

# **CORNEAL NERVE PATHOLOGY IN DIABETES**

A thesis submitted to the University of Manchester for the degree of  
Doctor of Philosophy in Medicine  
in the Faculty of Medical and Human Sciences

**2013**

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## **ABSTRACT**

The accurate detection and quantification of human diabetic somatic polyneuropathy (DSPN) are important to define at risk patients, anticipate deterioration, and assess new therapies. Current methods lack sensitivity, require expert assessment and have major shortcomings when employed to define therapeutic efficacy. In recent years, in vivo corneal confocal microscopy (IVCCM) has shown potential as a surrogate endpoint for DSPN.

This study aims to investigate fundamental aspects of IVCCM such as repeatability and optimal scanning methodology and establish changes in corneal nerve morphology in relation to the severity of DSPN and regeneration in response to normalisation of hyperglycaemia. Furthermore, it aims to provide a novel fully automated image analysis algorithm for the quantification of corneal nerve morphology and establish the diagnostic ability of CCM.

IVCCM shows high repeatability which is enhanced with more experienced observers. Central corneal innervation is comparable to adjacent peripheral innervation in mild diabetic neuropathy but the central cornea may be more sensitive to change. Corneal nerve loss is symmetrical and progressive with increasing neuropathic severity and corneal nerves show significant regenerative capacity following rapid normalisation of glycaemic control after simultaneous pancreas and kidney transplantation. The novel image analysis algorithm strongly correlates with human expert annotation and therefore represents a rapid, objective and repeatable means of assessing corneal nerve morphology. Automated image quantification may replace human manual assessment with high diagnostic validity for DSPN.



## **DECLARATION**

No portion of the work referred to in the thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning.

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## CONTRIBUTION

This section is to confirm that Ioannis Nikolaos Petropoulos, the author of this thesis, was actively involved and had a significant contribution in all chapters/studies presented and discussed in this thesis. Briefly, he consented and recruited a portion of the subjects, performed in vivo corneal confocal microscopy and relevant ophthalmic examinations, occasionally peripheral neuropathy assessment and performed all image analysis and quantification. In addition, he was responsible for collaborating with the department of Imaging Science and Biomedical Engineering and testing the novel fully automated image analysis algorithm presented herein. Finally, he performed the majority of statistical analysis, either on his own with knowledge gained through relevant modules during his MSc studies or in consultation with experts in medical statistics (Dr. Philip Morgan) and research team members with a qualification in clinical epidemiology (Dr. Uazman Alam). The following tasks were performed by other members of the research team:

- Electrodiagnostic studies by Dr Andrew Marshall, consultant neurophysiologist
- Ophthalmic examination were also performed by Dr. Mitra Tavakoli
- Peripheral neuropathy assessments and skin biopsies by Drs Uazman Alam, Omar Asghar and Hassan Fadavi
- Skin biopsy processing and analysis for the transplant study was by Drs Maria Jeziorska, Simon Forman and Luisa Nelson
- Patient recruitment and co-ordination by Georgios Ponirakis
- Software engineering by Drs Mohammad A Dabbah, Xin Chen and James Graham

- Blood and Urine sample collections and anthropometric measurements by the nursing staff in the Wellcome Trust Clinical Research Facility
- Haematology, immunology and clinical biochemistry analysis was performed and reported by the relevant departments under the directorate of Laboratory Medicine, Central Manchester University Hospitals, NHS foundation trust, UK

## **ALTERNATIVE THESIS FORMAT**

The author has been granted permission to submit this Ph.D. thesis in an alternative format by his supervisor Professor Rayaz A. Malik approved under the University of Manchester, Faculty of Medical and Human Sciences regulations, including sections which are in a format suitable for submission for publication or dissemination. The following chapters in this thesis have been published or will be submitted for publication:

- Chapter 3: Published in the journal *Cornea*, 2012
- Chapter 4: To be submitted for publication.
- Chapter 5: Published in the journal *Medical Image Analysis*, 2011
- Chapter 6: To be submitted for publication
- Chapter 7: To be submitted for publication
- Chapter 8: Published in the journal *Diabetes*, 2012

## **LIST OF ABBREVIATIONS**

AUC: area under the curve

BMI: body mass index

BP sys/dia: blood pressure systolic/diastolic

CI: confidence interval

CIDP: chronic inflammatory demyelinating polyneuropathy

CNBD: corneal nerve branch density

CNFD: corneal nerve fibre density

CNFL: corneal nerve fibre length

CoR: coefficient of repeatability

CP: cold induced pain

CS: corneal sensitivity

CT: cold threshold

CVD: cardiovascular disease

DAN: diabetic autonomic neuropathy

DCCT: diabetes control and complications trial

DM: diabetes mellitus

DPN: diabetic peripheral neuropathy

DSPN: diabetic somatic polyneuropathy

EDCS: epidemiology of diabetes complications study

HDL: high density lipoprotein

HIP: hot induced pain

HRT III RCM: Heidelberg Retinal Tomograph III Rostock Corneal Module

ICC: intra-class correlation coefficient

IENF: intra-epidermal nerve fibre

IENFD: intra-epidermal nerve fibre density  
IVCCM: in vivo corneal confocal microscopy  
LDL: low density lipoprotein  
LE: left eye  
LOA: limits of agreement  
LR: likelihood ratio  
LSCM: laser scanning confocal microscopy  
MMP: matrix metalloproteinase  
NB: nerve branch  
NBD: nerve branch density  
NCCA: non-contact corneal aesthiometer  
NCS: nerve conduction studies  
NDS: neuropathy disability score  
NF: nerve fibre  
NFD: nerve fibre density  
NF-kB: nuclear factor kappa B  
NFL: nerve fibre length  
NNT: neural network classifiers  
NO: nitric oxide  
NSP: neuropathy symptom profile  
NT: neurotrophins  
NCV: nerve conduction velocity  
OR: odds ratio  
PKC: protein kinase C  
PMNamp: peroneal motor nerve amplitude

QST: quantitative sensory testing

RAGE: receptor for advanced glycation end products

RE: right eye

RF: random forest

SAE: serious adverse event

SAR: serious adverse reaction

SD: standard deviation

SNF: small nerve fibre

SPK: simultaneous pancreas and kidney transplantation

SSCM: slit scanning confocal microscope

SSNamp: sural sensory nerve amplitude

TC: tortuosity coefficient

TSCM: tandem scanning confocal microscope

UKPDS: United Kingdom prospective diabetes study

VPT: vibration perception threshold

WT: warm threshold



## **DEDICATION**

“Ο αληθινός πλούτος είναι η γνώση”

This Thesis is dedicated  
to my beloved Parents and Grandparents, my first teachers and  
Anastasia.

## **ACKNOWLEDGEMENTS**

The completion of this project comes with happiness and much relief. However, this would not be possible without the support of many individuals.

First and above all I would like to thank Professor Rayaz A Malik for being a great mentor and the best supervisor somebody could wish to have. His passion for research and clinical excellence and his multipurpose support during this project have been a great source of motivation and inspiration.

The contribution of my co-supervisor Dr Mitra Tavakoli and Drs Uazman Alam, Omar Asghar, Hassan Fadavi, Andrew Marshall, Mohammad A Dabbah, Xin Chen, James Graham and Georgios Ponirakis to the series of studies presented here has been tremendous. Being part of this team did not only lay the groundwork for this thesis but also provided me with clinical experience to help me develop my research skills. I would also like to thank Dr Philip Morgan for his expert comments and advice as well as my course advisor Professor Andrew J M Boulton.

Both my family and close friends were very important in bringing all these together. Therefore I want to thank my parents, grandparents, my brother Christos, Anastasia Diangelaki, Georgios Athanasopoulos, Dimitrios Palaiologos, Spyros Sotiropoulos and Andreas Plakotakis.

Last but in no way least I would like to acknowledge the significant contribution of all patients who participated in this research and the generous support of the nursing staff at the Wellcome Trust Clinical Research Facility. Finally, I want to thank Olga, Georgios, Pantelis, Patrick, Maryam, Jasleen and Neophytos who in one way or another had an impact at various stages of my studies.

## **PREFACE**

Ioannis Nikolaos Petropoulos graduated with a BSc (hons) in Optometry from the Technological Educational Institution of Athens, School of Health Sciences and Disease Prevention, Greece in 2007 and practised as a pre-qualified optometrist between September 2006 and August 2007. Upon completion of undergraduate studies he studied for an MSc in Investigative Ophthalmology and Vision Sciences at the University of Manchester, UK from 2007 to 2008 where he undertook his first research project related to visual psychophysics and post-chiasma image processing and introduced to research methods and investigative techniques. In January 2009 he joined the cardiovascular research group at the School of Biomedicine, University of Manchester, UK as a clinical research fellow and commenced his PhD in “corneal nerve pathology in diabetes” under the supervision of Professor Rayaz A Malik and Dr. Mitra Tavakoli.

During his PhD he presented part of this work in regional, national and international conferences between 2009 and 2012, published and co-authored several scientific papers, presented at the President’s showcase in the American Diabetes Association 2011, won a travel award from Diabetes UK in 2011 and was nominated for the Nick Hales young investigator award in Diabetes UK 2013 for his research entitled “Corneal Confocal Microscopy Detects Neuropathy in Impaired Glucose Tolerance and Tracks Progression at Follow-up”. Towards the late stages of his PhD he also completed as a clinical investigator a pilot collaborative study with Eurolens Research, University of Manchester entitled “Analysis of the lid margin area of the upper eyelid of contact lens wearers with corneal confocal microscopy”.

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## **1. CHAPTER I-INTRODUCTION**



## **1.1 Definition, classification and aetiology of diabetes**

Diabetes mellitus (DM) is a heterogeneous group of metabolic disorders characterised by raised plasma glucose concentrations due to absolute (type 1 DM) or relative (type 2 DM) lack of insulin secretion, action or both; resulting in disturbances of fat, carbohydrate and protein metabolism. Diabetes is primarily categorised into two types with several subclasses, which differ by aetiology, onset, genetic predisposition, prevalence and degree of insulin insufficiency (1).

The nature of DM is insidious: certain genetic polymorphisms in the human leukocyte antigen class II (2) are associated with varying levels of susceptibility for type 1 DM in addition to environmental (3-4), seasonal (5-6) and nutritional factors (7-9). In contrast, type 2 DM is characterised by insulin resistance (10) which is strongly linked to unhealthy lifestyles (11) and the 'metabolic syndrome' (12-15) i.e. the clustering of factors such as central obesity, hypertension, lipid derangements and reduced physical activity. Type 2 DM is not a single entity but the consequence of a series of metabolic disturbances characterised by elevated fasting glucose and / or impaired glucose tolerance, which are independent predictors for the development of future disease (12; 16) and adverse outcomes (17-18).

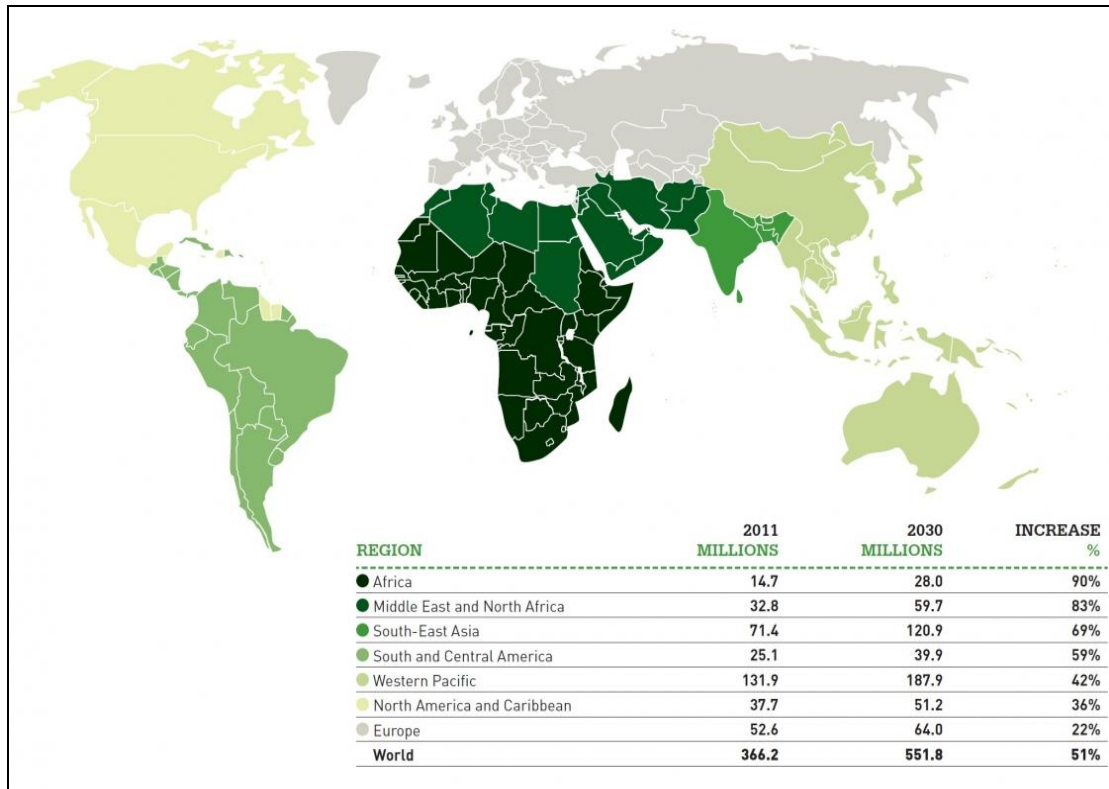
## **1.2 Epidemiology and costs of diabetes**

Diabetes is one of the most common chronic diseases with vast socioeconomic consequences, personally for those with the disease and globally on healthcare systems (19-21) (Figure 1-1). It is estimated that the

economic burden of diabetes is \$100 billion per annum in the United States alone and in the first years following diagnosis the average costs per patient are \$2,250 per year (21) which may increase substantially with co-existing cardiovascular disease (CVD) and modest metabolic control (22-24). Type 2 DM is the most common form and accounts for approximately 90% of the cases with diabetes worldwide (25). The global prevalence of diabetes has shown a rapid increase in the last decade from approximately 171 (26) million people in 2000 to 366 million in 2011 (27) and based on current incidence trends is projected to reach 552 million by 2030 dominated by large population countries such as Brazil, China and India (27). The San Antonio Heart Study has previously reported an up to three-fold increase in the incidence of type 2 DM over an 11 year period (28). From a public health point of view, population growth, ageing, urbanisation, low-middle income and increasing prevalence of obesity and physical inactivity have contributed significantly in the incidence and prevalence of the disease (26).

Type 1 DM is less frequent and varies by ethnic origin and latitude (29). Previous epidemiological research has showed a continuous increase in the annualised rates of type 1 DM (30-32). The EURODIAB (32) study reported a 2-5 % increase in 10-year incidence rates and young age, seasonality and geographic location as risk factors for disease onset. A more recent assessment of the 15-year incidence rates of childhood diabetes in Europe found an overall increase of 3.9% in incidence with the lowest rates in late childhood with females less affected than males. The DIAMOND study (30) found a 350-fold variation amongst 100 populations worldwide with the lowest

incidence in China and the highest in Sardinia and Finland (> 36.0 new cases per 100,000 per year).



**Figure 1-1** Adapted from the International Diabetes Federation (IDF) (25) and the IDF atlas 2011 (27).

### 1.3 Complications of Diabetes

The micro- and macro-vascular complications of diabetes are diverse in prevalence, severity, progression rates but have many common pathophysiological processes. Generally, the pathogenetic mechanisms leading to the development of complications may be classified into three broad categories: glycaemia-related such as abnormalities in the polyol pathway (33), vascular mechanisms including endothelial injury and damage in supporting

cells (34), abnormalities in platelet function (35), growth factors (36-37) and genetic predisposition (38).

### **1.3.1 Microvascular Complications**

Retinopathy, nephropathy and neuropathy complicate almost all patients with diabetes at some stage and may cause blindness, end-stage renal disease or lead to foot ulceration and amputation (39). Numerous studies have established an association between microangiopathy and glycaemia (40-45). The United Kingdom Prospective Diabetes Study (UKPDS) reported that every 1% reduction in mean haemoglobin A<sub>1c</sub> was associated with a substantial risk reduction for fatal or non fatal cardiovascular disease (CVD), amputation or cataract extraction (40). The Diabetes Control and Complications Trial (DCCT) (44) concluded that intensive glucose lowering intervention in type 1 DM slowed down the onset of microvascular complications with persistent benefits 14 years after trial completion (46) thus highlighting the role of 'metabolic memory' (47). Although poor glycaemic control is strongly related to adverse outcomes, co-existing conditions such as hypertension (41; 48), hypercholesterolaemia (49) and an unhealthy lifestyle (50-51) are independent risk factors for the manifestation of microvascular disease in DM.

### **1.3.2 Macrovascular Complications of Diabetes**

Macroangiopathy in DM results in ischaemic / coronary heart disease, stroke and amputation and is associated with a high risk of mortality. A Finnish population-based study found a significantly higher 7-year incidence of a major cardiovascular event in patients with DM compared to their non-diabetic peers.

The hazards ratio for death from coronary heart disease was comparable between subjects with DM without previous myocardial infarction and non-DM subjects with previous myocardial infarction suggesting the increased risk in DM and the need for aggressive treatment of cardiovascular risk factors in this group (52). A meta-analysis of 37 studies of type 2 diabetes and fatal myocardial infarction concluded that presence of DM was associated with significantly higher mortality compared to non-diabetic patients and females compared to males (53). Another study of all cause mortality in DM and controls concluded that the presence of DM was associated with a 30-fold increase in the probability of fatal cardiovascular disease (54).

Similarly, DM is the strongest risk factor for a fatal stroke (55-57) and erectile dysfunction (58-59) in the middle-aged population. Several studies have implicated elevated low density lipoprotein (LDL) cholesterol, systolic blood pressure (BP sys), HbA<sub>1c</sub>, fasting plasma glucose concentrations, duration of diabetes, male sex, excess body mass index (BMI) and cigarette smoking as independent risks for CVD (55-57; 60).

#### **1.4 Diabetic Neuropathy**

Somatic, central and autonomic nerve involvement in DM has been documented as early as 1953 by Garland (61) and later by others (62-64). Diabetic peripheral neuropathy (DPN) is a devastating complication of diabetes because of the debilitating symptoms it causes and the high associated risk for other complications particularly affecting the lower extremities. There are six major types of DPN: diabetic somatic polyneuropathy (DSPN), autonomic

neuropathy, nerve entrapment syndromes, proximal asymmetric mononeuropathy, truncal radiculopathy and cranial mononeuropathy (65).

DPNs differ by onset, severity of symptoms and underlying mechanisms (66).

The most common variety is DSPN which is a diffuse neuropathy affecting the unmyelinated (C fibres), thinly myelinated (A $\delta$  fibres) and myelinated sensory and motor (A $\beta$  fibres) nerves in a length-dependent and symmetric fashion (67-68). Epidemiologic studies have showed that DSPN is the cumulative effect of chronic hyperglycaemia and metabolic derangements (40; 69) and is the main initiating factor for foot ulceration and lower extremity amputation (70).

#### **1.4.1 Epidemiology and risk factors for diabetic somatic polyneuropathy**

Several studies have been designed to estimate the rates of prevalence, incidence and risk factors for DSPN. The Rochester Diabetic Neuropathy Study reported a prevalence of 54% and 45% for patients with type 1 and type 2 DM respectively, amongst 380 patients in total, with the majority however not exhibiting neuropathic symptoms (71). The San Luis Valley Study found that DSPN was present in 25.8 % of a community-based, mixed-background cohort of subjects with type 2 DM but was also detectable in subjects with pre-diabetes and in controls thus highlighting the need to exclude other causes of neuropathy (72). Age, duration of diabetes, higher HbA<sub>1c</sub>, lower fasting C-peptide, use of insulin, and the presence of retinopathy and nephropathy, were associated with neuropathy (73). Another community-based study, the Pittsburgh Epidemiology of Diabetes Complications Study (EDCS), estimated

an overall prevalence of 34% in type 1 DM (74) which was additionally related to low high density lipoprotein (HDL) cholesterol, longer standing DM, and overt macroangiopathy (75). A multinational study of type 1 DM, the EURODIAB IDDM Complications Study, assessed 3250 randomly selected subjects and reported 28% prevalence without significant variation across 16 countries. Age, weight, glycaemic control, smoking, severe ketoacidosis, microalbuminuria and retinopathy were positively correlated with DSPN (76). The UKPDS found a much lower prevalence of neuropathy amongst 2337 newly diagnosed type 2 DM patients (77) which was strongly related to glycaemia and systolic blood pressure (40; 78). Finally, the National Health and Nutrition Examination Survey examined 2873 people of whom 419 had DM and reported an overall prevalence of 14.8% in the general population and 28.5% amongst those with DM which was largely asymptomatic and related to age and ethnicity (79). A summary of the studies investigating the prevalence of DSPN and associated risk factors is presented in (Table 1-1).

The DCCT followed-up 1161 subjects with type 1DM who were randomised and assigned to intensive and usual glycaemic control categorised into primary and secondary prevention groups based on the duration of diabetes and the presence of complications. The 5-year cumulative incidence of neuropathic deficits (15-21%) and abnormal electrodiagnostic studies (40-52%) were significantly higher in the usual treatment group suggesting a link between hyperglycaemia and incidence of DSPN (44). The EDCS found that 15% of 453 patients free of DSPN at baseline subsequently developed neuropathy after 5 years. DSPN positively correlated with duration of DM, height, smoking, glycaemia and hypertension (80).

**Table 1-1** Prevalence and risk factors for DSPN

<b>Study (n)</b>	<b>Prevalence (type of DM)</b>	<b>Risk factors</b>
<b>Rochester Diabetic Neuropathy Study (n=359) (71)</b>	54% / 45% (Type 1/ Type 2 DM)	N/A
<b>San Luis Valley Study (n=277) (72-73)</b>	25.8% (Type 2 DM)	Age, male gender, HbA <sub>1c</sub> , duration, fasting C-peptide, exogenous insulin, retinopathy, nephropathy
<b>Pittsburgh Epidemiology of Diabetes Complications Study (n=366) (74-75)</b>	34% (Type 1 DM)	Low HDL, duration, current smoking, any macro-vascular disease
<b>EURODIAB IDDM Complications Study (n=3,250) (76)</b>	28% (Type 1 DM)	Age, weight, HbA <sub>1c</sub> , smoking, severe ketoacidosis, microalbuminuria, retinopathy
<b>United Kingdom Prospective Diabetes Study (n=2,337) (40; 77-78)</b>	5-7% (Type 2 DM)	HbA <sub>1c</sub> , hypertension
<b>National Health and Nutrition Examination Survey (n=419) (79)</b>	28.5% (Type 1 DM/Type 2 DM)	Age, ethnicity



A study looking at the 14-year cumulative benefits of prior intensive insulin treatment in the DCCT and the Epidemiology of Diabetes Interventions and Complications Study found a lower incidence of DSPN (22.0% vs. 28.0%) amongst patients assigned to the aggressive control arm of both studies compared to those who received conventional treatment (46). The UKPDS also concluded that intensive glucose control reduced the risk for microvascular complications, including DSPN by 25.0% overall but increased the incidence of hypoglycaemic episodes over a 10-year period (41). The EURODIAB IDDM complications study assessed the incidence of peripheral neuropathy during a mean 7 year period and found that 23.5% of subjects had developed neuropathy. Risk factors included duration of DM, baseline HbA<sub>1c</sub>, urinary albumin excretion rate, higher levels of total and LDL cholesterol, change in HbA<sub>1c</sub> during follow-up, smoking, BMI and presence of CVD at baseline (67). The San Luis Valley study found an overall incidence of 6.1 per 100 persons-years in a mean follow up period of 4.7 years (81). Insulin treatment, current smoking and a history of MI were positively related to the development of neuropathy. The Wisconsin Epidemiologic Study of Diabetic Retinopathy reported a 7.2%-9.9% (younger-older) cumulative 14-year incidence of lower extremity amputations which was associated with higher HbA<sub>1c</sub> levels, higher pulse pressure and more severe retinopathy (82). A summary of the studies investigating the incidence of DSPN is presented in Table 1-2.

**Table 1-2** Incidence and risk factors for DSPN

<b>Study (n)</b>	<b>Incidence (type of DM / years)</b>	<b>Risk factors</b>
<b>Diabetes Controls and Complications Trial (n=1,161) (44)</b>	15-21% / 40-52% (signs/NCS) (Type 1 DM/5 years)	Poor glycaemic control
<b>Pittsburgh Epidemiology of Diabetes Complications Study (n=453) (80)</b>	15% (Type 1 DM/5 years)	Duration, height, current smoking, HbA <sub>1c</sub> , hypertension
<b>Diabetes Controls and Complications Trial and Epidemiology of Diabetes Interventions and Complications Study (n=1,186) (46)</b>	22% v 28% (Type 1 DM/14years)	Poor glycaemic control
<b>EURODIAB IDDM Complications Study (n=3,250) (76)</b>	23.5% (Type 1 DM/7 years)	Duration, baseline and change in HbA <sub>1c</sub> , total and LDL cholesterol, current smoking, BMI, CVD at baseline, urine albumin excretion rate
<b>San Luis Valley Study (n=231) (81)</b>	6.1 per 100 persons- years (Type 2 DM/5 years)	Exogenous insulin, current smoking, CVD
<b>Wisconsin Epidemiologic Study of Diabetic Retinopathy (n=1,890) (82)</b>	7.2%-9.9% (14 years)	HbA <sub>1c</sub> , hypertension, severity of retinopathy

#### **1.4.2 Epidemiology of diabetic autonomic neuropathy, mononeuropathies and chronic inflammatory demyelinating neuropathy**

Detailed presentation of the prevalence, incidence and risk factors of other diabetic neuropathies is beyond the scope of this literature review however results from important studies will be discussed briefly. The prevalence of diabetic autonomic neuropathy (DAN) has been the subject of fewer epidemiologic studies. The Framingham Heart Study (83) assessed 1919 people (normal / impaired glucose tolerance and DM) from the Framingham Offspring Study and found that plasma glucose concentrations were inversely associated with reduced heart rate variability amongst all groups. The Pittsburgh Epidemiology study of Diabetes Complications Study III (84) evaluated 168 subjects with type 1 DM and found that hypertension, LDL and HDL cholesterol and gender (female) were significant risk factors for DAN.

A Finnish study (85) evaluated the occurrence of DAN in a total of 133 patients with type 1 DM and 144 control subjects by evaluating sympathetic and parasympathetic neuropathy. The frequency of parasympathetic neuropathy (4.9% vs. 2.2%) was greater in DM compared to controls at baseline and increased to 65.0% vs. 28.0% at 10 years follow up. The prevalence of sympathetic neuropathy did not differ at baseline but increased significantly to 24.4% vs. 9.0%. Similarly, combined neuropathy was no different at baseline and increased to 15.2% vs. 4.2% at 10 years follow-up. Glycaemia, higher insulin levels, and gender (female) were related to the presence of parasympathetic neuropathy and sympathetic dysfunction at 5 years was a

predictor of cardiovascular mortality at 10 years. On the contrary, the UKPDS did not find any difference in the incidence of DAN after 12 years follow-up between the intensive and conventional treatment groups, using heart rate response to deep breathing as an endpoint (41). The Steno-2 study, an open parallel trial of type 2 DM patients with microalbuminuria assigned to conventional or intensive treatment of modifiable risk factors for CVD estimated the prevalence and progression of DAN amongst other endpoints for a mean 7.8 years. DAN was present in 27% of the patients with microalbuminuria at baseline and increased to 50% in the conventional and 30% in the intensive treatment group at follow-up (86). Peripheral neuropathy was also measured as a secondary endpoint of microvascular disease and progression was comparable between the two groups (37 vs. 40 persons respectively) regardless of treatment assignment.

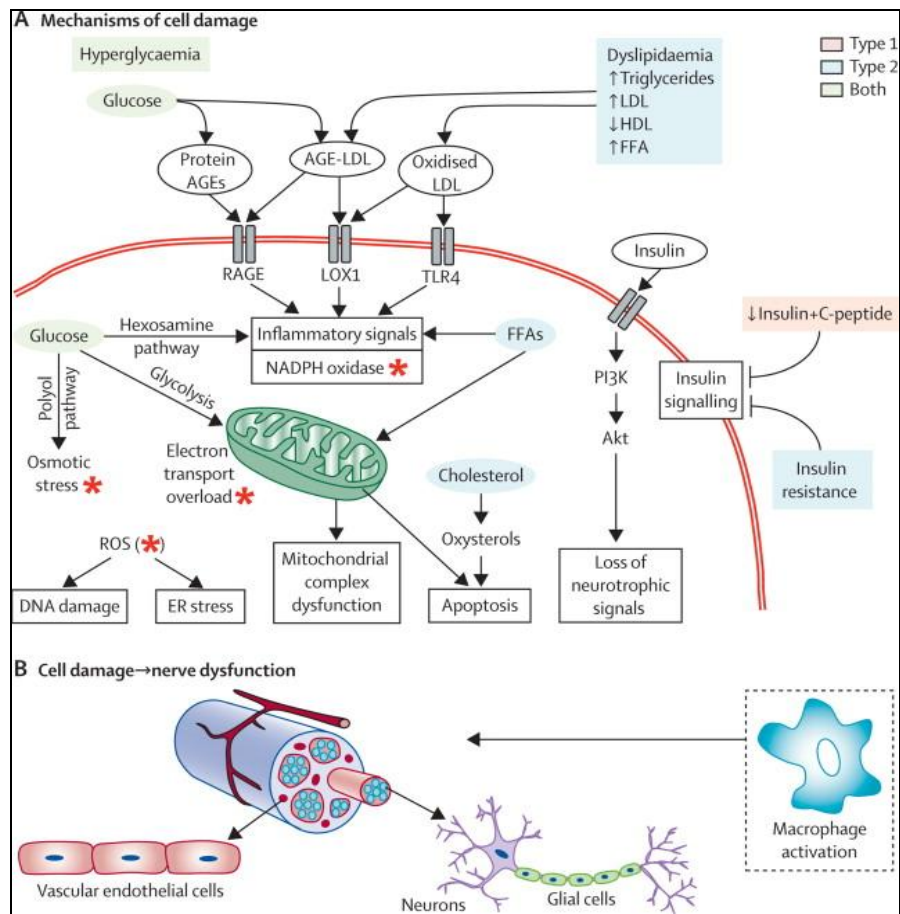
Prospective population-based studies of diabetic amyotrophy, various mononeuropathies and chronic inflammatory demyelinating polyneuropathy (CIDP) have been limited. A Rochester-based survey (71) estimated the prevalence of asymptomatic carpal-tunnel syndrome at 22% for type 1 DM and 29% for type 2 DM, however there were significantly fewer symptomatic cases (11% and 6% respectively). In the same study, ulnar and femoral cutaneous entrapment and cranial neuropathies affected about 1-2% of the cohort. Another hospital-based study (87) assessed the prevalence of various types of neuropathy in 800 type 1 and type 2 DM patients by interview and found a varying prevalence of pain (13%), loss of sensation (7%), neuropathic ulcers (2%), diabetic amyotrophy (0.8%), oculomotor palsy (0.1%) and truncal

neuropathy (0.1%). Age and duration of diabetes were identified as risk factors.

Based on the criteria previously defined by the American Academy of Neurology, the prevalence of probable and definite CIDP has been estimated to 1/100,000 persons in the Southeast England region (88) while another hospital-based, cross sectional study amongst 1125 subjects attending an electrophysiology clinic over a 14 month period found that 16.8% of the cases had DM and the prevalence of CIDP was 16.9% in diabetes and 1.8% in non-diabetic subjects (89). However the reported prevalence is highly variable and has not been extensively tested whether CIPD occurs more frequently in patients with DM or is a distinct entity. A recent study reviewed the medical records of 1581 patients in Olmsted County, Minnesota and identified 23 cases with diagnosed CIPD and median disease duration of 10 months. The prevalence was estimated at 8.9 / 100,000 persons and incidence at 1.6 / 100,000 persons-years. From 23 cases in total only 1 had overt diabetes and the authors concluded that DM is unlikely to be a significant risk factor for CIPD and misclassification of other forms of neuropathies in previous epidemiological studies is possible (90).

### **1.4.3 Pathogenesis of diabetic peripheral neuropathy**

DPN is a multi-factorial disease and mechanisms resulting in peripheral nerve injury may be divided into three broad categories: hyperglycaemia mediated effects (increased polyol pathway flux, oxidative stress, and glycation), vascular factors and deficiency of vascular and neuronal growth factors (Figure 1-2).



**Figure 1-2** Mechanisms of cell damage (A) and nerve dysfunction (B) in diabetic neuropathy in type 1 and type 2 DM. Schematic adapted from: Callaghan et al. (91).

### 1.4.3.1 Hyperglycaemia

Unequivocally, hyperglycaemia and microvascular disease play important roles in the pathogenesis and progression of the disease. As already discussed in section 1.4.1 several prospective trials, including the DCCT (44) and the UKPDS (41), have demonstrated a lower incidence of peripheral neuropathy with intensive glycaemic control. However, whilst a recent Cochrane review on the benefits of improved glycaemic control on neuropathy has shown a benefit in Type 1 diabetes it has failed to show a benefit in Type 2 diabetes although

even episodes of minimal hyperglycaemia have been related to DSPN (92). Thus, recent studies amongst persons with 'idiopathic small fibre neuropathy' and electrodiagnostic evidence of axonal damage have shown a high prevalence of impaired glucose tolerance (17). A study of 4 sural nerve biopsies investigating the mechanistic basis of neuropathy in impaired glucose tolerance, has shown the presence of the receptor for advanced glycation end products (RAGE) ligand N<sup>ε</sup>- (Carboxymethyl)lysine, the receptor itself and the transcription factor nuclear factor kappa B (NF-κB) in the perineurium, epineurial and endoneurial vessels (93).

The exact mechanism of how hyperglycaemia damages the nerves remains to be fully elucidated. However, accumulation of polyols through the aldose reductase pathway, oxidative stress, non-enzymatic glycation and protein kinase C (PKC) activation along with concurrent downregulation of critical neurotrophic factors have been implicated to play a central role. Each of these mechanisms will be briefly reviewed.

#### **1.4.3.1.1 Aldose reductase pathway**

Excessive flux of polyols, such as sorbitol, through the aldose reductase pathway has been associated with axonal damage in experimental diabetes which can be ameliorated with aldose reductase inhibitors (94-95). Whilst this relationship is consistent in animal models, it is not clear whether polyol hyperactivity plays a major role in human DPN. A study of sural nerve morphometry showed that sorbitol and fructose concentrations were higher and more variable in diabetic nerves compared to controls and that fructose, sorbitol and myoinositol concentrations were not related to clinical,

neurophysiological or pathological severity of neuropathy (96). Another study, found an inverse association between sorbitol concentration and myelinated nerve fibre density (97). It also appears that patients with diabetes and a higher set point of aldose reductase activity are more prone to complications (98) which may be further modulated by the activity of sorbitol dehydrogenase (99).

#### **1.4.3.1.2 Oxidative Stress and non-enzymatic glycation**

Increased free radical production and impaired free radical defences exert toxic effects on peripheral nerves and reduce NO and blood flow resulting in hypoxia and eventual ischaemic damage (100).  $\alpha$ -lipoic acid, is a powerful antioxidant scavenger of hydroxyl radicals, superoxide, and peroxy radicals and regenerates glutathione (101) and has been shown to improve neuropathic symptoms and signs (102). Non-enzymatic glycation of proteins has been demonstrated in diabetes and is associated with abnormal blood flow (103) and demyelination (95) through a range of cellular and sub-cellular alterations (104). Briefly, it may affect the function of matrix metalloproteinases (MMP), their tissue inhibitors -1 and -2, transforming growth factor- $\beta$  (105), epidermal growth factor autophosphorylation, activation of extra-cellular signal regulated kinases (106) and RAGE and its ligands (NF- $\kappa$ B and interleukin-6) (107).

#### **1.4.3.2 Vascular and growth factors**

Vascular disease has been implicated in the pathogenesis of diabetic neuropathy. Microthrombosis and microvessel occlusion in peripheral nerves,



endothelial duplication, smooth muscle proliferation, endoneurial capillary closure, basement membrane thickening and pericyte degeneration are amongst changes described previously (108-110). Malik et al. (111) have shown that microvascular changes develop early in patients with evidence of minimal neuropathy. In 1172 patients with type 1 DM from the EURODIAB prospective complications study, the incidence of neuropathy was related to modifiable CVD risk factors including a raised plasma triglyceride level, BMI, smoking and hypertension (67). Mechanisms of platelet dysfunction and microvessel endothelial injury have also been implicated in neuropathy and vascular disease (35) and thus a 12 week open label dose escalation study with an angiotensin converting enzyme inhibitor lisinopril, showed an improvement in electrophysiology and quantitative sensory testing (QST) (112). This was confirmed in a placebo controlled study with the ACEi Trandolapril (113). The appropriate blood pressure control in diabetes study showed no benefit (114), but a recent larger study (DEMAND) of Type 2 diabetic patients with hypertension has shown a significant benefit for reduced development and regression of neuropathy after treatment with an ACE inhibitor and a third generation dihydropyridine calcium channel blocker (115). The role of protein kinase C (PKC) has also been investigated in diabetic neuropathy. Specifically, 1, 2-diacylglycerol induced activation of PKC has been documented in experimental models of diabetes and inhibition of PKC- $\beta$  in diabetic rats has been shown to improve nerve blood flow (116). However, a Phase II, double-blind, placebo controlled, parallel-group trial of the PKC- $\beta$  inhibitor ruboxistaurin did not show a benefit for the primary end point of electrophysiology (117). As discussed previously, lipid derangements have

also been associated with the presence of DPN. Lipid lowering medications, such as statins and fibrates, may benefit patients with diabetes as they have been shown to prevent NF- $\kappa$ B induced protein-1 activation and upregulation of vascular endothelial growth factor (VEGF) mRNA (118).

A number of growth factors have been proposed in the pathogenesis of DPN. High plasma insulin like growth factor-1 levels prevent apoptotic cell death and neuroaxonal dystrophy in diabetic rats (119) but trials have failed to show a difference in humans (120). C-peptide has known effects on Na(+)/K(+)-ATPase activity, expression of neurotrophins, regulation of molecular species and DNA binding of transcription factors leading to apoptosis. Therefore C-peptide deficiency has emerged as a pathogenetic mechanism (121) with a recent study showing significant improvements in diabetic patients over a 6 month period of daily C-peptide administration (122). VEGF, originally described as an angiogenesis promoter in diabetic retinopathy, appears to directly stimulate growth, survival and axonal outgrowth of neurones and glial cells (123) and has thus been targeted as a potential therapy for neuropathy (124). Finally, the neurotrophin (NT) family (NT-4, nerve growth factor and brain derived neuronal growth factor) exerts direct effects on the nerves through morphological differentiation, enhancement of nerve regeneration and neurotransmitter stimulation in animals (125) but this has not been translated into humans(126).

#### **1.4.4 Diabetic peripheral neuropathy as a risk factor for foot ulceration**

Loss of sensory function and impaired sweating with loss of vasoregulation in diabetes plays a key role in the pathogenesis of foot ulceration and amputation (127) and several studies have demonstrated a link between DSPN and diabetic foot disease. A hospital based study of 42 patients with DM and 322 controls assessed peripheral neuropathy and vasculopathy with multiple endpoints and found that absence of Achilles tendon reflexes, loss of sensation and reduced transcutaneous oxygen pressure were significant factors for foot ulceration (128). Another cross-sectional study of 225 age-matched subjects with DM with and without prior foot ulceration reported reduced vibration perception, elevated plantar pressure, subjective symptoms of neuropathy, foot or toe deformity and poor diabetes control as risk factors (129). Prospective studies of risk factors for foot ulceration in diabetes have identified sensory loss assessed with a 10g monofilament, impaired vibration perception, a high neuropathy disability score (> 5 out of 10), plantar foot pressure, Charcot deformities, history of amputation and / or ulceration, insulin use, lower dorsal foot transcutaneous PO<sub>2</sub> and a 13 mmHg orthostatic BP fall as risk factors for foot ulceration.

#### **1.4.5 Clinical assessment of diabetic somatic polyneuropathy**

Several composite scores, symptom screening questionnaires, clinical examination techniques and simplified or modified versions of them have been used in research studies over the years. There are also a number of emerging

techniques which have shown potential as surrogate endpoints of DSPN and have been employed by research centres worldwide in longitudinal studies. The purpose of this section is to present an overview of current gold standard techniques and discuss the supporting evidence and limitations of emerging techniques focusing primarily on in-vivo corneal confocal microscopy (IVCCM).

#### **1.4.5.1 Symptom screening questionnaires**

Symptomatic DSPN may affect as many as 30-40% of patients with neuropathy at some stage. Patients commonly report sensory symptoms, such as pain in the distal extremities which may be experienced in the form of deep aching, burning, sharp or shooting, 'needle-like' pain as a result of nerve hyperactivity. Neuropathic symptoms and in particular pain, are difficult to accurately describe and purpose-designed questionnaires have been employed in clinical practice to assist patients to describe symptom quality and intensity and also to enable the clinician to appreciate the severity of neurologic deficits (65).

The Michigan Neuropathy Screening Instrument is a brief 15-item, sensory symptom questionnaire which in combination with physical examination (Michigan Diabetic Neuropathy Score) for signs of neuropathy provides good validity for the diagnosis of DSPN (130). Another screening questionnaire, the neuropathy symptom score (NSS), has also been developed and used to specifically assess diabetic neuropathy in a UK hospital-based study of 6,487 patients with DM (131). The Diabetic Neuropathy Symptom (DNS) score is an easy to perform, 4-items questionnaire and has previously shown good reproducibility and high predictive value when screening for DSPN and

strongly correlates with the NSS (132). The neuropathy symptom profile (NSP), is a less specific questionnaire to assess symptoms of DPN, weakness, autonomic and sensory neuropathy which is useful in diagnosing and staging severity of neuropathy (133). The use of visual analogue and verbal descriptive scales has been proposed as a more sensitive approach to grade pain with several advantages (134). The short-form McGill pain questionnaire, a combination of a 'present pain intensity' index and a visual analogue scale, has been validated across a variety of hospital-based patients and has shown sufficient sensitivity to statistically detect differences due to treatment (135) with high reliability which drops with ageing (136). Several other scores have been used to evaluate neuropathic symptoms: the clinical global impression-severity of illness and clinical global impression-improvement (137), NeuroQoL (138), the total symptoms score (139) and others.

#### **1.4.5.2 Quantitative sensory testing**

QST is used to identify a disturbance in sensory function by evaluating vibration perception and temperature sensation and induced pain mediated by unmyelinated (C-fibres) and thinly myelinated (A $\delta$ -fibres) nerves. It may be defined as a psychophysical procedure where the physical components are the stimuli and the psychological components the appreciation of the stimulus by the examinee (140). The advantages and limitations of QST are well established: it requires little expertise to perform and has reasonable sensitivity, specificity and reproducibility to diagnose and classify neuropathy at various anatomical sites (141). On the other hand, patient motivation, attention and expectation bias are common cofounders of the technique (142).

Moreover, factors such as room temperature, inter-stimulus intervals, gender, age and lifestyle may also affect the outcomes (143). The method of limits is commonly used in clinical practice to estimate stimulus perception and pain thresholds and studies have concluded that standardized procedures and appropriate testing environment are “safeguards” to maintain high reliability (144).

#### **1.4.5.2.1 Vibration perception threshold**

Vibration perception threshold (VPT) assesses large myelinated ( $A\beta$ ) nerve fibres, sensitivity of mechanoreceptors and transmission through the dorsal column spinal pathways (141). The typical vibratory stimulus used to quantify perception has a sinusoidal waveform of varying frequency and displacement (145). Earlier studies have shown that VPT is a reproducible and suitable measurement of diabetic neuropathy (146) and that VPT alone is a useful test to identify patients at risk for foot ulceration (147). Abbott et al. (148) has recently demonstrated a strong link between elevated VPT and increased odds for a first neuropathic foot ulcer.

#### **1.4.5.2.2 Thermal thresholds**

Temperature sensation is conducted via thinly myelinated ( $A\delta$ ) and small unmyelinated (C) nerve fibres and is transmitted in the crossed anterolateral spinothalamic tracts of the spinal cord (141). Separate warm and cold cutaneous thermoreceptors are known to exist (149). Thermal testing consists of controlled warm and cool stimuli presented in ascending and descending order respectively, delivered through a thermode. Thermal perception testing

quantifies warm and cool thresholds, hot and cold induced pain and temporal summation (145). Similar to VPT, alterations in thermal thresholds are reported in patients with DPN (146) and thermal perception testing is valuable to identify subclinical neuropathy (141), track progression and predict those at risk for foot ulceration (150). One study showed that temperature sensitivity is selectively affected when compared with VPT, suggesting that small nerve fibres (SNF) may be more vulnerable and the first to be involved in symptomatic DPN (151).

#### **1.4.5.3 Composite scores: the neuropathy disability score**

Validated composite scores, such as the neuropathy disability score (NDS) designed by Dyck and colleagues and later the neuropathy impairment score (152), have been employed in clinical trials of neuropathy. The NDS allows the assessment of an alteration in function of several classes of nerve fibres all of which can be affected by diabetes and is composed of four different testing categories: 1) pain sensation, 2) temperature (hot/cold) sensation, 3) vibration perception, and 4) absence/presence of the Achilles tendon reflex. Each category can be classified as abnormal or normal for each foot and the total NDS value can range from 0-10 (131). Abbott et al. (150) showed that NDS is a reliable screening tool for signs of neuropathy and an NDS>6 is an independent risk factor for diabetic foot ulceration which has also been confirmed by another study (153). Based on the findings from the Rochester Diabetic Neuropathy Study, a treatment effect of two points in the NIS in a clinical trial is considered as a clinically meaningful change (152).

#### **1.4.5.4 Nerve conduction studies**

Nerve conduction studies (NCS) of the peripheral nerves are non-invasive, reliable, objective and sensitive techniques and they have been the method of choice to diagnose DSPN and assess therapeutic benefit in clinical intervention trials (154). Electrophysiological tests measure conduction velocity of large sensory and motor nerves, the amplitude of the propagating neural signal, the density and synchrony of muscles activated by maximal nerve simulation and the integrity of neuromuscular transmission (155). There are several factors contributing to nerve conduction velocity (NCV) slowing: 1) stage of nerve demyelination, 2) mean diameter of the conducting axons, 3) internodal distance in the segment under study and 4) the nodal microenvironment (141).

A limitation is that maximal NCV only reflects a limited aspect of neuronal activity of a small subset of large diameter and heavily myelinated axons and is insensitive to early functional alterations in the small diameter sensory nerves (156). Nevertheless, the San Antonio consensus on diabetic neuropathy (157) and the Toronto Expert Neuropathy Committee (66) recommend the use of NCS along with other validated measures of DPN and SNF dysfunction to assess neuropathic deficits for clinical and epidemiological purposes. Previous studies have calculated the rate of decline in NCV and fibre loss for every 1 $\mu$ V loss in sural nerve amplitude. The DCCT found that sural and peroneal velocities diminished by 0.56 and 0.54 m/s per year respectively for a mean 5 years (158). Another study in patients with type 2 DM reported a 0.39 and 0.3 m/s rate of decline per year in sural and peroneal nerves respectively (159).



Others have showed that a loss of approximately 150 fibres / mm<sup>2</sup> corresponds to 1µV reduction in sural sensory nerve amplitude (SSNamp) while 1 µV reduction in peroneal motor nerve amplitude (PMNamp) corresponds to a loss of 200 fibres / mm<sup>2</sup> (160).

#### **1.4.5.5 Nerve and skin punch biopsy**

Morphologic evaluation of the myelinated or small unmyelinated nerve fibres requires whole nerve, fascicle or skin biopsy. Sural nerve biopsy has been commonly used in DPN (161-163) but it is associated with post-operative complaints which include pain in the biopsied area, allodynia and sensory disturbances (164). A number of parameters have been used as pathological endpoints in clinical trials including myelinated fibre density, regenerative cluster density, axonal atrophy and axo-glial dysjunction (165). As early as 1977, Behse et al. (161) reported electrophysiological abnormalities closely related to myelin alterations in sural nerve biopsies of patients with diabetic neuropathy. In another study vibration perception correlated with the total number of myelinated nerve fibres and thermal thresholds with median unmyelinated axon diameter but not with total unmyelinated nerve fibre number (162).

A 3 mm skin punch biopsy is a less invasive measurement of pathology in DPN as it enables the direct study of SNFs innervating the epidermis, autonomic fibres innervating sweat glands, blood vessels and arrector pilorum muscles. Its importance and role in clinical trials of diabetic neuropathy has been highlighted in several studies (166-168). A significant correlation has been found between intra-epidermal nerve fibre (IENF) density (IENFD) and

QST in diabetic patients with abnormal nerve conduction (169). Devigili et al. (170) showed superior diagnostic efficiency of skin biopsy compared with QST but failed to find a link between neuropathic pain severity and IENF loss. Casanova and co-workers (171) analysed the relationship between nociceptive laser evoked potentials or contact heat-evoked potentials and IENFD in patients with painful sensory polyneuropathy and concluded that in patients with pure sensory neuropathy IENFs were slightly reduced and morphologically abnormal compared to patients with mixed fibre neuropathy who showed a severe IENF loss. Moreover, studies have shown that assessment of IENFD with skin punch biopsy is a reliable, reproducible (172) and diagnostically sensitive method (173). It has been proposed that the rate of IENF regeneration before and after intervention could be utilised as an endpoint in clinical trials (174).

