The mechanistic basis of vascular and neural dysfunction in patients with diabetes:
The role of ethnic differences

A thesis submitted to the University of Manchester for the degree of
Doctor of Philosophy in Medicine (Diabetes)
in the faculty of Medical and Human Sciences

2014

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Institute of Human Development, University of Manchester
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The University of Manchester
Abstract of thesis submitted by Hassan Fadavi for the degree of Doctor of Philosophy and titled:

The mechanistic basis of vascular and neural dysfunction in patients with diabetes: The role of ethnic differences

May 2014

Abstract

Neuropathy is one of the main long term complications of diabetes affecting 30-50% of patients. It is the major contributing factor for foot ulceration with a lifetime risk which may be as high as 25%. Hence neuropathy leads to reduced pain and pressure perception, anatomic deformities and an impaired microcirculation. More specifically, unperceived minor trauma results in cutaneous injury which when combined with an inadequate pressure induced vasodilator response leads to tissue breakdown and ulceration. Once ulcers form, healing may be delayed or difficult to achieve, particularly if infection occurs in the deeper tissues and bone which can then lead to amputation. In the UK, South Asians (people originating from India, Pakistan and Bangladesh) have an excess mortality for coronary artery disease (CAD), stroke and end-stage renal disease when compared to white Europeans. However, it has been shown that South Asian people with type 2 diabetes in the UK are only one third as likely to have a foot ulcer compared with White European diabetic patients. This has been attributed to lower levels of peripheral neuropathy in Asians, but has not been systematically explored in detail.

In the present study, both neurological and vascular deficits in a group of South Asian and European patients with type II diabetes have been assessed. The results demonstrate that:

- South Asian diabetic patients have poorer glycaemic control, but paradoxically lower triglycerides. This finding may be relevant to the finding that they have a lower incidence of neuropathy, as triglycerides have been related to neuropathy and foot ulceration.
- South Asians compared to Europeans have better small fibre function and a trend for better structure (Intra epidermal nerve fibre density and corneal nerve morphology) and large fibre function assessed with nerve conduction studies.
- South Asians have higher foot skin oxygenation and hyperaemic blood flow response to heating.
- South Asians have a thicker epidermis and a trend for a better capillary density.

Therefore these alterations may protect South Asians from the development of foot ulceration.
Declaration

Neuropathy assessments in 70 patients from this cohort I have studied have been used by Patrick Green for his MRes (2012, University of Manchester) as part of a study to correlate the severity of neuropathy to retinopathy. With the agreement of Professor Malik (the supervisor for both projects), I shared the following data with him to use in his thesis including age, duration of diabetes, HbA1c, clinical neurological assessment of neuropathy including NDS, VPT and CCM results.

Hassan Fadavi
# Table of Contents

- Copyright statements.................................................................................2
- Abstract of thesis.......................................................................................3
- Declaration..................................................................................................4
- Table of Contents........................................................................................5
- Table of tables............................................................................................12
- Tables of figures.........................................................................................15
- Dedication..................................................................................................18
- Acknowledgement.......................................................................................19
- List of Abbreviations..................................................................................20
- About the Candidate..................................................................................22
- List of publications.....................................................................................23
- List of conference presentations...............................................................27

## CHAPTER 1  INTRODUCTION........................................................................... 29

1.1  INTRODUCTION .......................................................................................... 30

1.1.1  Diabetes, classification, diagnostic criteria and epidemiology........31

1.1.2  Ethnicity..................................................................................................32

1.1.3  Four ways to establish ethnicity:.......................................................32

1.1.4  Type 2 Diabetes in South Asian patients..........................................33

1.1.5  Genetic determinants..........................................................................36

1.1.6  Genetics and diabetic microvascular complications......................37

1.1.7  Genetic factors and diabetic neuropathy..........................................38

1.1.8  Genetic factors and diabetic nephropathy........................................38

1.1.9  Genetic factors and diabetic retinopathy..........................................39

1.1.10 Coronary heart disease........................................................................40

1.2  METABOLIC SYNDROME .......................................................................41
2.6 VARIABLES

2.6.1 Demographic and Anthropometric Variables

2.7 SYMPTOMS

2.7.1 Neuropathy Symptom Profile

2.7.2 McGill Pain Questionnaire

2.8 CLINICAL NEUROPATHY ASSESSMENT

2.8.1 The neuropathy disability score

2.9 LARGE FIBRE ASSESSMENT

2.9.1 Electrophysiology

2.9.2 Quantitative sensory testing

2.10 SMALL FIBRE ASSESSMENT

2.10.1 Thermal threshold assessments

2.11 AUTONOMIC ASSESSMENT

2.11.1 Neuropad

2.11.2 Punch skin biopsy

2.11.3 Light and electron microscopy

2.11.4 Image processing and morphological procedures

2.12 INTRAEPIDERMAL NERVE FIBRE DENSITY ASSESSMENT

2.13 IMAGE ANALYSIS

2.14 IMMUNOHISTOCHEMISTRY METHOD FOR WAX EMBEDDING SAMPLES

2.14.1 Usefulness of wax embedded samples for IENFD

2.15 LASER DOPPLER FLOWMETRY

2.15.1 Hyperaemic response and flare area

2.15.2 Corneal aesthesiometer

2.15.3 Corneal confocal microscopy

CHAPTER 3 RESULTS
Explanations for the lower prevalence of small fibre neuropathy in South Asian versus European patients with Type 2 diabetes ................................................................. 110

3.1 INTRODUCTION .................................................................................................. 112
3.2 RESEARCH DESIGN AND METHODS .............................................................. 112
3.2.1 Study Subjects ............................................................................................... 113
3.3 CLINICAL ASSESSMENTS ............................................................................... 113
3.4 COMPLICATIONS ASSESSMENTS ................................................................... 113
3.5 ASSESSMENT OF NEUROPATHY .................................................................... 114
3.6 SMALL FIBRE FUNCTION .................................................................................. 114
3.6.1 Intra epidermal nerve fibre density .............................................................. 114
3.6.2 Corneal confocal microscopy ...................................................................... 115
3.7 STATISTICAL ANALYSIS .................................................................................. 115
3.8 RESULTS ............................................................................................................ 116
3.8.1 Clinical signs and symptoms ....................................................................... 116
3.8.2 Neurophysiology ......................................................................................... 116
3.9 SMALL NERVE FIBRE FUNCTION ................................................................... 116
3.10 SMALL NERVE FIBRE STRUCTURE ............................................................. 117
3.10.1 Explanations for ethnic differences in corneal nerve fibre structure... ................................................................................................................................. 119
3.10.2 Explanations for ethnic differences in small and large fibre function. ................................................................................................................................. 120
3.11 CONCLUSION .................................................................................................. 124

Differences in neuronal and vascular function and structure between South Asian and Europeans patients with Type 2 diabetes ........................................................................ 126

3.12 INTRODUCTION ............................................................................................... 128
3.13 RESEARCH DESIGN AND METHODS .......................................................... 129
3.13.1 Selection of patients .................................................................................... 129
3.14 CLINICAL ASSESSMENT ................................................................. 130
3.15 VASCULAR STATUS .................................................................. 130
3.16 ASSESSMENT OF NEUROPATHY ........................................... 131
3.17 CORNEAL CONFOCAL MICROSCOPY ...................................... 131
3.18 BIOPSY .................................................................................. 132
3.18.1 Intra epidermal nerve fibre density ........................................ 132
3.18.2 Skin epidermal thickness and microangiopathy .................... 132
3.19 STATISTICS ANALYSIS ........................................................... 133
3.20 RESULTS ................................................................................ 133
3.21 NEUROPATHY ASSESSMENT ................................................... 135
3.22 VASCULAR FUNCTION ............................................................ 136
3.23 EPIDERMAL THICKNESS AND MICROANGIOPATHY ............... 136
3.24 EXPLANATION FOR ETHNIC DIFFERENCES IN MAXIMAL HYPERAEMIC RESPONSE .................................................................................................................. 138
3.25 CONCLUSION .......................................................................... 139

Longitudinal assessment of change in neuropathy in South Asian compared to Europeans patients with type 2 diabetes: A 5 years follow-up study ......................... 142

3.26 INTRODUCTION ...................................................................... 144
3.27 DESIGN OF STUDY .................................................................. 144
3.28 CLINICAL ASSESSMENT .......................................................... 145
3.29 STATISTICAL ANALYSES ......................................................... 145
3.30 RESULTS ................................................................................ 146
3.30.1 Change in clinical demographics .......................................... 146
3.30.2 Change in neuropathy evaluation ........................................ 146
3.30.3 Absolute difference in neuropathy during follow up .............. 149
3.31 EXPLANATION FOR LESS DETEORATION IN LARGE FIBRE FUNCTION ... 149
3.32 CONCLUSION .......................................................................... 151
Tables

Table 1. Prevalence of PAD in various studies ............................................. 45
Table 2. Association of risk factors with PAD .............................................. 46
Table 3. (a) Classification of the Diabetic Neuropathies based on clinical features ................................................................. 54
Table 4. Classification of Diabetic Neuropathy .......................................... 55
Table 5. Causes of small fibre neuropathy ................................................ 56
Table 6. Symptoms associated with small-fibre neuropathy in diabetic neuropathy ................................................................. 58
Table 7. The type size and function of different nerve fibres in the human body. ........................................................................ 59
Table 8. Advantage & disadvantages for different tests of diabetic neuropathy. ........................................................................ 60
Table 9. Grading of Diabetic Peripheral Neuropathy as defined by Diabetic Neuropathy Study Group of the European Association for the Study of Diabetes ........................................................................ 68
Table 10. Treatment options for painful diabetic neuropathy .................... 72
Table 11. Placebo response of trials of Duloxetine, Venlafaxine, Glyceryl Trinitrate/Sodium Valproate and Cannabinoids ........................................ 73
Table 12. Demographic and clinical characteristics of ethnic groups .......... 79
Table 13. Characteristics of participants and non-participants from baseline study of DAEMON ........................................................................ 83
Table 14. Characteristics of participants who did and did not undergo skin biopsy. ........................................................................ 84
Table 15. Summary of methods used in this project .................................... 108
Table 16. Demographic and clinical characteristics of ethnic groups .......... 118
| Table 17. Measures of neuropathy signs, symptoms, large nerve fibre function, small nerve fibre function, corneal nerve fibre structure and intra epidermal nerve fibre density by ethnicity. | 121 |
| Table 18. Analysis of covariance results for corneal nerve fibre length by ethnicity. | 122 |
| Table 19. Analysis of covariance results for sural nerve amplitude and heart rate variability to deep breathing by ethnicity. | 123 |
| Table 20. Demographics and clinical characteristics of patients and control subjects. | 134 |
| Table 21. Neuropathy assessment for diabetic patients and healthy controls. | 136 |
| Table 22. Vascular and neural assessment of diabetic patients and healthy control subjects. | 137 |
| Table 23. Analysis of covariance results for maximal hyperaemic response by ethnicity. | 138 |
| Table 24. Demographics and neuropathy measurements at the baseline and follow up visit in South Asian and European patients with type 2 diabetes. | 147 |
| Table 25. Change in measures of neuropathy from baseline to follow up visit. | 149 |
| Table 26. Analysis of covariance results for vibration perception threshold and sural nerve amplitude in South Asians and Europeans patients with type 2 diabetes. | 150 |
| Table 27. Analysis of covariance results for transcutaneous partial pressure by ethnicity. | 213 |
| Table 28. Analysis of covariance results for maximal hyperaemic response by ethnicity. | 214 |
| Table 29. Neuropad assessment (Normal, Patchy, Abnormal) in healthy controls, South Asians and Europeans. | 215 |
| Table 30. Correlation of structure assessment of neuropathy with functional tests. | 216 |
Table 31. Demographic, Corneal confocal microscopy and Langerhans cells (LCs) in South Asians compared to Europeans with T2DM............................218

Table 32. Correlations with Langerhans cell density........................................219
Tables of Figures

Figure 1. Regional variation in England in the incidence of major amputation in diabetes, expressed per $1 \times 10^3$ population with diabetes. ..................................................34

Figure 2. Prevalence of diagnosed diabetes by ethnic groups in the UK. Blue bar: men; Orange bar: women. ........................................................................................................34

Figure 3. Global prevalence of diabetes in 2011. Africa (AFR), Europe (EUR), Western Pacific (WP), South and Central of America (SACA), South East Asia (SEA), North America and Caribbean (NAC), Middle East and North Africa (MENA). ........................................................................................................35

Figure 4. Electron micrographs of dermal capillaries from a mildly neuropathic (left) and progressive thickening of basement membrane (BM)-red arrow-in severely neuropathic patient (right) (x 1400). ..................................................47

Figure 5. Schematic view of capillary structure ..................................................................................48

Figure 6. Pathophysiology of diabetic neuropathy .........................................................................53

Figure 7. DAEMON study schematic design. ..................................................................................83

Figure 8. Assessing NDS (a) Tendon hammer for Achilles tendon reflexes (b) Neurotip™ for superficial pain perception (c) 128 Hz tuning fork for vibration perception (d) Cool/hot rods for temperature sensation..................................................89

Figure 9. The Medtronic Keypoint electrophysiology system. ......................................................90

Figure 10. Neurothesiometer. ........................................................................................................91

Figure 11. TSA-II NeuroSensory Analyser (Medoc) .................................................................92

Figure 12. Method of Limits and position of the attached probe for the thermal threshold assessment. .................................................................................................93

Figure 13. Heart rate variability response to deep breathing using CASE IV....93

Figure 14. Neuropad (a) normal case, plaster is pink in 10 minutes (b) abnormal case, plaster remains blue (c) abnormal case where colour change is partial. 94

Figure 15. Punch skin biopsy procedure.................................................................95
Figure 16. Light microscopic section of epidermis and hypodermis outlining assessment of capillary density and epidermal thickness

Figure 17. Electronmicrograph of capillary structure of skin biopsy taken from the dorsum of left foot. (Magnification x1400) Outer layer of basement membrane (BM), pericyte area, endothelial area and luminal area are shown.

Figure 18. 50µm section of human skin immunostained for PGP9.5. IENF appeared positive for PGP9.5. Picture was taken at 20x magnification. Red arrows show IENF.

Figure 19. Laser Doppler Imaging system.

Figure 20. Laser doppler imaging, pre-hyperaemic response at baseline (top left image shows desired marked area without vein and hair and top right image shows no hyperaemic response at baseline) and after heating with the TCpO\textsubscript{2} probe (bottom left image marked area and bottom right, post- hyperaemic response LDI max (flux beneath probe).

Figure 21. Thermal probe for TCpO\textsubscript{2} assessment.

Figure 22. Post heating image of laser Doppler flowmetry. The mean perfusion unit within the region corresponding to the probe contact on the FLUX image (left) is the LDI max which is the red area in the post image. The internal area demarcated by the blue-green boundary (red arrow in right image) constitutes the LDI flare.

Figure 23. The non-nontact corneal aesthesiometer.

Figure 24. In vivo Corneal Confocal Microscopy and image of Bowman’s layer of cornea and corneal confocal machine used in this project (HRT III).

Figure 25. Bland-Altman plots for IENFD as an indication of agreement between repeated measurement of the same images on two occasions and by two observers.

Figure 26. Corneal confocal microscopy from South Asian (left) and European (right) patients with T2DM (white dots indicate Langerhans cells).
Dedication

This thesis is dedicated in the continuing memory of my beloved parents; Mohammad (1927-2006), Zahra (1936-2007); Rest in peace.
To my true friend, companion and wife, Mitra, who has always been encouraging for the last 12 years.
To my little beautiful daughter, Baran, who kept calling me in late afternoon; come home Daddy Please.
To all participants (patients and volunteers) who kindly agreed to enrol in this study enabling me to do this project.
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I would like to express my never-ending gratitude to my supervisor Professor Rayaz A Malik for helping me take my first steps in learning the craft and art of research. Throughout, all my PhD and thesis writing, he helped me with an enormous amount of guidance, encouragement, advice and good teaching. I have been extremely lucky to have a supervisor who cared so much about my work, and who responded to my questions and queries so promptly. I would have been lost without him.

I would like to express my warm and sincere thanks to my advisor, Professor Andrew J.M. Boulton who has been great to provide me the opportunity to attend the foot clinic and has given me encouragement and advice on effective presentation from the beginning of my PhD. I am privileged for having both as role models of dedication that will continue to inspire me for the years to come.

My sincere thanks are due to my co-supervisor Dr Caroline Abbott for helping me to recruit patients and teaching me the techniques to carry out this study.

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I am also grateful to the Neurophysiology department staff, Mrs Joanne Finnigan and Mrs Sarah Doyle, who helped me with the neurophysiology assessments of patients for this study.

I also thank my sisters, brother and my mother, father and brother in law, who all have been supportive and caring.

Last but not the least; I owe my loving thanks to my wife, Mitra for being such a good companion, best friend and wise advisor. She patiently looked after my little angel (Baran June) when I was writing this thesis.

I am grateful to the many patients who underwent clinical assessment and skin biopsy, enabling me to carry out much of the work described in this thesis.
# List of the most frequent used abbreviations in this thesis

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
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<tbody>
<tr>
<td>ADA</td>
<td>American Diabetes Association</td>
</tr>
<tr>
<td>AGE(s)</td>
<td>Advanced Glycation End product(s)</td>
</tr>
<tr>
<td>ANS</td>
<td>Autonomic Nervous System</td>
</tr>
<tr>
<td>BP</td>
<td>Blood pressure</td>
</tr>
<tr>
<td>C-BA</td>
<td>Cochet-Bonnet aesthesiometer</td>
</tr>
<tr>
<td>CCM</td>
<td>Corneal Confocal Microscopy</td>
</tr>
<tr>
<td>CDT</td>
<td>Cold Detection Threshold</td>
</tr>
<tr>
<td>CHD</td>
<td>Coronary Heart Disease</td>
</tr>
<tr>
<td>CIDP</td>
<td>Chronic Inflammatory Demyelinating Polyneuropathy</td>
</tr>
<tr>
<td>CIP</td>
<td>Cold Induced Pain</td>
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<tr>
<td>CS</td>
<td>Cold Sensation</td>
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<tr>
<td>CM</td>
<td>Centimetre</td>
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<td>CMT</td>
<td>Charcot-Marie-Tooth</td>
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<tr>
<td>DAEMON</td>
<td>Diabetes in Asians and Europeans: the Manchester study Of Neuropathy</td>
</tr>
<tr>
<td>DAN</td>
<td>Diabetic Autonomic Neuropathy</td>
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<td>DCCT</td>
<td>Diabetic Control and Complication Trial</td>
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<tr>
<td>DPN(s)</td>
<td>Diabetic Peripheral Neuropathies</td>
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<tr>
<td>DM</td>
<td>Diabetes Mellitus</td>
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<tr>
<td>DML</td>
<td>Distal Motor Latency</td>
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<td>DSPN</td>
<td>Diabetic sensory polyneuropathy</td>
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<tr>
<td>FDA</td>
<td>Food and Drug Administration</td>
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<tr>
<td>FEI</td>
<td>Federation of the Electronics Industry</td>
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<tr>
<td>OGTT</td>
<td>Oral Glucose Tolerance test</td>
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<td>HDLC</td>
<td>High-Density Lipoprotein Cholesterol</td>
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<td>HIF-1 α</td>
<td>Hypoxia inducible factor 1</td>
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<td>HIP</td>
<td>Heat Induced Pain</td>
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<tr>
<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
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<tr>
<td>HMSN</td>
<td>Hereditary Motor and Sensory Neuropathy</td>
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<tr>
<td>IGT</td>
<td>Impaired Glucose Tolerance</td>
</tr>
<tr>
<td>IENFD</td>
<td>Intraepidermal Nerve Fibre Density</td>
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<tr>
<td>IENBD</td>
<td>Intraepidermal Nerve branch Density</td>
</tr>
<tr>
<td>IENFL</td>
<td>Intraepidermal Nerve Fibre Length</td>
</tr>
<tr>
<td>IVCCM</td>
<td>In vivo Corneal Confocal Microscopy</td>
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<tr>
<td>ISFN</td>
<td>Idiopathic Small Fibre Neuropathy</td>
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<tr>
<td>JDRF</td>
<td>Juvenile Diabetes Research Foundation</td>
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<tr>
<td>LANDMARK</td>
<td>Longitudinal Assessment of Novel Ophthalmic Diabetic Markers</td>
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<tr>
<td>LDI</td>
<td>Laser Doppler Imaging</td>
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<tr>
<td>LDL</td>
<td>Low-density lipoprotein</td>
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<td>LEA</td>
<td>Lower Extremity Amputation</td>
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<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
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<td>MetS</td>
<td>Metabolic Syndrome</td>
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<tr>
<td>MESA</td>
<td>Multi Ethnic Study of Atherosclerosis</td>
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<td>MS</td>
<td>Meter/Second</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
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<tr>
<td>NCEP ATP</td>
<td>Adult Treatment Panel of the National Cholesterol Education Program</td>
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<tr>
<td>NIH</td>
<td>National Institutes of Health</td>
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<tr>
<td>NISLL</td>
<td>Neuropathy Impairment Score Lower limbs</td>
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<tr>
<td>CNFD</td>
<td>Corneal Nerve Fibre Density</td>
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<tr>
<td>CNBD</td>
<td>Corneal Nerve Branch Density</td>
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<td>CNFL</td>
<td>Corneal Nerve Fibre Length</td>
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<td>CNFT</td>
<td>Corneal Nerve Fibre Tortuosity</td>
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<td>NADH</td>
<td>Nicotinamide Adenine Dinucleotide</td>
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<td>NCCA</td>
<td>Non-Contact Corneal Aesthesiometer</td>
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<td>NDS</td>
<td>Neuropathy Deficit Score</td>
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<td>NSP</td>
<td>Neuropathy Symptom Profile</td>
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<td>NCS</td>
<td>Nerve Conduction Studies</td>
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<td>PAD</td>
<td>Peripheral Artery Disease</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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<td>PMNAP</td>
<td>Peroneal Motor Nerve Amplitude</td>
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<td>PMNCV</td>
<td>Peroneal Motor Nerve Conduction Velocity</td>
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<td>PVD</td>
<td>Peripheral vascular disease</td>
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<td>PN</td>
<td>Peripheral Neuropathies</td>
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<td>QSART</td>
<td>Quantitative sudomotor axon reflex test</td>
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<td>QST</td>
<td>Quantitative Sensory Testing</td>
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<td>SEM</td>
<td>Standard Error Mean</td>
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<td>SMRs</td>
<td>Standardised mortality ratios</td>
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<tr>
<td>SNAP</td>
<td>Sural Nerve Amplitude</td>
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<tr>
<td>SNCV</td>
<td>Sural Nerve Conduction Velocity</td>
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<td>SNS</td>
<td>Somatic nervous system</td>
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<td>SNP</td>
<td>Single nucleotide polymorphisms</td>
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<tr>
<td>SFN</td>
<td>Small Fibre Neuropathy</td>
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<tr>
<td>SSR</td>
<td>Sympathetic Skin Response</td>
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<td>SSIRs</td>
<td>Selective serotonin reuptake inhibitors</td>
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<td>TAOS</td>
<td>Total antioxidant status</td>
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<tr>
<td>TC</td>
<td>Tortuosity Coefficient</td>
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<td>TCpO₂</td>
<td>Transcutaneous partial pressure of oxygen</td>
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<tr>
<td>TTP</td>
<td>Thermal Threshold Perception</td>
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<td>UKPDS</td>
<td>United Kingdom Prospective Diabetes Study</td>
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<tr>
<td>V</td>
<td>Volts</td>
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<td>VDR</td>
<td>Vitamin D Receptor</td>
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<td>VEGF-A</td>
<td>Vascular Endothelial Growth Factor A</td>
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<td>VPT</td>
<td>Vibration Perception Threshold</td>
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<td>WHO</td>
<td>World Health Organization</td>
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<tr>
<td>WC</td>
<td>Waist Circumference</td>
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<tr>
<td>WT</td>
<td>Wild Type</td>
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<tr>
<td>WS</td>
<td>Warm Sensation</td>
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</table>
About The Candidate

The author is a physician with a special interest in Diabetes Mellitus and its complications, mainly peripheral neuropathy and vascular disease. He was awarded an MD degree in 2000 from Birjand University of Medical Sciences, Iran. He started working as a general practitioner and worked in hospital medicine before he moved to the UK. In 2007 he started his PhD part time under the supervision of Professor Rayaz A Malik, Professor Andrew JM Boulton and Dr Caroline A Abbott from the Department of Medicine and Dr Maria Jeziorska from the Department of Regenerative Medicine. During his PhD, he has participated actively in different projects funded by the NIH, JDRF and DUK, as a clinical research fellow while he was working on his PhD. He was awarded a NIHR BRC fellowship in 2008. The studies he has been involved in include:

- Diabetes UK: (present thesis), all assessments for this study were performed by the author apart from nerve conduction studies and corneal confocal microscopy which were done by a neurophysiologist and optometrist respectively. (Funded by DUK grant-08-147).

- Longitudinal assessment of novel ophthalmic diabetic markers (“LANDMark” study) (Funded by JDRF-17-2008-1031).

- Corneal Confocal Microscopy: a non-invasive surrogate for diabetic neuropathy (Funded by NIH grant-5R01-NS46259-03).

He has presented the results of his work in several national & international conferences including: American Diabetes Association (ADA), European Association for the Study of Diabetes (EASD), Neurodiab, International Diabetes Federation (IDF) and Diabetes UK. The details are presented in the list of publications.
List of Publications

**Book chapters:**


**Original papers (Peer reviewed)**


Reviews (Peer reviewed)


Published abstracts (Peer Reviewed)


List of Conference Presentations

A. Oral Presentations


2. **Fadavi H**, Tavakoli M, Petropoulos I, Chaturvedi N, Jeziorska M, Boulton AJM, Malik R, Abbott CA. Why is there a lower incidence of foot ulceration in South Asian compared to European patients with Type 2 diabetes? 22th Neurodiab meeting, Dresden, Germany, September 2012.


B) Poster Presentations


2. **Fadavi H**, Tavakoli M, Petropoulos I, Chaturvedi N, Boulton AJM, Malik RA, Abbott CA. Predisposing risk factors underlying the reduced risk of
foot ulceration in South Asian compared to European patient with diabetes. 70th ADA, Orlando, June 2010.


5. Fadavi H, Tavakoli M, Finnigan J, Doyle S, Boulton A JM, Malik RA, Abbott CA. Reduced small nerve fibre damage may underlie a lower incidence of foot ulceration in Asian compared to European patients with diabetes, 69th ADA New Orleans, June 2009.
Chapter 1  Introduction
1.1 Introduction

Diabetes mellitus (DM) presently affects around 347 million people worldwide and it is estimated that this will rise to 472 million by 2030. In 2010, in England 3,100,000 people were estimated to have diabetes (diagnosed or undiagnosed) accounting for 7.4% of the population. By 2030, it is forecast that this will rise to 4,603,363 people or 9.5% of the English population. Approximately half of this increase is attributable to the increasing age and ethnic group structure of the population and the other half is due to the rising prevalence of obesity. The prevalence of diabetes for all age groups worldwide was estimated to be 2.8% in 2000 and is predicted to rise to 4.4% by 2030. Diabetic neuropathy is the most common and costly complication of diabetes.

People of Indian Asian descent worldwide have one of the highest risks of type 2 diabetes; in the U.K., this is four times more prevalent than in the general population (1). Most studies have indicated that the risk of diabetes and cardiovascular disease is elevated worldwide in Indian Asians, reflecting their overall greater predisposition to cardiovascular disease (CVD) (2). However, risks of other diabetes-related complications, i.e., foot ulceration and amputation, also with a vascular basis, are substantially lower in Asians than white Europeans in the U.K., possibly due to less neuropathy (3). The main aim of the present thesis is to establish the underlying causes for this protection. In this chapter, a brief summary of the following sections has been presented:

- Diabetes, classification, diagnostic criteria and epidemiology
- Ethnicity & Type 2 Diabetes in patients from South Asian origin
- Genetic Determinants
- Genetics and diabetic microvascular complications
- Diabetic Neuropathy

Section 1
1.1.1 Diabetes, classification, diagnostic criteria and epidemiology

Diabetes mellitus can be defined as a group of metabolic diseases characterised by hyperglycaemia resulting from defects in insulin secretion, insulin action, or both. After a long duration of metabolic derangement, specific complications of diabetes (retinopathy, nephropathy, and neuropathy) may occur (4). Diabetes is primarily categorised into two types with several subclasses, which differ by aetiology, onset, genetics, prevalence and degree of insulin insufficiency (5).

Type 1 diabetes which accounts for 5-10% of all diabetes cases/diagnoses arises due to autoimmune destruction of beta cells leading a requirement of insulin for survival to prevent the development of ketoacidosis, coma and death. The rate of beta cell destruction is quite variable, being rapid in some individuals such as infants and children and slow in others (mainly adults) (4). A minority of patients with type 1 diabetes show no evidence of autoimmunity, and are thus sub-classified as having an idiopathic disease. This type of diabetes is more common in patients of African or Asian origin.

Type 2 diabetes constitutes 90-95% of the diabetes diagnoses. Type 2 diabetes patients have insulin resistance and usually have a relative insulin deficiency at least initially and often throughout their life time. The risk of developing this form of diabetes increases with age, obesity, and lack of physical activity (4).

For decades, the diagnosis of diabetes has been based on glucose criteria, either the fasting plasma glucose or the 75 grams oral glucose tolerance test. In 1997, the first expert Committee on diagnosis and classification of Diabetes Mellitus revised the diagnostic criteria and examined data from three epidemiologic studies that assessed retinopathy with fundus photography or direct ophthalmoscopy and measured glycaemia as fasting plasma glucose (FPG), 2-h plasma glucose by OGTT and HbA1c. The deciles of the three measures at which retinopathy began to increase were the same for each measure within each population. Furthermore, the glycaemic values above which retinopathy increased were similar among the populations (5). Prior Expert Committees have not recommended the use of the A1C for diagnosis of diabetes, in part due to a lack of standardisation of the assay. However, HbA1c assays are now highly standardised so that their results can be uniformly applied both temporally and across populations (6).
It is also estimated that the number of people with diabetes will rise from 171 million in 2000 to 366 million in 2030 (7). A study by Shaw JE et al (8) presented a gloomier projection estimating that the world prevalence of diabetes among 20–79 year old adults will increase from 6.4% in 2010 (affecting 285 million adults) to 7.7% by 2030 (affecting 439 million adults). The same study estimated that between 2010 and 2030, there will be a 69% increase in the number of adults with diabetes in developing countries and a 20% increase in developed countries.

Section 2

1.1.2 Ethnicity
The term ethnicity refers to common cultural traditions, geography, ancestry, religion and history. The term comes from the Greek word “ethnos”, which means “nation” or people. Common culture typically includes language, religion and diet. It also refers to the culture within a group of people with common or shared identity living within a larger mainstream group (9). In this study the term ‘European’ refers to people who originate from the UK, Wales, and Ireland. The term ‘South Asians’ refers to people from Pakistan, Bangladesh, India and Sri Lanka.

1.1.2.1 Reasons for ethnic differences in disease epidemiology
Although the exact cause of ethnic differences in disease epidemiology are not known, various factors which may contribute are as follows (10):

1. Differences in environmental exposure.
2. Genetic factors and pre-disposition to disease.
3. Variations in cultural and health behaviour.
4. Differing socio-economic positions of different ethnic groups.

1.1.3 Four ways to establish ethnicity:
1. Parental or preferably grandparental origin.
2. Self-identity permitting people to assert their own identity, which is in keeping with the patient centered approach adopted by the NHS.
3. Appearance although this is now discredited as it has poor reproducibility and serves a political rather than a scientific role.
4. Ancestral markers
In 2001, it was reported that half of the total minority ethnic population in the UK are South Asian (9).

1.1.4 Type 2 Diabetes in South Asian patients

Studies involving migrant South Asians in developed countries such as the United States, United Kingdom and Canada have reported a high prevalence rates of type 2 diabetes mellitus (T2DM) (11, 12). A number of factors including genetics, ethnicity, dyslipidaemia, migration, diet and lifestyle have been associated with the increased prevalence of T2DM in migrant South Asians. The prevalence of T2DM in migrant South Asians was the highest in the United Kingdom (11–33%) followed by Norway (14–28%), United States (18%), Singapore (12.8%) and Canada (10%) (12). These rates are significantly greater than the prevalence of the condition in native South Asians living in Pakistan (7.6%), India (7.1%) and Bangladesh (6.1%) (12). Hence the prevalence of T2DM ranges from 2% in rural Indian communities to 11% in urban Indian cities and up to 16% in migrant Indians living in the U.S., United Kingdom, or South Africa (13). In a study by Holman et al the incidence of amputations in adults with and without diabetes was determined from hospital episode statistics over 3 years from primary care trusts in the U.K. and this study found that diabetes continues to confer a very high relative risk of amputation. They also showed that in both people with and without diabetes, there was a ten-fold variation between different primary care trusts in the incidence of major amputation and an eight-fold variation in the incidence of all amputations (major and minor combined). The variation in incidence of major amputation is presented in figure 1 (14).
Figure 1. Regional variation in England in the incidence of major amputation in diabetes, expressed per $1 \times 10^3$ population with diabetes. Courtesy of Holman (14)

Figure 2. Prevalence of diagnosed diabetes by ethnic groups in the UK. Blue bar: men; Orange bar: women. Courtesy of Barnett (15)
A recent study which followed three ethnic groups for 20 years in the UK showed that approximately half of all South Asians, Africans and African-Caribbean people in the UK will develop diabetes by the age of 80, compared with only one in five people of European descent (16). The results showed that insulin resistance and truncal obesity account for the two-fold excess incidence of diabetes in Indian Asian and African Caribbean women, but not in men and they concluded that the excess diabetes risk in ethnic minority men remains unclear (17). The prevalence of diabetes, especially type 2 diabetes mellitus, continues to increase in the US and in all westernised countries. During the past four decades a five-fold increase of type 2 diabetes has been observed in the US (18). Asian Americans, people who emigrated from India, Pakistan, Bangladesh (South Asian) have the highest predisposition to develop diabetes (18).

Figure 3. Global prevalence of diabetes in 2011. Africa (AFR), Europe (EUR), Western Pacific (WP), South and Central of America (SACA), South East Asia (SEA), North America and Caribbean (NAC), Middle East and North Africa (MENA). Courtesy of International Diabetes Federation (IDF).
Section 3

1.1.5 Genetic determinants

Genetic factors are known to play a significant role in the pathogenesis of both type 1 and type 2 diabetes (19). Whilst a strong genetic predisposition to Type 2 diabetes is supported by twin studies (19), the genetics of diabetes is almost certainly complex, with a number of genes contributing to the overall risk (20). Some of the susceptibility genes for diabetes are likely to be specific to certain populations, while others may be more ‘universal’, or common to multiple ethnic groups. Transcription factor 7-like2 (TCF7L2) appears to be a strong predictor of T2DM in various ethnic groups, including South Asians. Some genetic polymorphisms have been found to prevent T2DM in certain population groups, but these might not offer a similar protection to all populations (12). Both type 2 diabetes and coronary heart disease (CHD) occur as a result of the complex interplay of genetic susceptibility and environmental factors (15). Genetic factors may underlie the inherited risk of type 2 diabetes and CHD in South Asians compared with Caucasians and there may be a higher prevalence of some risk alleles in specific ethnic groups (15). An example of an allele that may contribute to insulin resistance in people of South Asian descent is a polymorphism in the gene encoding plasma cell membrane glycoprotein-1 (PC-1), which affects insulin signalling by a direct interaction with the alpha subunit of the insulin receptor, blocking insulin action. The PC-1 K121Q polymorphism has been associated with a stronger inhibitory effect on the insulin receptor than the wild-type, and occurs with a significantly higher frequency in South Asian people in comparison to Caucasians (33 vs. 26%, respectively) (21). In another study mean LTL (leukocyte telomere length) was determined in 569 Caucasian, 103 South Asian, 70 Afro-Caribbean T2D patients between 24 and 92 years of age, 81 healthy Caucasian male students between 18 and 28 years of age and 367 healthy Caucasian men aged between 40 and 61 years, by real-time PCR. Plasma total antioxidant status (TAOS) was measured in the T2D patients by a photometric microassay and the patients were genotyped for the (uncoupling protein 2) UCP2 functional variants 866GA and A55V. Significant differences were found among the three ethnicities, after adjusting for age (p < 0.0001) (22). A shorter LTL was associated with the
presence of T2D which was partially attributed to the high oxidative stress in these patients. The association of the UCP2 (a gene involved in the mitochondrial production of reactive oxygen species) functional promoter variant with the shorter LTL implies a link between mitochondrial production of reactive oxygen species and shorter telomere length in T2D which could be partially attributed to the high oxidative stress in these patients (22). A number of variants may contribute to fasting glucose variation in people of South Asian descent which may confer risk of type 2 diabetes (23).

1.1.6 Genetics and diabetic microvascular complications

Some patients with good glucose control can develop diabetic microvascular complications, whilst others with poor control can escape these long-term complications (24). Type 2 diabetes results from impaired insulin secretion, insulin resistance in peripheral tissues and increased glucose production by the liver (25). Most individuals with type 2 diabetes are at high risk of developing serious complications including nephropathy, neuropathy, retinopathy and accelerated development of cardiovascular disease (26). Traditionally, chronic alterations in metabolic and/or haemodynamic factors have been recognised as the main cause of renal injury in patients with diabetes mellitus. However, in recent years, these conventional mechanisms have been considered to be only partially responsible for the development and/or progression of the microvascular complications. In a study of 200 Italian patients with type 2 diabetes the single nucleotide polymorphisms (SNP) for the PSMD9 gene was found to show a strong linkage with neuropathy, \( p \leq 0.0004 \) (27). An association between the genetic regulation of bone remodelling has also been demonstrated in patients with Charcot neuroarthropathy (28). Genetic variation in the tumour necrosis factor (TNF) receptor 2 gene (TNFRSF1B) superfamily number 1 B may also predispose to clinical neuropathy in patients with T2D (29), but it has also been associated with better glycosylated haemoglobin and elevated HDL cholesterol (29).

The predisposition to diabetic complications varies significantly between ethnic groups. Thus, the prevalence of diabetic nephropathy and retinopathy is higher in people of South Asian origin compared to Caucasians (30, 31). Furthermore, South Asian patients were more likely to require re-admission to treat clinical re-stenosis of
the index coronary artery lesion. However, there was no significant long-term difference in all-cause mortality between South Asian and European patients. Worse outcomes were reported in a cohort of 293 South Asian patients compared to 865 white European patients admitted for elective or urgent percutaneous coronary intervention (PCI) to de novo lesions (32).

1.1.7 Genetic factors and diabetic neuropathy
It has previously been suggested that genetic alterations in aldose reductase and the polyol pathway may play a critical role in the development of diabetic neuropathy (24). In a Finnish study, genetic analysis performed on 10 exons and the promoter region of the ALR2 (aldose reductase 2) gene for DNA sequence variants found one new polymorphism and confirmed three previously reported polymorphisms in diabetic patients with neuropathy (33). Genes encoding the enzymes (mitochondrial superoxide dismutase) Mn-SOD and extracellular superoxide dismutase (EC-SOD) have also been found to be associated with diabetic polyneuropathy (34). Recently, high levels of serum heat shock protein 27 (sHSP27) have been associated with protection from the development of diabetic neuropathy in patients with type 1 diabetes as high sHSP27 levels were associated with better nerve function and fewer neuropathic signs in subjects with normal glucose tolerance (NGT), impaired glucose tolerance (IGT) and T2DM (35).

1.1.8 Genetic factors and diabetic nephropathy
Between 20% and 40% of patients with diabetes ultimately develop nephropathy. The reason why not all patients with diabetes develop this complication has not been established, although differences in diagnostic criteria for diabetic nephropathy may provide a partial explanation. For example, proteinuria, glomerular filtration rate and renal biopsy will clearly show different prevalence rates, due to differing sensitivities of these methods to identify underlying pathology. Currently GWAS (genome-wide association studies) for the identification of susceptibility genes for common diseases have been initiated in many countries and they have already established some convincing associations with micro vascular complications (36).

Familial clustering of diabetic nephropathy in both type 1 (37) and type 2 (38) diabetes strongly suggests an association of genetic factors with diabetic
nephropathy. Several candidate genes, such as those for the renin–angiotensin system, have been shown to be linked to susceptibility to the disease in several independent cohorts (36, 39). The genes encoding some components of renin-angiotensin system, such as the angiotensin-converting enzyme (ACE), angiotensinogen (AGT), and angiotensin II receptor type I (AGTR1) have been reported to be the most probable candidate genes for diabetic nephropathy (36). However, the results of previous association and/or linkage studies for many genes and loci have not always been consistent, probably because of inadequate sample sizes and insufficient levels of statistical confidence, or because of variations in environmental conditions or ethnicity among the study populations (36). Hence, to date, whilst clinical and epidemiological studies suggest a genetic component for diabetic nephropathy, no specific gene has been associated with diabetic nephropathy (40). The prevalence rates of diabetic nephropathy show marked ethnic variations, and a genetic predisposition may constitute a major component in the development and/or progression of diabetic nephropathy (41). A recent study has shown that the vitamin D receptor (VDR) gene may be renoprotective in patients with type 1 diabetes (42).

1.1.9 Genetic factors and diabetic retinopathy
Diabetic retinopathy remains the most frequent cause of blindness in patients between 20 and 74 years of age. During the first two decades of the disease, nearly all patients with type 1 diabetes and more than 60% of patients with type 2 diabetes develop retinopathy (43). Ethnic differences in the prevalence of diabetic retinopathy may provide insights into the relative importance of genetic or environmental risk factors. The Multi-Ethnic Study of Atherosclerosis (MESA) study reported moderate differences in the prevalence of diabetic retinopathy among different ethnicities: 36.7% in African-Americans, 37.4% in Hispanics, 24.8% in whites, and 25.7% in Chinese-Americans (44). Discrepancies in risk factors such as diabetes duration, glycaemic control and hypertension did not explain the differences in prevalence of diabetes among these groups, which suggests that genetic or cultural factors may play a role in the pathogenesis of diabetic retinopathy (45). Furthermore, differential response to risk factors and treatments, ethnic differences and familial clustering strongly underlines the role of genetic factors in determining susceptibility to diabetic
retinopathy (45). An \((A_C)^n\) dinucleotide repeat polymorphism near the promoter region of aldose reductase 2 gene is associated with diabetic retinopathy in Chinese patients with type 2 diabetes (33). The authors found seven alleles, which were closely associated with an early onset of retinopathy (33). Another study in the Chinese population showed that the polymorphism \((WT/C (-12) G \text{ and } WT/C (-106) T)\) was strongly associated with diabetic retinopathy (34). Genome-Wide Association Studies have not been published in relation to diabetic retinopathy, although given past success of this approach in population based cohort settings, it may well provide a novel insight into genetic susceptibility to diabetic retinopathy (45).

1.1.10 Coronary heart disease

Coronary heart disease (CHD) rates have been shown to be high in several parts of the world, especially in people originating from the Indian subcontinent (46, 47). The earliest account of a higher prevalence of CHD in ethnic groups was presented in reports on Indians compared with other ethnic groups in Singapore in 1957. A higher prevalence was demonstrated at autopsy when CHD was defined by pathological evidence of coronary artery disease and myocardial involvement (47, 48). In a subsequent study in Fiji (49) CHD was found to be more prevalent in the Indian compared to Melanesian populations (49). In the UK, higher rates of CHD were first observed in 1971 and were predominantly reported in national data in Wales and England based on a population of Bangladeshi origin. Age standardised CHD mortality was 40% higher in both sexes of people of South Asians descent compared to Caucasians (1). South Asians were also younger at the time of their first myocardial infarction, had larger myocardial infarctions and more severe coronary artery disease at angiography, with a greater prevalence of subsequent heart failure (50-52). A retrospective sequential chart review of a South Asian and (non-South Asian) white population in Canada hospitalised with a primary diagnosis of congestive heart failure between 1997 and 1999 showed that South Asians were significantly younger, with a lower body mass index, more often diabetic, and less often smokers (51). Another study showed that the mortality rates from coronary artery disease in South Asians were two to three times higher than in Caucasians, irrespective of gender, religion, social class, dietary practices or country of residence (53, 54). Immigrants from other developing countries had lower SMRs (standardised
mortality ratios) for coronary heart disease. The explanation for the high CHD mortality in South Asians in both sexes from an early age suggests that there may be a common underlying mechanism (47) as the excess risk of CHD cannot be explained entirely by conventional risk factors (2, 47), despite the fact that such risk factors are more prevalent in South Asians (55). Thus South Asians have an increased risk of type 2 diabetes (1), insulin resistance, hyperinsulinaemia, hypertension and low plasma HDL cholesterol. Insulin resistance is known to underlie the risk of coronary artery disease, possibly through lipid-mediated and inflammatory mechanisms (53, 56). There is evidence to suggest that the presence of metabolic syndrome (MetS) predicts the future risk for T2D and coronary artery disease (CAD). MetS is a common and complex disorder combining obesity, dyslipidaemia, hypertension, and insulin resistance. There are studies which have shown that metabolic syndrome is associated with Apo A-I (Apolipoprotein A-I) polymorphism (57, 58). HDL has antioxidant, anti-inflammatory, and antithrombotic properties that contribute to its function as an anti-atherogenic agent. Recent studies in Caucasians have shown that HDL is not only ineffective as an antioxidant (54) but, paradoxically, appears to be pro-oxidant, and has been found to be associated with CAD. Several hypotheses have been forwarded for HDL becoming dysfunctional including Apolipoprotein A-I (Apo A-I) polymorphisms (54).

An association between (Adiponectin) ADIPOQ gene polymorphisms and CAD risk has been reported recently by Yang Y et al (59). Different SNPs of the ADIPOQ gene have been shown to be related to ethnicity and CAD risk. More specifically it has been discovered that different SNPs increase the risk in Asian populations and decrease the CAD risk in Caucasian populations (59). An association of UCP3 gene variant has also been observed with increased serum total and LDL-cholesterol levels, at baseline and during the 7 years follow-up period compared with those carrying the more common alleles.(60).

1.2 Metabolic syndrome

It is clear that individuals with metabolic syndrome (MetS) are at increased risk for the development of CHD and diabetes in particular (61). Metabolic syndrome consists of metabolic factors which are associated with a two-fold increased risk of
cardiovascular disease (CVD) and five-fold increase in the risk of diabetes (if not already present) within 5 years (62). Large variations exist in the prevalence of the MetS across countries and regions, ethnic groups, and gender. The prevalence of MetS is high and increasing, particularly in North and South American countries. The high prevalence, combined with a large number of people at risk for developing cardiovascular disease, T2DM, and other related disorders, suggests that the MetS may present a major worldwide public health challenge in the future (63). Five organisations have proposed definitions of the syndrome. Despite differences in specific criteria among the definitions, there is an agreement that the major characteristics of the syndrome include central obesity (except in one definition), elevated blood pressure, dyslipidaemia, and impaired glucose metabolism or insulin resistance. The NCEP ATP III definition of MetS, which is generally used in the US and Europe, considerably underestimates MetS in the South Asian population (64). Thus in the Chennai Urban Rural Epidemiology Study (CURES-34) the prevalence of MetS was estimated to be 25.8%, 23.2%, and 18.3% according to the International Diabetes Federation (IDF), World Health Organization (WHO), and NCEP ATP III definitions, respectively (65). There is an increasing belief that the definition of the metabolic syndrome by NCEP ATP III is not optimal for the identification of risks for T2DM or CHD, and does not identify the metabolic syndrome correctly in South Asians because the cut-off points of waist circumference (men >102 cm, and women, >88 cm) for diagnosis of abdominal obesity are not applicable to South Asians (66). The International Diabetes Federation’s (IDF) definition for MetS, which is ethnicity-specific, represents the best diagnostic tool for the identification of MetS in the South Asian population. Waist circumference (WC) cut-points are lower at >90 cm for men and >80 cm for women. Studies have shown that the optimal BMI for South Asians should be between 18.5 and 23 kg/m² (66). In a sample of 253 men and women aged between 35 and 69 years from general practice in the UK., mean plasma cholesterol concentrations and mean systolic blood pressures (10 mmHg) were lower in Bangladeshi than in European men and women; however, plasma fibrinogen concentrations were similar in both groups (67). Several studies have shown that insulin resistance is highly prevalent and occurs at an earlier age in South Asians than Caucasians (68-70).
1.3 Impaired glucose tolerance

There is evidence suggesting that Impaired Glucose Tolerance (IGT) causes neuropathy (71-73). Rates of IGT neuropathy (IGTN) are reported to vary between 8 and 30% although these figures remain controversial and require further research (74, 75). The neuropathy associated with IGT is milder than the neuropathy associated with diabetes mellitus (DM) (76). Impaired glucose tolerance was first shown to damage large fibres only (77); however, there is also evidence of impairment of small nerve fibres in IGT (76, 78). Injury to small nerve fibres results in pain and autonomic dysfunction (71). Although hyperglycaemia was at first proposed as a cause of neuropathy (78), the extent to which high glucose levels directly induce nerve injury as opposed to being a mere covariant with other equally or more important features (e.g., obesity, metabolic syndrome) is not clear (71).

1.4 Peripheral arterial disease

Peripheral arterial disease (PAD) in the legs, sometimes known as peripheral vascular disease, is caused by atheroma in the walls of the arteries, leading to insufficient blood flow to the muscles and other tissues. PAD causing arterial insufficiency is an important predictor of the outcome of ulceration of the foot in patients with diabetes (79). The prevalence of PAD in patients with DM has been estimated to be between 20% and 30% higher than in any other matched population (80, 81) and up to 38% in one cohort (82). The risk of developing PAD correlates with the duration and severity of DM (83). Patients with DM have symptomatic PAD more often than non-diabetic patients. In the Framingham cohort, the presence of DM increased the risk of claudication (a 3.5-fold increased risk in men and a 8.6-fold increased risk in women) (84). Diabetes mellitus also alters the distribution of the disease, and PAD in patients with DM involves the arteries below the knee more often than in non-diabetic patients (85). Finally, diabetic patients with PAD are more likely to present with tissue loss and are at a higher risk for amputation compared to patients without DM (86, 87).

Interestingly, there is an emerging body of evidence suggesting that the genetic background of an individual may play a significant role in the pathogenesis of PAD. In one study the prevalence of PAD was higher in African Americans even after
adjusting for age and other traditional risk factors for PAD. African Americans are at almost 50% increased risk of developing PAD compared to non-Hispanic white individuals and this difference is not explained by differences in conventional risk factors (88). On the other hand, Hispanic and Chinese individuals show a 50% reduced risk of developing PAD compared to non-Hispanic whites (89). Furthermore, PAD is lower in South Asian diabetic patients compared with African Caribbean and European patients (1, 90).

Diabetes causes significant pathological changes in major arteries, arterioles and capillaries (91). Peripheral arterial disease is a major manifestation of atherosclerosis characterised by atherosclerotic occlusive disease of the lower extremities (80). Endothelial dysfunction is now considered to be an early factor in atherogenesis (92) and it has also been recognised in the development of microvascular complications in diabetes (93). Typically, patients with peripheral arterial disease show symptoms like leg fatigue, pain on walking a short distance, but less than 20% of patients show intermittent claudication (94). Hyperglycaemia damages endothelial cells through at least three independent biochemical pathways: glucose-induced activation of protein kinase C (95), increased formation of advanced glycation end-products (96) and increased glucose flux through the aldose reductase pathway (97). The relevance of each of these pathways is supported by animal studies in which pathway-specific inhibitors prevent various hyperglycaemia-induced abnormalities (96, 98, 99).

PAD may be associated with diabetes and ulceration in approximately 30% of patients (100) and is a major determinant of ulcer healing. The incidence of foot ulceration varies from 5 to 7% in diabetic patients with neuropathy (101, 102), and 85% of amputations occur in patients with foot ulceration (103). Foot ulceration is also linked to higher cardiovascular mortality (104, 105). PAD is an important factor for morbidity and mortality particularly in elderly people (106-108). The prevalence of PAD is varied in different ethnic groups Table 1 (109). Recent studies have shown that Asian and Afro-Caribbean patients tend to have less foot ulceration and, correspondingly, fewer subsequent amputations compared to Europeans (110, 111). A difference in the rates of foot ulceration and amputation between black and white
American and Hispanic and non-Hispanic Americans has also been reported (112, 113).

Table 1. Prevalence of PAD in various studies

<table>
<thead>
<tr>
<th>Reference</th>
<th>Age (years)</th>
<th>Category</th>
<th>Prevalence of PAD (%)</th>
<th>Diagnostic criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beach (114)</td>
<td>50-70</td>
<td>Diabetes</td>
<td>22.0</td>
<td>ABI&lt;0.95</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NGT</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Fowkes (115)</td>
<td>55-74</td>
<td>General Population</td>
<td>18</td>
<td>ABI&lt;0.9 and/or intermittent claudication</td>
</tr>
<tr>
<td>Katsilambros (116)</td>
<td>All age</td>
<td>Diabetes</td>
<td>42</td>
<td>ABI&lt;0.9 and/or intermittent Claudication</td>
</tr>
<tr>
<td></td>
<td>groups</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beks (117)</td>
<td>50-74</td>
<td>NGT</td>
<td>7</td>
<td>ABI&lt;0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IGT</td>
<td>9.5</td>
<td></td>
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<td></td>
<td></td>
<td>NDD</td>
<td>15.1</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>KD</td>
<td>20.9</td>
<td></td>
</tr>
<tr>
<td>Premalatha (118)</td>
<td>≥ 20</td>
<td>NGT</td>
<td>3.5</td>
<td>ABI&lt;0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IGT</td>
<td>2.9</td>
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<tr>
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<td>NDD</td>
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<td>KD</td>
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<td></td>
<td>&gt;50</td>
<td>NGT</td>
<td>6.7</td>
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<td>IGT</td>
<td>10.0</td>
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<td>NDD</td>
<td>6.7</td>
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<td></td>
<td></td>
<td>KD</td>
<td>9.1</td>
<td></td>
</tr>
<tr>
<td>Adler (119)</td>
<td>25-65</td>
<td>NDD</td>
<td>1.2</td>
<td>ABI &lt;0.8 or absence of both dorsalis pedis and at least one posterior tibialis pulse or claudication.</td>
</tr>
<tr>
<td>AlZahrani (120)</td>
<td>50-80</td>
<td>Diabetes</td>
<td>61.4</td>
<td>ABI ≤0.9</td>
</tr>
</tbody>
</table>

Known diabetes (KD), newly diagnosed diabetes (NDD), Normal Glucose Tolerance (NGT), Impaired Glucose Tolerance (IGT), Ankle Brachial Index (ABI)

A variety of risk factors are associated with PAD and they are presented in Table 2.
### Table 2. Association of risk factors with PAD.

<table>
<thead>
<tr>
<th>Risk factors</th>
<th>Studied by</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypertension, diabetes, elevated serum cholesterol, LDL, Triglycerides, Fibrinogen, and hyperglycaemia</td>
<td>Cheng (121)</td>
</tr>
<tr>
<td>Diabetes</td>
<td>Papademetriou (122)</td>
</tr>
<tr>
<td>Serum Triglycerides</td>
<td>Katsilambros (116)</td>
</tr>
<tr>
<td>Triglyceride, low HDL, cholesterol, hypertension and smoking</td>
<td>Beach (114)</td>
</tr>
<tr>
<td>Microalbuminuria</td>
<td>Jager (123)</td>
</tr>
<tr>
<td>Homocysteine</td>
<td>Taylor (124)</td>
</tr>
<tr>
<td>Lipoprotein (a)</td>
<td>Wollesen (125)</td>
</tr>
</tbody>
</table>

Low density lipoprotein (LDL), High density lipoprotein (HDL)

### 1.5 Endothelial dysfunction in diabetes

Oxidative stress, induced by hyperglycaemia leads to early functional changes in the endothelial cell when no morphological changes can be detected. The changes are characterised by abnormal endothelium dependent and independent vasodilatory responses as well as changes in “cell-to-matrix and cell-to-cell interaction” (126, 127). Many studies suggest that an occlusive disease of the microcirculation doesn’t exist and the microcirculation (predominantly capillaries and arterioles) is impaired in the diabetic patients with increased vascular permeability and impaired autoregulation of blood and vascular tone (128). The initial changes are expressed as a microangiopathy (126), whereas advanced angiopathy which affects more proximal vessels is an outcome of hyperglycaemia and dyslipidaemia initiating pathology of the vessel wall (126).

### 1.6 Microangiopathy and diabetic neuropathy

Impaired nerve blood flow has been reported in diabetic neuropathy (129) and microvascular changes are known to occur in diabetic neuropathy (130). Type 2 diabetes is associated with reduced capillary blood flow due to endothelial-dependent and independent mechanism and this is related to the severity of neuropathy (131). Thickening of the capillary basement membrane is the hallmark of the diabetic microangiopathy (132) and skin capillary basement membrane thickness is increased with the severity of complications (133). Many studies have demonstrated a relationship between the severity of endoneurial microangiopathy and that of diabetic neuropathy (134-138).
Decreased nerve density and innervation of vessels can cause a decrease in endoneurial blood flow and ischaemia (139) assessed by laser Doppler (140). Structural changes such as thickening of the basement membrane also occur among diabetic patients and may result in capillary occlusion, although this has not been demonstrated morphometrically by evaluation of the capillary luminal area (141). Microangiopathy may decrease the elastic capacity of the vessel walls, limit vasodilator capacity, and also reduce exchange of nutrients and cellular migration, which decreases the ability to fight against infection (141).

Figure 4. Electron micrographs of dermal capillaries from a mildly neuropathic (left) and progressive thickening of basement membrane (BM)-red arrow- in severely neuropathic patient (right) (x 1400)

1.7 Structure of Arteries

1.7.1 Muscular arteries
Muscular arteries and their branches distribute blood to the organs and tissues of the body and are called distributing arteries. Contraction of the smooth muscle in their walls regulates the blood flow to the tissue and organs. The artery wall is comprised of three layers: tunica intima, tunica media and tunica adventitia from the lumen outwards, respectively.

1.7.2 Small arteries
Small arteries refer to arteries < 500 μm in diameter which are pre-arteriolar vessels and contribute actively to the pre-capillary resistance (142). Similarly to other arteries, the vascular wall of small arteries consists of three layers: the outer tunica
adventitia, a central tunica media, and the inner tunica intima (142). Despite the similarity in the structure in arterial microvessels, there are marked physiological differences between arterial microvessels greater than and smaller than 100-150 µm in inner diameter (143).

1.7.3 Arterioles
Arterioles are the small diameter blood vessels in the microcirculation that are characterised by sparse internal elastic lamina and larger muscle cells (usually one to two layers of smooth muscles). They branch out from an artery and empty into the capillaries which in turn empty into venules (144).

1.7.4 Capillaries
The capillaries are the smallest vessels in the circulation and serve a nutritional function (144). They are small, normally around 3-4µm, but some capillaries can be 30-40 µm in diameter. The largest capillaries are found in the liver. They are composed of a single layer of endothelium with an incomplete layer of pericytes (145).

Figure 5. Schematic view of capillary structure (146).

Section 4

1.8 Diabetic neuropathy
Diabetes mellitus (DM) is associated with several chronic complications, but without doubt neuropathy is the most common and troublesome (147-149), and likely to be
associated with considerable morbidity (painful neuropathy, neuropathic ulceration) and mortality (autonomic neuropathy), imposing a "huge economic burden for diabetes care" (150, 151). It is estimated that 50 to 75% of non-traumatic amputations are due to diabetic neuropathy (151, 152). Diabetic neuropathy leads to painful neuropathy (~20%), foot ulceration and amputation (23-fold increased relative risk in diabetic patients) (153). Furthermore, it has recently been shown to be an independent predictor for all-cause (hazard ratio = 4.4) and diabetes-related (hazard ratio = 11.82) mortality (154). Surprisingly, tight glycaemic control, a cornerstone for the management of diabetes, has been shown to limit progression of neuropathy in patients with Type 1 (155) but not in patients with Type 2 diabetes (156-159).

The diabetic neuropathies are a group of nerve disorders which increase with the duration of diabetes and poorer glycaemic control (160). A working definition of neuropathy is “the presence of symptoms and / or signs of peripheral nerve dysfunction in people with diabetes after the exclusion of other causes” (161). Although diabetic neuropathy develops in 60-90% of diabetic patients, symptoms only occur in 20-30% (162) and the condition can remain undiagnosed, particularly in its early stages (163). Good longitudinal studies in this condition are lacking; however, diabetic neuropathy has been reported to manifest itself in approximately 10% of patients within one year of diagnosis and the figure rises to more than 50% in patients with 25 years of diabetes (150, 164).

Given the high prevalence of diabetic peripheral neuropathy, surprisingly little is known about the distribution of symptom severity and its effect on healthcare cost (165). The annual cost of diabetic neuropathy has been estimated to be between 4.6 and 13.7 billion dollars in the US, which is approximately 27% of the overall direct medical cost of diabetes. However, non-medical and indirect costs (such as loss of productivity) are also likely to be substantial for people with DPN (166). In the UK, up to 17% of current NHS expenditure on diabetes can be attributed to the management of diabetic neuropathy (DN) and its complications (167). Interventions that successfully treat DN to prevent or delay its long-term outcomes such as foot ulceration and amputation have been shown to save substantial costs to health care payers (168). No similar cost effectiveness for neuropathy in diabetes has been
studied in the UK, however a study in the USA has estimated that the long-term complications of diabetic peripheral neuropathy (DPN) experienced by the population with established neuropathy (VPT>25) will cost approximately $14.7 billion over the next 10 years (169). As the cost of DPN is estimated to be 8 times greater in the USA than in the UK (169) it will cost approximately $2 billion in the UK. Therefore if individuals with early neuropathy could be identified, then preventative measures to prevent progression to more severe neuropathy could be implemented in these patients, potentially saving valuable resources and improving health outcomes. This is the only study that presented detailed estimates of the differences in healthcare treatment costs associated with increasing severity of diabetic neuropathy, taking into account confounding factors such as other diabetes-related complications. These models suggest that even subclinical neuropathic symptoms have significant healthcare cost implications not accounted for by covariance with other relevant predictors (168).

The cost of diabetic foot ulceration and amputations to the NHS each year is estimated to be around £650 million. Approximately £1 in every £150 the NHS spends is for diabetic foot ulceration and amputation (170).

Foot ulceration in diabetic patients has a lifetime incidence which may be as high as 25% (171). Once ulcers become infected, they can cause great morbidity, incurring considerable costs as a consequence of antibiotic use and prolonged in-patient stay (172). Therefore, foot ulceration poses a huge economic problem to the National Health Service, especially when it leads to amputation, which is 10-30 times more common in diabetic patients compared to the general population (172). The cost of care for diabetic patients with foot ulcers compared to diabetic patients without foot ulcers is 5.4 times higher in the year following the first ulcer episode and 2.8 times higher in the second year (173). Furthermore, costs for the treatment of the highest-grade ulcers are 8 times higher than for treating low-grade ulcers (173). Diabetic patients with foot ulcers require more frequent emergency department visits, are more commonly admitted to hospital, and require a longer stay in the hospital (173). Implementation of the team approach to manage diabetic patients with foot ulceration within a given region or health care system has been reported to reduce
long-term amputation rates from 82% to 62% (173). Limb salvage efforts may include aggressive therapy, such as revascularisation procedures and advanced wound healing modalities. However, these procedures are often not successful and tend to be extremely costly, hence improved screening and prevention programs with earlier interventions have been shown to be more effective in reducing long-term costs (173).

1.9 Definition
A practical definition of neuropathy as described earlier is “the presence of symptoms and/or signs of peripheral nerve dysfunction in people with diabetes after the exclusion of other causes” (161). Based on the Toronto expert committee paper, a different definition has been proposed for typical (diabetic sensorimotor polyneuropathy-DPNS) and atypical DPN. The typical DPN is chronic, symmetrical, length dependent and is thought to be the common variety (174) and develops on a background of hyperglycaemia (175). The atypical DPNs are different from DSPN in many characteristics such as “onset, course, manifestation, association and maybe putative mechanism” (175) and can develop at any time (175). Pain and autonomic symptoms are typical features; however, on the whole, atypical DPNs are less studied and their characterisation has not been fully developed (175, 176).

1.10 Pathophysiology of diabetic neuropathy
The pathophysiology of diabetic neuropathy is complex and multiple avenues of research have been explored to find the cause of diabetic neuropathy. The main mechanisms resulting in nerve damage are oxidative stress, advanced glycation end product (AGEs) formation, protein kinase C (PKC), and polyol pathway activation (177, 178). The polyol pathway was initially considered to play an important role in cataract formation in diabetic patients and only later it was found to potentially contribute to all the microvascular complications such as neuropathy, retinopathy, and nephropathy (178, 179). The role of PKC has recently also been subject to scientific scrutiny as PKC inhibitors have been shown to be effective in experimental diabetic neuropathy (97,177,180, 181). Increased activation of the diacylglycerol (DAG)-protein kinase C (PKC) signal transduction pathway has been identified in vascular tissues from diabetic animals, and in vascular cells exposed to elevated
glucose. Vascular abnormalities associated with glucose-induced PKC activation leading to increased synthesis of DAG include altered vascular blood flow, extracellular matrix deposition, basement membrane thickening, increased permeability and neovascularisation (182). AGEs form incessantly but at slow rates in the normal body. Their formation starts in early embryonic development and accumulates with time. However, their formation is markedly accelerated in diabetes because of the increased availability of glucose (183). A large body of evidence suggests that AGEs are important pathogenetic mediators of almost all diabetes complications (183). AGE formation leads to quenching of nitric oxide and impaired function of growth factors. During the past decade, many experimental studies have shown that these effects of AGE formation may play a role in the pathogenesis of micro- and macrovascular complications of diabetes, diabetic neuropathy and impaired wound healing. Moreover, in recent years several clinical studies have shown that glycation is an important pathway in the pathophysiology of those complications that predispose to the development of foot ulcers (184).

Aldose reductase catalyses the NADPH-dependent conversion of glucose to sorbitol, the first step in the polyol pathway. The pathway is completed by sorbitol dehydrogenase, which catalyses the NAD-linked oxidation of sorbitol to fructose. Thus, the polyol pathway results in conversion of glucose to fructose with stoichiometric utilisation of NADPH and production of NADH (185). All these pathways converge in the production of oxidative stress and in turn cause endothelial dysfunction, which reduces capillary blood flow leading to nerve dysfunction and damage (186). Pre-diabetes and metabolic syndrome are associated with increased oxidative stress (187). Both metabolic and vascular factors lead to direct neuronal damage and also result in vascular dysfunction which also drives nerve damage leading to diabetic neuropathy (188).
1.11 Classification of diabetic neuropathy

There are several different classifications for diabetic neuropathies based on the aetiology, anatomical or pathological features and clinical manifestations (189-191). Lyden et al, in 1893 proposed one of the earliest classifications of diabetic neuropathy and divided patients into three categories with hyperaesthetic (painful), paralytic (motor) and ataxic forms of neuropathy. In the same year Pryce classified diabetic neuropathy into sensory and patchy motor forms (189). In Table 3 and Table 4, two different classifications are presented (189, 190).
### Table 3. (a) Classification of the Diabetic Neuropathies based on clinical features

<table>
<thead>
<tr>
<th>Symmetry</th>
<th>Type of Neuropathy</th>
<th>Subtype</th>
</tr>
</thead>
<tbody>
<tr>
<td>Symmetrical</td>
<td>Diabetic polyneuropathy</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Diabetic autonomic neuropathy</td>
<td>Cardiovascular</td>
</tr>
<tr>
<td></td>
<td>Diabetic autonomic neuropathy</td>
<td>Gastrointestinal</td>
</tr>
<tr>
<td></td>
<td>Diabetic autonomic neuropathy</td>
<td>Genitourinary</td>
</tr>
<tr>
<td></td>
<td>Diabetic autonomic neuropathy</td>
<td>Other</td>
</tr>
<tr>
<td></td>
<td>Painful distal neuropathy with weight</td>
<td></td>
</tr>
<tr>
<td></td>
<td>loss, “diabetic cachexia”</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Insulin neuritis</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hypoglycaemic neuropathy</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Polyneuropathy after ketoacidosis</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Polyneuropathy with glucose impairment</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CIDP* in diabetes</td>
<td></td>
</tr>
<tr>
<td>Asymmetrical</td>
<td>Diabetic radiculoplexus neuropathies</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mononeuropathies</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Cranial neuropathies</td>
<td></td>
</tr>
</tbody>
</table>

**Classification of diabetic neuropathies based on anatomical features**

<table>
<thead>
<tr>
<th>Length-dependent diabetic polyneuropathy</th>
<th>-Distal symmetrical sensory polyneuropathy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-Large fibre neuropathy</td>
</tr>
<tr>
<td></td>
<td>-Painful symmetrical polyneuropathy</td>
</tr>
<tr>
<td></td>
<td>-Autonomic neuropathy</td>
</tr>
<tr>
<td>Focal and multifocal neuropathies</td>
<td>-Cranial neuropathies</td>
</tr>
<tr>
<td></td>
<td>-Proximal DN of the lower limbs</td>
</tr>
<tr>
<td></td>
<td>-Truncal neuropathies</td>
</tr>
</tbody>
</table>

*CChronic Inflammatory Demyelinating Polyneuropathy*

Diabetic neuropathy can be classified according to the system used in clinic: Sensorimotor Neuropathy, Autonomic Neuropathy and Focal Neuropathy (Table 4).
Diabetic Autonomic Neuropathy (DAN) is a serious and common complication of diabetes. Clinical manifestations of DAN include resting tachycardia, exercise intolerance, orthostatic hypotension, gastroparesis, erectile dysfunction, sudomotor dysfunction, impaired neurovascular function, brittle diabetes and hypoglycaemia unawareness. Cardiovascular autonomic neuropathy (CAN) is the most studied and clinically important form of DAN. The reduced cardiovascular autonomic function as measured by heart rate variability is strongly associated with a higher risk of silent myocardial ischemia and mortality (192). Since a consensus conference in 1992, three tests (R-R variation, Valsalva manoeuvre, and postural blood pressure testing) have been advocated for use in longitudinal testing of CAN (193).

It is important to exclude other, non-diabetes related conditions that may cause neuropathy in diabetic patients including alcoholism, HIV infection, vitamin deficiencies, monoclonal gammopathy, amyloidosis, vasculitic neuropathy and other inherited neuropathies such as Fabry disease and CMT (Table 5) (194).
### Table 5. Causes of small fibre neuropathy.

<table>
<thead>
<tr>
<th>Idiopathic</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Inherited</td>
<td>Familial amyloidosis</td>
</tr>
<tr>
<td></td>
<td>Autosomal recessive hereditary neuropathy</td>
</tr>
<tr>
<td></td>
<td>Hereditary sensory and autonomic neuropathy</td>
</tr>
<tr>
<td></td>
<td>Fabry disease</td>
</tr>
<tr>
<td></td>
<td>Ross syndrome</td>
</tr>
<tr>
<td></td>
<td>Friedreich's ataxia</td>
</tr>
<tr>
<td></td>
<td>Tangier disease</td>
</tr>
<tr>
<td>Acquired</td>
<td>Diabetes mellitus</td>
</tr>
<tr>
<td></td>
<td>Impaired glucose tolerance</td>
</tr>
<tr>
<td></td>
<td>Alcoholism</td>
</tr>
<tr>
<td></td>
<td>Systemic amyloidosis</td>
</tr>
<tr>
<td></td>
<td>Vasculitis</td>
</tr>
<tr>
<td></td>
<td>Sarcoidosis</td>
</tr>
<tr>
<td></td>
<td>Sjögren's disease</td>
</tr>
<tr>
<td></td>
<td>Systemic lupus erythematosus</td>
</tr>
<tr>
<td></td>
<td>Guillain-Barre syndrome</td>
</tr>
<tr>
<td></td>
<td>Antecedent viral infection</td>
</tr>
<tr>
<td></td>
<td>HIV</td>
</tr>
<tr>
<td></td>
<td>Antisulfatide antibodies</td>
</tr>
<tr>
<td></td>
<td>Hyperlipidemia</td>
</tr>
<tr>
<td></td>
<td>Complex regional pain syndrome</td>
</tr>
<tr>
<td></td>
<td>Paraneoplastic syndrome</td>
</tr>
<tr>
<td></td>
<td>Neurotoxic medication</td>
</tr>
</tbody>
</table>

#### 1.12 Clinical features and symptoms of diabetic neuropathy

Symptoms of diabetic neuropathy can be varied depending on which nerves are affected. Numbness, tingling, or pain in the feet appears early and is often symmetrical due to involvement of the sensory nerves, but later autonomic and motor nerves are also involved. The pain of diabetic neuropathy is most severe at
night and deprives patients of good sleep (195). Involvement of the motor system is not frequently seen at clinical examination, however, with the application of quantitative techniques, such as isokinetic dynamometry, both type 1 and type 2 diabetic patients exhibit weakness at the ankle and the knee (196). Muscle weakness is found only in diabetic patients with peripheral neuropathy, while non-neuropathic patients even with long-term diabetes have normal strength (196), therefore weakness is closely related to the severity of peripheral neuropathy. Furthermore, motor dysfunction may lead to an increased risk of developing a foot ulcer due to an alteration of the biomechanics of the feet caused by muscle atrophy (197) which may lead to foot ulceration and, ultimately to amputation (196). Small muscle wasting in the feet is believed to result in altered biomechanical properties of the foot that, together with the loss of sensation, dry skin, callus formation, and limited joint mobility, result in increased foot pressures and thereby increased risk of developing neuropathic foot ulcers (103). Limited joint mobility may further contribute to increased focal points of high pressure due to changes in connective tissue. Motor dysfunction can be detected as muscle weakness and may be accompanied by muscle atrophy in the lower extremities (196). This motor impairment is believed to be caused by diabetic neuropathy (DN) and is only seen in more advanced DN (160). Motor dysfunction occurs in 1-6% of diabetic patients, particularly in those with type 1 diabetes (160).
1.13 Diagnosis of Diabetic Neuropathy

Several different approaches have been employed to diagnose and evaluate the severity of neuropathic deficits and painful symptoms in diabetic neuropathy. To understand better the role of different tests, it is useful to briefly review the structure and function of the different types of nerves in the peripheral nervous system. Peripheral nerve fibres can be classified in terms of their size and the presence or absence of the myelin sheath into large (diameter 6-22 µm), medium and small fibres (1-5 µm) (198, 199). In the somatic nervous system (SNS), skeletal muscle control is mediated through large A-alpha (Aα) myelinated fibres. The sensations of touch, vibration and joint position sense are conducted via large myelinated Aα or Aβ fibres. Medium size myelinated A-gamma (Aγ) fibres carry motor function to muscle spindles. Cold perception and pain are carried along small thinly myelinated Aδ fibres, whilst warm sensation is conveyed by small unmyelinated C fibres (diameter 0.2-1.5 µm). Small fibres mediate sensation of temperature and pain and autonomic function such as sweating and heart rate variability. In contrast to the Sensory Nervous System (SNS), the Autonomic Nervous System (ANS) primarily functions by

Table 6. Symptoms associated with small-fibre neuropathy in diabetic neuropathy.

<table>
<thead>
<tr>
<th>Sensory symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pain</td>
</tr>
<tr>
<td>Paraesthesiae</td>
</tr>
<tr>
<td>Allodynia</td>
</tr>
<tr>
<td>Hyperalgesia</td>
</tr>
<tr>
<td>Mild impairment of vibration sense</td>
</tr>
<tr>
<td>Reduced thermal sensation</td>
</tr>
<tr>
<td>Loss of pinprick sensation</td>
</tr>
<tr>
<td>Sheet/sock intolerance</td>
</tr>
<tr>
<td>Restless legs syndrome</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Autonomic symptoms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increased or decreased sweating</td>
</tr>
<tr>
<td>Facial flushing</td>
</tr>
<tr>
<td>Skin discolouration</td>
</tr>
<tr>
<td>Dry eye symptoms (Sicca syndrome)</td>
</tr>
<tr>
<td>Sexual dysfunction</td>
</tr>
<tr>
<td>Gastrointestinal dysfunction</td>
</tr>
<tr>
<td>Bladder dysfunction (incontinence or retention)</td>
</tr>
<tr>
<td>Blurry vision due to difficulties in accommodation</td>
</tr>
</tbody>
</table>

...
conducting along thinly myelinated (preganglionic) or unmyelinated (postganglionic) neurones (199-201). A summary of nerve fibre types is presented in Table 7.

Table 7. The type size and function of different nerve fibres in the human body.

<table>
<thead>
<tr>
<th>Fibre type</th>
<th>Myelin</th>
<th>Function</th>
<th>Diameters(µm)</th>
<th>CV(m/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha(α)</td>
<td>Yes</td>
<td>Proprioception, somatomotor</td>
<td>12-22</td>
<td>70-120</td>
</tr>
<tr>
<td>Beta(β)</td>
<td>Yes</td>
<td>Touch, pressure</td>
<td>5-12</td>
<td>30-70</td>
</tr>
<tr>
<td>Gamma(γ)</td>
<td>Yes</td>
<td>Motor to muscle spindle</td>
<td>3-6</td>
<td>15-30</td>
</tr>
<tr>
<td>Delta(δ)</td>
<td>Thin</td>
<td>Pain, cold, touch</td>
<td>2-5</td>
<td>12-30</td>
</tr>
<tr>
<td>B</td>
<td>yes</td>
<td>Preganglionic autonomic</td>
<td>&lt;3</td>
<td>3-15</td>
</tr>
<tr>
<td>C</td>
<td>No</td>
<td>Thermal, mechanoreceptor</td>
<td>0.4-1.2</td>
<td>0.5-2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Postganglionic autonomic</td>
<td>0.3-1.3</td>
<td>0.7-2.3</td>
</tr>
</tbody>
</table>

1.1 Assessment of severity of neuropathic pain

The accurate assessment of the severity of painful symptoms in patients with diabetic neuropathy is very important, not just for the diagnosis but also to assess the benefits of treatment, especially with the potential for a large placebo effect as discussed elsewhere in this thesis. Many different questionnaires and scores have been developed or adopted to quantify neuropathic pain.

- McGill pain questionnaire
- Neuropathy Symptom Profile (NSP)
- Brief Pain Inventory for Diabetic Peripheral Neuropathy (BPI DPN)
- Neuropathic Pain Questionnaire (NPQ)
- Douleur Neuropathique Pain diagnostic questionnaire (DN4)
- Neuropathic Pain Symptom Inventory (NPSI)

The McGill Pain Questionnaire is the most frequently used questionnaire, but it was not developed originally for diabetic neuropathic pain. Recently, more specific scores have been developed for diabetic painful neuropathy and include the Brief pain inventory short form for diabetic peripheral neuropathy (BPI-PDN) (202). The BPI is a patient-completed numeric rating scale that assesses the severity of pain and its impact on daily functioning on a 7-item Pain Interference scale. The Neuropathic Pain Questionnaire (NPQ) was developed to provide a general assessment of neuropathic pain and discriminate between neuropathic and non-neuropathic pain (203). An additional diagnostic tool the pain diagnostic questionnaire (DN4) has been shown to distinguish neuropathic from nociceptive pain (204). Follow-up assessment
of pain in PDN can be undertaken using either the NPQ or the other recently
developed tool the Neuropathic Pain Symptom Inventory (NPSI), which is a self-
questionnaire designed to evaluate different symptoms of neuropathic pain (205). The NPSI includes ten descriptors that allow for the discrimination and quantification of clinically relevant aspects of neuropathic pain. It has been suggested that this pain questionnaire may be able to characterize subgroups of patients with neuropathic pain, and verify differential responses to pharmacologic or other treatment interventions. Finally, the Neuropathic Pain Scale has been designed specifically to monitor effects of therapy on neuropathic pain (137). A summary of the advantages and limitations of the most common methods of neuropathy assessment is presented in Table 8.

