Vitamin D and Diabetic Neuropathy

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The accurate assessment of human diabetic somatic polyneuropathy (DSPN) is important to define at risk patients, predict deterioration, and assess the efficacy of pathogenetic treatments. Corneal confocal microscopy (CCM) has been proposed as a surrogate endpoint for DSPN. Approximately 50% of patients with DSPN experience neuropathic pain or symptoms and the underlying reasons are not clearly elucidated. Vitamin D deficiency has been associated with diabetic complications including DSPN and diabetic retinopathy (DR). However there is a paucity of data regarding the interaction of vitamin D status with diabetic complications.

This thesis shows that CCM can readily detect small fibre neuropathy prior to large fibre involvement and assess rapidly progressive nerve fibre loss prior to conventional thermal threshold testing. CCM has a superior diagnostic capabilities compared to intra-epidermal nerve fibres and correlates better with nerve conduction studies. Patients with LADA have a greater prevalence of small fibre neuropathy compared to matched patients with type 2 diabetes. Vitamin D deficiency is highly prevalent in patients with diabetes and despite relatively aggressive replacement regimens are inadequate in raising vitamin D levels in a significant proportion of patients. Vitamin D deficiency is not associated with DR but there is a strong association between painful DSPN and vitamin D insufficiency and more so with overt deficiency.
DECLARATION

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CONTRIBUTION

This section is to confirm that Uazman Alam, the author of this thesis, was actively involved and had a significant contribution in all chapters/studies presented and discussed in this thesis. Briefly, he recruited the vast majority of subjects with type 1 diabetes and latent autoimmune diabetes, a large portion of control subjects and a smaller portion of subjects with type 2 diabetes. He consented subjects, performed peripheral neuropathy assessments and undertook skin biopsies in the majority of subjects in the included studies. He also partook in a portion of laboratory skin biopsy processing and undertook intra-epidermal nerve fibre analysis for all controls, subject with type 1 diabetes and latent autoimmune diabetes as the dual examiner (along with Dr Maria Jeziorska). For chapters 4, 5 and 7, which are analyses of vitamin D data, he designed the studies and undertook data extraction with co-researchers. He performed all of the statistical analyses in this thesis with knowledge gained through an MSc in Population Health, postgraduate certificate in statistics for clinical trials and masters level professional development modules in advanced epidemiology. Finally, he has written all the chapters of this thesis which have been reviewed by his supervisor, Professor Rayaz Malik. The following tasks were performed by other members of the research team:

- Electrodiagnostic studies by Dr Andrew Marshall, consultant neurophysiologist.
- Ophthalmic examinations were performed by Dr Ioannis Petropulos, Dr. Mitra Tavakoli and Maryam Ferdousi.
- Peripheral neuropathy assessments and skin biopsies were also performed by Dr Omar Asghar and Dr Hassan Fadavi.
- Skin biopsy processing was undertaken by Dr Maria Jeziorska, Simon Forman, Louisa Nelson, Aisha Meskiri, and Wendy Jones.
- Skin biopsy analyses were also Dr Maria Jeziorska and Professor Rayaz Malik.
- Patient recruitment was also conducted by Georgios Ponirakis, Professor Rayaz Malik and Dr Hassan Fadavi.
- Software engineering to develop image analysis was undertaken by Dr Mohammad A Dabbah, Dr Xin Chen and Dr 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.
- Vitamin D laboratory analyses were undertaken by the vitamin D laboratory at the Central Manchester Foundation Trust under the guidance of Dr Jacqueline Berry.
- Co-researchers assisting with trial design and data extraction on chapters 4, 5 and 7 were Professor J Kennedy Cruickshank, Dr Sara Al-Himdani, Dr Sophie Benoliel, Dr Yasar Amjad, Dr Agnes Chan, Dr Ravinder Jugdey and Dr Osman Najam.
ALTERNATIVE THESIS FORMAT

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

- Chapter 3: To be submitted for publication.
- Chapter 4: To be submitted for publication.
- Chapter 5: To be submitted for publication.
- Chapter 6: Published in the journal *Diabetic Medicine*, 2012.
- Chapter 7: Accepted for publication in the *Journal of Diabetes and its Complications*, 2013.
- Chapter 8: Submitted for publication in the journal *Diabetes and is currently being revised for resubmission*, 2013.
- Chapter 9: To be submitted for publication.
LIST OF ABBREVIATIONS

1,25(OH)$_2$D: 1,25 vitamin D: 1,25 hydroxyvitamin D

25(OH)D: 25(OH) vitamin D: 25 hydroxyvitamin D

Ab: antibody

ACCORD: Action to Control Cardiovascular Risk in Diabetes

ACR: albumin creatinine ratio

AE: adverse event

AGE: advanced glycation end products

ALADIN: Alpha Lipoic in Diabetic Neuropathy

Alb: Albumin

ALP: Alkaline phosphatise

BDR: background diabetic retinopathy

BMI: body mass index

BP: blood pressure

CCa$^+_{2+}$: corrected calcium

CCM: corneal confocal microscopy

CI: confidence interval

CNBD: corneal nerve branch density

CNFD: corneal nerve fibre density

CNFL: corneal nerve fibre length

CNFT: corneal nerve tortuosity coefficient

CNS: central nervous system

CBC: complete blood count

CKD: chronic kidney disease

CRGP: calcitonin gene-related peptide
CST: cold sensation threshold
CVA: Cerebrovascular accident
CVD: cardiovascular disease
DCCT: diabetes control and complications trial
DD: disc diameter
DM: diabetes mellitus
DNS: Diabetic Neuropathy Symptom Score
DPN: diabetic peripheral neuropathy
DR: diabetic retinopathy
DSPN: diabetic somatic polyneuropathy
EDIC: Epidemiology of Diabetes Interventions and Complications
eGFR: estimated Glomerular Filtration Rate
eNOS: endothelial nitric oxide synthase
fMRI: functional magnetic resonance imaging
GAD: anti-glutamic acid decarboxylase
HbA1c: glycated haemoglobin A1c
HDL: high density lipoprotein cholesterol
ICA: islet cell antibodies
IDF: International Diabetes Federation
IENF: intra-epidermal nerve fibre
IENFD: intra-epidermal nerve fibre density
IFN: interferon
IHD: ischaemic heart disease
IQR: interquartile range
IU: international units
LADA: latent autoimmune diabetes in adults

LANDMARK: Longitudinal Assessment of Ophthalmic Diabetic Neuropathy

LDL: low density lipoprotein cholesterol

LFT: liver function tests

LURIC study: Ludwigshafen risk and cardiovascular health study

MI: myocardial infarction

McGill VAS: McGill visual analogue score

NBF: nerve blood flow

NCCA: non-contact corneal aesthiometer

NCS: nerve conduction studies

NCV: nerve conduction velocity

NDR: no diabetic retinopathy

NDS: neuropathy disability score

NGF: nerve growth factor

NGSP: National Glycohaemoglobin Standardization Program

NHANES: National Health and Nutrition Examination study

NSP: neuropathy symptom profile

NCS: nerve conduction study

OGTT: oral glucose tolerance test

OR: odds ratio

PDR: proliferative diabetic retinopathy

PPDR: pre-proliferative diabetic retinopathy

PKC: protein kinase C

PMNCV: peroneal motor nerve conduction velocity

PMNamp: peroneal motor nerve amplitude
PVD: peripheral vascular disease
ROC: receiver operating characteristic
QST: quantitative sensory testing
RAGE: receptor for AGE
RAS: renin angiotensin aldosterone system
RCT: randomised clinical trial
RNFD: rapid nerve fibre decline
SD: standard deviation
SSNCV: sural sensory nerve conduction velocity
SSNAmpl: sural sensory nerve amplitude
TGF-β₁: transforming growth factor β₁
TRIG: triglycerides
TRPV1: transient receptor potential channel V1
T-CHL: total cholesterol
UE: urea and electrolytes
VAS: visual analogue score
RDA: recommended daily allowance
UKPDS: United Kingdom prospective diabetes study
VDR: vitamin D receptor
VEGF: vascular endothelial growth factor
VITAL: Vitamin D and Omega-3 Trial
VPT: vibration perception threshold
WST: warm sensation threshold
In the name of Allah, the beneficent, the merciful.

La-ilaha-iLLaLLah- Muhammadur-Rasulullah.

“There is no wealth like knowledge, no poverty like ignorance”.

“There is no knowledge and science like pondering and thought; and there is no prosperity and advancement like knowledge and science”.

Hazraat Ali bin Abu-Talib (RA), Fourth Caliph of Islam

DEDICATION

This Thesis is dedicated to my beloved mother,

my first and most important teacher.

She continues to be the most inspirational person in my life.
ACKNOWLEDGEMENTS

Even before I decided to practice medicine and become a physician, I always had a clear aspiration to undertake research. The completion of this PhD has brought together a number of years of work which started well before this project commenced. I have thoroughly enjoyed my time undertaking this novel work. It would not have been possible without the support of many individuals.

First and foremost, I would like to thank Professor Rayaz Malik who has been my friend and mentor for over ten years. He has guided me from a fourth year medical student working on peripheral nerve histology to my current role as a SpR in endocrinology and on the cusp of completing a PhD. He has been a continual source of personal encouragement and has a enthusiastic passion for research and clinical excellence which he projects on all his co-workers and students. A special mention must be given to Dr Kashif Khawaja who took an interest in me as a medical student on a paediatric placement when I said ‘I am interested in learning research’ and introduced Professor Malik and I in 2002.

My co-supervisor Dr Maria Jeziorska has spent much time and effort in providing me with skills of skin biopsy processing and analysis. Her help with reviewing chapters of the thesis and overall encouragement were invaluable. I would also like to thank my advisor Professor Andrew J M Boulton for allowing me to gain early research skills in the diabetic foot at the Diabetes Research Institute, University of Miami whilst an undergraduate medical student and for all his advice and support during the PhD.

Drs Ioannis Petropoulos, Omar Asghar, Hassan Fadavi, Maryam Ferdousi, Mitra Tavakoli, Andrew Marshall, Ravinder Jugdey, Georgios Ponirakis, Osman Najam, Yasar Amjad, Agnes Chan, Sara Al-Himdani, Sophie Benoliel, Cristiano Van Zeller,
and April Buazon have provided significant support to the series of studies presented and I am indebted for their efforts in this work. Dr Ioannis Petropoulos and Dr Salik Kakar require further mention, they have both provided me with detailed technical support of Microsoft office© Word.

Both my family and close friends were very important in bringing all this together. I must show an immense amount of gratitude to my wife, Iram who is expectant with our first child at the time of writing this thesis. She has encouraged me throughout the writing process and has been very patient and understanding of my work commitments, particularly as I have combined the thesis writing with working as an SpR. I would also like to thank my parents, my brother Kashif and two sisters, Afshaan and Saima, eldest niece Marihah and my nephew, Danyaal for all their support during this PhD and the many previous years.

I would like to acknowledge the significant contribution of all of the subjects who participated in this research and without their efforts none of this work would be possible. The support of the nursing staff at the Wellcome Trust Clinical Research Facility was of course invaluable. The Juvenile Diabetes Research Foundation and the National Institute of Health must be generously acknowledged for their financial and honourable commitment to these projects. Finally, I want to thank my close friends Ravinder Jugdey and Mohammed Qadir Hussain for their continual personal support and guidance.