#### **1.4.5.6 Assessment of autonomic neuropathy and sweat gland dysfunction**

DAN is a serious and common complication of diabetes which affects a variety of organ systems (gastrointestinal, genitourinary, sudomotor) and is associated with increased mortality (173). Conventional methods to evaluate autonomic system involvement include heart rate variability (HRV) in response to deep breathing and/or standing and the Valsalva manoeuvre (175). Other tests include the quantitative sudomotor axon reflex test (QSART), thermoregulatory sweat testing, adrenergic autonomic testing (144), sympathetic skin response (176) and the recently developed sudomotor function assessment test

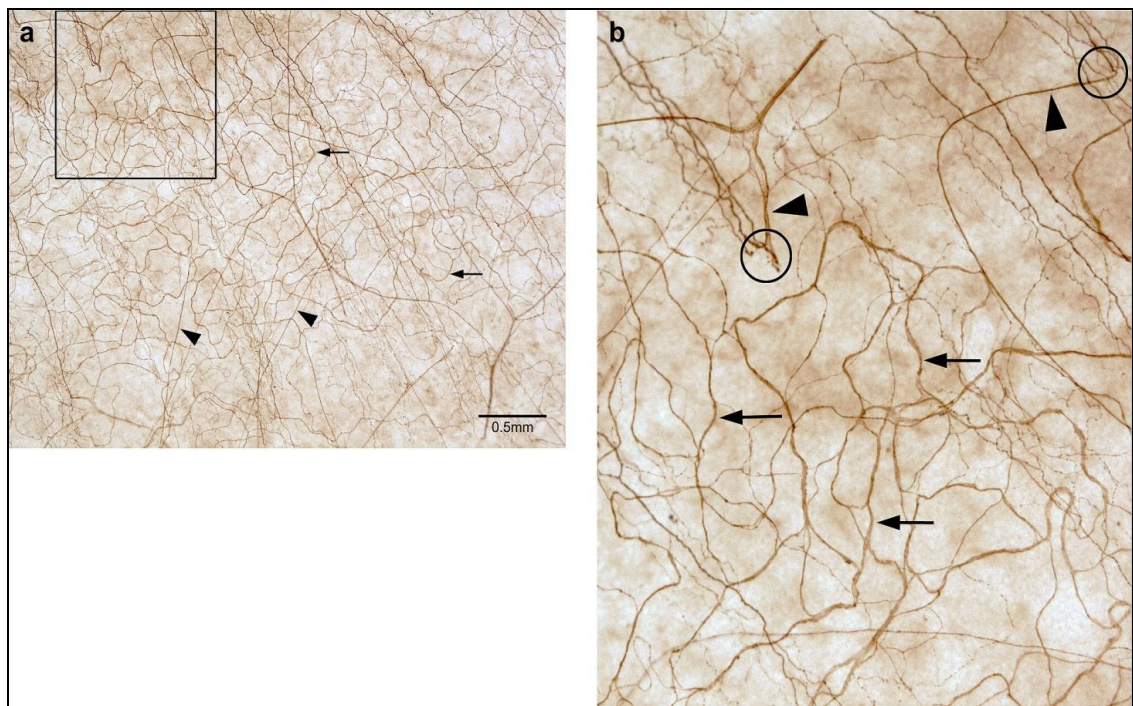
Neuropad® (177). Discussion of the evidence for each individual test is beyond the scope of this review of the literature.

#### **1.4.6 Ophthalmic markers of diabetic neuropathy**

##### **1.4.6.1 Corneal innervation: origin and distribution**

The cornea is the most densely innervated (~ 7000 nociceptors / mm<sup>2</sup>) and hence sensitive tissue in the human body (178). Corneal innervation not only exerts protective function but also has a role in regulating epithelial cell integrity, proliferation and wound healing through the release of several soluble growth factors and neurotrophins in response to infection, trauma and surgery (178-180) (Figure 1-3). Corneal nerves are primarily derived from the ophthalmic division of the trigeminal nerve, via the anterior ciliary nerves and a small proportion from the maxillary nerve. The superior cervical ganglion also provides some sympathetic innervation to the limbus and peripheral cornea (181). Studies using light (181-183), electron (184-186) and IVCCM (187-191) have previously described the distribution of the nerves in the human cornea. Approximately 70 – 80 trunks of large diameter (~ 6µm), myelinated nerves (Aδ) containing 900 – 1200 axons enter the cornea at the posterior to mid stroma and run forward and anteriorly in a radial fashion towards the centre. One millimetre from the limbus they lose their perineurium and myelin sheath giving rise to multiple branches that innervate the anterior stromal layers (Figure 1-4). They then penetrate the Bowman's membrane and turn in a 12-o'clock direction to form the subbasal nerve plexus (Figure 1-5 and Figure 1-6) in the interface between the Bowman's layer and the epithelium. Finally they

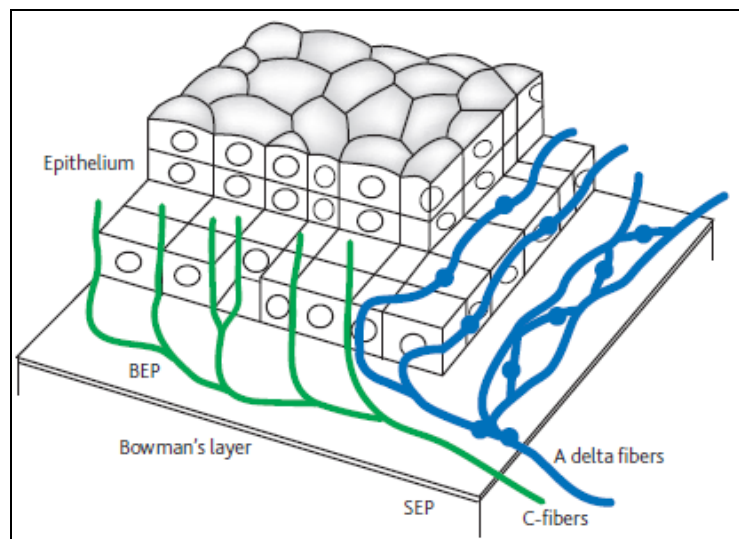
turn upwards in an almost 3-9 o' clock direction to terminate into the superficial layers of the epithelium (191-192).



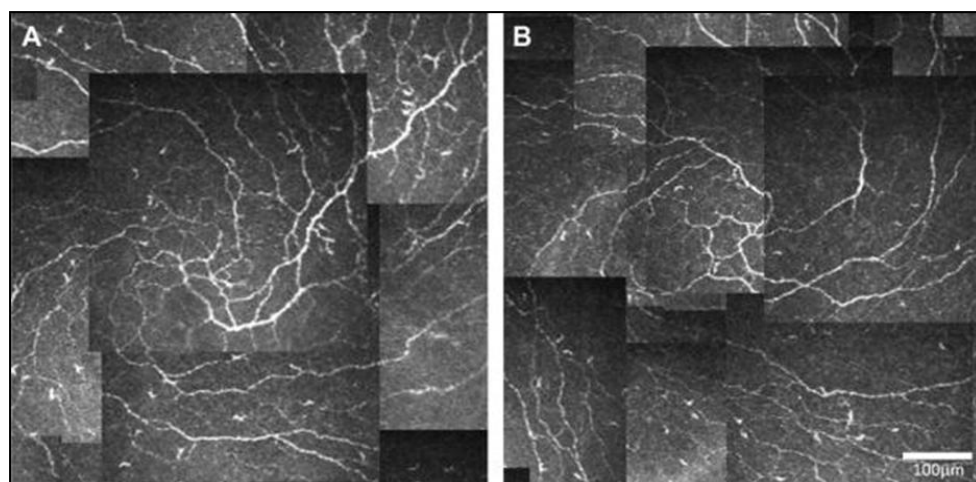
**Figure 1-3** Peripheral corneal innervation, focused on the subepithelial plexus. A. The subbasal plexus consists of modest numbers of straight or curvilinear fibres (arrowheads) and a dense, plexiform network of tortuous nerve fibres (arrows). B. subbasal straight or curvilinear fibres (arrowheads) penetrate Bowman's membrane (at open circles) to give rise to subbasal nerves. The tortuous nerve fibres (arrows) anastomose frequently and give the subepithelial plexus its highly characteristic plexiform appearance. Image adapted from Marfurt et al. (185).

Corneal sensitivity provides an important measure of corneal nerve function. Three different classes of receptors stimulate sensation in the cornea: mechanical (mechano-nociceptors), chemical (polymodal nociceptors), and thermal (cold receptors) (193-194). The mechanical receptors are primarily myelinated nerve fibres and constitute approximately 20% of corneal sensory fibres. Mechanical receptors mediate, sharp acute pain produced by touching the cornea. Polymodal nociceptors constitute 70% of the fibres and are

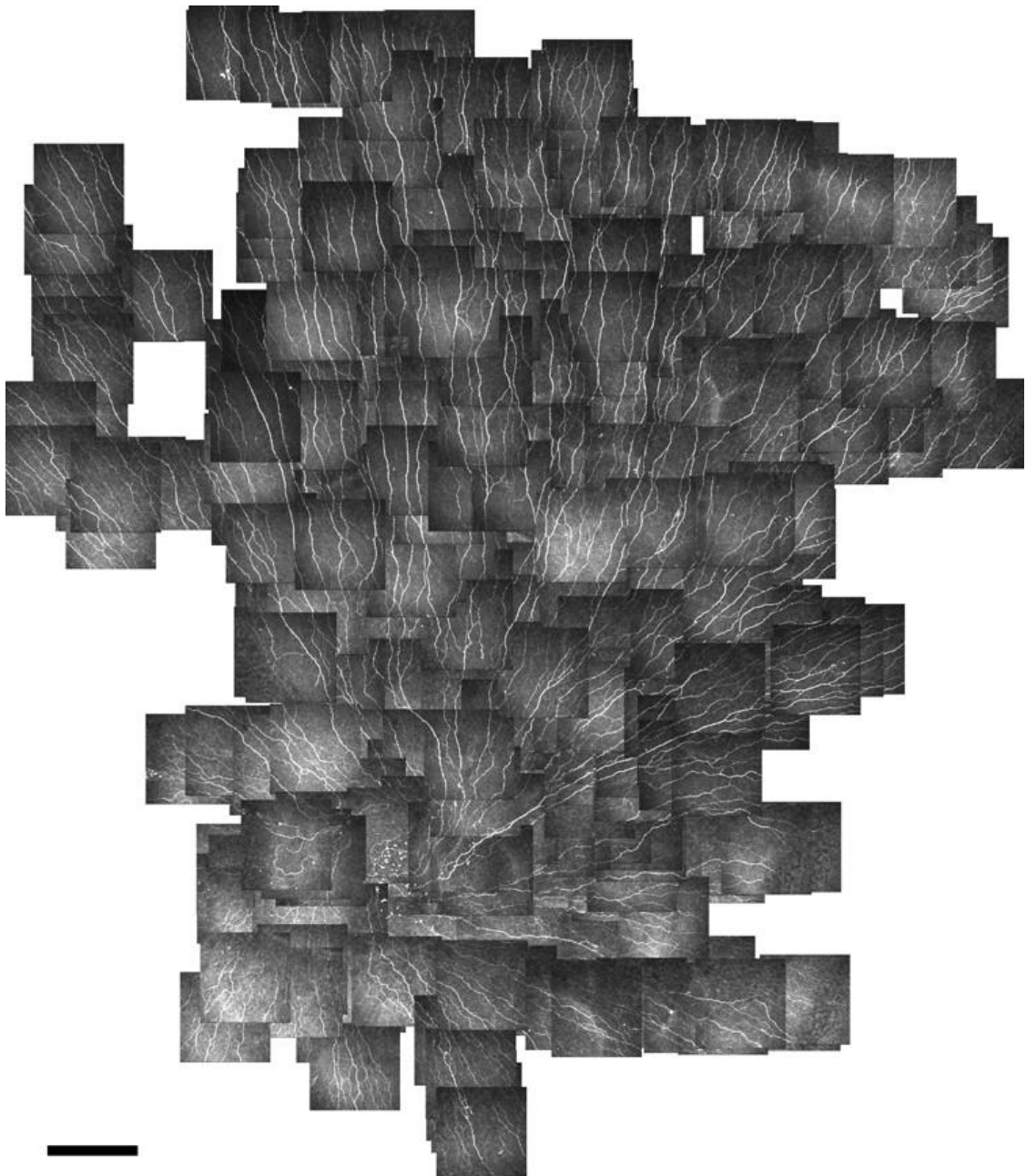
predominantly unmyelinated C fibres but with some myelinated A $\delta$  fibres which are stimulated by mechanical, thermal and chemical stimuli (194). Cold receptors constitute 10% of the nociceptors and are stimulated by moderate cooling of the cornea. Research has shown that sensitivity of the cornea decreases significantly over the age of 50 years, regardless of the area examined (195).



**Figure 1-4** Schematic representation of the organisation of the human corneal nerves [adapted from: Tavakoli and colleagues (196)].



**Figure 1-5** Montages of the inferior subbasal nerve plexus in the right (A) and left eye (B). Image adapted from Misra et al. (197).



**Figure 1-6** Montage of the whole human subbasal nerve plexus [Image adapted from: Patel and McGhee (187)].

#### **1.4.6.2 The role of corneal innervation in health and disease**

Corneal nerves provide multi-purpose support through the release of a variety of soluble neurochemicals which include substance P, calcitonin gene related peptide, pituitary adenylate cyclase-activating peptide, vasoactive intestinal

peptide, noradrenaline, serotonin, neuropeptide Y, met-enkephalin and galanin, cholecystokinin, brain natriuretic peptide, vasopressin, neurotensin and beta endorphin (178). In addition, several neurotrophins (198-199) (NT) (NT-3/4/5, brain derived growth factor, nerve growth factor) and extracellular molecules (200) (MMPs, serine protease plasmin) are expressed in the cornea. Dysfunction of corneal innervation due to topical surgery or infection can cause imbalance or impaired secretion of these factors which in combination with abnormal epithelial cell metabolism may result in neurotrophic keratitis (201-202). Neurotrophic corneal ulcers in patients with diabetes have been reported as early as 1977 by Hyndiuk and colleagues (203) and a growing number of studies have documented corneal nerve involvement in systemic disease.

#### **1.4.6.3 In vivo corneal confocal microscopy**

IVCCM is a relatively new technique which has evolved rapidly from an examination predominantly used in clinical research, to a diagnostic tool with a variety of ophthalmic and neurological applications. Its non-invasive nature has made it an ideal tool to examine all microstructures of the cornea, including the epithelial layer, Bowman's layer, stroma and endothelium. Historically, three types of IVCCM have been used: the tandem scanning confocal microscope (TSCM), the slit scanning confocal microscope (SSCM) (Nidek Confoscan 4, Nidek Technologies, Padova, Italy and Tomey Confoscan P4, Tomey, Erlangen, Germany) the latest laser scanning confocal microscope (LSCM) [Heidelberg Retina Tomograph III Rostock Corneal Module (HRT III RCM), Heidelberg GmbH, Germany]. Currently only the Nidek Confoscan 4

and HRT III RCM are commercially available. These microscopes operate under the same basic principles and provide an *en face* view of the corneal structure at very high magnification. However, there is a considerable difference in the image quality and hence resolution and clarity of cellular structures, making the HRT the instrument of choice as it provides images of high clarity for quantification of corneal nerve and stromal morphology.

#### **1.4.6.4 Corneal nerve morphology in systemic disease**

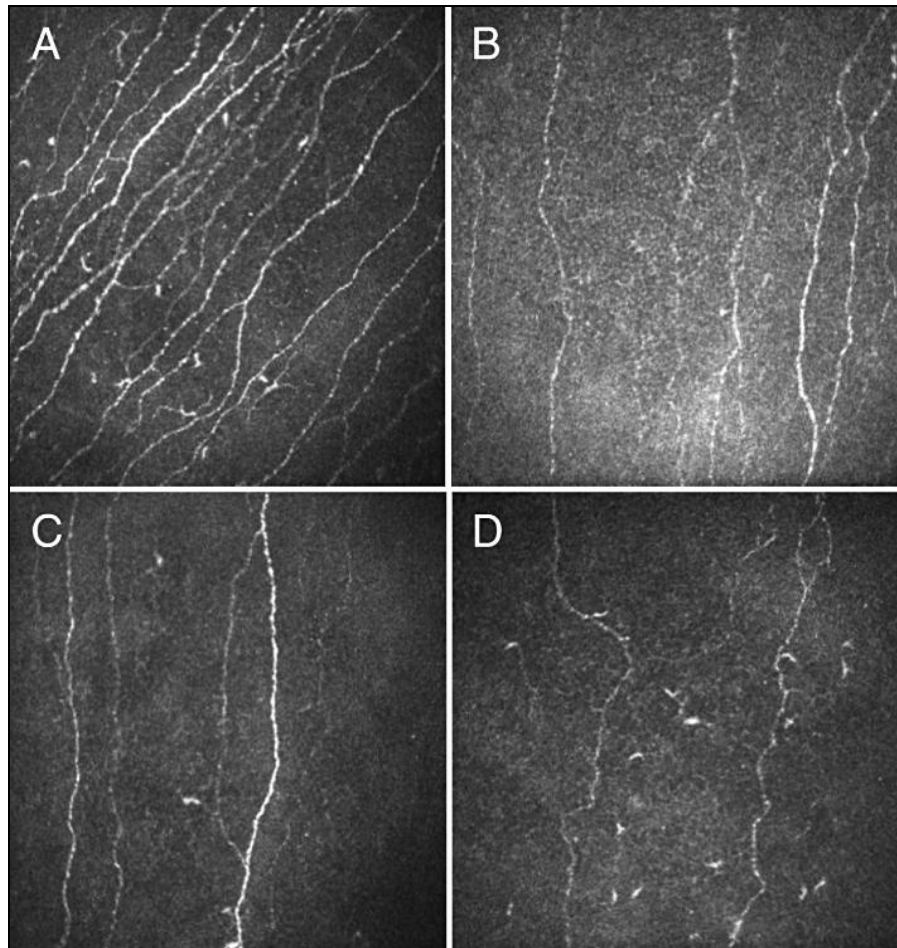
In recent years, IVCCM has been employed to describe novel pathological features in corneal innervation in ophthalmic and systemic disease. Therefore alterations, such as decreased density and increased tortuosity of nerves has been described in patients with dry eyes, and dry eyes related to primary Sjögren's syndrome compared to controls (204-205). IVCCM has been increasingly used to quantify various peripheral neuropathies and in particular DSPN (206). IVCCM has been shown to be an accurate non invasive method to diagnose nerve fibre damage in idiopathic small fibre neuropathy (207), Fabry disease (208), hereditary sensory and autonomic neuropathy (209), autoimmune neuropathy (210), Crohn's disease (211), non-length dependent small fibre neuropathy (212) chemotherapy-associated neuropathy (213) and Charcot-Marie-Tooth disease type 1A (214).

#### **1.4.6.5 Corneal nerve morphology in diabetic peripheral neuropathy**

In recent years, corneal innervation has attracted increasing research interest. An association between neurotrophic corneal ulcers and diabetes was



reported as early as 1977 (215). Subsequently, a reduction in corneal nerve density has been demonstrated in experimental diabetes ex-vivo (216). Lately, IVCCM has shown a capacity to diagnose and stratify the severity of DSPN (206; 217-218) (Figure 1-7).



**Figure 1-7** Corneal confocal microscopic image of Bowman's layer (A) control subject with normal corneal nerve density compared with images from diabetic patients with (B) mild, (C) moderate and (D) severe neuropathy. Image adapted from Tavakoli and colleagues (196).

Furthermore, reduced corneal nerve density correlates with IENFD loss (219), serum triglyceride level (207), HbA<sub>1c</sub> (220-221), systemic blood pressure (220) and the severity of diabetic retinopathy (222). IVCCM is more sensitive than IENFD in detecting early nerve repair in recipients of simultaneous pancreas

and kidney transplantation (223-224). Finally, recent studies have showed that IVCCM can be used to rule out DSPN with excellent sensitivity and specificity (218; 225). A summary of studies employing IVCCM to quantify corneal subbasal nerve morphology is presented in (Table 1-3).

#### **1.4.6.6 Quantification of subbasal nerve morphology using in vivo corneal confocal microscopy**

There is considerable inconsistency in the reported findings from studies employing IVCCM to quantify subbasal nerve morphology. Studies using LSCM compared to studies using TSCM and SSCM have reported higher densities of corneal nerves in the Bowman's layer. Other sources of variability include the absence of a uniform protocol in the number of images required to accurately evaluate morphological alterations and the relatively small IVCCM image size (50 x 50 $\mu$ m) in combination with the absence of location specific reference values. Furthermore, the absence of a fully-automated platform, free of inherent human observation biases also contributes to the variability.

Recent studies have reported high inter- and intra-observer repeatability in diabetes (229-230) and controls (231) as well as the prevalence of morphological symmetry in central corneal innervation patterns (231). A recent study has proposed a protocol to quantify the subbasal nerves (232).

**Table 1-3** Summary of corneal nerve quantitative assessment with IVCCM in systemic disease [adapted from: Tavakoli and colleagues (196)]

Cause of Neuropathy/Studies	n	CNFD (no./mm <sup>2</sup> )	CNBD (no./mm <sup>2</sup> )	CNFL (mm/mm <sup>2</sup> )
<b>Diabetes Mellitus (DM)</b>				
Tavakoli et al. (218)	101	24.1 ± 2.6	10.3 ± 1.7	4.9 ± 0.5
Messmer et al. (226)	67	16.5	17.5	10.2
Midena et al. (227)	42	2.2 ± 0.3 (no)	0.8±0.1	-
Chang et al. (228)	42	16.1 ± 5.7	24.9 ± 7.7	-
Quattrini et al. (219)	54	23.7 ± 3.2	7.31±1.98	3.94±0.63
Malik et al. (206)	18	27.8 ± 6.5	27.2 ± 13.2	7.5 ± 1.1
Rosenberg et al. (217)	23	3.1 ± 1.2	-	-
<b>Sjogren's Syndrome</b>				
Gemignani et al. (212)	1	12.5	-	-
Tuisku et al. (205)	20	Qualitative	-	-
Benitez del Castillo et al. (204)	21	20.1 ± 4.3	-	-
<b>Idiopathic Small Fibre Neuropathy (ISFN)</b>				
Tavakoli et al. (207)	17	24.7 ± 3.0	12.9±2.1	4.4 ± 0.6
Gemignani et al. (212)	3	20.8	-	-
<b>Impaired Glucose Tolerance (IGT)</b>				
Tavakoli et al. (207)	8	19.9 ± 3.2	10.7 ± 1.6	3.8 ± 0.5
<b>Chemotherapy-associated Peripheral Sensory Neuropathy</b>				
Ferrari et al. (213)	1	Qualitative	-	-
<b>Crohn's Disease</b>				
Geresara et al. (211)	1	16.4	-	-
<b>Fabry Disease</b>				
Tavakoli et al. (208)	22	29.5 ± 6.0	12.2 ± 3.3	7.0 ± 1.3
<b>Autoimmune Neuropathy</b>				
Lalive et al. (210)	1	Qualitative	-	-
<b>Hereditary Sensory and Autonomic Neuropathy (HSAN)</b>				
Mimura et al. (209)	3	Qualitative	-	-

\***CNFD**: corneal nerve fibre density, **CNBD**: corneal nerve branch density, **CNFL**: corneal nerve fibre length, **TC**: tortuosity coefficient

Research from several centres in Italy (233), Portugal (234), New Zealand (235) and the UK (236) has proposed various algorithms to enable fully automated assessment of corneal nerve morphology. The majority of studies

have defined sub-basal nerve density as the total number (no) of nerves per image, which allows quantification of the nerve density in an area (no/mm<sup>2</sup>) (206; 218; 226; 228). Others have presented their results as the number of nerves per image (227) or the total length of the nerves within a frame (237-238). Such variations in the definitions employed clearly cause difficulties in interpreting the results from different studies.

#### **1.4.6.7 Corneal sensitivity in diabetes**

Diminished corneal sensitivity (CS) in diabetes, with symmetrical involvement, was first described by Schwartz (239) and later by others (217; 240-242). Nielsen (241) reported that 83% of the diabetes cohort had reduced CS, which was related to reduced vibration perception. After the initial reports, more comprehensive studies have evaluated CS using a non-contact corneal aesthesiometer (NCCA). Murphy et al. (243) showed a gradual reduction in CS with increasing age in control subjects and diabetic patients using a NCCA. In other studies reduced CS has been associated with poor metabolic control (244) and the severity of polyneuropathy (245). A significant decline in CS was found in patients with diabetic retinopathy receiving argon laser photocoagulation, possibly as a result of physical damage to the ciliary nerves. The impairment was greater amongst those with type 2 DM (246). Pritchard and colleagues (247) showed that CS > 0.66 mbar using a NCCA may indicate the presence of neuropathy with reasonable sensitivity (70%) and specificity (75%).

## 1.5 References

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## **2. CHAPTER II-METHODS**

## **2.1 Synopsis**

The accurate detection and quantification of human DSPN are important to define at risk patients, anticipate deterioration, and assess new therapies. Current methods lack sensitivity (QST), require expert assessment (NCS) or are invasive (skin/nerve biopsy) and not routinely performed across health systems. A surrogate endpoint is defined as a biological 'marker' or laboratory measurement that is used in therapeutic trials as a substitute for a meaningful end point that is a direct measure of how a patient feels, functions, or survives and is expected to predict the effect of the therapy. Surrogates are thought to reflect the activity of the underlying process that leads to an adverse outcome. Thus, for diabetic neuropathy a valid surrogate is vital and ideally should quantify nerve damage in a rapid, reiterative and non-invasive manner to enable:

- a. Detection
- b. Anticipate progression / deterioration
- c. Assess therapeutic efficacy

## **2.2 Hypothesis and aims**

The primary hypothesis is that the assessment of corneal nerve morphology using IVCCM is a valid surrogate marker for human DSPN. This research aims to:

- i. Establish an optimal image acquisition method to assess corneal nerve fibre morphology: centre vs. adjacent periphery. Earlier studies have

used the central cornea to assess changes in corneal nerve structure in diabetes and in a range of peripheral neuropathies. A question arising from previous work is whether peripheral areas should also be sampled for an optimal result.

- ii. Establish whether IVCCM can assess corneal nerve morphology in a repeatable manner. This study aims to investigate the impact of inter-side (R v L), inter- and intra-observer variations when assessing corneal nerve structures.
- iii. Provide a novel fully automated image analysis algorithm to enable time-effective, less labour intensive, accurate assessment of corneal nerve fibre morphology. An automated system will eliminate inconsistencies through the application of objective criteria to evaluate nerve features and will significantly reduce the time for analysis making the technique suitable to clinical practice.
- iv. Establish that corneal nerve fibre damage as assessed with IVCCM and corneal sensation is present in patients with impaired glucose tolerance and diabetes with minimal evidence of neuropathy, thereby establishing its place in the diagnosis of DSPN.
- v. Establish that corneal nerve fibre damage visualised with IVCCM is progressive and symmetric in agreement with the natural history of the disease and relates to clinical (NDS, QST, NCS) and laboratory (intra-epidermal nerve fibre density) markers of somatic neuropathy in diabetes.
- vi. Estimate the prevalence of neuropathy measured with IVCCM and NCCA in a cohort of patients with type 1 and type 2 DM and compare

this with the results of neurological deficits (NDS); nerve electrophysiology, quantitative sensory testing (QST) and establish the relationship with risk factors.

- vii. Establish that improvement in glycaemic control by simultaneous pancreas and kidney transplantation (SPK), leads to an improvement in corneal nerve structure (IVCCM) and function (NCCA), thereby assessing the potential of IVCCM as a surrogate marker and end point for clinical trials of diabetic neuropathy.
- viii. Establish the validity of automated IVCCM image assessment to diagnose diabetic neuropathy.

### **2.3 Outcome Markers**

Previously established indicators of corneal nerve pathology which have been shown to be valid markers of peripheral neuropathy include corneal nerve fibre (CNFD) and branch density (CNBD), length (CNFL) and the tortuosity coefficient (TC):

- i. CNFD: the number of main nerve fibers /  $\text{mm}^2$ .
- ii. CNBD: the number of main nerve branches /  $\text{mm}^2$ .
- iii. CNFL: the sum of the length ( $\text{mm./mm}^2$ ) of all nerve structures.
- iv. TC: the tortuosity or non-linearity of the main nerve fibers.
- v. NCCA: corneal sensitivity measured as a functional correlate of sensory impairment in the corneal nerves.

## **2.4 Study Design**

Cross-sectional, investigator-masked, case-control observational study.

### **2.4.1 Patient Recruitment and Study Approval**

Participants with type 1 and type 2 DM were recruited from the Manchester Diabetes Centre at Manchester Royal Infirmary. Patients were given a patient information sheet where the nature, potential risks and benefits of the study were explained in detail (a sample patient information sheet can be found in Appendix 2) and at least 1 week to decide participation. Control subjects were recruited from the community in a similar fashion and often were relatives of study participants with diabetes or colleagues at the Manchester Royal Infirmary. This study adhered to the tenets of the Declaration of Helsinki and was approved by the North Manchester Research Ethics Committee (reference number: 09/H1006/38 and 08/H1004/1), the Scientific Advisory Board of the Manchester Wellcome Trust Clinical Research Facility (reference number: SUB309) and the local Research and Development office (copies of the forms can be found in Appendix 2).

### **2.4.2 Patient Enrolment**

Informed written consent was obtained from all subjects prior to their participation and they were given the chance to discuss any concerns about the experimental protocol and their safety with a trained member of the research team (a copy of the patient consent form can be found in Appendix 2). Upon enrolment, all control and diabetes participants underwent detailed screening by means of personal and medical history and a set of blood and

urine tests to determine their metabolic status and ensure eligibility for this study. The specific inclusion / exclusion criteria used are presented below. Appointments were offered to participants during normal working hours and if a participant could not complete the full visit assessment in a single visit a second visit was scheduled within a month.

#### **2.4.2.1 Recipients of simultaneous pancreas and kidney transplantation with type 1 diabetes mellitus**

Patients were informed about the nature and potential consequences of the study and they were given a relevant patient information sheet from a transplant surgeon at the Transplant Unit of the Manchester Royal Infirmary (a copy of the transplant patient information sheet can be found in Appendix 2). Patients willing to participate informed the surgeon who then contacted a member of the research team to schedule a visit.

Based on the hypothesis that rapid restoration of blood glucose levels through SPK could have a beneficial effect on the peripheral nervous system, and the small sensory nerves in particular, patients were assessed immediately after transplantation. During their baseline visit peripheral nerve status was determined through the same set of criteria and clinical tests employed for non-SPK participants (type 1 DM, type 2 DM and controls), in addition SPK recipients were assessed at 6 months and 12 months post transplantation.

#### **2.4.3 Inclusion criteria for enrolment**

All study subjects satisfied the following criteria prior to participation:



- i. Age 18-85
- ii. Signed informed written consent
- iii. Diagnosis of type 1 or type 2 DM
- iv. Willingness to comply with the experimental protocol (excluding skin biopsy which was voluntary and was indicated separately in the patient consent form)

#### **2.4.4 Exclusion Criteria**

Any of the following rendered a participant ineligible for inclusion:

- i. History of corneal trauma or any ocular surgery 12 months prior to enrolment date
- ii. Systemic disease known to affect the cornea
- iii. Any bilateral or unilateral ocular surface disease, infection or inflammation at the time of examination
- iv. History of malignant, infectious and or metabolic (other than diabetes but may co-exist e.g. hypothyroidism) disease, congestive heart failure and peripheral vascular disease
- v. Aetiology of peripheral neuropathy other than diabetes e.g. excess alcohol intake (defined for males > 21 units / week and females > 14 units / week), amyloidosis, hereditary sensory neuropathy, certain autoimmune disorders (e.g. Guillain-Barre syndrome), chronic kidney failure, connective tissue disease, liver failure, radiculopathy, vitamin deficiencies (e.g. vitamin B<sub>12</sub> deficiency).
- vi. Active diabetic foot ulcer or infection
- vii. Active participation in an interventional clinical trial

#### **2.4.4.1 Serious adverse events, serious adverse reactions and post-visit follow-up**

Serious adverse events (SAE) is any untoward medical occurrence that results in death, in a congenital anomaly or birth defect, persistent or significant disability / incapacity, in-patient hospitalisation for a prolonged period or prolongation of ongoing hospitalisation, is a medically important event (in accord with up-to-date clinical guidelines and judgement of the individual investigator) or is life threatening. Serious adverse reactions (SAR) are reactions of a subject to a medicinal product or drug, which do not constitute a SAE but suggest causality for the investigated product. This was an observational study and as such the occurrence of SAEs and / or SARs was highly unlikely. However, in the rare instance of a SAE and / or a SAR the standard procedures were followed according to 'Good Clinical Practice' as set out by the NHS nationally and the policies of local Trusts, Research and Development Divisions and Ethics Committees.

Briefly, in the unlikely case of significant ocular findings, which however did not constitute a SAE or a SAR, the participant was notified by the investigator and a post visit follow-up was scheduled at the investigator's discretion and participant's willingness. Skin biopsy of the dorsum of the left foot was performed in a sub-cohort of the participants with diabetes and controls. Following biopsy, if appropriate hygiene procedures are not taken, an infection may complicate the wound, which can lead to a SAE especially in patients with diabetes. For this reason, a leaflet with all relevant information and dressings

were given to the participant and a wound follow-up visit attended by a trained nurse was scheduled within 10 days from biopsy.

IVCCM is a highly specialised technique which requires considerable training (see section 2.6.3.3) prior to application by a qualified individual (optometrist or ophthalmologist). There are not known recorded risks associated with the use of IVCCM in humans. However, to perform an IVCCM scan requires local instillation of 2x drops / eye of oxybuprocaine hydrochloride 0.4 % and 1x drop / eye of a carbomer (polyacrylic acid 2.0 mg/g). Patients may occasionally exhibit symptoms of hypersensitivity to carbomers, oxybuprocaine or any component of the preparation. Ester-type anaesthetics are contraindicated only in premature babies due to immaturity of the enzyme system which metabolises the ester type local anaesthetics. Hypersensitivity may manifest with symptoms of transient blurring, transient burning and transient redness and is usually well-managed as opposed to persistent hypersensitivity reaction which constitutes a SAE. During this study no SAE, SARs or event likely to lead to a SAE (suspected unexpected serious adverse reactions) were reported.

#### **2.4.5 Discontinuation from the study**

Participants were discontinued from the study when any of the criteria set out below occurred:

- i. Illness preventing further participation.

- ii. Unacceptable SAEs or SARs as a result of participation to the study or other non-study reasons resulting in significant change in circumstances.
- iii. A decision from the participant to discontinue.
- iv. General or specific changes in the participant's medical condition (based on the judgement of the investigator) which make the participant not eligible for further investigation.
- v. Three consecutive missed visits.

## **2.5 Case definition of peripheral neuropathy**

Peripheral neuropathy in this study was defined according to the updated criteria set by the committee of the Toronto Diabetic Neuropathy Expert Group in 2010 (1). There is a difference between defining neuropathy for clinical purposes with the associated healthcare, social and economic consequences of the diagnosis and defining neuropathy for research and epidemiologic purposes. The guidelines suggest that definitions 3 'Confirmed DSPN' and 4 'Subclinical DSPN' are used in research. *Confirmed DSPN* requires the presence of an abnormality in electrodiagnostic studies of the nerves and the presence of a symptom or symptoms or a sign or signs of neuropathy; if NCS are normal then a validated measure of small fibre neuropathy may be used. The presence of *subclinical neuropathy* in the absence of signs or symptoms of DSPN is confirmed with an abnormal NCS result or with another validated measure of small fibre neuropathy. Briefly, presence of DSPN was defined as

follows (for more details on each experimental procedure see 2.6.2 Assessment of peripheral neuropathy):

- Clinical symptoms - an abnormal neuropathy symptom profile (NSP) with symptoms specific to DSPN (for the detailed questionnaire see Appendix 2- Neuropathy Symptom Profile).
- Clinical examination – a neuropathy disability score  $\geq 3/10$ .
- NCS - abnormal peroneal motor or sural sensory nerve conduction velocity and / or amplitude.
- Quantitative sensory testing (QST) - abnormal vibration perception threshold (VPT) or an abnormal thermal (hot or cold) perception threshold that is abnormal for the patient's age.

## **2.6 Study procedures**

All study participants underwent assessment of anthropometric factors, medical status (blood and urine testing) and peripheral neuropathy status.

### **2.6.1 Assessment of medical status and physical measurements**

Upon enrolment to the study participants underwent blood and urine testing to determine their medical (mainly cardiometabolic) status and exclude causes of peripheral neuropathy other than diabetes. Specifically, the percentage of glycated haemoglobin (HbA<sub>1c</sub>), total cholesterol (mmol/l), high HDL / LDL cholesterol (mmol/l), triglycerides (mmol/l), 25(OH)-Vitamin D ng/ml and Vitamin B<sub>12</sub> were measured. Renal status was determined by measuring the estimated glomerular filtration rate (eGFR) (ml/min/l), creatinine (mmol/l),

albumin-to-creatinine ratio (mg/mmol). Thyroid function [free T<sub>3</sub> (mu/l) and thyroid stimulating hormone (pmol/l)] and liver function tests [albumin (g/l), bilirubin (umol/l), total protein (g/l), ALT (U/l) and ALP (U/l)] were also assessed. Other causes of peripheral neuropathy were excluded by assessing antinuclear antibodies and undertaking electrophoresis. Blood and urine samples were destroyed following testing, typically within 7 days of collection, according to local laboratory protocols.

Physical measurements were performed by a senior research nurse using standard clinical protocols and included lying BP<sub>sys</sub>, BP<sub>dia</sub> (mmHg) and heart rate (bpm) using the Lifeline Automatic Digital Sphygmomanometer (Lifeline Ltd., Worthing, West Sussex, UK), height (cm), weight (kg), waist and hips circumference (cm), waist-to-hip ratio and BMI (kg/m<sup>2</sup>) based on the formula  $BMI = \text{weight} / \text{height}^2 \text{ (m}^2\text{)}$ . All blood pressure and resting heart rate measurements were performed in a quiet room using consistently the left arm at normal room temperature with the patient seated comfortably and the muscles relaxed. The patient was instructed not to smoke, eat, drink or ingest any adrenergic stimulants prior to measurements.

### **2.6.2 Clinical assessment of peripheral neuropathy**

Peripheral neuropathy was assessed by means of validated, gold standard clinical techniques. Details for each technique and definition of abnormality are presented below. All clinical procedures were carried out in a quiet room with appropriate temperature and illumination and with the patient lying on a bed

with their legs aligned horizontally. Results were recorded on purpose designed forms (Appendix 2).

### **2.6.2.1 Symptom screening questionnaires**

The symptom screening questionnaires employed in this study were completed by the examiner for each patient (Appendix 2). The neuropathy symptom profile (NSP) (2) was used to define symptoms related to diabetic neuropathy. It is composed of 38 questions in total, categorised in groups of symptoms related to weakness of head and neck, chest, upper limbs, lower limbs, sensory dysfunction and autonomic neuropathy. The short-form McGill questionnaire was used to assess symptoms of pain (3). It is subdivided in a) a pain descriptor section where the patient is asked to determine the location, type and severity of pain they may feel from a number of available descriptions such as shooting, throbbing, aching pain and others, b) a visual analogue scale where the patient is asked to assign a score from 0-10 for the type of pain they feel and c) a pain index where the patient is asked to characterise their painful symptoms if any, as 'no pain (0)', 'mild (1)', 'discomforting (2)', 'distressing (3)', 'horrible (4)' and 'excruciating (5)'.

### **2.6.2.2 Neuropathy deficit score**

The simplified version of the NDS was used to evaluate signs of neuropathy and stratify neuropathic severity in all study participants (4). The NDS is a clinical composite score composed of pin / prick, hot and cold and vibration sensation and Achilles tendon reflexes. Tests were performed on the great toes (pin / prick and vibration sensation) or on the dorsum (temperature

sensation) of both feet. The final score varies from 0-10 with 0 denoting no neuropathy and 10 denoting severe neuropathy. The neuropathy profiles based on the total NDS correspond to different stages of DSPN and are classified as: 'none' (NDS 0-2), 'mild' (NDS 3-5), 'moderate' (NDS 6-8) and 'severe' (NDS 9-10) neuropathy.

#### **2.6.2.2.1 Pain sensation**

A Neurotip™, which is composed of a sharp and a blunt end, was used to determine pain sensation (Figure 2-1). The Neurotip™ was first applied to the forearm to allow the patient to clearly distinguish between the two types of stimuli. It was then applied three times on each of the great toes of the foot and the patient was asked to determine if they felt a “sharp” or “blunt” sensation with the eyes closed ( $\geq 2/3$  correct responses=normal). The score for each foot was recorded (0: normal, 1: abnormal).



**Figure 2-1** Pin / prick sensation tested with a Neurotip™ during measurement of the NDS.



### 2.6.2.2.2 Vibration sensation

A tuning fork vibrates at 128Hz and was first tested on the patient's forearm both in vibrating and resting conditions to allow appreciation of the stimulus. The tuning fork was then applied three times to the great toe of each foot and the patient with the eyes closed is asked to determine when the fork is vibrating from two stimuli presented in random order ( $\geq 2/3$  correct responses=normal) (Figure 2-2). The score for each foot is recorded (0: normal, 1: abnormal).



**Figure 2-2** Testing vibration sensation with a 128 Hz tuning fork as part of the NDS

### 2.6.2.2.3 Temperature sensation

For this test one metal rod is placed in cold water and another in hot water. After about 30 seconds they are removed from the water and tested on the forearm of the patient. The rods are then applied alternatively on each foot and the patient with closed eyes is asked to determine the warm sensation (1<sup>st</sup> stimulus or 2<sup>nd</sup> stimulus') ( $\geq 2/3$  correct responses=normal). The score for each foot is recorded (0: normal, 1: abnormal) (Figure 2-3).



**Figure 2-3** Testing temperature sensation on the dorsum of foot as part of the NDS

#### **2.6.2.2.4 Achilles tendon reflex**

The examiner holds the plantar surface of the foot so that the Achilles tendon is under moderate tension and the tendon hammer is left to fall under its own weight onto the tendon (Figure 2-4). Reflex movement in the foot and contraction of the gastrocnemius muscle indicates normal function. If absent the patient is asked to pull their hands together in the reinforcement position just prior to hammer strike. The score is then recorded (0: normal, 1: present with reinforcement and 2: abnormal).



**Figure 2-4** Testing Achilles tendon reflexes with a tendon hammer as part of the NDS

### **2.6.2.3 Quantitative sensory testing**

#### **2.6.2.3.1 Vibration perception threshold**

VPT was measured with a Neurothesiometer (Horwell, Scientific Laboratory Supplies, Wilford, Nottingham, UK). The patient lies on the bed with the heels resting and a test stimulus and the probe is then balanced on the tip of the great toe under its own weight. The intensity of the stimulus is increased slowly from 0 to 50 volts by turning the dial on the Neurothesiometer and the patient is asked to determine when they first feel vibration. The procedure is repeated three times and the average of the measurements is calculated for each foot.

#### **2.6.2.3.2 Warm and cool detection thresholds**

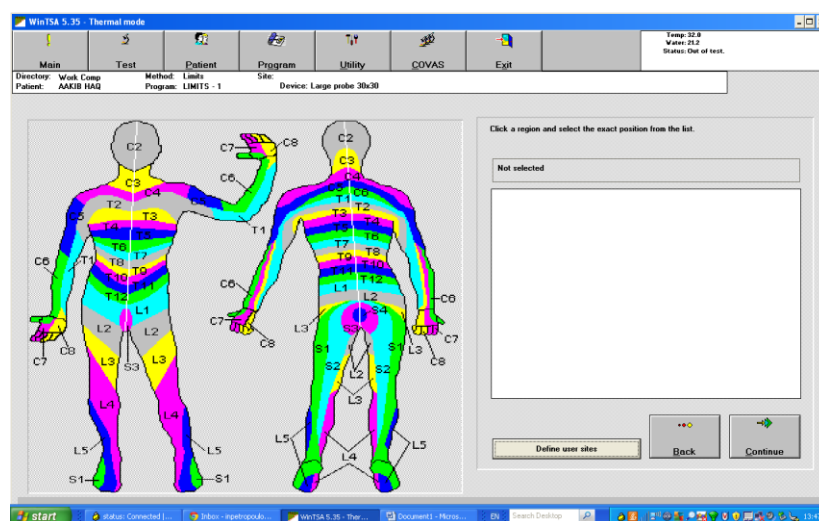
Thermal threshold testing was performed with the TSA II Neurosensory Analyser (Medoc Ltd., Ramat Yishai, Israel) with a thermode attached to the dorso-lateral region of the left foot. The examiner has full control of the procedure through a computer and purpose-built software (Figure 2-5 and Figure 2-6). First cold threshold (CT) is tested; the temperature gradually decreases and the patient is asked to determine the first time they feel a 'cooling' (threshold). When the patient replies, the examiner records the response and the test is repeated 4 times and the average is calculated. Warm threshold (WT) is tested using the same protocol (Figure 2-7).



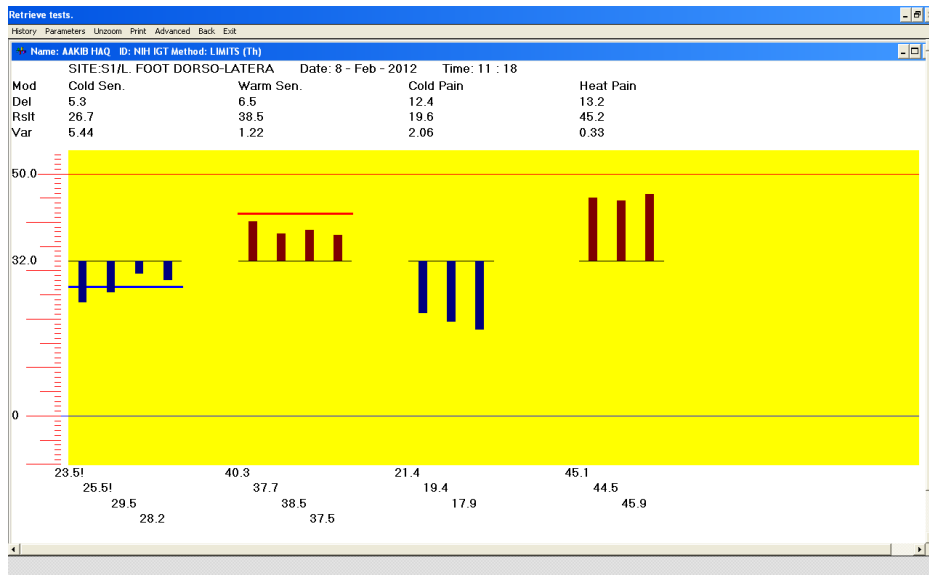
**Figure 2-5** Testing temperature sensation on the dorso-lateral region of the left foot

### 2.6.2.3.3 Hot and cold induced pain

Under the same conditions for CT and WT the patient is asked to determine the moment at which cold sensation becomes painful, intolerable or causes tingling (cold induced pain-CIP) on four occasions. The procedure is repeated to determine heat induced pain (HIP) (Figure 2-7).



**Figure 2-6** Selection of testing location user interface: the dorsolateral region on the left foot (S1) was tested in this study using the method of limits



**Figure 2-7** The user interface for thermal threshold and pain measurement

#### 2.6.2.4 Nerve conduction studies

NCS were undertaken according to local NHS trust protocols for electrodiagnostic testing of the peroneal and motor nerves by a consultant Neurophysiologist (Figure 2-8). Motor NCV (m/s), maximum M-wave amplitude (mV), baseline to peak and minimum F-wave latency (ms) of the peroneal nerve, amplitude of the sensory action potential, baseline to peak ( $\mu$ V), latency to onset (ms) and conduction velocity of the sural nerves were measured. Prior to and at the end of each examination the skin temperature was measured and if the temperature was below 31°C the limb was warmed. The nerves were stimulated by increasing the strength of the stimulus in steps until a maximal response was achieved. The stimulus strength was increased by 10-15% above maximal response to ensure a supra-maximal response. Motor response was not averaged while sensory responses were averaged using 3 but not more than 10 stimuli.



**Figure 2-8** Electrodiagnostic studies of the peroneal and sural nerves

Motor amplitude was measured from the baseline to the negative peak and reported to the nearest 0.1mV. Sensory amplitude was measured from baseline to the negative peak and reported to the nearest whole number. If a positive peak preceded the negative peak the amplitude was recorded from the base of the positive to the negative peak. Motor latency was measured at the take-off of the negative component of the M-wave. Sensory nerve latency was measured from the take-off of the negative component of the sensory nerve action potential. If a positive component preceded the negative component then the latency was measured at the peak of the negative component of the sensory nerve action potential. To measure F-wave latency, a minimum of 10 F-waves were measured and minimum F-latency was recorded. All NCVs were measured using onset latencies and NCV was reported to the closest 0.1 m/s.

