The Utility of Corneal Confocal Microscopy in the Detection and Follow-up of Small Fibre Neuropathy in Diabetes and Sexual Dysfunction

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Table of contents

Word Count: **38, 888**

List of Figures

Figure number in manuscript of result chapter showed in italics in brackets.

List of tables

Table number in manuscript of result chapter showed in italics in brackets.

Abstract

Diabetic peripheral neuropathy (DPN) is associated with significant morbidity due to resultant painful neuropathy, foot ulceration and lower limb amputation. Subjects with DPN are also at high risk of other vascular complications including erectile dysfunction (ED), diabetic retinopathy, nephropathy and premature cardiovascular disease. Although DPN involves both small and large nerve fibres, early damage occurs primarily to the small fibres. Previous studies have shown that corneal confocal microscopy (CCM) can quantify small nerve fibre neuropathy (SFN) in a reproducible manner.

We evaluated the role of SFN in the symptoms of ED in men with type 2 diabetes (T2DM). We showed that ED was associated with SFN rather than autonomic or large fibre neuropathy. In addition, CCM in patients with ED was comparable to IENFD, the current gold standard for assessment of small nerve fibre damage thus showing its reliability in assessing for SFN in patients with ED.

We also compared the prospective utility of CCM to the current 'gold-standards' for small (intraepidermal nerve fibre density [IENFD]) and large (nerve conduction studies [NCS]) nerve fibre assessments over a mean follow-up period of 6.5 years. Additionally, we undertook detailed neuropathy assessments, including clinical neuropathy measures and tests for autonomic nerve function. The changes in CCM were directly associated with changes in IENFD and autonomic nerve function but not NCS, showing a reliability in detecting SFN longitudinally. The deterioration in CCM was more pronounced than in IENFD which may suggest an increased sensitivity in detecting early deterioration nerve fibres using this method. The reduction in CCM parameters corresponded to progressive increases in albuminuria and reductions in estimated glomerular filtration rate, showing an association with alternate measures of microvascular disease.

Finally, in a further study, this thesis examined the association of different measures of neuropathy, especially CCM, with sexual function and ED to understand if SFN or low testosterone levels had a greater effect on sexual function in men with both Type 1 and Type 2 diabetes. Corneal nerve loss was associated with the severity of ED with a significant association between CCM measurements and both erectile function scores and frequency of early morning erections. Importantly, testosterone and free testosterone levels were not associated with any measures of sexual function, suggesting that SFN rather than low testosterone levels may be the major driver of sexual dysfunction in men with diabetes.

Current measures of SFN suffer multiple drawbacks. QST is highly subjective and has limited reproducibility, skin biopsy (IENFD) is invasive and NCS fail to identify small nerve fibre damage. CCM bridges this gap by virtue of being non-invasive, rapid, reproducible and reliable and as shown in this study it has advantage in assessing progression of DPN.

DECLARATION

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

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Contribution

Shaishav Shashikant Dhage, the author of this thesis, was actively involved in and made a significant contribution to all of the chapters/studies presented and discussed in this thesis. He co-authored the relevant study protocols for the research presented in this thesis. The author recruited participants and oversaw an informed consent process for the study participants. He also performed relevant clinical assessments, neuropathy assessments, administration of questionnaires and venepuncture for blood samples. In addition, he performed skin biopsies. He performed selected ophthalmic examination under guidance and supervision of Dr. Maryam Ferdousi. He separated samples for storage and kept a central log of sample identities with help of Mr. Jay Brown and biochemistry staff. He also co-ordinated the delivery of samples to relevant collaborators. The author performed statistical analysis for the results presented in this thesis.

Other members of the research team performed the following tasks:

- Patient recruitment and co-ordination was also done by Drs Jan Ho and Safwaan Adam;
- Clinical measurements and neuropathy assessments were also carried out by Drs Jan Ho and Safwaan Adam;
- Venepuncture was also performed by Drs Jan Ho and Safwaan Adam;
- Sample separation and processing for storage was also undertaken by Drs Jan Ho and Safwaan Adam;
- Electro diagnostic studies were undertaken by Dr Andrew Marshall, consultant neurophysiologist;
- Ophthalmic examinations were performed by Dr Maryam Ferdousi and Dr. Alise Kalteniece;
- Skin tissue biopsy histological analysis was undertaken by Dr Maria Jeziorska ;
- Biochemistry measurements presented in this thesis were done by the laboratory medicine department at Manchester University NHS Foundation Trust.

Alternative Thesis Format

The author, working in the University of Manchester, Faculty of Biology, Medicine and Health, has been granted permission by his supervisors Dr Handrean Soran, Prof. Rayaz A Malik and Dr. Rachelle Donn to submit this M.D. thesis in an alternative format under the regulations, including sections which are in a format suitable for submission for publication or dissemination. The following chapters in this thesis that have been published are under review or are being submitted for publication:

Chapter 4: Published in Diabetes Metabolism Research Review, November 2019

Chapter 5: Currently under review

Chapter 6: To be submitted for publication

Abbreviations

Aα	A –alpha large nerve fibres
Aβ	A – beta large nerve fibres
Að	A- delta large nerve fibres
ACCORD	Action to Control Cardiovascular risk in Diabetes
ACE	Angiotensin Converting Enzyme
ACR	Albumin Creatinine Ratio
ANOVA	Analysis of Variance
ARB	Angiotensin Receptor Blocker
AUC	Area Under Curve
BMI	Body Mass Index
BP	Blood Pressure
C Fibres	Type C small nerve fibres
CAN	Cardiac Autonomic Neuropathy(/Function)
CASS	Composite Autonomic Severity Score
CAT	Cardiac Autonomic testing
CCM	Corneal Confocal Microscopy
cGMP	cyclic Guanosine Monophosphate
CIDP	Chronic Inflammatory Demyelinating Polyneuropathy
CIP	Cold Induced Pain
CNBD	Corneal Nerve Branch Density
CNFD	Corneal Nerve Fibre Density

CNFL	Corneal Nerve Fibre Length
CNFT	Corneal Nerve Fibre Tortuosity
CNS	Central Nervous System
COMPASS	Composite Autonomic Symptom Scale
CPT/CT	Cold Perception Threshold
CS	Cold Sensation
DAN	Diabetic Autonomic Neuropathy
DB-HRV	Deep Breathing Heart Rate Variability
DCCT	The Diabetes Control and Complications Trial
DM	Diabetes Mellitus
DN	Diabetic Neuropathy
DNS	Diabetic Neuropathy Symptom Score
DPN	Diabetic Peripheral Neuropathy
EAA	European Academy of Andrology
EDB	Extensor Digitorum Brevis
eGFR	Estimated Glomerular Filtration rate
E/I ratio	Expiration/Inspiration ratio
EMAS-SFQ	European Ageing Male Study Sexual Function Questionnaire
FSH	Follicular Stimulating Hormone
GDS	German Diabetes Study Group
HbA1c	Glycosylated Haemoglobin

HDL-C	High Density Lipoprotein Cholesterol
HPLC	High Performance Liquid Chromatography
HRA	Health Research Authority
HR	Heart Rate
HRT	Heidelberg Retina Tomograph
IENFD	Intraepidermal Nerve Fibre Density
IENFL	Intraepidermal Nerve Fibre Length
IGT	Impaired Glucose Tolerance
IIEF	International Index of Erectile Function
IV-CCM	In vivo Corneal Confocal Microscopy
LDIflare	Laser Doppler Image Flare
LDL-C	Low Density Lipoprotein Cholesterol
LFA/RFA	Low Frequency (Sympathetic)/High, Frequency (Parasympathetic) Sympatho-vagal ratio
LH	Luteinising Hormone
LPA	Left Peroneal Amplitude
LPV	Left Peroneal Velocity
LSA	Left Sural Amplitude
LSV	Left Sural Velocity
LTA	Left Tibial Amplitude
LTV	Left Tibial Velocity

MDL	Mean dendritic length
MDRD	Modification of Diet Renal Disease
NCS	Nerve Conduction Studies
NDS	Neuropathy Disability Score
NEURODIAB	Diabetic Neuropathy Study Group Of The European Association For The Study Of Diabetes
NO	Nitric Oxide
NSP	Neuropathy Symptom Profile
NSS	Neuropathy Symptom Score
PBS	Phosphate buffered Saline
PDE5	Phosphodiesterase 5
PGP 9.5	Protein gene product 9.5
PMNA	Peroneal Motor Nerve Amplitude
PMNCV	Peroneal Motor Nerve Conduction Velocity
PNAP	Peroneal Nerve Amplitude
QST	Quantitative Sensory Testing
RCM	Rostock Cornea Module
SBP	Systolic Blood Pressure
SD	Standard Deviation
SHBG	Sex Hormone Binding Globulin
SPK	Simultaneous Pancreas Kidney

SNAP	Sural Nerve Amplitude
SSNCV/SNCV	Sural Sensory Nerve Conduction Velocity
TBS	Tris-buffered saline
T1D/T1DM	Type 1 Diabetes Mellitus
T2D/T2DM	Type 2 Diabetes Mellitus
TC	Tortuosity Coefficient
TG	Triglycerides
UKPDS	United Kingdom Prospective Diabetes Study
VPT	Vibration Perception Threshold
WIP	Warm Induced Pain
WPT/WT	Warm Perception Threshold
WS	Warm Sensation
Δ	Delta (representing percentage change)

“Do not lower your goals to the level of your abilities.

Instead, raise your abilities to the height of your goals. ”

Swami Vivekananda

Dedication

This thesis is dedicated to the fond memories of my

beloved father (late) Mr. Shashikant V. Dhage

and

our very special grand-mother (late) Mrs. Devakiamma

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far. I shall never be grateful enough for all their sacrifices for me and the hardships they have gone through for me. “A man with dreams needs a woman with vision”, my wife Arathy, has always provided that vision and fuel to my ambitions. She has been truly understanding, supportive and encouraging throughout my career. She has been a great motivator and an immense source of energy through the roller coaster of research and personal life. Without her help, support and sacrifice I would not have reached this far in life. I am very thankful to both of my sweet daughters, Shyamala and Nakshatra, for allowing me some of their time to be complete this thesis and I shall surely make it up to them. My sisters, Sanjivani and Sarojini have always been supportive and encouraging. I cannot express enough respect and gratitude towards my brother-in-law Dr. Vishal Amle, who has been a pillar of support for my mother and rest of the family back home after sad and untimely demise of my beloved father and my elder brother-in-law, Mr. Balasaheb Jadhav. Vishal`s this help has allowed me to fully concentrate on my research and clinical career. I am also very thankful to Mrs. A Savithry, nanny of my daughters for looking after them, which spared me some valuable time to complete this thesis.

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Preface

Shaishav Shashikant Dhage undertook his undergraduate medical training at the University of Pune, Maharashtra, India and graduated with MBBS degree in 2000. He was a gold medallist in microbiology and otorhinolaryngology. He completed his junior doctor rotations at Talegaon General Hospital, Talegaon D, Pune, India affiliated to Maharashtra Institute Of Medical Sciences and Research (MIMER), Talegaon D, Pune, India. He then worked as assistant clinical lecturer in General Internal Medicine (GIM) at MIMER, before moving to King Edward Memorial Hospital(K.E.M.), Mumbai to work in the department of Public Health and Social Medicine. He completed his post-graduation in GIM in 2008 with National Board of Examinations, New Delhi and was awarded Diplomate of National Board of Examinations (D.N.B Medicine). He was awarded Membership of National Academy of Medical Sciences (MNAMS). He was appointed as Clinical Assistant Professor of GIM at prestigious Amrita University of Medical Sciences, Kochi, Kerala, India in May 2008 and continued to serve there till Jan 2011, before moving to the UK.

He was appointed as a Clinical fellow at Wythenshawe Hospital, Manchester (then University Hospital South Manchester) on Royal College Of Physicians (RCP), London sponsored International Fellowship Programme (MTI), in Jan 2011. He completed his core medical training equivalent in 2012 and he was also awarded with MRCP in the same year. He gained his National Training Number (NTN) for postgraduate training in Endocrinology, diabetes and GIM in August 2013 and he completed his Speciality Certificate Examination in Endocrinology and Diabetes, in 2015. Between 2017 and 2019, he spent 27 months out of clinical programme (OOPR) to undertake research leading up to this thesis. Having finished his speciality training, he was awarded his Certificate of Completion of Training (CCT) in January 2020 and he is currently working as a consultant endocrinologist.

He is an experienced and astute clinician and always had a keen interest in evidence based medicine and research behind it. This interest prompted him to do a period of clinical research leading up to this thesis.

He has published 8 peer reviewed publications during his period of research. He has also presented his work at local, regional and national level conferences. He was also awarded a Travel Fellowship by American Society of Endocrinology, to attend Type 1 Diabetes Fellow Series and the ENDO 2018, Chicago, USA and another Travel Fellowship by American Society Of Endocrinology, to attend Early Career Forum and the Endo 2019, New Orleans, USA.

He has keen interest in medical education and is actively involved in teaching and also supervises medical students and junior doctors.

List of publications during period of registration:

1. Small fibre pathology is associated with erectile dysfunction in men with type 2 diabetes. Dhage S, Ho JH, Ferdousi M, Kalteniece A, Azmi S, Adam S, Marshall A, Jeziorska M, Donn R, Soran H, Malik RA. Diabetes Metab Res Rev. 2019 Dec 13:e3263. doi: 10.1002/dmrr.3263. [Epub ahead of print] PMID: 31833632

2. Efficacy and safety of PCSK9 monoclonal antibodies. Iqbal Z, Dhage S, Mohamad JB, Abdel-Razik A, Donn R, Malik R, Ho JH, Liu Y, Adam S, Isa B, Stefanutti C, Soran H. Expert Opin Drug Saf. 2019 Dec;18(12):1191-1201. doi: 10.1080/14740338.2019.1681395. Epub 2019 Nov 20. Review. PMID:31623472

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7. Effect of Roux-en-Y Bariatric Surgery on Lipoproteins, Insulin Resistance, and Systemic and Vascular Inflammation in Obesity and Diabetes. Yadav R, Hama S, Liu Y, Siahmansur T, Schofield J, Syed AA, France M, Pemberton P, Adam S, Ho JH, Aghamohammadzadeh R, Dhage S, Donn R, Malik RA, New JP, Jeziorska M, Durrington P, Ammori BA, Soran H. Front Immunol. 2017 Nov 15;8:1512. doi: 10.3389/fimmu.2017.01512. eCollection 2017. PMID:29187850

8. Diabetic Neuropathy Is Characterized by Progressive Corneal Nerve Fiber Loss in the Central and Inferior Whorl Regions. Ferdousi M, Kalteniece A, Petropoulos I, Azmi S, Dhage S, Marshall A, Boulton AJM, Efron N, Faber CG, Lauria G, Soran H, Malik RA. Invest Ophthalmol Vis Sci. 2020 Mar 9;61(3):48. doi: 10.1167/iovs.61.3.48. PMID: 32232351; PMCID: PMC7401481.

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1. Ho JH, Adam S, Azmi S, **Dhage SS**, Liu Y, Ferdousi M et al. Male sexual dysfunction and hypogonadism improves following bariatric surgery. Endocrine Abstracts. 2017 Nov. Available from, DOI: 10.1530/endoabs.50.P327

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9. Ho JH, Liu Y, Adam S, Azmi S, **Dhage SS**, Syed AA, Ammori BJ, Donn R, Malik RA, Yang X, Tsimikas S, Soran H.The Effect Of Metabolic Surgery On Lipoprotein (A), Oxidised Phospholipids And Biomarkers Of Lipoprotein Oxidation. (Oral Presentation, HEARTUK Annual Conference, Warwick, 2019)

10. Liu Y, **Dhage S**, France F, Adam S, Ho JH, Donn R, Durrington P, Soran H. Variation And Distribution Of Apolipoprotein E And Its Glycation In Plasma Of Type 2 Diabetes. (Poster Presentation at EAS Annual Meeting, Maastricht, 2019)

11. Ho JH, Liu Y, Adam S, Azmi S, **Dhage SS,** Syed AA, Ammori BJ, Donn R, Gibson MJ, Malik RA, Yang X, Durrington PN, Tsimikas S, Soran H. Divergent Changes In Lipoprotein (A) And Oxidised Phospholipids Following Metabolic Surgery. (Poster Presentation at EAS Annual Meeting, Maastricht, 2019)

12. Ho JH, Adam S Liu Y, , Azmi S, **Dhage SS**, Syed AA, Ammori BJ, Donn R, Malik RA, Yang X, Tsimikas S, Soran H.Reduction In Autoantibodies To Oxidised Ldl And Apob-Immune Complexes Following Metabolic Surgery (Poster Presentation at EAS Annual Meeting, Maastricht, 2019)

13. **Dhage SS**, Ho JH, Ferdousi M, Kalteniece A, Azmi S, Adam S, Marshall A, Jeziorska M, Donn R, Soran H, Malik RA. Small not large fibre pathology is associated with erectile

dysfunction in men with Type 2 diabetes. (Poster Presentation at Diabetes UK Annual Meeting, Liverpool, 2019)

14. **Dhage SS**, Adam S, Basu A. A rare case of hirsutism.(Poster Presentation at Society for Endocrinology BES, Glasgow, 2018)

15. Adam S, Liu Y, Siahmansur T, Ho JH, Azmi S, Siddals K, **Dhage SS**, Malik RA, Syed AA, Ammori BJ, Durrington PN, Donn R, Soran H. Novel insights into potential mechanisms by which bariatric surgery reduces cardiovascular disease risk. (Oral Presentation at British Obesity and Metabolic Surgery Society Annual Meeting, Telford, 2018) Prize winner for best oral communication.

16. Ho JH, Adam S, Azmi S, **Dhage SS**, Liu Y, Ferdousi M, Syed AA, Ammori BJi, Malik RA, Donn R, Soran H. The effects of bariatric surgery on obesity-related male sexual dysfunction. (Oral Presentation at British Obesity and Metabolic Surgery Society Annual Meeting, Telford, 2018)

Chapter 1: Introduction

Introduction

Diabetes Mellitus and its multiple chronic complications are a major public health challenge globally (1, 2). There are 3 main types of diabetes: Type 1 Diabetes, Type 2 Diabetes and Gestational diabetes (1). Other less common forms of diabetes include monogenic diabetes, latent autoimmune diabetes of adult onset (LADA) and secondary diabetes (3-7). Type 1 diabetes is characterised by relative or absolute insulin insufficiency due to autoimmune destruction of pancreatic beta cells. Whereas, type 2 diabetes is primarily driven by insulin resistance (1). Type 2 diabetes is the most prevalent form of diabetes comprising 91% of people with diabetes worldwide (2-6). The prevalence of diabetes mellitus is increasing, currently affecting 415 million adults and is projected to increase to 642 million by 2040 (1). Type 1 diabetes is less common, but the prevalence is also increasing annually by around 3 % (1). Approximately 86,000 children develop Type 1 diabetes every year around the world (1). Alarmingly, every 6 seconds a person dies from diabetes and related complications (5 million deaths in 2015) (8). The most common complication is diabetic neuropathy (DN) and affects 30 to 50% of patients (9). In the Western world 50% of all polyneuropathies are caused by diabetes (10). DN is the major cause of foot ulceration and can result in lower limb amputation (8-13). In addition to pain and ulceration DN leads to loss of employment through loss of mobility and independence, anxiety and depression (9, 10).

1.1 Definition and types of diabetic neuropathy

Diabetic neuropathy (DN) is comprised of a constellation of symptoms and/or signs resulting from somatic (sensory/motor) nerve and/or autonomic nerve dysfunction and can affect many organ systems (8, 10, 11). However, patients with DPN can be frequently asymptomatic (8, 10, 11). Diabetic neuropathies (Figure 1.1) should be a diagnosis of exclusion and are classified as follows :

1. Sensorimotor polyneuropathies:
    - Acute (e.g. Insulin neuritis) and chronic (diabetic polyneuropathy)
2. Focal and multifocal neuropathies:
    - Mononeuritis multiplex of cranial or truncal nerves
    - Diabetic amyotrophy

3. Autonomic neuropathies:
    - Cardiovascular, gastrointestinal or genitourinary.

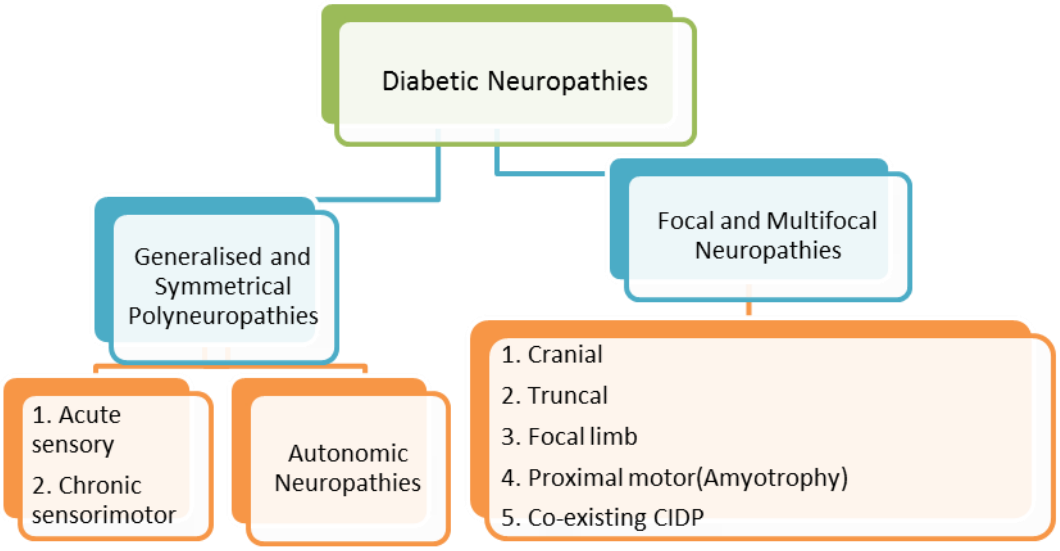


Figure 1.1. *Classification of Diabetic Neuropathies based on pattern of nerve involvement and clinical presentation. Adapted from Boulton et al Diabetes Care. 2005 (11).*

Dysfunction of different types of nerve fibres results in different clinical manifestations (Figure 1.2 and table 1.1, adapted from Tavee *et al* Cleve Clin J Med. 2009) (12, 13).

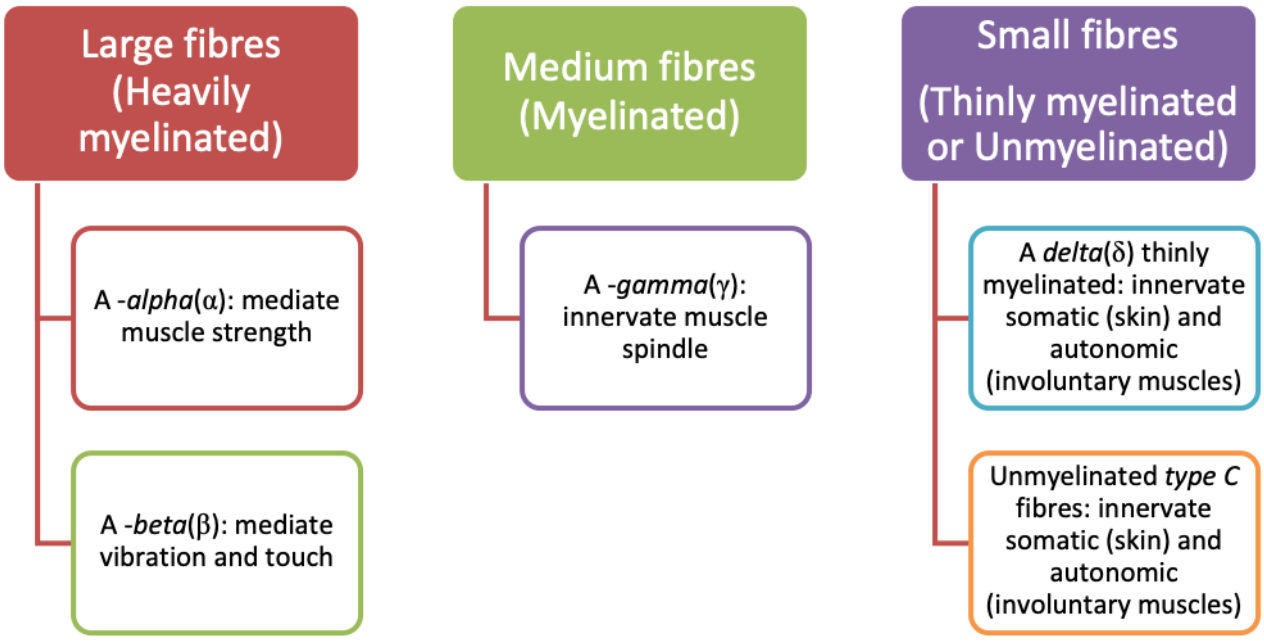


Figure 1. 2. *Classification of peripheral nerve fibres according to the type and pattern of myelination and innervation. Large and heavily myelinated A-alpha fibres mediate motor function while A-beta fibres mediate vibration and touch sensation (13). Medium sized myelinated A-gamma fibres innervate muscle spindles (13). Small fibres are thinly myelinated A-delta and unmyelinated type-C fibres, which innervate skin and autonomic nerves which innervate involuntary muscles, e.g. cardiac and smooth muscle (13).*

Small fibre involvement occurs early and primarily causes sensory and autonomic abnormalities (13). The most frequent presentation of DN is that of symptoms of pain, burning, tingling or numbness in the lower limbs (13). Overt autonomic small fibre dysfunction may result in symptoms of dry mouth, dry eyes, constipation, postural dizziness, sexual dysfunction, gastrointestinal and orthostatic hypotension (13). Small nerve fibre damage also contributes to altered tissue blood flow, ulceration and poor wound healing with gangrene and amputation (13). At the time of small fibre involvement, these patients usually have normal strength and reflexes and preserved touch, vibration and proprioception. Pinprick and temperature sensation is usually impaired and there may be hyperalgesia or allodynia. Postural hypotension with resting tachycardia indicates advanced autonomic involvement (12). Large nerve fibre damage results in defects of vibration, proprioception, postural instability, weakness and muscle wasting (13).

Table 1.1. Small and large fibre dysfunction in diabetic neuropathy

Small fibre neuropathy	**Large fibre neuropathy**
Earliest and commonest type of DN	Late onset and length dependent
Pain, burning, tingling, numbness, hyperalgesia and allodynia	Postural instability and imbalance, weakness
Dysesthesia, hyperesthesia, reduced pain and temperature sensation, autonomic dysfunction – dry eyes, dry mouth, diarrhoea, postural dizziness, orthostatic hypotension, sexual, genitourinary and gastrointestinal symptoms, anhidrosis	Muscle weakness and wasting, altered tendon reflexes, altered vibration and joint position
Abnormal quantitative sensory testing (QST), abnormal intraepidermal nerve fibre density (IENFD), abnormal autonomic function tests	Abnormal nerve conduction study (NCS) and electromyography (EMG)

***Table 1.1**. Small and large fibre dysfunction in diabetic neuropathy. Salient clinical features of small and large fibre neuropathy are outlined in the Table 13. DN: Diabetic neuropathy; EMG: Electromyography; IENFD: Intraepidermal Nerve Fibre Density; NCS: Nerve Conduction Studies; QST: Quantitative Sensory Testing.*

1.2 Assessment and evaluation of diabetic neuropathy

Figure 1.3. Methods to assess for diabetic peripheral neuropathy

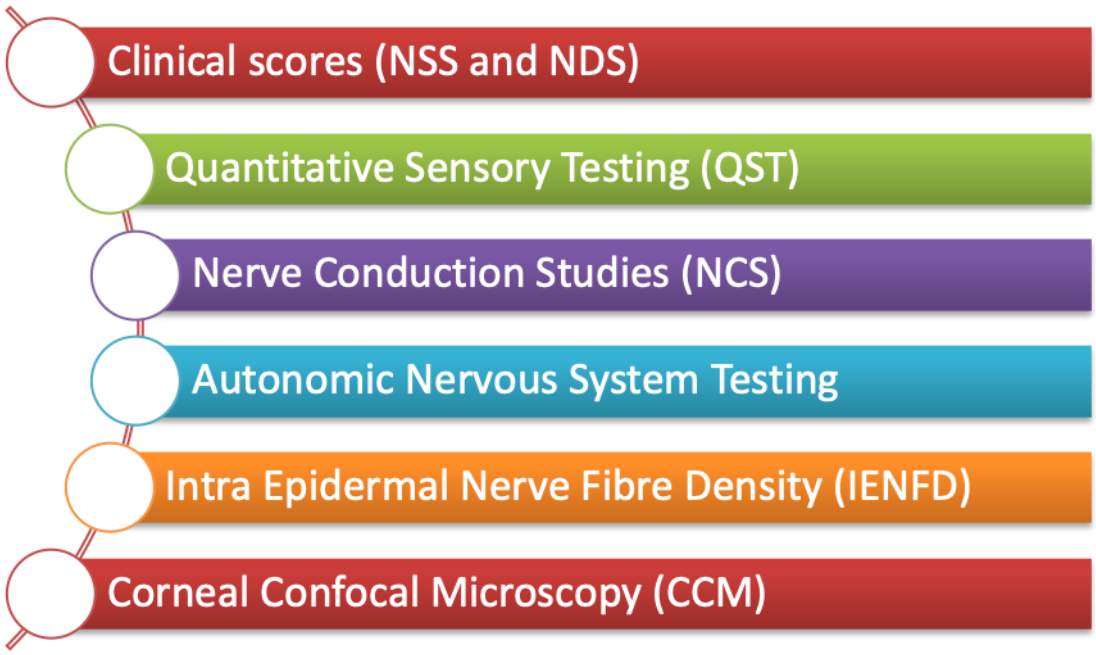


***Figure 1.3**. Methods to assess for diabetic peripheral neuropathy. NSS: Neuropathy Symptom Profile, NDS: Neuropathy Disability Score, QST: Quantitative Sensory Testing, NCS: Nerve Conduction Study, IENFD : Intra Epidermal Nerve Fibre Density, CCM: Corneal Confocal Microscopy.*

A careful medical history, physical examination and appropriate laboratory screening tests are key to excluding other causes of neuropathy in a subject with diabetes. The presentation of diabetic neuropathy can be quite variable and almost 50% of patients with diabetic neuropathy can be asymptomatic at the time of presentation (14, 15). Therefore, early and objective detection of neuropathy becomes very important to prevent morbidity (16).

Diabetic neuropathy can be assessed and quantified in various ways (Figure 1.3):

1.2.1 Clinical scores based on symptoms and neurological tests

These include assessment of symptoms with validated questionnaires such as the Neuropathy Symptom Score (NSS) and clinical examination to evaluate the sensory modalities of pinprick, vibration, light touch (using a monofilament) and proprioception. An NSS score of ≥1 is deemed to be abnormal (14). The Neuropathy Disability Score (NDS) is

based on an assessment of pin prick, temperature, vibration and ankle reflexes (Table 1.2) (17). It provides a score out of ten with scores of 0-2 (no DN), 2-4 (mild DN), 4-6 (moderate DN) and >6 (severe DN) (17).

Table 1.2. Modified Neuropathy Disability Score (NDS)

NDS parameters	**Scores (on either side)**
Pin prick	0 = present, 1=reduced or absent
Temperature(Cold tuning fork)	0 = present, 1=reduced or absent
Vibration sensation(128Hz tuning fork)	0 = present, 1=reduced or absent
Ankle reflex	0= present, 1= present with reinforcement 2= absent

***Table 1.2**. Modified Neuropathy Disability Score(NDS) is based on the results of assessment of sensations on either side. Severity of DN is assessed according to: 0 -2 points= No diabetic neuropathy (DN); 2-4 points= mild DN; 4-6 points=moderate DN; >6 points = severe DN. Adapted from - Yang Z et al (15).*

The neuropathy symptom profile (NSP) is another scoring system used to assess neurological symptoms, it contains 38 questions and a score ≥ 97.5 percentile is considered as abnormal (16). The diabetic neuropathy symptom score (DNS) is a modified and simpler scoring system. Four symptoms assessed in both legs and feet are – pain, ataxia, tingling and numbness (Table 1.3) (17). If a symptom is present more than once a week during the last 2 weeks it is scored as 1 and no symptoms are scored as 0. Any score ≥1 indicates presence of diabetic neuropathy (17).

Table 1.3. Diabetic Neuropathy Symptom score (DNS)

Symptoms in either legs or feet	Scores
Burning, aching pain or tenderness	1= present, 0=absent
Unsteadiness while walking	1= present, 0=absent
Prickling or tingling sensation	1= present, 0=absent
Numbness	1= present, 0= absent

Table 1.3. *Diabetic Neuropathy Symptom score (DNS). Maximum score: 4 points; 0 points = Peripheral Neuropathic Pain (PNP) absent; 1-4 points = PNP present (17).*

Multiple other scoring systems are being used all over the world for symptom and/ or sign screening for diabetic neuropathy (15). The disadvantages of clinical history and examination based scoring systems are that there is a high degree of intra-examiner and inter-examiner variability and inaccuracy and it only detects relatively advanced neuropathy (18).

1.2.2 Quantitative sensory testing (QST)

QST is a non-invasive psychophysical method of analysing human responses to painful or painless stimuli (19, 20). It is a reliable tool to test large and small nerve fibre function and is used to document sensory thresholds as determined by direct patient feedback. Thermal, mechanical and vibration sensation can be analysed using QST (19,20). Medoc products are most commonly used for thermal and vibration assessment and are comprised of integrated software and mission-specific hardware (21). The TSAII- Neurosensory analyser consists of a thermode based on Peltier elements which is capable of heating and cooling the skin to induce cold sensation, warm sensation, cold induced pain and warm induced pain (21). The vibratory sensory analyser- VSA 3000 measures sensory thresholds for vibration with a range of 0-130 microns at a rate of 0.1 to 4.0 microns/second (21).

The German Research Network on Neuropathic pain (DFNS) has proposed a validated QST protocol consisting of 13 parameters, which is being used in multiple centres internationally (Table 4) (19,20).

Table 1.4. Quantitative Sensory Testing parameters proposed by German Research Network on Neuropathic pain (DFNS)

Sensory Modality	**QST method/instrument**
Warm and cold detection and pain thresholds	Thermotest (e.g. Medoc)
Light touch detection	von Frey filaments
Vibration	Rydel-Seiffer tuning fork
Pain threshold for pin-prick	Pinprick stimulators of different intensities
Blunt pressure	Pressure algometer
Dynamic mechanical allodynia	Cotton wool, Q-tip and a standardised brush
Pin-prick stimuli	Temporal summation

Table 1.4. *Quantitative Sensory Testing parameters proposed by the German Research Network on Neuropathic pain (DFNS). This protocol consists of 13 different parameters for neuropathy assessment (Adapted from Rolke R et al (20)).*

QST is reasonably reproducible over several days and weeks in healthy subjects. However, reproducibility is dependent on factors such as training of the examiner and subsequent patient instructions, methodology of the assessment, baseline skin temperature, the number and site of stimuli and the interval and duration between tests (19). Large normative data sets with age and sex matched controls have recently been generated with QST to evaluate peripheral neuropathy and neuropathic pain (20, 22). There has been an effort to standardise the protocols and improve training to reduce variability (20, 23). Another potential shortcoming of QST is the inability to differentiate between central and peripheral causes of a sensory deficit. Other disadvantages include limited reliability with extreme temperatures (due to restrictions in the maximum and minimum temperatures allowed in order to prevent thermal injury) and patient factors which may comprise poor

cognition and concentration. Despite QST being a powerful research tool, it has not been recommended as the sole assessment method for small fibre neuropathy. However, it is very useful when used in conjunction with clinical examination and other investigations (24).

1.2.3 Nerve conduction studies (NCS)

Nerve Conduction Study (NCS) is an electrodiagnostic test used for the evaluation of motor and sensory nerves (25). It is often used in combination with needle electromyography (EMG), which also evaluates muscle function (25). Motor and sensory NCS along with F wave latency and H-reflex are the usual components of a NCS study (25). Nerve conduction velocity (NCV) is the most important measurement (25).

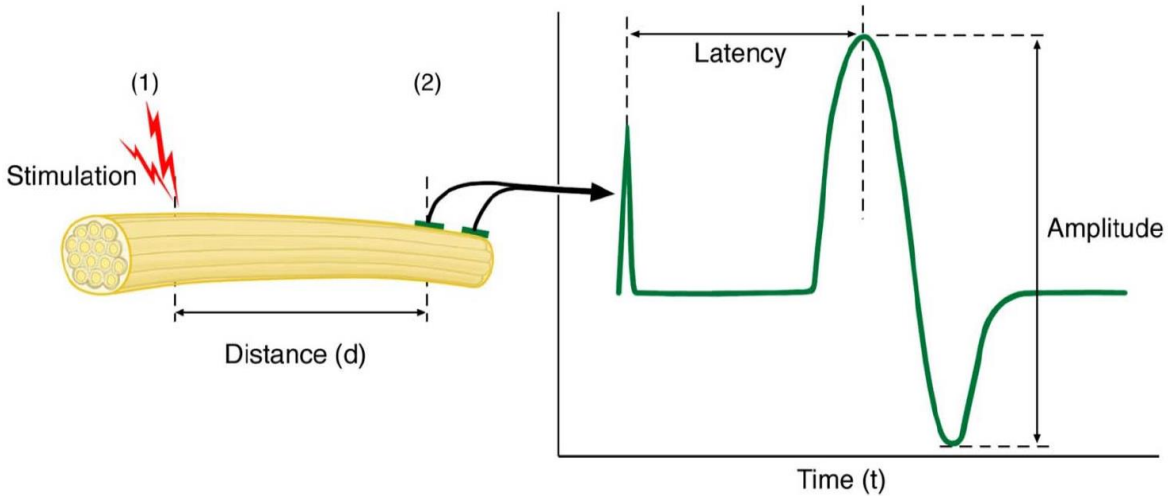


Figure 1.4. *Sensory nerve conduction study. (1): stimulation electrode site; (2): recording electrode site; (d): distance travelled by impulse; (t): time taken for travel by the impulse between (1) and (2); latency(ms): time taken for the impulse to travel between (1) and (2); Conduction velocity(m/s): the speed at which an impulse propagates (calculated as d/t); amplitude (microvolts): height of the response from baseline to peak. Adapted from(25).*

Figure 1.4 shows a diagrammatic representation of NCS in sensory nerves (25). In motor NCS (Compound Motor Action Potential-CMAP) the stimulation electrode is placed on a peripheral nerve and recording electrode is placed on the muscle innervated by this nerve, whereas in sensory NCS (Sensory Nerve Action Potential-SNAP) the recording electrode is placed on a sensory area innervated by sural nerve (25). Latency is the time taken for

propagation of the impulse from stimulation to the recording site and is measured in milliseconds (ms) (25, 26). The height of the response is measured as the amplitude in millivolts (mV) in motor NCS, however the amplitude is much smaller in sensory NCS and is measured in micro volts (μV)(25, 26).The F wave study utilises a supra-maximal stimulus to evaluate conduction velocity between the limb and spine (25, 26). A H reflex study also works on similar principles, however in this afferent impulses (those going to the spinal cord) are sensory but efferent impulses (those coming from spinal cord) are motor (25, 26).