**Addendum to the Acknowledgements**

My daughter, Inara, was born on Sunday 20th October 2013. I hope her life is full of happiness (with Allah’s will); she has brought immense happiness to my wife and I and my extended family.
PREFACE

Uazman Alam graduated with a BSc in Medical Science from the University of St. Andrews, Scotland, UK in 2001, before he went on to the University of Manchester, UK in 2001 to undertake Medicine (MBChB) and graduated from this in 2005. Part way through studying Medicine he took a year out to study for an MSc in Population Health (MPHe) in 2003 until 2004 and graduated from this in 2005. He has also completed other academic courses in Advanced Epidemiology and he has advanced his knowledge of statistics by completing a Post-Graduate Certificate in Statistics for Clinical Trials. Uazman has a plethora of work based clinical and research experience as well as experience of academic teaching. He has been actively involved in research from a fourth year medical student. The genesis of Uazman’s research education began working on peripheral nerve histology in experimental models of diabetes under the auspices of Professor Rayaz Malik. Uazman worked as a ‘houseman’ at the Pennine Acute Hospital NHS Trust in 2005 and rotated in A&E, chest medicine and general surgery. He successfully applied for a prestigious academic FY2 post at the North West Lung Centre, Wythenshawe Hospital and was involved in a randomised controlled trial on cough and inflammation which involved clinical assessment of subjects, undertaking flow volume loops, body plethysmography and laboratory analysis of sputum. From there, Uazman went on to complete his senior house officer training in South Yorkshire at Rotherham General Hospital and the Northern General Hospital (2007-2009). He is currently an SpR (ST5) in Endocrinology and Diabetes at Pennine Acute Hospital Trust.

Uazman’s interest in Diabetes and Endocrinology has its foundations as a 4th year medical student in 2003 working with Professor Rayaz Malik on animal models of
peripheral nerve histology and during his elective period at the Diabetes Research Institute, University of Miami in 2005. Uazman became involved in diabetes during this period of time and then advanced his interest in this speciality in 2009 when he was awarded a National Training Number in Endocrinology and Diabetes and the same year he took time out of programme as a Clinical Research Fellow to undertake a PhD. He undertook both clinical work and research at the Manchester Diabetes Centre, Manchester Royal Infirmary and Centre for Endocrinology and Diabetes, University of Manchester. The three years he spent working and researching at these latter two locations have allowed him to conduct research and extrapolate data for his PhD focusing on Longitudinal Assessment of Ophthalmic Diabetic Neuropathy Markers (LANDMARK) study and the role of vitamin D in diabetes under the supervision of Professor Rayaz A Malik.

During his PhD, he presented his work at regional, national and international conferences between 2010 and 2013 including the American Diabetes Association, NeuroDiab - Diabetic Neuropathy Study Group of the EASD, Diabetes UK and at the International Diabetes Federation’s World Diabetes Congress.

Uazman is a member of the Royal College of Physicians and a council member for Lipids, Metabolism and Vascular risk section of the Royal Society of Medicine. He is also an examiner for the University of Manchester Medical School and has been the assistant lead examiner for the final MB exams from 2010-2012 at the Manchester Royal Infirmary.
LIST OF PUBLICATIONS

Research Publications


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Editorials
LIST OF ABSTRACTS


Chapter I - INTRODUCTION
Diabetes Mellitus (DM) has been recognised for millennia and an Egyptian manuscript described a condition as “too great emptying of the urine” as early as 1550 BC. Around the same time, Indian physicians identified the disease and classified it as ‘madhumeha’ or honey urine noting that the urine would attract ants. However, it was not until the 18th century that Western physicians began to study diabetes and its complications (1). The islets of Langerhans were discovered in 1869 by the anatomist Paul Langerhan. The endocrine role of the pancreas and the existence of insulin were not fully clarified until the epochal discovery in 1921 when Frederick Banting and George Best demonstrated they could reverse induced diabetes in dogs, by extirpating the pancreas and then administrating an extract from the pancreatic islets of Langerhans of healthy dogs (2-3).

DM is a group of disorders which are characterised by hyperglycaemia due to an absolute or relative deficit in insulin production or action (4). The chronic hyperglycaemia of DM is associated with end-organ damage, dysfunction and failure, including the retina, kidney, nerves, heart and blood vessels (4). The International Diabetes Federation (IDF) estimates an overall prevalence of DM to be 366 million in 2011, and this is expected to rise to 552 million by 2030 (5).

1.1 Aetiopathology of Diabetes

DM is a heterogeneous group of metabolic disorders fall into two broad categories characterised by raised plasma glucose concentrations due to absolute (type 1 DM) or relative (type 2 DM) lack of insulin secretion, action or both and thus resulting in aberrations in fat, carbohydrate and protein metabolism. Diagnosis may be made through glucose tolerance test, fasting and/or random plasma glucose or glycated haemoglobin A1c (HbA1c) (Table 1-1).
Table 1-1 Diagnosis of DM

<table>
<thead>
<tr>
<th>Diagnostic Parameter</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Fasting Plasma Glucose</strong></td>
<td>≥7.0 mmol/l</td>
</tr>
<tr>
<td>2 hour plasma glucose ≥11.1 mmol/l during an OGTT</td>
<td>The test should be performed according to the World Health Organisation guidelines, using a glucose load containing the equivalent of 75 g anhydrous glucose dissolved in water.</td>
</tr>
<tr>
<td>In a patient with classic symptoms of hyperglycaemia, a random plasma glucose ≥11.1 mmol/l</td>
<td></td>
</tr>
<tr>
<td><strong>HbA1C ≥6.5% / 48mmol/mol</strong></td>
<td>Its role in diagnosis has been cemented by the American Diabetes Association, although some consider there is a further case for discussion in its precise role (6). The test should be performed in a laboratory using a method that is NGSP Certified and Standardized to the DCCT assay.</td>
</tr>
</tbody>
</table>

Adapted from Diagnosis and Classification of Diabetes Mellitus (7).

**Table key**

OGTT – Oral Glucose Tolerance Test.
DCCT – Diabetes Control and Complication Trial.
NGSP - National Glycohaemoglobin Standardization Program.

In type 1 DM, individuals are often metabolically normal before the disease is clinically manifest, but the process of autoimmune β-cell destruction can be detected before overt diabetes develops. The presence of this autoimmune disorder is characterised by the presence of auto-antibodies such as anti-glutamic acid decarboxylase (GAD), islet (ICA) or insulin antibodies (8). Type 1 DM is
associated with HLA DR3 and DR4 (9-11) and it has been proposed that an
interplay of a genetic predisposition with environmental factors may lead to an
autoimmune response (12). Environmental factors implicated include viral
infections, early childhood cow’s milk administration, malnutrition, and
vaccinations, which trigger an immune cascade leading to cross reactivity with
pancreatic β-cells, however to date no definitive causal link has been identified (12-
14). Furthermore, there is a putative link of vitamin D deficiency and the
occurrence of type 1 DM (15) with a positive impact of supplementation with
cholecalciferol (vitamin D₃) during the first year of life on the risk of developing type
1 DM (16) and a meta-analysis of five observational studies has confirmed this risk
reduction (17). Importantly in the Finnish Birth cohort study (16), children with
suspected rickets had a threefold increase in their risk of type 1 DM. There is a
striking geographical variation in the incidence of type 1 diabetes and seasonal
variation with the highest incidence of onset during the winter months and at
greater latitudes; thus suggesting that variations in vitamin D synthesis in the skin
and subsequent status may play a significant role in the pathogenesis (18-20). The
destruction of pancreatic β-cells is an autoimmune T-helper cell mediated disorder
and activated vitamin D (1,25(OH)₂D) has immunomodulatory actions in T-cells
and leads to a reduction in the induction of Th1 cytokines such as interferon (IFN) γ
(15; 21-23).

Type 2 DM has become an epidemic in Western society and virtually no health
care professional is without patients who have the disease. The majority of patients
with DM have type 2 DM (~90%) with only a small proportion having the
monogenic forms such as maturity onset diabetes of the young (24-25). Type 2 DM
results from defects in insulin secretion, but with a major contribution from insulin
resistance (7). There is subsequently an inadequate compensatory insulin secretary response resulting in hyperglycaemia. Numerous mechanisms have been implicated, including changes in fatty acid fractions, interplay of inflammatory cytokines and adipokines, mitochondrial dysfunction with insulin resistance, and glucotoxicity, lipotoxicity, and amyloid formation and deposition leading to β-cell dysfunction (26). Type 2 DM has a strong genetic component, however, only a small number of genes have been identified to date (26). A family history confers a 2-4 fold increase for type 2 DM. The lifetime risk of type 2 DM is approximately 38% and 60% if one or both parents have the disease respectively (27-28). This genetic predisposition is highlighted by the fact that monozygotic twins have higher concordance rates of type 2 DM than dizygotic twins (29-30). Vitamin D may play an important role in the pathogenesis of type 2 DM. Insulin sensitivity is improved significantly in adults with impaired fasting glucose who were randomised to calcium and vitamin D supplementation (31). Furthermore, baseline 25(OH) vitamin D levels in non-diabetic subjects were shown to predict future glycaemia and insulin resistance (32). In the meta-analysis by Parker et all (33), the risk of type 2 diabetes may be reduced by 55% in individuals with the highest levels of vitamin D. Despite the majority of cases of diabetes falling into the two broad aetiopathogenetic categories of type 1 and type 2 DM often classified on their clinical presentation. In some individuals this classification is not so well defined. It is common practice to classify individuals based on the following variables:

1. Age at onset of diabetes
2. The abruptness of hyperglycaemia
3. Presence of ketosis
4. Degree of obesity

5. Need for insulin at diagnosis

This however, does not always accurately classify the aetiology of diabetes.

1.2 Latent Autoimmune Diabetes in Adults (LADA)

Latent Autoimmune Diabetes in Adults (LADA) is an under diagnosed form of diabetes. Particularly, as the age at onset is later than in classical type 1 diabetes. The finding of auto antibodies is characteristic of type 1 DM but can be found in 10-30% of those who have phenotypically type 2 DM (34-35). It is this subset that is termed as LADA. Furthermore, the U.K. prospective diabetes study (UKPDS) demonstrated that approximately 10% of adults with type 2 diabetes have LADA (36). Although the prevalence may be as low as 3.6% of all newly diagnosed apparent type 2 diabetic patients (37). Despite the initial lack of requirement of exogenous insulin the underlying process is that of β-cell destruction as opposed to the insulin resistance of type 2 DM, it does however occur slower than the classical young onset type 1 DM (38).

1.2.1 Screening and Treatment in LADA

Insulin dependency can occur after a relatively short period of around 6 months or up to 10 years or more (39-40). The time to insulin therapy use can vary depending on local clinical judgement and use of laboratory GAD antibody testing (41). However, despite the variances, the median time to failure of oral medication and exogenous insulin use is less than 5 years (41). In fact, a clinical screening tool has been developed which can help to identify individuals with LADA (42). These criteria are based upon the clinical presentation of this subgroup of individuals. The five clinical features that are more common in LADA compared to type 2 DM are:
a. Age of onset less than 50 years
b. Acute symptoms (polyuria, polydipsia, osmotic visual changes etc)
c. BMI ≤25kg/m²
d. Personal history of autoimmune disorders
e. Family history of autoimmune disease

In one particular study (42) the presence of at least two of these distinguishing features (clinical risk score ≥2) had a 90% sensitivity and 71% specificity for identifying LADA. The negative predictive value for a LADA clinical risk score ≤1 was 99% (42). The most common diagnostic test for LADA is a GAD antibody assay (43). GAD antibodies may also be present in individuals with type 1 DM and thus generally an initial exogenous insulin free period is often considered a requirement for a definitive diagnosis of LADA. GAD antibodies are not found in patients with pure type 2 DM (37).

