extend the utility of this technique to clinical trials and to find an effective treatment for DPN which is the main factor predisposing diabetic patients to ulceration and subsequently to amputation. Therefore, due to the practicalities of CCM, it is time to move forward and employ this valuable alternative to conventional measures of small nerve fibres in clinical trials of DPN to assess the therapeutic efficacy of new drugs or treatments.

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APPENDICES

Appendix 1: Acknowledgement of Joint Authors and Verification of Permissions

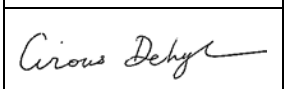
Statement of Contribution of Co-Authors for Thesis by Published Paper

The authors listed below have certified* that:

6. 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;
7. they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
8. there are no other authors of the publication according to these criteria;
9. 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
10. they agree to the use of the publication in the student's thesis and its publication on the QUT ePrints database consistent with any limitations set by publisher requirements.

Publication:

Dehghani C, Pritchard N, Edwards K, Russell AW, Malik RA, Efron N. Fully automated, semiautomated and manual morphometric analysis of corneal subbasal nerve plexus in individuals with and without diabetes. *Cornea* 2014; 33:696-702.

Contributor	**Statement of contribution***
Cirous Dehghani	Wrote the manuscript, experimental design, conducted experiments, data collection and data analysis
	
11/12/2014	
Nicola Pritchard*	LANDMark research design, conducted experiments, data collection and contribution to the manuscript
Katie Edwards*	LANDMark research design, conducted experiments, data collection and contribution to the manuscript
Anthony W Russell*	LANDMark research design, facilitated recruitment of participants, contribution to the manuscript
Rayaz A Malik*	LANDMark research design, contribution to the manuscript
Nathan Efron*	LANDMark research design, supervised the LANDMark project, contribution to the manuscript

Principal Supervisor Confirmation

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

Name: Professor Nathan Efron Signature:  Date: December 11, 2014

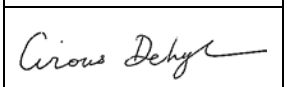
Statement of Contribution of Co-Authors for Thesis by Published Paper

The authors listed below have certified* that:

11. 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;
12. they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
13. there are no other authors of the publication according to these criteria;
14. 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
15. they agree to the use of the publication in the student's thesis and its publication on the QUT ePrints database consistent with any limitations set by publisher requirements.

Publication:

Dehghani C, Pritchard N, Edwards K, Vagenas D, Russell AW, Malik RA, Efron N. Morphometric stability of the corneal subbasal nerve plexus in healthy individuals: a 3-year longitudinal study using corneal confocal microscopy. *Invest Ophthalmol Vis Sci* 2014; 55:3195-3199.

Contributor	**Statement of contribution***
Cirous Dehghani  11/12/2014	Wrote the manuscript, conducted experiments, data collection and data analysis
Nicola Pritchard*	LANDMark research design, conducted experiments, data collection and contribution to the manuscript
Katie Edwards*	LANDMark research design, conducted experiments, data collection and contribution to the manuscript
Dimitrios Vagenas*	Assisted with data analysis
Anthony W Russell*	LANDMark research design, contribution to the manuscript
Rayaz A Malik*	LANDMark research design, contribution to the manuscript
Nathan Efron*	LANDMark research design, supervised the LANDMark project, contribution to the manuscript

Principal Supervisor Confirmation

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

Name: Professor Nathan Efron Signature:  Date: December 11, 2014

Statement of Contribution of Co-Authors for Thesis by Published Paper

The authors listed below have certified* that:

16. 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;
17. they take public responsibility for their part of the publication, except for the responsible author who accepts overall responsibility for the publication;
18. there are no other authors of the publication according to these criteria;
19. 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
20. they agree to the use of the publication in the student's thesis and its publication on the QUT ePrints database consistent with any limitations set by publisher requirements.