NCS can detect demyelinating and axonal neuropathies as well as the mixed forms. NCS are also able to characterise neuropathies in terms of symmetry, distribution (motor vs sensory or mixed), onset or duration (acute or chronic) and extent (proximal vs distal) (26). In primary axonal neuropathies, SNAP amplitudes are reduced, distal latency, F wave latency and conduction velocity are normal or only mildly affected (26). Whereas, in primary demyelinating neuropathies, distal and F wave latencies are prolonged and conduction velocities are slowed and SNAP amplitudes may be reduced with sural SNAP sparing (27).

Demyelination of large myelinated fibres in DN results in reduced nerve conduction velocity and axonal loss leads to reduced action potentials (28-31). A change in conduction velocity in asymptomatic patients does not predict the development of symptomatic DN (28). Whilst nerve conduction studies are highly objective and considered to be the gold standard for the diagnosis of diabetic neuropathy they have limited reproducibility (32). Furthermore, NCS assesses large myelinated nerve fibres and cannot diagnose small fibre neuropathy, which is the commonest and earliest form of diabetic neuropathy (33, 34). It also cannot be used for the early diagnosis of DN (28, 35).

1.2.4 Autonomic testing

Autonomic neuropathy in diabetes is a spectrum of disorders comprising- 1) Subclinical Autonomic dysfunction (AD), 2) peripheral diabetic autonomic neuropathy (DAN) and 3) end organ failure associated cardiac autonomic neuropathy (CAN) (8) . Autonomic neuropathy assessments in diabetes are based on 3 measures of heart rate variability (HRV) : exhalation/inhalation ratio(E/I) (> 1.17) from deep breathing, Valsalva ratio(>1.2) and 30:15 ratio(>1.03) from upright posture**(Ewing protocol, appendix 8.6 patient assessment form page 214)**. CAN is confirmed when all 3 of these ratios are abnormally low, whereas 2 of 3 ratios if abnormally low confirm DAN . Postural orthostatic

hypotension, DBHRV and software based low frequency area (sympathetic) and high frequency area (parasympathetic) i.e. LFA/RFA ratio are few other measures of autonomic neuropathy assessment. These could be abnormal in both DAN or CAN. Autonomic function testing includes cardiac autonomic function assessment of variation in heart rate in response to respiration (DB-HRV) and measurement of blood pressure (and pulse) on lying and standing. Tests of peripheral autonomic function include evaluation of sweat production in the feet using the sympathetic skin response (SSR), Neuropad or Sudoscan.

1.2.5 Nerve biopsy and Intraepidermal nerve fibre density (IENFD)

Sural nerve biopsy is not used as a diagnostic tool for diabetic neuropathy and should only be considered to exclude other causes of neuropathy e.g. vasculitis as it is invasive and carries a risk of infection, sensory loss and pain. Other drawbacks include an inability to differentiate between somatic and autonomic fibres and the need for specific operator training for tissue sample preparation and analysis which is both time-consuming and demanding (36). Intra epidermal nerve fibre density (IENFD) assessment in a skin biopsy is currently the most reliable and objective test for the diagnosis of small fibre neuropathy. It is minimally invasive, comparatively safe, reproducible and can differentiate between somatic and autonomic small nerve fibre damage (37). Skin biopsy is performed under local anaesthesia with a 3 to 6 mm punch from the dorsum of the foot or 10cm above the lateral malleolus, from the proximal thigh or from the site of symptoms (36, 37). Bright-field microscopy is used to quantify linear IEFND using specialist software. IEFND has a specificity of 95 to 97% and a sensitivity of 45 to 80%, in confirming the diagnosis of peripheral neuropathy of various aetiologies (38, 39). It has also demonstrated a positive predictive value of 92 % and a negative predictive value of 90% when used for neuropathy diagnoses (38, 39).

A skin biopsy can be repeated in the proximity of the previous biopsy site which can help with the assessment of progression or recovery of a neuropathy due to therapeutic interventions (37). However, skin biopsy requires specific resources, is time-consuming and is labour intense (40).

1.2.6 Corneal Confocal Microscopy (CCM) (Figure 1.5, 1.6, 1.7 and 1.8)

Over the last two decades, CCM has evolved from a research technique to become a tool for the assessment of diabetic and other peripheral neuropathies (40, 41). It may prove to be a valid biomarker for use in clinical trials of disease modifying treatments and interventions (41). It is important to highlight some key concepts to better understand the principles and use of CCM.

1.2.6.1 Corneal nerve architecture (Figure 1.5)

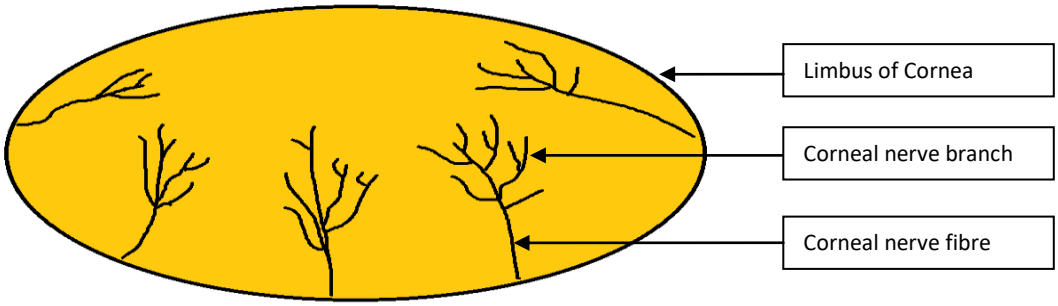


Figure 1. 5. *Corneal nerves and nerve fibres at the limbus. Corneal nerves run in a radial fashion from the limbus. Adapted from Muller et al (42).*

The human cornea is the most densely innervated surface tissue in the body (43). Most of the corneal nerve fibres are sensory and derived from the ophthalmic branch of the trigeminal nerve and sympathetic and parasympathetic fibres are derived from the superior cervical and ciliary ganglion, respectively (44). Nerve bundles enter the cornea at the periphery in a radial fashion (Figure 1.5) and run anteriorly in the stroma losing their myelin sheath (approximately 1mm from the limbus) (42). These nerve bundles are organised into three main groups: stromal nerves, sub-basal nerve plexus, sub-epithelial nerve plexus (44). These nerves are comprised of myelinated A-*delta* fibres, which respond to mechanical stimuli and unmyelinated type C fibres which respond to thermal and chemical stimuli (42, 44). CCM has been used to mainly image the sub-basal nerve plexus as the stromal nerves are randomly distributed in the stroma and the resolution of CCM is insufficient to image the sub-epithelial nerves. Other cellular components such as epithelial cells, endothelial cells, keratocytes and Langerhans cells can also be evaluated (45, 46).

1.2.6.2 Types of corneal confocal microscope and optimal image acquisition

The confocal microscopes bring two systems together at a joint focal point using 2 pinholes. This microscope eliminates the background information and allows one to focus on the particular area of interest (47). The white light passes through the condensing lens focusing on the cornea and passes through the first lens (47). The returning light passes through the objective lens and second pin hole before reaching the observer (47). The pinholes effectively act as spatial filters and help to image only the in-focus area (Figure 1.6). All three types of confocal microscope use the same optical principle.

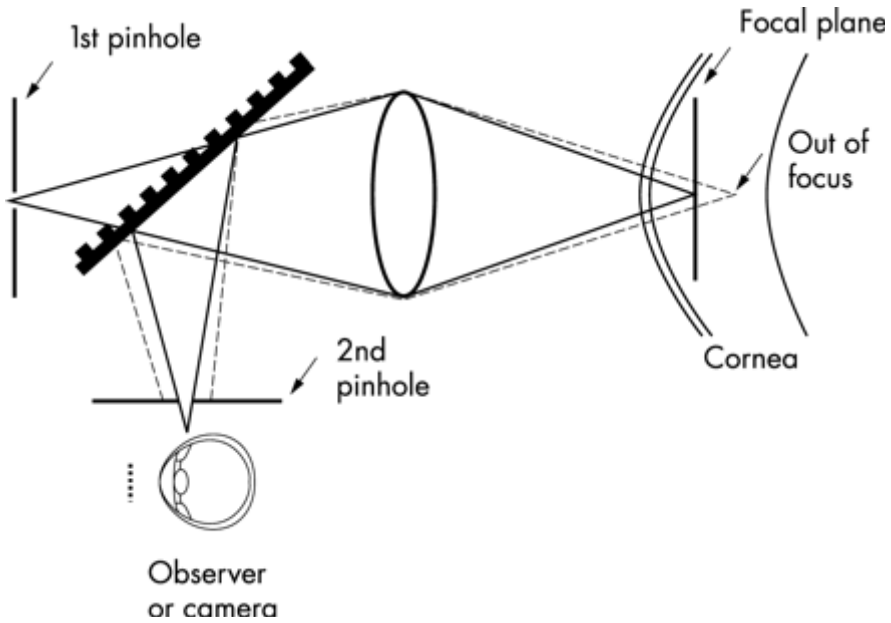


Figure 1.6. *Basic optical principles of a confocal microscope. The pinholes limit and prevent the scattered, out of focus light (shown as broken lines) from reaching the observer. This greatly improves resolution of the microscope (Adapted from Jalbert, I., et al.)(47).*

The image quality of CCM differs depending on the type of CCM used to acquire images due to differences in the light source, contrast and resolution (48). The three main types of CCM used are tandem-scanning based confocal microscopy, slit-scan based confocal microscopy and laser-scanning based microscopy. The Heidelberg Retina Tomograph (Heidelberg Engineering, Heidelberg, Germany) is the most widely used and well-established imaging systems for CCM (49). HRT was modified by Stave et al. by using a detachable objective system, called the Rostock Cornea Module (RCM), converting it into a high resolution confocal laser scanning microscope for the evaluation of the anterior segment of the eye

(50). Studies using HRT III with the RCM module for CCM have reported higher sub-basal nerve densities, when compared to studies using tandem-scanning and slit-scan based methods (48). CCM can also be used to examine microstructures in the cornea, sclera, limbal region, lacrimal glands, tear film and lymphatic tissue (51).

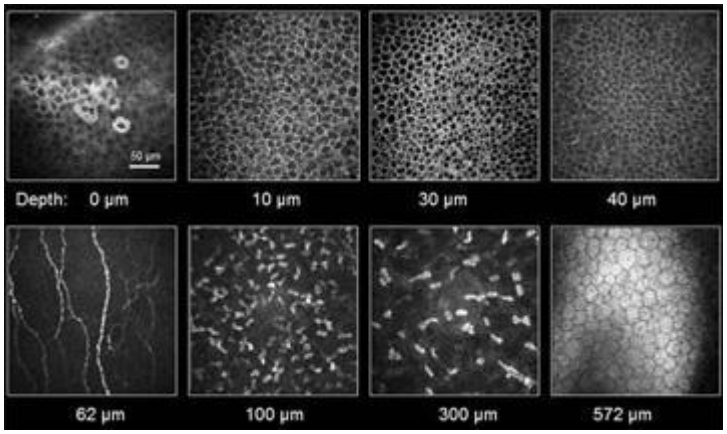
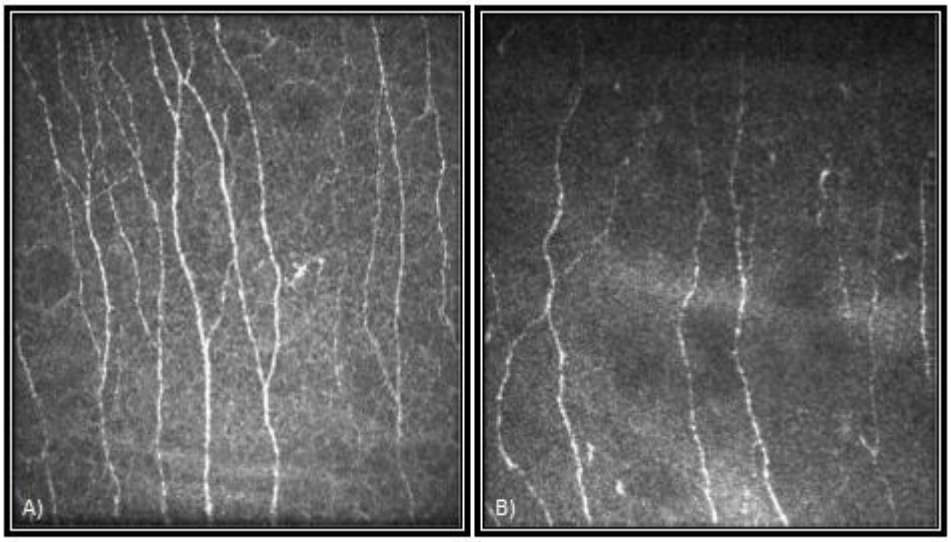


***Figure 1.7**. In vivo images of human corneal tissue with a confocal microscope. The four images in the top panel show a cross-sectional view through the corneal epithelium. The bottom panel, first image on left shows sub-basal plexus of nerves, anterior and posterior stroma and endothelium. (HRT Rostock Cornea Module [Web.] [cited 2015 12.10.](52).)*

1.2.6.3 Image quantification and analysis

The majority of studies utilising CCM to evaluate nerves have used up to 5 images per eye for representative quantitative analysis, but currently there is no consensus regarding the minimum number of images required for optimal results (53). The importance of selecting good quality CCM images cannot be over emphasised and is paramount for ensuring accuracy of results. Recently, Kalteniece *et al.* have published a method detailing image selection procedures to enable for a unified approach in image selection in multicentre studies that perform CCM (54) (Figure 1.8).



A) Healthy Volunteer and B) Patient with DM for 15 years. Adapted from Wang et al (94).

Figure 1.8. *Illustrates CCM images of the subbasal plexus of a healthy volunteer (A) and a patient with diabetes for 15 years. The white lines running parallel to each other vertically are the main nerve fibres and smaller branching lines are the nerve fibre branches. A simple visual inspection indicates a reduced number of main nerve fibres and branches in the patient with diabetes compared to the healthy volunteer. Decreased subbasal nerve fibre density is associated with reduced intraepidermal nerve fibre density (47,51,73).*

Stromal nerve quantification has not been widely undertaken using CCM due to their random orientation and sparse distribution, which contributes to inconsistencies in the quantification of these nerves (41, 55-57). The sub-basal plexus is commonly assessed due to superior and consistent visibility of nerves (55-57). The interface between Bowman`s layer and the corneal epithelium is identified using the RC module of the HRT III and images are taken at a depth of ~50 microns. Overall, the best quality images are selected manually and various parameters are measured using semi-automated analytical software (e.g. CCM image analysis tool v0.6, University of Manchester, UK)(55-57).

When assessing corneal nerves, the 4 most important measures are the corneal nerve fibre length (CNFL), corneal nerve fibre density (CNFD), corneal nerve branch density (CNBD), and the tortuosity coefficient (TC)(55). The CNFL is the total length of all nerve fibres and branches (mm per mm^2) in corneal tissue whilst the CNFD is the total number of major

nerve (per mm^2) of corneal tissue (55). The CNBD represents the total number of branches arising from major nerve trunks per mm^2 of corneal tissue (55). The TC is a coefficient denoting the degree of tortuosity of the major corneal nerves. These can be measured using manual or semi- or fully automated software (55).

Semi-automated image analysis has excellent reproducibility; however, it is labour-intensive and time-consuming. It can also be highly subjective depending on the level of experience of the examiner. Therefore, fully automated software has been developed to reduce variations and eliminate inconsistencies (41, 58-60). Scarpa *et al.* showed a strong correlation (r = 0.89 when using images from another centre and r = 0.94 when using images from their own centre) between results of images analysed using fully automated and manual software (59).

1.2.6.4 Clinical applications of CCM

Since the first report on the use of CCM in 1985 by Lemp *at al.*, significant progress has been made (61). CCM measurements have evolved considerably in corneal and ocular surface research, allowing translation from bench to bedside (62). It has rapidly evolved from solely being an assessment of the corneal apex to incorporating the whole ocular surface and from a qualitative assessment to an automated quantitative evaluation (41). It is a quick, non-invasive and easily reproducible method for evaluating corneal nerves (62). The main limitations of CCM are the small field of view and concerns about standardisation of image acquisition, interpretation and their quantification (62). However, it allows prompt diagnosis, disease follow-up and management of various neurological and ocular disorders (41, 62). It is currently a complimentary technique for clinical diagnosis and the management of various neurological conditions with a significant potential for adoption in the routine clinical assessment of neuropathy (62).

1.2.6.5 CCM in diabetic neuropathy

The corneal sub-basal plexus is an extension of the peripheral nervous system in the eye and possibly the only place in the human body where nerves can be visualised in real time with CCM. A relationship between neurotrophic corneal ulcers and diabetes was first reported in 1977 by Hyndiuk *et al.* (63). Rosenberg and colleagues demonstrated a reduction in corneal

sensitivity and a reduction in corneal nerve fibres in the sub-basal plexus of patients with type 1 diabetes and neuropathy (64). Since then, a number of studies have established the utility of CCM to quantify small fibre neuropathy in diabetes (65, 66).

Malik *et al.* demonstrated good sensitivity and specificity of CCM in quantifying early small nerve fibre damage (65, 66). CNFL has 93% specificity and 91 % sensitivity for identifying diabetic sensorimotor neuropathy (67) and has shown a significant correlation with cold detection thresholds, heart rate variability and laser Doppler imager flare (68). IENFD, the current 'gold-standard' for small nerve fibre damage on skin biopsy has been shown to correlate with CCM (69) and CCM and IENFD have comparable diagnostic performance for detecting patients with diabetic neuropathy (40).

1.2.6.6 The role of CCM in the diagnosis of DN

CCM can detect nerve damage in patients with recently diagnosed diabetes before clinical neuropathy develops (70, 71). Stem *et al*. evaluated the role of CCM in the detection of DN in 25 patients with type 1 diabetes, 18 with type 2 diabetes and 9 controls (72). None of the subjects with type 1 DM had evidence of neuropathy and of the subjects with type 2 DM, 10 had mild and 8 had severe neuropathy (73). Type 2 DM patients with severe neuropathy had fewer corneal nerves compared to controls (73). However, in participants with type 1 DM, without clinical DN, CNFL was 27 % lower than in controls (73). In addition, the diagnostic threshold of CNFL used to detect DN may be lower in type 1 DM compared to type 2 DM (67, 72).

Ahmed *et al*. investigated different approaches to determine the optimal diagnostic cut-off for diagnosing DN using CCM (67). They found that CNFL was the best parameter to evaluate DN and studied different values to determine a lower limit of normal (67). A value of < 14 mm/mm ^{2} provided a sensitivity of 85% and specificity of 84%, whilst a value of ≤11.5 mm/mm^2 optimised specificity (93%) and a value of < 15.8 mm/mm ^{2} optimised sensitivity (91%)(67). Accordingly, the authors proposed that CNFL values of between 11.5-15.8 mm/mm ^{2} represented patients who have subclinical DN and are at highest risk of progression to clinical DN (67). In another study, CNFL showed high sensitivity and specificity, whilst CNFD was more specific but less sensitive compared to IENFD (74). A

database of normative values of CCM has been published, but it has not yet been utilised in the clinical setting (75).

CNFL (r = -0.581; p<0.0001), CNFD (r = -0.475; p<0.0001) and CNBD (r = -0.511; p<0.0001) correlate with the severity of somatic neuropathy (74). In a study of 54 patients with diabetes and 15 controls, IENFD correlated with CNFD (r = 0.39; p = 0.001), IENFBD correlated with CNBD (r = 0.42; p = 0.001), but there was no correlation between IENFL and CNFL (69).

1.2.6.7 CCM in impaired glucose tolerance (IGT) and recent-onset type 2 diabetes

Zeigler *et al.,* from the German Diabetes Study group (GDS), conducted a study to determine presence of DN in patients with recent onset type 2 diabetes (70). Eighty-six recent onset type 2 DM and 48 age- and sex matched controls underwent CCM, IENFD, neurophysiology, QST and cardiac autonomic testing (70). CNFD and IENFD were reduced below the 2.5th percentile in 24 % and 14 % of subjects with diabetes, respectively. All CCM parameters, IENFD and NCV were significantly reduced in type 2 DM patients compared to controls (p<0.05)(70). 20.5% of subjects with type 2 DM had abnormal CNFD but concomitantly normal IENFD, whereas the converse was true in only 11 % of patients i.e. normal CNFD but abnormal IEFND. Therefore, CCM may be able to identify nerve damage in patients with recent onset diabetes, while IEFND could still be normal (70).

Impaired glucose tolerance is the earliest stage of dysglycemia before the development of type 2 DM. Its association with macrovascular disease, retinopathy and microalbuminuria has been documented, but whether it causes neuropathy is unclear. Patients with idiopathic small fibre neuropathy were found to have a high prevalence of IGT in one study (73). Subsequently two studies found evidence of DN in 11-13% of patients with IGT (76, 77), whilst Dyck *et al*. found no evidence of DN in subjects with IGT (78).

Asghar et al. studied 37 subjects with IGT and 20 age-matched controls by undertaking a comprehensive evaluation of neuropathy using neurological assessment, NCV, QST, cardiac autonomic testing (CAT), IENFD and CCM (79). Subjects with IGT had increased neuropathy symptoms (p <0.001), McGill pain index (p<0.001), neuropathy disability score (p=0.001), vibration perception threshold (VPT) (p=0.002) and warm and cold thresholds (p=0.006 and

p=0.03 respectively)(79). These subjects also had a reduction in IENFD (p=0.03) and a significant reduction in CNFD (p<0.001), CNBD (p=0.002) and CNFL (p=0.05). Indeed 40.5% of subjects with IGT had significant small fibre damage based on the observed reduction in CNBD (79).

In a similar study, Azmi et al have demonstrated the presence of greater small nerve fibre loss in subjects with IGT who later developed type 2 DM (80). Out of 37 subjects with IGT, ten developed type 2 DM over 3 years. These ten patients had a significantly lower CNFD (p=0.003), CNBD (p=0.04) and CNFL (p=0.04) at baseline and there was further deterioration in CNFL (p=0.006), IENFD (p=0.02) and mean dendritic length (MDL) (p=0.02) over the next 3 years (79). In 15 subjects who remained IGT and 5 subjects who reverted to NGT, CCM was normal but MDL (p<0.0001) was lower at baseline (80). Subjects with IGT showed a reduction in IENFD (p = 0.02), but no change in CCM or MDL, whilst the five participants who reverted to NGT showed an improvement in CNFL (p = 0.05); CNBD (p = 0.04) and CNFD (p = 0.05), but interestingly a decrease in IENFD (p = 0.02). This shows that worsening or improvement in glycaemic status is dynamically related to changes in CCM and IENFD (80).

There is therefore evidence supporting small fibre neuropathy in IGT, a predecessor of type 2 DM (79, 81-83). Pending further large validation studies, CCM could be used to diagnose and predict onset of neuropathy in recent onset type 2 DM and subjects with IGT.

1.2.6.8 The role of CCM in the detection of early nerve regeneration in DN

In an initial study, Mehra *et al* (84) showed a significant improvement in CNFD and CNFL, six months after simultaneous pancreas and kidney transplantation (SPK) in 20 patients with T1DM. Following this study, Tavakoli et al, evaluated 15 type 1 diabetes patients at baseline, 6 months and 12 months after SPK and compared them with 10 age and sex matched non-diabetic controls (85). The study showed that the NSP, the McGill pain index and the modified NDS were significantly higher in the diabetic patients (p= 0.005, 0.01, 0.003, respectively) than controls at baseline and there was no significant improvement in any of these parameters, 6 (P= 0.1, 0.9, 0.7 respectively) and 12 (p= 0.9, 0.9 and 0.8, respectively) months after SPK transplantation (85). The average heart rate variability, peroneal nerve conduction velocity and IENFD were significantly lower in diabetic subjects compared with controls at baseline and did not change significantly, 6 and 12 months after transplantation

(85). However, despite a markedly reduced CNFD (14.44 +/- 1.2 no./mm^2) and CNFL (11.35 +/- 1.04 mm/mm^2) at baseline, whilst there was no significant improvement at 6 months (15.22 +/- 1.53 and 13.35 +/- 1.50, p= 0.7 and 0.2 respectively), at 12 months there was a significant improvement (19.27 +/- 1.57 and 5.63 +/- 1.56, p=0.02 and 0.03)respectively (85). CNBD was significantly lower in patients with diabetes at baseline (21.46 no./mm^2, p <0.0001) and showed a significant improvement (p=0.03) at 6 and 12 months (p=0.008). IENFD did not improve at 12 months but showed a significant correlation with CNFD (r = 0.656, p< 0.0001), CNBD (r = 0 .709, p< 0.0001) and CNFL (r = 0.695, p< 0.0001) (85).

1.2.6.9 CCM in the diagnosis of diabetic autonomic neuropathy (DAN)

DAN is a highly prevalent complication in patients with diabetes and the presence of cardiac autonomic dysfunction is an independent risk factor for mortality in these patients (86). The prevalence of DAN varies depending on patient cohort, testing modality and criteria used for the diagnosis of autonomic dysfunction (87). It can be as low as 7.7 % in newly diagnosed type 1 DM (88) to as high as 90% in patients awaiting pancreas transplantation (89). Zeigler et al., reported abnormal autonomic function tests (more than 2 out of 6) in 25.3 % of type 1 DM and 34.3 % of type 2 DM patients (88). A clinical history and physical examination are not good indicators of early DAN (82, 86). Current tools for the evaluation of autonomic dysfunction through the assessment of cardiorespiratory reflexes are labour intensive and can be affected by concomitant drugs and disease (90, 91). Therefore, there is a need for developing an objective and reproducible surrogate diagnostic marker for the diagnosis of DAN (92). CNFL has been shown to correlate (r = 0.41; p<0.0001) significantly with heart rate variability, a marker of autonomic dysfunction (68).

Thirty four subjects with type 1 and type 2 diabetes and 18 healthy controls underwent assessment of the Composite Autonomic Symptom Scale (COMPASS), which has been shown to correlate with measures of autonomic dysfunction (93), cardiovagal function (heart rate variability, expiratory : inspiratory ratio, Valsalva ratio and Ewing 30:15 ratio), adrenergic function (blood pressure response to standing and Valsalva manoeuvre), sudomotor function (Sympathetic Skin Response [SSR]) and the 10- point Composite Autonomic Severity Score(CASS) was used to grade the severity and subtype of autonomic failure (90, 93). CNFD, CNBD and CNFL were reduced significantly in patients without and

with DAN compared to healthy volunteers and were lower in patients with compared to patients without DAN (90, 93). COMPASS and CASS scores were significantly correlated with CNFD (-0.754, -0.696), CNBD (-0.782, -0.744) and CNFL (-0.762, -0.721), respectively (90, 93). CCM also showed a very high sensitivity and specificity for diagnosing DAN (CNFD-sensitivity of 86% and specificity of 78%; CNBD- sensitivity of 100% and specificity of 56% ; CNFL-sensitivity of 86% and specificity of 78%) (90, 94). This study is consistent with other studies showing that CCM can detect subclinical DAN and somatic neuropathy (76, 95, 96).

1.3 Clinical manifestations of diabetic neuropathy

The clinical manifestations of DN vary depending on the type of nerve fibre and organ involved. Patients can be entirely asymptomatic or can present with limb threatening (foot ulcers and gangrene) or life threatening (silent cardiac ischaemic events) complications (97).

1.3.1 Distal symmetrical sensory-motor polyneuropathy (DSPN)

DSPN is the commonest form of DN. Sensory symptoms predominate, with positive (pain, paraesthesia, hyperesthesia, tingling, deep aching, burning and sharp stabbing) (98) and/or negative (numbness and hypoesthesia) symptoms (97). Abnormal proprioception, impaired muscle and joint position sense and strength results in an unsteady gait (99). Clinical examination reveals a glove and stocking distribution of sensory abnormalities with impaired pain, temperature and position sense (98, 99).

1.3.2 Cranial neuropathies

The most common cranial nerve palsies involve the third, fourth and 6th cranial nerves (100) and can be complete or partial, painless or painful and associated with ptosis, diplopia and sparing of the pupils (100, 101). Orofacial pain and burning may occur due to trigeminal nerve involvement (102).

1.3.3 Mononeuropathies and radiculopathies

Isolated involvement of practically all nerves is possible in diabetes e.g. carpal tunnel syndrome, ulnar, peroneal and sciatic neuropathy (103-105). These are usually acute in onset, can be painful and associated with weakness (104). Nerve entrapment is the commonest mechanism and occurs more commonly during periods of hypoglycaemia and hyperglycaemia, during insulin initiation or titration or during rapid weight loss (103-105).

Radiculopathies present with a subacute onset of pain followed by weakness or paralysis (106). Cervical, thoracic and lumbosacral radiculoneuropathies are a few examples (106, 107) and if multiple nerves are affected it is called mononeuritis multiplex.

1.3.4 Autonomic neuropathy

Diabetic autonomic neuropathy affects adrenergic, cholinergic and peptidergic fibres, which can be asymptomatic, subclinical or overtly symptomatic (11, 108).

Cardiovascular autonomic dysfunction results in postural hypotension, tachycardia, dizziness, exercise intolerance and syncope (8, 109, 110). It predisposes to silent myocardial ischaemia, stroke, nephropathy progression and perioperative morbidity (8).

Gastrointestinal autonomic neuropathy manifests with cyclical nausea, bloating, abdominal pain, painless nocturnal diarrhoea and constipation (8, 11, 108).

Genitourinary autonomic neuropathy can manifest with autonomic bladder dysfunction with urinary frequency, urgency, urinary retention and incontinence with recurrent urinary tract infection (11, 108, 111). Erectile dysfunction is common affecting ~60% of patients and is caused by autonomic neuropathy and endothelial dysfunction (110, 112) and in females pelvic autonomic neuropathy results in vaginal dryness and dyspareunia (110, 112). Diabetic sudomotor dysfunction results in hyperhidrosis, gustatory sweating and or anhidrosis with dry skin predisposing to foot ulceration (113).

1.3.5 Diabetic foot and related complications

DN is one of the most important factors in the development of foot complications (114). In the presence of DN even trivial trauma can initiate foot ulceration with delayed wound healing due to altered tissue blood flow (114). Sensory neuropathy impairs sensation, motor neuropathy creates abnormal pressure loading and autonomic neuropathy causes dry skin, fissures and predisposes to tissue breakdown (115, 116). Diabetic vasculopathy and endothelial dysfunction go hand in hand with DN (115, 116), starting the cascade of events resulting in foot ulceration and eventually amputation (114, 115).

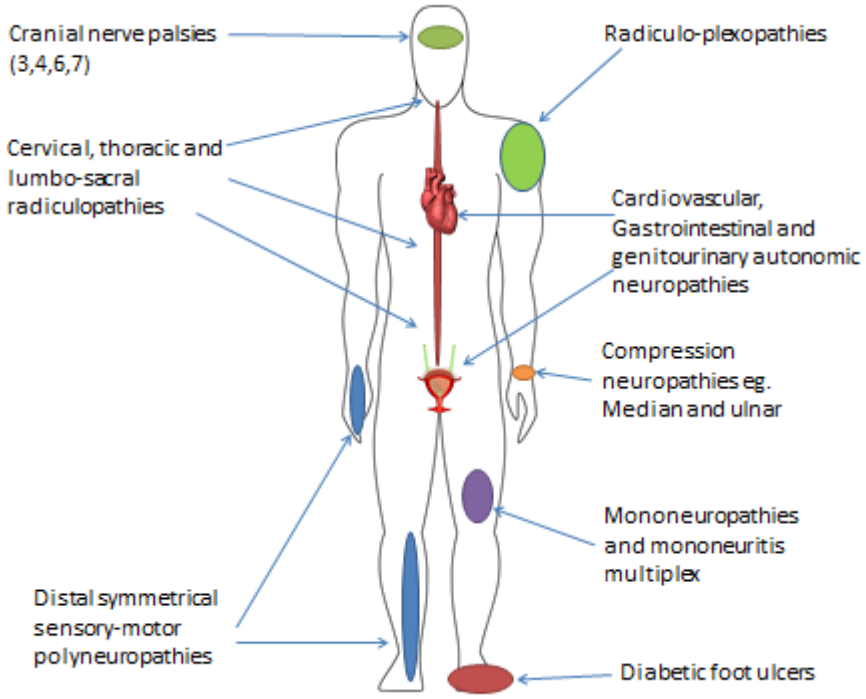


***Figure 1.9**. Clinical manifestations of diabetic neuropathy. Various clinical manifestations and presentations of the diabetic neuropathies.*

1.4 Sexual and erectile dysfunction in men with diabetes

Healthy and satisfying sexual function is an essential part of overall wellbeing, self-esteem, interpersonal relationship and indicates a good quality of life (117). Diabetes is a complex metabolic disorder which can affect sexual function (117, 118). Male sexual dysfunction in diabetes includes reduced libido, erectile dysfunction and disorders of ejaculation (117, 118). ED is very closely linked with other co-morbidities of diabetes like hypertension, atherosclerotic cardiovascular disease, hypogonadism, obesity, neuropathy, nephropathy and depression (117, 118). Endothelial dysfunction, vascular inflammation and atherosclerosis are common underlying mechanisms of ED (117, 118)(Figure 1.10).

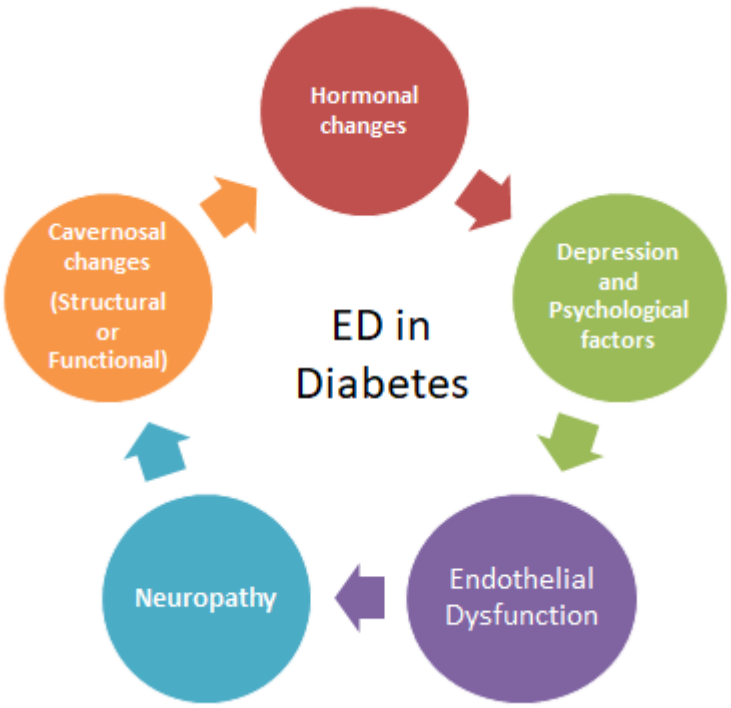


***Figure 1.10**. Mechanism of ED in Diabetes. This figure depicts various factors involved in the development of erectile dysfunction (ED) in diabetes patients.*

1.4.1 Physiology of penile erection

The physiology of erection is a complex interplay of psychogenic, hormonal and noradrenergic, non-cholinergic neurovascular mechanisms (119). Penile erection is mainly a vascular process, under the control of neurologic stimuli, an appropriate psychological mind-set and balanced hormonal influence (120).

Neural mechanisms involve both the central and peripheral nervous system, including both autonomic (sympathetic and parasympathetic) and somatic (motor and sensory) nerves supplying the shaft and the glans penis (119, 120). Penile erections can be psychogenic, reflexogenic and non-sexual like nocturnal erections. In psychogenic erection, integration of imaginative, visual, olfactory and tactile stimuli initiates a central process which relays the sensual input onto the thoracolumbar erection centre (T11-L2) (121). These neural impulses act on the vascular bed in the pelvis, redirecting blood to the corpora cavernosa (119-122). The increased blood flow and pressure in the penile lacunar spaces results in erection and reduced venous outflow by compression of venous outflow maintains the erection (119-

122). The sacral erection centre (reflex arc with sacral roots S2-S4) is responsible for reflex erections resulting from tactile stimuli to the penis and genital area (119-122).

Intact blood flow from the hypogastric arterial system and high intra-penile nitric oxide (NO) are vital for increasing blood flow and penile erection (123). NO is released from the endothelium of the corpora cavernosa and cavernous nerves in response to local physical or central sexual stimulation (119). NO activates soluble guanylyl cyclase resulting in an increase in cyclic guanosine monophosphate (cGMP) levels, which causes smooth muscle relaxation and arteriolar dilation leading to penile erection (119, 123)(Figure 1.11).