Individuals with LADA may require insulin at an earlier in the disease compared to those with type 2 DM. The slow process of β-cell destruction occurs to the point that oral agents poorly control hyperglycaemia. However, a number of interventions have been trialled to evaluate the possible beneficial effects of different classes of diabetes medications (44). There are a paucity of data on the effects of oral agents alone in LADA. Sulphonylurea treatments work by stimulating the β-cells to produce endogenous insulin. This class of medication has been associated with a more rapid progression to insulin treatment in those who are well controlled at baseline compared to other treatment groups (44). It is possible that β-cell failure is accelerated through the action of sulphonylureas. Furthermore, in the systematic
review by Brophy et al (44) individuals who were poorly controlled at baseline progressed to insulin treatment rapidly (60% in 2 years). There may be some beneficial effect on β-cell preservation in those with LADA who are treated with the now withdrawn rosiglitazone (when compared to sulphonylureas) and this may highlight the possibility that there is a degree of insulin resistance despite the autoimmune nature of the disorder (45-46). Other interventions have also been shown to have a protective effect on β-cell function. Li et al (47) undertook a randomised trial of insulin versus 1-α-hydroxyvitamin D₃ plus insulin and in a subgroup of those with a duration of diabetes less than 1 year they showed a significantly elevated fasting c-peptide and postprandial c-peptide in those who were treated with insulin and 1-α-hydroxyvitamin D₃, suggesting that β-cell function was better preserved. Indeed there is some evidence that vitamin D deficiency may be related to type 1 DM (48). Furthermore, vitamin D deficiency and receptor gene polymorphisms may be a risk factor for the development of type 1 DM and other forms of diabetes that have an autoimmune element to their pathogenesis (49-51). There is evidence to suggest that autoimmune diabetes presents a continuum of genetic susceptibility, which extends from a strong effect in childhood-onset type 1 DM to a more limited effect in LADA (52). The predominant imbalanced production of T helper 1 cells in part through the production of interleukin (IL) 12 in autoimmune diabetes is destructive to the pancreatic β-cell (52). Activated vitamin D may inhibit the production of IFN-γ, IL-2 and IL-12 (53) and thus possibly disrupting the T helper 1 mediated pathogenesis.

This highlights a possible opportunity to preserve β-cell function as there is a slower rate of β-cell decline than in the classical type 1 DM. Maximising β-cell
function through interventions may lead to better glycaemic control and hence reduce the rate of microvascular complications (54).

1.2.2 Auto Antibodies in LADA

Generally a diagnosis of LADA is based upon a phenotypically type 2 individual testing positive for GAD antibodies, as this is the most common antibody present in this cohort (55). Even in the absence of GAD antibodies some individuals may test positive for other β-cell auto-antibodies (56) conferring a diagnosis of LADA. Furthermore, other autoimmune markers such as ICA, and tyrosine phosphatase auto-antibodies (anti-IA2 or anti-ICA512) have been found in LADA. A combination of ICA and GAD antibodies and the levels of their titres have been shown by Lohmann et al 2001 (57) to help differentiate two clinical and phenotypical variants of LADA. Those who are ICA positive and have high titres of GAD antibodies are clinically more similar to individuals with type 1 diabetes whereas those who are negative for ICA and have low titres of GAD antibodies are clinically similar to individuals with type 2 diabetes (57). The levels of GAD antibody titres are not thought to predict disease progression (58) but a combination of thyroid peroxidase antibodies (which one study occurred at a possible prevalence of 29% (59)), anti-IA2 and C-peptide levels may predict β-cell failure (60).

Previous studies have shown co-existing coeliac disease in those with type1 DM with a rate of between 1-7% (61-62) and anti-gliadin antibodies may have a high prevalence in those with type 1 DM even without symptoms of coeliac disease (63). It seems that there is an increased frequency of humoral markers of autoimmunity and this is also mirrored in LADA (64). A number of non β-cell antibodies are associated with LADA and include anti-gliadin and thyroid
peroxidase antibodies (64). It has been suggested that a susceptibility to anti-gliadin antibodies may reflect changes in the mucosal barrier function and thus cause changes in antigen exposure (64).

Defining LADA through testing for auto-antibodies itself can pose a significant diagnostic challenge as these markers can change over time including absence of ICA and GAD antibodies after the presenting illness. Defining cut off levels of these antibodies for diagnosis is troublesome as there is a continuous spectrum of distribution in both diabetic and control populations. This also highlights the possibility of false positives, as these markers may be present in non-diabetic populations.

### 1.2.3 Microvascular complications in LADA

Historically, complications in LADA have been poorly studied. Baum et al (65), compared patients with LADA (n=14) versus type 1 DM (n=9) and type 2 DM (n=14) through clinical assessment, quantitative sensory testing (QST) and electrophysiology. Individuals with LADA had fewer features of diabetic neuropathy in particular DSPN in the early stages of the disease and were similar to the classical type 1 DM individuals who develop neuropathy at a later stage after diagnosis (65). In an epidemiological study (Freemantle study), increased prevalence of retinopathy compared with GAD negative controls with type 2 diabetes which was primarily attributable to poorer glycaemic control (66). All the studies relating to prevalence of LADA have a common limitation of small study size samples particularly for epidemiological studies. The LADA cohort requires further investigation with regard to the microvascular complications so that future care can be tailored to this group.
1.3 Complications of Diabetes

Diabetes is a major risk factor for macro and micro-vascular complications, such as strokes and amputations, nephropathy, retinopathy and neuropathy. These complications are a major cause of illness and an enormous economic burden especially with the increasing prevalence of diabetes in western society (67). Diabetes is a leading cause of end-stage renal disease in many developed countries accounting for up to 50% of patients receiving renal replacement therapy (67). Kidney disease is common at the time of diagnosis of DM with a prevalence of ~20% (68) but also accounts for 21% of deaths in type 1 DM and 11% of deaths in type 2 DM (69). Within 20 years after disease onset, nearly all adults with type 1 DM and more than 60% of adults with type 2 DM develop the complication of DR (70-71). In an analysis in the UK, it is believed that there is a prevalence of blindness of 4200 and an annual incidence of blindness of 1280 people with diabetes (72) and in a retrospective analysis (n=20,686) from 1990-2006 of national diabetic retinopathy screening program, the first retinal examination (baseline), 79% did not have retinopathy, 18% had non-proliferative retinopathy, and 2.9% had pre-proliferative retinopathy (73). In this same study after 10 years, patients without retinopathy at baseline had an increased incidence of pre-proliferative retinopathy (cumulative incidence 16.4%), sight-threatening maculopathy (1.2%), or proliferative retinopathy (1.5%) (73). In Wisconsin Epidemiologic Study, the 14-year rate of progression of retinopathy was 86%, regression of retinopathy was 17%, progression to proliferative retinopathy was 37% and incidence of macular oedema was 26% (74). Diabetic neuropathy is discussed in more detail below.
Diabetic patients from ethnic minorities have higher mortality rates and higher risk of diabetes complications (75). In the study by Lanting et al (75), additional adjustment for risk factors in most instances ethnic differences disappear. However, blacks and Hispanics in the U.S. and Asians in the U.K. have an increased risk of end-stage renal disease, and blacks and Hispanics in the U.S. have an increased risk of retinopathy (75). In terms of neuropathy, there appears to be a tendency for white individuals to have a higher prevalence of sensorimotor neuropathy than the groups they were compared (76). In a community based study in the U.K., painful DSPN was more prevalent in patients with type 2 diabetes, women, and people of South Asian origin despite a lower prevalence of neuropathy in the latter group (77).

1.3.1 Diabetic Neuropathy

The complication of neuropathy has been well documented since the end of the 19th century, when a physician by the name of Purdy concluded: ‘It is rare to meet with a case of diabetes in which there is not more or less nervous disturbance’ (78). Since those early days of investigation there has been extensive research of diabetic neuropathies. The most common presentation of neuropathy in diabetes is a DSPN and is considered to be the presence of symptoms and/or signs of peripheral nerve dysfunction in people with diabetes after the exclusion of other causes. DSPN is a symmetrical, length-dependent sensorimotor polyneuropathy attributable to metabolic and microvessel alterations as a result of chronic hyperglycaemia exposure and cardiovascular risk covariates (79). The occurrences of diabetic retinopathy and nephropathy in a given patient strengthen the case that the polyneuropathy is attributable to diabetes (79). The prevalence of DSPN in
diabetes can be as high as 50% (80) and symptomatic diabetic neuropathy can affect up to 30-40% of diabetic patients with neuropathy (81). Traditionally symptoms have been denoted as either positive (aching, burning, sharp or pressure pains) or negative (numbness or dead feeling). Both ‘negative and positive symptoms’ may coexist.

1.3.1.1 Prevalence of Distal Symmetrical Polyneuropathy

The prevalence of DSPN is thought to be around 30% (80; 82) and increases with the duration of the disease. When diabetes has been present for greater than 25 years, the prevalence rises to approximately 50% (82). The Rochester Diabetic Neuropathy Study reported a prevalence of 54% and 45% for patients with type 1 and type 2 DM respectively, amongst 380 patients in total, with the majority however not exhibiting neuropathic symptoms (83). The 1989 National Health Interview Survey consisted of a representative sample of 84,572 persons in the U.S.A. aged 18 years or over (84). Those with DM (n=2405) were identified and administered a questionnaire to define the prevalence of symptoms of sensory neuropathy which was found to be 30.2% among subjects with type 1 DM. This prevalence was 36.0% for men and 39.8% for women with type 2 DM, compared with 9.8 and 11.8% for non-diabetic men and women, respectively (84). Symptoms of sensory neuropathy affect 30-40% of diabetic patients and the prevalence of these symptoms increase with longer duration of diabetes; hypertension and hyperglycaemia (84). However, importantly there was neither a clinical or quantitative assessment of neuropathy in this study. In a survey of 10 English practices based in the community (85), 1077 subjects with diabetes were screened for neuropathy by eliciting two of the following five:
1. Neuropathic foot symptoms

2. Loss of light touch sensation

3. Impaired pin prick sensation

4. Absent ankle jerk reflexes

5. Vibration perception threshold greater than 97.5% of an age-standardised value.

There was an age and sex matched non-diabetic control group (n=480) (85). The prevalence of neuropathy was 16.3% in diabetic patients and 2.9% in non-diabetic subjects, yielding a prevalence odds of 6.75 (95% CI 3.87-11.79), p<0.001 (85).
# Table 1-2 Details of major epidemiological studies of DSPN

<table>
<thead>
<tr>
<th>Study</th>
</tr>
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<tbody>
<tr>
<td>Rochester Diabetic Neuropathy Study (n=380) (83)</td>
</tr>
<tr>
<td><strong>Prevalence (type of DM)</strong></td>
</tr>
<tr>
<td>54% / 45% (Type 1 (n=102) / Type 2 DM (n=278))</td>
</tr>
<tr>
<td><strong>Risk factors</strong></td>
</tr>
<tr>
<td>Not reported</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>San Luis Valley Study (n=277) (86-87)</td>
</tr>
<tr>
<td><strong>Prevalence (Type 2 DM)</strong></td>
</tr>
<tr>
<td>26%</td>
</tr>
<tr>
<td><strong>Risk factors</strong></td>
</tr>
<tr>
<td>Age, male sex, HbA1c, duration of disease, insulin use, lower fasting C-peptide, DR, diabetic nephropathy</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pittsburgh Epidemiology of Diabetes Complications Study (n=363) (88-89)</td>
</tr>
<tr>
<td><strong>Prevalence (Type 1 DM)</strong></td>
</tr>
<tr>
<td>34%</td>
</tr>
<tr>
<td><strong>Risk factors</strong></td>
</tr>
<tr>
<td>Duration of diabetes, HbA1c reduced HDL, current smoking, macrovascular disease</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dutch Population study (n=137) (90)</td>
</tr>
<tr>
<td><strong>Prevalence (Type 2 DM)</strong></td>
</tr>
<tr>
<td>53-63% Dependent on method of assessment (Type 2 DM)</td>
</tr>
<tr>
<td><strong>Risk factors</strong></td>
</tr>
<tr>
<td>Macrovascular disease</td>
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</table>