<table>
<thead>
<tr>
<th>Method</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical neurological examination</td>
<td>Simple, easy to use</td>
<td>Limited sensitivity &amp; repeatability</td>
</tr>
<tr>
<td>Nerve conduction studies</td>
<td>Sensitive, objective</td>
<td>Only large fibres assessed and need special equipment</td>
</tr>
<tr>
<td>Quantitative sensory tests(QST)</td>
<td>Evaluate both large and small fibres, reproducible</td>
<td>Subjective, need special equipment</td>
</tr>
<tr>
<td>Sympathetic skin response(SSR)</td>
<td>Simple, fast, objective</td>
<td>Semi-quantitative, low sensitivity, does not correlate with clinical or other features of small-fibre neuropathy</td>
</tr>
<tr>
<td>QSART</td>
<td>Sensitive, objective, reproducible, quantitative</td>
<td>Special equipment, time consuming</td>
</tr>
<tr>
<td>TST</td>
<td>Simple, sensitive, recognises pattern of anhidrosis</td>
<td>Unable to distinguish between post and preganglionic &amp; central lesions, discomfort, low specificity.</td>
</tr>
<tr>
<td>Skin biopsy</td>
<td>Quantitative, sensitive, specific</td>
<td>Invasive, histological technique, requires expertise for interpretation</td>
</tr>
<tr>
<td>Nerve biopsy</td>
<td>Useful in neuropathy with unclear aetiology, allows detailed morphometry</td>
<td>Invasive, painful, permanent nerve damage, discomfort after biopsy</td>
</tr>
<tr>
<td>CCM</td>
<td>Rapid, non-invasive, reiterative</td>
<td>Requires special equipment</td>
</tr>
<tr>
<td>Sudoscan</td>
<td>Simple, easy to use, sensitive, reproducible</td>
<td>Requires special equipment, not a substitute for conventional neuropathy testing</td>
</tr>
</tbody>
</table>
1.2 Nerve conduction studies
Nerve conduction velocity (NCV) is a key measure for the evaluation of large fibre neuropathies (206) but it is also important to consider a number of whole nerve electrophysiological parameters (e.g. NCV, F-waves, sensory and motor amplitudes) in patients who have normal nerve conduction velocity (207). Nerve conduction studies and electromyographic studies of large myelinated fibres are usually undertaken in their distal distribution. Dysfunction of or damage to small myelinated or unmyelinated fibres or location in areas inaccessible to conventional study methods require alternative assessment techniques. Indeed, a previous study showed that people of South Asian origin had a higher mean value for all motor and sensory NCVs and amplitudes compared with Europeans (3).

1.3 Quantitative Sensory Testing
1.3.1 Thermal thresholds
Abnormalities in heat pain thresholds reflect small fibre dysfunction and a number of instruments including CASE IV, Thermo-aesthesiometer and Medoc instruments have been used to quantify this parameter. In 498 type 2 diabetic patients and 434 control subjects an elevated warm threshold was the most frequent abnormality (60.2%) compared to an abnormal cold threshold (39.6%) and abnormal sural nerve conduction velocity (12.9%), and it was related to both symptoms and glycaemic control (208). However, a careful study of 59 diabetic patients with and without symptomatic neuropathy showed that unlike cold perception thresholds and Intra epidermal nerve fibre density (IENFD), warm perception thresholds did not differentiate diabetic patients with and without symptoms (209). Similarly, in a study of 191 diabetic patients there was no difference in heat pain thresholds between those with and without painful neuropathy (210).

1.4 Pain related evoked potentials
In a study of 57 diabetic patients with entirely normal electrophysiology, the latency was increased and amplitude was reduced in diabetic patients compared to healthy subjects for pain-related evoked potentials (PREP’s), elicited by nociceptive electrical stimulation of the skin here (211).
1.5 Nerve axon reflex/flare response

Stimulation of the nociceptive C fiber results in both orthodromic conduction to the spinal cord and antidromic conduction to other axon branches i.e. the axon reflex which can stimulate the release of peptides, such as substance P and calcitonin gene related peptide, resulting in vasodilation and increased permeability. Studies have shown that this neurovascular response mediated by the nerve axon reflex is reduced in diabetic neuropathic patients, correlates with other nerve function measurements and has reasonable sensitivity and specificity in identifying patients with diabetic neuropathy (127, 212). The Laser Doppler imaging (LDI) flare test evaluates 44°C heat-induced vasodilation (213) and is reduced in subjects with IGT (214), and type 2 diabetic patients with and without neuropathy (215, 216) but interestingly is normal in patients with type 1 diabetes of long duration (214). More longitudinal data and perhaps assessment after interventions when compared with established tests are necessary before these techniques can be recommended for clinical use.

1.6 Nerve biopsy

Nerve biopsy has traditionally been used to quantify myelinated nerve fibre density which is reduced and correlates with abnormalities in neurophysiology (217, 218) but may also predict development of future neurophysiological deficits (219). Few studies have quantified unmyelinated nerve fibre damage, but those that have shown that it precedes myelinated nerve fibre damage in sural nerve biopsies and therefore may be used to detect early DSPN (137). However, nerve biopsy is an invasive and highly specialized procedure which requires neurosurgical expertise to identify and perform, especially when a fascicular biopsy is required. Furthermore, electron-microscopy demands considerable expertise and there are very few centers which can perform quantification. It therefore cannot be advocated for diagnosing DSPN (220).

1.7 Skin biopsy

Skin biopsy, a minimally invasive procedure, allows morphometric quantification of intraepidermal nerve fibers (IENF) most commonly expressed as the number of IENF per length of section (IENF/mm) (221, 222). Intra- and inter-observer variability for
the assessment of IENF density demonstrates good agreement (222, 223), declines with age and does not appear to be influenced by weight or height (224). An international consortium of investigators has recently compiled a normative data base for IENFD in 550 participants and shown an effect of age, but no influence of height, weight or BMI (225). The blister technique is an alternative less invasive procedure which assesses innervation of the epidermis alone and shows good agreement with punch biopsy (226).

1.7.1 Diagnostic yield of IENF quantification

No study assessing the sensitivity and specificity of Intra epidermal nerve fibre density (IENFD) in patients with Diabetic Sensory Polyneuropathy (DSPN) is available. However, several studies in patients with Small Fibre Neuropathy (SFN) have included patients with DSPN. In 58 patients with pure SFN, a cut-off IENFD of \( \leq 8.8/\text{mm} \) at the ankle was associated with a sensitivity of 77.2% and a specificity of 79.6% (227). Similarly, in 67 patients with pure SFN, a sensitivity of 88% and a specificity of 88.8% have been reported (228). In a study of 210 patients with SFN, which included 65 diabetic patients, a specificity of 64%, and sensitivity of 78% has been reported for IENFD with a cut-off point of 10.3 fibers/mm (229). These findings suggest that the diagnostic yield of skin biopsy may depend on the reference and cut-off values selected and the definition of SFN adopted. IENFD correlates inversely with thermal thresholds. Whilst some have reported a closer correlation with warm and heat-pain thresholds (227, 230-232) compared to cooling thresholds (233, 234), others have reported the opposite, with a closer correlation with cold rather than heat detection thresholds (210, 235). A recent study has demonstrated no correlation between IENFD and the neuropathy symptom score, but interestingly an inverse correlation was demonstrated with the severity of pain assessed using the visual analogue score (VAS) (236). The correlation between quantitative sensory testing and IENF density therefore remains controversial.

The American Academy of Neurology, American Association of Neuromuscular and Electro diagnostic Medicine, and American Academy of Physical Medicine and Rehabilitation have concluded however that skin biopsy may be considered for the diagnosis of DSPN, particularly SFN, with a level C recommendation (237).
recently, under the auspices of the European Federation of the Neurological Societies and the Peripheral Nerve Society, revised guidelines on the use of skin biopsy concluded that IENF density is a reliable and efficient technique to confirm the clinical diagnosis of SFN with level A recommendation (238). Additional morphological features of IENFD include the branch density, length and mean dendritic length; all show an early reduction which progresses with neuropathic severity (239, 240). Several studies with serial skin biopsies in patients with small fibre neuropathy (SFN) have shown that axonal swellings predict a decline in IENF density (241-243). However, they occur not only in patients with SFN (244) but also in normal individuals (245) and isolated swellings with normal IENF densities have been observed in a variety of other neuropathies (245-248).

1.8 Non-contact corneal aesthesiometry

Evaluation of corneal sensitivity using the Cochet-Bonnet aesthesiometer (CB-A) represents a relatively quick clinical assessment technique, and has shown that corneal sensitivity correlates with the severity of diabetic neuropathy (249). Similarly, corneal sensitivity assessed using a non-contact corneal aesthesiometer (NCCA) was also significantly reduced in diabetic patients with minimal and more advanced diabetic neuropathy measured by the neuropathy disability score (NDS), compared with control subjects and there was a significant correlation between neuropathic severity and both NCCA and C-BA (249). Another recent study has confirmed that NCCA is a sensitive test for the diagnosis of minimal and more advanced diabetic neuropathy and concluded that it may serve as a useful surrogate marker for diabetic and other neuropathies (250).

1.9 Corneal confocal microscopy

The cornea is the most densely innervated tissue of the human body which contains both small myelinated Aδ and unmyelinated C fibres. Corneal confocal microscopy (CCM) is a non-invasive ophthalmic technique that has been shown to detect small sensory corneal nerve fiber loss in diabetic neuropathy (251), idiopathic small fibre neuropathy (252), and Fabry disease, a condition which characterized by painful neuropathy (253) by visualizing the sub basal nerve plexus in the sub-basal layer of the cornea. Corneal nerve fiber damage correlates with IENF loss and severity of
neuropathy in diabetic patients (239, 254) and is more marked in patients with painful diabetic neuropathy (239). There is also a correlation between loss of corneal nerve fibres and stage of diabetic retinopathy (255). Corneal confocal microscopy may also be more sensitive than IENFD in detecting early damage (239) and repair after simultaneous pancreas-kidney (SPK) transplantation (256, 257). Corneal nerve fiber density improves six months after combined pancreas/kidney transplantation (257). CCM has been shown to have high reproducibility (258), with reasonable sensitivity and specificity (259) for diabetic neuropathy. To enhance the practical application of this technique an automated image analysis system has also been developed recently to rapidly quantify corneal nerve pathology (260). A progressive loss of corneal sensation with increasing severity of neuropathy provides a functional correlate of corneal nerve fibre loss in diabetic patients (249, 250, 261). Therefore as CCM in non-invasive, it may be an ideal technique to assess alterations in small nerve fibre pathology in relation to PDN and progression or regression of neuropathic deficits.

1.10 Sudomotor dysfunction

Sudomotor deficits occur due to postganglionic cholinergic denervation and whilst symptoms are common they do not usually command much attention. Hyperhidrosis of the feet can be associated with coldness and can be followed by anhidrosis and vasomotor alterations. The most commonly used tests to evaluate sudomotor function are:

- Sympathetic skin response (SSR)
- Quantitative Sudomotor Axon Reflex Test (QSART)
- Thermoregulatory Sweat test (TST)
- Neuropad

1.10.1 Sympathetic skin response

Sympathetic skin response (SSR) assesses sudomotor and hence small fibre dysfunction. In an early study it failed to differentiate the presence or absence of neuropathy in a series of 337 diabetic patients (262). However, it has recently been shown to predict the risk of foot ulceration comparable with abnormalities in NDS and elevated vibration perception (263). It has also been shown to have a sensitivity
of 87.5% and a specificity of 88.2% for detecting diabetic autonomic neuropathy (264).

1.10.2 Quantitative sudomotor axon reflex
Quantitative Sudomotor Axon Reflex Testing (QSART) evaluates sudomotor function by assessing the local sweat response to iontophoresis of acetylcholine (265) and has been shown to be highly sensitive in the detection of distal SFN (266). QSART evaluates postganglionic axon function as opposed to the polysynaptic pathways assessed using SSR. In a series of 31 diabetic patients with early neuropathy it appeared to be better at detecting early neuropathy than SSR (267).

1.10.3 Thermoregulatory sweat test
Thermoregulatory Sweat Test (TST) is a sensitive qualitative test of sudomotor function that provides important information on the pattern and distribution of sweat loss. The presence of sweating causes a change in the indicator from brown to a violet colour whilst the subject is heated in a sweat cabinet (268). The TST provides reliable information about the distribution of neuropathic involvement and can be especially useful in the diagnosis of truncal radiculopathy and clinically significant autonomic neuropathy (268).

1.10.4 Neuropad
Neuropad is a simple visual indicator, and is semi-quantitative test for sudomotor function which uses a colour change to define the integrity of skin sympathetic cholinergic innervation. Neuropad responses have been shown to correlate with the modified NDS, QST, CAN and IENF loss and also show a relatively high sensitivity but lower specificity for detecting DSPN (269, 270). A recent study has shown that an abnormal Neuropad test in those with a normal NDS may predict the development of diabetic neuropathy after 5 years (271). This appears to reflect early small fibre involvement which is missed using NDS as a measure of neuropathy.

1.11 Sudomotor innervation
Recently, a novel stereologic technique has been applied in skin biopsies and showed a correlation between sweat gland nerve fibre density, neuropathic symptoms, neurological deficits and sweat production (272). However, morphometric
data in patients with diabetic SFN are limited and further studies are warranted.

1.11.1 Sudoscan
Sudomotor dysfunction is associated with increased risk of foot ulceration. Additionally, it has been shown that Cardiac autonomic Neuropathy (CAN) and the severity of peripheral neuropathy are also associated with foot ulceration (273). Sudoscan evaluates sudomotor function by measuring electrochemical skin conductance (ESC) resulting from an electrochemical reaction between sweat chloride and nickel electrodes after low DC (direct current) stimulation (274). Sweat glands are innervated by thin myelinated and unmyelinated sympathetic C fibres that may be affected early in the course of diabetes, as demonstrated in patients with pre-diabetes (274). It has good sensitivity (80%), specificity (95%) and reproducibility, which makes it a feasible alternative for assessing sudomotor dysfunction and autonomic neuropathy in diabetic patients. The test is quick and does not require special patient preparation and medical personnel training (275). Lower ESC on the feet was significantly associated with both increasing symptoms and physical abnormalities. Lower ESC at the feet was also shown to strongly correlate with increasing VPT and with a higher number of abnormal CAN results (274). Sudoscan can be used as a simple non-invasive test for screening diabetic neuropathy and sympathetic CAN along with conventional tests. It is not a substitute for conventional neuropathy testing but can alert the physician to perform more careful testing for autonomic neuropathy (274). A risk score for cardiac autonomic neuropathy (CAN) is calculated based on the results of conductance and biometric data such as age and BMI (276). The risk of CAN can be classified based on the scores as follows: no risk (≤25), moderate risk (25-50) and high risk (≥50) (276).

1.12 Magnetic resonance imaging
Magnetic resonance imaging (MRI) has been used to demonstrate that patients with diabetic peripheral neuropathy have a lower cross sectional spinal cord area than healthy controls in the cervical and thoracic regions (277). More recently MR has been employed to undertake imaging of peripheral nerves, and it has been shown to differentiate diabetic patients with and without neuropathy (278, 279). A summary of
the advantages and disadvantages of the most common methods of neuropathy assessment is provided in (Table 8).

1.13 Definition of small fiber neuropathy
Although the description of selective neuropathy of small nerve fibres is sufficient to conceptually define small fibre neuropathy (SFN), it is too non-specific for use in clinical and research environments (280). Attempts have been made in contemporary research to accurately determine SFN. One study defined SFN as two abnormal results on clinical examination, quantitative sensory testing (QST) and skin biopsy (228). Another classed patients as having predominant SFN if they were only found to have an abnormal distal leg skin biopsy (281). In 2010, an expert panel convened by the Diabetic Neuropathy Study Group of the European Association for the Study of Diabetes (NEURODIAB) agreed on a diagnostic criteria for SFN in diabetes, based on the distribution of signs and symptoms (Table 9) (282). Although the NEURODIAB definition encompasses SFN in diabetes, it has been suggested that it should also be applied when non-diabetic SFN is suspected as damage to small nerves is not disease specific (283, 284). Non-length dependent variants of SFN have been reported and it remains unclear how this definition can be applied in these patients (285). At present it is not possible to suggest criteria to define the severity of SFN in DPN. However, as normative ranges are established for the different tests of small fibre dysfunction and damage, it may be possible to devise a measure of severity using different percentiles or quartiles as cut-offs.

<table>
<thead>
<tr>
<th>Grade of SFN</th>
<th>Criterion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Possible</td>
<td>length-dependent symptoms, with or without clinical signs of small fibre damage</td>
</tr>
<tr>
<td>Probable</td>
<td>length-dependent symptoms, accompanied by clinical signs of small fibre damage with normal sural NC study</td>
</tr>
<tr>
<td>Definite</td>
<td>length-dependent symptoms, clinical signs of small fibre damage, normal sural NCS study, altered IENFD at the ankle with or without abnormal QST thermal thresholds at the foot</td>
</tr>
</tbody>
</table>

List of Abbreviations in this table: Small fibre neuropathy (SFN), Nerve conduction study (NCS), Intra epidermal nerve fibre density (IENFD), Quantitative sensory testing (QST)
1.14 Treatment of diabetic neuropathy

The ideal therapy should prevent or arrest the progressive loss of nerve fibres, improve symptoms, and have no side effects. Most trials report a 30% or 50% pain relief at best and it is therefore important that this information is conveyed to the patient when commencing treatment in order to manage expectations and minimise disappointment. Various algorithms have been proposed to manage patients with painful neuropathy based on best current evidence and the clinical experience of the investigators (286). Recently, a treatment algorithm defining the number needed to treat (NNT) and number needed to harm (NNH) for 50% pain relief has been formulated to compare the safety and effectiveness of current treatments for neuropathic pain syndromes (287). The treatment of PDN should focus on two strategies: treatment based on pathogenetic mechanisms and symptomatic treatment (pharmacological & non-pharmacological). The Diabetes Control and Complication Trial (DCCT) and the United Kingdom Prospective Diabetes Study (UKPDS) in Type 1 and Type 2 diabetic patients, respectively, showed that the progression of diabetic somatic and autonomic neuropathy is reduced in diabetic subjects that are treated intensively for normalization of blood glucose (288, 289). Therefore, the first step in the management of patients with diabetic peripheral neuropathy (DPN) is to have optimal control of blood sugar. Once pain develops, current treatment options do not address the underlying cause of nerve damage and at best achieve only partial alleviation of symptoms. According to studies in animal models and cultured cells, an elaborate causal pathway has been established providing a basis for the treatment of human diabetic neuropathy. However, due to limited translational studies we presently do not have an effective long term treatment (162). Despite there being many medications available for pain relief, e.g. Duloxetine, Pregabalin, Tricyclics, Selective serotonin reuptake inhibitors (SSRIs), Anticonvulsants and Opioids (162), they only provide alleviation of symptoms and in no way address the underlying nerve damage. There are limited data to suggest that antioxidant treatment with α-lipoic acid may be useful in alleviating neuropathic symptoms and deficits (290). Prostaglandin analogues may be effective by increasing endoneurial blood flow (291). The long term benefits of diet and exercise are not established as a recent study showed that they do not prevent neuropathy.
progression in IGT patients (292). Other options for treating PDN may be based on pathogenetic mechanisms of neuropathy and include aldose reductase inhibitors (293) and α-lipoic acid, which has been demonstrated to improve neuropathic symptoms and deficits (294). Many pharmaceutical and non-pharmaceutical treatments are available for the treatment of PDN (Table 10). There are three agents which are FDA-approved: Duloxetine (295), Pregabalin (296) and Tapentadol (297). Although non-steroidal anti-inflammatory drugs are prescribed commonly in PDN, there is no evidence that they are effective and they are contraindicated in patients with renal impairment (298). Similarly tricyclics have anti-cholinergic side effects and sedation limits their use, especially in elderly patients. Selective norepinephrine reuptake inhibitors (SNRIs) have fewer side effects than TCAs and duloxetine has received FDA approval for PDN treatment (295). A UK study comparing cost-effectiveness of the second-line use of Duloxetine in the treatment of PDN with the standard UK treatment showed that second-line use of duloxetine is a beneficial and cost-effective treatment strategy for diabetic peripheral neuropathic pain (299). Indeed, the National Institute for Health and Care Excellence (NICE) have recommended Duloxetine as a first-line intervention for diabetic painful neuropathy and in the most recent guidance it remains first line but amongst a choice from tricyclics, gabapentin and pregabalin. Anticonvulsants have been used in the management of neuropathic pain for many years, however, a recent analysis has found limited evidence for efficacy of this class of drugs in the treatment of PDN (300). Gabapentin is commonly prescribed, although Pregabalin has a higher potency and is a more effective analogue of Gabapentin and, moreover, the only other agent apart from Duloxetine and Tapentadol to have received FDA approval for the treatment of PDN. From the class of Antiarrhythmics, Mexilitine is a class 1B agent, which is a structural analogue of Lignocaine, but can be given orally. At low doses the risk of electrocardiographic side effects is low, but regular ECG monitoring is necessary and long-term use cannot be recommended (298). The use of opioids for neuropathic pain remains controversial, as studies have generally been small, yielded equivocal results, and have not established the long-term risk-benefit ratio (295). Capsaicin is an alkaloid found in chilli peppers and has been shown to be effective in PDN. However, the major clinical concern with this medication is that
topical capsaicin has been shown to produce complete or nearly complete
denervation of the epidermis in both control subjects and diabetic patients with a
significant reduction in regeneration in the latter (301). For patients with more
refractory PDN or those who suffer from significant side effects of pharmacotherapy
PDN can be treated non-pharmacologically using acupuncture (302) and frequency-
modulated electromagnetic neural stimulation (303). Recent data highlight the main
challenge with future trials assessing improvement in diabetic neuropathy, which is
the lack of significant worsening of neuropathy in the placebo group. The principle
that combining lower doses of agents which act on different pain pathways to
achieve better efficacy with fewer side effects, establishes a new paradigm for future
clinical trials in painful diabetic neuropathy and indeed the recent COMBO study has
shown that a combination of Duloxetine and Pregabalin may be superior to high
doses of each drug alone, although this did not reach significance (304).
### Table 10. Treatment options for painful diabetic neuropathy

<table>
<thead>
<tr>
<th>Mechanism of effect</th>
<th>Interventions</th>
<th>Drug</th>
<th>Dose. per day (mg)</th>
<th>Side effects</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optimal glycaemic control</td>
<td>Diet, OAT, insulin, exercise</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Contribution of patients &amp; Physician is required</td>
</tr>
<tr>
<td>Symptomatic treatment</td>
<td>Tricyclic antidepressants (TCAs)</td>
<td>Amitriptyline</td>
<td>20-150</td>
<td>+++</td>
<td>Sedation &amp; anticholinergic side effects</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Imipramine</td>
<td>25-150</td>
<td>+++</td>
<td>Sedation &amp; anticholinergic side effects</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Duloxetine</td>
<td>60-120</td>
<td>-</td>
<td>Approved by FDA</td>
</tr>
<tr>
<td></td>
<td>SSRIs</td>
<td>Gabapentin</td>
<td>900-3600</td>
<td>+</td>
<td>Side effects less than TCA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lamotrigine</td>
<td>200-400</td>
<td>+</td>
<td>Titration is required</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Carbamazepine</td>
<td>200-600</td>
<td>++</td>
<td>Agranulocytosis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pregabalin</td>
<td>300-600</td>
<td>-</td>
<td>FDA approved</td>
</tr>
<tr>
<td></td>
<td>Anticonvulsants</td>
<td>Mexilitene</td>
<td>Up to 900</td>
<td>+++</td>
<td>Potential cardiac side effects</td>
</tr>
<tr>
<td></td>
<td>Opioids</td>
<td>Tramadol</td>
<td>50-400</td>
<td>++</td>
<td>Sedation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Oxycodone</td>
<td>40-60</td>
<td>+++</td>
<td>Limit long-term use</td>
</tr>
<tr>
<td>Other agents</td>
<td>Capsaicin. Topical cream</td>
<td>α-Lipoic acid</td>
<td>600 mg IV 1200-1800 mg orally</td>
<td>-</td>
<td>No data on long term efficacy</td>
</tr>
<tr>
<td>Pathogenesis-based treatments</td>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Physical therapy</td>
<td>Electrical spinal cord stimulation</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Acupuncture</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Yoga</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Psychological support</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>FRN</td>
</tr>
</tbody>
</table>

Plus symbol (+) refers to the severity of symptoms (+ denotes minimal, +++ maximal). Food and drug administration (FDA), Intravenously (IV), Outdoor adventure training (OAT), Selective serotonin reuptake inhibitor (SSRI), Tricyclic antidepressant (TCA), Further research needed (FRN). Courtesy of Tavakoli M et al (186).

Other classes of drugs in recent trials such as duloxetine, venlafaxine, glyceryl trinitrate (GTN) and cannabinoids have also shown a significant placebo response (Table 11).
Glyceryl trinitrate (GTN)

Three key studies have demonstrated the efficacy of duloxetine in PDN with a mean percentage change from baseline in the placebo arm (PA) of 33%, 29% and 24% respectively (305-307). The duloxetine studies have also shown variability in the PA
with the percentage demonstrating 50% pain response ranging from 27-30%. Lacosamide, a new investigational drug in epilepsy and neuropathic pain slowly inactivates voltage-gated sodium channels and interacts with a collapsin response mediator protein-2. (311-313). In a phase 2 double blind randomised controlled trial of lacosamide the treatment arm achieved an average Likert pain scale score [(last observation carried forward (LOCF)] of 3.7±2.6 compared to a baseline of 6.6±1.6.(314). However, the PA achieved a Likert pain scale score (LOCF) of 4.5±2.6 compared to a baseline of 6.5±1.7. Despite this significant placebo effect lacosamide was significantly superior to the placebo but it raised questions regarding the repeatability of such an intervention given the extensive placebo effect. Thus in a recent study of 3 fixed-dose regimens, 68% of participants in the PA reported ‘feeling better’ on the patient global impression change (PGIC) evaluation compared to 69%, 81% and 83% on lacosamide 200mg, 400mg and 600mg respectively (315). Similarly, on the Likert pain scale the PA demonstrated a least square mean change of -1.6 from baseline and the only group which was significantly different from placebo was lacosamide 400mg.

In the only published trial of a medicinal cannabis-based product in the treatment of PDN, thirty subjects are randomised to either Sativex (tetrahydrocannabinol and cannabidiol) or placebo (309). The placebo effect was actually greater than Sativex with a reduction in all modalities of the pain diary score, indeed the mean reduction in the total pain score in the PA was 37% compared to 20% with active treatment. Such a significant placebo effect is worthy of further investigation, particularly when planning future trials of agents for PDN. The possible predictors of the placebo response have been assessed in three trials of lamotrigine using pooled data of 252 placebo subjects (222 had PDN) (316). A higher baseline pain score and a faster rate of recruitment were both identified as independent predictors of the placebo response. In an analysis of the Sativex trial, Selvarajah et al (309) suggested that depression may potentially be an important confounding factor as subjects with depression have a higher baseline pain score and consequently by entering a trial responds better to both the placebo and active drug. With depression being so common in PDN this confounding factor should clearly be accounted for, and exclusion by assessing depression scores, may not suffice. Indeed the placebo
response is a well-known entity in trials of depression (317). With regard to centers that recruit at a faster rate this may represent the fact that more of these subjects have intractable PDN and are therefore more eager to participate in novel treatments (316).

The placebo response is also thought to vary along the time course of a trial (318) hence the Food and Drug Administration (FDA) and other regulatory agencies require studies of at least 12 weeks for chronic pain (319). The premise being that a placebo effect is thought to stabilise after an initial period of several weeks (317, 320) with longer term trials producing a plateau. However an analysis of the placebo response variability in trials of PDN did not show such a plateau and variability may exist beyond 19 weeks (318).

A number of approaches have been suggested to separate the drug from the placebo response (317) and include use of a placebo run-in period (in order to cull placebo responders), flexible dosing, and the exclusion of subjects with mild pain (318, 320). However, the incorporation of these designs into clinical trials of pain has been shown to have little benefit. Indeed McQuay et al (321) conducted a review of the placebo response and concluded that the greatest determinants of the placebo response are in fact random factors. Thus the placebo effect is certainly one that should be considered prior to the initiation of any new study in PDN.

1.15 Non Pharmacological Treatment of Diabetic Neuropathy
Pharmacotherapy is the mainstay of therapy for the relief of painful diabetic neuropathy (322). Alternative non-pharmacological treatments such as acupuncture (323), transcutaneous electrical nerve stimulation (TENS) (324), spinal cord stimulation (325), percutaneous electrical nerve stimulation (PENS) (326) low intensity laser therapy (327) and monochromatic infrared light (328) are used in patients who are unresponsive or cannot tolerate pharmacotherapy, however the evidence for these approaches is limited and needs to be carefully reviewed.

1.16 Background
Diabetic neuropathy has a major microvascular basis, with functional and structural damage to the microvasculature leading to nerve dysfunction (329). In a recent study by Ylitalo K R et al (330), 9% of individuals from a population-based sample aged
≥40 years had peripheral neuropathy alone, 8.5% had PVD alone, and 2.4% had both. The obese group was more likely to have peripheral neuropathy, PVD, and both compared to non-obese subjects without cardiometabolic characteristics. The prevalence of diabetes is higher among the South Asian population and they have also been shown to have a higher prevalence of some diabetic complications, for example nephropathy and retinopathy (30, 31, 331, 332). However, despite the higher rates of ischaemic heart disease among the South Asian population, South Asians have been shown to have less neuropathy and lower rates of ulceration and amputation (111). The two major pathogenetic factors related to foot problems are diabetic neuropathy and peripheral vascular disease (PVD) (333). Several studies have shown abnormal endothelial function and vascular tone in the micro- and macrovasculature (334-336). Some small studies (110, 337) and a population-based cohort study (338) has shown that foot ulceration incidence in South Asian and Afro-Caribbean patients was significantly lower than in matched Europeans (1, 339). Whilst the traditional risk factors for the development of neuropathy are the degree of glycaemic control, duration of the disease and age (160), additional risk factors have been identified including vascular risk factors such as lipids, hypertension and obesity (340). During the past few years substantial data suggest that genetic factors may also play an important role in the pathogenesis of diabetic microvascular complications (341, 342). Thus some patients with well controlled hyperglycaemia may develop diabetic complications, whilst others with poorly controlled diabetes may escape complications (343). Of course these exceptions comprise a minority of the cases and it is important to remember that glycaemia is only one of many factors which influences the development, and progression of neuropathy. To date the role of ethnic differences in the development of neuropathy has not been fully studied. In this thesis, the author analyses the role of ethnic factors associated with diabetic neuropathy.

1.16.1 Aims
My aim was to define the basis for the ethnic differences in neuropathy by studying detailed nerve fibre and microvascular structure and function. In the first part of this study (DAEMON1, 2000-2005) which was conducted and led by Dr. Abbott, low rates of nerve dysfunction were shown in South Asians patients with T2DM
compared to their European counterparts. Therefore, the aim of present study (DAEMON 2, 2007-2012) was to investigate in details abnormalities in small fibres and vascular function and structure (H0-1, H0-2). The secondary aim of this study was to longitudinally study this cohort of patients (H0-3).

1.17 Hypotheses
The main hypothesis (null hypothesis-H0) of DAEMON 2 (present study) is:
Vascular and nerve abnormalities in South Asian patients do not differ from European patients with Type 2 diabetes.
The specific null hypotheses are as follows:
H0-1) Nerve fibres do not differ between South Asian and European patients with T2DM.
To investigate this immunohistological assessment of nerve fibres in skin biopsies and corneal nerve morphology was undertaken.
H0-2) Microvascular dysfunction does not differ between South Asian and European patients with T2DM.
To investigate this, laser Doppler imaging was used to assess capillary blood flow in South Asian and European patients with T2DM.
Furthermore, electron microscopic morphometric evaluation of capillaries in skin biopsies was assessed to quantify microangiopathy in these patients.
H0-3) There is no difference in the rate of changes of nerve function in South Asian and European patients with T2DM.
To investigate this, a follow-up study comparing large fibre assessment (electrophysiology and vibration perception threshold) was compared between the two groups. This follow-up study was based on inviting the patients who originally attended DAEMON I between 2000-2005 and DAEMON II (2007-2012).
1.18 Summary of Background Study related to the Present Study
(Parameters studied in DAEMON 1)

In DAEMON 1 which ran between 2000-2005 and was led by Abbott et al (3), 360 individuals with T2DM were selected from a population-based age- and sex-matched sample of 180 European (95 male and 85 female) and 180 South Asian (96 male and 84 female) patients with type 2 diabetes. The researchers collected and analysed demographic data, medical history including drug use, and lifestyle factors. They also measured HbA1c, fasting plasma glucose, lipid profile, serum γ-glutamyltransferase and fasting serum insulin. The methods employed were nerve conduction studies and vibration perception threshold to assess large fibres, thermal threshold assessment and heart rate variability response to deep breathing using CASE IV to assess small fibre function. Also Ankle Brachial Pressure Index (ABPI) and TCpO2 were measured using ankle and brachial pressure and the TCpO2 Machine (Novametrix Medical Systems, Wallingford). The South Asian population had a higher mean value for nerve conduction (p=0.003) and heart rate variability response to deep breathing (p=0.002) but did not differ from Europeans in terms of thermal threshold detection (p=ns). That study concluded that both somatic large fibre and autonomic small nerve fibre function is better in South Asian compared to European patients with diabetes, although thermal thresholds did not differ. Healthier peripheral vascular function was the key modifiable determinant of this protection from neuropathy in the South Asians, with an independent contribution from low smoking rates and shorter height. The demographic and clinical characteristics of this study are presented in Table 12 (3).
Table 12. Demographic and clinical characteristics of ethnic groups.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>South Asians</th>
<th>Europeans</th>
<th>P value-</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>180</td>
<td>180</td>
<td></td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>95/85</td>
<td>96/84</td>
<td>0.9</td>
</tr>
<tr>
<td>Age (years)</td>
<td>57.4 ± 9.9</td>
<td>59.1 ± 9.7</td>
<td>0.09</td>
</tr>
<tr>
<td>Duration diabetes (years)</td>
<td>9.4 ± 7.5</td>
<td>7.0 ± 5.7</td>
<td>0.001</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.61 ± 9.3</td>
<td>1.65 ± 9.8</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>77.2 ± 14.8</td>
<td>93.3 ± 19.3</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>29.7 ± 5.5</td>
<td>34.1 ± 6.5</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Waist (cm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>96.7 ± 13.1</td>
<td>106.6 ± 12.7</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Women</td>
<td>97.1 ± 12.8</td>
<td>102.7 ± 15.1</td>
<td>0.01</td>
</tr>
<tr>
<td>Hips (cm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>99.6 ± 8.1</td>
<td>105.4 ± 8.7</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Women</td>
<td>103.1 ± 11.3</td>
<td>111.0 ± 15.6</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Waist-to-hip ratio</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>0.97 ± 0.09</td>
<td>1.01 ± 0.07</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Women</td>
<td>0.94 ± 0.08</td>
<td>0.92 ± 0.08</td>
<td>0.2</td>
</tr>
<tr>
<td>Fasting plasma glucose (mmol/l)</td>
<td>7.7 ± 2.2</td>
<td>8.0 ± 2.3</td>
<td>0.2</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>8.1 ± 1.6</td>
<td>7.9 ± 1.7</td>
<td>0.3</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>4.3 ± 1.0</td>
<td>4.7 ± 1.1</td>
<td>0.004</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/l)</td>
<td>1.3 ± 0.3</td>
<td>1.3 ± 0.4</td>
<td>0.7</td>
</tr>
<tr>
<td>Fasting triglycerides (mmol/l)</td>
<td>1.4 (1.1, 1.9)</td>
<td>1.7 (1.2, 2.4)</td>
<td>0.005</td>
</tr>
<tr>
<td>Insulin (mU/l)</td>
<td>17.1 (9.6, 27.0.1)</td>
<td>18.2 (11.5, 28.6)</td>
<td>0.44</td>
</tr>
<tr>
<td>γ-Glutamyltransferase (U/l)</td>
<td>35.7 ± 34.4</td>
<td>58.7 ± 56.0</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Creatinine (µmol/l)</td>
<td>87.2 ± 31.2</td>
<td>85.9 ± 27.0</td>
<td>0.7</td>
</tr>
<tr>
<td>Urinary albumin-to-creatinine ratio</td>
<td>1.5 (0.7, 2.5)</td>
<td>1.4 (0.6, 3.0)</td>
<td>0.6</td>
</tr>
<tr>
<td>Retinopathy (%)</td>
<td>14</td>
<td>12</td>
<td>0.6</td>
</tr>
<tr>
<td>Nephropathy (%)</td>
<td>6</td>
<td>4</td>
<td>0.3</td>
</tr>
<tr>
<td>Hypertension (%)</td>
<td>54</td>
<td>74</td>
<td>0.0002</td>
</tr>
<tr>
<td>Myocardial infarction (%)</td>
<td>17</td>
<td>13</td>
<td>0.3</td>
</tr>
<tr>
<td>Angina (%)</td>
<td>23</td>
<td>17</td>
<td>0.2</td>
</tr>
<tr>
<td>Coronary artery bypass graft (%)</td>
<td>14</td>
<td>9</td>
<td>0.1</td>
</tr>
<tr>
<td>Resting heart rate (bpm)</td>
<td>76 ± 14</td>
<td>75 ± 13</td>
<td>0.8</td>
</tr>
<tr>
<td>Ankle systolic blood pressure (mmHg)</td>
<td>136 ± 22</td>
<td>142 ± 31</td>
<td>0.06</td>
</tr>
<tr>
<td>Brachial systolic blood pressure (mmHg)</td>
<td>123 ± 17</td>
<td>127 ± 17</td>
<td>0.02</td>
</tr>
<tr>
<td>ABPI ≤0.85 (%)</td>
<td>4.0</td>
<td>9.1</td>
<td>0.05</td>
</tr>
<tr>
<td>Lower limb vascular intervention (%)</td>
<td>3</td>
<td>9</td>
<td>0.03</td>
</tr>
<tr>
<td>Claudication (%)</td>
<td>7</td>
<td>15</td>
<td>0.01</td>
</tr>
<tr>
<td>TCPO₂ (mmHg)</td>
<td>61 ± 12</td>
<td>56 ± 13</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Pack-years smoked (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>74</td>
<td>36</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>≤20</td>
<td>14</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>Parameters</td>
<td>South Asians</td>
<td>Europeans</td>
<td>P value-&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>--------------------------------------------------------</td>
<td>--------------</td>
<td>-----------</td>
<td>---------------------</td>
</tr>
<tr>
<td>&gt;20</td>
<td>11</td>
<td>42</td>
<td></td>
</tr>
<tr>
<td>Drink alcohol ≥ once a week (%)</td>
<td>5</td>
<td>42</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Insulin (%)</td>
<td>19</td>
<td>23</td>
<td>0.4</td>
</tr>
<tr>
<td>Oral antidiabetic drugs (%)</td>
<td>87</td>
<td>76</td>
<td>0.007</td>
</tr>
<tr>
<td>Antihypertensive drugs (%)</td>
<td>61</td>
<td>77</td>
<td>0.001</td>
</tr>
<tr>
<td>Lipid-lowering drugs (%)</td>
<td>57</td>
<td>71</td>
<td>0.006</td>
</tr>
<tr>
<td>Other coronary heart disease drugs (%)</td>
<td>15</td>
<td>18</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Data are % prevalence, mean ± SD, or geometric means (25th, 75th percentiles). Courtesy of Abbott et al (3). *P value for ethnic difference.
Chapter 2

Patients and Methodology
2.1 Patients and methods

2.1.1 Introduction
A full range of clinical and neurological examinations were performed to assess the presence and severity of diabetic neuropathy and vascular changes in patients.

2.1.2 Patient recruitment
The original study (DAEMON 1- 2000-2005) was a cross-sectional survey of population-based sample of people with type 2 diabetes mellitus (T2DM), (n=360) of Europeans and South Asians drawn from eight primary care registers in Manchester, UK (3). In that study patients were selected from within ethnicity, gender and age stratified. All patients consented that they may be re-contacted for follow-up. It is important to note that patients taking part in the present study (DAEMON 2) were part of a cohort of patients that were recruited from DAEMON I led by Dr. Caroline Abbott (3).

Approximately 5 years (4.9 ± 0.6 years) after their initial visit, each participant was invited to re-attend The NIHR-Wellcome Trust Clinical Research Facility in Manchester for a single follow-up visit. Of the 360 subjects, 51 individuals declined to participate (via postal reply slip), 45 had moved away from the health authority, 81 did not respond and were untraceable and 28 patients had died. One-hundred and fifty-five individuals re-consented to participate in the follow-up study (response rate of 43%), with a mirroring ethnic split (Asian n=77-M: F 51:26); (European n=78-M: F 45:33). Differences between responders (n=155) and non-responders (n=206) were not significant for ethnicity, age, diabetes duration, BMI or HbA1c; however, a higher proportion of men re-consented (consenters - M: F 95:59; non-consenters - M: F 96:110; p<0.01). We tested all our models for effects of gender on neuropathy outcomes and there were none. Characteristics of participants and non-participants from DAEMON 1 study are demonstrated in Table 13. In skin biopsy subgroup there was no difference between those who underwent skin biopsy and did not undergo skin biopsy for age, duration of diabetes, BMI, HbA1c.

This study was approved by North Manchester Research Ethics Committee and by the University of Manchester Research Ethics Committee. We recruited 25 control subjects from the general population for this study.
Figure 7. DAEMON study schematic design.

Table 13. Characteristics of participants and non-participants from baseline study of DAEMON

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Follow up participants</th>
<th>Follow up non-participants</th>
<th>P value of participants vs. non-participants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Numbers</td>
<td>155</td>
<td>206</td>
<td>-</td>
</tr>
<tr>
<td>South Asian / European</td>
<td>77/78</td>
<td>103/102</td>
<td>ns</td>
</tr>
<tr>
<td>Male / Female</td>
<td>95/59</td>
<td>96/110</td>
<td>0.01</td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>57.4±6.1</td>
<td>58.9±10.4</td>
<td>ns</td>
</tr>
<tr>
<td>Duration of diabetes (yrs)</td>
<td>7.5±6.1</td>
<td>8.6±7.2</td>
<td>ns</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>31.2±5.0</td>
<td>32.4±7.2</td>
<td>ns</td>
</tr>
<tr>
<td>HbA₁c (%)</td>
<td>8.0±1.6</td>
<td>8.0±1.7</td>
<td>ns</td>
</tr>
<tr>
<td>HbA₁c (mmol/mol)</td>
<td>62.8±16.0</td>
<td>62.8±16.1</td>
<td>ns</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD. Body Max Index (BMI), glycated haemoglobin (HbA₁c).
Table 14. Characteristics of participants who did and did not undergo skin biopsy.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Participants (skin biopsy)</th>
<th>Participants (no skin biopsy)</th>
<th>P value *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Numbers</td>
<td>49</td>
<td>106</td>
<td>-</td>
</tr>
<tr>
<td>South Asian/European</td>
<td>24/25</td>
<td>53/53</td>
<td>-</td>
</tr>
<tr>
<td>Male/Female</td>
<td>34/15</td>
<td>61/45</td>
<td>0.1</td>
</tr>
<tr>
<td>Age (yrs)</td>
<td>61.9±7.8</td>
<td>62.9±9.4</td>
<td>0.5</td>
</tr>
<tr>
<td>Duration of Diabetes (yrs)</td>
<td>11.7±5.81</td>
<td>12.7±6.2</td>
<td>0.36</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>31.8±5.42</td>
<td>31.9±5.64</td>
<td>0.9</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>7.8±1.38</td>
<td>7.9±1.50</td>
<td>0.7</td>
</tr>
<tr>
<td>HbA1c (mmol/mol)</td>
<td>62.2±15.2</td>
<td>63.0±16.4</td>
<td>0.7</td>
</tr>
</tbody>
</table>

*P value between those who did and did not undergo skin biopsy. Body Max Index (BMI), glycated haemoglobin (HbA1c).

2.2 Ethical approval

Ethical approval of this study was obtained from the Central Manchester Local Research Ethics Committee (07/H1006/68). The research adhered to the tenets of the Declaration of Helsinki. Information sheets were sent to patients at least one week prior to appointment and the signed consent form was obtained on the attending day. For those participants who did not speak English, we sought help from an interpreter.

2.3 Identification of participants

2.3.1 Inclusion criteria

Type 2 diabetes diagnosed between the age of 30 and 39 without immediate need of insulin treatment, or at the age of 40 years and over regardless of initial treatment from both groups of South Asian and European patients who had previously participated in the DAEMON I study between 2000-2005. All patients underwent a detailed clinical history and examination to rule out any other cause of neuropathy.

2.3.2 Exclusion criteria

Patients were excluded from the study if they had any of following criteria:

1. Lower limb amputation (major).
2. Known HIV or hepatitis.
3. Both pulses on one foot non-palpable (for patients invited to undergo skin biopsy).
4. Ocular surgery, history of eye trauma and corneal dystrophy.
2.4 Study design

As described at the start of this chapter, the original study (DAEMON I, 2000-2005), was a cross-sectional survey of people with type 2 diabetes of European and South Asian origin from primary care in the UK. The sample was stratified for ethnicity and 5-year age groups in order to ensure a comparison of a representative sample of people with type 2 diabetes (3). The survey was also designed as a longitudinal follow up 4-5 years from the date of the baseline visit. In the baseline study (DAEMON 1) established techniques were used to assess neuropathy and the full details from this study are presented in Table 12, chapter 1. In that study the lower rates of small fibre neuropathy assessed with Heart rate variability response to deep breathing signalled the importance of doing further more robust tests of small fibre dysfunction in addition to large fibre assessment in South Asians and Europeans with type 2 diabetes. Furthermore, to study a microvascular contribution vascular function and capillary structure were assessed, as in DAEMON 1 there was evidence of less peripheral arterial disease in the South Asian diabetic population. Therefore in DAEMON 2 we performed a detailed assessment of neuropathy to establish whether the South Asian patients with T2DM had particularly less small fibre neuropathy and better vascular function and whether this was the explanation for the lower prevalence of neuropathy in South Asians.

2.4.1 Sample size of study

The original sample size of the study was established using corneal nerve fibre tortuosity in corneal images obtained by the Tomey Confoscan, a light based corneal confocal microscope (Diabetes UK, RD05/0003048). However with the new laser corneal confocal microscope (HRT III) used in this study (DAEMON 2), the study sample size was recalculated based on corneal nerve parameters measured using the HRT III corneal confocal microscope in our NIH study of a cohort of T2DM patients with mild neuropathy and control subjects. Corneal nerve fibre length (CNFL) was selected as the primary outcome as it is an early marker of small fibre neuropathy. It was calculated that 154 patients (77 in each ethnic group) would be sufficient with 80% power to detect a mean difference in CNFL of 3.20 (25.70 versus 22.50) and an SD of 7, at the 5% significance level with an effect size of 0.48.
2.5 Study site
Clinical tests were performed at the NIHR-Wellcome Trust Clinical Research Facility (NIHR-WTCRF), Manchester. Biopsy samples were analysed in the Immunohistology Laboratory in the Stopford building and also in the Electron Microscopy Unit in the Michael Smith Building at the University of Manchester.

2.6 Variables

2.6.1 Demographic and Anthropometric Variables
An information sheet was sent to all subjects no later than one week before their study appointment. Patients gave consent for the study on the day of arrival and their medication list was recorded. Blood pressure was measured based on an established protocol in the left arm in a semi-upright position three times and at least 10 minutes after arrival. Height, weight, waist and hip circumference were measured by a nurse and blood was collected for measuring glycated haemoglobin (HbA1c), fibrinogen and lipid profile.

2.6.1.1 Height measurement
The volunteer was asked to remove their shoes and stand with their back as straight as possible against the wall with their arms hanging loosely by their side. The head plate of the stadiometer (Holtain, United Kingdom) was lowered to rest gently on the volunteer’s head. The volunteer was then asked to move their head so that the Frankfort Plane was in a horizontal position (i.e. parallel to the floor). The Frankfort Plane is an imaginary line passing through the external ear canal and across the top of the lower bone of the eye socket, immediately under the eye. The volunteer was instructed to look slightly downwards bringing the top of their head to the highest point for measurement. The subject breathed deeply and stretched to their fullest height. The height was recorded in centimetres and millimetres.

2.6.1.2 Weight measurement
Each volunteer was asked to remove his/her shoes and stand with their feet together in the centre of the scale (SECA 770 Electronic Personal Scale, Germany) with their heels against the back edge of the scale. They were instructed to keep their hands
loosely by their sides with the head facing forward. The posture of the person was observed by a nurse. The measurement was recorded in kilograms and grams.

2.6.1.3 Waist measurement

The measurements were recorded under the subject’s clothes using a standard tape measure to measure the circumference of the waist. The subject was asked to stand up straight and breathe out gently, looking straight ahead with arms hanging loosely at their sides. Waist circumference was defined as the circumference at the level midway between the costal margins and the iliac crests. Measurements were recorded in centimetres and to the nearest even millimetre in mid expiration when the abdominal muscles were maximally relaxed.

2.6.1.4 Hip measurement

Subjects stood with arms by the side and feet together. The observers sat in front of the subject and fitted the measuring tape around the widest part of the hips, over the top of the greater trochanters of the femur. These were recorded in centimetres and to the even millimetre.

2.7 Symptoms

2.7.1 Neuropathy Symptom Profile

The neuropathic symptoms of patients were assessed using the modified Neuropathy Symptom Profile (NSP) questionnaire which was used in our previous study (344). The questionnaire consists of 38 questions for men and 36 questions for women evaluating symptoms of motor, sensory and autonomic neuropathy. The questionnaire can be found in Appendix 5.

2.7.2 McGill Pain Questionnaire

The McGill Pain Questionnaire is the most frequently used questionnaire to assess diabetic painful neuropathy (345, 346). The short form of the McGill Pain questionnaire (SF-MPQ) was administered and used to investigate the relationship between pain and neuropathic severity (347). To explain sensory pain the following words are provided: throbbing, shooting, stabbing, sharp, cramping, hot-burning, aching, heavy, and tender, splitting. The total score for this part (Pain Rating Index
(PRI) is 33. Other words (affective and total descriptors) are: tiring-exhausting, sickening, fearful, and punishing-cruel. Each word had to be given a mark from 0 to 3 according to the severity (none, mild, moderate, severe). The total number is 12. Visual test, whereby patients are asked to look at a straight line representing the severity of pain (left end indicates no pain and right end indicates maximum possible pain), and to tick the line and give a number to the point that they think corresponds to their present pain. The score ranges between 0 and 10. For the evaluative dimension of pain, patients were asked to choose a category among the following: no pain (0), mild (1), discomforting (2), distressing (3), horrible (4), and excruciating (5), the maximum score being 5. Therefore, the total score for this test is 60, as shown in Appendix 6.

2.8 Clinical neuropathy assessment

2.8.1 The neuropathy disability score
The neuropathy disability score (NDS) (Figure 8) quantifies abnormalities of vibration perception using a 128 Hz tuning fork, pin prick perception and temperature perception and the presence or absence of ankle reflexes. The NDS is measured over a range between 0 and 10. Thus it establishes different grades of neuropathic severity: NDS between 0-2 being classified as ‘no neuropathy’, 3-5 as ‘mild neuropathy’, 6-8 as ‘moderate neuropathy’, and 9-10 as ‘severe neuropathy’ (344, 348), as detailed in Appendix 4. The sharp and blunt edges of the Neurotip™ are randomly pressed against the plantar aspect of the hallux, and patients are required to distinguish between the painful and painless stimuli. The test was considered abnormal, if at least two responses out of three readings were incorrect (349). Temperature perception was impaired if there were at least two incorrect responses out of three readings on the dorsum of each foot (350).
2.9 Large fibre assessment

2.9.1 Electrophysiology

The Medtronic Keypoint™ EMG system (Figure 9) was used for nerve conduction studies whilst maintaining the skin temperature between 32-35 °C. The test was performed by a technician specialised in neurophysiology at the Department of Clinical Neurophysiology, Manchester Royal Infirmary. Motor nerve conduction velocity (m/s), amplitude (mv) baseline to peak and minimum F-wave latency (ms) of the peroneal nerve; amplitude of the sensory action potential, baseline to peak (µv), latency to onset (ms) and conduction velocity of the sensory sural nerve were evaluated (134). All assessments were performed according to a standardised protocol, using surface electrodes with a 9 mm diameter. Motor studies were recorded using a belly-tendon electrode placement. Motor nerve conduction was established in the peroneal nerve (Recording, Extensor Digitorum Brevis (EDB); Stimulation, Ankle (60mm proximal to active recording electrode), 5cm distal to the fibular head and 5cm proximal to the fibular head). Sensory nerve conduction was investigated in the sural nerve (recording, ankle; stimulation calf (100mm proximal to active recording electrode). The Distal Motor Latency (DML) reflects the time taken...
from supramaximal stimulation of the nerve 60mm proximal to the active recording electrode to the onset of the compound muscle action potential (i.e. the evoked motor response) (351).

![The Medtronic Keypoint electrophysiology system.](image)

**Figure 9. The Medtronic Keypoint electrophysiology system.**

### 2.9.2 Quantitative sensory testing

Quantitative sensory testing (QST) included vibration perception threshold (VPT) assessment with a Neurothesiometer (Horwell, Scientific Laboratory Supplies, Wilford, UK) (Figure 10) and thermal threshold assessment for warm sensation (WS), cold sensation (CS), cold induced pain (CIP) and heat induced pain (HIP) with a Medoc Neuro Sensory Analyser TSA-II (Figure 11 [(Medoc Ltd, Ramat Yishai 30095, Israel).]
The test was performed on the first toe of each foot, with the patient in a semi-recumbent position and with their heels resting on the examining bed. Before any measurements were taken, patients were first familiarised with the vibration sensation delivered by the equipment on their wrists. The probe was then placed on the end of the great toe and set to deliver repetitive mechanical indentation (vibrations) of the skin at a prescribed frequency and amplitude. The patient was instructed to close their eyes whilst the stimulations were gradually increased from zero. The patient was advised to indicate when they first detect a sensation of “buzzing” or vibration at the toe being tested. This was recorded three separate times and an average VPT was established. The VPT was measured in volts with a range between 0–50V (352). Severity of neuropathy was graded as ‘No neuropathy’ <10V; 10 <25V - Mild neuropathy’; 25-35V - ‘Moderate neuropathy’ and >35V - ‘Severe neuropathy’ (353).

2.10 Small fibre assessment

2.10.1 Thermal threshold assessments

Thermal Sensory Analyser II (TSA-II), NeuroSensory Analyser (Medoc Ltd., Ramat Yishai 30095, Israel) (Figure 11) is a computer-controlled device that can generate and record a response to a highly repeatable thermal stimulus, such as warmth, cold, heat-induced pain and cold-induced pain. A thermode was placed on the dorsolateral aspect of the foot and the TSA-II heats or cools the skin as needed. An initial adaptation temperature between 30°C and 32°C was established at the
beginning of the assessment where the patient feels neither warmth nor cold. Perception of cold and warm sensation is a measure of afferent function served by small-calibre myelinated (Aδ) and unmyelinated (C) nerve fibres, respectively.

Figure 11. TSA-II NeuroSensory Analyser (Medoc)

The method of limits was applied using a simple push-button response when the patient feels the relevant sensation, which is then recorded by the computer as the sensory threshold. Deviation from the normal range indicates peripheral nerve dysfunction. Each thermal sensation is tested 4 times and the average threshold is determined.

For measurement of Heat Induced Pain (HIP) and Cold Induced Pain (CIP) patients were asked to respond when they felt pain following the heat and cold stimuli, respectively. A normal detection threshold for warmth is approximately 1-2°C above the adaptation temperature (~35°C) with the perception of pain occurring at approximately 45°C, whilst cold detection is usually sensed at 1-2°C below the adaptation temperature. Cold pain occurs between 10-20°C in the normal population. All thermal thresholds were recorded using the Neuro Sensory Analyser on a visual analogue scale (Figure 12).
2.11 Autonomic assessment

The patient was instructed to sit comfortably on a reclining couch and disposable ECG electrodes were attached to the right and left subclavian region and the left wrist to enable continuous electrocardiogram (ECG) assessment. Autonomic function was assessed by measuring heart rate variability with deep breathing with the CASE IV machine (WR Medical Electronics, Inc, Stillwater, MN, USA), (Figure 13) connected to an ECG machine (see Appendix 8).

The patient was asked to breathe deeply for 8 cycles (80 seconds) in sequences of 5-second inspirations and 5-second expirations. The inspiratory and expiratory phases were standardised using visual and verbal cues given by the investigator. The patient was instructed to rest for 5 minutes without talking and a second breathing series was repeated. A HRV $\geq 15$ is considered to be normal; HRV $<10$ is considered to be pathological (354). Values between these 2 reference ranges are considered borderline (212).

![Figure 12. Method of Limits and position of the attached probe for the thermal threshold assessment.](image)

![Figure 13. Heart rate variability response to deep breathing using CASE IV.](image)
2.11.1 Neuropad

The Neuropad is a semi-quantitative test of sudomotor function (Neuropad®; micro Verbandstoffe GmbH, Wiehl-Drabenderhöhe, Germany), (Figure 14). This is a plaster which provides a semi-quantitative assessment of sweat production on the basis of a colour change of a cobalt II salt from blue to pink when it comes in contact with human sweat on the skin. In normal subjects the colour of the plaster changes from blue to pink in less than 10 minutes, but if there is an abnormality in sweat production due to small-fibre dysfunction, the plaster remains blue or patchy (Figure 14). The 10-minute cut-off for Neuropad provides a relatively high sensitivity of 87.5% and modest specificity of 80.0% for distal symmetric polyneuropathy and small-fibre dysfunction (355). It is also reported to have an 86% negative predictive value and a 50% positive predictive value for screening peripheral neuropathy (356).

Figure 14. Neuropad (a) normal case, plaster is pink in 10 minutes (b) abnormal case, plaster remains blue (c) abnormal case where colour change is partial.

2.11.2 Punch skin biopsy

After inspecting the dorsum of the foot, two points were chosen approximately 2 cm proximal to the metatarsal bone and two punch skin biopsies (3 mm) were taken from the dorsum of the foot under 1% Lidocaine (local anaesthesia) and the biopsy site was closed using 1-2 steri-strips Figure 15. One sample was cut into two pieces and half was processed for immunohistology and the other half for light and electron microscopy. The sample for immunohistology was fixed in 4% paraformaldehyde for 18-24 hours and after washing in buffer solution transferred to Industrial Methylated Spirit (IMS) at different concentrations (50%, 75%, 100%) using an orbital shaker and immersed in xylene before being embedded in wax (Appendix 13). The other sample was immediately immersed for 4 hours in 2.5% glutaraldehyde before being transferred to cacodylate buffer for further processing.
The whole sample was fixed in 4% paraformaldehyde for 18-24 hours, washed twice in buffer, immersed in different concentrations of sucrose (10%, 20%, 30%) for 2-4 hrs before being embedded in OCT and freezing at -80 °C.

Figure 15. Punch skin biopsy procedure.

2.11.3 Light and electron microscopy
For light and electron microscopic examination, fixation is applied immediately to maintain the ultra-structural features of the cells to allow accurate morphometric assessment. The fixation comprises primary and secondary fixative. Primary fixation enables fixation of cellular proteins which include proteolytic enzymes which may digest the cellular components, while secondary fixation preserves the lipid components of the cells such as the cellular membranes and myelin (357).

2.11.3.1 Preparation of the primary fixative (glutaraldehyde)
For the primary fixative we used 2.5% glutaraldehyde in sodium cacodylate buffer. This was prepared by adding 25ml of 0.2µ (Molar) cacodylate buffer (sodium cacodylate, agar scientific Ltd, Essex UK) and 5ml of glutaraldehyde (25%) plus 5g sucrose.

2.11.3.2 Primary fixation
Immediately after dissection, the samples were immersed in 2.5% glutaraldehyde in 1% cacodylate buffer (PH 7.4) at room temperature for 4 hours and then rinsed 6 times in cacodylate buffer and left overnight in buffer.
2.11.3.3 Preparation of the secondary fixative (osmium tetroxide)

The second fixative was prepared by breaking an ampoule containing 0.1g of crystalline Osmium tetroxide (Agar scientific Ltd, Essex, UK) in a fume cupboard and placed in a brown glass bottle to inhibit photo-degradation of the Osmium. 10 ml of distilled water was added to the crystals and the bottle was placed for 15 minutes in an ultrasonic mixing bath to dissolve the osmium.

2.11.3.4 Secondary fixation

The samples were placed in 1% Osmium tetroxide at room temperature for 3 hours and washed 8 times in distilled water.

2.11.3.5 Dehydration

As the embedding materials are water-insoluble, tissue water has to be removed completely to allow maximum infiltration by the resin. The samples were therefore serially dehydrated in increasing concentrations of ethanol as follows:

1- Two changes at 15 minutes in 50%, 70% ethanol.
2- Two changes at 15 minutes in 90% ethanol.
3- Two changes at 20 minutes in 100% ethanol.

2.11.3.6 Embedding in epon blocks

Propylene oxide mixes and dissolves resin readily, therefore after dehydration the tissue had 2 changes for 15 minutes in Propylene oxide (Propylene oxide, Agar scientific Ltd, Essex, UK) and was left in 1:1 Propylene oxide/ Epon for an hour and then in 1:3 Propylene oxide/ Epon overnight. Finally the tissue was left in 100% Epon (Spurr p/mix kit med 4221D-1, TAAB Laboratories Equipment Ltd, Berks, England) overnight. This process allowed the resin to infiltrate the tissue uniformly. The biopsy samples were then placed in rubber moulds and coded before pouring resin carefully into the mould, avoiding bubble formation. The moulds were kept in the oven for 48 hours at 60°C until the resin had polymerised.

2.11.3.7 Sectioning

The hardened resin-embedded tissue blocks were trimmed with a sharp razor blade to remove excess resin. Glass knives mounted on a Reichert mechanical advance
ultramicrotome (Reichert-Jung, Eindhoven, The Netherlands) were used to cut the blocks down to the tissue. Once the block surface was smooth, a new glass knife with a boat filled with distilled water was used to cut and collect 0.7 µm sections. The sections were picked up using metal forceps and placed on a drop of water on a glass slide, and stretched using a cotton stick immersed in trichloroethylene and left to dry on a hot plate for ~5 minutes.

Using a Diatome diamond knife (3mm ultra 35° knives, Diatome AG, Biel, Switzerland) with the knife boat filled with distilled water; ultra-thin sections (80-90 nm) were obtained on the Reichert microtome. The sections were stretched using trichloroethylene and collected on 200 mesh size copper grids. Grids were left on a piece of filter paper until dry and then stored in a dust free box.

**2.11.3.8 Staining**

Semi-thin sections were stained with 1% Toluidine blue (BDH Laboratory Supplies, Poole, UK) for 2 minutes. The slides were then rinsed in distilled water and dried. Staining of ultra-thin sections with metallic and slightly radioactive dyes is key to making tissue electron dense and providing contrast to enhance ultrastructural features. The stains used for this were lead citrate. The grids holding the sections were stained in droplets of lead citrate for 2 minutes, washed in three consecutive baths of distilled water and allowed to dry before storage in a dust free box.

**2.11.4 Image processing and morphological procedures**

**2.11.4.1 Light microscopy**

From each biopsy one suitable section was selected and photographed using a digital Kodak camera attached to a Leica WILD MPS32 photo light microscope (Leica, Cambridge, UK). Sections were photographed at 20x magnification and images (Figure 16) were saved for quantification using Image Analyser Pro-Plus software, version 4.1 (Media Cybernetics, Buckinghamshire UK).

Capillaries were visualised and counted in the hypodermis and the density was derived after measuring the area of hypodermis. The thickness of the epidermis was measured from each image using image analysis to quantify 5-8 randomly selected
tangential lengths across the epidermis Figure 16. Epidermal thickness was measured from just under the stratum corneum to the stratum basale.

![Figure 16. Light microscopic section of epidermis and hypodermis outlining assessment of capillary density and epidermal thickness.](image)

2.11.4.2 Electron microscopy

Ultra-thin sections were imaged in a Tecnai12 Biotwin transmission electron microscope (FEI, Eindhoven, The Netherlands) set at 100 kV accelerating voltage and filament emission of 30. The condenser settings and emissions were adjusted to achieve the highest contrast and an average of 5 capillaries, as shown in Figure 17 were photographed. Microvessels without a complete layer of pericytes or smooth muscle surrounding the endothelial cells favouring neither the large nor the small ones were photographed at magnification of x1400 using a camera connected to the electron microscope and the images were saved directly to the University server.

Each image was quantified with Image Processing and Analysis in Java (Image J software, National Institute of Health) using detailed morphometric techniques by tracing the luminal perimeter (luminal area, $\mu m^2$), outer endothelial cell perimeter and basement membrane perimeter ($\mu m$) including endothelial area and pericyte area.
From these measures the following morphological variables were derived: luminal area, endothelial area (outer endothelial area – luminal area), basement membrane area (basement membrane area – outer endothelial area). Pericyte nuclear numbers were counted directly from each capillary using electron micrograph at X1400 magnification.

![Electronmicrograph of capillary structure of skin biopsy taken from the dorsum of left foot, (Magnification x1400) Outer layer of basement membrane (BM), pericyte area, endothelial area and luminal area are shown.](image)

**2.12 Intraepidermal nerve fibre density assessment**

Skin samples were fixed in 4% paraformaldehyde in PBS buffer pH 7.6 at 4°C for 18-24 hours, washed in the same buffer 3 times for 5 minutes and then left in buffer on the oscillator overnight. The next step was cryoprotection, which was achieved by immersing samples in 10-30% sucrose in PBS buffer over 24 hours. The samples were individually placed in cryovials filled with OCT, frozen in liquid nitrogen and stored in a freezer at -80°C, until further requirement.

Each cryovial removed from the freezer was gently brought to room temperature and the skin sample was placed on the frozen OCT mount (Thermo Scientific Raymond Lamb, Walton, MA, USA) on a holder, properly orientated and cut into 50µm sections.
on a cryostat. All sections were collected in tissue culture plates filled with PBS buffer and then transferred to 96-well plates containing anti-freeze fluid, properly numbered and labelled and then stored in a freezer at -20 ºC.

Prior to immunostaining chosen sections (5 per case, selected from the medial part of the biopsy) were washed 3 times for 15 minutes in TBS buffer (pH 7.6) and then subjected to antigen retrieval in citrate buffer (pH 6.0) two times for 2 minutes at the lowest microwave setting. The sections were washed in TBS/Tween 20 3 times for 5 minutes followed by endogenous peroxidase blocking for 30 minutes (Dako Peroxidase Block, Dako, Glostrup, Denmark). After this, the sections were washed again in TBS/Tween 20 and transferred to 10% goat serum for 4 hours.

The nerve fibres were immunostained using Rabbit Anti-PGP9.5 Antibody (Millipore, Watford, UK; diluted 1:1000 in 5% goat serum (Vector Labs, Burlingame, CA, USA) in TBS/Tween 20 overnight) After being washed in TBS/Tween 20, the sections were incubated with biotinylated Goat Anti-Rabbit IgG (Vector Labs; 1:300 in 5% goat serum) for 2 hours, washed as before and incubated with HRP-Avidin D (Vector Labs; 1:300 in TBS) for 2 hours. After subsequent washing in TBS/ Tween 20, the presence of the antibody-antigen reaction was visualised with Vector SG chromogen (Vector Laboratories). After a final wash in distilled water the sections were spread with a brush on glass slides in aqueous mountant (Dako, Glostrup, Denmark) and coverslipped.

2.13 Image analysis

The immunostained sections from each case were observed under a Zeiss Axio Imager M2 microscope and intraepidermal nerve fibre density (IENFD) was calculated. Briefly, the nerves piercing the basement membrane of the epidermis were counted in all 5 sections stained per case at x200 magnification. All sections were photographed and the length of epidermis measured. IENFD was expressed as number/mm length of epidermis.
2.14 Immunohistochemistry method for wax embedding samples
2.14.1 Usefulness of wax embedded samples for IENFD

In general, paraffin-embedded tissue is not ideal for the quantification of IENFs and the best results are achieved with frozen thick sections (20-50 microns) (358-361). Only one study has been published from a Finnish group that was able to quantify IENF using paraffin-embedded tissue (362). The protocol detailed below is proposed for paraffin-embedded samples.

The half sample was fixed immediately after excision in 4% paraformaldehyde for 18-24 hours. This sample was then washed in distilled water (Appendix 13) and left in PBS overnight. The sample was subsequently processed in (25%, 50%, 75%, 100% IMS) before being washed in Xylene. After this it was put in plastic cassettes and left in molten wax in a vacuum oven with the optimum pressure of the oven, kept in 5 mm Hg and the wax was changed twice every 30 minutes. Ultimately, the samples were stored for further examination.
2.15 Laser Doppler flowmetry
A scanning Laser Doppler (Moor Instruments Ltd, Axminster, UK) (Figure 19) with a 2 mW near-infrared laser-scanning beam combined with a red visible aiming beam and Laser Doppler Imager Control Unit was used (further details in Appendix 9).

Figure 19. Laser Doppler Imaging system.
2.15.1 Hyperaemic response and flare area

The patient was asked to sit on a chair with the left foot under the Laser Doppler Imaging (LDI) unit. The dorsal skin temperature was between 31 - 35°C and was checked using an infrared thermometer. If the skin temperature was below 31°C, the foot was warmed using a warmed water bag or a hairdryer. The dorsal surface (3-5cm proximal to 1st and 2nd toes, free of surface veins) was cleaned by wiping the area with an alcohol wipe (4% isopropyl alcohol swab) and, if necessary, the sensor site was shaved. The chosen area (free of veins) was marked for LDI and Transcutaneous Oxygen Tension (TCpO₂) by a template ring with a marker pen. Baseline laser Doppler scanning was performed and the image was saved with the patient ID number to be processed with image processing software (Moor LDI Image Processing V3.01) to establish the baseline flux values. A TCpO₂ probe (Novametrix Medical Systems Inc., CT, USA) (Figure 21) was then applied using an adhesive ring attached to the membrane sensor and a drop of oxygen contact gel was placed on the surface of the membrane and attached to the dorsum of the foot in exactly the
same area where the baseline image had been taken by laser doppler flowmetry for 20 minutes to assess the skin oxygenation and to deliver the thermal stimulus, as the probe works at 44°C. With this technique, we measured the laser Doppler flare area. This is an established technique to assess small fibre neuropathy in diabetic patients and is a simple non-invasive test involving skin heating and measuring the size of the resulting axon reflex-mediated vasodilator (flare) response using a laser Doppler imager (363). This technique has been improved recently by Rayman et al (363) by heating with a bigger sized probe and a higher temperature of 47°C to induce a larger flare response. Figure 22 shows the flare area and also LDI max as described earlier in this thesis.

Figure 21. Thermal probe for TCpO₂ assessment.
Figure 22. Post heating image of laser Doppler flowmetry. The mean perfusion unit within the region corresponding to the probe contact on the FLUX image (left) is the LDI max which is the red area in the post image. The internal area demarcated by the blue-green boundary (red arrow in right image) constitutes the LDI flare.

2.15.2 Corneal aesthesiometer

Corneal sensitivity was quantified using a non-contact corneal aesthesiometer (NCCA; Glasgow, Caledonian University, UK), which uses a puff of air through a bore 0.5 mm in diameter lasting 0.9 second and exerts a force expressed in millibars. An electronic pressure sensor displays the force exerted (in millibars) (Figure 23). The stimulus jet was mounted on a slit lamp. It was positioned 1 cm from the eye, and the air jet was aligned to the centre of the cornea (364). The subject felt a sensation on the cornea, which is most commonly describing as being “cold” or a “breeze,” and acknowledged this sensation. Each subject was presented with a supramaximal stimulus, and a staircase method was employed by reducing the stimulus strength until the patient did not feel the jet. The jet was then increased to a threshold level and reduced to the point where the stimulus did not feel. The whole process was repeated three times to derive a threshold (249, 364). This test was performed by our optometrists.
2.15.3 Corneal confocal microscopy

Patients underwent examination with the Heidelberg Retinal Tomography (HRT III) (Rostock Cornea Module) in vivo corneal confocal microscopy (Figure 24). 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. The patient was instructed to fixate on a target with the eye not being examined. 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. The gel optically couples the objective lens to the cornea. Several scans of the entire depth of the cornea (Figure 24) were recorded by turning the fine focus of the objective lens backwards and forwards for approximately 2 minutes to acquire satisfactory images of all corneal layers providing two-dimensional images with a lateral resolution of approximately 2 µm/pixel and a final image size of 400 pixels. Images were obtained using the section mode which enables manual acquisition and storage of a single image at a time. For the purpose of our present study, we obtained high quality images 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 (Figure 24). These nerve fibre bundles contain unmyelinated fibres which run parallel to Bowman’s layer before dividing and turning upwards toward the surface to terminate as individual axons underneath the surface epithelium (365, 366). This has been confirmed using electron microscopy where nerve bundles containing unmyelinated axons were shown to penetrate Bowman’s membrane throughout the central and peripheral cornea at approximately 400 sites (367). Five images per patient from the centre of the cornea were selected and examined in a masked and randomised fashion (368).
Four corneal nerve parameters were quantified: (i) corneal nerve fiber density (CNFD) - the total number of major nerves/mm² of corneal tissue; (ii) corneal nerve branch density (CNBD) - the number of branches emanating from all major nerve trunks/mm² of corneal tissue and (iii) corneal nerve fiber length (CNFL) - the total length of all nerve fibers and branches (mm/mm²) within the area of corneal tissue; (iv) corneal nerve fibre tortuosity (CNFT). For quantification a digital, grip pen (Intuos, Wacom Technology Corp., Vancouver, WA) was used to trace the nerves manually. This test was performed by our optometrists.

Figure 24. In vivo Corneal Confocal Microscopy and image of Bowman’s layer of cornea and corneal confocal machine used in this project (HRT III).

A summary of the techniques used in this study to assess diabetic neuropathy are presented in Table 15.
Table 15. Summary of methods used in this project.

<table>
<thead>
<tr>
<th>Demographic</th>
<th>Symptoms</th>
<th>Large fibre</th>
<th>Small fibre</th>
<th>Vascular Assessment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, Gender, BP, DD, smoking, alcohol history, CVD history, BMI, Waist circumference, Hip circumference, PMH, lipid profile, fibrinogen, HbA1c</td>
<td>NSP, McGill questionnaire</td>
<td>NDS, VPT, NCS (peroneal, sural nerves)</td>
<td>HRV-DB, TPT(CS, WS, CIP, HIP), Neuropad, IENF, NCCA, CCM</td>
<td>LDI, iontophoresis, skin morphology (capillary density, epidermal thickness, BMT, lumen area, pericyte profile number, endothelial area)</td>
</tr>
</tbody>
</table>

Blood Pressure (BP), Cardiovascular disease (CVD), Body mass index (BMI), Past medical history (PMH), Neuropathy symptom profile (NSP), Neuropathy disability score (NDS), Vibration perception threshold (VPT), Nerve conduction study (NCS), Heart rate variability response to deep breathing (HRV-DB), Thermal perception threshold (TPT), Cold sensation (CS), Warm sensation (WS), Cold induced pain (CIP), Heat induced pain (HIP), Intra epidermal nerve fibre (IENF), Non-contact corneal aesthesiometry (NCCA), Corneal confocal microscopy (CCM), Laser Doppler imaging (LDI), Basement membrane thickness (BMT).
Chapter 3

Results
Section 1

Explanations for the lower prevalence of small fibre neuropathy in South Asian versus European patients with Type 2 diabetes.
Abstract:
OBJECTIVE: Low risk of foot ulceration in South Asian compared with European patients with Type 2 diabetes in the UK has been attributed to lower levels of neuropathy. We have undertaken a detailed study of corneal nerve morphology and neuropathy risk factors, to establish the basis of less small fibre neuropathy in Asians versus Europeans.

RESEARCH DESIGN AND METHODS: In a cross-sectional, population-based study, age- and sex-matched South Asians (n=77) and Europeans (n=78) with type 2 diabetes underwent detailed assessment of neuropathy symptoms and signs, quantitative sensory testing, electrophysiology, autonomic function testing, skin biopsy and corneal confocal microscopy (CCM). Multivariate linear regression analyses were used to determine factors accounting for ethnic differences in small fibre damage.

RESULTS: There was no difference in any measure of large fibre neuropathy, except better sural nerve amplitude in South Asians compared to Europeans. There was also no difference in thermal thresholds or intra epidermal nerve fibre density (IENFD); however, corneal nerve fibre length (22.0±7.9 vs. 19.3±6.3 mm/mm²; P<0.05), corneal nerve branch density (69.8±46.0 vs. 51.8±31.0 no./mm²; P<0.01) and heart rate variability with deep breathing (8.9±5.7 vs. 6.9±3.8; P<0.02), were significantly higher in South Asian vs. European patients. Lower triglyceride levels and less smoking in South Asians best explained the ethnic differences in corneal nerve fibre morphology (P value attenuated to P=0.28).

CONCLUSIONS: South Asians have less small fibre neuropathy than equivalent Europeans with type 2 diabetes. Classic modifiable risk factors for coronary heart disease may provide an explanation for this difference.
3.1 Introduction
Lower limb amputation, as a consequence of foot ulceration, is a major cause of morbidity and is associated with a high mortality in Type 2 diabetes, highlighting the importance of defining mechanisms and risk factors to enable targeted interventions for improving outcomes. It is therefore fascinating that migrant populations of South Asian descent to the UK, with their considerably elevated risk of type 2 diabetes and ischemic heart disease (369, 370), have substantially lower risks of foot ulceration and amputation (3- and 4-fold lower, respectively) compared with Europeans (111, 338, 371). Furthermore, recently the DISTANCE study showed that Asians in general, and South Asians in particular, in the US had a markedly reduced risk of lower extremity amputation (372). The underlying basis for this protection, however, is not clearly understood.

Peripheral neuropathy per se is one of the strongest risk factors for diabetic foot ulceration and amputation (373-376). Of relevance, we have previously shown that lower levels of clinical neuropathy in Asians accounted for approximately half of their reduced risk of foot ulceration (338). Furthermore, in a population-based sample of UK primary care patients with Type 2 diabetes, we demonstrated better large and small fibre function in South Asians vs. Europeans (3). This was largely accounted for by better peripheral vascular function and lower smoking rates in South Asians, rather than differences in glycemic control and hyperlipidaemia.

