LONGITUDINAL STUDIES IN METABOLIC NEUROPATHIES: DEVELOPMENT OF IMAGING BIOMARKERS

A thesis submitted to the University of Manchester for the degree of

Doctor of Philosophy in Medicine

In the Faculty of Medical and Human Sciences

2016

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SCHOOL OF MEDICINE

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Word Count 65,912

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The University of Manchester

Shazli Azmi

Degree: Doctor of Philosophy

September 2016

LONGITUDINAL STUDIES IN METABOLIC NEUROPATHIES: DEVELOPMENT OF IMAGING BIOMARKERS

Corneal Confocal Microscopy (CCM) is a non-invasive imaging technique to quantify small nerve fibre structure in patients with diabetic somatic and autonomic neuropathy and increasingly other metabolic, hereditary, toxic and inflammatory peripheral neuropathies.

This thesis establishes that CCM is indeed a powerful imaging technique which can identify early small fibre degeneration and regeneration in relation to the clinical phenotype of subjects with obesity, impaired glucose tolerance and Type1/2 diabetes.

We demonstrate a precise relationship between small fibre neuropathy and erectile dysfunction in subjects with Type 1 diabetes. We also demonstrate the utility of CCM in demonstrating relative protection from small fibre damage in Type 1 patients with extreme duration diabetes (medallists) at baseline and over 3 years and repair in patients undergoing simultaneous pancreas and kidney transplantation.

This thesis provides further evidence for the utility of CCM as a marker of early small fibre neuropathy by demonstrating nerve damage in subjects with morbid obesity with and without diabetes and explore the mechanisms underlying nerve damage at baseline and repair following bariatric surgery.

We also show that CCM can track dynamic changes in small fibre degeneration and regeneration in subjects with impaired glucose tolerance in relation to change in glucose tolerance status and following continuous subcutaneous insulin infusion in subjects with Type 1 diabetes.

DECLARATION

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

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CONTRIBUTION

This section is to confirm that Shazli Azmi, the author of this thesis, was actively involved and made a significant contribution to all chapters/studies presented and discussed in this thesis. In brief, she consented and recruited a portion of the type 1 diabetes subjects and all the bariatric subjects. She performed neuropathy assessments and skin biopsies. For chapter 4 she was involved in analysis of the skin biopsies. She performed all the statistical analysis in this thesis. Finally, she has written all the chapters of this thesis which have been reviewed by her supervisors Professor Rayaz Malik and Dr Maria Jeziorska.

The following tasks were performed by other members of the research team:

- Electrodiagnostic studies were undertaken by Dr Andrew Marshall, consultant neurophysiologist;

- Ophthalmic examinations were undertaken by Dr Maryam Ferdousi, Dr Ioannis Nikolaos Petropoulos and Dr Mitra Tavakoli;

- Peripheral neuropathy assessments and skin biopsies were also undertaken by Drs, Uazman Alam, Hassan Fadavi and Omar Asghar;

- Skin biopsy processing and analysis were undertaken by Drs Maria Jeziorska, Simon Forman, Louisa Nelson and Wendy Jones;

- Patient recruitment and co-ordination by Georgios Ponirakis;

- Software engineering by Drs Mohammad A Dabbah, Xin Chen and James Graham;

- Blood and urine sample collections and anthropometric measurements by the nursing staff in the Wellcome Trust Clinical Research Facility.

- Haematology, immunology and clinical biochemistry analysis was performed and reported by the relevant departments under the Directorate of Laboratory Medicine, Central Manchester University Hospitals, NHS Foundation Trust, UK.

- Laboratory tests for the bariatric study were processed by Tarza Siahmansur and Dr Yifen Liu.

ALTERNATIVE THESIS FORMAT

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

- Chapter 3: Published in Diabetes Care, 2015
- Chapter 4: Published in Diabetes Care, 2015
- Chapter 5: to be Submitted for publication
- Chapter 6: to be Submitted for publication
- Chapter 7: to be Submitted for publication
- Chapter 8: to be Submitted for publication

List of Abbreviations:

ACCORD: Action to Control Cardiovascular Risk in Diabetes

ACE: Angiotensin Converting Enzyme

ACR: Albumin Creatinine Excretion Ratio

AGEs: Advanced Glycation End-Products (AGEs)

ALA: Alpha-lipoic acid

ApoAI: Apolipoprotein A-I

ApoB: Apolipoprotein B

ARI: Aldose reductase inhibitors

BMI: Body Mass Index

BP: Blood Pressure

BPD: Biliopancreatic Diversion

BPI: Brief Pain Inventory

CCM: Corneal Confocal Microscopy

CIDP: Chronic Inflammatory Demyelinating Polyneuropathy

CIP: Cold Induced Pain

CNBD: Corneal Nerve Branch Density

CNE: Clinical Neurological Examination

CNFD: Corneal Nerve Fibre Density

CNFL: Corneal Nerve Fibre Length

CNFT: Corneal Nerve Fibre Tortuosity

CRP: C-reactive protein

CSII: Continuous Subcutaneous Insulin Infusion

CT: Cold Sensation Threshold

CV: Cardiovascular

DCCT: Diabetes Control and Complications Trial

DM: Diabetes Mellitus

DN: Diabetic Neuropathy

DN4: Doleur Neuropathique en 4

DPN: Diabetic Peripheral Neuropathy

DR: diabetic retinopathy

ECG: Electrocardiogram

ED: Erectile Dysfunction

EDIC: Epidemiology of Diabetes Interventions and Complications

EFNS: European Federation for Neurological societies

eGFR: Estimated Glomerular Filtration Rate

ESRF: End Stage Renal Failure

FDA: Food and Drug Administration

GABA: Gamma-aminobutyric Acid

GB: Laparoscopic Adjustable Gastric Banding

GLO1: Glyoxalase 1

GTN: Glyceryl Trinitrate

HbA1c: Haemoglobin A1c

HDL: High Density Lipoprotein

HRV: Heart rate variability

ICAM-1: Intercellular Adhesion Molecule 1

IENFD: Intra-epidermal Nerve Fibre Density

IGT: Impaired Glucose Tolerance

IL-6: Interleukin 6

ISDN: Isosorbide Dinitrate

LABS: Longitudinal Assessment of Bariatric Surgery

LDL: Low Density Lipoprotein

MDL – Mean Dendritic Length

mNDS: Modified Neuropathy Disability Score

MPI: McGill Pain Index

NA: Not Assessed

NC: Nerve Conduction

NCS: Nerve Conduction Studies

NCV: Nerve Conduction Velocity

NDS: Neuropathy Disability Score

NePIQoL: Neuropathic Pain Impact on Quality-of-Life Questionnaire

NGT: Normal Glucose Tolerance

NMDA: *N*-methyl-D-aspartate

NO: Nitric Oxide

NPQ: Neuropathic Pain Questionnaire

NPS: Neuropathic Pain Scale

NPSI: Neuropathic Pain Symptoms Inventory

NS: Not Significant

NSP: Neuropathy Symptom Profile

NSS: Neuropathy Symptom Score

NYHA: New York Heart Association

OGTT – Oral Glucose Tolerance Test

OxLDL: Oxidized LDL

PCSK9: Proprotein convertase subtilisin / kexin type 9

PDE5-I: Phosphodiesterase Type 5 Inhibitor

PENS: Percutaneous Electrical Nerve Stimulation

PGP: Protein Gene Product

PKC: Protein Kinase C

PMNA: Peroneal Motor Nerve Amplitude

PMNCV: Peroneal Motor Nerve Conduction Velocity

PN: Peripheral Neuropathy

PON1: Paraoxonase-1

QALYs: quality adjusted life years

QoL: Quality of Life

QST: Quantitative Sensory Testing

RAGE: Receptor For Advanced Glycation End Product

RCT: Randomised Control Trial

RYGB: Roux-en-Y Gastric Bypass

SAA: Serum Amyloid A

SD: Standard Deviation

SEM: Standard Error of the Mean

SFN: Small Fibre Neuropathy

SG: Sleeve Gastrectomy

SNAP: Sural Sensory Nerve Amplitude

SNCV: Sural Sensory Nerve Conduction Velocity

SNRI: Serotonin and noradrenaline re-uptake inhibitors

SOS: Swedish Obesity Study

SPK: Simultaneous Pancreases and Kidney Transplant

STAMPEDE: Surgical Treatment and Medications Potentially Eradicate Diabetes Efficiently Trial

T1DM: Type I Diabetes Mellitus

T2DM: Type 2 Diabetes Mellitus

TBS: Tris Buffered Saline

TC: Total Cholesterol

TCA: Tricyclic antidepressant

TENS: Transcutaneous Electrical Nerve Stimulation

TNF: Tumour necrosis factor

TRPV1: Transient Receptor Potential Vanilloid 1

UDNS: Utah Diabetic Neuropathy Study

UKPDS: United Kingdom Prospective Diabetes Study

VADT: Veteran's Affairs Diabetes Trial

VAS: Visual Analogue Scale

VA CSDM: Veterans Affairs Cooperative Study on Glycaemic Control

VAS-PR: Visual Analogue Pain Relief

VCAM-1: Vascular Cell Adhesion Molecule 1

VEGF: Vascular endothelial growth factor

VPT: Vibration Perception Threshold

WHO: World Health Organisation

WT: Warm Sensation Threshold

"My Lord! Increase me in knowledge."

20:114

ACKNOWLEDGEMENTS

I would like to thank Professor Rayaz Malik for being an amazing supervisor. He is one of most inspirational and motivating people in medicine and the experience of being able to work for him has been remarkable and provided me with great opportunities for which I am forever grateful.

I would also like to thank my supervisor, Dr Maria Jeziorska, for all her advice and guidance during the last few years. Her encouragement and the knowledge that I have gained from her has been invaluable.

I am grateful to my advisor, Professor Andrew J M Boulton who has provided me with excellent support and advice.

My colleagues who have become friends: Dr Maryam Ferdousi, who has provided me with endless help and support, Dr Ioannis Petropoulos, Georgios Ponirakis, Dr Hassan Fadavi, Dr Jonathan Schofield and Monirah Almurdhi. I would like to especially thank Dr Uazman Alam who encouraged me to undertake the PhD and guided me towards this.

My family without whom nothing in life would have been possible! Most importantly, my parents who are always there for me and given me the opportunity and encouragement to pursue anything that I want. My husband, Omair, who has been very understanding and supportive during this time especially as I am supposed to have been on maternity leave during the write up. My baby Ayana – Thank you so much for allowing me to have some of your time to be able to write up my thesis. I will make it up to you! My sister, Samia, who is always there giving me encouragement.

I would also like to acknowledge and thank everyone who volunteered their time to participate in this research as without them this would not have been possible as

well as the generous support provided by the staff at the Welcome Trust Clinical Research Facility.

PREFACE

Shazli Azmi studied Medicine (MBchB) at the University of Manchester and graduated in 2005. She did her foundation training at Manchester Royal Infirmary from 2005 and 2007 where she rotated through placements in renal transplant, cardiology, general medicine, general surgery, A&E, public health and dermatology. From there she went on to complete her Core Medical Training at Huddersfield Royal Infirmary and Calderdale Royal Hospital (2007-2009). Her interest in research began as a medical student where she was involved in research work in cardiovascular medicine for her project option.

Shazli developed an interest in Diabetes and Endocrinology while training as a junior doctor. She was awarded a National Training Number in Diabetes and Endocrinology in 2009. She has completed the Specialty Certificate Exam in Diabetes and Endocrinology and is due to commence her final months as a Specialist Registrar. She took time out of the training programme as a Clinical Research Fellow to undertake a PhD. She undertook clinical work and research at the Manchester Diabetes Centre, Manchester Royal Infirmary and Centre for Diabetes and Endocrinology, University of Manchester. She spent three years working on research projects for her PhD 'Longitudinal Studies in Metabolic Neuropathies: Development of Imaging Biomarkers' under the supervision of Prof. Rayaz Malik and Dr Maria Jeziorska.

During her PhD, she presented her work at regional, national and international conferences between 2013 and 2016. This includes the American Diabetes Association, Diabetes UK, NeuroDiab – Diabetic Neuropathy Study Group of the

EASD, as well as the European Association for the Study of Diabetes. She won a first prize for oral communication presented at the Society for Endocrinology Obesity Update Meeting in London 2015 for her work on obesity related neuropathy.

Shazli is a member of the Royal College of Physicians. She has an interest in academic teaching, having been involved in this throughout her career to date and has completed the Postgraduate Certificate in Medical Education.

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1. Chapter I – Introduction

1.1. Peripheral neuropathy (PN)

Peripheral neuropathy occurs as a consequence of damage to sensory, motor or autonomic nerves, either at the cell body or along the axon. Diabetes Mellitus (DM) is the primary cause of neuropathy in the western world. Other causes include alcohol, autoimmune diseases, vitamin deficiencies, drugs, infections or any trauma or pressure to the nerves. The focus of this thesis will be on neuropathy as a result obesity, impaired glucose tolerance and DM. The definition of diabetic polyneuropathy (DPN) in clinical practice is 'the presence of symptoms and/or signs of peripheral nerve dysfunction in people with diabetes after the exclusion of other causes' (1). The most common presentation is distal symmetrical polyneuropathy. Other presentations include: small fibre predominant neuropathy, radiculoplexopathy, autonomic neuropathy, radiculopathy and mononeuritis.

Patients may present with symptoms including numbness, tingling, pain or weakness. These can be divided into positive symptoms (paraesthesia or pain; burning, aching, sharp) or negative symptoms (numbness or a dead feeling). The symptoms are usually bilateral, start distally and spread proximally in a glove and stocking distribution. Neuropathic pain is one of the most disabling symptoms which can affect ~20 % of patients (2, 3). There are currently no food and drug administration (FDA) approved therapies to prevent, slow or arrest DPN, and management involves achieving good glycaemic control to halt the progression and symptomatic treatment. Current treatments available for symptomatic patients only modestly improve symptoms and their use is limited by side effects and drug interactions. Developing new treatments is essential, but has proven difficult without good surrogate endpoints for DPN.

Epidemiological studies show a DPN prevalence of 30 - 40% in patients with DM (1, 2, 4). This increases with the duration of disease and rises to approximately 50% in those patients who have had the disease from more than 20 years (2). The Rochester Diabetic Neuropathy Study reported the prevalence of neuropathy to be 54% in type 1 diabetes mellitus (T1DM) and 45% in type 2 diabetes mellitus (T2DM) (5). This number may in fact be greater as the diagnostic criteria for DPN

varies in reports and will not include those patients who are not diagnosed and therefore do not attend clinics. The prevalence of those with symptoms of polyneuropathy was much lower at 15% of T1DM and 13% of T2DM (5). There are reports of sensory symptoms affecting 30 – 40% of patients with diabetes and this prevalence increases with a longer duration of diabetes, hypertension and worsening hyperglycaemia (6). Patients with DPN are two to three times more likely to fall than patients without neuropathy. More than 80% of amputations occur following a foot ulcer or injury (1).

Identification of risk factors is paramount so that those patients who are at a higher risk for complications related to neuropathy can be targeted. This may include metabolic abnormalities, which lead to DPN even prior to a diagnosis of DM, as 5-7% of patients have DPN on diagnosis of T2DM (7). Small fibre dysfunction is an early manifestation of nerve injury and can progress to DPN (8, 9). Early detection and management are vital to reduce morbidity and mortality (10).

1.2. Pathogenesis of DPN

The pathogenesis of DPN is multifactorial with both metabolic and vascular mechanisms playing a role (11). Hyperglycaemia is one of the main factors attributed to the development of DPN. The Diabetes Control and Complications Trial (DCCT) showed that intensive glycaemic control in patients with type 1 DM prevented the progression of neuropathy (12). Of the studies undertaken to assess the benefits of intensive glucose control in type 1 DM, only 1 out of 7 failed to show that tighter glycaemic control was associated with a delayed progression of DPN. However this has not been replicated in major studies of type 2 DM and intensive glycaemic control including the United Kingdom Prospective Diabetes Study (UKPDS) (13) and Veterans Affairs Cooperative Study on Glycaemic Control (VA CSDM) (14). Hence suggesting that there are factors other than hyperglycaemia that contribute to the development and progression of neuropathy in T2DM. A recent Cochrane review reported on the effects of enhanced glucose control in 8 randomised controlled trials of patients with type 2 diabetes (Table 1.1) (15). However, only 4 of these investigated the outcome of peripheral neuropathy

and showed that enhanced glucose control reduced the incidence of clinical neuropathy, but this was not significant.

	Trial size	Clinical outcome	Other outcomes	Enhanced glucose control superior
UKPDS (13)	3867	No	QST	Yes
ACCORD (16)	10,251	Yes	No	No
Duckworth et al (17)	1791	Yes	No	No
Gaede et al (18)	160	No	QST	No
Shichiri et al (19)	110	No	QST, NCS	Yes
VA CSDM (14)	153	Yes	No	No
Tovi et al (20)	38	Yes	No	No
Kawamori et al (21)	50	No	NCS	Yes

Table 1-1 Clinical trials investigating effects of enhanced glucose control on neuropathy, adapted from Callaghan et al (22).

The EURODIAB IDDM complications study showed that DPN was related to glycaemic control and duration of disease. The 28% prevalence of DPN was significantly related to glycosylated haemoglobin (HbA1c) (p<0.001). The EURODIAB cohort, were followed up for a period of 7 years and approximately 25% developed neuropathy. The major factors associated with incident neuropathy included not only age, duration of diabetes and poor glycaemic control, but also hypertension, hyperlipidaemia, obesity and cigarette smoking (23).

These identified risk factors for DPN have been studied individually. Wiggin et al reported that patients with progressive neuropathy had higher triglyceride levels (24). Straub et al showed that a body mass index (BMI) greater than 26.5 was associated with a worse clinical neuropathy score in a cross-sectional study of 91 patients (25). Van Acker et al also showed DPN had an independent association with obesity, high-density lipoprotein (HDL) and triglyceride levels (26). Orchard et al demonstrated in a prospective study of 463 patients that hypertension had a significant impact on the development of distal symmetrical neuropathy (27).

The difference in the studies between type 1 and type 2 DM may highlight the differences in underlying mechanisms leading to neuropathy (2). In patients with type 2 DM the development of diabetes is preceded by pre-diabetes and metabolic syndrome for many years. These metabolic factors may play a role in the development of DPN as has been shown by the identification of early signs of small fibre damage in subjects with impaired glucose tolerance (IGT). These metabolic abnormalities may contribute a greater amount than glucose control in the development of neuropathy; hence solely controlling glucose will not improve neuropathy or halt its progression.

The main pathways involved in the pathogenesis of DPN are highlighted below.

1.2.1 Hyperglycaemia

Hyperglycaemia in patients with DM can lead to neuropathy via several pathways

- Excessive glycolysis causes an overload of the mitochondrial electron transport chain and hence generation of reactive oxygen species (28).

- Increased polyol pathway flux leads to an increase in sorbitol, cellular osmolarity and oxidative stress (29). Increased sorbitol accumulation in the peripheral nerve is thought to occur from increased conversion of glucose in the hyperglycaemic state via the enzyme aldose reductase. The high rate of flux may contribute to oxidative stress.
- Carbohydrates can form covalent bonds with proteins, lipids and nucleic acids leading to the formation of advanced glycosylation end products (AGEs) (30, 31). Extracellular AGEs bind to the receptor for AGE (RAGE), which activates pathways leading to oxidative stress (32).
- Increased glucose flux through the hexosamine pathway is related with inflammatory injury (33).

1.2.2. Dyslipidaemia

Several different pathways may contribute:

- Free fatty acids can induce nerve injury through promoting inflammatory cytokine release from adipocytes and macrophages (34).
- Low-density lipoprotein (LDL) can be modified by oxidation or glycation activating pathways leading to oxidative stress (35).
- Cholesterol may be oxidised to oxysterols, which can cause apoptosis in neurones (33, 36).

1.2.3. Impaired insulin signalling

Insulin promotes neuronal growth and survival, so in patients with diabetes, alteration to this pathway may contribute to neuropathy. Insulin resistance can occur in the neurones, which is controlled by the P13/Akt signalling pathway, a common mechanism for insulin resistance in other tissues (37).

1.2.4. Protein Kinase C (PKC) activation

In diabetes there is hyperactivity of Protein Kinase C (PKC) induced by 1,2 diacylglycerol which is associated with abnormalities in vascular function. In animal models, PKCβ inhibitors have been shown to play a role in improving nerve

conduction velocity and perfusion deficits, as well as promoting endothelium dependant relaxation (38, 39).

1.2.5. C-peptide

Patients with Type 1 Diabetes have a reduced c-peptide level. Reduced c-peptide is thought to promote neuropathy by several mechanisms:

- Reduction in sodium potassium ATPase activity (40).
- Reduction in endothelial nitric oxide synthase (40).
- Reduction in endoneurial blood flow (40).

Work assessing the complications in patients with long standing (>50 years) Type 1 Diabetes Mellitus in the Joslin 50 year medallist study (41) demonstrated a reduced microvascular complication rate in this group of patients, which was attributed to the presence of enriched protective factors. Residual C-peptide production in ~6% of patients may play a role in the protection from complications.

1.2.6. Obesity

Metabolic syndrome is a combination of central obesity, hypertension, raised cholesterol and impaired glucose tolerance. There are several mechanisms by which components of the metabolic syndrome are thought to lead to neuropathy. Obese patients have an increased visceral adiposity which leads to an increase in the plasma concentration of free fatty acids and release of adipokines, creating a pro-inflammatory state (42). Up regulation of the renin-angiotensin system occurs in obesity and is thought to contribute to type 2 diabetes (43) and hence may play a role in neuropathy and in fact there have been studies to show that angiotensin converting enzyme (ACE) inhibitors may improve neuropathy (44, 45).

1.3. Painful Diabetic Neuropathy

Pain is transmitted via small myelinated (*A delta*) or thinner unmyelinated (C) fibres (46). *A delta* fibres carry superficial pain which is a sharp/ prickling sensation, and deep pain which often manifests as a burning, itching or aching is transmitted via the slower unmyelinated C fibres.

Diabetes affects all levels of the nervous system, so the insult leading to neuropathic pain may occur anywhere from the brain to the peripheral nerves. Hence, the mechanism can be classified as either peripheral or central. There are several mechanisms implicated in the development of neuropathic pain and indeed more than one mechanism is likely to contribute to the symptoms in each individual. This may explain why the pain doesn't always respond to treatment and using combinations of treatments may provide a more therapeutic effect.

1.3.1 Peripheral Mechanisms

1.3.1.1 Transient receptor potential vanilloid 1 (TRPV1)

TRPV1 is activated by stimuli such as noxious heat. Upregulation of TRPV1 occurs in injured nerve fibres and this activation may lead to heat hyperalgesia (47, 48).

1.3.1.2 Ectopic electrical impulses

Damage to nerve fibres leads to a greater expression of sodium channels along the whole length of the fibre causing hyperexcitability and the production of ectopic electrical impulses (49). These impulses lead to the generation of electrical impulses at the dorsal horn (46). There may also be dysregulation of the synthesis of calcium and potassium channels along the axon (49).

1.3.1.3 Hyperglycaemia

Hyperexcitability of nerve fibres may occur secondary to hyperglycaemia per se. Misawa et al showed that there is a reduction in the refractory period in poorly controlled diabetic patients compared with well-controlled patients with diabetes and a control group (50). Hyperexcitability causes peripheral sensitization leading to an alteration in nociceptor processing causing an abnormal spontaneous electrical discharge. The spontaneous discharge can also be reflected on to adjacent axons throughout the nerve fibre and can impact on the dorsal root ganglion, leading to central sensitisation (51).

1.3.1.4   Sympathetic mediation

Sympathetically mediated pain occurs when there is abnormal transmission of information between sensory and sympathetic fibres in a phenomenon called ephaptic transmission. This is sympathetically mediated pain, as damaged nerves become more epinephrine sensitive (52).

1.3.2 Central Mechanisms

1.3.3.1   Gating theory

As mentioned earlier, C fibres transmit the deep pain that is associated with painful neuropathy. The impulses from C fibres and A**β**fibres (light touch and pressure sensation) enter the dorsal horn and are thought to play a role in controlling pain. If there are more impulses from C fibres then the gate opens and pain transmission occurs and if there are more impulses from A**β** fibres then the gate closes and there is no pain. Animal models show that the diabetic state leads A**β** fibres to synthesise substance P after injury, which strengthens the pain signal (53). Hence pain transmission is enhanced in response to even small stimuli like light touch, provoking an amplified pain response known as hyperalgesia. This is why some patients are unable to even tolerate the bed sheets touching their feet.

1.3.3.2   Spinal rewiring

Injury to peripheral nerves leads to release of substance P from the large afferent A fibres which are present in the dorsal column. Substance P is not normally released by the A fibres but instead by the C fibres which almost exclusively innervate the superficial laminae. The sprouting A fibres in this area cause the release of substance P rather than transmitting the usual non-noxious information. This leads to the generation of a signal that is perceived as mechanical allodynia (54, 55).

1.3.3.3   Central spinal sensitisation

Stimulation of the peripheral nerves leads to activation of post-synaptic N-methyl-D-aspartate (NMDA) receptors causing the release of the excitatory neurotransmitter glutamate. Glutamate release boosts post synaptic potentials causing synaptic potentiation. The prolonged exposure to synaptic potentiation leads to an alteration in NMDA receptors which leads to allodynia (56).

1.4.   Management of DPN

There are no FDA approved treatment options that alter disease progression in DPN and the mainstay of management revolves around symptomatic measures. Education plays a key role in the treatment, enabling patients to understand the chronic nature of the condition and importance of optimising metabolic factors.

The precedence in the management of DPN and reducing disease progression is to maintain good glycaemic control. Tight glycaemic control has been shown to reduce the progression of peripheral neuropathy in the DCCT (12). Boulton et al (57) assessed the benefits of continuous subcutaneous insulin infusion (CSII) in 9 patients with DPN and showed that after 4 months of CSII there was a significant improvement in the variability (M value) and overall glucose control, which was associated with an improvement in pain scores, motor nerve conduction velocity and vibration perception threshold (58).

It is not just overall improved glucose levels but also the fluctuations in glucose control which may elicit neuropathic pain. Oyibo et al showed that there was a greater glucose flux and glucose control in patients with painful neuropathy compared to the painless neuropathy (59). The current consensus is that achieving good glycaemic control as well as treating hypertension and hyperlipidaemia are important for DPN. Improvement in lifestyle and metabolic risk factors in patients with impaired glucose tolerance (IGT) has been shown to regenerate cutaneous small distal axons and reduce pain (60).

DPN affects patients in different ways and the support and input required will vary greatly between individuals highlighting the importance of a multi-disciplinary team approach to the management of DPN (61). Having specialist centres has been shown to have a positive impact (62).

1.4.1   Disease Modifying Treatment

1.4.1.1   Pancreas transplantation

Pancreas transplantation in patients with type 1 diabetes has been shown to improve nephropathy and retinopathy (63, 64). In a 10-year follow up of neuropathy post-transplant there was an improvement in sudomotor function in the hand and foot within one year, which was maintained throughout 10 years. However there was no impact on nerve conduction velocity or autonomic function (65, 66). Mehra et al and Tavakoli et al showed that 6 months post kidney pancreas transplant there was regeneration of corneal small nerve fibres (67). Islet cell transplantation is a less invasive approach and studies have shown an improvement in nerve conduction velocity and amplitude scores (68).

1.4.2.2.   Alpha-lipoic acid (ALA)

ALA is claimed to be a disease modifying therapy, however it has never been approved by the FDA as pivotal phase III studies have failed and therefore it is only marketed in certain countries (69). It is claimed to be an anti-oxidant, which targets the imbalance of oxidative stress and anti-oxidant defences. A meta-analysis by Ziegler et al reviewed the major trials of alpha lipoic acid including SYDNEY, ALADIN I and II, and NATHAN and showed that 600mg of intravenous ALA over 3 weeks significantly improved neuropathic symptoms. This large meta-analysis showed a positive impact on symptoms and progression in the short term, but longer term studies have failed (69).

1.4.2.3.   Aldose reductase inhibitors (ARI)

The only ARI which is currently marketed for DPN is Epalrestat in Japan and India. ARI's work by blocking the enzyme aldose reductase, which is involved in the

polyol pathway. Aldose reductase enzyme activity is increased by hyperglycaemia, which causes an accumulation of sorbitol and fructose in the nerves. The use of ARIs has not been successful with many agents being withdrawn in phase III, due to lack of efficacy or toxicity (70, 71).

Bril et al showed a significant improvement in summed motor nerve conduction velocities (NCV) (peroneal, tibial and median) at 12, 24 and 36 weeks and in peroneal NCV at 36 and 52 weeks. However there was no significant effect on sensory nerve function with Ranirestat when compared to placebo (72).

The Aldose Reductase Inhibitor-Diabetes Complications Trial was a 3 year trial on Japanese patients with mild DPN using Epalrestat which showed that this was effective in delaying the progression of diabetic neuropathy (73).

1.4.2.4. Vascular endothelial growth factor (VEGF)

VEGF is already known to play a role in retinopathy and angiogenesis. There is now also thought to be a neuroprotective mechanism promoting elongation of the neurites and proliferation of glial cells (74). Quattrini et al showed that in patients with progressive neuropathy there is a reduction in VEGF associated with intrae-pidermal nerve fibre loss found in skin biopsies from the dorsum of the foot (75). Ropper et al conducted a randomised double blind study of 50 patients which showed that those who had treatment with VEGF reported an improvement in symptoms compared to placebo however there was no improvement in nerve conduction or quantitative sensory examination (QST) (76).

1.4.2.5. ACE- inhibitors and calcium channel blockers

The DEMAND trial investigated the effects of combined manidipine and delapril and delapril alone compared to placebo (77). In 140 patients without neuropathy at inclusion 23.5% on the combined treatment went on to develop neuropathy at 3 years compared to 28.9% on delapril and 38.6% on placebo. The odds ratio between both groups and placebo was significant. This study also showed that of the 60 patients with neuropathy at inclusion, 33.3% had regression of neuropathy

compared to 28.9% on delapril and 8.3% on placebo after 3 years with significant odds ratios between the groups (77).

1.4.2 Symptomatic Treatment

1.4.2.6.　　　Tricyclic antidepressants (TCAs)

TCAs have been used as first line treatment for painful DPN since the 1970's (78). TCAs work through several mechanisms including monoamine re-uptake inhibition, antagonism of NMDA which mediate hyperalgesia and allodynia as well as blockade of sodium channels.　TCAs with balanced re-uptake inhibition of noradrenaline and serotonin are shown to work better than those that are mainly noradrenergic (79). Hence the most commonly used drugs in this class are amitriptyline and imipramine.

A systematic review by Saarto (80) concluded that TCAs were useful in the treatment of painful DPN. However, reviews by McQuay et al (81) place doubt on their position as a first line treatment. The main limitations are the anticholinergic side effects, which include sedation, blurred vision, dry mouth, orthostatic hypotension and cardiac arrhythmias and indeed caution must be taken when prescribing these to the elderly.　It is suggested that patients have an ECG to exclude prolongation of PR or QTc interval prior to treatment commencing in some centres (61).　As the side effects limit the compliance of TCAs it would be advised to start cautiously on a lower dose.　A suggested starting dose for both amitriptyline and imipramine is 25mg or 10mg in older patients.

1.4.2.7.　　　Serotonin and noradrenaline re-uptake inhibitors (SNRIs)

The mechanism of pain relief is by increasing the synaptic availability of 5-hydroxytryptamine and noradrenaline.　SNRIs have fewer side-effects compared to TCAs. Duloxetine is the only drug in this class that is licensed for use in DPN and since September 2011 has been licensed in 62 countries worldwide.

A review of 4 placebo controlled studies showed a significant reduction in pain severity and an improvement on brief pain inventory interference ratings (82). Adverse effects limit compliance with a withdrawal rate of 4.3-14.9% of patients on

duloxetine. The most commonly reported adverse events were nausea, somnolence, and headache however these are transient and not as severe as the adverse effects experienced by TCAs (82). The suggested effective doses were 60mg with maximal benefit at 120mg. Duloxetine also has anti-depressant properties, which is an added advantage to its use in DPN. Although a recent further analysis of the COMBO study showed that patients without depressive symptoms benefited the most from Duloxetine in terms of neuropathic pain relief (83).

Venlafaxine is used in doses of 150-225mg/day, however there is limited data to support its use in painful DPN (84). A double-blind placebo controlled study of 244 patients showed that venlafaxine extended release was effective and safe in relieving pain. The mean visual analogue pain relief (VAS-PR) scores in the high dose (150mg-225mg) were significantly lower than placebo at 6 weeks and the NNT was similar to those of TCAs and gabapentin (85).

1.4.2.8.   Anti-convulsant medication

1.4.2.8.1.   Gabapentin

Gabapentin is well established in the management of painful DPN and has been used for the treatment of partial seizures since 1994. Gabapentin is an analogue of the neurotransmitter gamma-aminobutyric acid (GABA), which has no effect on the receptor. It appears to inhibit voltage-activated calcium and sodium channels, suggesting analgesic effect at the spinal cord level.

Rowbotham et al conducted a placebo-controlled trial and showed that in 89 patients who received gabapentin they experienced a significant reduction in average daily pain scores compared to placebo (84). Similarly Backonja showed a significant decrease in daily pain score in 70 patients (p<0.01) and all secondary outcome measures of pain were significantly better with an improvement in the quality of life. Doses of 1800-3600mg/day have been shown to be effective (86). It has also been shown that treatment with gabapentin through the alleviation of pain improves sleep disturbance in DPN and hence improved mood and quality of life. The main side effects reported were dizziness, somnolence, ataxia, confusion and oedema (86).

1.4.2.8.2.   Pregabalin

Pregabalin is another GABA analogue with no effect on GABA receptors. It has a higher potency and is more effective than gabapentin and hence the only agent in this class of drugs with a licence for treatment of painful DPN. In fact this is the only other agent apart from duloxetine to have FDA approval for the treatment of painful DPN. Rosenstock et al showed significant improvement in the mean pain scores, sleep interference, mood disturbance and tension anxiety in a placebo-controlled randomised trial with 146 patients (87). A recent meta-analysis by Freeman et al reviewed seven randomised controlled trials across a range of doses and confirmed the safety and efficacy of pregabalin (88). Divided doses of 150-600mg/day are recommended for the treatment of painful DPN and in the analysis the NNT were 4.04 for 600mg/day and 5.99 for 300mg/day. As with TCA's, side-effects of pregabalin limit dose titration and include sedation, somnolence weight gain and pedal oedema.

1.4.2.8.3.   Carbamazepine

Carbamazepine has limited evidence with small single centre studies. Carbamazepine works by blocking voltage sensitive sodium channels, thus reducing neuronal excitability and has been shown to reduce pain induced by inflammatory mediators in animals. Quite an old and very small study by Rull et al (n=13) showed that 30-50% of patients improved compared to placebo (89). A review of anticonvulsants by McQuay shows that the studies gave conflicting results with two positive studies (Wilton and Rull et al) however the longest study (46 weeks) was negative (81). The limiting factor again here was the adverse effects that reduced the tolerability of these drugs.

Oxacarbazepine is a ketoderivative of carbamazepine. Beydoun et al conducted the largest randomised placebo-controlled study involving 347 patients, which showed that the mean weekly visual analogue scale (VAS) score improved significantly but there was no significant change in the mean VAS score from baseline (90). Dogra et al showed a significant reduction in VAS scores in 146 patients (91), however another study with similar numbers showed no significant change in VAS score from baseline (92).

1.4.2.8.4.　　Topiramate

Topiramate has several mechanisms of action – it works by blocking sodium channels and through interacting with GABA receptors to potentiate GABA activity. Edwards et al showed a significant reduction in the VAS and McGill Pain Questionnaire scores, however there was a 28% drop out rate from adverse events (93). An open label extension study reported by Donofrio et al also showed that there was effective pain relief but again a high discontinuation rate of 39.5% because of adverse events (94). Findings from three double-blind placebo trials showed no significant effect of topiramate versus placebo (95).

1.4.2.9.　　Anti-arrhythmic agents

Lidocaine, given intravenously, was first shown to be effective in a randomised, double-blind placebo trial by Kastrup (96). This was then further confirmed in a trial by Petersen which showed a significant beneficial effect of lidocaine infusion (5mg/kg over 30 minutes) compared with a saline infusion (97). However the main drawback is that there is no oral dosing available and the patients need to be monitored via electrocardiogram (ECG) during intravenous administration (98), limiting its use to a small number of patients with severe pain.

Lidocaine patches 5% are available and this will be discussed in topical treatments.

Mexiletine is a structural analogue of lidocaine, which can be administered orally. A review of seven controlled trials demonstrated only a modest analgesic effect (99). As there is equivocal data and regular ECGs are required for monitoring there is limited use of Mexiletene and it is not recommended.

1.4.2.10.　　*N*-methyl-D-aspartate receptor (**NDMA) antagonists**

Dextromethorphan is a low affinity NMDA receptor blocker that has been shown by Nelson et al to significantly reduce pain by 24% compared to placebo in a small trial (n=13) (100). Thisted et al showed in a multi-centre, open label study that there was a significant impact on pain in 36 patients with a combination of dextrometorphan and quinidine (101).

1.4.2.11. Opioids

Harati et al showed in a randomised controlled trial (RCT) that tramadol 200mg/day caused a significant reduction in pain when compared to placebo and in a 6-month open extension showed that this effect could be sustained up to 6 months. However 14.5% of patients dropped out because of adverse effects or insufficient analgesia. Tramadol works as a M*u* receptor antagonist and by inhibition of monoamine reuptake (102).

In a trial of 36 patients, oxycodone had a significant impact on the main daily pain and disability (103). A recent review reported that several randomised controlled trials have established the use of oxycodone at doses of 10mg-120mg (47).

These studies are limited and more extensive studies need to be done to evaluate the risks and benefits of using opioids. The main concerns faced when prescribing opioids is the side effect profile and dependence. Side-effects include constipation, nausea, vomiting and more worryingly sedation. Opioid dependence is also an issue in patients taking them long-term and hence there is a duty to monitor use to detect dependence. There has been some positive data in combining opioids with other medication which will be discussed later.

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1.4.3 Topical Treatments

1.4.3.1 Glyceryl Trinitrate (GTN)

GTN is a nitric oxide donor with local vasodilating properties. There are a limited number of trials assessing the use of topical nitrates. Yuen at al evaluated isosorbide dinitrate (ISDN) spray in a double- blind placebo-controlled cross-over study of 20 patients. They were randomised to use ISDN spray or placebo for 4 weeks followed by a 2-week washout and then their treatment was swapped. 50% of patients reported a benefit with the ISDN spray with reduced neuropathic pain overall and burning sensation. There was no difference in other sensory modalities with treatment. 18% of patients preferred the placebo spray and 32% were undecided. Following this the same group evaluated GTN patches in 18 patients and showed a 44% reduction in pain. However this was not a placebo-controlled study because of difficulty in obtaining placebo patches. The study found that when patients initially applied the patch to one leg there was a reduction of pain in that leg only, which may suggest a local mechanism of action (104, 105).

The results of these trials are promising but larger studies are required for these agents to be recommended.

1.4.3.2 Capsaicin

Capsaicin is a topical treatment that has been shown to be effective in painful DPN. It is a natural colloid which is extracted from chilli peppers which works by depleting substance P from the nerve terminals. A meta-analysis by Zhang et al concluded that topical capsaicin (0.075%) provided more effective relief in DPN than placebo (106). However, capsaicin was shown to be comparable to amitriptyline in a double blind study by Biesbroeck et al (107), although the patients with capsaicin had less serious adverse effects.

Mason et al reviewed 6 double blind placebo controlled trials (n=656) which showed that topical capsaicin was better than placebo at reducing pain. The pooled efficacy data was not as good as expected with the numbers needed to

treat being 5.7 for topical capsaicin (0.075%) over 8 weeks (108). However, topical capsaicin causes a marked reduction in intra-epidermal nerve fibre density (IENFD) regenerates over 6 weeks once treatment has been stopped (109). However, there is concern about using this in patients who already have IENFD loss i.e. patients with diabetes.