#### **2.6.2.5 Skin punch biopsy**

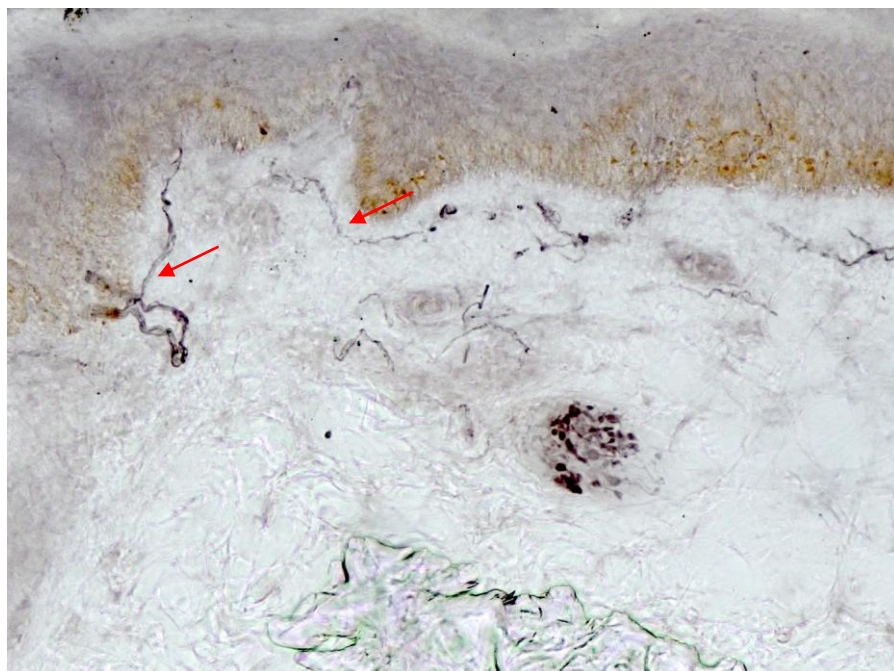
The patient was asked to rest in a semi-reclined position and the foot was inspected and 2 points 2 cm proximal to the metatarsal bone were chosen for the biopsy. After the skin was thoroughly cleaned with betadine, 1% lignocaine was injected subcutaneously. The skin was incised with a 3mm punch biopsy device. After two biopsy samples had been obtained the area was cleaned and the wounds were covered with steri-strips.

Samples after collection were immediately fixed in PBS-buffered paraformaldehyde for 18-24 hours, rinsed in Tris buffered saline and soaked in 33% sucrose (2-4h) for cryoprotection. Samples were embedded in optimum cutting temperature embedding compound (OCT), rapidly frozen in liquid nitrogen and cut into 50µm section using a cryostat microtome. For each case, 4 floating sections were selected to undergo melanin bleaching. The sections were protein blocked for 4h with a Tris-buffered saline solution of 5% normal swine serum, 0.5% powder milk and 1% Triton X-100. Samples were incubated overnight with 1:1200 Biogenesis polyclonal rabbit anti-human PGP9.5 antibody (Serotec Ltd, Oxford, England). Biotinylated swine anti-rabbit secondary antibody 1:300 (DakoCytomation Ltd., Ely, UK) was applied for 1h. Sections were quenched with 1% H<sub>2</sub>O<sub>2</sub> in 30% MeOH-PBS for 30 minutes and incubated with 1:500 HRP-Streptavidin (Vector Laboratories, Peterborough, England) for 1 hour. Nerve fibres were demonstrated using 3,3'-diaminobenzidine (DAB) chromogen (Sigma-Aldrich Ltd., Manchester, UK) and were mildly counterstained with eosin to allow better localisation of the



basement membrane to help identify nerve fibres passing through the basement membrane.

Nerves were immunolocalized using the pan-neuronal marker protein gene product 9.5 (PGP 9.5). Basement membrane length ( $\mu\text{m}$ ) was measured using computer image analysis (Nikon digital camera and Leica QWin standard V2.4 programme). IENFD i.e. the number of fibres per mm of basement membrane are expressed as no. /mm (Figure 2-9)



**Figure 2-9** Image of a skin biopsy sample obtained with light microscopy. Intra-epidermal nerve fibers (red arrows) are visible after immunolocalisation of the nerves with the pan-neuronal marker PGP 9.5

### **2.6.3 Ophthalmic assessment**

A history of previous or present ophthalmic conditions with a focus on corneal injuries, erosions, infections and surgical procedures or laser photocoagulation was obtained by the investigator and routine slit lamp biomicroscopy was performed to identify potential cofounders (Figure 2-10). Patients with an



active infection e.g. viral or bacterial keratitis or keratoconjunctivitis were excluded due to their known effects on the ocular surface and subbasal nerves and for causing radial neuritis (5). Subsequently, participants underwent scanning by means of in-vivo corneal confocal microscopy to estimate their subbasal nerve status and non-contact corneal aesthesiometry to assess corneal sensation.

### **2.6.3.1 Slit lamp biomicroscopy**

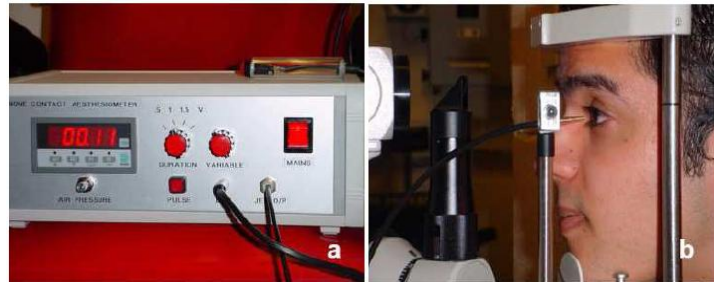
Initially the microscope was positioned at 0° and direct diffuse illumination with a wide slit (> 5mm) and low magnification was used for a gross assessment of the ocular surface, anterior eye segments and the lacrimal reflex. Direct focal illumination with a narrow slit (0.1 - 0.3 mm) at maximum magnification was used in the instance of a localised alteration or penetration by foreign body to allow appreciation of the magnitude of the defect. Indirect illumination with a decentred, narrow to medium slit (2 - 4 mm) and magnification set at 12-16x was used to examine objects in the direct vicinity of corneal areas with reduced transparency (e.g. infiltrates, scars, deposits, epithelial or stromal defects) (Figure 2-10). All observations were recorded on purpose designed forms (Appendix 2).



**Figure 2-10** Slit lamp biomicroscopy of the cornea before in-vivo corneal confocal microscopy

### **2.6.3.2 Non contact corneal aesthesiometry**

Corneal sensitivity was measured using a NCCA (constructed for the IHBI, Anterior Eye Lab by Kimble Dunster and Lincoln Hudson). The equipment is composed of a central unit from which the examiner controls (increase/decrease) stimulus intensity and from another instrument which is placed in a designated area in front of the slit lamp microscope and blows air gently onto the patient's corneal surface when a button is pressed by the examiner (Figure 2-11). The threshold was determined in a quiet dark room, at a controlled temperature (18-22 °C) using a staircase procedure. The equipment was calibrated at installation and by a trained medical equipment technician annually.



**Figure 2-11** Estimation of corneal sensation using NCCA

The examinee was asked to place their head in the head and chin-rests and the height of the table was adjusted for comfort. The patient was then instructed to look straight ahead and gaze at a fixation target. The probe emitting the air puff was moved forward at an approximate distance of 10mm from the cornea. The patient was initially given a high test stimulus to appreciate the feeling. The examiner then decreased the stimulus using the method of limits until this was just detectable by the subject (absolute threshold). The stimulus was again increased and repeated on 3 occasions. The average measurement was recorded and the procedure was repeated for the other eye.

### **2.6.3.3 In vivo corneal confocal microscopy**

Images of the corneal sub-basal nerve plexus were captured using the HRT III-RCM (Heidelberg Engineering GmbH, Heidelberg, Germany). Extensive training (> 2 months) was provided to a qualified study optometrist by a trained individual with considerable experience in IVCCM (> 4 years) to safely and successfully apply the technique to study subjects. Initial use of IVCCM (2 months) was supervised by the same trained individual to allow correction for minor inconsistencies in scanning methodology and ensure subject safety.



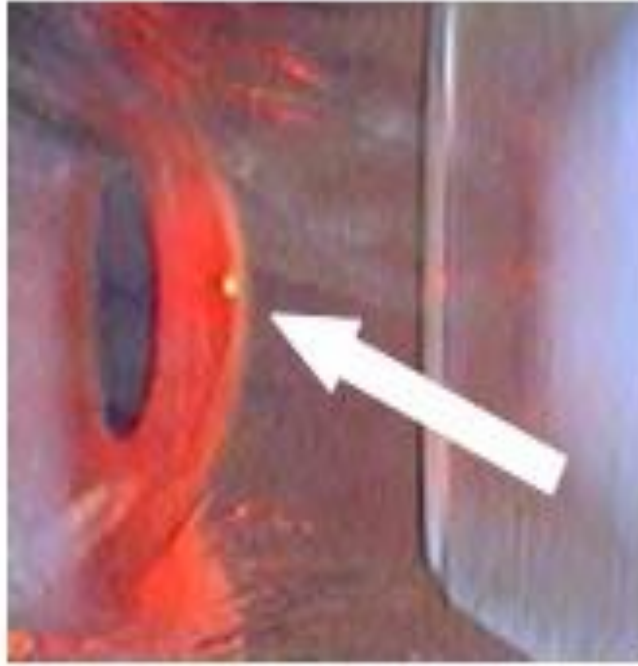
**Figure 2-12** in vivo corneal confocal microscopy

#### **2.6.3.3.1 Examination procedure**

Patient details were entered (study ID, gender, date of birth, full name) in purpose-built software (Heidelberg Eye Explorer, Heidelberg Engineering GmbH, Heidelberg, Germany) and the confocal microscope was prepared for examination. The camera was adjusted to the lowest position and the refraction of the objective lens of the laser scanning camera was set at +12 Dpt. A large homogeneous, bubble free, pea sized drop of viscotears (Carbomer 980, 0.2%; Novartis, UK) was applied on the lens tip. A TomoCap® (*polymethyl methacrylate*, Heidelberg Engineering GmbH, Heidelberg, Germany) was placed over the lens tip and pressed onto the microscope so that the distance from the cornea to the microscope was kept stable during the examination. A gel meniscus was formed between the back surface of the cap

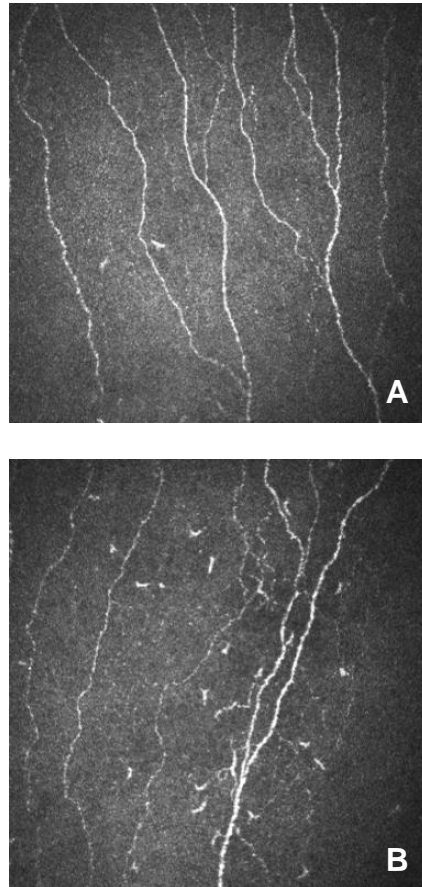
and the objective lens tip. The lens was then adjusted (by rotating the adjustment wheel) so that it was in focus. After a bright reflection (2<sup>nd</sup> focal plane) was observed on the screen and the depth value was between -150 µm and +150 µm, the depth value was reset to zero.

A drop of local anaesthetic (benoxinate hydrochloride 0.4%, Chauvin Pharmaceuticals Ltd., Essex, UK) was instilled in the eye to be examined to reduce the blinking reflex and a viscous tear drop (Carbomer, Viscotears®, Novartis Pharmaceuticals UK Ltd., Surrey, UK) was also placed on the eye to allow lubrication of the ocular surface. The image acquisition window, which allows control of the IVCCM and live imaging of the cornea was opened. The patient fixates on an outer fixation light with the eye that is not being examined. The CCD camera is also adjusted to allow imaging of the cornea and correct positioning of the TomoCap®. The laser scanning camera was then moved forward towards the patient's eye until it was 5-10 mm from the cornea. The position of the reflection from the laser beam was checked via the CCD camera (Figure 2-13). Images were captured using the "section" mode on the Heidelberg eye explorer.



**Figure 2-13** Positioning of the TomoCap® on the cornea: the red reflection (white arrow) seen through an external CCD camera is used as a topographic landmark to place the microscope approximately onto the corneal apex. Nerve features such as orientation are also used to determine the location.

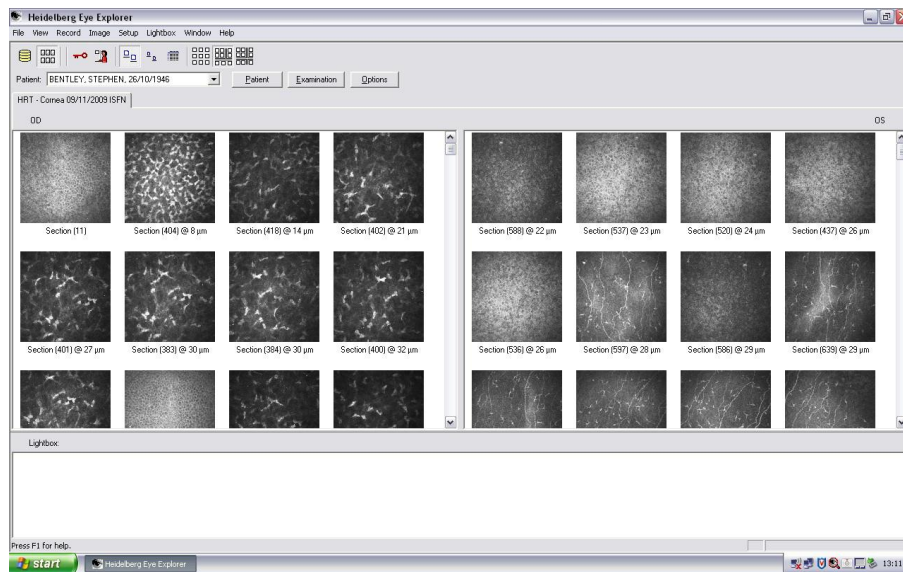
The patient was asked to open their eyes widely and the microscope was moved until minimal contact with the corneal surface was achieved. Movement of the adjustment wheel allows images to be captured at different depths. Care was taken to avoid pressure on the cornea (cornea appears flattened). When an adequate number of images had been captured from each layer the procedure was repeated for the other eye (Figure 2-14). There are several available options for image acquisition, a fixed sequence of images (sequence mode) or a series of 40 images at consecutive focal planes (volume mode) can be obtained.



**Figure 2-14** Images of the corneal subbasal nerves (A, B) using in vivo corneal confocal microscopy (HRT III-RCM)

In the “sequence” mode up to 100 images can be acquired with an adjustable frame rate (1-30 frames/sec). This mode also allows a movie to be recorded (duration 3-100 sec). A total depth of 85  $\mu\text{m}$  can be scanned with a 400  $\mu\text{m}$  field of view lens with the focal distance between two consecutive images set at approximately 2.1  $\mu\text{m}$ . The distance between each image was approximately 1  $\mu\text{m}$ ; therefore if the depth of Bowman’s is 10 $\mu\text{m}$ , approximately 10 images can be captured. For image analysis purposes 3-4 images / patient with a distance of approximately 2-3  $\mu\text{m}$  were selected. As Hertz et al. note (6) “the section mode of the corneal confocal microscope may

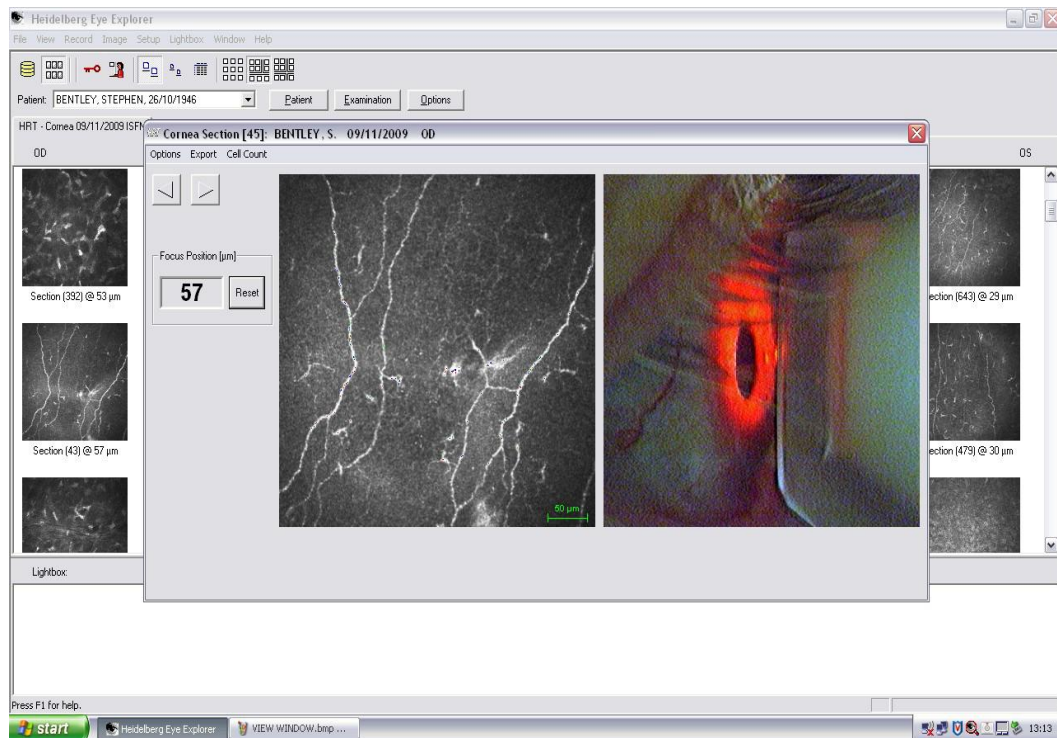
be more suitable to more experienced users as it enables manual focus on the structures of interest” (Figure 2-15).



**Figure 2-15** the Heidelberg eye explorer through which images could be previewed and exported for analysis

The displayed image was selected by the examiner for analysis of the corneal nerve parameters (Figure 2-16). Images were captured from all corneal layers but only images from the subbasal nerves were used for quantitative analysis in this study.

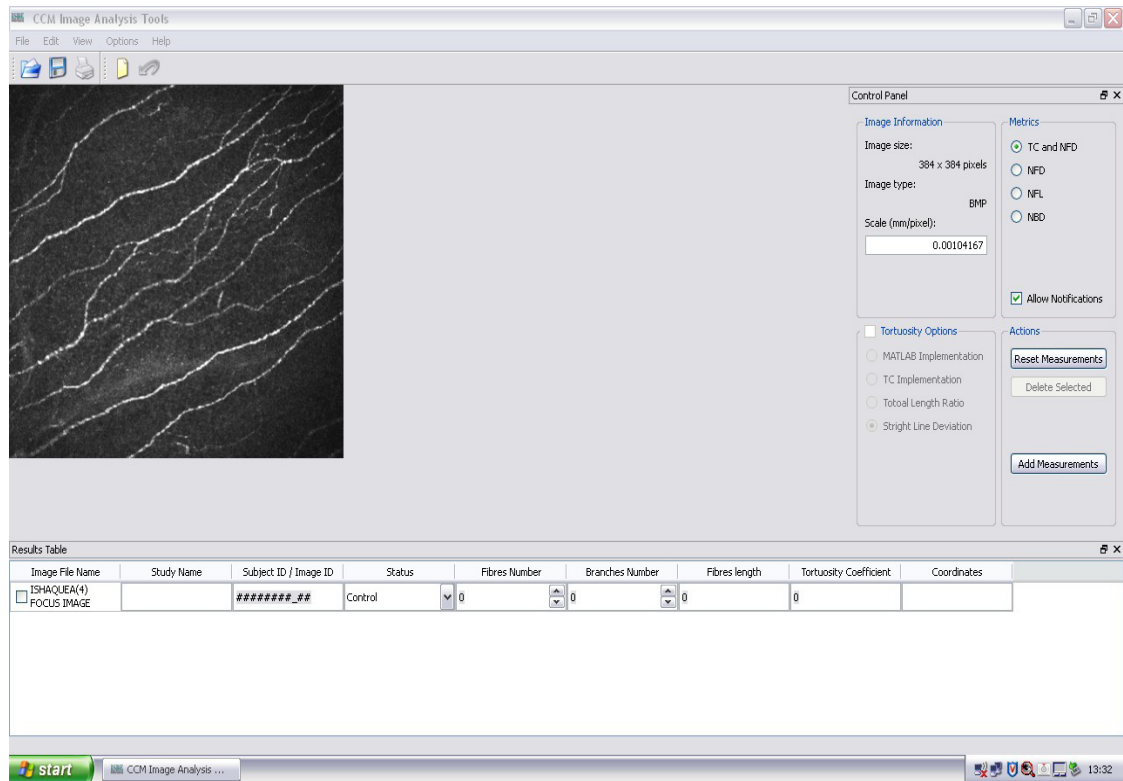




**Figure 2-16** A preview of an image from the corneal subbasal nerves on the Heidelberg eye explorer with a simultaneous view of the location of the image through a CCD camera.

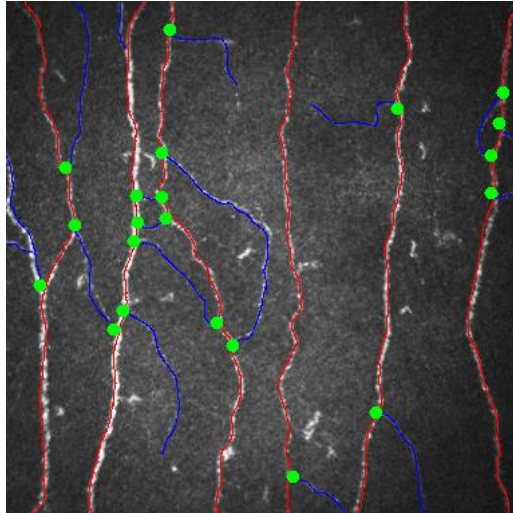
### 2.6.3.3.2 Manual image analysis

All images were analysed manually by the same examiner (Ioannis Nikolaos Petropoulos) using purpose-designed, proprietary software (CCMIATv0p6, M.A. Dabbah, Imaging Science and Biomedical Engineering, University of Manchester, Manchester, UK). The parameters of choice (CNFD, CNBD, CNFL and TC) as defined in 2.3 were measured by selecting the appropriate option in the software user menu (Figure 2-17).



**Figure 2-17** The user interface of corneal confocal microscopy manual image analysis tool v0p6 (M.A. Dabbah, Imaging Science and Biomedical Engineering, University of Manchester, Manchester, UK). The examiner selects the specific parameter they want to measure from the top right panel and upon completion they select “add measurements” to perform the calculations through an integrated algorithm. Calculations are then exported and saved for each subject

The image size is 384x384 pixels and each parameter is indicated with a different colour (Figure 2-18). Criteria for image selection were depth, focus position, location and contrast.

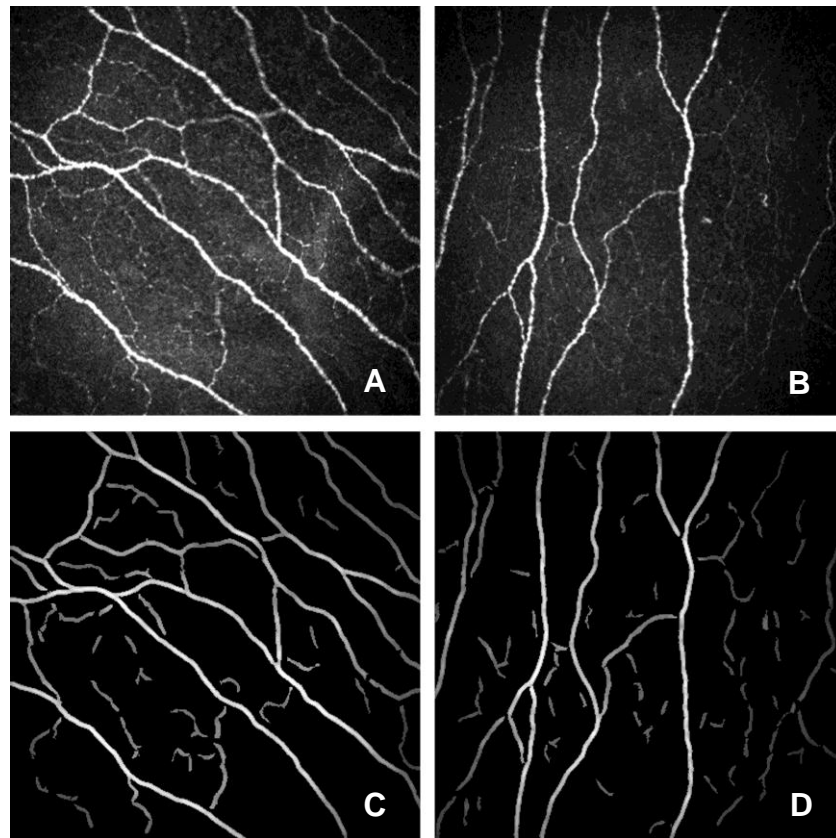


**Figure 2-18** A quantified IVCCM image using CCMIATv0p6 (M.A. Dabbah, Imaging Science and Biomedical Engineering, University of Manchester, Manchester, UK). The red colour corresponds to CNFD and the TC, the green dots highlight CNBD at the points of junction with main nerve fibers and CNFL, the length of nerve structures in the entire IVCCM image is highlighted under the red and blue colour. An integrated algorithm in the image analysis software performs all calculations which are exported per patient upon completion

### **2.6.3.3.3 Automated image analysis**

The automated corneal nerve fiber quantification framework consists of two steps: (1) IVCCM image enhancement and nerve fiber detection and (2) quantification of CNFD, CNBD, CNFL and TC (Figure 2-19). The detection of nerve fibers is a challenging task, as the nerve fibers often show poor contrast in the relatively noisy images. A dual-model feature descriptor combined with a neural network classifier is used to train the computer to distinguish the nerve fibers from the background (noise and underlying membranes). In the nerve fiber quantification process, all the end points and branch points of the detected nerve fibers are extracted and used to construct a connectivity map. Each segment in the connectivity map can then be connected and classified as

main nerve fibers or branches according to the nerve intensity, orientation and length.



**Figure 2-19** An illustration of the single-scale dual-model detector as presented by Dabbah et al. (6). Contrast enhancement and noise removal (c, d) of original IVCCM images (a, b) are essential steps for accurate nerve detection and quantification [from: Dabbah et al. 2011 (8)].

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### **3. CHAPTER III-REPEATABILITY OF IN VIVO CORNEAL CONFOCAL MICROSCOPY TO QUANTIFY CORNEAL NERVE MORPHOLOGY**

Author's contribution: Ioannis N Petropoulos contributed to the conception and study design, recruited subjects, acquired part of the data, analysed data, performed statistical analysis and wrote the manuscript which constitutes the basis for this chapter and was submitted and accepted for publication in the journal *Cornea*.

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### 3.1 Abstract

**PURPOSE** To establish intra- and inter-observer repeatability, agreement and symmetry of corneal nerve fibre morphology in healthy subjects using IVCCM.

**METHODS** 19 subjects underwent IVCCM (HRT III RCM) at baseline and 7 days apart. Bland-Altman plots were generated to assess agreement, and the intra-class correlation coefficient (ICC) and coefficient of repeatability (CoR) were calculated to estimate intra- and inter-observer repeatability for corneal nerve fibre density (NFD) (no./mm<sup>2</sup>), nerve branch density (NBD) (no./mm<sup>2</sup>), nerve fibre length (NFL) (mm/mm<sup>2</sup>) and nerve fibre tortuosity coefficient (TC). Symmetry between right and left eyes was also assessed.

**RESULTS** ICC and CoR for intra-observer were 0.66-0.74 and 0.17-0.64; for inter-observer were 0.54-0.93 and 0.15-0.85 and for symmetry 0.34-0.77 and 0.17-0.63, respectively. NBD demonstrated low repeatability.

**CONCLUSION** This study demonstrates good repeatability for the manual assessment of all major corneal nerve fibre parameters with the exception of nerve branch density, which highlights the difficulty in defining nerve branches and suggests the need for experienced observers or automated image analysis to ensure optimal repeatability.

### 3.2 Introduction

Detailed histological analysis of the human cornea prior to in-vivo corneal confocal microscopy (IVCCM) was only possible post-mortem using light and electron microscopy (1-2). Since the 1980's IVCCM has been used in ophthalmic research (3) and in clinical practice to assess corneal dystrophies and ectasias (4), acanthamoebal (5), fungal (6), bacterial (7) and viral (8) keratitis, the effects of contact lens wear (9), dry eye disease (10) and post surgical follow-up (11-12).

Real-time IVCCM has enabled the characterization of corneal nerves in healthy (13-14) and keratoconic (15) eyes. Recently, in vitro studies using state of the art immunohistochemical techniques have comprehensively investigated the architecture of the corneal nerves and described novel features (16-17). We and others have recently applied this technique to quantify corneal subbasal nerve fibres in a variety of peripheral neuropathies including diabetic neuropathy (18-21), idiopathic small fibre neuropathy (22), Fabry's disease (23), anti-myelin associated glycoprotein neuropathy (24), chemotherapy-associated peripheral sensory neuropathy (25), non-length dependent small fibre neuropathy (26) and type IV/V hereditary sensory and autonomic neuropathy (27). Quantification of corneal nerve morphology using 4 key parameters, namely nerve fibre density (NFD), nerve branch density (NBD), nerve fibre length (NFL) and the tortuosity coefficient (TC), has allowed the early detection of neuropathy and has enabled stratification of the severity of neuropathy (18; 28-29) as well as the assessment of repair following pancreas transplantation in diabetic patients (20). Tavakoli and colleagues (28)



recently reported high sensitivity (0.82) and moderate specificity (0.52) for the detection of diabetic neuropathy using IVCCM. However, there is considerable variability for the different corneal nerve parameters assessed due to the subjective criteria applied to identify each structure. Possible solutions include the adoption of internationally accepted criteria and rules to identify the different corneal nerve structures or the development of fully automated image analysis software (30-33). Two recent studies have demonstrated high repeatability of IVCCM, but focused primarily on NFL (34) (35). The aim of the present study was to establish intra-observer, inter-observer and between-eye repeatability and agreement in control subjects for each of the four key parameters used to quantify neuropathy.

### **3.3 Methods**

#### **3.3.1 Study Subjects**

19 randomly selected healthy subjects aged  $23.1 \pm 1.2$  years, without peripheral neuropathy and/or diabetes were studied. The study was approved by the North Manchester Research Ethics Committee and informed written consent was obtained from each subject. None of the subjects had a previous history of ocular surgery, contact lens wear, corneal infection or any other systemic disease known to affect the peripheral nervous system. Both eyes of each subject were examined by slit lamp biomicroscopy and confirmed to be clinically normal. None of the subjects was obese or had abnormal glycaemia or lipids. We used the Toronto consensus criteria (36) to exclude peripheral

neuropathy by assessing the NSP, NDS and QST (VPT, CT, WT, CIP and HIP).

### **3.3.2 In vivo corneal confocal microscopy**

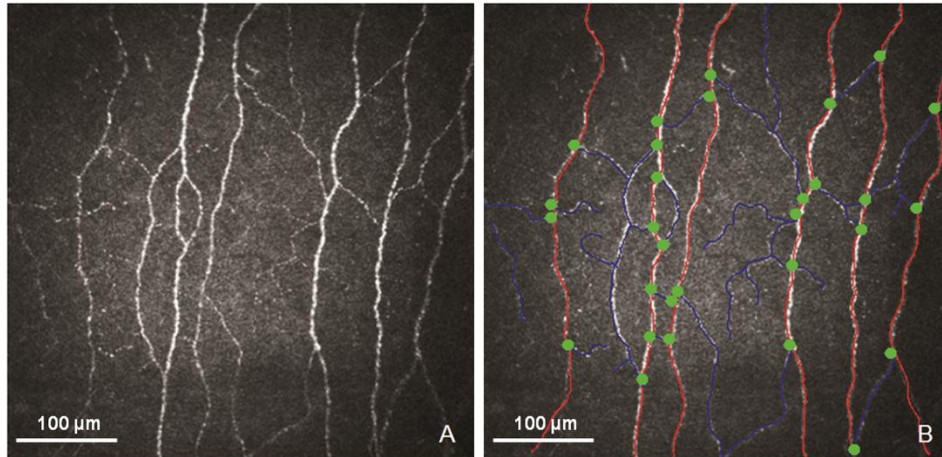
Nineteen subjects were scanned with a laser IVCCM (HRT III RCM) (Heidelberg Engineering GmbH, Heidelberg, Germany) on two occasions separated by a 1 week interval. This IVCCM uses a 670 nm wavelength helium neon diode laser, which is a class I laser and therefore does not pose any ocular safety hazard. A 63x objective lens with a numerical aperture of 0.9 and a working distance, relative to the applanating cap (TomoCap<sup>®</sup>, Heidelberg Engineering GmbH, Heidelberg, Germany) of 0.0 to 3.0 mm was used. The size of each two-dimensional image produced was 384  $\mu\text{m}$  x 384  $\mu\text{m}$  which has a 15° x 15° field of view and 10  $\mu\text{m}$ /pixel transverse optical resolution. HRT III RCM uses an entirely digital image capture system and all images are stored in an external hard drive.

A drop of 0.4% benoxinate hydrochloride (Chauvin Pharmaceuticals, Chefaro, UK) was used to anaesthetise each eye and Viscotears (Carbomer 980, 0.2%, Novartis, UK) were used as the coupling agent between the cornea and the applanating cap. All subjects were asked to fixate on an outer fixation light throughout the IVCCM scan and a CCD camera was used to image the cornea and correctly position the applanating cap to enable image capture strictly from the corneal apex. The overall examination took approximately 4 minutes for both eyes of each subject at each visit. All images were captured using the “section” mode in the Heidelberg Explorer of the HRT III RCM. The other two available modes are “volume” and “sequence”. As Hertz et al. (35) note, the

“volume” mode may have advantage when inexperienced examiners are using the technique. For the purposes of this study the same experienced examiner performed all IVCCM scans. There is no general consensus on optimal IVCCM image sampling. We captured 10 [5 left eye (LE), 5 right eye (RE)] images of high clarity at 1µm intervals from the central cornea of each subject.

### **3.3.3 Image analysis**

Two observers masked from each other analysed 380 IVCCM non-overlapping images, which were randomized prior to analysis. Observer 1 was experienced in the task of IVCCM image analysis (>2400 CCM images) and observer 2 had no previous experience of corneal nerve quantification. Criteria for image selection were depth, focus position and contrast. The images were manually analysed using proprietary, purpose-written software (CCMetrics<sup>®</sup> (31), M. A. Dabbah, ISBE, University of Manchester, Manchester, UK). The specific parameters measured per frame were those we have previously established (18): NFD (no./mm<sup>2</sup>), NBD (no./mm<sup>2</sup>), NFL (mm/mm<sup>2</sup>) and the TC (Figure1). NFD is defined as the total number of main nerve fibres (NF) per frame divided by the area of the frame in mm<sup>2</sup> (area = 0.16033585 mm<sup>2</sup>) (Figure 3-1). NBD is defined as the total number of main nerve branches (NB) (strictly nerve branches which stem from a NF) divided by the area of the frame. NFL is the total length of NFs, NBs as well as secondary nerve branches (nerve branches which stem from a NB) per frame. TC is a mathematical computation of the NF tortuosity as previously described by Kallinikos et al. (29), which is independent of the angle of the nerve in the image. A straight nerve equals a TC of zero and the TC increases with increasing tortuosity of the NF.



**Figure 3-1** Image (A) is an original image as captured with the HRT III RCM. Image (B) is an analysed image using CCMetrics<sup>®</sup> (31). NFD is measured under the red colour, which highlights the NFs, and an integrated algorithm measures the value. NBD is measured with the green dots that highlight the junction between NFs and NBs. NFL is the summation of the length of all the nerves highlighted under the blue and the red colour. TC-a measure of NF tortuosity-is measured simultaneously with NFD on each NF and is highlighted with the red colour. The method is identical to that previously described by Kallinikos et al. (29) and has been integrated into the current algorithm.

### 3.3.4 Statistical Analysis

Data analysis was performed using Microsoft Office Excel 2008 (Microsoft, WA, USA) and StatsDirect Version 2.7.7 (StatsDirect Ltd., Cheshire, UK) and the data are presented as mean  $\pm$  standard deviation (SD). The data were tested for normality prior to analysis and appropriate statistical techniques were employed. Differences between groups of measurements were assessed by means of a paired *t*-test. Power analysis was used to calculate the minimum sample size needed to detect an effect. The results showed that for 80%, 85% and 90% power 17, 19 and 21 subjects were required respectively. For the

purposes of the present analysis a 95% confidence interval (CI) was used and a  $p < 0.05$  was considered significant.

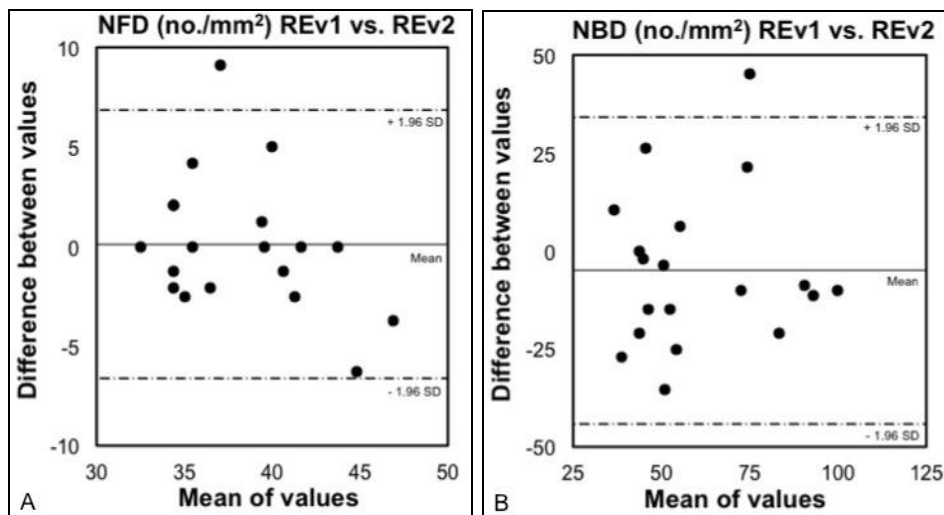
The intra-class correlation coefficient (ICC) was calculated to estimate the repeatability of the measurements between and within “occasions” and “observers”. The ICC can be used as an index of the correlation between repeated measures, i.e. as an index of repeatability (37). The ICC was considered excellent if 0.8-1 and very good if 0.60-0.79. CoR was also calculated as a percentage of an average measurement to estimate the repeatability of the sample. A CoR between 0-0.2 was considered good, 0.2-0.5 acceptable and  $>0.5$  poor. The means of the measurements were plotted against the differences between the measurements and the upper and lower limits of agreement were calculated ( $LOA = 1.96 + SD$ ,  $1.96 - SD$ ) as described by Bland and Altman (38) to appreciate the between-observer, within-subject and between-occasion agreement.

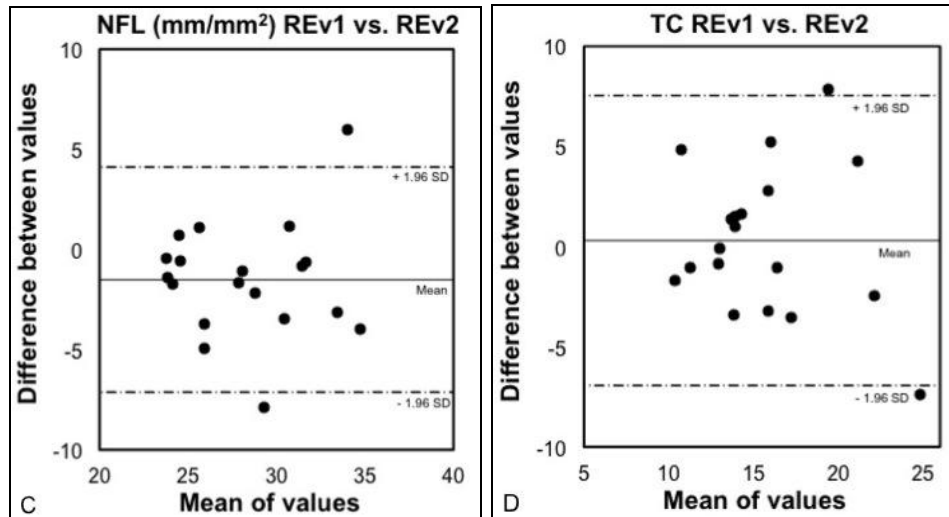
### **3.4 Results**

Subjects in this study had a body mass index of  $24.8 \pm 4.1$  kg/m<sup>2</sup>, hemoglobin A1c (%)  $5.5 \pm 0.2$ , low density lipoprotein cholesterol  $2.7 \pm 0.8$  mmol/mol, high density lipoprotein cholesterol  $1.5 \pm 0.3$  mmol/mol and serum triglycerides  $1.3 \pm 0.6$  mmol/mol. Subjects had no evidence of peripheral neuropathy: NDS 0, NSP 0, VPT  $3.3 \pm 1.3$  Hz, CT/WT  $28.6 \pm 2.4/36 \pm 1.8$  °C, CIP/HIP  $6.4 \pm 5.9/47.1 \pm 3.9$  °C.

### 3.4.1 Intra-observer repeatability

*Intra-observer repeatability* was assessed for each parameter using images from the same location and depth of the same eye on two separate occasions 7 days apart by the same observer (REv1 vs. REv2 for visit 1 and visit 2 respectively) (table 1). There were no significant differences ( $P>0.05$ , 95% CI) between the results from the first and the repeated scan. The mean of the values was plotted against the difference between them to derive the Bland-Altman plots Figure 3-2. The relevant ICC values were: NFD - 0.74 (Figure 3-2A), NBD - 0.61 (Figure 3-2B), NFL - 0.70 (Figure 3-2C) and TC - 0.66 (Figure 3-2D). The respective CoR values were: NFD - 0.17, NBD - 0.64, NFL - 0.19 and TC - 0.46. The mean difference  $\pm$  SD between the two assessments was  $0.1 \pm 3.6$  no./mm<sup>2</sup> (NFD),  $5.0 \pm 19.4$  no./mm<sup>2</sup> (NBD),  $1.5 \pm 2.8$  mm/mm<sup>2</sup> (NFL) and  $0.4 \pm 3.6$  (TC).

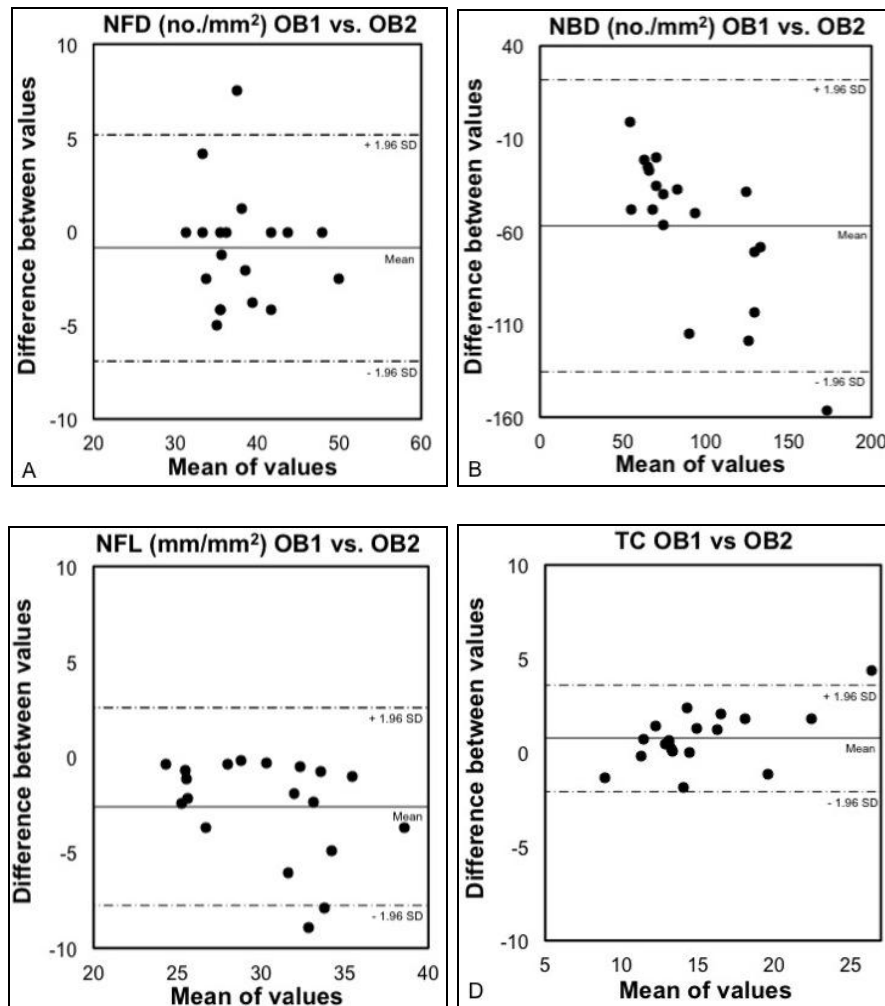




**Figure 3-2** Bland Altman plots for NFD (A), NBD (B), NFL (C) and TC (D) as an indication of agreement between repeated measurements of the same eye (REv1 vs. REv2) on two separate occasions [(visit 1 (v1) vs. visit 2 (v1))] assessed by the same examiner.

### 3.4.2 Inter-observer repeatability

*Inter-observer repeatability* refers to the assessment of corneal nerve parameters by two observers on images of the same eye from the same visit (table 1). Amongst the 4 parameters, only NBD showed a significant difference between observers ( $P < 0.0001$ , 95% CI). ICC values were: NFD - 0.82 (Figure 3-3A), NBD - 0.54 (Figure 3-3B), NFL - 0.66 (Figure 3-3C) and TC - 0.93 (Figure 3-3D) and respective CoR values were: NFD - 0.15, NBD - 0.85, NFL - 0.17 and TC - 0.18. The mean difference  $\pm$  SD between the two observers was  $1.1 \pm 3.1$  no./mm<sup>2</sup> (NFD),  $56.0 \pm 39.0$  no./mm<sup>2</sup> (NBD),  $2.7 \pm 2.6$  mm/mm<sup>2</sup> (NFL) and  $0.7 \pm 1.4$  (TC).



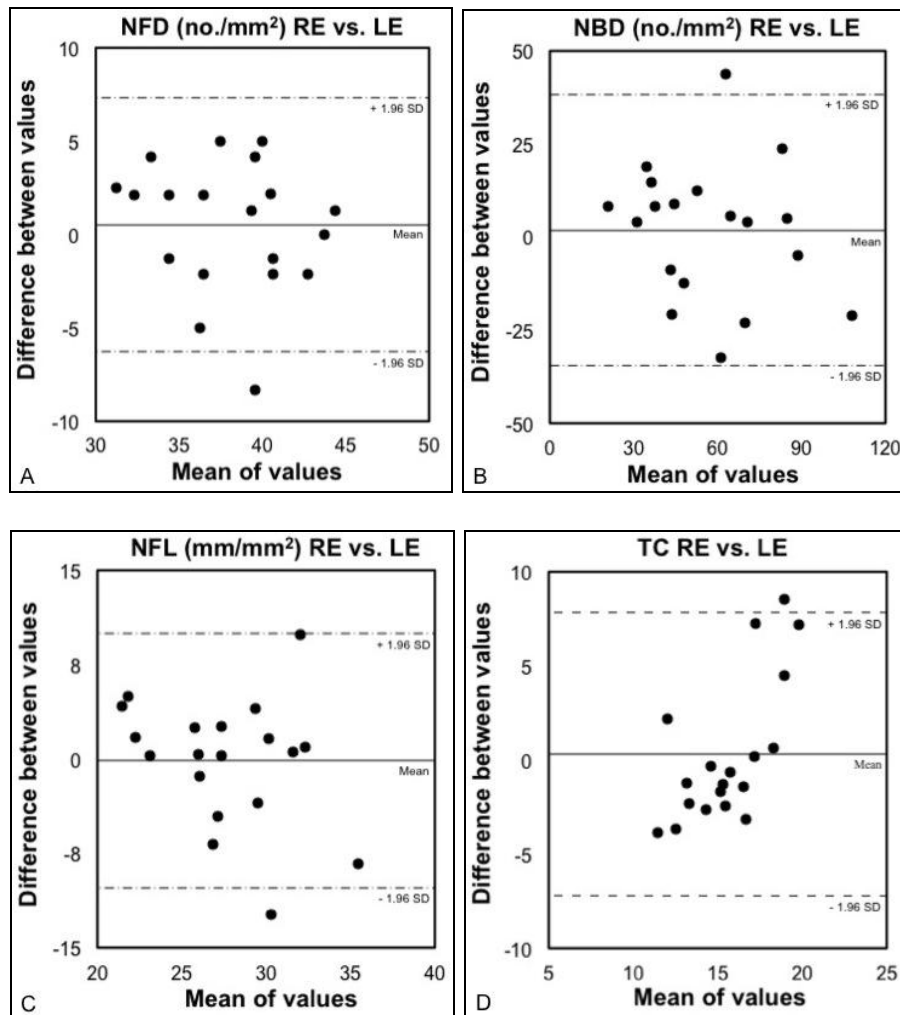
**Figure 3-3** Bland Altman plots for NFD (A), NBD (B), NFL (C) and TC (D) as an indication of agreement between measurements of the same eye (RE), scanned on the same occasion and analysed by two observers OB 1 and OB 2.

### 3.4.3 Symmetry

*Symmetry* of central corneal nerve morphology was assessed in images from the RE and LE, of the same individual, on the same occasion and quantified by the same examiner (table 1). There were no significant differences ( $P > 0.05$ , 95% CI) in corneal nerve morphology between the RE and LE. Calculated ICC values were: NFD - 0.77 (Figure 3-4A), NBD - 0.73 (Figure 3-4B), NFL - 0.45 (Figure 3-4C) and TC - 0.34 (Figure 3-4D). Respectively, CoR were: NFD -



0.17, NBD - 0.63, NFL -0.36 and TC - 0.48. The mean difference  $\pm$  SD between the RE and the LE was  $0.07 \pm 3.9$  no./mm<sup>2</sup> (NFD),  $1.28 \pm 18.1$  no./mm<sup>2</sup> (NBD),  $0.1 \pm 5.0$  mm/mm<sup>2</sup> (NFL),  $0.3 \pm 3.7$  (TC).