It is established that small fibre damage precedes large fibre damage in diabetic neuropathy (239, 377). Furthermore, small fibre damage results in loss of pain sensation and autonomic control of cutaneous blood flow (378, 379); both are permissive factors for foot ulceration. Perhaps of relevance, we have previously shown a higher prevalence of painful diabetic neuropathy in South Asians compared to Europeans (380) suggestive of less small fibre damage in this cohort.

Now, we hypothesise that South Asians with Type 2 diabetes will have less small fibre neuropathy than Europeans, as determined by detailed study of corneal nerve fibre structure. Furthermore we will determine which risk factors drive the ethnic difference in small fibre neuropathy.

3.2 Research design and methods
3.2.1 Study Subjects
This is a sub-study of a population-based bi-ethnic cohort of people with type 2 diabetes (n=360), half European and half of South Asian descent, recruited from eight primary care registers in Manchester, UK (3). Patients were selected from within ethnicity, gender and age stratified groups with type 2 diabetes from primary care in Greater Manchester (3). All patients consented that they may be re-contacted for follow-up.

Approximately 4-5 years (2007-2012) after their initial visit, each participant was invited to re-attend The NIHR-Wellcome Trust Clinical Research Facility, Manchester for a single follow-up visit. Exclusion criteria at follow-up were major lower limb amputation, psychiatric disorder, HIV, hepatitis, or terminal illness. Of the 360 subjects, 51 individuals declined to participate, 45 had moved away from the health authority, 81 did not respond and were untraceable and 28 patients had died. One-hundred and fifty-five individuals re-consented to participate in the follow-up study (response rate of 43%), with an equal ethnic split (South Asian n=77, European n=78). Differences between responders (n=155) and non-responders (n=206) were not significant for ethnicity, age, diabetes duration, BMI or HbA1c; however, a higher proportion of men re-consented (consenters - M:F 95:59; non-consenters - M:F 96:110; P<0.01). This study was approved by North Manchester Research Ethics Committee and by the University of Manchester Research Ethics Committee and written informed consent was obtained according to the declaration of Helsinki.

3.3 Clinical assessments
All subjects completed a questionnaire including detailed past medical history, current medication and lifestyle factors, including smoking behaviour. Self-assigned ethnicity was checked and cross validated with the country of birth. All South Asians were first generation migrants from India, Pakistan or Bangladesh. Height, weight, waist and hip circumference were measured once. Triplicate resting blood pressure readings were averaged using a standard protocol (3). Blood samples were assessed in central laboratories for HbA1c, total cholesterol, HDL-cholesterol, triglycerides and fibrinogen.

3.4 Complications assessments
Retinopathy and nephropathy were defined as a history of physician diagnosed disease. Cardiovascular Disease (CVD) was defined as either a physician diagnosed myocardial infarction, angina, or a positive response to the Rose angina questionnaire (381). Peripheral arterial disease was defined as a previous history of physician diagnosed claudication confirmed by a lower limb arteriogram or a positive response to the Edinburgh claudication questionnaire (382). Transcutaneous partial pressure of oxygen (TCpO$_2$) was measured on the dorsum of the left foot (3).

### 3.5 Assessment of neuropathy

Neurological symptoms of muscle weakness, sensation and autonomic neuropathy were evaluated using the Neuropathy Symptoms and Change validated questionnaire score (383) and the McGill pain analogue score was used to assess the severity of painful neuropathic symptoms. Neurological signs were assessed using the modified neuropathy disability score (NDS), evaluating vibration, pin-prick and temperature perception and ankle reflexes (3). Vibration Perception Threshold (VPT) was measured on the tip of the right and left hallux using a Neurothesiometer (Horwell, Scientific Laboratory Supplies, Wilford, Nottingham, UK); mean readings were used for analysis. Electrophysiological assessments of peroneal and sural nerves in the right lower limb were performed by a Neurophysiologist using a Dantec “Keypoint” electromyography system (Dantec Dynamics Ltd, Bristol, UK) with surface electrodes at standardised points (skin temperature $\geq$32°C).

### 3.6 Small fibre function

Cold sensation (A$\delta$ fibre) and warm sensation (C fibre) thresholds were determined with the MEDOC TSA II (Medoc Ltd., Ramat Yishai 30095, Israel) using the method of limits on the dorsum of the left foot, as previously described (351). Changes to heart rate in response to deep breathing (HRV-DB) was determined using the CASE IV system and ECG monitor, averaging two separate cycles of deep breathing (3). Peripheral cholinergic function (sudomotor function) was assessed using the Neuropad, on the plantar skin surface of both feet (269).

#### 3.6.1 Intra epidermal nerve fibre density

A 3-mm punch skin biopsy was taken from the dorsum of the foot and stained with PGP 9.5 to identify and count intra-epidermal nerve fibers and establish intra
epidermal nerve fibre density (IENFD), i.e. the number of fibers per millimeter of basement membrane (351).

3.6.2 Corneal confocal microscopy

Patients underwent examination with the Heidelberg Retina Tomograph (HRT III) in vivo corneal confocal microscope (IVCCM) using our established protocol (384). This test was performed in NIHR-Wellcome Trust Facility by Optometrists. Several scans of the entire depth of the cornea were recorded to provide en face two dimensional images with a lateral resolution of approximately 2 µm/pixel and final image size of 400 x 400 pixels of the sub-basal nerve plexus of the cornea. Five images per patient from the centre of the cornea were selected and examined in a masked and randomised fashion (368). Four corneal nerve parameters were quantified: (i) Corneal nerve fiber density (CNFD) - number of major nerves/mm² of corneal tissue; (ii) Corneal nerve branch density (CNBD) - number of branches emanating from all major nerve trunks/mm² of corneal tissue; (iii) Corneal nerve fiber length (CNFL) - length of all nerve fibers and branches (mm/mm²) within the area of corneal tissue; (iv) tortuosity – a mathematically derived index of the curvilinear deviation from the midline of each main nerve fiber (385). CNFD and CNFL are considered to reflect overall nerve fiber degeneration, whilst CNBD reflects nerve fiber regeneration, which is partially also captured by CNFL.

3.7 Statistical analysis

Neuropathy measures were compared between the ethnic groups as continuous variables using simple means. Normally distributed data were tested using Student’s t test, non-normally distributed data were either log transformed before analysis or the Mann-Whitney test was used. Categorical variables were compared as simple proportions and tested using chi-square test for significance. Multivariate linear regression models were constructed to investigate the effect of potential confounders previously shown to be associated with neuropathy, including glycaemic control, height, smoking, measures of hypertension, obesity and hyperlipidaemia and determine which factors could account for any ethnic differences in neuropathy measures.

The power size of the study was based on Corneal nerve fibre length (CNFL) as the primary outcome. It was calculated that 154 patients (77 in each ethnic group) would
be sufficient with 80% power to detect a mean difference of 3.20 (25.70 versus 22.50) with an SD of 7 at the 5% significance level and effect size of 0.48.

3.8 Results
We studied 155 type 2 diabetes patients (4.9±0.6 years post original visit) comprising 77 South Asians and 78 Europeans. South Asians were younger (P=0.05) and had a longer duration of diabetes (P=0.05) than Europeans (Table 16). South Asians were shorter (P=0.04) and had a lower BMI (P<0.0001). There was no difference in prevalence of clinically evident CVD or retinopathy and nephropathy. There was no difference in systolic and diastolic blood pressure; antihypertensive therapy use was lower in South Asians (P=0.05). Total cholesterol and HDL were comparable but TG levels (P=0.002) were lower in South Asians, despite comparable use of statins. South Asians had a higher HbA1c (P=0.01).

3.8.1 Clinical signs and symptoms
There was no difference in NDS or VPT between Europeans and South Asians (Table 17) and both groups had evidence of mild neuropathy. Painful diabetic neuropathy (significant signs plus at least one positive sensory symptom) did not differ between the groups, however painful neuropathy-like symptoms in the absence of clinical neuropathy (no signs but at least one positive sensory symptom) were more prevalent in South Asians (P<0.05).

3.8.2 Neurophysiology
Peroneal nerve conduction velocity, amplitude and F wave latency did not differ between groups. Sural NCV was comparable but South Asians had a significantly higher sural nerve amplitude (µA) than Europeans (12.3±8.7 vs. 8.4±5.9, P<0.01) (Table 17).

3.9 Small nerve fibre function
Thermal threshold assessments: cold sensation (25.4±4.6 vs. 25.4±4.6), warm sensation (41.6±4.0 vs. 41.7±3.8), cold-induced pain (5.0±6.7 vs. 4.1±5.4) and heat-induced pain (48.2±2.2 vs. 48.4±1.6) did not differ between South Asian and European patients (Table 17). There was no significant difference for the Neuropad response (%) (61.2±34.5 vs. 52.4±35.9) between South Asians and Europeans. Heart rate variability to deep breathing (HRV-DB) (beats/min) (8.9±5.7 vs. 6.9±3.8, P<0.05) was significantly higher in South Asians compared with Europeans.
3.10 Small nerve fibre structure

IENFD did not differ between South Asian and European diabetic patients. CNFD and tortuosity were comparable between the ethnic groups, however both CNFL (mm/mm²) (22.0±7.9 vs. 19.3±6.3; P<0.04) and CNBD (no./mm²) (69.8±46.0 vs. 51.8±31.0; P<0.01) were significantly higher in South Asians than Europeans (Table 17).
Table 16. Demographic and clinical characteristics of ethnic groups.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>South Asians</th>
<th>Europeans</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>( n ) (F/M)</td>
<td>77 (51/26)</td>
<td>78 (45/33)</td>
<td>0.27</td>
</tr>
<tr>
<td>Age (years)</td>
<td>61.2 ± 9.7</td>
<td>64.0 ± 8.0</td>
<td>0.05</td>
</tr>
<tr>
<td>Duration diabetes (years)</td>
<td>13.4 ± 6.7</td>
<td>11.4 ± 5.2</td>
<td>0.05</td>
</tr>
<tr>
<td>Height (m)</td>
<td>1.63 ± 8.7</td>
<td>1.67 ± 8.9</td>
<td>0.04</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>79.5 ± 14.1</td>
<td>95.2 ± 16.9</td>
<td>0.0001</td>
</tr>
<tr>
<td>BMI (kg/m(^2))</td>
<td>29.8 ± 4.7</td>
<td>34.3 ± 5.4</td>
<td>0.0001</td>
</tr>
<tr>
<td>Waist (cm)</td>
<td>103.5 ± 11.1</td>
<td>112.6 ± 12.1</td>
<td>0.0001</td>
</tr>
<tr>
<td>Hips (cm)</td>
<td>103.0 ± 13.0</td>
<td>113.1 ± 12.2</td>
<td>0.0001</td>
</tr>
<tr>
<td>Waist-to-hip ratio</td>
<td>1.0 ± 0.2</td>
<td>1.0 ± 0.1</td>
<td>0.47</td>
</tr>
<tr>
<td>HbA1(_c) (%)</td>
<td>8.2 ± 1.5</td>
<td>7.6 ± 1.4</td>
<td>0.01</td>
</tr>
<tr>
<td>HbA1(_c) (mmol/mol)</td>
<td>66 ± 16.4</td>
<td>60 ± 15.3</td>
<td>0.01</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)</td>
<td>3.9 ± 1.0</td>
<td>4.1 ± 0.9</td>
<td>0.4</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/l)</td>
<td>1.1 ± 0.2</td>
<td>1.1 ± 0.4</td>
<td>0.5</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.5 (1.1, 2.3)</td>
<td>2.1 (1.5, 3.0)</td>
<td>0.002</td>
</tr>
<tr>
<td>Fibrinogen (g/l)</td>
<td>3.7 ± 1.0</td>
<td>3.7 ± 0.8</td>
<td>0.6</td>
</tr>
<tr>
<td>Retinopathy (%)</td>
<td>34</td>
<td>29</td>
<td>0.58</td>
</tr>
<tr>
<td>Nephropathy (%)</td>
<td>15</td>
<td>7</td>
<td>0.16</td>
</tr>
<tr>
<td>Myocardial Infarction (%)</td>
<td>17</td>
<td>13</td>
<td>0.9</td>
</tr>
<tr>
<td>Angina (%)</td>
<td>30</td>
<td>20</td>
<td>0.2</td>
</tr>
<tr>
<td>CABG (%)</td>
<td>19</td>
<td>15</td>
<td>0.6</td>
</tr>
<tr>
<td>Resting Heart Rate (bpm)</td>
<td>75.5 ± 15.0</td>
<td>74.7 ± 11.79</td>
<td>0.7</td>
</tr>
<tr>
<td>Systolic blood pressure</td>
<td>132.5 ± 15.8</td>
<td>136.7 ± 16.9</td>
<td>0.3</td>
</tr>
<tr>
<td>Diastolic blood pressure</td>
<td>72.1 ± 9.8</td>
<td>71.4 ± 8.5</td>
<td>0.2</td>
</tr>
<tr>
<td>TCpO(_2) (mmHg)</td>
<td>62.0 ± 9.9</td>
<td>57.7±12.6</td>
<td>0.02</td>
</tr>
<tr>
<td>PAD (%)</td>
<td>7</td>
<td>20</td>
<td>0.06</td>
</tr>
<tr>
<td>LL angiography (n)</td>
<td>3</td>
<td>9</td>
<td>0.3</td>
</tr>
<tr>
<td>Pack-years smoked (n)</td>
<td>9.7 ± 17.3</td>
<td>20.0 ± 24.8</td>
<td>0.01</td>
</tr>
<tr>
<td>Insulin therapy (%)</td>
<td>20</td>
<td>30</td>
<td>0.23</td>
</tr>
<tr>
<td>Oral antidiabetic drugs (%)</td>
<td>93</td>
<td>91</td>
<td>0.76</td>
</tr>
<tr>
<td>Antihypertensive drugs (%)</td>
<td>65</td>
<td>81</td>
<td>0.05</td>
</tr>
<tr>
<td>Statin therapy (%)</td>
<td>78</td>
<td>89</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Data are % prevalence, mean ± SD, or geometric means (25\(^{th}\), 75\(^{th}\) percentiles).
* P value for ethnic difference. Coronary artery bypass graft (CABG); Transcutaneous partial pressure of oxygen (TCpO\(_2\)); Peripheral arterial disease (PAD); lower limb (LL).
3.10.1 Explanations for ethnic differences in corneal nerve fibre structure

Univariate analyses were used to explore the role of neuropathy risk factors, i.e. glycemic control, height, smoking, vascular function, measures of hypertension, obesity and hyperlipidemia as potential explanations for the ethnic difference in CNFL (Table 18). The variables with the greatest impact on attenuating the P value for the age- and diabetes duration-adjusted ethnic difference in CNFL (p=0.038) were weight (p=0.14), triglyceride levels (p=0.13) and pack-years smoked (p=0.12). There was no appreciable impact of height, glycemia or vascular factors (blood pressure, TCpO_2) on the CNFL model by ethnicity. In multivariate analyses, the combination of variables with the greatest impact on equalising estimated marginal means for CNFL in South Asians vs. Europeans were pack-years smoked and triglyceride levels. Together these two variables attenuated the ethnic differences in nerve structure, changing the age and diabetes duration-adjusted estimated marginal means to 20.9 (18.8-22.9) [95% CI] mm/mm^2 (Asian) and 19.3 (17.3-21.2) mm/mm^2 (European) and the P value to P=0.28 (Table 18). Triglycerides alone approached significance as an independent predictor of CNFL (P=0.047).

To further investigate the effect of smoking, we compared CNFL in never smokers, although numbers were small (P=ns). The ethnic difference did not remain in this sub-group (age and diabetes duration-adjusted estimated marginal means were 20.6 (17.7-23.5 [95% CI]) mm/mm^2 (South Asian n=30) and 21.8 (18.1-25.6) mm/mm^2 (European n=18); P=0.60). Asian ex-smokers, however, retained their high CNFL levels (21.2 (18.4–24.0) mm/mm^2) whereas European ex-smokers had lower CNFL levels (17.8 (15.8-19.9) mm/mm^2, P=0.05), despite the fact that pack-years smoked were very similar between these two sub-groups (P=0.95).

Linear modelling for CNBD by ethnicity showed similar results to CNFL. Variables with the greatest impact accounting for the ethnic difference in CNBD estimated marginal means were pack-years smoked and triglyceride levels, changing the age- and diabetes duration-adjusted means from 71.0 (60.9-81.1) [95% CI] no./mm^2 (Asian) and 51.9 (42.3-62.6) no./mm^2 (European), P=0.009 to 64.6 (53.7-75.5) no./mm^2 (Asian) and 51.8 (41.6-62.0) no./mm^2 (European), P=0.1.
3.10.2 Explanations for ethnic differences in small and large fibre function.

Only confounding factors with large attenuating effects on the South Asians versus Europeans estimated marginal means are shown in Table 19.

Weight had the greatest impact on the ethnic difference in sural nerve amplitude (Table 19). The final model with weight and height accounted for the majority of the ethnic difference in sural nerve amplitude (Asian = 10.7 (9.1-12.2) µA, European = 9.7 (8.2-11.2) µA, P=0.4). There were no additional effects of glycemia, vascular factors or triglyceride differences on the sural nerve amplitude model by ethnicity.

Univariate modelling of HRV-DB by ethnicity showed that adjustments for weight (P=0.31), TCpO\(_2\) (P=0.10) and use of anti-hypertensive therapy (P=0.14) attenuated the ethnic difference. The final model indicated that weight accounted for much of the ethnic variation in HRV-DB, with an additional effect of TCpO2 (Asian = 8.4 (7.3-9.5) beats/min, European = 7.6 (6.5-8.7) beats/min, P=0.38, (Table 19). There was no appreciable impact of height, glycaemia or triglyceride differences on the HRV-DB model by ethnicity.

It is important to note that potential parameters were adjusted for NFL (Table 18), sural nerve amplitude and heart rate variability response to deep breathing (Table 19). To look at which variables explain the South Asian versus European difference in outcomes (CNFL, Sural amplitude, HRV-DB) we can only choose variables which are significantly different between South Asians and Europeans, P≤0.05 (or possibly up to P= 0.1).

If there was no difference in a variable by ethnicity then it cannot possibly be used to explain apparent ethnic difference i.e. CNFL.
Table 17. Measures of neuropathy signs, symptoms, large nerve fibre function, small nerve fibre function, corneal nerve fibre structure and intra epidermal nerve fibre density by ethnicity.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>South Asian</th>
<th>European</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Neuropathy signs</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neuropathy Disability Score (0-10)</td>
<td>3.56±3.09</td>
<td>3.78±2.90</td>
<td>0.68</td>
</tr>
<tr>
<td>Vibration Perception Threshold (Volts)</td>
<td>13.94±9.93</td>
<td>17.77±11.46</td>
<td>0.17</td>
</tr>
<tr>
<td><strong>Neuropathy symptoms</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neuropathy Symptoms and Change (0-38)</td>
<td>6.78±6.45</td>
<td>5.67±5.24</td>
<td>0.3</td>
</tr>
<tr>
<td>McGill Dimension of Pain (0-5)</td>
<td>1.21±1.23</td>
<td>1.15±1.33</td>
<td>0.98</td>
</tr>
<tr>
<td>McGill Visual Analogue Score (0-10)</td>
<td>3.58±3.26</td>
<td>2.92±3.21</td>
<td>0.21</td>
</tr>
<tr>
<td>McGill Total pain rating index (0-60)</td>
<td>10.97±1.06</td>
<td>11.35±11.96</td>
<td>0.97</td>
</tr>
<tr>
<td>Painful neuropathy (%)</td>
<td>40</td>
<td>46</td>
<td>0.4</td>
</tr>
<tr>
<td>Painful symptoms, without neuropathy (%)</td>
<td>41</td>
<td>23</td>
<td>0.02</td>
</tr>
<tr>
<td><strong>Large fibre functional measures</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sural conduction velocity (m/s)</td>
<td>45.1 ± 6.9</td>
<td>45.1 ± 6.8</td>
<td>0.9</td>
</tr>
<tr>
<td>Sural Amplitude (µA)</td>
<td>12.3 ± 8.7</td>
<td>8.4 ± 5.9</td>
<td>0.004</td>
</tr>
<tr>
<td>Peroneal nerve conduction velocity (m/s)</td>
<td>45.3 ± 5.1</td>
<td>44.8 ± 5.6</td>
<td>0.7</td>
</tr>
<tr>
<td>Peroneal nerve Amplitude (mV)</td>
<td>4.4 ± 5.4</td>
<td>3.2 ± 1.8</td>
<td>0.1</td>
</tr>
<tr>
<td>Peroneal nerve F wave latency (m/s)</td>
<td>50.8 ± 7.1</td>
<td>53.1 ± 5.9</td>
<td>0.09</td>
</tr>
<tr>
<td><strong>Small fibre functional measures</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart rate variability deep breathing (bpm)</td>
<td>8.9 ± 5.7</td>
<td>6.9 ± 3.8</td>
<td>0.02</td>
</tr>
<tr>
<td>Neuropad colour change (%)</td>
<td>61.2 ± 34.5</td>
<td>52.4 ± 35.9</td>
<td>0.13</td>
</tr>
<tr>
<td>Cold Sensation threshold (°C)</td>
<td>25.4 ± 4.6</td>
<td>25.4 ± 4.6</td>
<td>0.78</td>
</tr>
<tr>
<td>Warm Sensation threshold (°C)</td>
<td>41.6 ± 4.0</td>
<td>41.7 ± 3.8</td>
<td>0.53</td>
</tr>
<tr>
<td>Cold Induced Pain (°C)</td>
<td>5.0 ± 6.7</td>
<td>4.1 ± 5.4</td>
<td>0.10</td>
</tr>
<tr>
<td>Heat Induced Pain (°C)</td>
<td>48.2 ± 2.2</td>
<td>48.4 ± 1.6</td>
<td>0.17</td>
</tr>
<tr>
<td><strong>Corneal confocal morphometry</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Corneal nerve fibre length (CNFL) (mm/mm²)</td>
<td>22.0 ± 7.9</td>
<td>19.3 ±6.3</td>
<td>0.04</td>
</tr>
<tr>
<td>Corneal nerve fibre density (CNFD) (no/mm²)</td>
<td>24.7 ± 8.2</td>
<td>25.1 ± 7.4</td>
<td>0.75</td>
</tr>
<tr>
<td>Corneal nerve branch density (CNBD) (no/mm²)</td>
<td>69.8 ± 46.0</td>
<td>51.8 ± 31.0</td>
<td>0.01</td>
</tr>
<tr>
<td>Corneal nerve fibre tortuosity coefficient</td>
<td>18.7 ± 4.7</td>
<td>20.1 ± 6.2</td>
<td>0.15</td>
</tr>
<tr>
<td>Intra-epidermal nerve fibre density (IENFD)</td>
<td>3.3 ± 2.0</td>
<td>2.5 ± 1.8</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Data are % prevalence, means ± SD, or geometric means (25th, 75th percentiles). *P value for ethnic difference.
Table 18. Analysis of covariance results for cornea l nerve fibre length by ethnicity.

<table>
<thead>
<tr>
<th>Adjustments factors</th>
<th>South Asian</th>
<th>European</th>
<th>P value *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unadjusted</td>
<td>22.0 (20.2-23.7)</td>
<td>19.3 (17.6-21.0)</td>
<td>0.037</td>
</tr>
<tr>
<td>Age+ diabetes duration</td>
<td>22.1 (20.2-23.9)</td>
<td>19.4 (17.6-21.1)</td>
<td>0.038</td>
</tr>
<tr>
<td>Height (p=0.33)</td>
<td>22.0 (20.1-23.8)</td>
<td>19.5 (17.7-21.3)</td>
<td>0.068</td>
</tr>
<tr>
<td>Weight (p=0.23)</td>
<td>21.8 (19.9-23.7)</td>
<td>19.7 (17.9-21.5)</td>
<td>0.14</td>
</tr>
<tr>
<td>Waist size (p=0.27)</td>
<td>21.8 (19.9-23.7)</td>
<td>19.5 (17.6-21.3)</td>
<td>0.10</td>
</tr>
<tr>
<td>HbA1c (p=0.001)</td>
<td>22.2 (20.4-23.9)</td>
<td>19.3 (17.6-21.0)</td>
<td>0.023</td>
</tr>
<tr>
<td>Triglycerides (p=0.047)</td>
<td>21.7 (19.9-23.6)</td>
<td>19.7 (17.9-21.4)</td>
<td>0.13</td>
</tr>
<tr>
<td>Diastolic BP (p=0.75)</td>
<td>22.1 (20.3-24.0)</td>
<td>19.4 (17.6-21.1)</td>
<td>0.037</td>
</tr>
<tr>
<td>TCpO2 (p=0.42)</td>
<td>22.2 (20.3-24.0)</td>
<td>19.3 (17.5-21.1)</td>
<td>0.03</td>
</tr>
<tr>
<td>Pack-Years Smoked (p=0.21)</td>
<td>21.2 (19.1-23.2)</td>
<td>18.9 (17.0-20.9)</td>
<td>0.12</td>
</tr>
<tr>
<td>Hypertensive therapy (p=0.78)</td>
<td>22.0 (20.1-24.0)</td>
<td>18.9 (17.1-20.8)</td>
<td>0.028</td>
</tr>
<tr>
<td>Weight (p=0.55) + pack years smoked (p=0.25)</td>
<td>21.0 (18.9-23.1)</td>
<td>19.1 (17.1-21.1)</td>
<td>0.24</td>
</tr>
<tr>
<td>Triglycerides (p=0.07) + pack years smoked (p=0.21)</td>
<td>20.9 (18.8-22.9)</td>
<td>19.3 (17.3-21.2)</td>
<td>0.28</td>
</tr>
</tbody>
</table>

* P value for significance of ethnic difference in the regression model after inclusion of specified adjustment factors. P value in parentheses shows significance of adjustment factor in the regression model. Transcutaneous partial pressure of oxygen (TCpO2).
Table 19. Analysis of covariance results for sural nerve amplitude and heart rate variability to deep breathing by ethnicity.

<table>
<thead>
<tr>
<th>Adjustments factors</th>
<th>Estimated marginal means (95% CI)</th>
<th>P value *</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sural nerve amplitude</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unadjusted</td>
<td>11.8 (10.1-13.4)</td>
<td>8.2 (6.6-9.9)</td>
</tr>
<tr>
<td><strong>Adjustment for age and diabetes duration</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age+ diabetes duration</td>
<td>11.9 (10.3-13.5)</td>
<td>8.5 (7.0-10.1)</td>
</tr>
<tr>
<td><strong>Models with age +diabetes duration adjustment and univariate associations with other risk factors:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Height (p&lt;0.0001)</td>
<td>11.3 (9.8-12.8)</td>
<td>9.1 (7.6-10.6)</td>
</tr>
<tr>
<td>Weight (p&lt;0.0001)</td>
<td>10.7 (9.1-12.3)</td>
<td>9.7 (8.1-11.2)</td>
</tr>
<tr>
<td><strong>Multivariate model:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Height (p=0.011) + Weight (p=0.004)</td>
<td>10.7 (9.1-12.2)</td>
<td>9.7 (8.2-11.2)</td>
</tr>
<tr>
<td><strong>Heart rate variability response to deep breathing</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unadjusted</td>
<td>8.9 (7.8-10.1)</td>
<td>6.9 (5.8-8.1)</td>
</tr>
<tr>
<td><strong>Adjustment for age and diabetes duration</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age+ diabetes duration</td>
<td>8.7 (7.7-9.8)</td>
<td>7.2 (6.1-8.3)</td>
</tr>
<tr>
<td><strong>Models with age and diabetes duration adjustment and univariate associations with other risk factors:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Height (p=0.63)</td>
<td>8.7 (7.6-9.8)</td>
<td>7.2 (6.1-8.3)</td>
</tr>
<tr>
<td>Weight (p=0.023)</td>
<td>8.4 (7.3-9.5)</td>
<td>7.6 (6.4-8.7)</td>
</tr>
<tr>
<td>TCpO₂ (p=0.12)</td>
<td>8.7 (7.6-9.7)</td>
<td>7.3 (6.2-8.4)</td>
</tr>
<tr>
<td>Triglycerides (p=0.55)</td>
<td>8.7 (7.6-9.8)</td>
<td>7.2 (6.1-8.4)</td>
</tr>
<tr>
<td>Hypertensive therapy (p=0.004)</td>
<td>8.5 (7.3-9.6)</td>
<td>7.2 (6.1-8.4)</td>
</tr>
<tr>
<td><strong>Multivariate model:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight (p=0.05) and TCpO₂ (p=0.26)</td>
<td>8.4 (7.3-9.5)</td>
<td>7.6 (6.5-8.7)</td>
</tr>
</tbody>
</table>

* P value for significance of ethnic difference in the regression model after inclusion of specified adjustment factors. P value in parentheses shows significance of adjustment factor in the regression model. Transcutaneous partial pressure of oxygen (TCpO₂).
3.11 Conclusion

In this detailed, population-based study, we show that South Asians have a lower prevalence of small fibre and cardiac autonomic neuropathy compared to Europeans with type 2 diabetes in the UK, despite low levels of clinical neuropathy in both ethnic groups. This is the first report of a lower prevalence of small nerve fibre structural abnormalities in South Asian compared to European patients with Type 2 diabetes and provides further evidence for a potential mechanism for the lower incidence of foot ulceration and amputation in South Asians compared to Europeans. We also show that classical risk factors for coronary heart disease, i.e. smoking, body weight and triglyceride levels, accounted for much of the structural differences in small nerve fibres between South Asians and Europeans, with negligible effects, surprisingly, from either vascular or glycemic factors.

Hyperglycemia has consistently been shown to be a strong risk factor for diabetic peripheral neuropathy in epidemiological studies of patients with Type 1 diabetes (386-388). However, in the present study, despite a worse HbA1c in South Asians, there was paradoxically less evidence of particularly small fibre neuropathy. Of relevance, the large Eurodiab study showed that vascular risk factors are as important as glycemic control in the development of diabetic neuropathy in patients with Type 1 diabetes (386). In one of the few longitudinal studies assessing the development of neuropathy in Type 2 diabetes, a higher HbA1c and a lower fasting and post glucose serum insulin and paradoxically, lower systolic and diastolic blood pressure were associated with the development of neuropathy (389). More recently elevated triglycerides have been associated with the development of neuropathy in a cohort of predominantly Type 2 diabetic patients (390). There is now a substantial body of experimental data linking dyslipidemia with neuropathy (391). Furthermore, obesity and hyperlipidaemia have also been shown to be important risk factors for early neuropathy in pre-diabetes (392). Of therapeutic relevance, an improvement in life style measures which led to an improvement in lipids resulted in an increase in IENFD (393). Thus, our findings that lower levels of smoking and triglycerides could explain the greater preservation of corneal nerve fibres in the South Asians are in agreement with these previous studies. Furthermore, the lower weight of the South Asians (body fat measure) was also the main driver of ethnic differences in sural
nerve amplitude (large fibre) and heart rate deep breathing (small, autonomic fibre). Weight and triglycerides are both indicators of adiposity and the imbalanced metabolic environment in obesity (390), and therefore may be proxy for other abnormal metabolic parameters such as oxidative stress or pro-inflammatory cytokines. Given that, a) small fibre damage results in loss of pain sensation and autonomic control of cutaneous blood flow (378, 379) and b) South Asians with Type 2 diabetes have a lower risk of foot ulceration and clinical neuropathy (338) the current detailed study using corneal confocal microscopy provide further evidence for the preservation of small fibres being key to the protection from foot ulceration and eventual amputation. The identification of cardiovascular risk factors attenuating this risk for the development of small fibre neuropathy, provides potentially important therapeutic targets for a major end point of diabetes (394). In addition, these data provide some support for the observations that triglycerides are important predictors of amputation (395) and may help explain the reduced incidence of lower extremity amputation in South Asians (372). It may also provide an explanation for the findings of the FIELD study which showed a remarkable ~50% reduction in minor amputations after 5 years of treatment with Fenofibrate (396). In light of these data, the role of lower triglycerides in reducing the risk of small fibre neuropathy in South Asians demands further evaluation.
Section 2

Differences in neuronal and vascular function and structure between South Asian and Europeans patients with Type 2 diabetes
Abstract

Objective: South Asians have been shown to have a lower incidence of foot ulceration compared to European patients with Type 2 diabetes (T2DM).

Methods: We have undertaken an assessment of neurological and vascular function together with skin biopsy and detailed light and electron- microscopy in a group of European (n=25) and South Asian (n=24) patients with T2DM and healthy volunteers (n=24).

Results: South Asians had a comparable age, HbA1c, cholesterol, HDL and blood pressure, but a longer duration of diabetes (P=0.02), lower BMI (P=0.01) and triglycerides (P=0.01), compared to Europeans. T2DM patients from South Asian and European ethnicity background demonstrated significant differences with control subjects but no difference between each other for the neuropathy symptom profile, neuropathy disability score, thermal threshold, heart rate variability response to deep breathing, intraepidermal nerve fibre density, Corneal nerve fibre density, Corneal branch density, Corneal nerve fibre length and corneal nerve fibre tortuosity. Epidermal thickness was significantly higher in South Asians patients compared to Europeans. The maximal hyperaemic response was significantly lower in Europeans compared to South Asians. There was no other significant difference in vascular density or any other measure of microangiopathy comparing South Asian to European patients with T2DM.

In conclusion differences in the BMI, triglycerides, maximal hyperaemic response and epidermal thickness may account for the protection from foot ulceration in South Asian compared to European patients with T2DM.
3.12 Introduction

People of South Asian (SA) origin have one of the highest risks of diabetes, and in the UK it is four times more common in South Asians than in the general population (1). Although diabetes increases the risk of peripheral vascular disease ten-fold (397), previous studies have shown that the risk of foot ulceration and amputation are three and four-fold, lower in South Asian compared to Europeans with diabetes (111, 398).

The pathophysiological factors involved in the development of foot ulceration in diabetes include neuropathy, microangiopathy and elevated static and dynamic pressure under the feet (399). South Asians have been shown to have a lower prevalence of neuropathy and this has been attributed to vascular differences (3) on the basis that microangiopathy is a major contributory factor in the aetiology of diabetic neuropathy (400). Furthermore, as a consequence of neuropathy, impaired pressure induced vasodilation (401) is a major contributor to the development of tissue ischaemia and ulceration. Several techniques are available to interrogate skin blood flow and oxygenation. Transcutaneous oxygen tension (TCpO₂) is a reliable indicator of the skin nutritional microcirculation and has for many years been used in clinical practice to evaluate the viability of the skin in patients with vascular disorders (402). Microangiopathy may contribute to skin hypoxia and develops early as shown by a reduction in the hyperaemic response in subjects at risk of developing type 2 diabetes (296). These functional alterations may initially be reversible but may become irreversible once structural alterations characteristic of diabetic microangiopathy become established (403). Thus a reduced microvascular hyperaemic response to local heating is present at diagnosis of T2DM, but can improve after improving glycaemic control (404). Heating the skin to a temperature of 44°C has been shown to induce maximal vasodilation (hyperaemic response) (405). Unlike the flare, LDI max is mediated by non-neurogenic means and reflects maximum microvascular hyperaemia (405). Laser Doppler flare, a measure of small fibre dysfunction, has also been shown to be abnormal in subjects with IGT and Type 2 diabetes (214).

Structural alterations such as basement membrane thickening in the capillaries and arterioles of patients with diabetes form the hallmark of diabetic microangiopathy
Abnormal neurogenic regulation of microvascular haemodynamics may contribute to the development of microangiopathy and manifest as increased basement thickening (409). A reduction in hyperaemia may also arise from basement membrane thickening and resultant mechanical restriction of vasodilation (410). Rayman et al showed that the hyperaemic response to skin trauma was impaired in patients with Type 1 diabetes and this was subsequently related to the severity of skin microangiopathy (411). Lamah et al used in vivo microscopy in 21 patients with intermittent claudication and 23 patients with rest pain or ischaemic ulceration and showed that skin capillary density of the foot was significantly reduced in patients with arterial ulceration compared to patients with claudication, and healthy subjects (412). Skin thickness may be a potential confounder when employing techniques such as TCpO₂ and hyperaemic response assessment using laser Doppler. In a study of patients with type 1 diabetes compared with control subjects, epidermal skin thickness did not differ significantly (413). However, a study used high resolution ultrasonography has shown a significant increase in skin thickness at the forearm, thigh and lower limb of diabetic patients with neuropathy compared to diabetic patients without neuropathy (414), and whilst it was not related to the duration of diabetes, age or HbA1c, it was associated with the severity of neuropathy (414). Chao et al, have recently used high-frequency ultrasonography and showed that patients with type 2 diabetes have thicker epidermal plantar skin than their non-diabetic counterparts but epidermal thinning of plantar skin occurred in those with diabetic neuropathy and ulceration, which increases the risk of tissue breakdown and ulceration formation (415).

To date, mechanistic studies have compared diabetic patients with control subjects. However there are no detailed clinico-pathological studies that identify potential underlying mechanisms to explain the protection in the risk of foot ulceration and amputation between South Asian and European patients with Type 2 diabetes.

### 3.13 Research design and methods

#### 3.13.1 Selection of patients

Forty nine (24 South Asian 25 European) patients with T2DM and 25 (11 South Asian and 14 European) control subjects were studied. All diabetic patients who
participated in DAEMON 2 (the results are presented in section 1 of this chapter) were asked to undergo skin biopsy. Control subjects were recruited from the general population who participated in our other studies. Those with a history of a systemic (apart from diabetes for the patient group) neurological condition, ocular trauma, wearing of contact lens and ocular surgery were excluded. This study was approved by North Manchester Research Ethics Committee and by the University of Manchester Research Ethics Committee and written informed consent was obtained according to the declaration of Helsinki.

3.14 Clinical assessment

All subjects completed a questionnaire including a detailed past medical history of medications, and lifestyle factors. Height, weight, waist and hip circumference were measured and triplicate resting blood pressure readings were averaged using a standard protocol. HbA1c, total cholesterol, HDL-cholesterol, triglycerides and fibrinogen were assessed. The full method of these techniques is presented in the methodology chapter.

Retinopathy and nephropathy were defined as a history of physician diagnosed disease. Cardiovascular Disease (CVD) was defined as either a physician diagnosed myocardial infarction, angina, or a positive response to the Rose angina questionnaire (381). Peripheral arterial disease was defined as a previous history of physician diagnosed claudication, confirmed by a lower limb arteriogram or a positive response to the Edinburgh claudication questionnaire (382). All participants filled out these questionnaires.

3.15 Vascular status

The transcutaneous partial pressure of oxygen was assessed on the dorsum of the left foot using a TCpO2 probe and a transcutaneous monitor [Novametrix Medical Systems Inc., CT, USA] (3). Laser Doppler analysis was performed on an area of skin 3-5cm proximal to the 1st and 2nd toes. The area was heated to 44°C using a probe, consisting of a centred metal thermode for 20 minutes and the laser head of the LDI [Moor Instruments Ltd., Axminster, UK] was perpendicularly aligned to the dorsum of the foot at a fixed distance of 45 cm and the flux, which is proportional to tissue blood flow was assessed pre- and post-heating. The region corresponding exactly to the size of heater probe was defined, and the mean flux, expressed in
arbitrary units (Flux), within that region was calculated (LDI max). For the LDI flare the region of interest on the post-image, demarcated by the edge of the flare, was drawn to calculate the area (cm$^2$) of the LDI flare.

3.16 Assessment of neuropathy

All patients and controls underwent a detailed neuropathy evaluation using the Neuropathy Symptom Profile (NSP) and the modified neuropathy disability score (mNDS) 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 as described in chapter of methodology (Chapter 2). Quantitative sensory testing a Neurothesiometer (Horwell, Scientific Laboratory Supplies, Wilford, Nottingham, U.K.) was used to assess cold sensation (CS) (A$\delta$ fibers) and warm sensation (WS) (C fibers) thresholds using the method of limits with the MEDOC TSA II (Medoc, Ramat Yishai, Israel) on the dorsum of the left foot (416). Computer aided sensory evaluation IV (CASE IV) was used to measure the heart rate response to deep breathing during two eight-cycle breathing series, interspersed by a five minute period of normal breathing. The acquired data was analysed by calculating the mean difference between the highest and lowest heart rate for five consecutive, artifact-free cycles in each eight-cycle series (3).

3.17 Corneal confocal microscopy

Patients underwent examination with the Heidelberg Retina Tomograph (HRT III) in vivo corneal confocal microscope (IVCCM) using our established protocol (368). Several scans of the entire depth of the cornea were recorded to provide en face two dimensional images with a lateral resolution of approximately 2 µm/pixel and final image size of 400 x 400 pixels of the sub-basal nerve plexus of the cornea. Five images per patient from the centre of the cornea were selected and examined in a masked and randomised fashion (368). Four corneal nerve parameters were quantified: (i) Corneal nerve fiber density (CNFD) - number of major nerves/mm$^2$ of corneal tissue; (ii) Corneal nerve branch density (CNBD) - number of branches emanating from all major nerve trunks/mm$^2$ of corneal tissue; (iii) Corneal nerve fiber length (CNFL) - length of all nerve fibers and branches (mm/mm$^2$) within the area of corneal tissue; (iv) tortuosity – a mathematically derived index of the curvilinear deviation from the midline of each main nerve fiber (385, 417).
3.18 Biopsy

3.18.1 Intra epidermal nerve fibre density

A 3 mm punch skin biopsy was taken ~ 2 cm proximal to the second metatarsal after local anaesthesia. The specimen was immediately fixed in PBS buffered 4% paraformaldehyde for 18-24 hours, rinsed in TBS and cryoprotected in OCT after immersing in 10-30% sucrose in PBS buffer. Biopsies were placed in frozen OCT mount (Thermo Scientific Raymond Lamb, Walton, MA, USA) and 50µm sections were cut on a cryostat (Model OTF; Bright Instruments, Huntington, UK). The nerve fibres were immunostained using rabbit anti-PGP9.5 antibody (Millipore, Watford, UK; diluted 1:1000 in 5% goat serum (Vector Labs, Burlingame, CA, USA) washed in TBS/Tween 20 and incubated with biotinylated goat anti-rabbit IgG (Vector Labs; 1:300 in 5% goat serum) for 2 hours, washed and incubated with HRP-Avidin D (Vector Labs; 1:300 in TBS) and visualised with SG chromogen (Vector Labs). The immunostained sections were viewed under a Zeiss AxioImager M2 microscope and intra epidermal nerve fibre density (IENFD- number/mm length of epidermis) was calculated according to established criteria (239).

3.18.2 Skin epidermal thickness and microangiopathy

Immediately after biopsy the samples were immersed in 2.5% glutaraldehyde in 1% cacodylate buffer (pH 7.4) at room temperature for 4 hours and then rinsed 6 times and left overnight in cacodylate buffer. The samples were placed in 1% Osmium tetroxide at room temperature for 3 hours, washed 8 times in distilled water and after dehydration placed in Propylene Oxide (Propylene oxide, Agar scientific Ltd, Essex, UK) and then left in 100% Epon (Spurr p/mix kit med 4221D-1, TAAB Laboratories Equipment Ltd, Berks, England) overnight before being placed in an oven for 48 hours at 60°C until the resin polymerised. The hardened resin-embedded tissue blocks were trimmed and sectioned. From each biopsy one suitable section was selected and photographed using a digital Kodak camera attached to a Leica WILD MPS32 photo light microscope (Leica, Cambridge, UK). Capillaries were visualised and counted in the hypodermis and the density was derived after measuring the area of hypodermis. The thickness of the epidermis was measured from each image to quantify 5-8 randomly selected tangential lengths across the epidermis. Ultrathin sections stained with lead citrate were imaged in a Tecnai12 Biotwin transmission electron microscope.
electron microscope (FEI, Eindhoven, The Netherlands). Five to eight dermal capillaries were imaged/biopsy to quantify the basement membrane area, lumen area, endothelial cell area and pericyte profile number/capillary.

3.19 Statistics analysis
Data have been analysed by using SPSS 20.0. Normally distributed data were tested using Student’s t test, non-normally distributed data were either log transformed before analysis or the Mann-Whitney test was used. ANOVA with Scheffe post-hoc tests was used to study differences between means. Categorical variables were compared as simple proportions and tested using chi-square test for significance. Univariate linear regression models were constructed to investigate the effect of potential confounders and determine which factors could account for any ethnic differences in the maximal hyperaemic response.
Post-hoc power calculation was calculated in 24 South Asian and 25 European patients from this study. To find 80% power for a mean difference of 1.31 µm² for lumen area with an SD 3.57, we needed 59 patients for each group to find 5% significance with an effect size of 0.4. Therefore, this morphometric study was underpowered.

3.20 Results
South Asians had a significantly lower height (P=0.006), weight (P=0.0001) and BMI (P=0.01) compared to Europeans. Waist (P=0.02) and hip (P=0.003) circumference were higher in Europeans but the waist hip ratio (WHR) (P=0.2) was comparable between South Asians and Europeans. The duration of diabetes (P=0.02) was shorter in South Asians compared to Europeans. Systolic (P=0.8) and diastolic (P=0.2) blood pressure, HbA1c (P=0.5) total cholesterol (P=0.4), HDL (P=0.8) and fibrinogen (P=0.4) were comparable but triglycerides were significantly lower in South Asians (P=0.01) compared to Europeans with T2DM. There was no significant difference for the prevalence of microvascular and macrovascular complications or the use of medication for lowering glucose, blood pressure or lipids.
<table>
<thead>
<tr>
<th>parameters</th>
<th>Controls (n=25)</th>
<th>South Asian (n=24)</th>
<th>European (n=25)</th>
<th>P value *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs)</td>
<td>58.0±1.0</td>
<td>61.3±8</td>
<td>62.5±8</td>
<td>0.6</td>
</tr>
<tr>
<td>DD (yrs)</td>
<td>-</td>
<td>13.6 ± 6.4</td>
<td>9.97±4.7</td>
<td>0.02</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.74±0.4</td>
<td>7.96±1.3</td>
<td>7.84±1.4</td>
<td>0.5</td>
</tr>
<tr>
<td>HbA1c (mmol/mol)</td>
<td>39.3 ± 4.6</td>
<td>63.5 ± 14.0</td>
<td>61.0 ± 16.3</td>
<td>0.5</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>165.4±9.3</td>
<td>162.8±8.5</td>
<td>169.6±8.1</td>
<td>0.006</td>
</tr>
<tr>
<td>Weight(kg)</td>
<td>76.6±17.6</td>
<td>79.2±12.9</td>
<td>96.9±16.6</td>
<td>0.0001</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>101.9±11.9</td>
<td>104.0±9.9</td>
<td>113.0±11.7</td>
<td>0.02</td>
</tr>
<tr>
<td>Hip circumference (cm)</td>
<td>93.3±14.0</td>
<td>99.9±18.3</td>
<td>114.7±12.3</td>
<td>0.003</td>
</tr>
<tr>
<td>Waist/hip ratio</td>
<td>1.09±0.1</td>
<td>1.10±0.3</td>
<td>1.01±0.1</td>
<td>0.2</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>28.7±6.1</td>
<td>29.9±4.7</td>
<td>33.7±5.5†</td>
<td>0.01</td>
</tr>
<tr>
<td>TG (mmol/l)</td>
<td>1.50(1.27,1.91)</td>
<td>1.70(1.20,2.60)</td>
<td>2.0(1.45,3.75) †</td>
<td>0.01</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>5.20(4.70,5.80)</td>
<td>3.60(3.10,4.30) ¶</td>
<td>4.10(3.60,4.80) ¶</td>
<td>0.4</td>
</tr>
<tr>
<td>HDL (mmol/l)</td>
<td>1.5±0.8</td>
<td>1.08±0.2‡</td>
<td>1.10±0.4‡</td>
<td>0.8</td>
</tr>
<tr>
<td>Fibrinogen (g/l)</td>
<td>-</td>
<td>3.74±1.1</td>
<td>3.54±0.7</td>
<td>0.4</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>135.4±19.3</td>
<td>134.8±16.7</td>
<td>136.0±19.8</td>
<td>0.8</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>75.3±10.8</td>
<td>72.1±9.8</td>
<td>73.4±10.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Retinopathy (%)</td>
<td>-</td>
<td>40</td>
<td>20</td>
<td>0.19</td>
</tr>
<tr>
<td>Nephropathy (%)</td>
<td>-</td>
<td>11</td>
<td>4</td>
<td>0.5</td>
</tr>
<tr>
<td>MI (%)</td>
<td>-</td>
<td>10</td>
<td>12</td>
<td>0.9</td>
</tr>
<tr>
<td>Angina (%)</td>
<td>-</td>
<td>26</td>
<td>20</td>
<td>0.7</td>
</tr>
<tr>
<td>CABG (%)</td>
<td>-</td>
<td>14</td>
<td>8</td>
<td>0.6</td>
</tr>
<tr>
<td>Stroke (%)</td>
<td>-</td>
<td>19</td>
<td>4</td>
<td>0.16</td>
</tr>
<tr>
<td>PAD (%)</td>
<td>-</td>
<td>6</td>
<td>12</td>
<td>0.6</td>
</tr>
<tr>
<td>LL angiography (n)</td>
<td>-</td>
<td>1</td>
<td>1</td>
<td>0.9</td>
</tr>
<tr>
<td>Pack years smoked (n)</td>
<td>-</td>
<td>7.7±12.6</td>
<td>19.8±26.4</td>
<td>0.08</td>
</tr>
<tr>
<td>Insulin therapy (%)</td>
<td>-</td>
<td>12</td>
<td>11</td>
<td>0.9</td>
</tr>
<tr>
<td>Oral anti-diabetic drugs (%)</td>
<td>-</td>
<td>88</td>
<td>95</td>
<td>0.5</td>
</tr>
<tr>
<td>Anti-hypertensive drugs (%)</td>
<td>-</td>
<td>80</td>
<td>63</td>
<td>0.3</td>
</tr>
<tr>
<td>Statin therapy (%)</td>
<td>-</td>
<td>82</td>
<td>83</td>
<td>0.9</td>
</tr>
</tbody>
</table>

Data expressed as Mean ± SD except in the case of skewed distributions where data are expressed as median (25th-75th) percentiles. *P value for ethnic differences. ¶P value ≤ 0.0001, †P value ≤ 0.001, ‡P value ≤ 0.05, between patients and controls. Myocardial infarction (MI), Coronary artery bypass graft (CABG), Peripheral artery disease (PAD), Lower limb (LL)
3.21 Neuropathy assessment

The results of neurological assessments are presented in Table 21. The neuropathy symptom profile (NSP) was significantly greater in South Asian (P=0.0001) and European (P=0.005) T2DM compared to control subjects, but did not differ between South Asians and Europeans with T2DM (P=0.2). The neuropathy disability score was significantly greater in South Asian (P=0.0001) and European (P=0.0001) T2DM compared to control subjects and also did not differ between South Asians and Europeans with T2DM (P=0.7). Cold sensation threshold was significantly greater in South Asian (P=0.001) and European (P=0.01) T2DM compared to control subjects and did not differ between South Asians and Europeans with T2DM (P=0.5). Warm sensation threshold was significantly greater in South Asian (P=0.0001) and European (P=0.0001) T2DM compared to control subjects and did not differ between South Asians and Europeans with T2DM (P=0.9). Cold induced pain threshold was significantly greater in South Asian (P=0.01) and European (P=0.005) T2DM compared to control subjects and did not differ between South Asians and Europeans with T2DM (P=0.6). Heat induced pain threshold was significantly greater in South Asian (P=0.0001) and European (P=0.0001) T2DM compared to control subjects and did not differ between South Asians and Europeans with T2DM (P=0.9). HRV-DB was significantly lower in South Asian (P=0.001) and European (P=0.0001) T2DM compared to control subjects and did not differ between South Asians and Europeans with T2DM (P=0.08). IENFD was significantly lower in South Asian (P=0.001) and European (P=0.001) T2DM compared to control subjects and did not differ between South Asians and Europeans with T2DM (P=0.18). CNFD was significantly lower in South Asian (P=0.0001) and European (P=0.0001) T2DM compared to control subjects and did not differ between South Asians and Europeans with T2DM (P=0.9). CNBD was no different in South Asian (P=0.3) and European (p=0.1) T2DM compared to control subjects and did not differ between South Asians and Europeans with T2DM (P=0.3). CNFL was significantly lower in South Asian (P=0.0001) and European (P=0.0001) T2DM compared to control subjects and did not differ between South Asians and Europeans with T2DM (P=0.6). CNFT was significantly greater in South Asian (P=0.001) and European (P=0.0001).
T2DM compared to control subjects and did not differ between South Asians and Europeans with T2DM (P=0.8).

### 3.22 Vascular function

Vascular function tests are presented in Table 22. Transcutaneous oxygen tension (TCpO$_2$) did not differ between South Asian (P=0.9) and European (P=0.17) T2DM compared to control subjects or between South Asians and Europeans with T2DM (P=0.3). The LDI max did not differ significantly between South Asian T2DM and control subjects (P=0.3) but was significantly higher compared to Europeans (P=0.03) with T2DM. LDI max was significantly different between South Asian and European (P=0.03). LDI flare did not differ between South Asians (P=0.2) and Europeans (P=0.3) with T2DM compared to control subjects nor between South Asians and Europeans with T2DM (P=0.9).

### 3.23 Epidermal thickness and microangiopathy

Epidermal thickness was significantly higher in South Asians compared to Europeans with T2DM (P=0.04). Dermal capillary density did not differ between South Asians and Europeans with T2DM (P=0.16). Dermal capillary basement membrane area (P=0.9), lumen area (P=0.2), endothelial cell area (P=0.4) and pericyte profile no./capillary (P=0.1) did not differ between South Asians and Europeans with T2DM.

### Table 21. Neuropathy assessment for diabetic patients and healthy controls.

<table>
<thead>
<tr>
<th>parameters</th>
<th>Controls (n=25)</th>
<th>South Asian (n=24)</th>
<th>European (n=25)</th>
<th>P value *</th>
</tr>
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<tbody>
<tr>
<td>NSP (0-38)</td>
<td>0.29±0.8</td>
<td>7.88±7.6¶</td>
<td>5.5±5.1¶</td>
<td>0.2</td>
</tr>
<tr>
<td>NDS (0-10)</td>
<td>0.44±0.6</td>
<td>3.26±2.5¶</td>
<td>3.0±2.4¶</td>
<td>0.7</td>
</tr>
<tr>
<td>Cold Threshold (°C)</td>
<td>28.1±1.7</td>
<td>25.5±2.8¶</td>
<td>26.0±2.8¶</td>
<td>0.5</td>
</tr>
<tr>
<td>Warm Threshold (°C)</td>
<td>36.7±3.0</td>
<td>41.4±2.8¶</td>
<td>41.3±3.6¶</td>
<td>0.9</td>
</tr>
<tr>
<td>Cold Pain Threshold (°C)</td>
<td>11.0±8.1</td>
<td>5.84±5.6‡</td>
<td>4.6±5.1‡</td>
<td>0.6</td>
</tr>
<tr>
<td>Heat Pain Threshold (°C)</td>
<td>44.6±2.7</td>
<td>48.4±1.9¶</td>
<td>48.4±1.5¶</td>
<td>0.9</td>
</tr>
<tr>
<td>IENFD (no./mm)</td>
<td>9.1±1.1</td>
<td>3.4±2.0¶</td>
<td>2.6±1.9¶</td>
<td>0.18</td>
</tr>
<tr>
<td>HRV-DB (beats/min)</td>
<td>14.4±5.8</td>
<td>8.5±5.1†</td>
<td>6.4±2.2†</td>
<td>0.08</td>
</tr>
<tr>
<td>NFD (no./mm$^2$)</td>
<td>36.9±6.6</td>
<td>24.7±6.9¶</td>
<td>27.1±8.5¶</td>
<td>0.9</td>
</tr>
<tr>
<td>NBD (no./mm$^2$)</td>
<td>88.8±45.2</td>
<td>72.5±36.7</td>
<td>63.8±31.1</td>
<td>0.3</td>
</tr>
<tr>
<td>NFL (mm/mm$^2$)</td>
<td>25.9±6.3</td>
<td>22.2±6.7¶</td>
<td>20.3±8.8¶</td>
<td>0.6</td>
</tr>
<tr>
<td>NFT (TC)</td>
<td>15.5±3.9</td>
<td>20.2±3.4¶</td>
<td>17.7±5.1¶</td>
<td>0.8</td>
</tr>
</tbody>
</table>

* P value for ethnic differences. ¶≤0.0001, †≤0.001, ‡≤0.05, between patients and controls. Neuropathy symptom profile (NSP), Neuropathy disability score (NDS), Intra epidermal fibre density (IENFD), Heart rate variability response to deep breathing (HRV-DB), Nerve fibre density (NFD), Nerve branch density (NBD), Nerve fibre length (NFL), Nerve fibre tortuosity (NFT).
Table 22. Vascular and neural assessment of diabetic patients and healthy control subjects.

| parameters                        | Controls (n=25) | South Asian (n=24) | European (n=25) | P value  
|-----------------------------------|----------------|--------------------|-----------------|-----------
| TCpO₂ (mmHg)                      | 61.2±9.8       | 59.9±10.9          | 55.1±12.1       | 0.3       
| Maximal hyperaemic response (flux)| 620.1±204.7    | 582.2±263.1        | 451.6±149.7†    | 0.03      
| LDI Flare area (cm²)              | 0.72(0.54,1.36)| 0.48 (0.31,0.88)   | 0.55(0.38,1.0)  | 0.9       
| Epidermal thickness (µm)          | -              | 40.8(36.98,47.40)  | 35.8(27.90,45.27)| 0.04      
| Capillary density (no.mm²)        | -              | 29.5(26.24,34.87)  | 28.6(22.07,31.71)| 0.16      
| Lumen area (µm²)                  | -              | 7.44±4.3           | 6.13±2.8        | 0.2       
| Endothelial cell area (µm²)       | -              | 28.4±10.7          | 26.3±9.4        | 0.4       
| Basement membrane area (µm²)      | -              | 109.3±33.1         | 109.8±34.4      | 0.9       
| Pericyte profile number (no.)     | -              | 3.68±1.2           | 4.37±1.5        | 0.1       

* P value for ethnic differences. †≤0.001 between patients and controls. Transcutaneous partial pressure oxygen (TCpO₂), Laser Doppler imaging (LDI).
### 3.24 Explanation for ethnic differences in maximal hyperaemic response

Weight, smoking and duration of diabetes had no impact on the ethnic differences for the maximal hyperaemic response. After adjustment for duration of diabetes, height and PAD were the only factors which attenuated the P values for the difference in maximal hyperaemic response between South Asians and Europeans with T2DM Table 23. Antihypertensive therapy, lower limb angiography, oral antidiabetic drugs, statin and insulin therapy, had no impact for differences in maximal hyperaemic response.

<table>
<thead>
<tr>
<th>Estimated Marginal means for maximal hyperaemic response (95% CI)</th>
<th>South Asian</th>
<th>European</th>
<th>P value *</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Maximal Hyperaemic response</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unadjusted</td>
<td>582.2(494.8,669.6)</td>
<td>451.6(365.9,537.3)</td>
<td>0.037</td>
</tr>
<tr>
<td><strong>Adjustment for different duration of diabetes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duration of diabetes</td>
<td>596.4(503.9,688.9)</td>
<td>439.8(351.2,528.4)</td>
<td>0.02</td>
</tr>
<tr>
<td><strong>Models adjusted for duration of diabetes and univariate associations with other risk factors</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Height</td>
<td>593.3(493.6,693.0)</td>
<td>442.7(347.7,537.7)</td>
<td>0.05</td>
</tr>
<tr>
<td>Weight</td>
<td>621.5(521.5,721.4)</td>
<td>416.7(321.6,511.8)</td>
<td>0.009</td>
</tr>
<tr>
<td>BMI</td>
<td>609.8(515.6,704.0)</td>
<td>427.5(337.5,517.4)</td>
<td>0.01</td>
</tr>
<tr>
<td>Waist circumference</td>
<td>630.1(536.7,724.0)</td>
<td>420.7(333.2,508.2)</td>
<td>0.003</td>
</tr>
<tr>
<td>Hip circumference</td>
<td>624.2(522.4,726.0)</td>
<td>457.3(355.4,559.0)</td>
<td>0.034</td>
</tr>
<tr>
<td>TG</td>
<td>606.8(508.3,705.2)</td>
<td>441.2(349.5,532.9)</td>
<td>0.02</td>
</tr>
<tr>
<td>Epidermal thickness</td>
<td>606.5(506.3,706.6)</td>
<td>435.8(345.6,526.6)</td>
<td>0.02</td>
</tr>
<tr>
<td>Capillary density</td>
<td>600.2(505.2,695.2)</td>
<td>436.3(345.5,527.1)</td>
<td>0.02</td>
</tr>
<tr>
<td>Lumen area</td>
<td>604.7(511.8,697.6)</td>
<td>445.5(358.9,532.1)</td>
<td>0.02</td>
</tr>
<tr>
<td>Pericyte number</td>
<td>611.7(516.8,706.5)</td>
<td>439.3(350.9,527.8)</td>
<td>0.014</td>
</tr>
<tr>
<td>Pericyte area</td>
<td>617.3(523.4,711.2)</td>
<td>434.3(346.6,522.1)</td>
<td>0.008</td>
</tr>
<tr>
<td>Weight</td>
<td>621.5(521.5,721.4)</td>
<td>416.7(321.6,511.8)</td>
<td>0.009</td>
</tr>
<tr>
<td>PAD</td>
<td>581.9(466.2,697.7)</td>
<td>433.0(342.9,523.2)</td>
<td>0.05</td>
</tr>
<tr>
<td>Antihypertensive therapy</td>
<td>670.0(548.9,791.2)</td>
<td>436.4(327.1,545.8)</td>
<td>0.01</td>
</tr>
<tr>
<td>LL angiography</td>
<td>604.6(507.8,701.5)</td>
<td>417.7(323.8,512.1)</td>
<td>0.009</td>
</tr>
<tr>
<td>Oral antidiabetic drugs</td>
<td>649.9(537.0,762.9)</td>
<td>453.3(350.8,555.8)</td>
<td>0.019</td>
</tr>
<tr>
<td>Statin therapy</td>
<td>663.0(547.8,778.3)</td>
<td>439.3(331.2,547.4)</td>
<td>0.01</td>
</tr>
<tr>
<td>Insulin therapy</td>
<td>603.7(509.8,697.7)</td>
<td>462.6(368.6,556.5)</td>
<td>0.04</td>
</tr>
</tbody>
</table>

*P value for ethnic difference between South Asian and European with type 2 diabetes. Body mass index (BMI), Triglyceride (TG), Peripheral artery disease (PAD), Lower limb angiography (LL).
3.25 Conclusion

Previous studies have reported a lower rate of foot ulceration and amputation in South Asians compared to Europeans with Type 2 diabetes (111, 398). In a large cohort of matched South Asian and European diabetic patients we have previously attributed this to lower rates of neuropathy in South Asians (3). Long term outcomes such as foot ulceration and amputation are determined by both neuropathy and vascular function (418). Therefore in the present study we have undertaken a detailed study to establish if differences in neuropathy and vascular function and capillary ultrastructure are already present in the early stages of disease in South Asians and Europeans with Type 2 diabetes.

We show that both South Asian and European patients with T2DM have evidence of mild neuropathy as evidenced by a significant abnormality in neuropathic symptoms, neurological deficits, warm and cold thresholds, cardiac autonomic function, corneal and intraepidermal nerve fibre morphology compared to control subjects. However, there was no significant difference between South Asian and European patients with T2DM for any parameter including the highly sensitive measures of small fibre neuropathy assessed using CCM, LDI flare or IENFD (419-421).

With regard to tissue perfusion and oxygenation whilst TCpO$_2$ was lower in diabetic patients it did not differ between South Asian and European patients with T2DM. The maximal hyperaemic response, a measure of the microvascular response to tissue injury (422, 423), was lower in diabetic patients but was better in South Asians, providing a potential mechanism of protection from injury and hence foot ulceration. Hence the response to tissue injury may become critical in those at higher risk of foot ulceration. This higher maximal hyperaemic response in South Asians was accounted for by a lower prevalence of PAD in combination with height. Height has been associated with neuropathy in a previous study (340) and of course the presence of PAD identifies those at higher risk of failure to heal an ulcer and subsequent amputation (424, 425). Small fibre neuropathy is thought to be more strongly associated with the development of foot ulceration than large fibre neuropathy (426). Weight (427) and smoking (340) are risk factors for diabetic neuropathy and both were lower in South Asian compared to European patients with T2DM.
Alterations to the structure of the underlying skin such as epidermal thickness, vascular density and microangiopathy may influence the interpretation of these aforementioned physiological tests of tissue perfusion and oxygenation. We show an increased epidermal thickness in South Asians compared to Europeans which represents a potential barrier to both TCpO$_2$ and blood flux measurements when using laser Doppler (428). Despite this both responses were better in South Asians, although in multiple regression analysis, epidermal thickness did not contribute to the overall differences in hyperaemic response or TCpO$_2$ between South Asians and Europeans. However, of relevance to ulceration a thicker epidermis may of course provide protection from pressure-induced damage, although we have assessed this from a biopsy on the dorsum of the foot and therefore cannot necessarily extrapolate this finding to the plantar aspect.

Structural alterations in the microvasculature may contribute to poorer skin blood flow and also explain the differences in TCpO$_2$ and the hyperaemic response. A lower capillary density has been reported in diabetic patients and rodents (429, 430). However, in the present study we find no significant difference in skin capillary density between South Asians and Europeans. Furthermore a range of ultra-structural alterations have been reported in the skin capillaries of diabetic patients including increased basement membrane thickness which was related to the severity of complications (423). A reduction in the capillary lumen area has also been shown in diabetic patients (423, 431) and predicts future deterioration in glucose tolerance (431) and the development of diabetes (136, 432). In the present study skin capillary lumen area was no different between South Asian compared to European patients with Type 2 diabetes. Thickening of the capillary basement membrane is the hallmark of diabetic microangiopathy (132). It has been associated with increased vascular permeability and impaired auto regulation of vascular tone (128) and occurs at a greater rate in poorly controlled patients with diabetes (433). In the present study despite moderate glycaemic control, capillary basement membrane area was comparable between South Asians and Europeans.

We observe some trends for differences, which may be relevant in the long term but may also reflect a lack of power of the present study. One may question the clinical relevance of the small differences we have observed between South Asian and
European patients with T2DM. However, these patients had a relatively short duration of diabetes and mild neuropathy, therefore any differences in terms of protection may become more relevant as neuropathy progresses and the risk of foot ulceration increases.
Section 3

Longitudinal assessment of change in neuropathy in South Asian compared to Europeans patients with type 2 diabetes: A 5 years follow-up study.
Abstract

Objectives: The prevalence of diabetic neuropathy, foot ulceration and amputation is lower in South Asian compared to European patients with diabetes in the UK. There is no study to show the progress of peripheral neuropathy between these two groups. The aim of present study was to assess the development and progression of diabetic neuropathy in relation to risk factors for this condition.

Methods: Neuropathy was assessed in 155 type 2 diabetic patients (T2DM) (77 South Asian and 78 European) at baseline (2000 to 2005) and at follow-up (2007 to 2012), with a mean ± SD follow-up of 4.9±0.6 years. All patients underwent a standardized assessment of neuropathic symptoms and deficits, quantitative sensory testing, autonomic-function testing and neurophysiology.

Results: There was no difference in the rate of progression of NSP, NDS, peroneal nerve conduction and amplitude, sural nerve conduction velocity and HRV-DB between South Asians and Europeans with T2DM over 5 years. However, the progression of neuropathy assessed with VPT and sural amplitude showed a slower worsening in South Asians compared to Europeans. Multivariate regression analysis show that lower weight, less cigarette smoking, lower triglycerides and alcohol consumption accounted for the lower rate of deterioration of neuropathy in South Asians compared to Europeans with T2DM.

Conclusion: This prospective study shows that the prevalence of neuropathy is lower and it progresses more slowly in South Asians compared to Europeans with T2DM, due to better cardiovascular risk factors.
3.26 Introduction

Diabetic polyneuropathy is one of the most common long-term complications of diabetes and underlies the development of painful neuropathy in ~21% of patients with diabetes (380). Longitudinal studies of diabetic neuropathy are limited but do show a progressive deterioration over time in patients with type 1 (155) and type 2 diabetes (434) and the placebo arm of several clinical trials in diabetic neuropathy show a monotonic worsening in electrophysiology and quantitative sensory testing (435).

Type 2 diabetes is 3-6 times (436) commoner in South Asians compared to Europeans (398) and it develops approximately 10 years earlier in South Asians compared to Europeans (437). Our previous studies have shown a lower prevalence of diabetic neuropathy and foot ulceration in cross-sectional studies of South Asians compared to Europeans with T2DM (3, 398). To our knowledge, there are no longitudinal studies comparing the natural history of neuropathy in South Asian and European diabetic patients in the UK. We hypothesised that there is no difference in the rate of changes of nerve function in South Asian and European patients with T2DM. Therefore, the aim of the present study was to assess the longitudinal change in neuropathy in relation to the risk factors which are known to influence the development and progression of neuropathy.

3.27 Design of study

We conducted a longitudinal cohort study of a population based sample of 78 European and 77 South Asians with T2DM originally assessed from DAEMON-1 (Diabetes in Asian and European Manchester study of Neuropathy). DAEMON 1 patients were originally selected from primary care after stratification for ethnicity and 5 year age groups of people with type 2 diabetes in Manchester, UK and studied between 2000-2005. For the longitudinal assessment, patients were re-invited and attended between 2007-2012 (DAEMON-2).

All original patients (n=360) were targeted to receive an invitation for a single follow-up visit because of the high expected drop-out rate. Out of this targeted group, 51 patients refused to attend, 28 patients died, 45 patients were untraceable and 81 patients did not respond. There were no differences between those who did and did
not participate in the follow up study for age, duration of diabetes and HbA1c, but there were fewer female participants in the follow up study.

Patients were assessed using a standardized protocol for neuropathic symptoms, signs, autonomic function and neurophysiology with a mean follow up of ~5 years.

**3.28 Clinical assessment**

All 155 patients underwent a detailed evaluation of demographic data and medical history including concurrent medication and lifestyle factors. Neurological deficits were assessed using the modified neuropathy disability score (NDS) which includes evaluation of vibration, pin prick, temperature perception as well as the presence or absence of ankle reflexes to establish the severity of neuropathy. Vibration perception threshold (VPT) was assessed at the right and left great toes in duplicate using a calibrated neuroaesthesiometer (Horwell; scientific Laboratory supplies, Nottingham, UK.) (438). The Computer-Aided sensory evaluator (CASE IV), (WR Medical Electronics, Inc, Stillwater, MN, USA) was used to measure heart rate variability response to deep breathing. The patient was asked to inhale and exhale deeply eight times in a row in the supine position while following the rhythm of a breathing cue and the changes in heart rate were displayed on an ECG monitor. Changes to heart rate in response to deep breathing (HRV-DB) was determined, averaging two separate cycle of deep breathing (3). Electrophysiological assessment of the peroneal and sural nerves was performed using a Dantec Keypoint system (Dantec Dynamics, Bristol, U.K.) equipped with a Dansk Industry Syndikat temperature regulator to keep limb temperature constantly, between 32°C and 35°C. Peroneal motor and sural sensory nerves were assessed in the left lower limb by a neurophysiologist. The motor study was performed using silver-silver chloride surface electrodes at standardized sites defined by anatomical landmarks, and recordings for the sural nerve were taken using antidromic stimulation over a distance of 100 mm (3, 351). The full techniques are presented in chapter 2.

**3.29 Statistical analyses**

SPSS for windows 20.0 was used to compute the results. Normally distributed data were tested using Student’s t test, non-normally distributed data were either log transformed before analysis or the Mann-Whitney test was used. Multivariate linear regression models were used to explore the role of potential confounders. Data are
presented as Mean ± SD. A $P$ value of less than 0.05 was considered statistically significant.

A Post-hoc power calculation indicated that we required 78 patients in each group (156 in total) based on equal variances for VPT and an estimated common SD of 10.64. The study had 80% power to detect a mean group difference of 4.8 or greater with an effect size of 0.45.

3.30 Results

3.30.1 Change in clinical demographics

There was no significant change in BMI, hip circumference, HbA1c, TG, systolic blood pressure in South Asians. Significant changes were observed in South Asian from baseline to follow up visit in weight ($P=0.02$), waist ($P=0.0001$), waist/hip ratio ($P=0.002$), cholesterol ($P=0.0001$), HDL ($P=0.0001$) and diastolic blood pressure ($P=0.004$). European patients also had significant differences for weight ($P=0.03$), BMI ($P=0.03$), waist ($p=0.0001$), hip circumference ($p=0.0001$), waist/hip ratio ($P=0.004$), cholesterol ($P=0.0001$), HDL ($P=0.0001$) and diastolic blood pressure ($P=0.0001$). Triglycerides and HbA1c did not show a significant difference in the follow up visit compared to baseline for both South Asians and Europeans.

3.30.2 Change in neuropathy evaluation

South Asians showed a significant increase in NSP ($P=0.0001$), NDS ($P=0.0001$), VPT ($P=0.002$), HRV-DB ($P=0.001$) and sural nerve amplitude ($P=0.03$), sural nerve conduction ($P=0.001$). Similarly, Europeans had significant differences for NDS ($P=0.0001$), VPT ($P=0.0001$), HRV-DB ($P=0.001$), sural amplitude ($P=0.0001$), sural nerve conduction ($P=0.0001$) in the follow up visit. Peroneal amplitude and conduction velocity did not change significantly in both cohorts.
Table 24. Demographics and neuropathy measurements at the baseline and follow up visit in South Asian and European patients with type 2 diabetes.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Baseline South Asian</th>
<th>Follow up South Asian</th>
<th>P value Baseline v follow-up (Asian)</th>
<th>Baseline European</th>
<th>Follow up European</th>
<th>P-value Baseline v follow up (European)</th>
<th>p-value Baseline (South Asian v European)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs)</td>
<td>55.9±10</td>
<td>61.2±10</td>
<td>-</td>
<td>58.9±8.0</td>
<td>64.0±8.0†</td>
<td>-</td>
<td>0.05</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>162.8±9.0</td>
<td>163.0±10.0</td>
<td>0.6</td>
<td>166.0±8.41</td>
<td>166.0±8.9†</td>
<td>0.9</td>
<td>0.02</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>78.3±14.04</td>
<td>79.9±14.0</td>
<td>0.02</td>
<td>91.2±15.8</td>
<td>93.0±17.0†</td>
<td>0.03</td>
<td>0.0001</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>29.4±4.7</td>
<td>30.0±5.2</td>
<td>0.1</td>
<td>32.9±4.8</td>
<td>33.7±5.5†</td>
<td>0.03</td>
<td>0.0001</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>95.9±12.9</td>
<td>103.5±11.0</td>
<td>0.0001</td>
<td>103.8±12.2</td>
<td>112.7±12.2†</td>
<td>0.0001</td>
<td>0.0001</td>
</tr>
<tr>
<td>Hip circumference (cm)</td>
<td>101.4±9.7</td>
<td>103.0±13.0</td>
<td>0.3</td>
<td>106.7±9.4</td>
<td>113.0±12.1†</td>
<td>0.0001</td>
<td>0.003</td>
</tr>
<tr>
<td>Waist/hip ratio</td>
<td>0.94±0.1</td>
<td>1.0±0.2</td>
<td>0.002</td>
<td>0.97±0.1</td>
<td>1.0±0.1</td>
<td>0.004</td>
<td>0.05</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>8.7±1.8</td>
<td>8.2±1.5</td>
<td>0.9</td>
<td>7.9±1.3</td>
<td>7.6±1.4†</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>HbA1c (mmol/mol)</td>
<td>71.6±17.0</td>
<td>66.1±16.4</td>
<td>0.9</td>
<td>62.8±15.0</td>
<td>59.6±15.8†</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>TG (mmol/l)</td>
<td>1.6(1.1,2.3)</td>
<td>1.5(1.1,2.3)</td>
<td>0.07</td>
<td>1.85(1.3,2.7)</td>
<td>2.1(1.5,3.0)†</td>
<td>0.07</td>
<td>0.05</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>4.4±0.91</td>
<td>3.9±1.0</td>
<td>0.0001</td>
<td>4.61±1.06</td>
<td>4.1±0.9</td>
<td>0.0001</td>
<td>0.1</td>
</tr>
<tr>
<td>HDL (mmol/l)</td>
<td>1.2±0.2</td>
<td>1.06±0.2</td>
<td>0.0001</td>
<td>1.26±0.32</td>
<td>1.1±0.4</td>
<td>0.0001</td>
<td>0.1</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>135.0±17.8</td>
<td>133.0±16.2</td>
<td>0.2</td>
<td>138.4±15.9</td>
<td>135.7±16.6</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>75.5±11.5</td>
<td>72.1±9.8</td>
<td>0.004</td>
<td>75.87±11.8</td>
<td>70.4±9.3</td>
<td>0.0001</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>P value</td>
<td>Mean ± SD</td>
<td>P value</td>
<td>Mean ± SD</td>
<td>P value</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td>--------------------------</td>
<td>-------------</td>
<td>---------</td>
<td>-------------</td>
<td>---------</td>
<td>-------------</td>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td><strong>Diabetes Duration (yrs)</strong></td>
<td>8.5±6.7</td>
<td>0.0001</td>
<td>13.4±6.7</td>
<td>0.0001</td>
<td>6.5±5.2</td>
<td>0.0001</td>
<td>11.4±5.2†</td>
</tr>
<tr>
<td><strong>NSP (0-38)</strong></td>
<td>1.98±1.96</td>
<td>0.0001</td>
<td>6.78±6.45</td>
<td>0.0001</td>
<td>1.96±2.25</td>
<td>0.0001</td>
<td>6.0±5.5</td>
</tr>
<tr>
<td><strong>NDS (0-10)</strong></td>
<td>2.5±2.6</td>
<td>0.0001</td>
<td>3.6±3.1</td>
<td>0.0001</td>
<td>2.5±2.63</td>
<td>0.0001</td>
<td>3.8±2.90</td>
</tr>
<tr>
<td><strong>VPT (v)</strong></td>
<td>12.8±9.87</td>
<td>0.002</td>
<td>15.3±9.97</td>
<td>0.0001</td>
<td>12.7±9.9</td>
<td>0.0001</td>
<td>17.3±11.3</td>
</tr>
<tr>
<td><strong>HRV-DB (bpm)</strong></td>
<td>11.5±7.9</td>
<td>0.001</td>
<td>8.9±5.7</td>
<td>0.001</td>
<td>8.3±3.4</td>
<td>0.001</td>
<td>6.9±3.7†</td>
</tr>
<tr>
<td><strong>Sural amplitude (µA)</strong></td>
<td>13.6±9.4</td>
<td>0.03</td>
<td>12.3±8.7</td>
<td>0.001</td>
<td>11.1±6.6</td>
<td>0.0001</td>
<td>8.4±5.7†</td>
</tr>
<tr>
<td><strong>Sural. SNCV (m/s)</strong></td>
<td>41.7±6.55</td>
<td>0.001</td>
<td>45.1±6.9</td>
<td>0.0001</td>
<td>42.1±5.2</td>
<td>0.0001</td>
<td>45.7±6.0</td>
</tr>
<tr>
<td><strong>Peroneal amplitude (mV)</strong></td>
<td>3.3±2.11</td>
<td>0.2</td>
<td>4.4±5.4</td>
<td>0.2</td>
<td>3.09±1.7</td>
<td>0.3</td>
<td>3.2±1.8</td>
</tr>
<tr>
<td><strong>Peroneal MNCV (m/s)</strong></td>
<td>44.6±5.6</td>
<td>0.9</td>
<td>44.6±5.6</td>
<td>0.2</td>
<td>43.6±5.9</td>
<td>0.2</td>
<td>44.8±5.6</td>
</tr>
<tr>
<td><strong>Alcohol intake (unit/day)</strong></td>
<td>1.1±2.7</td>
<td>0.04</td>
<td>0.5±1.7</td>
<td>0.0</td>
<td>4.2±3.8</td>
<td>0.08</td>
<td>3.9±5.43</td>
</tr>
<tr>
<td><strong>Insulin therapy (%)</strong></td>
<td>17</td>
<td>0.05</td>
<td>20</td>
<td>0.9</td>
<td>17</td>
<td>0.05</td>
<td>30</td>
</tr>
<tr>
<td><strong>Oral antidiabetic drugs (%)</strong></td>
<td>87</td>
<td>0.5</td>
<td>93</td>
<td>0.1</td>
<td>77</td>
<td>0.5</td>
<td>91</td>
</tr>
<tr>
<td><strong>Statin therapy (%)</strong></td>
<td>53</td>
<td>0.01</td>
<td>78</td>
<td>0.08</td>
<td>71</td>
<td>0.01</td>
<td>89</td>
</tr>
<tr>
<td><strong>Antihypertensive (%)</strong></td>
<td>65</td>
<td>0.9</td>
<td>65</td>
<td>0.9</td>
<td>80</td>
<td>0.9</td>
<td>81†</td>
</tr>
<tr>
<td><strong>Pack- years smoked (n)</strong></td>
<td>9.7±18.3</td>
<td>0.3</td>
<td>9.74±17.3</td>
<td>0.3</td>
<td>21.9±33.1</td>
<td>0.3</td>
<td>19.9±24.8</td>
</tr>
</tbody>
</table>

† P value ≤0.05 for follow up ethnic differences. (table 16 and 17 in chapter 3). List of Abbreviations: Blood Pressure (BP), Neuropathy Symptoms Profile (NSP), Neuropathy disability score (NDS), Vibration perception threshold (VPT), Body mass index (BMI), Motor Nerve Conduction Velocity (MNCV), Sensory Nerve Conduction Velocity (SNCV), Heart Rate Variability response to Deep Breathing (HRV-DB)
3.30.3 Absolute difference in neuropathy during follow up

There was no difference in the absolute change in NSP, NDS peroneal and sural nerve conduction velocity and peroneal amplitude between South Asians and Europeans with T2DM. There was a significant difference in the change in vibration perception threshold (P=0.01) between South Asians and Europeans. The VPT increased 1 volts/year in Europeans and 0.4 volts/year in South Asians. The rate of decline in sural nerve amplitude was -0.5 µv/year in Europeans and -0.3 µv/year in South Asians. Given that significant difference in variables such as age, duration of diabetes, alcohol intake, smoking, antihypertensive drugs between South Asians and Europeans were observed at baseline (Table 24), an adjustment for baseline factors was performed and are presented in Table 26.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>South Asians</th>
<th>Europeans</th>
<th>P value *</th>
</tr>
</thead>
<tbody>
<tr>
<td>NDS diff</td>
<td>1.1±2.1</td>
<td>1.3±2.0</td>
<td>ns</td>
</tr>
<tr>
<td>VPT diff</td>
<td>1.9±6.1</td>
<td>4.6±6.8</td>
<td>0.01</td>
</tr>
<tr>
<td>NSP diff</td>
<td>5.0±6.2</td>
<td>4.3±5.1</td>
<td>ns</td>
</tr>
<tr>
<td>HRV-DB diff</td>
<td>-2.9±5.7</td>
<td>-1.5±3.6</td>
<td>ns</td>
</tr>
<tr>
<td>Peroneal NCV diff</td>
<td>0.04±4.4</td>
<td>0.7±4.5</td>
<td>ns</td>
</tr>
<tr>
<td>Peroneal amplitude diff</td>
<td>0.8±1.9</td>
<td>0.6±1.4</td>
<td>ns</td>
</tr>
<tr>
<td>Sural NCV diff</td>
<td>3.5±8.6</td>
<td>3.6±7.5</td>
<td>ns</td>
</tr>
<tr>
<td>Sural Amplitude diff</td>
<td>-1.7±6.4</td>
<td>-2.7±4.1</td>
<td>0.004</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SD. * P value for ethnic differences.