The presumed advantage of topical capsaicin is the avoidance of drug interactions and apparently much fewer adverse effects. However the main side effects include a burning sensation and skin irritation at the site (damage to IENFD) and the maximum therapeutic effect may not occur until 4-6 weeks (presumably when all IENFD have been destroyed) and this is also coupled with the disadvantage of having to apply it 3 to 4 times a day, with questionable compliance.

1.4.3.3 Lidocaine

Lidocaine 5% is most commonly used in the treatment of post herpetic neuralgia. The only reported adverse effect is local irritation at the application site. Up to 4 plasters a day at the site of pain can be used in 12 hours. A study reported by Tesfaye et al showed that lidocaine was as effective as pregabalin in controlling pain and had no adverse effects (61). This has been recommended for use in the NICE guidelines.

1.4.4 Non-pharmacological agents

Through the course of DPN, many patients will exhaust the available medications and combinations and will continue to suffer from the symptoms. Pharmacological treatments are limited by their side-effects and hence limited compliance. This leads patients and clinicians to seek non-pharmacological methods to try and reduce their pain.

There are a number of therapies available which have been shown to be effective in a limited number of trials. Electrical stimulation has been shown to be effective and there are several methods of this which are reviewed below. The mechanism of action is presumed to be through stimulation of endogenous opioids at the spinal cord level, invoking the gating principle (46).

1.4.4.1   Transcutaneous electrical nerve stimulation (TENS)

Alvero et al reviewed three studies on the use of TENS in painful DPN (110). The first study by Kumar et al showed that in a group treated with electrotherapy alone (n=31), there was a 52% reduction in symptoms over 2-3 weeks (111). The second study showed that amitriptyline and TENS (n=26) together showed an overall reduction of symptoms by 66%, with amitriptyline alone leading to a 26% reduction of pain after 4 weeks (112). The third study by Alvero looked at the long term effectiveness of TENS using a questionnaire and telephone consultation (n=54). There was a 44% improvement in pain with continued benefit as patients used TENS for an average period of 1.7+/-0.3 years (113).

1.4.4.2   Percutaneous Electrical Nerve Stimulation (PENS)

PENS is a electroanalgesic therapy that amalgamates the benefits of TENS and electro acupuncture which works by using acupuncture like needles placed on the skin to stimulate peripheral sensory nerves that innervate the region of neuropathic pain. Hamza et al showed in a prospective crossover sham controlled 3 week study of 50 patients with DPN for more than 6 months that PENS provided effective short term relief from DPN (114) with increased levels of mood and activity and improved quality of sleep.

1.4.4.3   Frequency-modulated electromagnetic neural stimulation

This technique stems from the TENS family however has a more distinct and novel mechanism of action. There is a sequence of stimuli, which vary automatically in terms of pulse frequency, duration and voltage amplitude. This has been shown to improve symptomatic diabetic neuropathy with no effect on nerve conduction velocity (115).

1.4.4.4   Electrical Spinal Cord Stimulation

This involves implanting an electrode into a thoracic/lumbar disc space, which then stimulates endogenous opiate production; hence this is an invasive procedure. Tesfaye et al studied the effects on ten patients and showed a significant reduction

in pain assessed by the McGill pain questionnaire which was refractory to other treatments (116). This treatment is limited as it is only available in specialist centres and is invasive.

1.4.4.5   Low-intensity laser therapy

This is thought to work by increased release of serotonin and endorphins and possibly also has an anti-inflammatory effect. Zinman et al showed a reduction in pain scores through administration of bi-weekly therapy in 50 patients over 4 week (117).

1.4.4.6   Acupuncture

Acupuncture involves applying needles into the skin to relieve pain and has been shown to be beneficial and effective. Abuaisha et al showed that the benefits lasted for up to 6 months and 67% of patients stopped or reduced their medication (118). There is a high chance of a placebo response with the use of acupuncture but as it reduces the perceived pain, its use can still be justified, particularly as there are no obvious side effects. One limitation of its use is the need for specialist application of the needles.

1.4.5 Psychological treatments

Depression is common in patients with diabetes. Vileikyte et al conducted a longitudinal study, which showed depression to be a risk factor for neuropathy (119). It has been shown that improving patients' mood can have a positive impact on the quality of life in patients with type 2 DM (120).

1.4.6 Novel Treatments

Whilst there are several treatment options, there are limitations to the current therapies and in the long course of DPN it often leaves patients with limited options once the above have been exhausted. This leaves open the area for novel treatment options and there are several which are currently being developed. A recent paper reported that at least 50 new molecular entities had reached clinical development and 8 were in phase 3 trial (Table 1.2) (121). These novel therapies aim to act on the different mechanisms that cause neuropathic pain.

Drug	Drug Action	Originator Company
Dextromethorphan-quinidine combination	Glutamate antagonist	Avanir
NGX-4010 (capsaicin, dermal patch)	Vanilloid-receptor agonist	NeurogesX
Desvenlafaxine SR	SNRI	Wyeth
Lacosamide (SPM-927)	Amino acid anticonvulsant	Schwarz Pharma
Lamotrigine once daily	Anticonvulsant	GlaxoSmithKline
Oravescent fentanyl	Opioid agonist	Cephalon
Tramadol ER	Mu-opioid antagonist and SNRI	TheraQuest Biosciences
GW-406381	COX-2 inhibitor	GlaxoSmithKline

Table 1-2, Novel therapies being developed.

Transient receptor potential cation channel subfamily V member (TRPV1) is a non-selective cation channel expressed on C fibres, and antagonists of the TRPV1 receptor have been shown to relieve pain in rodent models. Capsaicin is a drug of this genre, which has shown benefit in several trials however its use is limited as it is only available in topical preparation and causes IENFD. Trials are currently in progress assessing an injectable form of capsaicin in other types of neuropathic pain (74). GRC17536 is a transient receptor potential ankyrin 1 (TRPA1) antagonist which is currently undergoing a 4 week double-blind placebo controlled phase 2 study (122) .

Tumour necrosis factor (TNF) alpha production is upregulated following nerve injury and levels are increased in DPN as opposed to non-painful neuropathies. There are higher levels of TNF alpha in diabetic patients as compared to non-diabetic patients and this is thought to be implicated in the development of diabetic micro and macroangiopathy (74). TNF alpha is a pro-inflammatory cytokine, which mediates hyperalgesia. There is evidence to show that using antibodies to TNF alpha and other cytokines show a reduction in hyperalgesia and allodynia. A reduction of TNF alpha can cause an improvement in neuropathy as shown by Sharma et al following administration of insulin and an antioxidant which causes a reduction in TNF-alpha and an improvement in experimental neuropathy (123). Several drugs used in diabetes such as the ACE-inhibitors (lisinopril (124) and trandalopril (125)) and the PPAR gamma agonist troglitazone (126) have all been shown to work against TNF-alpha and improve neuropathy.

There is a link between PKC-beta activation and neuropathy. Kamaei et al have implicated the role of PKC activation in DPN in experimental studies (127). Ruboxistaurin which is a PKC-beta inhibitor has been shown to cause a significant reduction in symptoms in 2 studies however due to a lack of benefit in neuropathy perse it has been withdrawn from further development (74). Differentiation and changes in expression of the sodium channels has been linked to the pain of neuropathy. There are several sodium channel isoforms that are specifically implicated in pain and therefore there is a role for medication against the specific isoforms of the sodium channel receptor (74).

Hyperglycaemia leads to the formation of the dicarbonyl metabolite methylglyoxal which is metabolised by glyoxalase 1 (GLO1) and GLO2 to the end product D-lactate (128). Peripheral nerves have low GLO1 activity so there may be accumulation of methylglyoxal. Bierhaus et al showed that a higher plasma methylglyoxal was present in patients with Type 2 diabetes because of decreased breakdown and increased formation from excessive glycolysis (128). Methylglyoxal causes modification of the voltage gated sodium channel $Na_v1.8$, which facilitates firing of nociceptive neurones causing hyperalgesia. It also promotes the slow inactivation of $Na_v1.7$ which is an essential voltage gated channel in nociceptive neurones. Several strategies have been identified which reduce methylglyoxal and therefore diabetes induced hyperalgesia. Other therapies that are currently being developed include glutamate antagonists, cytokine inhibitors, catecholamine modulators, COX inhibitors, acetylcholine modulators, adenosine receptor agonists and in the future gene-related therapies (121).

1.5   Diagnosis and Assessment

1.5.1   Neuropathy Symptoms

Several questionnaires have been developed to assess and quantify the patients' subjective view of neuropathic pain in the form of numeric rating scales. This enables a diagnosis to be made and may be used for monitoring treatment outcomes. These include the McGill Pain Questionnaire (129), which was not initially created for neuropathic pain but is widely used, as well as those that have been developed specifically for neuropathic pain: Brief Pain Inventory (BPI) (130), Neuropathic Pain Questionnaire (NPQ) (131), Neuropathic Pain Symptom Inventory (NPSI) and Doleur Neuropathique en 4 (DN4) (132). BPI, NPQ and NPSI are all self-administered questionnaires. BPI assesses the severity of pain and impact on daily functioning using a numeric rating scale. NPQ has an advantage of discriminating between neuropathic and non-neuropathic pain and hence may aid in diagnosis. The DN4 is a clinician administered questionnaire that includes both pain symptoms and items related to bedside examination.

Neuropathic Symptom Profile (NSP) consists of 38 questions divided into motor, sensory and autonomic symptoms. This has been validated in detecting neuropathy and staging severity (133). The Rochester Diabetic Neuropathy Study concluded that NSP when used in combination with another neurological examination is a valid tool to assess neuropathy (134).

It is important to assess quality of life (QoL) and this can be done with questionnaires that have been created and validated to assess the effect of neuropathy on QoL specifically. These include NeuroQol (135), Norfolk Quality of Life Scale (136), and Neuropathic Pain Impact on Quality of Life Questionnaire (NePIQol) (137). NeuroQol assesses physical symptoms and psychological function scales. The Norfolk QoL scale is a patient structured interview, which asks questions about symptoms related to small fibre, large fibre and autonomic nerve dysfunction as well as generic health status and general information questions. The NePIQol has 6 domains: psychological, physical, symptoms, personal care, relationships, and social/work activity providing a more detailed assessment of QoL.

The limitation to symptom assessment is that the severity of pain and response is completely subjective and an external observer cannot input into this. Furthermore, pain interpretation varies interpersonally depending on patients' experiences and psychological traits. The same type of pain may have a completely different meaning and severity to different people. Nevertheless the pain score can be monitored using the scales and if it improves then the outcome of the intervention is satisfactory, regardless of how exaggerated the pain interpretation may be.

1.5.2 Neuropathy Deficits

DPN can be confirmed with a combination of electrophysiology, sensory and autonomic function testing (1). The American Diabetes Association advises that all patients with diabetes should be screened for DPN annually by undertaking an assessment of:

- Pin prick
- Temperature

- Vibration perception (with 128 Hz tuning fork)
- 10g monofilament at the distal halluces
- Ankle reflexes

A positive result in more than one of the above tests has >87% sensitivity in detecting DPN (1). It is important to exclude other forms and causes of neuropathy such as chronic inflammatory demyelinating polyneuropathy (CIDP), B_{12} deficiency, hypothyroidism, and uraemia which may also occur in DM.

The Toronto Diabetic Expert Neuropathy group have stated that DPN can be defined by (138):

1. Confirmed DPN - Abnormal nerve conduction and a symptom or sign of neuropathy

2. Probably DPN – Two or more of the following signs or symptoms: neuropathic symptoms, decreased distal sensation, or decreased/absent ankle reflexes.

3. Possible DPN – Any of the following symptoms: decreased sensation, positive neuropathic sensory symptoms (e.g. 'asleep numbness' prickling/stabbing, burning or aching pain) predominantly in the toes, feet or legs OR signs – symmetric decrease of distal sensation or decreased/absent ankle reflexes

Once a diagnosis has been made there are very limited methods to track severity based on clinical signs and symptoms. One method which was proposed by Dyck (139) is:

Grade 0 – no abnormality of nerve conduction (NC)

Grade 1a – abnormality of NC

Grade 1b – NC abnormality plus neurological signs typical of DPN but without neuropathy symptoms.

Grade 2a – NC abnormality with or without signs and with typical neuropathic symptoms.

Grade 2b – NC abnormality, a moderate degree of weakness (e.g. 50%) of ankle dorsiflexion with or without neuropathy symptoms.

1.5.3 Neuropathy Disability Score (NDS)

The modified NDS (mNDS) is a screening tool used to identify neuropathy. This consists of testing sensory modalities, which include pain sensation (pin-prick), temperature perception (using hot and cold rods) and vibration (128 Hz tuning fork) which are scored as either normal (0) or reduced/absent (1). The Achilles reflex is scored as normal (0), present with reinforcement (1) or absent (2). Each leg is scored separately so the total maximal score is 10. A score of >8 indicates severe neuropathy, 6-8 moderate and 3-5 mild neuropathy.

The Rochester Neuropathy Study group validated the use of the original NDS in assessing severity of neuropathy (134).   mNDS has been shown to be a reliable and reproducible screening tool for neuropathy (140). Abbott et al showed that a mNDS of >6/10 was an independent risk factor for a new foot ulcer (140).

The key disadvantage of the mNDS is that is does not diagnose patients with small fibre neuropathy and those with sub-clinical large fibre neuropathy. It should therefore primarily be used to identify patients at increased risk of neuropathic ulceration.

1.5.4 Quantitative Sensory Testing (QST)

QST is a non-invasive technique that measures vibration (large fibres), warm and cold perception thresholds (small fibres). An electrode is placed on the patient's foot which delivers warm and cold stimuli, and the patient's response to the stimuli is then analysed. Vibration can be assessed using a Biosthesiometer. This device has a probe, which is placed on the distal hallux and vibrates at a rate of 100Hz and an amplitude of 0-50 volts. A vibration perception threshold (VPT) of greater than 25 volts is a strong predictor of foot ulceration (141).

QST is able to detect small nerve fibre damage which is an advantage compared to other tests such as nerve conduction studies (NCS) which only evaluate large fibre damage. QST has been shown to be a fairly sensitive method of detecting small fibre neuropathy, particularly in those patients with normal nerve conduction

studies (142), and it has also been shown to be a reliable and reproducible test of large and small fibre dysfunction (143). The main limitations lie in the fact that there are many different instruments that are available with varying specifications, algorithms and normal values hence caution must be exercised in selecting the appropriate test equipment (144) and in interpreting the results.

1.5.5 Nerve Conduction Studies (NCS)

NCS measure sensory and motor conduction velocity, amplitude and latency of the nerve fibres. NCS is considered a 'gold standard' test for neuropathy. Dyck et al compared NCS to an individual physician's clinical diagnosis of DPN and found that clinician's diagnosis was excessively variable and frequently inaccurate with an overestimation of DPN (145).

Furthermore, NCS assesses only large myelinated nerve fibres and hence using this alone will not identify small fibre neuropathy. The San Antonio consensus recommended the use of NCS to classify diabetic neuropathy alongside clinical examination, clinical symptoms, QST and autonomic function testing (146).

NCS are commonly used to assess the severity of DPN and are sensitive, specific, and reproducible and easily standardised (147). However they require a trained physician to conduct the test. Bril et al conducted a study of 205 patients which showed that sural nerve conduction correlated well to early mild DPN (148). This has an advantage of being able to identify patients who have less severe DPN and can be used in the assessment of treatments in clinical trials.

1.5.6 Skin Biopsy

Skin biopsy enables direct visualisation of thinly myelinated and unmyelinated nerve fibre damage and repair. It can be used to diagnose neuropathy, in particular small fibre neuropathy (149).

The most commonly used method is the 3mm punch skin biopsy which is minimally invasive when compared to sural nerve biopsy, which would be the alternative. The biopsy is taken from the distal leg and local anaesthetic is administered prior to doing the biopsy. The specimen is then fixed in either 2%

paraformaldehyde-lysine-periodate or Zamboni's (2% paraformaldehyde, picric acid) fixative for 24 hours at 4 ^{o}C and kept overnight in a cryoprotective solution. The sample provides on average 55, 50 µm thick sections which are immunostained using the antibody to protein gene product 9.5. The staining allows visualisation of the intra-epidermal nerve fibres (IENF) via light or confocal microscopy techniques (150).

The European Federation for Neurological societies (EFNS) recommends a 3 mm punch skin biopsy at the distal leg. The IENFD then needs to be quantified in at least three 50 µm thick sections per biopsy, which should be assessed by bright-field immunohistochemistry or immune fluorescence after staining with anti-PGP 9.5 antibodies (149).

There have been studies to correlate IENFD with the other measures of neuropathy. Pittenger et al showed a reduction in IENFD in patients with small fibre neuropathy with a sensitivity between 74-87.5%, and IENFD was inversely correlated with QST (151). An inverse correlation has also been shown between IENFD and duration of diabetes, neurological impairment score and results of sensory evaluation (151, 152).

The key advantage is that small fibre neuropathy which is missed on standard electrophysiological tests can be detected. The disadvantage is that, although only minimally invasive compared to sural nerve biopsy it is an invasive procedure that cannot be carried out routinely in patients'.

A summary of the common tests used to assess neuropathy are shown in table 1.3.

	Advantage	Disadvantage	Type of Nerve
NCS	Sensitive, specific, reproducible and easily standardised gold standard technique	Must be done by trained professional Only assesses large fibre damage	Large fibre
NDS	Good predictor for risk of ulceration	Does not detect small fibre damage or sub-clinical large fibre damage	Large fibre
QST	Reproducible and reliable test	Subjective	Large and small fibre
Skin Biopsy	Gold Standard, safe, reliable and reproducible	Invasive procedure	Small fibre
CCM	Quick, reproducible, non-invasive Can detect small fibre damage and track progression	Must be done by trained professional	Small fibre

Table 1-3. Summary of the advantages and disadvantages of neuropathy assessments.

1.5.7 Corneal Confocal Microscopy (CCM)

The cornea is one of the most densely innervated organs in the body (153).   It is supplied by the ophthalmic branch of the trigeminal nerve. In vivo corneal confocal microscopy allows real time visualisation of the corneal sub-basal nerve plexus. This enables small fibre damage and early neuropathy to be detected and hence might be an ideal surrogate end-point for DPN. CCM is able to demonstrate small fibre damage prior to any abnormality being detected in electrophysiology or QST and has been validated against the gold standard of IENFD (154). CCM has also been shown to detect early nerve fibre repair following pancreas transplantation (67). CCM is a quick, reproducible, non-invasive procedure, which therefore has the key advantage of use to monitor progression or regression of neuropathy in trials of new treatments for neuropathy.

1.6   Longitudinal Studies

Only a limited number of studies have assessed the natural progression of DPN. The initial studies defined DPN as either present or absent as opposed to tracking the severity.   Dyck et at (147) looked at longitudinal data from the Rochester Diabetic neuropathy cohort which showed that DPN got worse over 7 years and the rate of neuropathy deterioration was faster in patients who already had DPN compared to those who didn't. Amthor et al (155) studied 45 patients with type 1 diabetes over an 8 year period and showed that those with poor glycaemic control had a faster rate of progression of neuropathy. Patients whose HbA1c was <10% showed a tibial motor NCV reduction of 3.9 m/s compared to those with a HbA1c >10% who showed a reduction of 6.8 m/s. A 24 year prospective study of patients with type 1 diabetes showed that in a group with good glycaemic control there was faster median, ulnar, and peroneal nerve conduction velocity and median, ulnar and sural sensory nerve conduction velocities.   Impairment in QST and HRV developed faster in the group with poorer control(156).

Van de Poll-Franse (157) studied 486 patients with type 2 diabetes over 4 years. Assessment of neuropathy was based on the clinical neurological examination (CNE), which includes pin-prick, light touch, vibration sense and ankle jerks, where a score of >4 denotes DPN. The mean CNE score increased significantly during

the follow-up period. Of the patients without neuropathy at baseline, 21.3% progressed to a CNE score >4 after 3 years. It was found in those subjects who had neuropathy at baseline and in particular those with a CNE >14 there was a decrease in CNE score with a 'regression to the mean' effect.

Tesfaye et al studied 1172 patients from the EURODIAB study assessing DPN at baseline and approximately 7 years later (23). 24% of patients with type 1 diabetes without neuropathy at baseline developed neuropathy. Forrest et al (158) studied a population of 453 patients with childhood onset Type 1 Diabetes who did not have DPN at baseline. They reported that 15% of the patients developed DPN after 6 years giving an incidence rate of 2.8 per 100 person years. Partenen et al (159) found that in 132 patients with newly diagnosed type 2 diabetes the prevalence of DPN was 8.3% compared to 2.1% in the control group. After 10 years of follow up the prevalence of neuropathy determined by a reduction in sensory and motor nerve conduction was 41.9% in patients with Type 2 diabetes compared to 5.8% in the control group.

1.7 Impaired Glucose Tolerance (IGT) neuropathy studies

The relationship between IGT and DPN is still controversial. The San Luis Valley study included a large cohort of 856 subjects and found an increased prevalence of DPN in IGT subjects compared to age matched controls. However this study was conducted by nurses using a screening tool so the diagnosis of DPN was questionable (160).

Earlier studies from Singleton et al found that in a cohort of 107 patients referred with idiopathic DPN, 36 had IGT compare to 13 who had diabetes, suggesting that IGT may contribute to small fibre neuropathy (161). A study of 32 patients with IGT who underwent 3mm punch skin biopsy found that IENFD was reduced. They were then given diet and exercise advice and a repeat biopsy after 1 year showed a significant improvement in IENFD from the thigh but not the distal leg (162).

Conversely, Hughes et al found that in 50 consecutive subjects with DPN and 50 consecutive controls there was no significant difference in the prevalence of IGT and in the DPN group serum triglycerides were significantly higher (163). Fujimoto et al found that IGT subjects did not have nerve conduction abnormalities

compared to controls (164), although they did report an increase in retinopathy and nephropathy in IGT subjects.

More recently Dyck et al (165) showed there was a similar frequency of DPN in healthy subjects (1.7%) and subjects with impaired glycaemia (2.0%) and was only increased in those with Type 2 diabetes (7.8%). In a cohort of 393 subjects, Zeigler et al (166) found that there was a slightly increased prevalence of polyneuropathy in those with IGT (13%), compared to those with impaired fasting glycaemia (11.3%) and control subjects (7.4%) although this was not significant. There was a significant independent association between waist circumference and polyneuropathy, suggesting obesity was an important target for the prevention of diabetic polyneuropathy.

1.8   Obesity Related Neuropathy

There is a worldwide rapid increase in obesity prevalence such that it may be described as an epidemic. Obesity has more than doubled since 1980 such that in 2014 more than 1.9 billion adults were overweight and over 600 million were obese (167). In 2013, 42 million children under the age of 5 were overweight or obese (167).   The adverse consequences of this are emphasized when one considers that body mass index (BMI) is a powerful predictor of type 2 diabetes (T2DM), and cardiovascular (CV) morbidity and mortality (168, 169). World Health Organisation (WHO) estimates that approximately 171 million people were diagnosed with type2 DM in 2000 and this number will increase to 366 million by 2030 (170). Indeed despite the emphasis on malnutrition in the developing world, overweight and obesity are linked to more deaths than being underweight.

Overweight or obesity is the single most important predictor of T2DM (171). The relative risk of diabetes increased approximately 40 fold as BMI increased from less than 23 kg/m^2 to more than 35 kg/m^2 (171). NICE guidelines recommend that all patients with a BMI of 35 or over who have recent-onset T2DM be assessed for bariatric surgery. Of course the BMI may be lower in poorly controlled patients, those with co-morbidities or those of Asian origin.

1.8.1 Definition

Obesity and overweight are defined as abnormal or excessive fat accumulation that may impair health (167). The WHO definition of overweight is a BMI > 25 kg/m^2 and >30kg/m^2 is obesity. The National Institute of Health define morbid obesity as a BMI of 40 kg/m^2 or more or a BMI of 35 kg/m^2 or more in the presence of obesity related co-morbidities (172).

1.8.2 Medical Complications

There is a strong relationship between obesity and T2DM hence the rise in obesity corresponds to a rise in T2DM (173). The Nurses' Health Study reported an age-adjusted relative risk of 40 for diabetes in women with a BMI >31 kg/m^2 compared to women with a BMI <22 kg/m^2 (171). Obesity is associated with cardiovascular risk factors with BMI at age 18 and in midlife being positively associated with the occurrence of hypertension. The Swedish Obesity Study (SOS) found the baseline prevalence of hypertension in obese subjects to be 44-51% (174) and similarly the Longitudinal Assessment of Bariatric Surgery (LABS)-2 study reported that 68% of obese subjects had hypertension (175) and 63% of had dyslipidaemia. A meta-analysis reported that weight loss of 1kg was associated with a decrease in serum total cholesterol of 0.05mmol/l, LDL cholesterol by 0.02 mmol/l and increased HDL by 0.009 mmol/l (176).

Further risks associated with obesity include heart failure, atrial fibrillation and cerebrovascular disease (173). Other medical complications include gastro-oesophageal reflux, cholelithiasis, non-alcoholic fatty liver disease (173) and obstructive sleep apnoea (177).

1.8.3 Types of Surgery

Bariatric procedures can be divided into malabsorptive or restrictive. Malabsorptive interventions include Roux-en-Y Gastric Bypass (RYGB), which involves the creation of a gastric pouch and an intestinal bypass and is undertaken laparoscopically (Figure 1.1a). Biliopancreatic Diversion (BPD) involves a partial gastrectomy followed by reconstruction of the small intestine to divert the bile and

pancreatic juices to meet the food closer to the middle or distal small intestine. Restrictive procedures include laparoscopic adjustable gastric banding (LAGB) which involves placing a constricting ring just below the junction of the stomach and oesophagus and an inflatable balloon in the lining can be adjusted to regulate food intake (Figure 1.1c). More recently vertical sleeve gastrectomy (SG) has been introduced which involves a 70% vertical gastric resection creating a narrow canal but without intestinal bypass (Figure 1.1b).
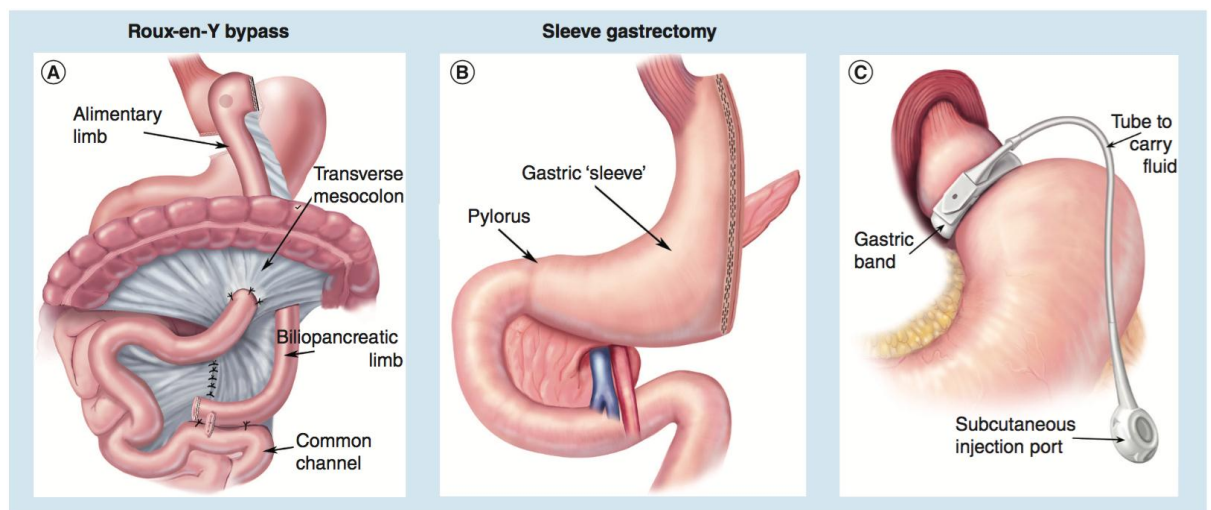
Figure 1-1. Types of bariatric surgery. (A) gastric bypass, (B) gastric sleeve and (C) gastric band.

1.8.4 Bariatric surgery effect on Type 2 Diabetes

The effect of bariatric surgery has been assessed in RCTs to determine the effect on weight loss, type 2 diabetes, as well as other co-morbidities. Schauer et al conducted a randomised, non-blinded, single-centre trial of 150 patients comparing the effect of intensive medical therapy verses bariatric surgery on glycaemic control in the Surgical Treatment and Medications Potentially Eradicate Diabetes Efficiently Trial (STAMPEDE) (178). The average starting Hba1C was 9.2+-1.5% and the primary end-point was the proportion of patients with an HbA1C of 6.0% or less after 12 months of treatment. 12% of patients in the medical therapy group reached the primary end point compared to 42% in the gastric bypass group (P<0.002) and 37% in the sleeve-gastrectomy group (P=0.003). It was noted that weight loss was greater in the bariatric surgery groups compared to those on medical therapy and those on medical therapy needed significantly more oral therapies to control blood pressure, lipids and glycaemia compared to the bariatric surgery group. All the patients in the gastric bypass group achieved the target HbA1c without any medications, whereas 28% of patients in the sleeve gastrectomy group required one or more glucose-lowering medications. Ikramuddin et al showed remission rates of 49% in a cohort undergoing RYGB and 19% in the control group at 12 months. The remission rates were less than in the STAMPEDE trial, but the end point used by Ikramiddin was more comprehensive with a composite goal of HbA1c less than 7.0%, low-density lipoprotein cholesterol less than 100 mg/dL, and systolic blood pressure less than 130 mm Hg (179). More recently Courcoulas et al and Halperin et al also noted significantly greater partial and complete remission in RYBG and LABG subjects at 12 months (180, 181). Two longer RCTs found that the bariatric surgery groups had significantly higher rates of type 2 diabetes remission at 2 years (182, 183) (table 1.4). A systematic review of 621 studies that included 135,326 patients found that 78.1% of diabetic patients had complete resolution and diabetes was improved or resolved in 86.6% of patients (184).

	Number of patients	Type of surgery	Length of Study	Main Outcome	Results
Schauer et al (178)	150	RYGB, SG	12 months	HbA1C <6% (with or without medication)	12% of patients achieved the primary outcome in the control group, compared to 42% in the RYGB group (P<0.002) and 37% in the SG group (P=0.003)
Dixon et al (183)	60	LAGB	24 months	HbA1C <6.2% and fasting plasma glucose <126mg/dL (7.0mmol/L) with no medication	Primary outcomes was achieved in 22(73%) in the SG group compared to 4(13%) in the control group.
Mingrone et al (182)	60	RYGB, BPD	24 months	Fasting glucose level of <100 mg per deciliter [5.6 mmol/l] and a HbA1C <6.5% in the absence of pharmacologic therapy	Primary outcome was achieved in no patients in the control arm compared to 75% in the RYGB group (P<0.001) and 95% in the BPD arm (P<0.001)
Ikramuddin et al (179)	120	RYGB	12	Composite goal of HbA1c <7.0%, low-density lipoprotein cholesterol less than 100 mg/dL, and systolic blood pressure less than 130 mm Hg	Primary outcome was achieved in 28 (49%) in the RYGB group compared to 11 (19%) in the control group (odds ratio [OR], 4.8; 95% CI, 1.9-11.7)
Courcoulas et al (180)	69	RYGB, LAGB	12	Feasibility and effectiveness measured by weight loss and improvements in glycaemic control	Partial and complete remission of T2DM occurred in 50% and 17%, respectively in the RYGB group and 27% and 23%, respectively, in the LAGB group (P <0.001 and P =0.047 between groups for partial and complete remission), with no remission in the control group
Halperin et al (181)	38	RYGB	12	Fasting plasma glucose< 126 mg/dL and HbA1c < 6.5%,	Primary outcome was achieved in 58% of RYGB patients compared to 16% in the control group (P=0.03)

Table 1-4. RCTs of bariatric surgery.

Although there are a number of RCTs, the data from these does not extend beyond 2 years with relatively small numbers of subjects being included. Therefore to appreciate the longer term outcomes of bariatric surgery we must look to non-RCT studies with a few large studies leading the way in outcomes of bariatric surgery. The Swedish Obesity Study (SOS) is one of the largest prospective controlled intervention studies involving 4047 obese subjects and commenced in 1987 (185). 2010 subjects underwent a bariatric intervention; vertical band gastroplasty (68%), gastric banding (19%) and Roux-en-Y gastric bypass (13%). The remaining 2037 made up a usual care control cohort. The primary outcome of this study was mortality with secondary outcomes looking at type 2 diabetes, weight loss, cardiovascular outcomes, and cancer. The maximal weight loss that was achieved in the surgical groups occurred at 1-2 years; gastric bypass 32%, vertical band gastroplasty 25% and banding 20%. This then stabilised by 10 years with weight loss from baseline being reduced to 25%, 16%, and 14% respectively. Overall at 20 years the weight loss in the surgery group was 18%, which was significantly reduced compared to -1% in the control group. A major drawback of this study is that the majority of patients underwent vertical band gastroplasty, which is now obsolete.

Patients who had bariatric surgery showed a reduction in mortality after a mean follow up of 10.9 years with 129 deaths in the control group vs 101 in the surgery group (hazard ratio 0.76, P=0.04 compare to controls) (186). When adjusted for sex, age and other risk factors the hazard ratio was 0.71 (P=0.01). The most common causes of death were myocardial infarction and cancer. The SOS also found that bariatric surgery was associated with a decrease in cardiovascular deaths and a lower incidence of cardiovascular events when compared to usual care (187). Bariatric surgery was found to be associated with reduced cancer incidence in women but not men (188).

In the first 6 years post-surgery those subjects undergoing bariatric surgery required more inpatient and non-primary care outpatient appointments compared to controls with inpatient stays being 1.7 and 1.2 days respectively (189). Subsequently from 7-20 years both groups had on average stay of 1.8 hospital days. The drug costs in this time period were lower in the surgery than control patients.

There was a major improvement in obesity related co-morbidities and in particular the subjects with T2DM had a 72% remission at 2 years (OR for remission 8.4, 5.4-12.5; P<0.001) with a reduction in this number to 36% at 10 years (OR 3.9, 11.6-7.3, P<0.001). In a subgroup analysis of the SOS, 505 subjects at baseline had Type 2 DM; 345 in the bariatric surgery group and 262 controls (190). At a mean follow up of 13.5 years there was a reduced incidence of myocardial infarction in the bariatric group but no effect on stroke incidence. Furthermore, the effect of surgery in reducing myocardial infarction was stronger in those with higher serum total cholesterol and triglycerides at baseline.

Interestingly in the cohort without T2DM, bariatric surgery was found to reduce the incidence for the development of T2DM with 6.8 cases/1000 developing T2DM in the surgery treated group compared to 25.4/1000 in the usual care group (191). Indeed Caballero et al show an improvement in glycaemia in both subjects with pre-diabetes and patients with T2DM undergoing laparoscopic gastric bypass (192). Current guidelines do not recommend bariatric surgery to prevent T2DM.

A long-term observational study from Utah compared mortality outcomes from 7295 obese subjects undergoing Roux-en-Y gastric bypass compared to 7295 matched controls (193). After a mean duration of 7.1 years they reported a 40% reduction in all-cause mortality and a 92% reduction in mortality related to diabetes, 56% for deaths from cardiovascular disease and a 60% reduction in cancer related deaths. The same group in Utah have reported a prospective study of 418 subjects who underwent gastric bypass compared to 2 control groups; one who were patients seeking to have bariatric surgery but did not have this done (n=417) and the other were comprised of randomly selected obese subjects not seeking any weight loss treatment (n= 321) (194). In the gastric bypass group there was a significant reduction in weight, diabetes remission, incidence of diabetes and cardiovascular outcomes over 6 years.

The LABS is a multi-centre observational study over 3 years (175). Of 2458 participants undergoing bariatric procedures; 1738 had RYGB, 610 LAGB and 110 other procedures. The majority of weight loss occurred at one year and after 3 years those with RYGB had lost 31.5% of weight and LAGB had lost 15.9%. Of the 774 participants with T2DM at baseline, 216 RYGB participants (67.5%) and 28

LAGB participants (28.6%) had partial remission at 3 years with the incidence of diabetes being 0.9% after RYGB and 3.2% after LAGB. In the RYGB participants, dyslipidemia resolved in 61.9% and hypertension resolved in 38.2% and in the LAGB participants 27.1% showed a remission for dyslipidemia and 17.4% for hypertension.

The RYGB hypertension remission rates were 38.2% in LABS-2 which was similar to the SOS remission rates of all surgery participants of 34% and 19% at 2 and 10 years of follow-up, respectively. The Utah Obesity Study reported hypertension remission of 53% and 42% at 2 and 6 years' follow-up. LABS-2 reported remission of dyslipidemia in 61.9% for RYGB with hyperlipidemia remission rates at 59.7% and hypertriglyceridemia remission at 85.8% in this group at 3 years. The Utah Obesity study noted similar rates of remission of hyperlipidemia with remission rates of 57% and 53% at 2 and 10 years respectively.

All obese people do not develop Type 2 Diabetes and in fact 10% of T2DM participants are thin (2 18,19). Post-bariatric surgery weight loss takes time and therefore does not explain the remission of T2DM, which occurs immediately after surgery. This suggests an alternative mechanism and an important role for gut hormone involvement has been implicated in the remission of type 2 diabetes. The gut hypothesis suggests that various gut peptides that may play a role including ghrelin, intestinal peptides such as GLP-1, neuropeptide YY or a decreased secretion of anti-incretin hormones (195, 196) which may improve insulin sensitivity and first phase of insulin secretion. There may be gut adaptation and a rise in the levels of gut hormones that promote satiety (197). These changes explain why different bariatric procedures have varying outcomes on remission of T2DM. One study investigated 4 procedures in 81 patients with T2DM – laparoscopic adjustable gastric banding (GB), intervention type Mason, gastric bypass (RYGB) and sleeve gastrectomy (SG) (198). They found that weight loss was similar amongst all types of bypass surgery but remission rates of T2DM differed with RYGB offering better remission rates. This has also been demonstrated in animal models by Rubio et al who found that bypass of the duodenum and upper jejunum in lean diabetic rats could render them euglycaemic with no change in weight (199).

1.8.5 Effects on Microvascular Complications

One of the largest retrospective population based surveys looked at 2580 participants undergoing bariatric surgery and 13371 obese control subjects who had no operative intervention. They report that surgery is associated with a significant decrease in microvascular events (adjusted HR 0.22 95% CI 10.09 to 0.49) and a 65% reduction in major macro and microvascular events (200). However the microvascular outcomes assessed were blindness in at least one eye, laser or retinal surgery, non-traumatic amputation or creation of a fistula for dialysis. These represent the end stage microvascular complications and therefore may well be underestimating the progression or improvement of microvascular disease. A further retrospective review of obese participants with T2DM who underwent bariatric surgery identified that in 67 subjects with complete retinal images pre-operatively and 12-18 months post operatively there was an improvement in 5 (7.5%), deterioration in 1 (1.5%) and no change in 61 (91%). 28 subjects who had preoperative retinopathy showed that 5 (17.8%) had an improvement, 1 (3.6%) deteriorated and there was no change in 22 (78.6%) subjects(201). The subset of patients undergoing RYGB with pre-operative albuminuria (n=32) demonstrated a 3.5 fold decrease in post-operative albumin creatinine ratio (ACR). Banks et al found in a case control study of 45 participants that retinopathy showed significant progression in the control group (p=0.03) but not in the group undergoing roux-en-Y gastric bypass(202). There was a significant trend in favour of surgery in improvement of glycaemic control. A recent systematic review and meta-analysis highlighted 4 primary studies, which were non-randomised case series. Of 148 participants, those with pre-existing diabetic retinopathy (DR) showed no change in 57.4+-18.5%, progression in 23.5+-18.7% and an improvement in 19.2+-12.9% (203). In those without pre-operative DR, 92.5+-7.4% remained disease free and 7.5+-% developed DR. These data are supported by a further smaller study looking at retinopathy (204).

The Swedish Obesity Study found that the cumulative incidence of microvascular complications was 41.8 per 1000 person years in the control group (OR, 6.3; 95% CI 12.2 to 15.9) compared to 20.6 per 1000 person years in the bariatric surgery

group (95% CI 17 to 24.9) (205). The end-points that were used were any micro or macrovascular diabetes complication requiring hospital or specialist outpatient treatment or that were associated with death during follow up identified through the Swedish Cause of Death Register and the Swedish National Patient Register.