**Figure 3-4** Bland Altman plots for NFD (A), NBD (B), NFL (C) and TC (D) as an indication of agreement between the RE and the LE.

**Table 3-1** intra- and inter-observer agreement and symmetry in RE vs. LE

<i>Measurements</i>	<b>NFD</b> (no./mm <sup>2</sup> )	<b>NBD</b> (no./mm <sup>2</sup> )	<b>NFL</b> (mm/mm <sup>2</sup> )	<b>TC</b>
<b>INTRAOBSERVER</b>				
RE VISIT 1	38.3 ± 3.9	58.1 ± 23.0	27.6 ± 4.0	15.8 ± 4.0
RE VISIT 2	38.2 ± 5.0	63.1 ± 21.7	29.1 ± 3.8	15.5 ± 4.6
<b>INTEROBSERVER</b>				
OBSERVER 1	38.2 ± 5.0	63.1 ± 21.7	29.1 ± 3.8	15.5 ± 4.6
OBSERVER 2	38.5 ± 5.4	120.0 ± 51.2	31.7 ± 4.8	14.7 ± 3.8
<b>SYMMETRY</b>				
RE VISIT 1	38.3 ± 3.9	58.1 ± 23.0	27.6 ± 4.0	15.8 ± 4.0
LE VISIT 1	37.8 ± 4.5	56.3 ± 26.4	27.8 ± 5.2	15.5 ± 1.8

\*Results are expressed as mean ± SD

### 3.5 Discussion

The quantification of corneal sub-basal nerves is a rapidly evolving area of special interest to both clinicians and scientists as a surrogate for diagnosing, assessing progression (18) and the benefits of therapeutic intervention in a range of peripheral neuropathies (39). Initial studies provided qualitative evidence of corneal nerve fibre alterations or reported changes in the architecture following surgery (11). In the context of using corneal nerve morphology as a surrogate for peripheral neuropathy, a more clearly defined approach has been developed to quantify four key parameters: NFD, NBD, NFL and TC (18; 28-29).

Whether individual anatomical variations and intra-/ inter-observer consistency influence the results remains unclear. A recent study has shown that NFL has a very high between observer and occasion repeatability in patients with type 2

diabetes (34) whilst another study showed that NFL had the best reproducibility and validity amongst all parameters in controls and patients with type 1 diabetes and suggested development of IVCCM should focus on the measurement of NFL due to its superiority to the other parameters (35). However, quantifying NFL alone limits the interpretation of corneal nerve damage and repair in the context of disease and particularly when assessing repair after treatment of peripheral neuropathy. Therefore we have undertaken a detailed assessment of the repeatability and agreement of the four main parameters we originally developed and applied (18-19; 29) in a range of peripheral neuropathies (22-23; 28).

In this study, corneal nerve fibre morphology showed consistency between the RE and LE. Whilst NFD and NFL achieved the highest values for intra and inter observer repeatability and agreement, NBD and TC showed less consistency. Across all assessments, NBD appeared to be the least repeatable parameter and this finding highlights the importance of accurately defining nerve branches and fibres. The correct identification of nerve branches in IVCCM images is especially difficult and mainly depends on background contrast, image clarity and observer experience and interpretation. In addition, Patel and McGhee showed for the first time a continuous centripetal movement of identifiable branch points in the human corneal subbasal nerve plexus of up to 26 $\mu$ m/week over a 6-week period which may also cause variability (40).

A common finding in IVCCM images is crossing, X-shaped nerve fibres running from the top to the bottom of the image or Y-shaped appearance of a nerve fibre and a branch. In the former case, interpretation is easy and is not

expected to vary between observers. However, in the latter case there is no standardised rule to-date to assist the analyser to correctly define the NF and the NB. Selecting either side to be the NF can affect the outcome, as the TC between nerves of the same individual varies. Individual criteria may include the thickness, the continuous pattern or the reflectivity of the main axon, which differs from that of the NB. In more complicated cases where the pattern is best described by a tree shape (more than one branch stemming from a NF) or an X shape with multiple branches, the variation will clearly increase and this may significantly affect NBD. Hence both NBD and TC have inherent liability for variability in repeated assessment, as this task is highly subjective, especially when different observers undertake the analysis.

Amongst the two most repeatable parameters, NFD was superior to NFL in all measurements. This finding contrasts that of Hertz et al. (35) who found NFL to be the most reliable of all IVCCM nerve parameters. NFL is defined as the sum of the total length of NFs and NBs per frame i.e. all nerve structures and may therefore be ideally used as a pan-corneal marker of peripheral neuropathy. However, high or low NFL does not capture concomitant degeneration and regeneration and may not be as sensitive as NBD hence limiting the interpretation of subbasal corneal nerve repair. Differences in image collection and sampling techniques may also affect the outcome.

The primary purpose of this study was to quantitatively evaluate the repeatability of quantifying sub-basal corneal nerves using IVCCM. Possible limitations of this study are the small sample size and the small area of the cornea chosen for analysis. Therefore, the assessment of IVCCM repeatability in multiple corneal areas should also be established. We have demonstrated

good intra and inter observer repeatability and consistency between RE and LE for NFD and NFL but have identified lower repeatability for NBD and NFT when deploying manual image analysis of corneal nerve fibre morphology. Both the latter parameters are however important to quantify corneal innervation as they add considerably to the interpretation of disease effect for both nerve degeneration and regeneration. The variability observed with the technique may be improved by applying predefined identification rules for the nerve fibres and their branches. A possible solution for both these issues may lie in the development of a fully automated image analysis system (31) which would eliminate inconsistencies, enhance repeatability, markedly reduce the analysis time and hence make IVCCM suitable for clinical practice.

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#### **4. CHAPTER IV-NO DIFFERENCE IN CENTRAL VERSUS ADJACENT PERIPHERAL CORNEAL INNERVATION USING IN VIVO CORNEAL CONFOCAL MICROSCOPY IN DIABETIC NEUROPATHY**

Author's contribution: Ioannis N Petropoulos contributed to the conception and study design, acquired part of the data, analysed data, performed statistical analysis and wrote the manuscript which constitutes the basis for this chapter. To-date this chapter has been submitted for publication.

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## 4.1 Abstract

**PURPOSE** Most studies employing IVCCM to assess DSPN acquire images from the central cornea, however, it is not established whether significant variation between central and adjacent peripheral areas exists.

**METHODS** 20 diabetic patients underwent clinical and metabolic evaluation together with a detailed assessment for severity of DPN. IVCCM was performed to capture images of the sub-basal nerves using two methodologies: a z-axis scan at the corneal apex (constant location) at different depth and a x- and y-axis scan (constant depth) from adjacent superior, temporal, nasal and inferior areas, keeping the depth constant.

**RESULTS** NFD ( $28.3 \pm 9.3$  v  $27.3 \pm 5.3$  no./mm<sup>2</sup>), NBD ( $54.2 \pm 26.8$  v  $67.5 \pm 28.1$  no./mm<sup>2</sup>), NFL ( $22.3 \pm 6.7$  v  $22.2 \pm 5.9$  mm/mm<sup>2</sup>) and the TC ( $18.1 \pm 5.7$  v  $16.8 \pm 4.0$ ) did not differ significantly in images derived from the central compared to adjacent peripheral images. There was a strong correlation between the different corneal nerve parameters assessed using the two different approaches (0.73-0.91,  $P < 0.0001$ ).

**CONCLUSION** Corneal subbasal nerve morphology between the central and adjacent peripheral areas is comparable. However, the central cornea may be more sensitive to pathological alterations in DSPN. Images captured from the corneal apex and adjacent peripheral areas are suitable for longitudinal assessment to define progression or indeed regression following intervention.

## 4.2 Introduction

Corneal confocal microscopy has rapidly established itself in the assessment of ocular and systemic disease. Research has shown that corneal sub-basal nerves reflect the integrity of somatic nerves in diabetic (1-3) and other peripheral neuropathies (4-6). In our earlier studies we have also shown that it can be used to assess the benefits of improved glycaemic control after SPK (7-8) as well as an improvement in risk factors associated with diabetic neuropathy (9). An association between corneal nerve alterations and previous glycaemic exposure (10), blood pressure (11), serum triglycerides and cholesterol (9) has also been reported.

Evaluation of DSPN using IVCCM shows high intra- and inter-observer and between occasion repeatability (12-15) and a recent report has proposed an image sampling methodology to accurately quantify the subbasal nerves (16). Alterations in corneal innervation have been most commonly assessed by acquiring images from the corneal apex with a z-axis scan. However, a study by Patel and McGhee (17) demonstrated that corneal nerves are arranged in a complex network that varies in the central compared to more peripheral areas of the cornea, a finding also supported by in vitro observations (18). Patel et al. (19) in another study using IVCCM reported moderate differences in NFD and tortuosity between nerves located centrally and the peripheral cornea.

However, it is not clear whether the variability between these areas will impact on the assessment of corneal nerve morphology, especially in longitudinal studies defining progression or indeed the benefits of therapeutic intervention. Given the rapid increase in the use of IVCCM as a diagnostic test for

peripheral neuropathy, defining the optimal image acquisition methodology is essential. We have therefore quantified whether the central and adjacent superior, temporal, nasal and inferior areas combined (depth constant-x and y axis scan) and a z-axis scan on the corneal apex (location constant) do or do not differ significantly in diabetic patients with neuropathy.

### **4.3 Methods**

#### **4.3.1 Study subjects**

Twenty subjects (10 males and 10 females) with diabetes mellitus underwent clinical, metabolic evaluation and an assessment for DPN based on the updated Toronto criteria (20). Informed written consent was obtained from all subjects. This study was approved by the North Manchester Research ethics committee and adhered to the tenets of the Declaration of Helsinki. Exclusion criteria were a history of corneal abrasion, ocular operations, systemic or ocular disease known to affect the cornea and non-diabetic causes of neuropathy.

#### **4.3.2 Clinical and peripheral neuropathy assessment**

All study participants underwent assessment of BMI, HbA<sub>1c</sub>, lipids and detailed assessment for DSPN based on the NDS (21), VPT using a Neuroesthesiometer (Horwell, Scientific Laboratory Supplies, Wilfrod, Nottingham, UK) and NCS (PMNCV and PMNamp) using a Dantec “Keypoint” system (Dantec Dynamics Ltd, Bristol, UK) equipped with a DISA temperature regulator to keep limb temperature constantly between 32-35°C.

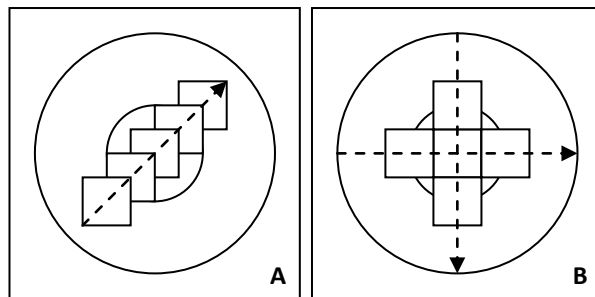
### **4.3.3 In vivo corneal confocal microscopy**

All study subjects were scanned with a laser IVCCM (HRT III RCM) (Heidelberg Engineering GmbH, Heidelberg, Germany) which uses a 670 nm wavelength helium neon diode laser, a class I laser which does not pose any ocular safety hazard. A 63x objective lens with a numerical aperture of 0.9 and a working distance, relative to the applanating cap (TomoCap<sup>®</sup>, Heidelberg Engineering GmbH, Heidelberg, Germany) of 0.0 to 3.0 mm was used. The size of each two-dimensional image produced was 384  $\mu\text{m}$  x 384  $\mu\text{m}$  which has a 15° x 15° field of view and 10  $\mu\text{m}$ /pixel transverse optical resolution. The HRT III RCM uses a digital image capture system and all images are stored in an external hard drive. A drop of 0.4% benoxinate hydrochloride (Chauvin Pharmaceuticals, Chelfaro, UK) was used to anaesthetise each eye and Viscotears (Carbomer 980, 0.2%, Novartis, UK) were used as the coupling agent between the cornea and the applanating cap. All subjects were asked to fixate on an outer fixation light throughout the IVCCM scan and a CCD camera was used to image the cornea and correctly position the applanating cap. The overall examination took approximately 5 minutes for both eyes of each subject and for this study a highly experienced examiner performed all IVCCM scans.

### **4.3.4 Image acquisition and analysis**

Two different techniques were employed to capture images using the “section” mode of the HRT III RCM. Images were acquired from the corneal apex (centre) with a z-axis scan during which the location was kept constant with a

change in depth, capturing 1 image every 1 $\mu$ m, starting from the point where subbasal nerves become visible (typically at ~50-70 $\mu$ m), and beyond (Figure 4-1A). Six images from both eyes were quantified. For comparing central v adjacent peripheral regions, images were captured with an x and y-axis scan at a constant depth (Figure 4-1B). Specifically, the applanating cap was placed on the corneal apex and the lens was focused at the depth where the subbasal nerves were optimally visualized and an image was captured. The examiner then moved the lens from the center in 4 directions keeping the depth constant: superior, inferior, nasal and temporal. 10 non-overlapping images from both eyes were used for analysis.



**Figure 4-1** Schematic representation of IVCCM scanning modes: images were generated with (A) a z-axis scan (constant location at ~50-70  $\mu$ m) and (B) x and y-axes scan (constant depth)

In total, 320 images were manually analysed by the same examiner who was masked to the eye, location of the image and patient details, using purpose-built software (CCMetrics (22), M.A.Dabbah, ISBE, University of Manchester, Manchester, UK). Corneal nerve morphology was quantified according to our previously established methodology (14) for NFD (no./mm<sup>2</sup>), NBD (no./mm<sup>2</sup>), NFL (mm/mm<sup>2</sup>) and TC. NFD is defined as the total number of main nerve fibres (NF) per frame divided by the area of the frame in mm<sup>2</sup> (area =

0.16033585 mm<sup>2</sup>) (Figure 1). NBD is defined as the total number of main nerve branches (NB) (strictly nerve branches which stem from a NF) divided by the area of the frame. NFL is the total length of NFs, NBs as well as secondary nerve branches (nerve branches which stem from a NB) per frame. TC is a mathematical computation of the NF tortuosity as previously described by Kallinikos et al. (23), which is unit-less and independent of the angle of the nerve in the image. A straight nerve equals a TC of zero and increases with increasing curvature of the NF.

#### **4.3.5 Statistical Analysis**

Data were analysed using StatsDirect statistical software (StatsDirect, version 2.7.7, StatsDirect Ltd., Cheshire, UK) and graphs were generated using Excel (Microsoft Corporation, USA). Differences between the two methodologies were assessed with a paired-t test or a Mann-Whitney U test depending on the distribution of the data. Two-way analysis of variance (ANOVA) or its non-parametric counterpart was used to assess differences between different corneal locations (superior vs inferior vs temporal vs nasal vs central). Finally, the intra-class correlation coefficient (ICC) was calculated to assess the agreement between the two methods across all parameters and plots were generated as first described by Bland and Altman (24).

#### **4.4 Results**

20 diabetic patients with signs of peripheral neuropathy were studied and results are presented below.



#### **4.4.1 Clinical and peripheral neuropathy assessment**

All participants in this study were confirmed to have diabetic neuropathy as defined by the Toronto Diabetic Neuropathy Expert Group (20). Participants had increased body mass index ( $29.4 \pm 5.0 \text{ Kg/m}^2$ ), good glycaemic control ( $\text{HbA1c} = 7.0 \pm 1.8 \%$ ), slightly elevated total cholesterol ( $5.0 \pm 0.9 \text{ mmol/l}$ ) and serum triglyceride levels ( $2.2 \pm 1.4 \text{ mmol/l}$ ). The NDS ( $2.1 \pm 2.8$ ), VPT ( $14.1 \pm 10.8 \text{ V}$ ), PMNCV ( $45.1 \pm 5.7 \text{ m/s}$ ), PMNamp ( $4.1 \pm 1.5 \text{ uV}$ ) were consistent with minimal diabetic neuropathy.

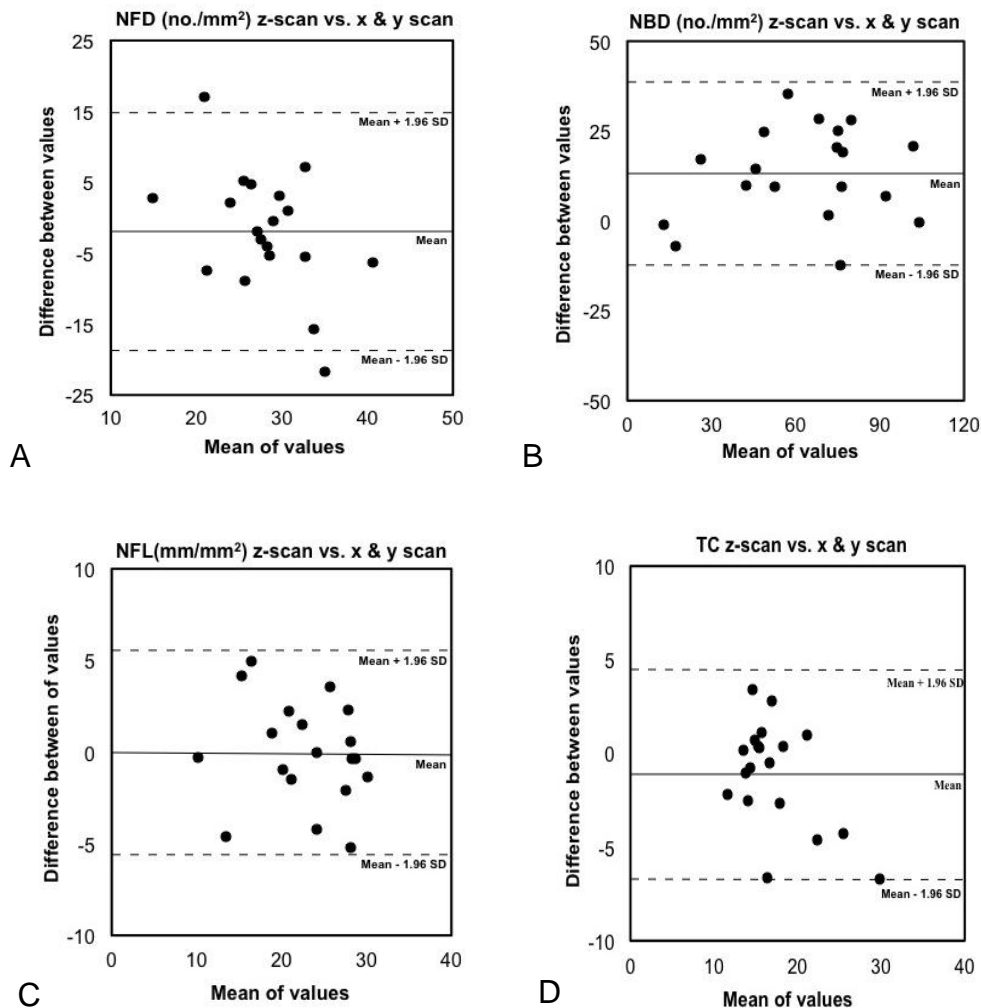
#### **4.4.2 Comparison between two IVCCM scanning methodologies to quantify corneal subbasal nerves**

There was no significant difference for NFD ( $P > 0.05$ ), NBD ( $P > 0.05$ ), NFL ( $P > 0.05$ ) and TC ( $P > 0.05$ ) between the two different acquisition methods (Table 1). There was high agreement between NBD, (ICC = 0.77) NFL (ICC = 0.89) and TC (ICC = 0.76), but poor agreement for NFD (ICC = 0.23) (Table 4-1 and Figure 4-2). The correlation coefficients NBD ( $r = 0.87$ ,  $P < 0.0001$ ), NFL ( $r = 0.91$ ,  $P < 0.0001$ ) and TC ( $r = 0.74$ ,  $P < 0.0001$ ). NFD ( $r = 0.21$ ,  $P > 0.05$ ), did not show a strong correlation between the two methods. This is in accord with in-vitro (18) and in-vivo (19) studies which found that peripheral innervation was sparser although more extreme peripheral sites were assessed.

**Table 4-1** Parameters estimated with a z-axis/constant location and x- and y-axis scan/constant depth

<i>Parameter</i>	<b>Z-axis scan</b>	<b>X- &amp; Y- axis scan</b>	<b>P Value</b>	<b>ICC</b>	<b>Spearman's <i>r</i></b>
<b>NFD (no./mm<sup>2</sup>)</b>	28.3 ± 9.3	27.3 ± 5.3	<i>P</i> > 0.05	0.23	0.21 ( <i>P</i> >0.05)
<b>NBD (no./mm<sup>2</sup>)</b>	54.2 ± 26.8	67.5 ± 28.1	<i>P</i> > 0.05	0.77	0.87 ( <i>P</i> <0.0001)
<b>NFL (mm/mm<sup>2</sup>)</b>	22.2 ± 6.7	22.2 ± 5.9	<i>P</i> > 0.05	0.89	0.91 ( <i>P</i> <0.0001)
<b>TC</b>	18.1 ± 5.7	16.8 ± 4.0	<i>P</i> > 0.05	0.76	0.74 ( <i>P</i> <0.0001)

\*Results are expressed as mean ± SD



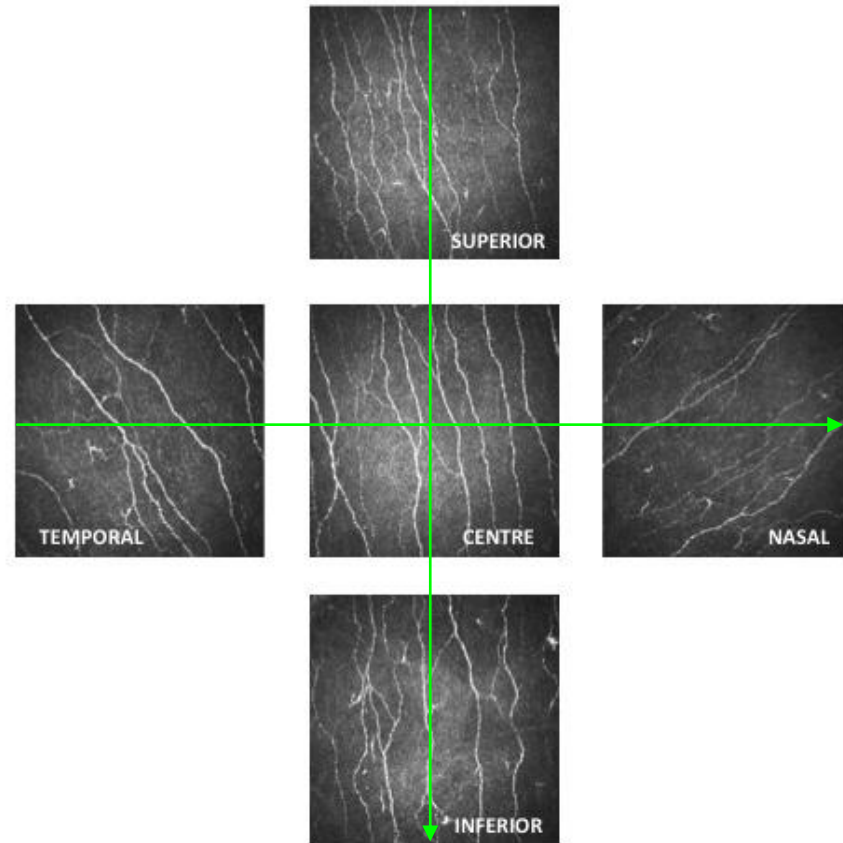
**Figure 4-2** Agreement plots between two methodologies to quantify corneal nerve morphology for NFD (A), NBD (B), NFL (C) and TC (D) as described by Bland and Altman (24).

#### 4.4.3 Variation by anatomical site

Central corneal nerve morphology estimated in an image of the corneal apex was compared individually with each of the adjacent peripheral sites (Figure 4-3). There was no significant difference between NFD, NBD, NFL and TC between peripheral and central sites, although the central cornea had the highest NFD, longest NFL and highest tortuosity. Amongst the peripheral areas examined, NFD was highest in the nasal cornea and lowest in the superior cornea. The inferior cornea contained the highest number of branches between all sites examined. NFL remained comparable in peripheral regions. Nerves in the central, superior and nasal areas were equally tortuous while the least tortuous nerves were found in the temporal and inferior cornea. Finally, nerves were almost vertically arranged centrally and tilted at an almost 140°-160° orientation at nasal and temporal sites. There were no differences related to orientation in superior and inferior sites (Table 4-2).

**Table 4-2** NFD, NBD, NFL and TC arranged by corneal site examined

	Temporal	Inferior	Nasal	Superior	Center
<b>NFD (no./mm<sup>2</sup>)</b>	27.4 ± 8.3	27.8 ± 7.5	28.6 ± 9.2	26.3 ± 10.7	29.6 ± 10.5
<b>NBD (no./mm<sup>2</sup>)</b>	59.7 ± 33.1	79.8 ± 50.7	71.7 ± 40.7	61.2 ± 30.4	64.8 ± 36.2
<b>NFL (mm/mm<sup>2</sup>)</b>	21.3 ± 7.3	22.7 ± 7.4	22.1 ± 6.9	20.9 ± 7.4	24.1 ± 6.7
<b>TC</b>	15.5 ± 4.8	16.8 ± 8.3	17.2 ± 8.4	17.2 ± 8.2	17.7 ± 5.1



**Figure 4-3** Image representation of a x- and y- axis scan from a patient with diabetes. The comparable densities of the nerves at all five locations are noticeable.

#### **4.5 Discussion**

Developments in corneal confocal microscopy have allowed high contrast digital imaging of the cornea in vivo and this has led to its adoption for clinical use in a number of different areas. The quantification of corneal sub-basal nerves is of special interest to both clinicians and scientists due not only to their importance in regulating epithelial cell integrity and their role in corneal wound healing in a number of ophthalmic conditions (25) but also increasingly as a surrogate for diagnosing, assessing progression and perhaps therapeutic

intervention in diabetes (1-2; 7) and in a range of peripheral neuropathies (4-6).

Quantification of corneal nerve fibre morphology has been undertaken by defining a range of morphological abnormalities including the density and length of nerves (1) as well as potentially pathologic alterations such as beading (26). In a study of the normal central and peripheral cornea, only the extreme peripheral locations differed significant in sub-basal nerve density and tortuosity, particularly in the superior, inferior and nasal mid-periphery (19). Previous studies have also adopted a qualitative approach to describe changes and define whether nerves are present, shorter or longer, following vitrectomy (27), whilst others have assessed the orientation and pattern of nerves and their appearance following laser in situ keratimileusis and penetrating keratoplasty (28). A more clearly defined quantitative approach has also been developed to identify the four key parameters of NFD, NBD, NFL and NFT (1; 23).

Amongst these established parameters, NFL has been found to have superior diagnostic validity for longitudinal assessment of DPN (29). NFL accounts for the length of all branches and fibres in a single frame and hence may serve as a pan-corneal marker of neuropathic alterations, while NFD and NBD focus entirely on nerve fibres and branches respectively. Both NFD and NFL have shown high agreement between eyes, occasions and observers whilst NBD and TC were found to be less reproducible (12-14). One of the widely acknowledged disadvantages of confocal microscopy is the relatively small field of view (40 $\mu$ m x 40 $\mu$ m) that allows only a small area of the cornea to be investigated. This limitation can be overcome by moving the IVCCM over a

stationary specimen but no study has assessed whether this would add significantly to the information we acquire through a z-in depth-scan of the central cornea as previous studies have done (1; 4-6; 14; 29).

The key to employing IVCCM in longitudinal studies is to ensure that corneal nerve morphology is comparable both at different depths and in adjacent peripheral areas. We have therefore undertaken a study to determine whether by using an alternative image sampling technique, which combines central and adjacent peripheral areas we detect more extensive damage of the subbasal nerves to what is already observed centrally. Our results show that both methodologies have comparable findings for NBD, NFL and TC. However, NFD showed poor correlation between the two methodologies despite a lack of significant difference. We found a marked reduction in central NFD in three cases which was not captured in the x- and y-axis scan using central and peripheral images combined. It suggests that the central cornea may be more sensitive to detect corneal nerve fibre loss in diabetic neuropathy. There was no significant difference between peripheral and central areas when images obtained with a x- and y-axis scan were compared with each other. Sample size, mild neuropathy and the lack of a control group limits generalisation of our findings, particularly in patients with more advanced peripheral neuropathy. Therefore, further research is required to establish this relationship in different neuropathic severity and also investigate the effect of age on this relationship. In conclusion, we show that the assessment of central corneal nerve pathology visualised with an in depth scan compared to a scanning methodology which includes adjacent peripheral areas in addition to central using IVCCM is comparable in diabetic patients with mild neuropathy. This therefore provides

confidence in the image acquisition approach to focus on the central cornea in longitudinal studies because of the relatively high inter- and intra-individual reproducibility of the scanning pattern and accuracy image sampling methodology.

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## **5. CHAPTER V-AUTOMATIC ANALYSIS OF DIABETIC PERIPHERAL NEUROPATHY USING MULTISCALE QUANTITATIVE MORPHOLOGY OF NERVE FIBRES IN CORNEAL CONFOCAL MICROSCOPY IMAGING**

Author's contribution : Ioannis N Petropoulos contributed to the study design, data acquisition, data analysis and revision of the manuscript. This chapter was published in the journal *Medical Image Analysis*.

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## 5.1 Abstract

Diabetic peripheral neuropathy is one of the most common long term complications of diabetes. In vivo corneal confocal microscopy (IVCCM) image analysis is a novel non-invasive technique which quantifies corneal nerve fibre damage and enables diagnosis of DPN. This paper presents an automatic analysis and classification system for detecting nerve fibres in IVCCM images based on a multi-scale adaptive dual-model detection algorithm. The algorithm exploits the curvilinear structure of the nerve fibres and adapts itself to the local image information. Detected nerve fibres are then quantified and used as feature vectors for classification using random forest (RF) and neural networks (NNT) classifiers. We show, in a comparative study with other well known curvilinear detectors, that the best performance is achieved by the multi-scale dual model in conjunction with the NNT classifier. An evaluation of clinical effectiveness shows that the performance of the automated system matches that of ground-truth defined by expert manual annotation.

## 5.2 Introduction

According to numerous clinical reports (1) diabetes is amongst the most challenging chronic health problems. For example, in the UK it is estimated that one in twenty people has diabetes, whether diagnosed or undiagnosed, and by 2025 four million people will have the condition. Damage to the peripheral nerves is one of the commonest long-term complications of diabetes occurring in at least 50% of patients with diabetes (2). As a consequence, about one in six diabetic patients have chronic painful neuropathy compared to one in 20 non-diabetic subjects (3). It is the main initiating factor for foot ulceration, Charcot's neuroarthropathy and lower extremity amputation. As 80% of amputations are preceded by foot ulceration, an effective means of detecting and treating neuropathy would have a major medical, social and economic impact. The development of new treatments to slow, arrest or reverse this condition is of paramount importance but is presently limited due to difficulties with end points employed in clinical trials (4). Therefore accurate detection and quantification of DPN are important to define at-risk patients, anticipate deterioration, and assess new therapies. Current methods are unsatisfactory, lacking sensitivity and requiring expert assessment, and focus only on large fibres (neurophysiology) or are invasive (skin/nerve biopsy). Diabetic neuropathy lacks a non-invasive surrogate for nerve damage (5). Recent research (6-8) using IVCCM suggests that this non-invasive, and hence reiterative, test might be an ideal surrogate endpoint for human diabetic neuropathy. The establishment of IVCCM as a surrogate for early diagnosis and an early biomarker for diabetic neuropathy could identify those at risk and

prompt more intense intervention including improved glycaemic, blood pressure and lipid control. Furthermore a sensitive surrogate endpoint would significantly lower hurdles to the development of disease-modifying therapeutics by enhancing the capacity to test therapeutic efficacy. The major advantage of IVCCM is the entirely non-invasive and relatively rapid (2 minutes) acquisition of images of small nerve fibres in patients. However, the major limitation preventing extension of this technique to wider clinical practice is that analysis of the images using interactive image analysis is highly labour-intensive and requires considerable expertise to quantify nerve pathology. To be clinically useful as a diagnostic tool, it is essential that the measurements be extracted automatically.

If an automatic IVCCM image analysis system is to be applied clinically, especially to define early degeneration or regeneration, then a key step is the automatic detection of low-contrast nerve fibres amongst image noise Figure 5-1. The literature on this topic is not extensive, although the problem has a superficial similarity to other, more widely investigated, applications, such as detection of blood-vessels in retinal images. Ruggeri et al. (9) and Scarpa et al. (10) describe a heuristic method that was adapted from retinal analysis. A number of methods have been developed to enhance the contrast of such linear structures. In a previous study (11), we used the 2D Gabor (12) filter to detect nerve fibres in IVCCM images. The filter is a band-pass filter that consists of a sinusoidal plane wave with a certain orientation and frequency, modulated by a Gaussian envelope. This spatial domain enhancement is based on the convolution of the image with the even-symmetric Gabor filter

that is tuned to the local nerve-fibre orientation. We subsequently extended this to form a dual-model detector (13), see section 5.5.

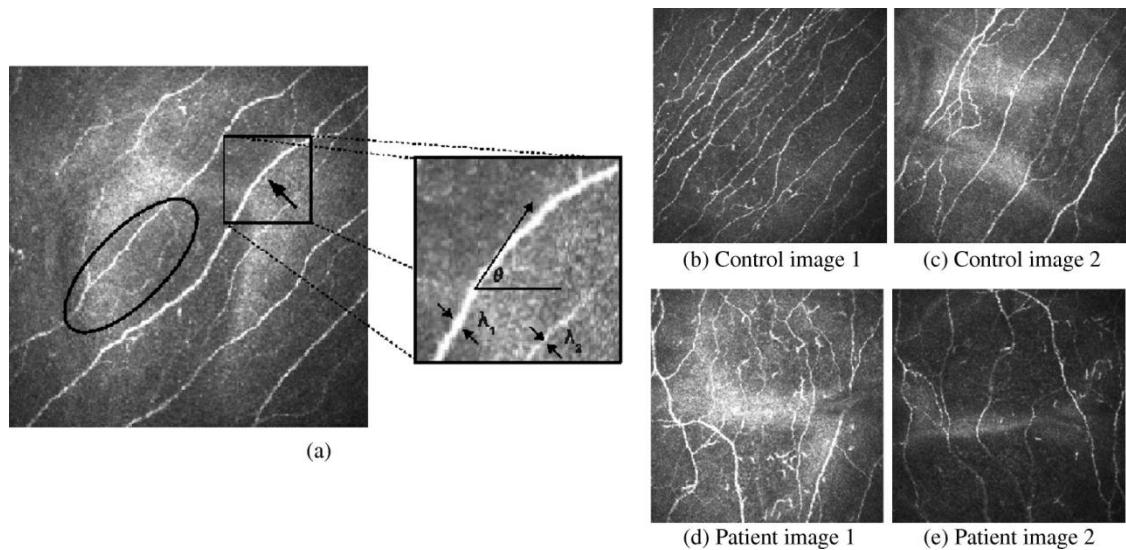
The automated system of analysing IVCCM images presented in this paper is an extension of our previous single scale dual-model fibre detector (13) (see appendix 1). The new detection algorithm uses the dual-model property in a multi-scale framework to generate feature vectors from localised information at every pixel. These vectors are then used to classify pixels using RFs (14) and NNTs (15).

In the remainder of the paper we introduce IVCCM imaging, the image characteristics and the metrics that have been used to quantify the nerve morphology by interactive image analysis (Sections 5.3 and 5.4). We describe the single-scale dual model filter (13) and its extension to multiple scales with pixel classification (Sections 5.5-5.7). In Section 5.8 we describe a comparative evaluation showing the improved performance of the multi-scale version over not only the single-scale filter but a number of other multi-scale detectors. We also demonstrate that the automatically detected fibres result in morphometric features equivalent to those generated by expert interactive analysis.

### **5.3 Corneal Confocal Microscopy**

The cornea is one of the body's most innervated tissues. The sub-basal nerve plexus runs parallel to the surface of the cornea in the Bowman's membrane, lying between the outer epithelial layer and the stroma. Bowman's layer is about 8–12  $\mu\text{m}$  thick, and the nerves may be imaged by confocal microscopy

using either a white-light source or a laser source. In this study laser confocal microscopy was used. Typical images are shown in Figure 5-1.



**Figure 5-1** (a) an example of an IVCCM image and nerve-fibre characteristics. (b)(e) Samples of IVCCM images from controls and patients, showing the effects of different imaging artifacts and neuropathy status.

### 5.3.1 IVCCM for imaging diabetic peripheral neuropathy

Recent studies suggest that small unmyelinated c-fibres may be the earliest to be damaged in diabetic neuropathy (16-18). The only techniques which allow a direct examination of unmyelinated nerve fibre damage are those of sural nerve biopsy with electron microscopy (18-19), and the skin-punch biopsy (20-22), but both are invasive procedures. However, our previous studies in patients with diabetic neuropathy have shown that IVCCM can be used to quantify early small nerve fibre damage and accurately quantify the severity of diabetic neuropathy (6-7). Moreover, we have shown that corneal nerve damage assessed using IVCCM relates to the severity of IENF loss in foot skin biopsies (23) and the loss of corneal sensation (24) in diabetic patients. IVCCM also detects early nerve fibre regeneration following SPK in diabetic



patients (25). Recently we have also shown that IVCCM detects nerve fibre damage in patients with Fabry disease (26) and idiopathic small fibre neuropathy (27) in the presence of normal electro-physiology and QST. CCM offers considerable potential as a surrogate marker, and hence as an endpoint for clinical trials in diabetic neuropathy.

### **5.3.2 Nerve fibre quantification**

Nerve fibres in IVCCM images appear as bright linear structures that flow in a predominant direction everywhere. However nerve fibres have their independent local orientation  $\theta$  Figure 5-1. They also have different dimensions of length and diameter  $\lambda$ . Longer nerve fibres with larger diameter are considered to be the main trunks while nerve fibres branching from the main trunks are considered to be secondary nerve fibres (or nerve branches) as shown in the square and the ellipse of Figure 5-1 respectively.

Previous analyses of IVCCM images have used manual delineation of the nerve fibres by experts (6-8). These studies have shown promising results in distinguishing control and patient groups using features such as NFL, NFD, NBD and tortuosity (NFT) of nerve fibres. Abnormal subjects usually have fewer nerve fibres than normal subjects and more tortuous structures as shown in Figure 5-1. This in turn affects the quantified metric that may provide a diagnosis of the neuropathy.

NFL, which we return to in Section 5.8, is defined as the total length of all nerve fibres visible in the IVCCM image per square mm. The total length is computed by tracing all the nerve fibres and nerve-branches in the image. This

number is then divided by the area of the field-of-view provided by the microscope to produce the NFL ( $\text{mm}/\text{mm}^2$ ) value.

### **5.3.3 Artefacts**

Although the process of capturing the images is relatively short and quick, saccadic eye movement is faster, which could result in motion or blurring effects of the nerve fibres. As shown in the image samples of Figure 5-1, the nerve fibres may also appear very faint due to differences of depth. The same nerve fibre could appear and disappear several times as it moves in and out of the focus plane. This movement will also affect the visual diameter and the brightness of the fibre. Since the cornea is a transparent spherical structure, illumination artefacts arise that result in low-frequency variation in image brightness and contrast. As shown in Figure 5-1d, CCM images also contain small bright structures (usually cells) that are not nerve fibres, which add to the challenge of identifying nerve fibres.

### **5.4 Linear-structure and feature detection**

Detection of curvilinear structures is a requirement in several applications of medical image analysis. A method of linear structure detection [Line Operator (LinOp)], originally developed for detection of asbestos fibres (28) has also been shown to be effective in detecting ducts in mammograms (29). LinOp exploits the linear nature of the structures to enhance their contrast by computing the average intensity of pixels lying on a line passing through the reference pixel for multiple orientations and scales. The largest values are chosen to correspond to the line, the strength of which is determined by the

difference with the average intensity of the similarly oriented square neighbourhood. Frangi et al. (30) used a multiscale decomposition of the Hessian matrix to detect and measure blood vessels in Digital Subtraction Angiography images. They derived a discriminant function based on the eigen values and eigen vectors that has maximum response for tube-like structures. The external energy is used to attract the curve towards points which have a high likelihood of lying on a central vessel axis.

The Monogenic signal (31) is a 2-dimensional (2D) generalization of the analytic signal, widely used in time-domain signal processing. There are several possible ways of extending this approach to multiple dimensions. The Monogenic signal approach makes use of the Riesz transform, and results in separating the signal into local amplitude (or “structure” corresponding approximately to image intensity) and local phase (corresponding to local changes). It has been used in extracting structure information (such as edge and ridge) from images in several medical image analysis applications (32-33). In a preliminary study (11) we used the 2D Gabor filter (12) to detect nerve fibres in IVCCM images. This spatial domain enhancement is based on the convolution of the image with the even-symmetric Gabor filter that is tuned to the local nerve-fibre orientation.

### **5.5 Single scale dual model enhancement**

All of the methods described in 5.4 are potential means of enhancing the linear nerve structures in the face of the image corruption outlined in Section 5.3.3. In Dabbah et al. (13) we reported on the performance of the single-scale dual-model detector in comparison with these methods. We showed that the

detectors specifically designed for detection of linear structures performed better than more general feature detectors, such as the monogenic filter. In particular the single-scale dual-model detector was superior to all of them. In this section we briefly describe the algorithm.

### 5.5.1 Nerve fibre contrast enhancement

The dual model consists of separate models of foreground and background, which adapt to local image conditions to cope with slowly varying illumination artefacts. The foreground model  $M_F$  is an even-symmetric and real-valued Gabor (12; 34) wavelet and the background model  $M_B$  is a 2D Gaussian envelope.

$$\mathcal{M}_F(x_\theta, y_\theta) = \left( \cos \left( \frac{2\pi}{\lambda} x_\theta + \phi \right) \right) \cdot \exp \left\{ -\frac{1}{2} \left( \frac{x_\theta^2}{\sigma_x^2} + \frac{y_\theta^2}{\sigma_y^2} \right) \right\} \quad (1)$$

$$\mathcal{M}_B(x_\theta, y_\theta) = \alpha \exp \left\{ -\frac{1}{2} \left( \frac{x_\theta^2}{\sigma_x^2} + \frac{y_\theta^2}{\sigma_y^2} \right) \right\} \quad (2)$$

$$x_\theta = x \cos \theta + y \sin \theta \quad (3)$$

$$y_\theta = -x \sin \theta + y \cos \theta \quad (4)$$

The  $x$  and  $y$  axes of the dual-model coordinate frame  $x_\theta$  and  $y_\theta$  are defined by a rotation of  $\theta$ , which is the dominant orientation of the nerve fibres in a particular region within the image (see Section 5.5.2).  $\lambda$  and  $\phi$  are the wavelength and the phase of the sinusoidal signal modulated by the 2D Gaussian envelope with  $x$  axis variance  $\sigma_x^2$  and  $y$  axis variance  $\sigma_y^2$ . The

aspect ratio of the Gaussian kernel is defined by  $\gamma$  and its magnitude is  $\alpha$ . This

dual-model is used to generate the positive response  $R_P = M_F + M_B$  and the negative response  $R_N = M_F - M_B$  that are applied to the original IVCCM image and can be represented as in equations (5) and (6) respectively.

$$\mathcal{R}_P(x_\theta, y_\theta) = \left[ \cos\left(\frac{2\pi}{\lambda}x_\theta + \phi\right) + \alpha \right] \cdot \exp\left\{-\frac{1}{2}\left(\frac{x_\theta^2}{\sigma_x^2} + \frac{\gamma^2 y_\theta^2}{\sigma_y^2}\right)\right\} \quad (5)$$

$$\mathcal{R}_N(x_\theta, y_\theta) = \left[ \cos\left(\frac{2\pi}{\lambda}x_\theta + \phi\right) - \alpha \right] \cdot \exp\left\{-\frac{1}{2}\left(\frac{x_\theta^2}{\sigma_x^2} + \frac{\gamma^2 y_\theta^2}{\sigma_y^2}\right)\right\} \quad (6)$$

The equations of  $R_P$  and  $R_N$  assume that the Gaussian envelopes of both responses are identical, i.e. they have the same variances  $\sigma_{(x,y)}^2$  and the same aspect ratio  $\gamma$ . The magnitude of the Gaussian envelope  $\alpha$  defines the threshold in which a nerve fibre can be distinguished from the background image. The value of  $\alpha$  can be set empirically to control sensitivity and accuracy of detection. The wavelength  $\lambda$  defines the frequency band of the information to be detected in the IVCCM image. Its value might be computed for a sub-region within the image that has significant variability of nerve-fibre width. However for simplicity,  $\lambda$  is chosen to be a global estimate of the entire image based on empirical results.

This in turn enhances the nerve fibres that are oriented in the dominant direction, and decreases anything that is oriented differently by increasing the contrast between the foreground and the noisy background, whilst effectively reducing noise around the nerve-fibre structure as shown in Figure 5-2. This pixel-wise operation adjusts the models to suit the local neighbourhood

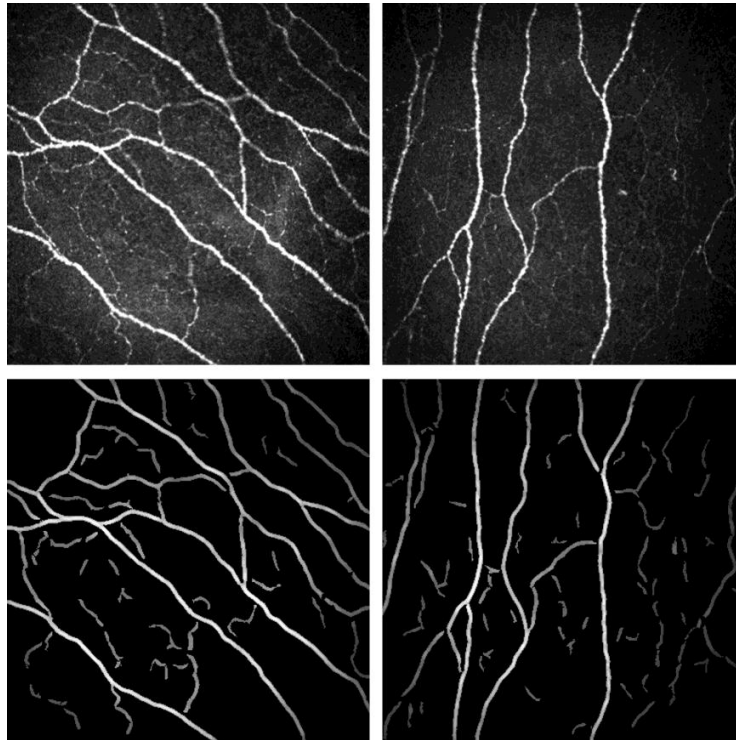
characteristics of the reference pixel at  $I(i, j)$  by modifying the parameters of the foreground and background models. The dot products of the models and the reference pixel's neighbourhood [Equations (7) and (8)] are then combined to generate the final enhanced value of this particular reference pixel  $g^{(i, j)}$  [Equation (9)].

$$\Gamma_p^{(i,j)} = \langle \mathbf{I}_\omega(i, j), \mathcal{R}_P \rangle \quad (7)$$

$$\Gamma_n^{(i,j)} = \langle \mathbf{I}_\omega(i, j), \mathcal{R}_N \rangle \quad (8)$$

$$\mathbf{g}^{(i,j)} = \frac{\Gamma_p^{(i,j)}}{1 + e^{(-2k\Gamma_n^{(i,j)})}} \quad (9)$$

The neighbourhood area,  $I_\omega(i, j)$  of the reference pixel  $(i, j)$  is defined by the width  $\omega$ .  $R_P$  and  $R_N$  are the responses from Equations (5) and (6).  $\langle . . . \rangle$  is the dot product operator. The sharpness of the transition of the enhanced image value at a particular pixel  $g^{(i, j)}$  is controlled by  $k$ . A larger  $k$  amounts to a sharper transition when  $\Gamma_n = 0$ .



**Figure 5-2** an illustration of the single-scale dual-model detector (Dabbah et al., 2010). The images in the top row are the original IVCCM images, and their response is shown in the bottom row.

### **5.5.2 Nerve fibre orientation estimation**

In CCM images, the nerve fibres flow in locally consistent orientations. In addition, there is a global orientation that dominates the general flow. This orientation field describes the coarse structure of nerve fibres. Using the least mean square (LMS) algorithm (35) the local orientation of the block centred at a certain pixel is computed as in Rao (36). Since the orientations vary at a slow rate, a low-pass Gaussian filter is applied globally in order to further reduce errors at near-nerve fibre and non-nerve fibre regions. The LMS produces a stable smooth orientation field in the region of the nerve fibres; however when applied on the background of the image, i.e. between fibres, the estimate is dominated by noise due to the lack of structure and uniform direction.

### **5.5.3 Nerve fibre extraction**

The response image is a map of the confidence at each pixel that it corresponds to a nerve fibre. The sharp transition of the dual model between background and foreground has resulted in useful characteristics in the response image, Figure 5-2. Well-defined nerve fibres are more likely to appear as connected structures, while noise and small undesired curvilinear structures will also be detected but usually manifested as ill-defined and disoriented small fragments. This makes the extraction of nerve fibres a trivial task, and the separation of noise and information becomes easier in the post-processing stages.