Publication:

Dehghani C, Pritchard N, Edwards K, Vagenas D, Russell AW, Malik RA, Efron N. Natural History of corneal nerve morphology in mild neuropathy associated with type 1 Diabetes: development of a potential measure of diabetic peripheral neuropathy. *Invest Ophthalmol Vis Sci* 2014; doi:10.1167/iovs.14-15605

Contributor	**Statement of contribution***
Cirous Dehghani  11/12/2014	Wrote the manuscript, conducted experiments, data collection and data analysis
Nicola Pritchard*	LANDMark research design, conducted experiments, data collection and contribution to the manuscript
Katie Edwards*	LANDMark research design, conducted experiments, data collection and contribution to the manuscript
Dimitrios Vagenas*	Assisted with data analysis
Anthony W Russell*	LANDMark research design, facilitated recruitment of participants, contribution to the manuscript
Rayaz A Malik*	LANDMark research design, contribution to the manuscript
Nathan Efron*	LANDMark research design, supervised the LANDMark project, contribution to the manuscript

Principal Supervisor Confirmation

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

Name: Professor Nathan Efron Signature:  Date: December 11, 2014

Appendix 2: Human Ethics Approval Certificate

University Human Research Ethics Committee

HUMAN ETHICS APPROVAL CERTIFICATE

NHMRC Registered Committee Number EC00171

Date of Issue: 3/5/12 (supersedes all previously issued certificates)

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

University Human Research Ethics Committee

HUMAN ETHICS APPROVAL CERTIFICATE

NHMRC Registered Committee Number EC00171

Date of Issue: 3/5/12 (supersedes all previously issued certificates)

Other Staff/Students:

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

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;

University Human Research Ethics Committee

HUMAN ETHICS APPROVAL CERTIFICATE

NHMRC Registered Committee Number EC00171

Date of Issue: 3/5/12 (supersedes all previously issued certificates)

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.

End of Document

Appendix 3: Participant Information and Consent Form

Princess Alexandra Hospital
Health Service District

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[1]

Associates: Prof Andrew Boulton[2], Prof Rayaz Malik[2], Prof John Prins[3], Dr Anthony Russell[3], Nicola Pritchard[1] , Katie Edwards[1] , AProf Andrew Cotterill[4]

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 hearts 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 Moavenshahididi and Nicola Pritchard to obtain Doctor or 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) sensations of pain/touch, 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 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 (50µl) to examine the proteins; this involves holding a tiny glass tube near the eye for a few seconds.

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. This test will be performed at PAH.

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 and PAH, Woolloongabba.

We expect the visit will be approximately 2 to 3 hours at IBHI at QUT, Kelvin Grove and another 1 hour at PAH 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/PAH (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 20-25ml, or 2-3 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 decardoxylase (GADAb) and antibodies to islet cells (ICAb) 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. These results will only be used for research purposes, and will be stored separately from the main body of study data to protect your (or their) privacy/confidentiality and anonymity, and 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 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) will be told about this new information and the researcher will discuss whether this new information affects you (or them).

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.

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 PAH and 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. Data is stored during the project in a re-identifiable format i.e. coded. Your (or their) name and contact details will be held separately to the study data to protect your (or their) privacy and anonymity. 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.

At completion of the project your (or their) data will be decoded, such that it will not be possible to determine which data belong to which participant. Data for this project will be kept for 15 years or 5 years after the last publication. Paper files will be shredded and electronic files will be carefully removed from their storage location (not just deleted).

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	Nicola Pritchard	Prof.Nathan Efron
Ph: 07 3138 6154	Ph: 07 3138 6414	Ph: 07 3138 6401
Email: katie.edwards@qut.edu.au	E-mail: n.pritchard@qut.edu.au	E-mail: 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	QUT Research Ethics Officer
Princess Alexandra Hospital Human Research Ethics Committee	Queensland University of Technology Human Research Ethics Committee
Ph: (07) 3240 5856	Ph: (07) 3138 2340
Email: PAH_Ethics_Research@health.qld.gov.au	E-mail: 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.