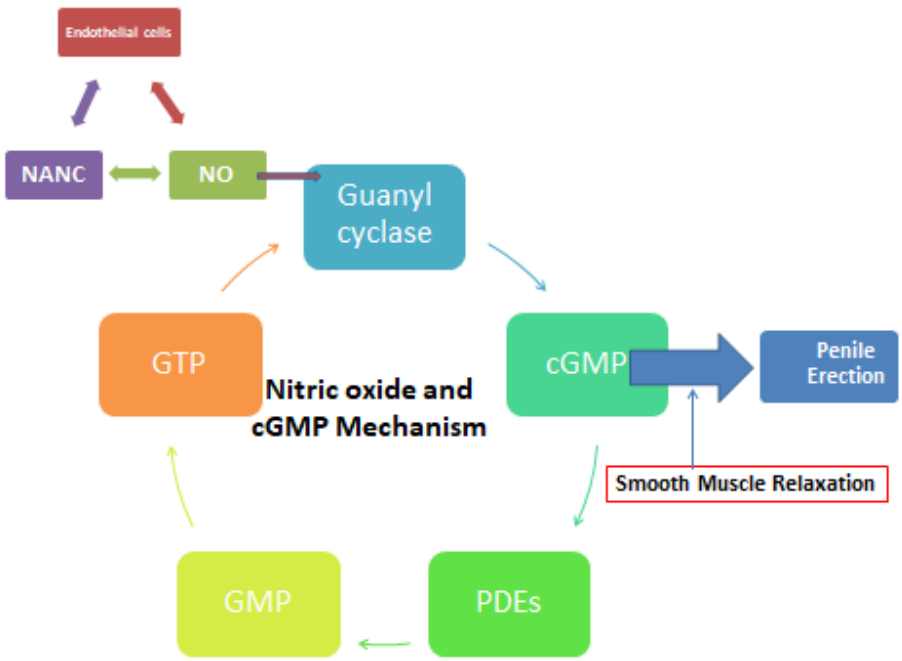


Figure 1.11. *Pathophysiology of the process of penile erection. This figure shows the chemical process involved in the process of penile erection. NANC: Noradrenergic noncholinergic , NO: Nitric oxide, c GMP: cyclic guanosine monophosphate, GMP: Guanosine monophosphate, GTP: Guanosine triphosphate, PDEs: Phosphodiesterase's*

Testosterone is vital for normal sexual function in men and studies in experimental animals and men show that testosterone deficiency is associated with erectile dysfunction (124). Testosterone not only boosts libido but also maintains normal levels of intra-penile nitric oxide synthase (125).

1.4.2 Erectile dysfunction in diabetes

Erectile dysfunction, decreased libido and abnormal ejaculation mainly affect men above 40 years of age (126). The prevalence of ED in men under 40 ranges from 1 to 10% (127), increasing to 20-40% in men aged 60-69 years and to 50-100 % in those above 70 years of age (128). The Massachusetts Male Aging Study (MMAS)study showed that the incidence of ED was 26 to 46 per 1000 man-years in men aged 60-69 years (129). It is predicted that ED will affect 322 million men worldwide by 2025 (130, 131). Diabetes is the commonest aetiology for ED and its prevalence ranges from 35 to 90 % in men with diabetes (131). The frequency of ED in diabetes increases with age, and the prevalence in one study increased from 6% in men aged 20 to 25 years to 52% in men aged 55 to 59 years (119). A longer duration of diabetes, suboptimal glycaemic control, peripheral neuropathy, autonomic neuropathy, retinopathy, hypertension, dyslipidaemia, smoking, sedentary life style and subnormal testosterone levels all contribute to an increased prevalence of ED in diabetes (131-133).

1.4.3 Mechanism of erectile dysfunction in diabetes

Organic, relational and psychological factors contribute to the development of ED (134) and include neurogenic, vasculogenic, endocrine and iatrogenic pathways (134) which mediate penile endothelial dysfunction and defective noradrenergic and cholinergic nerve signalling with increased cavernosal contractile sensitivity and impaired dilatory function (118).

Vascular abnormalities including penile smooth muscle and endothelial dysfunction and altered cavernosal haemodynamics play an important role in diabetes related ED (135).

Persistent hyperglycaemia leads to overproduction of advanced glycation end products (AGEs), increased hexosamine and protein kinase C (PKC) and increased flux through the polyol pathway, resulting in increased oxidative stress (136, 137) and overproduction of

reactive oxygen species (ROS) (136, 137). Endothelial dysfunction is complex, inducing micro-thrombosis, vasoconstriction and tissue inflammation which all contribute to ED (138, 139). An increase in activator protein 1 (AP-1) and nuclear factor kappa B (NF-kB) results in local tissue inflammation (138, 139), whereas reduced NO and increased tissue factor, plasminogen activator inhibitor-1 (PAF-1) and ET-1, leads to thrombosis and vasoconstriction (138, 139).

Neuropathy is a key factor in ED at all levels of the neural system and penile erection pathway (138). Studies have demonstrated evidence of large- and small-fibre as well as autonomic neuropathy in diabetic patients with ED (140-142). Indeed, some studies have identified cardiac autonomic and somatic neuropathy in ED and attributed failure of phosphodiesterase type-5 inhibitor therapy in patients with ED to the presence of small fibre and autonomic neuropathy (143-145).

Studies have shown that microangiopathy plays a more important role to ED in diabetes compared to macroangiopathy (146).

1.4.4 Pathophysiology of erectile dysfunction in diabetes and influence of cofactors

Obesity, hypertension, drug- treatment of hypertension, atherosclerosis, neuropathy, nephropathy, hypogonadism, depression and abnormalities of the lower urinary tract including penile structure play a substantial role in the pathophysiology of ED in DM (118).

1.4.5 Glycaemic control

Prolonged uncontrolled hyperglycaemia reduces NO activity, further reducing endothelium-dependent relaxation factors (147, 148). Men with poor glycaemic control and severe insulin resistance show a higher prevalence of ED (148), whereas intensive glycaemic control reduces the risk of developing ED up to 5 fold (148-150).

1.4.6 Metabolic syndrome and obesity in relation to hypogonadism and ED

Studies have shown a close link between metabolic syndrome, insulin resistance and ED (151, 152).Hermans et al, showed a close association of ED in T2DM with central adiposity,

metabolic syndrome and microangiopathy (153). Studies have also shown a higher relative risk of ED in diabetic patients with metabolic syndrome (154).

The underlying basis of hypogonadism in diabetes is not clear. Lower sex hormone binding globulin (SHBG) is closely linked to insulin resistance (155) and lower levels of SHBG may affect testosterone levels in DM and obesity (156). In obese men, visceral adipose tissue may have raised aromatase activity leading to increased conversion of testosterone to oestrogen, reducing serum testosterone levels (155). Reduced insulin activity in the hypothalamus as a consequence of insulin resistance has been proposed as a possible mechanism for hypogonadotropic hypogonadism in T2DM and obesity (156).

1.4.7 Autonomic and peripheral neuropathy

Autonomic neuropathy impairs parasympathetic activity, which is essential for smooth muscle relaxation in penile erectile tissue (157, 158). ED has been described as a forerunner of cardiac autonomic neuropathy and an independent cardiovascular risk factor (86, 159, 160). Sensory neuropathy impairs impulses from the shaft and glans to the reflexogenic erectile centre whereas motor neuropathy of pelvic pudendal nerves impairs contraction of cavernosal muscles, which is vital for preserving erection (86, 159, 160). In some cases dysfunction of penile nerves precedes somatic neuropathy (161).

1.4.8 Diabetic nephropathy

Diabetic nephropathy is the commonest cause of end stage renal failure in most countries (162). Albuminuria and reduced GFR are the hallmark of diabetic nephropathy (162). Albuminuria is associated with activation of proinflammatory and prothrombotic cytokines, increased levels of endothelin-1 and urotensin II which contribute to an imbalance between NO and NO synthase with vasoconstriction (163). Hence albuminuria is an independent risk factor for ED in diabetes (164).

1.4.9 Infections and penile structural diseases

Infections like chlamydia or cytomegalovirus are commoner in DM and lead to low grade inflammation, raised high sensitivity C-reactive protein (hs-CRP), fibrinogen levels and endothelial dysfunction (165). Balanitis is commoner in diabetes affecting 16 % compared to 5.8% of the general population (166). Pain and inflammation associated with balanitis impairs erectile function and sexual satisfaction (138). Peyronie`s disease and acquired phimosis impair erection (167-169) and their prevalence varies from 8.1-18.3 % and 12 %, respectively in diabetes (167-169).

1.4.10 Depression and psychological factors

Psychosexual factors and depression are associated with ED in diabetes (170-172). Bak et al, showed that symptoms of depression occurred in 42.5 % men with T2DM and ED compared to only 4.5% in controls (173). A number of studies have demonstrated a relationship between erectile dysfunction and depressive symptoms in men with T2DM (118, 174-176). Therefore psychological counselling is an important adjunct to the treatment of ED in diabetes (177).

1.4.11 Atherosclerosis and its effects on erectile function

There is increasing evidence of association between cardiovascular atherosclerotic disease (CVD) and ED (178, 179). ED precedes in onset by 5 years over other clinical manifestations of atherosclerotic cardiovascular disease including coronary artery disease, carotid and peripheral vascular disease(180). Hence ED represents “tip of iceberg” of atherosclerosis and related CVD (181). Vascular aetiology is the commonest cause of ED and it shares some common risk factors with atherosclerotic CVD including diabetes, hypertension, dyslipidaemia, smoking, obesity and sedentary life (182). Smaller sized penile arteries suffer earlier from atherosclerotic plaques and flow compromise due to obstruction as compared to larger coronary and carotid arteries (183). Therefore ED thus represents early clinical evidence of widespread atherosclerotic disease and endothelial dysfunction (183). Phosphodiesterase inhibitors (PDE5i) have shown positive effects on endothelial dysfunction including vasodilatation, thrombosis and inflammation (184).

1.4.12 Role of testosterone in erectile dysfunction

Though testosterone has well established role in increasing libido, its exact contribution to ED in men remains unclear. Animal data suggests that testosterone may be a vasodilator to penile vasculature and also contributes to penile vaso-occlusion which is vital to maintain erection (185, 186). Human studies have demonstrated possible direct and indirect evidence of vasodilatory effect of testosterone on cavernous vasodilation (187, 188), however further investigations are needed to clarify the effects of testosterone on erectile function, penile vasculature and higher centres of nervous system(189). Available evidence suggests that testosterone levels below normal limit are sufficient in most men to maintain erectile function (190).Erectile function is more likely to improve with testosterone therapy in men with severe degrees of hypogonadism (191-193). However, minimum testosterone levels to maintain erectile function remains unknown (190). Effectiveness of testosterone in ED is variable but superior to placebo and may improve response to PDE5i therapy (194-196). Therefore trial of testosterone treatment should be considered in hypogonadal men with documented low testosterone levels, unless there is any contraindication.

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Chapter 2: Hypothesis and Aims

Hypothesis and Aims

Diabetic peripheral neuropathy (DPN) is the commonest complication of diabetes, affecting both somatic and autonomic nerves. Small fibre neuropathy is the earliest and commonest type of diabetic neuropathy, primarily causing sensory symptoms and autonomic symptoms and deficits. Accurately diagnosing and quantifying the progression of DPN is the key to identify individuals who will progress to complications like foot ulceration, erectile dysfunction (ED) and to power clinical intervention trials.

Aims:

1. To evaluate the contribution of small and large fibre neuropathy to erectile dysfunction (ED) in men with type 2 diabetes.
2. To investigate the relationship between symptoms of sexual dysfunction, sex hormone levels and various measures of small fibre neuropathy.
3. To assess the longitudinal utility of different measures of neuropathy in DPN.

Chapter 3: Methodology

3.1 Preface

This chapter presents a comprehensive overview of the study design and assessments undertaken. As this thesis is being presented in journal format, this chapter overlaps with the methods section within each individual results chapter.

3.2 Study design

This thesis was completed using the results generated from three studies and the study design are described below.

3.2.1 Cross-sectional observational cohort study

Longitudinal assessment of novel ophthalmic diabetic markers, REC reference number: 09/H1006/38.

This was a cross-sectional observational cohort study of patients with type 2 diabetes mellitus. Patients were recruited from the Manchester University Hospital Diabetes centre. Study assessments were undertaken at the Manchester National Institute of Health Research / Wellcome Trust Clinical Research Facility (Manchester, UK). Study assessments included medical history, anthropometric measurements, neuropathy assessment, corneal confocal microscopy, skin biopsy, nerve conduction studies and laboratory measurements. Further details of each assessment are provided below. The ethical approval number of the study was 09/H1006/38 (see Appendix).

3.2.2 Prospective observational cohort study

Evaluation of corneal confocal microscopy as a surrogate endpoint for the identification and prediction of diabetic neuropathy (multinational study), REC reference: 16/NW/0729, IRAS project ID: 197851.

This was a prospective observational cohort study of patients with type 1 and type 2 diabetes mellitus. Patients were recruited from the Manchester University Hospital Diabetes centre. Study assessments were undertaken at the Manchester National Institute of Health Research/Wellcome Trust Clinical Research Facility (Manchester, UK). Study assessments

included medical history, anthropometric measurements, neuropathy assessment, corneal confocal microscopy, skin biopsy, nerve conduction studies and laboratory measurements at baseline and after a mean follow up period of 6.5 years. All the tests performed at baseline were repeated in the follow up study using the same protocol and equipment. Ethical approval number of the study was REC reference: 16/NW/0729, IRAS project ID: 197851 (see Appendix).

3.2.3. Cross-sectional observational cohort study 2

PROPANE study: Probing the Role of Sodium Channels in Painful Neuropathies, REC reference: 14/NW/0093, IRAS project ID: 143141.

This was a cross-sectional observational study of patients with type 1 and type 2 diabetes mellitus. Patients were recruited from the Manchester University Hospital Diabetes centre. Study assessments were undertaken at the Manchester National Institute of Health Research/Wellcome Trust Clinical Research Facility (Manchester, UK). Study assessments included medical history, anthropometric measurements, neuropathy assessment, sexual function assessment, corneal confocal microscopy and laboratory measurements. The ethical approval number for this study was REC reference: 14/NW/0093, IRAS project ID: 143141 (see Appendix).

Inclusion criteria for all 3 studies were:

- Aged 18 or above
- Type 1 diabetes mellitus or type 2 diabetes mellitus of any duration
- Ability to understand and cooperate with study procedures
- Able to provide informed consent

Exclusion Criteria for all 3 studies were:

- Neuropathy due to a non-diabetic cause (familial, alcoholic, nutritional, uremic etc)
- Current eye infection, corneal damage, or severe movement disorders which could preclude a safe CCM exam
- Allergy to Benoxinate hydrochloride 0.4% (ocular topical anaesthetic used for CCM)
- Dermatological and systemic disorders that might affect the cornea or skin
- Active diabetic foot ulceration or infection

Additional exclusion criteria for the PROPANE study were:

Patients known to have disease of the pituitary gland, testes or adrenal glands, or known to have cardiovascular disease, or on treatment for erectile dysfunction, or known to have conditions affecting androgen levels or causing erectile dysfunction and those with primary hypogonadism (LH > 9.4 U/L).

3.3 Ethics approval

These studies were conducted according to globally accepted standards of good clinical practice (as defined in the ICH E6 Guideline for Good Clinical Practice, 1 May 1996), in agreement with the Declaration of Helsinki (1964) and in keeping with local regulations. All 3 studies were approved by the Greater Manchester Central Research and Ethics Committee, the Manchester University National Health Service (NHS) Foundation Trust Research and Development office and the scientific Advisory Board of the Manchester National Institute of Health Research/ Wellcome Trust Clinical Research facility. All participants were provided with the patient information sheet (PIS) at least one week before the study visit and informed signed consent was obtained from all study participants prior to the study assessments. The study information sheet and consent form for the participants are attached in the appendix of this thesis (see Appendix). All study records are kept securely according to ICH guidelines for 10 years after closing the study.

3.4 Study procedures

Both type 1 DM and type 2 DM patients were consecutively recruited from the general diabetes clinic in the Manchester University Hospital Diabetes centre, during the respective study periods. All patients attended Wellcome trust research facility Manchester for study related visits after their recruitment from clinics.

3.4.1 Demographics and medical history

After informed consent all patients underwent a detailed medical history and anthropometric data were collected. Information regarding current and past co-morbidities, medication history and surgical procedures was collected during each study visit.

3.4.2 Anthropometric measurements

Clinical measurements included an assessment of height, weight, body mass index (BMI), blood pressure and heart rate, during each study visit. Blood pressure was measured using an automated blood pressure measuring device with appropriate cuff size. Three measurements of systolic and diastolic blood pressure were taken and the average of the last two readings was calculated.

3.4.3 Clinical neuropathy assessment

3.4.3.1 Assessment of neuropathy symptoms

The neuropathy symptom profile (NSP) was used to assess for symptoms of neuropathy. This questionnaire consists of 38 questions which cover symptoms relating to the sensory, motor and autonomic nervous system **(as in the appendix 8.6 page 201-211)**. The score 0 represents absence of any symptoms and maximum score of 38 represents the most severe symptoms of diabetic neuropathy.

3.4.3.2 Neuropathy Disability Score (NDS)

Assessment of neurological deficits was undertaken using the modified Neuropathy Disability Score (NDS). Each participant had both feet assessed while their eyes were closed to avoid visual bias during the examination. The maximum score for NDS is 10 with the severity of neuropathy was graded according to the NDS score (Table 3.1). Three sensory and 1 motor domain were tested:

1. Pain sensation: pin-prick examination using Neurotips (Owen Mumford Ltd, Oxford, UK). The forearm was used as a reference point. Patients with a blunted pain response scored 1 point for each foot respectively.
2. Vibration Sensation: using a tuning fork (128 Hz). Participants with blunted vibration sensation scored 1 point for each foot respectively.
3. Temperature Sensation: using ‘hot’ and ‘cold’ metal rods. Hot and cold water was used to warm or cool the metal rods respectively. Participants with blunted temperature sensation scored 1 point for each foot respectively.

4. Ankle Reflexes: using a reflex hammer, ankle reflexes were tested by asking patients to kneel on the examination couch with their ankles over the edge. If reinforcement (Jendrassik manoeuvre) was required to obtain an ankle reflex, then 1 point was added and if with reinforcement the reflex was absent, 2 points were given to the total score (per foot).

3.4.3.3 Quantitative Sensory Testing (QST)

QST was used to determine sensory and pain thresholds for cold and warm sensation using MEDOC TSA II Neurosensory Analyser (Ramat-Yishai, Israel) and vibration perception threshold using a Neurothesiometer (Horwell, Scientific Laboratory Supplies, Wilford, Nottingham, U.K.) (Figure 3.1). Both instruments use the method of limits algorithms.

TSA II Neurosensory Analyser consists of a thermal probe (thermode) attached to the main unit, which is capable of cooling or heating the probe depending on the stimulus. Participants used a computer mouse to record their responses to the stimuli. The whole unit is operated via a laptop on which manufacturer supplied software is pre-installed with mission-specific hardware.

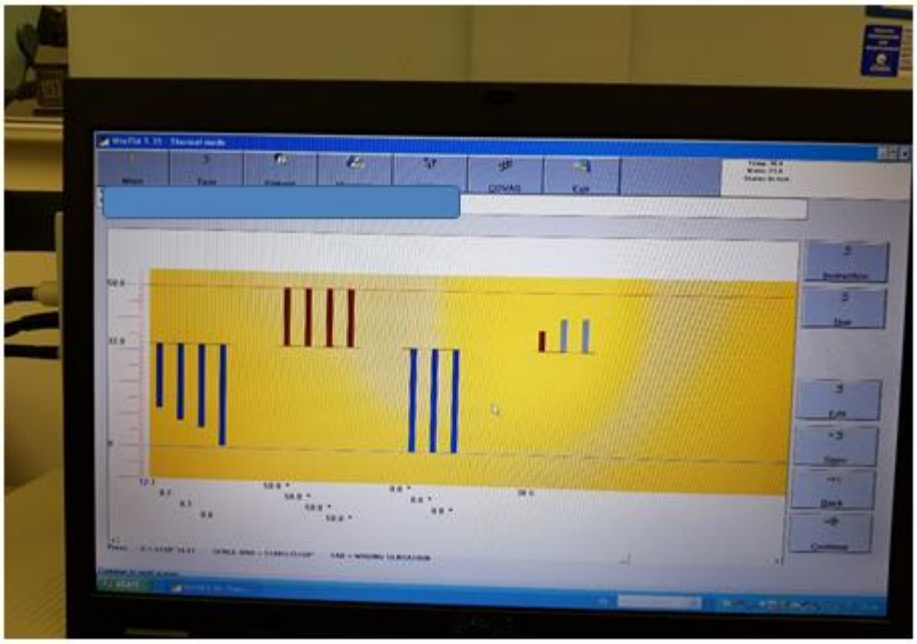

***Figure 3.1**. MEDOC TSA II Neurosensory Analyser for temperature threshold assessment. Bars in the figure show assessment of a patient for cold and warm temperature thresholds.*

The thermode was applied to the dorsum of non-dominant foot (S1 dermatome region) and the patient was asked to indicate the response by clicking the mouse (Figure 3.1 and 3.2). Thermal sensory element tests for four sub-sensory modalities:

1. Cold sensation (CS): 1-2 °C below adaptation temperature. This is the first moment the thermode felt cold (A-delta mediated sensation).
2. Warm sensation (WS): 1-2 °C above adaptation temperature. This is the first moment the thermode felt warm (C-fibre mediated sensation).
3. Cold induced pain (CIP): The normal threshold is approximately 10 °C . This is recorded as the moment of cold induced discomfort or pain (both C and A delta mediated sensation).
4. Warm induced pain (WIP): The normal threshold is approximately 45 °C. This is the point at which there is warm induced discomfort or pain (mostly C-fibre mediated sensation).

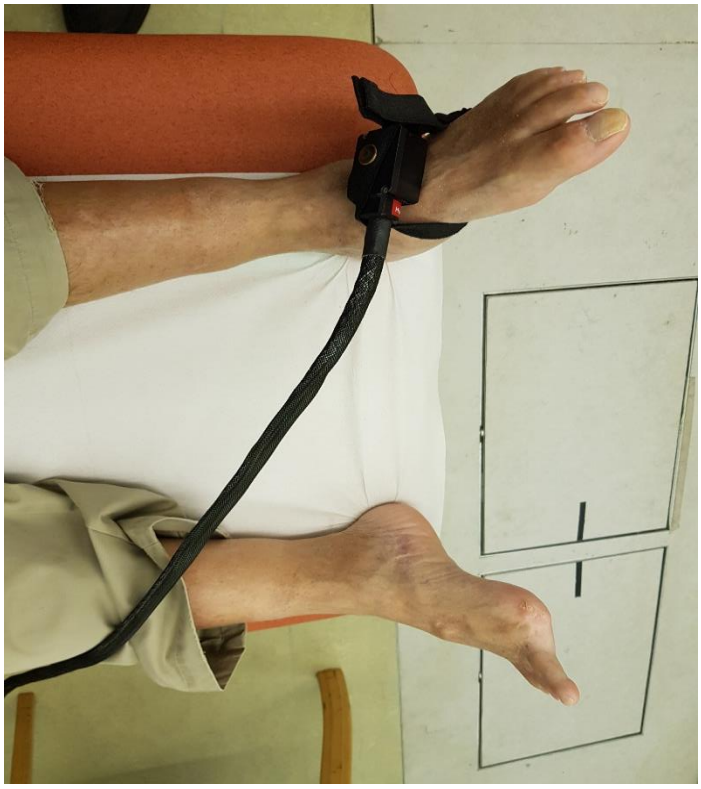

***Figure 3.2**. MEDOC TSA II Neurosensory Analyser for temperature threshold assessment, placement of the thermode on the non-dominant foot.*

Vibration perception threshold (VPT) was measured using a Neurothesiometer (Horwell Scientific, UK) and ranged from 0 to 50 volts, using the mean of 3 readings on each foot. The higher the voltage stimulus required to produce vibration sensation, the less perceptive the foot.

***Table 3.1** Neuropathy disability score*

	Right Foot			Left Foot		
Test	Normal	Reinforcement (ankle reflex only)	Abnormal	Normal	Reinforcement (ankle reflex only)	Abnormal
Pain	0		1	0		1
Vibration	0		1	0		1

Temperature	0		1	0		1
Ankle reflex	0	1	2	0	1	2
Total NDS						/10

Table 3.1 *NDS = Neuropathy disability score. 0 = normal, 1= abnormal (except for ankle reflex) 1 for ankle reflex = present with reinforcement, 2= abnormal ankle reflex.*

Table 3.2*. Severity of neuropathy based on the NDS Score*

Severity of neuropathy	NDS Score
Normal	0-2
Mild peripheral neuropathy	3-5
Moderate peripheral neuropathy	6-8
Severe peripheral neuropathy	9-10

Table 3.2*. Severity of neuropathy based on the NDS score.NDS= Neuropathy disability score*

3.4.3.4 Assessment of autonomic dysfunction

Autonomic symptoms were assessed by evaluating autonomic symptoms as part of the NSP and autonomic function was assessed using the ANX 3.0 Autonomic Nervous System Monitor (Figure 3.3) by evaluating resting and postural heart rate, breathing rate, blood pressure, and R-R variation using beat-to-beat heart rate variability as endorsed by the American Diabetes Association. Sudomotor function was assessed on the plantar aspect of both feet, using Neuropad (Trigocare, Germany). A change of colour from blue to pink indicates normal sweating.

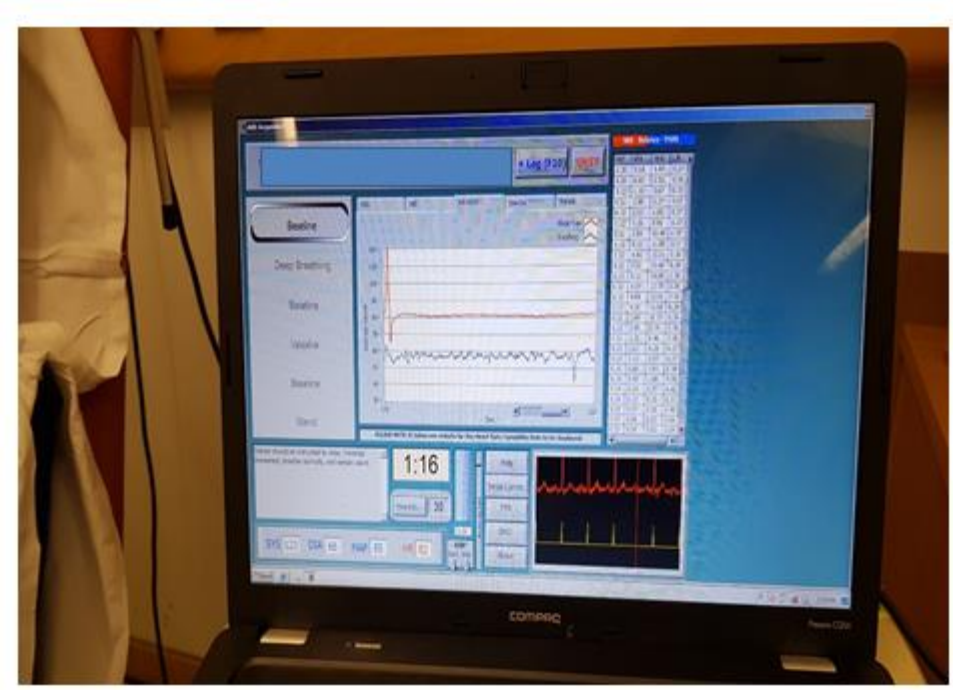

Figure 3.3. *Assessment of autonomic function on the ANSAR (ANX 3.0 Autonomic Nervous System Monitor), graphs on the screen showing real time patient assessment.*

3.4.3.5 In Vivo Corneal Confocal Microscopy

Real time assessment of small nerve fibres in the cornea was undertaken by Drs Maryam Ferdousi and Alise Kalteniece, using in vivo corneal confocal microscopy (IVCCM). All participants underwent assessment of both eyes, visualising the sub-basal layer of the cornea with the Rostock Cornea Module of the Heidelberg Tomograph HRT III Confocal microscope (Heidelberg Eye Explore, Heidelberg Engineering GmBH and Germany) lens of the microscope was disinfected using a medicated swab (Isopropyl alcohol 70%v/v, Swabs). Patients' eyes were anaesthetised with topical anaesthetic (0.4% benoxinate hydrochloride, Chauvin Pharmaceuticals Ltd., Essex, UK). Viscotears (Carbomer 980 0.2 %, Novartis, UK) was used as an eye lubricant as well as a coupling medium between the tip of the lens and the sterile TomoCap. The camera was adjusted to the lowest position and refraction of the objective lens was set at +12 dioptres. Optimal image acquisition was ensured with adjustment for depth and resolution of the camera.

Figure 3.4 *The window of image captured by the corneal confocal microscope. This figure illustrates real time capture of corneal nerve images using IVCCM (Courtesy of Dr. Maryam Ferdousi).*

The patient was asked to focus onto the white fixation light while the examiner aligned the lens onto the central cornea. The camera was then slowly advanced so that the TomoCap was in contact with the cornea. The camera was moved 1µm backward and forward at a time and images were acquired from different depths of the sub-basal layer. The pre-installed real-time image acquisition software on the laptop connected to the confocal microscope was used during this process. For each eye, three high quality images from each eye (6 for each patient) were selected from the sub-basal layer for exporting and final analysis as per a recently published protocol (1) (Figure 3.4).

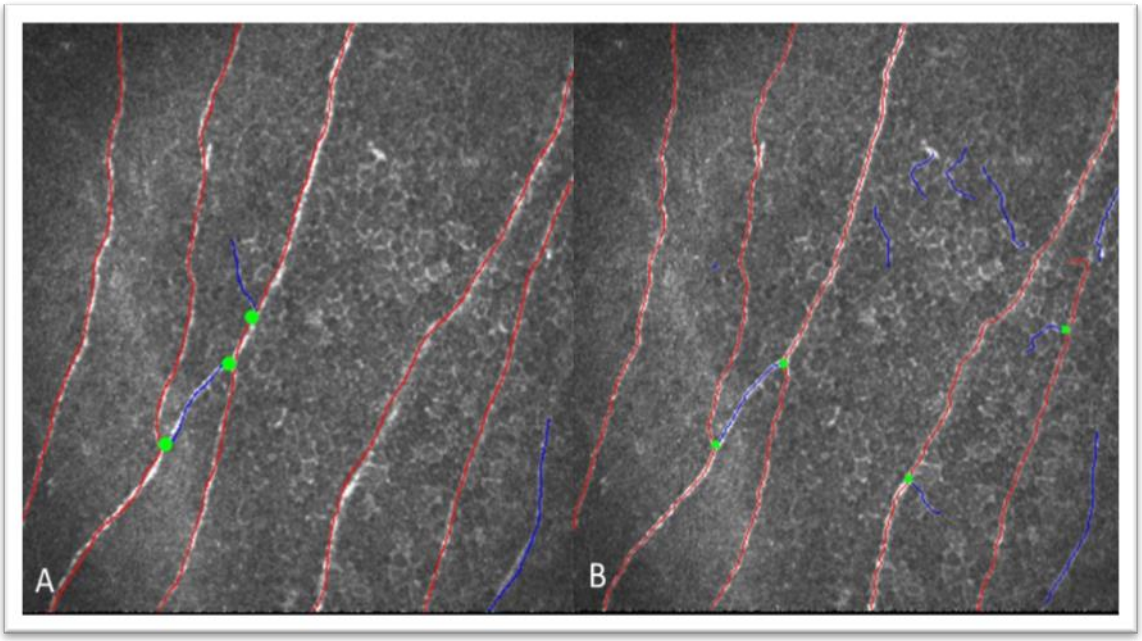

Figure 3.5. *This diagram shows CCM image analysis using automated and semi-automated software. Each nerve parameter is shown in a different colour. Red lines indicate main nerve fibres, blue lines show branches of nerve fibres and green dots indicate nerve junctions identifying branch points. A) Image analysis by semi-automated software CCMIAv0p6 (M.A. Dabbah, Imaging Science and Biomedical Engineering, The University of Manchester); B) Image analysis by automated software ACCMetrics32 (M.A. Dabbah, Imaging Science, The University of Manchester, 2010)(Courtesy Dr. Maryam Ferdousi).*

Images were analysed using specialised automated analytical software (ACCMetrics32, by M.A. Dabbah, Imaging Science and Biomedical Engineering, The University of Manchester, UK) (Figure 3.5). The corneal nerve parameters used for quantifying corneal small nerve fibres were as follows:

CNFD- Corneal Nerve Fibre Density- major nerves/mm2

CNBD- Corneal Nerve Branch Density – nerve branches/mm2

CNFL- Corneal Nerve Fibre Length – length of nerves/mm2

3.4.3.6 Nerve Conduction Studies (NCS)

The electrophysiological studies were performed on the non-dominant limb peroneal and sural nerves, using a Dantec "Keypoint" system (Dantec Dynamics Ltd, Bristol, UK) which is accredited by the American Association of Neuromuscular and Electrodiagnostic Medicine.

A DISA temperature regulator was used to maintain limb temperature between 32 to 35°C.

Surface electrodes (Silver chloride) of 9 mm diameter were used for all the studies and a standardised local protocol was followed. The stimulus used was supra-maximal and a belly-tendon electrode was used for motor assessment. The electrode placement was as shown in the Table 3.3.

The stimulus strength was increased until a maximal response was obtained and then further increased by 10 to 15% to produce a supramaximal response. Both sensory and motor amplitudes were measured from baseline to the negative peak. The sensory nerve action potential was reported to the nearest 0.1µV and the motor action potential was reported to the nearest 0.1 mV.

The specific parameters measured were as follows- sural nerve amplitude potential, latency and conduction velocity, peroneal nerve and tibial nerve amplitude potential, latency, F wave latency and conduction velocity.

All the electrophysiological assessments were undertaken by Dr Andrew Marshall, a consultant clinical neurophysiologist. The amplitude potentials were adjusted for the participant`s age and F-wave latency for their height. Values were considered as abnormal if they were greater than 99th percentile or less than 1st percentile in reference to the local healthy population database.

Table 3.3 *Electrode placement for Nerve Conduction Study (NCS)*

Motor/ Sensory Modality	Nerve	Recording	Stimulation
Motor nerve conduction	Peroneal	Extensor Digitorum Brevis (EDB)	Ankle (60mm proximal to active

			recoding electrode), 5cm proximal and 5cm distal to fibular head
Sensory Nerve Conduction	Sural	Ankle	Calf (140mm proximal to active recording electrode)

Table 3.3 *Electrode placement for Nerve Conduction Study (NCS). For motor nerves the peroneal nerve was tested on the non-dominant side and for sensory nerves the sural was tested.*

3.4.4 Skin biopsy and immunohistochemistry

All participants from the prospective observational study had a 3mm punch skin biopsy taken from their non-dominant foot. Local anaesthetic (1% lignocaine) was injected sub dermally in the area to be biopsied. Two samples were taken from each participant, from the dorsum of foot, 2 cm above the second metatarsal head. The biopsy site was closed and dressed using Steri-strips. The biopsy specimen was fixed in PBS-buffered 4% paraformaldehyde, immediately after the biopsy procedure. All the specimen were washed in Tris-buffered saline(TBS) and cryoprotected in sucrose. They were frozen in liquid nitrogen and stored at -80 °C.

The biopsy was cut into 50 µm sections on a cryostat microtome. Five floating sections per subject were immunostained for PGP9.5 neuronal marker using anti-human PGP 9.5 antibody (Abcam, Cambridge, UK). Non-specific protein binding and endogenous peroxidase activity were blocked by incubation in 5% goat serum and 0.3% hydrogen peroxide, respectively. The anti-PGP9.5 antibody (EMD Millipore, Billerica, MA, USA) was followed by goat anti-rabbit IgG and then by HRP-Streptavidin (both diluted 1 : 1000, Vector Laboratories, Peterborough, UK). SG chromogen (Vector Laboratories, Peterborough, UK) was used to demonstrate and visualise nerve fibres. Intraepidermal nerve fibre density

(IENFD) was quantified as the number of nerve fibres crossing the basement membrane of the epidermis and expressed as the number per millimetre length of epidermis.

The colour images were captured with an image analysis camera (Sony 2CCD, CCD-IRIS) and a computer program (Leica QWin Standard V2.4, Leica Microsystem Imaging Ltd, Clifton Road, Cambridge, CB1 3QH, England) was used to trace and quantify intra-epidermal nerve fibre morphology. Ideally, at least three sections per case were assessed and the average calculated. The biopsies were assigned a coded number and all nerve morphology assessments were performed blinded to the case diagnosis.

The follow up skin biopsy samples were taken from the same foot, in close proximity to the scar of the first examination and the same procedure was followed. IENFD was calculated by Dr. Maria Jeziorska (Senior Lecturer in Molecular Pathology in the Division of Diabetes, Endocrinology and Gastroenterology, School of Medical Sciences, Faculty of Biology, Medicine and Health at the University of Manchester). All the biopsies in the follow up study were performed by Dr. Shaishav S Dhage.

3.4.5 Assessment of sexual function

The European Male Ageing Study Sexual Function Questionnaire (EMAS-SFQ) was used to assess sexual function of the participants (2). The EMAS-SFQ is a 22 item questionnaire for men between ages 40 and 75 years, validated for its retest and internal consistency. For the assessment of overall sexual function, 3 sexual symptoms, most commonly associated with hypogonadism (3, 4) were selected: erectile function, frequency of sexual thoughts and frequency of morning erections. Previously established cut-offs based on validated scores for each individual question relating to these three sexual symptoms were used to divide participants into 2 groups, symptomatic and asymptomatic (Table 3.4) based on validated criteria (4).

Table 3.4. *EMS-SFQ questions and definitions of asymptomatic and symptomatic categories*

SFQ	Asymptomatic	Symptomatic
Were you able to get and keep an erection sufficient for sexual intercourse?	Usually or always	Never or sometimes
How often did you think about sex?	Once a week or more	2-3 times in the past month
How frequently did you awaken with a full erection in the past month ?	2-3 times in the past month	≤ 1 time in the past month

Table 3.4. *EMS-SFQ questions and definitions of asymptomatic and symptomatic categories, reproduced from Wu et al. (3)*

3.4.6 Laboratory measurements

Fasting venous samples were obtained between the hours of 0800 to 1000.

3.4.6.1 Separation of plasma and serum

Serum and EDTA-plasma were isolated by centrifugation at 3300rpm for 15minutes at 4°**C** within 2 hours of collection. Serum and plasma aliquots were stored at -20◦c or -80°**C** until analysed. Only one freeze-thaw cycle was used for each serum or plasma aliquot.

3.4.6.2 Glycated haemoglobin (HbA1c)

High performance liquid chromatography (HPLC) using a VARIANT II Turbo Haemoglobin Testing System (Bio-Rad D-100, Bio-Rad Laboratories, Hemel Hempstead, UK) in the Department of Biochemistry, Manchester University NHS Foundation Trust, was used to measure HbA1c on the day of study visit.

3.4.6.3 Sex hormones

Serum total testosterone, dihydrotestosterone, dehydroepiandrosterone sulphate and androstenedione levels were measured using liquid chromatography-tandem mass spectrometry in the validated clinical laboratory of the Department of Biochemistry at Manchester University NHS Foundation Trust (5, 6). Sex hormone binding globulin (SHBG), follicular stimulating hormone (FSH) and luteinising hormone (LH) were measured using electrochemiluminescence immunoassay (Roche Diagnostics, Basel, Switzerland) on Roche automated analysers (E170 platform). Serum free testosterone levels were derived from the patient`s albumin, SHBG and total testosterone using the mass action equation described by Vermeulen (7). A serum total testosterone level of less than 8 nmol/L or total testosterone level between 8 and 11 nmol/L with calculated free testosterone level less than 220pmol/L was considered as a “low testosterone” (3).