The coordinates of each detected nerve fibre are considered to be the central pixel along the width of the detected objects that appear as thick ridges flowing across the image. Hence, after the noise (small fragments) is removed in post-processing, the response images are converted to binary images using a global threshold. The remaining large fragments represent the detected nerve fibres and are thinned using the method of Zhang and Suen (37) to obtain the skeleton image (i.e. the one-pixel wide line).

### **5.6 Multi-resolution dual-model enhancement**

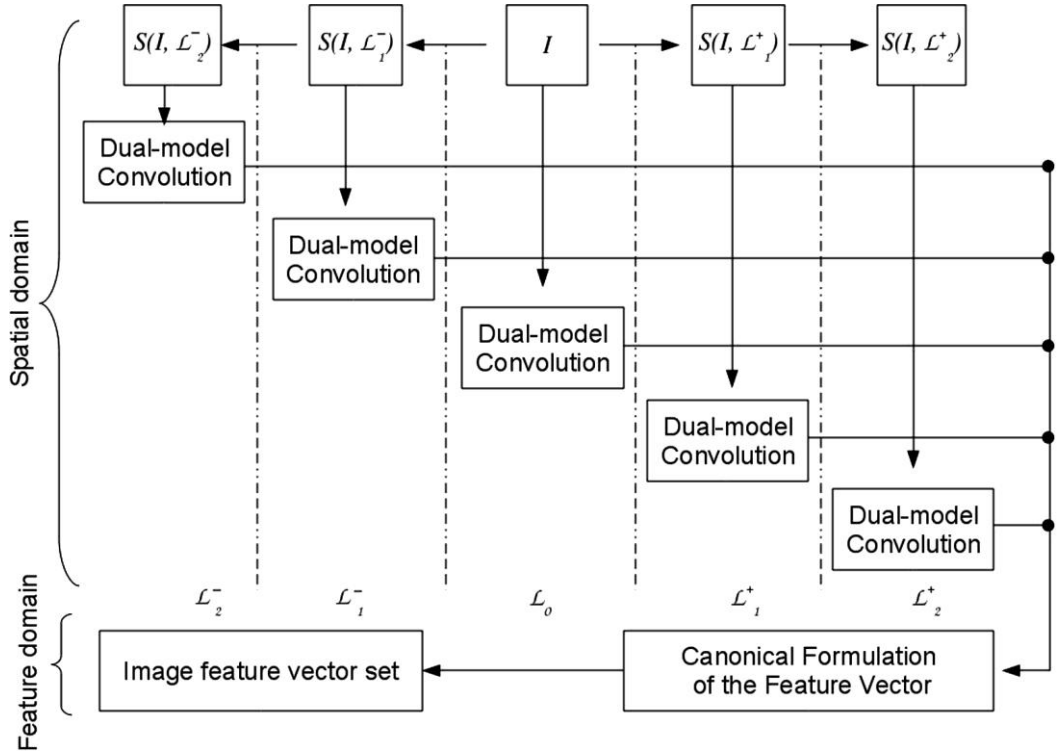
The single resolution detector described in Section 5.5 makes use of local orientations calculated on a regional basis and operates with a single wavelength parameter for the Gabor filter, thereby assuming a single width for all fibres. In this section we extend the model to multiple resolutions using a scale pyramid as shown in Figure 5-3. We also calculate responses over a



range of orientations, selecting the most appropriate scale and orientation of the response by pixel classification. There are three parameters of the Gabor filter that can vary in scale:  $\lambda$ , the wavelength of the sinusoid and  $\sigma_x$  and  $\sigma_y$ , the widths of the Gaussian envelope. To explore this scale space efficiently, we make use of the single-scale results, choosing values of  $k$ ,  $r_x$  and  $r_y$  at the original image scale to be the values used in the single-scale detector. Keeping these values constant we create a pixel pyramid by sub-sampling (with smoothing) and super-sampling (by interpolation) the original image. While super-sampling the image adds no new information to the pyramid, it has the effect of reducing the wavelength and Gaussian widths of the Gabor filter relative to the size of the image structure.

### 5.6.1 Image pyramid

Let us denote  $\mathcal{L}$  as a vector set of different scale (re-sampling levels) parameters. Each level  $l$  represents a set of estimated parameters used in the dual-model detection. The spatial frequency of the image structure (nerve fibres) in  $l$  is defined by the  $\lambda$ .



**Figure 5-3** A conceptual diagram illustrating the operation of the multi-scale dual-model detection algorithm. The images are convolved with the adaptable dual-model algorithm at different scales and then the responses are combined in the feature space to generate a feature vector for every pixel in the image. The  $S(\cdot)$  is the scaling function.

$$\mathcal{L} \triangleq \{l_L^-, l_{L-1}^-, \dots, l_0, \dots, l_{L-1}^+, l_L^+\} \in \mathbb{R}^3 | L \in \mathbb{Z}^+ \quad (10)$$

$$l_k^\pm \triangleq \{\lambda, \sigma_x, \sigma_y\} | k = 1, 2, \dots, 2L + 1 \quad (11)$$

For example  $l_1^- (\lambda)$  defines the wavelength of the Gabor filter's sinusoid at the super-sampled level 1.  $l_2^- (\sigma_x)$  defines the Gaussian spread in x of the Gabor filter at the sub-sampled level 2, etc. In our implementation  $L = 2$  and the pixel sampling is doubled (halved) between levels. At each level, eight values of orientation ( $\theta$ ) are explored. The specific values of  $\lambda$ ,  $\sigma_x$  and  $\sigma_y$  at level  $l_0$

were defined empirically in the single-scale detector to be  $\lambda = 9$ ,  $\sigma_x = 4$  and  $\sigma_y = 3$ .

### 5.6.2 Feature vector extraction

In order to generate the feature vector of each CCM image  $I$  we use the transform  $\mathcal{J}: \mathbb{R}^{M \times N} \rightarrow \mathbb{R}^{M \times N \times O \times S}$ , where  $M \times N$  are the dimensions of the image,  $O$  is the number of orientations used and  $S$  is the number of levels in the pyramid ( $2L + 1$ ). Analogous to the single-scale dual-model detection algorithm in Section 4, transform  $\mathcal{J}$  consists of two models: foreground model  $\mathcal{M}_f(x_\theta, y_\theta, I_k^\pm)$  and background model  $\mathcal{M}_B(x_\theta, y_\theta, I_k^\pm)$ . The difference between these models and those of the single scale [Equations (1) and (2)] is that they are a function of the different scales defined by the pyramid  $\mathcal{L}$ . Also, all orientations are computed at every pixel unlike the single model where orientation is locally estimated. Hence there are no equivalents of Equations (3) and (4) in this case.

$$\mathcal{M}_f(x_\theta, y_\theta, I_k^\pm) = \cos\left(\frac{2\pi}{\lambda_k}x_\theta + \phi\right) \cdot \exp\left\{-\frac{1}{2}\left(\frac{x_\theta^2}{\sigma_{xk}^2} + \frac{\gamma^2 y_\theta^2}{\sigma_{yk}^2}\right)\right\} \quad (12)$$

$$\mathcal{M}_B(x_\theta, y_\theta, I_k^\pm) = \alpha \cdot \exp\left\{-\frac{1}{2}\left(\frac{x_\theta^2}{\sigma_{xk}^2} + \frac{\gamma^2 y_\theta^2}{\sigma_{yk}^2}\right)\right\} \quad (13)$$

The adaptation of these two models across the complete range of scales and orientations defined by the pyramid  $\mathcal{L}$  should cover all of the relevant feature

space of the nerve fibres. By convolving them with the images to generate foreground and background responses  $R_f(\theta, \lambda)$  and  $R_B(\theta)$ , and finding the

difference  $\mathcal{G}_i$  between these responses we can generate the feature vector  $\mathcal{F}$  that describes the CCM image I.

$$\mathcal{R}_{\mathcal{F}}(\theta, l_k^{\pm}) = \mathbf{I} * \mathcal{M}_{\mathcal{F}}(x_{\theta}, y_{\theta}, l_k^{\pm}) \quad (14)$$

$$\mathcal{R}_{\mathcal{B}}(\theta, l_k^{\pm}) = \mathbf{I} * \mathcal{M}_{\mathcal{B}}(x_{\theta}, y_{\theta}, l_k^{\pm}) \quad (15)$$

$$\begin{aligned} \mathcal{G}_i &= \mathcal{R}_{\mathcal{F}}(\theta, l_k^{\pm}) - \mathcal{R}_{\mathcal{B}}(\theta, l_k^{\pm}) \\ &= \mathbf{I} * \left( \cos \left( \frac{2\pi}{\lambda_k} x_{\theta} + \phi \right) - \alpha \right) \cdot \\ &\quad \exp \left\{ -\frac{1}{2} \left( \frac{x_{\theta}^2}{\sigma_{xk}^2} + \frac{y_{\theta}^2}{\sigma_{yk}^2} \right) \right\} \end{aligned} \quad (16)$$

$$\mathcal{F} = \{\mathcal{G}_1, \mathcal{G}_2, \dots, \mathcal{G}_{O \times S}\} \quad (17)$$

$\alpha$  is the threshold parameters that is equivalent to the same parameter in the single-scale detector [Equations (5) and (6)]. However, in this multi-scale algorithm the logistic transition [Equation (9)] is replaced by the classification step of the generated feature vector  $\mathcal{F}$  in order to make the final decision.

### 5.6.3 Canonical form of the feature vector

Unlike the single-scale detector, the interpretation of the response is not trivial. Applying the transform  $\mathcal{T}$  using the pyramid  $\mathcal{L}$  generates longer feature vectors which raises the questions of how to interpret the response in the best possible way. Since these feature vectors are associated with certain orientations, frequencies and local regions of the image, the specific sequence

of features in the feature vectors is dependent on the order which these features are formulated. For example the order of the feature vector provides information about the local orientation of the fibre. This is useful to know, but irrelevant to classifying the pixel as belonging to the fibre or non-fibre classes. We need to generate the features in a canonical form, which means that similar pixels have similar feature vectors. In this case we wish the feature vectors to be orientation invariant. This can be achieved by assigning the first sample of the vector to its maximum value, corresponding to the predominant orientation, and then circularly shifting all samples by this offset.

$$\mathbf{f} \triangleq \mathcal{F}(i,j) = \{\mathcal{G}_1(i,j), \mathcal{G}_2(i,j), \dots, \mathcal{G}_{O \times S}(i,j)\} \quad (18)$$

$$\tau = t \mid \arg \max_{f_t}(\mathbf{f}) \quad (19)$$

$$\mathbf{f} \leftarrow \zeta(\mathbf{f} - \tau) \quad (20)$$

Where  $\mathbf{f}$  is the cyclic shift function;  $\mathbf{f}$  is the pixel-wise feature vector of  $\mathcal{F}$  at  $(i, j)$ .  $\tau$  is the number of shift cycles defined by the maximum value of the vector

$\mathbf{f}$ . This guarantees that responses of foreground and background models are canonically aligned in the newly formed feature vector and independent of the particular orientation of the models.

## 5.7 Nerve fibre classification

We consider three possible ways of using the feature vector  $\mathcal{F}$  to assign pixels  $(i, j)$  to the foreground or background classes.

### 5.7.1 Maximum projection

One simple way of interpreting the feature vector of each IVCCM image is by considering the maximum value of a particular sample among all different frequencies and orientations. Following the cyclic shift the first feature in the feature vector  $\mathcal{F}$  has the maximum value.

$$\mathbf{I}(i,j)_{Enh} = \begin{cases} f_t & \text{if } f_t \geq 0 | t = 0 \\ 0 & \text{Otherwise} \end{cases} \quad (21)$$

The scale and the orientation of this maximum value of  $\mathbf{f}$  is taken to be the frequency and orientation at a particular pixel  $(i, j)$  of the detected nerve fibre in the enhanced image  $I_{Enh}$ . Although this method is effective, efficient and easy to implement, it discards the rest of the sample responses at other orientations and scales, hence neglecting the possibility that combinations of features may be useful in correctly classifying pixels.

### 5.7.2 Scaled conjugate gradient neural network

We assign pixels to fibre or non-fibre classes by means of a multi-layer perception neural network trained using the conjugate gradient descent method. Conjugate gradient methods (CGM) (38) are general purpose second

order techniques that help minimise functions of several variables using the second derivatives of the function. They generally find a better way to a minimum than a first order technique (such as standard back-propagation), by proceeding in the direction which is conjugate to the directions of the previous steps of the error function. Thus the minimisation performed in one step is not partially undone by the next, as is the case with standard back-propagation and other gradient descent methods. The traditional CGM uses the gradient to compute a search direction. It then uses a line search algorithm to find the optimal step size along a line in the search direction (39).

### 5.7.3 Random forest classifier

The random forests (RF) machine learning algorithm is a classifier (14) that encompasses bagging (41) and random decision forests (42-43). RF became popular due to its simplicity of training and tuning while offering a similar performance to boosting. It is a large collection of decorrelated decision trees, which are ideal candidates to capture complex interaction structures in data. RF is supposed to be resistant to over-fitting of data if individual trees are sufficiently deep.

Consider a RF collection of tree predictors  $h(\mathbf{x}; \psi_u)$   $u = 1, \dots, U$ , where  $\mathbf{x}$  is a random sample of  $d$ -dimensions associated to random vector  $\mathbf{X}$  and  $\psi_u$  independent identically distributed random vectors. Given a dataset of  $N$  samples, the bootstrap training sample of tree  $h(\mathbf{x}; \psi_u)$  is used to grow the

tree by recursively selecting a subset of random dimensions  $\hat{d}$  such that  $\hat{d} \ll d$

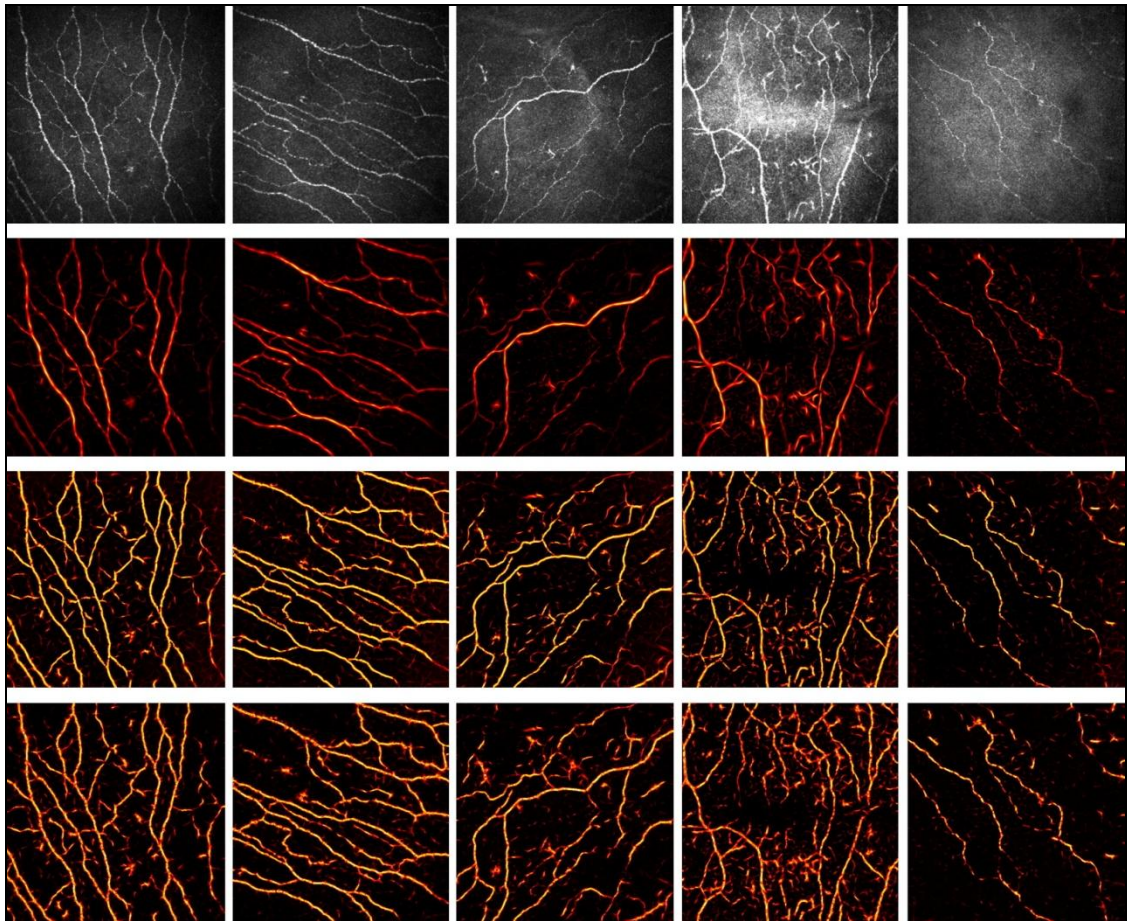
and picking the best split of each node based on these variables. Unlike conventional decision trees, pruning is not required.

$$\hat{c} = \text{majority vote}\{C_u(\mathbf{x})\}_1^U \quad (22)$$

To make a prediction for a new sample  $\mathbf{x}$ , the trained RF could then be used for classification by majority vote among the trees of the RF as shown in Equation (22), where  $C_u(\mathbf{x})$  is the class prediction of the  $u$ th RF tree. The

important parameters of the RF classifier were set as follows in this case. The number of trees in the forest should be sufficiently large to ensure that each input class receives a number of predictions: set to 1000. The number of variables randomly sampled at each branch: set to 5.





**Figure 5-4** An illustration of the multi-scale dual-model detection responses when using different pixel classification methods. The first row consists of the original IVCCM images. The following rows contain the response images when using maximum response, NNT and RF respectively. Responses are presented as heat maps, where brighter colours correspond to higher values. The best response is given when using NNT. The classifier successfully learnt the right balance of sensitivity and specificity (see Section 5.8). The RF has a far greater sensitivity than the maximum response but its higher sensitivity results in noisier detection. The improved response is most visible in regions of the image where the signal to noise ratio is low. The very bright region at the centre of the image in column 4 is an extreme example of a low-frequency illumination artefact. It is not clear visually whether any fibres are present there. The NNT and RF detectors identify more fibres with greater confidence.

## 5.8 Detection results and analysis

### 5.8.1 Database and experimental settings

The evaluation is conducted on a database of 521 IVCCM images captured using the HRT-III microscope from 68 subjects (20 controls and 48 diabetic patients). The images have a size of 384 x 384 pixels, 8-bit grey levels and are stored in BMP format. The resolution is 1.0417  $\mu\text{m}$  and the field of view is 400 x 400  $\mu\text{m}^2$  of the cornea. For each individual, several fields of view are selected manually from near the centre of the cornea that shows recognisable nerve fibres. Other than the processing inherent in the filters (described above), no additional pre-processing was applied to the images.

Using the NDS (44), the patients were categorised into four groups according to severity of neuropathy [non-neuropathic:  $0 \geq \text{NDS} \leq 2$  ( $n = 26$ ), mild:  $3 \geq \text{NDS} \leq 5$  ( $n = 9$ ), moderate:  $6 \geq \text{NDS} \leq 8$  ( $n = 10$ ) and severe:  $9 \geq \text{NDS} \leq 10$  ( $n = 3$ )].

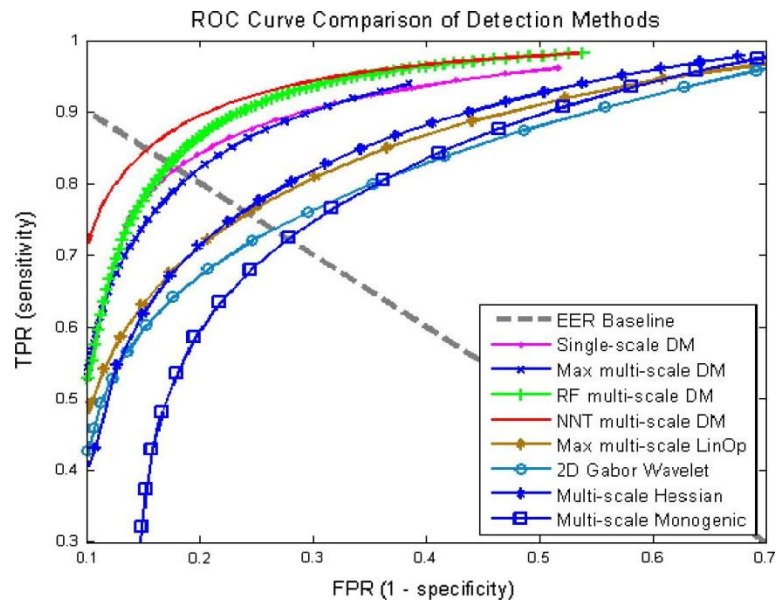
### 5.8.2 Nerve fibre detection performance

The evaluation of detecting nerve fibres is conducted against ground-truth data which has been generated by clinical experts using *CCMetrics* (*CCMetrics* is a purpose built interactive graphical interface which helps experts to manually delineate nerve fibres in IVCCM images). Each nerve fibre and branch is traced to generate a single-pixel wide line along the fibre centre, from which the parameters NFL, NFD, NBD and tortuosity can be derived. In automatic detection, the response images are thresholded and then thinned to one-pixel wide lines. These lines are then compared pixel by pixel to the ground-truth, a

true positive being scored if the detected pixel is within a three-pixel (3.14 lm) tolerance of ground-truth and a false positive if it is outside this tolerance. The evaluation is quantified in terms of true-positive rate (TPR or sensitivity) and false-positive rate (FPR or 1-specificity) defined at the operational point of the equal error rate (EER). The training of the NNT and RF was based on a single IVCCM image with ground-truth delineation. Once the classifier is trained using this single image, it is applied on the entire database and the results are obtained through a comparison with the ground-truth delineation of every test image.

The single-scale methods (Gabor wavelet and single scale dual model) were evaluated against their single-scale response, while the multi-scale methods (LinOp, Hessian and Monogenic filters) were evaluated against their maximum response. Figure 5-4 shows the response images in different IVCCM images arising from each of the three methods of pixel classification i.e. maximum response, NNT and RF. Both the RF and NNT classifiers are more sensitive than the maximum response method.

In our earlier study (13) we compared the single scale dual-model detector with the comparator methods described in Section 3, some of which are specifically designed to detect curvilinear structures, while others are more general feature detectors. In that comparison we used single-scale instantiations of all detectors, though some have multi-scale implementations. The dual model produced the best receiver operating characteristic (ROC) curves and EER classification rates. Here we repeat the evaluation using multi-scale versions of all detectors. Figure 5-5 shows the resulting ROC curves. The single-scale dual model detector is also included for comparison.



**Figure 5-5** ROC curves of nerve fibre detection for all different methods including the RF and NNT pixel classifiers of the multi-scale dual model. As shown the NNT has achieved the best performance followed by the RF classification. The single-scale dual-model algorithm has marginally outperformed the maximum response method.

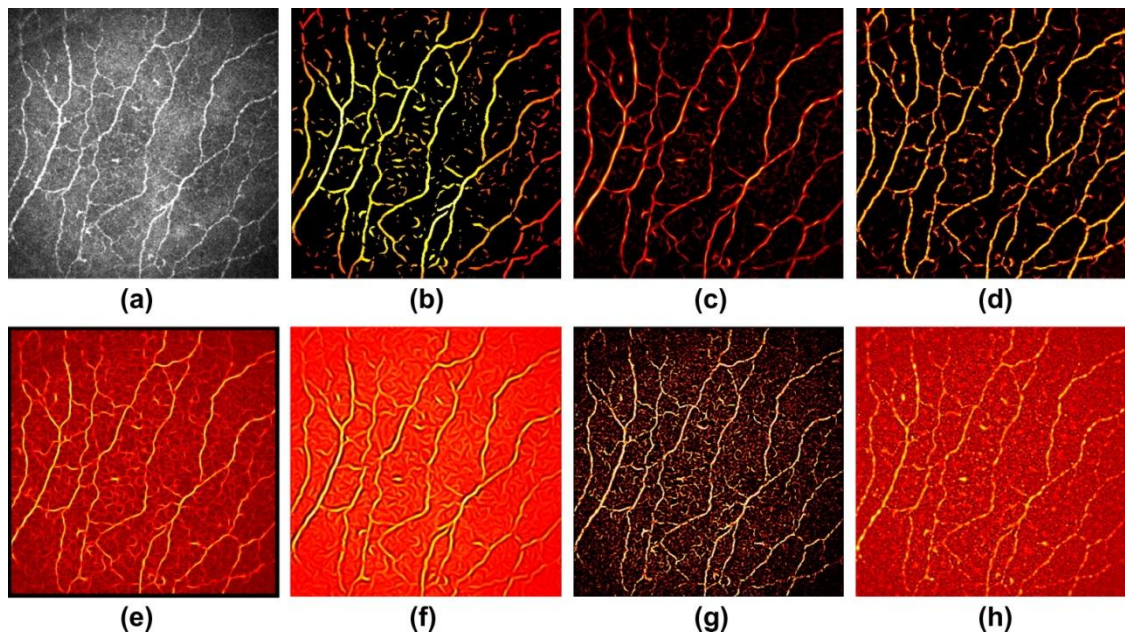
The single scale dual-model produces a better response than the multi-scale versions of the other methods. The maximum projection-scale version of the multi-scale dual model produced slightly worse results than the single-scale version, while both the RF and NNT versions generated improved results, more so in the case of the NNT classifier.

This may be due to the fact that the orientation estimate in the single scale model are locally smoothed, whereas those in the multi-scale, maximum response, model are not, and therefore subject to noise variations. The NNT and RF classifications are less sensitive to noisy orientation estimates because all orientations across scale are contributing to the solution.

Due to the second order derivative components in the Hessian and the Monogenic methods their responses are very sensitive to the background

noise. LinOp and the 2D Gabor methods, on the other hand were less sensitive to noise, but tended to include too much background.

Figure 5-6 provides a visual illustration of the responses of several of the detectors in the comparison. Figure 5-5 and Table 5-1 provide quantitative confirmation of the qualitative results shown in Figure 5-4 and Figure 5-6. The maximum response output of the multi-scale dual model achieves superior performance to the maximum response outputs of the Hessian and Monogenic filters, and matches the performance of the multi-scale LinOp. The multi-scale dual model using NNT pixel classification achieves the highest performance in detecting nerve fibres. It achieves highest sensitivity and specificity at the EER of 15.44%. We did not set out to conduct a comparison between the two classifiers used, rather to show that the classification method is capable of producing useful results. Using the particular (empirically set) parameters for these classifiers and this data set, the RF is more sensitive than the NNT, resulting in a noisier response Figure 5-4. The measured error rates for the NNT classifier shown in Table 5-1 (significant at the  $p < 0.05$  level) emphasise the superior performance achieved by the NNT classifier, here.



**Figure 5-6** A visual comparison between the responses of different detection methods for the original CCM image in (a). (b) is the single-scale dual-model response, (c) is the maximum response method for the multi-scale dual-model and (d) is its NNT counterpart. (e) is the LinOp response, (f) is the 2D Gabor Wavelet response, (g) the Hessian matrix response and (h) is the Monogenic signal response. The multi-scale dual model with NNT classification has the best performance followed by the single-scale dual-model. The Hessian and Monogenic responses suffer from a greater sensitivity to noise due the second derivative components in the algorithms. The LinOp and the 2D Gabor responses struggled to suppress the background. Responses are presented as heat maps, where brighter colours correspond to higher values.

### 5.8.3 Clinical utility using nerve fibre length analysis

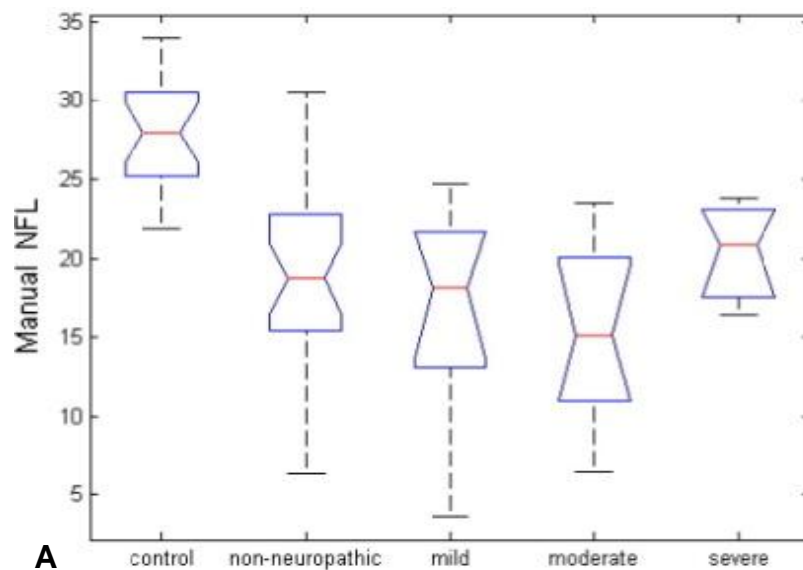
In studies using interactive measurements, NFL was shown to be the most sensitive of the IVCCM metrics to the presence of neuropathy as assessed by the current clinical techniques. Hence it is also used here to evaluate the similarity of the automatic analysis to the manual analysis. Figure 5-7 shows the distribution of NFL measurements in NDS groups made interactively by experts (a) and automatically (b). The manually and automatically generated

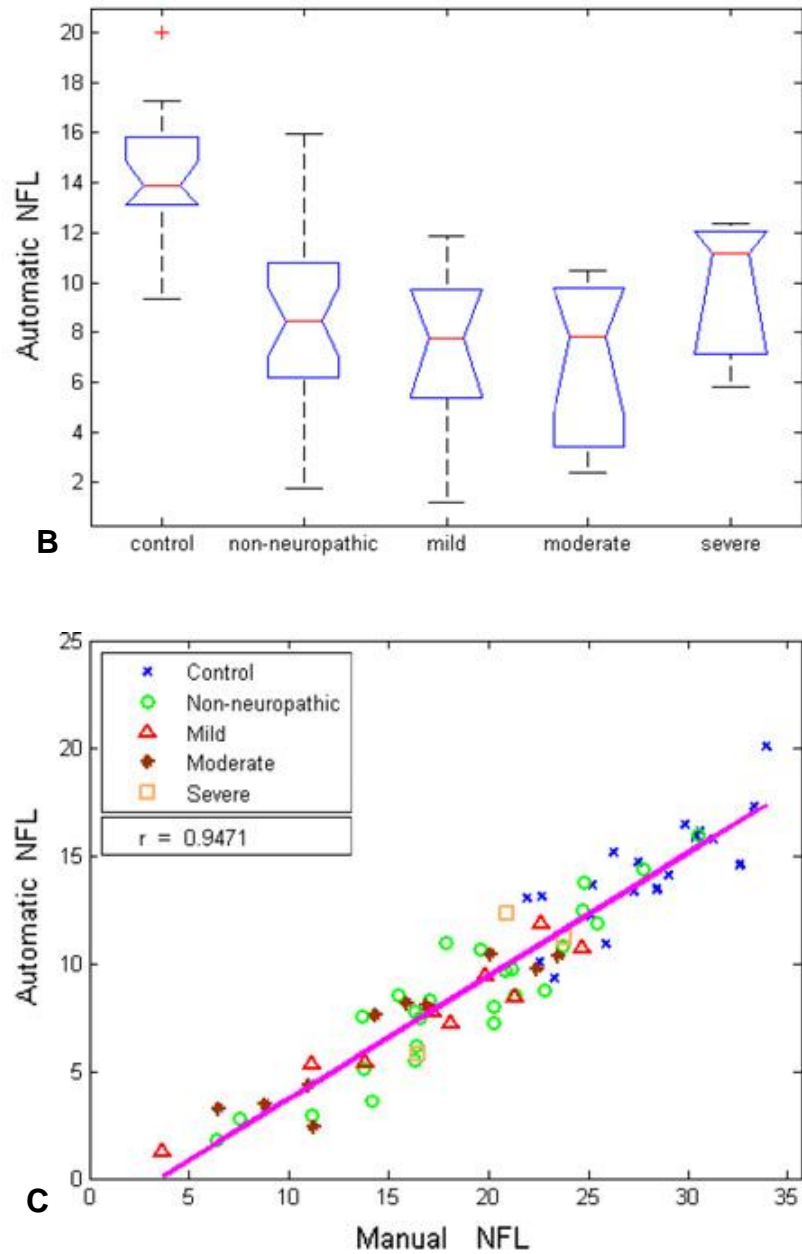


NFL distributions are very similar and strongly correlated (Figure 5-7c) with  $r = 0.95$ . They are both statistically significant in separating between the NDS groups: for the manual analysis ( $p = 0.03 \times 10^{-6}$ ), while the automatic has ( $p = 0.68 \times 10^{-6}$ ). However as shown in the scatter plot Figure 5-7c this statistical significance is not enough for classification of individual cases due to the overlapped distributions. This could be as a result of the limitation in using the NDS score, which is used as a diagnostic score and unstable for individual analysis. This result however could be improved by utilising the potential of the automatic analysis in utilising further metrics such as nerve fibre width.

**Table 5-1** A comparison of mean EER, its standard deviations, TPR (sensitivity) and FPR (1- specificity) of all detection methods. The table clearly shows that the multi-scale dual model with NNT classification results in the lowest error rate.

	Max	NNT	RF	Dual Model	LinOp	2D Gabor	Hessian	Monogenic
<b>EER (<math>\mu</math>)</b>	0.2056	0.1544	0.1746	0.1779	0.2265	0.2415	0.2314	0.2650
<b>EER (<math>\sigma</math>)</b>	0.1806	0.1083	0.1176	0.1058	0.1076	0.1074	0.1153	0.1258
<b>TPR (sensitivity)</b>	0.8135	0.8478	0.8290	0.8172	0.766	0.7212	0.7773	0.7240
<b>FPR (1-specificity)</b>	0.1940	0.1533	0.1747	0.1758	0.2489	0.2467	0.2527	0.2782





**Figure 5-7** A comparison between the manually and automatically obtained NFL in groups of different severity of neuropathy, as judged by NDS score. The manual (A) and automatic (B) box plots show strong similarity. Both are statistically significant ( $p \approx 0$ ) in separating the NDS groups detailed in Section 5.8.1. The scatter plot in (C) shows the strong correlation between them ( $r = 0.95$ ) and demonstrates the overlap between the groups according to the NDS categories.



## 5.9 Conclusion

The analysis of IVCCM images requires the identification of fibre-like structures in noisy images with low contrast. This is a requirement shared by a number of imaging applications in biology, medicine and other fields, and a number of methods have been developed and used in these various applications. In the present work we present a new multi-scale dual-model method to detect corneal nerve fibres in IVCCM images and we compare this with some more generic methods. In our evaluation the multi-scale dual-model with the NNT pixel classification has outperformed all other methods and obtained the lowest EER at 15.44%. A point worth noting is that the additional performance was achieved at the expense of a very small training burden. A single annotated image was used to provide training data for both the RF and NNT classifiers. This is a potentially important issue in the practical implementation of the method.

The clinical utility of the method was also evaluated by comparing our automatic detection against expert manual annotation of the images. We demonstrate equivalent results with the manual analysis which has previously demonstrated encouraging clinical performance for the stratification of neuropathic severity. Here we have used the NDS score, which is widely used clinically and is adequate for defining the clinical severity of neuropathy to assess the correspondence between manual and automatic detection. However, the NDS may not be adequate for a thorough assessment of clinical utility because it does not detect small fibre damage. Hence as CCM can evaluate small fibre damage, any assessment of the clinical utility of this test

may be limited. As noted in Section 5.3.1, the accepted gold standard for defining small fibre pathology can only be achieved by either nerve or skin biopsy, both of which are invasive and highly labour-intensive assessments. We are currently collecting a data set that will enable us to evaluate the IVCCM metrics with measures of loss of nerve fibres in skin biopsies.

In conclusion, the automated analysis produces equivalent results to manual analysis, while being a quicker and potentially more reliable and practical alternative due to its consistency and immunity to inter/intra-observer variability. The multi-scale detection method used here could, of course, be applied in other contexts as the detection of curvilinear structures is a requirement in a number of applications. The method is generic, requiring only the establishment of appropriate parameters for  $\lambda$ ,  $\sigma_x$  and  $\sigma_y$  at the resolution of the original image. The empirical values used in this application are quoted in Section 5.6.1.

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## **6. CHAPTER VI-CORNEAL NERVE LOSS IS SYMMETRICAL AND PROGRESSIVE WITH INCREASING SEVERITY OF DIABETIC NEUROPATHY**

Author's contribution: Ioannis N Petropoulos contributed to the conception and design of the study, acquired and analysed the data, performed statistical analysis and wrote the manuscript, which formed the basis for this chapter and was submitted for publication to the journal *Diabetes Care*.

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## 6.1 Abstract

**PURPOSE** To establish if corneal nerve fibre loss, detected using IVCCM, is symmetrical between RE and LE and progressive with increasing severity of diabetic neuropathy.

**METHODS** 111 patients with type 1 and type 2 diabetes mellitus and 47 age-matched healthy control subjects underwent detailed assessment and stratification into differing severity of diabetic neuropathy. In-vivo corneal confocal microscopy was performed in both eyes and CNFD, CNBD, CNFL and the TC were quantified.

**RESULTS** Diabetic patients were stratified into no neuropathy (n=50), mild neuropathy (n=26), moderate neuropathy (n=17) and severe neuropathy (n=18). All corneal nerve parameters were highly significant different in diabetic patients compared to controls and progressed with increasing severity of neuropathy. The reduction in CNFD, CNBD and CNFL was symmetrical in all groups except for patients with severe neuropathy.

**CONCLUSION** Corneal nerve fibre loss assessed using IVCCM is progressive and symmetrical with increasing severity of neuropathy, except in those with the most severe neuropathy. This is consistent with the symmetrical and progressive nature of diabetic somatic neuropathy.



## 6.2 Introduction

Diabetic sensorimotor polyneuropathy is a length-dependent, symmetrical neuropathy with initial involvement of sensory and autonomic nerve fibres, followed by motor nerve involvement (1-3). It is the most common long term complications of diabetes and is the main initiating factor for foot ulceration and lower extremity amputation (4) with substantial associated health care costs (5). Conventional techniques of electrophysiology and QST along with an assessment of neurological disability offer a relatively robust means of defining neuropathic severity (6) but have limitations in detecting the earliest stages of nerve damage (7-9).

IVCCM is a rapidly expanding technique to quantify the severity of neuropathy in DSPN (10). It has been used to demonstrate early and progressive nerve damage in diabetes and a range of other peripheral neuropathies (11-13) with good sensitivity and specificity (14). Recently, corneal nerve damage detected with IVCCM has been related to the level of previous glycemic exposure and blood pressure (15) and HbA1c even in healthy subjects (16). In a study of subjects with idiopathic small fibre neuropathy corneal nerve damage was associated with higher serum triglycerides (12). It has also shown significant nerve regeneration before improvement in a range of established measures of neuropathy including QST, neurophysiology and IENFD, following SPK (17) and after an improvement in glycaemia and cardiovascular risk factors for DSPN (18).

Corneal nerve fibre loss correlates with IENF loss (7) CNFL particularly, has shown superior discriminative capacity to diagnose DSPN (19). Recent studies show that quantification of corneal nerve morphology is highly reproducible and does not differ significantly between observers (20-21) and occasions (22) in subjects with diabetes and healthy individuals. As a functional correlate, corneal sensation has been found to decrease with increasing neuropathic severity (23).

Perkins and colleagues (2) and Bromberg and Jaros (24) have previously reported high inter-side symmetry of nerve conduction studies (NCS) consistent with the symmetrical nature of diabetic neuropathy. Whilst Petropoulos et al. (22) have shown that central corneal innervation is highly symmetrical between RE and LE of young healthy subjects, it is unknown whether the progressive corneal nerve loss in diabetic neuropathy maintains its symmetry in different stages of DSPN. This is relevant to further establish parallels in terms of pathophysiology between corneal and peripheral somatic nerve damage, but also has practical relevance when examining patients to allow examination of only one eye. The purpose of the present, cross-sectional, observational study was to establish whether corneal nerve damage is progressive and symmetrical with increasing severity of DSPN.

## **6.3 Methods**

### **6.3.1 Study subjects**

111 patients with diabetes and 47 age-matched control subjects were evaluated for the presence of DSPN based on the updated Toronto consensus

criteria (25). This research adhered to the tenets of the declaration of Helsinki and was approved by the North Manchester Research Ethics Committee. Informed written consent was obtained from all subjects prior to participation to the study.

### **6.3.2 Clinical assessment and evaluation of peripheral neuropathy**

All study participants underwent assessment of their clinical characteristics (BMI, HbA<sub>1c</sub>, lipid fractions, ACR and eGRF) and detailed evaluation of the symptoms and signs and of DSPN based on the McGill pain questionnaire which includes a visual analogue and a pain index scale, simplified NDS, VPT and NCS. The NDS, a scale of 0-10, was used to stratify the neuropathic severity of the study participants into *none* (0-2), *mild* (3-5), *moderate* (6-8) and *severe* (9-10) as described by others (26-27). It is composed of Achilles tendon reflex testing [present (0), reduced (1), absent (2)], temperature sensation [present (0), absent (1)], pin-prick sensation [present (0), absent (1)] and vibration perception scores of the great toe using a tuning fork [present (0), absent (1)].

VPT was tested using a Neuroesthesiometer (Horwell, Scientific Laboratory Supplies, Wilfrod, Nottingham, UK). Electro-diagnostic studies were undertaken using a Dantec “Keypoint” system (Dantec Dynamics Ltd, Bristol, UK) equipped with a DISA temperature regulator to keep limb temperature constantly between 32-35°C. Peroneal motor and sural sensory nerves were assessed in the left lower limb (calf-to-ankle) by a consultant neurophysiologist. The peroneal motor nerve study was performed using silver-silver chloride surface electrodes at standardised sites defined by

anatomical landmarks and recordings for the sural sensory nerve were taken using antidromic stimulation over a distance of 100mm.

### **6.3.3 In vivo corneal confocal microscopy and corneal sensation**

All study subjects were scanned with a laser IVCCM (Heidelberg Retinal Tomograph III Rostock Cornea Module [HRT III RCM] (Heidelberg Engineering GmbH, Heidelberg, Germany). This IVCCM uses a 670 nm wavelength helium neon diode laser, which is a class I laser and therefore does not pose any ocular safety hazard. A 63x objective lens with a numerical aperture of 0.9 and a working distance, relative to the applanating cap (TomoCap<sup>®</sup>, Heidelberg Engineering GmbH, Heidelberg, Germany) of 0.0 to 3.0 mm was used. The size of each two-dimensional image produced was 384  $\mu\text{m}$  x 384  $\mu\text{m}$  which has a 15° x 15° field of view and 10  $\mu\text{m}$ /pixel transverse optical resolution. HRT III RCM uses an entirely digital image capture system and all images are stored in an external hard drive.

A drop of 0.4% benoxinate hydrochloride (Chauvin Pharmaceuticals, Chefaro, UK) was used to anaesthetise each eye and Viscotears (Carbomer 980, 0.2%, Novartis, UK) were used as the coupling agent between the cornea and the applanating cap. All subjects were asked to fixate on an outer fixation light throughout the IVCCM scan and a CCD camera was used to image the cornea and correctly position the applanating cap onto the corneal apex. The overall examination took approximately 5 minutes for both eyes of each subject and in this study two experienced examiners performed all IVCCM scans. All images were captured using the “section” mode in the Heidelberg Explorer of the HRT III RCM. There is no consensus on optimal IVCCM image sampling but it has

been proposed that any number between 5 and 8 images will provide an acceptable level of accuracy to quantify the corneal subbasal nerve morphology (28). We selected and analysed 6 high clarity images/subject from the central subbasal nerve plexus captured by 1 $\mu$ m intervals at the z-axis. Criteria for image selection were depth, focus position and contrast. We also quantified CS using NCCA as described elsewhere (23).

#### **6.3.4 Image Analysis**

One examiner masked from the outcome of the medical and peripheral neuropathy assessment quantified the subbasal nerve morphology in 924 images of all study participants using semi-automated, purpose-written, proprietary software (CCMetrics<sup>®</sup>, M. A. Dabbah, ISBE, University of Manchester, Manchester, UK). The specific parameters measured per frame were those we have previously established (14): CNFD (no./mm<sup>2</sup>), CNBD (no./mm<sup>2</sup>), CNFL (mm/mm<sup>2</sup>) and (TC) (29) (Fig.1). CNFD is defined as the total number of main nerve fibres (NF) per frame divided by the area of the frame in mm<sup>2</sup> (area = 0.16033585 mm<sup>2</sup>) (Figure 1). CNBD is defined as the total number of main nerve branches (nerve branches which stem from a NF) divided by the area of the frame. CNFL is the total length of NFs and NBs per frame. TC is a mathematical computation of the NF tortuosity as previously described by Kallinikos et al. (29), which is independent of the angle of the nerve in the image. A straight nerve equals a TC of zero and the TC increases with increasing tortuosity of the NF.

### **6.3.5 Statistical analysis**

Statistical analysis was performed using StatsDirect for Windows (StatsDirect Ltd., Altrincham, Cheshire, UK) and OriginPro 8.5 (OriginLab Corporation, Northampton, USA) was used to plot the results. Prior to statistical analysis all the collected data were assessed for normality by relevant histograms and the Shapiro-Wilk test where appropriate. Differences between RE and LE and between groups [controls vs. none vs. mild vs. moderate vs. severe neuropathy] were tested by means of a paired student's *t*-test and one-way ANOVA or non-parametric ANOVA (Kruskall-Wallis) respectively and a  $P < 0.05$  was considered significant. Post-hoc analysis for multiple comparisons was performed using the Tukey (parametric) or the Conover-Inman test (non-parametric). The mean difference between the RE and LE for each of the IVCCM parameters was calculated to define the magnitude of asymmetry and the Spearman's rank test was used to investigate potential correlations between variables. Box and whisker plots (figure 1A and 1B) were generated for CNFD and CNFL, generally regarded as the most reliable parameters for evaluation of corneal nerve pathology (20), to allow visual assessment of the data.

## **6.4 Results**

### **6.4.1 Clinical and peripheral neuropathy assessment**

Among the 111 diabetic subjects, 61 (55%) were classified as having DSPN based on the case definition employed in this study. There was no significant difference in age, BMI and serum triglycerides, but HbA<sub>1c</sub> was significantly

increased in the diabetes cohort ( $P < 0.0001$ ) and was the highest in those with severe neuropathy ( $P < 0.001$ ). There was a trend for decreasing total cholesterol with increasing severity of neuropathy in diabetic patients compared to control subjects. There was an increase in ACR ( $P < 0.001$ ) and a significant reduction in eGFR in diabetic patients with moderate ( $P < 0.001$ ) and severe neuropathy ( $P < 0.001$ ) (Table 6-1). When differences were adjusted for type of diabetes, duration, gender and age, HbA<sub>1c</sub> tended to be higher in type 1 diabetes ( $P < 0.0001$ ) while eGFR correlated with duration of diabetes ( $P < 0.0001$ ) and age ( $P < 0.0001$ ).

Vibration perception although within the normal range ( $< 15$  V), was elevated in diabetic patients without neuropathy ( $P = 0.02$ ) and increased with increasing severity of neuropathy ( $P < 0.0001$ ). SSNamp ( $P < 0.01$ ) and SSNCV ( $P < 0.001$ ) showed a progressive decrease with increasing severity of neuropathy. Similarly, PMNamp and PMNCV also decreased, reaching significance ( $P < 0.0001$ ) in mild, moderate and severe neuropathy respectively (Table 6-1). A longer duration of diabetes and age correlated significantly with VPT ( $P < 0.0001$ ), PMNamp ( $P < 0.01$ ), SSNamp ( $P < 0.0001$ ), SSNCV ( $P < 0.0001$ ) and PMNCV ( $P < 0.0001$ ).