The SOS found that albuminuria developed in 246 control subjects and in only 126 of the bariatric group (HR, 0.37; 95% CI, 0.30 to 0.047) (206). Brethauer et al conducted a retrospective review of subjects undergoing bariatric surgery (RYGB n=162, LAGB n=32, VSG n=23) and reported that diabetic nephropathy regressed in 53% of participants and remained stable in the rest (47%) (207). Heneghan et al reported in 52 participants over a longer follow up period of 5 years and showed that 37.6% of participants had nephropathy at baseline which resolved in 58.3% of these subjects (208) and the incidence of microalbuminuria was 25%. A further study of 4 years duration in a small group of 25 patients showed that serum creatinine decreased by 16.2 +- 19.6 mmol and eGFR improved by 10.6 +- 15.5 (209). Hou et al studied changes in eGFR in 61 patients (210) who were divided into 4 groups; hyperfiltration (n=61) eGFR 146.4 +- 17.1 ml/min/1.73m^2, normal eGFR (n=127) 105.7 +- 17.1, chronic kidney disease (CKD) stage 2 n=39 (76.8 +- 16.7) and CKD stage 3 n=6 (49.5+-6.6). There was a reduction in eGFR in the hyperfiltration group and an increase in all other groups consistent with improvement. The data shows that overall there are improvements in nephropathy however again these are in small studies.

There are fewer studies assessing neuropathy. Schauer et al described the presence of diabetic neuropathy in 47 patients preoperatively (25%), and symptomatic improvement was reported by 50% of patients after surgery: 33% much improved, 17% improved, 39% no change, 7% worse, and 4% unknown (211). It is unclear as to how neuropathy was defined in these subjects and the reported improvement was through a questionnaire which assessed improvement of chronic diabetes related complications. We are lacking studies that assess objective markers of neuropathy. Muller-Stich et al reported in a small group of 12 patients who had documented pre-operative peripheral neuropathy that symptomatic neuropathy was reversible in 67% of patients (212). There was an improvement in neuropathy symptom score (NSS) from a median of 8 (range, 0-10) to 0 (range, 0-9) post-operatively (P=0.004) with 8 patients scoring an NSS of

0. Pre-operatively the median neuropathy disability score (NDS) was 6 (range, 2-8), which improved to 4 (range 0-8), post operatively (P=0.027). Conversely, individual case reports have identified the development of rare forms of neuropathy such as acute motor axonal neuropathy post bariatric surgery (213). There is also some focus on nutritional deficiencies that arise post-surgery that may lead to neuropathy including vitamin B12, copper and thiamine deficiency as well as osteomalacia (214-217). Indeed post bariatric surgery neuropathic pain has an incidence of 33% and can greatly affect quality of life. It is important that any nutritional deficiencies, lipid abnormalities and poor glycaemic control are identified early so the patient can be managed effectively (218).

The studies although small suggest good evidence for improvement of microvascular complications post bariatric surgery. The results on albuminuria and nephropathy are particularly encouraging as all studies show a positive effect even at 4-5 years duration. It is harder to interpret the evidence from retinopathy studies as overall it seems that early retinopathy remains stable and can improve, but it may progress in those with advanced retinopathy.

1.8.6 Cost effectiveness of Bariatric Surgery

There have been models to assess the cost benefit of bariatric surgery. A review of cost effectiveness studies identified 6 studies using three different models to predict the cost effectiveness of bariatric surgery. These included statistical models, Markov model and assumption based models which all showed surgery to be a cost effective method for the treatment of obesity (219). Furthermore Henteleff et al found gastric bypass and gastric banding are cost-effective methods of reducing mortality and diabetes-related complications in severely obese adults with diabetes (220). More recently Borisenko et al reports a saving of 8408 Euros with surgery generating an additional 0.8 years of life and 4.1 quality adjusted life years (QALYs) per patient, which equates to 32,390 QALYs and savings of 66 million Euros for their cohort in 1 year (221).

1.8.7 Conclusion

Bariatric surgery is an effective means of achieving weight loss and improving Type 2 Diabetes with an overall low complication rate. The cost of surgery can be offset by the decreased cost of complications and indeed improved quality of life in the future. Although studies in retinopathy and nephropathy are encouraging showing significant improvements in retinal images and urine albumin excretion, studies in neuropathy are more limited and lack clear end points. Therefore for neuropathy we advocate studies that use clear end points particularly focusing on small fibres by deploying the non-invasive ophthalmic technique of corneal confocal microscopy (222).

1.9   References

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2. Chapter II- Research Design And Methods

2.1 Hypothesis and Aims

Metabolic factors are implicated in the pathogenesis of neuropathy and by correcting these factors there may be an improvement in early neuropathy, which can be quantified using corneal confocal microscopy (CCM).

The aims of the research are:

i. To establish if continuous subcutaneous insulin infusion has an effect on neuropathy compared to multiple daily injection.

ii. To establish which measure of neuropathy, including CCM can be used to identify and track small fibre pathology in relation to glucose tolerance status To determine if neuropathy markers can predict which patients are at risk of developing type 2 diabetes mellitus.

iii. To establish neuropathy status in patients with Type 1 diabetes for more than 50 years and identify factors which have protected them from developing overt neuropathy.

iv. To assess if CCM can be used to identify patients with Type 1 Diabetes who have erectile dysfunction.

v. To establish if morbidly obese subjects awaiting bariatric surgery have neuropathy and the factors associated with the development of neuropathy.

vi. To assess if there is a change in neuropathy status post bariatric surgery in relation to changes in metabolic and lipid parameters.

2.2 Study Design

i. Prospective longitudinal observational study

ii. Prospective longitudinal observational study

iii. Prospective longitudinal observational study

iv. Retrospective cross sectional study

v. Prospective cross-sectional study

vi. Prospective longitudinal observational study of clinical intervention.

2.3 Methods

2.3.1 Study Approval

Ethical approval was obtained by the North Manchester and Salford and Trafford Research Ethics Committee, the Scientific Advisory Board of the Manchester Wellcome Trust Clinical Research Facility and the local Research and Development office. The studies adhered to the tenets of the Declaration of Helsinki and with Good Clinical Practice guidance. All participants were supplied with study literature at least 24 hours prior to written informed consent being obtained.

2.3.2 Study Recruitment

Informed written consent was obtained from all subjects prior to their participation. Participants had the opportunity to discuss any concerns about the study and participation with a trained member of the research team. The patient consent and study forms can be found in Appendix 1.

All participants underwent detailed screening of their personal and medical history as well as blood and urine tests to determine their metabolic status and ensure eligibility for this study.

2.3.2.1   Type 1 Diabetes Mellitus

Subjects were recruited from the Manchester Diabetes Centre at Manchester Royal Infirmary.

Patients with Type 1 DM were divided into 2 groups defined by the presence or absence of neuropathy according to the Toronto criteria. An individual was considered to have neuropathy if they met the following criteria:

1. Abnormal nerve conduction, based on a >2SD abnormality compared to age-matched controls;
2. A symptom or sign of neuropathy, defined as one or more of the following:
   - Diabetic neuropathy symptom score of 1 or more out of 4
   - Neuropathy disability score (NDS) of 3 or more out of 10.

2.3.2.2   Impaired Glucose Tolerance

IGT subjects were recruited from referrals attending Manchester Royal Infirmary for an oral glucose tolerance test from general practice. This cohort included male or female patients aged 18-85 who had impaired glucose tolerance (IGT) according to the 1999 WHO criteria; 'fasting venous glucose 6.1-7.0 mmol and 2 hour post glucose load 7.8-11.1 mmol.

2.3.2.2   Bariatric Study

Patients were recruited from the obesity clinic at Salford Royal Hospital. These patients had approval for bariatric surgery.

2.3.2.3   Controls

Control participants were recruited from the staff, students and associates of the University of Manchester and Manchester Royal Infirmary.

2.3.3 Type 1 Diabetes Mellitus and Impaired Glucose Tolerance Studies

2.3.3.1   Inclusion Criteria

Participants must satisfy the following conditions prior to inclusion in the study:

a)   Aged 14 to 85 years

b)   Signed written informed consent

c)   Impaired Glucose Tolerance, Type 1 diabetes, Type 2 diabetes or LADA (or absence of diabetes for the control group)

d)   Be willing to participate and comply with the experimental protocol.

2.3.3.2   Exclusion Criteria

Any of the following criteria rendered the participant ineligible for inclusion:

a)   History of corneal trauma or surgery (cataract surgery does not preclude enrolment unless surgery occurred in the 12 months prior to enrolment date)

b)   History of ocular disease or systemic disease which may affect the cornea

c)   Concurrent ocular disease, infection or inflammation

d)   History of systemic disease (e.g. malignant disease, congestive heart failure New York Heart Association Grade III or IV, major psychosis (i.e. schizophrenia or bipolar), certain autoimmune diseases – hypothyroidism, Addison's disease, vitiligo)

e)   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)

f)   Current or active diabetic foot ulcer or infection

g)   Participating in any other interventional (e.g. drug) research trial.

2.3.4 Bariatric Study

2.3.4.1   Inclusion criteria

•   Age: 20-75 years.

•   Patients scheduled for bariatric surgery.

•   Patients who have capacity and understanding for informed consent process.

2.3.4.2   Exclusion Criteria

Any of the following criteria rendered the participant ineligible for inclusion:

a)   History of corneal trauma or surgery (cataract surgery does not preclude enrolment unless surgery occurred in the 12 months prior to enrolment date)

b)   History of ocular disease or systemic disease, which may affect the cornea

c)   Concurrent ocular disease, infection or inflammation

d)   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, Addison's disease, vitiligo)

e)   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)

f)   Current or active diabetic foot ulcer or infection

g)   Participating in any other interventional (e.g. drug) research trial.

2.3.4.3 Schedule of visits for bariatric studies.

	Visit 1(baseline)	Visit 2 (3m)	Visit 3 (6m)	Visit 4 (12m)
Informed consent	✔			
Medical questionnaire	✔			
Height	✔			
Weight	✔		✔	✔
Waist circumference	✔		✔	✔
Blood pressure	✔		✔	✔
Fasting blood tests*	✔		✔	✔
Bio-impedence measurements	✔		✔	✔
Urinalysis	✔		✔	✔
Sex function questionnaire (male patients only)	✔		✔	✔
Nerve function tests [++]	✔		✔	✔

2.4 STUDY PROCEDURES

The following assessments were undertaken in all study participants. The dominant side was used where possible.

2.4.1 Blood and Urine Measurements

2.4.1.1 TIDM and IGT studies.

Blood and urine testing were undertaken by a research nurse. These included glycated haemoglobin (HbA1c), total cholesterol (mmol/l), high density lipoprotein cholesterol (HDL) (mmol/l), low density lipoprotein cholesterol (LDL) (mmol/l), triglycerides (mmol/l), 25 (OH)-Vitamin D (ng/ml), Vitamin B12, Thyroid Function [free T4 (mu/l) and thyroid stimulating hormone (mmol/l)], renal assessment [estimated glomerular filtration rate (eGFR) (ml/min/l), creatinine (mmol/l) and albumin creatinine ratio (ACR) (mg/mmol)], liver function tests [albumin (g/l), bilirubin (umol/l), total protein (g/l), ALT (U/l) and ALP (U/I)].

2.4.1.2 Bariatric study

The blood collected for the bariatric study was as follows:

- 3x 8.5ml gold top tube
- 2x10ml   purple top tube
- 1x2.5ml grey top tube   → To CTF LIPID lab
- 1X5ml   blue top tube
- 1X   urine container

A sample was collected for HbA1c.

2.4.1.2.1   Serum and plasma separation
The tubes were spun at 3300 rpm for 15 min at 4°C. The samples collected were: EDTA-plasma (purple top tubes) and serum (gold top tubes) in universals and flouride plasma (grey top tubes) and Sodium citrate plasma (blue top tubes) in bijou.

❖ **Serum**

- Serum was aliquoted into 6 eppendorfs of 0.25ml each.
- 5 tubes 2ml each
- These were stored at -20°C.

❖ **EDTA-Plasma**

- Plasma was aliquoted into 10 eppendorfs 0.25ml each.
- 2-4 tubes 2ml each
- The buffy coats were collected using sterile Pasteur pipette into 2 sterile tubes.
- These were all stored at -20°C.

❖ **Floride-Plasma**

- Floride-Plasma for glucose measurement was aliquoted into 2 eppendorfs 0.25ml each.
- These were stored at -20°C.

❖ **Sodium citrate -Plasma**

- Sodium citrate -Plasma was aliquoted into 2 eppendorfs 0.25ml each.
- These were stored at -20°C.

❖ **Urine sample**

- Urine was aliquoted into 2 eppendorfs 0.5ml each.
- These were stored at -20°C.

2.4.1.2.2   Glycated Haemoglobin (HbA1c)

HbA1c was measured by HPLC using a VARIANT II Turbo Hemoglobin Testing System (Bio-Rad Laboratories, Hemel Hempstead, UK) in the Department of Clinical Biochemistry at Central Manchester University Hospitals.

2.4.1.2.3   Total Cholesterol

3µl of sample was added to 20µl H_2O and 250µl reagent After enzymatic hydrolysis by cholesterol esterase, cholesterol is oxidized by cholesterol oxidase.

The released hydrogen peroxide reacts with 4-aminoantipyrine and phenol in the presence of peroxidase to form quinoneimine. The increase in absorption at 500 nm correlates with cholesterol concentration which was measured using Cobas Mira auto-analyzer (Horiba ABX- UK, Northampton, UK)

2.4.1.2.4 Triglyceride

3 µl of sample was added to 10 µl H2O and 290 µl reagent. Oxidation by glycerol-3-phosphate oxidase releases hydrogen peroxide, which generates quinoneimine from 4-aminoantipyrine and phenol in the presence of peroxidase. The increase in absorbance at 500 nm correlates with the triglyceride concentration which is measured using Cobas Mira auto-analyzer (Horiba ABX- UK, Northampton, UK)

2.4.1.2.5 High-density lipoprotein (HDL) cholesterol

3 µl of sample was added to 50 µl H2O, 250 µl of reagent 1 (*N,N*-Bis(2-hydroxyethyl)-2- aminoethanesulfonphonic acid, *N*-(2-hydroxy-3-Sulfopropyl)-3,5-dimethoxyaniline, sodium salt, cholesterol esterase, cholesterol oxidase, catalase and ascorbate oxidase), 83 µl of reagent 2 (*N,N*-Bis(2-hydroxyethyly)-2-aminoethanesulphonic acid, 4- aminoantipyrine, horse radish peroxidase, sodium azide and surfactants) and 12 µl H2O. When oxygen is present, cholesterol is oxidized by cholesterol oxidase and generated hydrogen peroxide reacts with 4-aminoantipyrine and N-(2-hydroxy-3-sulphopropyl)-3,5-dimethoxyaniline. The increase in absorbance at 600 nm correlates with the HDLholesterol concentration, which was measured using Cobas Mira auto-analyzer (Horiba ABX-UK, Northampton, UK).

2.4.1.2.6 Low-density lipoprotein (LDL) cholesterol Principle

LDL levels were calculated using the Friedewald formula:

LDL= total cholesterol – HDL-– Triglycerides/2.19

This formula is only accurate when serum triglycerides do not exceed 4.5 mmol/l.

2.4.1.2.7   Non-HDL cholesterol

This was calculated using the formula:

Non-HDL = total cholesterol – HDL

2.4.1.2.8   Apolipoprotein B (ApoB)

13 µl of sample was added to 30 µl of H2O, 200 µl of PBS polymer solution, 16.7 µl of anti- human apoB antibody and 53.3 µl of PBS. ApoB was measured immunoturbidimetrically. The immune complex formed was measured by turbidimetry where the signal generated correlates directly with the concentration of ApoB in the sample. The signal generated was measured at 340 nm using Cobas Mira auto-analyzer (Horiba ABX-UK, Northampton, UK)

2.4.1.2.9   Apolipoprotein A-I (ApoAI)

7µl of sample was added to 60µl H2O, 200µl of PBS Polymer solution, 23.3µl of purified immunoglobulins from rabbit antiserum (apoAI from human HDL immunogen) and 46.7 µl PBS.   ApoAI was measured using an immunoturbidimetric assay adapted for the Cobas-Mira auto-analyzer. The immune complex formed is measured by turbidimetry with the signal generated at 340 nm after 10 and 15 minutes using Cobas Mira auto- analyzer (Horiba ABX-UK, Northampton, UK) correlating directly with the concentration of apoAI in the sample.

2.4.1.2.10   Oxidized LDL (OxLDL)

µl of each calibrator, control and diluted sample was put into coated plate wells and 100 µl of assay buffer added to each well   This was incubated on plate shaker for 2h at room temperature. The reaction volume was discarded and 350 µl of wash buffer solution was added to each well. The solution was discarded and excess liquid removed using absorbent paper. 100 µl enzyme conjugate solution was added to each well. This was incubated on a plate shaker for 1h at room temperature..   200 µl 3.3',5,5'-tetramethylbenzidine was added and incubated for

15 minutes at room temperature. 50 µl Stop solution was added and the plate was put on shaker for 5 seconds. The optical density at 450 nm is read and the results calculated. The concentration of oxidized LDL was obtained by data reduction of the absorbance for the calibrators versus the concentration using cubic spline regression. The concentration of the samples was multiplied with the dilution factor

2.4.1.2.11 C-reactive protein (CRP)

CRP was measured by immunoturbidimetric assay. 2.5 µl of the sample was added to reaction buffer with CRP immunoparticles. The generated signal was measured at 340 nm after 10 and 15 minutes using Cobas Mira auto- analyzer (Horiba ABX-UK, Northampton, UK)

2.4.1.2.12 Cystatin C

The sample was added to the Cystatin Assay Buffer and Cystatin Antibody Reagent. The generated signal was measured at 570 nm after 10 and 15 minutes using Randox Daytona auto-analyser (Randox, Co. Antrim, UK)

2.4.1.2.13 Intercellular Adhesion Molecule 1 (ICAM-1)

This was measured using ELISA (R&D Systems Europe, Abingdon, UK) which measures ICAM-1, also known as CD54, a transmembrane protein that is upregulated on endothelial and epithelial cells at sites of inflammation.

2.4.1.2.14 Vascular Cell Adhesion Molecule 1 (VCAM-1)

VCAM-1 was measure using a kit (R&D Systems Europe, Abingdon, UK) which measures VCAM-1 (or CD106), a transmembrane molecule that mediates the adhesion of immune cells to the vascular endothelium during inflammation.

2.4.1.2.15 Interleukin 6 (IL-6)

Interleukin-6 measured by solid phase sandwich ELISA (R&D Systems Europe, Abingdon, UK).

2.4.1.2.16 Paraoxonase-1 (PON1) Activity

Serum PON-1 activity was determined by a semi-automated micro-titre plate method using paraoxon (*O,O*-Diethyl *O*-(4-nitrophenyl)phosphate as a substrate. The rate of generation of *p*- nitrophenol was determined at 25oC with the use of a continuously recording spectrophotometer at 405 nm using multiskan multisoft plate reader (Labsystems, Hampshire, UK). Activity was calculated as: PON1 activity (nmol / ml / min) = OD / min x 1390.7 x 1.714

2.4.1.2.17 Proprotein convertase subtilisin / kexin type 9 (PCSK9)

PCSK9 was measured using ELISA (R&D Systems Europe, Abingdon, UK) which was based on the antibody sandwich principle.

2.4.1.2.18 Serum Amyloid A (SAA)

SAA was measured using the human SAA solid-phase sandwich ELISA (ThermoFisher Scientific, Loughborough, UK).

2.4.1.2.19 3-Nitrotyrosine (3-NT)

3-NT was measured using quantitative sandwich ELISA (MyBioSource Inc. San Diego, CA, USA).

2.4.2 Body mass index (BMI)

This was measured as per the standard equation (mass/(height (kg/m^2)).

Weight was measured with a digital scale (Seca 701, Seca, Hamburg, Germany) to the nearest 0.1 kg and height to the nearest 0.1cm. This was measured with the participant's shoes removed and only wearing a light layer or clothing.

Height was measured with the participants shoes removed.

2.4.3 Blood Pressure

Blood pressure (BP) measurements were obtained with the use of an automated BP device (Dinamap pro 100v2, GE Medical Systems, Freiburg, Germany) with an appropriate cuff size. A minimum of two measurements of systolic and diastolic BP were made five minutes apart with the lowest reading recorded.

2.4.4 Bioimpedance – Bariatric Study Only

The patient had to take their shoes off and stand on the machine while holding the paddles in each hand. The information from this was then recorded and included fat mass, total body water, impedance, and basal metabolic rate.

2.4.5 Clinical assessment of peripheral neuropathy

2.4.5.1 Neuropathy Symptom Profile (NSP)

The NSP questionnaire consisted of 38 questions divided into motor, sensory and autonomic symptoms. A score was given out of 38 with 0 being no neuropathy and 38 the most severe neuropathy. If a symptom was deemed as present then the examiner gave a score of 1 for the symptom. Absence of a symptom was scored as 0.

2.4.5.2 Neuropathy Disability Score

NDS (figure 2.1) is a clinical scoring system obtained from a neurological examination, which includes:

- ❖ Vibration sensation
    - A 128Hz tuning fork was placed on the end of the big toe and the patient was asked if they felt the vibration on each foot 3 times. A score of 2/3 was considered normal.
- ❖ Pin-prick sensation
    - Pain sensation was evaluated using a Neurotip^{TM}. This device has a sharp and blunt end. Patients were required to distinguish between

sharp and blunt when the Neurotip^TM is placed on the pulp of their big toe on each foot. This is again done three times and a score of 2/3 is normal.

- ❖ Temperature perception
    - ▪ To evaluate this two metal rods are used. One rod is placed into cold water and the other hot water for 30 seconds before the procedure begins. The rods were then placed on the dorsum of each foot and the patient decides if the sensation was hot or cold. This was done three times and a score of 2/3 is normal.
- ❖ Ankle Reflexes
    - ▪ The Achilles tendon reflex was evaluated using a tendon hammer. A reflex was scored as 0 if normal, 1 if elicited on reinforcement and 2 if absent. This was done on both legs.

The score was added to give an NDS out of 10. (NDS 0-2: no neuropathy, 3-5: mild neuropathy, 6-8: moderate neuropathy and 9-10: severe neuropathy).

Figure 2-1. A. Measurement of temperature sensation using cold and warm rods B. Achilles tendon reflex C. Pain sensation using Neurotip D. Measurement of vibration perception using a tuning fork.

(Image from the Early Neuropathy Assessment website
http://research.bmh.manchester.ac.uk/ena/techniques/).

2.4.5.3　　10 g monofilament testing

A 10g monofilament was used to assess the patient's perception to soft touch. This was done at 10 sites on each foot. The filament is pressed against the skin at right angles for approximately one second with a force that makes the filament bend as shown in the figure below. At each site if the patient could feel the sensation then a score of 1 was given with a maximum score of 10, which implies no neuropathy.

2.4.5.4　　Vibration Perception Threshold (VPT)

Vibration perception threshold was measured using a biothesiometer (Horwell, Scientific Laboratory Supplies, Wilford, Nottingham, UK). The probe was placed on the pulp of the big toe.　The intensity of the stimulus was increased slowly from 0-50 Volts and the patient was then asked when they were first able to feel the vibration sensation.　This was repeated three times and recorded as an average

2.4.6　Quantitative Sensory Testing

a) Warm and cold thresholds were measured using the MEODOC TSA-11 Neurosensory analyser (Medoc Ltd. Ramat Yishai, Israel). The thermode was attached to the dorsum of the patients left foot.　To avoid tactile or pressure stimulation the probe was kept in contact with the skin for the entire duration of the test. The starting temperature (adaptation temperature) was 32°C. The thermode contacts the skin and a subject was asked to report a sensation of temperature change or heat pain. Cold sensation threshold (CT) was tested initially by a gradual decrease in the thermode temperature and the subject was asked to press the computer mouse button when they first become aware of a cold sensation. This was repeated a further three times and the same procedure was carried out to test warm sensation threshold (WT). The mean value was recorded. (Figure 2.2).

b) The participant was then asked to determine when the cold sensation becomes uncomfortable, painful or intolerable and this was measured four times (cold induced pain). This procedure was also repeated to determine heat induced pain.
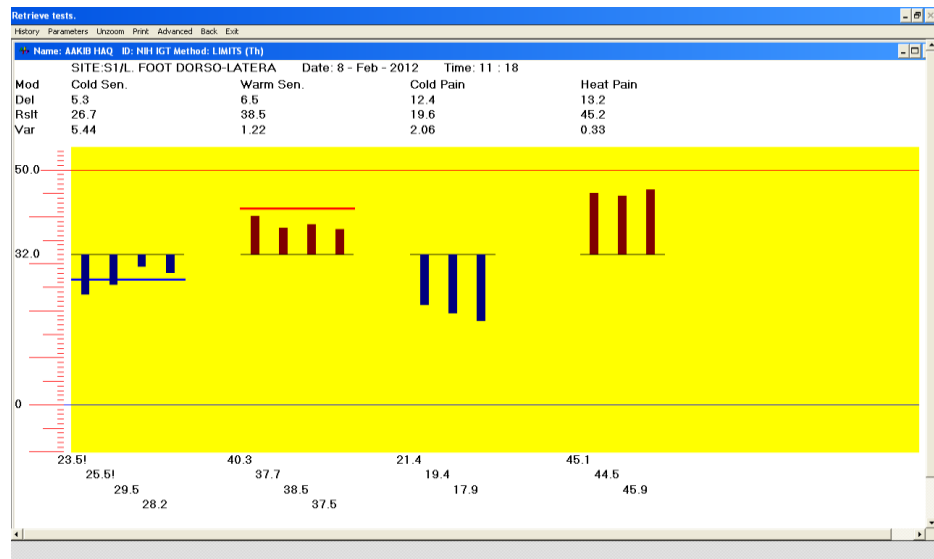
Figure 2-2. The onscreen display showing results from QST.

2.4.7 Autonomic Function Testing

Heart rate variability (HRV) was assessed with an ANX 3.0 autonomic nervous system monitoring device (ANSAR Medical Technologies Inc., Philadelphia, PA, USA).

2.4.8 Electrophysiology

Electro-diagnostic studies were undertaken using a Dantec "Keypoint" system (Dantec Dynamics Ltd, Bristol, UK) equipped with a DISA temperature regulator to keep limb temperature constantly between 32-35°C (Figure 2.3). This is shown in figure 2.3. Sural sensory nerve amplitude (SNA), sural sensory nerve conduction velocity (SNCV), peroneal motor nerve amplitude (PMNA) and peroneal motor nerve conduction velocity (PMNCV) 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 was taken using antidromic stimulation over a distance of 140mm. Radial sensory recordings are taken from the anatomical snuffbox using antidromic stimulation over a 100mm distance.

The strength of the stimulation was increased until a maximal response was obtained. The stimulus strength was increased 10-15% above the maximal stimulation to ensure a supramaximal response. The motor response was not averaged and the sensory responses were averaged using 3 but not more than 10 stimuli.

Motor amplitude was measured from the baseline to the negative peak and reported to the nearest 0.1mV. The sensory amplitude was measured from the baseline to the negative peak. If there was a positive preceding the negative, the amplitude is measured from the base of the positive peak to the negative peak. The sensory nerve action potential is reported to the nearest 0.1 microvolt.

Motor nerve latency was measured at the take-off of the negative component of the M wave. Sensory latency was measured from the take-off of the negative

component of the sensory nerve action potential. If there was a positive preceding the negative component, then the latency was measured at the peak of the positive component of the sensory nerve action potential. Latency was recorded to the nearest 0.1 ms.

All conduction velocities were measured using onset latencies and reported to the nearest 0.1 m/s. The distance used for measurement was the distance between the two sites of stimulation.

Figure 2-3. Nerve Conduction Studies.

2.4.9 Skin Punch Biopsy

Two 3mm punch biopsies were taken from the dorsum of the foot at the base of metatarsal head using aseptic technique. This is shown in figure 2.4. The area to be biopsied was infiltrated with 1% lidocaine. The specimen was fixed in 4% paraformaldehyde (Sigma-Aldrich) buffered in Tris Buffered Saline (TBS) for 24 hours at 4 degrees Celsius and kept overnight in a cryoprotective solution. 50 µm sections were cut on a cryostat and the sections rinsed in TBS. The anti-human pan-neuronal marker protein gene product 9.5 (PGP 9.5) antibody (Abcam, Cambridge, U.K) followed by goat anti rabbit (Vector UK). The next step was application of Avidin D (Vector, UK) conjugated to horseradish peroxidase (Vector, UK) followed by chromogen SG (Vector UK) and then washed in water to stop the SG reaction.

The nerve fibres that cross the dermal-epidermal junction are then counted. IENFD was obtained by dividing the number or nerves counted by the length of skin section examined. This is reported as no/mm.

Figure 2-4. Skin punch biopsy and immunohistochemistry testing.

2.4.10   Corneal Confocal Microscopy

The images of the corneal sub-basal nerve plexus were captured using the Heidelberg Retina Tomograph 3 with Rostock Cornea Module (Heidelberg Eye Explorer, Heidelberg Engineering GmBH, Heidelberg, Germany) (Figure 2.5). This is a laser-scanning confocal microscope which operates by scanning a laser beam spot of less than 1 µm in diameter sequentially over each point of the examined area. In order to scan the image, the laser beam spot must be deflected in two perpendicular directions. This is achieved using two scanning mirrors: a resonant scanner deflects the beam horizontally to produce a scan line and a galvanometric scanner deflects this scan line vertically, to produce a scan field. Descanning of reflected light is performed by the same two scanning mirrors. The reflected light is deflected to a detector, which is an avalanche photo diode (a point-like detector). The signal of the photo diode is digitized to form the image.

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

Figure 2-5. Corneal Confocal Microscopy

2.4.10.1   Examination Procedure

A large drop of Viscotears (Carbomer 980, 0.2 %; Novartis, UK) was placed onto the tip of the lens, avoiding air bubbles in the drop. A sterilized Tomocap (Heidelberg Eye Explorer, Heidelberg Engineering GmBH, Heidelberg, Germany) was then placed over the objective lens. The camera was positioned so that the optical axis of the camera runs perpendicular to the optical axis of the laser scanning camera. The patient details were entered into the software window. This included the study ID, full name, date of birth and gender.

The objective lens was focused onto the front surface of the Tomocap TM (bright white field) by rotating the adjustment wheel and the depth is then reset to zero.

The patient is seated comfortably and one drop of local anesthetic (benoxinate hydrochloride 0.4%, Chauvin Pharmaceuticals Ltd., Essex, UK) is placed into each eye to reduce the blinking reflex. Then a viscous tear-drop (Carbomer 980, 0.2 %; Novartis, UK was) was also placed in both eyes to lubricate the ocular surface.

The participant places their head in the head and chin rest, and the overall height of the instrument table was adjusted for comfort. The participant was asked to look straight ahead and gaze at the white fixation light with the eye that was not being examined. The camera was then moved forward until the cornea was about 15mm from the Tomocap. This was then aligned with the central cornea using the red reflex while the participant looked directly at the fixation light. The laser beam should fall in the centre of the pupil. The camera was slowly advanced until minimal contact with the cornea was achieved. The microscope was then focused forward through the whole cornea and images from all layers of central cornea were captured from both eyes.

2.4.10.2   Manual and Automated Image Analysis

For the purpose of image analysis, 6 images (3 per eye) from the sub basal nerve plexus considering the depth, quality and location were chosen. A purpose

designed software called CCMetrics (M.A. Dabbah, Imaging Science and Biomedical Engineering, The University of Manchester) was used to analyse images manually and another purpose designed software called ACCmetric (M.A. Dabbah, Imaging Science, The University of Manchester, 2010) was used to analyse images automatically. Nerve morphological parameters measured included corneal nerve fibre density (CNFD) (no. /mm^2), corneal nerve branch density (CNBD) (no./mm^2) and corneal nerve fibre length (CNFL) (mm/mm^2). (Figure 2.6)
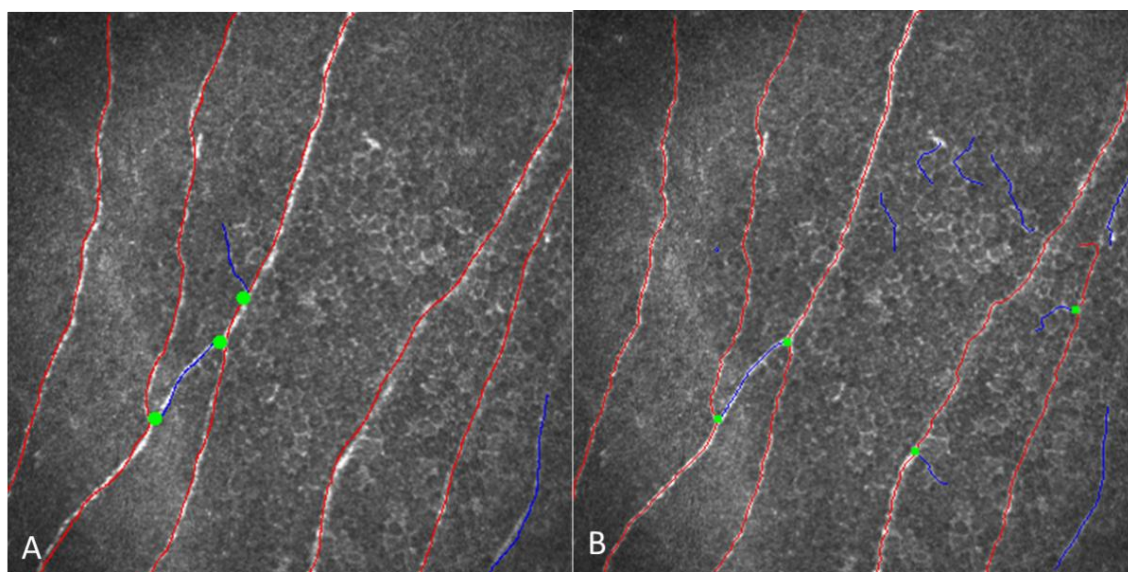
Figure 2-6. **A. CCM image analysed manually by semi-automated software (CCMIAv0p6) (M.A. Dabbah, Imaging Science, The University of Manchester). The red lines indicate main nerves, blue lines are branches and green spots show the junction between branches and main nerves. B. The same image analysed using fully automated software ACCMetrics32 (M.A. Dabbah, Imaging Science, The University of Manchester).**

3 Chapter III - Corneal Confocal Microscopy Shows An Improvement In Small Fibre Neuropathy In Subjects With Type 1 Diabetes On Continuous Subcutaneous Insulin Infusion Compared To Multi Day Injection.

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3.1 Abstract

Optimal glycaemic control has been shown at best to halt progression of diabetic peripheral neuropathy (DPN). Continuous subcutaneous insulin infusion (CSII) provides a means to achieve optimal glycaemic control. We studied the benefits of CSII on DPN.

49 subjects with Type 1 Diabetes Mellitus (18 on CSII and 31 on MDI) and 40 age-matched controls underwent assessment of vibration perception threshold (VPT), cold threshold (CT), warm threshold (WT), neurophysiology, intra-epidermal nerve fibre density (IENFD), corneal nerve fibre density (CNFD), branch density (CNBD) and fibre length (CNFL) at baseline and after 24 months.

At baseline, subjects on CSII and MDI were matched for duration of diabetes, HbA1c, blood pressure, total cholesterol, HDL, triglycerides and all measures of neuropathy. At 24 months, there was no significant change in HbA1c, BP or lipids in the CSII or MDI groups. Whilst there was no change in VPT, CT, WT, neurophysiology or IENFD over 24 months in any of the cohorts, there was a significant increase in CNFD ($P=0.05$), CNBD ($P=0.006$) and CNFL ($P=0.003$) and a significant decrease in HRV ($P=0.03$). There was no change in CNFD ($P=0.188$), CNBD ($P=0.215$) or CNFL ($P=0.687$) in the MDI group or controls (CNFD ($P=0.378$), CNBD ($P=0.877$), CNFL ($P=0.849$)).

Over 24 months, whilst T1DM patients on MDI and control subjects show no progression of neuropathy, patients treated with CSII show an improvement in small fibre morphology, which was detected using the novel non-invasive ophthalmic technique of corneal confocal microscopy.

3.2 Introduction

Epidemiological studies show that diabetic peripheral neuropathy (DPN) has a prevalence of 30% and has a significant clinical and economic impact [1]. Patients with DPN are two to three times more likely to fall and more than 80% of amputations occur following a foot ulcer or injury, for which DPN is a major risk factor [2]. DPN also results in pain which is one of the most disabling symptoms affecting ~20 % of patients [3, 4].

There are currently no FDA approved therapies to prevent, slow or arrest DPN and management therefore primarily involves achieving good glycaemic control to halt progression [5]. Other known modifiable and non-modifiable risk factors include hypertension, smoking and diabetes duration [6]. It has recently been shown that short term metabolic improvements in glycaemic control and serum triglyceride levels have an independent, additive and durable effect on restoration of nerve conduction [7].

The Diabetes Control and Complications Trial (DCCT) and Epidemiology of Diabetes Interventions and Complications (EDIC) studies have shown that improved glycaemic control halts the progression of neuropathy in Type 1 adults treated predominantly with a multiple daily injection regime (MDI) or Continuous Subcutaneous Insulin Injection (CSII). It is known that CSII compared to MDI results in an improvement in haemoglobin A1c (HbA1c), lifestyle flexibility and reduction in hypoglycaemic events [8, 9], but no benefit on hospital admissions for acute complications [10]. In a recent study of 32 adolescents, 6 months of CSII was associated with an improvement in carotid artery intima-media thickness and flow-mediated dilatation of the brachial artery, without an improvement in HbA1c [11]. In a study of 1604 adolescents followed over 8.6 years, an improvement in overall glycaemic control using either CSII or MDI showed a reduction in the incidence of retinopathy, microalbuminuria but no effect on neuropathy [12]. However, those treated with CSII compared to MDI showed a significant improvement in vibration and thermal thresholds [12]. In 26 patients with gastroparesis, CSII therapy resulted in a significant improvement in glycaemic

control, reduction in glycaemic variability and the number of hospital inpatient bed days, suggestive of an impact on autonomic neuropathy [13].

We have pioneered [14] the technique of corneal confocal microscopy (CCM) and shown that it can reproducibly [15] diagnose [16] and stratify [17] diabetic neuropathy. Furthermore we recently showed that it alone as opposed to QST, neurophysiology and IENFD is capable of detecting a significant improvement in DPN, after simultaneous pancreas and kidney transplantation (SPK) [18]. In the current study we have undertaken a comprehensive assessment of neuropathy employing all current end points and CCM in patients with T1DM treated with CSII or MDI over 24 months.

3.3 Research Design and Methods

3.3.1 Selection of patients

We assessed 49 subjects with Type 1 Diabetes Mellitus (T1DM) and 40 age matched controls. Of the subjects with T1DM, 18 were being treated with CSII and 31 with conventional MDI. Exclusion criteria were any history of neuropathy due to a non-diabetic cause, presence of severe diabetic neuropathy as indicated by a Neuropathy Disability Score (NDS) >8, current or active diabetic foot ulceration, and any history of corneal trauma or surgery, or history of ocular disease or systemic disease that may affect the cornea. This was an observational study, which was approved by the Central Manchester Research and Ethics Committee and written informed consent was obtained from all subjects prior to participation. This research adhered to the tenets of the declaration of Helsinki.

3.3.2 Assessment of Neuropathy

All tests were undertaken at baseline, 12 months and 24 months. All study participants underwent assessment of body mass index (BMI), blood pressure, HbA1c, lipid profile [total cholesterol, low density lipoprotein (LDL), high density lipoprotein (HDL) and triglycerides], albumin creatinine excretion ratio (ACR) and estimated glomerular filtration rate (eGFR). Symptoms of DPN were assessed using the Neuropathy Symptom Profile (NSP). Neurological deficits were

evaluated using the simplified NDS which is comprised of vibration perception, pin-prick, temperature sensation and presence or absence of ankle reflexes [19].

Vibration perception threshold (VPT) was tested using a Neurothesiometer (Horwell, Scientific Laboratory Supplies, Wilfrod, Nottingham, UK). Cold (CT) and warm (WT) thermal thresholds were established on the dorsolateral aspect of the left foot (S1) using the TSA-II NeuroSensory Analyser (Medoc Ltd., Ramat-Yishai, Israel).