3.4.6.4 Lipid profile

Cobas lipid panel (Cobas b 101) was used to quantitatively determine total cholesterol (TC), high density lipoprotein cholesterol (HDL-C) and triglycerides (TG). Roche Cobas c 702 (Cobas® 8000) analyser instruments in the Department of Biochemistry, Manchester University NHS Foundation Trust, were used to measure lipid profile on the day of study visit. A calculated value of low-density lipoprotein cholesterol (LDL-C) was provided by the Cobas b 101 system. TC, HDL-C and TG were determined by enzymatic method. A standard local protocol was followed.

3.5 Statistical analysis

This section provides an overview of the statistical analysis undertaken in this study. Detailed descriptions of the statistical analysis performed are provided within individual results chapters.

Statistical analyses were performed using SPSS for Mac (Version 23.0, IBM SPSS Statistics, Arnok, NY: IBM Corp.) and GraphPad Prism for Mac OS X (version 8.3.0, GraphPad Software, San Diego, California USA, www.graphpad.com). Figures were produced using the same software of GraphPad Prism. Data were tested for normality using the Shapiro-Wilk normality test and visualisation of histograms and Q-Q plots. Results were presented as mean $\pm$ standard deviation (SD) for parametric data and mean and interquartile range for non-parametric variables.

Continuous variables were compared between groups using the independent sample t-test and the Mann-Whitney U test was used for non-normally distributed data. The Chi-squared test was used for analysis of categorical data.

Continuous variables were compared between baseline and follow up visits using the paired t-test for normally distributed data and Wilcoxon matched-pairs signed rank test for non-normally distributed data. Ordinary one-way ANOVA was performed (Kruskal-Wallis test was used for non-normally distributed data) to compare between group differences of controls and baseline patient values. Post-hoc corrections for multiple comparison testing were done using Tukey's test.

Correlations between variables were assessed using Pearson's test for parametric and Spearman's analyses for non-parametric variables.

No attempt was made to adjust for missing data. The level of statistical significance was set at less than 0.05 for all analyses.

3.6 References

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Chapter 4 : Small fibre pathology is associated with erectile dysfunction in men with type 2 diabetes

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Author`s contribution : Shaishav S. Dhage researched the data(patient recruitment, clinical assessments, venous blood sample collection), analysed and interpreted data. In addition, he also researched the available literature and wrote the first draft of the manuscript. He also critically reviewed the final draft of the manuscript. The manuscript has formed the basis of this chapter.

Abstract

Aims

The aim of this study was to evaluate the contribution of small- and large-fibre neuropathy to erectile dysfunction (ED) in men with type 2 diabetes (T2D).

Methods

Measures of small and large fibre neuropathy were evaluated in 49 participants with T2D and 20 age-matched controls.

Results

ED was present in 59% of participants with T2D. There was no difference in age, duration of diabetes, blood pressure, lipid profile, vibration perception threshold (V) (14.3±7.8 vs 11.2±6.6, P=0.429), peroneal (41.4±8.2 vs 44.8±4.4, P=0.10) and sural (45.4±5.6 vs 47.1±5.8) nerve conduction velocities (m/s), cold (25.1±3.8 vs 26.2±2.9, P=0.815) and warm (43.2±4.0 vs 41.0±3.8) perception thresholds (^{0}C) and deep breathing-heart rate variability (18±8 vs 18±8) between participants with and without ED. However, intraepidermal nerve fibre density (no./mm^2)(4.6±2.8 vs 13.7±2.7, P<0.001), corneal nerve fibre density (no./ mm^2) (23.5±6.8 vs 31.3±8.2, P<0.001), corneal nerve fibre branch density (no/mm^2) (55.4±35.3 vs 97.7±46.4, P=0.004), corneal nerve fibre length (mm/mm^2) (17.6±6.8 vs 27.3±6.8, P<0.001) and sural (7.7±6.1 vs 14.6±6.7, P=0.003) and peroneal (2.5±2.0 vs 4.7±2.0, P=0.003) nerve amplitudes (μV), were significantly lower in participants with ED compared to those without ED.

Conclusion

ED affects almost 2/3 of men with T2D and is associated with small nerve fibre damage, but preserved nerve conduction and cardiac autonomic function. Corneal confocal microscopy may serve as a useful non-invasive imaging method to identify small fibre damage in patients with T2D and ED.

Key Words

Corneal Confocal Microscopy, Erectile dysfunction, Neuropathy, Small Fibre Neuropathy, Type 2 Diabetes

Abbreviations

AUC	Area Under Curve
BMI	Body Mass Index
CAN	Cardiac Autonomic Neuropathy
CCM	Corneal Confocal Microscopy
cGMP	cyclic Guanosine Monophosphate
CNFD	Corneal Nerve Fibre Density
CNBD	Corneal Nerve Fibre Branch Density
CNFL	Corneal Nerve Fibre Length
CT	Cold Threshold
DB-HRV	Deep Breathing Heart Rate Variability
DM	Diabetes Mellitus
ED	Erectile dysfunction
eGFR	Estimated Glomerular Filtration Rate
EMAS-SFQ	European Male Ageing Study Sexual Function Questionnaire
HbA1c	Glycosylated Haemoglobin
HDL-C	High Density Lipoprotein- Cholesterol
IENFD	Intraepidermal Nerve Fibre Density
IIEF	International Index of Erectile Function
LDL-C	Low Density Lipoprotein- Cholesterol
MDRD	Modification of Diet in Renal Disease
NSP	Neuropathy Symptom Profile
NO	Nitric oxide
NDS	Neuropathy Disability Score

PDE-5	Phosphodiesterase 5
QST	Quantitative Sensory Testing
SST	Sympathetic Skin Response
T1D	Type 1 Diabetes
T2D	Type 2 Diabetes
VPT	Vibration Perception Threshold
WT	Warm Threshold

Introduction

Erectile dysfunction (ED) is the persistent inability to achieve or maintain penile erection for satisfactory sexual intercourse (1). Diabetes mellitus(DM) is the commonest aetiology for ED (2) and it ranges in prevalence from 35 to 90% (2). Multiple risk factors including age, duration of diabetes, suboptimal glycaemic control, hypertension, dyslipidaemia, smoking, sedentary life style and subnormal testosterone levels have been associated with ED in diabetes (2-4). ED is also a marker of cardiovascular disease and has been associated with poorer generic and disease-specific quality of life among men with both type 1(T1D) and type 2 diabetes(T2D) (5-7).

Organic, relational and psychological factors contribute to ED and include neurogenic, vasculogenic, iatrogenic and endocrine pathways (8, 9), which mediate penile endothelial dysfunction and defective noradrenergic and cholinergic nerve signalling with increased cavernosal contractile sensitivity and impaired dilatory function (10). Vascular abnormalities including penile smooth muscle and endothelial dysfunction and altered cavernosal haemodynamics have been considered to play a major role in diabetes related ED (11), whilst the impact of neuropathy has been underestimated (12). Indeed, some studies have identified an association between cardiac autonomic and somatic neuropathy and ED and attributed failure of phosphodiesterase type-5 inhibitor therapy(PDE5) in patients with ED to the presence of small fibre and autonomic neuropathy (13-15).

The contribution of neuropathy to ED has been assessed using symptoms, elevated vibration perception and loss of reflexes (16), neurophysiology (17, 18), quantitative sensory testing (QST) and the sympathetic skin response (SSR) (19-23). Neurophysiology only assesses large fibres, QST is subjective (24), SSR is highly variable and a more objective measure like intraepidermal nerve fibre density (IENFD) is invasive. Corneal Confocal Microscopy (CCM) is a rapid, highly objective and easily reproducible technique that can quantify small nerve fibre damage in diabetes (25), comparable to IENFD (26). We have previously shown that patients with T1D and ED show greater small, large and autonomic nerve fibre damage (27).

In this study, we aimed to assess the relationship between different measures of small and large fibre as well as autonomic neuropathy with ED in an unselected cohort of men with T2D.

Methods

Participant selection

Forty-nine consecutive men with T2D were recruited from the Manchester University Hospital Diabetes Centre along with 19 age-matched healthy control participants. The control group comprised healthy volunteers without diabetes mellitus and were not on any regular medications for other co-morbidities. Patients with a history of neuropathy from another cause, corneal trauma or surgery, ocular disease, dermatological disorders and systemic disease that might affect the cornea or skin were excluded. This study has approval from the Central Manchester Research and Ethics Committee. Written informed consent was obtained from all individuals prior to participation.

Assessment of erectile function

Erectile dysfunction was identified from the Neuropathy Symptom Profile (NSP) defined by the inability to have penile erection not due to medication or prostate surgery (28).

Laboratory measurements

Glycosylated haemoglobin (HbA1c), total cholesterol, LDL-cholesterol, HDL-cholesterol, triglyceride, and creatinine were measured using standard laboratory methods in the Department of Biochemistry, Manchester University NHS Foundation Trust. Estimated glomerular filtration rate (eGFR) was calculated using the abbreviated Modification of Diet in Renal Disease (MDRD) equation: $186 \times (\text{creatinine} / 88.4)^{-1.154} \times (\text{age})^{-0.203} \times (0.742 \text{ if female}) \times (1.210 \text{ if black})$.

Assessment of neuropathy

The NSP was used to assess for symptoms of neuropathy. Neurological deficits were evaluated using the modified Neuropathy Disability Score, which is comprised of vibration perception, pinprick, temperature sensation and the presence or absence of ankle reflexes (28, 29). The vibration perception threshold (VPT) was established using a Horwell Neurothesiometer (Scientific Laboratory Supplies, Wilfrod, Nottingham, UK). Cold (CT) and warm (WT) perception thresholds were assessed on the dorsolateral aspect of the left foot using the TSA-II NeuroSensory Analyser (Medoc, Ramat-Yishai, Israel). Electrodiagnostic studies were undertaken using a Dantec Keypoint system (Dantec Dynamics, Bristol, UK). Sural sensory and peroneal motor nerve amplitude, conduction velocity and latency were assessed by a consultant neurophysiologist. The motor nerve study was performed using silver/silver chloride surface electrodes at standardised sites defined by anatomical landmarks, and recordings for the sural sensory nerve were taken using antidromic stimulation over a distance

of 100 mm. Deep breathing heart rate variability (DB-HRV) was assessed using an ANX 3.0 autonomic nervous system monitoring device (ANSAR Medical Technologies, Philadelphia, PA, USA).

Skin biopsy

A 3mm punch skin biopsy was performed 2 cm above the second metatarsal on the dorsum of the foot under local anaesthesia using 1% lidocaine. 50µm sections were stained using anti-human PGP9.5 antibody (Abcam, Cambridge, UK). SG chromogen (Vector Laboratories, Peterborough, UK) was used to demonstrate nerve fibres and IENFD was quantified using previously established criteria and expressed as number per millimetre (30).

Corneal confocal microscopy

Corneal confocal microscopy (CCM) (Heidelberg Retinal Tomograph III Rostock Cornea Module; Heidelberg Engineering, Heidelberg, Germany) was performed in all participants according to our previously established protocol (25). Six non-overlapping images from the centre of the cornea were selected per participant (three per eye). Corneal nerve fibre density (CNFD); the total number of major nerves per square millimetre of corneal tissue, corneal nerve branch density (CNBD); the number of branches emanating from the major nerve trunks per square millimetre of corneal tissue and corneal nerve fibre length (CNFL); the total length of all nerve fibres and branches [millimetre per square millimetre] within the area of corneal tissue were quantified (Figure 1 : representative CCM images). Analysis of corneal nerve morphology was performed using automated software, ACC Metrics (Manchester, UK) (31).

Statistical analyses

Statistical analyses were performed using SPSS for Mac (Version 23.0; IBM Corporation, New York, NY, USA). Data were tested for normality using the Shapiro–Wilk normality test. Continuous variables were compared between patients with and without ED using the independent samples t-test for normally distributed data, Mann-Whitney U test for non-normally distributed data, and chi-squared test for categorical data. ANCOVA was used for comparisons adjusted for age, beta-blocker and diuretic use. Data were presented as mean and standard deviation. Correlations between erectile dysfunction and other variables were assessed using point-biserial correlation. A *P*-value of less than 0.05 was considered to be statistically significant.

Results

Type 2 diabetes vs controls

Patients with T2D were well matched for age with the control group (62.0±8.1 vs 60.6±7.2, *P*=0.475). The prevalence of erectile dysfunction was 59.2% in patients with T2Ds, whilst none of the control participants reported symptoms of erectile dysfunction. Body mass index(BMI) (30.6±4.9 vs 27.1±3.9 kg/m^2, *P*=0.008) and HbA1c (57±13 vs 40±3 mmol/mol, *P*<0.001) were higher, and total cholesterol (4.0±1.0 vs 5.2±0.8 mmol/l, *P*<0.001), HDL-C (1.16±0.33 vs 1.55±0.40 mmol/l, *P*<0.001), and LDL-C (1.9±0.9 vs 2.9±0.7 mmol/l, *P*<0.001) were lower among patients with T2D compared to controls. Triacylglycerols (2.0±1.3 vs 1.7±0.7 mmol/l, *P*=0.221) and eGFR (77±17 vs 84±8 ml min^{-1} [1.73 $m]^{-2}$, *P*=0.085) did not differ between patients with type 2 diabetes and controls. Patients with T2D had a higher NSP score (4.4±4.8 vs 0.3±0.8, *P*<0.001), NDS (2.9±2.3 vs 0.9±1.2, *P*<0.001), VPT (13.0±7.4 vs 9.2±5.9, *P*=0.036), WT (42.3±4.0 vs 38.5±3.1 °C, *P*=0.001) and lower CT (42.3±4.0 vs 38.5±3.1 °C, *P*=0.002) and DB-HRV (18±8 vs 25±12 beats/min, *P*=0.027). Peroneal nerve amplitude (3.4±2.3 vs 5.1±1.7 mV, *P*=0.003) and peroneal nerve conduction velocity (42.8±7.0 vs 46.1±3.7 m/s, *P*=0.018) were lower in patients with type 2 diabetes but there was no difference in sural nerve amplitude (10.6±7.2 vs 12.6±6.3 μV, *P*=0.227) or sural nerve conduction velocity (46.1±5.7 vs 47.9±3.9, m/s *P*=0.161). IENFD (7.7±5.2 vs 15.9±3.2 no./mm, *P*=0.004), CNFD (26.7±8.3 vs 37.6±5.9 no./mm^2, *P*<0.001), CNBD (72.5±44.9 vs 93.9±30.1 no./mm^2, *P*=0.038) and CNFL (21.5±8.3 vs 26.6±4.4 mm/mm^2, *P*=0.003) were significantly lower in patients withT2D (supplementary table 1).

Type 2 diabetes with and without erectile dysfunction

There was no significant difference in age, BMI, duration of diabetes, HbA1c, lipid profile, blood pressure or eGFR in patients with T2D with and without erectile dysfunction (Table 1 and Figure 2). Both groups were well matched for the use of beta-blockers and diuretics.

NSP (6.0±5.1 vs 2.0±3.0, *P*<0.001) and NDS (3.4±1.9 vs 2.3±2.6, *P*=0.001) scores were significantly higher and IENFD (4.6±2.8 vs 13.7±2.7 no./mm^2, *P*<0.001), CNFD (23.5±6.8 vs 31.3±8.2 no./mm^2, *P*<0.001), CNBD (55.4±35.3 vs 97.7±46.4 no./mm^2, *P*=0.004), and CNFL (17.6±6.8 vs 27.3±6.8 mm/mm^2, *P*<0.001) were lower in patients with ED (Table 2 and Figure 1). VPT, CT, WT and DB-HRV did not differ significantly between T2D with and without ED. Sural (7.7±6.1 vs 14.6±6.7 μV, *P*=0.003) and peroneal (2.5±2.0 vs 4.7±2.0 mV, *P*=0.003) nerve amplitude was lower in patients with T2D and ED, but there was no significant difference in sural (45.4±5.6 vs 47.1±5.8 mV, *P*=0.530) and peroneal (41.4±8.2 vs 44.8±4.4 mV, *P*=0.101) nerve conduction velocity.

Correlations

There were significant correlations between ED and NSP (r=-0.418, P=0.003), CNFD (r=-0.466, *P*=0.001), CNBD (r=-0.468, *P*=0.001), CNFL (r=-0.578, *P*<0.001), IENFD (r=-0.853, *P*<0.001), sural (r=-0.477, *P*=0.001) and peroneal nerve (r=-0.478, *P*=0.001) amplitude. There was no correlation between ED and sural (r=-0.152, *P*=0.301) and peroneal (r=-0.246, *P*=0.095) nerve conduction velocity, NDS (r=0.256, *P*=0.076), VPT (r=0.207, *P*=0.159), CT (r=-0.160, *P*=0.277), WT (r=0.273, *P*=0.061) or DB-HRV (r=-0.005, *P*=0.977). There were no significant correlations between ED with age, BMI, duration of diabetes, systolic and diastolic BP, HbA1c, eGFR, total cholesterol, HDL, triacylglycerols and LDL(supplementary table 2).

Discussion

Almost 2/3 of men with T2D have erectile dysfunction, which was associated with small-fibre neuropathy rather than autonomic or large fibre neuropathy. Previous studies have reported an association between ED and symptomatic peripheral and autonomic neuropathy (32, 33). Unlike previous studies in men with T2D showing an association between ED and duration of diabetes, older age, suboptimal glycaemic control, hypertension, hyperlipidaemia and obesity (2, 33, 34) we found no correlation between ED and HbA1c, BMI, duration of diabetes, hypertension or lipid profile.

The physiology of erection is a complex interplay of psychogenic, hormonal and noradrenergic, non-cholinergic neurovascular mechanisms (35). Nitric oxide (NO) is released from the endothelium of the corpora cavernosa and cavernous nerves in response to local physical or central sexual stimulation (35). NO activates soluble guanylyl cyclase resulting in an increase in cyclic guanosine monophosphate (cGMP) levels (35), which causes smooth muscle relaxation and arteriolar dilation leading to penile erection (35). Thus penile erection involves both autonomic (sympathetic and parasympathetic) and somatic (motor and sensory) nerves supplying the shaft and glans of the penis (36). The aetiology of ED in diabetes is considered to be due to both vascular and neuronal dysfunction (2, 32, 33) and indeed PDE5 inhibitors promote NO release and mediate increased penile blood flow (33, 37, 38). The relationship between ED and neuropathy is complex, but studies have demonstrated evidence of large and small fibre neuropathy and autonomic neuropathy in diabetic patients with ED (21-23). In a cohort of 341 patients with ED the prevalence of neuropathy assessed using nerve conduction studies and QST was 38% in those with diabetes and 10% in those without diabetes. However, the prevalence of neuropathy among those with neurogenic (21%) compared to vasculogenic (23%) ED was comparable (20). Wellmer *et al.* showed no difference in neurological

examination or thermal sensory thresholds, but there was a difference in the capsaicin induced sensory-axon reflex and sural nerve amplitude between diabetic patients with and without ED (21). Studies have also shown an association between measures of cardiac autonomic neuropathy(CAN) and ED (13, 39). Penile vasotactile and thermal thresholds assessing somatic small fibre neuropathy have been found to be abnormal in diabetic patients with ED (40-42). More sophisticated tests including penile somatosensory evoked potentials (43) and corpus cavernosum electromyography (44) are abnormal in ED, but are not routinely available.

In our previous study, in patients with type 1 diabetes and ED, we demonstrated a global small and large fibre and autonomic neuropathy (27). These findings emphasize the importance of accurate and comprehensive phenotyping of neuropathy and ED severity before concluding that they are associated. A potential weakness in the current study is that NSP only identifies patients with severe ED and alternate questionnaires such as the international index of erectile function (IIEF) or the European Male Ageing Study Sexual Function Questionnaire (EMAS-SFQ) (45) which allow an assessment of mild and moderate ED may be more useful. Although, in a recent study from Japan, neuropathy defined by symptoms and loss of vibration perception and reflexes was associated with severe but not moderate or mild ED (16).

The management of ED in patients with T2D is challenging with a non-responder rate of over 50% for PDE 5 inhibitors, which may reflect a more severe neurogenic component for ED in these patients (46, 47). Indeed in a recent study the assessment of nocturnal penile tumescence and rigidity, which reflects predominantly neurogenic abnormalities, had an AUC of 0.860 in differentiating sildenafil responders from non-responders (46). CCM has been used to identify an association between ED and small fibre damage in subjects with obesity (45), T1D (27) and now T2D. The potential role of CCM as an objective marker for neurogenic abnormalities that may predict the response to therapy in ED warrants further study.

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Data availability

The datasets generated and analysed during the current study are available from the corresponding author on reasonable request.

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Duality of interest

The authors declare that there is no duality of interest associated with this manuscript.

Authors contributions

All authors were involved in revising the manuscript critically for important intellectual content and for final approval of the version to be published.

S Dhage and JHH were involved in acquisition of data, analysis and interpretation of data and wrote the manuscript.

S Dhage, S Azmi, S Adam, JHH, M Ferdousi and A Kalteniece recruited patients and controls.

S Azmi, S Adam and JHH contributed to acquisition and analysis of the data.

M Ferdousi and A Kalteniece performed confocal microscopy and S Azmi performed skin biopsies.

M Jeziorska analysed and reported skin biopsies.

A Marshall performed and analysed nerve conduction studies.

HS and RD contributed to conception, interpretation of the data, wrote and revised the manuscript.

RAM contributed to conception and design of the study, wrote and revised the manuscript and is principal investigator of the study.

RAM 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.

There are no conflicts of interest in relation to this work.

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Table 1. Clinical characteristics of patients with type 2 diabetes with and without erectile dysfunction and controls.

	Type 2 diabetes with ED (n=29)	*Type 2 diabetes without ED (n=20)*	*Controls (n=19)*	*P-value*
Age (years)	64.0±6.5	59.4±9.4	60.6±7.2	0.101
Duration of diabetes (years)	10.9±9.5	10.4±8.2	-	0.981
Hypertension (%)	44.8	60.0	0.0	0.296
BP (mmHg)	142±21/75±10	134±21/74±10	136±17/79±9	0.737/0.653
Beta-blocker or diuretic use (%)	20.7	20.0	0.0	0.625
HbA1c (mmol/mol)	58±16	54±8	40±2.8	0.569
BMI (kg/m^2)	30.7±4.6	30.4±5.3	27.1±3.9	0.668
eGFR (ml min^{-1} [1.73m]$^{-2}$)	73±18	81±14	84±8	0.359
Total cholesterol (mmol/l)	4.0±1.0	3.8±1.1	5.2±0.8	0.208
HDL-C (mmol/l)	1.19±0.34	1.12±0.30	1.55±0.40	0.714
Triglyceride (mmol/l)	2.1±1.2	1.9±1.3	1.7±0.7	0.274
LDL-C (mmol/l)	1.9±0.8	1.9±1.1	2.9±0.7	0.804

Data presented as mean±SD.

P-value is for comparison between participants with and without erectile dysfunction.

Table 2. Measures of neuropathy for patients with type 2 diabetes with and without erectile dysfunction and controls.

	Type 2 diabetes with ED (n=29)	*Type 2 diabetes without ED (n=20)*	*Controls (n=19)*	*P-value*
NSP (/38) [a]	6.0±5.1	2.0±3.0	0.3±0.8	<0.001
NDS (/10) [a]	3.4±1.9	2.3±2.6	0.9±1.2	0.001
VPT (V) [a]	14.3±7.8	11.2±6.6	9.2±5.9	0.429
Sural nerve amplitude (μV) [a]	6.8±4.3	13.3±5.8	12.6±6.3	<0.001
Sural nerve conduction velocity (m/s) [a]	44.0±6.0	47.1±5.8	47.9±3.9	0.170
Peroneal nerve amplitude (mV) [a]	2.9±2.1	4.7±2.0	5.1±1.7	0.009
Peroneal nerve conduction velocity (m/s) [a]	41.4±8.2	44.8±4.4	46.1±3.7	0.100
CT (℃) [a]	25.1±3.8	26.2±2.9	27.9±2.1	0.815
WT (℃) [a]	43.2±4.0	41.0±3.8	38.5±3.1	0.257
IENFD (no./mm) [a]	4.6±2.8	13.7±2.7	15.9±3.2	<0.001
CNFD (no./mm^2) [a]	23.5±6.8	31.3±8.2	37.6±5.9	<0.001
CNBD (no./mm^2) [a]	55.4±35.3	97.7±46.4	93.9±30.1	0.004
CNFL (mm/mm^2) [a]	17.6±6.8	27.3±6.8	26.6±4.4	<0.001
DB-HRV (beats/min) [a]	16 ± 4	18±8	25±12	0.841

Data presented as mean±SD.

[a]Adjusted for age and beta-blocker/diuretic use using ANCOVA

P-value is for comparison between participants with and without erectile dysfunction.

Figure 1. Comparison of corneal nerve parameters and intraepidermal nerve fibre density between patients with type 2 diabetes with and without erectile dysfunction and controls.

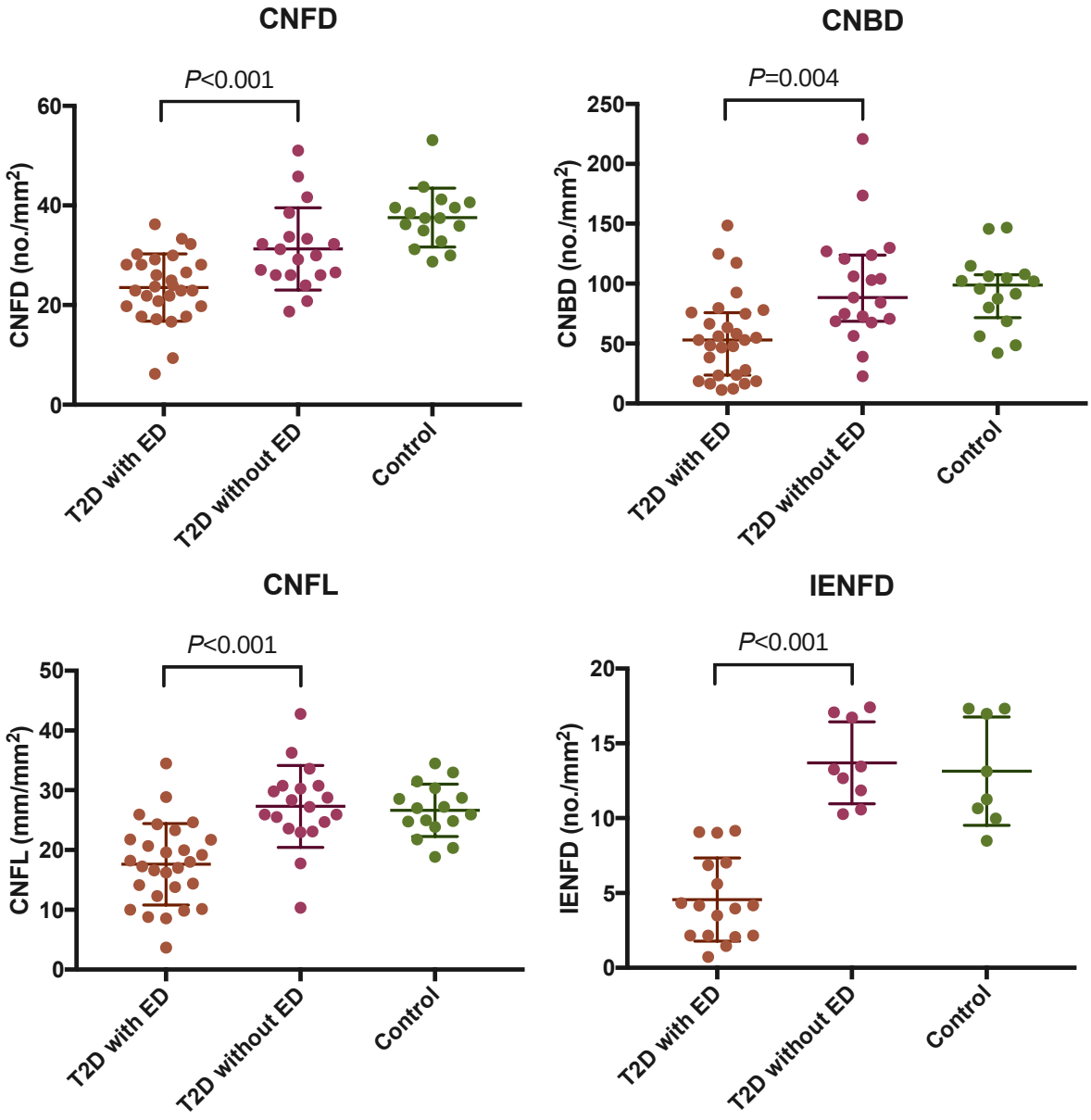
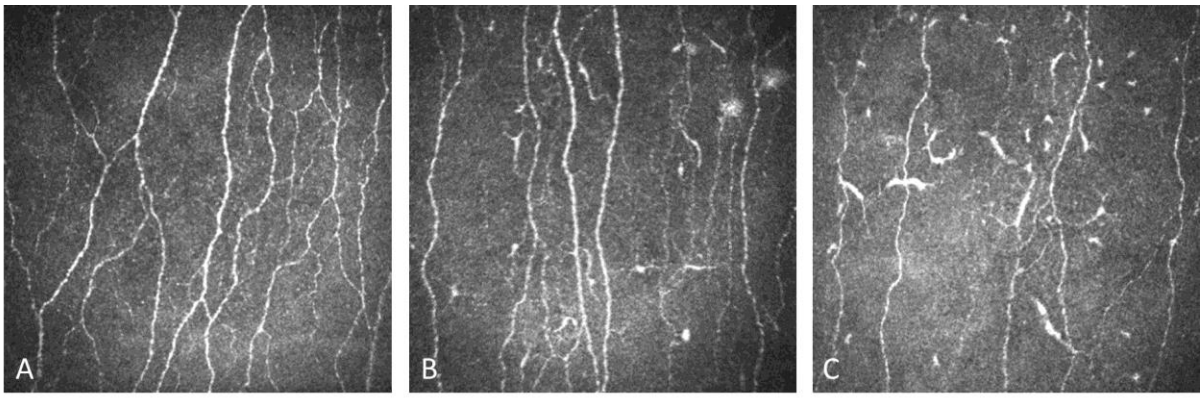


Figure 2. CCM images of corneal sub-basal nerves from a control participant (A), participant with T2DM and no ED (B) and participant with T2DM and ED.



Supplementary material for publication

Supplementary table 1. Clinical characteristics of patients with type 2 diabetes with and without erectile dysfunction and controls.

	Type 2 diabetes (n=49)	*Controls (n=19)*	*P-value*
Clinical characteristics			
Age (years)	62.1±8.1	60.6±7.2	0.475
BMI (kg/m^2)	30.6±4.9	27.1±3.9	0.008
Duration of diabetes (years)	9 (4–9)	-	
Hypertension (%)	51.0	0.0	<0.001
BP (mmHg)	139±21/74±10	136±17/79±9	0.558/0.053
Beta-blocker or diuretic use (%)	20.4	0.0	
Biochemistry			
HbA1c (mmol/mol)	57±13	40±2.8	<0.001
eGFR (ml min^{-1} [1.73m]$^{-2}$)	77±17	84±8	0.085
Total cholesterol (mmol/l)	4.0±1.0	5.2±0.8	<0.001
HDL-C (mmol/l)	1.16±0.33	1.55±0.40	<0.001
Triglyceride (mmol/l)	2.0±1.3	1.7±0.7	0.221
LDL-C (mmol/l)	1.9±0.9	2.9±0.7	<0.001
Neuropathy assessments			
NSP (/38) [a]	4.4±4.8	0.3±0.8	<0.001
NDS (/10) [a]	2.9±2.3	0.9±1.2	<0.001

VPT (V) [a]	13.0±7.4	9.2±5.9	0.036
Sural nerve amplitude (μV) [a]	9.5±5.9	13.0±6.1	0.041
Sural nerve conduction velocity (m/s) [a]	45.3±6.0	48.2±3.8	0.026
Peroneal nerve amplitude (mV) [a]	3.7±2.2	5.1±1.7	0.010
Peroneal nerve conduction velocity (m/s) [a]	42.8±7.0	46.1±3.7	0.018
CT (℃) [a]	25.6±3.4	27.9±2.1	0.002
WT (℃) [a]	42.3±4.0	38.5±3.1	0.001
IENFD (no./mm) [a]	7.7±5.2	15.9±3.2	0.004
CNFD (no./mm^2) [a]	26.7±8.3	37.6±5.9	<0.001
CNBD (no./mm^2) [a]	72.5±44.9	93.9±30.1	0.038
CNFL (mm/mm^2) [a]	21.5±8.3	26.6±4.4	0.003
DB-HRV (beats/min) [a]	18±8	25±12	0.027

Data presented as mean±SD.

P-value is for comparison between participants with type 2 diabetes and controls.

Supplementary table 2. Point-biserial correlation between erectile dysfunction and clinical characteristics in patients with type 2 diabetes.

	Erectile dysfunction
Clinical characteristics	
Age	r=0.281, *P*=0.051
BMI	r=0.029, *P*=0.841
Duration of diabetes	r=0.030, *P*=0.846
Systolic BP	r=0.168, *P*=0.249
Diastolic BP	r=0.043, *P*=0.769
Biochemistry	
HbA1c	r=0.155, *P*=0.287
eGFR	r=-0.257, *P*=0.178
Total cholesterol	r=0.102, *P*=0.490
HDL-C	r=0.100, *P*=0.500
Triglyceride	r=0.111, *P*=0.461
LDL-C	r=-0.015, *P*=0.924
Neuropathy assessments	

NSP	**r=0.418, *P*=0.003**
NDS	r=0.256, *P*=0.076
VPT	r=0.207, *P*=0.159
Sural nerve amplitude	**r=-0.477, *P*=0.001**
Sural nerve conduction velocity	r=-0.152, *P*=0.301
Peroneal nerve amplitude	**r=-0.478, *P*=0.001**
Peroneal nerve conduction velocity	r=-0.246, *P*=0.095
CT	r=-0.160, *P*=0.277
WT	r=0.273, *P*=0.061
IENFD	**r=-0.853, *P*<0.001**
CNFD	**r=-0.466 *P*=0.001**
CNBD	**r=-0.468, *P*=0.001**
CNFL	**r=-0.578, *P*<0.001**
DB-HRV	r=-0.005, *P*=0.977

Chapter 5: Corneal Confocal Microscopy Identifies Small Fibre Damage and Progression of Diabetic Neuropathy

Submitted for publication, currently under review process with the concerned journal.

Author`s contribution : Shaishav S. Dhage researched the data(patient recruitment, clinical assessments, venous blood sample collection, performed skin biopsies, assisted in fixation and storage of samples), analysed and interpreted data. In addition, he also researched the available literature and wrote the first draft of the manuscript. He also critically reviewed the final draft of the manuscript. The manuscript has formed the basis of this chapter.

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Corneal Confocal Microscopy Identifies Small Fibre Damage and Progression of Diabetic Neuropathy

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Abstract

Accurately quantifying the progression of diabetic peripheral neuropathy (DPN) is key to identify individuals who will progress to foot ulceration and to power clinical intervention trials. We have undertaken detailed neuropathy phenotyping to assess the longitudinal utility of different measures of neuropathy in patients with diabetes. Nineteen patients with diabetes (age 52.5±14.7 years, duration of diabetes 26.0±13.8 years) and 19 healthy controls underwent assessment of symptoms and signs of neuropathy, quantitative sensory testing, autonomic nerve function, neurophysiology, intra-epidermal nerve fibre density (IENFD) and corneal confocal microscopy (CCM) to quantify corneal nerve fibre density (CNFD), branch density (CNBD) and fibre length (CNFL). Mean follow-up was 6.5 years. Glycated haemoglobin ($p=0.04$), low-density lipoprotein-cholesterol (LDL-C)($p=0.0009$) and urinary albumin creatinine ratio ($p<0.0001$) improved. Neuropathy symptom profile ($p=0.03$), neuropathy disability score ($p=0.04$), vibration perception threshold ($p=0.02$), cold perception threshold ($p=0.006$), CNFD ($p=0.03$), CNBD ($p<0.0001$), CNFL ($p<0.0001$), IENFD ($p=0.04$), sural ($p=0.02$) and peroneal motor nerve conduction velocity ($p=0.03$) deteriorated significantly. Change (Δ) in CNFL correlated with ΔCPT ($p=0.006$) and ΔExpiration/Inspiration ratio ($p=0.002$) and ΔIENFD correlated with ΔCNFD ($p=0.005$), ΔCNBD ($p=0.02$) and ΔCNFL ($p=0.01$). This study shows worsening of diabetic neuropathy across a range of neuropathy measures, especially CCM, despite an improvement in HbA1c and LDL-C. It further supports the utility of CCM as a rapid, non-invasive surrogate measure of diabetic neuropathy.

Introduction

The natural history of diabetic peripheral neuropathy (DPN) is poorly defined with limited studies assessing progression of neuropathy (1). As a consequence clinical trials of disease modifying therapies in patients with diabetic neuropathy have not been able to identify the optimal neuropathy end points to adequately assess progression or improvement in DPN (2). Indeed, whilst the DCCT in patients with T1DM showed that intensive glycaemic control reduced the incidence of clinical DPN and nerve conduction abnormalities by 60% (3); in patients with T2DM, the UKPDS (4) and VA-CSDM trial (5) reported no effect on DPN and cardiac autonomic neuropathy and whilst the Kumamoto study (6) showed a prevention of nerve conduction slowing, the ACCORD trial (7) showed no effect on VPT over 6-years.

Quantitative sensory testing (QST) is relatively easy to perform but has limited reproducibility and a high degree of subjectivity (8). Nerve conduction studies (NCS) are the established 'gold standard' for evaluating DPN but require standardization in a clinical trial and cannot evaluate small fibres (9). Whilst small nerve fibre damage and repair can be identified by performing a skin biopsy and quantifying intra-epidermal nerve fibre density (IENFD), it is invasive and requires expertise (10-12). Other techniques for the assessment of small nerve fibres include microneurography, Laser doppler image flare (LDIflare), nociceptive-evoked potentials and electrochemical skin conductance, but have considerable variability and are not routinely available (13, 14). Corneal Confocal Microscopy (CCM) is a rapid non-invasive imaging technique for the quantitative assessment of small fibre damage. Several studies have shown that it has good diagnostic utility for sub-clinical DPN, predicts incident DPN (15, 16) and correlates with other measures of neuropathy (16). Furthermore, automated quantification of corneal nerve parameters allows rapid, unbiased and objective assessment of small fibre damage (17) with comparable diagnostic capability to IENFD (18, 19).