3.31 Explanation for less deterioration in large fibre function

Confounding factors with effect on the South Asian versus European estimated marginal means are shown in Table 26. After adjustment for age and duration of diabetes at baseline, the final model was weight in combination with smoking having the greatest impact for the difference in VPT between South Asians and Europeans (P=0.23). A lower TG in combination with less alcohol consumption in South Asians attenuated the P value for the sural nerve amplitude (P=0.18). Weight in combination with alcohol intake also had reduced the p value for sural nerve amplitude between South Asians and Europeans (P=0.2).
Table 26. Analysis of covariance results for vibration perception threshold and sural nerve amplitude in South Asians and Europeans patients with type 2 diabetes adjustment for baseline factors.

<table>
<thead>
<tr>
<th>Adjustment factors</th>
<th>South Asian</th>
<th>European</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unadjusted</td>
<td>15.3(13.8-16.7)</td>
<td>17.4(16.0-18.8)</td>
<td>0.036</td>
</tr>
<tr>
<td>Age+ Duration of diabetes</td>
<td>14.9(13.7-16.3)</td>
<td>17.0(15.7-18.3)</td>
<td>0.036</td>
</tr>
<tr>
<td>Weight</td>
<td>15.3(13.8-16.7)</td>
<td>16.8(15.4-18.1)</td>
<td>0.16</td>
</tr>
<tr>
<td>Height</td>
<td>15.2(13.8-16.6)</td>
<td>17.1(15.7-18.4)</td>
<td>0.07</td>
</tr>
<tr>
<td>Waist</td>
<td>14.8(13.5-16.3)</td>
<td>16.4(15.0-17.7)</td>
<td>0.14</td>
</tr>
<tr>
<td>BMI</td>
<td>15.2(13.8-16.6)</td>
<td>17.0(15.6-18.4)</td>
<td>0.08</td>
</tr>
<tr>
<td>Hips</td>
<td>14.7(13.3-16.0)</td>
<td>16.5(15.2-17.9)</td>
<td>0.07</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>15.1(13.8-16.5)</td>
<td>17.0(15.6-18.3)</td>
<td>0.06</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>15.2(13.8-16.6)</td>
<td>17.1(15.8-18.4)</td>
<td>0.05</td>
</tr>
<tr>
<td>Alcohol intake</td>
<td>15.11(13.7-16.5)</td>
<td>16.7(15.4-18.1)</td>
<td>0.01</td>
</tr>
<tr>
<td>Statin therapy</td>
<td>15.2(13.8-16.7)</td>
<td>17.3(15.8-18.7)</td>
<td>0.05</td>
</tr>
<tr>
<td>OHG</td>
<td>14.8(13.5-16.1)</td>
<td>17.2(15.9-18.5)</td>
<td>0.01</td>
</tr>
<tr>
<td>Antihypertensive drugs</td>
<td>14.9(13.6-16.3)</td>
<td>17.0(15.7-18.4)</td>
<td>0.02</td>
</tr>
<tr>
<td>Smoking</td>
<td>15.1(13.8-16.7)</td>
<td>16.8(15.5-18.1)</td>
<td>0.095</td>
</tr>
</tbody>
</table>

**Multivariate model:**
Weight+ smoking 15.3(13.9-16.7) 16.6(15.2-18.0) 0.23

<table>
<thead>
<tr>
<th>Adjustment factors</th>
<th>South Asian</th>
<th>European</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unadjusted</td>
<td>11.1(10.0-12.2)</td>
<td>9.2(8.1-10.4)</td>
<td>0.02</td>
</tr>
<tr>
<td>Age+ duration of diabetes</td>
<td>11.3(10.2-12.5)</td>
<td>9.5(8.3-10.6)</td>
<td>0.02</td>
</tr>
<tr>
<td>Weight</td>
<td>11.1(9.9-12.3)</td>
<td>9.7(8.5-10.9)</td>
<td>0.1</td>
</tr>
<tr>
<td>Height</td>
<td>11.1(9.9-12.2)</td>
<td>9.5(8.4-10.7)</td>
<td>0.06</td>
</tr>
<tr>
<td>Waist</td>
<td>11.2(10.0-12.4)</td>
<td>9.6(8.4-10.7)</td>
<td>0.06</td>
</tr>
<tr>
<td>BMI</td>
<td>11.3(10.2-12.5)</td>
<td>9.9(8.1-10.8)</td>
<td>0.02</td>
</tr>
<tr>
<td>Hips</td>
<td>11.4(10.2-12.5)</td>
<td>9.4(8.2-10.5)</td>
<td>0.01</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>11.3(10.0-12.4)</td>
<td>9.5(8.4-10.8)</td>
<td>0.08</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>11.3(10.2-12.4)</td>
<td>9.4(8.3-10.6)</td>
<td>0.03</td>
</tr>
<tr>
<td>Alcohol intake</td>
<td>11.2(10.0-12.3)</td>
<td>9.7(8.5-10.9)</td>
<td>0.1</td>
</tr>
<tr>
<td>OHG</td>
<td>11.4(10.3-12.5)</td>
<td>9.4(8.3-10.6)</td>
<td>0.02</td>
</tr>
<tr>
<td>Antihypertensive drugs</td>
<td>11.4(10.2-12.5)</td>
<td>9.4(8.3-10.6)</td>
<td>0.02</td>
</tr>
</tbody>
</table>

**Multivariate model:**
TG+ alcohol intake 11.0(9.8-12.3) 9.7(8.5-11.0) 0.18

*P value for ethnic differences. Body max index (BMI), Oral antidiabetic drugs (OHG), Triglyceride (TG).
3.32 Conclusion

We have previously shown that the incidence of foot ulceration and neuropathy is lower in South Asian compared to European patients with Type 2 diabetes (3, 398). This may be due to a lower overall prevalence of diabetic neuropathy per se or indeed a slower rate of progression in South Asians compared to Europeans with T2DM. There are very few studies which have assessed the natural history of nerve damage in people with diabetes in relation to risk factors. Indeed no studies to date have compared South Asian and European patients in the same study. Longitudinal data from the predominantly Caucasian Rochester cohort, supports the contention that the duration and severity of hyperglycaemia are related to the severity of neuropathy (439). In a study of people with Type 2 diabetes, 21% developed a significant neuropathy over 4 years (440). In a long-term follow-up study of another European cohort of patients with Type 2 diabetes, nerve conduction abnormalities in the legs and feet increased from 8% at baseline to 16% after 5 years and to 42% after 10 years (441). The present study was performed in a large cohort of South Asians and Europeans with T2DM who were evaluated in detail for neuropathy at baseline and after ~5 years. South Asian patients were younger and had longer duration of diabetes and were shorter with a lower BMI compared to Europeans. They also had lower triglycerides but comparable total cholesterol, HDL and blood pressure to Europeans with T2DM. Hypertriglyceridemia has been related to the progression of diabetic neuropathy (390). Microcirculatory function is impaired in diabetes and improves after the dyslipidaemia has been treated (442, 443). In a large cohort study of diabetic patients, triglyceride levels were an independent, stepwise risk factor for amputation, even after controlling for known socioeconomic, health behavioural and clinical factors (395). Of course a number of metabolic risk factors, such as glucose, lipids, blood pressure and BMI, have been shown to be related to the development of diabetic neuropathy (386, 444, 445). An improvement in glycaemic control slows the development and progression of diabetic neuropathy in people with Type 1 diabetes (446) but may have no (447) or minimal benefit (448) in people with Type 2 diabetes. Whilst combined improvement in weight, glycaemic control, lipids and blood pressure has shown significant improvements in retinopathy, nephropathy and autonomic neuropathy, no improvement was shown in vibration
perception (449, 450). Furthermore, recently lifestyle intervention, which improved weight, lipids, blood pressure and glycaemia in subjects with impaired glucose tolerance did not improve vibration perception and electrophysiology, measures of large fibre dysfunction, but did improve quantitative sudomotor axon reflex test and intra-epidermal nerve fibre density, measures of small fibre dysfunction and damage, respectively (393).

Thus we show differences in the progression of neuropathy in terms of worsening VPT and reduction in sural nerve amplitude. Consistent with a role of CV risk factors in neuropathy, indeed weight and smoking attenuated the difference in VPT between South Asians and Europeans. And of course smoking is associated with diabetic neuropathy and smokers with type 1 diabetes have been reported to have 3 times greater frequency of neuropathy (451).

Furthermore, sural nerve amplitude worsens in Europeans with T2DM over 5 years but remains virtually the same in the South Asians across the duration of the study (3). Indeed lower TG and less alcohol consumption attenuate the difference in amplitude between South Asians and Europeans with T2DM. These findings therefore provide a possible explanation for the lower rates of neuropathy in South Asians which have been documented in previous studies (3, 398). In relation to the consequences of a greater deterioration in VPT, it has of course previously been associated with an increased incidence of foot ulceration and amputation (445). An elevated VPT was an independent prognostic factor for foot ulceration in patients with established diabetic peripheral neuropathy (102).

We acknowledge certain limitations of the current study and they include the lack of more robust small fibre tests such as IENFD and CCM (239). Particularly as they have been shown to be relatively more responsive to metabolic alterations (351, 393). We found an improvement in the sural nerve conduction velocity at follow up, which raises questions about the reliability of the methods employed for neurophysiological assessment. A standard protocol was used to undertake the NCS in this study and physical factors such as the type of machine, electrodes, room temperature and skin temperature were the same. The key determining factor for these discrepant results was the level of expertise of the technician who performed the test at baseline (2000-2005) and follow up. No inter-intra observer study was
performed to assess the accuracy of those data at baseline, nor at follow up. This is one of the limitations of this study. Indeed a new study by Peter Dyck and colleagues from the Mayo clinic has suggested that the same clinical neurophysiologist should perform repeat nerve conduction studies of therapeutic trial patients. Indeed the differences in inter-observer judgment of abnormality decrease with use of common standard reference values and a defined percentile level of abnormality (452).

In conclusion we demonstrate significant worsening of metabolic and vascular risk factors in Europeans with Type 2 diabetes in association with worsening neuropathy. This may explain the lower overall prevalence of neuropathy which has previously been shown in South Asians (3), providing a possible explanation for the lower rates of foot ulceration and amputation in this group (398).
Chapter 4

DISCUSSION & CONCLUSION
It is estimated that 2.6 million people have DM in the UK and this is predicted to reach close to 4 million by 2025 (453). The first report focusing on diabetes among South Asian populations living in the UK came from the Southall survey, which showed that diabetes was more prevalent in South Asian people when compared to the European population (454). Indeed the prevalence of diabetes was four times higher in South Asian men compared to white men and twice as high in South Asian women compared to white women. Initially, it was thought that the higher rates of diabetes seen in South Asians in the UK may be due to the impact of migration to another country and as a consequence of the change in associated environmental factors or other as yet unidentified influences. Further studies examined this hypothesis and reported that migration from a rural to a nearby urban area in the same country had a similar impact (455). Therefore, these observations suggest that it was not migration to another country but moving from a rural to urban area which increased the risk of diabetes. Whilst genetics may predispose partially to the increased risk in South Asians, undoubtedly, environmental influences, such as sedentary lifestyle and carbohydrate-rich diets, contributed the most (12). Abate et al reported that the incidence of obesity, an important risk factor in the development of type 2 diabetes, is significantly lower in South Asian Indians compared to Europeans (18). However, the validity of this observation is questionable as the cut off values for obesity have recently been shown to be much lower in South Asians. Although, westernization and dietary changes with a lack of exercise may play a role in the increased prevalence of type 2 diabetes in migrant Indian Asians, various epidemiological studies have shown that these factors alone may not be sufficient to explain this trend (18). One important factor contributing to increased type 2 diabetes in South Asian Indians is ‘excessive’ insulin resistance compared to Europeans (456) in response to obesity (15), which is not considered in this study.

Among the many complications associated with diabetes, foot disease represents a significant and often challenging clinical problem (457). People with diabetes develop foot ulceration and amputation because of neuropathy (sensory, motor and autonomic deficits), ischaemia, or both (458). Interestingly, the risk of foot ulceration and amputation is substantially lower in South Asians
compared to white Europeans in the U.K., possibly due to less neuropathy (3, 111) or vascular disease (459). In Leicestershire between 1980 and 1985, the incidence of lower extremity amputation in South Asians (3.4/10000 patient years) was significantly lower compared to white Caucasian (14.2/10000) diabetic patients. Similarly, a lower incidence of lower extremity amputation was also reported in South Asians (0.4/10000) vs. Caucasians (1.5/10000 persons year) without diabetes (460).

In a study from Tanzania the incidence of foot ulceration was lower in Indian Asians compared with Africans and the latter group were significantly more likely to present with a first ulcer or to have gangrene of the lower limb at presentation, resulting in major amputation and subsequent longer hospital stay (461). A study in the UK has shown that South Asians and African Caribbean have less neuropathy, PAD, and foot deformities than Europeans (462). Although glycaemic control correlates with neuropathy, the cause of diabetic neuropathy is complex (463, 464). Indeed in our study, HbA1c in South Asians was actually worse than in Europeans, in keeping with a previous study (3). Dyslipidaemia is a major risk factor for cardiovascular disease and microvascular complications, including neuropathy in both type 1 and type 2 diabetes (463). Triglyceride levels were higher in European patients compared to South Asians and this may be relevant as in a large, multi-ethnic cohort of diabetic patients, elevated triglyceride levels were associated with an increased risk of amputation even after adjusting for a host of potential confounders (465).

The only potential caveat when interpreting our data is that the blood samples in this study were taken in a non-fasting state. In a recent study it has been suggested that the measurement of either total or HDL cholesterol or apolipoproteins may be performed in a non-fasting sample (466). In contrast to our findings, TG, HDL has been reported to be worse in South Asians compared to European (1). Of relevance, Dixon et al showed that South Asian patients were less likely to be prescribed a statin or antihypertensive drug treatment (467). Indeed in a previous study in this group (3), South Asians were less likely than Europeans to take lipid lowering medication. In a cohort of diabetic patients with mild to moderate diabetic neuropathy, elevated triglycerides correlated with a lower sural nerve myelinated fibre density,
independent of disease duration, age and glycaemic control. These data support the developing concept that hyperlipidaemia is instrumental in the progression of diabetic neuropathy (390). A similar study has shown that neuropathic patients had a significantly higher total and LDL cholesterol, and a higher prevalence of abnormal HDL and triglycerides (468). The extent of triglyceride elevation and HDL cholesterol suppression may be greater in women than in men (469). The impact of hyperlipidaemia on the microcirculation is less clear than in other areas of the arterial tree. There are reports of microcirculatory dysfunction which improves after the dyslipidaemia has been treated (442, 443). There is also a study which showed that the microcirculatory dysfunction is not related to hypercholesterolemia (470). A new study has shown that a 1 mg/dl increase in HDL was associated with a 1% decrease in any microvascular complications and for LDL, TG and non-HDL cholesterol, a 1 mg/dl increase resulted in increased risk by 0.2%, 0.1%, and 0.3%, respectively for any microvascular complication (471). This suggests that achieving established ADA goals for HDL, TG, and non-HDL-C may reduce the risk for microvascular events among patients with diabetes (471).

There are studies reporting a lower mean systolic but higher diastolic blood pressure in South Asians compared to whites (472). Furthermore, there is a difference amongst South Asians, with a higher blood pressure in Indians, a slightly lower blood pressure in Pakistanis, and a much lower blood pressure in Bangladeshi’s (472). A lower BMI has also been reported in several studies in South Asian patients (472-474). During a 9 year follow up study, body weight gain was more likely to occur in Caucasians compared to South Asian and Afro Caribbean subjects (474). In another study, BMI was lower and insulin sensitivity higher in subjects living in India compared with their siblings living in west London (475).

We found that the systolic and diastolic blood pressure were comparable but BMI was lower in South Asian compared to European diabetic patients, consistent with previous findings (3, 472, 476); although, a previous study did show that South Asians had more visceral fat and a different fat distribution compared to Whites (476), this was not performed in this study. Thus some studies (477, 478) now support the recent WHO initiative to revise the normal
limits of BMI in South Asians (479). South Asians in the United States have a lower body mass index (BMI) than non-Hispanic Whites, lower rates of tobacco usage and are less physically active (480). Similarly, we found a lower BMI and rates of smoking in South Asians compared to Europeans. Therefore in the present study, the lower BMI values in South Asian patients compared to their European counterparts may be an important factor in reducing the incidence of foot ulceration.

In our study HbA1c was significantly higher in South Asians compared to Europeans. Poorer glycaemic control, as demonstrated by higher levels of HbA1c in South Asians is consistent with the findings of several (31, 481, 482), but not all (474) studies. Although the processes of care indicators are similar in South Asian compared to European patients irrespective of the socioeconomic status (483) glycaemic control is worse suggesting inadequate intensification of therapy (481), indicating that better glycaemic control could be achieved if clinicians are encouraged to do so. Recently a study has indicated that 78% of patients with diabetic polyneuropathy had poor glycaemic control and an increasing diabetes duration was associated with greater sensory symptoms, amongst which the sensation of electric shock pain was present in 63% of patients (484). HbA1c is also related to a range of electrophysiological indicators of diabetic polyneuropathy (484). In the UKPDS independent of age, increased blood pressure and smoking a 1% increase in HbA1c was associated with a 28% increased risk of Peripheral vascular disease (PVD) (459). Neuropathy was more prevalent in Europeans compared to South Asians and the latter were less likely to have an abnormal vibration sensation (338). Loss of ankle reflexes in combination with reduced vibration sense has superior sensitivity and specificity for the detection of established DPN, compared with either, alone (485). An NDS of >6 indicates significant neuropathy (348) and equates with an increased risk of foot ulceration (486, 487). We used the NDS to quantify diabetic neuropathy in diabetic patients and it was comparable in South Asians compared to Europeans. NDS was previously found to be significantly better in South Asians compared to Europeans (338). Only 40% of diabetic patients with severe neuropathy were South Asian whilst 60% of patients with severe neuropathy were European.
Most previous studies have used subjective tests of neuropathy in South Asian and European patients. For the first time we have used more objective tests to assess neuropathy and vascular status in South Asians compared to Europeans. Quantitative sensory testing (QST) may help to predict feet at risk for ulceration but agreement is lacking as to which QST parameter is best suited for detection and follow-up. As diabetic neuropathy affects both large and small fibres complete sensory assessment requires the use of both thermal and vibratory testing (488). Impaired vibratory perception in diabetes mellitus was first observed by Williamson at the Manchester Royal Infirmary in 1905. Since then, Vibration perception threshold (VPT) has been routinely used to determine the degree of neuropathy in diabetic patients, with an increased VPT value reported to be one of the first clinical signs of a peripheral nerve disorder (489, 490). Vibration perception threshold can be used to identify at-risk diabetic patients but also those with early neuropathic deficits (489). High VPT values have been reported to be an independent risk factor for foot ulceration and possess a higher positive predictive value than the Semmes–Weinstein monofilament (SWF) test (491). The use of VPT for the diagnosis of neuropathy has been well validated by clinical studies with a sensitivity of 80% and specificity of 98% (492). Previous studies have also shown that for each 1 unit increase in VPT values at baseline, the hazard of the first foot ulcer increased by 5.6% (102). A correlation between VPT and electrophysiological parameters has been observed, indicating that both methods are valid, but the Neurothesiometer may be preferable in clinical practice due to the ease and rapidity of testing by this method (352). We found that South Asian had a slightly higher VPT compared to Europeans. Nerve conduction studies (NCS) are recommended in the clinical and epidemiological evaluation of diabetic polyneuropathy (DPN) (493). Reduced peroneal motor nerve conduction has been reported to be the best predictor of new foot ulcers (494). Nerve conduction measurements of two nerves (sural and peroneal) in our study showed a trend for better results with a significantly better amplitude in the sural nerve in South Asians compared to European patients, similar to a previous study (3). Sural nerve amplitude represents the
density of large myelinated axons (495), therefore a better sural amplitude in South Asians could be identifying less nerve damage. Some studies have found that sural nerve conduction velocity decreases with age, height (496, 497) and BMI (497) whilst other studies have found no association with this variable (498, 499). We found a significant inverse correlation between HbA1c and sural nerve conduction, HbA1c and sural nerve amplitude, and our results are compatible with a previous study (500) (Appendix). F wave latency measurement assesses conduction over the entire motor nerve, and is thus a sensitive method for detecting neuropathy (501). F wave response latency was comparable between South Asians and Europeans in our study. We performed nerve conduction studies on the left lower limb only as sural nerve conduction studies are symmetrically reduced in Diabetic peripheral neuropathy (DPN) (502).

Quantitative sensory testing (QST) of the thermal thresholds quantitatively assesses the function of somatic small fibres and their central connections (503). QST has been shown to be reasonably reproducible over a period of days or weeks in normal subjects (504). The number of examiners involved in the performance of QST may be important in the reproducibility as the study by Jamal and colleagues suggested that only one person should undertake QST (505) as the reproducibility may be different if several technicians are performing it (504). The increasing acknowledgment of QST as a diagnostic tool is evidenced by the American Diabetes Association endorsement of QST in 1992 as a valid test in epidemiologic studies and drug trials of diabetic neuropathy (504, 506). In addition, QST is also being used in research studies and drug trials of other types of neuropathy to monitor sensory nerve function (504). When establishing the normal values, researchers have found that age, sex, and the site of the stimulation can affect sensory thresholds (504). Age appears to be the most significant factor in determining sensory thresholds compared with the other factors such as gender and anthropometric parameters (507). Thermal threshold tests demonstrate a higher frequency of neuropathy than nerve conduction testing in clinically asymptomatic T2DM patients suggesting that small fibre dysfunction may precede large fibre dysfunction in diabetic neuropathy (508). Patients with diabetic neuropathy have altered thermal thresholds (458, 509, 510) that increase with worsening neuropathy and
eventually predict development of foot ulceration (511). In a previous study with the CASE IV system, cold detection and heat pain thresholds did not differ by ethnicity (3). In this study we used the Medoc Neuro-Sensory Analyser to assess cold/warm perception and cold/ warm induced pain and there was no difference between South Asian and European patients.

Autonomic small nerve fibres can be evaluated centrally by the assessment of cardiac autonomic function and peripherally by evaluating sudomotor function (201). We undertook an assessment of heart rate variability in response to deep breathing and Neuropad evaluation to assess differences in central and peripheral autonomic function, respectively between South Asians and Europeans. Heart rate variability (HRV) is a term which is used for a range of measures that assess autonomic influence on the heart. A reduced HRV positively correlates with obesity, poor aerobic fitness, and increasing age (512). We have shown that heart rate variability response to deep breathing is higher in South Asian compared to European diabetic patients. Sympathetic cholinergic denervation leads to diminished sweating and the overlying skin becomes dry, susceptible to fissures and subsequent ulceration (513).

Development and susceptibility to neuropathic foot ulceration in diabetic patients is closely related to autonomic neuropathy (514). Using clinical examination as a gold standard, Neuropad has been shown to have a 90% sensitivity and 74% specificity for the diagnosis of peripheral neuropathy (199). The Neuropad test has a validity comparable to that of NCS for the diagnosis of diabetic neuropathy, even though it evaluates sudomotor function (515) and NCS is a measure of large fibre function (206). Thus an abnormal Neuropad examination has been reported significantly more frequently in patients with nerve conduction impairment than in those with normal neurophysiological examination (515), reflecting both small and large fibre dysfunction (516). South Asians showed a higher percentage colour change compared to Europeans, but this was not significant. When it was considered to be normal, patchy or abnormal, South Asians showed fewer abnormal responses for Neuropad compared to Europeans (Appendix). The Neuropad can detect small fibre neuropathy (517) and whilst the response was lower in diabetic patients it did not differ significantly between South Asians and Europeans.
Structural evidence of small fibre neuropathy can be obtained by quantifying intra epidermal nerve fibres (IENF) in skin punch biopsies. It is less invasive than sural nerve biopsy and IENF density may even be more sensitive than sural nerve biopsy in identifying small fibre neuropathy (518). A significant correlation between IENFD and QST has been reported only when NCS was abnormal, and thus relies on the presence of significant neuropathy (519). South Asians had a non-significantly higher IENF density compared to Europeans and IENF density has been shown to be correlated inversely with warm threshold more so than cooling threshold and also showed a correlation with NDS and VPT similar to Lauria et al findings (243, 520) (Appendix). A reduction in IENF density in T1DM, T2DM patients with asymptomatic neuropathy has been reported (521). Although a significant relationship was observed between structural and functional tests, this was modest. This weak relationship is not surprising given that IENF density encompasses all nerve terminals and only a fraction of these are involved during functional testing (521). In diabetic rodents, “functional changes relating to sensory processing preceded structural changes in small fibre neuropathy or current microscopic techniques are not sufficiently sensitive to detect structural damage” (521, 522).

It is also possible that immunostaining with PGP9.5 does not identify early structural damage to IENF's, as PGP9.5 is a cytoplasmic enzyme (522). Non-contact corneal aesthesiometry (NCCA) has been used to assess corneal sensitivity in diabetic patients and it has been shown to be a sensitive test for the diagnosis of minimal and more advanced diabetic neuropathy (250). We assessed NCCA on the left eye, but reassuringly it was significantly correlated between the left and right eye. Corneal sensitivity was comparable between South Asians and Europeans.

The cornea may seem an unusual location to assess for the presence of small nerve fiber pathology in conditions that primarily affect the distal limbs, most often in a length-dependent manner. The cornea is however the most richly innervated (~7000 nociceptors per mm²) and hence sensitive tissue in the body (523). Among CCM parameters, CNFL (corneal nerve fibre length) best discriminated DSP cases from control subjects. The threshold value with optimal sensitivity and specificity for determining DSPN was a CNFL of ≤14.0
mm/mm² (sensitivity 85%, specificity 84%) (524), associated with positive and negative likelihood ratios of 5.3 and 0.18. A single threshold offers clinically acceptable operating characteristics, although a strategy that uses separate thresholds to respectively rule in and rule out DSP has excellent performance while minimizing unclassified subjects (525). Corneal nerve fibre density, nerve fibre length and nerve branch density decrease significantly with increasing NDS, compatible with the results of a previous study (524). Corneal confocal microscopy (CCM) can identify early small nerve fibre damage and accurately quantify the severity of diabetic neuropathy. NFL and NBD was significantly better in South Asians compared to Europeans whilst nerve fibre density was comparable between the two groups of patients with a trend for better nerve fibre tortuosity in South Asians.

Dysfunction of vascular smooth muscle, endothelial cells and perivascular nerves (526, 527) impairs cutaneous blood flow (363, 528). There are several different techniques to evaluate alterations in cutaneous blood flow (527). LDI max, the laser Doppler hyperaemic response of the skin microcirculation to local heating at 44 °C has been evaluated in the present study (405, 529). Although it represents predominantly the endothelial response to heating, local heating does cause an initial peak in cutaneous blood flow via a local sensory nerve reflex (517, 530, 531). An alternative method to evaluate endothelium dependent and independent vascular responses is to undertake Iontophoresis (517). We did evaluate it in a small subgroup of this study but the data are not presented as the cohort was not representative of the whole cohort due to age and duration of diabetes differences. The LDI flare depends on C-fibre function and has been undertaken in the present study. A reduction in LDI max has been reported in diabetic patients with and without microvascular complications compared to healthy control subjects (529). There has been little information regarding skin microcirculatory responses in South Asians and Europeans with diabetes. There was a significant difference for LDI max in South Asian compared to European diabetic patients and also in the biopsy sub-group. Previous studies have reported a ~50% reduction in lower extremity LDI max in diabetic patients compared to control subjects (532). This method has showed its potential usefulness in the clinical assessment of blood flow (533). The
hyperaemic response to local heating at the dorsum of the foot has shown that the maximum flow in diabetic patients with complications is lower than in uncomplicated patients (534). LDI flare has also been reported to be lower in Type 1 diabetic patients with microvascular complications and it correlated with duration averaged HbA1c and higher triglycerides (535). We did not find any difference for LDI flare in subgroup of patients that underwent skin biopsy. The LDI flare also detects early small-fibre dysfunction when conventional tests, including Computer Aided Sensory Evaluator IV (CASE IV), are normal (73). The test has good reproducibility and correlates with dermal nerve fibre density (536). A major limitation of laser Doppler flowmetry is that it is not possible to measure absolute perfusion values (i.e. cutaneous blood flow in ml/min relative to the volume or weight of tissue). Although some researchers have tried to convert millivolts to conventional blood flow units using theoretical calculations, this is not widely accepted. Measurements in most studies are expressed as arbitrary perfusion units (PU) or milli volts (1 PU = 10 mV) and are often referred to as flux rather than flow (537).

Local tissue hypoxia is clearly an important component of the development and progression of diabetic foot ulcers. Ischaemia resulting from peripheral vascular disease further complicates outcomes in patients who develop foot ulceration. These diabetic patients typically develop lower extremity atherosclerotic lesions in a more peripheral distribution when compared to non-diabetic patients. However, even limbs free from overt arterial insufficiency may suffer from a number of abnormalities in the micro-circulation that contribute to local tissue hypoxia (538) such as impaired vasodilation and physiological responses to hypoxia (538). TCpO2 is a valuable technique, which can assess the functional status of skin oxygenation and reflects tissue blood flow. Reduced values have been observed in diabetic patients and have been attributed to peripheral vascular disease (539). TCpO2 is directly related to skin oxygen delivery and the degree of hypoxia has been correlated with clinical symptoms of peripheral ischaemia (539, 540). Therefore, TCpO2 is currently being deployed to non-invasively investigate PVD and predict TCpO2 in the feet of 50 type 1 and 2 diabetes patients with foot ulcers. They showed that the ulcers on the feet of patients with TCpO2 greater than 25 mm Hg healed within 4–6 weeks while
ulcers on the feet of patients with TCpO₂ lower than 25 mmHg did not heal. The sensitivity and specificity for TCpO₂ were 85 and 92%, respectively, when a cut off level of 25 mmHg was used to determine ulcer healing (541). We have found that South Asians have better TCpO₂ compared to European diabetic patients. Previous studies have also shown that South Asians have a significantly higher TCpO₂ compared to Europeans (3).

Abnormalities in vascular reactivity in the micro and macrocirculation are well established in type 2 diabetes. Endothelial dysfunction is an important determinant of altered vascular reactivity and plays a major role in the genesis of micro and macrovascular complications in diabetes (93, 542). Reversible alterations in the microcirculation, consisting of increased capillary pressure, blood flow and endothelial permeability, can be detected at an early stage of diabetes mellitus. Irreversible structural modifications of the vascular wall, such as thickening of the basement membrane due to the extracellular accumulation of proteins occurs at a later stage (543). Based on experimental data, endothelial dysfunction may be directly related to hyperglycaemia (543). Although, other abnormalities in lipoprotein metabolism, generation of glycation end products, and increased oxidative stress may also be responsible for endothelial dysfunction in diabetes (543).

In the present study we have undertaken detailed histological quantification of the epidermal thickness and shown that it is significantly higher in South Asians compared to Europeans. An increase in skin thickness has been reported on the dorsum of the feet using high resolution ultrasonography in patients with diabetes mellitus (544). Sandby et al have previously shown that the thickness of the stratum corneum correlates positively with pigmentation and the epidermal thickness varied between 60 and 150 µm over the entire foot with the lowest value on the hallux (545). A study by Yudovsky et al showed that epidermal thickness in the area where the ulcer develops was significantly lower (98µm) compared to the surrounding regions (131 µm) (546). The epidermal thickness has been reported to be reduced by 15% in patients with diabetic foot ulceration compared to 9% in people with neuropathy. Ultrasound assessment has shown that epidermal thinning of plantar skin occurs in patients who have diabetic neuropathy and ulceration (415). However, Duffin et al (547) did not
find increased plantar skin thickness in young diabetic patients (415), whilst Hashemi et al, showed that plantar epidermal thickness was significantly thicker in type 2 diabetic patients without neuropathy compared to healthy subjects (548). In our study, epidermal thickness was not assessed in our healthy control subjects.

With regard to capillary pathology a number of studies have been performed in skin biopsies from diabetic patients (413, 549, 550). A previous study has shown that nerve capillaries show markedly greater pathology than skin and muscle capillaries. Nevertheless there was skin capillary pathology which worsened with severity of neuropathy (134). Abnormalities in capillary morphology, in particular thickening of the capillary basement membrane, have been proposed to contribute to tissue hypoxia, but few studies have correlated anatomic and haemodynamic variables in the same diabetic subjects (551). This is the first study to measure capillary density in the skin biopsy of South Asian and European diabetic patients. We have observed a non-significantly higher vascular density in South Asians compared to Europeans. In a study which used capillary video microscopy, it has been suggested that mechanisms other than reduced capillary density may be involved in limiting tissue blood flow, and may be dependent on microvascular vasodilation (552).

Previous studies in skin capillaries showed that basement membrane thickness was significantly higher in diabetic patients compare to non-diabetic patients which was related to the severity of complications and lumen area was also reduced in diabetic patients with peripheral neuropathy compared to those without peripheral neuropathy (423, 431). Decreased endoneurial capillary luminal area has been related to future deterioration in glucose tolerance (431) and the development of diabetes (136). Skeletal muscle capillary diameters decreased in rats with streptozotocin induced diabetes (529, 553) indicative of microvascular remodelling, reduced ‘capillary blood flow’ and skeletal muscle oxygenation (553). However, the mean capillary luminal diameter did not differ between young and old rats (554). There is speculation that the decrease in capillary luminal diameter occurs earlier than the decrease in the number of capillaries. In particular, the decrease in capillary luminal diameter presumably occurs during the early stages of microangiopathy in type 2 diabetes (432). In
the present study the lumen area did not differ between South Asians and Europeans. We measured skin capillary wall area which included the basement membrane and pericyte area and this also did not differ significantly between South Asians and Europeans. Pericytes are perivascular cells which were first recognized more than 100 years ago. They are found around the endothelial layer and are defined by their location within the basement membrane of capillaries (555) as seen by electron-microscopy (556). They have diverse functions and can sense angiogenic stimuli, guide sprouting vascular tubes, elicit endothelial survival functions, and even exhibit macrophage-like activities. Pericytes can produce vasoconstriction and vasodilation within capillary beds to regulate vascular diameter and capillary blood flow (556). Pericyte number per vessel was comparable between South Asians and Europeans.

Endothelial cells form a flat monolayer along the internal lumen of the blood vessels. In normal, physiological conditions endothelial cells are exposed to circulating blood glucose levels in the range of $\sim 3.6–5.8$ mmol/L, which is tightly regulated as part of metabolic homeostasis (557). The cells are metabolically active, and produce mediators that affect vascular tone, cell adhesion, clotting and fibrinolysis, ensuring fluidity of blood (557). Therapies to reduce hyperglycaemia, dyslipidaemia and insulin resistance may improve endothelial function and delay the onset of vascular complications. Novel therapeutic approaches designed to inhibit AGEs formation, reduce PKC activation, decrease inflammatory signals (558). Alterations in endothelial structure may reflect pathological changes and have been best characterised by alterations in endothelial number and size. We have found no difference in endothelial cell area between South Asian and European patients with diabetes.

A lower capillary density with reduced nutrient exposure to the metabolizing tissue has been reported in diabetic patients and rodents and has been proposed to contribute to insulin resistance (429, 430). Capillary density has also been found to be significantly lower in obese non-diabetic Caucasian and Pima Indian subjects. (559). Capillary density in South Asians in our study was comparable with European diabetic patients.

We showed that a lower level of smoking and triglycerides could explain the greater preservation of small fibre structure as evidenced by better corneal
nerves structure assessed using corneal confocal microscopy. Furthermore, the lower weight of South Asians was also the main driver of ethnic differences in sural nerve amplitude and heart rate variability response to deep breathing (large and small fibre). The current detailed study of corneal confocal microscopy provides further evidence for preservation of small fibres being key to the protection from foot ulceration (508). In addition, these data may support the basis on which triglycerides are important predictors of amputation (395) and may help to explain the reduced incidence of lower extremity amputation in South Asians (372). In the small skin biopsy subgroup, we found a better maximal hyperaemic response in South Asians for which height had the most impact on ethnic differences for LDI max. Of relevance, height is often cited as a key risk factor for neuropathy (340); it is therefore not surprising that the shorter height of South Asians contributes to their lower risk of neuropathy. Similarly, when adjusted for the duration of diabetes, PAD had impact on ethnic differences. Peripheral arterial disease (PAD) is twice as common in persons with diabetes as in persons without diabetes and is also a major risk factor for lower extremity amputation (560). Surprisingly, weight, smoking and duration of diabetes had no impact on the maximal hyperaemic response in the skin biopsy sub-group. However, in our larger cohort, weight in combination with smoking and NBD, HRV-DB (measure of small fibre) attenuated the P value for ethnic differences for TCpO$_2$ and maximal hyperaemic response. Given that small fibre neuropathy occurs early in diabetic neuropathy (561), it is important to perform small fibre tests in patients with diabetes.

The results of these studies have shown that South Asian diabetic patients have poorer glycaemic control, but lower triglycerides compared to Europeans. They have better small fibre function and structure and a higher foot skin oxygenation with a significantly better hyperaemic blood flow response to heating, despite a thicker epidermis. These alterations may protect South Asians from the development of foot ulceration.

Limitations of the current study include:

- The language barrier which may influence the assessment of symptoms, although we did use interpreters.
• The associated lifestyle risk factors including socioeconomic, behaviour, diet of patients and physical activity and foot care were not studied in this project.

• The coefficient of variation for many of the techniques applied in this thesis have not been established. Although, in relation to the skin biopsy study I did undertake an assessment of inter-observer and intra-observer differences for IENFD and these are presented in the supplementary results in the Appendix. Furthermore, we have previously established the coefficient of variation for CNFD, CNFL, CNBD and CNFT assessment and this data is published in Cornea 2013.

• No inter-intra observer studies were performed for nerve conduction studies, QST and AFT’s at baseline or follow up, which is a further limitation of this study. Indeed in a recent study by Peter Dyck and colleagues from the Mayo clinic has suggested that the same clinical neurophysiologist should perform repeat nerve conduction studies in therapeutic trials. Indeed the differences in inter-observer judgment of an abnormality decrease with the use of common standard reference values and a defined percentile level of abnormality (452).

Strengths of the study include:

• The strength of this study is that all individuals were recruited consecutively from a population based sample.

• We also undertook a comprehensive assessment of large fibre, small fibre and vascular changes. With regard to neurophysiology we chose to study two nerves (sural and peroneal nerves) which define peripheral neuropathy (495). In our previous study (3), radial, tibial and ulnar nerves were assessed and no differences were demonstrated between ethnic groups.
4.1 Future Directions

Identifying additional 'novel' risk factors in particular genetic factors that account for the ethnic differences in neuropathy is an important next step towards understanding why such differences exist.

As a new approach, in this group of diabetic patients we have also quantified the immune Langerhans cells (LCs) in the cornea using corneal confocal microscopy. This would be an important avenue to explore to define an immune mediated contribution to corneal nerve damage in South Asians compared to Europeans with diabetes. This new findings regarding Langerhans cells are summarised in supplementary data section in the appendix.

Based on the findings in this thesis, future studies should consider:

The role of socioeconomic status in explaining racial/ethnic disparities in diabetes complications. This may help to explain, and in some cases account for the disparities in diabetes complications (562).

The quality of care given to different ethnic groups compared to non-immigrant patients need to be studied.

Further detailed studies to investigate the pathophysiological changes in these ethnic groups are required to be studied not just for diabetic neuropathy, but also for the other microvascular (Nephropathy and Retinopathy) and macrovascular complications of diabetes.

The role of OMICs (e.g. genomics, proteomics or metabolomics) needs to be studied in details in these groups.

It is important to undertake further studies on the skin biopsies to define expression of VEGF-A, Hypoxia inducible factor-1α (HIF-1α) and use western blot analysis to establish protein expression of these factors as this may provide molecular insights into the physiological differences observed.

Quantification of the keratinocyte layer of the epidermis, given that it may act as a protective layer to reduce the risk of foot ulceration in South Asians is warranted.

An interventional study to assess the effect of treatment for risk factors such as triglycerides and smoking cessation in this group of patients may also be considered.
Chapter 5

APPENDIXES
Can lower rates of peripheral neuropathy in South Asians with diabetes be explained by enhanced micro vascular structure and function compared to Europeans?

We are asking you to participate in a research study to be conducted by Dr Caroline Abbott at the Manchester Royal Infirmary. This will be a follow-up of the original study that you were involved in a few years ago with Dr. Abbott at the Manchester Diabetes Centre. This leaflet explains why you are being invited to take part, 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?

We wish to discover why some people with diabetes develop nerve damage in their legs and feet. We have recently shown that South Asian people with type 2 diabetes living in the UK have lower levels of nerve damage than White Europeans with diabetes and we think that this might be linked to differences in blood flow in the legs. Therefore we need to investigate how blood flows and how the nerves work more thoroughly in these ethnic groups of people with diabetes. These 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 come to the Wellcome Trust Clinical Research Facility at the Manchester Royal Infirmary for just one visit for some tests that would take about 2 hours. We are happy to pay your travel costs.

You will be asked to complete a short questionnaire about your general health and we will take a blood sample from you (3 teaspoons of blood in a total of 4 tubes). We will measure your height, weight, waist size and blood pressure. We will measure the nerve function in your legs using simple clinical tests as used by your GP, standard electrophysiology tests, equipment to see how you feel cold and hot sensation, plus a special plaster to examine sweat formation on your foot. We will ask you some questions about any pain you have in your arms and legs due to nerve damage. We will also see how the nerves that control your heart are working by asking you to do simple deep breathing exercises whilst connected to an ECG. We will assess the circulation in your legs by placing a probe on top of your foot to measure your skin oxygen levels while you rest for 20 minutes. Then the Laser Doppler beam will pass over the skin of your foot to measure your blood flow. This beam does not heat your foot and you will not feel a thing. Finally, we will examine your eyes with a confocal microscopy to look at the nerves in your corneas. 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 jelly will be applied to the front of your eye and the microscope will be advanced such that it touches the jelly on the front of your eye. You will see a white light which does not harm your eye in any way but may cause a little discomfort but no pain.

**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 recognized risks of any of the procedures proposed for this study other than a risk of bruising or fainting after blood taking. If you have any problems, however, you should contact the Principal Investigator, Caroline Abbott, or the Research Nurse at the WTCRF, Pav Bhakar, at once.

**Are there any possible benefits?**

This study will provide you with the opportunity to undergo some important clinical tests to see how well the nerves and blood vessels in your legs are working as a result of your diabetes. The results will be sent to you and also (with your permission) to your doctor, Therefore, any problems may be picked up and your diabetes care may be improved as a result.

**Who will see the information about me?**

All information resulting from your participation in the study will be stored and analysed 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 CMMCUH 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 me 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, I 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, contact:*

Dr. Caroline Abbott (Lecturer) 
Tel. 0161 275 1226

Appendix 2 Information sheet (2)

Central Manchester and Manchester Children’s University Hospitals

Patient information sheet-Extra tests (Skin Biopsy and Iontophoresis)

Can lower rates of peripheral neuropathy in South Asians with diabetes be explained by enhanced micro vascular structure and function compared to Europeans?

We are asking you to participate in a research study to be conducted by Dr Caroline Abbott at the Manchester Royal Infirmary. This will be a follow-up of the original study that you were involved in a few years ago with Dr. Abbott at the Manchester Diabetes Centre. This leaflet explains why you are being invited to take part, 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?
We wish to discover why some people with diabetes develop nerve damage in their legs and feet. We have recently shown that South Asian people with type 2 diabetes living in the UK have lower levels of nerve damage than White Europeans with diabetes and we think that this might be linked to differences in blood flow in the legs. Therefore we need to investigate how blood flows and how the nerves work more thoroughly in these ethnic groups of people with diabetes. These 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 come to the Wellcome Trust Clinical Research Facility at the Manchester Royal Infirmary to undergo some extra tests to the ones you have already consented to. These extra tests will take about an hour and a half to complete and require you to sign a separate consent form. We are happy to pay your travel costs.

The extra tests:

1) We will apply 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 and blood vessels 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.

2) We will also perform an extra laser beam test to assess your circulation. We will tape two small chambers to the top of your foot and fill them with fluid. Whilst a current is passed between the fluids we will scan your skin with the laser Doppler to look at changes in skin blood flow. You may feel a very slight tingling sensation but it is not painful.

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 recognized risks of any of the procedures proposed for this study apart from very rare (<1/1000) infection at the biopsy site. If you have any problems, however, you should contact the Principal Investigator, Caroline Abbott, or the Research Nurse at the WTCRF, Pav Bhakar, at once.

**Are there any possible benefits?**

This study will provide you with the opportunity to undergo some important clinical tests to see how well the nerves and blood vessels in your legs are working as a result of your diabetes. The results will be sent to you and also (with your permission) to your doctor. Therefore, any problems may be picked up and your diabetes care may be improved as a result.

**Who will see the information about me?**

All information resulting from your participation in the study will be stored and analysed 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 CMMCUH 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 me 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, I 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, contact:

Dr. Caroline Abbott (Lecturer)  Tel. 0161 275 1226
Ms. Paven Bhakar (Study research nurse)  Tel. 0161 906 7503

Appendix 3. Consent Form

Central Manchester and Manchester Children’s University Hospitals

CONSENT FORM FOR PATIENT

Title of Project: Can lower rates of peripheral neuropathy in South Asians with diabetes be explained by enhanced microvascular structure and function compared to Europeans?

Investigators:
Dr. Caroline Abbott, Lecturer, PhD
Dr. Rayaz. A Malik, Senior Lecturer, Consultant Physician, MB ChB, MRCP, PhD.
Prof Nish Chaturvedi, Professor of Epidemiology, MBBS, MRCP, MFPHM, MD
Prof Andrew Boulton, MBBS, MRCP, MD, FRCP, DSc

Please initial box:

1. I confirm that I have read and I understand the information sheet dated……………….□
   (Version……..) for the above study and have had the opportunity to ask questions.
2. 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 □
3. I understand that sections of any of my medical notes may be looked at by responsible
   individuals from regulatory authorities where it is relevant to my taking part
   in research. I give permission for these individuals to have access to my records. □
4. I agree to take part in the above study. □
5. I agree that you may contact my GP regarding my participation in this study □
6. I also agree that you can contact me in the future to see how my circumstances have
   changed. □

……………………………. ……….……………….  …………………...
Name of Patient    Signature    Date
………………………………  ………………………….          ……………………..
Name of Person    Signature   Date

Appendix 4. Neuropathy Disability Score (NDS)

Participant’s Full Name:                             Date of Birth:
Date:
Investigator:

<table>
<thead>
<tr>
<th></th>
<th>Right</th>
<th></th>
<th>Left</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>Abnormal</td>
<td>Normal</td>
<td>Abnormal</td>
</tr>
<tr>
<td>Pain (pin-prick)</td>
<td>(0)</td>
<td>(1)</td>
<td>(0)</td>
<td>(1)</td>
</tr>
<tr>
<td>Vibration (tuning fork)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Temp. (Hot/cold rods)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Achilles Reflex:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal Reinforcement</td>
<td>(0)</td>
<td>(1)</td>
<td>Normal Reinforcement</td>
<td>(0)</td>
</tr>
<tr>
<td>Abnormal</td>
<td>(2)</td>
<td></td>
<td>Abnormal</td>
<td>(1)</td>
</tr>
<tr>
<td></td>
<td>(2)</td>
<td></td>
<td></td>
<td>(2)</td>
</tr>
</tbody>
</table>

Total NDS (/10) = □

VPT

Right:

Average

79
Appendix 5. Neuropathy Symptom Profile (NSP)

### Symptoms of Weakness

*Head and neck:*

“Do you experience these symptoms to an abnormal degree? Abnormal is beyond what is normal for you.”

<table>
<thead>
<tr>
<th></th>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Drooping of eyelids</td>
<td>☐</td>
<td>☐</td>
</tr>
<tr>
<td>2. Double vision (other than momentary)</td>
<td>☐</td>
<td>☐</td>
</tr>
<tr>
<td>3. Weakness in chewing</td>
<td>☐</td>
<td>☐</td>
</tr>
<tr>
<td>4. Weakness so you experience difficulty moving food in your mouth</td>
<td>☐</td>
<td>☐</td>
</tr>
<tr>
<td>5. Weakness in swallowing (more than occasionally)</td>
<td>☐</td>
<td>☐</td>
</tr>
<tr>
<td>6. Other weakness of head and neck</td>
<td>☐</td>
<td>☐</td>
</tr>
</tbody>
</table>

*Chest:*

“Do you experience these symptoms to an abnormal degree?”

<table>
<thead>
<tr>
<th></th>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>7. Weakness in speaking due to shortness of breath</td>
<td>☐</td>
<td>☐</td>
</tr>
<tr>
<td>8. Shortness of breath due to muscle weakness</td>
<td>☐</td>
<td>☐</td>
</tr>
<tr>
<td>9. Other weakness of the chest</td>
<td>☐</td>
<td>☐</td>
</tr>
</tbody>
</table>
### Upper Limbs:

“Do you experience these symptoms to an abnormal degree in one or both sides of your body?”

<table>
<thead>
<tr>
<th></th>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>10. Weakness of hands, e.g. when handling coins, using a key</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11. Weakness when straightening fingers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12. Weakness of fingers when clasping or grasping objects</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13. Weakness of the wrists</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14. Weakness of shoulders and upper arms (e.g. lift Objects from a high shelf, comb hair)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15. Other weakness in upper limbs</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Lower Limbs:

“Do you experience these symptoms to an abnormal degree in one or both sides of your body?”

<table>
<thead>
<tr>
<th></th>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>16. Weakness of the legs so that you slap your feet in Walking or cannot carry your weight on your heels</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17. Weakness of the legs so that you cannot walk on Your toes or forefoot</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18. Weakness of your thighs so that you have difficulty Climbing or descending stairs, getting up from a chair, sofa Or toilet seat, and in these acts you need to use your arms</td>
<td></td>
<td></td>
</tr>
<tr>
<td>19. Other weaknesses of the lower limbs</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Sensory Symptoms
“Do you experience these symptoms in one region or over the surface of your body to an abnormal degree? Do not include the brief symptoms of “prickling” or “asleep numbness” and discomfort which come from lying too long on an arm, or sitting or lying too long in one position on a leg.”

20. Decrease (or inability) to feel the surface features, Size, shape, or texture of what you touch?
   Yes          No
   If yes chose only one:
      In legs only (inc. feet) ☐
      In arms only (inc. hands) ☐
      In legs and arms only ☐
      In mouth, face, or head only ☐
      Other than any of the above ☐

21. Decreased (or inability) to recognize hot from cold?
   Yes          No
   If yes, choose only one:
      In legs only (inc. feet) ☐
      In arms only (inc. hands) ☐
      In legs and arms only ☐
      In mouth, face, or head only ☐
      Other than any of the above ☐

22. Decreased (inability) to feel pain, cuts, bruises, or injuries?
   Yes          No
   If yes, choose only one:
      In legs only (inc. feet) ☐
      In arms only (inc. hands) ☐
      In legs and arms only ☐
      In mouth, face, or head only ☐
      Other than any of the above ☐

23. A more or less continuous “dead feeling” like Novocain without prickling (tingling)?
   Yes          No
   If yes, choose only one:
      In legs only (inc. feet) ☐
      In arms only (inc. hands) ☐
      In legs and arms only ☐
      In mouth, face, or head only ☐
      Other than any of the above ☐
in arms only (inc. hands)
in legs and arms only
in mouth, face, or head only
Other than any of the above

24. A more or less continuous “prickling” or “tingling” feeling with or without an asleep dead feeling?

If yes, choose only one:
in legs only (inc. feet)
in arms only (inc. hands)
in legs and arms only
in mouth, face, or head only
Other than any of the above

25. Unusual sensitivity or tenderness when regions of the body are touched or when the hands or feet are used in manual activity?

If yes, choose only one:
in legs only (inc. feet)
In arms only (inc. hands)
In legs and arms only
in mouth, face, or head only
Other than any of the above

26. Sharp “jabbing” needle-like pains or pulse of pain (lasting seconds or a minute or two)

If yes, choose only one:
in legs only (inc. feet)
In arms only (inc. hands)
In legs and arms only
in mouth, face, or head only
Other than any of the above

27. Burning discomfort?

If yes, choose only one:
in legs only (inc. feet)
In arms only (inc. hands)
In legs and arms only
in mouth, face, or head only
Other than any of the above

28. Deep aching pain?
   If yes, choose only one:
   - in legs only (inc. feet)
   - In arms only (inc. hands)
   - In legs and arms only
   - in mouth, face, or head only
   Other than any of the above

29. Other pain?
   If yes, choose only one:
   - in legs only (inc. feet)
   - In arms only (inc. hands)
   - In legs and arms only
   - in mouth, face, or head only
   Other than any of the above

Autonomic Symptoms

“Do you experience these symptoms to an abnormal degree?”

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

30. Feel faint or actually faint, which only comes upon sitting or on standing, and which cannot be explained by use of blood pressure medication or psychologic stress (e.g. sight of blood)?

31. Repeated nausea or vomiting of undigested food, especially in the morning, which is not due to known stomach or gallbladder disease?

32. Persistent diarrhoea, especially at night which is not due to irritable bowel, or other bowel disease

33. Loss of bladder control, which is not due to
gynaecologic problems in women or prostate problems in men?

34. Loss of rectal control, with soiling which is not due to known rectal disease?  

35. Inability in men to have sexual erection which is not due to medication or prostate surgery?  

36. Inability in men to have emission of seminal fluid, which is not due to medication or prostate surgery?  

37. Dryness of the eyes, which is not due to use of medication or known eye disease?  

38. Dryness of the mouth, which is not due to use of medication or known mouth disease?
Appendix 6. Short-form of McGill pain questionnaire

Please select from the list below the words that you would use to describe your pain

<table>
<thead>
<tr>
<th></th>
<th>NONE</th>
<th>MILD</th>
<th>MODERATE</th>
<th>SEVERE</th>
</tr>
</thead>
<tbody>
<tr>
<td>THROBBING</td>
<td>0)</td>
<td>1)</td>
<td>2)</td>
<td>3)</td>
</tr>
<tr>
<td>SHOOTING</td>
<td>0)</td>
<td>1)</td>
<td>2)</td>
<td>3)</td>
</tr>
<tr>
<td>STABBING</td>
<td>0)</td>
<td>1)</td>
<td>2)</td>
<td>3)</td>
</tr>
<tr>
<td>SHARP</td>
<td>0)</td>
<td>1)</td>
<td>2)</td>
<td>3)</td>
</tr>
<tr>
<td>CRAMPING</td>
<td>0)</td>
<td>1)</td>
<td>2)</td>
<td>3)</td>
</tr>
<tr>
<td>GNAWING</td>
<td>0)</td>
<td>1)</td>
<td>2)</td>
<td>3)</td>
</tr>
<tr>
<td>HOT-BURNING</td>
<td>0)</td>
<td>1)</td>
<td>2)</td>
<td>3)</td>
</tr>
<tr>
<td>ACHING</td>
<td>0)</td>
<td>1)</td>
<td>2)</td>
<td>3)</td>
</tr>
<tr>
<td>HEAVY</td>
<td>0)</td>
<td>1)</td>
<td>2)</td>
<td>3)</td>
</tr>
<tr>
<td>TENDER</td>
<td>0)</td>
<td>1)</td>
<td>2)</td>
<td>3)</td>
</tr>
<tr>
<td>SPLITTING</td>
<td>0)</td>
<td>1)</td>
<td>2)</td>
<td>3)</td>
</tr>
<tr>
<td>TIRING-EXHAUSTING</td>
<td>0)</td>
<td>1)</td>
<td>2)</td>
<td>3)</td>
</tr>
<tr>
<td>SICKENING</td>
<td>0)</td>
<td>1)</td>
<td>2)</td>
<td>3)</td>
</tr>
<tr>
<td>FEARFUL</td>
<td>0)</td>
<td>1)</td>
<td>2)</td>
<td>3)</td>
</tr>
<tr>
<td>PUNISHING-CRUEL</td>
<td>0</td>
<td>1)</td>
<td>2)</td>
<td>3)</td>
</tr>
</tbody>
</table>

PRESENT PAIN INDEX

WHICH OF THE FOLLOWING WORDS EXPLAINS YOUR PRESENT PAIN?

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>NO PAIN</td>
</tr>
<tr>
<td>1</td>
<td>MILD</td>
</tr>
<tr>
<td>2</td>
<td>DISCOMFORTING</td>
</tr>
<tr>
<td>3</td>
<td>DISTRESSING</td>
</tr>
<tr>
<td>4</td>
<td>HORRIBLE</td>
</tr>
<tr>
<td>5</td>
<td>EXCRUCIATING</td>
</tr>
</tbody>
</table>

Your assessment of how bad your pain is. Please put a mark on this line to show how severe your pain is at the moment

---

NO PAIN WORST POSSIBLE PAIN
Appendix 7. Medical history assessments

An investigation into the ethnic differences in neuropathy in diabetes and the role of blood flow

Division of Cardiovascular & Endocrine Sciences
University of Manchester
3.30 Core Technology Facility
46 Grafton Street, Manchester, M13 9NT

Tel: 0161 275 1226

This questionnaire asks you about your medical history, your lifestyle and your background.
All your answers will be kept strictly confidential. It will not be possible to identify any individual person from the results of this study.

Please fill out the identification details below. When you return this questionnaire to us this page, which includes your name and address, will be removed. Please bring this with you to your appointment.

Surname __________________________________________

Forenames __________________________________________

Home address _______________________________________

_________________________________________________

_________________________________________________

Post Code _________    _________

Date of Birth:
Date of Hospital Visit:

General instructions
The answer to most questions can be indicated by simply ticking the appropriate box. For example:

Do you ride a bicycle regularly?  
Yes 1  No 2

Sometimes the questions ask you to fill in the actual number. e.g. How old were you when you started to smoke cigarettes regularly?  
20 yrs

When asked to write a few words you are given a box to write in—please use block letters. e.g. What is the name of your GP?  
DR A BLOGGS
DEMOGRAPHIC CHARACTERISTICS

1. Date of birth

2. Sex: Male 1 Female 2

3. What is your marital status? - Are you ...
   Single 1
   Married or living as married 2
   Divorced or separated 3
   Widowed 4
   Other 5

PAST MEDICAL HISTORY

4. Has a doctor ever told you that you have any diabetes damage in the back of your eyes [retinopathy]?
   Yes 1
   No 2
   Don’t know 9

   If yes,
   a) when did this first occur?

   b) Have you ever had laser therapy for retinopathy?

5. Have you ever been told that you have any kidney damage from the diabetes [nephropathy]?
   Yes 1
   No 2
   Don’t know 9

   a) If yes when did this first occur?

6. Have you ever been told by a doctor that you have had a heart attack?
7. Have you ever been told that you have had angina pectoris (chest pain due to heart disease)?

   Yes 1
   No 2
   Don't know 9

   a) If yes when did the first attack occur?

   mm yyy

   Don't know 9

8. Have you had a coronary angiogram (a dye test of the arteries of the heart) done?

   Yes 1
   No 2
   Don't know 9

   a) If yes when was this?

   mm yyy

   Don't know 9

9. Have you had an operation on the arteries of the heart? (Bypass graft or angioplasty)

   Yes 1
   No 2
   Don't know 9
10. Have you been told by a doctor that you have had a stroke?
   Yes 1
   No 2
   Don't know 9

   a) If yes when was the first stroke?
   /
   mm yyyy

   Don't know 9

11. Have you ever been told by a doctor that you have high blood pressure?
   Yes 1
   No 2
   Don't know 9

   a) If yes when were you first told this?
   /
   mm yyyy

12. Have you had a lower limb arteriogram done (a dye test of the arteries of the leg)
   Yes 1
   No 2
   Don't know 9

   a) If yes when?
   /
   mm yyyy

   Don't know 9

13. Have you been told by your doctor that you have peripheral vascular disease or intermittent claudication (bad circulation in your legs which makes it difficult or painful to walk)?
   Yes 1
   No 2
   Don't know 9

   a) If yes when was this diagnosed?
   /
   mm yyyy
14. When were you first told that you had diabetes?
   / mm yyyy
   Don’t know 9

15. Have you suffered from any other serious illnesses?
   Yes 1
   No 2
   Don’t know 9

If yes please specify giving dates
   a / mm yyyy
   Don’t know 9

   b / mm yyyy
   Don’t know 9

   c / mm yyyy
   Don’t know 9

SMOKING HABITS

16. Have you ever been a regular smoker?    
   of Cigarettes? Yes 1 (If ‘Yes’ to cigarettes go to Q16a)
   No 2

   of a Pipe? Yes 1 (now skip to Q25)
   No 2

   of Cigars? Yes 1 (now skip to Q25)
   No 2
(If you are a pipe or cigar smoker or a non-smoker skip to Q25)

16. a) Are you a:  
   - Current smoker? 1  →  (Go to next question [Q17])  
   - Ex-smoker? 2  →  (Skip to Q21 for ex-smokers)  
   - Never smoked? 3  →  (Skip to Q25)

**CURRENT SMOKERS**

17. About how many cigarettes a day do you usually smoke on weekdays - give the number
   
   If less than 1 (i.e. you inhale just a few puffs from one cigarette), put 0

18. About how many cigarettes a day do you usually smoke each day at the weekend - give the number - If less than 1, put 0

19. Do you mainly smoke
   - filter tipped cigarettes 1
   - plain or untipped cigarettes 2
   - hand rolled cigarettes 3

20. How old were you when you started to smoke cigarettes regularly - give your age
   
   For “can’t remember” put 99
   For “don’t smoke regularly” put 98

   (now go to question 25)

**EX-SMOKERS**

21. Did you smoke cigarettes regularly - that is at least one a day 1
   
   only occasionally 2
   
   never really smoked cigarettes, just tried them once or twice 3

**FOR EX-REGULAR SMOKERS (at least 1 cigarette/day)**

(if you never smoked regularly go to question 25)

22. About how many cigarettes did you smoke in a day - write the number

23. How old were you when you started to smoke cigarettes regularly? - write your age.  
   For can’t remember put 99

24. How old were you when you stopped smoking cigarettes regularly? - write your age.  
   For can’t remember put 99
DRINKING HABITS

25. Have you ever had a drink of wine, beer or spirits in your life?  
   Yes 1 [Blank]  
   (if Yes go to next question [Q25], if No go to Q26)  
   No 2 [Blank]

26. Thinking back to the last 12 months, please tick the box that best describes how often you usually drank each of the alcoholic drinks listed below - please exclude any non-alcoholic/low alcohol drinks except shandy

   | almost every day/wk | 3/4 days/wk | once/twice/wk | once/twice/months | every 2 months | once/twice/yr | never in past year | never in past year |
---|---------------------|-------------|---------------|-------------------|---------------|--------------|------------------|-------------------|
   a) wine | [Blank] | [Blank] | [Blank] | [Blank] | [Blank] | [Blank] | [Blank] | [Blank] |
   b) beer/lager/stout/cider | [Blank] | [Blank] | [Blank] | [Blank] | [Blank] | [Blank] | [Blank] | [Blank] |
   c) spirits/liqueurs | [Blank] | [Blank] | [Blank] | [Blank] | [Blank] | [Blank] | [Blank] | [Blank] |
   d) sherry/fortified wine | [Blank] | [Blank] | [Blank] | [Blank] | [Blank] | [Blank] | [Blank] | [Blank] |
   e) shandy | [Blank] | [Blank] | [Blank] | [Blank] | [Blank] | [Blank] | [Blank] | [Blank] |
   f) ‘alcopops’ | [Blank] | [Blank] | [Blank] | [Blank] | [Blank] | [Blank] | [Blank] | [Blank] |
   any other drinks? Write in names of other drinks and tick how often you drink them
   g) [Blank] | [Blank] | [Blank] | [Blank] | [Blank] | [Blank] | [Blank] | [Blank] | [Blank] |
   h) [Blank] | [Blank] | [Blank] | [Blank] | [Blank] | [Blank] | [Blank] | [Blank] | [Blank] |
For each group of alcoholic drinks that you have drunk in the last 12 months

27. On the days that you have a drink, how much do you usually drink on any one-day? Please enter the amount in the box:

a) Wine, (number of glasses)
   (incl. Sparkling wine)

b) Beer, lager, stout, cider (number of pints)
   (if half put 0.5)

c) Spirits/liqueurs (number of measures)
   (One measure=One shot)

d) Sherry or fortified wine
   (incl. Port, martini, campari, cinzano, dubonnet)

e) Shandy (number of pints)

f) Any other alcoholic drinks: state type and amount
   Name:
   1................................................
   2................................................

This section (Q's 28 – 31) to be completed by WOMEN only

MEN go to Q32

28- a) Have you EVER taken the combined
   (oestrogen/ progesterone) oral contraceptive pill?

   Yes 1
   No 2
   Don't know 9

b) If 'yes', how many years did you take the combined oral contraceptive pill? (add up if used on several different occasions. Less than 1 yr, put '1')

c) When did you last take the combined oral contraceptive pill?
   (put in today's date if still on it)
   / mm yyyy
29- a) Have you EVER taken the **progesterone only** oral contraceptive pill?  
   Yes 1  
   No 2  
   Don’t know 9

b) If ‘yes’, how many years did you take the **progesterone only** oral contraceptive pill? 
   (add up if used on several different occasions, if less than one year put one) 

   

c) When did you last take the **progesterone only** oral contraceptive pill?  
   (put today’s date if still on it)  
   mm yyyy

30- a) Have you had a menstrual period in the last 12 months?  
   (Including ‘periods’ you may have had whilst on hormone replacement therapy for those who had a period in past 12 months)  

   Yes 1  
   No 2

b). If ‘yes’, what was the date of the first day of your last menstrual period?  

   / /  
   dd mm yyyy

c) If ‘No period in the last 12 months’, were your periods stopped by:  
   Surgery 1  
   Chemotherapy or radiotherapy 2  
   Pregnancy or breastfeeding 3  
   No obvious reason/menopause 4  
   Other 5  
   (If other please specify) ..............................................................

31- a) Have you EVER taken hormone replacement therapy (HRT)?  
   Yes 1  
   (If No, go to Q32) No 2  
   Don’t know 9

b) If yes, how many years did you take HRT (add up if used on several different occasions, if less than one year put one)  

   Yrs.

c) When did you last take HRT?  
   (put today’s date if still on it)  
   mm yyyy
d) For those currently on HRT - Did your menstrual periods stop before you started HRT?
   Yes 1
   No 2
   Don't know 9

e) If yes, were your periods stopped by
   Surgery 1
   Chemotherapy or radiotherapy 2
   Pregnancy or breastfeeding 3
   No obvious reason / menopause 4
   Other 5

if other please specify

CARDIOVASCULAR HISTORY

32. Have you ever had any pain, discomfort, pressure or heaviness in your chest?
   Yes 1
   (Go to Q40) No 2

33. If yes, do you get these symptoms if you walk uphill or hurry?
   Yes 1
   Never walk uphill or hurry 2
   (Go to Q40) No 3

33 Do you get these symptoms if you walk at an ordinary pace of the level?
   Yes 1
   No 2

34. What do you do when you get this pain on walking?
   Do you continue walking? 1
   Do you stop or slow down? 2
   Do you take nitroglycerine (GTN)? 3

35. If you stand still or take GTN, what happens to the feeling in your chest?
   Pain is not relieved 1
   Pain is relieved 2

36. How long does it take for the pain to go away when you stand still?
   10 minutes or less 1
   More than 10 minutes 2

37 Please mark on the diagram (use X) where this discomfort is located
38 Have you ever had a severe pain across the front of your chest lasting for half an hour or more?

Yes 1

No 2

Office use only: AP Y AP N

LEGs

39 Do you get a pain or discomfort in your leg(s) when you walk?

Yes 1

No 2

I am unable to walk 3

(If you answered “Yes” to this question – please answer the following questions. Otherwise, skip to the last page).

40. Does this pain ever begin when you are standing still or sitting?

Yes 1

No 2

41. Do you get it if you walk uphill or hurry?

Yes 1

No 2

42. Do you get it when you walk at an ordinary pace on the level?

Yes 1

No 2

43. What happens to it if you stand still?

- Usually continues for more than 10 minutes 1
44. Where do you get this pain or discomfort? - Mark the place(s) with “X” on the diagram below.

- Usually disappears in 10 minutes or less

Office use only

Def. G1  Atyp. G1
Def. G2  Atyp. G2
No
CURRENT MEDICATION

Please list below the names of ALL medications you are currently taking. Make sure to include all medications including drops, inhalers, vitamins, ointments, and implanted devices e.g. contraceptive implants.

<table>
<thead>
<tr>
<th>Name of Medication(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

You are now finished. Thank you for your help.

Please remember to bring this questionnaire and ALL medications (including any ointments, inhalers etc.) with you to your appointment.
Appendix 8. Heart Rate variability response to Deep Breathing

• SWITCH ON CASE IV and Heart Rate Monitor
• From the ‘Main Menu’, SELECT ‘Tests Menu’

The default settings should be:

* Speed: 25
* Freeze: off
* Trigger mark: on
* Filter: on
* Height: maximum
* Beep: off (use the QRS volume switch on the rear panel of the Model 10 IT)
* Alarm: off
* Lead select: II

• Prepare patient for heart rate deep breathing test:

The patient, comfortable and relaxed, should recline on a couch with a single pillow. At least 5-10 minutes of supine rest are required before starting the test.

Placement of electrodes: First, clean skin with Alco wipes. Attach two disposable electrodes to the R and L sub clavicle regions, then connect the white + black electrode leads labelled ‘RN (right arm) and ‘LA’ (left arm). Place the reference disposable electrode at the left side (mid axillary level) and connect with the 3rd electrode lead ‘LL’

Placement of breathing cue: The patient must be able to see the up and down movements of the bar clearly.

Tell the patient: “For this part of the test, please take deep breaths in and out by following the movement of the bar. We will have a short practice first. I will tell you when to start and stop. The breaths should be as deep as you can.” Coordinate the patient’s breathing to the movement of the bar (bar goes up = inspiration / bar goes down = expiration). The practice session should consist of about two breaths, to be followed by two minutes rest.
It is very important that the patient breathes deeply and rhythmically, and is not distracted by noises (e.g. conversation, telephone ringing etc), moving papers, people walking around, etc.

- SELECT ‘Autonomic – Heart Rate Deep Breathing’ from CASE IV Menu
- SELECT ‘Return to Main Menu’ (Bypass ‘analyse data’ option)
- SELECT ‘Record HRDB Test’
- Tell the patient to start the deep breathing sequence beginning with an inspiration when the bar is next down
- Mark the beginning of this sequence by pressing the ‘M’ key (a blue line appears on screen).
- After 8 breathing cycles (80 secs) mark the end of the sequence with the ‘M’ key again (a 2nd blue line appears)
- Instruct the patient to rest for at least 5 minutes without talking. Check that the patient’s heart rate is stable.
- Ask the patient to start with a 2nd breathing series (8 cycles), marking the begin and the end again with a blue line (‘M’ key)
- Press ‘Q’ to quit the recording screen
- Analyse HRDB results:
  - SELECT ‘Accept recording and analyse’
  - For the 1st breathing series, choose the best 5 consecutive cycles, and mark the peaks and troughs with small yellow square, using the space bar (hint: using the up/down arrow keys the cursor jumps directly from peak to valley)
  - Repeat this marking procedure for the 2nd breathing cycle, after pressing the ‘T’ (toggle) key. This changes the colour of the mark to small red triangles.
  - Press ‘Q’ to quit [The printout of results (mean ratio of each eight-cycle series) will start automatically]
- SELECT ‘EXIT HRDB Software’
- Remove the electrodes from the patient and check if O.K.
  Store the print-outs in the CRF folder.
Appendix 9. LDI method for hyperaemic response

Prepare the left foot:
Dorsal skin temperature must be between 31 - 35°C. Check baseline skin temperature using the Infrared Thermometer. If below 31°C, warm the foot prior to testing using a hairdryer for 1-2 minutes (this should be held at least 30cm away from the skin surface of the foot). Re-check temperature. Clean and degrease the foot dorsal skin surface by wiping the area with an alcohol swab. If necessary shave the sensor site. Mark the area for LDI and TCpO\textsubscript{2} testing (area 3-5cm proximal to 1\textsuperscript{st} and 2\textsuperscript{nd} toes, free of surface veins) by marking around the disc template inner and outer circles with a black pen.

- Ask patient to put on sock (with hole exposing the dorsal surface) on the left foot, to maintain the correct skin temperature.
- Switch on the computer.
- Open Desktop program – “Moor Ionoto MEASURE”
- Press the first icon on LHS screen, this opens the shutter.
- Press the “Scan Settings Icon” (5\textsuperscript{th} icon along)
- Position the foot on platform to centre the laser beam on the marked dorsal site.
- Centre the box around the beam; click “Mark” to access area.
- Press “OK” to finish.
- To SCAN, click on “Measurement” at the top of the screen.
- Select “Image Scan”.
- Ensure the beam is in the centre of the marked area on the foot; then click on the “Play” button.
- Save the image. Click on ‘file’, ‘Save as’, double click on the data folder and type in the file name (eg. “001-a” for baseline assessment of patient “001-b” for hyperaemic response assessment). Click ‘OK’.
- In file ‘Notes’, Type in ‘Baseline’ (for 1\textsuperscript{st} LDI assessment) or ‘Hyperraemic Response’ (for 2\textsuperscript{nd} LDI assessment).
- Record foot skin temperature in CRF.

Processing LDI results:
• Open saved file.
• Click on ‘Options’, then ‘Load ROI’
• Select ‘test.roi’. This downloads a pre-set rectangular area (1cm x 1cm) with coordinates for the middle of the marked dorsal region, to be used for analysis of flux.

• To ensure you are within the marked dorsal region, enhance the photograph of the marked area by clicking on ‘Image Processing’, then click on ‘Photo Image’, then click on ‘Edge’. Adjust the position of the rectangle if necessary.

• Click on “Analysis” → “Statistics” → “Save File As…”

Record in the CRF (clinical record file) LDI (Baseline and Hyperaemic Response) mean flux (+ standard deviation) and peak flux.

1. Measure ‘Baseline’ LDI
2. Proceed with the TCpO2 test on dorsum of the foot (see below).
3. Immediately repeat the LDI on same site to assess ‘Hyperaemic Response’.
Appendix 10. TCPo$_2$ method

The TCPo$_2$ machine should be: Cleaned and re-membraned, once a month
Calibrated, every time machine is switched on

Applying the TCPo$_2$ sensor to the patient. Patient should be resting in semi-
recumbent position, legs horizontal and kept warm with a blanket, on couch.