Electro-diagnostic studies were undertaken using a Dantec "Keypoint" system (Dantec Dynamics Ltd, Bristol, UK) equipped with a DISA temperature regulator to keep limb temperature constantly between 32-35°C. Sural sensory nerve amplitude (SNAP), sural sensory nerve conduction velocity (SNCV), peroneal motor nerve amplitude (PMNA) and peroneal motor nerve conduction velocity (PMNCV) 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 was taken using antidromic stimulation over a distance of 100mm.

Heart rate variability (HRV) was assessed with an ANX 3.0 autonomic nervous system monitoring device (ANSAR Medical Technologies Inc., Philadelphia, PA, USA).

3.3.3 Skin Biopsy

A 3mm punch skin biopsy was taken from the dorsum of the foot, approximately 2 cm above the second metatarsal head under local anaesthesia (1% lidocaine). 50 µm sections were stained using anti-human PGP 9.5 antibody (Abcam, Cambridge, U.K) and nerve fibres were demonstrated using SG chromogen (Vector Laboratories, Peterborough, U.K). Intraepidermal nerve fibre density (IENFD) was quantified in accordance with established criteria and expressed as no/mm [20, 21].

3.3.4 Corneal Confocal Microscopy

Patients underwent examination with the CCM (Heidelberg Retinal Tomograph III Rostock Cornea Module, Heidelberg Engineering GmbH, Heidelberg, Germany) as per our previously established protocol [22]. All scans were performed by two purpose-trained optometrists. Five non-overlapping images/patient from the centre of the cornea were selected and quantified in a masked fashion [23]. Three corneal nerve parameters were quantified: Corneal nerve fibre density (CNFD) - the total number of major nerves/mm^2 of corneal tissue, Corneal nerve branch density (CNBD) - the number of branches emanating from all major nerve trunks/mm^2 of corneal tissue and Corneal nerve fibre length (CNFL) - the total length of all nerve fibres and branches (mm/mm^2) within the area of corneal tissue. Corneal nerve parameters were quantified using purpose designed automated software called Accmetrics [24].

3.4 Statistical Analysis

Analysis was carried out on SPSS for Mac (Version 19.0, IBM Corporation, New York, USA). All data are expressed as mean ± standard error of mean (SEM). The data was tested for normality by using the Shapiro Wilk Normality test and by visualising the histogram and normal Q-Q plot. To assess within and between group differences we used one was analysis of variance (non-parametric – Kruskal – Wallis). A significant p value was considered to be <0.05 (post hoc – Tukey).

3.5   Results

3.5.1   Baseline Demographic Factors

The participant's demographics are summarised in table 3.1. Subjects with Type 1 DM on MDI and CSII were age-matched with controls. The control group had a significantly lower HbA1c (P<0.0001) and higher cholesterol (P<0.0001) and LDL (P<0.001) than the diabetes group. There was no difference in blood pressure, eGFR, HDL, and triglycerides.

At baseline subjects on CSII and MDI did not differ for duration of diabetes (P=0.91), HbA1c (P=0.63), Blood Pressure (P= 0.79), total cholesterol (P=0.06), HDL (P=0.83), and triglycerides (P=0.49) and only LDL was significantly lower in MDI compared to CSII treated subjects (P=0.02)

	CONTROL BASELINE	CONTROL 24 MONTHS	MDI BASELINE	MDI 24 MONTHS	CSII BASELINE	CSII 24 MONTHS
Age (years)	53.2 ± 2.2		55.4 ± 2.9		49.9±3.3	
BMI (Kg/m^2)	26.4 ± 0.7	26.2 ± 1.1	27.4 ± 0.9	27.3 ± 0.7	26.0 ± 0.9	27.3 ± 1.4
Duration of Diabetes (years)	n/a		34.8 ± 3.1		35.2 ±3.6	
Blood Pressure (mmHg)	129.7 ± 2.6 / 72.7±1.6	116.9 ± 5.8/ 65.5 ± 3.1	136.5± 5.59 / 74.7 ± 2.3	127.9 ± 4.0 / 66.3±1.7	138.5 ± 4.1 / 71.7 ± 1.2	135.8 ± 6.3 / 68.6 ± 2.9
HbA1c (%) (mmol/mol)	5.5 ± 0.1 (37.1 ± 0.5)	5.2±0.4 (33.7 ± 0.7)	8.3 ± 0.3 (66.7 ± 2.8)#	8.2 ± 0.8 (66.2 ± 3.9)	8.1 ± 0.2 (64.2 ± 2.7)#	8.0 ± 0.2 (63.9 ± 2.3)
eGFR (ml/min/l)	84.3 ± 1.1	83.2 ± 1.8	79.8 ± 3.2	72.2 ± 3.9	84.8 ± 3.3	77.2 ± 3.9
Cholesterol (mmol/l)	5.1 ± 0.2	4.6 ± 0.2	4.1 ± 0.1#	4.2 ± 0.2	4.6 ± 0.3#	4.3 ± 0.3
HDL (mmol/l)	1.6 ± 0.1	1.6 ± 0.1	1.7 ± 0.1	1.8 ± 0.1	1.7 ± 1.3	1.4 ± 0.1
LDL (mmol/l)	2.7 ± 0.1	2.4 ± 0.2	1.8 ± 0.1#	1.9 ± 0.1	2.5 ± 0.2#	2.2 ± 0.3
Trig (mmol/l)	1.5 ± 0.1	1.3 ± 0.2	1.2 ± 0.2	1.0 ± 0.1	0.9 ± 0.2	1.1 ± 0.1

Table 3-1. Clinical and metabolic parameters in control and TIDM subjects at baseline and 24-month follow-up. Data are expressed as mean ± SEM, All symbols represent statistically significant differences using one-way ANOVA: #P < 0.0001; Baseline vs. Control.

3.5.2 Baseline Neuropathy Assessment

Subjects with Type 1 Diabetes on CSII and MDI had evidence of DPN, with all markers of neuropathy (NSP, NDS, VPT and Nerve Conduction Studies) being significantly impaired compared to the control population (Table 3.2). There was a significant impairment in CT and WT in the CSII and MDI treated groups, however there was no significant difference in cold and warm induced pain compared to control subjects. There was no difference in heart rate variability between any group. There was evidence of small fibre neuropathy with a significantly reduced IENFD in both CSII (P=0.04) and MDI (P=0.01) groups compared to controls (Table 3.3). There was a significant reduction in CNFD (P<0.0001), CNBD (P<0.0001) and CNFL (P<0.0001) in both CSII and MDI treated groups compared to control subjects. At baseline there was no significant difference between CSII and MDI groups for: VPT (P=0.51), CT (P=0.77), WT (P=0.35), CIP (P=0.54), WIP (P=0.22), peroneal nerve conduction velocity (P=0.66), IENFD (P=0.881), CNFD (P=0.34), CNBD (P=0.5) and CNFL (P=0.4).

3.5.3 Follow up Neuropathy Assessment

At 24 months, there was no significant change in HbA1c, BP or lipids in the CSII or MDI groups (Table 3.2). The CSII group showed a significant increase in CNFD (16.8 ±2.0 v 19.4±2.6, P=0.05), CNBD (17.6 ± 2.4 v 25.4±3.7, P=0.006) and CNFL (10.1±1.0 v 12.2±1.1, P=0.003) from baseline to 24 months (Table 3.3, Figure. 3.1). The MDI cohort showed no change in CNFD (20.1±1.6 v 18.6±1.8, P=0.188), CNBD (23.5±2.7 v 20.9±2.9, P=0.215) and CNFL (12.2±0.8 v 11.9±0.9, P=0.687) (Fig. 1). There was no change in controls in CNFD (29.8±1.2 v 29.2±1.9, P=0.378) CNBD (39.6±2.5 v 40.2±4.4, P=0.877) and CNFL (17.4±0.6 v 17.3±0.9, P=0.849) (Table 3.3, Figure 3.1). There was a significant reduction in DB-HRV in the CSII group (28.9 ± 4.5 v 22.5 ± 2.7, P=0.03). There was no change in NSP, NDS, QST, neurophysiology or IENFD during this time (Table 3.2).

	CONTROL BASELINE	CONTROL 24 MONTHS	MDI BASELINE	MDI 24 MONTHS	CSII BASELINE	CSII 24 MONTHS
NSP	0.1 ± 0.1	0.1 ± 0.1	4.8 ± 1.4*	5.0 ± 1.4	2.4 ± 1.2*	2.9 ± 1.2
NDS	0.6 ± 0.3	0.3 ± 0.2	3.7 ± 1.7#	3.7 ± 0.7	3.9 ± 0.9#	3.2 ± 0.9
VPT (V)	6.2 ± 1.3	6.3 ± 1.3	15.9 ± 2.5*	16.9 ± 2.2	14.6 ± 2.9*	15.2 ± 3.1
PMNCV (m/s)	48.7 ± 1.2	49.1 ± 1.2	40.5 ± 1.1*	40.7 ±1.1	38.8 ± 2.2*	38.4 ± 2.1
PMNA (mV)	6.3 ± 0.5	6.1 ± 0.5	3.1 ±0.5*	3.2 ± 0.2	2.7 ± 0.6*	2.5 ± 0.5
SNCV (m/s)	50.5 ± 0.8	50.1 ± 0.8	40.7 ± 1.4*	38.9 ± 1.3	43.3 ± 2.0*	40.4 ± 2.4
SNCA (μV)	18.3 ± 1.3	22.8 ± 1.9	7.4 ± 1.1*	7.2 ± 1.1	10.0 ± 2.3*	8.3 ± 2.0
DB-HRV	30.6 ± 2.3	27.1 ± 2.2	25.1 ± 3.7	20.0 ± 3.1	28.9 ± 4.5	22.5 ± 2.7†
CT (°C)	28.4 ± 0.3	28.1 ± 0.4	25.5 ± 0.9#	23.4 ± 1.4	22.9 ± 2.3#	22.0 ± 1.0
WT (°C)	37.1 ± 0.4	37.9 ± 0.8	39.5 ± 0.8#	41.3 ± 0.8	40.2 ± 1.2#	39.6 ± 1.1
Cold induced pain (°C)	11.6 ± 1.7	15.6 ± 2.4	8.5 ± 1.4	8.9 ± 1.6	7.7 ± 2.3	8.7 ± 2.4
Warm induced pain (°C)	43.9 ± 0.7	44.6 ±0.7	46.6 ± 0.6	47.6 ± 0.5	42.7 ± 3.7	44.0 ± 2.9

Table 3-2. Neuropathy assessment in control and subjects with T1DM on CSII and MDI at baseline and 24-month follow-up. Data are expressed as mean ± SEM, All symbols represent statistically significant differences using one-way ANOVA.*$P < 0.005$. #$P < 0.0001$; baseline vs. control; † $P<0.05$ baseline vs 24months

	CONTROL BASELINE	CONTROL 24 MONTHS	MDI BASELINE	MDI 24 MONTHS	CSII BASELINE	CSII 24 MONTHS
IENFD (no/mm)	9.7 ± 0.8	9.5 ± 0.7	5.8 ± 0.9*	4.9 ± 0.9	5.4 ± 0.93*	5.7 ± 1.1
CNFD (no./mm^2)	29.8 ± 1.2	29.2 ± 1.9	20.1 ± 1.6#	18.6 ± 1.8	16.8 ± 2.0#	19.4 ± 2.6~
CNBD (no./mm^2)	39.6 ± 2.5	40.2 ± 4.4	23.5 ± 2.7#	20.9 ± 2.9	17.6 ± 2.4#	25.4 ± 3.7~
CNFL (mm/mm^2)	17.4 ± 0.6	17.3 ± 0.9	12.2 ± 0.8#	11.9 ± 0.9	10.1 ± 1.0#	12.2 ± 1.1^

Table 3-3. Corneal nerve morphology and IENFD in control and subjects with T1DM on CSII and MDI at baseline and 24-month follow-up. Data are expressed as mean ± SEM, All symbols represent statistically significant differences using one-way ANOVA: *P < 0.05. #P < 0.0001 - baseline vs. control; ~P < 0.05, ^P < 0.005 - baseline vs 24 months.
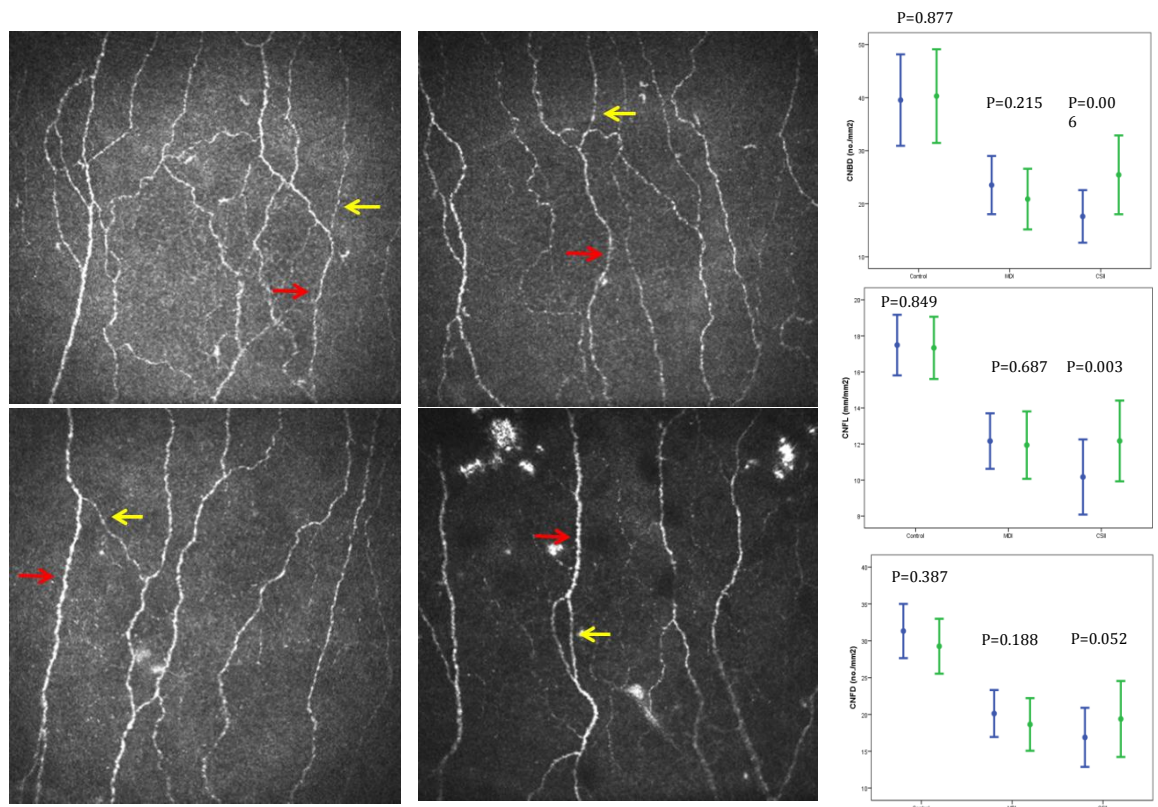
Figure 3-1. Corneal confocal images from patients with type 1 diabetes: a. CSII baseline, b. CSII 24 months, c. MDI baseline, d. MDI 24months. Red arrow = corneal nerve fibre, Yellow arrow = corneal nerve branch. e,f,g; Change in corneal nerve fibre morphological parameters in control subjects, patients with type 1 diabetes on MDI and CSII at baseline (blue) and 24 months (green). Data are expressed as mean ± SEM.

3.6 Discussion

Earlier studies demonstrated that initiation of CSII treatment was able to achieve near normal glycaemia with an improvement in nerve conduction [25-28]. Thus initiation of CSII improved nerve conduction velocity (NCV) by ~2.5m/s in 12 months with no change in subjects on conventional treatment [28]. Bertelsmann et al showed a modest improvement in thermal thresholds and neuropathic symptoms in subjects on CSII [29]. The primary objective was to evaluate the effect of CSII on painful neuropathy; the glycaemic control in these subjects was very poor and a significant improvement was observed during the study, which may have led to the improvement in symptoms. The positive effect on symptomatic neuropathy with CSII treatment has been confirmed in other studies [30]. In the present study NSP was significantly greater in both the CSII and MDI group at baseline and interestingly it was lower in the CSII group, suggestive of a possible impact on symptoms of CSII therapy *per se*, but did not change during the study. In a study of 9 subjects treated with CSII compared to 10 treated with MDI, after 12 months there was a significant improvement in overall glycaemic control in both groups, but with no difference in glycaemic excursion in the two groups. However, the average conduction velocity of the median, ulnar, and peroneal motor and median, ulnar and sural sensory nerves was significantly improved in the CSII (6.4%) compared to the MDI (1.3%) groups [31]. Our study had a similar level of sub-optimal glycaemic control in both the CSII and MDI groups which did not change over 24 months. Whilst the MDI group showed no significant change, the CSII group showed an improvement in all corneal confocal parameters. This improvement in corneal nerve morphology, with no change in any other measure of neuropathy echoes the results of our recent study in patients after SPK [18]. However, it is important to note that after SPK there was a significant improvement in HbA1c, indeed it was normalised and also those patients had a significantly greater amount of corneal nerve damage at baseline. The other markers of small fibre neuropathy assessed included QST and the gold standard measurement of IENFD which did not show any change, suggesting that

CCM may be more sensitive in detecting an improvement in small nerve fibre morphology.

The present study also did not find any change in NCS in either group, but this may not be unexpected given that glycaemic control did not change, and NCV may reflect a more acute effect of improved glucose control. Indeed animal studies have shown that short term CSII treatment improves nerve conduction velocity and abnormal myelinated nerve fibre morphology [32, 33]. Similarly Kronert et al showed that 4 weeks of CSII significantly improved motor and sensory nerve conduction velocity and autonomic nerve function, but this then deteriorated over the next 12 months with MDI [34]. The Oslo study found that there was an increase in nerve conduction velocity over 24 months in subjects treated with CSII who achieved near normoglycaemia [26]. The longest follow up was 36 months in a small number of patients with poorly controlled T1DM (n=11) confirming again that CSII had a positive effect on glucose control and showed a beneficial effect in the early stages of neuropathy evaluated by NCS [35]. In the present study there was a decrease in heart rate variability in the CSII group for which there is no clear explanation as there was no significant change in the MDI group.

Compared to previous studies assessing the effects of CSII on neuropathy, the main difference in the current study is that the subjects were already on CSII at baseline and we did not actively undertake any change in intervention. Furthermore, most of the earlier studies reported the short-term benefits of CSII therapy associated with an improvement in glycaemic control. However, in the present study glycaemic control in the CSII group was sub-optimal and comparable to those on MDI. We cannot therefore attribute the improvement in corneal nerve morphology to an improvement in glycaemic control and can only speculate that CSII may provide more stable blood glucose control which may in the future also provide benefits to neuropathy in patients with Type 2 Diabetes Mellitus [36]. Alternatively, earlier studies suggested that a lack of insulin and its resistance as well as reduction in IGFs (insulin-like growth factors) contribute to the development of DPN [37]. The direct role of insulin on neuropathy has been investigated in experimental studies and recently, Zochodne et al have shown that there may be a direct neurotrophic action of insulin on neurones and axons [38].

Thus insulin can directly impact axonal plasticity and regeneration as the intrathecal delivery of insulin and equimolar IGF-1, at levels that did not improve glycaemia, was shown to improve or reverse the slowing of motor and sensory conduction in diabetic rats [39, 40]. Toth et al also showed that intranasal insulin slowed the progression of experimental DPN whilst avoiding the side effects of subcutaneous insulin [41]. Similarly Singh al et al also found that the administration of low dose insulin in diabetic rats improved motor and sensory nerve conduction abnormalities in the sciatic nerve unilaterally without any impact on hyperglycaemia [42]. Therefore patients on CSII receiving continual small amounts of insulin show an improvement in small fibre neuropathy without an improvement in glycaemic control.

The main findings in this study are that T1DM patients treated with CSII show a significant improvement in corneal nerve morphology but no other measure of neuropathy over 24 months. Our study is of course not a randomised intervention study assessing the benefits of CSII compared to MDI, however, the observation that the improvement was observed without any change in glycaemia or other risk factors for neuropathy suggests that CSII may improve small fibre morphology through an independent neurotrophic effect of insulin. Furthermore, these data also provide further support for the use of CCM as a surrogate marker of diabetic neuropathy.

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4.   Chapter IV -Corneal Confocal Microscopy Identifies Small Fibre Neuropathy In Subjects With Impaired Glucose Tolerance Who Develop Type 2 Diabetes Mellitus

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4.1 Abstract

Impaired glucose tolerance (IGT) through to Type 2 diabetes mellitus (T2DM) is thought to confer a continuum of risk for neuropathy. Identification of subjects at high risk of developing T2DM and hence worsening neuropathy would allow identification and risk stratification for more aggressive management.

30 subjects with IGT and 17 age-matched controls underwent an OGTT, assessment of neuropathic symptoms and deficits, quantitative sensory testing, neurophysiology, skin biopsy and corneal confocal microscopy (CCM) to quantify corneal nerve fibre density (CNFD), branch density (CNBD) and fibre length (CNFL), at baseline and annually for 3 years.

10 subjects who developed T2DM had a significantly lower CNFD (P=0.003), CNBD, (P=0.04) and CNFL (P=0.04) compared to controls at baseline and a further reduction in CNFL (P=0.006), IENFD (P=0.02) and MDL (P=0.02) over 3 years. 15 subjects who remained IGT and 5 subjects who returned to normal glucose tolerance had no significant baseline abnormality on CCM or IENFD but had a lower MDL (P<0.0001) compared to controls. The IGT subjects showed a significant decrease in IENFD (P=0.02) but no change in MDL or CCM over 3 years. Those who returned to NGT showed an increase in CNFD (P=0.05), CNBD (P=0.04) and CNFL (P=0.05), but a decrease in IENFD (P=0.02), over 3 years.

CCM and skin biopsy detects a small fibre neuropathy in subjects with IGT who develop T2DM and also shows a dynamic worsening or improvement in corneal and intra-epidermal nerve morphology, in relation to change in glucose tolerance status.

4.2   Introduction

The International Diabetes Federation states that there are currently 316 million people with impaired glucose tolerance (IGT) which will increase to 471 million people by 2035 (1).   There is considerable debate as to whether these subjects should be considered to have a medical problem (2). However, in subjects with IGT, the risk of developing Type 2 Diabetes Mellitus (DM) ranges from 3.6-8.7% per year (3). Furthermore, IGT is also independently associated with the traditional microvascular complications of diabetes, including retinopathy, microalbuminuria and neuropathy (4). There appears to be a good rationale for identifying subjects with IGT, but there are limited data identifying subjects with IGT who may be at greatest risk for developing diabetes and its complications.

In relation to neuropathy, the specific focus of this study, the United Kingdom Prospective Diabetes Study showed that at the time of diagnosis of Type 2 DM, 5-7% of patients already had neuropathy (5) and longitudinal data from the Rochester cohort has shown that duration and severity of exposure to hyperglycaemia are related to the severity of neuropathy (6). In a recent study of patients with ~ 2 years of Type 2 diabetes, there was also evidence of a significant neuropathy (7). However, there is debate as to whether IGT is associated with neuropathy, with some studies showing evidence of neuropathy (8-12) whilst others do not (13-15). We have recently shown that a significant small fibre neuropathy occurred in 40.5% of 37 subjects with IGT(16). Interestingly a recent study evaluating electrochemical sweat conductance, a proxy for small fiber neuropathy, has shown that healthy subjects with an abnormal response have a significantly increased odds ratio for the development of IGT over 2 years (17). Of relevance, lifestyle modification has been shown to improve intra-epidermal nerve fibre density (11) and following chemical axotomy (18). We have previously shown an improvement in corneal nerve morphology following an improvement in glycaemic control, lipids and blood pressure (19), following simultaneous pancreas and kidney transplantation (20) and more recently in patients on CSII (21), suggesting a dynamic regenerative capacity of the small fibres in relation to metabolic change. We have undertaken a longitudinal study in subjects with IGT to assess whether baseline and follow up measures of neuropathy, particularly small

fibre neuropathy, relate to changes in glucose tolerance over 3 years.

4.3 Research Design and Methods

4.3.1 Selection of patients

We assessed 30 subjects with impaired glucose tolerance based on an oral glucose tolerance test (OGTT) (2 hours glucose 7.8-11.1 mmol) at Central Manchester and Manchester Children's University Hospital and 17 health control subjects. Exclusion criteria were any history of neuropathy due to a non-diabetic cause and any history of ocular pathology or systemic disease with corneal involvement. This study was approved by the Central Manchester Research and Ethics Committee and written informed consent was obtained from all subjects prior to participation. This research adhered to the tenets of the declaration of Helsinki.

4.3.2 Assessment of Neuropathy

All study participants underwent assessment at baseline, 12, 24 and 36 months. Participants underwent assessment of body mass index (BMI), blood pressure, OGTT, HbA1c, lipid profile [total cholesterol, low density lipoprotein (LDL), high density lipoprotein (HDL) and triglycerides], albumin creatinine excretion ratio (ACR) and estimated glomerular filtration rate (eGFR). Symptoms of DPN were assessed using the Neuropathy Symptom Profile (NSP). Neurological deficits were evaluated using the simplified neuropathy disability score (NDS), which is comprised of vibration perception, pin-prick, temperature sensation and presence or absence of ankle reflexes. Vibration perception threshold (VPT) was tested using a Neurothesiometer (Horwell, Scientific Laboratory Supplies, Wilfrod, Nottingham, UK). Cold (CT) and warm (WT) thresholds were established on the dorsolateral aspect of the left foot (S1) using the TSA-II NeuroSensory Analyser (Medoc Ltd., Ramat-Yishai, Israel).

Electro-diagnostic studies were undertaken using a Dantec "Keypoint" system (Dantec Dynamics Ltd, Bristol, UK) equipped with a DISA temperature regulator to keep limb temperature constantly between 32-35°C. Sural sensory nerve

amplitude (SNAP), sural sensory nerve conduction velocity (SNCV) and peroneal motor nerve conduction velocity (PMNCV) and amplitude (PMNA) were assessed by a consultant neurophysiologist.

4.3.3 Skin Biopsy

A 3mm punch skin biopsy was taken from the dorsum of the foot, approximately 2 cm above the second metatarsal head under local anaesthesia (1% lidocaine). 50 μm sections were stained using anti-human PGP 9.5 antibody (Abcam, Cambridge, UK) and nerve fibres were demonstrated using SG chromogen (Vector Laboratories Inc., UK). Intraepidermal nerve fibre density (IENFD) was quantified in accordance with established criteria and expressed as no./mm (22). Twenty Z-stack images per case were taken using a Zeiss AxioImager M2 microscope and mean dendritic length (MDL) (length of IENF from piercing the dermo-epidermal junction to its terminal in the epidermis) was manually traced and quantified using ImagePro 6.2 programme (MediaCybernetics, Marlow, UK).

4.3.4 Corneal Confocal Microscopy

Patients underwent examination with the CCM (Heidelberg Retinal Tomograph III Rostock Cornea Module, Heidelberg Engineering GmbH, Heidelberg, Germany) as per our previously established protocol (23). Six non-overlapping images/patient from the centre of the cornea were selected and quantified in a masked fashion. Three corneal nerve parameters were quantified: Corneal nerve fibre density (CNFD) - the total number of major nerves/mm^2 of corneal tissue, Corneal nerve branch density (CNBD) - the number of branches emanating from all major nerve trunks/mm^2 of corneal tissue and Corneal nerve fibre length (CNFL) - the total length of all nerve fibres and branches (mm/mm^2) within the area of corneal tissue. Analysis of the images was done using purposefully designed automated software called ACCmetrics (24).

4.4 Statistical Analysis

Analysis was carried out on SPSS for Mac (Version 19.0, IBM Corporation, New York, USA). All data are expressed as mean ± standard error of mean (SEM). The data was tested for normality by using the Shapiro Wilk Normality test and by

visualising the histogram and normal Q-Q plot. To assess within and between group differences we used one - way analysis of variance (non-parametric – Kruskal – Wallis). A significant p value was considered to be <0.05 (post hoc – Tukey).

4.5   Results

4.5.1. Baseline demographics and neuropathy assessments

The clinical characteristics are summarised in Table 4.1. The control and IGT subjects were age matched (62.3±1.8 v 60±2.1, P=0.2). Subjects with IGT had a significantly higher HbA1c (42.7±0.9 v 38.3±0.7, P<0.0001) and body mass index (BMI) (32.0±1.0 v 27.6±0.9, P=0.01) and lower HDL (1.2±0.1 v 1.7±0.1, P=0.03), but comparable total cholesterol, triglycerides, eGFR and blood pressure, compared to control subjects.

The IGT group had a significantly higher NSP (3.4±0.7 v 0.3±0.1, P<0.0001), NDS (2.9±0.5 v 1.1±0.3, P=0.03) and vibration perception threshold (16.2±2.1 v 8.4±1.5, P=0.02) compared to the control group. There was no significant difference in sural and peroneal nerve conduction velocity and amplitude between subjects with IGT and the control subjects.

There was no difference in IENFD, however MDL was significantly lower in the IGT group compared to controls (25.1±1.6 v 63.0±4.2, P<0.0001).   CNFD (24.4±1.3 v 30.7±1.5, P<0.0001) and CNFL (15.3±0.6 v 20.4±3.14, P=0.004) were significantly lower, but there was no difference in CNBD between subjects with IGT and control subjects.

There was no correlation between HDL and CCM measures at baseline (CNFL (r=0.2, P=0.2), CNBD (r=0.2, P=0.1), CNFD (r=0.2, P=0.3).

	Control N=17	Baseline N=30	P *	12months	24months	36months	P†
Age (years)	62.3±1.8	60±2.1	NS				
BMI (kg/m^2)	27.6±0.9	32.0±1.0	0.01	31.2±1.1	31.0±1.2	33.0±1.3	NS
HbA1c (mmol/mol)	38.3±0.7	42.7±0.9	<0.0001	44.0±1.2	43.0±1.5	44.2±2.0	NS
Cholesterol (mmol/l)	5.4±0.2	4.8±0.2	NS	4.8±0.2	4.5±0.2	4.6±0.2	NS
HDL (mmol/l)	1.7±0.1	1.2±0.1	0.03	1.2±0.1	1.2±0.1	1.3±0.1	NS
Triglycerides (mmol/l)	1.8±0.2	2.2±0.3	NS	2.2±0.3	2.0±0.5	1.8±0.3	NS
LDL (mmol/l)	3.0±0.2	2.6±0.2	NS	2.6±0.2	2.3±0.3	2.5±0.2	NS
eGFR (ml/min/l)	82.4±2.0	79.1±3.0	NS	78.8±3.4	74.9±3.5	74.3±4.3	0.03
Blood Pressure (mmHg)	136±4.3/75.9±2.5	129.2±3.4/72.9±2.1	NS	131.3±12.6/69.0±3.8	129.0±3.9/72.7±2.2	129.3±3.4/75.4±2.3	NS
NSP (/10)	0.3±0.1	3.4±0.7	<0.0001	2.78±0.7	3.5±0.8	2.8±0.6	NS
NDS (/10)	1.1±0.3	2.9±0.5	0.03	3.6±0.6	2.8±0.5	2.4±0.6	NS
VPT (V)	8.4±1.5	16.2±2.1	0.02	17.7±2.4	18.7±2.7	16.8±2.1	NS
CT (^{o}C)	27.9±1.1	25.5±1.4	NS	25.3±1.1	23.7±1.8	24.9±0.9	NS
WT (^{o}C)	39.5±1.1	39.8±1.0	NS	40.2±0.8	41.9±0.9	40.4±0.7	NS
SNCV (m/s)	49.0±1.1	49.7±1.4	NS	47.4±1.3	47.4±1.3	46.6±1.3	0.007
SNAP (μV)	15.3±1.8	11.3±1.2	NS	11.8±1.8	11.4±1.9	10.7±1.7	NS
PMNCV (m/s)	46.5±1.0	45.1±0.8	NS	44.4±0.8	44.9±0.8	44.7±0.9	NS
PMNA (mV)	5.2±0.4	4.4±0.4	NS	3.7±0.3	3.3±0.3	3.9±0.3	
IENFD (no./mm)	8.5±0.6	6.4±0.8	NS	6.5±4.1	NA	3.2±0.8	0.02
MDL (μm)	63.0±4.2	25.1±1.6	<0.0001	24.6±2.7	NA	22.9±3.1	NS
CNFD (no./mm^2)	30.7±1.5	24.4±1.3	<0.0001	22.6±1.5	27.4±1.5	24.4±1.2	NS
CNBD (no./mm^2)	37.0±2.7	33.8±2.9	NS	34.5±3.5	34.9±3.4	33.6±2.8	NS
CNFL (mm/mm^2)	20.4±3.14	15.3±0.6	0.0004	14.9±0.8	16.4±0.8	14.5±0.6	NS

Table 4-1. Data are expressed as mean ± SEM, All symbols represent statistically significant differences. * P value IGT baseline vs control, †IGT baseline vs 36 months. NS (not significant) NA (not assessed).

4.5.2 Longitudinal Assessments

Control subjects showed no significant change in metabolic parameters or neuropathy measures over 3 years (repeat skin biopsy not performed in control subjects). In subjects with IGT, BMI, HbA1c, lipids and blood pressure remained stable and there was a small but significant reduction in eGFR (79.1±3.0 v 74.3±4.3, P=0.03) over 3 years. The longitudinal data for the neuropathy assessments is presented in Table 4.1. There was no significant change in NSP, NDS, VPT or thermal thresholds. There was a significant reduction in sural nerve conduction velocity (49.7±1.4 v 46.6±1.3, P=0.007) and IENFD (6.4±0.8 v 3.2±0.8, p=0.02), but no change in MDL or CCM measures, from baseline to 36 months.

Change in Neuropathy measures in relation to change in glucose tolerance.

All subjects with IGT underwent an annual OGTT over 36 months, 10 developed Type 2 DM, 15 remained with IGT and 5 regressed to normal glucose tolerance (NGT) (Table 4.2). Figure 4.1 shows CCM images from each group.

In the 10 subjects who developed Type 2 DM, their baseline CNFD (20.0±2.2 v 30.7±1.5, P=0.003), CNBD (25.6±5.2 v 37.0±2.7, P=0.04) and CNFL (13.7±1.2 v 20.4±3.2, P=0.04) were significantly lower compared to control subjects. Over 36 months, there was a significant increase in HbA1c (42.4±1.0 v 50.3±1.4 P=0.02) and a significant decrease in CNFL (13.7±1.2 v 11.8±1.0 P=0.006), MDL (21.9±2.1 v 16.5±0.32, P=0.02) and IENFD (6.5± 1.2 v 3.9± 0.9, P=0.002), with no significant change in any other measure of neuropathy (Figure 4.2). Of the IGT subjects who had a significant (a CNFD value less than 2 standard deviations below the mean for controls) reduction in CNFD at baseline, 87.5% developed Type 2 Diabetes Mellitus and 12.5% remained IGT/ or reverted to NGT (P=0.007). In subjects who had a significant (a CNFL value less than 2 standard deviations below the mean for controls) reduction in CNFL, 100% developed Type 2 Diabetes (P<0.0001).

In the 15 IGT subjects who remained IGT, their baseline CNFD (28.6±1.5 v 30.7±1.5, P=0.33), CNBD (38.8±3.7 v 37.0±2.7, P=0.54) and CNFL (16.8±0.8 v 20.4±3.2, P=0.75) were comparable to controls. There was a significant reduction in IENFD (6.7±1.1 v 2.8±0.3, P=0.02) with no change in any measure of neuropathy over 36 months.

In the 5 subjects who became normal glucose tolerant, baseline CNFD (25.4±1.9 v 30.7±1.5, P=0.06), CNBD (29.3±5.6 v 37.0±2.7, P=0.07) and CNFL (15.7±1.3 v 20.4±3.2, P=0.24) did not differ from control subjects. However, there was a significant increase in CNFD (25.4±1.9 v 29.8±1.5 P=0.05), CNBD (29.3±5.6 v 44.9±6.2 P=0.04) and CNFL (15.7±1.3 v 17.2±0.9, P=0.05) There was a significant decrease in IENFD (6.5±1.1 v 3.0±0.4, P=0.02) with no significant change in any other measure of neuropathy over 36 months (Table 4.2 and Figure 4. 2).