Longitudinal studies of patients with diabetic neuropathy have been of relatively short duration and lacked detailed neuropathy phenotyping (20-23). In this study we compare the change in CCM and IENFD with symptoms, signs, QST, autonomic function and neurophysiology over 6.5 years in a cohort of patients with diabetes.

Results

Clinical and metabolic assessment (Table 1, 2)

Age (p=0.2), weight (p=0.9) and BMI (Body Mass Index) (p=0.5) did not differ significantly between patients and controls and also between patients at baseline and follow up. Systolic (p=0.9, p=0.37) and diastolic (p=0.5, p=0.08) blood pressure did not differ between controls and patients at baseline and between patients at baseline and follow up, respectively. HbA1c was significantly higher in patients with diabetes compared to controls at baseline (p=0.0002) and decreased significantly in patients at follow up (p=0.04). Low density lipoprotein cholesterol (LDL-C) was significantly lower in diabetic patients compared to controls at baseline (p=0.05) and decreased further at follow up (p=0.0009), whilst triglycerides did not differ between patients and controls at baseline (p=0.9) and did not change at follow up (p=0.9). eGFR did not differ significantly between diabetic patients and controls at baseline and decreased at follow up (p=0.004). Albumin creatinine ratio (ACR) was significantly higher in diabetic patients compared to controls at baseline (p<0.0001) and increased further at follow up (p<0.0001).

Neuropathy Assessments

Neuropathic symptoms and deficits (Table 1, 2, Figure 3)

Neuropathy symptom profile (NSP) (p=0.0005) and neuropathy disability score (NDS) (p<0.0001) were significantly higher in patients at baseline compared to controls and increased significantly (p=0.03, p=0.04, respectively) in patients at follow up.

Quantitative Sensory Testing (QST) (Table 1, 2, Figure 3)

Vibration perception threshold (VPT), cold perception threshold (CPT), warm perception threshold (WPT), cold induced pain (CIP), warm induced pain (WIP) and percentage colour change in Neuropad did not differ significantly (p>0.05) in patients at baseline compared to controls. Whilst VPT increased (p=0.02) and CPT (p=0.006) decreased significantly there was no change in WPT, CIP, WIP and Neuropad.

Electrophysiology (Table 1, 2, Figure 3)

Sural (p=0.01) and peroneal (p=0.007) nerve conduction velocity and peroneal nerve amplitude (p=0.004) were significantly lower in patients at baseline compared to controls. Sural (p=0.02) and peroneal (p=0.03) nerve conduction velocity decreased significantly, with no change in sural (p=0.75) or peroneal (p= 0.29) nerve amplitudes in patients at follow up.

Autonomic neuropathy (Table 1, 2, Figure 3)

Deep breathing heart rate variability (DB-HRV) was significantly lower in patients at baseline compared to controls (p=0.005). Expiration/inspiration (E/I) ratio (p=0.004), Valsalva ratio (p=0.001), and 30:15 ratio (p=0.003) increased significantly with no change in DB-HRV (p=0.67) and sympathetic low frequency area (LFa)/parasympathetic respiratory frequency area (RFa) ratio (p=0.42) at follow up.

IENFD (Table 1, 2, Figure 1 and 3)

Intraepidermal nerve fibre density (IENFD) was significantly lower in patients at baseline (p=0.04) compared to controls and decreased (p=0.04) in patients at follow up.

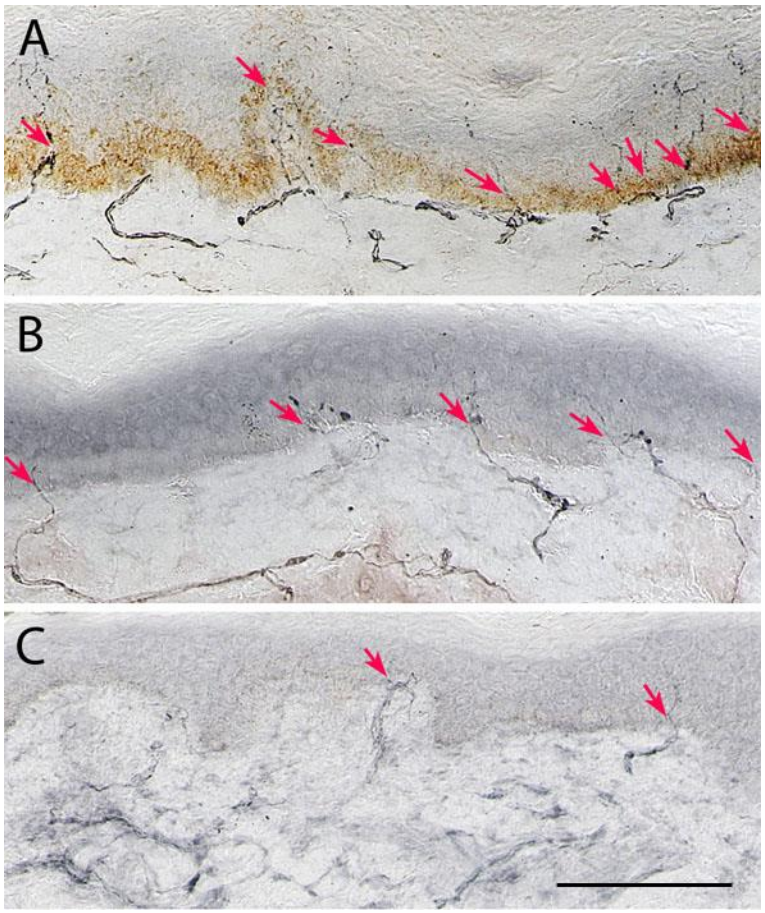

Figure 1. Representative images from skin biopsies from healthy control (A) and diabetes patient of similar age at baseline (B) and a follow-up visit after 6.5 years (C). Note numerous branching nerves reaching top layers of epidermis (A; red arrows) and sparse short single nerve and two dividing

nerves (red arrows) in epidermis of the baseline biopsy (B) and more difficult to discern shorter nerves in the follow-up biopsy (red arrows). Scale bar for A-C = 100 µm.

CCM (Table 1, 2, Figure 2 and 3)

Corneal nerve fibre density (CNFD)($p<0.0001$), Corneal nerve branch density (CNBD) ($p=0.009$) and Corneal nerve fibre length (CNFL) ($p=0.0007$) were significantly lower in patients at baseline compared to controls and CNFD ($p=0.03$), CNBD ($p<0.0001$) and CNFL ($p<0.0001$) decreased at follow up.

Associations between the change in clinical and neuropathy measures (Table 3, Figure 3)

ΔIENFD correlated with age (r= -0.56, p=0.01), BMI (r = -0.47, p=0.04), waist to hip ratio (r= - 0.66, p= 0.001), ΔE/I ratio (r=0.595, p= 0.0071) and ΔValsalva ratio (r= 0.59, p= 0.0078). ΔCNFD correlated with ΔVPT (r= -0.54, p=0.03), ΔDBHRV (r= 0.55, p=0.02) and ΔIENFD (r= 0.62, p= 0.005). ΔCNFL correlated with ΔCPT (r=0.66, p= 0.006), ΔE/I ratio (r= 0.68, p= 0.002) and ΔIENFD (r= 0.56, p= 0.014). ΔCNBD correlated with ΔVPT (r= -0.55, p=0.02) and ΔIENFD (r=0.53, p= 0.02). There was no correlation between change in HbA1c, lipids and neurophysiological parameters with change in CCM or IENFD (Supplementary Table 1).

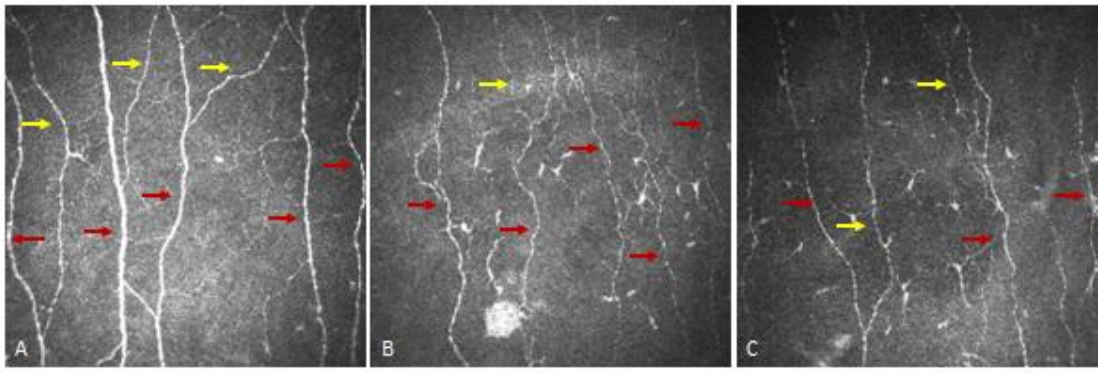

Figure 2. Corneal confocal microscopy image from a healthy control (A) and patient with diabetes at

baseline (B) and follow-up (C) showing a progressive loss of nerve fibres (red arrows main nerves, yellow arrows branches) in patients with diabetes.

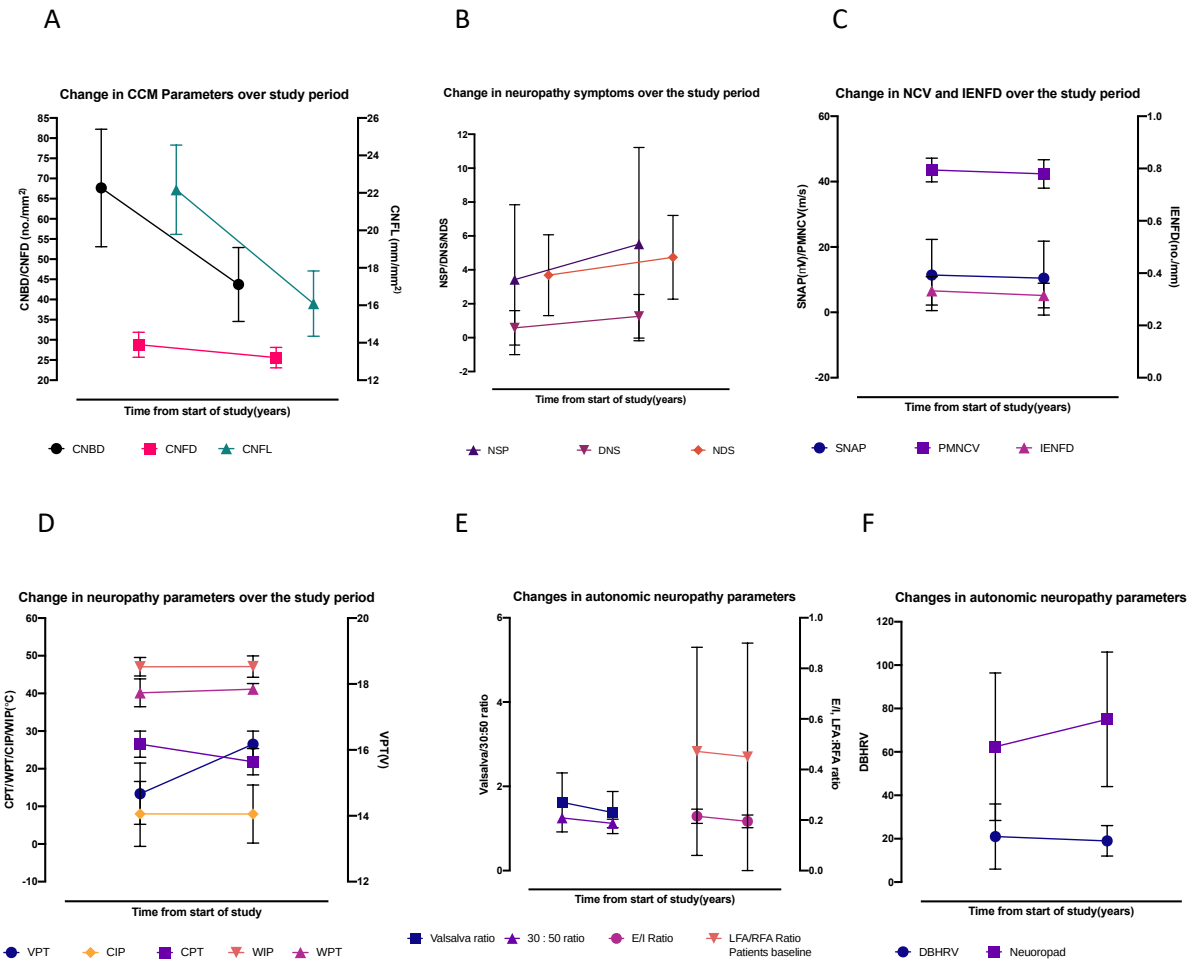

Figure 3. Percentage change from baseline values in CCM parameters (A), neuropathy symptoms (B), NCV and IENFD (C), quantitative sensory testing (D) and autonomic neuropathy (E,F).

Discussion

In this study we show a progressive worsening of diabetic neuropathy in diabetic patients despite an improvement in HbA1c and LDL cholesterol, although there was no correlation between change in HbA1c, and LDL cholesterol with change in any measure of neuropathy. In T1DM the DCCT showed that intensive glycaemic control reduced the incidence of DPN (3). However, in patients with T2DM, the UKPDS (4), VA-CSDM trial (5) and ACCORD (7) trials showed no effect of improved glycaemic control on DPN. A major problem in these clinical trials was the end points utilised to assess neuropathy including symptoms and signs of neuropathy and quantitative sensory testing, which were unable to accurately measure change in neuropathy (2).

Neurophysiology is considered to be the gold standard for the diagnosis of DPN and has been adopted as an endpoint in multiple clinical trials (9), but has failed to show a significant change in these trials (24). Indeed, our longitudinal data now shows a relatively small magnitude of reduction in peroneal and sural nerve conduction with no change in amplitudes over 6.5 years. It is therefore not surprising that most trials lasting 12-24 months show no change in neurophysiology.

Small fibre damage usually precedes large fibre damage and contributes to clinically meaningful end-points like painful diabetic neuropathy and foot ulceration due to altered skin blood flow and delayed wound healing (2). Skin biopsy with IENFD quantification is the current gold standard for the evaluation of small fibre damage (9) and whilst it is reliable and reproducible it is invasive and resource-intensive (11). CCM is a rapid, non-invasive and reproducible ophthalmic imaging technique which can be used to objectively quantify small fibre damage in a range of peripheral neuropathies (15, 25-29). We have previously shown comparable diagnostic utility of CCM and IENFD in diabetic neuropathy (19). Furthermore, in longitudinal studies reduced corneal nerve fibre length predicts incident DPN (30, 31) and those at risk of developing DPN (32). Indeed, CCM has shown corneal nerve regeneration 6 months after pancreas and kidney transplantation in T1DM with no change in quantitative sensory testing and an improvement in neuropathic symptoms and nerve conduction only after 24 and 36 months, respectively (21, 33). A recent study from Japan showed that an improvement in glycaemic control, body weight and blood pressure in patients with T2DM was associated with an improvement in corneal nerve fibres, neurophysiology and vibration perception over 4 years and correlated with a reduction in HbA1c (34).

Studies have also shown an association between CCM and LDIflare in healthy control subjects (35) and with LDIflare, cooling detection thresholds and HRV in patients with diabetes (16). In the present study CCM measures worsened with greater magnitude than IENFD and large fibre (VPT, CPT, sural and peroneal nerve conduction velocities) and autonomic (E/I ratio, Valsalva ratio and 30:15 ratio) measures of neuropathy. The worsening of corneal nerve fibre measures was associated with worsening of other small fibre measures including cold perception threshold, IENFD and autonomic neuropathy, but not neurophysiology. Indeed, a number of studies have shown corneal nerve loss in patients with diabetic autonomic neuropathy (36-38) and a correlation between CCM and a wide range of other measures of neuropathy including peroneal and sural nerve conduction (36) and both cold and warm perception thresholds (16, 39).

A limitation of this study is the relatively small number of patients assessed at follow up. However, the main strength of this study is the comprehensive phenotyping of diabetic neuropathy over 6.5 years, enabling a detailed comparison of the change in small and large fibre measures of diabetic neuropathy.

In conclusion, CCM identifies progressive nerve damage despite an improvement in glycaemic control and LDL cholesterol. Furthermore, corneal nerve loss was associated with a loss of IENFD and worsening of other measures of small fibre neuropathy. CCM is a rapid, non-invasive test to identify progression of neuropathy and may have greater utility than symptoms, signs, QST and nerve conduction studies in longitudinal follow-up studies and clinical trials of DPN.

Methods

Participant selection

Nineteen patients with diabetes (type 1 DM (n=15) and type 2 DM (n=4)), from the Manchester University Hospital Diabetes Centre and 19 age-matched healthy control participants were recruited and assessed between 2009 and 2011 and at follow up in 2017. The control group comprised of healthy volunteers without DM and were not on any regular medications for any co-morbidities. Patients with a history of neuropathy from any other cause, ocular disease, corneal trauma or surgery, systemic disorders affecting the skin or cornea were excluded. All the tests performed at baseline were repeated in the follow up study using the same protocol and equipment. This study has approval from the Health Research Authority (HRA), North West – Greater Manchester South Research Ethics Committee. Written informed consent was obtained from all individuals prior to participation. This research adhered to the tenets of the declaration of Helsinki.

Anthropometric and laboratory measurements

All participants underwent assessment of height, weight and body mass index (BMI). Glycated haemoglobin (HbA1c), total cholesterol, low-density lipoprotein (LDL)-cholesterol, triglycerides (TG), serum creatinine and urinary albumin creatinine ratio (ACR) were measured using routine laboratory methods in the Department of Biochemistry, Manchester University NHS Foundation Trust. Estimated glomerular filtration rate (eGFR) was calculated using the abbreviated Modification of Diet in Renal Disease (MDRD) equation: 186 x (creatinine/88.4) -1.154 x (age)-0.203 x (0.742 in females) x (1.210 if Afro-Caribbean race).

Assessment of neuropathy

The Neuropathy Symptom Profile (NSP) was used to assess the symptoms of neuropathy. The modified Neuropathy Disability Score (NDS) which is comprised of an assessment of vibration perception, pinprick, temperature sensation and presence or absence of ankle reflexes was used to evaluate neurological deficits. A Horwell Neurothesiometer (Scientific Laboratory Supplies, Wilford, Nottingham, UK) was used to establish the Vibration Perception Threshold (VPT). Cold (CT) and warm (WT) perception thresholds and cold (CIP) and warm induced pain (WIP) thresholds were tested on the dorsolateral aspect of left foot using the TSA-II NeuroSensory Analyser (Medoc, Ramat-Yishai, Israel). Electrodiagnostic nerve conduction studies (NCS) were undertaken using a Dantec Keypoint System (Dantec Dynamics, Bristol, UK), equipped with a DISA temperature regulator to keep the limb temperature constant at 32-35 °C. The ANX 3.0 autonomic nervous system monitoring device (ANSAR Medical Technologies, Philadelphia, PA, USA) was used to assess deep breathing heart rate variability (DB-HRV), sympathovagal balance via the sympathetic low frequency area (LFa)/parasympathetic respiratory frequency area (RFa) ratio, Expiratory/Inspiratory (E/I ratio), Valsalva ratio and 30:15 ratio. Sudomotor dysfunction was assessed by quantifying the percentage colour change after applying the Neuropad to the area over the base of the first metatarsal head using our previously established protocol and automated quantification (40).

Skin biopsy

Local anaesthetic (1% lignocaine) was applied to the dorsum of the foot, 2 cm above the second metatarsal head and two 3mm punch biopsies were performed. Sections of 50 µm were stained using anti-human PGP 9.5 antibody (Abcam, Cambridge, UK). SG chromogen (Vector Laboratories, Peterborough, UK) was used to demonstrate nerve fibres and IENFD was quantified using previously established criteria and expressed as the number per millimetre length of epidermis (41). The follow-

up skin biopsy was taken from the same foot, in close proximity to the first biopsy. IENFD was quantified by the same investigator in a masked fashion.

Corneal confocal microscopy (CCM)

CCM examination (Heidelberg Retinal Tomography III Rostock Cornea Module; Heidelberg Engineering, Heidelberg, Germany) was performed using our previously established protocol (42). Six non-overlapping images, three per eye, were selected from the centre of the cornea. Three corneal nerve parameters were quantified: Corneal nerve fibre density (CNFD): the total number of major nerve fibres per square millimetre of corneal tissue, corneal nerve fibre branch density (CNBD): the number of branches emanating from the major nerve trunks per square millimetre of corneal tissue and corneal nerve fibre length (CNFL): the total length of all nerve fibres and branches (millimetre per square millimetre) using manual quantification software (CCMetrics (Manchester, UK)) (43).

Statistical analyses

Statistical analyses were performed using GraphPad Prism for Mac OS X (version 8.3.0, GraphPad Software, San Diego, California USA, www.graphpad.com). Data were tested for normality using the Shapiro-Wilk normality test. All data are expressed as mean $\pm$ standard deviation (SD). Continuous variables were compared between baseline and follow up visits using the paired t-test for normally distributed data and Wilcoxon matched-pairs signed rank test for non-normally distributed data. Ordinary one-way ANOVA was performed (Kruskal-Wallis test was used for non-normally distributed data) to compare between group differences of controls and baseline patient values. Post-hoc corrections for multiple comparison testing was done using Tukey's test. Correlations were performed between the percentage change in IENFD and CCM parameters and other variables using Pearson's or Spearman's Rank Test according to the distribution of the data. A two-way p-value of less than 0.05 was considered to be statistically significant.

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Data Accessibility

The datasets generated and analysed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

The authors declare that there is no duality of interest associated with this manuscript.

Author Contributions

All authors were involved in revising the manuscript critically for important intellectual content and for final approval of the version to be published.

SD and MF were involved in acquisition of data, analysis and interpretation of data and wrote the manuscript.

SD, SA, JHH, MF and AK recruited patients for follow up.

SD, SA and JHH contributed to acquisition and analysis of the data.

SAz, UA, GP, IP and MF recruited patients at baseline.

SAz, UA performed skin biopsies for patients and controls at baseline and SD performed skin biopsies for all patients at follow up.

GP, IP and MF performed CCM for patients and controls at baseline. MF and AK performed CCM for patients at follow up.

AA processed skin biopsies on follow up patients. MJ analysed and reported skin biopsies for all patients and controls at baseline and follow up.

AM performed and analysed nerve conduction studies for all patients and controls at baseline and at follow up.

HS contributed to conception, interpretation of the data, wrote and revised the manuscript.

RAM contributed to conception and design of the study, wrote and revised the manuscript and is principal investigator of the study.

RAM 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.

There are no conflicts of interest in relation to this work.

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Table 1. Clinical and neuropathy parameters in control subjects and patients at baseline.

Variable	Controls (n=19)	Patients (Baseline) (n=19)	P value
Clinical and Laboratory Parameters			
Age (years)	47.4 ± 14.2	52.5 ± 14.7	0.20
Duration of Diabetes (years)	NA	26.0 ± 13.8	NA
Weight (kg)	80.65 ± 18.0	82.0 ± 19.8	0.9
BMI (kg/m^2)	27.5 ± 4.0	29.0 ± 5.7	0.50
BP (mmHg)	131 ± 23/74.0 ± 11.0	132 ± 21/ 71 ± 8	0.90/0.50
HbA1c (mmol/mol)	37.5 ± 3	63.5 ± 18.7	**0.0002**
Triglycerides (mmol/l)	1.4 ± 0.65	1.8 ± 1.7	0.9
LDL – C (mmol/l)	2.7 ± 0.92	2.23 ± 0.95	**0.05**
eGFR (ml min/ [1.73m]^2)	83 ± 7	82 ± 20	0.70
ACR (mg/mmol)	0.25 ± 0.07	7.5 ± 15.7	**< 0.0001**
Clinical Neuropathy and QST Measures			
NSP (/38)	0.15 ± 0.5	3.5 ± 4.5	**0.0005**
NDS (/10)	0.57 ± 1.01	3.70 ± 2.40	**<0.0001**
VPT (V)	7.5 ± 6.9	13.0 ± 8.0	0.06
CPT *(℃)*	28.4 ± 2.25	26.5 ± 3.5	0.51
WPT *(℃)*	36.9 ±2.24	40.0 ± 3.7	0.20
CIP *(℃)*	9.0 ± 8.25	8.0 ± 8.5	0.90

Variable	Controls (n=19)	Patients (Baseline) (n=19)	P value
Autonomic neuropathy measures			
DB-HRV (beats/min)	30 ± 12	21 ± 15	**0.005**
Neuropad (%)	91.0 ± 21	62.4 ± 34	0.13
Nerve Conduction studies			
SNAP (µV)	17.9 ± 9.7	11.41 ± 10.9	0.06
SNCV (m/s)	49.75 ± 4.45	43.5 ± 9	**0.01**
PNAP (mV)	6.0 ± 2.2	3.8 ± 1.9	**0.004**
PMNCV (m/s)	48.7 ± 4.1	43.53 ± 3.60	**0.0007**
Corneal Confocal Microscopy			
CNFD *(no./mm^2)*	37.7 ± 6.5	28.8 ± 6.5	**< 0.0001**
CNBD *(no./mm^2)*	96.5 ± 38.6	67.6 ± 30.2	**0.009**
CNFL *(mm/mm^2)*	27.24 ± 3.35	22.17 ± 4.95	**0.0007**
Skin Biopsy			
IENFD *(no./mm)*	9.8 ± 3.8	6.57 ± 4.32	**0.04**
WIP *(℃)*	45.0 ± 2.75	47.0 ± 2.5	0.15

BMI- Body Mass Index, BP- Blood pressure, HbA1c- Glycosylated haemoglobin, eGFR-estimated glomerular filtration rate, ACR- Albumin Creatinine Ratio, LDL-C- low density lipoprotein cholesterol, NSP- Neuropathy Symptom Profile, NDS- Neuropathy Disability score, VPT- Vibration Perception Threshold, DBHRV- Deep Breathing Heart Rate Variability, Sural nerve action potential (SNAP), Sural nerve conduction velocity (SNCV), Peroneal nerve amplitude (PNAP), Peroneal motor nerve

conduction velocity (PMNCV), CNFD- Corneal Nerve Fibre Density, CNBD- Corneal Nerve Branch Density, CNFL- Corneal Nerve Fibre Length, IENFD- Intraepidermal Nerve Fibre Density. Data is presented as mean ± standard deviation (SD). Bold values show statistically significant results. Continuous variables were compared between controls and baseline patient visits using the unpaired t-test for normally distributed data and Wilcoxon matched-pairs signed rank test for non-normally distributed data.

Table 2. Clinical and neuropathy parameters in patients at baseline and follow up.

Variable	Patients (Baseline) (n=19)	Patients (Follow up) (n=19)	P value
	Clinical and Laboratory Parameters		
Age (years)	52.5 ± 14.7	59.5 ± 15.6	NA
Duration of Diabetes (years)	26.0 ± 13.8	32.5 ± 13.8	NA
Weight (kg)	82.0 ± 19.8	81.75 ± 18	0.49
BMI (kg/m^2)	29.0 ± 5.7	28.7 ± 5.2	0.53
BP (mmHg)	132 ± 21/ 71 ± 8	127 ± 20 / 67 ± 9	0.37/0.08
HbA1c (mmol/mol)	63.5 ± 18.7	55.95 ± 12	**0.04**
Triglycerides (mmol/l)	1.8 ± 1.7	1.5 ± 1.05	0.9
LDL-C (mmol/l)	2.23 ± 0.95	1.88 ± 1.18	**0.0009**
eGFR *(ml min^{-1} [1.73m]$^{-2}$)*	82 ± 20	69 ± 21	**0.004**
ACR (mg/mmol)	7.5 ± 15.7	41.29 ± 123.6	**< 0.0001**

	Clinical Neuropathy Measures and QST		
NSP (/38)	3.50 ± 4.50	5.50 ± 5.70	**0.03**
NDS (/10)	3.68 ± 2.38	4.70 ± 2.50	**0.04**
VPT (V)	13.0 ± 8.0	18.0 ± 9.0	**0.02**
CPT *(℃)*	26.5 ± 3.5	21.8 ± 9.2	**0.006**
WPT *(℃)*	40.0 ± 3.7	41. 2 ± 4.8	0.38
CIP *(℃)*	8.0 ± 8.5	8.0 ± 7.7	0.81
WIP *(℃)*	47.0 ± 2.5	47.1 ± 2.8	0.622

Variable	Patients Baseline (n=19)	Patients Follow-up (n=19)	P value
Autonomic Neuropathy Measures			
DB-HRV (beats/min)	21.0 ± 15.0	19.0 ± 7.0	0.67
LFa/RFa	2.83 ± 2.47	2.7 ± 2.7	0.42
E/I Ratio	1.29 ± 0.17	1.17 ± 0.15	**0.004**
Valsalva ratio	1.62 ± 0.7	1.38 ± 0.50	**0.001**
30:15 ratio	1.25 ± 0.11	1.12 ± 0.10	**0.0003**
Neuropad (%)	62.4 ± 34	75.0 ± 31.0	0.47
Nerve Conduction Studies			
SNAP (μV)	11.41 ± 10.9	10.47 ± 11.31	0.75
SNCV (m/s)	43.5 ± 9	40.4 ± 7.4	**0.02**
PNAP (mV)	3.8 ± 1.9	3.45 ± 1.89	0.299
PMNCV (m/s)	43.53 ± 3.60	42.35 ± 4.34	**0.03**
Corneal Confocal Microscopy			
CNFD *(no./mm^2)*	28.8 ± 6.5	25.6 ± 5.2	**0.03**
CNBD *(no./mm^2)*	67.6 ± 30.2	43.7 ± 19	**<0.0001**
CNFL *(mm/mm^2)*	22.17 ± 4.95	16.1 ± 3.6	**<0.0001**
Skin Biopsy			
IENFD *(no./mm)*	6.57 ± 4.32	5.16 ± 3.7	**0.04**

BMI- Body Mass Index, BP- Blood pressure, HbA1c- Glycosylated haemoglobin, e GFR-estimated glomerular filtration rate, ACR- Albumin Creatinine Ratio, LDL-C- low density lipoprotein cholesterol, NSP- Neuropathy Symptom Profile, NDS- Neuropathy Disability score, VPT- Vibration Perception Threshold, CPT- Cold perception Threshold, WPT- Warm Perception Threshold, CIP-Cold induced pain, WIP- Warmth induced pain, DBHRV- Deep Breathing Heart Rate Variability, LFa/RFa ratio- low frequency area (sympathetic) and respiratory frequency area (parasympathetic) ratio, E/I- Expiration/Inspiration ratio, Sural nerve action potential (SNAP), Sural nerve conduction velocity (SNCV), Peroneal nerve amplitude (PNAP), Peroneal motor nerve conduction velocity (PMNCV), CNFD- Corneal Nerve Fibre Density, CNBD- Corneal Nerve Branch Density, CNFL- Corneal Nerve Fibre Length, IENFD- Intraepidermal Nerve Fibre Density. Data is presented as mean ± standard deviation (SD). Bold values show statistically significant results. Continuous variables were compared between baseline and follow up visits using the paired t-test for normally distributed data and Wilcoxon matched-pairs signed rank test for non-normally distributed data.

Table 3. Correlations between percentage change in small fibre pathology and other measures of diabetic neuropathy from baseline to follow up.

Variable	CNBD	CNFD	CNFL	IENFD
IENFD	**r = 0.53** **p = 0.02**	**r = 0.62** **p = 0.005**	**r = 0.56** **p = 0.01**	
NSP	r = -0.26 p = 0.29	r = -0.43 p = 0.08	r = -0.045 p = 0.86	r = -0.07 p = 0.76
NDS	r =- 0.13 p = 0.58	r = -0.43 p = 0.08	r =- 0.11 p = 0.66	r = -0.05 p = 0.82
CPT	r = 0.076 p = 0.77	r = 0.29 p = 0.26	**r = 0.66** **p = 0.006**	r = 0.27 p = 0.26
VPT	**r = -0.55** **p = 0.02**	**r = -0.54** **p = 0.03**	r = -0.08 p = 0.76	r = -0.12 p = 0.37

DB-HRV	r = - 0.19 p = 0.42	**r = -0.55** **p = 0.02**	r = -0.14 p = 0.57	r = -0.03 p = 0.87
LFA/RFA ratio	r = 0.26 p = 0.27	r = 0.09 p = 0.70	r = 0.017 p = 0.95	r = 0.13 p = 0.58
E/I ratio	r = 0.24 p = 0.32	r = 0.31 p = 0.21	**r = 0.68** **p = 0.002**	**r = 0.595** **p = 0.007**
Valsalva ratio	r = 0.41 p = 0.08	r = 0.14 p = 0.56	r = 0.25 p = 0. 32	**r = 0.59** **p = 0.008**

NSP- Neuropathy Symptom Profile, NDS- Neuropathy Disability score, DNS- Diabetic neuropathy symptom score, VPT- Vibration Perception Threshold, CPT- Cold perception Threshold, DB-HRV- Deep Breathing Heart Rate Variability, LFA/RFA ratio- low frequency area (sympathetic) and high frequency area (parasympathetic) ratio, E/I- Expiration/Inspiration ratio, CNFD- Corneal Nerve Fibre Density, CNBD- Corneal Nerve Branch Density, CNFL- Corneal Nerve Fibre Length, IENFD- Intraepidermal Nerve Fibre Density. Bold values show statistically significant results.

Chapter 6 : Male sexual dysfunction in diabetes : role of sex hormones and small fibre neuropathy

To be submitted for publication

Author`s contribution : Shaishav S. Dhage researched the data(patient recruitment, clinical assessments, venous blood sample collection), analysed and interpreted data. In addition, he also researched the available literature and wrote the first draft of the manuscript. He also critically reviewed the final draft of the manuscript. The manuscript has formed the basis of this chapter.

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Male sexual dysfunction in diabetes: role of sex hormones and small fibre neuropathy

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Abstract

Context

Pathophysiology of sexual dysfunction in diabetes is multifactorial. Testosterone and sex hormone levels are frequently abnormal in men with diabetes. Abnormal sex hormones are often a focus of attention in the diagnosis and management of sexual dysfunction in clinical practice. The role of small fibre neuropathy in sexual dysfunction in diabetes remains unestablished.

Aims/hypothesis

We aimed to investigate the relationship between symptoms of sexual dysfunction, sex hormone levels and various measures of small fibre neuropathy.

Methods

Sixty-four patients with diabetes (age 59.6 $\pm$ 8.9 years, duration of diabetes 13.9 $\pm$ 12.5 years) underwent assessment of sexual symptoms, quantitative sensory testing, autonomic neuropathy, corneal confocal microscopy(CCM) and sex hormone levels. Small fibre neuropathy assessment was done using quantitative sensory testing, autonomic neuropathy and CCM. Symptoms of sexual dysfunction were assessed using the European Male Ageing Study Sexual Function Questionnaire(EMAS). Measurement of sex hormone levels was done using mass spectrophotometry.

Results

Erectile dysfunction was present in 64% participants of whom 29 % reported severe erectile dysfunction. A reduced frequency of sexual erection was reported by 67% and 26% participants reported infrequent sexual thoughts. CNFD (Corneal nerve fibre density) and CNBD(Corneal nerve fibre branch density) both were significantly lower in the patients symptomatic for erectile dysfunction(p=0.01 and p=0.03 respectively) and those with infrequent sexual thoughts(p=0.003 and p=0.03 respectively). CNFL(Corneal nerve fibre length) was significantly low in those with erectile dysfunction(p=0.006) and reduced frequency of morning erections(p=0.01). The erectile function score correlated with CNFD

(r= -0.38, p=0.006), CNBD (r= -0.28, p=0.02), CNFL (r= -0.37, p=0.002) and frequency of early morning erections (r= -0.31, p=0.014) and the frequency of early morning erections correlated with CNFD (r=0.35, p= 0.006), CNBD (r=0.33, p=0.01), CNFL (r=0.33, p=0.008). Total serum testosterone (B= 1.291, p=0.038) and CNFL (B= 0.847, p =0.037) were independently associated with the symptom of erectile dysfunction.

Conclusions/interpretation

Prevalence of symptomatic sexual dysfunction is relatively high among men with diabetes. We report an association between measures of small fibre neuropathy(CCM), symptomatic erectile dysfunction and frequency of early morning erections. We found no association between testosterone and symptoms of sexual dysfunction. However, total serum testosterone and CNFL were independently associated with symptomatic erectile dysfunction. We thus demonstrate a specific relationship between small fibre neuropathy and symptoms of sexual dysfunction in men with diabetes. Early identification of small fibre neuropathy using CCM, may have therapeutic implications in the treatment of erectile dysfunction and late onset hypogonadism in men with diabetes, especially those with sub-optimal or no response to testosterone and or phosphodiesterase 5(PDE5) inhibitors. This merits important role of CCM in future interventional studies for treatment of small fibre neuropathy.

Introduction

Sexual dysfunction can present with reduced libido, retrograde ejaculation and erectile dysfunction(1) and it has a high prevalence in patients with type 1 diabetes mellitus (T1DM)(2, 3) and type 2 diabetes mellitus (T2DM)(4). Normal sexual function is a complex interplay between psychological, neurohormonal and vascular abnormalities as a consequence of metabolic dysfunction in diabetes mellitus(DM)(4-6). Studies have demonstrated an association between insulin resistance with androgen deficiency and disruption of the hypothalamic-pituitary-gonadal axis(3-5).

The latest European Academy of Andrology (EAA) guidelines recommend use of testosterone replacement therapy in men with symptomatic functional hypogonadism(7). Whilst, testosterone replacement therapy may improve sexual function in hypogonadal men(7, 8), there is limited evidence of its benefit in men with T2DM and low levels of testosterone, especially those who are obese and of older age(9).

Erectile dysfunction is also associated with small and large fibre neuropathy in patients with DM(7, 10). The penis is innervated by both autonomic (sympathetic and parasympathetic) and somatic (sensory and motor) nerves(10) and erection is mainly mediated through autonomic fibres. Quantitative sensory testing (QST) has demonstrated impaired sensation in the genital region of patients with ED(10-12). Measurement of the intraepidermal nerve fibre density (IENFD) using skin biopsy is the current gold standard for the diagnosis of small fibre neuropathy(13, 14). Corneal confocal microscopy (CCM) is a non-invasive, reliable and reproducible technique to assess small fibre neuropathy in diabetes with comparable diagnostic efficiency to IENFD(15, 16).We have recently shown that small fibre neuropathy diagnosed using CCM is associated with erectile dysfunction in men with T1DM, T2DM and severe obesity(17-19).