<table>
<thead>
<tr>
<th>Study</th>
</tr>
</thead>
<tbody>
<tr>
<td>EURODIAB IDDM Complications Study (n=3,250) (91)</td>
</tr>
<tr>
<td><strong>Prevalence (Type 1 DM)</strong></td>
</tr>
<tr>
<td>28%</td>
</tr>
<tr>
<td><strong>Risk factors</strong></td>
</tr>
<tr>
<td>Age, diabetes duration, weight, HbA1c, smoking status, severe ketoacidosis, microalbuminuria, retinopathy</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Study</th>
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</thead>
<tbody>
<tr>
<td>United Kingdom Prospective Diabetes Study (cross sectional analysis, n=2,337) (68; 92)</td>
</tr>
<tr>
<td><strong>Prevalence (Type 2 DM)</strong></td>
</tr>
<tr>
<td>5-7% Dependent on method of assessment (Type 2 DM)</td>
</tr>
<tr>
<td><strong>Risk factors</strong></td>
</tr>
<tr>
<td>HbA1c, hypertension</td>
</tr>
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<table>
<thead>
<tr>
<th>Study</th>
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</thead>
<tbody>
<tr>
<td>National Health and Nutrition Examination Survey (n=419) (93)</td>
</tr>
<tr>
<td><strong>Prevalence (Type 1 DM/Type 2 DM)</strong></td>
</tr>
<tr>
<td>28.5%</td>
</tr>
<tr>
<td><strong>Risk factors</strong></td>
</tr>
<tr>
<td>Age, ethnicity</td>
</tr>
</tbody>
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<th>Study</th>
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<tbody>
<tr>
<td>National Health and Nutrition Examination Survey (n=419) (93)</td>
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<tr>
<td><strong>Prevalence (Type 1 DM/Type 2 DM)</strong></td>
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<tr>
<td>13.3% in Controls</td>
</tr>
<tr>
<td><strong>Risk factors</strong></td>
</tr>
<tr>
<td>Age, ethnicity</td>
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</table>
1.3.1.2 Diabetic Neuropathy, Glycaemia Control and other risk factors

The role of tight glycaemic control in improving microvascular outcomes is irrefutable. The United Kingdom Prospective Diabetes Study (UKPDS) demonstrated a significant risk reduction for cardiovascular disease (CVD) and amputation for every 1% reduction in mean HbA1c (94). The Diabetes Control and Complications Trial (DCCT) is another landmark study conducted in type 1 DM (95). This prospective study evaluated 1441 subjects aged 13 to 39 years old who were followed up for 5 years (96). 726 had type 1 DM for 1 to 5 years and had no retinopathy at baseline (primary prevention cohort); 715 had type 1 DM for 1 to 15 years and had minimal to moderate non-proliferative retinopathy at baseline (secondary intervention cohort) (96). Intensive therapy reduced the development of confirmed clinical neuropathy by 64% in the combined cohorts after 5 years of follow-up (96). This was defined as a history or physical examination consistent with clinical neuropathy confirmed by either abnormal nerve conduction or autonomic nervous system testing. The prevalence of abnormal nerve conduction and abnormal autonomic nervous system function were reduced by 44% and 53% respectively (96). The Epidemiology of Diabetes Interventions and Complications (EDIC) Study (this assessed the 14-year cumulative benefits of prior intensive insulin treatment in the DCCT) found a lower incidence of DSPN (22.0% vs. 28.0%) amongst patients assigned to the intensive arm compared to the conventional arm (97). Therefore, the benefit of former intensive insulin treatment persisted for 13-14 years after DCCT closeout (97). These data introduced the concept of ‘metabolic memory’ in which provides significant evidence of a durable effect of prior intensive treatment on neuropathy (98).
The Eurodiab study (91), assessed the risk factors for the development of distal symmetric neuropathy in 1172 patients with type 1 DM. Neuropathy was assessed at baseline (1989 to 1991) and at follow-up (1997 to 1999), with a follow-up of 7.3±0.6 years. A standardised protocol for the evaluation of DSPN included clinical evaluation, quantitative sensory testing and autonomic-function tests. Serum lipids and lipoproteins, HbA1c, and the urinary albumin excretion rate were also measured. Duration of diabetes, HbA1c status, change in glycosylated haemoglobin value during the follow-up period, body-mass index and smoking were all independently associated with the incidence of neuropathy (91). Other than glycaemic control, the incidence of neuropathy is associated with potentially modifiable cardiovascular risk factors, including a raised triglyceride level, body-mass index, smoking and hypertension (91).

1.3.2 Painful Diabetic Neuropathy

DSPN has an insidious onset and is often indicated by sensory symptoms that start in the toes and then progress proximally to involve the feet and legs in a stocking distribution. DSPN results in the loss of sensation, which may lead to foot ulceration and eventual lower limb amputation, representing the commonest cause of hospitalisation amongst patients with DM (99). Approximately 50% of patients with DSPN experience neuropathic symptoms (100) and these symptoms include; burning pain, electrical or stabbing sensations, paraesthesiae, hyperaesthesia and a deep aching pain (Table 1.3).
Table 1-3 Abnormal sensations in DSPN

<table>
<thead>
<tr>
<th>Sensation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dysaesthesia</td>
<td>Spontaneous pain</td>
</tr>
<tr>
<td>Allodynia</td>
<td>Stimulus which does not usually evoke pain</td>
</tr>
<tr>
<td>Hyperalgesia</td>
<td>Exaggerated pain</td>
</tr>
<tr>
<td>Paraesthesia</td>
<td>Abnormal touch sensation</td>
</tr>
</tbody>
</table>

In a study conducted in the U.S.A., painful DSPN was associated with decrements in many aspects of patients’ lives: physical and emotional functioning and significant sleep disturbance (101) with a greater negative impact in patients with greater pain severity which has also been noted in other studies (100-101).

The precise reasons for the development of neuropathic pain despite loss of sensation is not clearly elucidated but suggests that aberrations in pain signalling in the peripheral and central nervous system occur (100). Capsaicin found in chilli peppers activates transient receptor potential channel (TRP) V1 on the peripheral nerve and topical application of capsaicin produces an analgesia effect at higher doses (102-103) and polymorphisms in TRPV1 can explain some of the variation in pain seen after neuropathy in patients (104). Alterations in ion channels have been noted in damaged peripheral nerve (105). Changes in sodium and calcium channel distribution and expression impact on the pattern of neuronal excitability (100) and inherited pain disorders are known to occur due to mutations in sodium channel NAV 1.7 (106). Voltage gated calcium channels induces release of neurotransmitter and subsequent to peripheral nerve injury the α2δ subunit is unregulated thus resulting in increased released of neurotransmitter such as glutamate and substance P (100; 107). The α2δ subunit is the target of both gabapentin and pregabalin and these drugs result a reduction in aberrant neurotransmitter release and subsequent pain relief (106; 108). Aβ nerve fibre
sprouting into dorsal horn, and reduced inhibition via descending pathways (109) together with axonal atrophy in peripheral nerves have been demonstrated in patients with painful diabetic neuropathy (110). Painful diabetic neuropathy has also been associated with significantly greater autonomic dysfunction than painless DPN (111). Central nervous system (CNS) involvement also seems to be a hallmark of DSPN but may actually be related to nocturnal exacerbations typically noted by sufferers which impairs sleep (100). A combination of central sensitisation, changes in inhibition of descending pathways and increased thalamic vascularity may all play a significant role in painful DSPN and the latter has been noted in studies of functional magnetic resonance imaging (fMRI) (112-113).

Currently there are two licenced treatments for the treatment of painful DSPN within the UK, namely pregabalin and duloxetine with amtriptylline also commonly used particularly within primary care. There is no definitive accepted cut off for a reduction in pain which is deemed clinically significant. However, an end-point of 50% improvement in pain relief is generally considered an adequate response to an intervention considering a placebo response may be as great as 35% (114). The treatment of painful DSPN remains inadequate and in recent studies in DSPN have shown a placebo effect which has approached and in the study by Selvarajah et al (115), a medicinal cannabis-based product even surpassed that of the active therapy, obscuring the precise treatment effect of the active treatment (116). There is an urgent need for novel therapeutic agents in the treatment of painful DSPN.

1.3.3 Pathogenesis of Diabetic Neuropathy

The pathogenesis of diabetic neuropathy is yet to be fully elucidated but is known to involve metabolic effects mediated directly and indirectly by hyperglycemia,
resulting in oxidative stress, accelerated polyol pathway metabolism, and generation of advanced glycation end-products (117-118). Both metabolic and microvascular factors are considered to play important roles (119-120). Chronic hyperglycaemia is a pivotal mediator. Even pre-diabetic or intermittent hyperglycaemia may be related to painful neuropathy (121). In one study, 25% of apparent idiopathic painful neuropathy and electrodiagnostic evidence of axonal injury with loss of epidermal nerve fibres had IGT (122). Diabetic neuropathy is initially a predominantly small fibre neuropathy which begins at the distal extremities moving proximally with time (123). In fact, around 56% of those presenting with the burning feet of idiopathic small fibre neuropathy have impaired glucose tolerance (124). Many individuals with small fibre neuropathy will only have mild, subclinical large fibre disease (125) and thus there is a cohort of those with small fibre neuropathy not detectable with traditional investigations.

The Diabetes Control and Complications Trial (96) and the United Kingdom Prospective Diabetes Study (126) have demonstrated that poor glycaemic control is related to increased prevalence of neuropathy and other microvascular complications. Improvement in glycaemic control may even prevent or slow elements of diabetic neuropathy (96). The results of the DCCT (96) were particularly important; this randomised, prospective study showed a significant reduction in the development and progression of clinical neuropathy (64%), motor nerve conduction velocity (44%) and autonomic dysfunction in type 1 diabetic patients with optimal glycaemic control.

After many years of research the precise mechanism of hyperglycaemia leading to diabetic neuropathy is still to be fully explained. The numerous pathological changes are mediated through competing or parallel pathological pathways;
glucose-induced activation of protein kinase C isoforms; increased formation of advanced-glycation end-products (AGE); and increased glucose flux through the aldose reductase pathway (127). Brief accounts of the postulated pathways which are the focus of therapeutic interventions are described below.