- Grasp a sensor adhesive ring by the coloured tad and peel it away from the
  paper-backing strip.
- Attach the adhesive ring properly to the membrane sensor – take care so
  that the adhesive ring does not touch the membrane (i.e. the membrane does
  not get covered or torn.)
- Grasp the ring by the coloured tab. Peel away the liner to expose the
  adhesive.
- Place a drop of transcutaneous sensor contact gel onto the centre of the
  sensor face.
- Press the sensor into place on the marked area of the left foot. Firmly press
  the edges of the adhesive ring to the skin, ensuring an airtight seal between
  the skin and sensor.
- Press run on the TCPo$_2$ machine. Start 20 minutes timer.
- Cover foot with light blanket to maintain overall foot skin temperature.
- After 20 minutes stabilisation, take the reading as the final TCPo$_2$ result.
- Measure and record dorsal skin surface temperature using the CASE IV
  thermal probe.

Record TCPo$_2$ and skin temperature in the CRF, Removing the TCPo$_2$ Sensor
from the Patient:

- Gently peel back the adhesive ring starting from the coloured tab.
- Wipe the face of the sensor membrane clean and remove any adhesive ring
  with an alcohol wipe before calibrating/or storing.
- Immediately repeat LDI method to obtain hyperaemic response.
Appendix 11. Skin biopsy protocol

Materials:
Two pots with PBS-buffered 4% Paraformaldehyde (PFA) and one pot Cacodylate buffer (2.5% Glutaraldehyde, 10% sucrose)

Biopsy Procedure:
- The patient will rest in semi-reclining position on the couch.
- Inspect the foot dorsum and choose 2 points approximately 2 cm proximal to the metatarsal bone.
- Clean the skin with betadine and inject 1% lidocaine. Wait 3-5 minutes and check the skin is insensitive to pinprick.
- Incise the skin with 3 mm punch biopsy device, lift the skin with forceps and cut the bottom with scissors.
- Stop the bleeding by pressing with gauze and close the wounds with steri-strips.

Cryo-preservation of skin biopsies
1st sample goes straight into 4% PFA. Second sample is cut into two pieces. First piece goes into another 4% PFA pot and second one goes into Cacodylate buffer pot.

Morphometry – capillary structure
Fix the tissue (half sample) immediately in glutaraldehyde fixative in cacodylate buffer and secondarily fixation in 1% osmium tetroxide.
Following below procedure:
Processing schedule for biopsy specimens for Embedding
- **Fixation:**
  - Three hours 2.5% Glutaraldehyde in Cacodylate buffer. Room temperature
  - Wash 6 times in Cacodylate buffer containing 10% Sucrose. Leave overnight at room temperature.
- **Post-fixation:**
  - 1% Osmium Tetroxide.
  - 3 hrs at room temperature.
- wash 8 times in distilled water.

- **Dehydration:**
  
  30% Ethanol - 2 changes of 15 minutes at room temperature
  50%        "          "       "      15    "        "      "         "
  90%        "       "      "         15     "        "       "        "
  100%       "       "       "         20     "        "       "        

- Propylene Oxide 2 changes of 15 minutes at room temperature.
- 1:1 Propylene Oxide/Epon (+Catalyst) mixture 1 hr at room temperature
- 1:3 Propylene Oxide/Epon (+Catalyst) mixture 1 hr at room temperature.
- Epon (+Catalyst) 24 hrs at room temperature.
- Polymerize at 60 °C for 2 days.

Store sample or cut with microtome straightaway.

**Recipes for Reagents for Tissue Processing**

**Cacodylate Buffer (500 mls)**

21.4 g Sodium Cacodylate
150 mls distilled water
40 mls 0.1 Hydrochloric acid

Make up to 500mls with distilled water

Add Sucrose 10% w/v

**Glutaraldehyde in Cacodylate Buffer**

62.5 ml Cacodylate buffer
20.0 ml 25% Glutaraldehyde
125 ml distilled water

**Osmium Tetroxide**

1% Osmium tetroxide w/v in distilled water [One ampoule containing 0.1g of crystalline osmium tetroxide (0.1g vial, Agar scientific Ltd, Essex UK) dissolved in 10 ml distilled water in Fume cupboard].

**Appendix 12. Processing tissue to wax**

**Fixing and Wax Embedding of Skin Biopsies (half sample)**

1. Fix skin biopsy in 4% Para formaldehyde for 24 hours.
2. Wash in 1xPBS at least 1 hour/overnight
3. Transfer to 25% IMS for 15 minutes on orbital shaker.
4. Transfer to 50% IMS for 15 minutes on orbital shaker.
5. Transfer to 75% IMS for 30 minutes on orbital shaker.
6. Transfer to 100% IMS for 30 minutes on orbital shaker.
7. Repeat step 6.
8. Transfer to Xylene for 30 minutes on orbital shaker.
9. Repeat step 8.
10. Label a pink cassette in pencil with biopsy details.
11. If specimen is small, place a blue sponge in the cassette.
12. Place biopsy on blue sponge, place another sponge on top of the biopsy and close the cassette.
13. Fill red dish with wax and place cassette in the dish.
14. Place the dish in the vacuum oven and close the door.
15. Switch the pump on and turn “open to release” clockwise, turn “vacuum anti-clockwise.
16. Leave in wax for 25 minutes.
17. Switch the pump off and turn to open. To release turn anti-clockwise, wait for vacuum to disperse and open the oven door.
18. Change the wax in the dish and repeat steps 15, 16 & 17.
19. Fill plastic mould with wax at embedding station.
20. Remove biopsy from the cassette and place in plastic mould. Orientate so cut side is facing down.
21. Put pink cassette (without lid) on top of the biopsy and press down.
22. Top up the mould with more wax and place on cold plate to set.
23. Once wax has set, store wax block at -20 °C overnight and ready to cut on Microtome.
24. Store block until required

**Appendix 13. Processing tissue to frozen**

**Tissue Processing for Immunohistology – nerve structure**
Fix skin biopsy in PBS-buffered 4% para formaldehyde for 18-24 hours.
1. Wash in PBS at least 1 hour/overnight.
2. Transfer to 10% Sucrose Solution for 4 hours at 4°C.
3. Transfer to 20% Sucrose Solution for 4 hours at 4°C.
4. Transfer to 30% Sucrose Solution for 4 hours at 4°C.

Skin biopsy can now be sectioned straight away into 50µm section using a cryostat (model OTF, Bright Instruments Ltd, Huntington, England) or stored at -80 °C using the following procedure:
5. Using 5ml bijou vial as template, make an aluminium foil container for the biopsy.
6. Pipette approximately 1cm deep layer of OCT into the bottom of the foil container.
7. Freeze OCT in liquid N\textsubscript{2} until just clear and liquid in the middle.
8. Place biopsy onto layer of OCT and add another 1 cm layer of OCT on top of the biopsy.
9. Freeze in liquid N\textsubscript{2} until OCT is completely white.
10. Fold over the top of the foil container and store the biopsies at -80 °C indefinitely

**Appendix 14. Cutting frozen biopsies on the Cryostat**

1. Remove the specimen from the freezer and allow to reach room temperature.
2. Transfer to a universal containing 30% sucrose in PBS on the oscillator to off the OCT.
3. Make a pyramids of OCT, then with the temperature at (-34-36) °C trim to form a platform for the specimen.
4. Hatch top for keying specimen and add 2drops of OCT
5. Quickly place skin biopsy into OCT with the epidermis at a 45ºC angle to the knife
6. Allow to freeze, making sure there is enough OCT covering the specimen
7. Collect 50µm sections in 24 well plates containing PBS
8. Transfer to 96 well plates containing 200µl antifreeze solution (2 biopsies per plate).
9. Place plates into plastic boxes in the -20 °C freezer.

**Anti-freeze solution**
300ml glycerol
300ml ethylene glycerol
300ml dH2O
100ml 2x phosphate buffer (33.35g sodium phosphate monobasic +7.7g sodium hydroxide/litre)

**Appendix 15: Immunohistochemistry intra epidermal nerve fibre staining Method**

**Immunohistochemistry intra epidermal nerve fibre staining**

Note: do not let the sections dry out between any of the steps.

1. Wash in TBS/Tween20 x 1 15 mins
   x2 5 mins
2. Microwave in Diva retrieval solution (200µl) 10 mins
   @25% power 25°C
3. Wash in TBS/Tween20 x3 5 mins
4. Block in Dako peroxidise block (100µl) 30 mins
5. Wash in TBS/Tween20 x3 5 mins
6. Block the sections 10% normal goat serum in TBS/Tween20 (100µl) 4 hours
7. Incubate overnight in anti-PGP9.5 1:1000 (Millipore) diluted in 5% goat serum in TBS/Tween20 (50µl)
8. Wash in TBS/Tween20 x1 20 mins
   x2 15 mins
9. Incubate in biotinylated goat anti-rabbit 1:1000 (vector) 2 hour
diluted in 5% goat serum in TBS (100µl)
10. Wash in TBS/Tween20 x1 20 mins
    x2 15 mins
11. Incubate in HRP-Streptavidin 1:1000 (vector) diluted 2 hour
    in TBS (100µl)
12. Wash in TBS/Tween20 20 mins
13. Wash in dH2O x2 15 mins
14. Visualise nerve fibres using SG 15 mins
   (Made as manufactures instructions)
15. Wash in dH₂O x2 5 mins
16. Transfer the section (2/3 per slide if possible) on a small amount of aqueous mountant.
17. Spread flat using a brush – try to get epidermis flat (check under the microscope).
18. Allow the sections to dry. Mount with a cover slip and aqueous mountant.
19. Dry in at 4°C overnight.
20. Allow the slides to come back to room temperature.
21. Draw round the edges of the cover slips with permanent mountant and allow drying in the fume hood.
22. Add the staining run on to the database and fill out the immune run form.
23. Measure epidermal length along the surface (µm) and the number of nerves using computer image analysis. Nerve fibre linear density i.e. the number of fibres per mm of epidermis is expressed as no/mm (IENFD).
5.1 Supplementary results from this study

5.1.1 Vascular and neural function in the whole group of this study

South Asians in the larger group of this study (77 South Asian and 78 European) showed a higher TcPO\(_2\) (62.0±10) vs. European (57.7±12), P=0.02. Similarly, South Asians had a higher maximal hyperaemic response (506.4±276.7) vs. Europeans (415.8±191.4), P=0.02, but the LDI flare was significantly lower in South Asians (0.47 (0.30, 0.75)) compared to Europeans (0.64 (0.40, 1.09)), P=0.05.

Univariate analyses were used to explore the role of neuropathy risk factors, i.e. height, obesity, glycaemic control, smoking, vascular function, hypertension, hyperlipidaemia as potential explanations for the ethnic differences in TcPO\(_2\). The variables with the greatest impact on attenuating the P value for the age and diabetes duration adjusted ethnic differences in TcPO\(_2\) were weight, PAD, pack years smoked, higher HRV-DB and CNBD. There was no appreciable impact of TG, glycaemia and blood pressure on the TcPO\(_2\) model by ethnicity.

In multivariate analyses the combination of variables with the greatest impact on equalising estimated marginal means for TcPO\(_2\) in South Asians versus Europeans were weight, HRV-DB and pack years smoked (P=0.58). In another regression model, we found that a combination of weight, HRV-DB and PAD reduced the P value to P=0.44 (Table 27).

Linear regression for the laser Doppler flare area after adjustment for age and duration of diabetes showed no significant differences between South Asians and Europeans (P=0.36) (Table 28).
Table 27. Association of covariance for transcutaneous partial pressure by ethnicity.

<table>
<thead>
<tr>
<th>Adjustments factors</th>
<th>South Asian (n=77)</th>
<th>European (n=78)</th>
<th>P Value *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unadjusted</td>
<td>62.0(59.4,64.5)</td>
<td>57.7(55.1,60.2)</td>
<td>0.019</td>
</tr>
<tr>
<td><strong>Adjustment for age and duration of diabetes</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age+ diabetes duration</td>
<td>62.3(59.6,64.9)</td>
<td>57.7(55.1,60.3)</td>
<td>0.019</td>
</tr>
<tr>
<td><strong>Models with age and diabetes duration adjustment and univariate associations with other risk factors:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Height</td>
<td>62.2(59.5,64.9)</td>
<td>57.7(55.0,60.4)</td>
<td>0.024</td>
</tr>
<tr>
<td>Weight</td>
<td>61.3(58.5,64.0)</td>
<td>58.6(55.9,61.4)</td>
<td>0.2</td>
</tr>
<tr>
<td>HbA1c</td>
<td>62.3(59.6,64.9)</td>
<td>57.7(55.0,60.3)</td>
<td>0.019</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>62.3(59.6,65.1)</td>
<td>57.7(55.0,60.4)</td>
<td>0.02</td>
</tr>
<tr>
<td>Systolic BP</td>
<td>62.4(59.7,65.0)</td>
<td>57.7(54.9,60.2)</td>
<td>0.015</td>
</tr>
<tr>
<td>Diastolic BP</td>
<td>62.2(59.5,64.8)</td>
<td>57.8(55.2,60.4)</td>
<td>0.024</td>
</tr>
<tr>
<td>NBD</td>
<td>62.3(59.2,65.3)</td>
<td>57.9(54.9,60.8)</td>
<td>0.05</td>
</tr>
<tr>
<td>NFL</td>
<td>62.3(59.2,65.3)</td>
<td>57.9(54.9,60.8)</td>
<td>0.048</td>
</tr>
<tr>
<td>HRV-DB</td>
<td>61.7(58.8,64.6)</td>
<td>57.9(54.9,60.8)</td>
<td>0.077</td>
</tr>
<tr>
<td>PAD</td>
<td>61.9(58.8,65.0)</td>
<td>57.9(55.1,60.7)</td>
<td>0.065</td>
</tr>
<tr>
<td>Pack-Years Smoked</td>
<td>62.2(59.1,65.4)</td>
<td>58.1(55.1,61.0)</td>
<td>0.067</td>
</tr>
<tr>
<td><strong>Multivariate models:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight+ HRV+ pack years smoked</td>
<td>60.8(57.3,64.3)</td>
<td>59.3(55.8,62.8)</td>
<td>0.58</td>
</tr>
</tbody>
</table>

*P value for ethnic difference. (BP): Blood pressure.
5.1.2 Repeatability of measurements

The inter-class correlation coefficient (ICC) (563) was calculated to estimate the repeatability of the IENFD measurements between and within “occasion” and “observers”. The ICC was considered excellent if 0.8 to 1 and very good if 0.6 to 0.79. The intra-class correlation coefficient values were 0.88 (95% confidence intervals: 0.64, 0.96) for intra-observer (same person in two occasion) and 0.95% (95% confidence intervals: 0.84, 0.98) for inter-observer (by two observers), which shows very good repeatability between-occasion and between observer agreement (Figure 25).

Table 28. Association of covariance for maximal hyperaemic response by ethnicity.

<table>
<thead>
<tr>
<th>Adjustments factors</th>
<th>South Asian (n=77)</th>
<th>European (n=78)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unadjusted</td>
<td>506.4(451.7,561.2)</td>
<td>415.8(362.5,469.1)</td>
<td>0.02</td>
</tr>
<tr>
<td>Adjustment for age and diabetes duration</td>
<td>Age+ diabetes duration</td>
<td>507.5(450.3,564.7)</td>
<td>409.7(353.2,466.1)</td>
</tr>
<tr>
<td></td>
<td>Models with age and diabetes duration adjustment and univariate associations with other risk factors:</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Height</td>
<td>509.9(451.7,568.0)</td>
<td>407.4(350.1,464.7)</td>
</tr>
<tr>
<td></td>
<td>Weight</td>
<td>516.9(456.7,577.1)</td>
<td>400.5(341.2,459.9)</td>
</tr>
<tr>
<td></td>
<td>Waist size</td>
<td>524.1(464.5,583.6)</td>
<td>420.6(360.6,480.6)</td>
</tr>
<tr>
<td></td>
<td>HbA1c</td>
<td>508.6(451.1,566.2)</td>
<td>408.6(351.8,465.3)</td>
</tr>
<tr>
<td></td>
<td>Triglycerides</td>
<td>519.9(461.6,578.2)</td>
<td>400.2(343.1,457.2)</td>
</tr>
<tr>
<td></td>
<td>Systolic BP</td>
<td>508.4(450.6,566.2)</td>
<td>407.8(350.4,465.1)</td>
</tr>
<tr>
<td></td>
<td>Diastolic BP</td>
<td>505.9(448.7,563.2)</td>
<td>411.2(354.7,467.6)</td>
</tr>
<tr>
<td></td>
<td>NBD</td>
<td>519.8(457.9,581.8)</td>
<td>434.8(375.6,494.1)</td>
</tr>
<tr>
<td></td>
<td>NFL</td>
<td>519.7(458.2,581.2)</td>
<td>434.9(376.1,493.8)</td>
</tr>
<tr>
<td></td>
<td>HRV-DB</td>
<td>509.9(448.8,571.1)</td>
<td>410.1(348.5,471.7)</td>
</tr>
<tr>
<td></td>
<td>PAD</td>
<td>481.7(416.3,547.2)</td>
<td>412.5(354.9,470.2)</td>
</tr>
<tr>
<td></td>
<td>Pack-Years Smoked</td>
<td>525.7(462.0,589.4)</td>
<td>422.9(362.6,483.2)</td>
</tr>
<tr>
<td></td>
<td>Multivariate models:</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PAD+NBD</td>
<td>489.7(418.6,560.9)</td>
<td>438.3(377.2,499.4)</td>
</tr>
</tbody>
</table>

*P value for ethnic difference
5.1.3 Neuropad
Sudomotor function was assessed using the Neuropad in 73 South Asian and 78 European patients with Type 2 diabetes. The percentage of people with a positive neuropad response was non-significantly higher in South Asian (61.2±34.5) compared to European (52.4±35.9) diabetic patients, P= 0.1 (Table 17). The Neuropad output was also assessed according to whether the colour change was normal (changed completely to pink), intermediate (partially changed to pink) or abnormal (stayed blue). Seventeen (11.25%) patients (10 (58.82%) Asian) and (7 (41.17%) of Europeans) had a normal result. 122 (80.79%) of patients 60 (49.18%) South Asian and 63 (51.63%) Europeans had a patchy result and 11(7.33%) of patients (4(36.36% ) Asian and 7 (63.63% ) European patients had an abnormal neuropad results (Table 29).

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Control</th>
<th>South Asian (n=74)</th>
<th>European (n=76)</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>18 (52.94%)</td>
<td>10 (13.51%)</td>
<td>7 (9.21%)</td>
<td>17 (22.97%)</td>
</tr>
<tr>
<td>Patchy</td>
<td>16 (47.05%)</td>
<td>60 (81.08%)</td>
<td>62 (83.57%)</td>
<td>122 (81.33%)</td>
</tr>
<tr>
<td>Abnormal</td>
<td>0</td>
<td>4 (5.40%)</td>
<td>7 (9.21%)</td>
<td>11 (7.33%)</td>
</tr>
</tbody>
</table>
5.1.4 Structure function correlations for small fibre neuropathy

Correlations between structural (NFD, NBD, NFL, NFT, IENFD) and functional (CS, WS, CIP, HIP, HRV-DB, Neuropad) tests are presented in Table 30. Corneal confocal microscopy measures showed a significant correlation with functional tests of neuropathy to a varying degree, whilst IENFD only correlated significantly with warm sensation in both South Asian and European T2DM. These findings add to the literature regarding the relationship between structural and functional tests of small fibre neuropathy. In particular NFD, NBD, NFL correlated well with measure of small fibre function, making these potential predictive biomarkers.

Table 30. Correlation of structure assessment of neuropathy with functional tests

<table>
<thead>
<tr>
<th>Parameters</th>
<th>CS (°C)</th>
<th>WS (°C)</th>
<th>CIP (°C)</th>
<th>HIP (°C)</th>
<th>HRV-DB (beats/min)</th>
<th>Neuropad (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NFD(no./mm²)</td>
<td>r=0.263**</td>
<td>r=-0.297**</td>
<td>r=0.291**</td>
<td>r=-0.303**</td>
<td>r=0.279**</td>
<td>r=0.242**</td>
</tr>
<tr>
<td>NBD(no./mm²)</td>
<td>r=0.275**</td>
<td>r=-0.259**</td>
<td>r=0.321**</td>
<td>r=-0.294**</td>
<td>r=0.249**</td>
<td>r=0.263**</td>
</tr>
<tr>
<td>NFL(mm/mm²)</td>
<td>r=0.247**</td>
<td>r=-0.381**</td>
<td>r=0.225**</td>
<td>r=-0.448**</td>
<td>r=0.457**</td>
<td>r=0.272**</td>
</tr>
<tr>
<td>NFT(TC)</td>
<td>r=-0.018</td>
<td>r=-0.076</td>
<td>r=0.036</td>
<td>r=-0.150</td>
<td>r=-0.019</td>
<td>r=-0.035</td>
</tr>
<tr>
<td>NCCA (mbar)</td>
<td>r=-0.243**</td>
<td>r=0.293**</td>
<td>r=-0.109</td>
<td>r=0.191*</td>
<td>r=-0.252**</td>
<td>r=-0.192*</td>
</tr>
<tr>
<td>IENFD(no./mm)</td>
<td>r=0.215</td>
<td>r=-0.265*</td>
<td>r=-0.045</td>
<td>r=-0.030</td>
<td>r=0.150</td>
<td>r=0.074</td>
</tr>
</tbody>
</table>

Spearman’s correlation between structural and functional tests of neuropathy. (*) P value is ≤0.05, (**) P value <0.001.

We also found a significant inverse correlation between HbA1c and sural nerve conduction (r=-0.250, P=0.001), HbA1c and sural nerve amplitude (r=-0.187, P=0.01) and our results are compatible with a previous study (500). A significant correlation between IENFD and QST has been reported only when NCS was abnormal, and thus relies on the presence of significant neuropathy (519). IENFD correlated inversely with warm threshold more so than cooling threshold and also showed a correlation with NDS (r=-0.339, P=0.02) and VPT (r=-0.340, P=0.02) similar to the findings of Lauria et al (243, 520).

5.2 Langerhans Cells

Immune mechanisms have been proposed to play a role in the development of diabetic neuropathy. A previous study from our group showed increased
Langerhans cells (LCs) in diabetic patients particularly in the earlier phases of neuropathy and corneal nerve damage, suggestive of an immune mediated contribution to corneal nerve damage in diabetes. However, with progression of nerve damage, diabetic patients with moderate and severe neuropathy showed a reduction in the LC density (564). Further studies are needed to explore the role of Langerhans cells in diabetic neuropathy and particularly any differences between South Asians and Europeans. The LC density was significantly greater in both South Asian and European patients with T2DM (Figure 26) compared to control subjects. Furthermore the LC density was greater in South Asians compared to Europeans with T2DM (Table 31). There was a significant correlation between LC density and HbA1c, CNFD and CNBD (Table 32).
### Table 31. Demographic, Corneal confocal microscopy and Langerhans cells (LCs) in South Asians compared to Europeans with T2DM

<table>
<thead>
<tr>
<th>Parameters</th>
<th>South Asians T2DM (group1)</th>
<th>European T2DM (group2)</th>
<th>Asian Control (group3)</th>
<th>European Control (group4)</th>
<th>P value (1 vs 2)</th>
<th>P value (1 vs 3)</th>
<th>P value (2 vs 4)</th>
<th>P value (3 vs 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>60</td>
<td>68</td>
<td>10</td>
<td>11</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Age (years)</td>
<td>62.5±9.3</td>
<td>64.2±8.5</td>
<td>54.8±10.1</td>
<td>49.0±6.7</td>
<td>0.9</td>
<td>0.07</td>
<td>0.001</td>
<td>0.8</td>
</tr>
<tr>
<td>Gender (F/M)</td>
<td>20/40</td>
<td>28/48</td>
<td>5/5</td>
<td>5/6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>7.9±1.3</td>
<td>7.7±1.5</td>
<td>-</td>
<td>-</td>
<td>0.1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Duration of diabetes (years)</td>
<td>13.3±5.8</td>
<td>12.1±6.7</td>
<td>-</td>
<td>-</td>
<td>0.2</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NDS (0-10)</td>
<td>3.4±3.0</td>
<td>3.7±2.8</td>
<td>-</td>
<td>-</td>
<td>0.3</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>CNFD (no./mm^2)</td>
<td>24.5±8.1</td>
<td>25.0±7.5</td>
<td>37.5±8.7</td>
<td>38.6±4.9</td>
<td>0.7</td>
<td>0.001</td>
<td>&lt;0.001</td>
<td>0.7</td>
</tr>
<tr>
<td>CNBD (no./mm^2)</td>
<td>70.6±45.9</td>
<td>51.1±31.2</td>
<td>90.0±47.8</td>
<td>103.1±41.1</td>
<td>0.007</td>
<td>0.2</td>
<td>&lt;0.001</td>
<td>0.4</td>
</tr>
<tr>
<td>CNFL (mm/mm^2)</td>
<td>22.1±7.9</td>
<td>19.3±6.3</td>
<td>26.1±6.7</td>
<td>28.1±5.4</td>
<td>0.03</td>
<td>0.1</td>
<td>&lt;0.001</td>
<td>0.4</td>
</tr>
<tr>
<td>CNFT (TC)</td>
<td>18.7±4.8</td>
<td>20.1±6.2</td>
<td>15.8±4.6</td>
<td>15.2±2.0</td>
<td>0.1</td>
<td>0.08</td>
<td>&lt;0.001</td>
<td>0.7</td>
</tr>
<tr>
<td>NCCA (mbars)</td>
<td>0.99±0.79</td>
<td>1.02±0.75</td>
<td>0.74±0.56</td>
<td>0.50±0.22</td>
<td>0.5</td>
<td>0.1</td>
<td>0.007</td>
<td>0.4</td>
</tr>
<tr>
<td>LC density (no./mm^2)</td>
<td>74.4±75.8</td>
<td>53.9±63.8</td>
<td>18.5±13.7</td>
<td>15.2±13.9</td>
<td>0.02</td>
<td>0.004</td>
<td>0.01</td>
<td>0.7</td>
</tr>
<tr>
<td>LC(%) (Presence/Absence)</td>
<td>96.7% (58/2)</td>
<td>97.1% (66/2)</td>
<td>100% (10/0)</td>
<td>81.8% (9/2)</td>
<td>0.5</td>
<td>0.1</td>
<td>0.007</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Data are presented as Mean ± SD. Langerhans cells (LC).
Table 32. Correlations with Langerhans cell density (Spearman’s)

<table>
<thead>
<tr>
<th>Variable</th>
<th>r</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>0.131</td>
<td>0.1</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>0.164</td>
<td>0.04</td>
</tr>
<tr>
<td>Duration of diabetes (years)</td>
<td>0.128</td>
<td>0.04</td>
</tr>
<tr>
<td>NDS (0-10)</td>
<td>0.151</td>
<td>0.06</td>
</tr>
<tr>
<td>CNFD (no./mm²)</td>
<td>-0.284</td>
<td>0.007</td>
</tr>
<tr>
<td>CNBD (no./mm²)</td>
<td>-0.184</td>
<td>0.02</td>
</tr>
<tr>
<td>CNFL (mm/mm²)</td>
<td>-0.148</td>
<td>0.07</td>
</tr>
<tr>
<td>CNFT (TC)</td>
<td>-0.007</td>
<td>0.9</td>
</tr>
<tr>
<td>NCCA (mbars)</td>
<td>-0.09</td>
<td>0.3</td>
</tr>
</tbody>
</table>

Figure 26. Corneal confocal microscopy from South Asian (left) and European (right) patients with T2DM (white dots indicate Langerhans cells).
Chapter 6

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Published papers:
During my PhD, I have been working as clinical research fellow and following papers were published from our group with my name as co-authors.
Longitudinal assessment of neuropathy in type 1 diabetes using novel ophthalmic markers (LANDMark): Study design and baseline characteristics


Aims: Corneal nerve morphology and corneal sensation threshold have recently been explored as potential surrogate markers for the evaluation of diabetic neuropathy. We present the baseline findings of the 'Longitudinal Assessment of Neuropathy in type 1 Diabetes using novel ophthalmic Markers' (LANDMark) study.

Methods: The LANDMark study is a 4-year, two-site, natural history study of three participant groups: type 1 diabetes with neuropathy (T1W), type 1 diabetes without neuropathy (T1WO) and control participants without diabetes or neuropathy. All participants undergo a detailed annual assessment of neuropathy including corneal nerve parameters measured using corneal confocal microscopy and corneal sensitivity measured using non-contact corneal aesthesiometry.

Results: 76 T1W, 166 T1WO and 154 control participants were enrolled into the study. Corneal sensation threshold was significantly higher (i.e., sensitivity was lower) in T1W (1.0 ± 1.1 mbars) than T1WO (0.7 ± 0.7 mbars) and controls (0.6 ± 0.4 mbars) (p < 0.001), with no difference between T1WO and controls. Corneal nerve fibre length was lower in T1W (14.0 ± 6.4 mm/mm²) compared to T1WO (19.1 ± 5.8 mm/mm²) and controls (23.2 ± 6.3 mm/mm²) (p < 0.001). Corneal nerve fibre length was lower in T1WO compared to controls.

Conclusions: The LANDMark baseline findings confirm a reduction in corneal sensitivity only in Type 1 patients with neuropathy. However, corneal nerve fibre length is reduced in Type 1 patients without neuropathy with an even greater deficit in Type 1 patients with neuropathy.

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1. Introduction

Diabetic neuropathy is a significant and prevalent complication, which leads to morbidity in the form of painful feet, foot ulceration and lower extremity amputation [1]. Currently there are no disease modifying therapies approved by the USA Food and Drug Administration for diabetic neuropathy. Poor glycaemic control [2] and vascular risk factors [3] have been shown to be significant risk factors in the development of neuropathy. However, improved glycaemic control has been demonstrated to prevent progression of diabetic neuropathy in type 1 but not type 2 diabetes [4,5].

The few longitudinal studies of diabetic neuropathy that have been undertaken show a progressive deterioration of neuropathy over time in patients with type 1 [19] and type 2 [20] diabetes, and the placebo arms of several clinical trials of diabetic neuropathy show a monotonic worsening in electrophysiology and quantitative sensory testing [21]. We are conducting a longitudinal assessment of patients with type 1 diabetes using novel ophthalmic markers (LANDMark), the aims of which are to (a) observe the natural history of diabetic neuropathy using these novel non-invasive ophthalmic tests, (b) prospectively characterize changes in structural (CCM) and functional (NCCA) corneal parameters in individuals with and without diabetic neuropathy, (c) determine the predictive validity of CCM and NCCA, and (d) identify demographic and metabolic risk factors associated with these changes over time. In this paper, we describe the study design and summarize the baseline characteristics of participants enrolled in the LANDMark study.

2. Materials and methods

2.1. Study design

The LANDMark study is an investigator-masked, prospective, longitudinal, controlled, natural history (observational) study conducted at two sites–Brisbane, Australia and Manchester, UK. A minimum cohort of 404 participants were intended to be enrolled (202 per site) according the inclusion/exclusion criteria, and followed annually for four subsequent years (i.e. 5 examinations in total). All participants were examined at baseline and will be examined annually thereafter. Ethical clearance was granted by partner hospitals, universities and other relevant national research ethics committees in Australia and the UK. Written informed consent was obtained from all participants and the study is being carried out in accordance with the principles of the Declaration of Helsinki as revised in 2000.

2.2. Setting and participants

Individuals with type 1 diabetes (age range 14–80 years) were recruited from the Centre for Diabetes and Endocrine Research at Princess Alexandra Hospital and Mater Hospitals in Brisbane, and the Manchester Diabetes Centre, Manchester Royal Infirmary, as well as community and other clinics at both sites. Sample size was determined using the expected effect size in the two primary outcome variables – corneal nerve fibre length and corneal sensation threshold – taking into account at least 40% attrition and multisite variability. Individuals were excluded if they had a history of ocular trauma or surgery, ocular disease or systemic disease affecting the cornea; systemic disease other than diabetes (e.g. malignant disease, congestive heart failure, major psychosis); history of neuropathy of non-diabetic cause, current diabetic foot ulcer or infection or participation in any interventional research trial. A group of non-diabetic control participants was also included and two specific exclusion criteria applied were: (a) positive glutamic acid decarboxylase antibody status, or (b) neuropathy according to the Toronto criteria [3].
An individual was considered to have neuropathy if they met the following ‘Toronto criteria’ [3]: (a) abnormal nerve conduction, based on age-matched controls at the site, and (b) a symptom or sign of neuropathy, defined as one or more of the following: (i) diabetic neuropathy symptom score of \( \geq 1/4 \) [22], or (ii) neuropathy disability score of \( \geq 3/10 \) [23].

2.3. **Masking and retention**

The order of testing was randomized where practical, and all procedures were conducted by trained individuals according to standard operating procedures. Investigators were masked as to the group assignment of participants. All data analyses were performed in a masked fashion. For example, a masked investigator analyzed CCM images in large batches, with the images only being identifiable by number codes; thus, these measurements were made without reference to NCMA, neuropathy results or participant history, which otherwise could have compromised masking. Retention of individuals was maximized through strategies of flexibility, accessibility, personalized attention, and feedback.

2.4. **Corneal nerve morphology**

Images of the sub-basal nerve plexus of the cornea were captured from one eye (the side of hand dominance) of each participant using a CCM (Heidelberg Retinal Tomograph III with Rostock Cornea Module; Heidelberg Engineering GmbH, Germany), after anaesthetizing the cornea. For each participant, a minimum of three of the clearest images at the level of the sub-basal nerve plexus of the central cornea were analyzed using semi-automated software (CCMetrics, University of Manchester) to calculate CNFL in mm/mm\(^2\) and corneal nerve branch density (CNBD) in number/mm\(^2\) [24].

2.5. **Corneal sensation threshold**

Corneal sensation threshold was measured using an NCMA designed and constructed for the Institute of Health and Biomedical Innovation based on the original work of Murphy et al. [25]. The eye on the side of hand dominance was measured using a stimulus duration of 0.9 s in a quiet room without distractions. The participant was instructed to fixate on a target, the nozzle tip distance was positioned 10 mm from the central cornea and sensation threshold was determined using a modified Garcia-Perez staircase technique, according to the procedure described previously by Pritchard et al. [18].

2.6. **Measures of neuropathy**

Symptoms were assessed using the diabetic neuropathy symptom score [22], where 0 indicates no neuropathy and 1–4 points indicates increasing severity of neuropathy.

Neurological deficits were evaluated by determining the neuropathy disability score [23], which involved measurement of vibration perception, pin prick perception, temperature perception and the presence or absence of ankle reflexes using a tendon hammer applied to both lower limbs. A score is derived ranging from 0 to 10, whereby a neuropathy disability score of \( \leq 2 \) indicates no neuropathy.

The Neuropad® (miro Verbandstoffe GmbH, Wiehl, Germany) [26] was applied to the plantar aspect of the big toe and after 10 min, the appearance was assessed. A 100% pink pad indicated a normal, non-neuropathic response and an abnormal response was indicated when the pad remained blue or was patchy.

The monofilament test was performed using a 10-g nylon filament and was applied to 3 pre-determined points on the sole of the foot on the hand-dominant side.

Vibration thresholds were measured using a Medoc VSA-3000 Vibratory Sensory Analyzer (Medoc Advanced Medical Systems, Ramat-Yishai, Israel) in Brisbane, and a Horwell Neuroaesthesiometer (Scientific Laboratory Supplies, Nottingham, UK) in Manchester. Assessments were made on the hand-dominant side of the dorsolateral aspect of the foot. Warm and cold sensation and pain thresholds were determined at both sites using the Medoc TSA-II NeuroSensory Analyzer.

Nerve conduction studies were performed using a NeuroPack S1 EMG/Evoked Potential Measuring System (Nihon Kohden, Tokyo, Japan) in Brisbane and a Dantec Keypoint System (Dantec Dynamics Ltd., Bristol, UK) in Manchester. Peroneal and sural nerve conduction velocities, amplitudes and latencies were recorded on the hand-dominant side in Brisbane (87% right) and the left side in Manchester.

2.7. **Diabetic retinopathy**

Retinal fundus images (3-field) were taken at each visit with a dilated pupil and each image was graded by an ophthalmologist according to the Early Treatment of Diabetic Retinopathy Study scale [27] or the National Screening Committee, UK.

2.8. **Skin punch biopsy**

Intraepidermal nerve fibre density was performed on a subset of 207 individuals at the Manchester site. Two punch skin biopsies (3 mm in diameter) were performed on the dorsum of the foot approximately 2 cm above the second metatarsal head under local anaesthesia (1% lignocaine). The biopsy site was closed using Steri-strips, and the specimen was fixed, cryoprotected and processed as previously described [15]. Intraepidermal nerve fibre linear density, i.e. the number of fibres per millimetre of epidermal length, expressed as intraepidermal nerve fibres per millimetre, was recorded.

2.9. **Risk factors**

Blood pressure, body mass index, waist circumference, alcohol and tobacco consumption, renal function, \( \text{HbA_1c} \), lipid profile and red blood cell \( \text{B}_{12} \) and folate were assessed.

2.10. **Statistical methods**

Descriptive statistics for the total cohort and for each group are presented. For quantitative variables, data are presented as mean ± standard deviation unless otherwise stated. Differences between quantitative variables with a normal distribution were tested using a generalized linear model or with an analysis of variance (IBM SPSS Statistics Version 19, Armonk,
NY, USA). Posthoc tests were performed using the Tukey HSD post hoc test. The non-parametric Kruskal–Wallis test was used to analyze non-normally distributed data. The Chi-squared test was used to analyze categorical data.

3. Results

Of 449 individuals recruited with type 1 diabetes mellitus and healthy controls, 396 were deemed to be eligible for participation in the LANDMark study. Table 1 shows the baseline demographic and clinical characteristics for the three groups studied: ‘type 1 diabetes with neuropathy’ (T1W), ‘type 1 diabetes without neuropathy’ (T1WO) and ‘no diabetes or neuropathy’ controls. Approximately half (53%) of the commencing cohort were Caucasian of European descent and the remaining 47% were of Asian, South East Asian, Middle Eastern, or other ethnic origin.

3.1. Clinical measures

Clinical measures relating to the three groups are shown in Table 1. Both age and duration of diabetes were 14 years greater in T1W versus T1WO. Supine systolic and diastolic blood pressure was 13 and 2 mmHg greater in T1W than T1WO, respectively. Waist circumference was 6 cm greater in T1W than T1WO. A significantly higher proportion of T1W had retinopathy (75%) compared with T1WO (45%).

Height, body mass index, alcohol consumption and number of cigarettes per day did not differ between groups. There were no significant differences in any clinical measures between the T1WO and controls.

Antihypertensive or antianginal medications were being taken by 45% of T1W, 17% of T1WO and 4% of controls. Approximately double the number of T1W took analgesics and anti-inflammatory medicine compared to T1WO and controls.

Table 1 – Clinical demographics of the LANDMark cohort at baseline (mean ± standard deviation, range [in parenthesis], n), of the following groups of study participants: type 1 diabetes with neuropathy (T1W), type 1 diabetes without neuropathy (T1WO) and control participants without diabetes or neuropathy.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>T1W (A)</th>
<th>T1WO (B)</th>
<th>Controls (C)</th>
<th>P Group difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Site n (Brisbane/Manchester)</td>
<td>48/26</td>
<td>100/68</td>
<td>60/94</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Sex (n/%F)</td>
<td>33/45%</td>
<td>83/49%</td>
<td>84/54%</td>
<td>0.346</td>
</tr>
<tr>
<td>Age (years) (20–80)</td>
<td>57 ± 11</td>
<td>43 ± 16</td>
<td>46 ± 15</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>74</td>
<td>168</td>
<td>154</td>
<td>A vs. B, C</td>
</tr>
<tr>
<td>Duration of diabetes (years) (1–58)</td>
<td>34 ± 16</td>
<td>20 ± 15</td>
<td>n/a</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>74</td>
<td>165</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Height (cm) (143–189)</td>
<td>170 ± 9</td>
<td>170 ± 10</td>
<td>168 ± 10</td>
<td>0.169</td>
</tr>
<tr>
<td></td>
<td>74</td>
<td>167</td>
<td>151</td>
<td></td>
</tr>
<tr>
<td>Weight (kg) (46–172)</td>
<td>81 ± 21</td>
<td>77 ± 15</td>
<td>74 ± 16</td>
<td>0.016</td>
</tr>
<tr>
<td></td>
<td>74</td>
<td>167</td>
<td>151</td>
<td>A vs. C</td>
</tr>
<tr>
<td>Weight (kg) (18–48)</td>
<td>28 ± 6</td>
<td>26 ± 4</td>
<td>26 ± 5</td>
<td>0.033</td>
</tr>
<tr>
<td></td>
<td>74</td>
<td>167</td>
<td>151</td>
<td>A vs. B, C</td>
</tr>
<tr>
<td>Waist circumference (cm) (67–139)</td>
<td>96 ± 16</td>
<td>89 ± 13</td>
<td>89 ± 14</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>73</td>
<td>161</td>
<td>148</td>
<td>A vs. B, C</td>
</tr>
<tr>
<td>Systolic BP (mmHg) (supine) (85–207)</td>
<td>136 ± 22</td>
<td>123 ± 17</td>
<td>122 ± 17</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>73</td>
<td>166</td>
<td>150</td>
<td>A vs. B, C</td>
</tr>
<tr>
<td>Diastolic BP (mmHg) (supine) (54–101)</td>
<td>75 ± 10</td>
<td>71 ± 8</td>
<td>72 ± 9</td>
<td>0.031</td>
</tr>
<tr>
<td></td>
<td>73</td>
<td>166</td>
<td>150</td>
<td>A vs. B, C</td>
</tr>
<tr>
<td>Cigarettes (number per day) (0–30)</td>
<td>2 ± 5</td>
<td>1 ± 3</td>
<td>1 ± 3</td>
<td>0.326</td>
</tr>
<tr>
<td></td>
<td>71</td>
<td>162</td>
<td>143</td>
<td></td>
</tr>
<tr>
<td>Alcohol (units per week) (0–56)</td>
<td>6 ± 9</td>
<td>5 ± 6</td>
<td>4 ± 6</td>
<td>0.146</td>
</tr>
<tr>
<td></td>
<td>67</td>
<td>159</td>
<td>140</td>
<td></td>
</tr>
<tr>
<td>Retinopathy (n)</td>
<td>15</td>
<td>67</td>
<td>n/a</td>
<td>&lt;0.001 (Chi²)</td>
</tr>
<tr>
<td>No apparent retinopathy</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mild non-proliferative</td>
<td>19</td>
<td>41</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Moderate non-proliferative</td>
<td>19</td>
<td>15</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Severe non-proliferative</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proliferative</td>
<td>3</td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 2 – Metabolic profile of the LANDMark cohort at baseline (mean ± standard deviation, range [in parenthesis], n), of the following groups of study participants: type 1 diabetes with neuropathy (T1 W), type 1 diabetes without neuropathy (T1WO) and control participants without diabetes or neuropathy.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>T1W (A)</th>
<th>T1WO (B)</th>
<th>Controls (C)</th>
<th>P</th>
<th>Group difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>HbA1c (%)</td>
<td>8.6 ± 1.8</td>
<td>8.0 ± 1.2</td>
<td>5.5 ± 0.3</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>Albumin:creatinine ratio (mg/mmol)</td>
<td>28 ± 76</td>
<td>5 ± 36</td>
<td>1 ± 2</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>4.8 ± 1.2</td>
<td>4.6 ± 0.9</td>
<td>5.2 ± 1.0</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
<td>1.7 ± 0.6</td>
<td>1.6 ± 0.4</td>
<td>1.5 ± 0.4</td>
<td>0.013</td>
<td></td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.4 ± 1.0</td>
<td>1.1 ± 0.6</td>
<td>1.3 ± 0.7</td>
<td>0.003</td>
<td></td>
</tr>
<tr>
<td>eGFR (mL/min)</td>
<td>65 ± 23</td>
<td>81 ± 14</td>
<td>83 ± 10</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>HbA1c (mmol/mol)</td>
<td>70 ± 19</td>
<td>64 ± 13</td>
<td>37 ± 4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>4.8 ± 1.2</td>
<td>4.6 ± 0.9</td>
<td>5.2 ± 1.0</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
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<td>1.5 ± 0.4</td>
<td>0.013</td>
<td></td>
</tr>
<tr>
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<td>1.3 ± 0.7</td>
<td>0.003</td>
<td></td>
</tr>
<tr>
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<td>5 ± 36</td>
<td>1 ± 2</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
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<td>4.6 ± 0.9</td>
<td>5.2 ± 1.0</td>
<td>&lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>HDL (mmol/L)</td>
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<td>1.6 ± 0.4</td>
<td>1.5 ± 0.4</td>
<td>0.013</td>
<td></td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.4 ± 1.0</td>
<td>1.1 ± 0.6</td>
<td>1.3 ± 0.7</td>
<td>0.003</td>
<td></td>
</tr>
</tbody>
</table>

3.2. Metabolic profile

Compared to T1WO, T1W had 0.6% higher HbA1c, 0.6 mg/mmol higher albumin creatinine ratio, 16 mL/min lower estimated glomerular filtration rate, 0.2 mmol/L higher total cholesterol and 0.3 mmol/L higher triglycerides. HbA1c was higher and triglycerides were lower in T1WO compared to controls (Table 2).

3.3. Neuropathy assessment

All measures of neuropathy indicated greater disease severity in T1W compared to T1WO and controls, with the exception of warm pain threshold, which did not differ between groups (Table 3). Neuropathy measures were significantly more severe in the T1WO group than in controls in respect of neuropathy disability score, cold threshold, vibration threshold, peroneal and sural conduction velocity and monofilament sensitivity.

3.4. Ophthalmic measures

Outcomes of ophthalmic assessments are shown in Fig. 1. Corneal sensitivity threshold (mbars) was significantly higher in T1W (1.0 ± 1.1) than T1WO (0.7 ± 0.7) and controls (0.6 ± 0.4) (p < 0.001), with no difference between T1WO and controls (Tukey HSD, p = 0.482). Corneal nerve fibre length (mm/mm²) was significantly lower in T1W (14.0 ± 6.4) than T1WO (19.1 ± 5.8) and controls (23.2 ± 6.3) (p < 0.001); post hoc testing showed that CNFL in T1WO was significantly lower than in controls (Tukey HSD p < 0.001). CNBD (branches/mm²) was significantly lower in T1W (40.1 ± 32.1) than T1WO (61.7 ± 37.2) and controls (83.5 ± 45.8) (p < 0.001). Furthermore post hoc testing showed T1WO had a significantly lower CNBD compared to controls (Tukey HSD p = 0.001).

3.5. Site differences

Duration of diabetes, blood pressure, height, alcohol consumption and ethnicity were different between the two sites, regardless of group. All metabolic test parameters showed differences between sites, with the exception of urine albumin-creatinine ratio and HDL cholesterol. Most measures of neuropathy showed more severe disease at the Manchester site compared with the Brisbane site. Warm and cold sensation threshold and cold pain threshold were not different between sites. Regardless of group, corneal nerve sensitivity threshold was 0.2 mbars higher (i.e. poorer sensitivity) and corneal nerve fibre length was 4 mm/mm² greater (i.e. better nerve morpholo) at the Manchester site compared to the Brisbane site, representing a 30% and 22% difference, respectively.

4. Discussion

The primary objective of the LANDMark study is to observe the natural history of diabetic neuropathy, applying two novel, non-invasive ophthalmic tests, which may serve as useful markers of this potentially debilitating condition. We present the baseline findings at the two study sites, Brisbane, Australia and Manchester, UK. The LANDMark study cohort of 396 participants was recruited over approximately 1.5 years. The application of strict inclusion and exclusion criteria has
Table 3 - Neuropathy findings of the LANDMark cohort at baseline (mean ± standard deviation, range [in parenthesis], n), of the following groups of study participants: type 1 diabetes with neuropathy (T1 W), type 1 diabetes without neuropathy (T1WO) and control participants without diabetes or neuropathy.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>T1W (A)</th>
<th>T1WO (B)</th>
<th>Controls (C)</th>
<th>P Group difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetic neuropathy symptom score</td>
<td>1.5 ± 1.3</td>
<td>0.2 ± 0.6</td>
<td>0.1 ± 0.3</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>(0–4)</td>
<td>0–4</td>
<td>0–4</td>
<td>0–3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>167</td>
<td>144</td>
<td></td>
</tr>
<tr>
<td>Neuropathy disability score</td>
<td>4.2 ± 3.2</td>
<td>1.1 ± 1.8</td>
<td>0.3 ± 0.8</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>(0–10)</td>
<td>0–10</td>
<td>0–8</td>
<td>0–5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>73</td>
<td>166</td>
<td>149</td>
<td></td>
</tr>
<tr>
<td>Cold sensation threshold (°C)</td>
<td>20.6 ± 9.4</td>
<td>27.1 ± 4.6</td>
<td>28.5 ± 2.3</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>0.0–31.3</td>
<td>6.0–31.5</td>
<td>18.3–33.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>69</td>
<td>165</td>
<td>150</td>
<td></td>
</tr>
<tr>
<td>Warm sensation threshold (°C)</td>
<td>42.9 ± 4.1</td>
<td>37.8 ± 3.7</td>
<td>37.1 ± 3.4</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>33.9–50.0</td>
<td>33.0–47.5</td>
<td>28.5–47.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>69</td>
<td>165</td>
<td>150</td>
<td></td>
</tr>
<tr>
<td>Cold pain threshold (°C)</td>
<td>5.1 ± 7.5</td>
<td>12.0 ± 9.3</td>
<td>11.4 ± 8.8</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>0–26.2</td>
<td>0–29.0</td>
<td>0–29.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>71</td>
<td>162</td>
<td>149</td>
<td></td>
</tr>
<tr>
<td>Warm pain threshold (°C)</td>
<td>48.8 ± 2.2</td>
<td>46.0 ± 3.4</td>
<td>45.3 ± 3.9</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>36.7–50.0</td>
<td>36.9–50.0</td>
<td>19.9–50.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>71</td>
<td>162</td>
<td>149</td>
<td></td>
</tr>
<tr>
<td>Vibration thresholda (Hz)</td>
<td>30.1 ± 30.4</td>
<td>8.7 ± 10.5</td>
<td>7.0 ± 8.1</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>1.1–130.0</td>
<td>0.7–47.7</td>
<td>0.7–42.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>47</td>
<td>100</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>Vibration thresholdb (V)</td>
<td>27.3 ± 13.5</td>
<td>9.2 ± 7.6</td>
<td>5.2 ± 4.5</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>3.5–51.0</td>
<td>2.0–35.0</td>
<td>0.5–27.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>26</td>
<td>66</td>
<td>89</td>
<td></td>
</tr>
<tr>
<td>Peroneal conduction velocity, ankle to Fem (m/s)</td>
<td>35.3 ± 8.4</td>
<td>45.6 ± 4.8</td>
<td>48.8 ± 4.9</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>19.0–50.9</td>
<td>20.0–57.4</td>
<td>21.1–60.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>162</td>
<td>143</td>
<td></td>
</tr>
<tr>
<td>Sural conduction velocity, calf to anklec (m/s)</td>
<td>30.9 ± 11.3</td>
<td>42.2 ± 5.7</td>
<td>48.1 ± 6.1</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>5–50</td>
<td>31–54</td>
<td>31–61</td>
<td></td>
</tr>
<tr>
<td></td>
<td>70</td>
<td>161</td>
<td>144</td>
<td></td>
</tr>
<tr>
<td>Neuropad (n)</td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.001 (Chi²)</td>
</tr>
<tr>
<td>Abnormal</td>
<td>8</td>
<td>6</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Patchy</td>
<td>30</td>
<td>39</td>
<td>31</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>35</td>
<td>121</td>
<td>114</td>
<td></td>
</tr>
<tr>
<td>Monofilamenta (responses/3)</td>
<td>2.3 ± 1.0</td>
<td>2.9 ± 0.5</td>
<td>3.0 ± 0.2</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>0–3</td>
<td>0–3</td>
<td>0–3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>97</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>Monofilamentc (responses/10)</td>
<td>6.6 ± 4.0</td>
<td>8.7 ± 3.0</td>
<td>10 ± 0.0</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td></td>
<td>0–10</td>
<td>0–10</td>
<td>0–10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>61</td>
<td>86</td>
<td></td>
</tr>
<tr>
<td>Intraepidermal nerve fibre densityd (number/mm)</td>
<td>4.47 ± 4.77</td>
<td>6.98 ± 4.68</td>
<td>10.29 ± 3.29</td>
<td>0.006</td>
</tr>
<tr>
<td></td>
<td>0–11.6</td>
<td>2.85–16.24</td>
<td>4.96–16.63</td>
<td></td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>48</td>
<td>33</td>
<td></td>
</tr>
</tbody>
</table>

a Brisbane – Medoc VSA-3000 (Hz).
b Manchester–Horwell Bioesthesiometer (volts).
c Manchester – where there was no response, the following substituted data were used: Peroneal CV 20 m/s; sural CV 31 m/s, reflecting lowest values recorded in this laboratory.
d Manchester – where there was no response, the following substituted data were used: Peroneal CV 19 m/s; sural CV 5 m/s, reflecting lowest values recorded in this laboratory.
* Brisbane – responses out of 3.
† Manchester – responses out of 10.
§ Data from Manchester only.
** Kruskal–Wallis test.
†† Mann–Whitney U test.

resulted in a valid cohort, as judged by comparison of these baseline findings with those of diabetic participants examined in other large clinical trials. For example, in the LANDMark study, HbA1c was 8.6% at baseline compared to 8.3% in the Diabetes Control and Complications/Epidemiology of Diabetes Interventions and Complications Study [28]. Similarly, total cholesterol was 4.4 mmol/L in the present study versus 4.8 mmol/L (185 mg/dl) in the study of Lorbeer et al. [29]. Controls in the LANDMark cohort had a mean HbA1c of 5.6% compared to 5.3% in the study of Lorbeer et al. [29].
4.1. Between-group differences in ophthalmic measures

CNFL was greater in the controls compared to patients with type 1 diabetes (23.2 vs. 13.8–19.1 mm/mm²). The measures of CNFL and CNBD reported in our control group are similar to those reported by researchers using the same technology in individuals without diabetes [30–32]. Several authors have reported corneal nerve parameters of individuals with diabetes using the same Heidelberg technology as used in this study [33–35]. CNFL of diabetic participants in the present study (17.4 mm/mm²) is similar to that reported by Ahmed et al. [33] and Hertz et al. [34] (9.5–16.7 mm/mm²), but higher than reported by Ishibashi et al. [35], Tavakoli [36] and Messmer et al. [37] (9.7–11.4 mm/mm²). This discrepancy is likely due to differences in sample sizes, severity of neuropathy of the cohorts examined, acquisition mode using CCM, number of images analyzed per participant, field of view of the acquisition lens, operator technique and software applied to analyze images.

Corneal sensation threshold in the present study (average 0.61 mbars in controls) was similar to that reported in other studies, using similar instrumentation, investigating healthy individuals without diabetes [14,38–40]. The corneal sensation threshold for T1WO in the current study (0.95 mbars), was similar to that reported by Murphy et al. [40], but somewhat lower than reported by other studies, where measures were on average 1.5 mbars [17,38,39]. This discrepancy may be due to diabetic participants in those studies having a greater severity of neuropathy.

4.2. Limitations of the study

Some site differences are to be expected due to recruitment and cohort differences, instrumentation and investigator preferences, despite common standard operating procedures adopted by both sites. Observer and laboratory differences are not uncommon in multi-centre studies and in fact broaden the sample to represent real-world data. These differences are viewed as improving the generalisability of the outcomes of the study. The generalisability (external validity) of the trial findings should be sound given the large population and the range of ethnicities and participant ages at the two sites.

The variables expected to show differences between sites, but analyzed together, include all nerve conduction results and corneal sensation thresholds. The variables that were not analyzed together – due to differences in instrumentation and technique of these tests – include monofilament and vibration sensation threshold.
Participant age and duration of diabetes were each 2 years greater at the Manchester site than the Brisbane site. We would have expected these differences to influence the severity of neuropathy; however, this was not the case. Another limitation of the LANDMark study is that, despite our best efforts, we were unable to ensure masking of all clinical measurement procedures. Intermittent failure to mask the investigators from the diabetic and/or neuropathic status of the individuals being examined may be a source of potential bias.

In conclusion, CCM and NCCA have the potential to supplement the clinical repertoire of endocrinologists and neurologists, perhaps with increased involvement from eye care professionals, in diagnosing and managing diabetic neuropathy.

**Conflicts of interest**

All authors declare that they have no conflicts of interests.

**REFERENCES**


Corneal Confocal Microscopy Detects Neuropathy in Subjects with Impaired Glucose Tolerance

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Key Words:
Confocal Microscopy, Complication(s), Neuropathy, Neuropathy Complications
Corneal Confocal Microscopy Detects Neuropathy in Subjects with Impaired Glucose Tolerance

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Word Count: 1,323

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LIST OF ABBREVIATIONS

CCM: corneal confocal microscopy
CNBD: corneal nerve branch density
CNFD: corneal nerve fiber density
CNFL: corneal nerve fiber length
CT: cold threshold
HRVdb: heart rate variability deep breathing
IENFD: intra epidermal nerve density
IGT: impaired glucose tolerance
IGTN: impaired glucose tolerance neuropathy
ISFN: idiopathic small fiber neuropathy
NCS: nerve conduction studies
NDS: neuropathy disability score
NSP: neuropathy symptom profile
SD: standard deviation
VPT: vibration perception threshold
WT: warm threshold
ABSTRACT

Objective Impaired glucose tolerance (IGT) represents one of the earliest stages of glucose dysregulation and is associated with macrovascular disease, retinopathy and microalbuminururia, but it is unclear whether it causes neuropathy.

Research Design and Methods 37 subjects with IGT and 20 age-matched controls underwent a comprehensive evaluation of neuropathy by assessing symptoms, neurological deficits, nerve conduction studies (NCS), quantitative sensory testing, heart rate variability deep breathing (HRVdb), skin biopsy and corneal confocal microscopy (CCM).

Results Subjects with IGT had a significantly increased NSP (P<0.001), McGill pain index (P<0.001), NDS (P=0.001), VPT (P=0.002), WT (P=0.006) and CT (P=0.03) with a reduction in IENFD (P=0.03), CNFD (P<0.001), CNBD (P=0.002) and CNFL (P=0.05), but no significant difference in sensory and motor nerve amplitude and conduction velocity or HRVdb.

Conclusions Small fibre neuropathy is present in subjects with IGT and can be readily detected using CCM.
INTRODUCTION

The association between IGT and peripheral neuropathy was first highlighted when subjects with idiopathic small-fibre neuropathy (ISFN) were found to have an unexpectedly high prevalence of IGT (1). Subsequently in the population-based San Luis Valley (2) and MONICA/KORA Augsburg (3) studies, neuropathy occurred in 26-28% of patients with diabetes, 11-13% in IGT and 4-8% in control subjects. In contrast, Dyck and colleagues (4) did not find an increased prevalence of neuropathy amongst subjects with impaired glycemia.

Establishing neuropathy in IGT is important as it may provide insights into the early pathogenetic components of diabetic neuropathy, and highlights that neuropathy may occur with minimal metabolic derangement. The detection of peripheral neuropathy in IGT remains challenging, especially as the majority of studies have used a combination of symptoms and neurologic signs, which are large-fiber weighted. An increasing body of evidence suggests a predominantly small-fiber neuropathy with a significant reduction in IENFD and minimal large-fiber involvement in subjects with IGT (5). CCM, a novel surrogate measure of small-fiber neuropathy, has been shown to detect early small fibre damage in diabetic patients (6; 7). The purpose of this study was to undertake a comprehensive assessment of neuropathy in subjects with IGT using symptoms and neurological deficits, neurophysiology, quantitative sensory testing and in particular skin biopsy and CCM, as sensitive measures of small fibre neuropathy.
METHODS

Study Subjects

37 subjects aged 30-75 years with IGT (Oral Glucose Tolerance Test: 2-hour glucose = 7.8-11.1 mmol/l) and 20 age-matched control subjects with normal glucose tolerance were studied. Subjects with any other cause of peripheral neuropathy, active corneal disease or surgery and chronic contact lens use were excluded. 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.

Clinical and Peripheral Neuropathy Assessment

All subjects underwent assessment of systolic (sys) and diastolic (dia) blood pressure (BP), BMI, HbA1c, lipid fractions (total cholesterol, LDL, HDL and triglycerides) and estimated glomerular filtration rate. Signs and symptoms of neuropathy were assessed using the neuropathy symptom profile (NSP), neuropathy disability score (NDS), vibration perception threshold (VPT) (Horwell Scientific Laboratory Supplies, Wilford, Nottingham, UK) and cool (CT) and warm (WT) thresholds (Medoc Ltd., Ramat-Yishai, Israel). Sural sensory nerve amplitude and conduction velocity and peroneal motor nerve amplitude and conduction velocity were assessed. HRVDB was assessed with an ANX 3.0 autonomic nervous system monitoring device (ANSAR Medical Technologies Inc., Philadelphia, PA, USA). Sudomotor function was
assessed using Neuropad (miro Verbandstoffe, Wiehl-Drabenderhole, Germany).

**Skin Biopsy**

A 3-mm punch skin biopsy was obtained from the dorsum of the foot ~2 cm above the second metatarsal head after local anesthesia (1% lidocaine) and IENFD was quantified in accord with established criteria (8).

**Corneal Confocal Microscopy**

Patients underwent examination with a CCM (Heidelberg Engineering GmbH, Heidelberg, Germany) and three established corneal nerve parameters (CNFD, CNBD and CNFL) were quantified in a masked fashion as previously described (9).

**Statistical analysis**

SPSS for Mac (Version 19.0, IBM Corporation, New York, USA) was used for descriptive and frequency statistics. An Unpaired *t* test (or non parametric Mann-Whitney *U* test) was used to study differences between means. All data are expressed as mean ± standard error of mean and a *P*<0.05 was considered significant.

**RESULTS**

**Clinical assessment**

Subjects with IGT and controls were matched for age and the 2-hour glucose for the OGTT was significantly greater (9.2±1.0 vs. 6.5±0.6, *P*=0.001).
Subjects with IGT had a higher BMI (31.7±1.0 vs. 27.9±1.2, P=0.02) and HbA1c (6.0±0.2 / 43.9±1.0 vs. 5.4±0.1 / 36.0±0.3 mmol/mol, P<0.001) with a lower total (4.8±0.2 vs. 5.5±0.2 mmol/l, P=0.02) and HDL (1.2±0.1 vs. 1.7±0.1 mmol/l, P<0.001) cholesterol, compared to controls. There was no difference in BP sys/dia (132/78 vs. 137/79 mmHg, P=0.5) LDL cholesterol (2.1±1.1 vs. 3.2±0.6 mmol/l, P=0.1) and triglycerides (2.8±1.0 vs. 1.7±0.9 mmol/l, P=0.2).

**Neuropathy assessment**

NSP (4.1±1.0 vs. 0.5±0.2, P<0.001), the McGill pain index (2.8±0.3 vs. 0.2±0.1, P<0.001), NDS (2.9±0.5 vs. 0.6±0.2, P=0.001), VPT (15.9±2.3 vs. 6.5±1.1, P=0.002), and WT (40.6±0.8 vs. 37.6±0.6, P=0.006) were significantly increased, whilst CT (24.9±1.3 vs. 27.5±0.6, P=0.03), Neuropad (%) response (71.0±2.8 vs. 93.0±5.6, P=0.05), IENFD (6.3±0.6 vs. 9.1±0.7, P=0.03), CNFD (27.6±1.2 vs. 37.4±1.6, P< 0.001), CNBD (55.8±6.0 vs. 89.2±8.4, P=0.02) and CNFL (22.1±1.2 vs. 25.7±1.2, P=0.05) were significantly decreased in the IGT group compared to controls (figure 1).

There was no significant difference in sural sensory nerve amplitude (14.0±1.4 vs. 16.6±1.9, P=0.2) conduction velocity (49.9±0.9 vs. 49.9±1.0, P=0.8), and peroneal motor nerve amplitude (4.6±0.4 vs. 5.3±0.5, P=0.1) and conduction velocity (45.6±0.7 vs. 47.5±0.7, P=0.1) or HRVdb (9.5±6.9 vs. 11.9±6.9, P=0.09).

Under the assumption that CNFD is normally distributed in controls and IGT (Shapiro-Wilk W Test, P>0.05) and based on a cut-off point = 2 standard deviations (SD) from the control average (CNFD=24.0 no./mm²), subjects with IGT were re-stratified into two groups: those without (IGT) (n=22)
Neuropathy in Impaired Glucose Tolerance

(CNFD>24.0 no./mm²) and those with IGT neuropathy (IGTN) (n=15 (40.5%)) (CNFD<24.0 no./mm²). There was significantly greater self-reported pain intensity (McGill Pain Index, P=0.04) and reduction in CNBD (P=0.02) and CNFL (P<0.001) in subjects with IGTN compared to IGT (figure 1E).

DISCUSSION

A recent study has shown a significant reduction in IENFD and abnormal corneal nerve morphology in patients with Type 2 diabetes of short duration, suggesting that neuropathy may be an early complication (10), and of course longitudinal data from the Rochester cohort (11) suggest that duration and severity of exposure to hyperglycemia are related to the severity of neuropathy. In the present study we show a significant increase in neuropathic symptoms, consistent with the MONICA/KORA Augsburg Surveys (3), which also found a threefold increase in neuropathic pain in subjects with IGT. We also show a significant alteration in sudomotor function whilst cardiac autonomic function and electrophysiology were normal, similar to a previous study in subjects with IGT demonstrating an abnormal sympathetic skin response but normal electrophysiology and standard autonomic function tests (12). These latter findings are similar to a large population based study, which also showed no electro-diagnostic abnormalities in subjects with IFG or IGT (13). However, we demonstrate a significant abnormality in VPT, and warm and cold thresholds similar to the San Luis Valley study (2), which also reported impaired VPT in 11.2% of subjects with IGT compared to 3.5% in controls. Previous studies have demonstrated a reduction in IENFD in subjects with IGT, which improved after
lifestyle intervention (5), suggesting that this early abnormality may be amenable to treatment. We now confirm a significant reduction in IENFD in subjects with IGT. In addition we also demonstrate a significant abnormality in corneal nerve morphology using the non-invasive technique of CCM and indeed show that 40.5% of subjects with IGT have significant small fibre damage based on CNFD reduction. CCM provides a unique opportunity to non-invasively and rapidly assess unmyelinated C-fibers in vivo, which has important diagnostic (6) and prognostic (8) implications. In conclusion, the present study extends our previous findings showing the utility of CCM in identifying small fibre damage in diabetic and other peripheral neuropathies (14). It also strengthens the argument that small fibre neuropathy occurs in subjects with IGT.
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Author's Contribution

O.A. recruited patients, researched data, performed analysis and wrote the manuscript, I.N.P. researched data, performed analysis and wrote the manuscript, U.A. researched data and contributed to analysis, W.J. research data, M.J. research data, A.M. performed all electrodiagnostic studies, G.P. researched data, H.F. researched data, A.J.M.B. reviewed the manuscript and is a co-investigator, M.T. researched data, reviewed the manuscript and is a co-investigator and R.A.M. designed the study, reviewed the manuscript and is the principal investigator of the study.

R.A.M. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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No conflict of interest related to this work for any of the authors.
FIGURES

Figure 1. Skin punch biopsies immunostained for PGP9.5 (A, C) and corneal confocal microscopy images (B, D) from a healthy control vs. a subject with IGT and graphs showing the distribution of CNFD (E), CNBD (F) and CNFL (G) in controls vs. IGT. In (C) compared to (D) a significant reduction in corneal nerve fibers (yellow arrows) and nerve branches (red arrows) is observed, which mirrors the reduction in the same subject in intra-epidermal nerve fibers (yellow arrows) reaching the upper levels of epidermis in (B) compared to (A). The sub-epidermal nerve plexus is also visible (purple arrowhead). Data points in (E, F, G) represent actual corneal sub-basal nerve parameters in controls (n = 20) vs. IGT (n = 37). Purple dashed lines represent group averages and the blue dashed line in (E) represents a cut-off for “risk of neuropathy” (ITGN).
Repeatability of In Vivo Corneal Confocal Microscopy to Quantify Corneal Nerve Morphology

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Purpose: To establish intraobserver and interobserver repeatability, agreement, and symmetry of corneal nerve fiber (NF) morphology in healthy subjects using in vivo corneal confocal microscopy.

Methods: Nineteen subjects underwent in vivo corneal confocal microscopy (Heidelberg Retinal Tomograph III Rostock Cornea Module) at baseline and 7 days apart. Bland–Altman plots were generated to assess agreement, and the intraclass correlation coefficient and coefficient of repeatability were calculated to estimate intraobserver and interobserver repeatability for corneal NF density (numbers per square millimeter), nerve branch density (NBD; numbers per square millimeter), NF length (millimeters per square millimeter), and NF tortuosity coefficient. Symmetry between the right and left eyes was also assessed.

Results: Intraclass correlation coefficient and coefficient of repeatability for intraobserver repeatability were 0.66 to 0.74 and 0.17 to 0.64, for interobserver repeatability 0.54 to 0.93 and 0.15 to 0.85, and for symmetry 0.34 to 0.77 and 0.17 to 0.63, respectively. NBD demonstrated low repeatability.

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

Key Words: In vivo corneal confocal microscopy, diabetic neuropathy, repeatability

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Detailed histological analysis of the human cornea before in vivo corneal confocal microscopy (IVCCM) was only possible postmortem using light and electron microscopy. Since the 1980s, IVCCM has been used in ophthalmic research and in clinical practice to assess corneal dystrophies and ectasias; Acanthamoeba keratitis; the effects of contact lens wear; dry eye disease; and postsurgical follow-up. Real-time IVCCM has also enabled the characterization of corneal nerves in healthy and keratoconic 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. We and others have recently applied this technique to quantify corneal subbasal nerve fibers (NFs) in a variety of peripheral neuropathies including diabetic neuropathy, idiopathic small fiber neuropathy, Fabry disease, anti–myelin-associated glycoprotein neuropathy, chemotherapy-associated peripheral sensory neuropathy, non–length-dependent small fiber neuropathy, and type IV/V hereditary sensory and autonomic neuropathy. Quantification of corneal nerve morphology using 4 key parameters, namely, NF density, nerve branch density (NBD), NF length (NFL), and the tortuosity coefficient (TC), has allowed the early detection and stratification of peripheral neuropathy and also the assessment of repair after simultaneous pancreas and kidney transplantation. Tavakoli et al 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 because of 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. Two recent studies have demonstrated high repeatability of IVCCM but focused primarily onNFL. Hence, the aim of this study was to establish intraobserver, interobserver, and between-eye repeatability and agreement in control subjects for each of the 4 key parameters used to quantify neuropathy.
METHODS

Study Subjects

Nineteen randomly selected healthy subjects aged 23.1 ± 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 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 glucose or lipid levels. We used the Toronto consensus criteria to exclude peripheral neuropathy by assessing the neuropathy symptom profile, neuropathy deficit score, quantitative sensory testing for vibration perception threshold, cold and warm thresholds, and cold-induced and heat-induced pain.

Corneal Confocal Microscopy

All subjects were scanned with a laser IVCCM [Heidelberg Retinal Tomograph III Rostock Cornea Module (HRT III RCM); Heidelberg Engineering GmbH, Heidelberg, Germany] on 2 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 ×63 objective lens with a numerical aperture of 0.9 and a working distance, relative to the applanating cap (TomoCap; Heidelberg Engineering GmbH), of 0.0 to 3.0 mm was used. The size of each 2-dimensional image produced was 384 × 384 µm, which has a 15 × 15–degree field of view and 10 µm per 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, United Kingdom) was used to anesthetize each eye, and Viscotears (0.2% carbomer 980; Novartis UK) was 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 [charge-coupled device] 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 ~4 minutes for both eyes of each subject at each visit. All images were captured using the “section” mode in the Heidelberg Eye Explorer of the HRT III RCM. The other 2 available modes are “volume” and “sequence.” As Hertz et al 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 1-µm intervals from the central cornea of each subject.

Image Analysis

Two observers masked from each other analyzed 380 IVCCM nonoverlapping images, which were randomized before analysis, to assess interobserver repeatability. Observer 1 was experienced in the task of IVCCM image analysis (>2400 images), and observer 2 had no previous experience of corneal nerve quantification. Observer 1 quantified the relevant IVCCM images to assess intraobserver repeatability and symmetry masked for the visit and eye examined. Criteria for image selection were depth, focus position, and contrast. The images were manually analyzed using proprietary purpose-written software (CCMetrics; M. A. Dabbah, imaging science and biomedical engineering, University of Manchester, Manchester, United Kingdom). The specific parameters measured per frame were those we have previously established: NFD (numbers per square millimeter), NBD (numbers per square millimeter), NFL (millimeters per square millimeter), and the TC (Fig. 1). NFD is defined as the total number of main NFs per frame divided by the area of the frame in square millimeters (area = 0.16033585 mm²; Fig. 1). NBD is defined as the total number of main nerve branches (NBs; strictly branches that

FIGURE 1. A, An original image as captured with the HRT III RCM. B, An analyzed image using CCMetrics. NFD is measured under the red color, 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 red colors. TC—a measure of NF tortuosity—is measured simultaneously with NFD on each NF and is highlighted with the red color. The method is identical to that previously described by Kallinikos et al and has been integrated into the current algorithm.
stem from an NF) divided by the area of the frame. NFL is the total length of NFs, NBs, and secondary NBs (branches that stem from an 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.

Statistical Analysis

Data analysis was performed using Microsoft Office Excel 2008 (Microsoft, WA) and StatsDirect version 2.7.7 (StatsDirect Ltd, Cheshire, United Kingdom), and the data are presented as mean ± SD. The data were tested for normality before 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 considered significant.

The intraclass 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, that is, as an index of repeatability.37 The ICC was considered excellent if 0.8 to 1 and very good if 0.60 to 0.79. Coefficient of repeatability (CoR) was also calculated as a percentage of an average measurement to estimate the repeatability of the sample. A CoR between 0 and 0.2 was considered good, 0.2 to 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 (limits of agreement: 1.96 ± SD, 1.96 − SD), as described by Bland and Altman,38 to appreciate the between-subject, within-subject, and between-occasion agreement.

RESULTS

Subjects in this study had a body mass index of 24.8 ± 4.1 kg/m², hemoglobin A1c level of 5.5% ± 0.2%, low-density lipoprotein cholesterol level of 2.7 ± 0.8 mmol/mol, high-density lipoprotein cholesterol level of 1.5 ± 0.3 mmol/mol, and serum triglyceride level of 1.3 ± 0.6 mmol/mol. Subjects had no evidence of peripheral neuropathy: neuropathy deficit score, 0; neuropathy symptom profile, 0; vibration perception threshold, 3.3 ± 1.3 Hz; cold threshold/warm threshold, 28.6 ± 2.4/36 ± 1.8°C; and cold-induced pain/heat-induced pain, 6.4 ± 5.9/47.1 ± 3.9°C.

Intraobserver repeatability was assessed for each parameter using images from the same location and depth of the same eye on 2 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 scan and the repeated scan. The mean of the values was plotted against the difference between them to derive the Bland–Altman plots (Fig. 2). The relevant ICC values were as follows: NFD, 0.74 (Fig. 2A); NBD, 0.61 (Fig. 2B); NFL, 0.70 (Fig. 2C); and TC, 0.66 (Fig. 2D). The respective CoR values were as follows: NFD, 0.17; NBD, 0.64; NFL, 0.19; and TC, 0.46. The mean differences (±SD) between the 2 assessments were as follows: NFD, 0.1 ± 3.6 numbers per square millimeter; NBD, 5.0 ± 19.4 numbers per square millimeter; NFL, 1.5 ± 2.8 mm/mm²; and TC, 0.4 ± 3.6.

Interobserver repeatability refers to the assessment of corneal nerve parameters by 2 observers on images of the same eye from the same visit (Table 1). Among the 4 parameters, only NBD showed a significant difference between observers (P < 0.0001, 95% CI). The ICC values were as follows: NFD, 0.82 (Fig. 3A); NBD, 0.54 (Fig. 3B); NFL, 0.66 (Fig. 3C); and TC, 0.93 (Fig. 3D). The respective CoR values were as follows: NFD, 0.15; NBD, 0.85; NFL, 0.17; and TC, 0.18. The mean differences (±SD) between the 2 observers were as follows: NFD, 1.1 ± 3.1 numbers per square millimeter; NBD, 56.0 ± 39.0 numbers per square millimeter; NFL, 2.7 ± 2.6 mm/mm²; and TC, 0.7 ± 1.4.

The 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.

### Table 1. NFD, NBD, NFL, and TC to Assess Intraobserver and Interobserver Repeatability, Agreement, and Symmetry Between the RE and the LE

<table>
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<th>NFD (No./mm²)</th>
<th>NBD (No./mm²)</th>
<th>NFL (mm/mm²)</th>
<th>TC</th>
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<td><strong>Intraobserver</strong></td>
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<tr>
<td>RE visit 1</td>
<td>38.3 ± 3.9</td>
<td>58.1 ± 23.0</td>
<td>27.6 ± 4.0</td>
<td>15.8 ± 4.0</td>
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<tr>
<td>RE visit 2</td>
<td>38.2 ± 5.0</td>
<td>63.1 ± 21.7</td>
<td>29.1 ± 3.8</td>
<td>15.5 ± 4.6</td>
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<tr>
<td><strong>Interobserver</strong></td>
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<tr>
<td>Observer 1</td>
<td>38.2 ± 5.0</td>
<td>63.1 ± 21.7</td>
<td>29.1 ± 3.8</td>
<td>15.5 ± 4.6</td>
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<tr>
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<td>31.7 ± 4.8</td>
<td>14.7 ± 3.8</td>
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<tr>
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<tr>
<td>RE visit 1</td>
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<td>58.1 ± 23.0</td>
<td>27.6 ± 4.0</td>
<td>15.8 ± 4.0</td>
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<tr>
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<td>56.3 ± 26.4</td>
<td>27.8 ± 5.2</td>
<td>15.5 ± 1.8</td>
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</table>

Results are expressed as mean ± SD.
the LE. The calculated ICC values were as follows: NFD, 0.77 (Fig. 4A); NBD, 0.73 (Fig. 4B); NFL, 0.45 (Fig. 4C); and TC, 0.34 (Fig. 4D). The respective CoR values were as follows: NFD, 0.17; NBD, 0.63; NFL, 0.36; and TC, 0.48. The mean differences (±SD) between the RE and the LE were as follows: NFD, 0.07 ± 3.9 numbers per square millimeter; NBD, 1.28 ± 18.1 numbers per square millimeter; NFL, 0.1 ± 5.0 mm/mm²; and TC, 0.3 ± 3.7.

**DISCUSSION**

The quantification of corneal subbasal 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.¹⁸ Initial studies provided qualitative evidence of corneal NF alterations or reported changes in the architecture after 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 the 4 key parameters: NFD, NBD, NFL, and TC.¹⁸,²⁸,²⁹

Whether individual anatomical variations and intraobserver and interobserver consistency influence the results remains unclear. A recent study has shown that NFL has a very high between-observer and between-occasion repeatability in patients with type 2 diabetes,⁴⁴ whereas another study showed that NFL had the best reproducibility and validity among all parameters in controls and patients with type 1 diabetes and suggested that the development of IVCCM should focus on the measurement of NFL because of its superiority over 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 4 main parameters we originally developed and applied¹⁸,¹⁹,²⁹ in a range of peripheral neuropathies.²²,²³,²⁸

In this study, corneal NF morphology showed consistency between the RE and the LE. Although NFD and NFL achieved the highest values for intraobserver and interobserver 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 NBs and NFs. The correct identification of NBs in IVCCM images is especially difficult and mainly depends on background contrast, image clarity, and observer experience and interpretation. In addition, Patel and McGhee\(^40\) showed for the first time a continuous centripetal movement of identifiable branch points in the human corneal subbasal nerve plexus of up to 26 µm per week over a 6-week period, which may also cause variability.