In this study, we aimed to investigate the relationship between sexual dysfunction, sex hormone levels and small nerve fibre morphology in men with T1DM and T2DM.

Materials and methods

Participant selection

Sixty-four male patient (10 with type 1 DM and 54 with type 2 DM) were recruited from the diabetes clinic at Manchester University Hospital Diabetes centre between 2014 and 2016. Patients known to have cardiovascular disease, on treatment for erectile dysfunction, disease of the pituitary gland, testes or adrenal glands, or known to have conditions affecting androgen levels or primary hypogonadism(LH > 9.4 U/L) were excluded from the study(20).Patients with peripheral neuropathy due to any other cause apart from diabetes, or those with corneal disease or eye surgery due to corneal disease, or cancer treated with radiotherapy or chemotherapy were excluded from the study. This study was approved by the Greater Manchester Central Research and Ethics Committee. Written and informed consent was obtained from all individuals prior to participation in the study.

Assessment of sexual function

The European Male Ageing Study Sexual Function Questionnaire (EMAS) was used to assess sexual function in the participants(21). Sexual function was assessed using 3 sexual symptoms: erectile function, frequency of sexual thoughts and frequency of morning erections. Previously established cut-offs based on validated scores for each individual question relating to the three sexual symptoms were used to divide participants into symptomatic and asymptomatic groups(22, 23).

Neuropathy assessment

Neurological deficits were evaluated using the modified Neuropathy Disability Score (NDS), comprised of assessment of vibration perception, pinprick, temperature sensation and ankle reflexes(24).The vibration perception threshold (VPT) was established using a Horwell Neurothesiometer (Scientific Laboratory Supplies, Wilford, Nottingham, UK). Cold (CT) and warm (WT) perception thresholds were assessed on the dorsolateral aspect of the left foot using the TSA-II NeuroSensory Analyser (Medoc, Ramat-Yishai, Israel). Deep breathing heart rate variability (DB-HRV) was assessed using an ANX 3.0 autonomic nervous system monitoring device (ANSAR Medical Technologies, Philadelphia, PA, USA).

Corneal Confocal Microscopy (CCM)

Corneal confocal microscopy (CCM) (Heidelberg Retinal Tomograph III Rostock Cornea Module; Heidelberg Engineering, Heidelberg, Germany) was performed in all participants according to our previously established protocol(14). Six non-overlapping images from the centre of the cornea were selected per participant (three per eye). Corneal nerve fibre density (CNFD); the total number of major nerves per square millimetre of corneal tissue, corneal nerve branch density (CNBD); the number of branches emanating from the major nerve trunks per square millimetre of corneal tissue and corneal nerve fibre length (CNFL); the total length of all nerve fibres and branches [millimetre per square millimetre] within the area of corneal tissue were quantified. Analysis of corneal nerve morphology was performed using automated software (ACC Metrics, Manchester, UK)(25).

Laboratory measurements

Fasting venous blood samples were obtained between the hours of 0800 to 1000. Glycosylated haemoglobin (HbA1c) was measured using standard laboratory methods in the Department of Biochemistry, Manchester University Foundation Trust. For other blood tests, serum or plasma isolated within 2 hours of collection, was stored at 4°C or -20 °C until analysed. Each serum aliquot was stored for a maximum of 2 years and underwent one freeze-thaw cycle only. Liquid chromatography-tandem mass spectrometry in a validated clinical laboratory was used to determine serum total testosterone, dihydrotestosterone, dehydroepiandrosterone and androstenedione(26, 27). Electrochemiluminescence immunoassay (Roche diagnostics) using the Roche automated analyser (E170 platform) was used to measure sex hormone binding globulin (SHBG), luteinising hormone (LH) and follicular stimulating hormone (FSH). Serum free testosterone levels were calculated using the mass action equation by Vermeulen, utilising total serum testosterone, SHBG and albumin levels(28). Total testosterone level of less than 8nmol/L or total testosterone level between 8 and 11 nmol/L with calculated free testosterone level of < 220pmol/L(<0.220nmol/L) was considered as a low testosterone level(23).

Statistical analyses

Statistical analyses were performed using SPSS for Mac(Version 23.0, IBM SPSS Statistics, Arnok, NY: IBM Corp.) and GraphPad Prism for Mac OS X (version 8.3.0, GraphPad Software,

San Diego, California USA, www.graphpad.com). Data were tested for normality using the Shapiro-Wilk normality test. Continuous variables were compared between groups using the independent sample t-test and the Mann-Whitney U test was used in case of non-normally distributed data. The chi-squared test was used for analysis of categorical data. Correlations between variables were assessed using Spearman`s analyses. Multifactorial regression was used to assess independent risk factors for different measures of sexual dysfunction. Variables chosen for regression model were based on potential predicted influential factors. All data are expressed as Mean ± Standard deviation (SD). No attempt was made to adjust for missing data. The level of statistical significance was set at less than 0.05 for all analyses.

Results

Forty-one out of sixty-four (64%) participants had erectile dysfunction of whom 29% reported severe erectile dysfunction. A reduced frequency of sexual erection was reported by 67% and 26% of subjects reported infrequent sexual thoughts. The median overall satisfaction score was 1 which corresponds to moderate dissatisfaction. The median testosterone level was 10.9 nmol/L (95 % confidence interval 9.7-12.3 nmol/L). The total testosterone level was less than 8nmol/L in 14 (22%) subjects, between 8 to 11 nmol/L in 19 (30%) subjects and was normal in 31 (48%) subjects. Furthermore, late onset hypogonadism (LOH) was defined by the presence of symptoms and low (T <8 nmol/L) or moderate (T- 8-11 nmol/L) with free testosterone (<0.220nmol/L) and was present in 23.45% of the participants.

Erectile dysfunction scores (Table 1)

Clinical, biochemical and sex hormone parameters did not differ significantly between the symptomatic and asymptomatic groups, except for a higher HbA1c (p=0.03) in the asymptomatic group. There was no significant difference in NDS, VPT, CPT, WPT and DB-HRV between symptomatic and asymptomatic groups. However, CNFD (p=0.01), CNBD (p=0.003) and CNFL(p=0.006) were significantly lower in the symptomatic compared to the asymptomatic group (Table 4). The erectile function score correlated with CNFD (r= -0.38,

p=0.006), CNBD (r= -0.28, p=0.02), CNFL (r= -0.37, p=0.002) and frequency of early morning erections (r= -0.31, p=0.014) (Supplementary table). There was no correlation between the erectile function score and total or free testosterone or any other measure of neuropathy.

Frequency of sexual thoughts (Table 2)

There was no significant difference in clinical, biochemical, sex hormone measures and NDS, VPT, CPT, WPT and DB-HRV between those with infrequent sexual thoughts and normal frequency of sexual thoughts. CNFD (p=0.03) and CNBD (p=0.03) were significantly lower, but CNFL (p=0.11) did not differ between those with infrequent sexual thoughts compared to a normal frequency of sexual thoughts. There was no correlation between CCM parameters and frequency of sexual thoughts (Table 4). The frequency of sexual thoughts correlated with the frequency of early morning erections (r=0.42, p=0.001)(Supplementary table).

Frequency of morning erections (Table 3)

There was no significant difference in clinical and biochemical parameters, NDS, VPT, CPT, WPT and DB-HRV between those with infrequent and normal frequency of early morning erections. The total testosterone levels (p=0.04), CNBD(p=0.01) and CNFL (p=0.01) were lower in patients with infrequent compared to a normal frequency of early morning erections. The frequency of early morning erections correlated with CNFD (r=0.35, p= 0.006), CNBD (r=0.33, p=0.01), CNFL (r=0.33, p=0.008)(Table 4), frequency of sexual thoughts score (r=0.42, p=0.001) and erectile function score (r= -0.31, p=0.014)(Supplementary table).

Relationship between clinical demographics, biochemistry, sex hormone levels, neuropathy measures and symptoms of hypogonadism (Table 5)

Multifactorial regression was used to assess independent risk factors for different measures of sexual dysfunction. Total serum testosterone (B= 1.291, p=0.038) and CNFL (B= 0.847, p =0.037) were independently associated with the symptom of erectile dysfunction. Parameters of frequency of morning erections and frequency of sexual thoughts did not show any significant association with any of the variables.

Discussion

This is the first study to simultaneously assess the relationship between sexual function, detailed measures of small/large fibre and autonomic neuropathy and sex hormone levels in men with diabetes. Symptomatic sexual dysfunction was present in almost two thirds of the cohort studied and was relatively high compared to other studies of men with diabetes reflecting the methodology used to assess ED and population studied (29). We have used exactly the same methods as in the EMAS study and our population had a similar age (21). However, the overall satisfaction scores were lower and distress related to sexual function was higher in our cohort of patients with diabetes compared to the EMAS study cohort of community-dwelling men aged 40-79 years (21).

Furthermore, in the EMAS study only 2.1% had late onset hypogonadism defined by symptoms and a low testosterone level, whereas in the present study 16 % were symptomatic for reduced frequency of morning erections, 20% for reduced frequency of sexual thoughts and 18% for erectile dysfunction alone; with low testosterone levels. As in previous studies, we report that a significant proportion of patients (23.45% in current cohort) have low testosterone levels in men with diabetes (30-33).

Multitude of non specific symptoms could be associated with potential testosterone deficiency in ageing men (23).Studies have shown relatively high prevalence of most specific sexual symptoms of androgen deficiency in ageing men with normal testosterone levels(34-36). To overcome this limitation and to avoid overdiagnosis of late onset hypogonadism, Wu et al, proposed diagnostic criteria of multiple symptoms(minimum 3) with testosterone level of < 8 nmol/L (23).Wu et al confirmed syndromic association between low total and free testosterone levels and 3 sexual symptoms of erectile dysfunction, decreased frequency of sexual thoughts and decreased frequency of morning erections (23). In the current study we have used the same diagnostic criteria for the diagnosis of late onset hypogonadism in patients with diabetes. However, testosterone and free testosterone levels were not associated with any of the 3 measures of sexual function, in this cohort, suggesting that low testosterone level are not the major driver of sexual dysfunction in men with diabetes.

Several studies have demonstrated an association between diabetic neuropathy and ED(30-

33) . However, we now demonstrate significantly greater corneal nerve loss in relation to the different measures of severity of ED and a significant correlation between CCM parameters with erectile function scores and the frequency of early morning erections. Indeed, none of the QST and autonomic neuropathy parameters were associated with sexual dysfunction scores. Multifactorial liner regression was used to assess independent risk factors for different symptoms of sexual dysfunction. Total serum testosterone and CNFL were independently associated with the symptom of erectile dysfunction. This further supports the data from our recent studies showing greater corneal nerve loss and a specific relationship between small fibre neuropathy and erectile dysfunction in patients with T1DM (17) , T2DM (18) and severe obesity (19).The frequency of sexual thoughts did not correlate with CCM parameters and may reflect that it has a more complex psychological and hormonal basis.

A limitation of this study is the relatively small cohort of patients studied. However, the detailed phenotyping for the severity and characteristics of ED and hormonal status has allowed us to re-emphasize the importance of assessing small fibre neuropathy in diabetic patients with ED (29, 37) . Indeed patients with a poor response to first line therapy such as PDE5 inhibitors (38)may well have greater small fibre neuropathy. Therefore, CCM may prove to be useful in rapidly and non-invasively identifying non-responders for more rapid referral for alternative treatments for ED.

Table 1. Comparison of clinical demographics, biochemistry, sex hormone levels and neuropathy measures between patients with and without **symptoms of erectile dysfunction**.

	Asymptomatic (n= 22)	**Symptomatic (n = 42)**	**p-value**
Clinical characteristics			
Age (years)	57.7 ± 9.2	61.0 ± 8.7	0.15
Duration of diabetes (years)	11.2 ± 8.4	15.6 ± 14	0.27
Hypertension (n, %)	14 (60%)	21(48%)	0.47
Antihypertensives (n)	14 (0-2)	21 (0-3)	0.63
Biochemistry			
HbA1c (mmol/mol)	64. 4 ± 19.75	53.40 ± 12.32	**0.03***
eGFR (ml/min)	76.95 ± 16.7	76.40 ± 16.2	0.85
Triglyceride (mmol/l)	1.7 ± 0.82	1.72 ± 0.83	0.94
LDL-C (mmol/l)	1.95 ± 0.61	1.84 ± 0.60	0.28
Sex hormones			
Low testosterone (n(%))	7 (30.4%)	8 (18.6%)	0.367
Total testosterone (nmol/L)	10.61 ± 4.4	12.38 ± 5.7	0.21
Sex hormone binding globulin (nmol/L)	25.14 ± 13	23.9 ± 12.35	0.94
Albumin (gm/L)	36.8 ± 3.5	38.2 ± 3	0.11
Free testosterone (nmol/L)	0.266 ± 0.09	0.314 ± 0.10	0.08
Luteinising hormone (mIU/L)	8.4 ± 5.5	8.3 ± 5.2	0.89
Follicle-stimulating hormone (mIU/L)	7.2 ± 5.4	9 ± 9.1	0.71
Dihydrotestosterone (nmol/L)	0.76 ± 0.42	1.03 ± 0.70	0.16
Measures of neuropathy			

NDS (/10)	4.2 ± 2.9	3.7 ± 2.4	0.41
VPT (V)	17.36 ± 12.1	18.51 ± 12.6	0.70
CPT (°C)	28.8 ± 1.88	29.2 ± 1.38	0.75
WPT (°C)	41.5 ± 4.39	42.31 ± 4.2	0.14
CNFD (no./mm^2)	27. 52 ± 4.8	24.06 ± 5.6	**0.01***
CNBD (no./mm^2)	73.6 ± 35.36	50.88 ± 23.4	**0.003***
CNFL (mm/mm^2)	25.85 ± 5.7	21.96 ± 4.8	**0.006***
DB-HRV (beats/min)	16 ± 10	17 ± 7	0.41

Data are presented as mean and standard deviation. Independent t-test was performed for normally distributed variables and Mann-Whitney U test for non-parametric variables when comparing asymptomatic and symptomatic groups. *$p<0.05$ is considered statistically significant.

Abbreviations: HbA1c- Glycosylated haemoglobin, eGFR-estimated glomerular filtration rate, LDL-C-Low density lipoprotein cholesterol, NDS- Neuropathy Disability Score, VPT-Vibration perception threshold, CPT- Cold perception threshold, WPT- Warm perception threshold, CNFD- Corneal nerve fibre density, CNBD-Corneal nerve fibre branch density, CNFL- Corneal nerve fibre length, DB-HRV-deep breathing-heart rate variability.

Table 2. Comparison of clinical demographics, biochemistry, sex hormone levels and neuropathy measures between patients based on **frequency of sexual thoughts**.

	Normal frequency of sexual thoughts (n=49)	**Reduced frequency of sexual thoughts (n= 15)**	**p-value**
Clinical characteristics			
Age (years)	60 ± 8.9	57 ± 8.7	0.32
Duration of diabetes (years)	14.15 ± 13.2	13.27 ± 10.4	0.95
Hypertension (n)	24 (48.9%)	8 (53.30%)	0.99
Antihypertensives (n)	24 (1-3)	8 (1-2)	0.4
Biochemistry			
HbA1c (mmol/mol)	58.41 ± 20.09	65.83 ± 16.35	0.09
eGFR (ml/min)	77.94 ± 15.3	74 ± 15.8	0.35
Triglyceride (mmol/l)	1.6 ± 0.80	1.8 ± 0.88	0.46
LDL-C (mmol/l)	2.13 ± 0.53	2.08 ± 0.72	0.45
Sex hormones			
Low testosterone (n(%))	11 (22.45%)	3 (20%)	0.90
Total testosterone (nmol/L)	11.8 ± 5.4	11.9 ± 4.7	0.90
Sex hormone binding globulin (nmol/L)	25.7 ± 13	21.93 ± 11.16	0.30
Albumin (gm/L)	37.5 ± 3.2	38.2 ± 3.5	0.52
Free Testosterone (nmol/L)	0.293 ± 0.1	0.318 ± 0.1	0.28
Luteinising hormone (mIU/L)	8.23 ± 5.3	8.5 ± 5.2	0.81
Follicle-stimulating hormone (mIU/L)	8.39 ± 7.9	9.0 ± 8.2	0.72
Dihydrotestosterone (nmol/L)	0.93 ± 0.64	0.99 ± 0.56	0.79
Measures of neuropathy			

NDS (/10)	4 ± 3.1	3.1 ± 3.3	0.23
VPT (V)	17.6 ± 11.48	17.07 ± 12.9	0.36
CPT (°C)	29.1 ± 1.45	29.3 ± 0.93	0.18
WPT (°C)	41.5 ± 3.9	43.04 ± 4.6	0.31
CNFD (no./mm^2)	26.07 ± 5.3	22.54 ± 5.7	**0.03***
CNBD (no./mm^2)	63.52 ± 29.5	48.6 ± 28.2	**0.03***
CNFL (mm/mm^2)	24 ± 5.3	21.5 ± 5.6	0.11
DB-HRV (beats/min)	16 ± 9	18 ± 5	0.31

Data are presented as mean and standard deviation. Independent t-test was performed for normally distributed variables and Mann-Whitney U test for non-parametric variables when comparing asymptomatic and symptomatic groups. $p<0.05$ is considered statistically significant. **Abbreviations**: HbA1c- Glycosylated haemoglobin, eGFR-estimated glomerular filtration rate, LDL-C- Low density lipoprotein cholesterol, NDS- Neuropathy Disability Score, VPT-Vibration perception threshold, CPT-Cold perception threshold, WPT- Warm perception threshold, CNFD- Corneal nerve fibre density, CNBD-Corneal nerve fibre branch density, CNFL- Corneal nerve fibre length, DB-HRV- deep breathing-heart rate variability.

Table 3. Comparison of clinical demographics, biochemistry, sex hormone levels and neuropathy measures between patients based on **frequency of morning erections**.

	Normal frequency of morning erections (n= 22)	**Reduced frequency of morning erections (n=42)**	**p-value**
Clinical characteristics			
Age (years)	57.8 ± 9.2	61.07 ± 8.7	0.15
Duration of diabetes (years)	11.24 ± 8.5	15.6 ± 14	0.27
Hypertension, n(%)	10 (43.5%)	25 (58%)	0.60
Antihypertensives (n)	10 (1-2)	23 (1-3)	0.47
Biochemistry			
HbA1c (mmol/mol)	63.57 ± 24	56.5 ± 16.45	0.40
eGFR (ml/min)	76 ± 16.5	77.5 ± 15	0.84
Triglyceride (mmol/l)	1.6 ± 0.65	1.74 ± 0.90	0.70
LDL-C (mmol/l)	2.3 ± 0.87	1.99 ± 0.54	0.16
Sex hormones			
Low testosterone, n(%)	6 (26%)	7 (16%)	0.05
Total testosterone(nmol/L)	9.6 ± 3.5	12.93 ± 5.6	**0.04***
Albumin (gm/L)	36.9 ± 3.4	38.0 ± 3.2	**0.11**
Sex hormone binding globulin (nmol/L)	19.4 ± 7.4	27.4 ± 13.8	**0.03***
Free Testosterone (nmol/L)	0.278 ± 0.10	0.309 ± 0.1	**0.28**
Luteinising hormone (mIU/L)	8.0 ± 4	8.4 ± 5.8	0.80
Follicle-stimulating hormone (mIU/L)	8.0 ± 7.8	8.8 ± 8.0	0.31
Dihydrotestosterone (nmol/L)	0.77 ± 0.33	1.04 ± 0.71	0.33
Measures of neuropathy			

NDS (/10)	3.2 ± 3.1	4.14 ± 3.2	0.32
VPT (V)	16.65 ± 12.4	17.8 ± 11.5	0.55
CPT (°C)	28.8 ± 1.8	29.3 ± 1	0.79
WPT (°C)	41.5 ± 4.8	42 ± 3.8	0.58
CNFD (no./mm^2)	27 ± 5.9	24.38 ± 5.2	0.07
CNBD (no./mm^2)	74.6 ± 34.2	52.88 ± 24.7	**0.01***
CNFL (mm/mm^2)	25.87 ± 6	22.35 ± 4.8	**0.01***
DB-HRV (beats/min)	17 ± 9	17 ± 8	0.88

Data are presented as mean and standard deviation. Independent t-test was performed for normally distributed variables and Mann-Whitney U test for non-parametric variables when comparing asymptomatic and symptomatic groups. *$p<0.05$ is considered statistically significant.

Abbreviations: HbA1c- Glycosylated haemoglobin, eGFR-estimated glomerular filtration rate ,LDL-C-Low density lipoprotein cholesterol, NDS- Neuropathy Disability Score, VPT-Vibration perception threshold, CPT- Cold perception threshold, WPT- Warm perception threshold, CNFD- Corneal nerve fibre density, CNBD-Corneal nerve fibre branch density, CNFL- Corneal nerve fibre length, DB-HRV-deep breathing-heart rate variability.

Table 4. Correlations between CCM measures with sexual function assessments, serum testosterone levels and neuropathy measures

	CNFD	**CNBD**	**CNFL**
Frequency of sexual thoughts score	r = 0.10 p = 0.42	r = 0.16 p = 0.18	r = 0.08 p = 0.53
Erectile function assessment score	**r = -0.38** **p = 0.006***	**r = - 0.28** **p = 0.02***	**r = - 0.37** **p = 0.002***
Frequency of early morning erections score	**r = 0.35** **p = 0.006***	**r = 0.33** **p = 0.01***	**r = 0.33** **p = 0.008***
VPT	**r = - 0.24** **p = 0.04***	**r = - 0.35** **p = 0.004***	**r = - 0.41** **p = 0.0006***
CPT	r = 0.12 p = 0.32	**r = 0.35** **p = 0.004***	**r = 0.365** **p = 0.003***
WPT	r = -0.07 p = 0.54	**r = -0.31** **p = 0.012***	**r = -0.28** **p = 0.021***
DBHRV	r = 0.01 p = 0.94	**r = 0.21** **p = 0.13***	**r = 0.30** **p = 0.03***
NDS	r = -0.09 p = 0.44	r = -0.15 p = 0.23	r = -0.25 p = 0.05
HbA1c	r = 0.04 p = 0.77	r = 0.16 p = 0.31	r = 0.04 p = 0.78
Serum Testosterone	r = 0.04 p = 0.74	r =0.062 p = 0.63	r = 0.06 p = 0.64
Free testosterone	r = -0.023	r = 0.020	r = -0.001

	p = 0.86	p = 0.87	p = 0.99

Correlations between variables were assessed using Spearman's analyses.* $p<0.05$ is considered statistically significant.

Abbreviations: HbA1c- Glycosylated haemoglobin, NDS- Neuropathy Disability Score, VPT-Vibration perception threshold, CPT- Cold perception threshold, WPT- Warm perception threshold, CNFD-Corneal nerve fibre density, CNBD-Corneal nerve fibre branch density, CNFL- Corneal nerve fibre length, DB-HRV- deep breathing-heart rate variability.

Table 5. Association of clinical demographics, biochemistry, sex hormone levels and neuropathy measures with symptoms of erectile dysfunction

Variable	**Co-efficient (B)**	**p Value**
Age	0.948	0.252
Duration of Diabetes	1.095	0.109
Total Testosterone	**1.291**	***0.037**
CNFL	**0.847**	***** **0.038**
HbA1c	0.948	0.07
LDL-C	0.462	0.23

Multifactorial regression was used to assess independent risk factors for different measures of sexual dysfunction. Variables chosen for regression model were based on potential predicted influential factors. .* $p<0.05$ is considered statistically significant.

Abbreviations: CNFL- Corneal nerve fibre length, HbA1c- Glycosylated haemoglobin, LDL-C- Low density lipoprotein cholesterol.

Supplementary table 1. Correlations of measures of sexual function with serum testosterone levels and neuropathy measures

	Frequency of sexual thoughts score	**Erectile function assessment score**	**Frequency of early morning erection score**
NDS	r = 0.12 p = 0.32	r = -0.004 p = 0.97	r = -0.164 p = 0.21
VPT	r = 0.15 p = 0.24	r = 0.07 p =0.57	r = -0.11 p = 0.40
CPT	r = 0.011 p = 0.73	r = -0.14 p = 0.26	r = 0.08 p = 0.50
WPT	r = - 0.08 p = 0.49	r = 0.14 p = 0.26	r = - 0.05 p = 0.66
DBHRV	r = -0.043 p = 0.76	r = 0.077 p = 0.60	r = 0.065 p = 0.65
HbA1c	**r = - 0.34** **p = 0.03***	r = - 0.016 p = 0.92	r = - 0.004 p = 0.98
Testosterone	r = -0.33 p = 0.80	r = 0.11 p = 0.38	r = -0.143 p = 0.28
Free Testosterone levels	r = -0.056 p =0.67	r = 0.189 p = 0.146	r = -0.037 p = 0.78
Frequency of sexual thoughts score		r = - 0.15 p =0.23	**r = 0.42** **p = 0.001***
Erectile function assessment score	r = -0.15 p = 0.23		**r = - 0.31** **p = 0.014***
Frequency of early morning erection score	**r = 0.42** **p = 0.001***	**r = -0.31** **p = 0.014***	

Correlations between variables were assessed using Spearman's analyses. *p<0.05 is considered statistically significant.

Abbreviations: HbA1c- Glycosylated haemoglobin, NDS- Neuropathy Disability Score, VPT-Vibration perception threshold, CPT- Cold perception threshold, WPT- Warm perception threshold, DB-HRV-deep breathing-heart rate variability.

Supplementary table 2: Questions regarding sexual symptoms within the EMAS sexual function questionnaire and definitions of asymptomatic and symptomatic response categories.

	Asymptomatic	**Symptomatic**
Were you able to get and keep an erection sufficient for sexual intercourse?	Usually or always	Never or sometimes
How often did you think about sex?	Once a week or more	2–3 times in the past month
How frequently did you awaken with a full erection in the past month?	2–3 times in the past month	≤1 time in the past month

The definitions of asymptomatic and symptomatic response categories are based on validated published criteria.

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Data Accessibility

The datasets generated and analysed during the current study are available from the corresponding author on reasonable request.

Conflict of Interest

The authors declare that there is no duality of interest associated with this manuscript.

Author Contributions

All authors were involved in revising the manuscript critically for important intellectual content and for final approval of the version to be published.

SD and JHH were involved in acquisition of data, analysis and interpretation of data and wrote the manuscript.

SD, SA, JHH, MF and AK recruited patients.

SD, SA and JHH contributed to acquisition and analysis of the data.

MF and AK performed CCM for patients.

HS contributed to conception, interpretation of the data, wrote and revised the manuscript.

RAM contributed to conception and design of the study, wrote and revised the manuscript and is principal investigator of the study.

RAM 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.

There are no conflicts of interest in relation to this work.

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Chapter 7: Discussion

Discussion

Preface

Diabetes Mellitus (DM) is a major public health challenge with an predicted global prevalence by 2040, in approximately 10 % of the general population (1). Diabetic neuropathy (DN) is the most common complication of diabetes with a lifetime prevalence of 50% in patients with diabetes (2). DN presents with a spectrum of disorders from being asymptomatic to a constellation of symptoms and/or signs resulting from somatic (sensory and/or motor) nerve and/or autonomic nerve dysfunction, affecting many organ systems (3-5). Small fibre neuropathy is the earliest and commonest type of DN, affecting thinly myelinated A-delta and unmyelinated type-C fibres (6). Small fibre dysfunction precedes large fibre damage, resulting in neuropathic symptoms of pain, tingling and numbness in a length dependent manner (6, 7). Autonomic small fibre damage causes symptoms of postural dizziness, orthostatic hypotension, dry mouth, dry eyes, gastrointestinal, genitourinary, and erectile dysfunction (6, 7). Early diagnosis and assessment of progression of small fibre neuropathy may allow risk stratification and risk factor modification to limit advanced complications like diabetic foot ulcers, cardiac autonomic, gastrointestinal and sexual dysfunction (6, 7).

Small fibre neuropathy plays a key role in neuropathic pain and foot ulceration by contributing to abnormal blood flow, low skin oxygen tension, poor wound healing, ulceration, gangrene and amputation (6, 7). The usual presenting symptoms of small fibre neuropathy are pain, burning, tingling, numbness, hyperalgesia and allodynia in ~30% of patients with diabetes (6, 7). Crude touch, vibration sensation and proprioception are usually preserved in the early stages, with normal muscle strength and reflexes (6, 7). Autonomic small fibre neuropathy becomes clinically detectable only in advanced stages with symptoms such as postural orthostatic hypotension, resting tachycardia, anhidrosis, diabetic gastropathy and genitourinary symptoms including erectile and sexual dysfunction (6, 7).

Complications as a consequence of foot ulceration including infection and gangrene are a major source of morbidity in patients with diabetes (6, 7). About 50 % of patients with DN

can be asymptomatic at the time of presentation, hence early detection of DN in an objective manner is important to prevent this morbidity (6-8).

Sexual dysfunction is another source of distress in patients with type 1 and type 2 diabetes (9-11). Sexual dysfunction in men with diabetes ranges from 30-90% and results in disorders of libido, ejaculatory problems and erectile dysfunction (9). Females with diabetes suffer from disorders of sexual desire, arousal, vaginal lubrication, orgasms, satisfaction, and dyspareunia (12). Based on a meta-analysis, the overall prevalence of sexual dysfunction in women with type 2 diabetes was 68.6% (12). Normal sexual function is a complex interplay of various factors including psychological, neurohormonal and vascular, all of which are vulnerable to metabolic dysfunction resulting from DM (13, 14). There is limited evidence of benefits from testosterone replacement in improving sexual dysfunction in these patients except in hypogonadal men with diabetes (14-17). Role of DN especially small fibre dysfunction in the pathogenesis and treatment of sexual dysfunction has remained under investigated. Hence it is important to understand and address the role of factors like small fibre neuropathy, contributing to sexual dysfunction in diabetes.

External genitalia are innervated by both autonomic (sympathetic and parasympathetic) and somatic (sensory and motor) nerves via the inferior hypogastric plexus and branches of the pudendal nerve, respectively (18). Pain, temperature, touch-pressure and position sense are four major somatic sensations, which are transmitted through both both large (Aα and Aβ) and small (Að and C) fibres (18). All these sensations influence one's ability to respond to sexual response or sexual pleasure (12, 18, 19). Diabetic neuropathy thus contributes to the disorders of arousal, erectile dysfunction, lubrication, ejaculation, orgasm and overall sexual dysfunction (12, 18).

Erectile dysfunction is the most studied component of sexual dysfunction in patients with DM. Erectile dysfunction has been associated with both small and large fibre neuropathy (18). All of the four major somatic sensations can be assessed using quantitative sensory tests (QST) (18, 20). Whilst, most QST tests are used to assess sensations on the hands and feet, a few studies have used them to assess sensation in the genital region (18, 21, 22). Erection is mainly mediated through small fibres, hence using QST is more relevant in the assessment of ED than using neurophysiological studies which quantify large fibres (23, 24). However, QST is subjective and remains highly variable (25).

Measurement of intraepidermal nerve fibre density (IENFD) using skin biopsy is the current gold standard for the diagnosis of small fibre neuropathy in diabetes (26, 27). Corneal confocal microscopy (CCM) is a non-invasive, reliable and reproducible technique to assess small fibre neuropathy in diabetes with comparable diagnostic efficiency to IENFD (28, 29).

Small fibre pathology is associated with erectile dysfunction in men with type 2 diabetes

Erectile dysfunction has complex pathophysiology and is considered to be a marker of microvascular and cardiovascular disease, especially in men with diabetes (30, 31). Whilst, vascular abnormalities play a major role in diabetes related ED, some studies have demonstrated autonomic and somatic small fibre neuropathy in ED (32, 33). In this study we investigated the relationship between various measures of small and large fibre neuropathy and autonomic neuropathy in men with type 2 diabetes, with and without ED. We used neurophysiology (NCS) and vibration perception threshold (VPT) to assess large fibres and deep breathing heart rate variability (DB-HRV) to assess autonomic neuropathy. QST, IENFD and CCM were used for comprehensive assessment of small fibre damage. Unlike previous studies in men with type 2 diabetes (24, 34, 35), we found no correlation between ED and HbA1c, duration of diabetes, BMI, hypertension or lipid profile. However, we demonstrate significantly reduced IENFD and CCM parameters, confirming significant small fibre neuropathy with relative preservation of cardiac autonomic function (CAN) and neurophysiology, unlike previous studies where a strong association between ED and CAN was reported (33, 36). This is also in contrast to our previous study in type 1 diabetes and ED, where we demonstrated a more global neuropathy, involving small, large and autonomic fibres alike (25). Thus, in this study we demonstrate an association between small nerve fibre injury and erectile dysfunction.

Until recently, the impact of peripheral neuropathy in ED has been underestimated (32). Organic, relational and psychological factors contribute to ED, involving neurogenic, vasculogenic and endocrine pathways (37, 38). Although, some studies have identified association of somatic and autonomic neuropathy with ED, investigations and management

are usually directed to the assessment of sex hormones, cardiovascular and other risk factors, neuropathy remains rather neglected (18, 23, 39, 40).

Sensory impulses from the shaft and glans of penis are relayed into the reflexogenic centre in the spinal cord via pudendal nerves (34, 40). Contraction of bulbocavernosus and ischiocavernosus muscles contribute to the reduced venous outflow and help in maintenance of erection (34, 40). Pelvic floor muscles innervated from the motor part of pudendal nerve plexus are also important in the process of erection and ejaculation (34, 40). The vascular endothelium and autonomic nerves around penile arteries and corpora cavernosa contribute to the synthesis and release of nitric oxide (NO) which is vital for maintaining penile erection (41, 42). Abnormal penile thermal and vasotactile thresholds in diabetic patients with ED, attributed to small fibre neuropathy have been reported in some studies (21, 43, 44). Lefaucheur et al reported a strong correlation between clinical evaluation of ED and penile thermal testing (45). Our study clearly demonstrates widespread small fibre neuropathy is patients with T2DM and ED.

More sophisticated tests to measure local neurological damage in erectile dysfunction include penile somatosensory evoked potentials, bulbocavernous reflex, penile thermal sensory thresholds and corpus cavernosum electromyography (21, 43, 44, 46-49). However, these are not routinely available and lack reproducibility and normative data. Studies have shown a lack of correlation between neurophysiology and the severity of ED, principally because it assesses only large fibre integrity (33). QST is highly subjective and IENFD is too invasive to be incorporated into the routine evaluation of men with ED (46). Being non-invasive, rapid, highly objective and easily reproducible, CCM has the potential to bridge the gap in quantification of small fibre damage in patients with ED. In our current study both CCM and IENFD correlated well with ED. CCM has shown high sensitivity and specificity in the identification of autonomic neuropathy in diabetes (47) and we have also demonstrated a correlation between CCM and IENFD (53). We thus reinforce the utility of CCM in identification and quantification of small fibre neuropathy. Relative preservation of large fibres and CAN in the current study further argue for the utility of CCM in the identification of small fibre damage in relation to the major clinical outcome of ED. This will need confirmation by further studies.

Management of ED in diabetes is challenging as over 50 % patients do not adequately respond to PDE5 inhibitors (48, 49). A more severe neurogenic component to their ED may account for this suboptimal response (50). CCM could be used to rapidly identify this particular subgroup of potential non-responders to help in the decision making to deploy alternative treatments. CCM could also be utilised in prospective studies to analyse the impact of therapeutic interventions to improve small fibre neuropathy and assess the impact on ED.

Corneal Confocal Microscopy Identifies Small Fibre Damage and Progression of Diabetic Neuropathy

Clinical signs and symptoms in combination with QST and neurophysiology are the current standard of care measures for the assessment of DN. Small fibre neuropathy is plays a key role in complications like neuropathic pain and foot ulceration (51) and develops even in subjects with impaired glucose tolerance (51). There are limited studies utilising a comprehensive assessment of neuropathy to assess the progression of DN. This leaves gaps in the knowledge required to design and identify the end-points in clinical trials of disease modifying treatments for DN (52). QST suffers from lack of reproducibility, IENFD is too invasive for regular long term follow up and neurophysiology cannot assess small nerve fibres (2, 53-55). CCM has the potential to be used in long term follow up studies to delineate the natural history of DN better (56). Studies comparing CCM with IENFD have been relatively short in duration and mostly cross-sectional (57-60). In this study we compared CCM with detailed measures of neuropathy including QST and IENFD to assess progression of DN.

Besta criteria and revised Diabetic Neuropathy Study Group Of The European Association For The Study Of Diabetes (NEURODIAB) criteria are the two major criteria often used to define small fibre neuropathy. Both of these criteria are based on abnormal clinical signs and symptoms of small fibre damage, in addition to abnormal QST and or abnormal IENFD, in the presence of a normal nerve conduction study (NCS) (2, 61). These criteria emphasise the importance of a combined clinical, functional and structural approach to the diagnosis of small fibre neuropathy (52). However sensory symptoms and clinical signs are subjective,

and their reliability cannot be assessed directly (52). Though QST is a valid test, it`s diagnostic accuracy is not as good as IENFD (52). IENFD quantification is the most reliable and current gold standard, however it is resource intense, time consuming and expensive (62). CCM has shown diagnostic comparability to IENFD with the added advantage of being rapid, non-invasive and unbiased (28). CCM may thus play an important role in the diagnosis and follow up of patients with DN. Identification of new sodium channel gene mutations have opened up possibilities of various new targets for the treatment of neuropathies (63-65). CCM could play a crucial role as an accurate and reliable end-point to assess disease modifying treatments (66). Hence assessing the role of CCM in accurately quantifying the DN progression is very important.

In this study, we hypothesised that CCM is more sensitive than QST, IENFD and other accepted measures to assess the progression of DN. Detailed phenotyping of DN over a period of 6.5 years enabled a comprehensive comparison of the change in small and large fibre measures of DN and is the main strength of this study. We demonstrate worsening of DN despite improvement in glycaemic control and dyslipidaemia. Interestingly, there was also significant worsening of eGFR and albumin creatinine ratio (ACR) which indicates progression of diabetic nephropathy. CCM measures worsened to a greater degree than IENFD and other measures of large fibre and autonomic neuropathy. Worsening of CCM and IENFD was associated with worsening of autonomic function. The main limitation of this study was the relatively small number of patients assessed at follow up as patients refused to undergo a repeat skin biopsy. Given the rapidity, reliability and non-invasive nature of CCM in identifying progression of DN, it should have a greater role in future longitudinal studies and clinical trials.