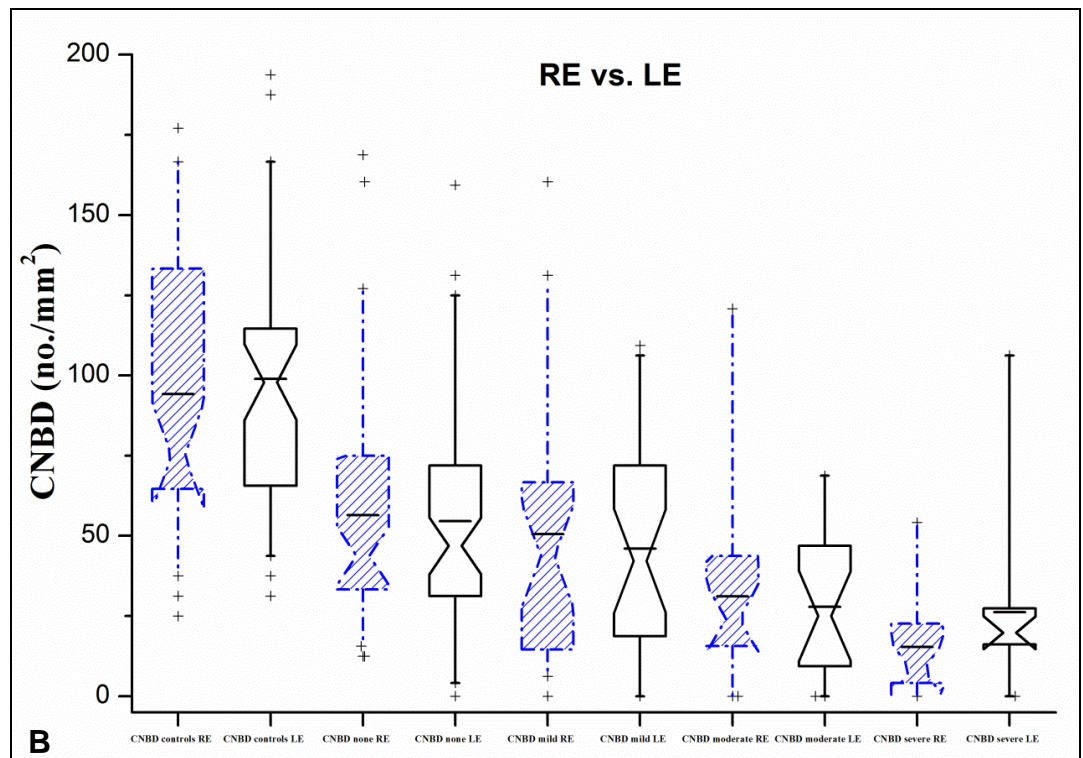
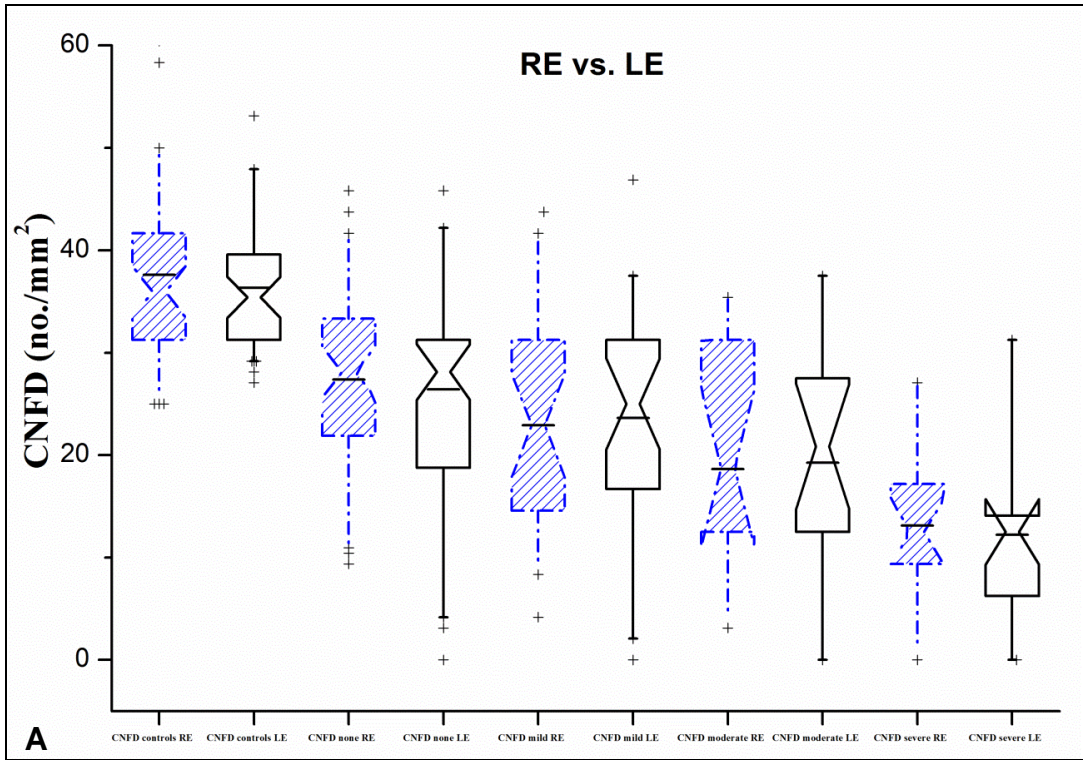
#### **6.4.2 Assessment of neuropathy by in vivo corneal confocal microscopy and corneal sensitivity**

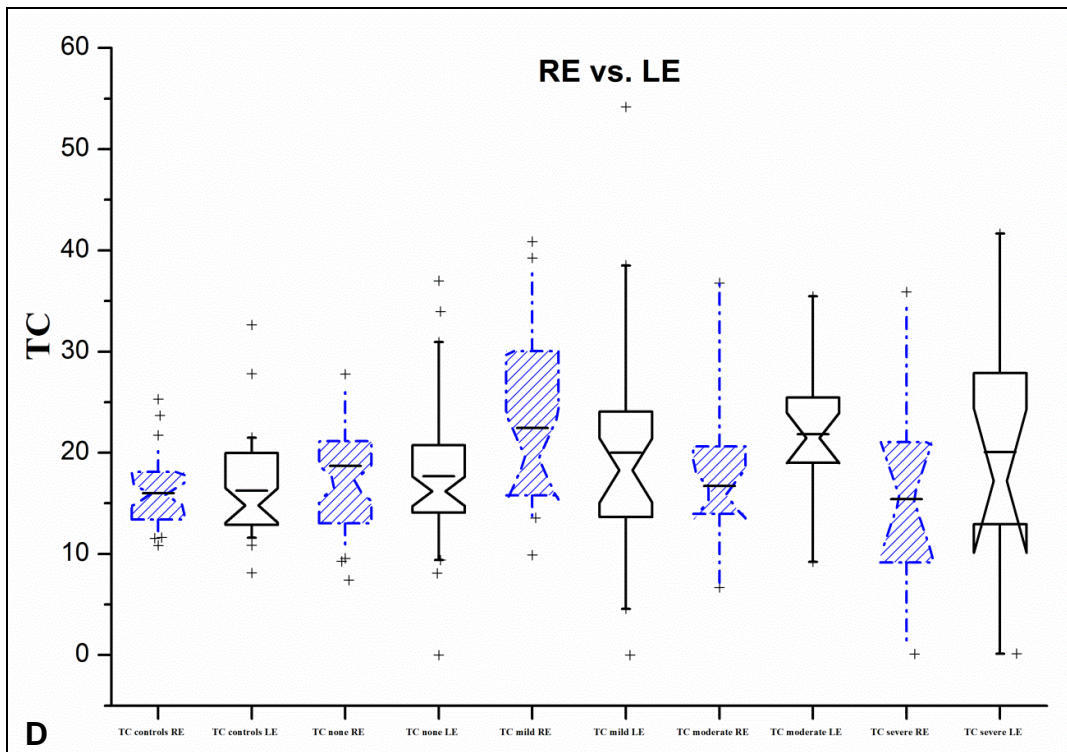
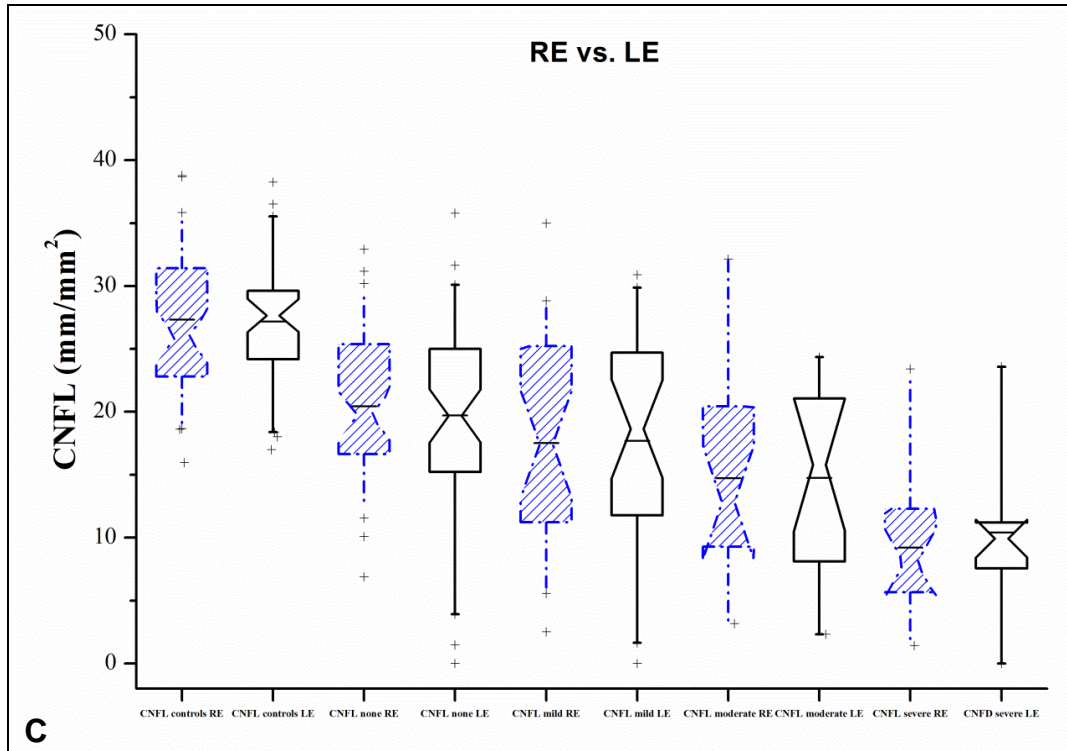
CNFD ( $P < 0.001$ ), CNBD ( $P < 0.001$ ) and CNFL ( $P < 0.001$ ) demonstrated a progressive and significant reduction between controls and diabetic patients with increasing severity of neuropathy (Table 6-2, Figure 6-1a and b). Corneal sensation thresholds increased gradually and symmetrically in diabetic

patients with increasing severity of neuropathy compared to controls ( $P < 0.001$ ) (Table 6-2). There were no differences attributed to type of diabetes, gender and age.

There were no significant differences between the RE and the LE in CNFD, CNBD, CNFL, TC and NCCA for any stage of DSPN confirming symmetrical corneal nerve damage across all groups (Figure 6-1A, B, C and D). There was a strong correlation between RE and LE in CNFD in controls ( $r = 0.51$ ,  $P < 0.05$ ), and diabetic patients without ( $r = 0.64$ ,  $P < 0.001$ ), mild ( $r = 0.89$ ,  $P < 0.001$ ), moderate ( $r = 0.83$ ,  $P < 0.001$ ) but not severe neuropathy ( $r = 0.47$ ,  $P > 0.05$ ). Likewise, CNFL was significantly correlated in controls ( $r = 0.60$ ,  $P < 0.001$ ), none ( $r = 0.67$ ,  $P < 0.001$ ), mild ( $r = 0.92$ ,  $P < 0.001$ ) and moderate ( $r = 0.92$ ,  $P < 0.001$ ) neuropathy but not severe neuropathy ( $r = 0.45$ ,  $P > 0.05$ ). CNBD, although more variable between groups showed a significant correlation in controls ( $r = 0.57$ ,  $P < 0.05$ ), none ( $r = 0.72$ ,  $P < 0.001$ ), mild ( $r = 0.85$ ,  $P < 0.001$ ), moderate ( $r = 0.61$ ,  $P < 0.01$ ) and severe ( $r = 0.52$ ,  $P < 0.05$ ) neuropathy. Finally, TC was correlated between RE and LE in controls ( $r = 0.53$ ,  $P < 0.05$ ), none neuropathy ( $r = 0.51$ ,  $P < 0.05$ ) and severe neuropathy ( $r = 0.50$ ,  $P < 0.05$ ) (Table 6-3). The associated mean differences are presented in Table 6-3.







**Figure 6-1** box and whisker plots of the prevalence of symmetrical morphology in different stages of DSPN in the RE (dashed blue) and LE (solid black) for (A) CNFD, (B) CNBD, (C) CNFL and (D) TC.



**Table 6-1** Clinical and Peripheral Neuropathy Status

Variables	Controls	None	Mild	Moderate	Severe
<b>Number</b>	47	50	26	17	18
<b>Type 1 Diabetes (%)</b>	N/A	40 (81)	18 (69)	15 (88)	16 (88)
<b>Duration of Diabetes (y)</b>	N/A	23 ± 14	31 ± 16	41 ± 14	34 ± 13
<b>Age (y)</b>	52 ± 13.2	44.2 ± 15.6	56.8 ± 12.2	59.6 ± 12.8	53.2 ± 14.5
<b>HbA1c (%) ‡</b>	5.6 ± 0.3	7.9 ± 1.7 ¶	7.9 ± 1.2 ¶	7.9 ± 1.3 ¶	9.5 ± 2.8
<b>BMI (Kg/m<sup>2</sup>)</b>	26.6 ± 4.3	26.9 ± 5.3	27.5 ± 4.7	27.4 ± 4.0	23.5 ± 7.0
<b>ACR (mg/mmol) ‡</b>	1.1 ± 1.0	1.1 ± 0.9	1.2 ± 0.9	4.4 ± 5.1	10.8 ± 11.4
<b>eGFR (ml/min/l) ‡</b>	84.9 ± 7.2	81.8 ± 19.3	79.2 ± 19.4	57.8 ± 28.3	68.7 ± 17.4
<b>Total Cholesterol (mmol/l) †</b>	5.3 ± 0.9	4.2 ± 1.0	4.4 ± 1.0	4.4 ± 1.1	4.0 ± 0.9
<b>Triglycerides †</b>	1.7 ± 0.8	1.4 ± 0.8	1.3 ± 0.8	1.5 ± 0.9	1.4 ± 0.5
<b>NDS ‡</b>	0	0.7 ± 0.9 ¶	4.0 ± 0.7 §	7.2 ± 0.9	9.75 ± 0.5
<b>VPT (V) †</b>	6.6 ± 5.1	9.0 ± 7.1 ¶	17.9 ± 13.3 §	25.7 ± 8.6	33.1 ± 12.1
<b>SSNamp (mV) †</b>	14.3 ± 11.2	11.1 ± 6.5 ¶	8.4 ± 6.8 ¶	4.8 ± 3.1 §	2.4 ± 1.2 §
<b>SSNCV (m/s) ‡</b>	49.9 ± 4.4	45.6 ± 5.1 ¶	43.2 ± 5.1 ¶	39.8 ± 5.6 §	43.6 ± 4.1
<b>PMNamp (mV) ‡</b>	5.5 ± 2.0	5.7 ± 7.9 ¶	2.9 ± 2.1 §	1.5 ± 1.0 §	1.4 ± 1.3 §
<b>PMNCV (m/s) ‡</b>	48.1 ± 3.1	43.0 ± 4.8 ¶	40.5 ± 4.8 ¶	36.4 ± 5.6 §	33.2 ± 6.0

Results are expressed as mean ± SD, Statistically significant differences using ANOVA: \* P<0.05, † P<0.01, ‡ P<0.001, ¶ Post hoc results significantly different from control subjects, § Post hoc results differ significantly from no neuropathy (none) group, || Post hoc results differ significantly from the mild neuropathy group

**Table 6-2** In Vivo Corneal Confocal Microscopy & NCCA for right and left eye in different stages of peripheral neuropathy

Variables	Controls	None	Mild	Moderate	Severe
<b>CNFD (no./mm<sup>2</sup>)</b>					
RE ‡	37.6 ± 8.2	27.4 ± 8.9 ¶	22.9 ± 10.5 §	18.6 ± 10.1 §	13.1 ± 7.3
LE ‡	36.3 ± 6.2	26.4 ± 10.1 ¶	23.6 ± 11.3 ¶	19.2 ± 10.4 §	13.1 ± 9.6
<b>CNBD (no./mm<sup>2</sup>)</b>					
RE ‡	94.2 ± 44.8	56.4 ± 35.7 ¶	50.5 ± 43.3 ¶	36.0 ± 28.2 §	13.7 ± 16.1 ~
LE ‡	98.9 ± 39.4	54.6 ± 36.5 ¶	46.0 ± 34.5 ¶	28.8 ± 20.5 §	25.5 ± 26.2
<b>CNFL (mm/mm<sup>2</sup>)</b>					
RE ‡	27.3 ± 5.8	20.4 ± 5.9 ¶	17.5 ± 8.1 ¶	14.7 ± 7.9 §	9.2 ± 5.7 ~
LE ‡	27.2 ± 4.9	19.7 ± 7.5 ¶	17.7 ± 8.9 ¶	14.7 ± 7.3 §	10.3 ± 5.7
<b>TC</b>					
RE *	16.6 ± 3.3	18.7 ± 10.7	22.4 ± 8.5 §	16.2 ± 7.8	14.2 ± 9.8
LE *	16.2 ± 4.7	17.7 ± 6.6	20.0 ± 10.4	20.7 ± 8.3 §	18.7 ± 12.1
<b>NCCA (mbar)</b>					
RE †	0.6 ± 0.4	0.8 ± 0.7	0.9 ± 0.6 §	1.1 ± 0.5	3.4 ± 4.1
LE †	0.6 ± 0.5	0.9 ± 0.7 ¶	1.0 ± 0.8 §	0.9 ± 0.4	5.1 ± 5.3

Results are expressed as mean ± SD, Statistically significant differences between groups using ANOVA: \* P<0.05, † P<0.01, ‡ P<0.001, ¶ Post hoc results significantly different from control subjects, § Post hoc results differ significantly from no neuropathy (none) group, || Post hoc results differ significantly from the mild neuropathy group, ~ Post hoc results differ significantly from the moderate neuropathy group.

**Table 6-3** Mean difference and inter-side correlation of IVCCM parameters

Variable	Control	None	Mild	Moderate	Severe
<b>CNFD (no./mm<sup>2</sup>)</b>					
Mean of Differences	1.27	0.97	-0.71	-0.61	3.33
Spearman's <i>r</i>	0.51*	0.64‡	0.89‡	0.83‡	0.47 (NS)
<b>CNBD (no./mm<sup>2</sup>)</b>					
Mean of Differences	-8.33	1.84	4.53	3.12	-9.3
Spearman's <i>r</i>	0.57*	0.72‡	0.85‡	0.61†	0.52*
<b>CNFL (mm/mm<sup>2</sup>)</b>					
Mean of Differences	0.16	0.73	-0.17	-0.1	-1.18
Spearman's <i>r</i>	0.60‡	0.67‡	0.92‡	0.92‡	0.45 (NS)
<b>TC</b>					
Mean of Differences	-0.22	-1.02	2.42	-4.45	-4.21
Spearman's <i>r</i>	0.53†	0.51*	0.22 (NS)	0.19 (NS)	0.50*

Statistically significant inter-side correlations using paired *t*-test: \* P<0.05, † P<0.01, ‡ P<0.001. Not significant (NS).

## 6.5 Discussion

DSPN is characterized by progressive distal and symmetrical sensory and autonomic nerve damage with eventual motor nerve involvement (3). It is hypothesized that the initial injury occurs in the thinly myelinated A $\delta$ - or myelinated C-fibres where morphological alterations can be assessed with skin biopsy (30). Although, NCS is the preferred endpoint for diagnosis and assessment of outcome in clinical intervention trials, it is limited to large nerves (2). IVCCM has emerged as a powerful technique to detect and stratify human DSPN as it allows direct, non-invasive visualisation of the corneal subbasal nerves (10). Corneal innervation shares anatomical similarities with intra-

epidermal innervation (31) and corneal nerve fibre loss has been found to reflect intra-epidermal nerve fibre loss (7).

Observational studies employing IVCCM to evaluate peripheral neuropathy have reported on the concurrent validity (19), reproducibility (20-21) and optimisation of image selection (28). In a previous study (22) we showed that corneal innervation patterns between RE and LE in healthy subjects are symmetrical with the exemption of branching which showed wider limits of agreement. It is unknown however whether corneal nerve loss remains symmetrical in DSPN of varying severity. A robust test to diagnose DSPN should not only be able to detect changes but also have comparable properties to the clinical presentation and current endpoints of choice i.e. symmetrical involvement (2; 30). This also has important practical relevance when undertaking IVCCM as symmetrical involvement will enable examination of one eye only, reducing the examination time.

This study shows that DSPN, as detected by gold standard clinical and electrophysiological testing, is paralleled by significant and progressive corneal nerve fibre loss, which is highly symmetrical between RE and LE. Specifically, we demonstrate a dramatic stepwise reduction in CNFD, CNFL and CNBD with an increase in TC using the latest 3<sup>rd</sup> generation IVCCM in diabetic patients with increasing severity of neuropathy compared to control subjects. This confirms and extends our findings using the less sensitive 2<sup>nd</sup> generation IVCCM (14). We have also found a significant increase in corneal sensation thresholds with increasing severity of neuropathy (23). The progressive loss of corneal nerve fibres is symmetrical and there is no difference between the right and left eye at any stage of neuropathic severity. Correlation between the right

and left eye was highly significant amongst control subjects and diabetic patients with increasing severity of neuropathy, except in patients with severe neuropathy. This may reflect variability and perhaps the patchy nature of central corneal nerve damage in advanced neuropathy which has been shown recently in a small whole corneal nerve mapping study in a diabetic patient with severe neuropathy (32).

A study by Perkins et al. (2) and an earlier study by Bromberg and Jaros (24) found high inter-side symmetry of NCS in patients with varying degree of DSPN. However, Perkins et al. (2) reported differences in each NCS parameter per nerve as a mean of the whole study cohort, regardless of the severity of neuropathy. To our knowledge no previous studies have assessed whether small fibre involvement in DSPN is symmetrical. A potential limitation and a source of variation is the use of NDS which is large fibre weighted to classify the severity of neuropathy. Thus this may lead to variability when comparing to our findings using IVCCM, a small fibre measure, and may explain the large variation in corneal nerve measures amongst the different groups of neuropathic severity.

In conclusion, we confirm and extend our previous findings (14) in a large cohort of diabetic patients using the latest 3<sup>rd</sup> generation IVCCM with optimal image clarity. We show that DSPN results in progressive corneal nerve fibre loss, which is highly symmetrical except in patients with severe neuropathy.

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**7. CHAPTER VII-VALIDITY OF DETECTION OF  
DIABETIC SOMATIC POLYNEUROPATHY BY IN VIVO  
CORNEAL CONFOCAL MICROSCOPY: HUMAN  
EXPERT VERSUS FULLY AUTOMATED  
QUANTIFICATION**

Author's contribution: Ioannis N Petropoulos contributed to the conception and experimental design, collected and analysed data, performed statistical analysis and wrote the manuscript which formed the basis for this chapter.

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## 7.1 Abstract

**Objective** To assess the performance of a novel automated quantification algorithm of IVCCM images compared to human expert quantification and evaluate the validity of three primary nerve morphometric parameters which have been deployed to diagnose and stratify the severity of DSPN.

**Methods** 186 patients with type 1 and type 2 DM representing a wide spectrum of neuropathic severity and 55 age-matched controls were assessed with gold standard clinical endpoints and underwent bilateral IVCCM. CNFD, CNBD and CNFL were quantified with expert manual and fully-automated analysis. The areas under the curve (AUC), optimal thresholds, odds ratios (OR), positive and negative likelihood ratios for the diagnosis of DSPN were calculated for both quantification methods.

**Results** Neuropathy was detected in 100 (53%) patients with DM. A progressive and significant reduction of manual / automated CNFD ( $P < 0.0001$ ) / ( $P < 0.0001$ ), CNBD ( $P = 0.0005$ ) / ( $P = 0.0002$ ) and CNFL ( $P = 0.0002$ ) / ( $P < 0.0001$ ) was found with increasing neuropathic severity. The two quantification methods were highly correlated for CNFD ( $r = 0.9$ ,  $P < 0.0001$ ) and CNFL ( $r = 0.89$ ,  $P < 0.0001$ ) and CNBD ( $r = 0.75$ ,  $P < 0.0001$ ). Manual CNFD and automated CNFL were associated with the highest AUC (0.84/0.84), sensitivity/specificity [(0.79/0.78) & (0.77/0.74)] and OR (16.5 and 12.9 respectively) to diagnose neuropathy.

**Conclusion** Diabetic peripheral neuropathy is paralleled by significant corneal nerve loss detected with IVCCM. Fully automated corneal nerve quantification offers an objective and robust means of assessing neuropathic deficits.

## 7.2 Introduction

Diabetic neuropathy is heterogeneous in nature and can vary by symptoms, pattern of involvement and risk covariates (1). DSPN is the most common type and the main initiating factor for foot ulceration and amputation, with vast medical, personal, social and economic implications in the US (2), the UK (3) and the rest of the world (4-5). Cardiometabolic clustering and chronic glycaemic exposure, are common risk factors for the onset and progression of the disease (6-7). The UKPDS (8) found that DSPN was present in excess of 10% at diagnosis of type 2 DM while the EURODIAB IDDM Complications Study (9) has reported prevalence as high as 28%. Another UK-wide hospital population study (10) reported that DSPN was prevalent in 23% type 1 DM and 32% type 2 DM patients and that prevalence increased with age and duration of diabetes. The Seattle prospective diabetic foot study found a 50% overall prevalence of neuropathy and a 20% incidence at follow up amongst subjects without DSPN at baseline (7). Finally, the Rochester Diabetic Neuropathy Study (11) reported a 45-54% prevalence of neuropathy, which differed by types of diabetes and severity.

Signs and symptoms together with electrodiagnostic studies are the current endpoints of choice to diagnose DSPN and assess therapeutic benefit in clinical intervention trials (12). These tests predominantly assess large fibre deficits, yet the earliest deficits for DSPN occur in the small unmyelinated C and thinly myelinated A $\delta$ -nerve fibres(13). These early small fibre deficits can be evaluated using quantitative sensory testing by establishing warm and cold sensory thresholds. However, the assessment of thermal thresholds is liable to variability and have limited reproducibility. More accurate quantification of

small fibre deficits can be established by evaluating the IENFD obtained from skin biopsy (14). However, skin biopsy is an invasive and costly technique and is not routinely available across health care systems. We (15) pioneered the use IVCCM and showed that this rapid non-invasive ophthalmic technique could accurately quantify changes in the human subbasal nerve plexus of patients with diabetes. These studies showed that alterations in the subbasal corneal nerves occur early, and progress with neuropathic severity (16) and are paralleled by significant IENF loss (17). Recent studies have shown that chronic glycaemic exposure (18) even in subjects without overt diabetes (19), blood pressure (18) and elevated serum triglycerides (20) are strong risk factors for corneal subbasal nerve loss. Furthermore, early reinnervation of the cornea has been shown in recipients of SPK (21-22).

Concerns regarding the use of IVCCM focus on its ability to diagnose DSPN, reproducibility of the technique and the absence of an automated image analysis system for corneal nerve quantification. The latter is essential to make IVCCM an objective and rapid technique which can be used in the clinic and be deployed as a surrogate end point in clinical trials of diabetic and other neuropathies. However, recent studies have reported high repeatability of IVCCM between observers and occasions (22-24) and have also confirmed symmetrical loss of nerve fibres in diabetic patients with neuropathy (24). We (25-26) and others (27-28) have developed automated assessment of the corneal subbasal nerves and previously we have proposed an algorithm, which concurrently uses a dual model feature descriptor and a neural network classifier to train the computer to distinguish nerve fibres from the background (25). In the present study we have undertaken a cross-sectional, observational

case-control study to assess the performance of a fully automated algorithm compared to manual human expert quantification in diabetic patients with varying severities of neuropathy. Additionally, we have evaluated the diagnostic validity of the three primary morphometric parameters which have been deployed to diagnose and stratify the severity of human DSPN in relation to established clinical endpoints.

### **7.3 Methods**

#### **7.3.1 Study subjects**

186 patients with type 1 and type 2 DM and 55 age-matched controls ( $50.4 \pm 14.1$  v  $51.7 \pm 11.4$ ) were assessed between 2010 and 2011. Informed written consent was obtained from all participants prior to their enrolment to the study. This research adhered to the tenets of the declaration of Helsinki and was approved by the North Manchester Research Ethics Committee.

#### **7.3.2 Medical status assessment**

All participants underwent assessment of their cardiometabolic (HbA<sub>1c</sub>, HDL and LDL cholesterol, triglycerides, BMI) and renal status (eGFR, ACR). Thyroid function, vitamin B<sub>12</sub> level and electrophoresis were performed to exclude other causes of peripheral neuropathy.

#### **7.3.3 Peripheral neuropathy assessment**

We undertook a uniform investigator-masked protocol to prospectively evaluate signs and symptoms of peripheral neuropathy. The NDS, a scale of 0-10, was initially used to stratify patients as “with signs of neuropathy” (>2) or

“without signs of neuropathy” (0-2); controls did not exhibit symptoms or signs of peripheral neuropathy (Table 1 and 2). The NSP was employed to assess symptoms of neuropathy. Vibration perception threshold was evaluated on the great toe of both feet with a Neuroesthesiometer (Horwell Scientific Laboratory Suppliers, Wilford, UK). CT and WT, CIP and HIP induced pain were established on the dorsolateral aspect of the left foot using the TSA-II NeuroSensory Analyser (Medoc Ltd., Ramat-Yishai, Israel) using the method of limits.

Electro-diagnostic studies were undertaken using a Dantec “Keypoint” system (Dantec Dynamics Ltd, Bristol, UK) equipped with a DISA temperature regulator to keep limb temperature constantly between 32-35°C. Peroneal motor and sural sensory nerves were assessed in the left lower limb (calf-to-ankle) by a consultant neurophysiologist. The specific parameters were peroneal compound muscle action potential (PMNamp) and conduction velocity (PMNCV) and sural sensory nerve action potential amplitude (SSNmap) and conduction velocity (SSNCV). The peroneal motor nerve study was performed using silver-silver chloride surface electrodes at standardized sites defined by anatomical landmarks and recordings for the sural sensory nerve were taken using antidromic stimulation over a distance of 100mm. 11 patients from the diabetes cohort did not agree to undergo NCS. These patients were not excluded from the study but were not considered when NCS results were assessed.



### **7.3.4 Study definition of peripheral neuropathy**

The Toronto Diabetic Neuropathy Expert Group (1) recommendation was followed to define “Confirmed DSPN: the presence of an abnormality of NCS and a symptom or symptoms or a sign or signs of neuropathy. In the absence of an abnormal NCS, a validated measure of SFN should be used” and “Subclinical DSPN: the presence of no signs or symptoms of neuropathy confirmed with an abnormal NCS or a validated measure of SFN”. In the absence of a universally accepted, validated protocol to define a definitely abnormal result we have used a mean  $\pm$  2SD cut-off based in our diabetes cases database for NCS and QST.

### **7.3.5 In vivo corneal confocal microscopy**

Study subjects were scanned with a laser IVCCM (HRT III RCM) (Heidelberg Engineering GmbH, Heidelberg, Germany) as described previously (24) (see also Chapter 3, section 3.3.2). Briefly, this IVCCM uses a 670 nm wavelength helium neon diode laser, which is a class I laser and therefore does not pose any ocular safety hazard. A 63x objective lens with a numerical aperture of 0.9 and a working distance, relative to the applanating cap (TomoCap<sup>®</sup>, Heidelberg Engineering GmbH, Heidelberg, Germany) of 0.0 to 3.0 mm was used. The size of each two-dimensional image produced was 384  $\mu$ m x 384  $\mu$ m which has a 15° x 15° field of view and 10  $\mu$ m/pixel transverse optical resolution. HRT III RCM uses an entirely digital image capture system. The overall examination took approximately 5 minutes for both eyes of each subject and in this study two experienced examiners performed all IVCCM scans. All images were captured using the “section” mode in the Heidelberg

Explorer of the HRT III RCM. Prior to IVCCM, corneal sensation was assessed as a functional correlate using non-contact corneal aesthesiometry (NCCA).

### **7.3.6 Manual Image Analysis**

One examiner masked from the outcome of the medical and peripheral neuropathy assessment quantified the subbasal nerve morphology in 1506 images of all study participants using purpose-written, proprietary software (CCMetrics<sup>®</sup>, M. A. Dabbah, Imaging Science Biomedical Engineering, University of Manchester, Manchester, UK). During a bilateral IVCCM scan > 100 images / patient were captured. We selected 6 images equally divided between right and left eyes; any number of 5-8 images allows subbasal corneal nerve quantification with a relatively high accuracy given the size of the frame and the variability observed (29). Criteria for image selection were depth, focus position and contrast. The specific parameters measured per frame were: CNFD (no./mm<sup>2</sup>), CNFL (mm/mm<sup>2</sup>) and CNBD (no./mm<sup>2</sup>) in accord with our previously published data (24).

### **7.3.7 Automated image analysis**

Automated corneal nerve fiber quantification consists of two steps: (1) IVCCM image enhancement and nerve fiber detection and (2) quantification of CNFD, CNBD and CNFL. The detection of nerve fiber is a challenging task, as the nerve fibers often show poor contrast in the relatively noisy images. As described in our earlier work (25-26), a dual-model feature descriptor combined with a neural network classifier was used to train the computer to distinguish nerve fibers from the background (noise and underlying connective

tissue). In the nerve fiber quantification process, all the end points and branch points of the detected nerve fibers are extracted and used to construct a connectivity map. Each segment in the connectivity map can then be connected and classified as main nerve fibers or branches according to the nerve intensity, orientation and length.

### **7.3.8 Statistical analysis**

At 0.05 level of significance and power of 90% a minimum sample of 78 participants with diabetic neuropathy (determined by the reference standard, NCS/QST/NDS) was required to determine sensitivity of IVCCM. Specificity was determined by recruiting an equal number of subjects without neuropathy. Statistical analysis was performed using StatsDirect for Windows (version 2.7.9, StatsDirect Ltd., Cheshire, UK) and STATA 12 for Windows (Stata Corporation, Texas, USA). Appropriate statistical methods were employed based on the distribution of the data. Correlation analysis was performed to assess the strength of the relationship between automated and manually generated variables. A linear regression model was employed to investigate the consistency of the responses from the fully automated algorithm for a given manual estimate of CNFD, CNBD and CNFL respectively. The intra-class correlation coefficient (ICC) was calculated as a measure of reliability i.e. reproducibility of the automated image analysis algorithm over repeated assessment of the dataset. One-way analysis of variance (ANOVA) or non-parametric Kruskal-Wallis were used to evaluate within and between group differences in manual and automated IVCCM responses based on the case definition of neuropathic severity in this study. Overall the *P* value was

maintained at 0.05 for multiple comparisons (Bonferoni adjustment or Conover-Inman pairwise comparisons) and a  $P < 0.05$  was considered significant.

ROC analysis was performed and ROC curves were generated for all IVCCM related parameters to identify the point closest to the upper left corner of the ROC graph which concurrently optimised sensitivity and specificity. The Wilcoxon estimate of the AUC was calculated directly by an extended trapezoidal rule and a confidence interval was constructed using DeLong's variance estimate (30). We also calculated the diagnostic OR, positive (+LR) and negative likelihood ratios (-LR) associated with the point, which concurrently optimized sensitivity and specificity to estimate the strength of the relationship between two measures of DSPN. Confidence intervals (CI) for likelihood ratios were generated using the approach to binomial proportions suggested by Gart and Nam (31). The diagnostic validity of IVCCM was assessed with respect to four measures of DSPN (PMNamp, SSNamp, PMNCV or WT). A  $\chi^2$  test was the method of choice to compare the AUCs generated for all IVCCM parameters.

## **7.4 Results**

### **7.4.1 Medical status and DSPN assessment**

Detailed medical and DSPN assessment results for subjects with diabetes and controls are presented in Table 7-1. Diabetic patients with "neuropathy" compared to those "without neuropathy" and control subjects had a significantly higher ACR ( $P < 0.0001$ ), BP<sub>sys</sub> ( $P = 0.0003$ ), VPT ( $P < 0.0001$ ), WT ( $P = 0.0005$ ), CT ( $P = 0.0004$ ), CIP ( $P < 0.0001$ ), and a significantly lower eGFR ( $P < 0.0001$ ), PMNCV ( $P < 0.0001$ ), SSNCV ( $P < 0.0001$ ), PMNamp ( $P$

< 0.0001) and SSNamp ( $P < 0.0001$ ). Subjects with DSPN had a longer duration of diabetes ( $34.4 \pm 17.3$  v  $24.2 \pm 21.2$ ,  $P = 0.01$ ) but there was no difference in age between controls and the entire diabetes cohort ( $50.7 \pm 14.9$  v  $51.7 \pm 11.4$ ,  $P > 0.05$ ) although diabetic patients “with DSPN” tended to be older than these “without DSPN” ( $55.3 \pm 12.4$  v  $47.3 \pm 15.6$ ,  $P=0.001$ ). Metabolic control and BMI were comparable between the diabetic patients with and without neuropathy but higher compared to controls ( $HbA_{1c}$ ,  $P < 0.0001$ ) and (BMI,  $P < 0.05$ ). Diabetic patients with / without neuropathy had generally higher systolic BP ( $P < 0.0001$ ), VPT ( $P < 0.0001$ ), WT ( $P = 0.002$ ), CT ( $P = 0.0003$ ), PMNCV ( $P < 0.0001$ ), SSNCV ( $P < 0.0001$ ) and SSNamp ( $P < 0.0001$ ). Total cholesterol was similar between the two groups with diabetes, and paradoxically lower compared to controls ( $P < 0.0001$ ). However, the vast majority of patients with diabetes in this study were on a cholesterol-lowering medication.

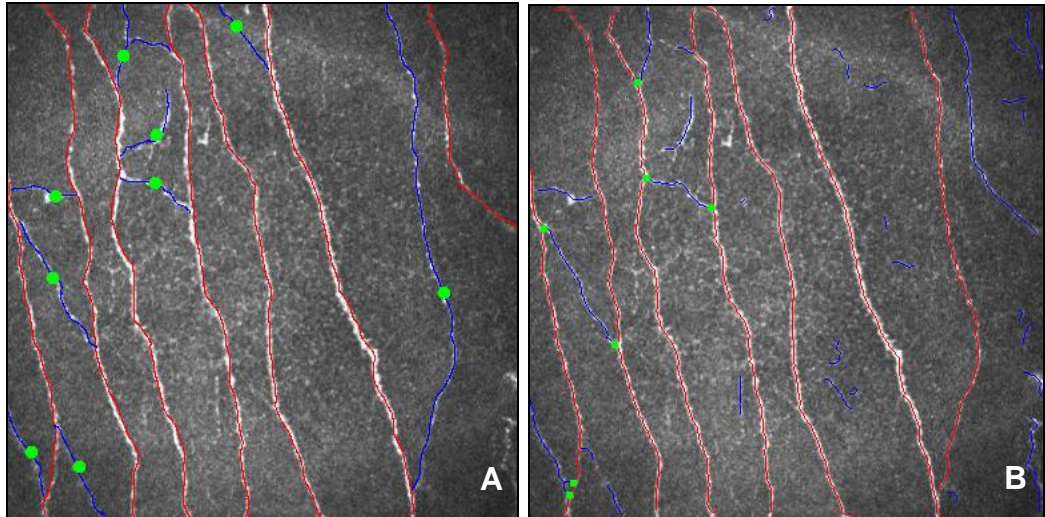
#### **7.4.2 Manual and Automated assessment of DSPN with IVCCM**

Diabetic subjects “with DSPN” compared to diabetic subjects “without DSPN” and controls had significantly lower manually  $CNFD_M$  ( $P < 0.0001$ ),  $CNBD_M$  ( $P = 0.0005$ ),  $CNFL_M$  ( $P = 0.0002$ ) and automatically quantified  $CNFD_A$  ( $P < 0.0001$ ),  $CNBD_A$  ( $P = 0.0002$ ) and  $CNFL_A$  ( $P < 0.0001$ ) parameters. Diabetic patients “without DSPN” had significantly lower  $CNFD_M$  ( $P < 0.0001$ ),  $CNBD_M$  ( $P = 0.0006$ ),  $CNFL_M$  ( $P = 0.0003$ ) and  $CNFD_A$  ( $P < 0.0001$ ),  $CNBD_A$  ( $P = 0.0003$ ) and  $CNFL_A$  ( $P < 0.0001$ ) compared to control subjects. Furthermore, changes detected using automated image quantification were associated with a stronger significance level. NCCA showed a significant elevation in the

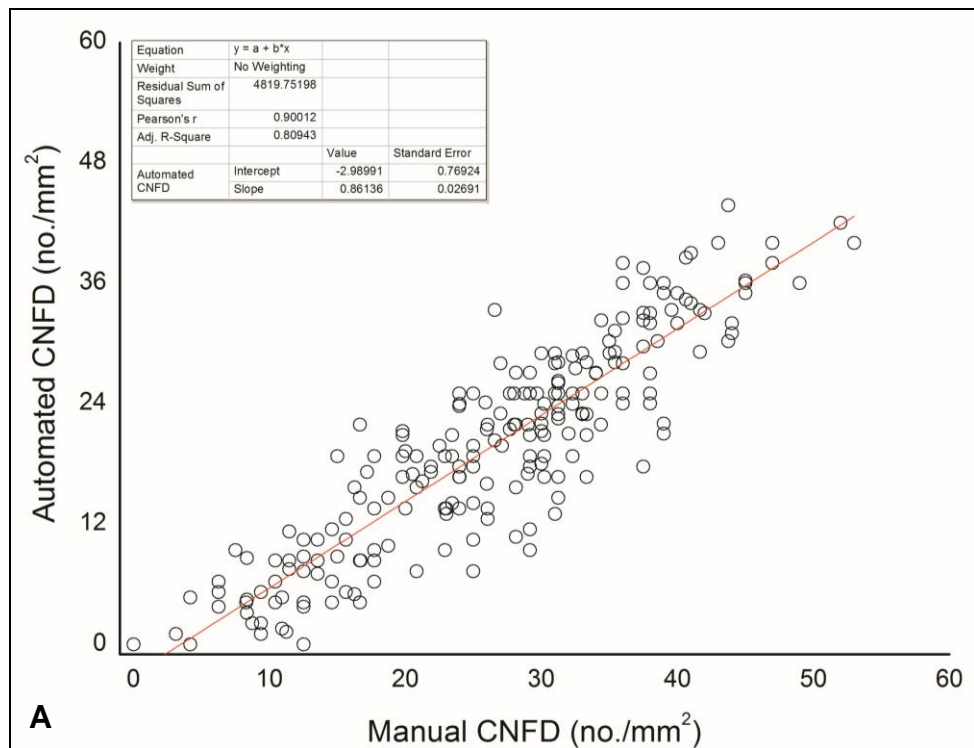
corneal sensation threshold in diabetic subjects and control subjects ( $P = 0.004$ ). All results are presented in detail in Table 7-1.

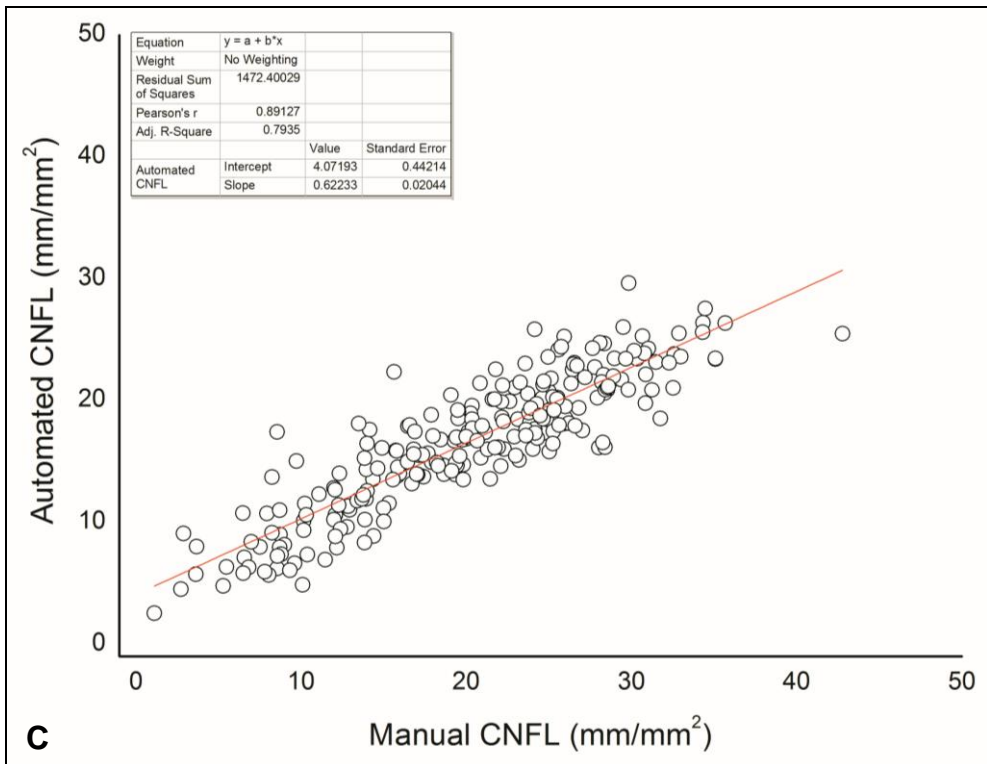
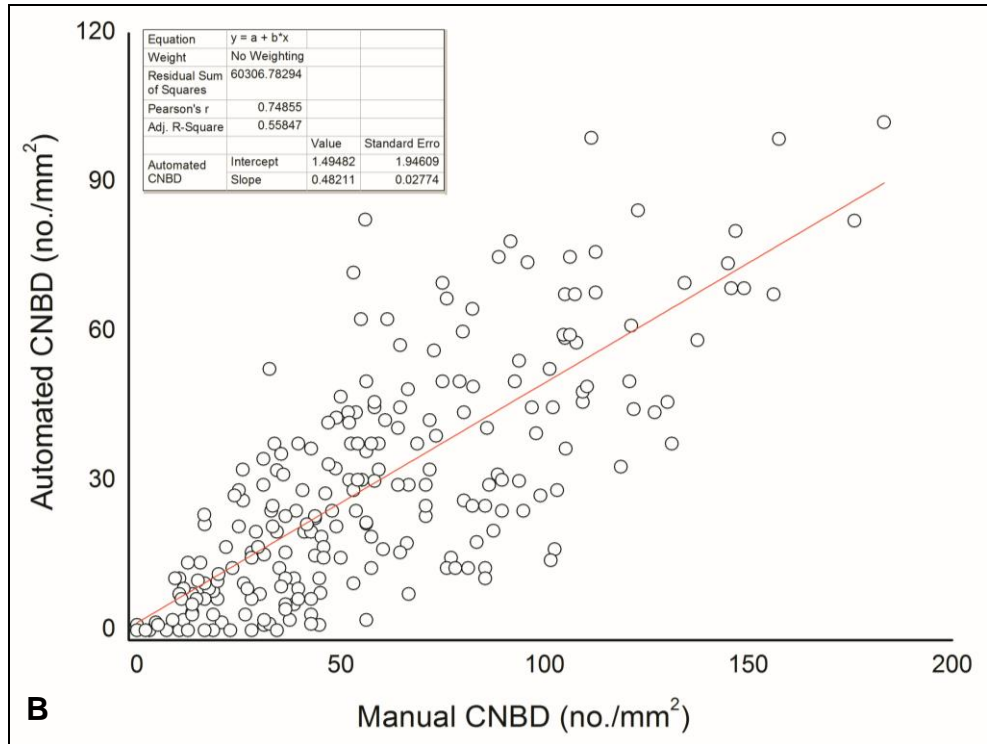
### **7.4.3 Manual versus automated IVCCM image analysis**

The automated value was lower than the manually generated value for corneal nerve morphology parameters. However, the manual and automated results were strongly correlated for CNFD (adjusted  $R^2 = 0.81$ ,  $r = 0.90$   $P < 0.0001$ ), CNBD (adjusted  $R^2 = 0.58$ ,  $r = 0.75$   $P < 0.0001$ ) and CNFL (adjusted  $R^2 = 0.79$ ,  $r = 0.89$   $P < 0.0001$ ) (Figure 7-2A, B and C). Upon reevaluation of the same dataset the reproducibility of the automated algorithm was excellent (ICC = 0.98) across all IVCCM parameters. Moreover, automated quantification significantly enhanced the image analysis time. Each image required a maximum of 22 seconds to be processed automatically while manual analysis was estimated at 2-7 minutes per image depending on the density of the nerves. Sample analysed images are presented in Figure 7-1.



**Figure 7-1** An IVCCM image of a control subject analysed using (A) manual expert and (B) fully-automated image analysis to quantify corneal subbasal nerve morphology in DSPN. Accurate fully-automated quantification is achieved through background noise elimination and nerve fibre contrast enhancement using a multi-scale dual model with neural network classification. Use of either quantification method results in the detection of almost identical structures in the image.