A common finding in IVCCM images is crossing X-shaped NFs running from the top to the bottom of the image or Y-shaped appearance of an NF and an NB. In the former case, interpretation is easy and is not expected to vary between observers. However, in the latter case there is no standardized rule to-date to assist the analyzer to correctly define the NF and the NB. Selecting either side to be the NF can affect the outcome because 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 (>1 branch stemming from an 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.

Among the 2 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, that is, all nerve structures and may therefore be ideally used as a pan-corneal marker of peripheral neuropathy. However, high or low NFL

![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 analyzed by 2 observers (OB 1 and OB 2).](image-url)
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 assessing subbasal 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 intraobserver and interobserver repeatability and consistency between the RE and the LE for NFD and NFL but have identified lower repeatability for NBD and TC when deploying manual image analysis of corneal NF morphology. Both the latter parameters are however important to quantify corneal innervation because 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 NFs and their branches. A possible solution for both these issues may lie in the development of a fully automated image analysis system,\textsuperscript{31} which would eliminate inconsistencies, enhance repeatability, markedly reduce the analysis time, and hence make IVCCM suitable for clinical practice.

ACKNOWLEDGMENTS

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REFERENCES

Corneal Nerve Loss Detected With Corneal Confocal Microscopy Is Symmetrical and Related to the Severity of Diabetic Polyneuropathy

OBJECTIVE—To establish if corneal nerve loss, detected using in vivo corneal confocal microscopy (IVCCM), is symmetrical between right and left eyes and relates to the severity of diabetic neuropathy.

RESEARCH DESIGN AND METHODS—Patients (n = 111) with type 1 and type 2 diabetes and 47 age-matched healthy control subjects underwent detailed assessment and stratification into no (n = 50), mild (n = 26), moderate (n = 17), and severe (n = 18) neuropathy. IVCCM was performed in both eyes and corneal nerve fiber density (CNFD), branch density (CNBD), and fiber length (CNFL) and the tortuosity coefficient were quantified.

RESULTS—All corneal nerve parameters differed significantly between diabetic patients and control subjects and progressively worsened with increasing severity of neuropathy. The reduction in CNFD, CNBD, and CNFL was symmetrical in all groups except in patients with severe neuropathy.

CONCLUSIONS—IVCCM noninvasively detects corneal nerve loss, which relates to the severity of neuropathy, and is symmetrical, except in those with severe diabetic neuropathy.

Diabetic sensorimotor polyneuropathy (DSPN) is a length-dependent, symmetrical neuropathy with initial involvement of sensory and autonomic nerve fibers (NFs), followed by motor nerve involvement (1). It is the most common long-term complication of diabetes and is the main initiating factor for foot ulceration and lower extremity amputation with substantial associated healthcare costs (2). Conventional techniques of electrophysiology and quantitative sensory testing along with an assessment of neurological disability offer a relatively robust means of defining neuropathic severity (3) but have limitations in detecting the earliest stages of nerve damage (4,5).

In vivo corneal confocal microscopy (IVCCM) is a rapidly expanding technique to quantify the severity of neuropathy in DSPN (6). It has been used to demonstrate early nerve damage in diabetics and a range of other peripheral neuropathies (7,8) with good sensitivity and specificity (9). Recently, corneal nerve damage detected with IVCCM has been related to the level of previous glycemic exposure and blood pressure (10) and HbA1c even in healthy subjects (11). In a study of subjects with idiopathic small fiber neuropathy, corneal nerve damage was associated with higher serum triglycerides (8). It has also shown significant nerve regeneration before improvement in a range of established measures of neuropathy, including quantitative sensory testing, neurophysiology, and intraepidermal NF density, after simultaneous pancreas and kidney transplantation (12) and after an improvement in glycemia and cardiovascular risk factors for DSPN (13).

Corneal NF loss correlates with intraepidermal NF loss (4), and corneal NF length (CNFL), particularly, has shown superior discriminative capacity to diagnose DSPN (14). Recent studies show that quantification of corneal nerve morphology is highly reproducible and does not differ significantly between observers (15) and occasions (16) in subjects with diabetes and healthy individuals. As a functional correlate, corneal sensation has been found to decrease with increasing neuropathic severity (17).

Perkins et al. (18) and Bromberg and Jaros (19) have previously reported high interside symmetry of nerve conduction studies (NCS) consistent with the symmetrical nature of diabetic neuropathy. Whilst Petropoulos et al. (16) have shown that central corneal innervation is highly symmetrical between right eyes (REs) and left eyes (LEs) of young healthy subjects, it is unknown whether 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 if corneal nerve loss, detected using IVCCM, is symmetrical between REs and LEs with increasing severity of diabetic neuropathy.
RESEARCH DESIGN AND METHODS

Study subjects
Patients (n = 111) with diabetes and 47 age-matched control subjects were evaluated for the presence of DSPN based on the updated Toronto consensus criteria (20). 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 in the study. Participants were excluded if they had a positive history of malignant connective tissue or infectious disease, deficiency of vitamin B₁₂ or folate, chronic renal failure, liver failure, active diabetic foot ulcers, or family history of peripheral neuropathy. Participants were also excluded if they had active ocular disease, systemic disease known to affect the cornea other than diabetes, or chronic corneal pathologies.

Clinical assessment and evaluation of peripheral neuropathy
All study participants underwent assessment of their clinical characteristics (BMI, HbA₁c, lipid fractions, albumin-to-creatinine ratio [ACR], and estimated glomerular filtration rate [eGFR]) and detailed evaluation of signs of DSPN based on the simplified neuropathy disability score (NDS), vibration perception threshold (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 and 10), as described elsewhere (21). It is composed of Achilles tendon reflex testing (present [0], reduced [1], or absent [2]), temperature sensation (present [0] or absent [1]), pin-prick sensation (present [0] or absent [1]), and vibration perception scores of the great toe using a tuning fork (present [0] or absent [1]).

VPT was tested using a neurothesiometer (Horwell; Scientific Laboratory Supplies, Wilford, Nottingham, U.K.). Electrodiagnostic studies were undertaken using a Dantec Keypoint system (Dantec Dynamics, Ltd., Bristol, U.K.) equipped with a DISA temperature regulator to keep limb temperature constantly between 32 and 35°C. Peroneal motor and sural sensory nerves were assessed in the left lower limb (calf to ankle) to estimate sural sensory nerve amplitude (SSNamp), sural sensory nerve conduction velocity (SSNCV), peroneal motor nerve amplitude (PMNamp), and peroneal motor nerve conduction velocity (PMNCV) by a consultant neurophysiologist. 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 100 mm.

IVCCM 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) by a purpose-trained optometrist. 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 63× objective lens with a numerical aperture of 0.9 and a working distance relative to the illuminating cap (TomoCap; Heidelberg Engineering GmbH) of 0.0–3.0 mm was used. The size of each two-dimensional image produced was 384 × 384 μm, which has a 15° × 15° field of view and 10 μm/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, U.K.) was used to anesthetize each eye, and Viscoatears (Carbomer 980, 0.2%; Novartis Pharmaceuticals, Surrey, U.K.) were used as the coupling agent between the cornea and the illuminating cap. All subjects were asked to fixate on an external fixation light throughout the IVCCM scan, and a charge-coupled device camera was used to image the cornea and correctly position the illuminating cap onto the corneal apex. The overall examination took ~5 min 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 five and eight images will provide an acceptable level of accuracy to quantify the corneal subbasal nerve morphology (22). We selected and analyzed six high-clarity images per subject from the central subbasal nerve plexus captured by 1-μm intervals at the z-axis using the “section” mode. Criteria for image selection were depth, focus position, and contrast.

Corneal nerve loss and diabetic polyneuropathy
Corneal nerve loss was evaluated using a purpose-built noncontact corneal aesthesiometer (NCCA) (Anterior Eye Laboratory, Queensland University of Technology, Brisbane, Australia) as described elsewhere (17).

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 semiautomated, purpose-written, proprietary software (CCMetrics; M.A. Dabbah, Imaging Science and Biomedical Engineering, University of Manchester, Manchester, UK). The specific parameters measured per frame were those we have previously established (9): corneal NF density (CNFD; n/mm²), corneal nerve branch density (CNBD; n/mm²), CNFL (mm/mm²), and tortuosity coefficient (TC) (23) (Fig. 1). CNFD is defined as the total number of main NFs per frame divided by the area of the frame in mm² (area = 0.1603385 mm²) (Fig. 1). CNBD is defined as the total number of main nerve branches (nerve branches that stem from a NF) divided by the area of the frame. CNFL is the total length of NFs and nerve branches per frame. TC is a mathematical computation of the NF tortuosity as previously described by Kallinkos et al. (23), 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.

Statistical analysis
Statistical analysis was performed using StatsDirect for Windows (StatsDirect Ltd., Altrincham, Cheshire, U.K.), and OriginPro version 8.5 (OriginLab Corporation, Northampton, MA) 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. Under the assumptions of a 0.05 type 1 error and power of 0.95, we calculated that a minimum sample of 68 patients with diabetes/neuropathy was required to detect a statistically meaningful effect. The recruitment continued and reached 111 patients in total until each group contained at least 17 patients. Differences between REs and LEs and between groups (controls vs. none vs. mild vs. moderate vs. severe neuropathy) were tested by means of a paired Student t test and one-way ANOVA or nonparametric 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 (nonparametric). The mean difference between the REs and LEs for each of the IVCCM parameters was calculated to define the magnitude of asymmetry, and the Spearman rank test was used to investigate the strength of the relationship between the variables. Box and whisker plots (Fig. 1A–D) were generated for CNFD, CNBD, CNFL, and TC to allow visual assessment of the data.

**RESULTS**

**Clinical and peripheral neuropathy assessment**

Among the 111 diabetic subjects, 61 (55%) were classified as having DSPN based on the case definition used in this study. There was no significant difference in age, BMI, and serum triglycerides, but HbA$_1c$ was significantly increased in the diabetes cohort ($P < 0.0001$) and was the highest in those with severe neuropathy ($P < 0.001$). Paradoxically, there was a trend for decreasing total cholesterol with increasing severity of neuropathy in diabetic patients compared with 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 1). When differences were adjusted for type of diabetes, duration, sex, and age, HbA$_1c$ tended to be higher in type 1 diabetes ($P < 0.0001$) whereas 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 decline with increasing severity of neuropathy. Similarly, PMNamp and PMNCV also decreased, reaching significance ($P < 0.0001$) in mild, moderate, and severe neuropathy, respectively (Table 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 ($P < 0.0001$).

**IVCCM and corneal sensation**

CNFD ($P < 0.001$), CNBD ($P < 0.001$), and CNFL ($P < 0.001$) demonstrated a significant reduction between control subjects and diabetic patients with increasing severity of neuropathy (Table 2 and Fig. 1). Corneal sensation thresholds increased gradually and symmetrically in diabetic patients with increasing severity of neuropathy compared with control subjects ($P < 0.001$) (Table 2). There were no differences attributed to type of diabetes, sex, 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 study subjects (Fig. 1A–D). Spearman correlation coefficients and the associated mean differences for each subject group and
Corneal nerve loss and diabetic polyneuropathy

Table 1—Clinical and peripheral neuropathy status

<table>
<thead>
<tr>
<th>Variable</th>
<th>Controls</th>
<th>None</th>
<th>Mild</th>
<th>Moderate</th>
<th>Severe</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>47</td>
<td>50</td>
<td>26</td>
<td>17</td>
<td>18</td>
</tr>
<tr>
<td>Type 1 diabetes (%)</td>
<td>N/A</td>
<td>40 (81)</td>
<td>18 (69)</td>
<td>15 (88)</td>
<td>16 (88)</td>
</tr>
<tr>
<td>Duration of diabetes (years)</td>
<td>N/A</td>
<td>23 ± 14</td>
<td>31 ± 16</td>
<td>41 ± 14</td>
<td>34 ± 13</td>
</tr>
<tr>
<td>Age (years)</td>
<td>52 ± 13.2</td>
<td>44.2 ± 15.6</td>
<td>56.8 ± 12.2</td>
<td>59.6 ± 12.8</td>
<td>53.2 ± 14.5</td>
</tr>
<tr>
<td>HaA1c (%)‡</td>
<td>5.6 ± 0.3</td>
<td>7.9 ± 1.7‡</td>
<td>7.9 ± 1.2‡</td>
<td>7.9 ± 1.3‡</td>
<td>9.5 ± 2.8‡</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>26.6 ± 4.3</td>
<td>26.9 ± 5.3</td>
<td>27.5 ± 4.7</td>
<td>27.4 ± 4.0</td>
<td>23.5 ± 7.0</td>
</tr>
<tr>
<td>ACR (mg/mmol)‡</td>
<td>1.1 ± 1.0</td>
<td>1.1 ± 0.9</td>
<td>1.2 ± 0.9</td>
<td>4.4 ± 5.1‡</td>
<td>10.8 ± 11.4‡</td>
</tr>
<tr>
<td>eGFR (ml/min/L)‡</td>
<td>84.9 ± 7.2</td>
<td>81.8 ± 19.3</td>
<td>79.2 ± 19.4</td>
<td>57.8 ± 28.3‡</td>
<td>68.7 ± 17.4‡</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)†</td>
<td>5.3 ± 0.9</td>
<td>4.2 ± 1.0</td>
<td>4.4 ± 1.0</td>
<td>4.4 ± 1.1</td>
<td>4.0 ± 0.9</td>
</tr>
<tr>
<td>Triglycerides†</td>
<td>1.7 ± 0.8</td>
<td>1.4 ± 0.8</td>
<td>1.3 ± 0.8</td>
<td>1.5 ± 0.9</td>
<td>1.4 ± 0.5</td>
</tr>
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<td>NDS‡</td>
<td>0</td>
<td>0.7 ± 0.9‡</td>
<td>4.0 ± 0.7</td>
<td>7.2 ± 0.9‡</td>
<td>9.75 ± 0.5‡</td>
</tr>
<tr>
<td>VPT (V)†</td>
<td>6.6 ± 5.1</td>
<td>9.0 ± 7.1</td>
<td>17.9 ± 13.3§</td>
<td>25.7 ± 8.6</td>
<td>33.1 ± 12.1§</td>
</tr>
<tr>
<td>SSNamp (µV)†</td>
<td>14.3 ± 11.2</td>
<td>11.1 ± 6.5</td>
<td>8.4 ± 6.8</td>
<td>4.8 ± 3.1§</td>
<td>2.4 ± 1.2§</td>
</tr>
<tr>
<td>SSNCV (m/s)‡</td>
<td>49.9 ± 4.4</td>
<td>45.6 ± 5.1</td>
<td>43.2 ± 5.1</td>
<td>39.8 ± 5.6§</td>
<td>43.6 ± 4.1§</td>
</tr>
<tr>
<td>PMNamp (µV)‡</td>
<td>5.5 ± 2.0</td>
<td>5.7 ± 7.9</td>
<td>2.9 ± 2.1§</td>
<td>1.5 ± 1.0§</td>
<td>1.4 ± 1.3§</td>
</tr>
<tr>
<td>PMNCV (m/s)‡</td>
<td>48.1 ± 3.1</td>
<td>43.0 ± 4.8</td>
<td>40.5 ± 4.8</td>
<td>30.4 ± 2.6</td>
<td>43.2 ± 6.0</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SD. Statistically significant differences using ANOVA. *P < 0.05. †P < 0.01. ‡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.

Conclusions—DSPN is characterized by progressive distal and symmetrical sensory and autonomic nerve damage with eventual motor nerve involvement (1). It is hypothesized that the initial injury occurs in the thinly myelinated Aδ- or myelinated C-fibers where morphological alterations can be assessed with skin biopsy (24). Although NCS is the preferred end point for diagnosis and assessment of outcome in clinical intervention trials, it is limited to large nerves (18). IVCCM has emerged as a powerful technique to detect and stratify human DSPN as it allows direct, noninvasive visualization of the corneal subbasal nerves (6). Corneal innervation shares anatomical similarities with intraepidermal innervation, and corneal NF loss has been found to reflect intraepidermal NF loss (4).

Observational studies using IVCCM to evaluate peripheral neuropathy have reported on the concurrent validity (14), reproducibility (9, 15), and optimization of image selection (22). In a previous study (16), we showed that corneal innervation patterns between REs and LEs in healthy subjects are symmetrical, with the exception of branching, which showed wider limits of agreement. It is unknown, however, whether corneal nerve loss remains symmetrical in DSPN of varying severity.

Table 2—IVCCM and NCCA for REs and LEs in different stages of peripheral neuropathy

<table>
<thead>
<tr>
<th>Variable</th>
<th>Controls</th>
<th>None</th>
<th>Mild</th>
<th>Moderate</th>
<th>Severe</th>
</tr>
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<tr>
<td>CNFD (n/mm²)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RE‡</td>
<td>37.6 ± 8.2</td>
<td>27.4 ± 8.9</td>
<td>22.9 ± 10.5§</td>
<td>18.6 ± 10.1§</td>
<td>13.1 ± 7.3</td>
</tr>
<tr>
<td>LE‡</td>
<td>36.3 ± 6.2</td>
<td>26.4 ± 10.1</td>
<td>23.6 ± 11.3</td>
<td>19.2 ± 10.4§</td>
<td>13.1 ± 9.6</td>
</tr>
<tr>
<td>CNBD (n/mm²)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RE‡</td>
<td>94.2 ± 44.8</td>
<td>56.4 ± 35.7</td>
<td>50.5 ± 43.3</td>
<td>36.0 ± 28.2§</td>
<td>13.7 ± 16.1**</td>
</tr>
<tr>
<td>LE‡</td>
<td>98.9 ± 39.4</td>
<td>54.6 ± 36.5</td>
<td>46.0 ± 34.5</td>
<td>28.8 ± 20.5§</td>
<td>25.5 ± 26.2</td>
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<tr>
<td>CNFL (mm/mm²)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RE‡</td>
<td>27.3 ± 5.8</td>
<td>20.4 ± 5.9</td>
<td>17.5 ± 8.1</td>
<td>14.7 ± 7.9§</td>
<td>9.2 ± 5.7**</td>
</tr>
<tr>
<td>LE‡</td>
<td>27.2 ± 4.9</td>
<td>19.7 ± 7.5</td>
<td>17.7 ± 8.9</td>
<td>14.7 ± 7.3</td>
<td>10.3 ± 5.7</td>
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<tr>
<td>TC</td>
<td></td>
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<tr>
<td>RE*</td>
<td>16.6 ± 3.3</td>
<td>18.7 ± 10.7</td>
<td>22.4 ± 8.5§</td>
<td>16.2 ± 7.8</td>
<td>14.2 ± 9.8</td>
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<tr>
<td>LE*</td>
<td>16.2 ± 4.7</td>
<td>17.7 ± 6.6</td>
<td>20.0 ± 10.4</td>
<td>20.7 ± 8.3</td>
<td>18.7 ± 12.1</td>
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<tr>
<td>NCCA (mbar)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RE‡</td>
<td>0.6 ± 0.4</td>
<td>0.8 ± 0.7</td>
<td>0.9 ± 0.6§</td>
<td>1.1 ± 0.5</td>
<td>3.4 ± 4.1</td>
</tr>
<tr>
<td>LE‡</td>
<td>0.6 ± 0.5</td>
<td>0.9 ± 0.7</td>
<td>1.0 ± 0.8$</td>
<td>0.9 ± 0.4</td>
<td>5.1 ± 5.3</td>
</tr>
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</table>

Results are expressed as mean ± SD. Statistically significant differences between groups using ANOVA. *P < 0.05. †P < 0.01. ‡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. mbar, millibar.
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 end points of choice i.e., symmetrical involvement (18,24). 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 for the first time that DSPN, as detected by gold standard clinical and electrophysiological testing, is paralleled by significant corneal NF loss, which is highly symmetrical between REs and LEs except in those with severe neuropathy. Specifically, we demonstrate a dramatic stepwise reduction in CNFD, CNFL, and CNBD with an increase in TC in diabetic patients with increasing severity of neuropathy compared with control subjects. This confirms and extends our findings using the less sensitive second-generation IVCCM (9). We have also found a significant increase in corneal sensation thresholds with increasing severity of neuropathy (17). The relationship between the right and left corneal innervation patterns was highly significant among 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 (25).

A study by Perkins et al. (18) and an earlier study by Bromberg and Jaros (19) found high interside symmetry of NCS in patients with varying degrees of DSPN. However, Perkins et al. (18) 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 fiber involvement in DSPN is symmetrical. A potential limitation and a source of variation is the use of NDS, which is large fiber weighted to classify the severity of neuropathy. Thus, this may lead to variability when comparing to our findings using IVCCM, a small fiber measure, and may explain the large variation in corneal nerve measures among the different groups of neuropathic severity.

In conclusion, we confirm and extend our previous findings (9) in a large cohort of diabetic patients using the latest third-generation IVCCM with optimal image clarity. We show that DSPN results in gradual and significant corneal NF loss with more advanced neuropathy, which is highly symmetrical except in patients with severe neuropathy.

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I.N.P. designed the study, researched data, performed analysis, and wrote the manuscript. U.A., H.F., O.A., and A.M. researched data. P.G. collected data and performed analysis.

G.P. researched data and contributed to discussion. A.J.M.B. reviewed the manuscript. M.T. researched data and reviewed the manuscript. R.A.M. designed the study, reviewed and revised the manuscript, and was the principal investigator of the study. R.A.M. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

References


Rapid Automated Diagnosis of Diabetic Peripheral Neuropathy With In Vivo Corneal Confocal Microscopy

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PURPOSE. To assess the diagnostic validity of a fully automated image analysis algorithm of in vivo confocal microscopy images in quantifying corneal subbasal nerves to diagnose diabetic neuropathy.

METHODS. One hundred eighty-six patients with type 1 and type 2 diabetes mellitus (T1/T2DM) and 55 age-matched controls underwent assessment of neuropathy and bilateral in vivo corneal confocal microscopy (IVCCM). Corneal nerve fiber density (CNFD), branch density (CNBD), and length (CNFL) were quantified with expert, manual, and fully-automated analysis. The areas under the curve (AUC), odds ratios (OR), and optimal thresholds to rule out neuropathy were estimated for both analysis methods.

RESULTS. Neuropathy was detected in 53% of patients with diabetes. A significant reduction in manual and automated CNBD (P < 0.001) and CNFD (P < 0.0001), and CNFL (P < 0.0001) occurred with increasing neuropathic severity. Manual and automated analysis methods were highly correlated for CNFD (r = 0.9, P < 0.0001), 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, sensitivity/specificity and OR to rule out neuropathy.

CONCLUSIONS. Diabetic peripheral neuropathy is associated with significant corneal nerve loss detected with IVCCM. Fully automated corneal nerve quantification provides an objective and reproducible means to detect human diabetic neuropathy.

Keywords: corneal confocal microscopy, diabetic neuropathy, diabetes

Diabetic sensorimotor polyneuropathy (DSPN) is a frequent complication of diabetes affecting up to 53% of people with diabetes.1 Diagnosis of the condition is important to define at-risk patients, anticipate deterioration, and assess new therapies. Neuropathic symptoms and signs, together with electrophysiological studies are the endpoints of choice to diagnose DSPN and assess therapeutic outcomes.2 Although these tests offer a robust means of assessing neuropathy, they predominantly focus on large fiber deficits, yet the earliest alterations occur in the small unmyelinated C- and thinly myelinated Aδ-nerve fibers.3 Small fiber neuropathy can be evaluated using quantitative sensory testing of thermal thresholds or skin biopsy to quantify intra-epidermal nerve fiber density (IENFD). However, the assessment of thermal thresholds is subjective and therefore liable to variability,4 while skin biopsy is an invasive and costly technique, which is not routinely available across healthcare systems.5

We have pioneered the use of IVCCM and shown that this rapid, noninvasive ophthalmic technique can accurately quantify changes in the human subbasal nerve plexus of patients with diabetes.6 Alterations in the subbasal corneal nerves occur early, increase with neuropathic severity,7 and are paralleled by significant IENF loss.8 Recent studies have shown that chronic glycemic exposure,9 even in subjects without overt diabetes,10 hypertension,11 and elevated serum triglycerides,12 are strong risk factors for corneal subbasal nerve loss. Furthermore, early reinnervation of the cornea has been shown in recipients of simultaneous pancreas and kidney transplantation (SPK).12,13 It is important to note that other ocular diseases, such as dry eyes,14 atopic keratoconjunctivitis,15 epithelial membrane basement dystrophies,16 cystic corneal disorders,17 and other conditions18 may also affect corneal innervation, and should therefore be excluded in any study using IVCCM in DSPN.

Concerns regarding the use of IVCCM have focused on the reproducibility19,20 of the technique, its ability to prospectively assess neuropathy, and the absence of an automated image analysis system to allow objective corneal nerve quantification. The latter is essential to eliminate inconsistencies, produce comparable outcomes across centers, and enable the deployment of IVCCM for diagnosis, and as a surrogate endpoint in clinical trials of diabetic neuropathy. Previous studies21–23 have proposed a variety of quantification algorithms, which differ by methodology and detection properties. In our recent work,25...
we described an algorithm that concurrently uses a dual-model feature descriptor and a neural network classifier to distinguish nerve fibers from the background and presented an evaluation of its performance against other available detection methods. The aim of the present study was to assess the diagnostic validity of a fully automated image analysis algorithm of in vivo confocal microscopy images in quantifying corneal subbasal nerves to diagnose diabetic neuropathy.

**METHODS**

**Study Subjects**

One hundred eighty-six patients with diabetes mellitus (108 male/78 female) and 55 age-matched control subjects (28 male/27 female) (50.4 ± 14.1 vs. 51.7 ± 11.4 years) were assessed for the presence and severity of DSPN between 2010 and 2011 based on the updated Toronto consensus criteria.²

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. Subjects were excluded if they had a positive history of malignancy, connective tissue or infectious disease, deficiency of vitamin B₁₂ or folate, chronic renal failure, liver failure, active diabetic foot ulceration, and/or family history of peripheral neuropathy. Control subjects were excluded from the study if they had evidence of neuropathy or risk factors likely to cause neuropathy. All subjects were also assessed for the presence of corneal lesions by means of relevant history and slit-lamp examination. Subjects were excluded if they had active ocular disease (e.g., severe dryness), systemic disease known to affect the corneal subbasal innervation, other than diabetes or chronic corneal pathologies (cystic corneal disorders, epithelial basement membrane dystrophies).

**Medical Status Assessment**

All participants underwent assessment of their cardiometabolic [glycated hemoglobin (HbA1c), total cholesterol (TC), triglycerides and body mass index (BMI)] and renal status [estimated glomerular filtration rate (eGFR) and albumin to creatinine ratio (ACR)].

**Peripheral Neuropathy Assessment**

The neuropathy disability score (NDS), a scale of 0 to 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 elsewhere²¹ (Tables 1, 2). The neuropathy symptom profile (NSP) was employed to assess symptoms of neuropathy. Vibration perception threshold (VPT) was evaluated on the hallux of both feet with a Neurothesiometer (Horwell Scientific Laboratory Suppliers, Wilford, UK). Cool and warm thermal (CT/WT) thresholds and cold- and heat-induced pain (CIP/HIP) were established on the dorsolateral aspect of the left foot (S1) with a TSA-II

### TABLE 1. Medical and Peripheral Neuropathy Status

<table>
<thead>
<tr>
<th>Variable</th>
<th>Controls, n = 55, NDS = 0</th>
<th>DSPN (−), n = 86, NDS ≤ 2</th>
<th>DSPN (+), n = 100, NDS &gt; 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Duration of diabetes</td>
<td>N/A</td>
<td>24.2 ± 21.2</td>
<td>34.4 ± 17.3</td>
</tr>
<tr>
<td>HbA₁c, %/mmol/mol ‡</td>
<td>5.5 ± 0.3/34 ± 3.3</td>
<td>7.7 ± 1.6/61 ± 17.5 §</td>
<td>7.9 ± 1.6/63 ± 17.5 §</td>
</tr>
<tr>
<td>BMI, Kg/m²</td>
<td>25.6 ± 4.6</td>
<td>27.2 ± 5.2</td>
<td>27.6 ± 5.8</td>
</tr>
<tr>
<td>TC, mM‡</td>
<td>5.1 ± 0.9</td>
<td>4.3 ± 1.2 §</td>
<td>4.4 ± 0.9 §</td>
</tr>
<tr>
<td>Triglycerides, mM</td>
<td>1.5 ± 0.8</td>
<td>1.5 ± 0.9</td>
<td>1.4 ± 0.9</td>
</tr>
<tr>
<td>eGFR, mL/min/L†</td>
<td>85.8 ± 7.8</td>
<td>81.8 ± 18.2</td>
<td>70.0 ± 24.5 §</td>
</tr>
<tr>
<td>ACR, mg/mmol ‡</td>
<td>1.0 ± 1.4</td>
<td>2.9 ± 1.3</td>
<td>18.8 ± 11.3 §</td>
</tr>
<tr>
<td>BP, systolic†/diastolic, mm Hg</td>
<td>122 ± 16/70 ± 8.8</td>
<td>130 ± 18/87 ± 9.1</td>
<td>138 ± 23/72/8 ± 8</td>
</tr>
<tr>
<td>VPT, V ‡</td>
<td>5.8 ± 4.6</td>
<td>9.2 ± 6.5 §</td>
<td>22.3 ± 12.6 §</td>
</tr>
<tr>
<td>WT†/CT†, °C</td>
<td>37.0 ± 3.0/28.2 ± 2.2</td>
<td>59.6 ± 3.9/27.0 ± 9.2 §</td>
<td>42.7 ± 4.6/20.8 ± 9.2 §</td>
</tr>
<tr>
<td>HIP/CIP†, °C</td>
<td>44.8 ± 2.9/11.9 ± 9.2</td>
<td>45.5 ± 6.6/9/10.7</td>
<td>46.9 ± 7.3/4.1/6.2 §</td>
</tr>
<tr>
<td>PMNCV, m/s‡</td>
<td>48.8 ± 3.3</td>
<td>43.7 ± 4.7 §</td>
<td>39.2 ± 6.1 §</td>
</tr>
<tr>
<td>SSNCV, m/s†</td>
<td>51.0 ± 4.8</td>
<td>46.4 ± 5.8 §</td>
<td>42.2 ± 6.4 §</td>
</tr>
<tr>
<td>PMNamp, µV ‡</td>
<td>5.2 ± 1.8</td>
<td>4.5 ± 5.2</td>
<td>2.4 ± 2.1 §</td>
</tr>
<tr>
<td>SSNamp, µV ‡</td>
<td>20.0 ± 9.7</td>
<td>12.5 ± 7.8 §</td>
<td>6.5 ± 6.6 §</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SD, statistically significant differences using ANOVA/Kruskal-Wallis. N/A, not applicable for this group.

* *P < 0.05.

† P < 0.0001; post hoc results for DSPN (+) significantly different from § control subjects and || DSPN (−).

### TABLE 2. IVCCM Assessment of DSPN Status

<table>
<thead>
<tr>
<th>Variable</th>
<th>Controls, NDS = 0</th>
<th>DSPN (−), NDS ≤ 2</th>
<th>DSPN (+), NDS &gt; 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manual IVCCM quantification</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CNFDₙ, no./mm²‡</td>
<td>37.2 ± 6.7</td>
<td>26.7 ± 8.5 §</td>
<td>20.5 ± 9.5 §</td>
</tr>
<tr>
<td>CNBₙ, no./mm²‡</td>
<td>92.7 ± 38.6</td>
<td>54.9 ± 35.7 §</td>
<td>48.7 ± 33.2 §</td>
</tr>
<tr>
<td>CNFLₙ, mm²/mm²‡</td>
<td>26.4 ± 5.6</td>
<td>20.3 ± 6.7 §</td>
<td>16.7 ± 7.6 §</td>
</tr>
<tr>
<td>Automated IVCCM quantification</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CNFDₙ, no./mm²‡</td>
<td>30.0 ± 6.9</td>
<td>20.1 ± 8.7 §</td>
<td>14.4 ± 8.9 §</td>
</tr>
<tr>
<td>CNBₙ, no./mm²‡</td>
<td>50.4 ± 24.7</td>
<td>31.4 ± 25.6</td>
<td>20.1 ± 18.7 §</td>
</tr>
<tr>
<td>CNFLₙ, mm²/mm²‡</td>
<td>21.2 ± 5.5</td>
<td>17.1 ± 4.5 §</td>
<td>13.7 ± 5.2 §</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SD, statistically significant differences using ANOVA/Kruskal-Wallis. no., number; mbar, millibar.

* *P < 0.05.

† P < 0.01.

‡ P < 0.0001; post hoc results for diabetes DSPN (+) significantly different from § control subjects and ¶ DSPN (−).
Automated Detection of Diabetic Neuropathy

Nerve conduction studies (NCS) were undertaken by a consultant neurophysiologist (AM) as previously described.24 Peroneal motor nerve amplitude (PMNamp) and conduction velocity (PMNCV) and sural sensory nerve amplitude (SSNamp) and conduction velocity (SSNCV) were assessed. The diabetes cohort included 11 patients that did not agree or were unable to undergo NCS. These patients were not excluded from the study, but were not considered when NCS results were assessed.

Study Definition of Peripheral Neuropathy

The Toronto Diabetic Neuropathy Expert Group2 recommendation was followed to define "Confirmed DSPN: the presence of an abnormality of NCS and a symptom or symptoms of a sign or signs of neuropathy. In the absence of an abnormal NCS, a validated measure of small fiber neuropathy 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 small fiber neuropathy." To define an abnormal result for NCS and QST we have used a mean ±2 SD cutoff based on our control population.

In Vivo Corneal Confocal Microscopy

All study subjects were scanned with a laser IVCCM (Heidelberg Retinal Tomograph III Rostock Cornea Module [HRT III RCM]; Heidelberg Engineering GmbH, Heidelberg, Germany) as described elsewhere.20 The overall examination took approximately 5 minutes for both eyes of each subject, and in this study two experienced optometrists performed all IVCCM scans. All images were captured using the "section" mode and prior to scanning corneal sensation was assessed using noncontact corneal aesthesiometry (NCCA) as described elsewhere.25

Manual Image Analysis

During a bilateral IVCCM scan more than 100 images per patient were typically captured from all corneal layers. Six subbasal images from right and left eyes were selected for analysis. Criteria for image selection were depth, focus position, and contrast. A single experienced examiner (INP), masked from the outcome of the medical and peripheral neuropathy assessment, quantified 1506 images of all study participants using purpose-written, proprietary software (CCMetrics, MA Dhabah; Imaging Science and Biomedical Engineering, University of Manchester, Manchester, UK). The specific parameters measured per frame were: CNFD (no./mm²), CNFL (mm/mm²), and CNBD (no./mm²) in accord with our previously published protocol.20

Automated Image Analysis

Automated corneal nerve fiber quantification consists of two steps: (1) IVCCM image enhancement and nerve fiber detection, and (2) quantification of the three morphometric parameters. As described in our earlier work,22,23 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 was then connected and classified as main nerve fibers or branches.

Statistical Analysis

Statistical analysis was performed using StatsDirect for Windows (version 2.7.9; StatsDirect Ltd., Cheshire, UK) and STATA 12 for Windows (Stata Corporation, College Station, TX, USA) was used to generate the receiver operating characteristic curves (ROC). Correlation analysis was performed to assess the strength of the relationship between automated and manually generated variables. Linear regression analysis was used to assess the consistency of the responses from the fully automated algorithm for a given manual estimate. The intraclass correlation coefficient (ICC) was calculated as a measure of reliability of the automated image analysis algorithm over repeated assessment of the dataset. One-way ANOVA (nonparametric Kruskal-Wallis) were used to evaluate within and between group differences. P value was maintained at 0.05 for multiple comparisons (Bonferroni adjustment or Conover-Inman pairwise comparisons) and a P less than 0.05 was considered significant.

Receiver operating characteristic curves analysis was performed for all corneal nerve parameters to identify the point closest to the upper left corner of the ROC graph, which concurrently optimized sensitivity and specificity and the AUC, OR, and positive (LR) and negative likelihood ratios (LR) associated with the point were calculated. The diagnostic validity of IVCCM was assessed in relation to four established measures of DSPN (PMNamp, SSNamp, PMNCV, and WT). A χ² test was used to compare the AUCs generated for all IVCCM parameters.

RESULTS

Medical Status and DSPN Assessment

Detailed medical and DSPN assessment results for subjects with diabetes and controls are presented in Table 1. Diabetic sensorimotor polyneuropathy(+) compared with DSPN(−) and controls had a lower eGFR (P < 0.0001), higher ACR (P < 0.0001), systolic blood pressure (BP) (P = 0.0003), VPT (P < 0.0001), WT (P = 0.0005), and lower CT (P = 0.0004), CIP (P < 0.0001), PMNCV (P < 0.0001), SSNamp (P < 0.0001), and SSNamp (P < 0.0001). Diabetic sensorimotor polyneuropathy(+) subjects had a longer duration of diabetes (54.4 ± 17.3 vs. 24.2 ± 21.2, P = 0.01) and were older compared with DSPN(−) (55.3 ± 12.4 vs. 47.3 ± 15.6, P = 0.001). Metabolic control and BMI were significantly different between controls (HbA 1c, P < 0.0001; BMI, P < 0.05) and patients with diabetes, but comparable between DSPN(+) and DSPN(−). Total cholesterol (TC) was similar between the two groups with diabetes, and lower compared with controls (P < 0.0001), which is likely due to statin used in the diabetes cohort.

Manual and Automated Assessment of DSPN With IVCCM

Diabetic sensorimotor polyneuropathy(+) compared with DSPN(−) and controls had significantly lower manually quantified CNFDa (P < 0.0001), CNBDa (P = 0.0005), CNFLa (P = 0.0002), and automatically quantified CNFDb (P < 0.0001), CNBDb (P = 0.0002), and CNFLb (P < 0.0001) parameters. A significant reduction was also detectable between DSPN(−) and controls in CNFDa (P < 0.0001), CNBDb (P = 0.0006), CNFLb (P = 0.0003), and CNFDb (P < 0.0001), CNBDb (P = 0.0003), and CNFLb (P < 0.0001). Changes detected using automated image quantification were associated with a stronger significance level. Noncontact corneal aesthesiometry showed a significant elevation in the
was the point where sensitivity were equally 4.6. There were 95 (51%) patients 0.3 (95% CI 0.2–0.5) (Fig. 2A). Similarly, 2.1 (95% CI 1.5–3.0) and 2074 less than 2.6 (95% CI was associated with 0.74 sensitivity and 0.63 LR. When an abnormal SSNamp was associated with the highest AUC (0.79) and specificity (0.78) were concurrently optimized and associated with the highest AUC = 0.84, OR = 16.5, +LR = 4.6 (95% confidence interval [CI] 5.0–6.9), and −LR = 0.3 (95% CI 0.2–0.4). The corresponding point for automated analysis was CNFDa less than 14.7 no./mm2 with sensitivity (0.76) and specificity (0.72) and AUC = 0.80, OR = 11.0, +LR = 5.4 (95% CI 2.4–4.9), and −LR = 0.3 (95% CI 0.2–0.5) (Fig. 2A). Similarly, CNFLa and CNFLA were associated with an AUC of 0.82 and 0.84 respectively, +LR = 5.25 (95% CI 2.5–11.0) and −LR = 0.35 (95% CI 0.2–0.5) (Fig. 2). SSNamp Less Than 5.5 μV. When an abnormal SSNamp result was used as an indicator of neuropathy, the number of normal cases increased to 72 (40%). Automatically quantified CNFLa was associated with the highest AUC (0.77) and the highest OR = 5.1. A CNFLa less than 16.1 mm/mm2 optimized sensitivity (0.72) and specificity (0.66) with +LR = 2.1 (95% CI 1.6–2.9) and −LR = 0.4 (95% CI 0.3–0.6). A CNFLa less than 19.1 mm/mm2 optimized sensitivity (0.68) and specificity (0.67), but was associated with a 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). Both CNFDa and CNFDAl were equally capable in ruling out neuropathy. Both CNBDa and CNBDAl showed limited ability to differentiate between cases with and without neuropathy.

PMNCV Less Than 42 M/S. There were 96 (54%) diabetic patients who had an abnormal PMNCV result. Automatically quantified CNFDa was associated with the highest AUC (0.79) and a CNFLa less than 16.0 mm/mm2 optimized sensitivity (0.74) and specificity (0.71) with OR = 7.2, +LR = 2.6 (95% CI 1.9–3.8), and −LR = 0.3 (95% CI 0.2–0.5). A CNFLa less than 19.7 mm/mm2 was associated with 0.74 sensitivity and 0.65 specificity, 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 CNFDa and CNFDAl showed comparable AUC, OR, LR, and sensitivity/specificity to rule out neuropathy.

WT Greater Than 42°C. There were 95 (51%) patients with diabetes who had an abnormal WT greater than 42°C. Both CNFDa and CNFDAl were associated with the highest AUC and modest OR. Specifically, a CNFDa less than 24.0/ mm2 optimized sensitivity (0.65) 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 CNFDa and a WT was 61 (64%), while 35 (37%) had reduced CNFDa with a normal WT result. All CNFDa, CNFLa, and CNFDAl values were comparable, but were associated with slightly lower AUC and OR while sensitivity and specificity remained modest (Table 3).

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.
Severity stratification of DSPN. At present, a major literature supports the use of IVCCM in the diagnosis and treatment of DSPN. Recent studies using IVCCM, have reported an increased occurrence of large fibers and previous research has shown that small nerve fibers are affected first. An objective, noninvasive surrogate of this was not as high as for CNFD and CNFL. Corneal nerve branch density showed a significant positive correlation between manual and automated assessment, but the correlation between manual and automated assessment was not as high as for CNFD and CNFL.

Table 3. Validity and Associated Probabilities of DSPN Detection Using Manual and Automated IVCCM Parameters Quantification

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

Outcomes. The prevalence of DSPN, in the diabetic population varies from 10% to 53%. However, only a few studies have used objective endpoints to estimate the rates of neuropathy and this may explain the reported variability. Dyck and colleagues found that when NCS was used in combination with a functional abnormality to diagnose DSPN, a significant decline in eGFR, and found that a significant decline in eGFR, and poor metabolic control.

Automated image analysis is a labor-intensive task, where a human investigator applies subjective criteria to define a nerve and its density, branching, length when automated analysis was used, which is however consistent. The detection of nerve structures in IVCCM images is a challenging task: Nerve fibers often show poor contrast on a relatively noisy background due to microscope properties and underlying structures. As described in our earlier work, 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 labor-intensive task, where a human investigator applies subjective criteria to define a nerve and an overestimation with less experience has been described.

 Investigators 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 HbA1c, BP, and triglycerides with the density of corneal innervation. This study assessed 188 subjects with diabetes, but no other identifiable cause of neuropathy, and found that a significant decline in eGFR, increased ACR, and systolic BP were associated with neuropathy. Both diabetes groups with DSPN (+), DSPN (–) had modest to poor metabolic control.

Corneal confocal microscopy provides the unique opportunity to repeatedly and reliably visualize 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. At present, a major drawback is the absence of an automated analysis system, which would eliminate inconsistencies and make the technique suitable to a clinical setting. This study assessed, for the first time, the performance and validity of a novel fully-automated image analysis algorithm compared with manual human expert analysis in relation to multiple gold standard clinical endpoints used to define neuropathy.

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 fibers often show poor contrast on a relatively noisy background due to microscope properties and underlying structures. As described in our earlier work, 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 labor-intensive task, where a human investigator applies subjective criteria to define a nerve and an overestimation with less experience has been described. In this study, we found a significant and progressive reduction in nerve density, branching, length between diabetic patients with and even without DSPN, and controls using either quantification method.

Corneal nerve branch density showed a significant positive correlation between manual and automated assessment, but this was not as high as for CNFD and CNFL. Corneal nerve branch density, a measurement of nerve branches directly connected to nerve fibers, has been reported to be highly variable and appears to have modest validity in diagnosing.
find that a CNFL less than or equal to 27.8 no./mm² and less than or equal to 20.8 no./mm² as the values with the highest validity to define disease status among patients with mild and more severe neuropathy respectively. Ahmed et al. 34 found that a CNFL less than or equal to 14.0 mm/mm² was the value with the highest validity to rule in DSPN. We assessed the performance of manual and automated IVCCM quantification to identify patients “with” or “without” neuropathy based on gold standard measures of peripheral nerve damage. We found that CNFDₐ, CNFDₐ, CNFLₐ, and CNFLₐ were associated with the highest sensitivity and specificity to diagnose DSPN when PMNamp was used as the primary measure of neuropathy. Corneal nerve branch density showed less but acceptable validity in diagnosing DSPN and CNBDₐ had a significantly higher AUC and OR compared with CNBDₐ. When other endpoints of DSPN were used, such as SSNamp and PMNCV, the diagnostic validity of IVCCM remained high and CNFLₐ was consistently associated with the highest AUC and OR among all parameters. We observed a significant decline in sensitivity and specificity when an abnormality in WT was used as the primary marker of neuropathy. One would expect the opposite since warm detection is mainly mediated by small nerve fibers, and previously we have shown an association between IENFD and corneal nerve morphology. More recently CNFL has been related to three different measures of small fiber neuropathy. This is likely for two main reasons: NCS offer a robust and objective means of assessing neuropathy, while WT is a subjective measurement of small fiber function. Cassanova et al. in their study found that even patients with no IENFs had consistent responses in WT and concluded that it is possible for partially damaged nerve endings to still generate a propagated action potential. We speculate that a similar association may exist for the corneal subbasal nerves.

The validity of fully automated corneal nerve quantification was comparable and in several cases exceeded the performance of human expert assessment in ruling out DSPN. A CNFLₐ between 14.6 mm/mm² and 16.1 mm/mm² was the value consistently associated with the highest AUC and OR given the case definition employed. Both CNFDₐ (18.7–25.4 no./mm²) and CNFDₐ (14.7–19.7 no./mm²) also showed excellent performance with high OR, but were slightly more variable.

This study has several strengths and limitations. The 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 disease duration 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 cutoff points selected for the diagnosis of DSPN by IVCCM are comparable with the previous studies of Ahmed et al. 34 and Tavakoli et al.; 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 have compared IVCCM with several objective and subjective 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 that has been validated in a large cohort of patients in this and other studies. Moreover, inter- and intraobserver estimation of the parameter in highly innervated corneas has shown moderate reproducibility. The relevance of corneal nerve branching to DSPN is not clear. In our recent study, of the 1-year effects of SPK transplantation in type 1 DM recipients, we found a significant and stable increase before an improvement in any other measure of regeneration.
Automated Detection of Diabetic Neuropathy

with diabetes and varying degrees of DSPN. Our results are cross-sectional and ongoing longitudinal studies will determine the ability of IVCCM to predict the development and progression or regression of DSPN. Recent data generated from wide-field assessment of the subbasal plexus have suggested that both central and inferior whorl nerve density may be reduced early and therefore future studies should explore this further.

In conclusion, we show that diabetic peripheral neuropathy is paralleled by a significant and progressive reduction in central CNFD and CNFL. 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 practice and research centers worldwide.

Acknowledgments

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References


Diabetic neuropathy is associated with increased morbidity and mortality. To date, limited data in subjects with impaired glucose tolerance and diabetes demonstrate nerve fiber repair after intervention. This may reflect a lack of efficacy of the interventions but may also reflect difficulty of the tests currently deployed to adequately assess nerve fiber repair, particularly in short-term studies. Corneal confocal microscopy (CCM) represents a novel noninvasive means to quantify nerve fiber damage and repair. Fifteen type 1 diabetic patients undergoing simultaneous pancreas–kidney transplantation (SPK) underwent detailed assessment of neurologic deficits, quantitative sensory testing (QST), electrophysiology, skin biopsy, corneal sensitivity, and CCM at baseline and at 6 and 12 months after successful SPK. At baseline, diabetic patients had a significant neuropathy compared with control subjects. After successful SPK there was no significant change in neurologic impairment, neurophysiology, QST, corneal sensitivity, and intraepidermal nerve fiber density (IENFD). However, CCM demonstrated significant improvements in corneal nerve fiber density, branch density, and length at 12 months. Normalization of glycemia after SPK shows no significant improvement in neuropathy assessed by the neurologic deficits, QST, electrophysiology, and IENFD. However, CCM shows a significant improvement in nerve morphology, providing a novel noninvasive means to establish early nerve repair that is missed by currently advocated assessment techniques. Diabetes 62:254–260, 2013

Diabetic polyneuropathy is one of the most common long-term complications of diabetes and underlies the development of painful neuropathy in 21% of both type 1 and type 2 diabetic 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 fibers and improve diabetic neuropathy. Even in the Diabetes Control and Complications Trial (DCCT) and follow-up Epidemiology of Diabetes Interventions and Complications (EDIC) study, improved glycemic control only delayed the progression of clinical diabetic neuropathy and indeed nerve conduction studies at closeout showed no significant risk reduction (3). Furthermore, the Steno-2 study demonstrated that although multifactorial 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, neurologic 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 simultaneous pancreas–kidney transplantation (SPK) (6) and intraepidermal nerve fiber density (IENFD) 2.5 years after SPK (7). Neuropathy is of course extremely severe at this stage, as evidenced by severe intraepidermal nerve fiber depletion in pancreas transplant recipients, suggesting either a point of no return or the need for long-term follow-up to identify posttransplant nerve fiber regeneration (8). However, IENFD and corneal nerve morphology have been shown to improve in subjects with impaired glucose tolerance neuropathy (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. Although current end points 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 prespecified primary end points such as neuropathic deficits and electrophysiology (13). The assessments of neurologic symptoms and deficits have recently been shown to have poor diagnostic reproducibility (14). Although electrophysiology correlates with large fiber damage, it does not assess small fibers, which are the earliest to be damaged (15) and demonstrate repair even in advanced neuropathy (12). Nerve fiber morphology in sural nerve biopsies (16) and IENFD in skin-punch biopsies (17) can accurately quantify nerve fiber damage and repair, but both are invasive procedures.

We and others (18,19) have used corneal confocal microscopy (CCM) to detect subclinical diabetic neuropathy and relate it to the severity of somatic neuropathy (20) and IENFD (21) with good sensitivity and specificity (20). This led us to propose that CCM, a noninvasive and reiterative test, might be an ideal surrogate end point 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 fiber density (CNFD) and length 6 months after SPK (23), but at that time we did not compare CCM with established end points of diabetic neuropathy. In the current study we have compared CCM with neurologic deficits, quantitative sensory testing (QST), electrophysiology, and IENFD at baseline and 6 and 12 months after SPK to help define the measures that may best detect an improvement in diabetic neuropathy after intervention.

RESEARCH DESIGN AND METHODS

Selection of patients. Fifteen type 1 diabetic patients were evaluated at baseline and 6 and 12 months after SPK and compared with 10 age-sex-matched nondiabetic healthy control subjects. The healthy volunteers were recruited from the general population. Both patients and control subjects underwent full neurologic and medical assessments. Those patients with any history of systemic (apart from diabetes for patient group) or neurologic conditions or history of ocular trauma and those wearing contact lens or those who had 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.

Assessment of neuropathy. All patients and control subjects underwent a detailed evaluation of neurologic symptoms according to the neuropathy symptom profile (NSP), and the McGill pain analog score was used to assess the severity of painful neuropathy. Neurologic deficits were assessed using the modified neuropathy disability score (NDS), which includes evaluation of vibration, touch, 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 on the first toe using a Neurothesiometer (Horwell, Scientifical Laboratory Supplies, Welwyn, U.K.), cold sensation (CS) (Aβ fibers) and warm sensation (WS) (C fibers) thresholds using the method of limits with the MEDOC TSA II (Medoc, Ramat Yishai, Israel) on the dorsum of the left foot (24).

Computer-Aided Sensory Evaluator (CASE IV) was used to measure the heart rate response to deep breathing. In this test, the patient was asked to inhale and exhale deeply eight times in a row in the supine position while 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 5-min period of normal breathing. The acquired data were analyzed 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, Bristol, U.K.) equipped with a Dansk Industri Syntal screen and driving electronics to keep the limb temperature constantly between 32°C and 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 standardized sites defined by anatomical landmarks, and recordings for the sural nerve were taken using antidromic stimulation over a distance of 100 mm. Corneal sensitivity. Corneal sensitivity was quantified using a noncontact corneal aesthesiometer (CACC) (Glasgow Caledonian University, Glasgow, Scotland, U.K.), which uses a puff of air through a bore 0.5 mm in diameter lasting 0.9 ms and exerting a force expressed in millibars (mbar) (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 center of the cornea. Each subject was presented with a supramaximal stimulus, and the staircase method was used 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%.

CCM. Patients underwent examination with the Heidelberg retina tomograph III in vivo corneal confocal microscope. The subject’s eyes were anesthetized 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 peripheral corneal rim to exclude any movement 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 backward and forward for ~2 min using the section mode, which enables manual acquisition and storage of single images of all corneal layers. This provides an en face two-dimensional image with a lateral resolution of ~2 μm/pixel and final image size of 400 x 400 pixels of the subbasal 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 fiber bundle contains unmyelinated fibers, 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 center of the cornea were selected and examined in a masked and randomized fashion (27). Three corneal nerve parameters were quantified: 1) CNFD, the total number of major nerves per square millimeter of corneal tissue; 2) corneal nerve branch density (CNBD), the number of branches emanating from all major nerve trunks per mm² of corneal tissue; and 3) corneal nerve length (CNFL), the total length of all nerve fibers and branches (mm²/mm²) within the area of corneal tissue. CNFD and CNFL are considered to reflect overall nerve fiber degeneration, whereas CNBD reflects nerve fiber regeneration, which is partially also captured by CNFL.

Skin biopsy and 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 3% sucore (2–4 h) for cryoprotection. It was then embedded in optimal cutting temperature–embedding compound, rapidly frozen in liquid nitrogen, and cut into 50-μm sections using a cryostat (model OCT; Bright Instruments, Huntingdon, U.K.). Four floating sections per subject were subjected to melanin bleaching (0.22% potassium permanganate and potassium hydroxide for 3 min), blocked with 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 1:200 Biogenes polyclonal rabbit anti-human PGP9.5 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 then incubated with 0.03% H2O2 in 30% hydrogen peroxide (30 min) before a 1-h 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 control subjects consisted of replacing the anti-PGP9.5 antibody with rabbit immunoglobulin (DakoCytomation) at the same dilution. Non-SPK control subjects, which showed no immunostaining. IENFD, i.e., the number of fibers per millimeter of basement membrane, was quantified in accord with established criteria and techniques and expressed as number per millimeter (28).

Statistics. SPSS 16.0.5.0 for Windows was used to compute the results. Analysis included descriptive and frequency statistics. All data are expressed as means ± SEM. A paired sample t test was used to test whether a sample mean of a normally distributed interval variable) differed between control subjects and diabetic patients at baseline and at follow-up 6 and 12 months after SPK.

RESULTS

The clinical characteristics and detailed assessment of neuropathy in diabetic patients and age-matched control subjects are summarized in Table 1. BMI was non-significantly lower in diabetic patients and showed an increase after SPK. HbA1C was higher in diabetic patients compared with control subjects 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 triglycerides were comparable between diabetic patients and control subjects, and remained unchanged after SPK. The estimated glomerular filtration rate was lower in diabetic patients at baseline (P = 0.02) and did not change significantly at 6 and 12 months after SPK.

Symptoms and neurologic 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 with control subjects and did not show a significant change at 6 (P = 0.9) or 12 (P = 0.9) months after transplantation. The modified NDS was significantly
Clinical demographic results in control subjects and type 1 diabetic patients undergoing SPK at baseline and follow-up visits at 6 and 12 months

<table>
<thead>
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<th>Parameter</th>
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<th>Baseline</th>
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<th>12 months</th>
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<tr>
<td>n (female/male)</td>
<td>10 (3/7)</td>
<td>15 (5/10)</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Age (years)</td>
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<td>47 ± 3</td>
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<td>—</td>
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<tr>
<td>Diabetes duration (years)</td>
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<td>27 ± 3.5</td>
<td>—</td>
<td>—</td>
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<tr>
<td>BMI (kg/m²)</td>
<td>27 ± 1</td>
<td>22 ± 2</td>
<td>25.5 ± 1</td>
<td>25.5 ± 1</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.7 ± 0.1</td>
<td>7.4 ± 0.8</td>
<td>5.9 ± 0.3</td>
<td>5.9 ± 0.4</td>
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<td>Cholesterol (mmol/L)</td>
<td>5.1 ± 0.2</td>
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<td>4.3 ± 0.3</td>
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<tr>
<td>HDL (mmol/L)</td>
<td>1.5 ± 0.1</td>
<td>1.3 ± 0.2</td>
<td>1.5 ± 0.2</td>
<td>1.6 ± 0.2</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.3 ± 0.2</td>
<td>1.4 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>1.03 ± 0.1</td>
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<tr>
<td>Estimated glomerular filtration rate (mL/min/L)</td>
<td>86.22 ± 2.13</td>
<td>60.53 ± 8.64†</td>
<td>64.0 ± 7.5</td>
<td>66.0 ± 6.19</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SEM in diabetic patients and control subjects unless otherwise indicated. All symbols represent statistically significant differences using paired sample t test. *P < 0.01. †P < 0.02 (baseline vs. control).

(P = 0.003) greater at baseline compared with 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 2).

**Quantitative sensory tests.** VPT was significantly greater in diabetic patients compared with 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 with 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 with 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.

**Autonomic function.** Average heart rate variability was significantly lower in diabetic patients compared with 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.

**Electrophysiology.** Peroneal nerve conduction velocity and amplitude were significantly lower in diabetic patients compared with 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. Sural nerve conduction velocity and amplitude were significantly lower in diabetic patients compared with 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 (Table 2).

**IENFD.** IENFD was significantly lower in diabetic patients compared with control subjects at baseline (P < 0.0001) and did not show a significant improvement 12 months after transplantation (P = 0.9) (Fig. 1 and Table 3).

**Corneal sensation.** The corneal sensation threshold was significantly greater in diabetic patients compared with control subjects at baseline (P = 0.03) and did not change at 6 (P = 0.9) or 12 (P = 0.9) months after transplantation (Table 3).

**CCM.** Representative images from a diabetic patient at baseline show a marked reduction in subbasal corneal nerves with a progressive repair at 6 and 12 months after SPK. CNFD was significantly lower in diabetic patients compared with control subjects at baseline (P = 0.003), 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 with control subjects at baseline (P < 0.0001) and did not improve at 6 months (P = 0.2) but reached statistical significance at 12 months (P = 0.02).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control subjects</th>
<th>Baseline</th>
<th>6 months</th>
<th>12 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSP (0–38)</td>
<td>0</td>
<td>6.7 ± 1.8†</td>
<td>7.6 ± 2.2</td>
<td>7.3 ± 2.0</td>
</tr>
<tr>
<td>NDS (0–10)</td>
<td>0.3 ± 0.2</td>
<td>4.6 ± 0.9†</td>
<td>5.0 ± 1.1</td>
<td>5.4 ± 0.7</td>
</tr>
<tr>
<td>McGill pain index</td>
<td>1.7 ± 0.6*</td>
<td>1.9 ± 0.8</td>
<td>1.5 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>VPT (volts)</td>
<td>6.7 ± 1.8</td>
<td>19.4 ± 3.7*</td>
<td>17.4 ± 3.3</td>
<td>16.9 ± 3.4</td>
</tr>
<tr>
<td>CS (°C)</td>
<td>29.3 ± 0.4</td>
<td>17.5 ± 3.1*</td>
<td>19.8 ± 2.9</td>
<td>20.0 ± 2.7</td>
</tr>
<tr>
<td>WS (°C)</td>
<td>38.1 ± 0.8</td>
<td>43.7 ± 1.4*</td>
<td>43.8 ± 1.2</td>
<td>42.3 ± 1.1</td>
</tr>
<tr>
<td>Heart rate variability (average bpm)</td>
<td>15.3 ± 2.1</td>
<td>7.1 ± 1.7*</td>
<td>5.7 ± 1.7</td>
<td>4.9 ± 2.1</td>
</tr>
<tr>
<td>Sural nerve conduction velocity (m/s)</td>
<td>47.9 ± 0.5</td>
<td>40.6 ± 2.2*</td>
<td>41.5 ± 1.6</td>
<td>41.8 ± 1.9</td>
</tr>
<tr>
<td>Sural amplitude (µA)</td>
<td>20.7 ± 3.4</td>
<td>5.1 ± 0.9*</td>
<td>5.1 ± 0.9</td>
<td>4.0 ± 0.6</td>
</tr>
<tr>
<td>Peroneal nerve conduction velocity (m/s)</td>
<td>47.7 ± 0.9</td>
<td>35.9 ± 1.8*</td>
<td>37.7 ± 1.2</td>
<td>38.5 ± 1.8</td>
</tr>
<tr>
<td>Peroneal amplitude (mV)</td>
<td>12.2 ± 0.9</td>
<td>2.4 ± 0.4‡</td>
<td>1.9 ± 0.4</td>
<td>1.7 ± 0.3</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SEM in diabetic patients and control subjects. All symbols represent statistically significant differences using paired sample t test. *P < 0.05. †P < 0.01. ‡P < 0.001 (baseline vs. control; 6 months vs. baseline; 12 months vs. baseline).
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 ($P = 0.695$, $r < 0.0001$).

**DISCUSSION**

The natural history of nerve damage in patients with type 1 diabetes is not entirely clear. Longitudinal data from the Rochester cohort support 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 glycemic 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 as small fiber evaluation to diagnose neuropathy (11). However, there is no clear consensus as to the critical end points, which should be used 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, normoglycemia can stop the progression of diabetic glomerulopathy, but does not reverse it (36,37). Similarly, pancreas transplantation alone can limit further reduction in glomerular filtration rate (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 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 type 1 diabetic patients there was no improvement in IENFD 21–40 months post-SPK (7). However, most patients receiving transplantation had severe nerve fiber damage as evidence by marked depletion of intraepidermal nerve fibers (8).

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 ($P = 0.695$, $r < 0.0001$).

**FIG. 1.** A: Skin biopsies immunostained for PGP9.5. Healthy control (A) shows numerous intraepidermal nerve fibers (red arrowheads) reaching upper levels of epidermis with a well-developed subepidermal nerve plexus (yellow arrowheads) in a healthy subject (A) compared with scant subepidermal and minimal intraepidermal nerve fibers in the diabetic patient both at baseline (B) and at follow-up (C). Scale bar = 100 μm. B: IENFD in control subjects and in diabetic patients at baseline and 12 months after SPK. Data are mean ± SEM. (A high-quality digital representation of this figure is available in the online issue.)
predominantly nociceptive C fibers (43,44). Indeed, CCM has been applied to evaluate diabetic neuropathy (19,20), idiopathic small fiber neuropathy (45), and Fabry disease (46). We have shown that corneal nerve damage assessed using CCM relates to the severity of intraepidermal nerve fiber loss (21) and is related to a loss of corneal sensitivity (25) in diabetic neuropathy. CCM detects very early small-fiber damage even in subjects with an elevated HbA1c, still within the normal range (18), and HbA1c levels 7–10 years before CCM correlate with the severity of nerve damage (47). Furthermore, an improvement in HbA1c by optimizing medical therapy (10) and pancreas transplantation (23) led to corneal nerve regeneration, shown using CCM. However, in these studies the evaluation of neuropathy was limited to CCM.

The present study allowed us to evaluate the relative ability of CCM to detect nerve fiber repair compared with all other established measures for assessing neuropathy, including neurologic deficits, QST, neurophysiology, and IENFD. The results demonstrate a severe neuropathy in diabetic patients before SPK as evidenced by significant abnormalities in electrophysiology, QST, IENFD, and corneal nerve fibers, 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. This improvement confirms our previous work (23) indicating an early nerve-fiber repair process with the restoration of euglycemia, followed by a significant improvement in nerve-fiber density and nerve-fiber 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 euglycemia (5–8,48–51). However, these studies focused heavily on electrophysiology and quantitative sensory assessment, which predominantly assessed large fiber function. It is relevant that where small fiber 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

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control subjects</th>
<th>Baseline</th>
<th>6 months</th>
<th>12 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCCA (mbars)</td>
<td>0.56 ± 0.1</td>
<td>1.78 ± 0.42*</td>
<td>1.83 ± 0.73</td>
<td>1.84 ± 0.89</td>
</tr>
<tr>
<td>CNFD (no./mm²)</td>
<td>35.77 ± 1.53</td>
<td>14.44 ± 1.20†</td>
<td>15.22 ± 1.63</td>
<td>19.27 ± 1.57*</td>
</tr>
<tr>
<td>CNBD (no./mm²)</td>
<td>100.92 ± 13.1</td>
<td>21.46 ± 3.78‡</td>
<td>36.85 ± 6.04*</td>
<td>43.02 ± 6.48†</td>
</tr>
<tr>
<td>CNFL (mm/mm²)</td>
<td>27.93 ± 1.26</td>
<td>11.35 ± 1.04‡</td>
<td>13.35 ± 1.50</td>
<td>15.63 ± 1.56*</td>
</tr>
<tr>
<td>IENFD (no./mm)</td>
<td>9.77 ± 1.24</td>
<td>2.03 ± 0.61‡</td>
<td>—</td>
<td>2.31 ± 1.17</td>
</tr>
</tbody>
</table>

Data are presented as mean ± SEM in diabetic patients and control subjects. Note that skin biopsy was not performed at 6 months. All symbols represent statistically significant differences using paired sample t test. *P < 0.05. †P < 0.01. ‡P < 0.001 (baseline vs. control; 6 months vs. baseline; 12 months vs. baseline).

FIG. 2. CCM images from Bowman’s layer of cornea: a control subject (A) and patient with type 1 diabetes at baseline (B) and at 6 (C) and 12 (D) months after SPK. The red arrows indicate main nerve fibers, and yellow arrows indicate branches. (A high-quality color representation of this figure is available in the online issue.)
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 as a result of lesser severity of damage (52).

We now confirm and extend the results of our previous study using the latest generation Heidelberg retina tomograph III, which provides enhanced small fiber imaging and detects earlier nerve fiber repair, particularly reflected in the increase in nerve branch density, followed by significant improvements in nerve fiber density and length. We believe these data provide further support for the need to study small fibers as surrogate markers and end points in intervention trials of diabetic neuropathy. An important issue with regard to the utility of CCM 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 CCM 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 normoglycemia, whereas other peripheral nerves do not. Nevertheless, CCM appears to represent a promising noninvasive and hence repetitive test with high sensitivity, which may represent an ideal surrogate end point for assessing the benefits of pancreas transplantation and indeed other therapies in clinical trials of human diabetic neuropathy.

ACKNOWLEDGMENTS
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No potential conflicts of interest relevant to this article were reported.
M.T. researched and analyzed the data and wrote the manuscript. M.M.-P. and T.A. were the transplant surgeons.

L.N.P. researched data and analyzed CCM images. H.F., O.A., and U.A. undertook clinical and neurological assessment, skin biopsy, and QST. G.P. was the study coordinator. M.J. undertook IENFD assessments. A.M. undertook neurophysiology. N.E. reviewed and revised the manuscript. A.J.B. reviewed and revised the manuscript. R.A.M. supervised the project, undertook IENFD assessment, and reviewed and revised the manuscript. R.A.M. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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REFERENCES
ABSTRACT: Although unmyelinated nerve fibers are affected in Charcot–Marie–Tooth type 1A (CMT1A) disease, they have not been studied in detail due to the invasive nature of the techniques needed to study them. We established alterations in C-fiber bundles of the cornea in patients with CMT1A using non-invasive corneal confocal microscopy (CCM). Methods: Twelve patients with CMT1A and 12 healthy control subjects underwent assessment of neuropathic symptoms and deficits, electrophysiology, quantitative sensory testing, corneal sensitivity, and corneal confocal microscopy. Results: Corneal sensitivity, corneal nerve fiber density, corneal nerve branch density, corneal nerve fiber length, and corneal nerve fiber tortuosity were significantly reduced in CMT1A patients compared with controls. There was a significant correlation between corneal sensation and CCM parameters with the severity of painful neuropathic symptoms, cold and warm thresholds, and median nerve CMAP amplitude. Conclusions: CCM demonstrates significant damage to C-fiber bundles, which relates to some measures of neuropathy in CMT1A patients.

Corneal Confocal Microscopy in CMT1A Patients

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CORNEAL CONFOCAL MICROSCOPY DETECTS SMALL-FIBER NEUROPATHY IN CHARCOT–MARIE–TOOTH DISEASE TYPE 1A PATIENTS


CMT1A typically have uniform slowing of nerve conduction velocities (NCVs) consistent with a demyelination, but they also develop reduced amplitudes of compound motor and sensory nerve action potentials (CMAPs and SNAPs, respectively) and progressive muscle weakness suggestive of axonal degeneration. Although these changes have been attributed to primary demyelination with secondary axonal degeneration of the large myelinated fibers, it is of interest that there is no correlation between motor or sensory NCVs and neurologic disability in patients with CMT1A.

Although small-fiber dysfunction has been demonstrated in CMT using quantitative sensory testing (QST) and sympathetic skin response (SSR), preservation of cerebral potentials after laser and electrical stimulation suggested preservation of small fibers in a patient with CMT. Sural nerve biopsy studies have demonstrated degeneration and regeneration of the unmyelinated fibers but because this procedure is invasive, it is undertaken less frequently, particularly with the development of genetic analyses. However, pathological studies allow definitive assessment of the primary pathology and also provide insights into underlying pathogenetic mechanisms. As an alternative, less invasive approach, a detailed immunohistological and electron microscopic study of dermal nerves in skin biopsies from patients with CMT1A demonstrated shortening of the internodal length with a loss of Meissner corpuscles and accumulation of intra-axonal mitochondria, suggestive of axonal pathology. Comparable pathology has also been demonstrated recently in dermal nerves of foot skin pad and peripheral nerves in a variety of animal models of CMT.

There is now increasing study of the potential for corneal confocal microscopy (CCM) as a means to quantify C-fiber pathology in peripheral neuropathies. Several detailed morphometric and immunohistological investigations have demonstrated that the sub-basal nerve fiber bundles assessed by CCM are predominantly nociceptive C-fibers.
Indeed, this approach has been applied to evaluate diabetic neuropathy, idiopathic small-fiber neuropathy, Fabry disease, and a series of conditions that cause small nerve fiber damage, including hereditary sensory and autonomic neuropathy (HSAN), autoimmune neuropathy, Crohn disease, and neuropathy associated with chemotherapy. We have also shown that corneal nerve damage assessed using CCM relates to the severity of intraepidermal nerve fiber loss, is related to a loss of corneal sensitivity, and can detect early small nerve fiber regeneration after pancreas transplantation in diabetic patients.

CCM may therefore provide a non-invasive means to determine whether there is small-fiber pathology in patients with CMT. We have undertaken a detailed evaluation of neuropathy using conventional neurophysiology and QST in addition to CCM and non-contact corneal aesthesiometry (NCCA) to quantify nerve damage in patients with CMT1A.

**METHODS**

**Selection of Patients.** Twelve patients with CMT1A (6 men and 6 women, average age 43.0 ± 3.5 years) and 12 healthy control subjects (7 men and 5 women, average age 43.0 ± 3.5 years) were studied. All patients were confirmed to have the PMP22 duplication by dose analysis.

The study was approved by the ethics committee of Central Manchester, and written informed consent was obtained in accordance with the Declaration of Helsinki. All patients were diagnosed and referred from the Department of Genetic Medicine, Central Manchester University Hospital NHS Foundation Trust.

**Assessment of Neuropathy.** All patients and controls underwent a detailed evaluation of their neurological symptoms according to the neuropathy symptom profile (NSP). The McGill Pain Analogue score was used to assess the severity of pain. Neurological deficits were assessed using the neuropathy disability score (NDS) and included 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, NDS 9–10 = severe neuropathy). Vibration perception threshold (VPT) was measured using a neurothesiometer (Horwell, Scientific Laboratory Supplies, Wilford, Nottingham, UK). Quantitative sensory testing included assessment of cold sensation (CS) and cold-induced pain (CIP) to assess Aδ fibers and warm sensation (WS) and heat-induced pain (HIP) to assess C-fibers on the dorsum of the left foot, using a quantitative sensory testing device (TSA II; Medoc, Ltd., Ramat Yishai, Israel). Electrodiagnostic studies were undertaken using a Keypoint system (Dantec Dynamics, Ltd., Bristol, UK) equipped with a DISA temperature regulator to keep limb temperature constant between 32° and 35°C. Full electrophysiological assessment of motor (median, ulnar, fibular) and sensory (radial, sural) nerves was performed on the right limb. Motor studies were performed using silver–silver chloride surface electrodes at standard sites defined by anatomical landmarks. Compound muscle action potential (CMAP) amplitudes were taken from baseline to negative peak. Motor nerve conduction velocities were calculated after distal and proximal stimulation. Sensory studies were recorded using a bar electrode (cathode–anode distance 3 cm) placed at standard sites. Recordings for sural and radial nerves were taken using antidromic stimulation over distances of 140 and 100 mm, respectively.

**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 0.5 mm in diameter lasting 0.9 second and exerts a force expressed in millibars. An electronic pressure sensor displays the force exerted (in millibars). The stimulus jet is mounted on a slit lamp. It is positioned 1 cm from the eye, and the air jet is aligned to the center of the cornea. The subject feels a sensation on the cornea, which is most commonly describing as being “cold” or as a “breeze,” and acknowledges this sensation. Each subject is presented with a supramaximal stimulus, and the staircase method is employed by reducing the stimulus strength until the patient does not feel the jet. This is then increased to a threshold level and reduced to the point where the stimulus is not felt. The whole process is repeated 3 times to derive a threshold. The coefficient of variation for NCCA was 5.6%.

**Corneal Confocal Microscopy.** Patients underwent examination with a retinal tomography device (HRT III; Rostock Cornea Module; Heidelberg Engineering, Heidelberg, Germany) for in vivo corneal confocal microscopy. The subject’s eyes were anesthetized using a drop of 0.4% benoxinate hydrochloride, and ViscoTears were applied on the front of the eye for lubrication. The patient was instructed to fixate on a target with the eye that was not being examined. 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. The gel optically couples the objective lens to the cornea. Several scans of the entire depth of
the cornea were recorded by turning the fine focus of the objective lens backward and forward for approximately 2 minutes to acquire satisfactory images of all corneal layers providing en face two-dimensional images with a lateral resolution of approximately 2 μm/pixel and a final image size of 400 pixels × 400 pixels. Images were obtained using the section mode, which enabled manual acquisition and storage of a single image at a time. For purposes of this study, we obtained high-quality images 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, because it is the location of the main nerve plexus that supplies the overlying corneal epithelium. These nerve fiber bundles contain unmyelinated fibers which run parallel to the Bowman layer before dividing and turning upward toward the surface to terminate as individual axons underneath the surface epithelium.33,34 This has been confirmed using electron microscopy, where nerve bundles containing unmyelinated axons were shown to penetrate the Bowman membrane throughout the central and peripheral cornea at approximately 400 sites.35 Five images per patient from the center of the cornea were selected and examined in a masked and randomized fashion.36

Table 1. Clinical and neurological assessments in control subjects and CMT1A patients.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>CMT1A</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No (females/males)</td>
<td>12 (5/7)</td>
<td>12 (6/6)</td>
<td>—</td>
</tr>
<tr>
<td>Age (years)</td>
<td>43.0 ± 3.5</td>
<td>43.0 ± 3.5</td>
<td>—</td>
</tr>
<tr>
<td>NDS (0–10)</td>
<td>9.1 ± 0.4</td>
<td>9.0 ± 2.1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>NSP (0–38)</td>
<td>0</td>
<td>6.3 ± 1.9</td>
<td>0.001</td>
</tr>
<tr>
<td>McGill Pain Analogue (0–10)</td>
<td>3.6 ± 0.3</td>
<td>33.3 ± 4.4</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>VPT (V)</td>
<td>28.2 ± 0.5</td>
<td>18.6 ± 6.3</td>
<td>0.01</td>
</tr>
<tr>
<td>CS (°C)</td>
<td>37.9 ± 1.0</td>
<td>41.4 ± 2.3</td>
<td>0.12</td>
</tr>
<tr>
<td>WS (°C)</td>
<td>11.0 ± 2.4</td>
<td>5.7 ± 3.2</td>
<td>0.26</td>
</tr>
<tr>
<td>CIP (°C)</td>
<td>45.3 ± 0.9</td>
<td>47.6 ± 1.2</td>
<td>0.19</td>
</tr>
</tbody>
</table>

Data expressed as mean ± SEM.

Statistics. SPSS (version 16.05.0) for Windows was used to compute the results. Analysis included descriptive and frequency statistics. All data are expressed as mean ± SEM. One-way analysis of variance (ANOVA) with Scheffé post hoc test was used to study differences between means. Pearson correlation tests were used to analyze correlations between potentially related variables.

RESULTS
The clinical characteristics and detailed assessment of neuropathy in CMT1A patients and their matched controls are summarized in Table 1.

Symptoms and Neurological Deficits. Neuropathic symptoms assessed with the NSP were significantly increased in CMT1A patients (P < 0.0001), as was the severity of pain according to the McGill Pain Analogue (P = 0.001). The neuropathy deficit score (NDS) was significantly increased, consistent with a severe neuropathy in all 12 patients (9.1 ± 4.0, P < 0.0001).

Quantitative Sensory Tests. Vibration perception threshold (VPT) (P < 0.0001) and CS (P = 0.01) were significantly increased in patients compared with control subjects. However, CIP, WS, and HIP findings did not differ significantly from those of control subjects.

Electrophysiology. Sural SNAPs and fibular CMAPs were not elicited in any of the cases. Low-voltage, slowed radial and median SNAPs were obtained in only 4 cases. As expected, upper limb motor nerve conduction in all patients with CMT1A showed diffuse and uniform slowing of conduction velocity (<30 m/s) along with prolongation of distal and F-wave latencies. Upper limb CMAP amplitudes were significantly reduced (Table 2).

Corneal Sensation. Corneal sensitivity was significantly reduced in CMT1A patients compared with control subjects (P = 0.01) (Table 3).