Male sexual dysfunction in diabetes: role of sex hormones and small fibre neuropathy

Sexual dysfunction in men with diabetes comprises of disorders of libido, erectile function and ejaculation (9). Normal sexual function, especially penile-erection requires a complex interaction between various factors including hormonal, vascular, neurological and psychological (10, 11). Evaluation of vascular, neurological and psychological factors can be challenging and may not be possible in a single clinic visit (11). Hormonal aetiology is comparatively easy to evaluate in clinical settings with a morning testosterone sample and receives more attention as compared to other factors responsible for sexual and erectile dysfunction (11).Testosterone deficiency is an important part of the problem. Prevalence of testosterone deficiency in men with diabetes can be as high as, up to 40%, demonstrated by various studies (67). Recent studies suggest effectiveness of testosterone as monotherapy in those with mild ED (67) and it can improve response to PDE5 therapy in non-responders (68, 69). However, in men with moderate to severe ED, testosterone may be more effective in improving sexual desire rather than erectile function (67). This highlights the importance of investigating other factors of sexual dysfunction including neurological.

In this study, we investigated the relationship between sexual dysfunction, sex hormone levels and small nerve fibre pathology in a cohort of men with diabetes.

We demonstrate significant corneal nerve loss in relation to the different measures of severity of ED and a significant correlation between CCM parameters with erectile function scores and the frequency of early morning erections. This supports a central role of small fibre neuropathy in erectile function. We report significant correlation between CCM parameters and measures of QST and autonomic neuropathy. However, none of the QST and autonomic neuropathy parameters were associated with sexual dysfunction scores. Though significant proportion of men had low testosterone levels, total and free testosterone levels were not associated with any of the 3 major symptoms of sexual dysfunction. However, testosterone and CNFL were independently and significantly associated with symptom of erectile dysfunction. This suggests low testosterone levels are not a major driver in sexual dysfunction in patients with diabetes. Hence normalizing testosterone levels may not be beneficial in all patients with sexual dysfunction in diabetes.

Cross sectional design of the study and relatively small number of patients are the major limitations of the study. However, CCM can identify potential non-responders to conventional therapy and facilitate rapid referral to alternative treatment options.

Limitations

Even though each of the studies were sufficiently powered, relatively small sample sizes in chapters 4, 5 and 6 was an important limitation. This could have influenced the outcomes in these studies and limited our assessment of associations to define underlying mechanisms.

Elimination of confounding factors was not possible due to the observational nature of all the studies. Chapter 5 did not have a control group in the follow up study and chapter 6 did not have a healthy control group for comparison.

In chapter 4 a single question from the neuropathy symptom score (NSP) was used to define ED, which may have identified more severe ED and therefore mild to moderate ED may have been missed.

In chapter 5, many participants opted out of a repeat skin biopsy on follow up due to the invasive nature of the procedure. This was a major limitation, leading to an overall small number of patients in the study.

In chapter 6, patients with both T1DM and T2DM were included in the study. However, the number of patients with T1DM was small compared to T2DM and the overall number of T1DM participants was too small to draw any conclusions about phenotyping for the severity, characteristics of sexual dysfunction and hormonal status of these patients.

Future Work

The work in this thesis reinforces the role of CCM as an important objective and reproducible biomarker in the assessment of small fibre neuropathy in patients with both T1DM and T2DM. We have shown that CCM is independently associated with symptoms and severity of erectile dysfunction. In the longitudinal study using CCM, we have shown that CCM is a rapid, non-invasive test to identify progression of neuropathy and may have greater utility than clinical symptoms, signs, QST and nerve conduction studies. Corneal nerve loss was associated with a loss of IENFD and worsening of other measures of small fibre neuropathy.

CCM could be deployed as a potential surrogate for the assessment of progression of small fibre neuropathy and in therapeutic drug trials of DN. Objective quantification of the severity of DN using CCM could help in identifying patients at high risk of complications and potential non-responders to conventional treatments for ED.

We have shown an association between corneal nerve loss with increasing albuminuria and reduced eGFR. CCM may help to predict the onset and progression of these and other microvascular complications and this needs to be evaluated in further studies.

ED is a potential surrogate of cardiovascular disease and related morbidity and mortality. Early identification of underlying small fibre damage in ED using CCM could explain the link with increased CV outcomes as autonomic neuropathy has been shown to predict CV events. The role of CCM in the evaluation of onset and progression of macrovascular disease is another area for potential future studies.

Retinal photographs for annual screening of diabetic retinopathy has revolutionised screening, early diagnosis and management of retinopathy in diabetes. Early identification and early intervention is the key to prevent blindness and related morbidity. CCM has similar potential to work for early identification of diabetic neuropathy which can be incorporated into annual screening programmes. This will help for identification of subclinical DPN and will potentially help to prevent related morbidity and complications including foot ulcers, foot infections and amputations. CCM thus has a great potential of translational link between research and clinical use.

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Chapter 8: Appendix

Study related documents

8.1 Ethical approval NIH Study

NHS

National Research Ethics Service
North Manchester Research Ethics Committee
Room 181
1st Floor
Gateway House
Piccadilly South
Manchester
M60 7LP

Telephone: 0161 237 2166
Facsimile: 0161 237 2383

20 August 2009

Dr Rayaz Malik
Senior Lecturer / Consultant Physician
University of Manchester
Cardiovascular & Endocrine Sciences
Core Technology Facility (3rd floor)
46 Grafton Street
Manchester
M13 9NT

Dear Dr Malik

Study Title: **Longitudinal assessment of novel ophthalmic diabetic markers**
REC reference number: **09/H1006/38**

Thank you for your letter of 14 August 2009, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Ethical review of research sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

For NHS research sites only, management permission for research ("R&D approval") should be obtained from the relevant care organisation(s) in accordance with NHS research governance arrangements. Guidance on applying for NHS permission for research is

This Research Ethics Committee is an advisory committee to North West Strategic Health Authority

The National Research Ethics Service (NRES) represents the NRES Directorate within the National Patient Safety Agency and Research Ethics Committees in England

available in the Integrated Research Application System or at http://www.rdforum.nhs.uk. *Where the only involvement of the NHS organisation is as a Participant Identification Centre, management permission for research is not required but the R&D office should be notified of the study. Guidance should be sought from the R&D office where necessary.*

Sponsors are not required to notify the Committee of approvals from host organisations.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Letter from Sponsor	1.0	06 May 2009
Covering Letter		20 April 2009
REC application	5.6	25 March 2009
Funding letter		02 October 2008
Investigator CV	Andrew Boulton	21 April 2009
Investigator CV	Rayaz Malik	06 November 2007
Investigator CV	Mitra Tavakoli	30 March 2009
Investigator CV	Andrew Marshall	25 April 2009
Letter of invitation to participant		
BioBank manual of operations	March 2009	29 April 2009
Participant Information Sheet: BioBank Participant and Consent Form	1	23 April 2009
BioBank Withdrawal of Consent Form	1	30 March 2009
BioBank participant summary form	1	29 April 2009
BioBank Blood Collection Form	1	29 April 2009
BioBank Tissue Collection Form	1	29 April 2009
BioBank Equipment Record Form	1	29 April 2009
BioBank Specimen Transfer Form	1	29 April 2009
BioBank Specimen Destruction Form	1	29 April 2009
BioBank researcher specimen distribution form	1	29 April 2009
Response to Request for Further Information		16 July 2009
Statistician Comments		26 June 2009
Revised application from	5.6	
Protocol	August 2009	
Participant Information Sheet: Information Sheet A	3	12 August 2009
Participant Information Sheet: Youth Participants Information Sheet A	1	10 August 2009
Participant Consent Form	3	13 August 2009
Assent form for children aged 14 - 16	1	13 September 2009
Response to Request for Further Information		14 August 2009

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating

Procedures for Research Ethics Committees in the UK.

After ethical review

Now that you have completed the application process please visit the National Research Ethics Service website > After Review

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

We would also like to inform you that we consult regularly with stakeholders to improve our service. If you would like to join our Reference Group please email referencegroup@nres.nosa.nhs.uk.

09/H1006/38	**Please quote this number on all correspondence**

Yours sincerely

Stephen Tebbutt

Mr Ken Cook
Chair

Email: stephen.tebbutt@northwest.nhs.uk

Enclosures: "After ethical review – guidance for researchers"

Copy to: Dr Karen Shaw, Head of the Research Office, The University of Manchester

8.2 Ethical approval for PROPANE

Study

NHS
Health Research Authority
National Research Ethics Service

NRES Committee North West - Greater Manchester East
3rd Floor, Barlow House
4 Minshull Street
Manchester
M1 3DZ

Telephone: 0161 625 7820

05 March 2014

Professor Rayaz A Malik, Honorary Consultant Physician
Centre for Endocrinology & Diabetes, Institute of Human Development
Core Technology Facility (3rd Floor)
46 Grafton Street
Manchesster
M13 9NT

Dear Professor Malik

Study title:	**PROPANE STUDY: Probing the Role of Sodium Channels in Painful Neuropathies**
REC reference:	**14/NW/0093**
IRAS project ID:	**143141**

Thank you for your letter of 04 March 2014, responding to the Committee's request for further information on the above research and submitting revised documentation. The further information has been considered on behalf of the Committee by the Vice Chair.

We plan to publish your research summary wording for the above study on the HRA website, together with your contact details, unless you expressly withhold permission to do so. Publication will be no earlier than three months from the date of this favourable opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to withhold permission to publish, please contact the REC Manager, Elaine Hutchings, on nrescommittee.northwest@nhs.net.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Ethical review of research sites

NHS sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

A sentence should be added to the information sheet to specify the genes which will be looked at.

You should notify the REC in writing once all conditions have been met (except for site approvals from host organisations) and provide copies of any revised documentation with updated version numbers. The REC will acknowledge receipt and provide a final list of the approved documentation for the study, which can be made available to host organisations to facilitate their permission for the study. Failure to provide the final versions to the REC may cause delay in obtaining permissions.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned. Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

Sponsors are not required to notify the Committee of approvals from host organisations

Registration of Trials

All clinical trials (defined as the first four categories on the IRAS filter page) must be registered on a publically accessible database within 6 weeks of recruitment of the first participant (for medical device studies, within the timeline determined by the current registration and publication trees).

There is no requirement to separately notify the REC but you should do so at the earliest opportunity e.g. when submitting an amendment. We will audit the registration details as part of the annual progress reporting process.

To ensure transparency in research, we strongly recommend that all research is registered but for non-clinical trials this is not currently mandatory.

If a sponsor wishes to contest the need for registration they should contact Catherine Blewett (catherineblewett@nhs.net), the HRA does not, however, expect exceptions to be made. Guidance on where to register is provided within IRAS.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Covering Letter		04 February 2014
Evidence of insurance or indemnity		22 January 2014
GP/Consultant Information Sheets	1	04 February 2014
Investigator CV	Rayaz Malik	
Investigator CV	Andrew Marshall	13 January 2014
Investigator CV	Mitra Tavakoli	30 October 2013
Investigator CV	Hassan Fadavi	14 January 2014
Letter from Sponsor		22 January 2014
Letter of invitation to participant	1	04 February 2014
Other: Email with additional information		05 February 2014
Participant Consent Form	2	26 February 2014
Participant Information Sheet	2	26 February 2014
Protocol	2	26 February 2014
Questionnaire: Neuropathic pain scale		
Questionnaire: Pre-RODS		
Questionnaire: SF-36 (tm) Health Survey		
Questionnaire: Health state		
Questionnaire: HADS		
Questionnaire: Small Fibre Symptoms Inventory Questionnaire		
Questionnaire: VAS		
REC application	143141	04 February 2014
Response to Request for Further Information		04 March 2014

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The NRES website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Feedback

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the website.

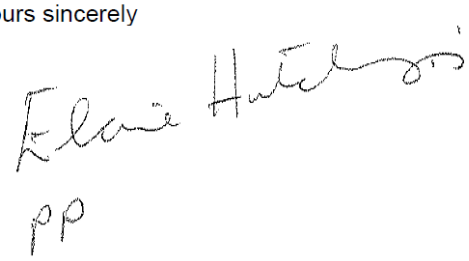
Further information is available at National Research Ethics Service website > After Review

14/NW/0093	**Please quote this number on all correspondence**

We are pleased to welcome researchers and R & D staff at our NRES committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

With the Committee's best wishes for the success of this project.

Yours sincerely



pp

Mr Francis Chan
Chair

Email:nrescommittee.northwest-gmeast@nhs.net

Enclosure: "After ethical review – guidance for researchers"

Copy to: *Ms Lynne Macrae, University of Manchester*

Lorraine Broadfoot,
Central Manchester University Hospitals NHS Foundation Trust

8.3 Ethical approval for NIHDP3 Study

Health Research Authority

North West - Greater Manchester South Research Ethics Committee
3rd Floor, Barlow House
4 Minshull Street
Manchester
M1 3DZ

Telephone: 0207 104 8002

Please note: This is the favourable opinion of the REC only and does not allow you to start your study at NHS sites in England until you receive HRA Approval

11 November 2016

Professor Andrew J Boulton
Chief Investigator
The University of Manchester
Cardiovascular and Endocrine Sciences, Manchester Royal Infirmary, Oxford Road
Manchester
M139WL

Dear Professor Boulton

Study title:	**Evaluation of corneal confocal microscopy as a surrogate endpoint for the identification and prediction of diabetic neuropathy (multinational study)**
REC reference:	**16/NW/0729**
IRAS project ID:	**197851**

Thank you for responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Vice-Chair, Dr Nader Touqan and Dr Joel Sanderson.

We plan to publish your research summary wording for the above study on the HRA website, together with your contact details. Publication will be no earlier than three months from the date of this opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to make a request to postpone publication, please contact the REC Assistant, Miss Ewa Grzegorska, nrescommittee.northwest-gmsouth@nhs.net.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Conditions of the favourable opinion

The REC favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements. Each NHS organisation must confirm through the signing of agreements and/or other documents that it has given permission for the research to proceed (except where explicitly specified otherwise).

Guidance on applying for NHS permission for research is available in the Integrated Research Application System, www.hra.nhs.uk or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of management permissions from host organisations

Registration of Clinical Trials

All clinical trials (defined as the first four categories on the IRAS filter page) must be registered on a publically accessible database within 6 weeks of recruitment of the first participant (for medical device studies, within the timeline determined by the current registration and publication trees).

There is no requirement to separately notify the REC but you should do so at the earliest opportunity e.g. when submitting an amendment. We will audit the registration details as part of the annual progress reporting process.

To ensure transparency in research, we strongly recommend that all research is registered but for non-clinical trials this is not currently mandatory.

If a sponsor wishes to contest the need for registration they should contact Catherine Blewett (catherineblewett@nhs.net), the HRA does not, however, expect exceptions to be made. Guidance on where to register is provided within IRAS.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Ethical review of research sites

NHS sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document	*Version*	*Date*
Evidence of Sponsor insurance or indemnity (non NHS Sponsors only) [Sponsor Insurance]		26 August 2016
GP/consultant information sheets or letters	2	31 October 2016
IRAS Application Form [IRAS_Form_02112016]		02 November 2016
IRAS Application Form XML file [IRAS_Form_02112016]		02 November 2016
IRAS Checklist XML [Checklist_02112016]		02 November 2016
Letter from sponsor [Sponsor Letter]		26 August 2016
Letters of invitation to participant [Invitation letter]	1	16 August 2016
Letters of invitation to participant [Letter to participants]	2	29 October 2016
Other [Email clarification re: US SHHS Grant Details]		28 September 2016
Other [Neuropathy assessment form]	1	06 August 2016
Other [Ophthalmic assessment form]	1	06 August 2016
Participant consent form [Consent form]	3	16 August 2016
Participant consent form [Biobank consent form]	1	06 August 2016
Participant information sheet (PIS) [patient information sheet]	4	29 October 2016
Research protocol or project proposal	4.0	29 October 2016
Summary CV for Chief Investigator (CI) [Chief investigator]		
Summary CV for student [Alise Kalteniece's CV]		
Summary CV for student [Shaishav Dhage's CV]		
Summary CV for supervisor (student research) [Rayaz Malik's CV]		
Summary CV for supervisor (student research) [Handrean Soran's CV]		
Validated questionnaire [Assessment Form]	1	06 August 2016
Validated questionnaire [McGill Pain]	1	06 August 2016
Validated questionnaire [NSP]	1	06 August 2016
Validated questionnaire [EMAS]	1	06 August 2016
Validated questionnaire [FSFI]	1	06 August 2016

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document *"After ethical review – guidance for researchers"* gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The HRA website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

User Feedback

The Health Research Authority is continually striving to provide a high quality service to all applicants and sponsors. You are invited to give your view of the service you have received and the application procedure. If you wish to make your views known please use the feedback form available on the HRA website: http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance/

HRA Training

We are pleased to welcome researchers and R&D staff at our training days – see details at http://www.hra.nhs.uk/hra-training/

16/NW/0729	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project.

Yours sincerely

On behalf of
Professor Sobhan Vinjamuri
Chair

Email: nrescommittee.northwest-gmsouth@nhs.net

Enclosures: "After ethical review – guidance for researchers"

Copy to: Ms Lynne Macrae

Dr Lynne Webster,
Central Manchester and Manchester Children's Hospitals NHS Trust

8.4 Patient consent form

IRAS Project ID: 197851

Central Manchester and Manchester Children's University Hospitals NHS Trust

Participant Study Number:

CONSENT FORM

Title of Project: Evaluation of corneal confocal microscopy as a surrogate endpoint for the identification and prediction of diabetic neuropathy (multinational study)

Investigators:
Prof. Rayaz. A Malik, Consultant Physician, MB ChB, FRCP, PhD.
Dr Handrean Soran, Consultant Physician, MD, FRCP.
Prof. Andrew Boulton, Consultant Physician, MBBS, MD, FRCP, DSc.
Dr. Andrew Marshall, Consultant Clinical Neurophysiologist BSc, BM CHB, MRCP
Dr Maria Jeziorska, Senior Lecturer in Pathology

		Please initial box:
1.	I confirm that I have read and I understand the information sheet dated [*16.08.2016*] (Version [3]) 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 relevant sections of my medical notes and data collected during the study may be looked at by individuals from the University of Manchester, from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this 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.	☐
7.	I understand that this study requires two small samples of skin to be removed from the top of the foot which may leave a small scar that will fade over time. I agree to have this procedure undertaken.	☐
8.	I permit that blood samples taken may be stored for use in the future studies subject to approval from a suitable ethics committee.	☐

.....	.....	.....
Name of Patient	Signature	Date
.....	.....	.....
Name of Person taking consent	Signature	Date

1 copy for patient; 1 copy for researcher

Consent form – 16/08/2016, Version 3

8.5 Biobank patient consent form

IRAS Project ID: 197851

Central Manchester University Hospitals NHS
NHS Foundation Trust

wellcome trust

Participant Information and Consent Form for Biobank

Principal Investigator:
Professor Andrew Boulton
Professor Rayaz Malik
Dr Handrean Soran
Dr Andrew Marshall
Dr Maria Jeziorska

1. Introduction

As a participant in the study entitled "Evaluation Of Corneal Confocal Microscopy As A Surrogate Endpoint For The Identification And Prediction Of Diabetic Neuropathy (Multinational Study)," you are also invited to donate blood to the LANDMARK BioBank. The purpose of this biobank is to collect and store blood and skin tissue for future research purposes. Your involvement entails: donation of blood and a skin biopsy which can be stored in the tissue bank and allowing access to your relevant medical records.

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

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

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

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

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

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

2. What is the LANDMARK BioBank?

The LANDMARK BioBank is part of a project sponsored by JDRF and University of Manchester that collects and stores tissue and blood for future research purposes. These samples will be labelled with a unique code and when a researcher wishes to study certain aspects of diabetes, he/she can apply to the BioBank for samples of tissue, blood or other biological samples. The researcher will not know who donated the samples (i.e. the samples are 'de-identified'). These

samples can only be given to researchers with your permission and the BioBank will require written evidence of ethical approval before researchers are allowed access to samples.

Associated with the BioBank is the collection of the clinical study data acquired during the LANDMARK study and other relevant medical history and clinical information of people who have donated tissue and or blood. This information is stored in a secure clinical database.

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

3. What is the purpose of sample collection?

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

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

An additional blood sample (20-25 mls, or 2-3 tubes) from you will be collected and the sample will undergo processing into the plasma and serum components as well as DNA, which will be stored frozen to allow future research to be undertaken in diabetes. You can donate a blood samples to the BioBank, even if you are not donating a skin biopsy. If you consent to having a blood sample collected it will be done at a time that is convenient to you; for example, rather than having blood collected at your study visit, it can be arranged for the blood samples to be collected at another time.

You are about to have a skin biopsy to remove tissue to do some tests. Not all the tissue is needed for assessing the nerves by examination under the microscope. It is this 'left over' tissue that we invite you to donate to the Bio Bank. The amount stored will be approximately 1.5mm in diameter.

5. Are there any risks involved?

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

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

We would also like to collect other relevant information about you and store it on a secure computer database, so that we can link your tissue samples to the data collected in the study and other ongoing information about your health (e.g. HbA1c levels). This information will be obtained from health care providers, for example medical practitioners, consultants and hospitals and will be stored in the secure database. This information will remain strictly confidential and protected by a password. Only authorised BioBank staff from University of Manchester will be able to access this information. All information will be dealt with and stored in accordance with State and Federal Legislation and National Health & Medical Research Council National Statement 2007. Your information will not be used other than for the purpose of this protocol. The BioBank may be asked to supply proof of your consent.

6. What are the benefits involved?

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

7. What if I change my mind?

Your participation in this project is voluntary and the choice to donate your tissue and or blood is entirely up to you. No matter what you decide, it will not affect your care in any way. You are under no obligation to donate blood/tissue. If you change your mind at any time, please inform the principle Investigator Professor Rayaz Malik or Dr Handrean Soran.

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

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

10. Who will use my tissue?

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

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

We encourage you to call us with any concerns or questions you may have. You can contact principle investigator of this project Professor Rayaz Malik at rayaz.a.malik@manchester.ac.uk, or Dr Handrean Soran at 0161 276 4066 (secretary).

12. What else do I need to know?

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

13. Consent

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

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

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

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

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

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

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

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

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

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

Participant's name (printed) _____

Signature_____ Date _____

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

Name of parent/guardian of participant (printed) _____

Signature_____ Date _____

Name of witness to participant's signature (printed) _____

Signature_____ Date _____

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

Researcher's name (printed) _____

Signature_____ Date _____

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

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

14. Who else can I contact?

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

Future Contact Consent

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

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

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

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

Name of 1st nominee if I cannot be contacted _____

Relationship to me: _____

Address _____

_____

Phone _____

Name of 2nd nominee if I cannot be contacted _____

Relationship to me: _____

Address _____

_____

Phone _____

Signature: _____ Date _____

Witness name _____

Witness Signature _____ Date _____

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

Investigator full name _____

Investigator signature _____ Date: _____

8.6 Patient assessment forms

IRAS Project ID: 197851

Central Manchester University Hospitals NHS
NHS Foundation Trust

Check List:

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

Name of Patient:

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

Medical history		
Hypertension	stroke	High cholesterol
Heart problems	Breathing problems	
Other health issues:		
Medication		
Beta blockers	Warfarin	Synthrome
Aspirin	Clopidogrel	ACE inhibitor
A2RB	Statin	Fibrates
Other anti-hypertensive drugs		
Neuphropathy		
Exclusion criteria		

Assessment form-06/08/2016 (Version 1)

History of corneal trauma or surgery (NB cataract surgery does not preclude enrolment unless surgery occurred in the 12 months prior to enrolment date)	History of ocular disease or systemic disease which may affect the cornea	Concurrent ocular disease, infection or inflammation
History of systemic disease (e.g. malignant disease, congestive heart failure NYHA Grade III or IV, major psychosis (i.e. schizophrenia or bipolar), certain autoimmune diseases – hypothyroidism, Addisons, vitiligo)	Warfarin or Aspirin & Clopidrogrel	Participating in any other interventional (e.g. drug) research trial.
History of neuropathy due to non-diabetic cause e.g. alcoholism, amyloidosis, autoimmune disorders, chronic kidney failure, connective tissue disease, infectious disease (e.g., Lyme disease, HIV/AIDS, hepatitis B, leprosy), liver failure, radiculopathy, vitamin deficiencies (e.g. pernicious anaemia, B12 deficiency)		
The following exclusion criteria apply to Group 5 (non-diabetic participants without neuropathy): Diabetes and GADAb positive		

IRAS Project ID: 197851

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

PHYSICAL MEASUREMENTS:

Height (cm) (no shoes):	
Weight (Kg) (no shoes):	
Waist (cm):	
Hips (cm):	

Brachial blood pressure – (mmHg):

			Average
Systolic pressure lying :			
Diastolic pressure lying:			
Heart rate lying:			
Systolic pressure standing :			
Diastolic pressure standing:			
Heart rate standing:			

IRAS Project ID: 197851

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

NEUROPATHY SYMPTOM PROFILE

Symptoms of Weakness

Head and neck:

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

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

Chest:

"Do you experience these symptoms to an abnormal degree?

	Yes	No
7. Weakness in speaking due to shortness of breath	☐	☐
8. Shortness of breath due to muscle weakness	☐	☐
9. Other weakness of the chest	☐	☐

Upper Limbs:

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

	Yes	No
10. Weakness of hands, *eg.* when handling coins, using a key	☐	☐
11. Weakness when straightening fingers	☐	☐
12. Weakness of fingers when clasping or grasping objects	☐	☐
13. Weakness of the wrists	☐	☐
14. Weakness of shoulders and upper arms (e.g. lift	☐	☐

objects from a high shelf, comb hair)

15. Other weakness in upper limbs ☐ ☐

Participant's Full Name: Date of Birth: Date:

Investigator:

NEUROPATHY SYMPTOM PROFILE / continued

Lower Limbs:

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

	Yes	No
16. Weakness of the legs so that you slap your feet in walking or cannot carry your weight on your heels	☐	☐
17. Weakness of the legs so that you cannot walk on your toes or forefoot	☐	☐
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	☐	☐
19. Other weaknesses of the lower limbs	☐	☐

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."

	Yes	No
20. Decrease (or inability) to feel the surface features, size, shape, or texture of what you touch?	☐	☐
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?	☐	☐
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

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

NEUROPATHY SYMPTOM PROFILE / continued

	Yes	No
22. Decreased (inability) to feel pain, cuts, bruises, or injuries?	☐	☐
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)?	☐	☐
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	☐	
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

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

NEUROPATHY SYMPTOM PROFILE / continued

	Yes	No
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	☐	

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

NEUROPATHY SYMPTOM PROFILE / continued

Autonomic Symptoms

"Do you experience these symptoms to an abnormal degree?"

	Yes	No
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?	☐	☐

IRAS Project ID: 197851

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

DNS-score and guidelines

The questions should be answered 'yes' (positive: 1 point) if a symptom occurred more than once in a week during the last 2 weeks or 'no' (negative: no point) if it did not.

1. Are you suffering of unsteadiness in walking?
   (ie. need for visual control, increase in the dark, walk like a drunk man, lack of contact with floor)

   ☐ *Yes(1)* ☐ *No (0)*

2. b Do you have a burning, aching pain or tenderness at your legs or feet?
   (ie. occurring at rest or at night, not related to exercise, exclude claudicatio intermittens)

   ☐ *Yes(1)* ☐ *No (0)*

3. Do you have prickling sensations at your legs and feet?
   (ie occurring at rest or at night, distal>proximal, stocking glove distribution)

   ☐ *Yes(1)* ☐ *No (0)*

4. Do you have places of numbness on your legs or feet?
   (ie. distal>proximal, stocking glove distribution)

   ☐ *Yes(1)* ☐ *No (0)*

Total score _____/4

Maximum score: 4 points;
0 points = PNP absent;
1-4 points = PNP present.

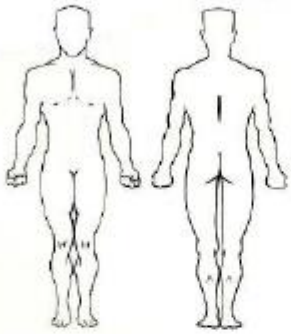
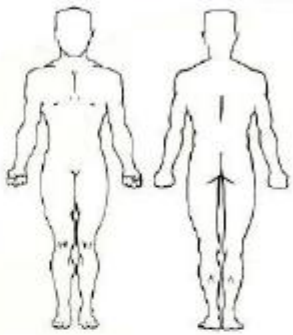
Short Form McGill Pain Questionnaire

Initials:	Date:	Visit ID:	ID:

Please descibe any pain you have today, or most days. Choose tthe two most significant if you have more than two.

Please leave the shaded cells blank.

Pain Location & Descriptors

Pain 1	Pain 2
Write location of pain _____	_____
Indicate pain location on torso	
	
Pain 1	Pain 2

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

Throbbing	None	Mild	Moderate	Severe		None	Mild	Moderate	Severe	
Shooting	None	Mild	Moderate	Severe		None	Mild	Moderate	Severe	
Stabbing	None	Mild	Moderate	Severe		None	Mild	Moderate	Severe	
Sharp	None	Mild	Moderate	Severe		None	Mild	Moderate	Severe	
Cramping	None	Mild	Moderate	Severe		None	Mild	Moderate	Severe	
Gnawing	None	Mild	Moderate	Severe		None	Mild	Moderate	Severe	
Hot-Burning	None	Mild	Moderate	Severe		None	Mild	Moderate	Severe	
Aching	None	Mild	Moderate	Severe		None	Mild	Moderate	Severe	
Heavy	None	Mild	Moderate	Severe		None	Mild	Moderate	Severe	
Tender	None	Mild	Moderate	Severe		None	Mild	Moderate	Severe	
Splitting	None	Mild	Moderate	Severe		None	Mild	Moderate	Severe	
Tiring-Exhausting	None	Mild	Moderate	Severe		None	Mild	Moderate	Severe	
Sickening	None	Mild	Moderate	Severe		None	Mild	Moderate	Severe	
Fearful	None	Mild	Moderate	Severe		None	Mild	Moderate	Severe	
Punishing-Cruel	None	Mild	Moderate	Severe		None	Mild	Moderate	Severe	
				Total					Total	

Continued over page...

Assessment form-06/08/2016 (Version 1)

Short Form McGill Pain Questionnaire (cont)

Initials	Date:	ID:

Pain 1	Pain 2
Visual Analogue Scale	
Please place a vertical line on the line below that best represents the pain you have on a from no pain to worst possible pain. NO PAIN — WORST POSSIBLE PAIN Total	NO PAIN — WORST POSSIBLE PAIN Total
Pain Index	
Please circle one word from the index below that best describes your pain. 0 No Pain 1 Mild 2 Discomforting 3 Distressing 4 Horrible 5 Excruciating Total	0 No Pain 1 Mild 2 Discomforting 3 Distressing 4 Horrible 5 Excruciating Total
Comments	
Please write any comments you have below: *Thanks for completing the questionnaire.*	
Investigator	

Neuropathy Disability Score

	Right		Left	
	Normal (0)	Abnormal (1)	Normal (0)	Abnormal (1)
Pain (pin-prick)	☐	☐	☐	☐
Vibration (tuning fork)	☐	☐	☐	☐
Temp. (hot/cold rods)	☐	☐	☐	☐

Achilles Reflex:	Normal (0)	Reinforcement (1)	Abnormal (2)	Normal (0)	Reinforcement (1)	Abnormal (2)
	☐	☐	☐	☐	☐	☐

Total NDS (/10) = ☐

VPT **Average**

Right:				
Left:				

Neuropad

Left: ☐ **Right:** ☐

Dominant hand:

Right: ☐ **Left:** ☐

10 gram monofilament:

Right: ☐ **Left:** ☐

MEDOC on left foot:

CT: ☐ **WT:** ☐ **CIP:** ☐ **WIP:** ☐

Cardiac autonomic dysfunction assessments:

DB-HRV ☐ **E/I ratio** ☐

LFA/RFA ratio ☐ **Valsalva ratio** ☐

30:15 ratio ☐

Baseline sitting BP ☐ **Standing BP** ☐

Baseline sitting DP		**Standing DP**	
Baseline sitting HR		**Standing HR**	

EMAS Sexual Function Questionnaire (for male participants)

Section A

This asks about some general background information

Please tick the ONE statement that best describes your circumstances IN THE LAST 4 WEEKS.

I have been living with my wife	☐	1
I have been cohabitating with my partner	☐	2
I have a sexual partner but we did not live together	☐	3
I did not have a sexual partner	☐	4

If you had a sexual partner in the last 4 weeks please answer all questions in this booklet.

If you did NOT have a sexual partner in the last 4 weeks, please skip the next two questions and go straight to Section B.

1 In general, would you say that the health of your partner is:

Excellent	☐	4
Very good	☐	3
Good	☐	2
Fair	☐	1
Poor	☐	0

2 How satisfied have you been with your general (non-sexual) relationship with your partner?

Very satisfied	☐	4
Moderately satisfied	☐	3
		2

About equally satisfied and dissatisfied	
Moderately dissatisfied	
Very dissatisfied	

Section B

This section asks about your sexual drive or sexual desire.

Please tick the ONE response that best describes you IN THE LAST 4 WEEKS.

1. 1 How often did you think about sex? This includes times of just being interested in sex, daydreaming or fantasizing about sex, as well as times when you wanted to have sex.

Not at all		0
Once in the last month		1
2-3 times in the last month		2
Once a week		3
2-3 times a week		4
4-6 times a week		5
Once a day		6
More than once a day		7

2 How would you rate your level of sexual desire?

Very low/none at all		0
Low		1
Moderate		2
High		3
Very high		4

3 Are you worried or distressed by your current level of sexual drive/desire?

Not at all worried or distressed		0
A little bit worried or distressed		1
Moderately worried or distressed		2
Very worried or distressed		3
Extremely worried or distressed		4

4 Compared with a year ago, has your sexual drive/desire changed?

Increased a lot		+2
Increased moderately		+1
Neither increased nor decreased		0
Decreased moderately		-1
Decreased a lot		-2

Section C

This section asks about the frequency of your sexual activities.

If you DID NOT have a sexual partner in the last 4 weeks please skip Questions 5 and 6 and go straight to Question 7.

Please tick the ONE response that best describes you IN THE LAST 4 WEEKS.

5 How many times have you attempted sexual intercourse?

Not at all	☐	0
Once in the last month	☐	1
2-3 times in the last month	☐	2
Once a week	☐	3
2-3 times a week	☐	4
4-6 times a week	☐	5
Once a day	☐	6
More than once a day	☐	7

6 Apart from when you attempted sexual intercourse, how frequently did you engage in activities such as kissing, fondling, petting etc?

Not at all	☐	0
Once in the last month	☐	1
2-3 times in the last month	☐	2
Once a week	☐	3
2-3 times a week	☐	4
4-6 times a week	☐	5
Once a day	☐	6
More than once a day	☐	7

7 How often did you masturbate?

Not at all		0
Once in the last month		1
2-3 times in the last month		2
Once a week		3
2-3 times a week		4
4-6 times a week		5
Once a day		6
More than once a day		7

2. 8 Are you worried or distressed by the overall frequency of your sexual activities (including intercourse, kissing etc and masturbation)?

Not at all worried or distressed	0	Skip Question 8A and go straight to Question 9
A little bit worried or distressed	1	
Moderately worried or distressed	2	
Very worried or distressed	3	
Extremely worried or distressed	4	

8A If you **ARE** worried or distressed by the current frequency of your sexual activities, do you consider it to be

Too frequent		1
Not frequent enough		2

3.
4. 9 Compared with a year ago, has the overall frequency of your sexual activities changed?
5.

Increased a lot		+2
Increased moderately		+1
Neither increased nor decreased		0
Decreased moderately		-1
		-2

Decreased a lot ☐

6.

Section D

This section asks about your ability to have an erection. It is common for men to experience erectile problems. This may mean that one is not always able to get or keep an erection that is rigid enough for satisfactory sexual activity (including sexual intercourse and masturbation).

Please tick the ONE statement or response that best describes you IN THE LAST 4 WEEKS.

10 You are

Always able to get and keep an erection which would be good enough for sexual intercourse	☐	1
Usually able to get and keep an erection which would be good enough for sexual intercourse	☐	2
Sometimes able to get and keep an erection which would be good enough for sexual intercourse	☐	3
Never able to get and keep an erection which would be good enough for sexual intercourse	☐	4

11 Are you worried or distressed by your current ability to have an erection?

Not at all worried or distressed	☐	0
A little bit worried or distressed	☐	1
Moderately worried or distressed	☐	2
Very worried or distressed	☐	3
Extremely worried or distressed	☐	4

12 Compare with a year ago, has your ability to have an erection changed?

Increased a lot	☐	+2
Increased moderately	☐	+1
Neither increased nor decreased	☐	0
		-1

Decreased moderately	
Decreased a lot	

Section E

This section asks about your feelings of orgasm or climax leading to ejaculation of semen in response to any sexual stimulation (including intercourse or masturbation).

Please tick the ONE response that best describes you IN THE LAST 4 WEEKS.

13 When you had sexual stimulation, how often did you have the feeling of orgasm or climax?

No sexual intercourse/masturbation	☐	0
Almost never/never	☐	1
A few times (much less than half the time)	☐	2
Sometimes (about half the time)	☐	3
Most of the time (much more than half the time)	☐	4
Almost always/always	☐	5

14 How satisfied have you been with your sense of control over the timing of your orgasm? (Not being satisfied with 'timing' can mean either taking too long to climax or climaxing too early in the course of sexual activity)

Extremely **satisfied**	☐ 4	Skip Question 14A and go straight to Question 15
Highly **satisfied**	☐ 3	Skip Question 14A and go straight to Question 15
Moderately **satisfied**	☐ 2	
Slightly **satisfied**	☐ 1	
Not at all **satisfied**	☐ 0	

14A If you are not extremely or highly satisfied, do you climax

Too early	☐	1
Too late	☐	2

See next page for Question 15.

15 Are you worried or distressed by your current orgasmic experience?

Not at all worried or distressed	0
A little bit worried or distressed	1
Moderately worried or distressed	2
Very worried or distressed	3
Extremely worried or distressed	4

16 Compared with a year ago, has the enjoyment of your orgasmic experience changed?

Increased a lot	+2
Increased moderately	+1
Neither increased nor decreased	0
Decreased moderately	-1
Decreased a lot	-2

Section F

This section asks about your morning erections. Men may awaken with an erection although this can vary from day to day.