**Figure 7-2** Correlation between manual and fully automated measurements for (A) CNFD, (B) CNBD and (C) CNFL



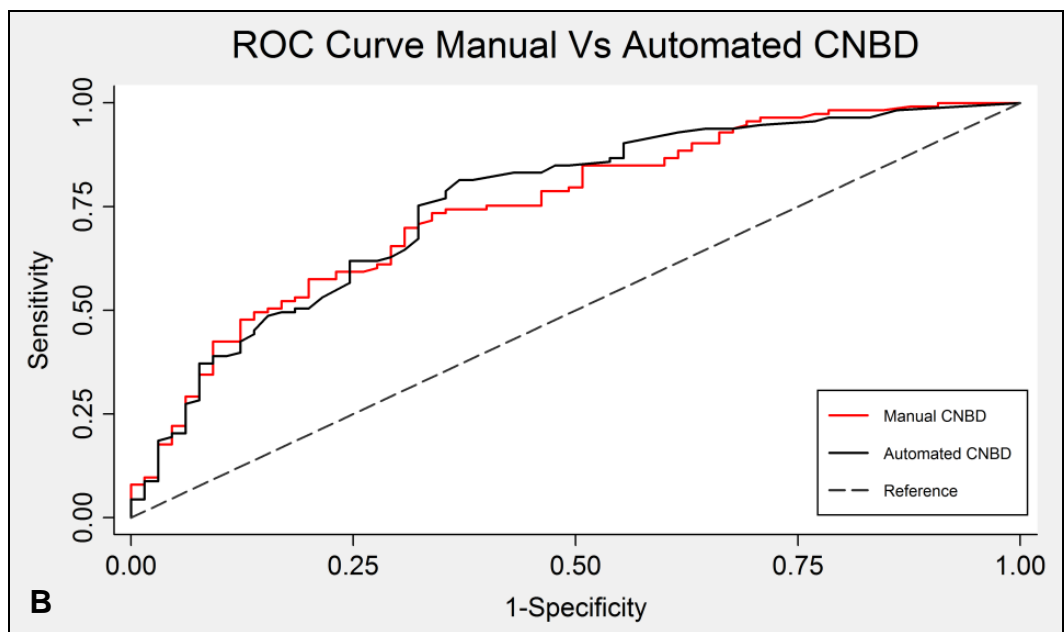
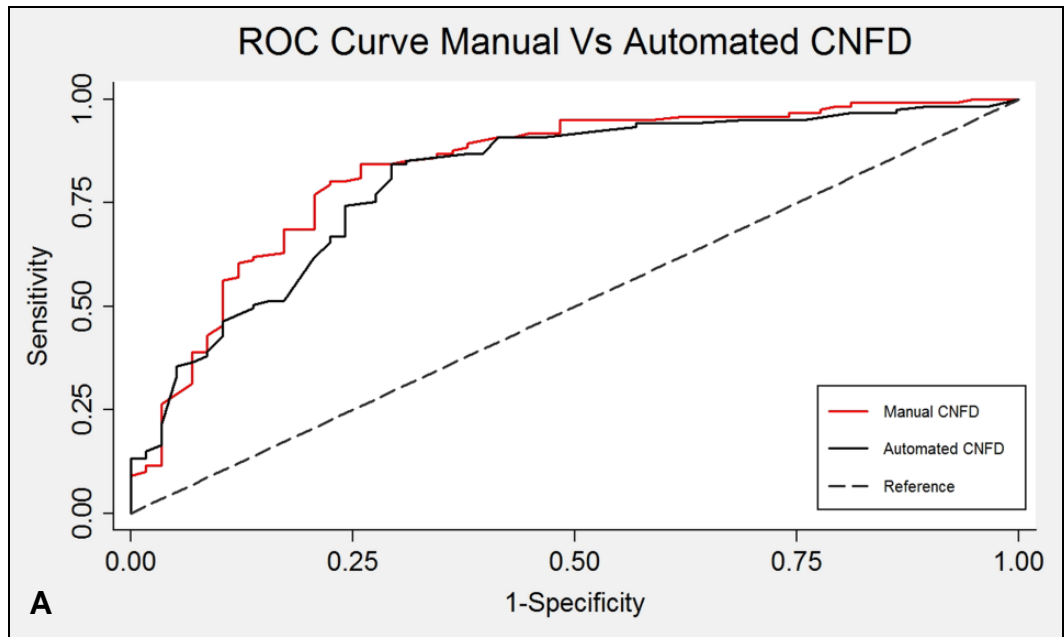
#### **7.4.4 Validity of IVCCM-related nerve morphometric parameters to diagnose DSPN: Human expert versus fully automated quantification**

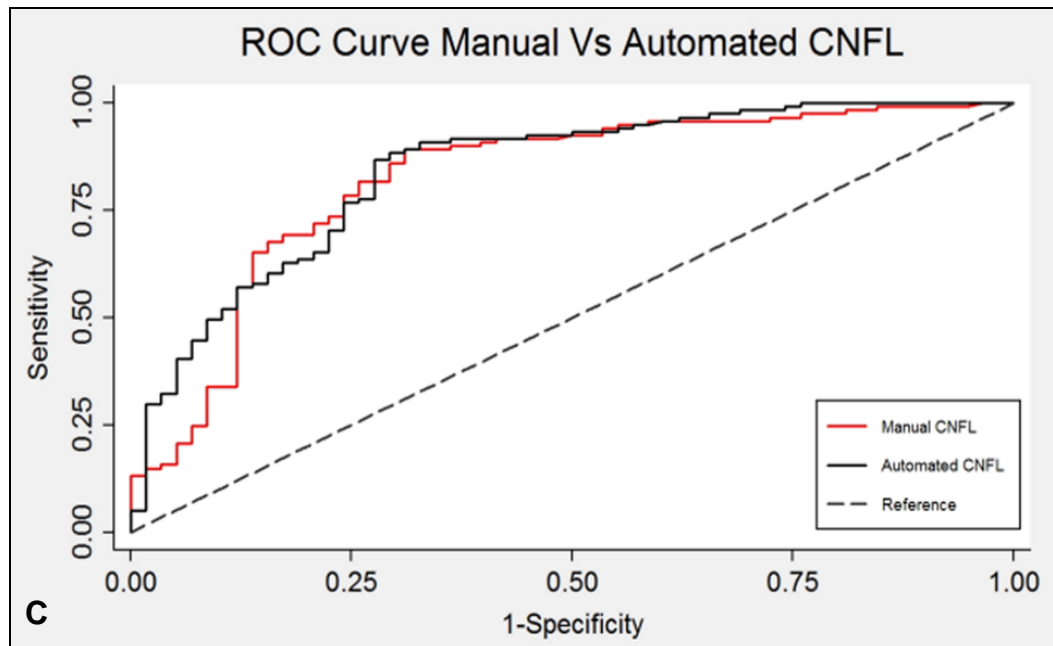
ROC curves were inspected for concurrent optimization of sensitivity and specificity and the associated AUCs were calculated for manual and automated IVCCM parameters with respect to the definition of “neuropathy” (Table 7-3).

##### ***PMNamp < 1.4 uV***

178 patients with diabetes had a valid NCS result. 53 (30%) of diabetic patients had neuropathy based on an abnormality of PMNamp. A CNFD<sub>M</sub> < 18.7 no./mm<sup>2</sup> was the point where sensitivity (0.79) and specificity (0.78) were concurrently optimized with the highest AUC 0.84, OR 16.5, +LR 4.6 (95% CI 3.0 – 6.9) and -LR 0.3 (95% CI 0.2 – 0.4). The corresponding point for automated analysis was CNFD<sub>A</sub> < 14.7 no./mm<sup>2</sup> with sensitivity (0.76) and specificity (0.72) and AUC 0.80, OR 11.0, +LR 3.4 (95% CI 2.4 – 4.9) and -LR 0.3 (95% CI 0.2 – 0.5) (Figure 3a). Amongst the 53 patients with abnormal PMNamp, 41 (77%) had an abnormal CNFD<sub>M</sub>, while in the cohort without a defect only 21 (17%) had an abnormal CNFD<sub>M</sub>. Results for automated analysis were comparable with the number of diabetic patients with an abnormal CNFD<sub>A</sub> in the absence of an abnormal PNamp showing a slight increase to 29 (22%). Similarly, CNFL<sub>M</sub> and CNFL<sub>A</sub> were associated with an AUC of 0.82 and 0.84 respectively, +LR of 3.23 (95% CI 2.3 – 4.6) and -LR 0.33 (95% CI 0.2 – 0.5). Manual and automated CNBD showed a significantly worse (P = 0.01) performance and more variability amongst patients with diabetes and were

associated with lower OR of 5.9 (95% C.I. 2.7 – 13.1) +LR of 2.2 (95% CI 1.7 – 3.1) and -LR of 0.4 (95% CI 0.2 – 0.5). CNBD<sub>A</sub> was however superior to CNBD<sub>M</sub> as it was associated with a higher AUC (0.79) and OR of 9.2 (Figure 7-3) (supplementary material Appendix 13).





**Figure 7-3** ROC curves for manual (solid black) and automated (red) CNFD (A), CNBD (B) and CNFL (C). CNFD and CNFL showed with the highest validity to diagnose DSPN with comparable AUCs (no significant difference). Manual CNFD and automated CNFL were associated with the highest OR.

### ***SSNamp < 5.5 uV***

When an abnormal SSNamp result was used as an indication of neuropathic deficits in the diabetes cohort, the number of cases with a defect increased to 72 (40%) (Table 7-3). CNFL<sub>A</sub> was associated with the highest AUC (0.77) and the highest OR 5.1. A CNFL<sub>A</sub> < 16.1 mm/mm<sup>2</sup> was the point where sensitivity (0.72) and specificity (0.66) optimized with +LR 2.1 (95% CI 1.6 – 2.9) and -LR 0.4 (95% CI 0.3 – 0.6). CNFL<sub>M</sub> < 19.1 mm/mm<sup>2</sup> was the corresponding point, which optimized sensitivity (0.68) and specificity (0.67) but was associated with a significantly (P = 0.01) lower AUC (0.70) and OR 4.6 and comparable +LR 2.1 (95% CI 1.5 – 3.0) and - LR 0.5 (95% CI 0.3 – 0.7). Based on the parameter with the highest OR from the 72 patients with an abnormal

SSNamp, 52 (72%) had an abnormal CNFL<sub>A</sub>. There was no significant difference between CNFD<sub>M</sub> and CNFD<sub>A</sub> in AUCs, ORs, +LR and – LR (results shown in table). CNBD<sub>A</sub> showed a significantly ( $P = 0.02$ ) greater AUC compared to CNBD<sub>M</sub> (0.70 v 0.65) but both had comparably poor OR (2.1 v 2.1), and + LR (1.4 v 1.9, 95% CI 1.0 – 1.9 v 1.6 – 2.5) and -LR (0.7 v 0.7 95% CI 0.3 – 0.7 v 0.3 – 0.6).

### ***PMNCV < 42 m/s***

96 (54%) of diabetic patients had an abnormal PMNCV result. CNFL<sub>A</sub> was the parameter associated with the highest AUC (0.79) and a CNFL<sub>A</sub> < 16.0 mm/mm<sup>2</sup> optimized sensitivity (0.74) and specificity (0.71) with a diagnostic OR 7.2, +LR 2.6 (95% CI 1.9 – 3.8) and - LR = 0.3 (95% CI 0.2 – 0.5). A CNFL<sub>M</sub> < 19.7 mm/mm<sup>2</sup> was associated with 0.74 sensitivity and 0.63 sensitivity, AUC 0.73, OR 4.8, +LR 2.0 (95% CI 1.6 – 2.6) and -LR 0.4 (95% CI 0.3 – 0.6). Both CNFD<sub>M</sub> and CNFD<sub>A</sub> showed higher diagnostic OR (8.2 and 7.8 respectively) but lower AUC (0.74) compared to CNFL<sub>A</sub>. Based on CNFL<sub>A</sub>, amongst the 93 patients with neuropathy, 71 (76%) also had a lower CNFL; while the percentage with reduced CNFL in the absence of an abnormal PMNCV fell to 23 (27%). CNBD<sub>M</sub> and CNBD<sub>A</sub> showed inferior performance associated with lower AUC, OR, + LR and - LR (detailed results are shown in Table 7-3).

### ***WT > 42°C***

95 (51%) diabetic patients had abnormal WT and 93 (49%) were within normal limits. When an abnormal WT, was used to define neuropathy the performance of IVCCM decreased significantly and none of the parameters reached an

AUC > 0.70. CNFD<sub>M</sub> and CNFD<sub>A</sub> were associated with the highest AUC and modest OR. Specifically, a CNFD<sub>M</sub> < 24.0 no./mm<sup>2</sup> optimized sensitivity (0.63) and specificity (0.62) and was associated with AUC 0.69, OR 2.9, + LR 1.6 (95% CI 1.2 – 2.1) and -LR 0.7 (95% CI 0.5 – 0.8). The number of patients with an abnormal CNFD<sub>M</sub> and WT < 42°C was 61 (64%), while 35 (37%) had reduced CNFD<sub>M</sub> with a normal WT result. CNFD<sub>A</sub>, CNFL<sub>M</sub> and CNFL<sub>A</sub> showed comparable performance but were associated with slightly lower AUC and OR while sensitivity and specificity remained modest. CNBD<sub>M</sub> and CNBD<sub>A</sub> remained lower than all other parameters (results are shown in Table 7-3).

**Table 7-1** Medical and peripheral neuropathy status

Variable	Controls ( <i>n</i> = 55)	Diabetes without DSPN ( <i>n</i> = 86)	Diabetes with DSPN ( <i>n</i> = 100)
	(NDS = 0)	(NDS ≤ 2)	(NDS > 2)
Duration of Diabetes	N/A	24.2 ± 21.2	34.4 ± 17.3
HbA <sub>1c</sub> (%) ‡	5.5 ± 0.3	7.7 ± 1.6 ¶	7.9 ± 1.6 ¶
BMI (Kg/m <sup>2</sup> ) *	25.6 ± 4.6	27.2 ± 5.2	27.6 ± 5.8 ¶
Total cholesterol (mmol/l) ‡	5.1 ± 0.9	4.3 ± 1.2 ¶	4.4 ± 0.9 ¶
Triglycerides (mmol/l)	1.5 ± 0.8	1.5 ± 0.9	1.4 ± 0.9
eGFR (ml/min/l) ‡	85.8 ± 7.8	81.8 ± 18.2	70.0 ± 24.5 ¶§
ACR (mg/mmol) ‡	1.0 ± 1.4	2.9 ± 1.3	18.8 ± 11.3 ¶§
BP (mm Hg) Systolic † / Diastolic	122 ± 16 / 70 ± 8.8	130 ± 18 ¶ / 71 ± 9	138 ± 23 ¶§ / 72 ± 8
NSP	0	1.9 ± 3.0	5.6 ± 6.2
VPT (V) ‡	5.8 ± 4.6	9.2 ± 6.5 ¶	22.3 ± 12.6 ¶§
WT † / CT † (°C)	37.0 ± 3.0 / 28.2 ± 2.2	39.6 ± 3.9 ¶ / 27.0 ± 9.2 ¶	42.7 ± 4.6 § / 20.8 ± 9.2 ¶§
HIP / CIP ‡ (°C)	44.8 ± 2.9 / 11.9 ± 9.2	45.5 ± 6.6 / 9.8 ± 10.7	46.9 ± 7.3 / 4.1 ± 6.2 ¶§
PMNCV (m/s) ‡	48.8 ± 3.3	43.7 ± 4.7 ¶	39.2 ± 6.1 ¶§
SSNCV (m/s) ‡	51.0 ± 4.8	46.4 ± 5.8 ¶	42.2 ± 6.4 ¶§
PMNamp (uV) ‡	5.2 ± 1.8	4.5 ± 3.2	2.4 ± 2.1 ¶§
SSNamp (uV) ‡	20.0 ± 9.7	12.5 ± 7.8 ¶	6.5 ± 6.6 ¶§

Results are expressed as mean ± SD, statistically significant differences using ANOVA / Kruskal-Wallis: \* P<0.05, ‡ P<0.01, † P<0.001, ‡ P < 0.0001 Post hoc results for diabetes “with signs of DSPN” significantly different from ¶ control subjects and § diabetes “without signs of DSPN”. N/A: not applicable.

**Table 7-2** IVCCM assessment of DSPN status

Variable	Controls	Diabetes without DSPN	Diabetes with DSPN
	(NDS = 0)	(NDS ≤ 2)	(NDS > 2)
<b>Manual IVCCM quantification</b>			
CNFD <sub>M</sub> (no./mm <sup>2</sup> ) ‡	37.2 ± 6.7	26.7 ± 8.5 ¶	20.5 ± 9.5 ¶§
CNBD <sub>M</sub> (no./mm <sup>2</sup> ) †	92.7 ± 38.6	54.9 ± 35.7 ¶	48.7 ± 33.2 ¶
CNFL <sub>M</sub> (mm/mm <sup>2</sup> ) †	26.4 ± 5.6	20.3 ± 6.7 ¶	16.7 ± 7.6 ¶§
<b>Automated IVCCM quantification</b>			
CNFD <sub>A</sub> (no./mm <sup>2</sup> ) ‡	30.0 ± 6.9	20.1 ± 8.7 ¶	14.4 ± 8.9 ¶§
CNBD <sub>A</sub> (no./mm <sup>2</sup> ) †	50.4 ± 24.7	31.4 ± 25.6 ¶	20.1 ± 18.7 ¶§
CNFL <sub>A</sub> (mm/mm <sup>2</sup> ) ‡	21.2 ± 3.5	17.1 ± 4.5 ¶	13.7 ± 5.2 ¶§
<b>Corneal Sensation</b>			
NCCA (mbar) ¿	0.7 ± 0.5	0.9 ± 0.8 ¶	1.5 ± 2.1 ¶

Results are expressed as mean ± SD, Statistically significant differences using ANOVA / Kruskal-Wallis: \* P<0.05, ¿ P<0.01, † P<0.001, ‡ P < 0.0001 Post hoc results for diabetes “with signs of DSPN” significantly different from ¶ control subjects and § diabetes “without signs of DSPN”.

**Table 7-3** Validity of DSPN detection using manual and fully automated IVCCM quantification

Definition of DSPN	IVCCM value (sensitivity / specificity)	AUC	Odds Ratio (95% C. I.)	+LR (95% C. I.)	-LR (95% C. I.)
<b>PMNamp (&lt; 1.4 uV)</b>					
CNFD <sub>M</sub>	18.7 (0.79 / 0.78)	0.84	16.5 (7.0 – 39.9)	4.6 (3.0 – 7.0)	0.3 (0.2 – 0.4)
CNFD <sub>A</sub>	14.7 (0.76 / 0.72)	0.80	11.0 (4.8 – 24.8)	3.4 (2.4 – 4.9)	0.3 (0.2 – 0.5)
CNBD <sub>M</sub>	41.7 (0.73 / 0.68)	0.75	5.9 (2.7 – 13.1)	2.3 (1.7 – 3.1)	0.4 (0.2 – 0.6)
CNBD <sub>A</sub>	14.9 (0.74 / 0.73)	0.79	9.2 (4.1 – 21.4)	2.9 (2.1 – 4.7)	0.3 (0.2 – 0.5)
CNFL <sub>M</sub>	15.8 (0.77 / 0.76)	0.82	9.8 (4.4 – 22.0)	3.2 (2.3 – 4.6)	0.3 (0.2 – 0.5)
CNFL <sub>A</sub>	14.6 (0.77 / 0.74)	0.84	12.9 (5.5 – 31.8)	3.3 (2.4 – 4.6)	0.2 (0.1 – 0.4)
<b>SSNamp (&lt; 5.5 uV)</b>					
CNFD <sub>M</sub>	23.1 (0.72 / 0.67)	0.74	4.7 (2.3 – 10.0)	1.9 (1.5 – 2.6)	0.4 (0.3 – 0.6)
CNFD <sub>A</sub>	18.9 (0.73 / 0.56)	0.72	5.1 (2.4 – 11.1)	1.9 (1.5 – 2.5)	0.4 (0.2 – 0.6)
CNBD <sub>M</sub>	47.1 (0.61 / 0.56)	0.65	2.1 (1.1 – 4.9)	1.4 (1.0 – 1.9)	0.7 (0.5 – 1.0)
CNBD <sub>A</sub>	23.4 (0.63 / 0.54)	0.70	2.1 (1.1 – 4.2)	1.4 (1.0 – 1.9)	0.7 (0.5 – 0.9)
CNFL <sub>M</sub>	19.4 (0.68 / 0.67)	0.70	4.6 (2.3 – 9.3)	2.1 (1.5 – 3.0)	0.5 (0.3 – 0.7)
CNFL <sub>A</sub>	16.1 (0.72 / 0.66)	0.77	5.1 (2.5 – 10.4)	2.1 (1.6 – 2.9)	0.4 (0.3 – 0.6)
<b>PMNCV (&lt; 42.0 m/s)</b>					
CNFD <sub>M</sub>	25.4 (0.78 / 0.70)	0.74	8.2 (4.1 – 17.3)	2.6 (1.9 – 3.7)	0.3 (0.2 – 0.5)
CNFD <sub>A</sub>	19.7 (0.80/ 0.61)	0.74	7.8 (3.7 – 16.7)	2.2 (1.7 – 3.0)	0.3 (0.2 – 0.4)
CNBD <sub>M</sub>	49.0 (0.69 / 0.61)	0.68	3.7 (1.9 – 7.2)	1.8 (1.3 – 2.5)	0.5 (0.4 – 0.7)
CNBD <sub>A</sub>	24.9 (0.68 / 0.52)	0.67	2.4 (1.2 – 4.6)	1.4 (1.1 – 1.9)	0.6 (0.4 – 0.9)
CNFL <sub>M</sub>	19.7 (0.74/ 0.63)	0.73	4.9 (2.4 – 9.7)	2.0 (1.5 – 2.8)	0.4 (0.3 – 0.6)
CNFL <sub>A</sub>	16.0 (0.74 / 0.71)	0.79	7.2 (3.5 – 14.7)	2.6 (1.8 – 3.8)	0.4 (0.3 – 0.5)
<b>WT (&gt; 41 °C)</b>					
CNFD <sub>M</sub>	24.0 (0.63 / 0.62)	0.69	2.9 (1.5 – 5.3)	1.7 (1.3 – 2.3)	0.6 (0.4 – 0.8)
CNFD <sub>A</sub>	17.3 (0.63 / 0.60)	0.67	2.5 (1.4 – 4.6)	1.5 (1.2 – 2.1)	0.6 (0.5 – 0.8)
CNBD <sub>M</sub>	47.2 (0.65 / 0.55)	0.65	2.1 (1.2 – 3.8)	1.4 (1.1 – 1.9)	0.7 (0.5 – 0.9)
CNBD <sub>A</sub>	22.9 (0.60 / 0.58)	0.64	2.1 (1.1 – 3.9)	1.4 (1.1 – 2.0)	0.7 (0.5 – 0.9)
CNFL <sub>M</sub>	19.2 (0.63 / 0.61)	0.67	2.7 (1.5 – 5.0)	1.6 (1.2 – 2.2)	0.6 (0.4 – 0.8)
CNFL <sub>A</sub>	15.9 (0.61 / 0.61)	0.68	2.3 (1.3 – 4.2)	1.5 (1.1 – 2.1)	0.7 (0.5 – 0.9)



## 7.5 Discussion

Diabetic peripheral neuropathy is the main initiating factor for foot ulceration and amputation and is associated with heavy morbidity, reduced quality of life and poor healthcare outcomes (32). The prevalence of DSPN, in the diabetic population varies from 10% - 54% (7-11). No or few studies have used objective endpoints to estimate the rates of neuropathy and this may explain the reported variability. Dyck and colleagues (33) found that when NCS was used in combination with a functional abnormality to diagnose DSPN as opposed to conventional clinical examination, twice as many patients were detected. Electrodiagnostic studies are the gold standard to diagnose neuropathy but they are limited to large fibres and previous research has shown that small nerve fibres are affected first (13). An objective, non-invasive surrogate of small fibre damage, such as IVCCM (16), is therefore desirable to diagnose neuropathy early and define those at risk.

All previous studies have identified age, duration of diabetes, renal status, BP, cardiometabolic control and anthropometric parameters as risk factors for the onset and severity of DSPN. Recent studies using IVCCM, have reported an association between levels of HbA<sub>1c</sub>, BP and triglycerides and corneal innervation (18-20). This study assessed 188 subjects with diabetes but no other identifiable cause of neuropathy and found a significant decline in eGFR and increased ACR and systolic BP, which were associated with neuropathy. Modest to poor metabolic control was common between both diabetes groups. Corneal confocal microscopy provides the unique opportunity to repeatedly and reliably visualise the corneal nerves adjacent to Bowman's membrane. An increasing body of literature supports the use of IVCCM in the diagnosis and

severity stratification of DSPN. A major drawback is the absence of an automated analysis system, which would eliminate inconsistencies and make the technique suitable in a clinical setting. This study assessed for the first time the performance and validity of a fully automated image analysis algorithm compared to manual human expert quantification using multiple diagnostic criteria for neuropathic deficits.

We found that both methods of image quantification were highly correlated primarily for CNFD and CNFL but also CNBD. We detected a slight underestimation of corneal nerve density and length when automated analysis was used, which was however consistent. The detection of nerve structures in IVCCM images is a challenging task: nerve fibres often show poor contrast on a relatively noisy background due to microscope properties and underlying structures. As described in our earlier work (25), the algorithm operates through a combination of detection methods and predefined criteria, mainly nerve-specific characteristics such as orientation and axon reflectivity, to construct a connectivity map and distinguish a nerve structure from noise. In contrast, manual image analysis is a labour-intensive task, where a human investigator applies subjective criteria to define a nerve and an overestimation with less experience has been described (24). Peripheral neuropathy as detected with clinical examination and NCS in this study was reflected on all IVCCM parameters, which showed a significant and progressive reduction in nerve density, branching and length between diabetic patients with DSPN and moreover even without DSPN and controls using either quantification method. CNBD did show a significant positive correlation between manual and automated assessment, but this was not as high as for CNFD and CNFL.

CNBD, a measurement of nerve branches connected to nerve fibres, has been reported to be highly variable and appears to have modest validity in diagnosing neuropathy (22; 34). Moreover, inter- and intra- observer estimation of the parameter in highly innervated corneas has shown moderate reproducibility (24). The relevance of corneal nerve branching to DSPN is not clear: in our recent study (35) of the 1-year effects of SPK transplantation in type 1 DM recipients, we found a significant and stable increase indicative of an active regeneration process. In contrast, unpublished 12 month follow-up data of patients with pre-diabetes / early type 2 DM from our centre showed an increase in CNBD despite a significant decrease in CNFD indicating a dynamic role for branches. In this study, automated analysis of CNBD was more capable of staging neuropathy but importantly with less variability compared to manual human analysis.

Recently, two studies have assessed the validity of IVCCM in diagnosing DSPN. Tavakoli et al. (16) has reported a CNFD  $\leq 27.8$  no./mm<sup>2</sup> and  $\leq 20.8$  no./mm<sup>2</sup> as the values with the highest validity to define disease status amongst patients with mild and more severe neuropathy respectively. Ahmed et al. (34) in contrast found that a CNFL  $\leq 14.0$  mm/mm<sup>2</sup> was the value with the highest validity to rule in DSPN. We assessed the performance of manual and automated IVCCM quantification after we classified patients as “with” or “without” neuropathy based on established measures of peripheral nerve damage. We found that CNFD<sub>M</sub>, CNFD<sub>A</sub>, CNFL<sub>M</sub> and CNFL<sub>A</sub> were the parameters associated with the highest sensitivity and specificity to diagnose DSPN. When PMNamp was used as the primary marker of neuropathy, a CNFD<sub>M</sub>  $< 18.7$  was the value which concurrently optimized sensitivity (0.79)

and specificity (0.78) with a high AUC of 0.84 and diagnostic OR = 16.1 (95% C.I. 7.0 – 39.9). A CNFL<sub>A</sub> < 14.6 mm/mm<sup>2</sup> was associated with an equal AUC of 0.84, diagnostic OR = 12.9 (95% C.I. 5.5 – 31.8) with sensitivity of 0.77 and specificity 0.74. CNBD showed less but acceptable validity in diagnosing DSPN and CNBD<sub>A</sub> had a significantly higher AUC and OR compared to CNBD<sub>M</sub>.

When other measures of DSPN were used, such as SSNamp and PMNCV, the diagnostic validity of IVCCM remained high and CNFL<sub>A</sub> was consistently associated with the highest AUC and OR amongst all parameters. We observed a decline in sensitivity and specificity when an abnormality on WT was used as the primary marker of neuropathy. One would expect the opposite since warm detection is mainly mediated by small nerve fibres, and previously an association between IENFD and corneal nerve status was shown (17). This is likely for two main reasons: NCS offer a robust and objective means of assessing neuropathy; on the other hand, WT is a subjective measurement of small fibre function. Cassanova et al. (36) in their study found that even patients with no IENFs had consistent responses in WT, despite a good correlation overall. They note that it is possible for partially damaged nerve endings to still be able to generate a propagated action potential because epidermal and dermal nerves may be implicated in the process. We speculate that a similar association may exist for the corneal nerves. The validity of fully automated IVCCM quantification was comparable and in several cases exceeded the performance of human expert assessment in diagnosing DSPN. A CNFL<sub>A</sub> between 14.6 mm/mm<sup>2</sup> and 16.1 mm/mm<sup>2</sup> was the value consistently associated with the highest AUC and OR given the case definition employed.

CNFD<sub>M</sub> (18.7 – 25.4 no./mm<sup>2</sup>) and CNFD<sub>A</sub> (14.7 – 19.7 no./mm<sup>2</sup>) also showed excellent performance with high OR but were slightly more variable.

This study has several strengths and limitations. Strengths of this study are the detailed clinical assessment by gold standard clinical techniques of a relatively large number of participants with diabetes, representing a wide range of duration of diabetes and neuropathic severity. Moreover, the same highly trained individuals performed all examinations for the 241 participants of this study ensuring consistency of the results. Our findings and cut-off points selected for the diagnosis of DSPN by IVCCM are comparable with the previous studies of Ahmed et al. (34) and Tavakoli et al. (16); slight differences could be due to the case definition of neuropathy employed in each study, the number of patients investigated and the disease severity in each group. We do not provide IENFD assessment, despite the fact that IENFD is the gold standard method to evaluate skin denervation in diabetes and as such relevant to IVCCM. We have however compared IVCCM related parameters to several objective and subjective valid markers of DSPN with significant findings for the validity of the technique. There are no directly comparable published results for the fully automated algorithm employed in this study, therefore we cannot exclude the possibility that another system may be superior to the one presented here. This is to date the only available purpose-built, automated corneal nerve quantification system which has been validated in a large cohort of patients with diabetes and varying degrees of DSPN. Finally, our results are cross-sectional and ongoing longitudinal studies (37) will determine the ability of IVCCM to predict the development and progression or regression of DSPN.

In conclusion, we show that diabetic peripheral neuropathy is paralleled by significant and progressive nerve loss detected by corneal confocal microscopy and that CNFD and CNFL are the parameters associated with the highest validity. We have validated a rapid fully automated analysis system to quantify alterations to replace human manual quantification. The use of this system will clearly enhance reproducibility, eliminate inconsistencies and make the technique suitable to clinical practise worldwide.

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## **8. CHAPTER VIII-CORNEAL CONFOCAL MICROSCOPY DETECTS EARLY NERVE REGENERATION IN DIABETIC NEUROPATHY AFTER SIMULTANEOUS PANCREAS AND KIDNEY TRANSPLANTATION**

Author's contribution: Ioannis N Petropoulos collected and analysed data and revised the manuscript which formed the basis for this chapter. This work was published in the journal *Diabetes* in 2013.

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## 8.1 Abstract

Diabetic neuropathy is associated with increased morbidity and mortality. To date limited data in subjects with impaired glucose tolerance and diabetes demonstrate nerve fibre repair. This may reflect a lack of efficacy of the interventions but may also reflect difficulty of the tests currently deployed to adequately assess nerve fibre repair, particularly in short term studies. IVCCM represents a novel non-invasive means to quantify nerve fibre damage and repair. 15 type 1 diabetic patients undergoing SPK underwent detailed assessment of neurological deficits, QST, electrophysiology, skin biopsy, corneal sensitivity and IVCCM at baseline and at 6 and 12 months after successful SPK. At baseline diabetic patients had a significant neuropathy compared to control subjects. Following successful SPK transplantation there was no significant change in neurological impairment, neurophysiology, quantitative sensory testing, corneal sensitivity and IENFD. However IVCCM demonstrated significant improvements in CNFD, CNBD and CNFL at 12 months. Normalization of glycaemia following SPK shows no significant improvement in neuropathy assessed by the neurological deficits, QST, electrophysiology and IENFD. However, IVCCM shows a significant improvement in nerve morphology, providing a novel non-invasive means to establish early nerve repair, missed by currently advocated assessment techniques.

## 8.2 Introduction

Diabetic polyneuropathy is one of the commonest long-term complications of diabetes and underlies the development of painful neuropathy in 21% of both Type 1 and Type 2 DM patients (1). It is the main initiating factor for foot ulceration and lower extremity amputation (2). At present we have no treatment to repair nerve fibres and improve diabetic neuropathy. Even in the DCCT and follow up EDIC study, improved glycemic control only delayed the progression of clinical diabetic neuropathy and indeed NCS at closeout showed no significant risk reduction (3). Furthermore the STENO-2 study demonstrated that whilst multi-factorial intervention showed an improvement in retinopathy, nephropathy and cardiac autonomic neuropathy, there was no benefit for somatic neuropathy (4). Even in the most dramatic example of “curing” type 1 diabetes with pancreas transplantation, in 115 patients followed over 10 years, neurological function, nerve conduction studies, and autonomic function were only prevented from worsening and failed to show an improvement (5). This is in keeping with the lack of improvement in heart rate variability, 43 months after SPK (6) and IENFD 2.5 years after SPK (7). Neuropathy is of course extremely severe at this stage, as evidenced by severe intra-epidermal nerve fibre depletion in pancreas transplant recipients, suggesting either a point of no return or the need for long-term follow-up to identify post-transplant nerve fibre regeneration (8). However, IENFD and corneal nerve morphology have been shown to improve in subjects with impaired glucose tolerance neuropathy (IGTN) (9) and in patients with Type 2 diabetes (10), respectively, after improvement in metabolic risk factors.

To establish efficacy of a new treatment, ideally an improvement in diabetic neuropathy has to be shown. Whilst current endpoints have a good ability to diagnose diabetic neuropathy (11), their ability to define a therapeutic response may have significant limitations (12). This may indeed be a major reason why clinical trials in human diabetic neuropathy have failed to reach pre-specified primary end points such as neuropathic deficits and electrophysiology (13). The assessments of neurological symptoms and deficits have recently been shown to have poor diagnostic reproducibility (14). Although, electrophysiology correlates with large fibre damage, it does not assess small fibres, which are the earliest to be damaged (15) and demonstrate repair even in advanced neuropathy (12). Nerve fibre morphology in sural nerve biopsies (16) and IENFD in skin-punch biopsies (17) can accurately quantify nerve fibre damage and repair, but both are invasive procedures.

We and others (18-19) have employed corneal confocal microscopy to detect subclinical diabetic neuropathy, relate it to the severity of somatic neuropathy (20) and IENFD (21) with good sensitivity and specificity (21). This led us to propose that IVCCM, a non-invasive and reiterative test might be an ideal surrogate endpoint for evaluating therapeutic efficacy in clinical trials of human diabetic neuropathy (22). In a preliminary study we have previously shown a significant improvement in corneal nerve fibre density and length 6 months after SPK (23), but at that time we did not compare IVCCM with established endpoints of diabetic neuropathy. In the present study we have compared IVCCM with neurological deficits, QST, electrophysiology and IENFD at

baseline, 6 and 12 months after SPK to help define the measures which may best detect an improvement in diabetic neuropathy after intervention.

### **8.3 Methods**

#### **8.3.1 Study subjects**

15 Type 1 diabetic patients were evaluated at baseline (within 3 days from SPK transplantation) and 6 and 12 months after SPK transplantation and compared with 10 age/gender matched non-diabetic healthy control subjects. The healthy volunteers were recruited from general population. Both patients and controls underwent full neurological and medical assessments and those with any history of systemic (apart from diabetes for patient group), neurological conditions, history of ocular trauma, wearing contact lens and ocular surgery were excluded. The study was approved by the Central Manchester Ethics committee, and written informed consent was obtained according to the declaration of Helsinki.

#### **8.3.2 Assessment of Neuropathy**

All patients and controls underwent a detailed evaluation of neurological symptoms according to the NSP and the McGill pain analogue score was used to assess the severity of painful neuropathy. Neurological deficits were assessed using the NDS which includes evaluation of vibration, pin prick and temperature perception as well as the presence or absence of ankle reflexes to establish the severity of neuropathy: NDS 0–2, no neuropathy; NDS 3–5, mild neuropathy; NDS, 6–8, moderate neuropathy; and NDS, 9–10, severe neuropathy. Quantitative sensory testing included an assessment of Vibration

Perception Threshold (VPT), measured using a Neurothesiometer (Horwell, Scientific Laboratory Supplies, Wilford, Nottingham, UK), CT (A $\delta$  fibres) and WT (24) (c fibres) sensation thresholds using the method of limits with the MEDOC *TSA II* (Medoc Ltd., Ramat Yishai 30095, Israel) on the dorsum of the left foot.

CASE IV was used to measure the heart rate response to deep breathing (HRDB). In this test the patient was asked to inhale and exhale deeply eight times in a row in the supine position, whilst following the rhythm of a “breathing cue”, and the changes in heart rate were displayed on an ECG monitor. Two eight-cycle breathing series were completed interspersed by a five minute period of normal breathing. The acquired data were analysed by calculating the mean difference between the highest and lowest heart rate for five consecutive, artifact-free cycles in each eight-cycle series.

Electro-diagnostic studies were undertaken using a Dantec “Keypoint” system (Dantec Dynamics Ltd, Bristol, UK) equipped with a DISA temperature regulator to keep limb temperature constantly between 32-35°C. Peroneal motor and sural sensory nerves were assessed in the right lower limb by a consultant neurophysiologist. The motor study was performed using silver-silver chloride surface electrodes at standardised sites defined by anatomical landmarks and recordings for the sural nerve were taken using antidromic stimulation over a distance of 100mm.

### **8.3.3 Corneal Sensitivity**

Corneal sensitivity was quantified using a non-contact corneal aesthesiometer (NCCA) (Glasgow, Caledonian University, UK) which uses a puff of air through



a bore 0.5mm in diameter lasting 0.9 seconds and exerting a force expressed in millibars (mbars)(25). The stimulus jet is mounted on a slit lamp and is positioned 1 cm from the eye and the air jet is aligned to the centre of the cornea. Each subject was presented with a supramaximal stimulus and the staircase method was employed by reducing the stimulus strength until the patient did not feel the jet on three occasions, to establish the threshold. The coefficient of variation for NCCA was 5.6%.

#### **8.3.4 In vivo corneal confocal microscopy**

Patients underwent examination with the HRT III RCM IVCCM. The subject's eyes were anaesthetised using a drop of 0.4% Benoxinate hydrochloride and Viscotears were applied on the front of the eye for lubrication. A drop of viscoelastic gel was placed on the tip of the objective lens and a sterile disposable Perspex cap was placed over the lens allowing optical coupling of the objective lens to the cornea. The patient was instructed to fixate on a target with the eye not being examined. Several scans of the entire depth of the cornea were recorded by turning the fine focus of the objective lens backwards and forwards for approximately 2 minutes using the section mode which enables manual acquisition and storage of single images of all corneal layers. This provides en face two dimensional images with a lateral resolution of approximately 2  $\mu\text{m}/\text{pixel}$  and final image size of 400 x 400 pixels of the sub-basal nerve plexus of the cornea from each patient and control subject. This layer is of particular relevance for defining neuropathic changes since it is the location of the main nerve plexus that supplies the overlying corneal epithelium. Each nerve fibre bundle contains unmyelinated fibres which run

parallel to Bowman's layer before dividing and terminating as individual axons underneath the surface epithelium (26). Five images per patient from the centre of the cornea were selected and examined in a masked and randomised fashion (27). Three corneal nerve parameters were quantified: (i) CNFD - the total number of major nerves/mm<sup>2</sup> of corneal tissue; CNBD - the number of branches emanating from all major nerve trunks/mm<sup>2</sup> of corneal tissue and (iii) CNFL - the total length of all nerve fibers and branches (mm/mm<sup>2</sup>) within the area of corneal tissue. CNFD and CNFL are considered to reflect overall nerve fiber degeneration, whilst CNBD reflects nerve fiber regeneration which is partially also captured by CNFL.

### **8.3.5 Skin biopsy immunohistochemistry**

A 3-mm punch skin biopsy was taken from the dorsum of the foot ~2 cm above the second metatarsal head after local anesthesia (1% lidocaine). The biopsy site was closed using Steri-strips, and the specimen was immediately fixed in PBS-buffered 4% paraformaldehyde. After 18–24 h, it was rinsed in Tris-buffered saline and soaked in 33% sucrose (2–4 h) for cryoprotection. It was then embedded in OCT (optimum cutting temperature embedding compound), rapidly frozen in liquid nitrogen, and cut into 50 µm sections using a cryostat (model OTF; Bright Instruments, Huntington, UK). Four floating sections per subject were subjected to melanin bleaching (0.25% KMnO<sub>4</sub> for 15 min followed by 5% oxalic acid for 3 min), a 4-h protein block with a Tris-buffered saline solution of 5% normal swine serum, 0.5% powdered milk, and 1% Triton X-100, and overnight incubation with 1:1,200 Biogenesis polyclonal rabbit anti-human PGP 9.5 antibody (Serotec, Oxford, U.K.). Biotinylated swine anti-rabbit

secondary antibody (1:300; DakoCytomation, Ely, U.K.) was then applied for 1 h; sections were quenched with 1% H<sub>2</sub>O<sub>2</sub> in 30% MeOH-PBS (30 min) before an 1-hour incubation with 1:500 horseradish peroxidase–Streptavidin (Vector Laboratories, Peterborough, U.K.). Nerve fibers were demonstrated using 3, 3'-diaminobenzidine chromogen (Sigma-Aldrich, Manchester, U.K.). Sections were mildly counterstained with eosin to better localize the basement membrane to identify nerve fibers passing through it. Negative controls consisted of replacing the anti-PGP9.5 antibody with rabbit immunoglobulin (DakoCytomation) at a concentration matching that of the primary antibody which showed no immunostaining. IENFD, i.e., the number of fibers per millimeter of basement membrane were quantified in accord with established criteria and techniques and expressed as number per millimeter (28).

### **8.3.6 Statistical analysis**

SPSS 16.5.0 for Windows was used to compute the results. Analysis included descriptive and frequency statistics. All data are expressed as mean  $\pm$  standard error of mean (SEM). Paired sample t-test used to test whether a sample mean (of a normally distributed interval variable) significantly differs between controls and diabetic patients before SPK and for 6 months and 12 months.

## **8.4 Results**

The clinical characteristics and detailed assessment of neuropathy in diabetic patients and age matched controls are summarized in Table 8-1. Diabetic patients undergoing SPK (age:  $47.0 \pm 3.0$  yrs., duration of diabetes:  $27.0 \pm 3.5$

yrs.) were assessed. BMI was non-significantly lower in diabetic patients and showed an improvement after SPK. HbA1c was higher in diabetic patients compared to controls and improved into the normal range at 6 and 12 months after SPK, but this was not statistically significant. The total cholesterol was significantly lower ( $P=0.01$ ) in diabetic patients and remained the same at 6 and 12 months after SPK. Both HDL and serum triglycerides were comparable between diabetic patients and control subjects, and remained unchanged after SPK. Estimated Glomerular Filtration Rate (eGFR) was lower in diabetic patients at baseline ( $P=0.02$ ) which showed a non-significant improvement at 6 and 12 months after SPK.

**Table 8-1** Clinical demographic results in control subjects and T1DM patients undergoing SPK at baseline and follow-up visits at 6 and 12 months

Parameter	Controls	Baseline (0 months)	6-months	12-months
n (F/M)	10 (3/7)	15 (5/10)	15	15
Age (yrs.)	47±3	47±3	-	-
Diabetes duration (yrs.)	0	27±3.5	-	-
BMI (kg/m <sup>2</sup> )	27±1	22±2	25.5±1	25.5±1
HbA <sub>1c</sub> (%)	5.7±0.1	7.4 ± 0.8	5.9±0.3	5.9±0.4
Cholesterol (mmol/L)	5.1±0.2	4.0±0.3*	4.3±0.3	4.5±0.3
HDL (mmol/L)	1.5±0.1	1.3±0.2	1.5±0.2	1.6±0.2
Triglycerides (mmol/L)	1.3±0.2	1.4±0.1	1.2±0.1	1.03±0.1
eGFR (ml/min/l)	86.22±2.13	60.53±8.64*	64.0±7.5	66.0±6.19

Data are means ± SEM in diabetic patients and control subjects. \* $P < 0.05$  (baseline v control)

#### 8.4.1 Symptoms and neurological deficits

Neuropathic symptoms as assessed with the NSP were significantly greater in diabetic patients than in control subjects at baseline ( $P=0.005$ ), but there was

no significant improvement at 6 ( $P=0.1$ ) or 12 ( $P=0.9$ ) months after transplantation. The McGill Pain index was significantly ( $P=0.01$ ) greater at baseline compared to control subjects and did not show a significant change at 6 ( $P=0.9$ ) or 12 ( $P=0.9$ ) months after transplantation. The modified Neuropathy deficit score (mNDS) was significantly ( $P=0.003$ ) greater at baseline compared to control subjects indicating a mild to moderate neuropathy, and did not change significantly at 6 ( $P=0.7$ ) or 12 ( $P=0.8$ ) months after transplantation (Table 8-2).

#### **8.4.2 Quantitative sensory testing**

Vibration perception threshold was significantly greater in diabetic patients compared to control subjects at baseline ( $P=0.01$ ) and did not change significantly at 6 ( $P=0.1$ ) or 12 ( $P=0.6$ ) months after transplantation. CS was significantly greater in diabetic patients compared to control subjects at baseline ( $P=0.004$ ) and did not change significantly at 6 ( $P=0.5$ ) or 12 ( $P=0.5$ ) months after transplantation. WS was significantly greater in diabetic patients compared to control subjects, at baseline ( $P=0.005$ ) and did not change significantly at 6 ( $P=0.9$ ) or 12 ( $P=0.4$ ) months after transplantation.

#### **8.4.3 Autonomic function**

Average heart rate variability (HRV) was significantly lower in diabetic patients compared to control subjects at baseline ( $P=0.01$ ) and did not change significantly at 6 ( $P=0.9$ ) or 12 ( $P=0.8$ ) months after SPK transplantation.

#### **8.4.4 Electrophysiology**

Peroneal nerve conduction velocity and amplitude were significantly lower in diabetic patients compared to control subjects, at baseline ( $P=0.0001$ ,  $P=0.0001$ , respectively) and did not change significantly at 6 ( $P=0.6$ ,  $P=0.5$ ) or 12 ( $P=0.3$ ,  $P=0.2$ ) months after transplantation. SSNCV and SSNamp were significantly lower in diabetic patients compared to control subjects, at baseline ( $P=0.003$ ,  $P=0.001$ , respectively) and did not change significantly at 6 ( $P=0.7$ ,  $P=0.9$ ) or 12 ( $P=0.6$ ,  $P=0.3$ ) months after transplantation.

#### **8.4.5 Intra-epidermal nerve fibre density**

IENFD was significantly lower in diabetic patients compared to control subjects at baseline ( $P<0.0001$ ) and did not show a significant improvement, 12 months after transplantation ( $P=0.9$ ) (Table 8-3, Figure 8-1).

#### **8.4.6 Corneal sensation**

The corneal sensation threshold was significantly greater in diabetic patients compared to control subjects at baseline ( $P=0.03$ ), and did not change at 6 ( $P=0.9$ ) or 12 ( $P=0.9$ ) months following transplantation (Table 8-3).