	Control N=17		Normal Glucose Tolerance N=5		Impaired Glucose Tolerance N=15		Type 2 Diabetes Mellitus N=10	
	Baseline	36months	Baseline	36months	Baseline	36months	Baseline	36months
HbA1c (mmol/mmol)	38.3±0.7	37.7±0.9	41.4±2.3	40.5±2.4	42.8±1.2	42.3±2.3	42.4±1.0	50.3±1.4~
NSP (/10)	0.3±0.1	0.4±0.2	3.6±1.4†	1.8±1.3	4.0±1.3†	4.2±1.2	2.5±0.9†	3.0±1.3
NDS (/10)	1.1±0.3	1.4±0.2	3.8±2.0*	4.8±2.2	2.5±0.6*	2.3±0.5	3.5±1.4*	1.6±1.6
VPT (V)	8.4±1.5	9.1±1.8	19.3±3.5*	24.2±7.1	13.5±3.4*	13.3±2.6	16.9±3.3*	17.3±3.5
Sural Velocity (m/s)	49.0±1.1	46.0±1.6	47.9±2.6	44.2±3.2	50.8±1.4	46.3±1.9	50.1±2.3	47.1±2.0
Sural Amplitude (μV)	15.3±1.8	13.2±2.7	8.8±2.6	6.3±2.0	12.5±1.5	11.3±1.7	11.0±2.5	12.0±3.4
Peroneal Velocity (m/s)	46.5±1.0	45.0±1.4	44.1±2.4	44.5±2.2	45.9±1.2	45.7±2.5	44.7±1.2	44.1±1.0
Peroneal Amplitude(mV)	5.2±0.4	5.3±0.3	3.2±0.6	3.2±0.6	3.8±0.4	3.8±0.4	4.6±0.6	4.2±0.6
CT (^{o}C)	27.9±1.1	27.6±0.4	22.9±1.0	20.3±3.9	29.2±1.6	26.5±0.6	24.8±2.5	25.0±1.4
WT (^{o}C)	39.5±1.1	38.8±0.6	42.8±1.9	41.5±2.5	38.8±1.7	40.1±1.2	39.6±1.1	40.1±1.0
IENFD (no./mm)	8.5±0.6	-	6.5±1.1	3.0±0.4~	6.7±1.1	2.8±0.3~	6.5±1.2	3.9±0.9~
MDL (μm)	63.0±4.2		25.1±3.7†	27.5±4.2	27.9±2.1†	29.3±3.8	21.9±2.1†	16.5±0.3~
CNFD (no./mm^2)	30.7±1.5	29.7±1.4	25.4±1.9	29.8±1.5*	28.6±1.5	27.9±1.3	20.0±2.2^	18.3±1.7
CNBD (no./mm^2)	37.0±2.7	39.0±3.2	29.3± 5.6	44.9±6.2#	38.8±3.7	38.7±3.1	25.6±5.2#	21.8±3.9
CNFL (mm/mm^2)	20.4±3.14	19.2±0.9	15.7±1.3	17.2±0.9*	16.8 ±0.8	16.2±0.6	13.7±1.2#	11.8±1.0+

Table 4-2. Neuropathy assessments at baseline and 36 months in subjects who reverted to normal glucose tolerance, remained with Impaired Glucose Tolerance or developed Type 2 diabetes at 36 months.Data are expressed as mean ± SEM, All symbols represent statistically significant differences.   ~P=0.02, #P=0.04, *P=0.05, ^P=0.003, +P=0.0006, †P<0.0001, baseline vs. control or baseline vs 36 months.
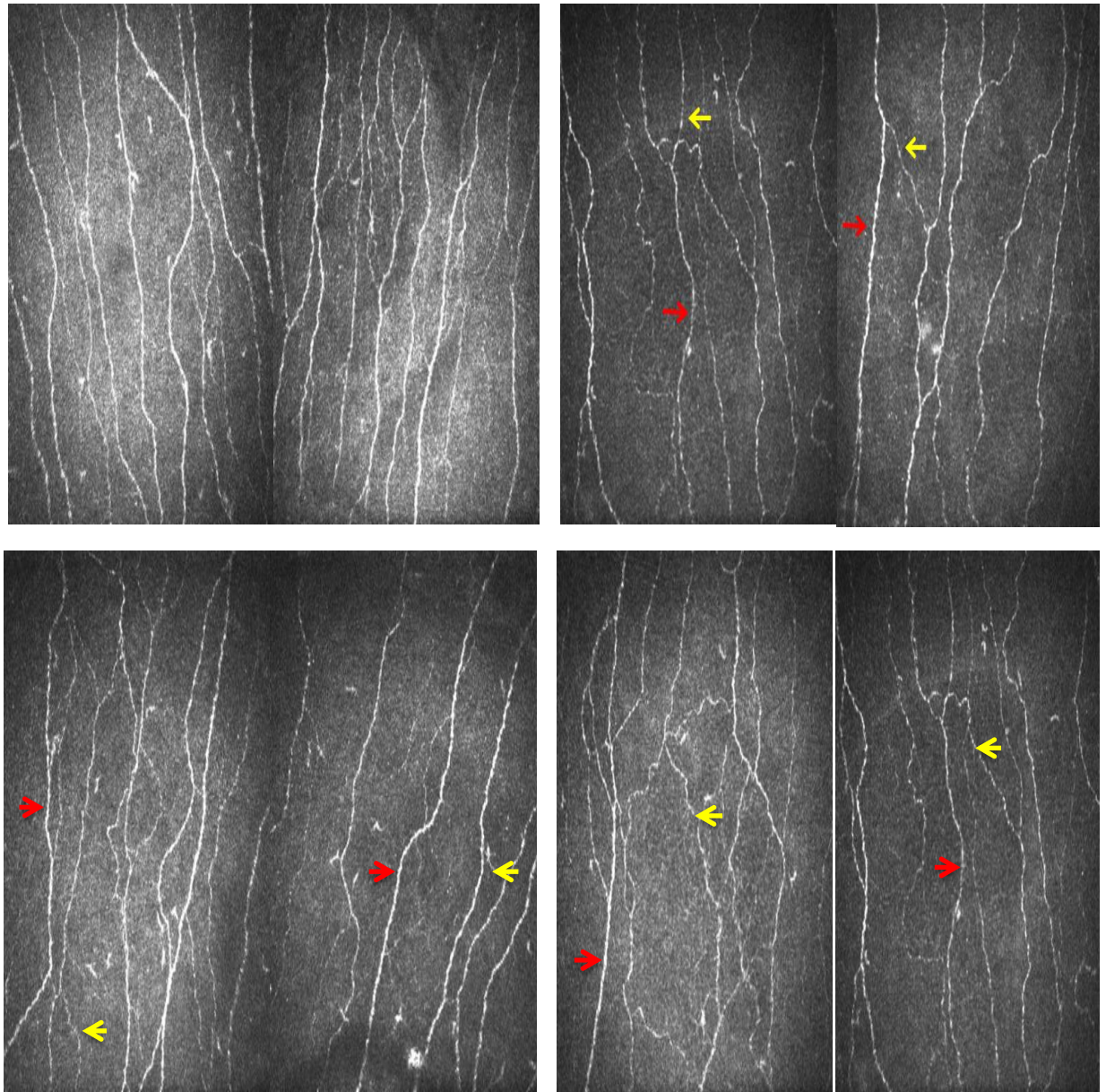
Figure 4-1. Corneal Confocal Images from a) control subject at baseline, b) control subject at follow up, c) IGT subject who developed Type 2 Diabetes at baseline d) IGT subject who developed Type 2 Diabetes at follow up, e) IGT subject who remained IGT at baseline, f) IGT subject who remained IGT at follow up, g) IGT subject who reverted to NGT at baseline, h) IGT subject who reverted to NGT at follow up. Red arrow = corneal nerve fibre, Yellow arrow = corneal nerve branch.
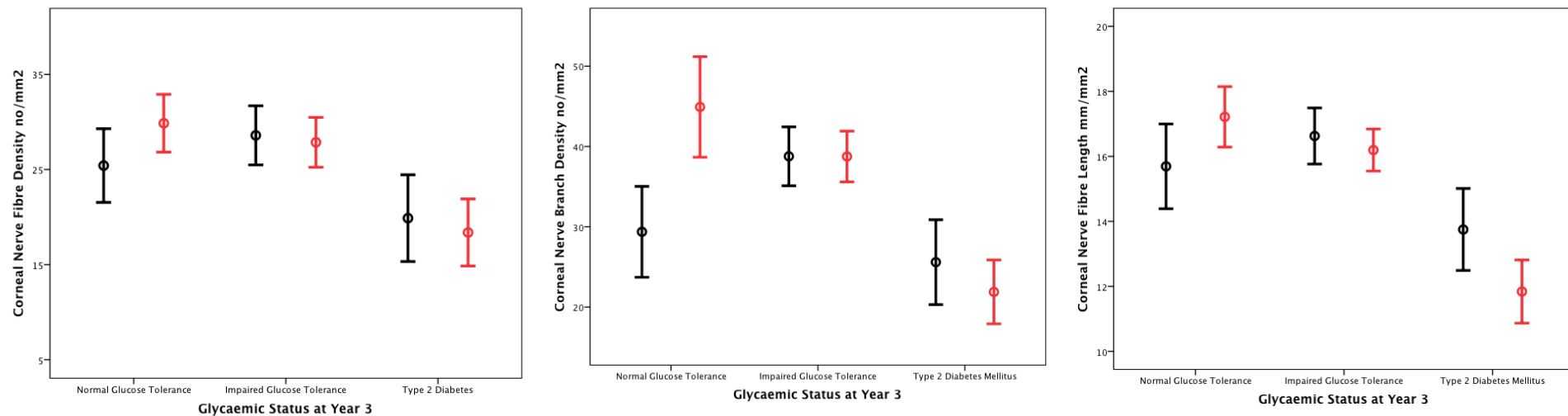
Figure 4-2. Change in corneal nerve fibre morphological parameters in subjects at baseline (black) and 36 months (red).

4.6   Discussion

The association between of peripheral neuropathy (PN) and IGT remains controversial. Hughes et al found that in 50 consecutive subjects with PN and 50 consecutive controls there was no significant difference in the prevalence of IGT, but in the PN group serum triglycerides were significantly higher (25). Fujimoto et al showed that subjects with IGT had comparable nerve conduction studies, but had a greater prevalence of retinopathy and nephropathy compared to control subjects (26). More recently Dyck et al (27) showed that the frequency of PN was comparable in healthy subjects (1.7%) and subjects with impaired glycaemia (2.0%) and was only increased in those with Type 2 diabetes (7.8%). In a cohort of 393 subjects, Zeigler et al (28) found that there was an increased prevalence of polyneuropathy in those with IGT (13%), compared to those with impaired fasting glycaemia (IFG) (11.3%) and control subjects (7.4%), although this was not significant.   These findings may be attributed to the fact that neuropathy was diagnosed by assessing predominantly large fibres (13, 29). Indeed there are accruing data to suggest that there is an increased prevalence of painful symptoms (30-32) and evidence of a small fibre neuropathy in subjects with IGT (10, 11, 16, 32). Thus small fibre neuropathy may be the earliest change in the spectrum of peripheral neuropathy, with injury beginning in the small myelinated Aδ and unmyelinated C fibers, which over time progresses to affect larger nerves (33).

Whilst IENFD is accepted as the gold standard for quantifying IENF pathology, interestingly, Pittenger et al showed that MDL was reduced before IENFD, in subjects with metabolic syndrome, and may therefore be an early marker of sensory neuropathy (34). Our data supports these findings, as MDL was significantly reduced, whilst IENFD was comparable in the IGT cohort, compared to controls at baseline. Furthermore, MDL appears to be more responsive to changes in glucose tolerance status with a further worsening in only those IGT subjects who developed Type 2 diabetes, whilst IENFD showed a reduction in all three groups.

In relation to causal factors, Pittenger et al also reported a correlation between peripheral neuropathy and HDL (34). In the present study we show that HDL was lower in the IGT group compared to the controls, however this was not associated with lower CCM measures. In an 18 week open-label trial, Boyd at al showed that treatment with topiramate resulted in a significant improvement in MDL at the forearm and proximal leg and an increase in IENFD at the proximal leg (35)

Smith et al have shown that a 1-year diet and lifestyle intervention programme leads to an increase in IENFD (11). However, the much larger Da Qing study showed that lifestyle intervention over 6 years reduced the incidence of severe retinopathy, but had no impact on neuropathy, although the end point was monofilament insensitivity (36). More recently a six month twice weekly individualised exercise programme significantly improved the rate of cutaneous nerve regeneration in a capsaicin nerve ablation model (18). Our recent study in patients with Type 1 DM undergoing simultaneous pancreas kidney transplantation showed that corneal confocal microscopy can detect small fibre regeneration as early as 6 months post-surgery (37). And we have also shown that improvement in glycaemia as well as blood pressure and lipids leads to corneal nerve regeneration (19). This leads to the notion that if there is an improvement in glycaemia then it may improve neuropathy. In the present study we show that subjects with IGT have evidence of small fibre neuropathy as evidenced by a greater prevalence of painful symptoms and abnormalities in corneal confocal microscopy as well as a reduction in MDL, in keeping with our recent study (16). However, we now show that patients who progress to Type 2 diabetes have worse baseline corneal nerve morphology and MDL, at a time when they are diagnosed with IGT. This is in keeping with a recent study showing that subjects with normal glucose tolerance, but with abnormal electrochemical sweat conductance have a significantly increased odds ratio for the development of IGT (17). Furthermore, subjects who progressed to Type 2 diabetes mellitus also showed a further significant reduction in CNFL and MDL. In subjects who remained with IGT there was no baseline loss nor was there any change over time. And in subjects who reverted to normal glucose tolerance, the baseline CCM values did not differ significantly from controls and indeed there was a significant increase in all CCM parameters. Whilst this is a small study, the detailed quantification, particularly of the small fibres,

provides insights into the dynamic relationship between small fibre damage and repair in relation to overall glucose tolerance status.

We confirm the data from our previous study showing that small fibre neuropathy, detected using CCM is prevalent in subjects with IGT (16). More importantly, both CCM and MDL appear to be early and dynamic markers of small fibre neuropathy, which may allow risk stratification of subjects with IGT who are likely to progress to Type 2 diabetes.

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5. Chapter V - Diabetic Neuropathy: Lessons From Longitudinal Studies In Medallists And Extreme Phenotypes Of Patients With Type 1 Diabetes Mellitus

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5.1 Abstract

Patients with Type 1 Diabetes Mellitus (T1DM) of more than 50 years (medallists) represent a unique cohort of individuals with apparent protection from the long-term microvascular complications.

90 patients with T1DM: 34 medallists, 36 patients with end stage renal failure (ESRF), awaiting simultaneous pancreas kidney transplantation (SPK), 20 diabetes duration (to SPK) matched patients with T1DM and 20 age matched control participants underwent a comprehensive assessment of neuropathy at baseline and annually over 3 years.

Medallists demonstrate relative protection from small fibre neuropathy as evidenced by a better intra-epidermal nerve fibre density (P=0.05), corneal nerve fibre density (CNFD) (P=0.02), branch density (CNBD) (P=0.05) and length (CNFL) (P=0.03), compared to the SPK group. Over 3 years of follow up medallists showed no change in any measure of neuropathy; T1DM showed a significant worsening in CNFD (P=0.001), CNBD (P=0.013) and CNFL (P=0.001), whilst there was a significant improvement in CNFD (P=0.01), CNFL (P=0.001), neuropathy symptom profile (P=0.04) and peroneal nerve conduction velocity (P=0.05) after SPK.

Medallists demonstrate protection and no progression in small fibre neuropathy, whilst patients with T1DM show deterioration and patients undergoing SPK show early small nerve fibre regeneration. Corneal confocal microscopy is a sensitive measure for detecting nerve fibre degeneration and regeneration in human diabetic neuropathy.

5.2   Introduction

The majority of adults with Type 1 Diabetes Mellitus (T1DM) will develop varying degrees of diabetic retinopathy, nephropathy and neuropathy (1). Optimal glycaemic control may limit the development and progression of these complications, but does not reverse it (2, 3).

There is a unique group of patients with extreme duration (>50 years) T1DM who develop no or minimal long- term cardiac and microvascular complications. In recognition of this Diabetes UK award the Alan Nabarro medal to patients with more than 50 years of T1DM and the RD Lawrence medal to those with more than 60 years of T1DM, and these long-term survivors are referred to as 'medallists'. Early studies showed that there may be less microvascular complications in these patients, but were primarily focused on nephropathy and retinopathy (4). Several studies have explored factors which may predict long term survival of patients with T1DM and in particular what protects these patients from developing complications (5). The Golden Years Study found a relative protection from diabetic nephropathy and large vessel disease which was associated with elevated high density cholesterol (HDL) (6). The Joslin medallist study reported proliferative diabetic retinopathy and neuropathy in approximately 50% of their patients with 50-60 years of TIDM (7). A higher HDL and lower triglycerides as well as residual insulin production, but not HbA1c or diabetes duration, predicted protection from microvascular complications in this cohort (8). Similarly in a recent analysis of 325 individuals with more than 50 years of T1DM from Canada, a lower burden of both microvascular and macrovascular complications was associated with current physical activity, higher quality of life, and higher HDL (9). We have demonstrated minimal evidence of diastolic dysfunction and cardiac fibrosis in a cohort of medallists despite poor glycaemic control, but they had a raised HDL (10). We have also recently demonstrated no structural or functional abnormality on cardiac MRI in medallists (11).

To our knowledge there have been no previous detailed studies assessing neuropathy in the medallist group. We have undertaken a comprehensive

assessment of small and large fibre neuropathy in a group of medallists and compared them with a cohort of patients with T1DM awaiting simultaneous pancreas kidney transplantation (SPK) and a diabetes duration matched cohort of patients with Type 1 DM, at baseline and annually over 3 years.

5.3 Research Design and Methods

5.3.1 Selection of patients

We assessed 90 patients: Medallists with T1DM (n=34), T1DM patients with ESRF undergoing simultaneous pancreas kidney transplantation (SPK) (n=36); patients with T1DM who were diabetes duration matched to the SPK group (T1DM) (n=20) and an age matched control group (n=20). The patients were recruited from Central Manchester and Manchester Children's University Hospital. Exclusion criteria were any history of neuropathy due to a non-diabetic cause and any history of corneal trauma or surgery, or systemic or ocular disease that may affect the cornea. The Central Manchester Research and Ethics Committee approved this study and written informed consent was obtained from all subjects participating in the study. This research adhered to the tenets of the declaration of Helsinki.

5.3.2 Assessment of Neuropathy

All tests were undertaken at baseline, 12, 24, and 36 months. Study participants underwent assessment of body mass index (BMI), blood pressure, HbA1c, lipid profile [total cholesterol, low density lipoprotein (LDL), high density lipoprotein (HDL) and triglycerides], albumin creatinine excretion ratio (ACR) and estimated glomerular filtration rate (eGFR). Symptoms of DPN were assessed using the Neuropathy Symptom Profile (NSP). Neurological deficits were evaluated using the modified neuropathy disability score (NDS) (12). Vibration perception threshold (VPT) was tested using a Neurothesiometer (Horwell, Scientific Laboratory Supplies, Wilfrod, Nottingham, UK). Cold (CT) and warm (WT) thresholds were assessed on the foot using the TSA-II NeuroSensory Analyser (Medoc Ltd., Ramat-Yishai, Israel). Sural sensory nerve amplitude (SNAP), sural sensory nerve conduction velocity (SNCV), Sural sensory nerve latency, peroneal

motor nerve amplitude (PMNA), Peroneal motor nerve latency and peroneal motor nerve conduction velocity (PMNCV) were assessed by a consultant neurophysiologist using a Dantec "Keypoint" system (Dantec Dynamics Ltd, Bristol, UK). Heart rate variability (HRV) was assessed with an ANX 3.0 autonomic nervous system monitoring device (ANSAR Medical Technologies Inc., Philadelphia, PA, USA).

5.3.3 Skin Biopsy

A 3mm punch skin biopsy was taken from the dorsum of the foot, approximately 2 cm above the second metatarsal head under local anaesthesia (1% lidocaine). 50 µm sections were stained using anti-human PGP 9.5 antibody (Abcam, Cambridge, U.K) and nerve fibres were demonstrated using SG chromogen (Vector Laboratories, Peterborough, U.K). Intraepidermal nerve fibre density (IENFD) was quantified in accordance with established criteria and expressed as no/mm (13).

5.3.4 Corneal Confocal Microscopy

Patients underwent examination with the CCM (Heidelberg Retinal Tomograph III Rostock Cornea Module, Heidelberg Engineering GmbH, Heidelberg, Germany) according to our established protocol (14). Six non-overlapping images/patient (3 per eye) from the centre of the cornea were selected and quantified in a masked fashion. Three corneal nerve parameters were quantified: Corneal nerve fibre density (CNFD) - the total number of major nerves/mm^2 of corneal tissue, Corneal nerve branch density (CNBD) - the number of branches emanating from the major nerve trunks/mm^2 and Corneal nerve fibre length (CNFL) - the total length of all nerve fibres and branches (mm/mm^2) within the area of corneal tissue. Automated analysis of corneal nerve morphology was performed using automated software (ACCMetrics) (15).

5.4   Statistical analyses

Analyses were carried out on SPSS for Mac (Version 19.0, IBM Corporation, New York, USA). All data are expressed as mean ± standard deviation (SD). The data

was tested for normality by using the Shapiro Wilk Normality test and by visualising the histogram and normal Q-Q plot. To assess within and between group differences we used one-way analysis of variance (non-parametric – Kruskal – Wallis). A significant *p* value was considered to be <0.05 (post hoc – Tukey).

5.5 Results

5.5.1 Baseline data

Medallists' vs Controls (Tables 5.1 and 5.2)

There was no significant difference between anthropomorphic measurements between the two groups. The medallist's had a significantly lower cholesterol (P<0.001) and LDL (P<0.001) and higher HbA1c (P<0.001) with a significantly lower eGFR (P=0.005) and higher ACR (P=0.01). NSP, NDS, VPT, sural and peroneal nerve latencies were significantly higher and sural and peroneal nerve amplitudes and conduction velocities were lower (P<0.001 for all), whilst CT (P<0.001), WT (P=0.002), CIP (P=0.004) were higher and HRV (P=0.006), IENFD (P=0.02), CNFD (P<0.001), CNBD (P<0.001) and CNFL (P<0.001) were significantly lower in the medallist group compared to controls (Table 5.2).

Medallists' vs T1DM (Tables 5.1 and 5.2)

Age (P=0.01), systolic blood pressure (P=0.001) and ACR (P=0.03) were higher and eGFR was lower in medallists compared to patients with T1DM (P<0.0001). All other clinical and metabolic variables including HbA1c and lipids were comparable. There was no significant difference in NSP or NDS, but VPT (P<0.0001) and sural and peroneal nerve conduction studies were abnormal and cold threshold (P=0.007), HRV (P<0.0001) were lower in the medallists compared to T1DM. CNFD (P=0.02) and CNFL (P=0.003) were significantly lower in the medallists compared to the T1DM group.

Medallists' vs SPK (Tables 5.1 and 5.2)

Age (P<0.001), duration of diabetes (P<0.001), BMI (P=0.002), HDL (P=0.003), systolic blood pressure (P=0.05) and number of cigarettes smoked/day (P=0.05) were higher and height (P=0.04) was lower in medallists compared to the SPK cohort. There was no difference in alcohol consumption, HbA1c, total cholesterol, LDL and triglycerides between the two groups. There was no significant difference in NSP, NDS, VPT, sural and peroneal nerve conduction studies, cold and warm sensory thresholds or HRV between medallists and SPK. However, IENFD

(P=0.05), CNFD (P=0.02), CNBD (P=0.05) and CNFL (P=0.03) were significantly higher in the medallists compared to the SPK group.

Retinopathy (Table 5.1)

26/36 SPK patients, compared to 16/34 medallists (P=0.04) and 4/20 T1DM patients had diabetic retinopathy.

Nephropathy (Table 5.1)

The T1DM group had a normal eGFR, whilst the medallists (P<0.001) and SPK group (P<0.001) had a significantly lower eGFR, compared to control.

	Control (n=20)	Type 1 DM (n=20)	Medallist (n=34)	SPK (n=36)
Age (years)	62·3±5·7	49·8±9·9	63·6±8·6	48·6±9·2*
Gender (F/M)	8/12	9/11	18/16	11/25
Smoking (cigarette/day)	0·4±1·6	1·4±4·5	1·3±4·6	1·9±3·4
Alcohol Consumption (units/week)	5·7±8·5	7·5±10·7	4·3±7·3	6·6±9·9
Duration of Diabetes (years)	N/A	29·7±1·2*	56·1±4·7	32·3±10·5*
Blood Pressure (mm Hg)	135±20·7/78·8±9·3	129±15·52*/69·9±8·0	147·7±18·9/71·4±8·9	131·8±22·3/73·9±10·5
Height (cm)	169·3±10·9	169·3±8·47	164·1±9·4	168·0±18·1♦
BMI	26·6±2·9	25·9±3·9	27·5±4·6	23·6±5·3^
HbA1c DCCT (%)	5·1±0·3*	8·4±1·6	8·1±1·2	8·4±1·6
(IFCC) (mmol/mol)	(36·9±3·0)*	(68·5±18·0)	(64·9±12·2)	(69·1±16·9)
ACR (mg/mmol)	0·3±0·2♦	1·3±2·5♦	10·0±3·2	N/A
eGFR (ml/min/l)	80·3±7·8^	89·1±4·2*	64·5±3·9	14·6±2·4*
Diabetic retinopathy (no (%))	0 (0%)*	4 (20%)♦	16 (47%)	26 (72%)♦
Cholesterol (mmol/l)	5·3±0·8*	4·4±0·7	4·4±0·9	3·9±1·0
HDL (mmol/l)	1·6±0·3	1·8±0·3	1·9±0·6	1·3±0·6^
LDL (mmol/l)	3·0±0·7*	2·1±0·6	2·1±0·7	2·1±0·8
Triglycerides (mmol/l)	1·7±0·6	1·0±0·5	1·2±0·8	1·2±0·6

Table 5-1. Demographic data for all groups of participants. Medallist vs Control, Medallist vs Type 1 DM, Medallist vs SPK: *P<0.0001, ^P<0.005, ♦P<0.05.

	Control (n=20)	Type 1 DM (n=20)	Medallist (n=34)	SPK (n=36)
NDS	1·2±1·5*	3·6±2·8^	5·6±2·7	5·2±3·7
NSP	0·3±0·6*	3·4±4·8	3·9±4·4	5·3±5·9
VPT (volts)	9·7±6·7*	10·5±8·3*	25·3±13·7	22·6±13·5
Sural Latency (ms)	2·9±0·3*	3·3±0·5◆	4·1±0·8	4·1±0·9
Sural Amplitude (µv)	14·3±7·1*	10·9±7·5*	2·9±2·5	2·7±2·7
Sural Velocity (m/s)	48·9±5·4*	43·4±5·6^	33·7±7·3	34·4±7·9
Peroneal Latency (ms)	4·3±0·6*	4·5±0·8◆	5·6±1·4	5·8±2·0
Peroneal Amplitude (mV)	4·9±1·5*	3·1±1·7*	1·5±1·4	1·2±1·3
Peroneal Velocity (m/s)	45·7±3·1*	41·6±3·4◆	35·6±8·2	31·1±9·0
Cold Threshold ($^{\circ}$C)	27·6±2·1*	25·0±7·0◆	22·3±7·3	16·8±10·7
Warm Threshold ($^{\circ}$C)	37·8±2·9^	39·4±3·8	40·9±4·2	43·8±4·9
DB-HRV (beats/min)	23.4±11·6◆	23·4±14·9*	16·3±4·8	13·9±14·7
IENFD (no./mm)	8·8±2·5◆	5·3±4·3	4·0±3·7	1·9±2·3◆
CNFD (no./mm^2)	29·0±6·5*	20·4±8·0◆	14·7±8·5	9·4±5·8◆
CNBD (no./mm^2)	34·0±13·7*	22·9±10·8	18·4±17·8	9·8±8·5◆
CNFL (mm/mm^2)	16·6±3·1*	12·4±3·5^	9·9±4·6	7·2±3·0◆

Table 5-2. Neuropathy assessments for all groups of participants. Medallist vs Control, Medallist vs Type 1 DM, Medallist vs SPK: *P<0.0001, ^P<0.005, ◆P<0.05.

5.5.2 Longitudinal follow up in controls

There was no significant change in any of the measures of neuropathy in the control group over 36 months (Table 5.3, Figures 5.1 and 5.2).

	Baseline	12 Months	24 Months	36 Months	P value
NDS	1.2±1.5	1.4±1.8	0.2±0.5	0.5±0.9	NS
NSP	0.3±0.6	0.4±1.4	0.2±0.4	0.2±0.7	NS
VPT (volts)	9.7±6.7	9.6±7.7	10.2±5.9	9.0±7.3	NS
Sural Latency (ms)	2.9±0.3	3.1±0.3	2.5±1.3	2.9±0.3	NS
Sural Amplitude (uV)	14.3±7.1	12.7±5.1	14.1±6.4	12.0±6.4	NS
Sural Velocity (m/s)	48.9±5.4	46.2±4.9	45.6±4.9	47.0±4.8	NS
Peroneal Latency (ms)	4.3±0.6	4.5±0.5	4.4±0.7	4.1±0.6	NS
Peroneal amplitude (m/s)	4.9±1.5	4.7±1.7	4.85±1.1	4.8±1.8	NS
Peroneal velocity (m/s)	45.7±3.1	45.5±2.8	44.8±4.5	45.8±3.7	NS
Cold Threshold (˚C)	27.6±2.1	29.8±9.6	25.2±6.6	27.7±1.9	NS
Warm Threshold (˚C)	37.8±2.9	39.1±4.2	40±3.9	38.4±2.3	NS
HRV	23.4±11.6	21.1±20.2	14.0±7.0	20.0±12.0	NS
IENFD (no./mm)	8.8±2.5		9.3±4.86		NS
CNFD (no./mm^2)	29.0±6.5	28.8±4.8	28.6±5.1	28.8±5.3	NS
CNBD (no./mm^2)	34.0±13.7	37.4±7.6	35.9±11.1	35.7±13.7	NS
CNFL (mm/mm^2)	16.6±3.1	16.7±2.2	16.9±2.0	16.8±2.1	NS

Table 5-3. Longitudinal follow up of neuropathy in the control group.

5.5.3 Longitudinal follow up in Type 1 DM

There was a significant reduction in the T1DM group in CNFD (P=0.001), CNBD (P=0.013) and CNFL (P=0.001) over 36 months (Table 5.4, Figures 5.1 and 5.2).

	Baseline	12 Months	24 Months	36 Months	P value
NDS	3.5±2.8	3.6±2.4	3.6±2.4	2.2±3	NS
NSP	3.4±4.8	4.0±6.5	5.2±8.8	5±8.5	NS
VPT (volts)	10.5±8.3	14.2±11.3	14.2±11.3	13.2±10.7	NS
Sural Latency (ms)	3.3±0.5	3.3±0.4	3.4±0.52	3.4±0.49	NS
Sural Amplitude (uV)	10.9±7.5	10.0±6.4	10.9±5.6	9.8±5.8	NS
Sural Velocity (m/s)	43.4±5.6	43.7±4.9	41.9±5.6	41.2±5.3	NS
Peroneal Latency (ms)	4.5±0.8	4.5±0.7	4.2±1.1	4.4±0.6	NS
Peroneal amplitude (m/s)	3.1±1.7	2.9±1.9	2.8±1.7	3.4±1.8	NS
Peroneal velocity (m/s)	41.6±3.4	39.5±11.0	39.4±10.3	41.7±3.5	NS
Cold Threshold (˚C)	25.0±7.0	25.4±6.0	25.4±6.0	23.6±8.3	NS
Warm Threshold (˚C)	39.4±3.8	40.6±4.3	40.6±4.3	40.7±4.7	NS
HRV	23.4±14.9	22.6±6.2	21.0±11.0	21.0±10.0	NS
IENFD (no./mm)	5.3±4.3		4.7±3.9		NS
CNFD (no./mm^2)	20.4±8.0	19.4±7.6	18.9±7.4	15.3±7.9	0.001
CNBD (no./mm^2)	22.9±10.8	21.2±12.0	20.6±12.4	16.7±13.4	0.013
CNFL (mm/mm^2)	12.4±3.5	12.7±3.3	11.8±3.6	10.1±3.5	0.001

Table 5-4. Longitudinal follow up of neuropathy in the T1DM group.

5.5.4 Longitudinal follow up in medallists

There was no significant change in any of the measures of neuropathy in the medallist group over 36 months (Table 5.5, Figures 5.1 and 5.2).

	Baseline	12 Months	24 Months	36 Months	P value
NDS	5.6±2.7	5.8±2.7	5.8±2.7	5.5±3.1	NS
NSP	3.9±4.4	5.1±4.0	4.7±3.6	4.5±4.9	NS
VPT (volts)	25.3±13.7	28.2±13.1	28.2±13.1	24.8±13.9	NS
Sural Latency (ms)	4.1±0.8	4.2±0.8	4.2±0.8	4.2±0.7	NS
Sural Amplitude (uV)	2.9±2.5	1.9±2.6	2.9±2.6	2.6±2.5	NS
Sural Velocity (m/s)	33.7±7.3	35.1±7.0	34.4±7.2	33.9±6.5	NS
Peroneal Latency (ms)	5.6±1.4	5.7±1.3	5.4±1.5	5.2±1.8	NS
Peroneal amplitude (m/s)	1.5±1.4	1.5±1.2	1.8±1.5	1.6±1.4	NS
Peroneal velocity (m/s)	35.6±8.2	33.2±7.9	35.7±8.5	33.9±9.7	NS
Cold Threshold (˚C)	22.3±7.3	20.2±9.2	20.2±9.2	20.8±5.9	NS
Warm Threshold (˚C)	40.9±4.2	42.7±3.9	42.7±3.9	43.6±4.5	NS
HRV	16.3±4.2	9.4±12.2	7.0±4.0	13±7	NS
IENFD (no./mm)	4.0±3.7		3.5±2.7		NS
CNFD (no./mm^2)	14.7±8.5	14.0±9.0	13.4±7.7	13.8±8.9	NS
CNBD (no./mm^2)	18.4±17.8	19.1±17.5	16.3±14.2	17.5±15.9	NS
CNFL (mm/mm^2)	9.9±4.6	10.2±4.6	9.1±4.3	9.6±4.5	NS

Table 5-5. Longitudinal follow up of neuropathy in the medallist group.

5.5.5 Longitudinal follow up in SPK

There was a significant improvement in NSP (P=0.04), PMNCV (P=0.05), CNFD (P=0.01) and CNFL (P=0.001) in the SPK group over 36 months (Table 5.6, Figures 5.1 and 5.2).

	Baseline	12 Months	24 Months	36 Months	P value
NDS	5.2±3.7	5.6±3.6	3.1±3.6	4.7±3.7	NS
NSP	5.3±5.9	5.1±6.8	2.5±4.2	3.1±7.2	0.04
VPT (volts)	22.6±13.5	21.1±14.3	19.5±12.5	22.8±17.1	NS
Sural Latency (ms)	4.1±0.9	3.2±1.8	3.9±0.9	3.8±0.9	NS
Sural Amplitude (uV)	2.7±2.7	2.5±2.6	3.7±2.9	4.7±3.8	NS
Sural Velocity (m/s)	34.4±7.9	37.2±8.6	37.0±8.9	37.8±8.2	NS
Peroneal Latency (ms)	5.8±2.0	5.6±1.8	5.4±1.7	4.6±1.5	NS
Peroneal amplitude (m/s)	1.2±1.3	1.3±1.3	1.3±1.2	1.6±1.4	NS
Peroneal velocity (m/s)	31.1±9.0	33.1±10.3	34.5±10.3	38.7±8.2	0.05
Cold Threshold (˚C)	16.8±11.7	16.8±12.2	17.1±12.1	17.6±12	NS
Warm Threshold (˚C)	43.8±4.9	43.4±4.9	42.8±5.1	40.9±4	NS
HRV	13.9±14.7	10.1±7	11±9	11±7	NS
IENFD (no./mm)	1.9±2.3		2.3±2.7	3.0±1.9	NS
CNFD (no./mm^2)	9.4±5.8	12.2±8.4	12.5±6.6	14.4±5.0	0.01
CNBD (no./mm^2)	9.8±8.5	13.2±13.2	12.7±8.9	14.9±6.6	NS
CNFL (mm/mm^2)	7.2±3.0	8.3±3.9	8.9±3.5	10.3±2.0	0.001

Table 5-6. Longitudinal follow up of neuropathy in the SPK group.
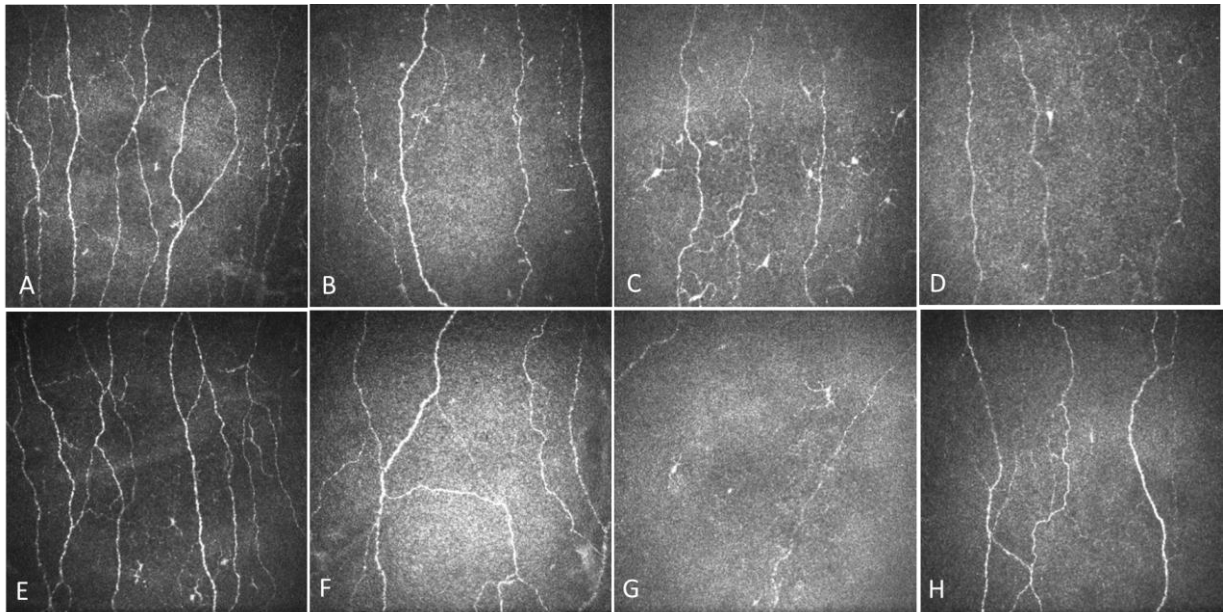
Figure 5-1. Corneal confocal microscopy images of a control at baseline (A) and at 36 months (E), a medallist at baseline (B) and at 36 months (F), a patient with T1DM at baseline (C) and at 36 months (G), and a patient prior to SPK (D).
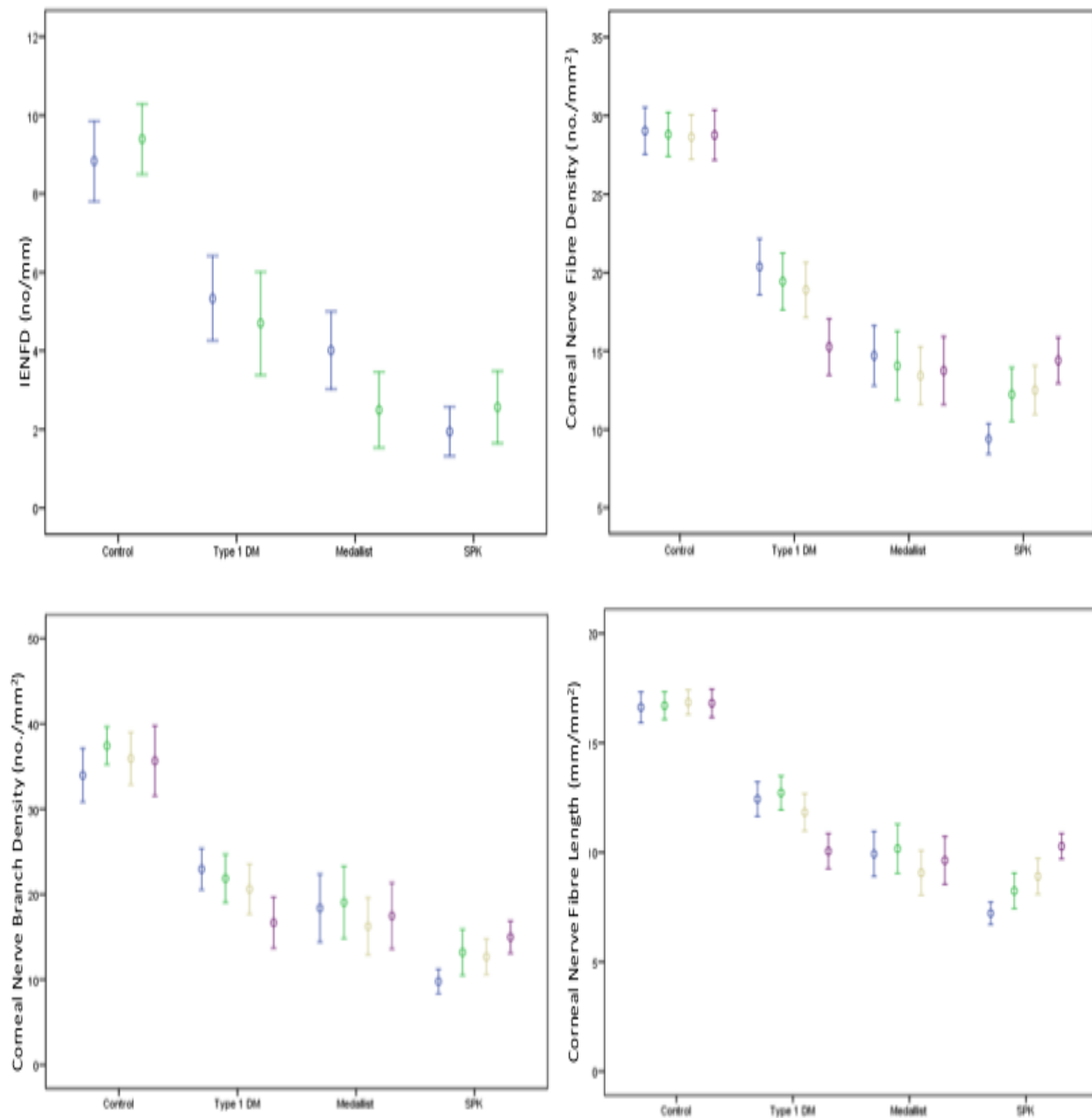
Figure 5-2. Intra-epidermal Nerve Fibre Density at baseline and 24 months, Corneal nerve fibre density, branch density and length at baseline, 12 months, 24 months, and 36 months in control, Type 1 DM, Medallist and SPK groups. Data are expressed as mean ± SEM.

5.6 Discussion

In medallists we demonstrate evidence of a moderately severe large fibre neuropathy with a relative preservation in functional and structural measures of small fibre neuropathy. This suggests that the medallist group, despite an extreme duration of diabetes are partially protected from the development of small fibre neuropathy, perhaps explaining the long-term survival of these individuals (9), given the recent findings that the burden of microvascular disease is a major determinant of future cardiovascular disease (16).

During the 1970s several reports defined the characteristics of the 'long term survivor' of Type 1 diabetes. This included a low prevalence of smoking, good metabolic control, regular physical activity, frequent medical contact, low insulin dose and normal or below weight BMI (17). Paz Guevara et al found a low prevalence of micro and macrovascular complications and 50% had no evidence of 'neuropathy' (18), which was attributed to meticulous day to day care of their illness. The Steno reported that 53% of their extreme duration patients had no major complications, especially retinopathy (19). Oakley et al demonstrated a low prevalence of neuropathy (15/92) in a group of patients with T1DM for 40 years (4), which was attributed to a lack of obesity, but not glycaemic control. Indeed in the present study we find no association between glycaemic control and neuropathy, instead the medallists were shorter, smoked less cigarettes and had a higher HDL.