Please tick the ONE response that best describes you IN THE LAST 4 WEEKS.

17 How frequently did you awaken with a full erection?

Not at all	0
Once in the last month	1
2-3 times in the last month	2
Once a week	3
2-3 times a week	4
4-6 times a week	5
Once a day	6
More than once a day	7

18 Are you worried or distressed by the frequency of your morning erections?

Not at all worried or distressed	☐	0
A little bit worried or distressed	☐	1
Moderately worried or distressed	☐	2
Very worried or distressed	☐	3
Extremely worried or distressed	☐	4

7.
8. 19 Compared with a year ago, has the frequency of your morning erections changed?

Increased a lot	☐	+2
Increased moderately	☐	+1
Neither increased nor decreased	☐	0
Decreased moderately	☐	-1
Decreased a lot	☐	-2

Section G

Considering the answers you have already given above, we would like to know what you think about the quality of your overall sex life.

Please tick the ONE response that best describes you IN THE LAST 4 WEEKS.

20 How satisfied have you been with your overall sex life?

Very satisfied	☐	4
Moderately satisfied	☐	3
About equally satisfied and dissatisfied	☐	2
Moderately dissatisfied	☐	1
		0

Very dissatisfied ☐

21 How worried or distressed have you been about your overall sex life?

Not at all worried or distressed	☐	0
Slightly worried or distressed	☐	1
About equally worried/not worried or distressed/not distressed	☐	2
Moderately worried or distressed	☐	3
Very worried or distressed	☐	4

22 Compared with a year ago, has your overall sexual satisfaction changed?

Increased a lot	☐	+2
Increased moderately	☐	+1
Neither increased nor decreased	☐	0
Decreased moderately	☐	-1
Decreased a lot	☐	-2

Female Sexual Function Index (FSFI)

INSTRUCTIONS: These questions ask about your sexual feelings and responses during the past 4 weeks. Please answer the following questions as honestly and clearly as possible. Your responses will be kept completely confidential. In answering these questions the following definitions apply:

Sexual activity can include caressing, foreplay, masturbation and vaginal intercourse.

Sexual intercourse is defined as penile penetration (entry) of the vagina.

Sexual stimulation includes situations like foreplay with a partner, self-stimulation (masturbation), or sexual fantasy.

CHECK ONLY ONE BOX PER QUESTION.

Sexual desire or interest is a feeling that includes wanting to have a sexual experience, feeling receptive to a partner's sexual initiation, and thinking or fantasizing about having sex.

1. Over the past 4 weeks, how **often** did you feel sexual desire or interest?

- ☐ Almost always or always
- ☐ Most times (more than half the time)
- ☐ Sometimes (about half the time)
- ☐ A few times (less than half the time)
- ☐ Almost never or never

2. Over the past 4 weeks, how would you rate your **level** (degree) of sexual desire or interest?

- ☐ Very high
- ☐ High
- ☐ Moderate
- ☐ Low
- ☐ Very low or none at all

Sexual arousal is a feeling that includes both physical and mental aspects of sexual excitement. It may include feelings of warmth or tingling in the genitals, lubrication (wetness), or muscle contractions.

3. Over the past 4 weeks, how **often** did you feel sexually aroused ("turned on") during sexual activity or intercourse?

- ☐ No sexual activity
- ☐ Almost always or always
- ☐ Most times (more than half the time)
- ☐ Sometimes (about half the time)
- ☐ A few times (less than half the time)
- ☐ Almost never or never

4. Over the past 4 weeks, how would you rate your **level** of sexual arousal ("turn on") during sexual activity or intercourse?

- ☐ No sexual activity
- ☐ Very high
- ☐ High
- ☐ Moderate
- ☐ Low
- ☐ Very low or none at all

5. Over the past 4 weeks, how **confident** were you about becoming sexually aroused during sexual activity or intercourse?

- ☐ No sexual activity
- ☐ Very high confidence
- ☐ High confidence
- ☐ Moderate confidence
- ☐ Low confidence
- ☐ Very low or no confidence

6. Over the past 4 weeks, how **often** have you been satisfied with your arousal (excitement) during sexual activity or intercourse?

- ☐ No sexual activity
- ☐ Almost always or always
- ☐ Most times (more than half the time)
- ☐ Sometimes (about half the time)
- ☐ A few times (less than half the time)
- ☐ Almost never or never

7. Over the past 4 weeks, how **often** did you become lubricated ("wet") during sexual activity or intercourse?

- ☐ No sexual activity
- ☐ Almost always or always
- ☐ Most times (more than half the time)
- ☐ Sometimes (about half the time)
- ☐ A few times (less than half the time)
- ☐ Almost never or never

8. Over the past 4 weeks, how **difficult** was it to become lubricated ("wet") during sexual activity or intercourse?

- ☐ No sexual activity
- ☐ Extremely difficult or impossible
- ☐ Very difficult
- ☐ Difficult
- ☐ Slightly difficult
- ☐ Not difficult

9. Over the past 4 weeks, how often did you **maintain** your lubrication ("wetness") until completion of sexual activity or intercourse?

- ☐ No sexual activity
- ☐ Almost always or always
- ☐ Most times (more than half the time)
- ☐ Sometimes (about half the time)
- ☐ A few times (less than half the time)
- ☐ Almost never or never

10. Over the past 4 weeks, how **difficult** was it to maintain your lubrication ("wetness") until completion of sexual activity or intercourse?

- ☐ No sexual activity
- ☐ Extremely difficult or impossible
- ☐ Very difficult
- ☐ Difficult
- ☐ Slightly difficult
- ☐ Not difficult

11. Over the past 4 weeks, when you had sexual stimulation or intercourse, how **often** did you reach orgasm (climax)?

- ☐ No sexual activity
- ☐ Almost always or always
- ☐ Most times (more than half the time)
- ☐ Sometimes (about half the time)
- ☐ A few times (less than half the time)
- ☐ Almost never or never

12. Over the past 4 weeks, when you had sexual stimulation or intercourse, how **difficult** was it for you to reach orgasm (climax)?

- ☐ No sexual activity
- ☐ Extremely difficult or impossible
- ☐ Very difficult
- ☐ Difficult
- ☐ Slightly difficult
- ☐ Not difficult

13. Over the past 4 weeks, how **satisfied** were you with your ability to reach orgasm (climax) during sexual activity or intercourse?

- ☐ No sexual activity
- ☐ Very satisfied
- ☐ Moderately satisfied
- ☐ About equally satisfied and dissatisfied
- ☐ Moderately dissatisfied
- ☐ Very dissatisfied

14. Over the past 4 weeks, how **satisfied** have you been with the amount of emotional closeness during sexual activity between you and your partner?

- ☐ No sexual activity
- ☐ Very satisfied
- ☐ Moderately satisfied
- ☐ About equally satisfied and dissatisfied
- ☐ Moderately dissatisfied
- ☐ Very dissatisfied

15. Over the past 4 weeks, how **satisfied** have you been with your sexual relationship with your partner?

- ☐ Very satisfied
- ☐ Moderately satisfied
- ☐ About equally satisfied and dissatisfied
- ☐ Moderately dissatisfied
- ☐ Very dissatisfied

16. Over the past 4 weeks, how **satisfied** have you been with your overall sexual life?

- ☐ Very satisfied
- ☐ Moderately satisfied
- ☐ About equally satisfied and dissatisfied
- ☐ Moderately dissatisfied
- ☐ Very dissatisfied

17. Over the past 4 weeks, how **often** did you experience discomfort or pain during vaginal penetration?

- ☐ Did not attempt intercourse
- ☐ Almost always or always
- ☐ Most times (more than half the time)
- ☐ Sometimes (about half the time)
- ☐ A few times (less than half the time)
- ☐ Almost never or never

18. Over the past 4 weeks, how **often** did you experience discomfort or pain following vaginal penetration?

- ☐ Did not attempt intercourse
- ☐ Almost always or always
- ☐ Most times (more than half the time)
- ☐ Sometimes (about half the time)
- ☐ A few times (less than half the time)
- ☐ Almost never or never

19. Over the past 4 weeks, how would you rate your **level** (degree) of discomfort or pain during or following vaginal penetration?

- ☐ Did not attempt intercourse
- ☐ Very high
- ☐ High
- ☐ Moderate
- ☐ Low
- ☐ Very low or none at all

MANCHESTER 1824

Central Manchester University Hospitals NHS
NHS Foundation Trust

Ophthalmic markers

Ophthalmic record sheet

Participant's Full Name:		Date of Birth:
Date of Visit:	Investigator(s):	Study & Visit ID:

IF NOT PART OF A TRIAL

Patient referred from:	Hospital No.:
Address details:	

Medical History:

Type of Diabetes:

Duration of Diabetes:

Family History of Diabetes (quote parental/maternal side):

Other systemic disease (e.g. Heart Failure, Liver Failure, Hep B, HIV+, Vit. Deficiencies, Alcohol abuse, MS, Connective Tissue Disease, SLE, psoriasis):

Medication (quote reason e.g. hypertension, cholesterol, diabetes, other CVD-related etc.):

Ocular History:

History of previous ocular disease (e.g. systemic, infections) / trauma:

History of operations (quote year, eye, type of operation):

History of contact lens use (quote type and frequency):

History of retinopathy (official grading):

For Transplant Study:

Date of Transplant:

Type of Transplant:

Duration on Renal Dialysis:

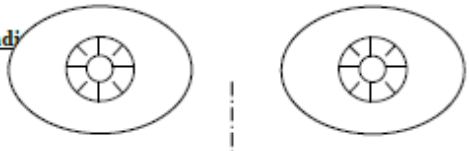
Smoking: per day

Drinking: units per week

Ophthalmic Examinations:

OD OS

Slit Lamp Biomicroscopy (draw findi



Comments: Cormments:

Corneal Aesthesiometry:

	NCCA (mbar)	CB-A
OD		
OS		

Puppilometry (ensure 10 min. dark adaptation before examination):

Tear Tests:

BUT:

Schirmer Test:

Corneal Confocal Microscopy (HRT III-RCM)

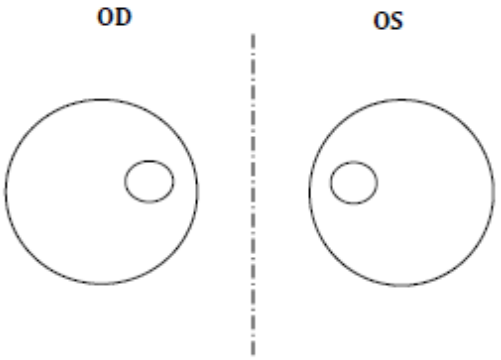
	Epithelium	Bowman's Layer/Nerve Plexus	Stroma	Endothelium
OD				
OS				

Corneal nerve parameters (values):

	NFD (no./mm^2)	NBD (no./mm^2)	NFL (mm/mm^2)	NFT
OD				
OS				

Fundoscopy (Mydriatic/Non-Mydriatic) and Ophthalmoscopy (**draw findings**):

Comments:



Comments:

Additional Notes

8.7 Published copy of manuscript

Small fibre pathology is associated with erectile dysfunction in men with type 2 diabetes

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RESEARCH ARTICLE

WILEY

Small fibre pathology is associated with erectile dysfunction in men with type 2 diabetes

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Abstract

Aims: The aim of this study was to evaluate the contribution of small and large fibre neuropathy to erectile dysfunction (ED) in men with type 2 diabetes (T2D).

Methods: Measures of small and large fibre neuropathy were evaluated in 49 participants with T2D and 20 age-matched controls.

Results: ED was present in 59% of participants with T2D. There was no difference in age, duration of diabetes, blood pressure, lipid profile, vibration perception threshold (V) (14.3 ± 7.8 vs 11.2 ± 6.6, $P = .429$), peroneal (41.4 ± 8.2 vs 44.8 ± 4.4, $P = .10$) and sural (45.4 ± 5.6 vs 47.1 ± 5.8) nerve conduction velocities (m/s), cold (25.1 ± 3.8 vs 26.2 ± 2.9, $P = .815$) and warm (43.2 ± 4.0 vs 41.0 ± 3.8) perception thresholds (°C), and deep breathing heart rate variability (18 ± 8 vs 18 ± 8) between participants with and without ED. However, intraepidermal nerve fibre density (no./mm^2) (4.6 ± 2.8 vs 13.7 ± 2.7, $P < .001$), corneal nerve fibre density (no./mm^2) (23.5 ± 6.8 vs 31.3 ± 8.2, $P < .001$), corneal nerve fibre branch density (no./mm^2) (55.4 ± 35.3 vs 97.7 ± 46.4, $P = .004$), corneal nerve fibre length (mm/mm^2) (17.6 ± 6.8 vs 27.3 ± 6.8, $P < .001$), and sural (7.7 ± 6.1 vs 14.6 ± 6.7, $P = .003$) and peroneal (2.5 ± 2.0 vs 4.7 ± 2.0, $P = .003$) nerve amplitudes were significantly lower in participants with ED compared with those without ED.

Conclusion: ED affects almost 2/3 of men with T2D and is associated with small nerve fibre damage but preserved nerve conduction and cardiac autonomic function. Corneal confocal microscopy may serve as a useful non-invasive imaging method to identify small fibre damage in patients with T2D and ED.

KEYWORDS

corneal confocal microscopy, erectile dysfunction, neuropathy, small fibre neuropathy

Abbreviations: AUC, area under curve; BMI, body mass index; CAN, cardiac autonomic neuropathy; CCM, corneal confocal microscopy; cGMP, cyclic guanosine monophosphate; CNFD, corneal nerve fibre density; CNBD, corneal nerve branch density; CNFL, corneal nerve fibre length; CT, cold threshold; DB-HRV, deep breathing heart rate variability; DM, diabetes mellitus; ED, erectile dysfunction; eGFR, estimated glomerular filtration rate; EMAS-SFQ, European Male Ageing Study Sexual Function Questionnaire; HbA1c, glycosylated haemoglobin; HDL-C, high-density lipoprotein cholesterol; IENFD, intraepidermal nerve fibre density; IIEF, International Index of Erectile Function; LDL-C, low-density lipoprotein cholesterol; MDRD, Modification of Diet in Renal Disease; NSP, Neuropathy Symptom Profile; NO, nitric oxide; NDS, neuropathy disability score; PDE-5, phosphodiesterase 5; QST, quantitative sensory testing; SST, sympathetic skin response; T1D, type 1 diabetes; T2D, type 2 diabetes; VPT, vibration perception threshold.

Rayaz A. Malik and Handrean Soran contributed equally as joint main authors.

Tweet – Erectile dysfunction in individuals with T2DM is associated with small fibre neuropathy, identified by corneal confocal microscopy @ENAgroup@ manchesterCVresearch @FBMH_UoM @WCMQatar

1 | INTRODUCTION

Erectile dysfunction (ED) is the persistent inability to achieve or maintain penile erection for satisfactory sexual intercourse.[1] Diabetes mellitus (DM) is the commonest aetiology for ED,[2] and in patients with DM it ranges in prevalence from 35% to 90%.[2] Multiple risk factors including age, duration of diabetes, suboptimal glycaemic control, hypertension, dyslipidaemia, smoking, sedentary life style, and subnormal testosterone levels have been associated with ED in diabetes.[2-4] ED is also a marker of cardiovascular disease and has been associated with poorer generic and disease-specific quality of life among men with both type 1 (T1D) and type 2 diabetes (T2D).[5-7]

Organic, relational, and psychological factors contribute to ED and include neurogenic, vasculogenic, iatrogenic, and endocrine pathways,[8,9] which mediate penile endothelial dysfunction and defective noradrenergic and cholinergic nerve signalling with increased cavernosal contractile sensitivity and impaired dilatory function.[10] Vascular abnormalities including penile smooth muscle and endothelial dysfunction and altered cavernosal haemodynamics have been considered to play a major role in diabetes related ED,[11] whilst the impact of neuropathy has been underestimated.[12] Indeed, some studies have identified an association between cardiac autonomic and somatic neuropathy and ED and attributed failure of phosphodiesterase type-5 (PDE5) inhibitor therapy in patients with ED to the presence of small fibre and autonomic neuropathy.[13-15]

The contribution of neuropathy to ED has been assessed using symptoms, vibration perception and reflexes,[16] neurophysiology,[17,18] quantitative sensory testing (QST), and the sympathetic skin response (SSR).[19-23] Neurophysiology only assesses large fibres, QST is subjective,[24] SSR is highly variable, and a more objective measure like intraepidermal nerve fibre density (IENFD) is invasive. Corneal confocal microscopy (CCM) is a rapid, highly objective, and easily reproducible technique that can quantify small nerve fibre damage in diabetes,[25] comparable with IENFD.[26] We have previously shown that patients with T1D and ED show greater small, large, and autonomic nerve fibre damage.[27]

In this study, we aimed to assess the relationship between different measures of small and large fibre as well as autonomic neuropathy with ED in an unselected cohort of men with T2D.

2 | METHODS

2.1 | Participant selection

Forty-nine consecutive men with T2D were recruited from the Manchester University Hospital Diabetes Centre along with 19 age-matched healthy control participants. The control group comprised healthy volunteers without DM and were not on any regular medications for other co-morbidities. Patients with a history of neuropathy from another cause, corneal trauma or surgery, ocular disease, dermatological disorders, and systemic disease that might affect the cornea or skin were excluded. This study has approval from the Central Manchester Research and Ethics Committee. Written informed consent was obtained from all individuals prior to participation.

2.2 | Assessment of erectile function

ED was identified from the Neuropathy Symptom Profile (NSP) defined by the inability to have penile erection not due to medication or prostate surgery.[28]

2.3 | Laboratory measurements

Glycosylated haemoglobin (HbA1c), total cholesterol, low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), triglyceride, and creatinine were measured using standard laboratory methods in the Department of Biochemistry, Manchester University NHS Foundation Trust. Estimated glomerular filtration rate (eGFR) was calculated using the abbreviated Modification of Diet in Renal Disease (MDRD) equation: $186 \times (\text{creatinine}/88.4)^{-1.154} \times (\text{age})^{-0.203} \times (0.742 \text{ if female}) \times (1.210 \text{ if black})$.

2.4 | Assessment of neuropathy

The NSP was used to assess for symptoms of neuropathy. Neurological deficits were evaluated using the modified Neuropathy Disability Score (NDS), which is composed of vibration perception, pinprick, temperature sensation, and the presence or absence of ankle reflexes.[28,29] The vibration perception threshold (VPT) was established using a Horwell Neurothesiometer (Scientific Laboratory Supplies, Wilfrod, Nottingham, UK). Cold (CT) and warm (WT) perception thresholds were assessed on the dorsolateral aspect of the left foot using the TSA-II NeuroSensory Analyser (Medoc, Ramat-Yishai, Israel). Electrodiagnostic studies were undertaken using a Dantec Keypoint system (Dantec Dynamics, Bristol, UK). Sural sensory and peroneal motor nerve amplitude, conduction velocity, and latency were assessed by a consultant neurophysiologist. The 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. Deep breathing heart rate variability (DB-HRV) was assessed using an ANX 3.0 autonomic nervous system monitoring device (ANSAR Medical Technologies, Philadelphia, PA, USA).

2.5 | Skin biopsy

A 3-mm punch skin biopsy was performed 2 cm above the second metatarsal on the dorsum of the foot under local anaesthesia using 1% lidocaine; 50-μm sections were stained using anti-human PGP9.5 antibody (Abcam, Cambridge, UK). SG chromogen (Vector Laboratories, Peterborough, UK) was used to demonstrate nerve fibres, and IENFD was quantified using previously established criteria and expressed as number per millimetre.[30]

2.6 | Corneal confocal microscopy

CCM (Heidelberg Retinal Tomograph III Rostock Cornea Module; Heidelberg Engineering, Heidelberg, Germany) was performed in all participants according to our previously established protocol.[25] Six nonoverlapping images from the centre of the cornea were selected per participant (three per eye). Corneal nerve fibre density (CNFD), the total number of major nerves per square millimetre of corneal tissue; corneal nerve branch density (CNBD), the number of branches emanating from the major nerve trunks per square millimetre of corneal tissue; and corneal nerve fibre length (CNFL), the total length of all nerve fibres and branches (millimetre per square millimetre) within the area of corneal tissue were quantified (Figure 1: representative CCM images). Analysis of corneal nerve morphology was performed using manual software, CCMetrics (Manchester, UK).[31]

2.7 | Statistical analyses

Statistical analyses were performed using SPSS for Mac (Version 23.0; IBM Corporation, New York, NY, USA). Data were tested for normality using the Shapiro-Wilk normality test. Continuous variables were compared between patients with and without ED using the independent samples *t* test for normally distributed data, Mann-Whitney *U* test for nonnormally distributed data, and chi-squared test for categorical data. ANCOVA was used for comparisons adjusted for age, beta-blocker, and diuretic use. Data were presented as mean and standard deviation. Correlations between ED and other variables were assessed using point-biserial correlation. A *P* value of less than .05 was considered to be statistically significant.

3 | RESULTS

3.1 | T2D vs controls

Patients with T2D were well matched for age with the control group (62.0 ± 8.1 vs 60.6 ± 7.2, *P* = .475). The prevalence of ED was 59.2% in patients with T2D, whilst none of the control participants reported symptoms of ED. Body mass index (BMI) (30.6 ± 4.9 vs 27.1 ± 3.9 kg/m^2, *P* = .008) and HbA1c (57 ± 13 vs 40 ± 3 mmol/mol, *P* < .001) were higher, and total cholesterol (4.0 ± 1.0 vs 5.2 ± 0.8 mmol/L, *P* < .001), HDL-C (1.16 ± 0.33 vs 1.55 ± 0.40 mmol/L, *P* < .001), and LDL-C (1.9 ± 0.9 vs 2.9 ± 0.7 mmol/L, *P* < .001) were lower among patients with T2D compared with controls. Triglycerides (2.0 ± 1.3 vs 1.7 ± 0.7 mmol/L, *P* = .221) and eGFR (77 ± 17 vs 84 ± 8 mL/min [1.73 m]$^{-2}$, *P* = .085) did not differ between patients with T2D and controls. Patients with T2D had a higher NSP score (4.4 ± 4.8 vs 0.3 ± 0.8, *P* < .001), NDS (2.9 ± 2.3 vs 0.9 ± 1.2, *P* < 0.001), VPT (13.0 ± 7.4 vs 9.2 ± 5.9, *P* = .036), WT (42.3 ± 4.0 vs 38.5 ± 3.1°C, *P* = .001) and lower CT (42.3 ± 4.0 vs 38.5 ± 3.1°C, *P* = .002), and DB-HRV (18 ± 8 vs 25 ± 12 beats/min, *P* = .027). Peroneal nerve amplitude (3.4 ± 2.3 vs 5.1 ± 1.7 mV, *P* = .003) and peroneal nerve conduction velocity (42.8 ± 7.0 vs 46.1 ± 3.7 m/s, *P* = .018) were lower in patients with T2D, but there was no difference in sural nerve amplitude (10.6 ± 7.2 vs 12.6 ± 6.3 μV, *P* = .227) or sural nerve conduction velocity (46.1 ± 5.7 vs 47.9 ± 3.9, m/s *P* = .161). IENFD (7.7 ± 5.2 vs 15.9 ± 3.2 no./mm, *P* = .004), CNFD (26.7 ± 8.3 vs 37.6 ± 5.9 no./mm^2, *P* < .001), CNBD (72.5 ± 44.9 vs 93.9 ± 30.1 no./mm^2, *P* = .038), and CNFL (21.5 ± 8.3 vs 26.6 ± 4.4 mm/mm^2, *P* = .003) were significantly lower in patients with T2D (Table S1).

3.2 | T2D with and without ED

There was no significant difference in age, BMI, duration of diabetes, HbA1c, lipid profile, blood pressure, or eGFR in patients with T2D with and without ED (Table 1 and Figure 2). Both groups were well matched for the use of beta-blockers and diuretics.

NSP (6.0 ± 5.1 vs 2.0 ± 3.0, *P* < .001) and NDS (3.4 ± 1.9 vs 2.3 ± 2.6, *P* = .001) scores were significantly higher and IENFD (4.6 ± 2.8 vs 13.7 ± 2.7 no./mm^2, *P* < .001), CNFD (23.5 ± 6.8 vs 31.3 ± 8.2 no./mm^2, *P* < .001), CNBD (55.4 ± 35.3 vs 97.7 ± 46.4 no./mm^2, *P* = .004), and CNFL (17.6 ± 6.8 vs 27.3 ± 6.8 mm/mm^2, *P* < .001) were lower in patients with ED (Table 2 and Figure 1). VPT, CT, WT, and DB-HRV did not differ significantly between T2D with and without ED. Sural (7.7 ± 6.1 vs 14.6

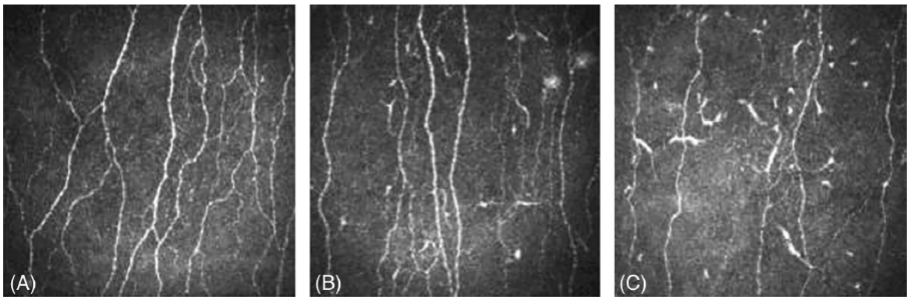

FIGURE 1 CCM images of corneal sub-basal nerves from (A) control participant, (B) participant with T2DM and no ED, (C) participant with T2DM and ED. CCM, corneal confocal microscopy; ED, erectile dysfunction; T2DM, type 2 diabetes mellitus

± 6.7 μV, $P = .003$) and peroneal (2.5 ± 2.0 vs 4.7 ± 2.0 mV, $P = .003$) nerve amplitude was lower in patients with T2D and ED, but there was no significant difference in sural (45.4 ± 5.6 vs 47.1 ± 5.8 mV, $P = .530$) and peroneal (41.4 ± 8.2 vs 44.8 ± 4.4 mV, $P = .101$) nerve conduction velocity.

3.3 | Correlations

There were significant correlations between ED and NSP ($r = -.418$, $P = .003$), CNFD ($r = -.466$, $P = .001$), CNBD ($r = -.468$, $P = .001$), CNFL

TABLE 1 Clinical characteristics of patients with type 2 diabetes with and without erectile dysfunction and controls

	Type 2 Diabetes with ED (n = 29)	Type 2 Diabetes without ED (n = 20)	Controls (n = 19)	*P* Value
Age (years)	64.0 ± 6.5	59.4 ± 9.4	60.6 ± 7.2	.101
Duration of diabetes (years)	10.9 ± 9.5	10.4 ± 8.2	-	.981
Hypertension (%)	44.8	60.0	0.0	.296
BP (mmHg)	142 ± 21/75 ± 10	134 ± 21/74 ± 10	136 ± 17/79 ± 9	.737/.653
Beta-blocker or diuretic use (%)	20.7	20.0	0.0	.625
HbA1c (mmol/mol)	58 ± 16	54 ± 8	40 ± 2.8	.569
BMI (kg/m^2)	30.7 ± 4.6	30.4 ± 5.3	27.1 ± 3.9	.668
eGFR (mL/min [1.73 m]$^{-2}$)	73 ± 18	81 ± 14	84 ± 8	.359
Total cholesterol (mmol/L)	4.0 ± 1.0	3.8 ± 1.1	5.2 ± 0.8	.208
HDL-C (mmol/L)	1.19 ± 0.34	1.12 ± 0.30	1.55 ± 0.40	.714
Triglyceride (mmol/L)	2.1 ± 1.2	1.9 ± 1.3	1.7 ± 0.7	.274
LDL-C (mmol/L)	1.9 ± 0.8	1.9 ± 1.1	2.9 ± 0.7	.804

Note: Data presented as mean ± SD. *P* value is for comparison between participants with and without erectile dysfunction.
Abbreviations: BMI, body mass index; ED, erectile dysfunction; eGFR, estimated glomerular filtration rate; HbA1c, glycosylated haemoglobin; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.

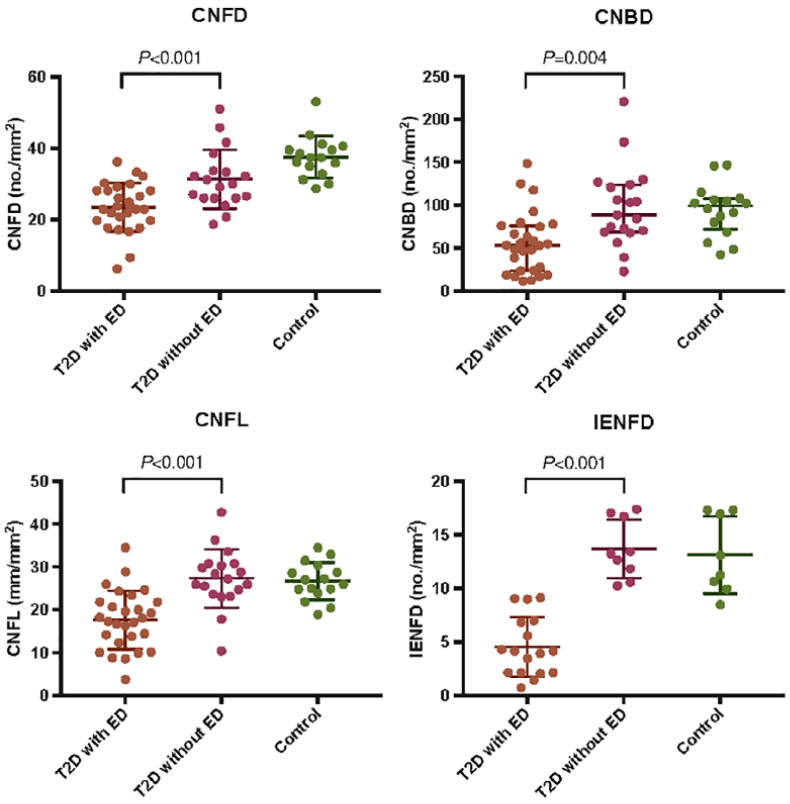

FIGURE 2 Comparison of corneal nerve parameters and intraepidermal nerve fibre density between patients with type 2 diabetes with and without erectile dysfunction and controls. CNBD, corneal nerve branch density; CNFD, corneal nerve fibre density; CNFL, corneal nerve fibre length; IENFD, intraepidermal nerve fibre density; ED, erectile dysfunction; T2D, type 2 diabetes

TABLE 2 Measures of neuropathy for patients with type 2 diabetes with and without erectile dysfunction and controls

	Type 2 Diabetes with ED (n = 29)	Type 2 Diabetes without ED (n = 20)	Controls (n = 19)	*P* Value
NSP (/38)[a]	6.0 ± 5.1	2.0 ± 3.0	0.3 ± 0.8	<.001
NDS (/10)[a]	3.4 ± 1.9	2.3 ± 2.6	0.9 ± 1.2	.001
VPT (V)[a]	14.3 ± 7.8	11.2 ± 6.6	9.2 ± 5.9	.429
Sural nerve amplitude (μV)[a]	6.8 ± 4.3	13.3 ± 5.8	12.6 ± 6.3	<.001
Sural nerve conduction velocity (m/s)[a]	44.0 ± 6.0	47.1 ± 5.8	47.9 ± 3.9	.170
Peroneal nerve amplitude (mV)[a]	2.9 ± 2.1	4.7 ± 2.0	5.1 ± 1.7	.009
Peroneal nerve conduction velocity (m/s)[a]	41.4 ± 8.2	44.8 ± 4.4	46.1 ± 3.7	.100
CT (°C)[a]	25.1 ± 3.8	26.2 ± 2.9	27.9 ± 2.1	.815
WT (°C)[a]	43.2 ± 4.0	41.0 ± 3.8	38.5 ± 3.1	.257
IENFD (no./mm)[a]	4.6 ± 2.8	13.7 ± 2.7	15.9 ± 3.2	<.001
CNFD (no./mm^2)[a]	23.5 ± 6.8	31.3 ± 8.2	37.6 ± 5.9	<.001
CNBD (no./mm^2)[a]	55.4 ± 35.3	97.7 ± 46.4	93.9 ± 30.1	.004
CNFL (mm/mm^2)[a]	17.6 ± 6.8	27.3 ± 6.8	26.6 ± 4.4	<.001
DB-HRV (beats/min)[a]	18 ± 8	18 ± 8	25 ± 12	.841

Note: Data presented as mean ± SD. *P* value is for comparison between participants with and without erectile dysfunction.
Abbreviations: CNBD, corneal nerve branch density; CNFD, corneal nerve fibre density; CNFL, corneal nerve fibre length; CT, cold threshold; DB-HRV, deep breathing heart rate variability; ED, erectile dysfunction; IENFD, intraepidermal nerve fibre density; NDS, Neuropathy Disability Score; NSP, neuropathy symptom profile; VPT, vibration perception threshold; WT, warm threshold.
[a]Adjusted for age and beta-blocker/diuretic use using ANCOVA.

(r = –.578, P < .001), IENFD (r = –.853, P < .001), sural (r = –.477, P = .001), and peroneal nerve (r = –.478, P = .001) amplitude. There was no correlation between ED and sural (r = –0.152, P = .301) and peroneal (r = –.246, P = .095) nerve conduction velocity, NDS (r = .256, P = .076), VPT (r = .207, P = .159), CT (r = –.160, P = .277), WT (r = .273, P = .061), or DB-HRV (r = –.005, P = .977). There were no significant correlations between ED with age, BMI, duration of diabetes, systolic and diastolic BP, HbA1c, eGFR, total cholesterol, HDL, triglycerides, and LDL (Table S2).

4 | DISCUSSION

Almost 2/3 of men with T2D have ED, which was associated with small fibre neuropathy rather than autonomic or large fibre neuropathy. Previous studies have reported an association between ED and symptomatic peripheral and autonomic neuropathy.[32,33] Unlike previous studies in men with T2D showing an association between ED and duration of diabetes, older age, suboptimal glycaemic control, hypertension, hyperlipidaemia, and obesity,[2,33,34] we found no correlation between ED and HbA1c, BMI, duration of diabetes, hypertension, or lipid profile.

The physiology of erection is a complex interplay of psychogenic, hormonal and noradrenergic, noncholinergic neurovascular mechanisms.[35] Nitric oxide (NO) is released from the endothelium of the corpora cavernosa and cavernous nerves in response to local physical or central sexual stimulation.[35] NO activates soluble guanylyl cyclase resulting in an increase in cyclic guanosine monophosphate (cGMP) levels,[35] which causes smooth muscle relaxation and arteriolar dilation leading to penile erection.[35] Thus, penile erection involves both autonomic (sympathetic and parasympathetic) and somatic (motor and sensory) nerves supplying the shaft and glans of the penis.[36] The aetiology of ED in diabetes is considered to be due to both vascular and neuronal dysfunction[2,32,33] and indeed PDE5 inhibitors promote NO release and mediate increased penile blood flow.[33,37,38] The relationship between ED and neuropathy is complex, but studies have demonstrated evidence of large and small fibre neuropathy and autonomic neuropathy in diabetic patients with ED.[21-23] In a cohort of 341 patients with ED, the prevalence of neuropathy assessed using nerve conduction studies and QST was 38% in those with diabetes and 10% in those without diabetes. However, the prevalence of neuropathy among those with neurogenic (21%) compared with vasculogenic (23%) ED was comparable.[20] Wellmer et al showed no difference in neurological examination or thermal sensory thresholds, but there was a difference in the capsaicin-induced sensory axon reflex and sural nerve amplitude between diabetic patients with and without ED.[21] Studies have also shown an association between measures of cardiac autonomic neuropathy (CAN) and ED.[13,39] Penile vasotactile and thermal thresholds assessing somatic small fibre neuropathy have been found to be abnormal in diabetic patients with ED.[40-42] More sophisticated tests including penile somatosensory evoked potentials[43] and corpus cavernosum electromyography[44] are abnormal in ED but are not routinely available.

In our previous study, in patients with T1D and ED, we demonstrated a global small and large fibre and autonomic neuropathy.[27] These findings emphasize the importance of accurate and comprehensive phenotyping of neuropathy and ED severity before concluding that they are associated. A potential weakness in the current study is that NSP only identifies patients with severe ED and alternate

questionnaires such as the International Index of Erectile Function (IIEF) or the European Male Ageing Study Sexual Function Questionnaire (EMAS-SFQ),[45] which allow an assessment of mild and moderate ED may be more useful. Although, in a recent study from Japan, neuropathy defined by symptoms and loss of vibration perception and reflexes was associated with severe but not moderate or mild ED.[16]

The management of ED in patients with T2D is challenging with a nonresponder rate of over 50% for PDE5 inhibitors, which may reflect a more severe neurogenic component for ED in these patients.[46,47] Indeed, in a recent study, the assessment of nocturnal penile tumescence and rigidity, which reflects predominantly neurogenic abnormalities, had an area under curve (AUC) of 0.860 in differentiating sildenafil responders from nonresponders.[46] CCM has been used to identify an association between ED and small fibre damage in subjects with obesity,[45] T1D,[27] and now T2D. The potential role of CCM as an objective marker for neurogenic abnormalities that may predict the response to therapy in ED warrants further study.

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DATA ACCESSIBILITY

The data sets generated and analysed during the current study are available from the corresponding author on reasonable request.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest associated with this manuscript.

AUTHORS CONTRIBUTIONS

All authors were involved in revising the manuscript critically for important intellectual content and for final approval of the version to be published. S. Dhage and J.H. Ho were involved in acquisition of data, analysis, and interpretation of data and wrote the manuscript. S. Dhage, S. Azmi, S. Adam, J.H. Ho, M. Ferdousi, and A. Kalteniece recruited patients and controls. S. Azmi, S. Adam, and J.H. Ho contributed to acquisition and analysis of the data. M. Ferdousi and A. Kalteniece performed confocal microscopy, and S. Azmi performed skin biopsies. M. Jeziorska analysed and reported skin biopsies. A. Marshall performed and analysed nerve conduction studies. H. Soran and R. Donn contributed to conception and interpretation of the data and wrote and revised the manuscript. R.A. Malik contributed to conception and design of the study and wrote and revised the manuscript and is principal investigator of the study. R.A. Malik 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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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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