Table 2. Electrophysiology in control subjects and CMT1A patients.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>CMT1A</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sural amplitude (μm)</td>
<td>21.0 ± 4.7</td>
<td>No response</td>
<td>—</td>
</tr>
<tr>
<td>Sural velocity (m/s)</td>
<td>47.9 ± 2.6</td>
<td>No response</td>
<td>—</td>
</tr>
<tr>
<td>Fibular amplitude (mV)</td>
<td>5.6 ± 0.8</td>
<td>No response</td>
<td>—</td>
</tr>
<tr>
<td>Fibular velocity (m/s)</td>
<td>48.5 ± 1.5</td>
<td>No response</td>
<td>—</td>
</tr>
<tr>
<td>Median amplitude (mV)</td>
<td>9.0 ± 0.6</td>
<td>3.3 ± 0.7</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Median velocity (m/s)</td>
<td>53.7 ± 0.7</td>
<td>21.8 ± 1.9</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Median latency (ms)</td>
<td>3.0 ± 0.2</td>
<td>10.3 ± 0.8</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Median F-wave (ms)</td>
<td>27.3 ± 0.9</td>
<td>63.5 ± 4.6</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Ulnar amplitude (mV)</td>
<td>8.9 ± 0.7</td>
<td>3.8 ± 0.6</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Ulnar velocity (m/s)</td>
<td>59.9 ± 1.7</td>
<td>20.9 ± 2.5</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Ulnar latency (ms)</td>
<td>2.4 ± 0.1</td>
<td>6.2 ± 0.8</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Ulnar F-wave (ms)</td>
<td>28.2 ± 0.9</td>
<td>63.2 ± 5.3</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Data expressed as mean ± SEM.
Corneal Confocal Microscopy. CNFD ($P = 0.01$; Fig. 2a), CNBD ($P = 0.02$; Fig. 2b), CNFL ($P < 0.0001$; Fig. 2c), and CNFT ($P = 0.004$; Fig. 2d) were significantly reduced in CMT1A patients compared with control subjects (Figs. 1 and 2 and Table 3). There was no difference in corneal nerve parameters between males and females (CNFD: $P = 0.8$; CNBD: $P = 0.6$; CNFL: $P = 0.9$; CNFT: $P = 0.5$; NCCA: $P = 0.4$).

Correlations. Both NDS and NSP showed no significant correlations with NCCA or corneal nerve morphology. However, the severity of pain as judged by the McGill Pain Analogue score correlated significantly with NCCA, CNFD, and CNFL (Table 4). There was no significant correlation with VPT, but NCCA correlated significantly with CS and WS. CCM demonstrated a correlation with CS and WS and reached statistical significance between NFD and CS. Both median and ulnar nerve conduction velocity showed no association with NCCA or CCM. However, NCCA correlated significantly with ulnar nerve amplitude and showed borderline significance with median nerve amplitude. Although CCM did not correlate with ulnar amplitude, both CNBD and CNFL showed a significant correlation with median nerve CMAP amplitude.

**DISCUSSION**

The diagnosis of CMT has evolved from a purely clinical approach supported by electrophysiology to the currently employed combined clinical/genetic approach. Despite advances in the identification of many of the causative genes for CMT, accurate diagnosis of CMT still requires a detailed knowledge of the clinical and genetic subtypes and their frequencies in different populations. CMT1A is caused by a 1.4-Mb duplication of the PMP22 gene on 17p11.2.\(^2,3^7\) Electrophysiology is an essential component of the diagnosis of CMT and allows patients to be classified into two types: CMT1 (demyelinating) and CMT2 (axonal) subtypes using upper limb NCVs (median or ulnar nerves), where an NCV $< 38$ m/s (commonly around 20 m/s) is strongly suggestive of CMT1A. In this study we have undertaken a systematic evaluation of neuropathy assessing symptoms, neurological deficits, conventional neurophysiology, quantitative sensory testing, NCCA, and CCM in patients with CMT1A. The results confirm a significant neuropathy with a markedly elevated NDS and a significant deficit of myelinated nerve fiber function as evidenced by absent lower limb CMAPs and SNAPs and markedly reduced upper limb nerve conduction velocity and amplitudes. In addition, we have demonstrated a significant increase in the cold threshold suggestive of A$\delta$, thinly myelinated fiber deficits.

With regard to small-fiber deficits, patients with CMT1A have clinical evidence of moderate painful

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>CMT1A</th>
<th>$P$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCCA (mbar)</td>
<td>0.4 ± 0.1</td>
<td>1.4 ± 0.3</td>
<td>0.01</td>
</tr>
<tr>
<td>NFD (number/mm$^2$)</td>
<td>39.5 ± 1.4</td>
<td>31.6 ± 2.6</td>
<td>0.01</td>
</tr>
<tr>
<td>NBD (number/mm$^2$)</td>
<td>60.1 ± 11.8</td>
<td>29.9 ± 4.7</td>
<td>0.02</td>
</tr>
<tr>
<td>NFL (mm/mm$^2$)</td>
<td>26.7 ± 1.3</td>
<td>15.8 ± 1.5</td>
<td>$&lt;0.0001$</td>
</tr>
<tr>
<td>NFT (TC)</td>
<td>14.1 ± 0.7</td>
<td>11.1 ± 0.6</td>
<td>0.004</td>
</tr>
</tbody>
</table>

Data expressed as mean ± SEM.

**FIGURE 1.** CCM images of the sub-basal layer of cornea. (a) Control subject. (b) CMT1A patient. Red arrows: main nerve fibers; yellow arrows: branches. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]
neuropathic symptoms, which is probably related to a reduction of the Aδ afferents. We have also shown an elevated NSP and McGill Pain score, which correlated with damage to corneal nerve fibers. Although quantitative sensory testing of small fibers did not demonstrate a significant

**Table 4.** Correlations between corneal sensitivity and corneal nerve morphology with neurological parameters in CMT1A in CMT1A patients.

<table>
<thead>
<tr>
<th></th>
<th>NDS</th>
<th>McGill Pain</th>
<th>VPT</th>
<th>CS</th>
<th>WS</th>
<th>Median nerve amplitude</th>
<th>Median nerve velocity</th>
<th>Ulnar nerve amplitude</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCCA</td>
<td>r</td>
<td>0.314</td>
<td>0.779</td>
<td>0.452</td>
<td>-0.997</td>
<td>0.974</td>
<td>-0.800</td>
<td>0.320</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>0.377</td>
<td>0.008</td>
<td>0.261</td>
<td>0.003</td>
<td>0.026</td>
<td>0.056</td>
<td>0.536</td>
</tr>
<tr>
<td>NFD</td>
<td>r</td>
<td>-0.194</td>
<td>-0.799</td>
<td>-0.300</td>
<td>0.954</td>
<td>-0.932</td>
<td>0.758</td>
<td>-0.486</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>0.545</td>
<td>0.002</td>
<td>0.470</td>
<td>0.046</td>
<td>0.068</td>
<td>0.080</td>
<td>0.328</td>
</tr>
<tr>
<td>NBD</td>
<td>r</td>
<td>-0.510</td>
<td>-0.513</td>
<td>-0.078</td>
<td>0.769</td>
<td>-0.646</td>
<td>0.898</td>
<td>-0.768</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>0.090</td>
<td>0.088</td>
<td>0.854</td>
<td>0.231</td>
<td>0.354</td>
<td>0.015</td>
<td>0.075</td>
</tr>
<tr>
<td>NFL</td>
<td>r</td>
<td>-0.522</td>
<td>-0.734</td>
<td>-0.220</td>
<td>0.911</td>
<td>-0.793</td>
<td>0.842</td>
<td>-0.689</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>0.062</td>
<td>0.007</td>
<td>0.001</td>
<td>0.089</td>
<td>0.207</td>
<td>0.036</td>
<td>0.150</td>
</tr>
</tbody>
</table>
increase in the thresholds for warmth and heat pain, our studies did show a significant abnormality in corneal sensation and corneal nerve fiber morphology.

The cornea contains myelinated Aδ fibers, which respond primarily to mechanical stimuli, and unmyelinated C-fibers, which respond to thermal and chemical stimuli.19 We scanned the subbasal layer of the central cornea to image corneal C-fibers. Stromal nerves representing Aδ fibers could not be consistently imaged in all patients. Quantitative analysis of stromal nerves imaged using CCM is recognized to be difficult,39 especially with regard to objectively distinguishing between normal from abnormal nerves.39–41 In a previous sural nerve biopsy study the number of unmyelinated axons/Schwann cells was reduced in patients with CMT1A.14 We have now demonstrated significant pathology of the corneal C-fiber bundles located in the sub-basal layer using CCM together with reduced corneal sensation in patients with CMT1A. Importantly, these findings correlate closely, particularly with the severity of painful neuropathic symptoms and small-fiber deficits as well as median and ulnar nerve CMAP amplitude, with the latter reflecting axonal integrity. This is the first time that a correlation between C-fiber pathology and clinical severity of neuropathy has been established in CMT1A. Thus, unlike QST, sural nerve biopsy, and dermal skin biopsy, NCCA and CCM can be used as rapid, non-invasive objective tools for clinical assessment and quantification of small-fiber abnormalities in CMT1A. The explanation of these findings may lie in roles of PMP22 in processes other than myelination.42 PMP22 overexpression in myelinating Schwann cells produces abnormal growth and differentiation, resulting in defective myelin stability and turnover.43 We hypothesize that similar PMP22 overexpression in non-myelinating Schwann cells could also be the basis of the observed defects in C-fiber bundles in CMT1A. This would be consistent with previous observations in sural nerve biopsies,14 where a reduction in the number of axons/Schwann cell profile has been demonstrated, thus providing insights into the biological role(s) of PMP22 and pathological mechanisms involving its protein.

Furthermore, these observations add to our published studies demonstrating the clinical utility of CCM in the assessment of patients with a range of peripheral neuropathies, including diabetic neuropathy,22,44 Fabry disease,41 and idiopathic small-fiber neuropathy.23 These findings provide the basis for further studies to define whether there are differences in corneal nerve morphology of patients with CMT that primarily affect axons. Ideally, detailed ultrastructural morphological study of the corneas of patients with CMT1 should also be undertaken to be absolutely certain that all fibers assessed using CCM are indeed unmyelinated. We acknowledge that this is a preliminary study, and further studies are required in a larger group of patients to compare corneal nerve morphology in patients with CMT1 and CMT2 of differing severity of neuropathy. Therefore, CCM may aid in earlier diagnosis and in longitudinal or interventional studies in patients with CMT.

This work was supported by the National Institutes of Health (R105991) and from the NIHR Manchester Biomedical Research Centre and Wellcome Trust Clinical Research Facility.

REFERENCES


Article: Complications

Corneal confocal microscopy detects improvement in corneal nerve morphology with an improvement in risk factors for diabetic neuropathy


Division of Cardiovascular Medicine, University of Manchester and Manchester Royal Infirmary, *Pennine Acute Hospitals NHS Trust, Manchester, UK and †Institute of Health and Biomedical Innovation, Queensland University of Technology, Brisbane, Queensland, Australia

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Abstract

Aim We have assessed whether corneal confocal microscopy can be used to detect alterations in nerve morphology following an improvement in risk factors associated with diabetic neuropathy.

Methods Twenty-five patients with diabetes with mild to moderate neuropathy and 18 control subjects underwent corneal confocal microscopy to quantify corneal nerve fibre (density, branch density, length and tortuosity) at baseline and after 24 months from first visit. This was not planned as an intervention trial and was simply an observational follow-up.

Results At baseline, nerve fibre density (18.8 ± 2.1 vs. 46.0 ± 3.8 number/mm², P = 0.001), nerve branch density (6.9 ± 1.5 vs. 35.6 ± 6.7 number/mm², P < 0.0001), nerve fibre length (8.3 ± 0.9 vs. 13.5 ± 0.8 mm/mm², P < 0.0001) and nerve fibre tortuosity (19.8 ± 1.6 vs. 22.7 ± 2.2, P < 0.05) were significantly lower in patients with diabetes than in control subjects. At follow-up, glycaemic control (HbA1c 64 ± 3 to 58 ± 2 mmol/mol, P = 0.08), total cholesterol (4.9 ± 0.2 to 4.2 ± 0.2 mmol/l, P = 0.01), systolic blood pressure (145.8 ± 4.9 to 135.9 ± 3.7 mmHg, P = 0.09) and diastolic blood pressure (77.8 ± 2.7 to 70.8 ± 2.5, P = 0.03) improved. Nerve fibre density (24.1 ± 2.0, P = 0.05), nerve branch density (11.1 ± 1.3, P < 0.01) and nerve fibre tortuosity (22.6 ± 1.5, P = 0.05) increased significantly, with no change in nerve fibre length (8.4 ± 0.5). Improvement in nerve fibre density correlated significantly with the improvement in HbA1c (r = −0.51, P = 0.008). Via four multifactorial regressions, this confirms the negative association between HbA1c and nerve fibre density (P = 0.02).

Conclusions This study shows that corneal confocal microscopy may be employed in longitudinal studies to assess progression of human diabetic neuropathy and also supports the hypothesis that improvements in risk factors for diabetic neuropathy, in particular HbA1c, may lead to morphological repair of nerve fibres.


Keywords corneal confocal microscopy, corneal nerves, diabetic neuropathy, risk factors

Introduction

Diabetic polyneuropathy is among the most common long-term complications of diabetes and is the main precipitating factor for foot ulceration and lower extremity amputation [1]. Furthermore, a clinical history of diabetic neuropathy has recently been shown to be one of only three independent risk factors to predict mortality in patients with Type 2 diabetes [2]. Longitudinal studies are limited, but show a deterioration of neuropathy over time in both Type 1 [3] and Type 2 diabetes [4], and the placebo arms of several clinical trials in diabetic neuropathy show a monotonic worsening in electrophysiology and quantitative sensory testing [5].

The Diabetes Control and Complications Trial and the recent Epidemiology of Diabetes Interventions and Complications study have confirmed the immediate and durable benefit of improved glycaemic control on neuropathy in patients with Type 1 diabetes [3]. However, in Type 2 diabetes, the Veterans Affairs Diabetes Trial demonstrated no impact of improved glycaemic control on somatic neuropathy and, in fact, a slight
worsening of autonomic neuropathy [6]. Furthermore, multiple clinical trials in diabetic neuropathy have demonstrated limited or no efficacy of various pathogenetically relevant interventions [1]. These data have prompted several investigators to question the reliability of the endpoints currently employed in trials of human diabetic neuropathy [5]. This has led to the suggestion that the lack of significant improvement in clinical trials of diabetic neuropathy is because of the lack of deterioration in the placebo arm of several clinical trials, possibly attributable to an improvement in the risk factors for human diabetic neuropathy [5].

The link between neuropathy and cardiovascular risk factors, such as lipids, blood pressure and BMI, has been established in a study of patients with Type 1 diabetes followed over 7 years [7]. Whilst multiple risk factor intervention has demonstrated a significant improvement in retinopathy, nephropathy and autonomic neuropathy, no benefit was seen for somatic neuropathy [8]. Similarly, in a lifestyle intervention study to improve weight, lipids, blood pressure and glycaemia in subjects with impaired glucose tolerance, vibration perception and electrophysiology did not improve, whilst quantitative sudomotor axon reflex test and intra-epidermal nerve fibre density, a functional and structural measure of small fibre damage, did [9]. Thus, quantification of small fibre pathology may be a key to assessing degeneration and regeneration of nerves in patients with diabetes. However, techniques such as nerve and skin biopsy are invasive and cannot be readily deployed in natural history or intervention studies.

Recently, we have shown that corneal confocal microscopy accurately detects corneal small nerve fibre damage, which is directly related to the level of severity of neuropathy [10–12] and intra-epidermal nerve fibre density in skin biopsy [13]. Furthermore, corneal confocal microscopy can detect nerve

<table>
<thead>
<tr>
<th>Table 1 Clinical detail of control subjects and patients with diabetes at baseline and at 24 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
</tr>
<tr>
<td>n</td>
</tr>
<tr>
<td>Age (years)</td>
</tr>
<tr>
<td>Sex (female/male)</td>
</tr>
<tr>
<td>Duration of diabetes</td>
</tr>
<tr>
<td>Type of diabetes (Type 1/Type 2)</td>
</tr>
<tr>
<td>Neuropathy Impairment Score</td>
</tr>
<tr>
<td>Vibration perception threshold (volts)</td>
</tr>
<tr>
<td>HbA1c (mmol/mol)</td>
</tr>
<tr>
<td>HbA1c (%)</td>
</tr>
<tr>
<td>Total cholesterol (mmol/l)†</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
</tr>
<tr>
<td>HDL (mmol/l)</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
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<tr>
<td>Diastolic blood pressure (mmHg)†</td>
</tr>
<tr>
<td>Nerve fibre density (number/mm²)†</td>
</tr>
<tr>
<td>Nerve branch density (number/mm²)†</td>
</tr>
<tr>
<td>Nerve fibre length (mm/mm²)</td>
</tr>
<tr>
<td>Nerve fibre tortuosity (tortuosity coefficient)†</td>
</tr>
</tbody>
</table>

Significant improvement at follow-up compared with baseline; *P ≤ 0.05; †P ≤ 0.03; ††P ≤ 0.01.
To test the hypothesis that corneal confocal microscopy may be useful in monitoring the progression of human diabetic neuropathy, we have evaluated corneal nerve fibre morphology at baseline and follow-up after approximately 24 months in relation to change in glycaemia, lipids and blood pressure.

Subjects and methods

Twenty-five patients with diabetes attending the Manchester Diabetes Centre and 18 healthy volunteers without diabetes underwent assessment of neuropathy using the Neuropathy Impairment Score and vibration perception threshold. Patients were excluded if they had a neuropathy of any other cause, absent pedal pulses, wore contact lenses or had a history of corneal trauma or surgery. The protocol was approved by the local research ethics committee of the Greater Manchester Health Authority and all subjects gave written informed consent. This study was not planned as an intervention trial and was simply an observational follow-up.

The patients underwent corneal confocal microscopy examination with a Tomey Confoscan confocal microscope Model P4 (Erlangen, Germany) applying our established methodology [12,13]. Several scans of the entire depth of the cornea were recorded and 3–5 high-quality images with best resolution of the sub-basal nerve plexus were acquired from the centre of the cornea. The investigator who examined the cornea and who undertook morphometric measurements of the images was masked with respect to the identity of the patients and medical and neurological results of subjects. The following variables were quantified to define corneal nerve fibre damage and repair: (1) nerve fibre density—the total number of major nerves per mm²; (2) nerve fibre length—the total length of all nerve fibres and branches per mm²; (3) nerve branch density—the number of branches emanating from each nerve trunk per mm²; (4) nerve fibre tortuosity is mathematically derived from the images [10]. Measures 1 and 3 were determined using morphometric software incorporated within the Tomey instrument, measure 2 was determined using third-party image analysis software (Scion Image for Windows; Scion Corporation, Frederick, MA, USA) and measure 4 was calculated using a MATLAB function (Mathworks, version 6.5; MATLAB, Mathworks, USA) that was created for this purpose [10]. To estimate the error in measuring nerve fibre density, nerve fibre length and nerve branch density, we acquired images and determined each of these variables on 15 subjects on two occasions, separated by at least 48 h. The coefficient of variation of these measures was: 12% for nerve fibre density, 9% for nerve fibre length and 24% for nerve branch density.

![FIGURE 2](image_url) Change in HbA1c (P = 0.08), cholesterol (P = 0.01), systolic (P = 0.09) and diastolic (P = 0.03) blood pressure from baseline to follow-up.
Statistical analysis

SPSS 11.05.0 for Windows (SPSS Inc., Chicago, IL, USA) was used to compute the results. Data are expressed as mean ± SEM and the analysis includes descriptive and frequency statistics. ANOVA with Scheffe post-hoc tests was used to study differences between means. The association between the change in various risk factors and nerve fibre density, nerve fibre length, nerve branch density and nerve fibre tortuosity as the dependent variables were assessed by use of a multifactorial regression, via the regression coefficients and their confidence intervals.

Results

Twenty-five people with diabetes (15 with Type 1 and 10 with Type 2) aged 52 ± 2 years with mild to moderate neuropathy (Neuropathy Impairment Score 10.3 ± 2.8 and vibration perception threshold 16.86 ± 3.05) and 18 age-matched (57.0 ± 3.0 years) control subjects were studied. The people with diabetes were attending a specialist diabetes centre to improve overall glycaemic control and cardiovascular risk factors. Corneal nerve morphology and glycaemic control (HbA1c), total cholesterol, HDL, triglycerides, systolic and diastolic blood pressure were assessed at baseline and after 24 months. This was not planned as an intervention trial and was simply an observational follow-up.

Baseline

At baseline, nerve fibre density (18.8 ± 2.1 vs. 46.0 ± 3.8 number/mm², \( P = 0.001 \)), nerve branch density (6.9 ± 1.5 vs. 35.6 ± 6.7 number/mm², \( P < 0.0001 \)), nerve fibre length (8.3 ± 0.9 vs. 13.5 ± 0.8 mm/mm², \( P < 0.0001 \)) and nerve fibre tortuosity (19.8 ± 1.6 vs. 22.7 ± 2.2, \( P < 0.05 \)) were significantly reduced in subjects with diabetes compared with control subjects (Table 1).

Follow-up

Clinical metabolic variables

An improvement was demonstrated from baseline to follow-up in glycaemic control (HbA1c, 64.60 ± 3.34 to 58.72 ± 2.53 mmol/mol, \( P = 0.08 \)), total cholesterol (4.9 ± 0.2 to 4.2 ± 0.2 mmol/l, \( P = 0.01 \)), systolic blood pressure (145.8 ± 4.9 to 135.9 ± 3.7 mmHg, \( P = 0.09 \)) and diastolic blood pressure (77.8 ± 2.7 to 70.8 ± 2.5 mmHg, \( P = 0.03 \)), with a non significant decrease in triglycerides (1.8 ± 0.2 vs. 1.5 ± 0.2 mmol/l) and no change in HDL (1.4 ± 0.1 vs. 1.3 ± 0.1 mmol/l) (Table 1, Fig. 1).

Corneal nerve morphology

Nerve fibre density (18.8 ± 2.1 to 24.1 ± 2.0, \( P < 0.05 \)), nerve branch density (6.9 ± 1.5 to 11.1 ± 1.3, \( P < 0.01 \)) and nerve fibre tortuosity (19.8 ± 1.6 to 22.6 ± 1.5, \( P < 0.05 \)) increased...
significantly, with no change in nerve fibre length (8.3 ± 0.9 to 8.4 ± 0.5) (Table 1, Figs 2 and 3).

**Correlation/regression between risk factors and corneal confocal microscopy**

The increase in nerve fibre density was significantly associated with the reduction in HbA1c from baseline to follow-up [Pearson’s correlation coefficient = −0.51 (95% CI −0.76 to −0.13), *P* = 0.008] (Fig. 4). To assess the role of a change in all the risk factors under consideration, a multifactorial regression was carried out in turn for each of the corneal confocal microscopy variables, whereby the outcome of this regression was defined as the change from baseline to 24 months in each, and the potential risk factors for neuropathy progression, which were included in the regression simultaneously. The change from baseline to 24 months of the variables and the type of diabetes was inserted into the regression analyses to adjust for potential confounding factors, such as type of diabetes. The results of these four regressions are shown in Table 2 and demonstrate a significant association between HbA1c and nerve fibre density (coefficient −3.4, 95% CI −6.24 to −0.57, *P*-value = 0.02).

**Discussion**

The natural history of nerve damage in people with diabetes is not determined. Longitudinal data from the Rochester cohort supports the contention that the duration and severity of hyperglycaemia are related to the severity of neuropathy [15]. In a study of people with Type 2 diabetes, 21% developed a significant neuropathy over 4 years [16]. In a long-term follow-up study of another cohort of people with Type 2 diabetes, nerve conduction abnormalities in the legs and feet increased from 8% at baseline to 16% after 5 years and to 42% after 10 years [4]. However, it is important to note that each of these studies used

![FIGURE 4](image.png) Correlation between change in nerve fibre density with change in HbA1c (*r* = −0.52, *P* = 0.008).

**Table 2** Regression estimates for risk factors, with outcome as change in nerve fibre density, nerve branch density, nerve fibre length and nerve fibre tortuosity between baseline and 24 months

<table>
<thead>
<tr>
<th>Risk factor</th>
<th>Coefficient*</th>
<th>95% CI</th>
<th><em>P</em>-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nerve fibre density</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>−3.40</td>
<td>−6.24 to −0.57</td>
<td>0.02</td>
</tr>
<tr>
<td>Total cholesterol (mmol)</td>
<td>−0.64</td>
<td>−5.41 to 4.12</td>
<td>0.78</td>
</tr>
<tr>
<td>Triglyceride (mmol)</td>
<td>−1.28</td>
<td>−6.20 to 3.65</td>
<td>0.59</td>
</tr>
<tr>
<td>HDL (mmol)</td>
<td>2.68</td>
<td>−11.81 to 17.16</td>
<td>0.70</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>−0.10</td>
<td>−1.06 to 0.31</td>
<td>0.31</td>
</tr>
<tr>
<td>Nerve branch length</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>−0.81</td>
<td>−4.39 to 2.77</td>
<td>0.64</td>
</tr>
<tr>
<td>Total cholesterol (mmol)</td>
<td>0.98</td>
<td>−5.04 to 6.99</td>
<td>0.74</td>
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<tr>
<td>Triglyceride (mmol)</td>
<td>0.74</td>
<td>−5.48 to 6.95</td>
<td>0.80</td>
</tr>
<tr>
<td>HDL (mmol)</td>
<td>−2.84</td>
<td>−21.13 to 15.44</td>
<td>0.75</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>−0.01</td>
<td>−0.27 to 0.25</td>
<td>0.95</td>
</tr>
<tr>
<td>Nerve fibre length</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>−329.74</td>
<td>−1478.02 to 818.54</td>
<td>0.55</td>
</tr>
<tr>
<td>Total cholesterol (mmol)</td>
<td>−619.79</td>
<td>−2548.23 to 1308.65</td>
<td>0.51</td>
</tr>
<tr>
<td>Triglyceride (mmol)</td>
<td>2.91</td>
<td>−1990.29 to 1996.11</td>
<td>0.998</td>
</tr>
<tr>
<td>HDL (mmol)</td>
<td>2389.66</td>
<td>−3475.09 to 8254.41</td>
<td>0.40</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>16.68</td>
<td>−66.65 to 100.01</td>
<td>0.68</td>
</tr>
<tr>
<td>Nerve fibre tortuosity</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>0.28</td>
<td>−1.68 to 2.25</td>
<td>0.77</td>
</tr>
<tr>
<td>Total cholesterol (mmol)</td>
<td>0.14</td>
<td>−3.16 to 3.43</td>
<td>0.93</td>
</tr>
<tr>
<td>Triglyceride (mmol)</td>
<td>−1.28</td>
<td>−4.69 to 2.13</td>
<td>0.44</td>
</tr>
<tr>
<td>HDL (mmol)</td>
<td>−2.90</td>
<td>−12.93 to 7.14</td>
<td>0.55</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>0.07</td>
<td>−0.08 to 0.21</td>
<td>0.35</td>
</tr>
</tbody>
</table>

*Estimates adjusted for effect of type of diabetes.
different clinical tests to evaluate neuropathy and the focus was on large fibres. A number of metabolic risk factors, such as glucose, lipids, blood pressure and BMI, have been shown to be related to the development of diabetic neuropathy [7,17,18]. An improvement in glycaemic control improves diabetic neuropathy in people with Type 1 diabetes [3] but may have no [6] or minimal benefit [19] in people with Type 2 diabetes. Whilst combined improvement in weight, glycaemic control, lipids and blood pressure has shown significant improvements in retinopathy, nephropathy and autonomic neuropathy, no improvement was shown in vibration perception [8,20]. Furthermore, recently lifestyle intervention, which improved weight, lipids, blood pressure and glycaemia in subjects with impaired glucose tolerance did not improve vibration perception and electrophysiology, measures of large fibre dysfunction, but did improve quantitative sudomotor axon reflex test and intra-epidermal nerve fibre density, measures of small fibre dysfunction and damage, respectively [9].

Our recent studies using corneal confocal microscopy demonstrate that corneal small nerve fibre damage can be detected prior to abnormalities in electrophysiology and quantitative sensory testing [13]. Furthermore, it shows progression with the severity of neuropathy and has good sensitivity and specificity for defining those at risk of neuropathy and foot ulceration, using a relatively crude measure of neuropathic severity, the Neuropathy Deficit Score [12]. Our studies also show that corneal confocal microscopy quantifies small nerve fibre damage as accurately as intra-epidermal nerve fibre assessment using skin biopsy [13]. Added to this, we have recently shown that corneal confocal microscopy can detect early nerve fibre repair following pancreas transplantation [14].

In the present study, an improvement in HbA1c was associated with an improvement in nerve fibre density but not nerve branch density or nerve fibre tortuosity. Because of the multiple testing undertaken and the non-significance of the other risk factors with the other three corneal confocal microscopy outcomes, this requires cautious interpretation. We have no obvious explanation for the lack of this association with nerve branch density and nerve fibre tortuosity, but it is consistent with our previous data following pancreas transplantation, which also showed no improvement in these variables [14]. A major limitation of the current study is the small size of the study and also the lack of randomization and placebo control. Therefore, a larger randomized study with active intervention is required to confirm our findings. Nevertheless, the present data suggest that corneal confocal microscopy may be a convenient non-invasive technique to assess progression of nerve damage and potentially assess the effects of therapeutic intervention in future clinical trials of human diabetic neuropathy.

Competing interests

Nothing to declare.

Acknowledgments

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References


Corneal confocal microscopy: A novel means to detect nerve fibre damage in idiopathic small fibre neuropathy

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A B S T R A C T

Patients with idiopathic small fibre neuropathy (ISFN) have been shown to have significant intraepidermal nerve fibre loss and an increased prevalence of impaired glucose tolerance (IGT). It has been suggested that the dysglycemia of IGT and additional metabolic risk factors may contribute to small nerve fibre damage in these patients.

Twenty-five patients with ISFN and 12 aged-matched control subjects underwent a detailed evaluation of neuropathic symptoms, neurological deficits (Neuropathy deficit score (NDS); Nerve Conduction Studies (NCS); Quantitative Sensory Testing (QST) and Corneal Confocal Microscopy (CCM)) to quantify small nerve fibre pathology.

Eight (32%) patients had IGT. Whilst all patients with ISFN had significant neuropathic symptoms, NDS, NCS and QST except for warm thresholds were normal. Corneal sensitivity was reduced and CCM demonstrated a significant reduction in corneal nerve fibre density (NFD) (P<0.0001), nerve branch density (NBD) (P<0.0001), nerve fibre length (NFL) (P<0.0001) and an increase in nerve fibre tortuosity (NFT) (P<0.0001). However, these parameters did not differ between ISFN patients with and without IGT, nor did they correlate with BMI, lipids and blood pressure.

Corneal confocal microscopy provides a sensitive non-invasive means to detect small nerve fibre damage in patients with ISFN and metabolic abnormalities do not relate to nerve damage.

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Introduction

Patients with impaired glucose tolerance (IGT) or impaired fasting glucose (IFG) are at substantially increased risk of developing diabetes and cardiovascular disease (Unwin et al., 2002). The incidence of microalbuminuria (Barr et al., 2006) and retinopathy (Wong et al., 2005) is higher in patients with IFG and IGT compared to subjects with normal glucose metabolism. The evidence that there is a similar link between IGT and neuropathy is less clear. The majority of studies supporting this contention have demonstrated a greater than expected prevalence of impaired glucose tolerance (IGT) in patients with idiopathic small fibre neuropathy (ISFN) (Smith et al., 2001; Smith et al., 2006; Sumner et al., 2003) with an improvement in IENF following an improvement in weight, lipid and glucose levels with diet and exercise in ISFN patients with IGT (Smith et al., 2006). However, recently the design, interpretation and hence the contributory role of IGT in the development of nerve damage in ISFN has been questioned (Dyck et al., 2007). Furthermore, a European study has not shown as high a prevalence of IGT in patients with ISFN (Nebuchennykh et al., 2008), suggesting that the higher prevalence of IGT in the previous US studies may well have reflected the higher background incidence of IGT in the US population.

If patients are selected for having IGT only as opposed to ISFN, then a recent population based study from Germany has shown that the prevalence of polyneuropathy assessed using the Michigan Neuropathy Screening Instrument was only slightly increased in individuals with IGT compared to those with NGT (Ziegler et al., 2008). More detailed studies in IGT subjects have shown no abnormality in either nerve conduction velocity and amplitude (Cappellari et al., 2005) or sural nerve myelinated nerve fibre density (Sundkvist et al., 2000).

Although, a recent study in 46 subjects with IGT demonstrated a significantly greater abnormality for heart rate variability and a higher frequency of hyperesthesia and hypoesthesia as well as increased heat detection thresholds (Putz et al., 2009), suggestive of predominantly small fibre damage.

With regard to causation, ISFN patients with normal glucose levels have recently been shown to have a significantly higher total...
and LDL cholesterol, and a higher prevalence of abnormal HDL and triglycerides (Smith et al., 2008), suggesting that dyslipidaemia may be as important as glucose dysmetabolism in the development of neuropathy (Smith and Singleton, 2008). This notion is supported in Type 1 diabetic patients, as in addition to hyperglycaemia; BMI, lipids and blood pressure have also been shown to contribute to the development of neuropathy (Tesfaye et al., 2005). Studies also suggest that the earliest damage in patients with ISFN or metabolic syndrome may occur in the small fibres, and accumulating evidence suggests IENF loss predate large fibre damage, in early diabetic polyneuropathy (Loseth et al., 2008; Umapathi et al., 2007). Hence assessment of IENF in skin biopsies has been endorsed recently in the detection of distal small fibre sensory polyneuropathy (Ebenzer et al., 2007; England et al., 2009). However, this is an invasive procedure and cannot be advocated for use in patients with minor metabolic abnormalities and no overt evidence of neuropathy.

Previously we have shown that corneal confocal microscopy (CCM), a non-invasive means of quantifying small fibre damage in patients with diabetic neuropathy (Kallimikos et al., 2004; Malik et al., 2003) may relate to intraepidermal nerve fibre loss (Quattrini et al., 2007). It also relates to a loss of corneal sensitivity (Tavakoli et al., 2007) and detects early small nerve fibre regeneration following pancreas transplantation in patients with Type 1 diabetes (Mehra et al., 2007). We have also recently shown that CCM detects nerve damage in patients with Fabry disease (Tavakoli et al., 2009).

In the present study patients with ISFN underwent CCM in addition to conventional neurophysiology and quantitative sensory testing. Furthermore nerve fibre damage was related to a range of metabolic risk factors for nerve damage.

**Methods**

The study was approved by Central Manchester Ethics committee and written informed consent was obtained from each patient according to the declaration of Helsinki.

**Patient description**

Twenty-five patients referred with ISFN from the Department of Neurology and 12 age-matched healthy volunteers were studied. All patients were examined carefully by a neurologist and other causes of neuropathy were excluded based on a detailed medical history, no family history of neuropathy, toxin exposure and normal renal function, complete blood count, thyroid function, B12 levels and plasma electrophoresis. An Oral Glucose Tolerance Test (OGTT) was performed according to ADA and WHO guidelines in all patients and Impaired Glucose Tolerance (IGT) was defined on the basis of a 2-h plasma glucose ≥ 7.8 and <11.1 mmol/l. Fasting lipids and blood pressure were also evaluated.

**Assessment of neuropathy**

All patients and control subjects underwent a detailed evaluation of neurological symptoms using the Neuropathy Symptom Profile (NSP) (Dyck et al., 1986; Young et al., 1993). Neurological deficits were assessed using the neuropathy disability score (NDS) (Abbott et al., 2005) which included tuning fork vibration perception, pin prick perception and temperature perception as well as the presence or absence of ankle reflexes to give a combined score between 0 and 10. Electrodiagnostic studies were undertaken using a Dantec “Key-point” system (Dantec Dynamics Ltd, Bristol, UK) equipped with a DISA temperature regulator to keep limb temperature constantly between 32 °C and 35 °C. Electrophysiological studies were performed in Sural, Peroneal, Tibial, Radial and Ulnar nerves. All studies were performed according to a standardised protocol using surface electrodes of 9 mm diameter. Stimulation was supra-maximal and motor studies were recorded using a belly-tendon electrode placement. Motor nerve conduction was investigated in the following nerves: Ulnar (Recording, Abductor Digitii Minimi (ADM); Stimulation, Wrist (60 mm proximal to active recording electrode), 4 cm distal to elbow and 5 mm proximal to elbow) and Peroneal (Recording, Extensor Digitorn Brevis (EDB); Stimulation, Ankle (60 mm proximal to active recording electrode), 5 cm distal to the fibular head and 5 cm proximal to the fibular head). Sensory nerve conduction was investigated in the following nerves: Sural (recording, ankle; stimulation calf (140 mm proximal to active recording electrode)) and Radial (recording, anatomical snuffbox; stimulation, wrist (80 mm proximal to active recording electrode)). The DML reflects the time taken from supra-maximal stimulation of the nerve 60 mm proximal to the active recording electrode to the onset of the compound muscle action potential (i.e. the evoked motor response). Vibration Perception Threshold (VPT) was measured using a Neurothesiometer (Horwell, Scientific Laboratory Supplies, Wilford, Nottingham, UK). Quantitative sensory testing included warm sensation and heat induced pain (WS, HIP) to assess C fibres and cold sensation and cold induced pain (CS, CIP) to assess Aδ fibres, using the MEDOC TSA II (Medoc Ltd., Ramat Yishai 30095, Israel) device on the dorsum of the left foot. Sensory thresholds were determined by the method of limits.

**Corneal sensitivity**

Corneal sensitivity was quantified using a non contact corneal aesthesiometer (NCCA, Glasgow, Caledonian University, UK) which uses a puff of air on the centre of the cornea, lasting 0.9 s and exerting a force expressed in millibars (mbars). 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. The sensation described by the subject varies with subjects most commonly describing a “cold” sensation and others a “breeze” like sensation which is not regarded as unpleasant (Tavakoli et al., 2007). The coefficient of variation for NCCA is 5.6%

**Corneal confocal microscopy**

Patients underwent examination with a Tomey Confoscan corneal confocal microscope model P4 (Erlangen, Germany). One eye of each subject was selected at random for examination and anaesthetized with one drop of benoxinate hydrochloride 0.4% (oxybuprocaine hydrochloride, Minims). The objective lens of the confocal microscope was disinfected (isopropyl alcohol 70% v/v, Swabs). A large drop of Viscoatears liquid gel (carbomer 940, Ciba Vision Ophthalmics) was applied onto the tip of the lens and advanced forwards until the gel touched the cornea, allowing optical but not physical contact between the objective lens and corneal epithelium during the examination. 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 min to acquire satisfactory images of all corneal layers providing en face two dimensional images with a lateral resolution of approximately 1 to 2 μm and final image size of 768 pixels × 576 pixels. Our recently published study compared five different regions of the cornea and demonstrated that the density in the centre was the highest and was only reduced in the extreme periphery (Patel et al., 2009). Therefore we selected three to five high quality images of Bowman’s layer from the centre of the cornea in 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. The images were coded and the observer quantified the abnormalities in a randomised and masked fashion.

We have established four parameters as potential indicators of corneal nerve fibre damage and repair (Malik et al., 2003; Quattrini et al., 2007): (i) Corneal nerve fibre density (CNFD), the total number of major
nerve density/mm² of corneal tissue; (ii) Corneal nerve fibre length (CNFL), the total length of all nerve fibres and branches (mm²/mm²) of corneal tissue; and (iii) Corneal nerve branch density (CNBD), the number of branches emanating from major nerve trunks/mm² of corneal tissue and (iv) Corneal nerve fibre tortuosity (NFT). Measures (i) and (iii) were determined using morphometric software incorporated within the Tomy instrument. Measure (ii) was determined using third party image analysis software (Scion Image for Windows, Scion Corporation, Frederick, MD, USA) and measure (iv) was calculated using a MATLAB function (MATLAB, Mathworks, USA, version 6.5) that was created for this purpose (Kallinikos et al., 2004). To estimate the error in measuring CNFD, CNFL and CNBD, we acquired images and determined each of these parameters in 15 subjects on two occasions separated by at least 48 h. The coefficient of variation of these parameters was: 12% for CNFD, 9% for CNFL and 24% for CNBD.

Statistics

SPSS 11.05.0 for Windows was used to compute the results. Analysis included descriptive and frequency statistics. All data are expressed as mean ± SEM. One-way analysis of variance (ANOVA) with Scheffe Post-hoc tests was used to study differences between groups. The Pearson test was used to analyze correlations between potentially related variables.

Results

Twenty-five patients (age: 60.2 ± 3.3) with ISFN and 12 healthy volunteers (age: 59.3 ± 3.9) were studied. The clinical characteristics of ISFN patients and controls are summarized in Table 1.

Glucose tolerance test

The OGTT demonstrated that 8 (32%) patients (age: 63 ± 3.6 years) had IGT (2 h: 8.8 ± 0.8 mmol/l) and 17 patients (age: 57.5 ± 3.1 years) had NGT (2 h: 5.5 ± 0.4 mmol/l).

Symptoms and neurological deficits

Neuropathic symptoms as assessed by the NSP were significantly increased in ISFN patients with IGT (11.3 ± 2.8, P < 0.002) and NGT (10.7 ± 1.8, P < 0.0001) compared to controls, but no difference was observed between patients with NGT and IGT. The severity of neurological deficit according to the NDS was significantly increased in patients with IGT (4.9 ± 1.8, P < 0.01) and NGT (3.9 ± 0.9, P = 0.01) compared to controls, but with no difference between IGT and NGT.

Electrophysiology

By definition i.e. ‘idiopathic small fibre neuropathy’ ISFN patients had normal sensory (Sural and Radial) and motor (Peroneal, Tibial and Ulnar) nerve electrophysiology and only sural nerve conduction velocity was marginally but significantly reduced (Table 2).

Quantitative sensory testing

Vibration perception threshold did not differ significantly between patients with IGT (22.8 ± 6.2) and NGT (17.6 ± 3.4) compared to controls (13.3 ± 2.4). The threshold for cold sensation (CS), Cold Induced Pain (CIP) and heat induced pain (HIP) did not differ significantly between patients and control subjects. However, the threshold for warm sensation (WS) was increased in ISFN patients with NGT (P < 0.005) but not in those with IGT (P = 0.08) compared to controls.

Corneal nerve sensitivity

Although the threshold of corneal sensitivity was increased in patients, it did not differ significantly in those with IGT (1.4 ± 0.3) and NGT (1.2 ± 0.2) compared to controls (0.8 ± 0.1) (Table 3).

Corneal nerve morphology

In patients with ISFN there was a highly significant reduction in CNFD (P < 0.0001) (Table 3, Fig. 1a), CNBD (P < 0.0001) (Fig. 1b) and

### Table 1
Clinical assessments.

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>ISFN patients with NGT</th>
<th>ISFN patients with IGT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>12</td>
<td>17</td>
<td>8</td>
</tr>
<tr>
<td>Sex (female/male)</td>
<td>9/3</td>
<td>5/12</td>
<td>2/6</td>
</tr>
<tr>
<td>Age (years)</td>
<td>59.3 ± 3.9</td>
<td>57.5 ± 3.1</td>
<td>63 ± 3.6</td>
</tr>
<tr>
<td>Fasting glucose (mmol/l)</td>
<td>&lt; 5.5</td>
<td>5.3 ± 0.1</td>
<td>5.6 ± 0.2</td>
</tr>
<tr>
<td>2 h glucose (mmol/l)  ≤ 7.7</td>
<td>5.5 ± 0.4</td>
<td>8.8 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>&lt; 25</td>
<td>26.3 ± 4.2</td>
<td>31.6 ± 2.8</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>147 ± 7</td>
<td>139 ± 5.5</td>
<td></td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>82 ± 5.5</td>
<td>72.5 ± 3.5</td>
<td></td>
</tr>
<tr>
<td>Total cholesterol (mmol)</td>
<td>4.7 ± 0.4</td>
<td>5.1 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>TG (mmol)</td>
<td>2.9 ± 0.8</td>
<td>2.9 ± 0.3</td>
<td></td>
</tr>
</tbody>
</table>
CNFL \((P<0.0001)\) (Fig. 1c) with an increase in CNFT \((P < 0.0001)\) compared to controls. However, there was no difference for any parameter between ISFN patients with IGT compared to NGT (Fig. 2).

Correlation

NCCA correlated with NPS \((r = 0.657, P < 0.002)\) and NDS \((r = 0.598, P = 0.007)\). Corneal NFD correlated significantly with NSP \((r = -0.47, P = 0.03)\), NDS \((r = -0.50, P = 0.02)\), sural conduction velocity \((r = 0.59, P = 0.007)\) but not WS \((r = -0.38, P = 0.13)\). Similarly, corneal NBD correlated significantly with NSP \((r = -0.83, P = 0.01)\), NDS \((r = -0.48, P = 0.03)\), sural conduction velocity \((r = 0.63, P = 0.004)\) but not WS \((r = -0.38, P = 0.13)\). NFL correlated with NSP \((r = -0.46, P = 0.04)\), NDS \((r = -0.58, P = 0.007)\), sural conduction velocity \((r = 0.55, P = 0.01)\) and WS \((r = -0.49, P = 0.04)\). CNFD correlated with other measures of corneal nerve pathology \((NBD (r = 0.92, P < 0.0001), NFL (r = 0.90, P < 0.0001)\) and NFT \((r = -0.59, P = 0.009)\). CNFD did not correlate with either fasting \((r = -0.396, P = 0.180)\) or 2 h glucose levels in the OGTT \((r = -0.034, P = 0.911)\), BMI \((r = -0.293, P = 0.382)\), total cholesterol \((r = -0.208, P = 0.539)\), triglycerides \((r = -0.529, P = 0.116)\) or systolic \((r = -0.316, P = 0.542)\) and diastolic \((r = 0.471, P = 0.345)\) blood pressure. Although NBD \((r = -0.653, P = 0.04)\) and NFL \((P = -0.618, P = 0.06)\) correlated with the triglycerides this was an inverse correlation (Table 4).

Discussion

Patients with ISFN have been shown to have significant small nerve fibre pathology using the novel non-invasive technique of CCM. Whilst we confirm the significantly increased prevalence of IGT (32%), comparable to previous studies in patients with ISFN, suggesting that IGT may be important in the genesis of nerve damage in this condition (Cappellari et al., 2005; Novella et al., 2001; Singleton et al., 2001a,b).
our data differ from recent data in a cohort of Norwegian patients with ISFN, where only 13% had IGT (Nebuchennykh et al., 2008). A potential reason for this difference may be that we studied only 25 subjects which formed a small sample of all the patients with ISFN in the clinic and therefore there was potential for selection bias.

However, in patients selected for having IGT as opposed to ISFN, the prevalence of polyneuropathy is only slightly increased in individuals with IGT compared to those with NGT (Ziegler et al., 2008). Subjects with IGT consistently show no abnormality in either nerve conduction velocity and amplitude (Cappellari et al., 2005) or sural nerve myelinated nerve fibre density (Sundkvist et al., 2000; Thrainsdottir et al., 2003). Although in a recent study, subjects with IGT demonstrated both autonomic and small nerve damage in early diabetic polyneuropathy (Loseth et al., 2008; Umaphati et al., 2007). Hence assessment of IENF in skin biopsies has been endorsed recently in the detection of distal small fibre sensory polyneuropathy (Ebenazer et al., 2007; England et al., 2009).

As expected patients with ISFN have intact large fibre function, although the NDS a neurological exam was abnormal as was sural nerve conduction velocity. The warm threshold, suggestive of a small fibre deficit, was significantly increased in patients with ISFN. However, the key finding of this study is that we have shown significant small nerve fibre pathology using the rapid and non-invasive technique of CCM, providing a novel diagnostic means to diagnose small fibre damage in patients with ISFN. These data are supported by our recent findings of abnormal CCM findings in patients with Fabry disease (Tavakoli et al., 2009).

CCM may also allow one to undertake more readily further studies to address the mechanic basis of ISFN in relation to risk factors. Thus with regard to caustion of nerve damage in ISFN patients, the early focus was in relation to impaired glucose tolerance (Singleton et al., 2001a,b). However, in a recent study a significantly higher total and LDL cholesterol, and a higher prevalence of abnormal HDL and triglycerides (Smith et al., 2008) has been demonstrated in normo-glycaemic patients with ISFN, suggesting that factors such as dyslipidaemia may be as important in the development of neuropathy in these patients (Smith and Singleton, 2008). Similarly in a recent population based study which did not preselect patients with ISFN polyneuropathy was associated with not only diabetes but also waist circumference (Ziegler et al., 2008). Furthermore, not only hyperglycaemia but also BMI, lipids and blood pressure have been shown to contribute independently to the development of neuropathy in Type 1 diabetes (Tefsaye et al., 2005). In the present study although the incidence of IGT was higher in patients with ISFN than one might expect in the general population, the premise that IGT may cause nerve damage is not sustained as despite marked corneal nerve damage in patients with ISFN this did not differ between patients with IGT and NGT. Furthermore there was also no correlation between corneal nerve fibre loss and a range of metabolic parameters, except triglycerides with NBD and NFL. Although, clearly a lack of correlation does not necessarily mean a lack of association and therefore these conclusions must be guarded due to the small sample size studied.

In conclusion CCM provides a novel non-invasive means to demonstrate significant small fibre abnormalities in patients with ISFN. This study questions the role of impaired glucose tolerance in the genesis of this condition. Larger cross sectional or longitudinal mechanistic studies are warranted and CCM may facilitate this.

Acknowledgments

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References


Table 4

<table>
<thead>
<tr>
<th>NSP</th>
<th>NDS</th>
<th>Sural velocity</th>
<th>WS</th>
<th>Two-hour glucose</th>
<th>BMI</th>
<th>Total cholesterol</th>
<th>TG</th>
</tr>
</thead>
<tbody>
<tr>
<td>NBD</td>
<td>r = -0.473*</td>
<td>r = -0.504*</td>
<td>r = 0.594**</td>
<td>r = -0.375</td>
<td>r = -0.03</td>
<td>r = -0.293</td>
<td>r = -0.208</td>
</tr>
<tr>
<td>P = 0.03</td>
<td>P = 0.02</td>
<td>P = 0.007</td>
<td>P = 0.13</td>
<td>P = 0.91</td>
<td>P = 0.382</td>
<td>P = 0.539</td>
<td>P = 0.116</td>
</tr>
<tr>
<td>NFD</td>
<td>r = -0.832*</td>
<td>r = -0.483*</td>
<td>r = 0.631**</td>
<td>r = -0.376</td>
<td>r = -0.254</td>
<td>r = -0.517</td>
<td>r = -0.215</td>
</tr>
<tr>
<td>P = 0.01</td>
<td>P = 0.03</td>
<td>P = 0.004</td>
<td>P = 0.13</td>
<td>P = 0.41</td>
<td>P = 0.104</td>
<td>P = 0.526</td>
<td>P = 0.001</td>
</tr>
<tr>
<td>NFL</td>
<td>r = -0.462*</td>
<td>r = -0.581**</td>
<td>r = 0.549*</td>
<td>r = -0.484*</td>
<td>r = 0.210</td>
<td>r = -0.212</td>
<td>r = -0.362</td>
</tr>
<tr>
<td>P = 0.04</td>
<td>P = 0.007</td>
<td>P = 0.01</td>
<td>P = 0.04</td>
<td>P = 0.49</td>
<td>P = 0.531</td>
<td>P = 0.275</td>
<td>P = 0.057</td>
</tr>
<tr>
<td>NCCA</td>
<td>r = 0.657**</td>
<td>r = 0.598**</td>
<td>r = -0.381</td>
<td>r = 0.205</td>
<td>r = -0.414</td>
<td>r = -0.401</td>
<td>r = -0.010</td>
</tr>
<tr>
<td>P = 0.002</td>
<td>P = 0.007</td>
<td>P = 0.19</td>
<td>P = 0.447</td>
<td>P = 0.15</td>
<td>P = 0.221</td>
<td>P = 0.978</td>
<td>P = 0.525</td>
</tr>
</tbody>
</table>

*Correlation is significant at 0.01 level. **Correlation is significant at 0.05 level.

NSP: Neuropathy Symptom Profile; NDS: Neuropathy Deficit Score; WS: Warm Sensation; TG: Triglyceride.


Chapter 6
Optimal Measures of Small Fiber Neuropathy in Diabetic Polyneuropathy

M. Tavakoli, H. Fadavi, and R.A. Malik

6.1 Introduction and Objectives

Recently it has been proposed that “If nerve conduction (NC) is normal, a validated measure (with class 1 evidence) of small fiber neuropathy (SFN) may be used” to define and quantify the severity of diabetic sensory-motor polyneuropathy (DSPN) [1]. NC assesses large myelinated nerve fiber function and has been used as an end point in clinical trials of human diabetic neuropathy, based on relative ease of quantification, reproducibility, and reasonable sensitivity and specificity [2]. However, recent data have demonstrated minimal worsening [3] and improvements [4] in electrophysiology in placebo and epidemiological cohorts with little relation to other measures of small fiber and autonomic function in diabetic patients [5].

Small fibers constitute 79.6 % [6] to 91.4 % [7] of peripheral nerve fibers. Damage to this class of fibers underlies the symptoms of painful diabetic neuropathy, which are typically distal, symmetrical, and associated with nocturnal exacerbation. The descriptors used by patients to describe the symptoms can be variable but often include the following: prickling, aching, and burning pain with intermittent sharp stabbing electric-shock-like pains and on examination one can elicit dysesthesia and allodynia. In addition to these troublesome symptoms, dysfunction and damage to this class of fibers are also key to the genesis of foot ulceration through the effect on sudomotor function [8], pressure-induced vasodilation [9, 10], and of course heat and pain perception [11]. Moreover, an increasing body of data shows that small fiber damage may precede large fiber damage in diabetic neuropathy [12–14].

Therefore it appears pertinent to address whether any definition of DSPN should include a measure of small fiber neuropathy. Issues that arise before we can adopt...
the assessment of SFN to diagnose DSPN include establishing the reproducibility, sensitivity, specificity, and accuracy but also the practical viability of any proposed test. For the purposes of this review, we will consider the available evidence for established and emerging measures of “small fiber damage” to diagnose and stratify the severity of DSPN.

6.2 Quantitative Sensory Testing

6.2.1 Thermal Thresholds

Abnormalities in heat-pain thresholds reflect small fiber dysfunction, and a number of instruments including CASE IV, thermoesthesiometer, and Medoc instruments have been used to quantify this parameter. In 498 type 2 diabetic patients and 434 control subjects, an elevated warm threshold was the most frequent abnormality (60.2 %) compared to an abnormal cold threshold (39.6 %) and abnormal sural nerve conduction velocity (12.9 %), and it was related to both symptoms and glycemic control [15]. However, a careful study of 59 diabetic patients with and without symptomatic neuropathy showed that unlike cold perception thresholds and IENFD, warm perception thresholds did not differentiate diabetic patients with and without symptoms [14]. Similarly, in a study of 191 diabetic patients, there was no difference in heat-pain thresholds between those with and without painful neuropathy [16].

6.2.2 Pain-Related Evoked Potentials

In a study of 57 diabetic patients with entirely normal electrophysiology, the latency was increased and amplitude was reduced for pain-related evoked potentials (PREPs), elicited by nociceptive electrical stimulation of the skin [17].

6.2.3 Nerve Axon Reflex/Flare Response

Stimulation of the nociceptive C fiber results in both orthodromic conduction to the spinal cord and antidromic conduction to other axon branches, i.e., the axon reflex (Fig. 6.1) which can stimulate the release of peptides, such as substance P and calcitonin gene-related peptide, resulting in vasodilation and increased permeability. Studies have shown that this neurovascular response mediated by the nerve axon reflex is reduced in diabetic neuropathic patients, correlates with other nerve function measurements, and has reasonable sensitivity and specificity in identifying
patients with diabetic neuropathy [18, 19]. The LDI flare test evaluates 44 °C heat-induced vasodilation [20] and is reduced in subjects with impaired glucose tolerance (IGT) [21] and type 2 diabetic patients with and without neuropathy [22, 23] but interestingly is normal in patients with type 1 diabetes of long duration [21].

More longitudinal data and perhaps assessment after interventions when compared with established tests are necessary before these techniques can be recommended for clinical use.

### 6.3 Skin Biopsy

Skin biopsy, a minimally invasive procedure, allows morphometric quantification of intraepidermal nerve fibers (IENF) most commonly expressed as the number of IENF per length of section (IENF/mm) [24, 25] (Fig. 6.2). Intra- and interobserver variability for the assessment of IENF density demonstrates good agreement [25, 26], declines with age, and does not appear to be influenced by weight or height [27]. An international consortium of investigators has recently compiled a normative database for IENFD in 550 participants and shown an effect of age, but no influence of height, weight, or BMI [28]. The blister technique is an alternative less invasive procedure which assesses innervation of the epidermis alone and shows good agreement with punch biopsy [29].
No study assessing the sensitivity and specificity of IENF in DSPN is available. However, several studies in SFN have included patients with DSPN. In 58 patients with pure SFN, a cutoff IENF density of \( \leq 8.8 \) per mm at the ankle was associated with a sensitivity of 77.2 % and a specificity of 79.6 % [30]. Similarly, in 67 patients with pure SFN, a sensitivity of 88 % and a specificity of 88.8 % have been reported [31]. In a study of 210 patients with SFN, which included 65 diabetic patients, the Z-scores and 5th percentile provided the highest specificity (98 and 95 %, respectively) but a very low sensitivity (31 and 35 %, respectively) compared to the ROC analysis (specificity 64 %, sensitivity 78 %) [32]. These findings suggest that the

**6.3.1 Diagnostic Yield of IENF Quantification**

![Fig. 6.2 Skin biopsy with PGP 9.5 immunostaining for IENF showing normal IENF (→) in a control subject (top) and absence of IENF with only dermal nerve fibers (→) in a diabetic patient with severe neuropathy (bottom)](image_url)
diagnostic yield of skin biopsy may depend on the reference and cutoff values
selected and the definition of SFN adopted. IENF density correlates inversely with
thermal thresholds. While some have reported a closer correlation with warm and
heat-pain thresholds [30, 33–35] compared to cooling thresholds [36, 37], others
have reported the opposite, with a closer correlation with cold rather than heat
detection thresholds [16, 38]. A recent study has demonstrated no correlation
between IENFD and the neuropathy symptom score, but interestingly an inverse
correlation was demonstrated with the severity of pain assessed using the VASmax
[39]. The correlation between quantitative sensory testing (QST) and IENF density
therefore remains controversial.

The American Academy of Neurology, American Association of Neuromuscular
and Electrodiagnostic Medicine, and American Academy of Physical Medicine and
Rehabilitation have concluded however that skin biopsy may be considered for the
diagnosis of DSPN, particularly SFN, with a level C recommendation [40]. More
recently, under the auspices of the European Federation of the Neurological
Societies and the Peripheral Nerve Society, revised guidelines on the use of skin
biopsy concluded that IENF density is a reliable and efficient technique to confirm
the clinical diagnosis of SFN with level A recommendation [41].

Additional morphological features of IENFs include the branch density, length,
and mean dendritic length; all show an early reduction which progresses with neu-
ropathic severity [13, 42]. Several studies with serial skin biopsies in patients with
SFN have shown that axonal swellings predict a decline in IENF density [43–45].
However, they occur not only in patients with SFN [46] but also in normal individu-
als [47], and isolated swellings with normal IENF densities have been observed in a
variety of other neuropathies [47–50].

6.4 Diabetic Neuropathy

In patients with diabetic neuropathy, the prevalence of abnormal NC, QST, and
IENF was comparable [39]. However, IENF density was significantly reduced in
patients with normal NC, suggesting early damage to small nerve fibers [12, 14].
Although, a recent study has shown comparable abnormalities in electrophysiology,
thermal thresholds and loss of IENF in diabetic patients with mild neuropathy [39].
There is an inverse correlation between IENF density and the severity of DSPN,
deﬁned by the neurological disability score [13, 34, 51] and the neuropathy impair-
ment score [14]. Additionally, IENF density appears to be lower in diabetic patients
with painful compared to painless neuropathy [13, 34, 52]. A 1-year diet and exer-
cise intervention program in patients with SFN and IGT led to increased IENF
density [53]. However, no change was observed in 18 diabetic patients after simul-
taneous pancreas/kidney (SPK) transplantation [54]. This may reﬂect the marked
IENF loss at baseline [55], particularly in diabetic patients undergoing SPK and the
slower regeneration rate of IENF in diabetic patients [56]. These data suggest that
IENF loss is an early feature of diabetes, progresses with increasing neuropathic severity, and may improve with appropriate intervention.

A considerable body of experimental data has been generated recently to show that IENF loss may be an early morphological marker of small fiber damage in animal models of diabetes. A loss of epidermal innervation similar to that observed in diabetic patients has been observed in rodent models of both type 1 and type 2 diabetes, and several therapeutics have been reported to prevent reductions in intraepidermal nerve fiber density in these models [57]. Several studies have assessed cutaneous innervation in mouse footpad [58–60] and showed a reduction in intraepidermal innervation of both flank and footpad skin [61]. There is high interobserver agreement when two experts use the protocol used in humans to quantify the density of IENFs [62]. In a study in nonhuman primates with naturally occurring obesity and type 2 diabetes, hypertrophic epidermal nerve fibers were found in monkeys with short-time hyperglycemia; however, a severe reduction of nerve fibers was demonstrated in those with a duration of diabetes exceeding 8 years [63]. In diabetic mice, although the total epidermal innervation appears unchanged in early diabetes, staining for peptidergic fibers is significantly reduced [64]. These early changes may have a functional relevance, as previous studies in rodents demonstrate behavioral deficits prior to quantifiable intraepidermal nerve fiber loss [65]. Thus IENF density can be reliably quantified in the footpad of healthy and neuropathic rats and interestingly correlates significantly with tail nerve conduction velocity [62]. These findings support the use of IENF quantification as an outcome measurement in experimental neuropathies.

6.5 Nerve Biopsy

Nerve biopsy has traditionally been used to quantify myelinated nerve fiber density which is reduced and correlates with abnormalities in neurophysiology [66, 67] but may also predict development of future neurophysiological deficits [68]. Few studies have quantified unmyelinated nerve fiber damage, but some have shown that it precedes myelinated nerve fiber damage in sural nerve biopsies and therefore it may be used to detect early DSPN [7]. However, nerve biopsy is an invasive and highly specialized procedure which requires neurosurgical expertise to identify and perform, especially when a fascicular biopsy is required. Furthermore, electron microscopy demands considerable expertise and there are very few centers which can perform quantification. It therefore cannot be advocated for use to diagnose DSPN [69].
6.6 Corneal Confocal Microscopy

Corneal confocal microscopy (CCM) is a noninvasive ophthalmic technique that has been shown to detect small sensory corneal nerve fiber loss in diabetic neuropathy (Fig. 6.3) [70], idiopathic small fiber neuropathy and IGT patients [71], and Fabry disease, a condition which is characterized by painful neuropathy [72], by visualizing the subbasal nerve plexus in Bowman’s layer of the cornea. Corneal nerve fiber damage correlates with IENF loss and severity of neuropathy in diabetic patients [13, 73] and is more marked in patients with painful diabetic neuropathy [13]. A correlation between loss of corneal nerve fibers and the stage of diabetic retinopathy has also been demonstrated [74]. CCM may also be more sensitive than IENFD in detecting early damage [13] and repair after SPK transplantation [55, 75]. Thus corneal nerve fiber density improves 6 months after combined pancreas/kidney transplantation [75]. CCM has been shown to have high reproducibility [76], with reasonable sensitivity and specificity [77]. To enhance the practical application of this technique, an automated image analysis system has also been developed recently to rapidly quantify corneal nerve pathology [78]. A progressive loss of corneal sensation with increasing severity of neuropathy provides a functional correlate of corneal nerve fiber loss in diabetic patients [79–81].

Therefore as CCM is noninvasive, it may be an ideal technique to assess alterations in small nerve fiber pathology in relation to PDN and progression or regression of neuropathic deficits.

Fig. 6.3 Corneal confocal microscopy image of a control subject (right panel) with normal corneal nerve (→) density compared to an image from a diabetic patient with severe neuropathy and marked loss of corneal nerve fibers (left panel)
6.7 Sudomotor Dysfunction

6.7.1 Sympathetic Skin Response

Sympathetic skin response (SSR) assesses sudomotor and hence small fiber dysfunction. In an early study it failed to differentiate the presence or absence of neuropathy in a series of 337 diabetic patients [82]. However, it has recently been shown to predict the risk of foot ulceration comparable with abnormalities in NDS and elevated vibration perception [83]. It has also been shown to have a sensitivity of 87.5 % and a specificity of 88.2 % for detecting diabetic autonomic neuropathy [84].

6.7.2 Quantitative Sudomotor Axon Reflex Testing

Quantitative sudomotor axon reflex testing (QSART) evaluates sudomotor function by assessing the local sweat response to iontophoresis of acetylcholine [85] and has been shown to be highly sensitive in the detection of distal SFN [86]. QSART evaluates postganglionic axon function as opposed to the polysynaptic pathways assessed using SSR. In a series of 31 diabetic patients with early neuropathy, it appeared to be better at detecting early neuropathy than SSR [87].

6.7.3 Neuropad

The neuropad test is a simple visual indicator test which uses a color change to define the integrity of skin sympathetic cholinergic innervation. Neuropad responses have been shown to correlate with the modified NDS, QST, CAN, and IENF loss with relatively high sensitivity but lower specificity for detecting DSPN [88, 89]. A recent study has shown that an abnormal neuropad test in those with a normal NDS may predict the development of diabetic neuropathy after 5 years [90]. This appears to reflect early small fiber involvement which is missed using NDS as a measure of neuropathy.

6.7.4 Sudomotor Innervation

Recently, a novel stereologic technique has been applied in skin biopsies and showed a correlation between sweat gland nerve fiber density, neuropathic symptoms, neurological deficits, and sweat production [91]. However, morphometric data in patients with diabetic SFN are limited and further studies are warranted.
6.8 Definition of SFN

Given the overwhelming evidence for the involvement of small fibers in the early and late phases of peripheral nerve damage in diabetic patients, we propose to grade SFN as follows: (1) possible, presence of distal symmetrical symptoms and/or clinical signs of small fiber damage; (2) probable, presence of distal symmetrical symptoms, clinical signs of small fiber damage, and normal or abnormal sural NC study; and (3) definite, presence of length-dependent symptoms, clinical signs of small fiber damage, normal or abnormal sural NC study, and/or abnormal QST thermal thresholds at the foot and reduced IENF density at the ankle.

At present it is not possible to suggest criteria to define the severity of SFN in DPN. However, as normative ranges are established for the different tests of small fiber dysfunction and damage, it may be possible to devise a measure of severity using different percentiles or quartiles as cutoffs.

References

Diabetes is the most common cause of peripheral neuropathy, and painful diabetic neuropathy (PDN) affects approximately 30% of diabetic patients with neuropathy. It is extremely distressing for the patient and poses significant management difficulties because no treatment provides total relief, and side effects of therapy are a major limiting factor for titrating therapy. Understanding the pathogenesis of diabetic neuropathy may lead to the development of new treatments to prevent nerve damage, and a better understanding of the mechanisms that modulate pain may lead to more effective relief of painful symptoms. We provide an update on the pathogenesis, diagnosis, and treatment of PDN.

Introduction
Diabetic neuropathy, the most common cause of peripheral neuropathy, causes substantial morbidity and increases mortality. It is defined by “the presence of symptoms and/or signs of peripheral nerve dysfunction in people with diabetes after the exclusion of other causes” and may occur in 50% to 90% of patients depending on the criteria used for diagnosis [1•]. Painful diabetic neuropathy (PDN), which is extremely distressing and significantly reduces patients’ quality of life [2], occurs in 15% to 30% of diabetic patients with neuropathy [3]. Hyperglycemia is clearly important in the genesis of nerve damage, and recent studies suggest that even minimal perturbations in blood glucose in those with impaired glucose tolerance (IGT) may lead to the development of small nerve fiber damage and neuropathic pain [4,5].

Pathophysiology
The pathophysiology of neuropathic pain in diabetic neuropathy is complex and not well understood. The literature focusing on specific mechanisms relating to PDN is limited when compared with data available on the molecular processes leading to nerve damage in diabetes mellitus.

The International Association for the Study of Pain defined neuropathic pain as “pain initiated or caused by a primary lesion or dysfunction in the nervous system” [6]. Therefore, any lesion in the peripheral and/or central nervous system can potentially cause pain. In short, the pathophysiology of diabetic neuropathy includes increased oxidative stress, advanced glycation, polyol accumulation, decreased nitric oxide, and impaired (Na+/K+)-ATPase. Not only do nerve cells die more readily in a hyperglycemic environment, but repair mechanisms are also defective. Reduced levels of a range of neurotrophic factors, including nerve growth factor and insulin-like growth factor, are downregulated in experimental diabetes (Fig. 1). It is important to remember that even short-term hyperglycemia may be sufficient to induce oxidative stress and nerve cell dysfunction/death leading to neuropathy in experimental diabetes, but the degree and magnitude of hyperglycemia may not necessarily be the same in diabetic patients [7].

With regard to the central and peripheral mechanisms leading to neuropathic pain, many of the postulated abnormalities are derived principally from models of nondiabetic painful neuropathy and very few have been confirmed in diabetic patients. Thus, it is known that pain transmission in peripheral nerves occurs via the small A-δ and C-nerve fibers [8], but the source of pain may be central and/or peripheral because diabetes affects all levels of the nervous system, from the peripheral nerves to the brain (Fig. 2) [9].

Peripheral mechanisms
Pathology
Specific pathological changes in sural nerve biopsies have thus far not been attributed to the development of PDN [10]. However, the temporal course of nerve damage has been delineated from cross-sectional studies in which degeneration and demyelination are balanced in the early
stages of neuropathy by axonal regeneration and remyelination. It has been suggested that degenerating nerve fibers and those that exhibit impaired regeneration may generate inappropriate excitation impulses, which are perceived as pain and paresthesiae. With disease progression and a reduction in regenerative capacity, nerve fiber loss predominates, leading to sensory loss [10]. Progressive nerve fiber loss has been attributed to a reduction in vascular endothelial growth factor expression in the foot skin of diabetic patients with increasing neuropathic severity [11]. It has been demonstrated that patients with painful neuropathy show a decreased threshold for a cold stimulus and no difference for a heat pain stimulus compared with those without pain, which suggests that A-δ myelinated nerve fibers rather than C fibers may be important in the genesis of pain [8,12]. In one skin biopsy study, significant intraepidermal nerve fiber (IENF; C-fiber) loss occurred in patients with PDN [8], yet in another study diabetic patients with and without PDN showed no difference in IENF density [13]. This suggests that the basis of pain is
likely to be complex, with subtle involvement of at least these two classes of fibers and perhaps additional peripheral molecular alterations such as altered keratinocyte expression of the transient receptor potential vanilloid 1 in patients with diabetic neuropathy [14•].

**Hyperexcitability**

Hyperglycemia leads to an increase in axonal excitability and a reduction in the refractory period in poorly controlled diabetic patients, compared with well-controlled diabetic patients (HbA1c < 7%) and nondiabetic subjects [15]. Furthermore, patients with established PDN have smaller action potentials and decreased recovery cycles of excitability compared with control subjects [16]. In diabetic neuropathy, ongoing damage results in hyperexcitability of primary afferent nociceptors (peripheral sensitization), and continued sensitization alters nociceptor processing, leading to spontaneous discharge of the neurons. Sensitization is characterized by a lowered activation threshold with an exaggerated response to a given stimulus, and abnormal spontaneous activity. Peripheral sensitization leads to hyperexcitability in central neurons (central sensitization) and generation of spontaneous impulses within the axon as well as the dorsal root ganglion of these peripheral nerves [17].

**Ion channels**

In chronic neuropathic pain, ion channel expression is altered across a range of ion channels, including sodium, potassium, and calcium channels. Thus, along sites of nerve fiber injury, sodium channels accumulate, facilitating hyperexcitability, and discharge of ectopic electrical impulses contributes to the generation of electrical impulses to the dorsal horn [18].

**Sympathetic alterations**

Damaged peripheral nerves become epinephrine-sensitive and result in sympathetically mediated pain. In animal models, abnormal transmission of information occurs from one axon to another via a phenomenon called ephaptic transmission or “cross-talk.” When these connections occur between sensory and sympathetic nerve fibers, sympathetically mediated pain arises [19,20]. Not only do the nerves become more sensitive to epinephrine but the blood levels of norepinephrine are also increased in patients with PDN, compared with diabetic patients with or without diabetic neuropathy. Patients with PDN also have local selective sympathetic denervation in their feet evidenced by increased norepinephrine spillover assessed using positron emission tomography [21]. Furthermore, sympathetically mediated peripheral vasoconstriction is also impaired in patients with PDN, resulting in inappropriate local blood flow regulation [22].

**Central involvement**

Central mechanisms at the level of the spinal cord are considered to be very important in the development and perception of neuropathic pain. This may be particularly relevant at the level of the dorsal root ganglia where afferent and sympathetic neurons couple. When peripheral nerves are injured, local sympathetic fibers sprout terminals that surround large afferent A fibers, which release substance P—generating signals that are misinterpreted as mechanical allodynia. Also, hyperexcitable peripheral nerve fibers lead to hyperexcited central fibers through the stimulation of the postsynaptic N-methyl-D-aspartate receptors. Glutamate has an excitatory effect on postsynaptic neurons, and its release enhances postsynaptic potentials leading to synaptic potentiation, augmenting the perception of normal stimuli resulting in allodynia [19,23].

**Clinical Features**

Thirty percent to 40% of patients with diabetic neuropathy have painful symptoms [24]. It is most commonly associated with distal symmetrical neuropathy affecting the lower limbs (especially toes and feet), and patients present with burning, stabbing, and tingling sensations. Patients with distal symmetric neuropathy also have negative symptoms such as loss of sensation and balance. Pain is also a prominent feature of the focal neuropathies, such as the entrapment neuropathies, and can be particularly troublesome in diabetic amyotrophy or lumbosacral plexopathy.

To help clinicians quantify the magnitude of pain and to allow patients to describe their symptoms, several questionnaires have been developed to profile the distribution, severity, and frequency of symptoms; however, the main limitation of these methods is that there is no accepted cutoff for the level of pain [24]. The most frequently used questionnaire is the McGill Pain Questionnaire; however, it was not designed specifically for PDN [19]. Recently, more specific scores for PDN have been developed. These include the Brief Pain Inventory (BPI) and the Pain Diagnostic Questionnaire (DN4). The BPI assesses the severity of pain and its impact on daily functioning on a seven-item pain interference scale that can be completed by the patient [25,26]. The DN4 compares pain syndromes associated with nervous or somatic lesions [27].

**Diagnosis**

Standard measures of neuropathy such as nerve conduction studies and vibration perception thresholds (VPTs) can be used to detect abnormalities of nerve function, but they focus on large nerve fibers; as discussed earlier, pain is generated and mediated by small C and A-δ fibers. Quantitative sensory testing (QST), including thermal threshold assessment for cold sensation (A-δ fibers) and warm sensation (C fibers), can assess small-fiber dysfunction but is highly subjective and lacks precision and accuracy, making reproducibility difficult. Thus, Sorensen et al. [8] showed no difference in VPT between diabetic patients with painful and painless neuropathy. Although Gore et al. [28]
showed that patients with pain were more likely to have an abnormal cold threshold compared with those without pain, the former group also had more severe neuropathy evidenced by higher VPT and absent reflexes. In general, QST and nerve conduction studies cannot distinguish painful and painless diabetic neuropathy. One of the limitations of QST has been the lack of normative data; however, normative data were recently published for QST [29] and may allow detection and quantification of negative symptoms and positive sensory symptoms (such as allodynia), both of which are present in patients with PDN [30].

The only techniques that allow a direct examination of thinly myelinated and unmyelinated nerve fiber damage and repair are the invasive techniques of sural nerve biopsy with electron microscopy [31], and skin-punch biopsy [32]. IENF density can be used to evaluate small-fiber involvement in diabetic neuropathy [33]. However, there is no clear consensus on the role of IENF loss in patients with painful and painless neuropathy. Yet, some recent studies suggest that more refined morphometric evaluation of epidermal nerve fiber morphology, such as axonal swellings [34] and alterations in branching [35], may be associated with neuropathic pain. A recent study also showed a marked impairment in the cutaneous response to iontophoresed Ach in patients with painful compared with painless diabetic neuropathy, suggesting that alterations in tissue blood flow may modulate signals generating pain in the periphery [36••]. Recently, we have shown that the novel noninvasive technique of corneal confocal microscopy can detect small-fiber neuropathy in diabetic patients by visualizing small nerve fibers in Bowman’s layer of cornea [36••, 37]. Furthermore, it may be more sensitive than IENF density in detecting early damage [38] and repair after pancreas transplantation [39]. Thus, this may be an ideal technique to accurately quantify small nerve fiber morphology, especially in a reiterative manner following patients with exacerbations and remissions of PDN. We have recently shown that corneal confocal microscopy can be used to demonstrate more advanced small nerve fiber damage in patients with Fabry disease, a hereditary condition with pure small-fiber neuropathy (unpublished data).

Treatment
Intensive blood glucose control should form the cornerstone of diabetic neuropathy treatment. For the treatment of PDN, the degree of glycemic flux rather than the overall blood sugar control may be more important [40]. Indeed, even a minor flux of glucose in patients with IGT may generate pain, and a study in patients with IGT showed that diet and exercise improved glucose tolerance and pain [41••]. However, the long-term benefits of diet and exercise are not established—a recent study showed it does not prevent neuropathy progression in IGT patients [42]. Other options for treating PDN may be based on pathogenetic mechanisms of neuropathy and include aldose reductase inhibitors [38] and α-lipoic acid, which has demonstrated improvement in neuropathic symptoms and deficits [43]. Many pharmaceutical and nonpharmaceutical treatments are available for PDN (Table 1). The standard treatment strategy is often first-line tricyclic antidepressants (TCAs; amitriptyline), second-line anticonvulsants (gabapentin), and third-line opioid-related treatment. However, only two agents are approved by the US Food and Drug Administration (FDA): duloxetine [44] and pregabalin [45].

Although NSAIDs are prescribed commonly in PDN, there is no evidence that they are effective, and they are contraindicated in patients with renal impairment [33]. TCAs have traditionally been first-line therapy for PDN, but anticholinergic side effects and sedation limit their use, especially in elderly patients. Selective serotonin reuptake inhibitors have fewer side effects than TCAs, and duloxetine has received FDA approval for PDN treatment [44]. Furthermore, a recent United Kingdom–based economic model suggests that second-line use of duloxetine is a beneficial and cost-effective treatment strategy for diabetic peripheral neuropathic pain [35]. Anticonvulsants have been used in the management of neuropathic pain for many years; however, a recent analysis has found limited evidence for efficacy with this class of drugs in PDN [46]. Gabapentin is most commonly prescribed, although pregabalin, a higher-potency and more effective analog of gabapentin, is the only agent (in addition to duloxetine) approved by the FDA for treatment of PDN. Of the antiarrhythmics, mexiletine is a class 1B agent that is a structural analog of lidocaine; unlike lidocaine, it can be administered orally. At low doses the risk of electrocardiographic side effects is low; regular electrocardiographic monitoring is necessary, and long-term use cannot be recommended [33].

The use of opioids for neuropathic pain remains controversial, as studies have generally been small, yielded equivocal results, and have not established the long-term risk–benefit ratio [19,44]. Capsaicin, an alkaloid found in chilli peppers, has been shown to be effective in PDN. However, a major clinical concern is that topical capsaicin has been shown to produce complete or near-complete denervation of the epidermis in both control subjects and diabetic patients, with a significant reduction in regeneration in the latter group [47].