The Golden Years Study involves ~400 participants and has shown that although 35.7% of participants had an elevated urinary albumin creatinine ratio, they had a low risk of significant renal deterioration and only 43% had previously undergone laser photocoagulation for diabetic retinopathy (6), however neuropathy was not assessed. This cohort had a normal body mass, low insulin dose and elevated HDL. Similarly, the Joslin Medallist study has reported that 46.8% had no significant microvascular complications (8), but no objective assessment of neuropathy was undertaken. A more recent study reported that 42.6% of medallists remain free from proliferative diabetic retinopathy, 86.9% from nephropathy and 39.4% from neuropathy, which was evaluated using the Michigan

Screening Instrument, a relatively crude clinical measure of neuropathy (7). They also undertook a longitudinal assessment of retinopathy and showed that in those without proliferative diabetic retinopathy, 96% had no evidence of retinopathy progression over a 17-year follow up. However, these studies have lacked detailed phenotyping, particularly of neuropathy and therefore cannot define the true degree of protection from this complication. This is important given that the burden of microvascular complications has recently been shown to predict cardiovascular outcomes better than glucose, blood pressure and lipid control, which was attributed to autonomic neuropathy (16).

This the first study to undertake objective assessment of large and small fibre neuropathy in a medallist cohort. The Steno group compared survivors of more than 40 years of T1DM to those who died within 35 years (19). In the present study we have compared medallists with specific groups of patients with T1DM representing extreme phenotypes, such that the SPK group has already developed ESRF requiring transplantation, despite a much shorter duration of diabetes and the T1DM comparator represents patients with a comparable duration of diabetes to the SPK group, but without overt complications.

Previous studies in medallists have shown that a higher HDL was associated with protection from the development of retinopathy and nephropathy (6), and higher triglycerides and insulin requirement (8) with lower residual insulin production (20) were found in medallists with microvascular complications. In the present study HDL is higher, whilst total cholesterol and triglycerides were comparable between medallists and the other groups of patients. Insulin increases the activity of lipoprotein lipase and decreases serum triglycerides in patients with T1DM (21), hence HDL may be higher because of an elevated lipoprotein lipase/hepatic lipase ratio and higher HDL has been shown to protect against the development of albuminuria (22).

Tight glycaemic control can prevent the development and progression of microvascular complications (2, 3). The Epidemiology of Diabetes Interventions and Complications (EDIC) and Diabetes Control and Complications Trial (DCCT) (2) showed a lower incidence of diabetic neuropathy in patients who were in the intensive glycaemic control arm. The medallists in the present study developed

Type 1 DM at a time when tight glycaemic control was not standard practice and will have been difficult to achieve. So it is unlikely that this group had 'good' preceding glycaemic control and indeed there were no differences in HbA1c between the medallists and patients undergoing SPK.

The EURODIAB study identified age, duration of diabetes, glycaemic control, height, the presence of background or proliferative retinopathy, cigarette smoking and HDL to predict the development of neuropathy in a cohort of patients with T1DM (23). Indeed in the present study we have shown that patients awaiting SPK had worse small fibre neuropathy and were taller and had a lower HDL.

This is the first longitudinal study to undertake detailed phenotyping of neuropathy in a cohort of medallists and shows that there is no progression of neuropathy over 36 months. This is in contrast to the cohort of patients with T1DM with a shorter duration of diabetes who showed a worsening of small fibre neuropathy, identified using CCM, but no change in neurophysiology and other quantitative sensory tests. We have previously shown that CCM has comparable sensitivity and specificity to IENFD in identifying peripheral neuropathy (13) and can predict the development of diabetic neuropathy (24), and more recently foot ulceration and Charcot foot (25). In contrast the SPK group showed an improvement in CCM, supporting our previous studies showing small nerve fibre regeneration at 6 (26) and 12 (27) months following SPK, after continuous subcutaneous insulin infusion (28) and after treatment with ARA 290 (29). Additionally we now show an improvement in peroneal nerve conduction velocity and the neuropathy symptom profile at 36 months, which are important patient outcomes in clinical trials of diabetic neuropathy (30).

Medallists with extreme duration Type 1 Diabetes appear to be partially protected from the development and progression of diabetic neuropathy, particularly affecting the small fibres. Corneal confocal microscopy is an ophthalmic imaging technique, which could act as a surrogate end point for assessing nerve degeneration and regeneration in clinical trials of diabetic neuropathy.

5.7   References

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6.   Chapter VI - Small Fibre Neuropathy In Patients With Type 1 Diabetes And Erectile Dysfunction

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6.0   Abstract

To identify the contribution of small and large fibre neuropathy to erectile dysfunction (ED) in patients with type 1 diabetes mellitus.

70 patients (29 without ED and 41 with ED) with type 1 diabetes and 34 age-matched controls underwent a comprehensive assessment of large and small fibre neuropathy.

The prevalence of ED in patients with type 1 diabetes was 59.4%. After adjusting for age, participants with type 1 diabetes and ED had a significantly higher neuropathy symptom profile (5.33±0.89 v 1.82±1.15, P=0.03) and vibration perception threshold (18.25±1.89 v 10.70±2.43, P=0.02) with a lower sural nerve amplitude (5.04±1.11 v 11.67±1.53, P=0.002), peroneal nerve amplitude (2.11±0.36 v 4.68±0.5, P<0.0001) and peroneal nerve conduction velocity (34.84±1.45 v 41.92±2.01, P=0.01) compared to those without ED. There was also evidence of a marked small fibre neuropathy (SFN) with impaired cold threshold (19.68±1.4 v 27.34±1.79, P=0.003), warm threshold (42.93±0.76 v 38.98±0.92, P=0.005), heart rate variability (21.46±3.08 v 29.95±3.72, P=0.001) and reduced intra-epidermal nerve fibre density (2.82±0.7 v 5.94±0.74, P=0.008), corneal nerve fibre density (12.58±1.5 v 23.94±2.01, P<0.0001), corneal nerve branch density (12.65±2.46 v 31.63±3.31, P<0.0001) and corneal nerve fibre length (8.30±0.71 v 14.52±0.96, P<0.0001). ED correlated significantly with measures of both large and small fibre neuropathy.

SFN is prominent, associated with ED and can be objectively quantified using corneal confocal microscopy (CCM) in patients with type 1 diabetes. Identification of these patients may allow us to identify those less likely to respond to conventional therapies such as phosphodiesterase type 5 inhibitors and who should therefore be considered for daily or higher doses, combinations or indeed alternative therapies such as intraurethral alprostadil or penile prosthesis.

6.1   Introduction

Erectile Dysfunction (ED) in patients with Type 1 diabetes mellitus poses a major clinical problem and was associated with poorer diabetes related quality of life in the DCCT/EDIC cohort, particularly in those with other complications including neuropathy (1). It is principally mediated by impaired cavernosal vasodilatation due to a defect in non-adrenergic-non-cholinergic nerve signalling, penile endothelial dysfunction and venocclusive disease; however, the relative contributions of each may differ between type 1 and type 2 diabetes (2).

Earlier reports focused primarily on patients with type 2 diabetes and ED and demonstrated abnormalities in quantitative sensory testing (QST) and sympathetic skin responses (3-7). More recent studies in patients with type 1 diabetes from the DCCT and EDIC cohorts have shown that cardiovascular autonomic neuropathy predicts the development of erectile dysfunction and peripheral neuropathy (DPN) is a major risk factor for ED (8, 9). Furthermore, immediate or delayed failure of therapy for ED has been attributed to severe erectile dysfunction at presentation, worsening of endothelial dysfunction and the presence of a significant neuropathy (10, 11). The relative contribution of the different underlying mechanisms for failed treatment of ED may differ, arguing for a more precise diagnostic and tailored therapeutic approach.

QST can identify small fibre neuropathy, however, the subjective nature and high variability has limited wider use and indeed the Neuropathic Pain Specialist Interest Group consensus statement on QST cautions on the interpretation of results in relation to the clinical context (12). More objective measures of small fibre neuropathy include skin biopsy with assessment of intra-epidermal nerve fibre density (IENFD) (15), but this procedure is invasive, requires considerable laboratory expertise for analysis and has not been evaluated in patients with ED. Corneal confocal microscopy (CCM) is a rapid non-invasive ophthalmic examination technique, which objectively evaluates small fibre neuropathy in patients with diabetes (13, 14) and is comparable to skin biopsy in the diagnosis of diabetic neuropathy (15, 16). We have undertaken a comprehensive assessment of small and large fibre neuropathy, particularly focusing on small fibre neuropathy

evaluated using a comprehensive battery of tests including autonomic function, skin biopsy and CCM in a group of men with type 1 diabetes with and without ED.

6.2 Methods

6.2.1 Selection of patients

We assessed 70 patients with type 1 diabetes from the Central Manchester University Hospital Diabetes Centre and 34 age matched control participants. Exclusion criteria were any history of neuropathy due to a non-diabetic cause, current or active diabetic foot ulceration, and any history of corneal trauma or surgery, or history of ocular disease or systemic disease that may affect the cornea. The Central Manchester Research and Ethics Committee approved this study and written informed consent was obtained from all subjects prior to participation. This research adhered to the tenets of the declaration of Helsinki.

6.2.2 Erectile Dysfunction

Patients were assessed using the Neuropathy Symptom Profile Questionnaire (NSP), which specifically includes questions about sexual function (17).

6.2.3 Assessment of Neuropathy

All study participants underwent assessment of body mass index (BMI), blood pressure, HbA1c, lipid profile [total cholesterol, low density lipoprotein (LDL), high density lipoprotein (HDL) and triglycerides] and estimated glomerular filtration rate (eGFR). NSP was used to assess the symptoms of DPN. Neurological deficits were evaluated using the modified neuropathy disability score (NDS), which is comprised of vibration perception, pinprick, temperature sensation and presence or absence of ankle reflexes (18). Vibration perception threshold (VPT) was tested using a Neurothesiometer (Horwell, Scientific Laboratory Supplies, Wilfrod, Nottingham, UK). Cold (CT) and warm (WT) thresholds were established on the dorsolateral aspect of the left foot (S1) using the TSA-II NeuroSensory Analyser (Medoc Ltd., Ramat-Yishai, Israel). Electro-diagnostic studies were undertaken using a Dantec Keypoint system (Dantec Dynamics Ltd, Bristol, UK), equipped

with a DISA temperature regulator to keep limb temperature constantly between 32-35°C. Sural sensory nerve amplitude, sural sensory nerve conduction velocity, sural sensory nerve latency, peroneal motor nerve amplitude, peroneal motor nerve latency and peroneal motor nerve conduction velocity 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 was taken using antidromic stimulation over a distance of 100mm. Heart rate variability (HRV) was assessed with an ANX 3.0 autonomic nervous system monitoring device (ANSAR Medical Technologies Inc., Philadelphia, PA, USA).

6.2.4 Skin Biopsy

A 3mm punch skin biopsy was taken from the dorsum of the foot, approximately 2 cm above the second metatarsal head under local anaesthesia (1% lidocaine). 50 μm sections were stained using anti-human PGP 9.5 antibody (Abcam, Cambridge, U.K) and nerve fibres were demonstrated using SG chromogen (Vector Laboratories, Peterborough, U.K). IENFD was quantified in accordance with established criteria and expressed as number per millimetre (16).

6.2.5 Corneal Confocal Microscopy

Patients underwent examination with the CCM (Heidelberg Retinal Tomograph III Rostock Cornea Module, Heidelberg Engineering GmbH, Heidelberg, Germany) as per our previously established protocol (19). Six non-overlapping images/patient (3 per eye) from the centre of the cornea were selected and quantified in a masked fashion. Three corneal nerve parameters were quantified: Corneal nerve fibre density (CNFD) - the total number of major nerves/mm^2 of corneal tissue, Corneal nerve branch density (CNBD) - the number of branches emanating from the major nerve trunks/mm^2 of corneal tissue and Corneal nerve fibre length (CNFL) - the total length of all nerve fibres and branches (mm/mm^2) within the area of corneal tissue. Analysis of corneal nerve morphology was performed using automated software (ACCMetrics) (20).

6.3   Statistical Analysis

Analysis was carried out on SPSS for Mac (Version 19.0, IBM Corporation, New York, USA). All data are expressed as mean ± standard error of mean (SEM). The data was tested for normality by using the Shapiro Wilk Normality test and by visualising the histogram and normal Q-Q plot. To assess within and between group differences we used one-way analysis of variance (non-parametric – Kruskal – Wallis). In comparison between type 1 diabetes with and without ED the analysis of variance (ANCOVA) was used for age adjustment.   A significant p value was considered to be <0.05 (post hoc – Tukey).

6.4   Results

6.4.1 Control vs Type 1 Diabetes (Tables 6.1 and 6.2)

The control group were age matched to those with type 1 diabetes (45.4±2.6 v 46.2±1.7, P=0.77). The prevalence of ED in patients with type 1 diabetes was 59.4% and 5.9% in age matched control subjects. There was no difference in BMI, blood pressure, smoking and alcohol consumption between the two groups. Subjects with type 1 diabetes had a significantly higher HbA1c (7.7±0.2 (58.9±2.1) v 5.6±0.1 (38.0±0.7), P<0.0001) and a lower cholesterol (4.2±0.1 v 5.1±0.1, P<0.0001) and LDL (2.1±0.1 v 2.1±0.1, P<0.0001).

Patients with type 1 diabetes had a significantly higher NSP (3.9±0.7 v 0.2±0.1, P<0.0001), NDS (3.6±0.4 v 0.7±0.2, P<0.0001) and VPT (16.4±1.6 v 6.2±0.9, P<0.0001) and lower sural sensory nerve amplitude (7.5±1.0 v 17.9±1.5, P<0.0001) and velocity (39.7±1.1 v 49.0±0.6, P<0.0001) and peroneal amplitude (3.1±0.4 v 6.2±0.3, P<0.0001) and velocity (37.5±1.2 v 48.8±0.7, P<0.0001) compared to controls. Patients with type 1 diabetes had a significantly higher warm perception threshold (41.4±0.6 v 37.6±0.7) and a significantly lower cold perception threshold (22.7±1.0 v 28.2±0.4, P<0.0001), HRV (25.1±2.4 v 31.0±2.2, P<0.0001), IENFD (4.3±0.5 v 10.5±0.7, P<0.0001), CNFD (16.9±1.2 v 30.1±1.2, P<0.0001), CNBD (19.8±2.0 v 37.1±2.7, P<0.0001) and CNFL (10.7±0.6 v 17.1±0.6, P<0.0001), compared to controls (Figures 6.1 and 6.2).

6.4.2 Type 1 Diabetes Participants with ED vs Without ED (Tables 6.1 and 6.2)

Type 1 diabetes participants without ED were younger than those with ED (41.78±2.3 v 57.05±1.85) (Table 6.1). There was no difference in blood pressure, BMI, HbA1c and lipid profile, but eGFR was significantly lower (P<0.0001) in patients with ED. After adjusting for age, both groups had a comparable HbA1c. Type 1 diabetes patients with ED had a higher NSP (5.33±0.89 v 1.82±1.15, P=0.03) and VPT (18.25±1.89 v 10.70±2.43, P=0.02) with a lower sural nerve amplitude (5.04±1.11 v 11.67±1.53, P=0.002), peroneal nerve amplitude (2.11±0.36 v 4.68±0.5, P<0.0001) and peroneal nerve conduction velocity (34.84±1.45 v 41.92±2.01, P=0.01) compared to patients without ED.

WT (42.93±0.76 v 38.98±0.92, P=0.005) was higher, whilst CT (19.68±1.4 v 27.34±1.79, P=0.003), DB-HRV (21.46±3.08 v 29.95±3.72, P=0.001), IENFD (2.82±0.7 v 5.94±0.74, P=0.008), CNFD (12.58±1.5 v 23.94±2.01, P<0.0001), CNBD (12.65±2.46 v 31.63±3.31, P<0.0001) and CNFL (8.30±0.71 v 14.52±0.96, P<0.0001) were all significantly lower patients with ED compared to patients without ED (Figure 6.1 and 6.2).

ED correlated significantly with NSP (r=0.561, P<0.0001), NDS (r=0.452, P<0.0001), VPT (r=0.619, P<0.001), CT (r=-0.488, P<0.0001), WT (r=0.496, P<0.0001), sural amplitude (r=-0.655, P<0.0001), sural velocity (r=-0.548, P<0.0001), peroneal amplitude (r=-0.685, P<0.0001), peroneal velocity (r=-0.635, P<0.0001), IENFD (r=-0.603, P<0.0001), CNFD (r=-0.641, P<0.0001), CNBD (r= -0.552, P<0.0001), and CNFL (r=-0.657, P<0.0001).

There was no correlation between ED and BMI (r=-0.011, P=0.926), BP (r=0.025, P=0.828 / r=-0.004, P=0.975), HbA1c (r=-0.174, P=0.169), cholesterol (r=0.020, P=0.874), HDL (r=-0.051, P=0.689), LDL(r=0.001, P=0.994) or triglycerides (r=-0.004, P=0.978).

	Control (n=34)	Type 1 diabetes no ED (n=29)	Type 1 diabetes with ED (n=41)	P=
Age (years)	45.4±2.6	41.78±2.3	57.05±1.85	0.7
Blood Pressure (mmHg)	136.9±3.0±75.2±1.8	133±3.1/70.5±1.9	139±3.9/73.2±1.5	0.8/0.7
HbA1C (IFCC)	5.6±0.1(38.0±0.7)*	7.97±0.34	7.56±0.28	0.7
Duration of Diabetes (years) $	N/A	28.78±2.29	28.05±1.82	0.8
BMI (Kg/m2)	26.4±0.6	26.84±0.88	26.49±0.67	0.7
eGFR (ml/min/l)	85.2±1.2	87.44±1.39	66.61±3.73	<0.0001
Smoking (cigarettes per day)	0.3±0.3	0.87±0.6	1.24±0.73	0.4
Alcohol (units per week)	6.9±1.9	3.75±1.43	7.21±1.89	0.4
Cholesterol (mmol/l)	5.1±0.1 *	4.16±0.18	4.14±0.15	0.8
HDL (mmol/l)	1.4±0.1	1.49±0.08	1.5±0.07	0.6
Triglycerides (mmol/l)	1.5±0.1^	1.26±0.16	1.21±0.11	0.9
LDL (mmol/l)	2.9±0.1*	2.11±0.17	2.09±0.11	0.9
Erectile Dysfunction (yes %)	5.9	59.4		

Table 6-1. Background demographic factors and clinical parameters for control v type 1 diabetes mellitus with erectile dysfunction (ED) vs type 1 diabetes without ED. $ adjusted for age using analysis of covariance (Ancova), *P<0.001, ^P<0.05, +P<0.005 control vs type 1 diabetes mellitus.

	Control (n=34)	Type 1 diabetes no ED (n=29)	Type 1 diabetes with ED (n=41)	P=
Neuropathy Symptom Profile$^\$$	0.2±0.1*	1.82±1.15	5.33±0.89	0.03
Neuropathy Disability Score$^\$$	0.7±0.2*	2.80±0.68	4.13±0.55	0.1
Vibration Perception Threshold (V)$^\$$	6.2±0.9*	10.70±2.43	18.25±1.89	0.02
Left Sural Latency (ms)$^\$$	2.9±0.0*	3.34±0.19	3.82±0.14	0.08
Left Sural Amplitude (mV)$^\$$	17.9±1.5*	11.67±1.53	5.04±1.11	0.002
Left Sural Velocity (m/s)$^\$$	49.0±0.6*	42.63±1.92	37.91±1.39	0.07
Left Peroneal Latency (ms)$^\$$	4.2±0.1*	4.67±0.39	5.57±0.29	0.09
Left Peroneal Amplitude (mV)$^\$$	6.2±0.3*	4.68±0.5	2.11±0.36	<0.0001
Left Peroneal Velocity (m/s)$^\$$	48.8±0.7*	41.92±2.01	34.84±1.45	0.01
Cold Perception Threshold (^{o}C)$^\$$	28.2±0.4*	27.34±1.79	19.68±1.4	0.003
Warm Perception Threshold (^{o}C) $^\$$	37.6±0.7*	38.98±0.92	42.93±0.76	0.005
IENFD (no./mm)$^\$$	10.5±0.7*	5.94±0.74	2.82±0.7	0.008
CNFD (no./mm^2)$^\$$	30.1±1.2*	23.94±2.01	12.58±1.5	<0.0001
CNBD (no./mm^2)$^\$$	37.1±2.7*	31.63±3.31	12.65±2.46	<0.0001
CNFL (mm/mm^2)$^\$$	17.1±0.6*	14.52±0.96	8.30±0.71	<0.0001
HRV$^\$$	31.0±2.2*	29.95±3.72	21.46±3.08	0.001

Table 6-2. Neuropathy assessments for control v type 1 diabetes mellitus with erectile dysfunction (ED) vs Type 1 diabetes without ED. $ adjusted for age using analysis of covariance (Ancova), *P<0.001, ^P<0.05, +P<0.005, control vs type 1 diabetes mellitus.
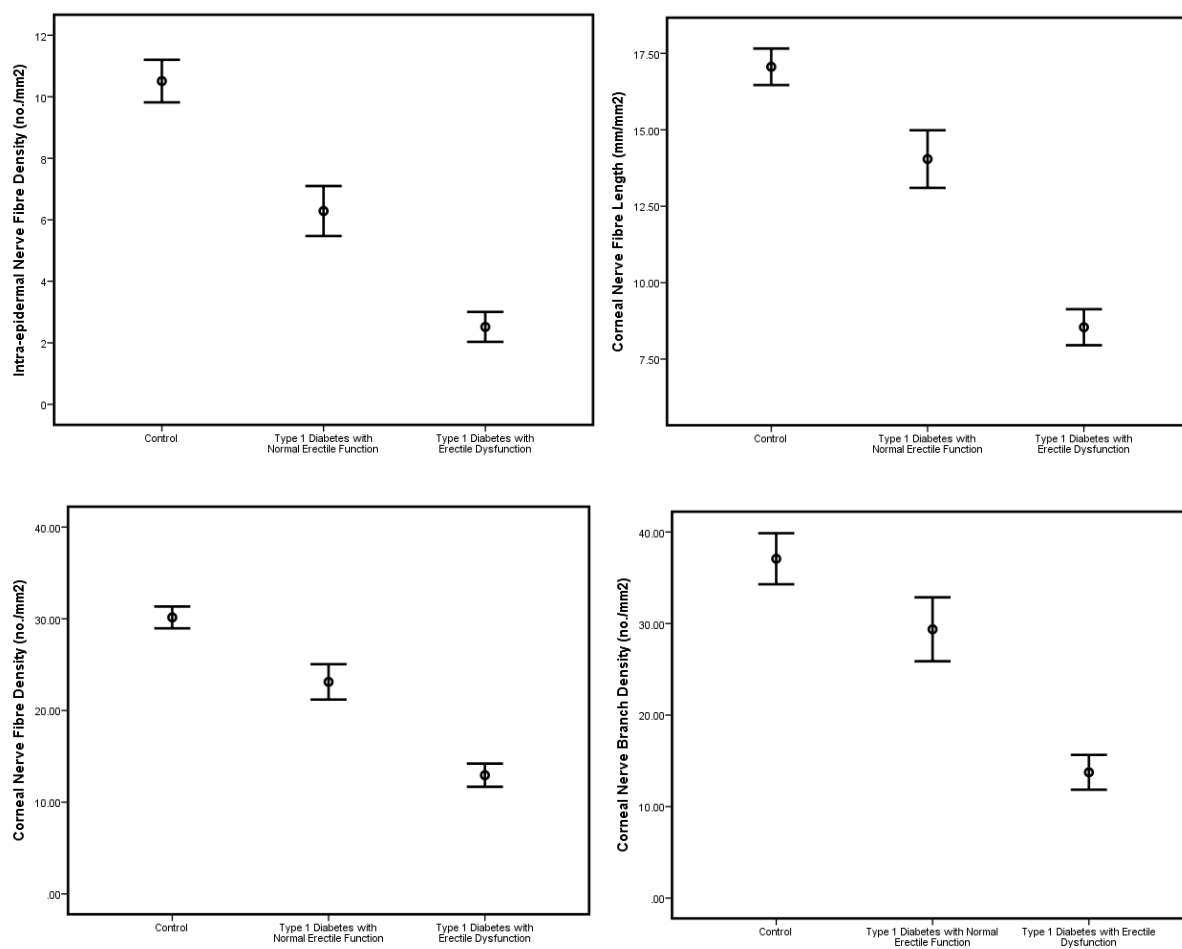
Figure 6-1. IENFD and CCM data from control subjects, T1DM with normal erectile function and T1DM with ED. Data are expressed as mean ± SEM.
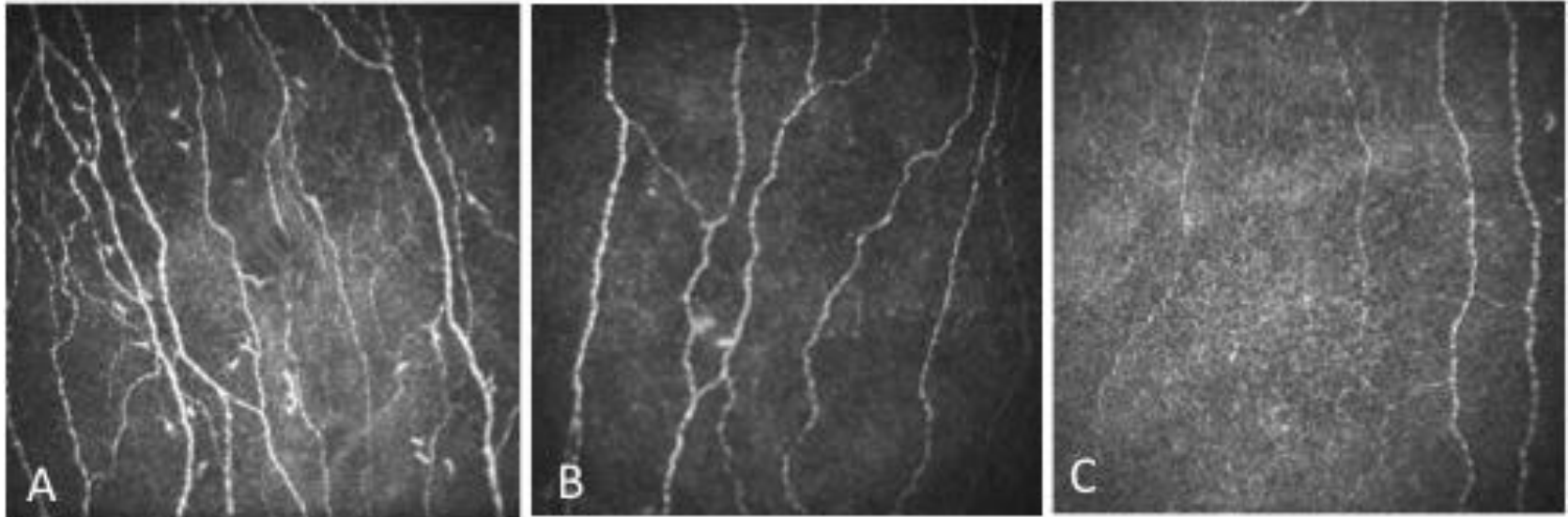
Figure 6-2. CCM images of corneal sub-basal nerves of; A) a healthy subject; B) a subject with T1DM no ED; C) a subject with T1DM and ED.

6.5 Discussion

We have demonstrated a high prevalence of ED in patients with type 1 diabetes, with evidence of large and particularly small fibre and autonomic neuropathy in those with ED. The majority of prevalence studies of ED have not distinguished between type 1 and type 2 diabetes, and have in fact focused primarily on patients with type 2 diabetes (21). The UroEdic study showed that 55% of men with type 1 diabetes had decreased libido and 34% suffered from ED (22). The self-reported prevalence of ED in a group of men with Type 1 DM aged 43 years or older was 47.1% (23). Age and duration of diabetes affect the prevalence of ED (24), and of course differences in methodology to diagnose ED and population characteristics also leads to the variability in the reported prevalence rates of 35-75%(21, 24). Whilst the duration of diabetes, poor glycaemic control, hypertension, hyperlipidaemia and obesity have been associated with ED in type 2 diabetes (25), our study in type 1 diabetes did not find a correlation between ED and HbA1c, BMI, hypertension or duration of diabetes.

The most significant correlations are with age and the presence of symptomatic peripheral and autonomic neuropathy (23, 24). Despite this in patients with ED vascular function is commonly assessed, and neuropathy less so. Indeed in patients with diabetic polyneuropathy, there is impairment of sensory impulses from the shaft and glans of the penis to the reflexogenic erectile centre and impaired pudendal nerve innervation of the pelvic floor muscles, limiting the contraction of the bulbo-cavernous and ischio-cavernous muscles, which normally contribute to the reduction in venous outflow from the cavernous bodies and maintain an erection (21). Furthermore, as parasympathetic activity is involved in achieving an erection, autonomic neuropathy is strongly associated with ED (21). Nitric Oxide (NO) plays a key role in maintaining penile erection (26) and is synthesised and released via the endothelium and autonomic nerves of the penile arteries and corpus cavernosum (26). Certain populations are less responsive to phosphodiesterase type 5 inhibitor (PDE5-I) therapy, which is the first line in management of ED (27), these include patients with diabetes and presumed more severe neuropathy, severe neurological damage from procedure such as radical prostatectomy and severe vascular disease (11, 27). PDE5-I's require a minimum

amount of NO production, which will not be synthesised by severely damaged nerves. It has been suggested that therapeutic strategies to promote NO synthesis and availability may improve erectile function and if used in combination with PDE5-I may make them more effective in patients who are less responsive (27).

A large study of 341 patients with ED reported peripheral neuropathy in 38% of patients with diabetes and 10% of non-diabetic patients using NCS and QST (4). The majority of patients in this report found to have a vascuolgenic basis for ED, based on the nocturnal tumescence test had neuropathy (4). Similarly others have found impaired thermal thresholds, capsaicin-induced sensory axon-reflex vasodilatation and sural nerve amplitude in patients with ED (5, 6). Blaeustein et al undertook QST of the penis and showed that non-diabetic patients with ED had impaired thermal and vibration perception thresholds and also showed that patients with type 1 diabetes and ED had a large and small fibre neuropathy (7). This is consistent with our findings of a significant large and small fibre neuropathy in patients with type 1 diabetes with ED compared to patients without ED and control subjects. More specific neurological evaluation for ED includes the bulbocavernous reflex, penile thermal sensory thresholds, the corpus cavernosum electromyogram and somatosensory evoked potentials, which are highly specialised and lack reproducibility with no age adjusted normal values to aid in diagnosis. However, the central role of small fibre dysfunction is evidenced by the strong correlation between penile thermal sensory testing and clinical evaluation of erectile dysfunction (28) and the lack of correlation between neurophysiology and severity of ED determined using the International Index of Erectile Function (8).

We demonstrate widespread autonomic and small fibre damage as evidenced by a reduction in IENFD in foot skin biopsies and corneal nerve fibre abnormalities using CCM in subjects with type 1 diabetes and ED. Indeed we have previously demonstrated the very high sensitivity and specificity for identifying diabetic autonomic neuropathy using CCM (29). Furthermore, IENFD and CCM abnormalities correlated with ED. Because IENFD is invasive, it is not practical to deploy this in the diagnostic workup of patients with ED. Alternatively, CCM is a non-invasive objective method to quantify small nerve fibre damage, using an unbiased automated image analysis technique (30, 31) which correlates with IENFD (32), and in the present study correlates significantly with ED.

The diagnosis and management of ED in patients with diabetes is challenging with a greater failure rate of therapies for ED (33). The identification of more extensive small fibre damage using CCM may allow us to identify those patients with ED, who are less likely to respond to conventional therapies such as PDE5-I and who should therefore be considered for daily or higher doses, combinations or indeed alternative therapies such as intraurethral alprostadil or penile prosthesis (34, 35).

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7. Chapter VII - The Effect of Obesity on Neuropathy

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7.1 Abstract

Obese subjects have risk factors for the development of neuropathy. BMI, impaired glucose tolerance, hypertriglyceridemia, and waist circumference have all been linked to neuropathy. The mechanisms by which obesity causes neuropathy need to be further investigated.

31 with morbid obesity awaiting bariatric surgery and 31 age matched control participants underwent comprehensive neuropathy assessment and laboratory blood tests for lipid, lipoprotein, vascular and inflammation markers.

Obese subjects had a significantly higher BMI (49.3±1.4 v 27.6±0.8, P<0.0001), and waist circumference (133.3±2.9 vs 92.5±2.6, P<0.0001). They also had a lower cholesterol (4.3±0.2 v 5.1±0.2, P=0.002), HDL (1.0±0.01 v 1.4±0.1, P<0.0001), PON1 (69.7±13.5 v 175.3±18.9, P<0.0001), ApoA1 (139.2±4.6 v 164.6±6.2, P=0.002) and ApoB (75.0±4.1 v 89.8±4.0, P=0.006) with a higher ICAM (190.3±10.3 v 156.1±9.1, P=0.001), VCAM (424.4±20.0 v 349.6±11.3, P=0.006), SAA (102.2±7.2 v 46.1±8.8, P<0.0001), CRP (7.6±0.9 v 3.4±0.7, P=0.001), IL-6 (9.2±3.4 v 3.9±1.5, P=0.002) and cystatin-c (0.9±0.01 v 0.7±0.01, P<0.0001) compared to control subjects.

Obese participants had a significantly higher NSP (4.3±1.1 v 0.5±0.2, P<0.0001), VPT (11.7±1.7 v 5.1±0.6, P<0.0001) and WT (40.4±0.6 v 37.1±0.5, P<0.0001) with a lower CT (25.6±1.1 v 28.2±0.5, P=0.018). They had a significantly lower sural amplitude (11.3±2.1 v 23.2±1.9, P<0.0001), peroneal amplitude (3.8±0.5 v 5.7±0.4, P=0.006), HRV (23.7±3.2 v 43.3±9.8, P=0.027), CNFD (26.0±1.0 v 39.5±1.1, P<0.0001), CNBD (58.6±4.8 v 104.6±6.8, P<0.0001), and CNFL (18.4±0.8 v 28.7±0.9, P<0.0001) compared to control subjects. Multiple regression analysis showed BMI (r=-0.605, P=0.029) to be significantly associated with CNFL, but not HDL, HbA1c or waist circumference (F (4,40)= 9.580, P<0.0001).

Subjects with morbid obesity were sub-divided into those with and without small fibre neuropathy based on CNFL less than 2 standard deviations of the control CNFL. Obese subjects with small fibre neuropathy were found to have significantly higher triglycerides (1.7±0.3 v 1.1±0.2, P=0.02), PCSK9 (1076.5±61.4 v 856.2±133.9, P=0.043) and 3-NT (108.7±5.2 v 83.2±6.2, P=0.011) with a lower

PON1 (36.4±12.7 v 83.2±20.5, P=0.024). PCSK9 correlated significantly with CNFL (r=-0.564, r=0.018) and multiple regression analysis showed that 3-NT was significantly associated with CNFL (r=-0.898, P=0.05).

Obese subjects with normoglycaemia have a significant small fibre neuropathy. There are a number of underlying metabolic abnormalities, which warrant further study, as they may provide insights into the mechanisms of diabetic neuropathy.

7.2 Introduction

Obesity is a worldwide epidemic with 1.9 billion overweight adults and over 600 million obese individuals in 2014 (1). This is a major public health challenge placing an economic burden on health systems with overweight and obesity being the fifth leading cause for global deaths (2). For patients the consequences include increased disability and an impaired quality of life along with a higher risk of developing type 2 diabetes, cardiovascular disease, stroke, dyslipidaemia and musculoskeletal disease (3).

In patients with diabetes, peripheral neuropathy (PN) is associated with increased rates of foot ulceration (4). As 80% of amputations are preceded by foot ulceration, an effective means of treating neuropathy would have a major medical, social and economic impact (5). There are currently no FDA approved treatments for the management of PN and hence identification of at risk individuals is paramount. The United Kingdom Prospective Diabetes Study showed that 5-7% of patients with type 2 diabetes mellitus already had PN at the time of diagnosis (6). Hence, the pathogenesis of PN is multifactorial and not limited to hyperglycaemia. Body mass index (BMI), impaired glucose tolerance (IGT), hypertriglyceridemia, and waist circumference, a part of the metabolic syndrome, have been associated with neuropathy (7-10). Symptomatic distal symmetrical polyneuropathy is more common in metabolic syndrome, independent of glycaemic status (11).

The mechanisms by which obesity can cause neuropathy therefore warrant study. We have undertaken a comprehensive assessment of large and small fibre neuropathy in obese patients awaiting bariatric surgery. We have further evaluated possible vascular and inflammatory markers as well as lipoproteins to provide insights into the pathogenesis of PN in subjects with morbid obesity.

7.3   Research Design and Methods

7.3.1   Selection of patients

62 subjects; 31 with morbid obesity awaiting bariatric surgery and 31 age matched control participants were studied. The obese subjects were recruited from the obesity clinic at Salford Royal Hospital and the control group were recruited from members of staff at the University of Manchester and their acquaintances. Exclusion criteria were any history of neuropathy due to a non-diabetic cause, any history of corneal trauma or surgery, or history of ocular disease or systemic disease that may affect the cornea. This study was approved by the Central Manchester Research and Ethics Committee and written informed consent was obtained from all subjects prior to participation. This research adhered to the tenets of the declaration of Helsinki.

7.3.2   Blood Pressure and Anthropometric Measurements

All patients underwent measurement of blood pressure, BMI and waist circumference.

7.3.3   Neuropathy Assessments

Symptoms of DPN were assessed using the neuropathy symptom profile (NSP). Neurological deficits were evaluated using the modified NDS which is comprised of vibration perception, pin-prick, temperature sensation and presence or absence of ankle reflexes (12). Vibration perception threshold (VPT) was tested using a Neurothesiometer (Horwell, Scientific Laboratory Supplies, Wilfrod, Nottingham, UK). Cold (CT) and warm (WT) thresholds were assessed on the dorsolateral aspect of the left foot (S1) using the TSA-II NeuroSensory Analyser (Medoc Ltd., Ramat-Yishai, Israel).

7.3.4 Nerve Conduction Studies

Electro-diagnostic studies were undertaken using a Dantec "Keypoint" system (Dantec Dynamics Ltd, Bristol, UK) equipped with a DISA temperature regulator to keep limb temperature constantly between 32-35°C. Sural sensory nerve amplitude (SNAP), sural sensory nerve conduction velocity (SSNCV), Sural sensory nerve latency, peroneal motor nerve amplitude (PMNA), Peroneal motor nerve latency and peroneal motor nerve conduction velocity (PMNCV) 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 was taken using antidromic stimulation over a distance of 100mm.

7.3.5 Heart Rate Variability

Heart rate variability (HRV) was assessed with an ANX 3.0 autonomic nervous system monitoring device (ANSAR Medical Technologies Inc., Philadelphia, PA, USA).

7.3.6 Corneal Confocal Microscopy

Patients underwent examination with a CCM (Heidelberg Retinal Tomograph III Rostock Cornea Module, Heidelberg Engineering GmbH, Heidelberg, Germany) as per our previously established protocol (13). Six non-overlapping images/patient (3 per eye) from the centre of the cornea were selected and quantified in a masked fashion. Three corneal nerve parameters were quantified: Corneal nerve fibre density (CNFD) - the total number of major nerves/mm^2 of corneal tissue, Corneal nerve branch density (CNBD) - the number of branches emanating from the major nerve trunks/mm^2 of corneal tissue and Corneal nerve fibre length (CNFL) - the total length of all nerve fibres and branches (mm/mm^2) within the area of corneal tissue. Automated analysis of corneal nerve morphology was performed using purposefully designed software (ACCMetrics) (14).