Over 80% of amputations follow a foot ulcer or injury (82), early recognition of at risk individuals and appropriate foot care may result in a reduced incidence of ulceration and consequently amputation. However, the study by Lincon et al (128) has challenged this notion with an observer-blind, randomised controlled trial to assess the effect of a foot care education programme in the secondary prevention of foot ulcers. Intervention was associated with improved foot care behaviour, however there was no evidence that a programme of targeted education was associated with clinical benefit over and above usual care (128). The ever increasing prevalence of DM and consequently lower limb amputation in the western world (80) highlights the need to understand and accurately assess DSPN. Figure 1.1 highlights the main mechanisms contributing to DSPN. Cellular factors derived from the bone marrow may produce chimeric cells in peripheral nerves of diabetic animals thus elicit nerve injury and cellular components from the bone marrow have an influence on the nerve pathology in diabetes (129).
Polyol pathway (Figure 1.2) hyperactivity has been postulated as a mechanism linking hyperglycaemia to neuropathy (130). Animal models have consistently demonstrated an association between increased polyol pathway flux and a reduction in nerve conduction velocity, which may be ameliorated with aldose reductase inhibitors (131). It is thought that hyperglycaemia leads to sorbitol accumulation in the peripheral nerve due to increased conversion from glucose, via the enzyme aldose reductase. This is evidenced by elevated sorbitol in diabetic nerves (132-133) however, it is now thought that this accumulation may actually not be as important as previously considered (134). The high rate of flux through the pathway may contribute to oxidative stress (134). This pathway has been the focus of much of the therapeutic interventions that have been trialled. Aldose

Schematic adapted from Yagihashi et al (129)
reductase inhibitors have shown an improvement in nerve conduction velocity and some improvement in nerve fibre count (135) but have consistently failed in phase III clinical trials (136). It is postulated that the role of the polyol pathway in humans perhaps is not as crucial as described in animal models. Furthermore, there may be a considerable heterogeneity in the level of the polyol pathway metabolites as shown by numerous studies (137-140).

**Figure 1-2 Polyol Pathway in DSPN**

![Polyol Pathway Diagram](image)

1.3.3.2 Non-enzymatic glycation

Glucose and other sugars can form covalent bonds with proteins (non-enzymatic glycation) which has been documented in diabetes (141). Advanced Glycation End products have also been identified that may contribute to oxidative stress and interplay with other pathways (134). AGEs can quench nitric oxide and thus lead to impaired tissue blood flow (142).

1.3.3.3 Oxidative stress

In diabetes, there is increased free radical production due to hyperglycaemia and a reduced ability to neutralize free radicals. This is partly the result of non-enzymatic glycation and polyol pathway hyperactivity (134). There is acceleration in free-radical formation that may be associated with a deficiency of antioxidant and detoxification pathways (134). With polyol pathway hyperactivity, the ability to neutralize free radicals is reduced, as there is NADH depletion (143). Oxidative
stress impairs nerve function by either a direct toxic effects or by reducing nitric oxide. This in turn may impinge on blood flow in endoneurial capillaries.

A powerful antioxidant (alpha lipoic acid) which scavenges hydroxyl radicals, superoxide and peroxy radicals and generates glutathione has shown some benefit (144) and the Alpha Lipoic in Diabetic Neuropathy (ALADIN) study demonstrated an improvement of neuropathic symptoms with 3 weeks of intravenous treatment with alpha lipoic acid (145) although with such a short duration of intervention a considerable placebo effect is likely. The ALADIN II study which comprised of 5 days of intravenous treatment with alpha lipoic acid followed by 2 years of oral treatment showed a significant improvement in sural and tibial nerve conduction parameters (146). However, the more recent study by Nathan I study (147), which was a 4 year randomized double-blind parallel-group trial enrolling 460 diabetic patients with mild to moderate DSPN who were randomly assigned to oral treatment with 600 mg alpha lipoic acid once daily ($n = 233$) or placebo ($n = 227$) did not achieve the primary end point. However, importantly the primary end point did not deteriorate significantly in placebo treated subjects and brings into question the reoccurring dilemma of the lack of placebo decline in trials of human DSPN (148).

1.3.3.4 Protein Kinase C (PKC) activation

Hyperactivity of Protein Kinase C (PKC) by 1,2-diacylglycerol inducement occurs in diabetes and is associated with abnormalities in vascular function. It can improve Na+-K+-ATPase activity, which is normally suppressed (134). PKC inhibitors have been shown to correct nerve conduction velocity and perfusion deficits and to protect endothelial dependent relaxation in animal models (149-150). A PKC-β
inhibitor, Ruboxistaurin, has been studied in randomised placebo controlled trials and failed in its primary outcome measure (151-152).

1.3.3.5 Renin-Angiotensin-Aldosterone System (RAS)

A small open label study of lisinopril in hypertensive subjects (n=13) showed improvements in nerve conduction measures over 12 weeks (153). One double blind randomised controlled study has found benefit of trandolopril in normotensive patients with DM and mild neuropathy over a one year period (154). The mechanism of action is thought to be mediated through protection from nerve hypoxia.

1.3.3.6 C-Peptide, Complications and DSPN

C-peptide deficiency is an important contributing factor to the characteristic functional and structural abnormalities of the peripheral nerves (155). C-peptide binds to cell membranes, resulting in stimulation of endothelial nitric oxide synthase (eNOS) and Na+, K+ -ATPase (Figure 1.3) (156). In the Joslin 50-Year Medallist Study (157), protection from complications (retinopathy, nephropathy and neuropathy) was thought to be due to the presence of enriched protective factors against microvascular complications. It was also noted that there was residual C-peptide production (6% of subjects) which may itself play an important role (157).

In two double-blind, placebo-controlled studies in type 1 DM patients, C-peptide replacement or placebo was given with the patients’ regular insulin therapy (158-159). Sensory NCV assessed in the sural nerve and showed a significant improvement. The two studies had different populations: In the first study, patients were at an early stage of nerve dysfunction with an average age of 29 years and approximately 10 years diabetes duration. There was an increase after 3 months
which amounted to 2.7 m/s, corresponding to an 80% correction of the initial conduction velocity deficit in these patients (158). In the second study, patients presented with sensory NCV in the sural nerve that was at baseline on average 2.6 SD below normal after correction for body height thus suggesting that some patients had quite marked nerve conduction deficits prior to the 6 months of intervention (159). Further detailed large randomised controlled trials of sufficient quality and length are required to truly assess the effectiveness of C-peptide as a therapeutic intervention. Figure 1.3 shows the postulated mechanism by which C-peptide may exert an effect on nerve structure and function.

Figure 1-3 Putative effect of C-peptide on peripheral nerve structure and function

Adapted from Ekberg et al (156).

1.3.3.7 Treatments for DSPN based on pathogenetic mechanisms

Treatments based on the pathogenetic mechanisms of DSPN have dramatically failed at the clinical trial stage (82; 136; 160-162). In part, the failure of these
treatments may be due to inadequate trial design, inappropriate surrogate end points and a lack of decline in the placebo arms of clinical trials (148). Currently, there is a single treatment which is licensed only in Japan (163), although its clinical effectiveness is debatable. There are no licensed treatments in the UK. Treatments based on their putative mechanism and effects in RCTs are detailed in Table 1.4.

1.4 Treatment of DSPN based on the putative pathogenetic mechanisms and outcome of clinical trials.

<table>
<thead>
<tr>
<th>Abnormality</th>
<th>Compound</th>
<th>Aim of treatment</th>
<th>Status of RCTs</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Polyol pathway↑</strong></td>
<td>Aldose reductase inhibitors</td>
<td>Nerve sorbitol↓</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sorbinil/Tolrestat/</td>
<td>Withdrawn (AE)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Zenarestat/Lidorestat</td>
<td>Withdraw</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Zopolrestat</td>
<td>No published clinical trials</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ponarestat</td>
<td>Ineffective</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Epalrestat</td>
<td>Marketed in Japan</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ranirestat</td>
<td>In phase III trials (157)</td>
<td></td>
</tr>
<tr>
<td><strong>Oxidative stress↑</strong></td>
<td>α-Lipoic acid</td>
<td>Oxygen free radicals↓</td>
<td>Effective in RCTs, Nathan I (147) – ineffective at primary endpoint</td>
</tr>
<tr>
<td><strong>Nerve hypoxia↑</strong></td>
<td>ACE inhibitors</td>
<td>NBF↑</td>
<td>Effective in one RCT</td>
</tr>
<tr>
<td><strong>Protein kinase C↑</strong></td>
<td>Protein kinase C-β inhibitor</td>
<td>NBF↑</td>
<td>RCTs ongoing,</td>
</tr>
<tr>
<td><strong>C-peptide↓</strong></td>
<td>C-peptide</td>
<td>NBF↑</td>
<td>Some data available, Studies ongoing</td>
</tr>
<tr>
<td><strong>Neurotrophism↓</strong></td>
<td>Nerve growth factor</td>
<td>Nerve regeneration, growth↑</td>
<td>Ineffective</td>
</tr>
</tbody>
</table>

Adapted from Boulton et al: Diabetes Neuropathies (162).
AE adverse event, NBF nerve blood flow, RCT randomised clinical trial.
1.4 Vitamin D deficiency

It estimated that vitamin D deficiency affects one billion people worldwide (164). Previous estimates of vitamin D deficiency in the UK suggest it may affect up to 87% of adults, depending on the season (165). Considerable attention has recently been focused on the role of vitamin D deficiency on cardiovascular health (161; 166-167). The vitamin D receptor (VDR) is known not only to be expressed in bone but ubiquitously in other tissues and cells including lymphocytes, cardiomyocytes, endothelium, pancreatic β-cells, and lipid laden macrophages (foam cells) (168). 25(OH) vitamin D is used to determine vitamin D status as it accurately represents body stores and has a half-life of approximately 15 days (169-170), whereas the active form (1,25(OH)₂ vitamin D) has a short half life 15 hours and can alter over a 24 hour period (169).

Current ‘healthy’ or sufficient levels of 25(OH) vitamin D are suggested to be >30ng/dl although this advice is based on data derived from bone metabolic health (171) (Table 1.5) and there is no definitive consensus on optimal levels (164). Vitamin D deficiency is defined by some experts as a 25(OH) vitamin D of less than 20 ng/ml (172-174), however, 25(OH) vitamin D levels are inversely associated with parathyroid hormone levels until 25(OH) vitamin D reaches 30 to 40 ng/ml at which point parathyroid hormone levels reach their nadir (175-177).
1-5 Serum 25(OH) vitamin D concentrations and status

<table>
<thead>
<tr>
<th>25(OH) vitamin D Concentration</th>
<th>25(OH) vitamin D status</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;10ng/ml</td>
<td>Severely Deficient</td>
</tr>
<tr>
<td>10–&lt;20ng/ml</td>
<td>Deficient</td>
</tr>
<tr>
<td>20–&lt;30ng/ml</td>
<td>Insufficient</td>
</tr>
<tr>
<td>≥30ng/ml</td>
<td>Adequate</td>
</tr>
<tr>
<td>≥150ng/ml</td>
<td>Possible toxicity</td>
</tr>
</tbody>
</table>

1.4.1 Vitamin D deficiency, Hyperlipidaemia and Hypertension

1.4.1.1 Hyperlipidaemia

Vitamin D and cholesterol share a common metabolic pathway through 7-dehydrocholesterol and this metabolite is a precursor for both. Lower levels of 25(OH) vitamin D have been associated with lower high density lipoprotein cholesterol (HDL) and hyper triglyceridaemia (178). Statin therapy has shown to raise vitamin D levels (179) and a study in macrophages from obese hypertensive patients with DM established that culturing with 1,25(OH)₂ vitamin D suppressed foam cell formation by reducing oxidized low-density lipoprotein cholesterol (LDL) uptake (180). The deletion of the vitamin D receptor in macrophages in the same study accelerated foam cell formation induced by modified LDL (180). 25(OH)
vitamin D is sequestered in adipose tissue and this may partly explain the low levels associated with obesity by reducing the release of this inactive form into the circulation for transformation to active vitamin D (181) as well as being independently associated with HDL and metabolic syndrome (178).

1.4.1.2 Hypertension

Vitamin D is implicated in hypertension by its role as a negative regulator of the RAS and in experimental studies, VDR knockout mice have elevated renin, angiotensin and aldosterone, suggesting that vitamin D may be a potent inhibitor of the RAS axis (182). The NHANES III study (183) described a lower blood pressure in those in the highest deciles of 25(OH) vitamin D and vitamin D$_3$ with calcium supplementation has been shown to reduce blood pressure compared to calcium supplementation alone (184). However, the largest interventional study was the Women's Health Initiative (n = 36 282), which was designed to evaluate the effects of 400 IU of daily vitamin D$_3$ with calcium supplementation on fracture and cancer risk when compared to placebo showed no change in blood pressure or incident hypertension over 7 years (185).