For patients with more refractory PDN or those who suffer from significant side effects of pharmacotherapy, PDN can be treated nonpharmacologically using acupuncture [18,48] and frequency-modulated electromagnetic neural stimulation [49].

Conclusions
At present, apart from improving glycemic control, there is no licensed treatment for diabetic neuropathy. Thus, defining the pathogenesis of diabetic neuropathy may provide insights into the development of nerve damage and repair
to prevent diabetic neuropathy and regenerate nerves that have been damaged. Although there are many approaches to the treatment of PDN, achieving greater than 50% relief is rare, and side effects limit dose titration. Thus, improvement in the understanding of the pathogenesis of pain in diabetic neuropathy may lead to new, better-targeted treatments with greater efficacy and fewer side effects.

Disclosures
Dr. Malik has been a consultant for Pfizer and Boehringer Ingelheim.

References and Recommended Reading
Papers of particular interest, published recently, have been highlighted as:

- Of importance
-• Of major importance


Table 1. Treatment options for painful diabetic neuropathy

<table>
<thead>
<tr>
<th>Mechanism of effect</th>
<th>Therapy/class of drug</th>
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<th>Dose/day, mg</th>
<th>Side effects*</th>
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<td>Optimal glycemic control</td>
<td>Diet, OAT, insulin, and exercise</td>
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<td>25–150</td>
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<td>SSRIs</td>
<td>Duloxetine</td>
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<td>Gabapentin</td>
<td>900–3600</td>
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<td>Fewer side effects than TCAs</td>
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<td>Carbamazepine</td>
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<td>Antiarrhythmics</td>
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*Plus symbols refer to the severity of symptoms (+ denotes minimal, +++ denotes maximal). FDA—US Food and Drug Administration; IV—intravenously; OAT—outdoor adventure training; SSRI—selective serotonin reuptake inhibitor; TCA—tricyclic antidepressant.


This study details a novel mechanism of pain generation in the periphery, which suggests that pain may arise from sites other than neurons.


Provides the first immunohistologic evidence in humans of a progressive reduction in intraepidermal nerve fibers with increasing neuropathic severity, which relates to a reduction in vascular endothelial growth factor expression, providing a therapeutic option for diabetic neuropathy.


This study confirms that loss of intraepidermal nerve fibers occurs in patients with idiopathic small-fiber neuropathy and impaired glucose tolerance, but additionally shows that IENF may repair with improved metabolic control.


Novel insights on diagnosis, cause and treatment of diabetic neuropathy: focus on painful diabetic neuropathy

Mitra Tavakoli, Omar Asghar, Uazman Alam, Ioannis N. Petropoulos, Hassan Fadavi and Rayaz A. Malik

Abstract: Diabetic neuropathy is common, under or misdiagnosed, and causes substantial morbidity with increased mortality. Defining and developing sensitive diagnostic tests for diabetic neuropathy is not only key to implementing earlier interventions but also to ensure that the most appropriate endpoints are employed in clinical intervention trials. This is critical as many potentially effective therapies may never progress to the clinic, not due to a lack of therapeutic effect, but because the endpoints were not sufficiently sensitive or robust to identify benefit. Apart from improving glycaemic control, there is no licensed treatment for diabetic neuropathy, however, a number of pathogenetic pathways remain under active study. Painful diabetic neuropathy is a cause of considerable morbidity and whilst many pharmacological and nonpharmacological interventions are currently used, only two are approved by the US Food and Drug Administration. We address the important issue of the ‘placebo effect’ and also consider potential new pharmacological therapies as well as nonpharmacological interventions in the treatment of painful diabetic neuropathy.

Keywords: diabetic neuropathy, diagnosis, painful diabetic neuropathy, nonpharmacological treatment, pharmacological

Introduction
Diabetic neuropathy is extremely distressing and significantly reduces the patients’ quality of life [Zelman et al. 2006; Mojaddidi et al. 2005]. Hyperglycaemia is clearly important in the genesis of nerve damage and recent studies suggest that even minimal perturbations in blood glucose in those with impaired glucose tolerance (IGT) may lead to the development of both small [Green et al. 2010; Tavakoli et al. 2010] and large [Sahin et al. 2009] nerve fibre damage and neuropathic pain [Smith and Singleton, 2008].

Diagnosis of diabetic neuropathy
Several different approaches have been employed to diagnose and evaluate the severity of neuropathic deficits in diabetic neuropathy. The neuropathy disability score and 10 g monofilament have been recommended as screening tools in general practice to detect those at risk of foot ulceration [Abbott et al. 2002]. However, data to suggest that the 10 g monofilament may not be reliable [Booth and Young, 2000] or optimal for identifying those at risk of foot ulcers [Miranda-Palma et al. 2005] have been conveniently ignored. A more important point relates to the inappropriate use of the 10 g monofilament to diagnose ‘neuropathy’ as it will only detect advanced large fibre neuropathy. Hence, a ‘normal test’ may falsely reassure practitioners when in fact the patient may have mild neuropathy or indeed involvement of the small fibres. Furthermore, because effective intervention must be aimed at a stage when there is a capacity for the nerve to repair, i.e. in the subclinical or mild neuropathy, it is important to reliably quantify small fibre damage. Quantitative sensory testing (QST) including a thermal threshold assessment for cold sensation (A-δ fibres) and warm sensation (c fibres) assesses small fibre dysfunction and therefore can detect early neuropathy, but are highly subjective with low reproducibility [Boulton et al. 2004] and hence have shortcomings when employed to define
therapeutic efficacy in clinical intervention trials [Mojaddidi et al. 2005]. Indeed small fibre abnormalities as assessed by intraepidermal nerve fibre (IENF) density and the Quantitative Sudomotor Axon Reflex Test (QSART) and not neurophysiology or QST, improved after lifestyle intervention in patients with IGT neuropathy [Smith et al. 2006]. Diabetic patients with minimal neuropathy (normal electrophysiology and quantitative sensory tests) show significant unmyelinated fibre [Malik et al. 2005] and IENF damage [Loseth et al. 2008; Quattrini et al. 2007; Umapathi et al. 2007]. Direct examination of these fibres can be undertaken in sural nerve [Malik et al. 2005, 2001] or skin-punch [Smith et al. 2005; Sumner et al. 2003] biopsies, however, both are invasive procedures. Recently, we have shown that corneal confocal microscopy (CCM), a novel noninvasive technique can detect small fibre neuropathy in diabetic patients by visualizing the subbasal nerve plexus in Bowman’s layer of the cornea [Quattrini et al. 2007; Hossain et al. 2005]. CCM may also be more sensitive than IENF density (IENFD) in detecting early damage [Quattrini et al. 2007] and repair after pancreas transplantation [Mehra et al. 2007; Boucek et al. 2005]. We have also demonstrated a progressive loss of corneal sensation with increasing severity of neuropathy, providing a functional correlate of corneal nerve fibre loss [Tavakoli et al. 2007]. With regard to painful neuropathy, more severe IENF loss [Sorensen et al. 2006b] and reduction in both IENF and corneal nerve fibre length [Quattrini et al. 2007] has been related to symptoms, suggestive of a pathological basis for painful diabetic neuropathy (PDN). As CCM is noninvasive it may be an ideal technique to assess alterations in small nerve fibre pathology in relation to PDN and progression of neuropathic deficits. In our recent study of patients with idiopathic small fibre neuropathy (ISFN) and IGT we have demonstrated significant corneal nerve damage [Tavakoli et al. 2010]. We have also shown that CCM as opposed to thermal thresholds can be used to demonstrate small nerve fibre damage in patients with Fabry disease, a condition characterized by painful neuropathy [Tavakoli et al. 2009].

A summary of the advantages and limitations of the present techniques to quantify nerve fibre damage in diabetic neuropathy is presented in Table 1.

**Treatment of diabetic neuropathy**

The ideal therapy should prevent or arrest the progressive loss of nerve function and improve symptoms with minimal side effects. However, current treatment options do not address the underlying cause of nerve damage. Furthermore, recent data highlight the main challenge with future clinical trials assessing improvement in diabetic neuropathy to be the lack of significant worsening of neuropathy in the placebo group [Dyck et al. 2007]. At present, apart from improving glycaemic control there is no licensed treatment for diabetic neuropathy. We have not provided a detailed review of all treatments but we have focused on three areas which are still being actively pursued.

**Aldose reductase inhibitors**

The aldose reductase theory remains viable in experimental diabetic neuropathy [Oates, 2008], however, its translation to man has been disappointing [Schemmel et al. 2009; Hamada and Nakamura, 2004]. Some of the aldose reductase inhibitors (ARIs) were withdrawn due to toxicity (Tolrestat, Sorbinil, Zenerastat), others due to a lack of efficacy [Schemmel et al. 2009; Chalk et al. 2007]. However, it also appears that reliance on nerve sorbitol as a means to assess aldose reductase inhibition may well have lead to an underestimation of the doses needed for clinical efficacy and an overestimation of drug safety margins [Oates, 2008]. Furthermore, the choice of clinical endpoints and the magnitude of response required to prove efficacy has been questioned recently [Dyck et al. 2007].

Epalrestat, the only ARI in use, is currently only licensed for use in Japan [Ramirez and Borja, 2008; Hotta et al. 1996]. In a small study of 39 type 2 diabetic patients Epalrestat prevented progression of peripheral neuropathy, but surprisingly this was related to a reduction in the production of advanced glycation endproducts (AGEs) [Kawai et al. 2009], suggesting cross talk between two important pathogenetic pathways of diabetic neuropathy. In a recent study from India, Epalrestat improved motor and sensory nerve conduction velocities (NCVs) and the vibration perception threshold (VPT) [Sharma and Sharma, 2008]. Longer-term benefits have also been demonstrated with Epalrestat in a 3-year trial, reporting improvements in motor NCV, F-wave latency and VPT as well as neuropathic symptoms, particularly in patients with better glycaemic control and less overt microvascular
complications [Hotta et al. 2006]. In 30 diabetic patients with mild to moderate neuropathy, median, tibial and sural NCV and wrist and ankle F-waves improved over 6 months and were associated with increased nodal Na\(^+\) [Misawa et al. 2006]. The only other ARI that remains in clinical trials is Ranirestat (AS-3201), which has been shown to prevent sural nerve accumulation of sorbitol and fructose with an improvement in sural NCV [Bril and Buchanan, 2004]. In a double-blind, placebo-controlled biopsy trial, sensory nerve function improved at 12 weeks, whilst motor NCV and VPT improved at 60 weeks [Bril and Buchanan, 2006]. In one of the largest ARI trials to date, 549 patients randomized to a 52-week, multiple-dose, placebo-controlled, double-blind study demonstrated a significant improvement in summed motor NCV (peroneal, tibial and median) at 12, 24 and 36 weeks and in peroneal NCV at 36 and 52 weeks, but with no improvement in neurological examination, QST or symptoms of neuropathy [Bril et al. 2009].

**Antioxidants**

Oxidative stress and impaired antioxidant defence mechanisms have been implicated as major pathogenic components of diabetic polyneuropathy [Shay et al. 2009]. Intravenous alpha-lipoic acid (ALA), an antioxidant and a free-radical scavenger, has been shown to improve symptomatic diabetic neuropathy [Ziegler et al. 2004; Evans et al. 2002]. The SYmptomatic Diabetic NEuropathyY (SYDNEY) trial [Ametov et al. 2003] demonstrated an improvement in neuropathic symptoms in patients treated with ALA. A very short (4-week) study in 14 type 2 diabetic patients demonstrated that 400 mg daily ALA over 4 weeks improved reactive oxygen metabolites (ROMs) and high density lipoprotein-cholesterol (HDL-C) [Gianturco et al. 2009]. In a small study of nine type 1 diabetic patients, ALA (600 mg/day twice) in combination with benfotiamine (300 mg/day twice) downregulated markers of ROM, reduced hexosamine activity by 40%, prostacyclin synthase activity by 70% and normalized AGE formation [Du et al. 2008]. However, in a double-blind, randomized, placebo-controlled study of an oral controlled-release formulation of ALA in 40 type 1 diabetic adolescents, no significant effects were observed on markers of oxidative damage [Huang and Gitelman, 2008]. Tankova and coworkers investigated the effect of 600 mg/day intravenous ALA for 10 days followed by 60 days of oral ALA in 23 patients and showed improvements in clinical signs of oculomotor, trochlear and abducent nerve mononeuropathies (double vision, ptosis) with an improvement in the Total Symptom Score (TSS) and an effect on peripheral and autonomic neuropathy [Tankova et al. 2005]. The SYDNEY 2 trial assessed the effects of 600, 1200 and 1800 mg oral ALA versus placebo in 181 diabetic patients over 5 weeks and showed an improvement in the TSS, neuropathy symptoms and change score (NSC), neuropathy impairment score (NIS) and patients’ global assessment of efficacy [Ziegler et al. 2006].

**Vascular endothelial growth factor**

Vascular endothelial growth factor (VEGF) is regarded as a potent stimulator of angiogenesis and vasculogenesis in health and disease [Carmeliet, 2003] and acts through binding to the tyrosine kinase receptors VEGFR-1 and VEGFR-2 [Storkebaum et al. 2004]. Numerous studies have demonstrated the central role of

<table>
<thead>
<tr>
<th>Method</th>
<th>Advantage</th>
<th>Disadvantage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical/neurological examination</td>
<td>Simple, easy to use</td>
<td>Not sensitive or reproducible</td>
</tr>
<tr>
<td>Electrophysiology</td>
<td>Sensitive, objective</td>
<td>Assesses only large fibres</td>
</tr>
<tr>
<td>Quantitative sensory tests (QST)</td>
<td>Evaluates both large and small nerve fibres</td>
<td>Requires special equipment</td>
</tr>
<tr>
<td>Sympathetic skin response (SSR)</td>
<td>Simple, fast, objective</td>
<td>Subjective, low reproducibility, low sensitivity, time-consuming</td>
</tr>
<tr>
<td>Quantitative sudomotor axon reflex test (QSART)</td>
<td>Sensitive, objective, reproducible</td>
<td></td>
</tr>
<tr>
<td>Autonomic testing</td>
<td>Objective, quantitative</td>
<td></td>
</tr>
<tr>
<td>Nerve/skin biopsy</td>
<td>Quantitative, sensitive</td>
<td></td>
</tr>
<tr>
<td>Corneal confocal microscopy/Corneal aesthesiometry</td>
<td>Rapid, noninvasive, reiterative and quantitative</td>
<td></td>
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</tbody>
</table>
VEGF in the pathogenesis of diabetic retinopathy (DR) and diabetic macular oedema (DMO). Increased VEGF levels and VEGFR-2 expression have been found in models of experimental diabetes [Gilbert et al. 1998]. Tissue hypoxia is known to trigger an increase in VEGF content [Shweiki et al. 1992] and increased VEGF activity causes angiogenesis in DR [Aiello et al. 1994].

To date, there are two US Food and Drug Administration (FDA)-approved anti-VEGF agents, pegaptanib (Macugen, OSI/Eyetech, Melville, NY, USA) and ranibizumab (Lucentis, Genentech, Inc., South San Francisco, CA, USA), for use in DR but also DMO. Pegaptanib targets the VEGF165 isoform and has been found to inhibit VEGF’s actions on endothelial mitogen activity and vascular permeability [Ishida et al. 2003]. Ranibizumab is a recombinant, humanized, antibody fragment that binds all VEGF isoforms [Nicholson and Schachat, 2010]. Bevacizumab (Avastin, Genentech, Inc.) is currently being evaluated in clinical trials to prove its safety and efficacy for intraocular use [Nicholson and Schachat, 2010]. In 40 type 2 diabetic patients with proliferative retinopathy, a single intravitreal injection of Bevacizumab in addition to standard laser treatment resulted in complete regression of proliferative change in 87.5% compared with only 25% in the sham group at week 6, however, by week 16 recurrence of Proliferative Diabetic Retinopathy (PDR) meant that the complete regression rate was identical in the two groups [Mirshahi et al. 2008]. Similarly in DMO, the benefits of triamcinolone and bevacizumab versus bevacizumab compared with standard macular laser photocoagulation have been shown to be short lived with no correlation between reduction in macular thickness and visual acuity [Faghihi et al. 2008].

In the nervous system, VEGF also appears to have a beneficial effect as it has been shown to be neuroprotective promoting elongation of neurites and proliferation of nonneural cells [Storkebaum et al. 2004]. Experimental studies report upregulation of VEGF in response to ischaemia [Samii et al. 1999] and a key role of VEGF in maintaining neuronal integrity and preventing hypoxic death of Schwann cells [Schartzberger et al. 2000]. Intramuscular administration of an engineered zinc finger protein activator of VEGF-A has been shown to prevent sensory and motor nerve conduction velocity deficits [Price et al. 2006]. More recently it has been shown to correct endogenous VEGF-A protein levels in L4/5 dorsal root ganglia and protect against mechanical allodynia [Pawson et al. 2009]. Topical application of VEGF has also been previously shown to accelerate diabetic wound healing in experimental models of diabetes [Galiano et al. 2004].

The role of VEGF in human diabetic neuropathy has not been explored fully and data remains somewhat confusing. Thus, Quattrini and colleagues demonstrated a significant reduction in VEGF expression in skin biopsies from the dorsum of the foot which was related to the severity of neuropathy in diabetic patients [Quattrini et al. 2008]. However, serum VEGF level have been found to be increased in diabetic patients with symptomatic neuropathy [Deguchi et al. 2009]. Bevilacqua and coworkers assessed 10 diabetic and 10 nondiabetic subjects and showed an increase in plasma VEGF during frequency modulated electromagnetic simulation (FREMS), providing a mechanistic basis for the beneficial effect of FREMS on NCV [Bevilacqua et al. 2007], but also highlighting the potential for variability when assessing serum VEGF levels. In a randomized, double-blinded study intramuscular gene transfer at eight standardized sites adjacent to the sciatic, peroneal, and tibial nerves was undertaken in 39 diabetic patients who received plasmid VEGF (VEGF-1/VEGF-A or VEGF-2/VEGF-C) whilst 11 received placebo over 6 months. An improvement in neuropathic symptoms was observed but without an effect on nerve conduction or quantitative sensory examination [Ropper et al. 2009]. Although an increased incidence of side effects was observed this was not characterized by increased oedema or haemorrhage, as might have been predicted recently with the demonstration of increased capillary leakage following VEGF treatment [de Leeuw et al. 2008]. Thus, the demonstration of a clinical benefit for diabetic neuropathy after VEGF treatment is at best, limited.

**Painful diabetic neuropathy**

**Assessment of severity of neuropathic pain**

The accurate assessment for the presence and severity of painful symptoms in patients with diabetic neuropathy is very important, not just to ensure a correct diagnosis but also to assess the benefits of treatment, especially with the potential for a large placebo effect as discussed elsewhere in this review. Many different questionnaires and scores have been developed or adopted to quantify neuropathic pain. The McGill Pain Questionnaire is the most frequently used questionnaire, but it
was not developed originally for diabetic neuropathic pain. Recently, more specific scores have been developed for diabetic painful neuropathy and include the Brief Pain Inventory short form for peripheral diabetic neuropathy (BPI-PDN) [Sorensen et al. 2006b]. The BPI is a patient-completed numeric rating scale that assesses the severity of pain and its impact on daily functioning on a 7-item pain interference scale. The Neuropathic Pain Questionnaire (NPQ) was developed to provide a general assessment of neuropathic pain and discriminate between neuropathic and nonneuropathic pain [Gore et al. 2007]. An additional diagnostic tool, the pain diagnostic questionnaire (DN4), has been shown to distinguish neuropathic from nociceptive pain [Calkins and Backonja, 2007]. Follow-up assessment of pain in PDN can be undertaken using either the NPQ or the other recently developed tool, the Neuropathic Pain Symptom Inventory (NPSI), which is a self-questionnaire designed to evaluate different symptoms of neuropathic pain [Kelly et al. 2005]. The NPSI includes 10 descriptors that allow for the discrimination and quantification of clinically relevant aspects of neuropathic pain. It has been suggested that this pain questionnaire may be able to characterize subgroups of patients with neuropathic pain, and verify differential responses to pharmacologic or other treatment interventions. Finally, the Neuropathic Pain Scale has been designed specifically to monitor effects of therapy on neuropathic pain [Malik et al. 2005].

**Treatment of painful diabetic neuropathy**

Small fibre damage is an essential prerequisite for the development of PDN. However, additional alterations which include both peripheral and central sensitization make the treatment of this condition difficult. Hence, whilst many approaches have been advocated for the treatment of PDN, achieving >50% relief is rare and side effects limit dose titration. Thus, an improvement in the understanding of the pathogenesis of pain in diabetic neuropathy may lead to new more targeted treatments, with better efficacy and less side effects. Whilst the traditional approach has been to change or substitute treatments, owing to a lack of efficacy or side effects, a growing body of recent data suggests that combining lower doses of agents which act on different pain pathways may achieve better efficacy with fewer side effects [Baron et al. 2009; Zin et al. 2009; Hanna et al. 2008]. This establishes a new paradigm for future clinical trials in PDN [Backonja et al. 2006]. The goal of this review is not to exhaustively detail all of the studies in PDN as several recent excellent reviews and analyses provide this [Noble et al. 2010; Wiffen et al. 2010; Moore et al. 2009; Ziegler, 2008a, 2008b]. Tables 2, 3 and 4 provide a summary of available treatments.

**Placebo effect in double-blind trials of painful diabetic neuropathy**

A recognized but unaddressed issue in trials of PDN is the ‘placebo effect’ and merits consideration [Wymer et al. 2009; Katz et al. 2008; Quessy and Rowbotham, 2008; Turner et al. 1994]. Quessy and Rowbotham have suggested that the true mean pain score reduction in trials of PDN is around 26–27% [Quessy and Rowbotham, 2008]. Several recent studies in PDN have shown a placebo effect which has approached and even surpassed that of the active therapy, obscuring the precise treatment effect of the active treatment (Table 5).

Pregabalin has been studied extensively in parallel cohort designed studies. In a meta-analysis of pregabalin in acute and chronic pain [Moore et al. 2009], a subgroup analysis in PDN trials showed that those achieving at least 50% pain reduction in the placebo arm (PA) was 23%, 26% and 25% and discontinuation due to a lack of efficacy was 7%, 8% and 14% in combined studies of 150 mg (n = 2 studies), 300 mg (n = 4 studies) and 600 mg (n = 6 studies), respectively. Although discontinuation was greater than in the treatment arm (TA) it still suggests that subjects gain a sufficient benefit to continue with the sham treatment. In a study of patients with postherpetic neuralgia and PDN comparing oxycodone against placebo with a dose titration of Pregabalin, a greater response rate (~50% pain reduction) was observed in the PA in three of the four groups with pregabalin dose titration [Zin et al. 2009]. Furthermore, prior to the initiation of Pregabalin, 34.5% of patients in the PA compared with 23.2% in the Oxycodone arm obtained at least 50% pain reduction.

Duloxetine, a dual reuptake inhibitor of serotonin and noradrenaline [Schuessler, 2006], has been placed as a first-line therapy for PDN in the 2010 National Institute for Health and Clinical Excellence (NICE) guidance on pharmacological management of painful neuropathy. However, three key studies have demonstrated the efficacy of Duloxetine in PDN with a mean percentage change from baseline in the PA of 33%, 29% and 24%, respectively [Raskin et al. 2006; Wernicke et al. 2009; Hanna et al. 2009].
The Duloxetine studies have also shown variability in the PA with the percentage demonstrating 50% pain response ranging from 26% to 30%. Lacosamide, a new investigational drug in epilepsy and neuropathic pain, slowly inactivates voltage-gated sodium channels [Errington et al. 2008; Sheets et al. 2008; Bretin et al. 2006]. In a phase 2 double-blind randomized controlled trial the Lacosamide arm achieved an average Likert pain scale score (last observation carried forward [LOCF]) of 3.7 ± 2.6 compared with a baseline of 6.6 ± 1.6 [Rauck et al. 2007]. However, the PA achieved a Likert pain scale

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**Table 2. Pathogenetic treatment options for diabetic neuropathy.**

<table>
<thead>
<tr>
<th>Mechanism of effect</th>
<th>Class of drug</th>
<th>Drug</th>
<th>Dose per day (mg)</th>
<th>Side effects</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glycaemic control</td>
<td>Tricyclic antidepressants [TCAs]</td>
<td>Amitriptyline [Max et al. 1992]</td>
<td>20–150</td>
<td>+++</td>
<td>Sedation and anticholinergic side effects</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Imipramine</td>
<td>25–150</td>
<td>+++</td>
<td>Sedation and anticholinergic side effects</td>
</tr>
<tr>
<td>Glycaemic control</td>
<td>Selective serotonin reuptake inhibitors [SSRIs]</td>
<td>Duloxetine [Raskin et al. 2006]</td>
<td>60–120</td>
<td></td>
<td>Approved by FDA</td>
</tr>
<tr>
<td>Oxidative stress</td>
<td>Anticonvulsants</td>
<td>Gabapentin [Wiffen et al. 2010]</td>
<td>900–3600</td>
<td>+</td>
<td>Somnolence</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lamotrigine [Wiffen et al. 2010]</td>
<td>200–400</td>
<td>+</td>
<td>Nausea, headache</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Carbamazepine [Wiffen et al. 2010]</td>
<td>200–600</td>
<td>++</td>
<td>Agranulocytosis</td>
</tr>
<tr>
<td>Aldose reductase [AR] inhibition Improved blood flow</td>
<td>Epalrestat</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Antiarrhythmics</td>
<td>Pregabalin [Freeman et al. 2008]</td>
<td>300–600</td>
<td></td>
<td>FDA approved, pedal oedema</td>
</tr>
<tr>
<td></td>
<td>Opioids</td>
<td>Tramadol [Freeman et al. 2007]</td>
<td>50–400</td>
<td>++</td>
<td>Sedation, constipation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Oxycodeone [Zin et al. 2009]</td>
<td>40–60</td>
<td>+++</td>
<td>Sedation, constipation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Glyceryl trinitrate (GTN) [Agrawal et al. 2009]</td>
<td></td>
<td></td>
<td>Headache, tolerance</td>
</tr>
</tbody>
</table>

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2006; Goldstein et al. 2005]. The Duloxetine studies have also shown variability in the PA with the percentage demonstrating 50% pain response ranging from 26% to 30%. Lacosamide, a new investigational drug in epilepsy and neuropathic pain, slowly inactivates voltage-gated sodium channels and interacts with a collapsin response mediator protein-2 [Errington et al. 2008; Sheets et al. 2008; Bretin et al. 2006]. In a phase 2 double-blind randomized controlled trial the Lacosamide arm achieved an average Likert pain scale score (last observation carried forward [LOCF]) of 3.7 ± 2.6 compared with a baseline of 6.6 ± 1.6 [Rauck et al. 2007]. However, the PA achieved a Likert pain scale
score (LOCF) of 4.5 ± 2.6 compared with a baseline of 6.5 ± 1.7. Despite this marked placebo effect, Lacosamide was superior to the placebo, but it raised questions regarding the repeatability of such an intervention, given the extensive placebo effect. Thus, in a recent study of three fixed-dose regimens, 68% of participants in the PA reported ‘feeling better’ on the patient global impression change (PGIC) evaluation compared with 69%, 81% and 83% on Lacosamide 200, 400 and 600 mg, respectively [Wymer et al. 2009]. Similarly, on the Likert pain scale the PA demonstrated a least-squares mean change of /C0 1.6 from baseline and the only group which was significantly different from placebo was Lacosamide 400 mg.

In the only published trial of a medicinal cannabis-based product in the treatment of PDN, thirty subjects were randomised to either Sativex (tetrahydrocannabinol and cannabidiol) or placebo [Selvarajah et al. 2009]. The placebo effect was actually greater than with Sativex, with a reduction in all modalities of the pain diary score; indeed the mean reduction in the total pain score in the PA was 37% compared with 20% with active treatment. Such a significant placebo effect is worthy of further investigation, particularly when planning future trials of agents for PDN. The possible predictors of the placebo response have been assessed in three trials of Lamotrigine using pooled data of 252 placebo subjects (222 had PDN) [Irizarry et al. 2009].

A higher baseline pain score and a faster rate of recruitment were both identified as independent predictors of the placebo response. In an analysis of the Sativex trial, Selvarajah and colleagues suggested that depression may potentially be an important confounding factor as subjects with depression have a higher baseline pain score and consequently by entering a trial respond better to both the placebo and active drug [Selvarajah et al. 2009]. With depression being so common in PDN this confounding factor should clearly be accounted for, however, exclusion by assessing depression scores may not suffice [Vileikyte et al. 2009]. Indeed the placebo response is a well-known entity in trials of depression [Dworkin et al. 2005]. Also centres that recruit at a faster rate may introduce bias as they may represent those centres where more of the subjects have intractable PDN and are therefore more eager to participate in novel treatments [Irizarry et al. 2009].

### Table 4. Placebo response of trials of duloxetine, venlafaxine, glyceryl trinitrate/sodium valproate and cannabinoids.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Drug</th>
<th>Number in placebo group</th>
<th>Length of trial (weeks)</th>
<th>Percentage change from baseline of pain scores</th>
<th>Percentage of placebo responders</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goldstein et al. [2005]</td>
<td>Duloxetine</td>
<td>115</td>
<td>12</td>
<td>33%</td>
<td>26% (50% pain improvement)</td>
</tr>
<tr>
<td>Raskin et al. [2006]</td>
<td>Duloxetine</td>
<td>116</td>
<td>12</td>
<td>29%</td>
<td>30% (50% pain improvement)</td>
</tr>
<tr>
<td>Wernicke et al. [2006]</td>
<td>Duloxetine</td>
<td>108</td>
<td>12</td>
<td>24%</td>
<td>27% (50% pain improvement)</td>
</tr>
<tr>
<td>Rowbotham et al. [2004]</td>
<td>Venlafaxine</td>
<td>81</td>
<td>6</td>
<td>27%</td>
<td>34% (50% pain improvement)</td>
</tr>
<tr>
<td>Selvarajah et al. [2009]</td>
<td>Sativex</td>
<td>14</td>
<td>12</td>
<td>37%</td>
<td>64% (30% pain improvement)</td>
</tr>
<tr>
<td>Agrawal et al. [2009]</td>
<td>Sodium valproate + GTN</td>
<td>GTN + placebo = 20</td>
<td>12</td>
<td>36%</td>
<td>n/a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Placebo + Sodium valproate = 20</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Placebo + placebo = 21</td>
<td></td>
<td>6%</td>
<td></td>
</tr>
</tbody>
</table>

### Table 5. Nonpharmacological symptomatic treatment options for diabetic neuropathy.

<table>
<thead>
<tr>
<th>Mechanism of effect</th>
<th>Type of treatment</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Physical therapy</td>
<td>Electrical spinal cord stimulation</td>
<td>Highly invasive</td>
</tr>
<tr>
<td></td>
<td>Transcutaneous electrical nerve stimulation [TENS]</td>
<td>Limited data</td>
</tr>
<tr>
<td></td>
<td>Percutaneous electrical nerve stimulation [PENS]</td>
<td>Limited data</td>
</tr>
<tr>
<td></td>
<td>Magnetic field therapy</td>
<td>Limited data</td>
</tr>
<tr>
<td></td>
<td>Low-intensity laser therapy [LILT]</td>
<td>Limited data</td>
</tr>
<tr>
<td></td>
<td>Monochromatic near-infrared treatment [MIRE]</td>
<td>Limited data</td>
</tr>
<tr>
<td></td>
<td>Dressings</td>
<td>Limited data</td>
</tr>
<tr>
<td></td>
<td>Acupuncture</td>
<td>Limited data</td>
</tr>
<tr>
<td></td>
<td>Yoga</td>
<td>Limited data</td>
</tr>
<tr>
<td>Others</td>
<td>Psychological support</td>
<td>Limited data</td>
</tr>
</tbody>
</table>
The placebo response is also thought to vary along the time course of a trial [Quessy and Rowbotham, 2008], hence the FDA and other regulatory agencies require studies of at least 12 weeks for chronic pain [Rappaport, 2007]. The premise being that a placebo effect is thought to stabilize after an initial period of several weeks [Katz et al. 2008; Dworkin et al. 2005] with longer-term trials producing a plateau. However, an analysis of the placebo response variability in trials of PDN did not show such a plateau and variability may exist beyond 19 weeks [Quessy and Rowbotham, 2008].

A number of approaches have been suggested to separate the drug from the placebo response [Dworkin et al. 2005] and include use of a placebo run-in period (in order to cull placebo responders), flexible dosing and the exclusion of subjects with mild pain [Katz et al. 2008; Quessy and Rowbotham, 2008]. However, the incorporation of these designs into clinical trials of pain has shown little benefit. Indeed McQuay conducted a review of the placebo response and concluded that the greatest determinants of the placebo response are in fact random factors [McQuay, 2008]. Thus, the placebo effect is certainly one that should be considered prior to the initiation of any new study in PDN. Perhaps the current double-blind parallel randomised controlled designs in trials of PDN require modification, to minimise the placebo effect and limit obscuration of the true treatment effect.

**Novel approaches for the treatment of painful diabetic neuropathy**

Given the limitations of current treatments of PDN in relation to limited therapeutic effect, significant side effects and the placebo effect, we briefly review novel mechanisms and agents currently in development which may have therapeutic potential in PDN.

**Transient receptor potential vanilloid receptor 1 (TRPV1).** TRPV1 is a nonselective cation channel abundantly expressed in C-fibres [Wong and Gavva, 2009] and several observations suggest a potentially important role in the modulation of TRPV1 as a therapeutic for pain. Capsaicin can cause desensitization of TRPV1 channels and relieves pain in humans [Knotkova et al. 2008] and pain behaviour in animals [Szallasi and Blumberg, 1999, 1993]. Similarly, antagonists at the TRPV1 receptor relieve pain behaviour in rodent models of inflammation [Joshi et al. 2009], osteoarthritis and cancer. Furthermore, TRPV1 knockout mice display reduced sensitivity to noxious stimuli [Christoph et al. 2008]. Of the TRPV1 agonists, capsaicin is the most widely studied in PDN. Its use has been limited to topical administration, due to its very narrow therapeutic index and undesirable side effects following systemic administration. Topical administration however is not without its drawbacks; unlike its more potent analogue Resiniferatoxin (RTX), Capsaicin evokes an excitatory response prior to desensitization and this has been postulated as one of the reasons for noncompliance during clinical trials. Twenty two patients with chronic severe PDN were randomized to 0.075% topical Capsaicin or vehicle for 8 weeks and reported a significant improvement in pain intensity (16% versus 4.1%) and pain relief (44.6% versus 23.2%) in the Capsaicin group [Tandan et al. 1992]. A follow-up open-label study by the same group noted improvement or complete cure of pain in 50% of patients. A similar study reported a 90% improvement in symptoms in those treated with Capsaicin [Scheffler et al. 1991]. A larger study found topical Capsaicin to be equivalent in efficacy to Amitriptyline, but with a better safety profile [Biesbroeck et al. 1995]. The Capsaicin Study Group carried out a multicentre, double-blind, vehicle-controlled trial of topical capsaicin in 252 patients with painful DPN and reported a significant reduction in pain scores versus vehicle [The Capsaicin Study Group, 1991]. In each of these trials, capsaicin was well tolerated, the most common side effect being that of localized burning which improved over the duration of the trial. A systematic review of Capsaicin trials of six randomised controlled trials in neuropathic pain and the relative benefit of topical Capsaicin compared to placebo produced a number needed to treat (NNT) of 5.7 [Mason et al. 2004]. A higher concentration dermal patch has been tried recently in patients with human immunodeficiency virus (HIV)-related painful neuropathy and showed efficacy, tolerability and safety for at least 12 weeks [Simpson et al. 2008a, 2008b]. However, topical Capsaicin produces a uniform epidermal nerve fibre injury with a marked reduction in IENFD which takes ~50 days to recover [Polydefkis et al. 2004]. Thus, it is not surprising that it works for 12 weeks. However, given that IENFD loss is already considerable in diabetic patients, may be related to the genesis of pain [Sorensen et al. 2006a] and demonstrates slower rates of regeneration [Polydefkis et al. 2004], the authors would not recommend this treatment for patients with PDN.
This is particularly relevant in diabetes as small fibres regulate skin vasomotor responses and sweating, disturbance of which may predispose to ulceration [Demiot et al. 2006; Fromy et al. 2002]. There are several ongoing phase 2 and 3 trials investigating the use of injectable preparations of capsaicin in a variety of pain syndromes and an excellent review of trials using Capsaicin in other pain syndromes can be found elsewhere [Knotkova et al. 2008]. The results of clinical trials with topical RTX in patients with PDN have never been published. Although several different molecules selectively antagonize the TRPV1 receptor and many have been tested in vitro and in rodents, there are very limited data in humans [Chizh et al. 2007]. Some clinical trials were terminated early due to hyperthermia whilst [Gavva et al. 2008] the results of other phase 1 trials are yet to be published [Madej et al. 2009]. Thus, the clinical potential for TRPV1-related treatment remains to be explored in DPN.

Tumour necrosis factor α. Tumour necrosis factor (TNF)-α is one of a group of pro-inflammatory cytokines which mediate hyperalgesia in a diverse range of inflammatory and neuropathic conditions [Uceyler and Sommer, 2008]. TNF-α production is upregulated following nerve injury and linked to hyperalgesia, whilst cytokine inhibitors and anti-inflammatory cytokines have an analgesic effect. TNF-α and Interleukin-2 (IL2) levels are increased in painful neuropathies compared with nonpainful neuropathies [Uceyler et al. 2007]. TNF-α may also be involved in the pathogenesis of experimental diabetic neuropathy [Sato et al. 2003]. Although the precise mechanism is not clear, TNF-α level is higher in the serum of diabetic patients compared with normal individuals and it has been implicated in the development of diabetic microangiopathy and macroangiopathy [Katsuki et al. 1998]. In a recent study of gluteal fat biopsies from obese patients, adiponectin was shown to be a modulator of local vascular tone by increasing nitric oxide bioavailability, but this capacity was lost in obesity by the development of adipocyte hypertrophy, leading to hypoxia, inflammation (increased TNF receptor 1) and oxidative stress [Greenstein et al. 2009]. Hypertriglyceridaemia has recently been associated with the development of diabetic [Vincent et al. 2009; Wiggin et al. 2009] and idiopathic small fibre [Tavakoli et al. 2010; Smith and Singleton, 2008] neuropathy and can of course induce TNF-α production [Liu et al. 2008]. In experimental diabetes a reduction in NCV occurs following the administration of TNF-α [Satoh et al. 2003] which improves following the administration of the antioxidant N-acetylcysteine [Sagara et al. 1996]. The administration of insulin and antioxidant therapy results in a reduction of TNF-α and an improvement in neuropathy [Sharma et al. 2007]. Furthermore, experiments using antibodies to TNF-α and other cytokines have shown a marked reduction in hyperalgesia and mechanical alldynia [Schafer et al. 2001]. TNF-α also induces the upregulation of cyclooxygenase-2 (COX-2) and associated immuno-inflammatory substances including prostaglandin E2 (PGE2), IL6 and calcitonin gene related peptide (CGRP) [Ma and Quirion, 2006] and appears to be involved in the regulation of nerve growth factor (NGF) [Takei and Laskey, 2008].

Several commonly prescribed drugs in diabetes such as angiotensin-converting enzyme (ACE) inhibitors and beta blockers have been shown to have anti-TNF properties in vivo and in vitro [Madej et al. 2009]. And indeed both Lisinopril [Reja et al. 1995] and Trandalopril [Malik et al. 1998], have been shown to ameliorate diabetic peripheral neuropathy. In animal studies, Gliclazide [Qiang et al. 1998a], and Troglitazone [Qiang et al. 1998b] have been shown to inhibit TNF-α and ameliorate neuropathy. A large questionnaire-based retrospective study of 33,000 Japanese patients with diabetes found a reduction in reported symptoms of neuropathy in those treated with Troglitazone [Satoh et al. 2003]. Although Troglitazone has been withdrawn both Pioglitazone and Rosiglitazone are currently prescribed for improving glycaemic control. Anticytokine treatment such as Infliximab, thalidomide and lenalidomide have been successfully used in the treatment of complex regional pain syndromes [Bernateck et al. 2007; Schwartzman et al. 2003]. However, there are case reports of infliximab-induced acute sensory motor neuropathy, which merits caution [Fauire et al. 2010]. Hence, the potential for these therapies in the treatment of PDN, especially if they only need to be administered intermittently, is worthy of exploration.

Protein kinase-C inhibitors. The link between protein kinase C (PKC)-β activation, endoneurial ischaemia and neuropathy is well established and prompted the now-aborted Ruboxistaurin (PKC-β inhibitor) clinical trial programme in diabetic neuropathy. A randomized control trial
of Ruboxistaurin versus placebo showed no overall differences between the groups in terms of the primary endpoint (vibration detection threshold) but there was a significant reduction in symptom scores in the Ruboxistaurin treated group [Vinik et al. 2005]. In a more recent but relatively small study Ruboxistaurin improved skin blood flow and improved the neuropathy symptom score [Casellini et al. 2007]. However, PKC comprises a group of key regulatory enzymes which modulate neuronal function, the synthesis and release of neurotransmitters and the regulation of receptors. Experimental studies have implicated the role of PKC activation in the development of neuropathic pain and PDN [Kamei et al. 2001]. In postchemotherapy painful neuropathy, increased phosphorylation of other PKC isoforms (gamma/epsilon) has been demonstrated in the thalamus and periaqueductal grey which can be inhibited and is associated with pain relief after supraspinal administration of the PKC specific inhibitor calphostin C [Norcini et al. 2009]. In diabetic mice activation of Ca$^{2+}$-dependent PKC in the spinal cord has been shown to contribute to the development of mechanical allodynia [Honda et al. 2007]. Furthermore, a recent study has shown the fascinating interaction between several different pain pathways with PKC as a central mediator, as enkephalin-mediated activation of the presynaptic delta-opioid receptor prevents increased neuronal Na (v) 1.7 in dorsal root ganglion (DRG) via inhibition of PKC [Chattopadhyay et al. 2008]. Hence, the future role of PKC in PDN needs to be explored.

**Sodium channel antagonists.** The important role of voltage-gated sodium channels has been established primarily in experimental studies which have causally linked changes in sodium channel expression and modulation to channel gating properties or current density in nociceptor neurones and different pain states [Dib-Hajj et al. 2009a, 2009b]. The sodium channel isoforms Na (v) 1.3, Na (v) 1.7, Na (v) 1.8, and Na (v) 1.9 are particularly important in the pathophysiology of pain. Thus, gain-of-function mutations in SCN9A, the gene encoding Na (v) 1.7, has been linked to a twofold increase in firing frequency following depolarization of DRG neurones [Estacion et al. 2009] providing a basis for the association with inherited erythromelalgia [Fischer et al. 2009; Han et al. 2009] and paroxysmal extreme pain disorder, while loss-of-function mutations in SCN9A has been linked to complete insensitivity to pain.

Nonspecific sodium channel blockers such as Mexilitene, a class Ib sodium channel antagonist, are used for the treatment of neuropathic pain and cardiac arrhythmias. As a general rule, most treatment guidelines recommend Mexilitene as a third-line agent [Dworkin et al. 2007] owing to its limited efficacy and side effects. A meta-analysis of 19 trials using either lidocaine or Mexilitene found that both of these drugs were superior to placebo and equal to morphine, gabapentin, Amitriptyline, and amantadine for neuropathic pain [Tremont-Lukats et al. 2005]. No major adverse events were reported in the clinical trials and the most common side effects were drowsiness, fatigue, nausea and dizziness. A Cochrane database review also reached the same conclusions [Challapalli et al. 2005]. European guidelines however do not recommend the use of Mexilitene for DPN [Attal et al. 2006]. In order to overcome the problems associated with the use of Mexilitene, a group in Stanford has identified factors which may help to identify those patients who may tolerate chronic Mexilitene therapy [Carroll et al. 2008]. A fascinating study in a girl with inherited erythromelalgia suggests that some patients with this condition may show a favourable response to Mexilitene due to a use-dependent effect on mutant Na (v) 1.7 channels [Choi et al. 2009].

These data suggest that future strategies which may prove to be more efficacious must involve isoform-specific blockers of these channels, facilitated by studies of ion-channel pathophysiology to define specific abnormalities in ionic conductance and allow tailored pharmacologic blockade or modulation [Kuwabara and Misawa, 2008].

**Nonpharmacological treatment of diabetic neuropathy**

Whilst pharmacotherapy is the mainstay of therapy for the relief of PDN [Max et al. 1992]. Alternative nonpharmacological treatments such as acupuncture [Abuaisha et al. 1998], transcutaneous electrical nerve stimulation (TENS) [Kumar and Marshall, 1997], spinal cord stimulation [Tesfaye et al. 1996], percutaneous electrical nerve stimulation (PENS) [Hamza et al. 2000], low-intensity laser therapy (LILT) [Zinman et al. 2004] and monochromatic infrared light [Leonard et al. 2004] are used in patients who are unresponsive or cannot tolerate...
pharmacotherapy, however the evidence for these approaches is limited and needs to be carefully reviewed.

**Acupuncture.** Acupuncture, developed in Chinese medicine in the fifth century BC was first brought into Europe in the 17th century [Hsu, 1996]. Its major attraction is that it is relatively inexpensive, painless and free from side effects. Its efficacy in diabetic painful neuropathy is supported by a small number of clinical trials which has facilitated its acceptance in pain clinics in most countries [Andersson and Lundeberg, 1995]. The mechanism of action of acupuncture remains unclear [Eshkevari and Heath, 2005; Eshkevari, 2003], however it has been proposed that acupuncture stimulates A-δ and c afferent fibres in muscles which activates the spinal cord, midbrain and hypothalamus leading to the release of endorphins in the peripheral circulation and CSF and inducing analgesia via enkephalins which block neuropathic pain [Eshkevari, 2003; Abuaiasha et al. 1998]. A small study in diabetic patients with painful neuropathy demonstrated a significant improvement in pain relief and the ability to sleep at night [Abuaisha et al. 1998]. Another study claimed multiple benefits using wrist ankle acupuncture, but also claimed to improve blood sugars and lipids, lower blood viscosity and restore the ‘function of peripheral nerve cells’ [Jiang et al. 2006]. Electroacupuncture (EA) has been shown to be efficacious via the release of a number of neuropeptides depending on the frequency of stimulation, with 2 Hz releasing enkephalin, b-endorphin and endomorphin, whilst 100 Hz releases dynorphin [Han, 2004]. EA has also been shown to decrease substance P and increase beta endorphin levels [Lee et al. 2009]. Beyond simple symptom relief the potential for this therapy has also been explored in a small study of diabetic patients with diabetic gastroparesis and demonstrated an improvement in gastric emptying time as well as the Gastroparesis Cardinal Symptom Index (GCSI) [Wang et al. 2008].

**Electrical spinal cord stimulation.** Spinal cord stimulation has been used over the last 40 years [Shrivastav and Musley, 2009] and the original idea was pioneered in the late 1960s by Dr Norman Dhealy, a neurosurgeon, who implanted the first dorsal column stimulator in a patient suffering from terminal metastatic cancer. Currently, it is widely used for the management of different types of chronic neuropathic or intractable pain [Shrivastav and Musley, 2009].

A simplistic explanation for the mode of action of electrical spinal cord stimulation (ESCS) is that of the production of an electrical field on the dorsal horns of the spinal cord. However, recent data generated in an experimental mononeuropathic model using tactile thresholds has demonstrated that ESCS may activate the descending serotonergic pathways, thus inhibiting spinal nociceptive processing [Song et al. 2009]. Two small studies have shown ESCS to be effective in 6/10 patients with chronic intractable PDN, to achieve relief within 3 months of implantation followed by continued relief for a mean of 3.3 years in the six patients who achieved an initial response. The expense and invasive nature precludes recommendation as a routine option for treatment [Chong and Hester, 2007; Daousi et al. 2005], however a cost-effectiveness analysis of ESCS has shown that whilst the cost was greater than for conventional pain therapy in the first 2.5 years, it became less after this period especially as 15% of patients were able to return to work [Kumar et al. 2002].

**Transcutaneous electrical nerve stimulation.** In a study of diabetic patients with mild to moderate neuropathic pain, TENS showed an improvement in pain, numbness and allodynia [Forst et al. 2004]. Another study showed cyclic doses of electrical stimulation through a contact stocking electrode may be effective in alleviating neuropathic pain. However, pain relief was maintained after discontinuation of therapy [Armstrong et al. 1997; Kumar and Marshall, 1997], questioning the direct efficacy of this treatment and invoking the ‘placebo effect’. In a recent analysis from the American Academy of Neurology the effectiveness of TENS was assessed from two Class II studies and given a Level B recommendation for the treatment of PDN [Dubinsky and Miyasaki, 2010].

**Percutaneous electrical nerve stimulation.** Three weeks of treatment with PENS showed a significant improvement in neuropathic pain for more than 6 months, questioning the direct benefit of this treatment and again raising the intriguing role of the ‘placebo effect’. In addition to decreasing extremity pain, PENS therapy improved physical activity, sense of well-being and quality of sleep while reducing the requirement of oral nonopioid analgesic medication [Hamza et al. 2000].
**Magnetic field therapy.** Magnetic field therapy has been employed in a range of medical problems including arthritis, chronic pain, wound healing, insomnia and headaches [Colbert et al. 2009]. A placebo-controlled trial has shown that transcranial magnetic stimulation is effective for the treatment of postherpetic neuralgia and central poststroke pain [Khedr et al. 2005]. Static magnetic field therapy delivered by wearing a constant multipolar sole in the shoe significantly reduced neuropathic pain in a multicentre parallel group study [Weintraub et al. 2003]. Repetitive transcranial magnetic stimulation at the prefrontal [Borckardt et al. 2007], motor [Andre-Obadia et al. 2008] and somatosensory cortex [Topper et al. 2003] has been shown to provide relief for disabling and refractory neuropathic pain. However, pulsed low-frequency electromagnetic fields delivered in a repetitive and cumulative manner failed to show a benefit in neuropathic pain [Weintraub et al. 2009]. Frequency-modulated electromagnetic neural stimulation (FREMS), however, has been shown to increase microvascular blood flow and provide pain relief, but needs to be confirmed in larger study [Conti et al. 2009].

**Low-intensity laser therapy.** LILT has been shown to be beneficial in several pain models including patients with neuropathic pain [Chow et al. 2009]. The exact mechanism of pain relief is not established but increased release of serotonin and endorphin as well as anti-inflammatory effects have been suggested. There is only one clinical trial in which the administration of biweekly therapy over 4 weeks in 50 diabetic patients showed a reduction in weekly mean pain scores [Zinman et al. 2004].

**Monochromatic near-infrared treatment.** Several studies have shown that temporary application of monochromatic near infrared photo energy (MIRE; Anodyne Therapy System) increases foot sensitivity and reduces neuropathic pain [Harkless et al. 2006; DeLellis et al. 2005; Leonard et al. 2004; Prendergast et al. 2004] and this has been attributed to the release of nitric oxide [Goldberg, 2005].

**Nerve decompression.** Decompression of nerves in the lower extremity has been claimed to improve sensation and provide pain relief and has therefore been proposed for use in patients with neuropathy who have failed conventional medical treatment [Valdivia et al. 2005]. However, a systematic review showed that only class IV studies supported the utility of this therapy, hence the Therapeutics and Technology Assessment Subcommittee of the American Academy of Neurology has concluded that this treatment is unproven (Level U) until prospective randomized controlled trials with standard definitions and outcome measures of neuropathy are undertaken [Chaudhry et al. 2006].

**Dressings.** Whilst a number of dressings including OpSite, Fixomull and Lycra® have been used to treat patients with PDN [Troy, 2002], the evidence for their efficacy is limited. In a small study of 33 patients with PDN OpSite was applied to one and then the other painful leg for 4 weeks each. Pain assessed by visual analogue scale was significantly reduced in the OpSite-treated limbs which was associated and a significant improvement in contact discomfort, sleep, mood, appetite and mobility with a reduction in paracetamol intake [Foster et al. 1994]. Given the limited evidence base and the fact that OpSite film can result in an increased risk of fungal and bacterial infection [Strickland, 1997], this treatment is not recommended.

**Exercise.** Specific exercise such as tai chi chuan has been shown to improve fasting blood glucose and peripheral NVCs in patients with type 2 diabetes [Hung et al. 2009; Orr et al. 2006]. Patients with mild to moderate neuropathic pain have demonstrated an improvement in symptoms after 30–40 minutes of yoga for 40 days [Malhotra et al. 2002]. In a study of 149 patients with type 2 diabetes, 40 days of yoga therapy improved blood glucose in 104 patients, particularly those with a short duration of diabetes and good glycaemic control [Nayak and Shankar, 2004; Jain et al. 1993].

**Psychological therapy.** Depression among diabetic patients is reported to be almost double compared with nondiabetic patients [Yoshida et al. 2009; Egede et al. 2002; Anderson et al. 2001]. In a recent longitudinal study, neuropathy itself has been shown to be a risk factor for depressive symptoms by generating pain and unsteadiness, with the latter being particularly related to a perception of diminished self-worth due to an inability to perform normal social roles [Vileikyte et al. 2009]. Thus, psychological treatment may be another nonpharmacological treatment for this group of patients and has been shown to have a positive effect on the quality of
life and emotional well-being in diabetic patients [Yalcin et al. 2008].

**Conflict of interest statement**
The authors have no conflicts of interest to disclose.

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Symptoms of painful diabetic neuropathy (PDN) occur in 30–40% of patients with diabetic neuropathy. It is most commonly associated with distal symmetrical neuropathy affecting the lower limbs (especially toes and feet), and patients present with burning, stabbing and tingling sensations. PDN is extremely distressing and significantly reduces the quality of life of patients. Hyperglycaemia is clearly important in the genesis of nerve damage, and recent studies suggest that even minimal perturbations in blood glucose in those with impaired glucose tolerance (IGT) may lead to the development of small nerve fibre damage and neuropathic pain.

The causes and consequences of diabetic neuropathy are complex and not well understood. Several hypotheses have been advocated in an attempt to explain the pathophysiology of diabetic neuropathy and include a combination of increased oxidative stress, advanced glycation, polyol accumulation, decreased nitric oxide and impaired sodium–potassium(+/−)–adenosine triphosphate (ATPase). Paradoxically, a lack of treatment for underlying nerve damage has improved our understanding of the natural history of PDN because, although nerve damage may initiate PDN, it is clear that as nerve damage progresses pain may diminish.

Diagnosis of Painful Diabetic Neuropathy

Standard measures of neuropathy such as nerve conduction studies and vibration perception thresholds (VPTs) can be used to detect abnormalities of nerve function, but they focus on large nerve fibres. However, pain is generated and mediated by small c and a δ fibres. Thus, it is no surprise that VPT does not differ between diabetic patients with painful and painless neuropathy. Quantitative sensory testing (QST) including thermal threshold assessment for cold sensation (aδ fibres) and warm sensation (c fibres) can assess small fibre dysfunction, but is highly subjective and lacks precision and accuracy, which makes reproducibility difficult. Gore et al. showed that patients with pain were more likely to have an abnormal cold threshold compared with those without pain; however, the former group also had more severe neuropathy, which was evidenced by a higher VPT and absent reflexes. In general, QST and nerve conduction studies (NCS) cannot distinguish between painful and painless diabetic neuropathy. One of the limitations of QST is the lack of normative data; however, normative data have recently been published for QST and may allow detection and quantification not only of negative symptoms but also of positive sensory symptoms, such as allodynia, both of which are present in patients with PDN.

The only techniques that allow direct examination of thinly myelinated and unmyelinated nerve fibre damage and repair are sural nerve biopsy with electron microscopy and skin-punch biopsy, but both are invasive. Intra-epidermal nerve fibre density can be used to evaluate small fibre involvement in diabetic neuropathy. However, there is no clear consensus on the role of intra-epidermal nerve fibre (IENF) loss in patients with painful and painless neuropathy. However, some recent studies do suggest that more refined morphometric evaluation of epidermal nerve fibre morphology, such as axonal swellings and alterations in branching, may be associated with neuropathic pain. A recent study also showed a marked impairment in the cutaneous response to iontophoresed acetylcholine (ACh) in patients with painful neuropathy compared with painless diabetic neuropathy, suggesting that alterations in tissue blood flow may modulate signals generating pain in the periphery. Recently, we have shown that the novel non-invasive technique of corneal confocal microscopy can detect small fibre neuropathy in diabetic patients by visualising small nerve fibres in Bowman’s layer of cornea. Furthermore, it may be more sensitive than IENF density in detecting early damage and repair after pancreas transplantation. Thus, this may be an ideal technique to accurately quantify small nerve fibre morphology, especially in a reiterative manner following patients with exacerbations and remissions from PDN. We have recently shown that corneal confocal microscopy (CCM) can be used to demonstrate more advanced small nerve fibre damage in patients with Fabry disease, a hereditary condition with pure small fibre neuropathy (unpublished data).
Assessment of the Severity of Neuropathic Pain
Assessing the severity of painful symptoms of patients is important, not only for diagnosis but also to assess the benefits of treatments. Many different questionnaires and scores have been developed or adopted to quantify neuropathic pain. The McGill Pain Questionnaire is the most frequently used questionnaire, but it was not originally developed for PDN. Recently, more specific scores have been developed for diabetic painful neuropathy and include the brief pain inventory short form for DPN (BPI-DPN). The BPI is a patient-completed numerical rating scale that assesses the severity of pain and its impact on daily functioning on a seven-item pain interference scale. The Neuropathic Pain Questionnaire (NPQ) was developed to provide a general assessment of neuropathic pain and discriminate between neuropathic and non-neuropathic pain. The pain diagnostic questionnaire (DN4) is another diagnostic tool that has been shown to compare pain syndromes associated with nervous or somatic lesions.

Follow-up assessment of pain in PDN can be undertaken using either the NPQ or the other recently developed tool, the Neuropathic Pain Symptom Inventory (NPSI), which is a self-questionnaire designed to evaluate different symptoms of neuropathic pain. The NPSI includes 10 descriptors that allow for the discrimination and quantification of clinically relevant aspects of neuropathic pain. It has been suggested that this pain questionnaire may be able to characterise subgroups of neuropathic pain patients and verify differential responses to pharmacological or other treatment interventions. Finally, the Neuropathic Pain Scale (NPS) has been specifically designed to monitor effects of therapy on neuropathic pain.

Treatment of Painful Diabetic Neuropathy
The ideal therapy should prevent or arrest the progressive loss of nerve function and improve symptoms with minimal side effects. However, once pain develops, current treatment options do not address the underlying cause of nerve damage and at best achieve partial alleviation of symptoms, due to significant adverse effects. The treatment of PDN can be focused on three different strategies: treatment based on pathogenetic mechanisms, symptomatic treatments and/or physical and non-pharmacological treatments. Table 1 provides a brief summary of available treatments.

Treatments Based on Pathogenetic Mechanisms
Intensive glycaemic control is the first priority for both prevention and management of PDN. Recent studies suggest that even minor perturbations in blood glucose in those with IGT may lead to the development of small nerve fibre damage and neuropathic pain. 

Pancreas Transplantation
The replacement of functioning islet β-cells by pancreas transplantation has been considered to be the most logical treatment for patients with diabetes to normalise blood glucose and ameliorate long-term complications. Although pancreas transplantation takes approximately five years to prevent progression and 10 years to reverse the lesions of diabetic nephropathy, a recent study has demonstrated an improvement and/or stabilisation of diabetic retinopathy after a median follow-up of only 17 months. For diabetic nephropathy, the largest and longest follow-up series to date has shown that pancreas transplantation improved sudomotor function in the hand and foot

Table 1: Treatment Options for Painful Diabetic Neuropathy

<table>
<thead>
<tr>
<th>Mechanism of Effect</th>
<th>Treatment Method</th>
<th>Drug</th>
<th>Dose per Day (mg)</th>
<th>Side Effects</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Optimal glycaemic control</td>
<td>Diet, insulin, exercise</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Contribution of patients and physician is required</td>
</tr>
<tr>
<td>Pathogenetic treatments</td>
<td>Pancreas transplantation</td>
<td>–</td>
<td>–</td>
<td>Immunosuppression</td>
<td>Limited to small centres for selected patients</td>
</tr>
<tr>
<td></td>
<td>Alpha-lipoic acid</td>
<td>600 intravenous</td>
<td>–</td>
<td>–</td>
<td>No data on long-term efficacy</td>
</tr>
<tr>
<td></td>
<td>Alpha-lipoic acid</td>
<td>1,200–1,800 orally</td>
<td>–</td>
<td>–</td>
<td>Only licensed in Germany</td>
</tr>
<tr>
<td></td>
<td>Aldose reductase inhibitors</td>
<td>Epalrestat</td>
<td>–</td>
<td>–</td>
<td>Only licensed in Japan</td>
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<tr>
<td></td>
<td>ACE inhibitors</td>
<td>Trandalopril</td>
<td>–</td>
<td>–</td>
<td>More studies needed</td>
</tr>
<tr>
<td>Pharmacological symptomatic treatment</td>
<td>TCAs</td>
<td>Amitriptyline</td>
<td>20–150</td>
<td>+++</td>
<td>Sedation and anticholinergic side effects</td>
</tr>
<tr>
<td></td>
<td>Imipramine</td>
<td>25–150</td>
<td>+++</td>
<td>–</td>
<td>Sedation and anticholinergic side effects</td>
</tr>
<tr>
<td></td>
<td>SSRIs</td>
<td>Duloxetine</td>
<td>60–120</td>
<td>–</td>
<td>Approved by the FDA</td>
</tr>
<tr>
<td></td>
<td>Anticonvulsants</td>
<td>Gabapentin</td>
<td>900–3,600</td>
<td>+</td>
<td>Fewer side effects than TCA</td>
</tr>
<tr>
<td></td>
<td>Lamotrigine</td>
<td>200–400</td>
<td>+</td>
<td>–</td>
<td>Titration is required</td>
</tr>
<tr>
<td></td>
<td>Carbamazepine</td>
<td>200–600</td>
<td>++</td>
<td>–</td>
<td>Agranulocytosis</td>
</tr>
<tr>
<td></td>
<td>Pregabalin</td>
<td>300–600</td>
<td>–</td>
<td>++</td>
<td>FDA-approved, pedal oedema</td>
</tr>
<tr>
<td></td>
<td>Antiarrhythmics</td>
<td>Mexiletine</td>
<td>Up to 900</td>
<td>+++</td>
<td>Potential cardiac side effects</td>
</tr>
<tr>
<td></td>
<td>Opioids</td>
<td>Tramadol</td>
<td>50–400</td>
<td>++</td>
<td>Sedation</td>
</tr>
<tr>
<td></td>
<td>Oxycodeone</td>
<td>40–60</td>
<td>+++</td>
<td>–</td>
<td>Limited-long-term use</td>
</tr>
<tr>
<td></td>
<td>Topical agents</td>
<td>Capsaicin cream</td>
<td>Topical</td>
<td>–</td>
<td>Denervation</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Topical nitrates</td>
<td>Topical spray</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Physical therapy</td>
<td>Electrical spinal-cord stimulation</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Highly invasive</td>
</tr>
<tr>
<td></td>
<td>Acupuncture</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Further research needed</td>
</tr>
<tr>
<td></td>
<td>Yoga</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Further research needed</td>
</tr>
<tr>
<td>Others</td>
<td>Psychological support</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Further research needed</td>
</tr>
</tbody>
</table>

ACE = angiotensin-converting enzyme; FDA = US Food and Drug Administration; SSRIs = selective serotonin re-uptake inhibitors; TCAs = tricyclic antidepressants; + = severity of side effect.
within one year – which was maintained throughout follow-up for 10 years – but had no impact on nerve conduction velocity.\textsuperscript{27,28} Autonomic function did not improve,\textsuperscript{24} and this has been confirmed by another study.\textsuperscript{29} Although in a recent study we have shown that corneal nerves regenerate six months after transplantation,\textsuperscript{18} there is a case report of painful neuropathy worsening after successful transplantation.\textsuperscript{30} However, this treatment option is limited due to a shortage of donated organs, the complications of the procedure and the risks of long-term immunosuppressive therapy. An alternative, less invasive approach is islet cell transplantation, which has recently been shown to improve nerve conduction velocity scores and skin n-carboxymethyl-lysine (CML) and receptor for advanced glycation end-product (RAGE) expression, but without evidence of intra-epidermal or sweat gland reinnervation four years after the procedure.\textsuperscript{31}

### The use of opioids for neuropathic pain remains controversial, as studies have generally been small, yielded equivocal results and have not established the long-term risk–benefit ratio.

#### Alpha-lipoic acid

Alpha-lipoic acid (ALA) (thioctic acid) is a powerful antioxidant, and several studies – including the SYDNEY2 trial – have demonstrated an improvement in neuropathic symptoms and deficits.\textsuperscript{32} Results of a meta-analysis provided evidence that treatment with ALA 600mg/day intravenously over three weeks is safe and significantly improves both positive neuropathic symptoms and neuropathic deficits to a clinically meaningful degree in diabetic patients with symptomatic polyneuropathy.\textsuperscript{33} The only disadvantage of ALA is that it must be given intravenously to achieve maximum benefit.\textsuperscript{34} Of relevance, a recent analysis of two large placebo-controlled clinical intervention trials that included ALA over four years (Nathan 1, Viatris) and a recent analysis of two large placebo-controlled clinical intervention trials that included ALA over four years (Nathan 1, Viatris) and a protein kinase C-β inhibitor over one year, which had failed to establish efficacy for each compound, showed that most of the endpoints, which included electrophysiology and QSTs, failed to show monotonic worsening in the placebo arm.\textsuperscript{35}

#### Aldose Reductase Inhibitors

Aldose reductase inhibitors block the enzyme aldose reductase, which has a role in the metabolism of blood glucose via the polyol pathway and may reduce the risk of diabetic neuropathy. Epalrestat is the only aldose reductase inhibitor that has been licensed in Japan.\textsuperscript{36} A recent three-year study showed that epalrestat was effective in slowing down the development of neuropathy as measured by changes in nerve conduction compared with controls; however, there was no significant difference in pain between the treated and untreated groups.\textsuperscript{37}

#### Angiotensin-converting Enzyme Inhibitors

Angiotensin-converting enzyme (ACE) inhibitors have some protective effect against microvascular complications in diabetes, including neuropathy.\textsuperscript{27} In a placebo-controlled study, the ACE inhibitor trandolapril showed an improvement in electrophysiology over 12 months in normotensive patients with mild diabetic neuropathy, but had no impact on QST or painful symptoms.\textsuperscript{38}

### Symptomatic Treatments

#### Pharmacological

The standard treatment strategy is often first-line tricyclic antidepressants, second-line anticonvulsants and third-line opioid-related treatment. However, only two agents are US Food and Drug Administration (FDA)-approved: duloxetine\textsuperscript{39} and pregabalin.\textsuperscript{40}

#### Antidepressants

Tricyclic antidepressants (TCAs) have traditionally been first-line therapy for PDN; however, anticholinergic side effects and sedation limit their use, especially in elderly patients. Selective serotonin re-uptake inhibitors (SSRIs) have fewer side effects than TCAs. Most studies with TCAs have enrolled a small number of patients, with still fewer completing the treatment regimens. The most extensively prescribed antidepressant for PDN is amitriptyline, but fewer than 150 patients with PDN have been studied in controlled trials.\textsuperscript{41,42} The major problem remains the frequency of predictable side effects, which include drowsiness and lethargy, and the anticholinergic side effects, particularly dry mouth and postural hypotension. There were also significant problems in defining patients with PDN and the outcome measures employed in each of these trials, thus comparisons cannot be made between studies to determine which drug may have been most beneficial. Duloxetine is the only drug from this class of medications that has received FDA approval for PDN treatment.\textsuperscript{39} Furthermore, a recent UK-based economic model suggests that second-line use of duloxetine is a beneficial and cost-effective treatment strategy for diabetic peripheral neuropathic pain.\textsuperscript{14}

#### Anticonvulsants

Anticonvulsants have been used in the management of neuropathic pain for many years; however, a recent analysis has found limited evidence for efficacy with this class of drugs in PDN.\textsuperscript{43} Gabapentin is most commonly prescribed, although pregabalin, a higher potency and more effective analogue of gabapentin, is the only other agent apart from duloxetine to have received FDA approval for the treatment of PDN.

#### Antiarrhythmics

Mexiletine is a class 1B agent that is a structural analogue of lignocaine, but, unlike lidocaine, can be given orally. The results of the Mexiletine Study Group showed that mexiletine at a dosage of 675mg daily reduced PDN, and the effect of this drug appears to have a rapid onset.\textsuperscript{44} At low doses the risk of electrocardiographic (ECG) side effects is low; however, regular ECG monitoring is necessary and long-term use cannot be recommended.\textsuperscript{12}

#### Opioid-related Treatment

The use of opioids for neuropathic pain remains controversial, as studies have generally been small, yielded equivocal results and have not established the long-term risk–benefit ratio.\textsuperscript{45} Short-term studies provide only equivocal evidence regarding the efficacy of opioids in reducing the intensity of neuropathic pain, whereas intermediate-term studies demonstrate significant efficacy of opioids over placebo, which is likely to be clinically important. Reported adverse events of opioids, such as sedation and constipation, are common but not life-threatening. Further randomised controlled trials are needed to establish long-term efficacy, safety (including addiction potential) and effects on quality of life.\textsuperscript{45}
Topical Agents

Capsaicin
This is an alkaloid found in chilli peppers and has been shown to be effective in PDN. However, the major clinical concern with this medication is that topical capsaicin has been shown to produce complete or nearly complete denervation of the epimides in both control subjects and diabetic patients, with a significant reduction in regeneration in the latter.46

Topical Nitrates
A double-blind, placebo-controlled, cross-over study with isosorbide dinitrate spray (a nitric oxide donor with local vasoatingilizing properties) showed a significant reduction in overall pain and burning discomfort over four weeks compared with controls, although the lack of a placebo effect was not consistent with most other studies in PDN.47

Physical and Non-pharmacological Treatments
For patients with more refractory PDN or who suffer from significant side effects of pharmacotherapy, there are some non-pharmacological options. Use of acupuncture is supported by some studies,48,49 however, a placebo-controlled study has not been performed. Frequency-modulated electromagnetic neural stimulation50 has been reported to provide long-term relief for some patients with painful symptoms.

Conclusion
PDN is a common, difficult to manage, distressing and disabling complication of diabetes. Apart from improving glycaemic control, we have no licensed treatment for diabetic neuropathy. Although we have many approaches to the treatment of PDN, achieving >50% relief is rare and side effects limit dose titration. Currently, there are only two FDA-approved medications for PDN. Thus, prevention, early diagnosis and improvement in the understanding of the pathogenesis of pain in diabetic neuropathy may lead to new, more targeted treatments with greater efficacy and fewer side effects.
Short Report

An assessment of the accuracy and usability of a novel optical wound measurement system

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Abstract

Aims Measurement of wound size can predict healing and provide information to guide treatment. This study assesses a novel optical wound imaging system that creates a three-dimensional image of the ulcer.

Methods Using a new camera-based digital system and traditional elliptical wound measurements, 36 foot ulcers from 31 patients (aged 44–94 years, median 70 years) were examined during a 12-week period at two centres. Median diabetes duration was 18 years (range 6–56 years). Seventeen percent had Type 1 diabetes, 93% had peripheral neuropathy and 57% had peripheral artery disease. Twenty-five were reviewed consecutively, resulting in 76 ulcer examinations. Median ulcer size was 94 mm², with size ranging from 3.1 to 2195 mm².

Results Pearson, Spearman and Kendall rank coefficients showed a strong correlation (in all cases \( P < 0.001 \)) between digital measurements of wounds against traditional hand-measured estimates. Intra-observer variation of wound length using digital elliptical measurement (DEM) gave a coefficient of variation of \( \text{coefficient} < 3.0\% \). Interobserver variation of wound length using DEM was \( < 6.5\% \). Variation from a standard known-size wound area was \( < 8.0\% \) across 30 trials.

Conclusions This study shows a strong correlation between digital and traditional measurement techniques. The system can be easily deployed in routine clinical practice, providing an objective visual record, allowing remote in-depth analysis.

Keywords diabetic foot, imaging, measurement, ulcer care

Abbreviations 3D, three-dimensional; DAM, digital area measurement; DEM, digital elliptical measurement; EM, elliptical measurement

Introduction

Diabetic foot ulceration is a serious complication associated with significant morbidity and mortality [1]. Up to 80% of diabetes-related amputations are preceded by foot ulceration, and the economic burden of treatment expenses is high, with annual costs in the UK estimated at £157 million [2]. Wound measurement has a central role in the successful management of foot ulcers [3]. Regular assessment of wound size predicts healing and provides information used to guide treatment decisions [3,4]. It facilitates communication between healthcare professionals and can aid patient education, leading to improved compliance with treatment [5]. Effective use of such information is dependent on acquisition of accurate and reproducible data [6]. It is also vital for research into agents and treatments for wound healing that quantification of wound size and depth is undertaken, and that such quantification should be free of observer bias.

Current methods used for wound measurement in clinical practice are centred around simple ruler techniques or wound tracing. These approaches are limited by subjective interpretation and significant interobserver variability [7]. Ruler-based methods may not produce a reliable measure of wound surface area, although this is improved by elliptical measurement techniques involving calculation of surface area using the formula for an ellipse [8]. The elliptical wound measurement method is based on the assumption that wound shape can be modelled as an ellipse. The (visually) longest axis of the wound

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The simplicity and speed of the elliptical method have led to its relative popularity amongst clinicians, and this was the primary driver of its use in our study.

The assumption of modelling wound shape as an ellipse has been challenged for wounds of > 40 cm² [9]. More complex methods of wound measurement, such as planimetry and stereophotogrammetry, often require technical expertise beyond that available in the routine clinical environment and are mainly limited to use in specialist centres [6,8]. There is currently a need for more accurate methods of wound measurement that can be used practically by all healthcare professionals in a variety of settings [5,8].

Patients and methods

This study was carried out with all necessary local ethical approvals. After a full description of the nature of the study was given, participants gave written consent granting full use of the images for research purposes. We aimed to determine the accuracy and performance of a novel wound imaging system that uses optical methods to create a three-dimensional (3D) image of a diabetic foot ulcer. The new system uses standard techniques and equipment akin to routine wound photography and are increasingly difficult to satisfy. In all cases

Absolute system accuracy was estimated by imaging a fabricated wound of known size from five viewpoints and performing six independent sessions of digital area measurement (DAM). DAM consisted of marking the boundary in the 3D model allowing an area (mm²) to be calculated. Intra-observer repeatability of DAM was estimated by repeating digital measurement five times on six representative wounds and observing variation. Interobserver variation of DAM was estimated by having five clinicians repeat measurements three times on three different representative wound images.

Results

Pearson, Spearman and Kendall correlations between length, width and elliptical area measurement of DEM under clinician control against traditional hand-measured EM are presented in Table 1. These imply increasingly less strict assumptions on the (unknown) underlying statistical distribution of wound size and are increasingly difficult to satisfy. In all cases P < 0.001 (N = 76).

Figure 1 gives Bland–Altman plots for the difference between EM- and DEM-measured elliptical area, width and length. The primary observation is that the discrepancy in wound area estimation increases with overall wound size. This is unsurprising given the equation for elliptical wound area includes a squared term. It may also indicate that on large circumferential wounds it may not be possible to image the whole wound from a single viewpoint.

Intra-observer variation of wound length and with using DEM gave a coefficient of variation of < 3.0%. Variation from the standard known-size wound area was < 8.0% across 30 trials. Interobserver variation of wound length was < 6.5% and of wound width < 10.0%.

Discussion

This study has shown a strong correlation between DEM and traditional EM techniques. With no known ‘gold standard’ approach to the accurate measurement of wounds, direct

<p>| Table 1 Correlation of digital elliptical measurement with elliptical measurement |
|---------------------------|---------------------|---------------------|---------------------|---------------------|---------------------|</p>
<table>
<thead>
<tr>
<th>Measurement</th>
<th>Pearson</th>
<th>Spearman</th>
<th>Kendall</th>
<th>Min. error</th>
<th>Max. error</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length</td>
<td>0.938</td>
<td>0.922</td>
<td>0.801</td>
<td>0.01 mm</td>
<td>14.95 mm</td>
</tr>
<tr>
<td>Width</td>
<td>0.948</td>
<td>0.906</td>
<td>0.735</td>
<td>0.02 mm</td>
<td>14.96 mm</td>
</tr>
<tr>
<td>Elliptical area</td>
<td>0.961</td>
<td>0.929</td>
<td>0.791</td>
<td>0.51 mm²</td>
<td>886.7 mm²</td>
</tr>
</tbody>
</table>

Area (mm²) = Length (mm) × Width (mm) × 0.25 × π

Digital measurements were obtained by clinicians at the two centres using a camera and a small disposable optical target placed next to the wound. Image processing was performed in an automated manner by the developers of the system using a PC software package. The software produced calibrated-colour 3D models of the ulcers and surrounding region. Off-line DEM was then performed on the 3D models by clinicians using interactive tools.

Area (mm²) = Length (mm) × Width (mm) × 0.25 × π

The primary observation is that the discrepancy in wound area estimation increases with overall wound size. This is unsurprising given the equation for elliptical wound area includes a squared term. It may also indicate that on large circumferential wounds it may not be possible to image the whole wound from a single viewpoint.
analysis of accuracy on real wounds is impossible. However, the very low variation in measurements obtained from the standard known-size area in a fabricated wound indicates a high level of absolute accuracy from the digital imaging system. The creation of digital image measurements should reduce the subjective element of measurement found in physical techniques. Both inter- and intra-observer variation of wound dimensions during DEM was small, suggesting good repeatability of measurement. It is noted that, as with EM, there is a level of subjectivity in DEM as the clinician must choose axes of measurement. It is believed that DEM offers an advantage over EM, as the wound may be more thoroughly inspected from several viewpoints prior to performing elliptical measurement. The choice of length axis was most consistent, having an interobserver coefficient of variation of <6.5%.

Using these digital constructs offers several advantages over current wound measurement techniques. They are non-invasive, reducing infection risk. The system is simple to use and can be easily deployed in routine clinical practice. Fast processing allows near-immediate display and interaction with the 3D wound models, which, displayed on a standard PC monitor, gives a detailed, full-colour representation of the wound. This provides an objective visual record that allows remote in-depth analysis of the wound. The 3D wound models can also provide valuable information about depth and volume of a wound that is not conveyed in conventional wound measurement and allows detailed monitoring of wound progression.

Digital images are useful for electronic transmission and provide significant potential for telemedicine and remote diagnosis [10]. A multidisciplinary approach to management
Assessment of a novel optical wound measurement system • F. L. Bowling et al.

of the diabetic foot ulcer is currently accepted as the most effective strategy in treatment [11]. This is facilitated by the sharing of electronic images between healthcare professionals. Limitations of the current data include a small sample size and the fact that the elliptical method of measurement underestimates wound area in small wounds [8].

In summary, creation of a 3D wound image using this optical wound measurement system produces accurate and reproducible data. Digital images are ideal for electronic transmission and can provide reliable monitoring of wounds over time.

Competing interests
J.A.P. is employed by, has shares in, and is a Director of Eykona Technologies Ltd. R.W.D. is a Director of, and has shares in Eykona Technologies Ltd.

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