7.3.7 Laboratory Measurements

7.3.7.1   Glycated Haemoglobin (HbA1c)

HbA1c was measured by HPLC using a VARIANT II Turbo Hemoglobin Testing System (Bio-Rad Laboratories, Hemel Hempstead, UK) in the Department of Clinical Biochemistry at Central Manchester University Hospitals.

7.3.7.2   Total Cholesterol

3µl of sample was added to 20µl H_2O and 250µl reagent. After enzymatic hydrolysis by cholesterol esterase, cholesterol is oxidized by cholesterol oxidase. The released hydrogen peroxide reacts with 4-aminoantipyrine and phenol in the presence of peroxidase to form quinoneimine. The increase in absorption at 500 nm correlates with cholesterol concentration which was measured using Cobas Mira auto-analyzer (Horiba ABX- UK, Northampton, UK)

7.3.7.3   Triglyceride

3 µl of sample was added to 10 µl H_2O and 290 µl reagent. Oxidation by glycerol-3-phosphate oxidase releases hydrogen peroxide, which generates quinoneimine from 4-aminoantipyrine and phenol in the presence of peroxidase. The increase in absorbance at 500 nm correlates with the triglyceride concentration which is measured using Cobas Mira auto-analyzer (Horiba ABX- UK, Northampton, UK)

7.3.7.4   High-density lipoprotein (HDL) cholesterol

3 µl of sample was added to 50 µl H_2O, 250 µl of reagent 1 (N,N-Bis(2-hydroxyethyl)-2- aminoethanesulfonphonic acid, N-(2-hydroxy-3-Sulfopropyl)-3,5-dimethoxyaniline, sodium salt, cholesterol esterase, cholesterol oxidase, catalase and ascorbate oxidase), 83 µl of reagent 2 (N,N-Bis(2-hydroxyethyly)-2-aminoethanesulphonic acid, 4- aminoantipyrine, horse radish peroxidase, sodium azide and surfactants) and 12 µl H_2O. When oxygen is present, cholesterol is oxidized by cholesterol oxidase and generated hydrogen peroxide reacts with 4-aminoantipyrine and N-(2-hydroxy-3-sulphopropyl)-3,5-dimethoxyaniline. The

increase in absorbance at 600 nm correlates with the HDLholesterol concentration, which was measured using Cobas Mira auto-analyzer (Horiba ABX-UK, Northampton, UK).

7.3.7.5 Low-density lipoprotein (LDL) cholesterol

LDL levels were calculated using the Friedewald formula:

LDL = total cholesterol – HDL – Triglycerides/2.19

This formula is only accurate when serum triglycerides do not exceed 4.5 mmol/l.

7.3.7.6 Apolipoprotein B (ApoB)

13 µl of sample was added to 30 µl of H_2O, 200 µl of PBS polymer solution, 16.7 µl of anti- human apoB antibody and 53.3 µl of PBS. ApoB was measured immunoturbidimetrically. The immune complex formed was measured by turbidimetry where the signal generated correlates directly with the concentration of apoB in the sample. The signal generated was measured at 340 nm using Cobas Mira auto-analyzer (Horiba ABX-UK, Northampton, UK)

7.3.7.7 Apolipoprotein A-I (ApoAI)

7µl of sample was added to 60µl H_2O, 200µl of PBS Polymer solution, 23.3µl of purified immunoglobulins from rabbit antiserum (apoAI from human HDL immunogen) and 46.7 µl PBS. ApoAI was measured using an immunoturbidimetric assay adapted for the Cobas-Mira auto-analyzer. The immune complex formed is measured by turbidimetry with the signal generated at 340 nm after 10 and 15 minutes using Cobas Mira auto- analyzer (Horiba ABX-UK, Northampton, UK) correlating directly with the concentration of apoAI in the sample.

7.3.7.8 Oxidized LDL (OxLDL)

25 µl of each calibrator, control and diluted sample was put into coated plate wells and 100 µl of assay buffer added to each well   This was incubated on plate shaker

for 2h at room temperature. The reaction volume was discarded and 350 μl of wash buffer solution was added to each well. The solution was discarded and excess liquid removed using absorbent paper. 100 μl enzyme conjugate solution was added to each well. This was incubated on a plate shaker for 1h at room temperature. 200 μl 3.3',5,5'-tetramethylbenzidine was added and incubated for 15 minutes at room temperature. 50 μl Stop solution was added and the plate was put on shaker for 5 seconds. The optical density at 450 nm is read and the results calculated. The concentration of oxidized LDL was obtained by data reduction of the absorbance for the calibrators versus the concentration using cubic spline regression. The concentration of the samples was multiplied with the dilution factor

7.3.7.9 C-reactive protein (CRP)

CRP was measured by immunoturbidimetric assay. 2.5 μl of the sample was added to reaction buffer with CRP immunoparticles. The generated signal was measured at 340 nm after 10 and 15 minutes using Cobas Mira auto- analyzer (Horiba ABX-UK, Northampton, UK)

7.3.7.10 Cystatin C

The sample was added to the Cystatin Assay Buffer and Cystatin Antibody Reagent. The generated signal was measured at 570 nm after 10 and 15 minutes using Randox Daytona auto-analyser (Randox, Co. Antrim, UK)

7.3.7.11 Intercellular Adhesion Molecule 1 (ICAM-1)

This was measured using ELISA (R&D Systems Europe, Abingdon, UK) which measures ICAM-1, also known as CD54, a transmembrane protein that is upregulated on endothelial and epithelial cells at sites of inflammation.

7.3.7.12   Vascular Cell Adhesion Molecule 1 (VCAM-1)

VCAM-1 was measure using a kit (R&D Systems Europe, Abingdon, UK) which measures VCAM-1 (or CD106), a transmembrane molecule that mediates the adhesion of immune cells to the vascular endothelium during inflammation.

7.3.7.13   Interleukin 6 (IL-6)

Interleukin-6 measured by solid phase sandwich ELISA (R&D Systems Europe, Abingdon, UK).

7.3.7.14   Paraoxonase-1 (PON1) Activity

Serum PON-1 activity was determined by a semi-automated micro-titre plate method using paraoxon (O,O-Diethyl O-(4-nitrophenyl)phosphate as a substrate. The rate of generation of p- nitrophenol was determined at 25oC with the use of a continuously recording spectrophotometer at 405 nm using multiskan multisoft plate reader (Labsystems, Hampshire, UK). Activity was calculated as: PON1 activity (nmol / ml / min) = OD / min x 1390.7 x 1.714

7.3.7.15   Proprotein convertase subtilisin / kexin type 9 (PCSK9)

PCSK9 was measured using ELISA (R&D Systems Europe, Abingdon, UK) which was based on the antibody sandwich principle.

7.3.7.16   Serum Amyloid A (SAA)

SAA was measured using the human SAA solid-phase sandwich ELISA (ThermoFisher Scientific, Loughborough, UK).

7.3.7.17   3-Nitrotyrosine (3-NT)

3-NT was measured using quantitative sandwich ELISA (MyBioSource Inc. San Diego, CA, USA).

7.4   Statistical Analysis

Analysis was carried out on SPSS for Mac (Version 19.0, IBM Corporation, New York, USA). All data are expressed as mean ± standard error of mean (SEM). The data was tested for normality by using the Shapiro Wilk Normality test and by visualising the histogram and normal Q-Q plot. To assess within and between group differences we used one-way analysis of variance (non-parametric – Kruskal – Wallis). A significant p value was considered to be <0.05 (post hoc – Tukey).

7.5 Results

7.5.1 Demographic and anthropometric measurement

The obese and control cohorts were matched for age (46.2±16. vs 45.1±1.5, P=0.59). The obese group had a significantly higher BMI (49.3±1.4 v 27.6±0.8, P<0.0001), weight (136.9±4.8 vs 77.6±2.9, P<0.0001) and waist circumference (133.3±2.9 vs 92.5±2.6, P<0.0001) (Table 7.1).

7.5.2 Lipid, lipoprotein, vascular and inflammation markers

The obese group had a lower total cholesterol (4.3±0.2 v 5.1±0.2, P=0.002), HDL (1.0±0.01 v 1.4±0.1, P<0.0001), PON1 (69.7±13.5 v 175.3±18.9, P<0.0001), ApoA1 (139.2±4.6 v 164.6±6.2, P=0.002) and ApoB (75.0±4.1 v 89.8±4.0, P=0.006) with a higher ICAM (190.3±10.3 v 156.1±9.1, P=0.001), VCAM (424.4±20.0 v 349.6±11.3, P=0.006), SAA (102.2±7.2 v 46.1±8.8, P<0.0001), CRP (7.6±0.9 v 3.4±0.7, P=0.001), IL-6 (9.2±3.4 v 3.9±1.5, P=0.002) and cystatin-c (0.9±0.01 v 0.7±0.01, P<0.0001) but no difference in NT-3 or PCSK9 compared to control subjects (Table 7.2).

7.5.3 Neuropathy assessments

The obese group had a significantly higher NSP (4.3±1.1 v 0.5±0.2, P<0.0001), VPT (11.7±1.7 v 5.1±0.6, P<0.0001) and WT (40.4±0.6 v 37.1±0.5, P<0.0001) with a lower CT (25.6±1.1 v 28.2±0.5, P=0.018). They had a significantly lower sural amplitude (11.3±2.1 v 23.2±1.9, P<0.0001), peroneal amplitude (3.8±0.5 v 5.7±0.4, P=0.006), HRV (23.7±3.2 v 43.3±9.8, P=0.027), CNFD (26.0±1.0 v 39.5±1.1, P<0.0001), CNBD (58.6±4.8 v 104.6±6.8, P<0.0001), and CNFL (18.4±0.8 v 28.7±0.9, P<0.0001) compared to control subjects (Table 7.3).

7.5.4 Associations

BMI correlated significantly with CT (r=-0.409, P=0.002), WT (r=-0.516, P<0.0001), CNFD (r=-0.654, P<0.0001), CNBD (r=-0.622, P<0.0001) and CNFL (r=-0.725, P<0.0001). Waist circumference correlated significantly with CT (r=-0.516,

P<0.0001), WT (r=-0.590, P<0.0001), CNFD (r=-0.665, P<0.0001), CNBD (r=-0.324, P<0.0001) and CNFL (r=-0.756, P<0.0001). HDL correlated significantly with CNFD (r=0.456, P=0.001), CNBD (r=0.444, P=0.001), and CNFL (r=0.494, P=0.001). Multiple regression analysis showed BMI (r=-0.605, P=0.029) to be significantly associated with CNFL, but not HDL, HbA1c or waist circumference (F (4,40)= 9.580, P<0.0001).

	Control	Obese	P
Age (years)	45.1±1.5	46.2±1.6	NS
Smoking (no. per day)	0.6±0.4	1.3±0.9	NS
Alcohol (units per week)	2.8±1.1	1.5±0.9	NS
Height (cm)	167.1±1.9	166.9±1.8	NS
Weight (kg)	77.6±2.9	136.9±4.8	<0.0001
Waist circumference (cm)	92.5±2.6	133.3±2.9	<0.0001
BMI (Kg/m^2)	27.6±0.8	49.3±1.4	<0.0001
HbA1c (%)	5.6±0.1	5.6±0.1	NS
IFCC (mmol/mol)	37.4±0.7	37.9±1.1	NS
BP (systolic/diastolic mmHg)	127.5±3.7/73.3±1.6	129.8±3.6/72.6±1.9	NS
eGFR (ml/min/l)	83.9±1.8	81.8±4.4	NS

Table 7-1. Background demographic factors for control and obese participants.

	Control	Obese	P
Cholesterol (mmol/l)	5.1±0.2	4.3±0.2	0.002
Triglyceride	1.5±0.1	1.4±0.1	NS
HDL	1.4±0.1	1.0±0.0	<0.0001
LDL	2.9±0.2	2.6±0.2	NS
ApoAI (mg/dl)	164.6±6.2	139.2±4.6	0.002
ApoB (mg/dl)	89.8±4.0	75.0±4.1	0.006
Ox LDL (U/l)	39.5±2.3	38.6±2.1	NS
Cystatin C (mg/l)	0.7±0.0	0.9±0.0	<0.0001
PON1 activity (nmol/ml/min)	175.3±18.9	69.7±13.5	<0.0001
CRP (mg/l)	3.4±0.7	7.6±0.9	0.001
IL-6 (pg/ml)	3.9±1.5	9.2±3.4	0.002
ICAM (ng/ml)	156.1±9.1	190.3±10.3	0.001
VCAM (ng/ml)	349.6±11.3	425.4±20.0	0.006
SAA (ug/ml)	46.1±8.8	102.2±7.2	<0.0001
3-NT (umol/l)	86.6±6.7	90.7±5.0	NS
PCSK9 (ng/ml)	1003.6±82.8	1044.4±98.4	NS

Table 7-2. Lipid, lipoprotein, vascular and inflammation markers for control and obese participants.

	Control	Obese	P
NSP	0.5±0.2	4.3±1.1	<0.0001
NDS	0.4±0.1	1.6±0.4	NS
VPT (Volts)	5.1±0.6	11.7±1.7	<0.0001
Sural Latency (ms)	2.8±0.01	2.9±0.2	NS
Sural Amplitude (uV)	23.2±1.9	11.3±2.1	<0.0001
Sural Velocity (m/s)	51.2±0.9	49.1±2.0	NS
Peroneal latency (ms)	4.2±0.1	4.2±0.3	NS
Peroneal amplitude (m/s)	5.7±0.4	3.8±0.5	NS
Peroneal velocity (m/s)	49.5±0.7	46.6±1.2	NS
CT (^{O}C)	28.2±0.5	25.6±1.1	0.018
WT (^{O}C)	37.1±0.5	40.4±0.6	<0.0001
HRV (beats per min)	43.3±19.8	23.7±3.2	0.027
CNFD (no/mm^2)	39.5±1.1	26.0±1.0	<0.0001
CNBD (no/mm^2)	104.6±6.8	58.6±4.8	<0.0001
CNFL (mm/mm^2)	28.7±0.9	18.4±0.8	<0.0001

Table 7-3. Neuropathy assessments for control and obese participants.

7.5.5 Obese subjects with small fibre neuropathy

The subjects with morbid obesity were divided into those with and without small fibre neuropathy based on CNFL less than 2 standard deviations of the control CNFL (Table 7.4).

Obese subjects with small fibre neuropathy were found to have significantly higher triglycerides (1.7±0.3 v 1.1±0.2, P=0.02), PCSK9 (1076.5±61.4 v 856.2±133.9, P=0.043) and 3-NT (108.7±5.2 v 83.2±6.2, P=0.011) with a lower PON1 (36.4±12.7 v 83.2±20.5, P=0.024).

3-NT correlated significantly with CNFD (r=-0.735, P=0.001), CNBD (r=-0.479, P=0.05) and CNFL (r=-0.610, P=0.009) and PCSK9 correlated significantly with CNFL (r=-0.564, r=0.018).

Multiple Regression Analysis showed that 3-NT was significantly associated with CNFL (r=-0.898, P=0.05).

	Normal	SFN	P
BMI (kg/m^2)	48.9±1.9	49.4±2.4	NS
IFCC (mmol/mol)	38.4±1.8	37.3±1.5	NS
Cholesterol (mmol/l)	3.9±0.3	4.4±0.2	NS
Triglyceride (mmol/l)	1.1±0.2	1.7±0.3	0.02
HDL (mmol/l)	1.0±0.07	1.0±0.07	NS
LDL (mmol/l)	2.5±0.2	2.6±0.2	NS
ApoAI (mg/dl)	138.9±8.8	138.1±6.5	NS
ApoB (mg/dl)	66.8±4.1	76.4±7.8	NS
oxLDL (U/l)	35.2±2.9	40.2±2.8	NS
Cystatin C (mg/l)	0.9±0.06	0.9±0.02	NS
PON1 activity (nmol/ml/min)	83.2±20.5	36.4±12.7	0.024
CRP (mg/l)	6.8±1.6	7.7±1.1	NS
IL-6 (pg/ml)	14.8±7.6	4.8±0.9	NS
ICAM (ng/ml)	195.7±13.9	185.5±16.7	NS
VCAM (ng/ml)	461.7±31.9	393.9±24.9	NS
SAA (ug/ml)	109.1±8.5	91.1±12.4	NS
3-NT (umol/l)	83.2±6.2	108.7±5.2	0.011
PCSK9 (ng/ml)	856.2±133.9	1076.5±61.4	0.043

Table 7-4. BMI, lipid, lipoprotein, vascular and inflammation markers obese patients with and without small fibre neuropathy (SFN)

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7.6 Discussion

We report the presence of a significant neuropathy, particularly affecting the small fibres in patients with obesity awaiting bariatric surgery. Small nerve fibre neuropathy may predate large fibre neuropathy (15) and can occur in subjects with impaired glucose tolerance (16). Herman et al previously also reported significant small fibre dysfunction in morbidly obese patients (17). Large fibre involvement with a reduction in the amplitude of the tibial and peroneal nerves and decreased sensory amplitude has also been reported in obese participants (18). Thermal sensory thresholds have been related to hyperinsulinemia and reduced insulin sensitivity and BMI correlates with sensory and mixed nerve amplitudes but not nerve conduction velocity (19).

Most previous reports have studied obese patients with type 2 diabetes mellitus, whilst we now show that obese subjects with normoglycaemia awaiting bariatric surgery have a significant small fibre neuropathy and autonomic neuropathy. BMI, waist circumference and HDL correlated significantly with all CCM parameters, whilst there was no association with HbA1c or blood pressure. One of the first studies to identify the contribution of obesity to neuropathy was undertaken by Pirart et al (20) and Straub et al showed that obese patients with T2DM had worse neuropathy than the lean group (7), independent of duration of diabetes, glycaemic control, cholesterol, triglycerides and blood pressure. They suggested that improving BMI might improve neuropathy. The Utah Diabetic Neuropathy Study (UDNS) also reported that obesity and hypertriglyceridemia significantly increased the risk of DPN, independent of glucose control (21). Multivariate analysis showed that obesity and hypertriglyceridemia were related to small fibre neuropathy assessed using IENFD and hyperglycaemia was related to large fibre neuropathy (21). Central obesity has also been associated with cardiac autonomic neuropathy in subjects with impaired glucose tolerance (22).

In patients with type 2 diabetes, several large studies including UKPDS (23) have failed to confirm a benefit on neuropathy through improved glycemic control (24). Indeed in the Action to Control Cardiovascular Risk in Diabetes (ACCORD) study the MNSI score, vibration sensation and loss of ankle jerks did not improve with intensive glycemic management (25). Similarly in the Veteran's Affairs Diabetes

Trial (VADT) trial there was no significant effect on peripheral neuropathy in patients in the intensive glycaemic treatment group (26). In the multifactorial intervention study from the Steno, whilst retinopathy, nephropathy and autonomic neuropathy improved, somatic neuropathy did not (27). In the recent ADDITION Europe study, in newly diagnosed patient with T2DM, multi-risk factor reduction over 5 years failed to improve diabetic neuropathy (28). This suggests that the development and hence treatment of diabetic neuropathy is complex and the process of nerve damage starts early. Indeed we and others have previously demonstrated a significant small fibre neuropathy in subjects with metabolic syndrome or IGT (21, 29-32). This report extends this finding to include normoglycaemic patients with obesity.

There is a scarcity of studies relating obesity to neuropathy in a non-diabetic population. Mechanisms suggested for the development of obesity related neuropathy include mechanical compression of somatic and autonomic nerves by adipose mass and the direct metabolic effect on nerves (7). Up regulation of the renin-angiotensin system occurs in obesity and may play a role in neuropathy as previous studies have shown that ACE inhibitors improve neuropathy (33-35).

We demonstrate a significantly reduced PON1 activity in subjects with obesity compared with controls. Furthermore those patients with worse CNFL in the obese group had significantly lower PON1. PON1 is a high-density lipoprotein associated with anti-oxidant/glycation properties. PON1 genotypes have been shown to increase the risk of developing microalbuminuria and retinopathy (36). Abbott et al demonstrated lower PON specific activity in Type 1 and 2 diabetic patients with clinical neuropathy via increased lipid peroxidation (37). There is an increasing body of evidence to suggest an important role for oxidized and glycated LDL in the pathogenesis of neuropathy (38). Thus the Ox-LDL/apoB ratio has been associated with peripheral neuropathy in Japanese patients with type 2 diabetes (39) and elevated triglycerides correlate with myelinated nerve fibre loss, independent of disease duration, age and glycaemic control (40).

Obese patients have increased visceral adiposity, which causes an increase in the plasma concentration of free fatty acids and release of adipokines, resulting in endothelial dysfunction and inflammation, which may play a role in the

pathogenesis of peripheral neuropathy. The circulating levels of ICAM are increased in PN (41) and correlate with peroneal nerve function (42). IL-6 levels have been shown to correlate with dorsal sural NCS (43). SAA is both an inflammatory protein and apolipoprotein, which directly mediates obesity related inflammation and correlates with BMI and decreases with weight loss (44). This study confirms the inflammatory state of obese patients with CRP, IL-6, ICAM, VCAM and SAA all being significantly higher. We did not however demonstrate further increased inflammation in subjects with a small fibre neuropathy.

Serum Cystatin C, is a sensitive marker of kidney function and has been found to be higher in diabetic patients with PN (45) and we also show an increase in obese subjects.

The EURODIAB study reported a significant association between cholesterol and fasting triglycerides in the development of diabetic peripheral neuropathy and cardiac autonomic neuropathy (46). In particular raised LDL, hypertriglyceridemia and increased BMI were associated with an increase in the cumulative incidence of PN. Wiggin et al found that factors which predicted the progression of PN over 1 year included elevated triglycerides at baseline (40). They hypothesised that dyslipidaemia may explain why patients with type 2 diabetes develop PN early on in the disease course when compared to those with type 1 diabetes. The mechanism involved may be through oxidative stress induced by dyslipidaemia as in previous experimental rat models a high fat diet alone can increase oxidative stress and induce neuropathy (47). Furthermore, in the recent DISTANCE study where 28,701 diabetic patients were followed over 10 years, the triglyceride level was an independent, stepwise risk factor for non-traumatic lower extremity amputation (48). We also report a significantly higher level of triglycerides in obese patients who had small fibre neuropathy.

TNF-Alpha has been implicated in the pathogenesis and progression of several neuropathies and indeed TNF-alpha inhibitors have been shown to block the development of PN in rats (49). In obesity and type 2 diabetes, TNF-alpha causes an increase in free fatty acids, cholesterol, stimulation of hepatic lipid synthesis and secretion and inhibition of lipoprotein lipase (49). Our report shows a significantly lower HDL and ApoA1 in the obese group, which is in keeping with the

suggestion that they may play a role in the development of PN. Furthermore HDL correlated significantly with corneal nerve pathology. The mechanism for the HDL mediated protection from neuropathy is unclear, but it has been shown to prevent TNF-alpha induced apoptosis of human vascular endothelial cells through inhibition of CPP32-like protease activity (50). A further study speculates that TNF-alpha is directly responsible for the upregulation of hepatic ApoA1 production which inhibits neutrophil activation in inflammation.

PCSK9 is an important regulator of LDL receptor expression such that a high expression of PCSK9 is positively associated with LDL (51). The CODAM study found no relationship between PCSK9 and glucose metabolism however its relationship with non-HDL cholesterol and apolipoprotein B may be modified in type 2 diabetes suggesting a role for this in type 2 diabetes (51). In our cohort we demonstrate significantly higher PCSK9 levels in obese patients with small fibre neuropathy and a significant correlation between PCSK9 and CNFL.

Nitrotyrosine (NT) has been considered as a potential biomarker for diabetic peripheral neuropathy in experimental animals and indeed previously plasma NT has been shown to correlate with diabetes associated endothelial dysfunction (52). It has been detected in several systemic autoimmune conditions (53). We show elevated 3-NT in obese patients with small fibre neuropathy and a correlation with corneal nerve loss. Sciatic nerve NT concentrations have been shown to correlate with motor and sural nerve conduction velocity and myelin thickness (54). In a recent study the improvement in neuropathy following RYG-B was associated with improvements in nitrotyrosine, as opposed to OxLDL or HbA1c(55).

In summary we demonstrate a significant small fibre neuropathy in obese patients without type 2 diabetes. BMI, waist circumference and HDL but not HbA1C were significantly correlated with all CCM parameters. Furthermore we identify a higher level of triglycerides, PCSK9 and 3-NT and a lower PON1 in obese patients with small fibre neuropathy and PCSK9 and 3-NT levels correlate with CNFL. We demonstrate a small fibre neuropathy is subjects with morbid obesity and identify a number of underlying metabolic abnormalities, which warrant further study, as they may provide insights into the mechanisms of diabetic neuropathy and may also

explain the lack of benefit on neuropathy followed an improvement in glycaemic control.

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8. Chapter VIII - Corneal Confocal Microscopy Shows An Improvement In Small Fibre Neuropathy In Obese Subjects Post Bariatric Surgery

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8.1 Abstract

Bariatric surgery can lead to remission of type 2 diabetes in obese patients. The effect on microvascular complications, in particular neuropathy has not yet been established.

42 morbidly obese patients who underwent bariatric surgery had comprehensive neuropathy assessments at baseline, 6 and 12 months post-surgery.

Obese subjects with type 2 diabetes (n=25) had reductions in BMI (49.6±1.8 v 37.5±1.7 v 34.4±1.2, P<0.0001), HbA1c (57.3±3.4 v 41.2±2.9 v 38.4±1.9, P<0.0001), systolic (135.7±3.3 v 121.9±3.8 v 118.7±3.5, P=0.002) and diastolic (73.6±3.6 v 66.6±2.9 v 69.0±2.6, P=0.008) blood pressure at 6 and 12 months after bariatric surgery. There was a significant and progressive improvement in NSP (4.6±0.9 v 2.7±1.1 v 0.7±0.3, P=0.001), NDS (2.1±0.4 v 1.4±0.6 P=0.02), CNFD (24.2±1.4 v 25.5±2.3 v 28.1±1.3, P=0.019), CNBD (34.1±3.5 v 39.5±4.7 v 42.3±3.7, P=0.048) and CNFL (14.9±0.8 v 16.4±1.0 v 16.9±0.7, P<0.009). The latter was significant at 6 months (P=0.02).

Obese subjects without type 2 diabetes (n=17) also demonstrated a significant reduction in BMI (50.1±2.2v 38.6±2.8 v 33.7±2.5, P<0.0001), HbA1C (38.5±1.3 v 38.6±2.8 v 33.7±2.5, P<0.0001) and diastolic blood pressure (71.8±3.0 v 70.6±5.7 v 66.5±2.2, P=0.02). There was a significant and progressive improvement in NSP (4.0±1.6 v 3.4±1.2 v 0.3±0.2, P=0.01), NDS (1.7±0.6 v 0.9±0.5 v 0.07±0.1, P=0.04), CNFD (23.7±1.4 v 24.1±1.7 v 27.3±1.7, P=0.02), CNBD (27.7±3.3 v 31.1±4.9 v 33.6±3.8, P=0.05) and CNFL (13.8±0.6 v 14.1±0.8 v 15.5±0.8, P<0.02).

Bariatric surgery leads to an improvement in symptoms and small nerve fibre structure which can be identified using CCM. These data strengthen the argument that CCM is a surrogate marker for identifying early improvement in neuropathy, advocating its use in clinical trials of new therapies for diabetic neuropathy.

8.2 Introduction

Sixty-five percent of the world's population now live in countries where overweight and obesity kills more people than being underweight (1). This is a major public health challenge which is associated with morbidity and mortality and places considerable economic burden on health care systems (1).

Obesity is a powerful predictor of type 2 diabetes and cardiovascular morbidity and mortality (2-4). The World Health Organisation estimates that approx. 171 million people were diagnosed with type2 DM in 2000 and this number will increase to 366 million by 2030 (5). The management of type 2 diabetes and obesity is paramount and even though several new medications have been approved which improve both glycaemic control and weight, bariatric surgery is becoming the treatment of choice in a large group of patients. This is reflected in the NICE recommendation that all patients with type 2 diabetes and a BMI >35 should be assessed for bariatric surgery.

Bariatric surgery results in remission of type 2 diabetes in the majority of patients (6-8), however the impact on the complications, which may have already developed remains to be determined. The Swedish Obesity Study showed that the incidence of microvascular complications was lower in patients undergoing bariatric surgery compared to those undertaking lifestyle interventions (9). Whilst another study has demonstrated a 80% lower risk of incident microvascular disease following bariatric surgery (10), the end-points assessed were crude as retinopathy was assessed from the incidence of blindness in at least one eye, laser or retinal surgery, neuropathy via the incidence of non-traumatic amputation and nephropathy via the incidence of creating a fistula for dialysis.

Other reports focused on retinopathy and nephropathy have shown an improvement or no progression following bariatric surgery (11-14). With regard to neuropathy a recent study has reported no change in nerve conduction studies one year after bariatric surgery in patients with type 2 diabetes (15), whilst others have shown an improvement in symptoms of neuropathy (16, 17). Studies assessing objective markers or large and small fibre neuropathy are lacking. Conversely, individual case reports have identified the development of acute

Guillain-Barre like demyelinating (18) and motor axonal (19, 20) neuropathy following bariatric surgery. There is also concern that vitamin B12, copper and thiamine deficiency arising post-surgery may result in neuropathy (21-24). Contrary to the report of improved neuropathic symptoms, following bariatric surgery, surprisingly neuropathic pain has an incidence of 33% (25). This is the first study to investigate the effect of bariatric surgery on neuropathy employing detailed phenotyping to quantify large and small fibre neuropathy.

8.3   Research Design and Methods

8.3.1 Selection of patients

42 subjects with obesity (17 without and 25 with Type 2 Diabetes) were recruited from Salford Royal Hospital. They were assessed at baseline and then 6 months and 12 months following surgery. The exclusion criteria were any history of neuropathy other than diabetes, any history of corneal trauma or surgery, or history of ocular disease or systemic disease that may affect the cornea.

This study was approved by the Central Manchester Research and Ethics Committee and written informed consent was obtained from all subjects prior to participation. This research adhered to the tenets of the declaration of Helsinki.

8.3.2 Blood Pressure, Anthropometric and Laboratory Assessments

All study participants underwent assessment of body mass index (BMI), blood pressure, HbA1c, lipid profile [total cholesterol, low density lipoprotein (LDL), high density lipoprotein (HDL) and triglycerides], albumin creatinine excretion ratio (ACR) and estimated glomerular filtration rate (eGFR).

8.3.3 Assessment of Neuropathy

Neuropathy Symptom Profile (NSP) was used to assess the symptoms of neuropathy. Neuropathy Disability Score (NDS) was assessed and is comprised of vibration perception, pin-prick, temperature sensation and presence or absence of ankle reflexes (26). Vibration perception threshold (VPT) was tested using a Neurothesiometer (Horwell, Scientific Laboratory Supplies, Wilfrod, Nottingham, UK). Cold (CT) and warm (WT) thresholds were assessed on the dorsolateral aspect of the left foot (S1) using the TSA-II NeuroSensory Analyser (Medoc Ltd., Ramat-Yishai, Israel).

8.3.4 Nerve Conduction Studies

'Electro-diagnostic studies were undertaken using a Dantec "Keypoint" system (Dantec Dynamics Ltd, Bristol, UK) equipped with a DISA temperature regulator to

keep limb temperature constantly between 32-35°C. Sural sensory nerve amplitude, sural sensory nerve conduction velocity, peroneal motor nerve amplitude and peroneal motor nerve conduction velocity 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 was taken using antidromic stimulation over a distance of 140mm. Radial sensory recordings are taken from the anatomical snuffbox using antidromic stimulation over a 100mm distance. The strength of the stimulation was increased until a maximal response was obtained. The stimulus strength was increased 10-15% above the maximal stimulation to ensure a supramaximal response. The motor response was not averaged and the sensory responses were averaged using 3 but not more than 10 stimuli. Motor amplitude was measured from the baseline to the negative peak and reported to the nearest 0.1mV. The sensory amplitude was measured from the baseline to the negative peak. If there was a positive preceding the negative, the amplitude was measured from the base of the positive peak to the negative peak. The sensory nerve action potential was reported to the nearest 0.1 microvolt. Motor nerve latency was measured at the take-off of the negative component of the M wave. Sensory latency was measured from the take-off of the negative component of the sensory nerve action potential. If there was a positive preceding the negative component, then the latency was measured at the peak of the positive component of the sensory nerve action potential. Latency was recorded to the nearest 0.1 ms. all nerve conduction velocities were measured using onset latencies and reported to the nearest 0.1 m/s. The distance used for measurement was the distance between the two sites of stimulation.

8.3.5 Autonomic Neuropathy

Heart rate variability (HRV) was assessed with an ANX 3.0 autonomic nervous system monitoring device (ANSAR Medical Technologies Inc., Philadelphia, PA, USA).

8.3.6 Corneal Confocal Microscopy

Patients underwent examination with a CCM (Heidelberg Retinal Tomograph III Rostock Cornea Module, Heidelberg Engineering GmbH, Heidelberg, Germany) as per our previously established protocol (27).   Six non-overlapping images/patient (3 per eye) from the centre of the cornea were selected and quantified in a masked fashion. Three corneal nerve parameters were quantified: Corneal nerve fibre density (CNFD) - the total number of major nerves/mm^2 of corneal tissue, Corneal nerve branch density (CNBD) - the number of branches emanating from the major nerve trunks/mm^2 of corneal tissue and Corneal nerve fibre length (CNFL) - the total length of all nerve fibres and branches (mm/mm^2) within the area of corneal tissue. Automated analysis of corneal nerve morphology was performed using purposefully designed software (ACCMetrics) (28).

8.4 Statistical Analysis

Analysis was carried out on SPSS for Mac (Version 19.0, IBM Corporation, New York, USA). All data are expressed as mean ± standard error of mean (SEM). The data was tested for normality by using the Shapiro Wilk Normality test and by visualising the histogram and normal Q-Q plot. To assess within and between group differences we used one-way analysis of variance (non-parametric – Kruskal – Wallis). A significant p value was considered to be <0.05 (post hoc – Tukey).

8.5 Results

8.5.1 Demographic Factors

8.5.1.1 Obese with Type 2 Diabetes (Table 8.1)

Obese subjects demonstrated significant reductions in BMI (49.6±1.8 v 37.5±1.7 v 34.4±1.2, P<0.0001), HbA1c (57.3±3.4 v 41.2±2.9 v 38.4±1.9, P<0.0001), systolic (135.7±3.3 v 121.9±3.8 v 118.7±3.5, P=0.002) and diastolic (73.6±3.6 v 66.6±2.9 v 69.0±2.6, P=0.008) blood pressure at 6 and 12 months after bariatric surgery.

25 participants had type 2 diabetes at baseline and at 1 year 20 were in remission (P<0.0001).

16 patients were taking anti-hypertensive medication at baseline and at 1 year only 6 remained on treatment (P<0.0001).

8.5.1.2 Obese (Table 8.1)

Obese subjects also demonstrated a significant reduction in BMI (50.1±2.2v 38.6±2.8 v 33.7±2.5, P<0.0001), HbA1C (38.5±1.3 v 38.6±2.8 v 33.7±2.5, P<0.0001) and diastolic blood pressure (71.8±3.0 v 70.6±5.7 v 66.5±2.2, P=0.02) at 6 and 12 months after bariatric surgery

8 patients were taking anti-hypertensive medication at baseline and at 1 year only 1 patient remained on treatment (P<0.0001).

8.5.2 Types of Surgery

8.5.2.1 Obese with Type 2 Diabetes

19 patients underwent Roux – en – Y Gastric Bypass (RYGB). 4 patients had a Mini Bypass or Omega Loop Bypass and 7 patients had a Gastric Sleeve.

8.5.2.2 Obese

7 patients underwent RYGB. 3 patients had a Mini Bypass or Omega Loop Bypass and 7 patients had a Gastric Sleeve.

8.5.3   Neuropathy Assessments

8.5.3.1 Obese Type 2 Diabetes (Table 8.2)

There was a significant and progressive improvement in NSP from baseline to 6 months and 12 months (4.6 ± 0.9 v 2.7 ± 1.1 v 0.7 ± 0.3, P=0.001). There was also a significant improvement in NDS at 12 months (2.1 ± 0.4 v 1.4 ± 0.6 P=0.02).

There was no significant change in any nerve conduction parameters over 12 months.

There was a significant improvement in CNFD (24.2 ± 1.4 v 25.5 ± 2.3 v 28.1 ± 1.3, P=0.019), CNBD (34.1 ± 3.5 v 39.5 ± 4.7 v 42.3 ± 3.7, P=0.048) and CNFL (14.9 ± 0.8 v 16.4 ± 1.0 v 16.9 ± 0.7, P<0.009) and the latter was significant at 6 months (P=0.02). (Figure 8.1)

8.5.3.2 Obese (Table 8.2)

There was a significant and progressive improvement in NSP (4.0 ± 1.6 v 3.4 ± 1.2 v 0.3 ± 0.2, P=0.01) and NDS (1.7 ± 0.6 v 0.9 ± 0.5 v 0.07 ± 0.1, P=0.04) from baseline to 6 months and 12 months.

There was no significant change in any nerve conduction parameters over 12 months.

There was a deterioration in WT (39.7 ± 0.8 v 39.97 ± 1.8 v 41.5 ± 1.05, P=0.05). There was significant improvement in CNFD (23.7 ± 1.4 v 24.1 ± 1.7 v 27.3 ± 1.7, P=0.02), CNBD (27.7 ± 3.3 v 31.1 ± 4.9 v 33.6 ± 3.8, P=0.05) and CNFL (13.8 ± 0.6 v 14.1 ± 0.8 v 15.5 ± 0.8, P<0.02). (Figure 8.1)

	Obese with Type 2 Diabetes				Obese			
	Baseline	6 Months	12 Months	P	Baseline	6 Months	12 Months	P
Age	51.7±1.5	-	-	-	46.2±1.6	-	-	NS
BMI (Kg/m^2)	49.6±1.8	37.5±1.7	34.4±1.2	<0.0001	50.1±2.2	38.6±2.8	33.7±2.5	<0.0001
IFCC (mmol/mmol)	57.3±3.4	41.2±2.9	38.4±1.9	<0.0001	38.5±1.3	34.9±0.8	33.9±0.6	0.002
Systolic BP (mmHg)	135.7±3.3	121.9±3.8	118.7±3.5	0.002	123.9±3.4	117.4±6.1	115.3±4.5	NS
Diastolic BP (mmHg)	73.6±3.6	66.6±2.9	69.0±2.6	0.008	71.8±3.0	70.6±5.7	66.5±2.2	0.02
Duration of diabetes (years)	9.0±0.7	-	-	-	-	-	-	-
Patients with type 2 diabetes	25	-	5	P<0.0001	-	-	-	-
Patients on anti-hypertensives	16	-	6	P<0.0001	8	-	1	P<0.0001

Table 8-1. Demographic and Anthropometric data in obese patients with and without type 2 diabetes mellitus at baseline, 6 months and 12 months.