1.4.2 Vitamin D deficiency and Macrovascular Complications

The Framingham offspring study longitudinally followed up individuals (n=1739) with no prior history of cardiovascular disease in this cohort (167). During the follow up period (mean 5.4 years) a composite of cardiovascular events was assessed and after multivariate adjustment those with 25(OH) vitamin D of <15ng/ml had a hazard ratio of 1.62 (95% confidence interval (CI) 1.11-2.36, p=0.01) for incident cardiovascular events compared to 25(OH) vitamin D ≥15ng/ml (167). This increased risk was even more evident in those with hypertension (167). Vitamin D
deficiency has been related to cardiac disease in the form of coronary artery calcification, myocardial infarction, stroke and congestive cardiac failure (170). The Ludwigshafen risk and cardiovascular health study (LURIC study) assessed a consecutive cohort of 3258 individuals scheduled for coronary angiography (186) for their vitamin D status. Sudden cardiac death and death due to heart failure were independently and inversely associated with 25(OH) vitamin D, and CVA was related to both 25(OH) vitamin D and 1,25(OH)₂ vitamin D levels (186). Furthermore, the NHANES III study subgroup (n=3408) analysis supported these findings as 25(OH) vitamin D was inversely associated with all-cause mortality over a mean period of 7.3 year (187). Compared to individuals with 25(OH) vitamin D of ≥40ng/ml, those with 25(OH) vitamin D of <10ng/ml the adjusted risk was approximately 83% higher (187). A study vitamin D deficiency was associated with an increased amputation risk in veterans with peripheral arterial disease (188).

1.4.3 Vitamin D and Diabetic Neuropathy

The effective treatment of DSPN remains a major challenge and anticonvulsants and anti-depressants remain the mainstay of symptom relief, but provide no benefit for underlying nerve damage. However, increasing data suggest that vitamin D may have not only comparable analgesic but also play a pivotal role in the peripheral nervous system and in particular diabetic neuropathy (189-192). Vitamin D deficiency may occur in a significant proportion of UK patients; with diabetes ~90% classified as being insufficient (<30ng/ml) with severe deficiency in over 30% (<10ng/ml) (193). Nerve Growth Factor (NGF) and neuronal Ca2+ homeostasis, both may play a neuroprotective role in the peripheral nerve have been linked to vitamin D experimental studies through the regulation of
neurotrophins (194). NGF is depleted in experimental diabetes (195). Sciatic nerve NGF was preserved in animals exposed to a vitamin D analogue (CB1093) (194) and another vitamin D analogue (MC903) is known to increase NGF synthesis (196). Also in the peripheral nervous system, VDR are found in predominantly nociceptive neurons of dorsal root ganglia and their expression is influenced by ovarian hormones and a significant increase in VDR expression has been observed in DRG neurons of diabetic rats (197).

Observational studies have demonstrated a significant link between vitamin D deficiency and DSPN (190-191). In the National Health and Nutrition Examination Survey (NHANES) 2001-2004, an unweighted sample of 591 subjects with diabetes demonstrated a significant association between vitamin D deficiency and both paraesthesiae (odds ratio 2.12; 95% CI 1.17–3.85) and numbness (odds ratio 2.04; 95% CI 1.18–3.52) after adjusting for confounders (191). Similarly in a cross sectional study by Shebab et al (190), vitamin D deficiency was an independent risk factor for DSPN assessed using the neuropathy symptom score, as well as clinical and electrophysiological measures of DSPN.

Vitamin D deficiency has been implicated in numerous pain syndromes (198-199). Vitamin D supplementation may improve musculoskeletal pain and non-traumatic back pain as shown in some studies (200), although the data on vitamin D deficiency prevalence and non-specific musculoskeletal pain is not as compelling (201). The data supporting a benefit of vitamin D treatment in painful DSPN is as yet limited. However, in a non-randomised non-placebo controlled but prospective study with cholecalciferol (vitamin D₃) at a mean dose of 2059 IU daily for 3 months in painful DSPN an improvement of ~50% on the Visual Analogue (pain) Scores (VAS) was observed (202). Furthermore Valensi et al (203), showed use of
a compound (QR-333) containing a vitamin D analogue in a double blind randomised controlled trial reduced the severity of numbness, jolting pain, and irritation in subjects with painful DSPN although QR-333 also contained an aldose reductase inhibitor compound, which may have had an impact on the pathogenetic mechanisms causing neuropathy. The largest observational study of patients with in the community in northwest England (n = 15,692) has shown that the prevalence of painful symptoms and painful DSPN was 34 and 21% respectively (77). Additionally, despite a lower prevalence of neuropathy in South Asians compared to Europeans and Afro-Caribbeans, painful symptoms were greater in South Asians (77). These differences may partly be explained in relation to vitamin D deficiency as these groups have been shown to have excess vitamin D deficiency (204). Thus the role of vitamin D supplementation in painful DSPN requires further study in terms of mechanisms but also demands good quality randomised controlled trials.

1.4.4 Vitamin D and Diabetic Retinopathy

There is a possible direct link between vitamin D deficiency and the development and progression of diabetic retinopathy. Not only have immunostaining in animal models shown that vitamin D receptor (VDR) is expressed in the ganglion cells, the inner and outer plexiform layer and the photoreceptor layer (205) but VDR dependent calcium binding proteins have been isolated in the photoreceptor layer of the human retina (206). In experimental models of ischaemic retinopathy, calcitriol is a potent inhibitor of retinal neovascularisation (207). Other experimental studies show similar findings with administration of active vitamin D having a partially protective effect on diabetic retinopathy of diabetic rats, through a
mechanism which may involve inhibition of vascular endothelial growth factor (VEGF) and transforming growth factor (TGF)-β1 expressions in the retinal tissues (208).

Furthermore, retinoblastoma tissue expressing VDR has shown reduction of growth with subsequent apoptosis of the retinoblastoma cells after supplementation with vitamin D (209). VDR genotypes have been linked with the cumulative prevalence of diabetic retinopathy (210). The VDR gene in the French population, FokI and TaqI single nucleotide polymorphisms have been associated with DR (211-212). In a Turkish study, there was a demonstrated inverse correlation between worsening diabetic retinopathy and lower activated vitamin D in a population of 66 subjects (213). Cross-sectional studies in children and adults with DM, show positive associations with vitamin D deficiency and DR (214-216). The NHANES III study showed an association between severity of diabetic retinopathy and prevalence of vitamin D deficiency, but the findings were inconclusive about the existence of a significant relationship (216). These findings were also mirrored in a smaller cross sectional study of type 1 DM and controls and those with proliferative diabetic retinopathy having the lowest vitamin D status (215). Further evaluation of this possible important relationship is required.

1.4.5 Vitamin D and Diabetic Nephropathy

Vitamin D therapies for renal disease have been used for over a half century and are likely to be utilised for many more years (217). Vitamin D has paracrine functions through local activation by 1-alpha-hydroxylase and thus maintain vascular function, cardiomyocyte health, and abrogate inflammation and insulin resistance (218). Low serum 25(OH) vitamin D status is a common complication in
patients with chronic kidney disease (CKD) and the severity of deficiency increases with the progression of CKD (219). The RAS plays a critical role in the development of diabetic nephropathy, and blockade of the RAS is currently used for treatment of diabetic nephropathy (220). Vitamin D plays a significant role as a RAS inhibitor (220). In an experimental study, combination of vitamin D analogues and RAS inhibitors prevented renal injury in diabetic nephropathy (220) and a vitamin D analogue (paricalcitol) has shown an improvement in microalbuminuria in a human study through a mechanism related to inhibition of RAS (221).

1.5 Clinical Evaluation and Investigations of Neuropathy

The assessment of DSPN is a common clinical entity facing the general physician. A simple approach to the assessment of neurological deficits is essential and can be undertaken using the neuropathy disability score (NDS). Further evaluation includes symptom questionnaires, quantitative sensory testing, electrophysiology, autonomic tests and/or skin biopsy. Sural sensory nerve biopsy is a well established method of accurately assessing neuropathy. However, due to the invasive nature of the investigation it is not commonly used and is reserved for diagnoses that are inconclusive, often when a vasculitic phenomena is a differential (222). Sensory nerve biopsy will not be considered further in this thesis for this reason. A thorough examination should include evaluation of large myelinated nerve fibres (Aβ) by evaluating vibration perception, small myelinated (Aδ) fibres by evaluating sharp pain and cold sensation and small unmyelinated (C) nerve fibres by evaluating warm and pain perception. Types of nerve fibres in peripheral nervous system are shown in table 1.6.
1-6 Type of nerve fibres in the peripheral nervous system

<table>
<thead>
<tr>
<th>Myelination</th>
<th>Motor</th>
<th>Sensory</th>
<th>Autonomic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Myelinated</td>
<td>Myelinated</td>
<td>Thinly myelinated</td>
</tr>
<tr>
<td>Fibre type</td>
<td>Aα</td>
<td>Aα/Aβ</td>
<td>Aδ</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Function</th>
<th>Motor control</th>
<th>Coarse Touch Proprioception Vibration perception</th>
<th>Cold perception Pain</th>
<th>Warm perception Pain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cardiovascular control (heart rate, blood pressure), genitourinary and sexual function diaphoresis and gastrointestinal control.</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1.5.1 Diagnosis of Diabetic Distal Symmetrical Polyneuropathy

A number of approaches are employed to diagnose and evaluate the severity of neuropathic deficits and painful symptoms in diabetic neuropathy. In a clinical environment, bedside screening instruments are usually sufficient in screening for neuropathy (136). The diagnosis of DSPN can only be made after a careful history and clinical examination. The consensus is all patients with diabetes should be screened annually for DSPN by examining:

1. Pinprick
2. Temperature
3. Vibration perception (using a 128-Hz tuning fork)
4. Ankle reflexes
5. 10g monofilament pressure sensation at the distal halluces.
A combination of more than one test has >87% sensitivity in detecting DSPN (4). The modified neuropathy disability score (NDS) is a screening instrument for signs of neuropathy. It comprises three sensory modalities and one assessment of reflexes: 1) pain sensation (pin prick), 2) temperature sensation (hot and cold rods), 3) vibration perception (128Hz tuning fork), and 4) absence/presence of Achilles tendon reflexes. Thus the total maximum abnormal score possible is 10. A score of 3-5 is regarded as evidence of mild neuropathic signs, 6-8 as moderate and a score of 9 or 10 as severe signs of neuropathy. The original NDS in the Rochester Diabetic Neuropathy study (223) and more recently a study by Abbott et al, (224) using the modified NDS showed this evaluation to be reliable and reproducible as a screening instrument of peripheral neuropathy. An increasing NDS and Vibration Perception Thresholds (VPT) along with abnormal plantar foot pressures can be used in identifying those at risk of foot ulceration (225). An NDS >6 was an independent risk factor for diabetic foot ulceration (224). Also the loss of vibration perception and 10g monofilament alone can accurately predict the risk for future foot ulceration (82) which is an important factor in the morbidity that can be associated with diabetes. A NDS of >6 has however been shown to be a better predictor than the 10g monofilament and is only second to past or present history of ulceration (224). The NDS does not diagnose the cohort with small fibre neuropathy or with subclinical large fibre disease and should be used as a guide to the risk of neuropathic ulceration.