**Table 8-2** Clinical neuropathy evaluation in control subjects and T1DM patients undergoing SPK at baseline and follow-up visits at 6 and 12 months

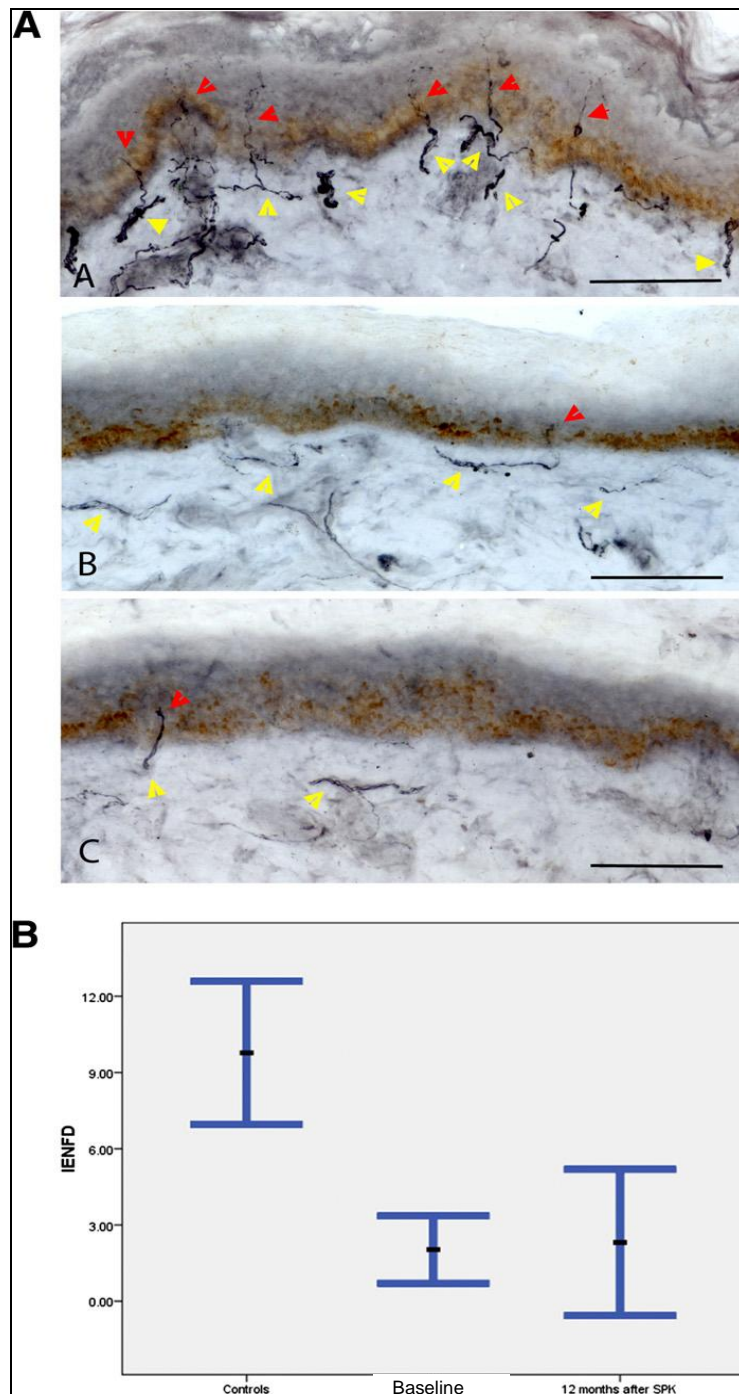
Parameter	Controls	Baseline	6-months	12-months
<b>(0 months)</b>				
NSP (0-38)	0	6.7 ± 1.8†	7.6 ± 2.2	7.3 ± 2.0
NDS (0-10)	0.3 ± 0.2	4.6 ± 0.9†	5.0 ± 1.1	5.4 ± 0.7
McGill Pain Index	0	1.7 ± 0.6*	1.9 ± 0.8	1.3 ± 0.5
VPT (V)	6.7 ± 1.8	19.4 ± 3.7*	17.4 ± 3.3	16.9 ± 3.4
CT (°C)	29.3 ± 0.4	17.5 ± 3.1†	19.8 ± 2.9	20.0 ± 2.7
WT (°C)	38.1 ± 0.8	43.7 ± 1.4†	43.8 ± 1.2	42.3 ± 1.1
HRV (average- bpm)	15.3 ± 2.1	7.1 ± 1.7†	5.7 ± 1.7	4.9 ± 2.1
SSNCV (m/s)	47.9 ± 0.5	40.6 ± 2.2†	41.5 ± 1.6	41.8 ± 1.9
SSNAmp (µA)	20.7 ± 3.4	5.1 ± 0.9†	5.1 ± 0.9	4.0 ± 0.6
PMNCV (m/s)	47.7 ± 0.9	35.9 ± 1.8‡	37.7 ± 1.2	38.5 ± 1.8
PMNAmp	12.2 ± 0.9	2.4 ± 0.4‡	1.9 ± 0.4	1.7 ± 0.3

Data are means ± SEM in diabetic patients and control subjects. Statistically significant difference using paired sample t-test: \**P* < 0.05; †*P* < 0.01; ‡*P* < 0.001. (Baseline v control; 6 months v baseline; 12 months v baseline)

**Table 8-3** Corneal sensitivity, corneal nerve morphology and IENFD in control subjects and T1DM patients at baseline and following SPK at 6 (no skin biopsy) and 12 months

Parameter	Controls	Baseline	6-months	12-months
<b>(0 months)</b>				
NCCA (28)	0.56 ± 0.1	1.78 ± 0.42*	1.83 ± 0.73	1.84 ± 0.89
CNFD (no./mm <sup>2</sup> )	35.77 ± 1.53	14.44 ± 1.20‡	15.22 ± 1.63	19.27 ± 1.57 *
CNBD (no./mm <sup>2</sup> )	100.92 ± 13.1	21.46 ± 3.78‡	36.85 ± 6.04 *	43.02 ± 6.48†
CNFL (mm/ mm <sup>2</sup> )	27.93 ± 1.26	11.35 ± 1.04‡	13.35 ± 1.50	15.63 ± 1.56*
IENFD (no/mm)	9.77 ± 1.24	2.03 ± 0.61‡	-	2.31 ± 1.17

Data are means ± SEM in diabetic patients and control subjects. Statistically significant difference using paired sample t-test: \**P* < 0.05; †*P* < 0.01; ‡*P* < 0.001. (baseline v control; 6 months v baseline; 12 months v baseline)

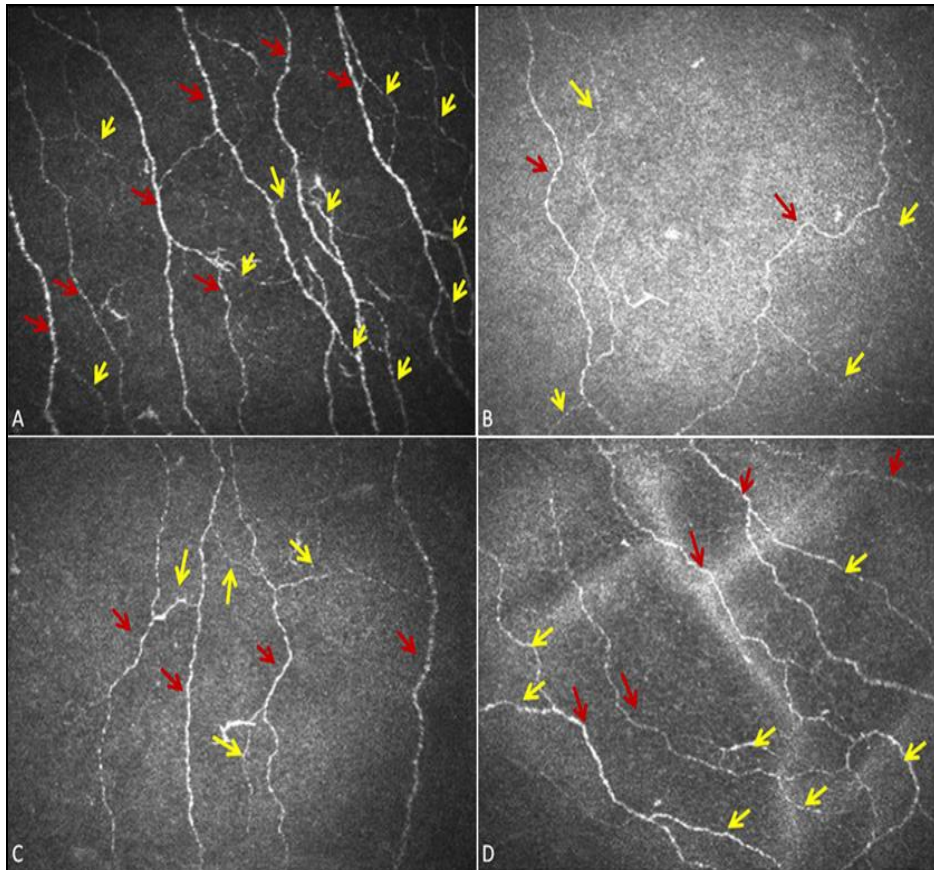


**Figure 8-1** (A) Skin biopsies immunostained for PGP9.5. Healthy control (a) shows numerous IENFs (red arrowheads) reaching upper levels of epidermis. Baseline (b) and 12 months (c) after SPK. Note well developed sub-epidermal nerve plexus (yellow arrowheads) in a healthy subject (a) compared to scant sub-epidermal and minimal IENFs in the diabetic patient both at baseline (b) and at follow up (c). Scale bar = 100  $\mu$ m. (B) IENFD in control subjects and in diabetic patients at baseline and 12 months after SPK. Data are presented as Mean  $\pm$  SEM.

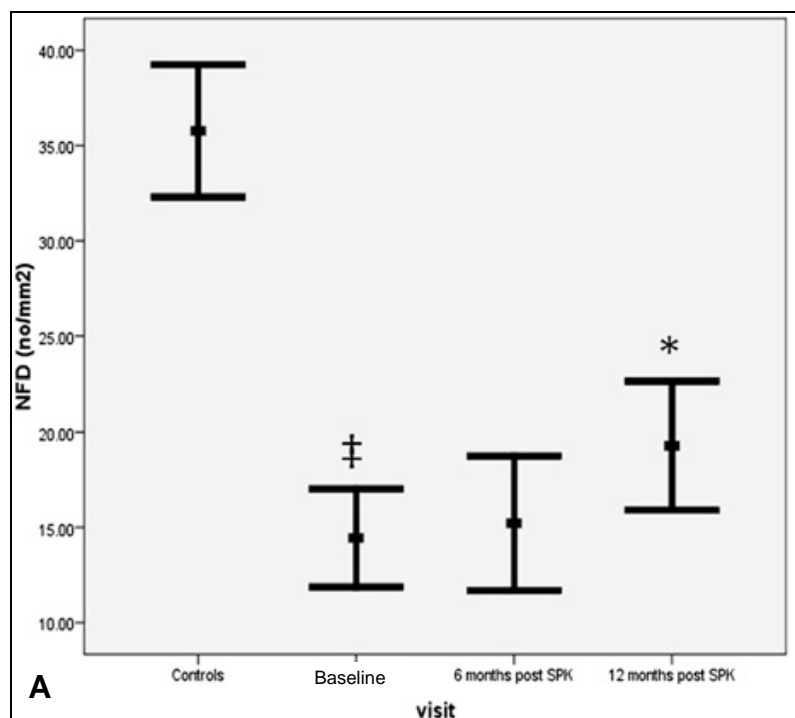


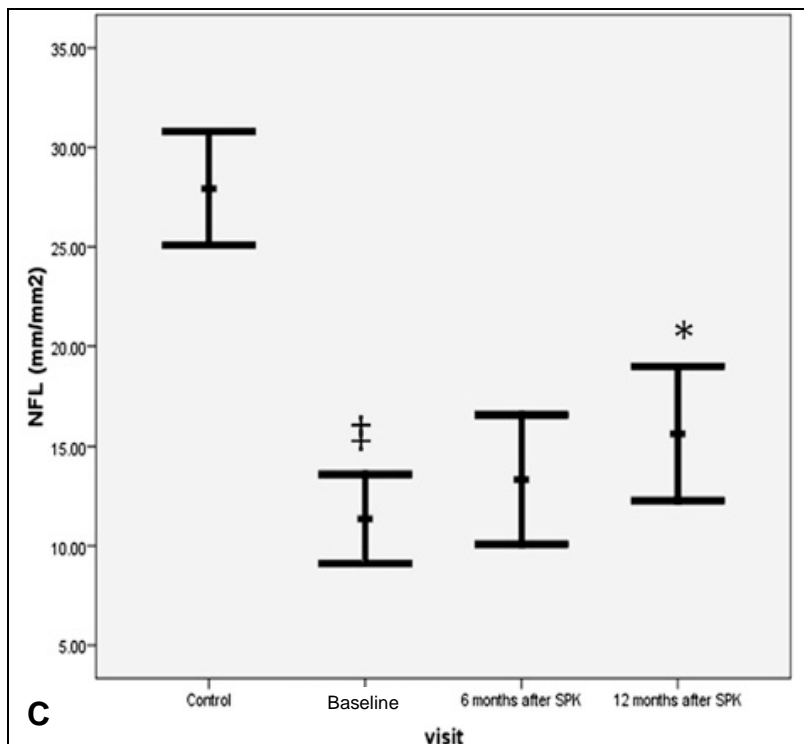
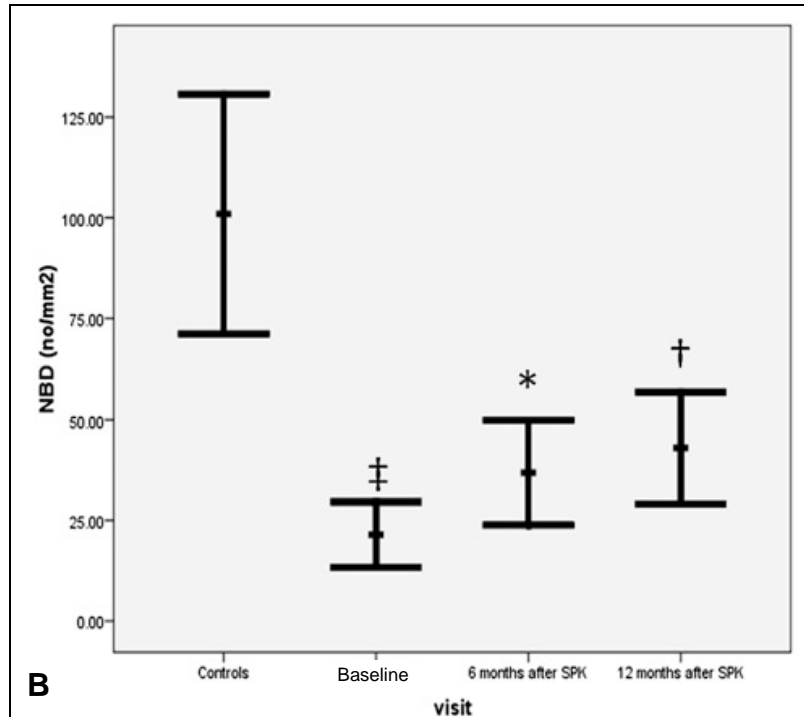
#### **8.4.7 In vivo corneal confocal microscopy**

Representative images from a diabetic patient at baseline show a marked reduction in sub-basal corneal nerves with a progressive repair at 6 and 12 months following SPK (Figure 8-2). CNFD was significantly lower in diabetic patients compared to control subjects at baseline ( $P < 0.0001$ ), did not improve at 6 months ( $P = 0.7$ ), but reached significance at 12 months ( $P = 0.02$ ). Similarly CNFL was significantly lower in diabetic patients compared to control subjects at baseline ( $P < 0.0001$ ), did not improve at 6 months ( $P = 0.2$ ) but reached statistical significance at 12 months ( $P = 0.03$ ). CNBD was significantly lower in diabetic patients compared to control subjects at baseline ( $P < 0.0001$ ), but showed a significant improvement at 6 months ( $P = 0.03$ ) and continued to improve significantly ( $P = 0.008$ ) at 12 months. Although, IENFD did not show an improvement at 12 months, it showed a significant correlation with corneal nerve parameters including CNFD ( $P = 0.656$ ,  $r < 0.0001$ ), CNBD ( $P = 0.709$ ,  $r < 0.0001$ ) and CNFL ( $P = 0.695$ ,  $r < 0.0001$ ) (Figure 8-3, Table 8-3).



**Figure 8-2** CCM images from Bowman's layer of cornea: a control subject (A), patient with T1DM at baseline (B), 6 months (C) and 12 months (D) after SPK. The red arrows indicate main nerve fibres and yellow arrows indicate branches.





**Figure 8-3** (A) Corneal nerve fibre density (B) corneal nerve branch density (C) corneal nerve fibre length in diabetic patients at baseline and at 6 and 12 months after SPK transplantation. (Statistically significant different using ANOVA: \* $P < 0.05$ ; † $P < 0.01$ ; ‡ $P < 0.001$ . (baseline v control; 6 months v baseline; 12 months v baseline))

## 8.5 Discussion

The natural history of nerve damage in patients with Type 1 diabetes is not entirely clear. Longitudinal data from the Rochester cohort supports the contention that the duration and severity of exposure to hyperglycemia are related to the progression and hence severity of neuropathy rather than its onset (29). In type 1 diabetes the development of diabetic neuropathy has been related not only to glycaemic control but also to conventional cardiovascular risk factors such as hypertension and lipids (30). The Toronto consensus identified clinical and neurophysiologic evaluation combined with quantitative sensory and autonomic function testing as well small fibre evaluation to diagnose neuropathy (11). However, there is no clear consensus as to the critical endpoints which should be employed to define the benefits of therapeutic intervention.

The 'cure' for Type 1 diabetes is via pancreas transplantation, which normalizes blood glucose. Over the past 20 years, the survival and mortality of SPK transplants has improved significantly (31), therefore it provides the ideal intervention to assess whether the long-term complications of diabetes are reversible. Some studies show that retinopathy can deteriorate in 10–35% of patients with unstable eye disease immediately after pancreas transplantation, but benefits do become apparent after several years (32-33). Other studies demonstrate an improvement and/or stabilization of diabetic retinopathy after a median follow up of only 17 months (34-35). For Nephropathy, normoglycaemia can stop the progression of diabetic glomerulopathy but does not reverse it (36-37). Similarly, pancreas transplantation alone can limit further

reduction in eGFR (33) and SPK protects the graft kidney from developing diabetic nephropathy (38).

With regard to neuropathy, pancreas transplantation has previously been shown to improve nerve conduction and motor and sensory action potentials in the upper but not the lower limb as well as sudomotor function (5), within 1 year, but with no impact on autonomic function (5-7). SPK transplantation has been shown to improve gastric emptying and symptoms related to gastroparesis compared with kidney transplantation alone (39), although gastrointestinal symptoms and autonomic deficits do not correlate with each other. In a recent study in 18 T1DM patients there was no improvement in IENFD; 21-40 months post SPK (7). However, most patients receiving transplantation had severe nerve fibre damage as evidenced by marked depletion of intra-epidermal nerve fibres (8).

Whilst nerve conduction studies and quantitative sensory testing are useful and well validated measures to help diagnose and assess progression of diabetic neuropathy, their utility in evaluating a therapeutic response may be limited (40). More detailed and reproducible measures, which accurately quantify small fibre neuropathy via skin or nerve biopsy may be more sensitive but are invasive (15-17). There is now an increasing literature on the potential for IVCCM to quantify c-fibre pathology in peripheral neuropathies (18; 41-42). Detailed morphometric and immunohistological studies have demonstrated that the sub-basal nerve fibre bundles studied by IVCCM are predominantly nociceptive C fibers (43-44). Indeed IVCCM has been applied to evaluate diabetic neuropathy (19-20), idiopathic small fibre neuropathy (45) and Fabry disease (46). We have shown that corneal nerve damage assessed using

IVCCM relates to the severity of intraepidermal nerve fiber loss (21) and is related to a loss of corneal sensitivity (25) in diabetic neuropathy. Corneal confocal microscopy detects very early small fibre damage even in subjects with an elevated HbA1c, still within the normal range (18) and HbA1c levels 7-10 years prior to IVCCM correlate with the severity of nerve damage (47). Furthermore, an improvement in HbA1c by optimising medical therapy (10) and pancreas transplantation (23) led to corneal nerve regeneration, shown using IVCCM. However in these studies the evaluation of neuropathy was limited to IVCCM.

The present study allowed us to evaluate the relative ability of IVCCM to detect nerve fibre repair compared with all other established measures for assessing neuropathy, including neurological deficits, QST, neurophysiology and IENFD. The results demonstrate a severe neuropathy in diabetic patients prior to SPK as evidenced by significant abnormalities in electrophysiology, QST, IENFD and corneal nerve fibres, confirming previous studies (5-8). However, despite this considerable baseline damage, we now show a significant improvement in corneal nerve branch density within 6 months of transplantation, confirming our previous work (23), indicating an early nerve fibre repair process with the restoration of euglycaemia, followed by a significant improvement in nerve fibre density and nerve fibre length 12 months after SPK. This is in contrast to all other standard measures of neuropathy, including detailed QST, autonomic function, electrophysiology and IENFD, all of which failed to show an improvement 12 months after SPK. These findings support previous studies in diabetic neuropathy where at best a prevention of progression in nerve damage was shown only after several years of euglycaemia (5-8; 48-51).

However, these studies focused heavily on electrophysiology and quantitative sensory assessment which predominantly assessed large fibre function. It is of relevance that where small fibre function was assessed in the form of sudomotor function, a significant improvement was demonstrated within 1 year of SPK (5; 7). The main limitations of this study are the small number of subjects studied, the possibility of false positive results based on the number of comparisons, the lack of sudomotor testing given its previous improvement in these patients and the lack of blinding given that all patients were known to have had a SPK during the follow up period. Furthermore, with regard to the lack of improvement in IENFD this may reflect the location of the skin biopsy as we assessed this on the dorsum of the foot, whereas a previous study (9) has shown that proximal IENFD assessment in the thigh is more responsive to intervention. Similarly for neurophysiological assessment it has been suggested that upper limb neurophysiology may show a better response to intervention due to lesser severity of damage (52).

We now confirm and extend the results of our previous study using the latest generation HRT-III which provides enhanced small fibre imaging and detects earlier nerve fibre repair, particularly reflected in the increase in nerve branch density, followed by significant improvements in nerve fibre density and length. We believe these data provide further support for the need to study small fibres as surrogate markers and end points in intervention trials of diabetic neuropathy. An important issue with regard to the utility of IVCCM or indeed any surrogate end point has to be that these alterations in corneal nerve morphology predict deterioration of neuropathy and ultimately clinically meaningful outcomes such as foot ulceration. An alternative interpretation of

this data could of course be that IVCCM is measuring something unique that is not an accurate biomarker of how other peripheral nerves are faring or indeed that corneal nerves respond well to restoration of insulin and normoglycaemia, whereas other peripheral nerves do not. Nevertheless, corneal confocal microscopy appears to represent a promising non-invasive and hence reiterative test with high sensitivity, which may represent an ideal surrogate endpoint for assessing the benefits of pancreas transplantation and indeed other therapies in clinical trials of human diabetic neuropathy.



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## **9. CHAPTER IX-CONCLUSION**

## **9.1 Introduction**

The accurate detection and quantification of human DSPN are important to define at risk patients, anticipate deterioration, and assess new therapies. Current methods lack sensitivity (QST), require expert assessment and are large-fibre weighted (NCS) or are invasive (skin/nerve biopsy) and therefore not routinely performed across healthcare systems. In recent years, IVCCM has been proposed as a surrogate endpoint of human DSPN as it allows non-invasive visualisation of the corneal subbasal nerve plexus. The evaluation of corneal subbasal nerve morphology has previously shown considerable diagnostic potential for a number of peripheral neuropathies (1-5) and in particular DSPN (6-8). Moreover, corneal nerve loss parallels IENF loss (9) and is directly associated with cardiometabolic clustering (3; 10-11). International, multi-centre prospective studies currently underway will investigate the utility of IVCCM to assess the status and progression of DSPN in patients with type 1 and type 2 DM (12). Moreover, recent studies using IVCCM have reported significant corneal nerve alterations in models of experimental diabetes (13) and have also shown the therapeutic benefits of a vasopeptidase inhibitor in peripheral neuropathy and corneal innervation, providing a mechanistic basis for the observed changes (14).

## **9.2 Repeatability of in vivo corneal confocal microscopy to quantify corneal nerve morphology (Chapter 3)**

The purpose of this study was to assess the repeatability of IVCCM between occasions and observers and the prevalence of bilateral morphological symmetry in healthy subjects, all fundamental aspects of a valid diagnostic

tool. Repeatability and agreement was consistently high for CNFD and CNFL and modest and occasionally poor for CNBD and TC. Hence, both CNBD and TC have inherent liability for variability in repeated assessment, especially when different observers undertake the analysis. Furthermore, the accurate estimation of CNBD is confounded by the structural complexity of the subbasal nerve plexus and is therefore subject to interpretation errors by less experienced observers. This highlights the need for a standardised protocol in assessing nerve morphometric parameters but may also indicate a highly dynamic role for CNBD in health and disease which should be investigated further. This study demonstrates that repeatable assessment of corneal innervation is possible but has also identified lower repeatability for certain parameters which may be improved through the application of fully-automated image analysis software. In addition, symmetrical differences between RE and LE were minimal, a significant finding which provides morphological support for CCM as the primary property of a peripheral neuropathy is of symmetrical damage.

### **9.3 No difference in corneal nerve morphology between central and adjacent peripheral areas using in vivo corneal confocal microscopy (Chapter 4)**

Previous studies employing IVCCM to assess the status of DSPN have used the central cornea (corneal apex) for quantification. However, the subbasal nerve plexus is a structure, which extends to inferior, superior, temporal and nasal areas, where little is known regarding the variability of corneal nerve morphology. This study aimed to investigate whether images from adjacent



peripheral areas in addition to central (depth constant) compared to a z-scan on the corneal apex (location constant) were comparable in subjects with mild DSPN. There was no significant difference for CNFD, CNBD, CNFL and TC estimated using either scanning method. Slight, but non significant variations attributed to the scanning location were found when images were assessed individually. This study showed comparable nerve morphology between central and adjacent peripheral areas in mild DSPN with the exception of three subjects with a significant reduction in central CNFD, which was not reflected in the periphery. Hence, images captured from the corneal apex and adjacent periphery are suitable for longitudinal assessment to define progression or regression following intervention.

#### **9.4 Automated analysis of diabetic peripheral neuropathy using multi-scale quantitative morphology of nerve fibres in corneal confocal microscopy imaging (chapter 5)**

A fully automated image analysis system is required to make IVCCM a suitable endpoint for the longitudinal assessment of DSPN. The purpose of this study was to develop a novel automated analysis and classification algorithm for detecting nerve fibres in IVCCM images, which are characterised by low contrast and noise. Background work has shown that the dual-model detection algorithm significantly improves error rate and signal-to-noise ratio over competitor methods, successfully differentiates patients with diabetes from controls and strongly correlates with human expert annotation (see appendix 1-Dual model automatic detection of nerve fibres in corneal confocal microscopy images). The algorithm used in this study exploits the curvilinear

structure of the nerve fibres and adapts itself to the local image information; subsequently nerves are used as feature vectors for classification using neural network and random forest classifiers. The algorithm is finally compared to other well-established curvilinear detectors and the best performance is achieved by a multi scale dual-model algorithm in combination with a neural network classifier. Initial clinical effectiveness evaluation shows that the automated system matches the ground truth set by human expert image annotation. This study provides a fully automated quantification algorithm which has been extensively compared to alternative methods and tested for its clinical capacity in a diverse population of diabetic patients with varying degrees of neuropathy. This algorithm is a significantly faster and potentially more reliable alternative to manual expert analysis due to its immunity from inter- and intra-observer variability.

#### **9.5 Corneal nerve loss is symmetrical and progressive with increasing severity of diabetic neuropathy (chapter 6)**

DSPN is characterised by progressive, distal and symmetrical sensory nerve damage. The primary objective of this study was to assess whether corneal nerve loss is symmetrical and progressive with increasing neuropathic severity. The results indicated that CNFD, CNBD and CNFL were symmetrically and progressively reduced with increasing severity of neuropathy and reflected the progressive functional decline observed using gold standard techniques such as NDS and QST, except in those with severe neuropathy. A similar but less significant relationship was observed for the tortuosity coefficient and corneal

sensitivity. Corneal nerve changes parallel somatic changes and are symmetrical in accord with the natural history of peripheral neuropathy.

### **9.6 Validity of detection of diabetic somatic polyneuropathy by in vivo corneal confocal microscopy: human expert versus fully automated quantification (Chapter 7)**

This study was performed to assess the performance of the novel fully automated image analysis algorithm described in chapter 5 (and appendix 1) in a cohort of patients with type 1 and type 2 DM, compare its performance to standard human expert quantification and assess the validity of three corneal nerve morphometric parameters to diagnose and stratify the severity of DSPN. There was a progressive and consistent nerve loss using either quantification system and there was a very strong positive correlation between manual and automated analysis for CNFD and CNFL and a less strong but significant correlation for CNBD. Both manual and automated CNFD and CNFL were associated with a very high diagnostic odds ratio to diagnose neuropathy. The diagnostic effectiveness of corneal nerve morphometric parameters declined significantly when subjective endpoints were used to define the presence of DSPN. This study demonstrated significant evidence in favour of adopting a fully automated image analysis algorithm which offers a robust and objective means of assessing neuropathic deficits. To date this is the only automated analysis system, which has been validated against a relatively large cohort of patients with diabetic peripheral neuropathy.

## **9.7 Corneal Confocal Microscopy Detects Early Nerve Regeneration in Diabetic Neuropathy Following Simultaneous Pancreas and Kidney Transplantation (Chapter 8)**

The final study presented in this thesis was designed to assess the benefits of improved glycaemic control and renal function in DSPN. Previous studies have failed to show regression of neuropathy in clinical trials which may reflect insufficiency of the interventions employed, but also the difficulty of current techniques to assess nerve fibre repair. Recipients of simultaneous pancreas and kidney transplantation underwent detailed evaluation of their peripheral neuropathy status using neurophysiology, QST, skin biopsy and IVCCM at baseline, 6 and 12 months. At 6 months there was no change in any of the endpoints except CNBD. However, at 12 months IVCCM showed a statistically significant increase in CNFD, CNBD and CNFL while there was no change in any of the other measures of neuropathy. This study demonstrated that normalisation of glycaemia following successful SPK results in a significant improvement in corneal nerve morphology which is not detectable with conventional clinical testing. Moreover, IVCCM showed capacity to detect regression of neuropathy and may be employed as a surrogate endpoint in clinical intervention trials.

## **9.8 Study Limitations**

Several limitations exist and should be acknowledged. A major and common limitation in all studies presented here is the cross-sectional design. IVCCM has not been prospectively evaluated to define the capacity to predict the development and progression of neuropathy. A large longitudinal study of

IVCCM and corneal sensitivity as surrogate markers of DSPN is currently ongoing to establish their prognostic value (12).

A second limitation is the absence of skin biopsy results for the majority of the subjects at this stage, which limits interpretation against the gold standard test for small fibre neuropathy. Estimation of IENFD is the gold standard technique to assess sensory nerve alterations and this should be directly compared to corneal nerve changes and peripheral nerve dysfunction described here to allow a more accurate appreciation of the structure to function relationship in diabetic peripheral neuropathy.

Third, the absence of a uniform image acquisition and analysis protocol makes it difficult to compare the results of the current studies to those generated by other investigators previously (8). A recent study has assessed the value of estimating 95% confidence intervals of epidermal nerve fibres per mm for the number of skip sections to be evaluated and for confidently judging normality or abnormality (15). The authors concluded that the variability of differences decreased progressively with increasing numbers of skip sections evaluated. Although a study by Vagenas and colleagues (16) estimated that any number between 5-8 IVCCM generated images are required for accurate quantification, which is in agreement with the acquisition and analysis protocol employed in this study, this has not been weighted for neuropathic severity.

Fourth, the transplant study (chapter 8) presents preliminary results of a 12-month period following SPK. Although significant morphological repair was detected by IVCCM this was not accompanied by functional improvement in this study. Undoubtedly, restoration of peripheral nerve function is of paramount importance and a future study should show that regression of

neuropathy detected by IVCCM precedes meaningful changes in somatic polyneuropathy. Moreover, SPK transplantation represents an extreme example of therapeutic intervention in diabetes and is of course only in patients with Type 1 diabetes. Further evidence from clinical intervention trials of other therapies for diabetic neuropathy are required. Fifth, the automated algorithm presented here has been to-date the only purpose-built algorithm validated against a large cohort of patients with diabetes representing various degrees of neuropathy. Recent studies (17-18) have proposed alternative quantification systems which however have not been validated for clinical effectiveness nor have they been directly compared to the algorithm proposed here. We cannot exclude the possibility that any of these systems may show comparable or even superior properties to the present algorithm.

Finally, this study does not provide a mechanistic basis for the observed changes. This is important as the human cornea is an avascular and hence immune privileged tissue and therefore not directly exposed to vascular risk factors. However, the corneal subbasal nerve plexus is a terminal point of the ophthalmic branch of the trigeminal nerve. It is possible that the observed alterations are the result of vascular-mediated effects which occur earlier in the pathway. A recent study employing IVCCM in experimental models of diabetes has attributed changes observed in the diabetic rat corneal nerves to acetylcholine-induced vascular relaxation in the posterior ciliary artery (14). Such an association may exist and should also be investigated in humans. Furthermore, a number of neurotrophins and growth factors are expressed in the human and animal cornea and play an important role in providing trophic and multipurpose support for tissue growth and maintenance. It is documented

that deprivation of these factors in disease states may result in neurotrophic keratitis and corneal ulceration (19). It is not established whether an association also exists with diabetic peripheral neuropathy. A convenient means to sample for these growth factors arises from the tears and analysis of tear growth factor content may provide insights.

### **9.9 Future work**

Further work is required to define the prognostic value of the corneal nerve morphometric parameters established in this study. Diabetic peripheral neuropathy is a common complication of diabetes associated with heavy morbidity and is the main initiating factor for foot ulceration and subsequently amputation (20). Implementation of IVCCM in diabetic neuropathy screening could assist the timely diagnosis and management of individuals at risk. A pilot, community-based study, investigating the effects of IVCCM implementation in diabetic neuropathy screening on the rates of foot ulceration or referrals to hospital specialised services would provide valuable evidence on the utility of the technique. Lately, studies have reported an association between corneal nerve loss detected with IVCCM and the severity of diabetic retinopathy (21) or retinal nerve fibre layer thinning and diabetic peripheral neuropathy in the absence of detectable retinopathy (22). A prospective study is needed to define whether corneal nerve loss precedes the onset of diabetic retinopathy, nephropathy and detectable somatic neuropathy. Although corneal nerve changes in DSPN are well documented, little is known regarding the pathophysiological mechanisms behind these changes. The corneal endothelium plays an important physiological role in the maintenance and

trophic support of the corneal layers. Future studies should investigate the relationship between hypoxia-induced alterations, detectable as disruption of the endothelial cell layer and morphological cell changes, and the status of DSPN and corneal subbasal innervation.

### **9.10 Conclusion**

In conclusion, this thesis has investigated fundamental aspects of IVCCM such as the optimal scanning methodology and the reproducibility of the technique and has provided a morphological reference for future studies. Corneal nerve loss is symmetrical and progressive and parallels somatic polyneuropathy but corneal nerves also show significant regenerative capacity following normalisation of glycaemic control by SPK transplantation. The novel image analysis algorithm described here will likely enhance the use of the technique worldwide and make it suitable for rapid clinical screening. Automated image quantification may replace human manual assessment with high diagnostic validity for diabetic neuropathy.



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## **11. APPENDIX 1 - PUBLICATIONS**

## **12. APPENDIX 2 - STUDY RELATED DOCUMENTS**

## 12.1 Patient information sheet - Ophthalmic markers of diabetic neuropathy



Central Manchester and Manchester   
Children's University Hospitals  
NHS Trust

### STUDY INFORMATION SHEET

#### Ophthalmic markers of diabetic neuropathy

We are asking you (or your child or the person you are responsible for) to participate in a research study to be conducted by Professor Rayaz Malik and Professor Andrew Boulton at the Central Manchester Foundation Trust (CMFT) & University of Manchester. This leaflet explains the benefits and possible discomforts of your/their participation and what we would like you (or your child or the person you are responsible for) to do during the study. If you (or your child or the person you are responsible for) are willing to take part you or your child or the person you are responsible for will be asked to sign a consent (or if child assent) form and you will be given a copy to keep.

#### WHY IS THIS STUDY BEING DONE?

The study is being carried out to develop a new test to examine nerve damage in diabetic patients and to follow the progression of the nerve damage (neuropathy) over a period of four years. The results will help us to understand how nerve damage develops and how we might help repair this nerve damage.

#### WHAT ARE WE ASKING YOU TO DO?

We wish to invite you (or your child or the person you are responsible for) to the Wellcome Trust Clinical Research Facility (WTCRF) and department of clinical neurophysiology at CMFT for a detailed assessment of nerve damage.

We will ask you (or your child or the person you are responsible for) to undergo a measurement of your/their height, weight and blood pressure. A non-fasting blood sample (25-35mls) and urine (approximately 10mls) will be collected to assess liver, kidney, thyroid function, glucose control, and standard antibodies tests. These will help us exclude other causes of nerve damage.

We will ask you (or your child or the person you are responsible for) to complete a questionnaire about pain in your (or their) legs, and we will test your (or their) ability to sense pain/touch, vibration and temperature using a pointed tip, a tuning fork and warm and cool metal rods in addition to reflexes in your knees and ankles.

The speed the nerves conduct messages will also be tested to assess nerve damage using nerve conduction studies, which are a well-established method of assessing nerve damage. This takes about 20 minutes and may cause minor short lived discomfort when the nerve is stimulated and causes the muscle to twitch involuntarily. A test of your (or their) ability to feel different sensations will be done using instruments that can measure when you (or they) just notice sensations of cool,

Study Information Sheet Version 5, Date: 26/04/2011

warm and vibration on the foot. Another test that can reveal damage to the nerves is a standard ECG tracing of the heart during deep breathing and a change in blood pressure in standing up.

Sensitivity of your (or their) cornea will be assessed by giving an air puff stimulus to the front of your (or their) eye with no direct contact and asking you (or them) whether the air can be felt. A corneal confocal microscope (CCM) will be used to examine the number of nerves in the front part of the eye. A drop of anaesthetic is applied to numb the front of the eye which will sting for 1 or 2 seconds only. Then a gel on the lens of the camera touches the front of the eye for 1-2 minutes whilst we record images of the cornea. We will also use a standard fundus camera which does not need drops to dilate your (or their) pupils to collect pictures from the back of your eye (the retina).

You (or they) will also be asked to undergo a skin biopsy which will require a separate consent form. We will inject some local anaesthetic (to numb) the skin on the top of your (or their) foot and remove two small pieces of skin (3mm each) to allow us to study the nerves which provide sensation to your foot. The biopsy area will be covered with a dressing and we will review the foot 1 week later. You (or they) will be left with a small scar which will fade over 6 months and will be barely visible at 1 year.

The study visit will take approximately 1.5-2 hours. You (or they) will not be paid for participation in this research, but will be provided transport to and from WTCRF (e.g. taxi will be provided or reasonable travel expenses will be paid).

Because we aim to monitor the progression of neuropathy over 4 years, after the first visit, we will arrange for repeat examination at 12, 24 and 36 and 48 months.

#### **DO I HAVE TO TAKE PART?**

No, this is voluntary. If you (or they) would prefer not to take part you do not have to give a reason. Your (or their) doctor would not be upset and your (or their) treatment would not be affected.

#### **WHAT ARE THE POSSIBLE RISKS OF TAKING PART?**

There are no recognised risks of any of the procedures proposed for this study apart from very rarely one person in a thousand can develop infection at the biopsy site. If you (or they) have any problems you (or they) should let the doctor know at once.

#### **ARE THERE ANY POSSIBLE BENEFITS?**

During the study your (or their) condition will be assessed in detail. The knowledge gained from this study may affect the tests employed to diagnose nerve damage and also which treatment you (or they) receive in the future. It will also help ensure that future patients are offered a more accurate diagnosis and receive the most effective treatment available. A summary of the results will be provided to you (or them) on request to the investigators.

#### **WHO WILL SEE THE INFORMATION ABOUT ME?**

All information resulting from your (or their) participation in the study will be stored and analyzed in a computer and will be treated confidentially. A number will identify you (or them) in the computer. The study records will not be made available in any form to anyone other than authorized representatives of the Health Authority. Individuals responsible for audit and monitoring on behalf of the University and NHS Trust will have access for this purpose.

Your (or their) confidentiality will be maintained in accordance with the Data Protection Act, 1984. If the results of this study are published, your (or their) identity will remain confidential.

Study Information Sheet Version 5, Date: 26/04/2011

### **COMPENSATION IN CASE OF INJURY**

In the unlikely event that something does go wrong and you (or they) are harmed during the research and this is due to someone's negligence then you (or they) may have grounds for legal action for compensation against University of Manchester and/or NHS Trust, but you (or they) may have to pay your (or their) legal costs. The normal National Health Service complaints mechanisms will still be available to you (or them).

The University of Manchester has cover for no fault compensation for bodily injury, mental injury or death where the injury resulted from a trial or procedure you (or they) received as part of the trial. This would be subject to policy terms and conditions. Any payment would be without legal commitment.

### **WHAT IF THERE IS A PROBLEM?**

If you (or they) have any concerns regarding this study, please contact the research team in the first instance who will do their best to address them. If you (or they) do not wish to contact the research team directly, or if you (or they) want to make a formal complaint, please contact the University Research office on 0161 2757583 or 0161 2758093 or by email to [research-governance@manchester.ac.uk](mailto:research-governance@manchester.ac.uk).

### **WHAT DO I DO NOW?**

Please sign the enclosed reply slip and return it to us as soon as possible in the pre-paid envelope, so we know whether or not you (or they) are happy to take part in the study. If you (or your child or the person you are responsible for) are interested, we will call you on the telephone in about one week to answer any questions you (or they) may have, and we can arrange a suitable appointment for you to visit us. Thank you very much for considering taking part in our research. Please discuss this information with your family, friends or GP if you wish.

For further information or appointments or if you (or they) want any further information concerning this project or 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 our diabetes research nurse, Ms. Karthi Balakrishnan on 0161 276 6706, or the following people:

Prof. Rayaz Malik  
Ph: 0161 275 1196  
E-mail: [rayaz.a.malik@manchester.ac.uk](mailto:rayaz.a.malik@manchester.ac.uk)

Dr. Mitra Tavakoli  
Ph: 07930453389  
E-mail: [Mitra.tavakoli@manchester.ac.uk](mailto:Mitra.tavakoli@manchester.ac.uk)

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

## 12.2 Patient information sheet – Detecting early nerve repair after transplantation in patients with type 1 diabetes

Central Manchester and Manchester  
Children's University Hospitals  
NHS Trust



### PATIENT INFORMATION SHEET

#### Detecting early nerve repair after transplantation in patients with type 1 diabetes

We are asking you to participate in a research study to be conducted by Dr. Rayaz Malik and Mr. Titus Augustine and Mr. Neil Parrott at the Manchester Royal Infirmary. This leaflet explains why your doctor is undertaking this study, the benefits and possible discomforts of your participation and what we would like you to do during the study. If you are willing to take part you will be asked to sign this consent form and you will be given a copy to keep.

#### **WHY IS THIS STUDY BEING DONE?**

Most diabetic patients undergoing Pancreas / Kidney Transplant suffer from severe nerve damage (neuropathy), mainly in their legs and feet. We wish to develop a new test for neuropathy which will be used to detect early nerve repair and recovery after transplantation.

The results will hopefully improve our understanding of the cause of neuropathy in general, and help in the development of new treatments for this condition for all people with diabetes.

#### **WHAT ARE WE ASKING YOU TO DO?**

We wish to invite you to the Wellcome Trust Clinical Research Facility in Central Manchester Foundation Trust Hospitals where the presence and severity of neuropathy in your legs will be assessed. At each visit, we will perform the following tests/assessments:

Height, weight and blood pressure (20 minutes).

Blood tests including: cholesterol profile, kidney, liver, bone profile and glucose control will be assessed on every visit. Thyroid function, vitamins B12 and D, folic acid and antinuclear antibody will be assessed only once. The blood volume we require is 25-35 mls (5 minutes).

Clinical neurological evaluation of the lower limbs. This will include completing a symptom questionnaire and assessment of sensitivity to temperature, vibration and touch. Cardiac autonomic function testing (40 minutes).

Corneal confocal microscopy. Using a special camera, we will look at the nerves in the front of your eye (cornea). This is a painless procedure that takes only one minute (each eye). A drop of local anaesthetic will be applied to the front of your eye to numb this part to reduce your blinking during the test period. Some jell will be applied to the front of the microscope lens and this will be advanced such that the jell touches the front of your eye. You will see a white light which does not harm your eye in any way and there is no pain associated with this. The images captured from your cornea will be analysed and some of them will be used also in another project that we are trying to develop a programme to analyse corneal images automatically, however all the images will be masked and your name and details will be covered (40 minutes).

Nerve conduction studies which are a common, well-established method of assessing nerve function in the arms and legs. Testing takes approximately 15 minutes and there are no after effects. The test involves stimulating a nerve with controlled electrical pulses and recording a response from a muscle. Stimulating the nerve will cause the muscle to twitch involuntarily. The sensation produced is well tolerated but a short-lived discomfort may occur.

Skin biopsy. This will involve applying a local anaesthetic to the skin on the top of your foot and then remove two small pieces of skin (3mm each) to enable us to study the nerves which provide sensation to your foot. This will produce minimal discomfort as we will numb the area using local anaesthetic prior to the biopsy. You will be left with a small scar which will fade over a period of 6 months and will be barely visible at 1 year. This test should take no longer than 20 minutes.

Because we will monitor the progression of neuropathy over three years, after the first visit, we will arrange for repeat examination at 6, 12, 24 and 36 months.

#### **DO I HAVE TO TAKE PART?**

No, this is voluntary. If you would prefer not to take part you do not have to give a reason. Your doctor would not be upset and your treatment would not be affected.

#### **WHAT ARE THE POSSIBLE RISKS OF TAKING PART?**

There are no recognised risks of any of the procedures proposed for this study apart from very rarely one person in a thousand can develop infection at the biopsy site. If you have any problems you should let the doctor know at once.

#### **ARE THERE ANY POSSIBLE BENEFITS?**

During the study your condition will be assessed in detail. The knowledge gained from this study may affect the tests employed to diagnose nerve damage and also which treatment you receive in the future. It will also help ensure that future patients are offered a more accurate diagnosis and receive the most effective treatment available. A summary of the results will be provided to you on request to the investigators.



## **WHO WILL SEE THE INFORMATION ABOUT ME?**

All information resulting from your participation in the study will be stored and analyzed in a computer and will be treated confidentially. A number will identify you in the computer. The study records will not be made available in any form to anyone other than authorized representatives of the Health Authority. Your confidentiality will be maintained in accordance with the Data Protection Act, 1984. If the results of this study are published, your identity will remain confidential.

## **COMPENSATION IN CASE OF INJURY**

In the unlikely event that something does go wrong and you are harmed during the research and this is due to someone's negligence then you may have grounds for a legal action for compensation against University of Manchester and/or NHS Trust, but you may have to pay your legal costs. The normal National Health Service complaints mechanisms will still be available to you.

The University of Manchester has cover for no fault compensation for bodily injury, mental injury or death where the injury resulted from a trial or procedure you received as part of the trial. This would be subject to policy terms and conditions. Any payment would be without legal commitment.

## **WHAT DO I DO NOW?**

Please sign the enclosed reply slip and return it to us as soon as possible in the pre-paid envelope, so I know whether or not you are happy to take part in the study. If you are interested, we will call you on the telephone in about one week to answer any questions you may have, and we can arrange a suitable appointment for you to visit us. Thank you very much for considering taking part in our research. Please discuss this information with your family, friends or GP if you wish.

If you have any questions, please contact:

Mr. Titus Augustine (Consultant Transplant and Endocrine Surgeon) Tel. 0161-276 5496

Mr. Neil Parrott (Consultant, General, Endocrine and Transplant Surgeon) Tel. 0161-276 5496

Prof. Rayaz Malik (Consultant Physician) Tel. 0161 275 1196

Prof. Andrew Boulton (Consultant Physician and Professor of Medicine) Tel. 0161 276 4406/4452

Dr. Mitra Tavakoli (Post-Doctoral Clinical Research Fellow) Tel. 0161 901 1466

## 12.3 Patient consent form

Central Manchester and Manchester  
Children's University Hospitals

NHS Trust



The University of Manchester

Participant Study Number:

Participant identification Number:

### CONSENT FORM

**Title of Project: Ophthalmic markers of diabetic neuropathy**

#### Investigators:

**Prof. Rayaz. A Malik**, Consultant Physician, MB ChB, FRCP, PhD.

**Prof. Andrew Boulton**, Consultant Physician, MBBS, MD, FRCP, DSc.

**Dr. Andrew Marshall**, Consultant Clinical Neurophysiologist BSc, BM CHB, MRCP

**Dr. Mitra Tavakoli**, Post-Doctoral Research Fellow BSc (Hons), MSc, PhD

**Please initial box:**

I confirm that I have read and I understand the information sheet dated...26/04/2011  
(Version 5...) for the above study and have had the opportunity to ask questions.

I understand that my participation is voluntary and that I am free to withdraw at any time,  
without giving any reason, without my medical care or legal rights being affected

I understand that sections of any of my medical notes may be looked at by responsible  
individuals from University of Manchester and NHS Trust where it is relevant to my  
taking part in research. I give permission for these individuals to have access to my records.

I agree to take part in the above study.

I agree that you may contact my GP regarding my participation in this study

I also agree that you can contact me in the future to see how my circumstances have  
changed.

I understand that this study requires two small samples of skin to be removed from the  
top of the foot. I agree to have this procedure undertaken.

.....  
Name of Patient

.....  
Signature

.....  
Date

.....  
Name of Person  
taking consent

.....  
Signature

.....  
Date

*1 for participant; 1 for researcher*

## 12.4 Physical measurements and peripheral neuropathy assessment forms

### Check List:

Surrogate markers of diabetic neuropathy ( IGT- Diabetes- Transplant- JDRF)

Name of Patient:

	Date	Notes
Travel costs		
Information sheet		
Consent forms		
NCS		
NDS		
VPT		
Neuropad		
Medoc		
Blood pressures		
Blood samples		
NCCA		
CCM		
Fundus camera		
Skin biopsy		

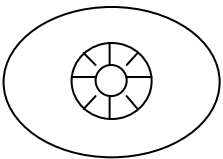
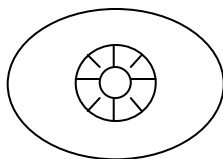
<b><u>Medical history</u></b>		
Hypertension	Stroke	High cholesterol
Heart problems	Breathing problems	
Other health issues:		
<b><u>Medication</u></b>		
Beta blockers	Warfarin	Synthrome
Aspirin	Clopidogrel	ACE inhibitor
A2RB	Statin	Fibrates
Other anti-hypertensive medication		
<b><u>Neuropathy</u></b>		

<b>Exclusion criteria</b>		
Severe systemic diseases (e.g. congestive cardiac failure, rheumatoid disease, SLE)	Chronic renal impairment (serum creatinine >250 umol/l)	Known peripheral vascular disease (e.g. previous bypass surgery, angioplasty or claudication)
Alcohol intake >21 units per week (males) or >14 units/week (females)	Non-diabetic peripheral neuropathy	Aspirin and Clopidogrel

## 12.5 Ophthalmic examination sheet



### Ophthalmic markers Ophthalmic record sheet

Participant's Full Name:	Date of Birth:	
Date of Visit:	Investigator(s):	
	Study & Visit ID:	
<b><u>IF NOT PART OF A TRIAL</u></b>		
Patient referred from:	Hospital No.:	
Address details:		
Medical History :		
Type of Diabetes:		
Duration of Diabetes:		
Family History of Diabetes (quote parental/maternal side):		
Other systemic disease (e.g. Heart Failure, Liver Failure, Hep B, HIV <sup>+</sup> , Vit. Deficiencies, Alcohol abuse, MS, Connective Tissue Disease, SLE, psoriasis):		
Medication (quote reason e.g. hypertension, cholesterol, diabetes, other CVD-related etc.):		
Ocular History:		
History of previous ocular disease (e.g. systemic, infections) / trauma:		
History of operations (quote year, eye, type of operation):		
History of contact lens use (quote type and frequency):		
History of retinopathy (official grading):		
For Transplant Study:		
Date of Transplant:		
Type of Transplant:		
Duration on Renal Dialysis:		
Smoking:	per day	
Drinking:	units per week	
<b>Ophthalmic Examinations:</b>		
<u>Slit Lamp Biomicroscopy (draw findings):</u>		
Comments:	<div style="display: flex; justify-content: space-around; align-items: center;"> <div style="text-align: center;"> <p><b>OD</b></p>  </div> <div style="border-left: 1px dashed black; height: 80px; margin: 0 10px;"></div> <div style="text-align: center;"> <p><b>OS</b></p>  </div> </div>	Comments:



Additional Notes

**13. APPENDIX 3-SUPPLEMENTARY MATERIAL**