	Obese with Type 2 Diabetes				Obese			
	Baseline	6 months	12 months	P	Baseline	6 months	12 months	P
Neuropathy Symptom Profile	4.6±0.9	2.7±1.1	0.7±0.3	0.001	4.0±1.6	3.4±1.2	0.3±0.2	0.01
Neuropathy Disability Score	2.1±0.4	2.9±0.8	1.4±0.6	0.02	1.7±0.6	0.9±0.5	0.07±0.1	0.04
Sural Latency (ms)	2.8±0.1	3.04±0.1	3.1±0.07	NS	3.1±0.2	2.9±0.2	2.9±0.1	NS
Sural Amplitude (uV)	10.3±2.1	7.1±0.8	7.6±1.6	NS	8.7±2.04	13.2±3.1	12.6±1.3	NS
Sural Velocity (m/s)	49.2±1.6	46.3±1.2	45.9±1.2	NS	46.6±2.8	48.5±3.2	46.9±0.8	NS
Peroneal latency (ms)	4.0±0.1	4.1±0.2	4.2±0.2	NS	4.5±0.6	4.9±0.9	3.9±0.2	NS
Peroneal amplitude (uV)	3.5±0.3	3.2±0.5	3.3±0.5	NS	3.9±0.82	3.6±1.2	3.4±0.5	NS
Peroneal velocity (m/s)	45.4±1.2	45.4±0.9	45.1±0.9	NS	45.3±1.9	40.7±5.5	47.4±1.0	NS
Radial Amplitude (uV)	33.9±2.7	36.5±3.4	37.4±3.6	NS	42.3±5.9	45±7.0	49.5±4.4	NS
Radial velocity (m/s)	62.3±1.0	61.5±1.2	59.9±1.2	NS	63.1±0.9	61.1±1.7	64.9±1.5	NS
Cold Threshold (^{O}C)	23.2±1.5	23.1±1.6	24.4±1.4	NS	24.7±1.7	24.4±2.3	25.9±0.8	NS
Warm Threshold (^{O}C)	39.8±0.8	42.1±0.9	41.2±0.9	NS	39.7±0.8	39.97±1.8	41.5±1.05	0.05
DB-HRV (beats per min)	17.0±2.3	16.5±1.8	16.4±1.4	NS	20.9±3.9	22.2±7.2	21.6±3.04	NS
CNFD (no./mm^2)	24.2±1.4	25.5±2.3	28.1±1.3	0.019	23.7±1.4	24.1±1.7	27.3±1.7	0.02
CNBD (no./mm^2)	34.1±3.5	39.5±4.7	42.3±3.7	0.048	27.7±3.3	31.1±4.9	33.6±3.8	0.05
CNFL (mm/mm^2)	14.9±0.8	16.4±1.0	16.9±0.7	0.009	13.8±0.6	14.1±0.8	15.5±0.8	0.02

Table 8-2. Neuropathy assessments in obese patients with and without type 2 diabetes mellitus at baseline, 6 months and 12 months.
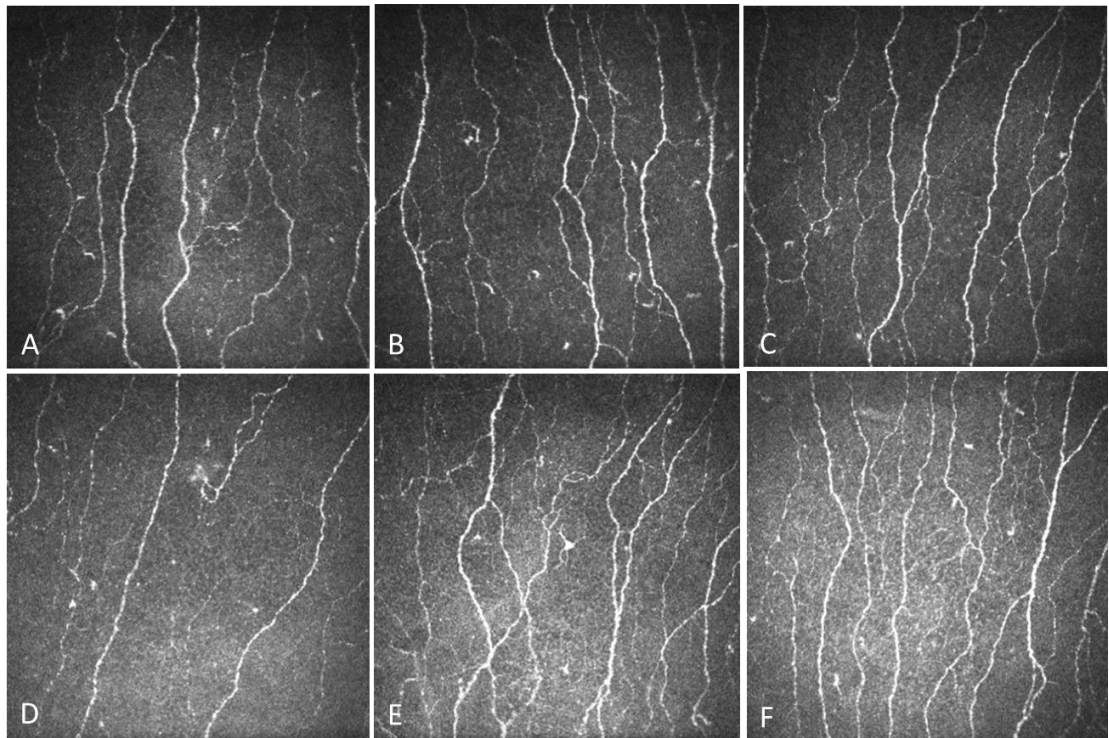
Figure 8-1 CCM images from: an obese patient without diabetes at baseline (a), 6 (b) and 12 (c) months post bariatric surgery and an obese subject with type 2 diabetes at baseline (d), 6 (e) and 12 (f) months post bariatric surgery.

8.6   Discussion

This report is the first to show that there is an improvement in structural measures of small fibre neuropathy in obese patients with and without T2DM after bariatric surgery. Indeed CCM detected an improvement in corneal nerve fibre length as early as 6 months after bariatric surgery in patients with type 2 diabetes. The improvement in small fibre neuropathy is accompanied by a significant improvement in neuropathic symptoms which also occur as early as 6 months and an improvement in autonomic neuropathy in obese patients with diabetes after bariatric surgery. However, we find no improvement in quantitative sensory threshold testing and indeed we observed a worsening in the warm threshold in obese patients with no change in a range of electrophysiological tests in the upper and lower limbs, consistent with a recent study (15).

Obese patients with and without T2DM demonstrated an improvement in BMI, HbA1c and blood pressure and 80% of patients achieved remission from T2DM 1 year after surgery. Remission was classified as having an HbA1c below normal for the diagnosis of diabetes and no active pharmacological therapy as per the American Diabetes Association consensus (29). Similarly, 62.5% of obese patients with T2DM and 87.5% of subjects with obesity had remission from treatment for hypertension.

One of the earliest studies demonstrating a benefit of bariatric surgery on microvascular endpoints was by Pories et al who showed that in their cohort none had progressed to develop microvascular complications using the crude end points of renal failure, blindness or amputation (30). More recently, Coleman et al reported a 29% reduction in the risk of incident microvascular disease 7 years after bariatric surgery compared to those who did not have surgery (31). The main driver for this risk reduction in microvascular complications was retinopathy, as neuropathy was assessed through ICD diagnosis codes. Very few studies have assessed the effect of bariatric surgery on DPN which is remarkable in view of the morbidity caused by DPN. However, retinal and renal data is relatively easy to collect as many of the tests are standard of care, whilst for neuropathy, specific

questionnaires and neurological testing has to be undertaken. Schauer et al reported symptomatic improvement in 50% of patients after surgery: 33% much improved, 17% improved, 39% no change, 7% worse, and 4% unknown (16). The definition of neuropathy in this group was unclear with complications being assessed via a generic questionnaire on chronic diabetes related complications. Again, objective markers of neuropathy were not assessed. Muller-Stich et al found an improvement in symptomatic neuropathy in a small group of 12 patients (17) with the neuropathy symptom score (NSS) showing an improvement from a median of 8 (range, 0-10) to 0 (range, 0-9) post-operatively, with 8 patients scoring an NSS of 0. Pre-operatively the median neuropathy disability score (NDS) was 6 (range, 2-8), which improved to 4 (range 0-8), post operatively. However, Miras et al found that 1 year after RYGB patients with type 2 diabetes demonstrated no change in nerve conduction parameters (15). There is also concern with regards to nutritional deficiencies that arise post-surgery that may lead to neuropathy including vitamin B12, copper and thiamine deficiency as well as osteomalacia (21-24). A retrospective study of 435 patients found that peripheral neuropathy occurred more frequently after bariatric surgery and malnutrition was the most important factor (32). Hence it is important that nutritional deficiencies need to be identified early to prevent the development or worsening of neuropathy in such patients (25). In our bariatric cohort nutritional deficiencies were assessed for and corrected if present prior to and after surgery. Post bariatric surgery neuropathic pain has an incidence of 33% and can greatly affect the quality of life (25). However, we report an improvement in the neuropathy symptom profile following bariatric surgery.

Singleton et al showed an improvement in IENFD after a programme of diet and exercise in patients with IGT which was independent of weight loss or change in metabolic factors (33). Indeed we have previously shown in patients with type 1 diabetes who have undergone simultaneous pancreas kidney (SPK) transplantation that CCM shows and improvement in small fibre neuropathy as early as 6 months after surgery (34). In patients with impaired glucose tolerance CCM was also able to show dynamic changes in small fibre neuropathy related to changes in the glycemic status (35). Accordingly IENFD (33) and CCM have been

suggested as possible surrogate markers in trials of new therapies for diabetic neuropathy(36).

We demonstrate that neuropathy, particularly small nerve fibres regenerate, once metabolic abnormalities are corrected after bariatric surgery and occurs as early as 6 months post-surgery. This study strongly advocates the use of CCM as a biomarker for assessing nerve repair and neuropathy.

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9. Chapter IX - Discussion

9.1   Introduction

Diabetic peripheral neuropathy (DPN) is one of the most common complications of diabetes mellitus with a prevalence of 30% which increases with the duration of diabetes and has a significant clinical and economic impact (1). DPN is a major risk factor for foot ulcers, which are responsible for 80% of amputations (2) and also results in neuropathic pain which impacts on sleep, mood and quality of life in ~20 % of patients (3, 4).

The UKPDS showed that 5-7% of patients with type 2 diabetes had evidence of neuropathy at the time of diagnosis (5). Furthermore studies have shown the presence of neuropathy, primarily small fibre, in patients with impaired glucose tolerance (6-9). Hence, the pathogenesis of PN is multifactorial and not limited to hyperglycaemia. BMI, IGT, hypertriglyceridemia, and waist circumference, which are part of the metabolic syndrome, have been associated with neuropathy (10-13).

There are currently no FDA approved therapies to prevent, slow or arrest DPN and management therefore primarily involves achieving good glycaemic control to halt progression (14). Other known modifiable and non-modifiable risk factors include hypertension, smoking, triglycerides and diabetes duration (15).

Nerve conduction studies are a measure of large fibre neuropathy. However, small nerve fibre dysfunction may predate large fibre neuropathy (16), and small nerve fibres may be affected by impaired glycaemia and large nerve fibres may become affected by overt diabetes mellitus (17). This places emphasis on the importance of assessing small fibre neuropathy in metabolic disease as crude assessments of large fibre neuropathy will lead to under-diagnosis and incorrect classification of patients. The gold standard test for the diagnosis of small fibre neuropathy is IENFD. However as this is invasive it is not practical to perform routinely and is not easily undertaken repeatedly in clinical trials. CCM is a non-invasive objective method to quantify small nerve fibre damage, using an unbiased automated image analysis technique (18, 19) which correlates with IENFD (20). Previous studies have shown the potential of using CCM as a marker for the diagnosis of several peripheral neuropathies, particularly DPN (21-24).

We now extend the use of this novel tool further to diagnose neuropathy in obesity as well as dysglycaemic states and to assess its utility in longitudinal studies monitoring small nerve fibre degeneration and regeneration. Earlier reports have shown that CCM shows an improvement in small fibre neuropathy following simultaneous pancreas kidney transplantation (SPK) (21, 25). IENFD has also been shown to improve after diet and exercise intervention in patients with IGT (6). These studies lend support for the utility of assessing small fibres to identify nerve regeneration and more specifically CCM as a surrogate maker that detects nerve repair in clinical trials.

CCM can also be used to identify patients with sub-clinical small fibre neuropathy who are at risk of developing overt peripheral neuropathy (26). Due to the lack of effective treatments currently available and the lack of benefit to established neuropathy, it is paramount to establish early neuropathy so that control of conventional risk factors can prevent neuropathy and reduce the morbidity associated with it.

9.2　Corneal Confocal Microscopy Shows an Improvement in Small Fibre Neuropathy in Subjects with Type 1 Diabetes On Continuous Subcutaneous Insulin Infusion Compared to Multi Day Injection

Earlier studies demonstrated that initiation of CSII treatment achieved near normal glycaemia with an improvement in nerve conduction (27-30). Downie et al reported a reduction in the incidence of retinopathy, microalbuminuria and neuropathy in 1604 adolescents followed up over 8.6 years and an improvement in vibration and thermal thresholds in patients treated with CSII compared to MDI (31).

The present study reports an improvement in small nerve fibre morphology as assessed by CCM in patients on CSII as opposed to MDI treatment, which was independent of glycaemic control, as this was comparable in both groups. One difference in this study compared to previous studies was that the patients were already on CSII treatment as opposed to studying the effect of initiating CSII.

Experimental studies have shown the potential of a direct neurotrophic action of insulin on neurones and axons (32). Thus insulin can directly impact axonal plasticity and regeneration, as the intrathecal delivery of insulin and equimolar IGF-1, at levels that did not improve glycaemia, was shown to improve or reverse the slowing of motor and sensory nerve conduction in diabetic rats (33, 34). The effects seen in the present study may therefore be a result of the effect of small doses of continuous insulin via CSII. Thus CSII may improve small fibre morphology though an independent neurotrophic effect of insulin.

9.3   Small Fibre Neuropathy in Patients with Type 1 diabetes and Erectile Dysfunction

The diagnosis and management of ED in patients with diabetes is challenging with a greater failure rate of therapies for ED (35). Patients with diabetes and presumed worse neuropathy are less responsive to phosphodiesterase type 5 inhibitors (PDE5-I), which are first line therapy in the management of ED (36). This is thought to occur because PDE5-I's require a minimum amount of nitric oxide (NO) production, which cannot be synthesised by severely damaged nerves. Hence in this group it is though that therapeutic strategies to promote NO synthesis and availability may improve erectile function if used in combination with PDE5-I (36).

The pathogenesis of ED involves both vascular and neurogenic mechanisms, but the role of neuropathy has been under investigated. Previous studies report the prevalence of peripheral neuropathy to vary from 35-75%, which reflects the different methodologies used to identify neuropathy in these populations (37, 38). The majority of prevalence studies have not distinguished between type 1 and type 2 diabetes with the majority focussing on type 2 diabetes. We have demonstrated a high prevalence of ED in patients with type 1 diabetes and report the presence of large and small fibre neuropathy in patients with ED and type 1 diabetes. In particular there is wide spread autonomic and small fibre neuropathy which can be demonstrated by reduced HRV, IENFD in foot skin biopsies and corneal nerve fibre abnormalities using CCM in subjects with Type 1 diabetes and

ED. In a previous report, QST of the penis showed impaired thermal and vibration perception thresholds in non-diabetic patients with ED and those with diabetes and ED had both large and small fibre neuropathy (39). Indeed the most significant correlations with ED are age and the presence of symptomatic peripheral and autonomic neuropathy (37, 40). The involvement of parasympathetic activity in maintaining an erection means it is not surprising that autonomic neuropathy is strongly associated with ED (38). In our report markers of small fibre neuropathy, IENFD and CCM correlated with ED. CCM represents a non-invasive objective method to quantify small fibre damage and allows us to identify those patients with more extensive nerve damage and hence those less likely to respond to conventional therapies. These patients can therefore be considered for daily higher does, combination therapy or alternative therapies such as intraurethral alprostadil or penile prosthesis (41, 42)

9.4 Corneal Confocal Microscopy identifies small fibre neuropathy in subjects with Impaired Glucose Tolerance who develop Type 2 Diabetes Mellitus

It is debated as to whether patients with IGT are at risk from the complications of type 2 diabetes and hence need intervention. However, the UKPDS showed that 5-7% of patients with type 2 diabetes had evidence of neuropathy at the time of diagnosis (5). Whilst some studies have shown no association between IGT and neuropathy (43-45), others have demonstrated neuropathy, in particular small fibre neuropathy (6-9, 46). We have recently shown that a significant small fibre neuropathy occurred in 40.5% of subjects with IGT (11).

IENFD is the gold standard measure of small fibre pathology, although Pittinger at al showed that mean dendrite length (MDL) was reduced before IENFD in metabolic syndrome (47). We also report a reduction in MDL but not IENFD in subjects with IGT at baseline compared to controls. MDL appears to be more responsive to changes in glucose status showing a reduction in those patients who go on to develop diabetes whereas IENFD showed a reduction in all groups.

Smith et al have shown that a 1-year diet and life style intervention can improve IENFD (6), and a further study following lifestyle intervention over 6 months reported a reduced incidence of severe retinopathy, but no impact on neuropathy (48). This is clearly confounded by the neuropathy end-point chosen which was monofilament insensitivity, a crude large fibre measure of neuropathy. We have previously shown that the small fibres are affected earlier in IGT and markers of large fibre neuropathy may not detect neuropathy in this cohort of patients.

We also show that those patients who progress to Type 2 diabetes have worse baseline corneal nerve morphology and MDL. This is in keeping with a recent study showing that subjects with normal glucose tolerance, but with abnormal electrochemical sweat conductance have a significantly increased odds ratio for the development of IGT (49). This allows us to identify those subjects who are at risk of developing type 2 diabetes and target them for intervention to reduce their metabolic risk factors for developing complications. Furthermore, subjects who progressed to Type 2 diabetes showed a further significant reduction in CNFL and MDL. In subjects who remained with IGT there was no baseline loss nor was there any change over time. And in subjects who reverted to normal glucose tolerance, the baseline CCM values did not differ significantly from controls and indeed there was a significant increase in all CCM parameters over 36 months. We have shown a dynamic relationship between small nerve fibre damage and repair in relation to overall glucose tolerance status.

9.5 Diabetic Neuropathy: Lessons from Longitudinal Studies in Medallists and Extreme Phenotypes of Patients with Type 1 Diabetes Mellitus

Patients with type 1 diabetes mellitus for >50 years (medallists) represent a unique cohort of patients who develop no or minimal long- term cardiac and microvascular complications. There has been interest in this group of patients with earlier studies showing less microvascular complications, in particular retinopathy and nephropathy (50-52). However the effect on neuropathy has not been studied in detail. We demonstrate evidence of a moderately severe large fibre neuropathy

with a relative preservation in functional and structural measures of small fibre neuropathy. The medallists in our cohort also had a higher HDL, in support of earlier reports (51) (53). Keenan et al reported a higher HDL, lower triglycerides as well as residual insulin production, but no difference in HbA1c or diabetes duration in those protected from microvascular complications in this cohort (54). Insulin increases the activity of lipoprotein lipase and decreases serum triglycerides in patients with T1DM (55), hence HDL may be higher because of an elevated lipoprotein lipase/hepatic lipase ratio and higher HDL has been shown to protect against the development of albuminuria (56).

This is the first study to undertake detailed baseline and longitudinal phenotyping of neuropathy in medallists and shows that there is no progression of neuropathy over 36 months. This is in contrast to a cohort of patients with T1DM with a shorter duration of diabetes who showed a worsening of small fibre neuropathy, identified using CCM, but no change in other neuropathy markers. In subjects who underwent SPK there was significant damage at baseline but an improvement in CCM parameters followed by an improvement in symptoms of neuropathy as well as peroneal nerve conduction velocity. This supports our earlier work showing regeneration of small nerve fibres in patients at 6 (25) and 12 (21) months after SPK and 24 months after CSII infusion (57). Therefore CCM appears to be a novel surrogate end-point for assessing nerve degeneration and regeneration in trials of diabetic neuropathy.

9.6   The Effect of Obesity on Neuropathy

Studies showing the direct effect of obesity on the nervous system are limited. Herman et al reported the presence of significant SFN in morbidly obese patients (58). Other studies have shown that patients with type 2 diabetes and obesity have a neuropathy independent of hyperglycaemia (59) (10) (60). Multivariate analysis showed that obesity and hypertriglyceridemia were related to small fibre neuropathy assessed using IENFD, whilst hyperglycaemia was related to large fibre neuropathy (60). We now show that obese subjects with normoglycaemia awaiting bariatric surgery have evidence of small fibre neuropathy using CCM as well as autonomic neuropathy. BMI, waist circumference and HDL correlated

significantly with all CCM parameters. We also did not find an association between small fibre neuropathy and HbA1C in keeping with the studies of patients with type 2 diabetes, suggesting an independent effect of glucose on neuropathy.

Furthermore, we have also investigated the effects of lipoproteins and inflammatory markers on the pathogenesis of neuropathy in obesity. There was a significantly reduced PON1 activity in subjects with obesity compared with controls and patients with worse CNFL had significantly lower PON1. Whilst previously PON1 genotypes have been shown to increase the risk of developing microalbuminuria and retinopathy (61), we now show an association with neuropathy.

Obese patients have increased visceral adiposity, which causes an increase in the plasma concentration of free fatty acids and release of adipokines, resulting in endothelial dysfunction and inflammation which may play a role in the pathogenesis of peripheral neuropathy. Indeed, the obese cohort had a pro-inflammatory state with significantly higher IL-6, ICAM, VCAM and SAA compared to the controls.

Triglycerides were also significantly elevated in obese patients who had worse small fibre neuropathy. The EURODIAB study reported a significant association between cholesterol and fasting triglycerides in the development of diabetic peripheral neuropathy and cardiac autonomic neuropathy in patients with Type 1 diabetes (15). In particular raised LDL, hypertriglyceridemia and increased BMI were associated with an increase in the cumulative incidence of PN. Wiggin et al found that factors which predicted the progression of PN over 1 year included elevated triglycerides and decreased peroneal motor conduction velocity at baseline (62).

The CODAM study found no relationship between PCSK9 and glucose metabolism however its relationship with non-HDL cholesterol and apolipoprotein B may be modified in type 2 diabetes suggesting a role for this in type 2 diabetes (63). In our cohort we found significantly higher PCSK9 levels in obese patients with corneal nerve fibre loss and a significant correlation between PCSK9 and CNFL.

Nitrotyrosine (NT) has been considered as a potential biomarker for diabetic peripheral neuropathy however this has not yet been evaluated (64). Its presence has been detected in several pathological and systemic autoimmune conditions (65). Previously plasma NT has been shown to correlate with endothelial dysfunction (64). We now show elevated 3-NT in obese patients with small fibre neuropathy and a correlation with all corneal confocal markers. Sciatic nerve NT concentrations have been shown to correlate with motor and sural nerve conduction velocity and myelin thickness (66).

We show the presence of a small fibre neuropathy in obese patients who do not have type 2 diabetes and BMI, waist circumference and HDL but not HbA1C were significantly correlated with all CCM parameters. Furthermore we identify a higher level of triglycerides, PCSK9 and 3-NT and a lower PON1 in obese patients with small fibre neuropathy. PCSK9 and 3-NT levels correlate with a reduction in CNFL and may represent biomarkers for small fibre neuropathy. These abnormalities may also represent pathogenic pathways for neuropathy, which needs to be validated in larger studies.

9.7   Corneal Confocal Microscopy Shows An Improvement In Small Fibre Neuropathy In Obese Subjects Post Bariatric Surgery.

Bariatric surgery has been shown to be superior to medical management for the treatment of type 2 diabetes (67-69). Cost effectiveness analysis have led to the NICE recommendation that all patients with type 2 diabetes and BMI >35 be considered for bariatric surgery. Previous reports have shown nephropathy and retinopathy, either remain stable or improve post-bariatric surgery (70-73). However there are very limited studies of the benefits on neuropathy using objective markers. We report an improvement in small fibre neuropathy in obese subjects with and without type 2 diabetes post bariatric surgery, which occurs as early as 6 months and this is associated with an improvement in neuropathic symptoms (NSP).

A recent study reported no change in nerve conduction studies one year post-bariatric surgery in patients with type 2 diabetes (74), although others have shown an improvement in symptoms of neuropathy (75, 76). Conversely, there has been concern over the development of acute Guillain-Barre like demyelinating (77) and motor axonal (78, 79) neuropathy following bariatric surgery. Nutritional deficiencies arising after bariatric surgery may also lead to neuropathy (80-83) with post bariatric surgery neuropathic pain being reported in 33% of subjects (84). However, in our cohort we show an improvement in small fibre structure and symptoms, which would be expected given the amelioration of obesity and metabolic as well as inflammatory abnormalities.

9.8   Study Limitations

The main limitation of the first 4 chapters is that they are observational studies. We have aimed to control for the risk factors for neuropathy when making comparison between groups. However, our studies provide real world data, which has translational value in obesity, dysglycaemia and diabetes. The method for diagnosis ED in chapter 6 could be a potential limitation and may have in fact under-diagnosed the condition. Although similar diagnostic criteria have been used in previous studies.

The bariatric studies lack data on IENFD, which is considered to be the current gold standard technique in assessing small nerve fibres. Although, major weight loss and alterations in skin has been forwarded as a potential limitation for the use of skin biopsies in such studies. Given the previous data from our group showing that IENFD correlates with CCM we believe that IENFD assessment is not essential.

Chapter 8 lacks a non-surgical control group undergoing weight loss after medical management. This group is however, not a good comparator as the results achieved in relation to metabolic remission via bariatric surgery are superior to medical management.

9.9   Future work

These data strengthen the argument that CCM is a surrogate marker for identifying early neuropathy and improvement following an improvement in metabolic risk factors. It also strengthens the use of CCM in clinical trials of new therapies for diabetic neuropathy as it shows early degeneration and regeneration of nerve fibres.

The improvement in CCM parameters and symptoms with CSII treatment provides a good rationale for the use of CSII to manage patients with symptomatic neuropathy.   This needs to be further investigated with a larger randomized controlled trial of CSII vs MDI and the mechanism of benefit needs to be evaluated, particularly in relation to the effect of insulin.

The use of CCM as a potential marker for the benefits of therapy in ED needs to be extended to include other groups of patients in particular type 2 diabetes, IGT and obesity. It also needs to be incorporated into studies where patients with ED are commenced on therapy to assess whether the extent of nerve damage at baseline can predict the efficacy of the intervention. Furthermore, the effect of bariatric surgery on ED should be assessed.

Medallists have been shown to have a relative protection from the development and particularly the rate of progression of small fibre neuropathy. The factors that lead to this protection need to be interrogated in detail and we have bio-banked serum and plasma to undertake such studies using the genomic/proteomic/metabolomic platforms.

The bariatric studies need evaluation in larger studies over a longer duration of follow up to monitor the effect on neuropathy in the context of nutritional and vitamin deficiencies which may develop in some patients. More detailed sub-group analysis of patients who relapse in terms of weight loss may provide important insights into the dynamic nature of degeneration and regeneration of small fibres. We also need to identify those patients in whom neuropathy improves to investigate what baseline factors and markers if any can predict the improvement in these patients.   This work will further add to the search for a biomarker for neuropathy which is paramount considering the impact that this has on patients

and healthcare systems. Establishing biomarkers as well as a surrogate end point for small fibre neuropathy in necessary for clinical trials as there are no current FDA approved treatments for neuropathy. There are already studies evaluating the feasibility of CCM in detecting early neuropathy in patients undergoing diabetic retinal screening in the community. The impact of identifying these patients and implementing risk factor reduction strategies in the prevention and early reversal of neuropathy needs to be studied in cost effectiveness analysis.

10.0 References

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10. Chapter X – Appendix – Study Related Documents

10.1 Patient Information Sheet for Studies in Chapters 3-6

MANCHESTER
1824
The University of Manchester

Central Manchester and Manchester NHS
Children's University Hospitals
NHS Trust

STUDY INFORMATION SHEET

Ophthalmic markers of diabetic neuropathy

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

WHY IS THIS STUDY BEING DONE?

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

WHAT ARE WE ASKING YOU TO DO?

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

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

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

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

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

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

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

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

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

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

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

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

WHO WILL SEE THE INFORMATION ABOUT ME?
All information resulting from your (or their) participation in the study will be stored and analyzed in a computer and will be treated confidentially. A number will identify you (or them) in the computer. The study records will not be made available in any form to anyone other than authorized representatives of the Health Authority. Individuals responsible for audit and monitoring on behalf of the University and NHS Trust will have access for this purpose.
Your (or their) confidentiality will be maintained in accordance with the Data Protection Act, 1984. If the results of this study are published, your (or their) identity will remain confidential.

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COMPENSATION IN CASE OF INJURY

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

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

WHAT IF THERE IS A PROBLEM?

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

WHAT DO I DO NOW?

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

For further information or appointments or if you (or they) want any further information concerning this project or if you (or they) have any medical problems which may be related to your (or their) involvement in the project (for example, any side effects), you can contact our diabetes research nurse, Ms. Karthi Balakrishnan on 0161 276 6706, or the following people:

Prof. Rayaz Malik
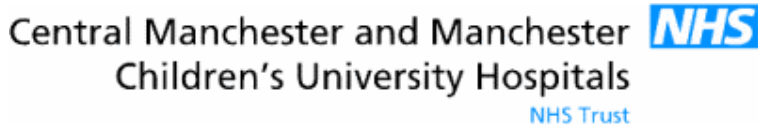Ph: 0161 275 1196
E-mail: rayaz.a.malik@manchester.ac.uk

Dr. Mitra Tavakoli
Ph: 07930453389
E-mail: Mitra.tavakoli@manchester.ac.uk

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

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10.2 Patient Consent form for Studies in Chapters 3-6

MANCHESTER 1824

The University of Manchester

Central Manchester and Manchester NHS Children's University Hospitals

NHS Trust

Participant Study Number:

CONSENT FORM

Title of Project: Ophthalmic markers of diabetic neuropathy

Investigators:

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

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

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

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

Please initial box:

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

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

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

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. I agree to have this procedure undertaken.

☐

……………………………..   ………………………..   ………………....

Name of Patient   Signature   Date

…………………………….   ………………………..   ……………....

Name of Person   Signature   Date

taking consent

1 for patient; 1 for researcher

10.3 Patient Information Sheet for Bariatric Studies in Chapters 7-8

Study Title

Changes in paraoxonase activity, HDL properties and inflammatory markers in post-bariatric surgery patients, type 1 diabetics with and without nephropathy, type 2 diabetics, and during an oral glucose tolerance test

- You are being invited to take part in a research study to look at cholesterol (fat) metabolism and cardiovascular health.

- This sheet provides you with the information about the study and how it involves you.

- Before you decide it is important for you to understand why the research is being done and what it will involve.

- Please take time to read the following information carefully before deciding on whether to take part or not.

Introduction

Apart from weight loss, bariatric surgery is thought to have major impact on fat metabolism and nerve function. Changes to the levels of cholesterol (a type of fat) in the bloodstream have profound effects on the health of the heart and blood vessels. Some forms of cholesterol are beneficial to the body while others are harmful. The mechanisms by which these effects occur are not clear. One of the enzymes which can increase the effectiveness of the good cholesterol is called Paraoxonase 1 (or PON1).

In this study, we try to determine which factors affect PON1 activity and we assess nerve function before and after bariatric surgery.

What is the purpose of this study?

The purpose of this study is to relate changes of PON1 activity with function of blood vessels and to evaluate nerve function before and after bariatric surgery. This will help us to understand why obese individuals with and without diabetes have an increased risk of vascular diseases and how the risk can be reduced. It helps us to understand how weight loss may influence changes in nerves and their structure and function.

Why have I been chosen?

You have been scheduled for bariatric surgery and therefore suitable for the study. We hope to confirm that weight loss after the surgery results in favourable changes to PON1 that in turn is associated with lower cardiovascular risk. We also examine other changes following bariatric surgery including sexual function and nerve function.

What will I have to do if I take part?

If you agree to take part, we will confirm that you have understood the study and that you meet with the study criteria. You will be asked to sign a consent form for the study and you will need to attend the Wellcome Trust Clinical Research Facility (in Manchester) for 3 visits.

Your first visit will be arranged before the bariatric surgery. During this visit, we will review your medical and medication history. You will have a brief physical examination that includes measurements of height, weight, waist circumference and blood pressure. We will perform an ECG test to assess the health of your heart and measure your blood vessel stiffness using non-invasive methods.

You will be asked to attend for all the visits having fasted overnight (for at least 10 hours) so that fasting blood samples can be taken from you. A total of 60ml (about 12 teaspoons) of blood will be taken from you. Most of the blood samples will be analysed in the Manchester laboratory. A small sample of serum or plasma will be retained for future research at the end of the study. In addition, a small frozen anonymised blood sample will be sent to Australia for further tests. The tests to be carried out in the Australian laboratory are for research and not for clinical/ diagnostic purposes. The tests will provide more information on the relationship between fat metabolism and diabetes. When the analyses are completed, the samples will be destroyed. You will be asked to give a sample of urine for analysis. Male participants will complete a sexual function questionnaire.

Nerve Function Tests consist of:

- Short questionnaire on pains (if any) in your legs.

- Nerve conduction study. Nerves in your legs are stimulated resulting in momentary muscle twitching. This may cause minor fleeting discomfort. Your ability to sense different temperatures and vibration in your lower legs will be measured.

- Corneal sensitivity is assessed with an air puff stimulus to the front of your eyes with no direct contact.

- A corneal confocal microscope (CCM) will be used to examine the number of nerves in the front part of the eye. A drop of anaesthetic is applied to numb the front of the eye. This allows a gel on the lens of the camera to touch the front of the eye for 1-2 minutes whilst we record images of the cornea.

We ask for your consent to take a small sample of fat from inside your abdomen during your bariatric surgery when you are under general anaesthesia.

Visits 2 and 3 will take place 6 and 12 months after your weight loss surgery. All the measurements will be repeated during these visits.

What are the possible risks of taking part?

The blood samples will be taken by an experienced doctor or nurse and the only risk involved may be some bruising at the puncture site. Very rarely there may be a mild infection at the biopsy site. This happens approximately once in every 25 procedures (a rate of 4%). If this occurs, a short course of antibiotics from your GP will resolve it. There are no risks involved in the nerve function tests.

Are there any possible benefits?

There are no immediate benefits to you. However, the knowledge gained from this study will help us to develop better tests in assessing nerve damage. It will improve our knowledge of factors leading to heart disease and develop new therapies to prevent it.

Will I be paid for taking part in the study?

No. But your travel expenses will be reimbursed. You have the option of receiving £20 as a single payment for each visit, or you can be reimbursed at each visit on the production of taxi receipt for attending and we will arrange a taxi (paid for by the research team) for your return after your visit.

Do I have to take part?

No, taking part is entirely voluntary. If you do not wish to take part you do not have to give a reason and in no way will your future treatment be affected.

What will happen to my clinical and personal information?

All the clinical information you provide will be encoded (so that your personal details such as name and address are secure) and stored securely. This information will not be revealed to anyone other than the researchers and your GP if you wish the latter to be informed. We would ask your permission to inform your GP of any clinically relevant abnormalities identified during the study.

Complaints

If you have any concerns about any aspect of this study, you should ask to speak with the researchers who will do their best to answer your questions (see below). If you remain unhappy and wish to complain formally, you can do this through the NHS Complaints Procedure or the Patient Advisory Liaison Service (PALS). Details can be obtained from the hospital.

What do I do now?

If you have any questions please contact:

- Dr Reza Zadeh, Dr Rahul Yadav or Dr Adam Greenstein. Cardiovascular Research Group, University of Manchester, Core Technology Facility (3rd floor), 46 Grafton Street, Manchester, M13 9NT). Tel: 0161 275 1229.

Alternatively, you can contact the doctor whose clinics you are attending:

- Dr Handrean Soran, Consultant Physician, Department of Medicine, Manchester Royal Infirmary, Oxford road, Manchester, M13 9WL. Tel: 0161 276 4066 (secretary).

- Mr Basil Ammori, Consultant Surgeon, Department of Surgery, Hope Hospital, Stott Lane, Salford M6 8HD. Tel: 0161 789 7373.

Thank you for taking the time to read this and considering taking part in our research. Please discuss this information with your family, friends or GP, if you wish. You will have at least 24 hours to read this information leaflet. After this time, we will contact you again to see if you are still interested in taking part.

10.4 Patient Consent Forms for Bariatric Studies in Chapter 7-8

Title of Study

Changes in paraoxonase activity, HDL properties and inflammatory markers in post-bariatric surgery patients, type 1 diabetics with and without nephropathy, type 2 diabetics, and during an oral glucose tolerance test

To be completed by the patient: **Please initial the boxes**

1. I confirm that I have read and understood the patient information sheet [version 3.0. 16.06.2012] provided for the study and I have had the opportunity to ask questions and have had these answered satisfactorily.

2. I understand that my participation is voluntary and that I am free to withdraw at any time 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 NHS trust or regulatory authorities, where it is relevant to my taking part in this research. I give my permission for these individuals to have access to my records.

4. I agree to serum and plasma being retained and stored as a gift to Manchester University and used for future ethically approved research at the end of the study.

5. I agree to fat biopsy samples being retained and stored as a gift to Manchester University and used for future ethically approved research at the end of the study.

6. I consent to my general practitioner being informed of my participation in the study and of any clinically relevant information.

7. I agree for my anonymised blood samples to be transferred to Australia for research purpose.

8. I give my consent to take part in the above study including:

a. blood tests ☐

b. Fat biopsy during bariatric surgery ☐

c. Retention and storage of biopsy tissue ☐

d. Nerve function tests ☐

Name……………………………………… Date of Birth…………………………

Signature………………………………………… Date…………………………………….

To be completed by the investigator or physician or nurse taking consent:

I confirm that I have fully explained and discussed with the patient the nature and purpose of the above study.

Name………………………………….Position………………… (e.g. Investigator)

Signature…………………………… Date……………………

Signature of physician if consent was witnessed by a nurse………………………

10.5 Study Assessment Forms

Check List:

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

Name of Patient:

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

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

Nephropathy

Exclusion criteria

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

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:

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

Page 1 of 5

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

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 □

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	Yes	**No**

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

If yes, choose only one:

in legs only (inc. feet) ☐

in arms only (inc. hands) ☐

in legs and arms only ☐

in mouth, face, or head only ☐

other than any of the above ☐

23. A more or less continuous "dead feeling" like novocain without prickling (tingling)? ☐ Yes ☐ No

If yes, choose only one:

in legs only (inc. feet) ☐

in arms only (inc. hands) ☐

in legs and arms only ☐

in mouth, face, or head only ☐

other than any of the above ☐

24. A more or less continuous "prickling" or "tingling" feeling with or without an asleep dead feeling? ☐ Yes ☐ No

If yes, choose only one:

in legs only (inc. feet) ☐

in arms only (inc. hands)

in legs and arms only

in mouth, face, or head only

other than any of the above

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

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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 ☐

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? ☐ ☐

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

Investigator:

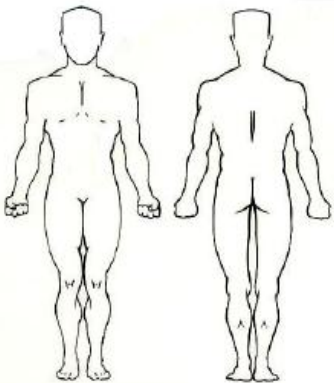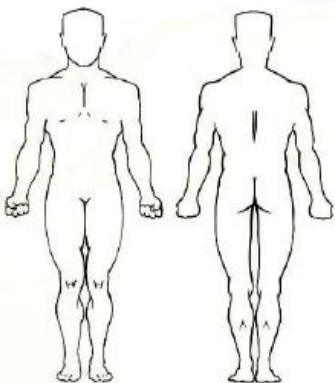**DNS-score and guidelines**

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

	Pain 1					Pain 2				
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-Buming	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-Exhauting	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	

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	NO PAIN	WORST POSSIBLE PAIN

	Total			Total	

Pain Index

Please circle one word from the index below that best describes your pain.

0	No Pain		0	No Pain
1	Mild		1	Mild
2	Discomforting		2	Discomforting
3	Distressing		3	Distressing
4	Horrible		4	Horrible
5	Excruciating		5	Excruciating

Total			Total	

Comments

Please write any comments you have below:

Thanks for completing the questionniare.

Investigator	

Neuropathy Disability Score

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

Achilles Reflex:

	☐	☐	☐	☐	☐	☐
	Normal	Reinforcement	Abnormal	Normal	Reinforcement	Abnormal
	(0)	(1)	(2)	(0)	(1)	(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 [ ]

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:

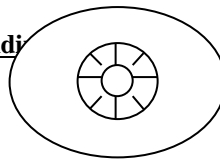Type of Transplant:

Duration on Renal Dialysis:

Smoking: per day

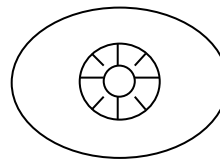Drinking: units per week

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:
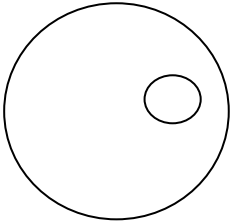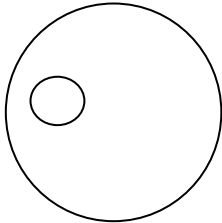
Corneal Confocal Microscopy (HRT III-RCM)

	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)**:

OD   **OS**

Comments: Comments:

Additional Notes