Two major end-points are used particularly when assessing neuropathy for either research purposes or quantifying the benefits of an intervention (81). These are:

a. Assessment of symptoms in defining efficacy of pain relief agents.

b. Assessment of neurological deficits in defining efficacy of nerve repair agents.
An alteration of symptoms does not necessarily constitute an improvement in nerve function itself and changes in nerve ultrastructure may not equate to improved nerve function. The criteria which is used to determine the effectiveness of an intervention on symptoms is thus varied and lacks any specialist consensus.

1.5.1.1 Neuropathy Symptom Questionnaires

Diabetic patients usually find it difficult to describe the neuropathic pain they experience and thus numerous questionnaires and scoring systems have been developed to quantify the subjective feeling of neuropathic pain. Some of the more commonly used questionnaires are discussed below.

Neuropathy Symptom Profile (NSP) assesses neuropathy in general but has been validated in diabetes (226). It comprises of 38 questions with further subsections which are subdivided on anatomy. It is useful in detecting neuropathy and staging severity (226). The Rochester Diabetic Neuropathy Study (227) concluded that NSP, combined with other neurological examinations is a valid means to assess neuropathy.

The McGill Pain Questionnaire is the most frequently used questionnaire, however is not specific for diabetic neuropathy. This questionnaire is a valid measure of neuropathic pain symptoms (228). A more recently developed questionnaire, the Diabetic Neuropathy Symptom Score (DNS) is a simple and easily reproducible test in clinical practice. It is a 4-item questionnaire and the DNS score has been shown to differentiate between individuals with and without diabetic neuropathy (229) but is used more as a screening questionnaire. These questionnaires, however valid at assessing symptoms are unable to differentiate accurately between large and small nerve fibre deficit.
1.5.1.2 Quantitative Sensory Testing (QST)

QST involves the delivery of a stimulus which is reproducible and controlled, has an established normal range and allows for serial evaluation of a standardised stimuli (81). The biothesiometer is a QST of vibration and the other widely used QST is that of thermal and pain thresholds (136). Both can track progression (230) and predict those at risk of ulceration (231). The advantages and limitations of QST are established. The most important advantage is that it requires little expertise to perform and has a reasonable sensitivity, accuracy and reproducibility when evaluating different stages of neuropathy at multiple anatomical sites (136). The main limitations are variability and subjectivity of the test, patient motivation, attention and expectation bias (232). Thermal testing consists of graded and standardised warm and cold stimuli delivered through a thermode attached to the foot. Thermal testing is able to quantify warm and cold sensation thresholds, temperature induced pain and temporal summation. Room temperature, inter-stimulus intervals, gender, age and lifestyle may also affect the outcomes (233). However, QST including thermal threshold assessment for cold sensation (Aδ fibres) and warm sensation (C fibres) can assess small fibre dysfunction but are subjective, lack accuracy and reproducibility (136; 232).

The San Antonio consensus (234-235) and now the Toronto consensus (79) states that nerve conduction studies and symptoms and/or signs should be used as a definitive diagnosis of DSPN. However, a significant limitation is that this approach primarily assesses large myelinated nerve fibres (235) and only then the neural activity of a specific small subset of large, myelinated fibres (236). Despite this obvious draw back nerve electrophysiology is used as a surrogate marker in clinical trials (237).
1.5.1.3 **Electrophysiology**

Electrophysiological procedures in the form of nerve conduction studies (NCS) are one of the fundamental tests in the diagnosis of diabetic neuropathy and are particularly used in clinical trials as a measure of effectiveness. Indeed the San Antonio and now the Toronto consensus on diabetic neuropathy recommends the use of electrophysiological testing along with other tests such as QST and autonomic function to assess clinical deficits of neuropathy (79, 235). The procedure is not invasive, but objective and is highly sensitive and therefore accurately measures and classifies DSPN. The drawback is an inability to target the fibre types most commonly affected by diabetes (small fibres) as it assesses large myelinated fibres (238) and also has limited ability to demonstrate regeneration and repair. Electrophysiological tests measure conduction of both sensory and motor nerves, the amplitude of the propagating neural signal, the density and synchrony of muscles activated by maximal nerve simulation and the integrity of neuromuscular transmission (236). There are several factors contributing to nerve conduction velocity (NCV) slowing: 1) stage of nerve demyelination, 2) mean diameter of the conducting axons, 3) internodal distance in the segment under study and 4) the nodal microenvironment (136).

In a study by Dyck et al, (239) when compared to nerve conduction studies, individual physicians clinical diagnosis was highly variable with a propensity to over diagnose DSPN. Although considered a ‘Gold Standard’ test, confounding factors can lead to decreased sensitivity and specificity (240). Gender, weight, age and height may all affect the results of NCS with the latter two variables having the most significant effect (240). In a 10-year follow-up study of newly diagnosed patients with type 2 DM, NCV slowing and decrease in sensory and motor
amplitudes were reported in 16.7% of the patients at 5 years and 41.9% at 10 years, indicating an increase in the severity of DPN with time (241). Although more recent experimental studies have shown significant variability in measures of nerve conduction with up to 56.4% variability in motor amplitude in serial measures over a 4.5 month period (242).

Abnormal NCV is known to correlate with morphologic features in small fibre neuropathy and QST (243) and is still used as an end point in interventional/observational trials of DSPN.

1.5.1.4 Skin Biopsy

The only techniques which allow a direct examination of thinly myelinated and unmyelinated nerve fibre damage and repair, are those of sural nerve biopsy with electronmicroscopy (119; 244) and the newly refined skin-punch biopsy (124; 245). This technique allows a reliable and reproducible means of assessing the small (C) fibres which previously were limited to nerve biopsies, in particular that of the sural nerve (246). Intraepidermal nerve fibres (IENF) are stained using immunohistochemistry with the antibody to protein gene products 9.5 (120). The densities are calculated in at least three sections as the number of IENF per length of the section (IENF/mm) (247). According to the European Federation of Neurological Societies/Peripheral Nerve Society Guideline on the use of skin biopsy in the diagnosis of small fibre neuropathy, single IENF crossing the dermal–epidermal junction should be counted, whereas secondary branching is excluded from quantification (247). Intra- and inter observer variability, and inter laboratory agreement on IENF counts has been assessed with good reliability scores obtained (kappa values > or = 0.90) (248).
Previous studies have associated reduced IENF density with small fibre neuropathy and more so with painful DSPN (123; 125) with excellent sensitivity (123). There is increased small fibre loss at >5 years duration of diabetes with shortening of IENF at all durations (123). Also reduced rates of nerve regeneration have been seen in individuals with diabetes but without signs or symptoms of disease or evidence of neuropathy (249). Unsurprisingly skin biopsy analysis may be more sensitive than sural nerve conduction studies for diagnosing small fibre neuropathy (250). The advantages of this method of assessing small fibres are:

1. Quantitative
2. Reproducible
3. Can be used for longitudinal assessment
4. Can be used at multiple sites

However, there are also major disadvantages as the technique is limited significantly by its availability and is certainly not commonly used in clinical practice, it is invasive and requires potentially intensive complicated laboratory analysis.

1.5.1.5 Corneal Confocal Microscopy (CCM)

The cornea is one the most densely innervated organs in the body (251). Most corneal nerves are derived from the ophthalmic branch of the trigeminal nerve and constitute both A\(\delta\) and C fibres. Approximately 70 – 80 trunks of large diameter (~6\(\mu\)m), myelinated nerves (A\(\delta\)) containing 900 – 1200 axons enter the cornea at the stroma and run anteriorly towards the midpoint of the cornea. The nerves penetrate the Bowman’s membrane and form the subbasal nerve plexus (Figure 1.3) in the
boundary between the Bowman’s layer and the epithelium and finally terminate in the superficial layers of epithelium.

Figure 1-4 Montage of the whole human subbasal nerve plexus [Image adapted from: Patel and McGhee (252).]
Recently developed, CCM is a rapid, reiterative, non-invasive procedure which allows microscopic examination of the cellular and nerve structures of the cornea. Conventional techniques of electrophysiology and quantitative sensory testing along with an assessment of neurological disability offer a relatively robust means of defining neuropathic severity (136). However, they have major shortcomings when they are employed to define therapeutic efficacy in clinical intervention trials (253). New therapies in the treatment of diabetic neuropathy have been hampered by the lack of clinically relevant end points (136) as historically quantification of large nerve fibres were used to diagnose neuropathy and not in assessing nerve regeneration or a response to an intervention (254). CCM may provide an ideal surrogate end-point and has been shown to demonstrate corneal nerve fibre pathology which is correlated with DSPN (255-257). Numerous studies have looked at the relationship of corneal nerve pathology and diabetic neuropathy suggesting it may be used as a surrogate marker of small fibre neuropathy (258-263). Significant changes can be detected in corneal nerve fibre density, branching, and tortuosity which relate to the severity of DSPN (256; 264). CCM has also been shown to detect the benefits of therapeutic intervention with pancreas transplantation after 6 months of the intervention (265) and more recently with simultaneous pancreas and kidney transplantation after 12 months (266). This highlights the ability of this modality to be potentially used in assessing therapeutic interventions. It has been argued that however significant the changes to nerve structure this may not necessarily equate to alterations in function. However, corneal sensitivity is reduced and is directly related to the severity of the neuropathy in diabetes (267). This modality has shown significant potential as a surrogate test of nerve function.
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2 CHAPTER II - METHODS
2.1 Synopsis

The ability to define the ‘at risk’ patient, anticipate deterioration, and assess new therapies is of paramount importance in DSPN and there is also an urgent need for ‘new’ surrogate endpoints for this condition. Current techniques lack the sensitivity (QST), require expert assessment or target inappropriate nerve fibre types (NCS), are invasive (skin biopsy) and are not routinely performed across health systems. A surrogate endpoint needs to reliably reflect the activity of the underlying disease process, which ultimately causes significant morbidity such as foot ulceration and lower limb amputation. Vitamin D has long been linked with bone metabolic disease however, more recently there have been postulated links to DM and its complications. There is a subsequent need to assess the role of vitamin D in diabetic microvascular complications, the effectiveness of therapeutic interventions and the prevalence of vitamin D deficiency in patients with DM.

2.2 Hypothesis and aims

The primary hypothesis of this study is corneal nerve morphology using CCM is a valid surrogate marker for human DSPN and the vitamin D status has a definitive role in the diabetic microvascular complications of DR and DSPN. This research aims:

i. To delineate subjects who have rapidly progressive DSPN and assess whether this may be discerned through the novel modality of CCM. It will also aim to assess corneal nerve fibre damage visualised with CCM and evaluate if it is progressive in nature and in agreement with the natural history of the disease relating to clinical (NDS, QST, NCS) and laboratory (intra-epidermal nerve fibre density) markers of DSPN.
ii. To establish whether subjects with LADA have a different neuropathic phenotype than those with type 2 DM. We aim to establish the ability of CCM to discernibly validate small fibre changes compared to conventional small and large fibre neurological tests.

iii. To estimate the prevalence of neuropathy measured with CCM and Non Contact Corneal Aesthesiometry (NCCA) in a cohort of patients with type 1 DM and compare this with the results of neurological deficits (NDS); nerve electrophysiology, quantitative sensory testing (thermal thresholds and VPT), IENF quantitative analysis and establish the relationship with risk factors.

iv. To establish the local prevalence of vitamin D deficiency and its association with the constituents of metabolic syndrome and cardiovascular disease risk which may also drive DSPN.

v. To establish the improvement in vitamin D status through the effects of 3 different vitamin D replacement regimens (using combinations of ergocalciferol, cholecalciferol and calcium carbonate/cholecalciferol) for the treatment of vitamin D deficiency.

vi. To establish any phenotypic and metabolic differences between groups with mild neuropathy with and without painful DSPN and to compare the results of neurological deficits (NDS); nerve electrophysiology, quantitative sensory testing (thermal thresholds and VPT), IENF quantitative analysis and vitamin D status.

vii. To establish the relationship of vitamin D status to DR alongside conventional risk factors such as duration of diabetes, glycaemic control, lipids and blood pressure.
2.3 Study Design

1. Chapter III – Prospective longitudinal, investigator-masked observational study.

2. Chapter IV, V – Prospective cross-sectional, investigator-masked observational study.

3. Chapter VI – Retrospective cross-sectional, case-control study

4. Chapter VII – Retrospective analysis of clinical therapeutic intervention

5. Chapter VIII – Prospective cross-sectional, investigator-masked observational study.

6. Chapter IX – Retrospective cross-sectional study

2.4 METHODS: Neuropathy Studies - Chapters 3, 4, 5 & 8

Ethical approval was obtained prior to the initiation of the study by the North Manchester and Salford and Trafford Research Ethics Committee, and written informed consent was obtained according to the Declaration of Helsinki. This study is conducted in accordance with the stipulations stated in the Declaration of Helsinki and along with Good Clinical Practice guidance. All participants were supplied with study literature at least 24 hours prior to informed consent being obtained.

2.4.1 Patient Enrolment

Informed written consent was obtained from all subjects prior to their participation and they were provided with the opportunity to discuss any concerns about the
experimental protocol and their safety with a trained member of the research team (a copy of the patient consent and study forms can be found in Appendix 2). Upon enrolment to the study, all control and diabetes participants underwent detailed screening by means of personal and medical history and a set of blood and urine tests to determine their metabolic status and ensure eligibility for this study. The specific inclusion / exclusion criteria used are presented below. Appointments were offered to participants during normal working hours and if a participant could not complete the full visit assessment in a single visit, a second visit was scheduled within a month.

Patients with type 1 and 2 DM and LADA were recruited from the Manchester Diabetes Centre and general medical clinics at the Manchester Royal Infirmary, Manchester, UK. Controls are recruited from the University of Manchester and via social networks of the study investigators.

### 2.4.2 Inclusion Criteria for Chapters 3, 4, 5 & 8

Participants were expected to satisfy the following criteria prior to inclusion in the study:

a) Aged 14 to 80 years

b) Signed written informed consent

c) Type 1 or 2 DM or LADA

d) Absence of diabetes for the control group

e) Be willing to participate and comply with the experimental protocol.
2.4.3 Exclusion Criteria for Chapters 3, 4, 5 & 8

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.

2.4.4 Questionnaire Administration

The NSP, DNS and McGill Pain Questionnaire were either administered by a researcher or they were self-completed. When questionnaires were self completed, careful instructions were provided to the patient on how to score the questionnaire in order to avoid any misinterpretation.
2.4.5 Neuropathy Disability Score (NDS)

The NDS is derived from a basic neurological examination testing vibration, pin-prick and temperature perception in addition to deep tendon reflexes. A score ranging between 0 and 10 is calculated and the degree of neuropathy is categorised as no neuropathy [0-2], mild neuropathy [3-5], moderate neuropathy [6-8] and severe [9-10] (Figure 2.1).

Pain sensation (C fibres) was tested using a neurotip. Sharp and blunt ends were applied and the patient then distinguished the sharp sensation. Vibration sensation was tested using a 128 Hz tuning fork. The vibrating fork was first applied on the subject’s forearm/sternum and was then applied three times to the bony prominence of the first hallux of each foot. Two correct responses were deemed as being normal (Figure 2.2). Temperature sensation was tested using two metal rods separately placed in hot and cold water (Figure 2.3). The patient was then asked to ascertain the correct sensation and two or more correct responses were deemed as being normal. Deep tendon reflexes were assessed by the achilles tendon reflex. If absent, subjects were asked to ‘reinforce’ the reflex. The score was then recorded (0: normal, 1: present with reinforcement and 2: abnormal). All procedures were repeated for the opposite foot.
**Figure 2-1 The Neuropathy Disability Score**

<table>
<thead>
<tr>
<th>Neuropathy Disability Score (NDS)</th>
<th>Right</th>
<th>Left</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Vibration Perception Threshold</strong>&lt;br&gt;128-Hz tuning fork; apex of big toe:&lt;br&gt;normal = can distinguish vibrating/ not vibrating</td>
<td></td>
<td></td>
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<tr>
<td><strong>Temperature Perception on Dorsum of the Foot</strong>&lt;br&gt;Use tuning fork with beaker of ice/warm water</td>
<td>Normal = 0&lt;br&gt;Abnormal = 1</td>
<td></td>
</tr>
<tr>
<td><strong>Pin-Prick</strong>&lt;br&gt;Apply pin proximal to big toe nail just enough to deform the skin;&lt;br&gt;trial pair = sharp, blunt;&lt;br&gt;normal = can distinguish sharp/not sharp</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Achilles Reflex</strong>&lt;br&gt;Present = 0&lt;br&gt;Present with reinforcement = 1&lt;br&gt;Absent = 2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NDS Total out of 10

From Boulton, A.J.M (1).

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

The Neuropad is an early detection test to ascertain the hydrosis status (moisture content) of the sole of the foot (Figure 2.4). Those with chrome, nickel and/or cobalt allergy were excluded from the test. Shoes and socks were removed at least 5 minutes prior to application of the neuropad on the ball of the small toe. After 10 minutes the colour of the pad was assessed and a gross % assessment was made. 100% pink is considered normal whilst the pad remaining blue or patchy is considered abnormal (Figure 2.4).

Figure 2-4 Neuropad: a) 100% pink- normal response, b) no change- abnormal and c) patchy- abnormal
2.4.7 Heart Rate Variability to Deep Breathing

Computer-aided sensory evaluator IV (CASE IV) was used to measure the heart rate response to deep breathing. In this test the patient was asked to inhale and exhale deeply six to eight times in a row in the supine position, whilst following the rhythm of a “breathing cue” in the form on an LED light. The changes in heart rate are displayed on an ECG monitor. One eight-cycle breathing series was completed and then a subsequent six-cycle breathing series interspersed by two minutes of normal breathing. The acquired data was analysed by calculating the mean difference between the highest and lowest heart rate for five consecutive, artefact-free cycles in each series.

2.4.8 Quantitative Sensory Testing

The patient was made comfortable in a semi-recumbent position, with heels resting on the bed. The patient was familiarised with the sensation of vibration from the neurothesiometer at the wrist/forearm. The tip of the neurothesiometer was rested on the great toe (right and left). The amplitude of vibration for the probe was increased from zero, by turning the dial slowly, whilst the patient has their eyes closed. The patient was instructed to say “Yes” when he/she first detects the slightest “buzzing” or vibration sensation and this is recorded as the vibration perception threshold which was repeated three times on each test site. VPT is measured in volts and has a range between 0-50 volts.

Thermal tests were performed with a Medoc TSA 2001 device (Medoc Ltd, Minneapolis, MN). 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 is controlled by a computer
software program (MEDOC TSA II, Medoc Ltd, Ramat Yishai 30095, Israel) (Figure 2.5). The room temperature ranged between 18-22°C as per standard protocol. The thermode contacts the skin and a subject is asked to report a sensation of temperature change or heat pain. Cold sensation threshold (CST) is tested initially by a gradual decrease in the thermode temperature and the subject is asked to press the computer mouse button when they first become aware of a cold sensation. This was repeated a further three times and a similar procedure was carried out to test warm sensation threshold (WST). The mean value is recorded.

![Figure 2-5 The user interface for thermal threshold and pain measurement](image)

2.4.9 Electrophysiology

All nerve conduction studies were performed by a consultant neurophysiologist (Dr Andrew Marshall) using an MS92a EMG machine (Medelec Limited, Old Woking Surrey UK) using surface stimulation and recording electrodes), maintaining skin temperature at 31°C (Figure 2.6). Any deviations from the standard temperature
were recorded. Lower limb nerves were assessed, namely, motor peroneal and tibial nerves and the sensory sural nerve. Nerve conduction velocity (m/s), maximum amplitude (mV), and baseline to peak and minimum F-wave latency (ms) were recorded for the motor nerves. For the sensory nerve, baseline to peak amplitude of the sensory action potential (μV), latency to onset (ms) and conduction velocity were recorded. Graded stimulation of nerves was undertaken by increasing the strength of the stimulus until a maximal response was achieved and then the stimulus was increased by 10-15% above maximal response to ensure a supra-maximal response.

Figure 2-6 Electrodiagnostic studies of the peroneal and sural nerves

Motor amplitude was measured from the baseline to the negative peak. The amplitude was reported to the nearest 0.1 mV. Sensory amplitude of the sensory potential 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 was reported to the nearest whole number.

2.4.10 Skin Biopsies and Immuno-histology

A sub-cohort of participants underwent a 3-mm punch skin biopsy from the dorsum of the foot; 2 cm above the second metatarsal head after local anaesthesia (1% lidocaine). The biopsy site was closed using Steristrips, and the specimen was immediately fixed in PBS-buffered 4% paraformaldehyde for 18-24 h, washed in Tris-buffered saline, cryoprotected in sucrose, frozen in liquid nitrogen and stored at -80°C and subsequently cut into 50-μm sections on a cryostat microtome. Five floating sections per subject were immunostained for PGP 9.5 neuronal marker. Briefly, the non-specific protein binding and endogenous peroxidase activity were blocked by incubation in 5% goat serum and 0.3% hydrogen peroxide, respectively. The anti-PGP9.5 antibody (Milipore 1:1000; Billerica, MA) was followed first by goat anti-rabbit IgG and then by HRP-Streptavidin (both diluted 1:1000, both from Vector Laboratories, Peterborough, UK). Nerve fibres were visualised by SG chromogen (Vector Laboratories). Intraepidermal nerve fibre density (IENFD) was calculated as the number of nerve fibres crossing the basement membrane of the epidermis and expressed per millimetre length of epidermis (Figure 2.7).

IENFD were assessed by two independent assessors as per European Federation of Neurological Societies/Peripheral Nerve Society Guideline on the use of skin biopsy in the diagnosis of small fibre neuropathy (2).
Figure 2-7 Skin biopsies stained for PGP9.5. Healthy control (A) shows numerous long intraepidermal nerve fibres and a well-developed subepidermal nerve plexus, compared with short intraepidermal nerves and less pronounced subepidermal plexus in subjects with DM (B & C) (Bar = 100 µm).

2.4.11 Corneal sensitivity

Corneal sensitivity was measured using an NCCA (constructed for the IHBI, Anterior Eye Lab by Kimble Dunster and Lincoln Hudson). It uses a puff of air lasting 0.9s expressed through a bore of 0.5-mm diameter with an electronic pressure sensor, which displays the force exerted in millibars (3). The patient places their head in the head and chin-rests and the NCCA table and equipment are adjusted for comfort. The patient was instructed to look straight ahead and gaze at the fixation target. The stimulus jet was mounted on a slit lamp positioned 1 cm from the eye and aligned to the centre of the cornea. Corneal sensitivity was assessed by the patient being administered a high test stimulus (Figure 2.8). Thus the patient is familiarised with ‘normal’ stimulus. The stimulus was then decreased until it is just detectable by the subject (absolute threshold). The stimulus was subsequently increased and repeated on 3 occasions with an average
measurement derived. This was recorded and the procedure repeated for the alternate eye.

**Figure 2-8 Estimation of corneal sensation using NCCA**

2.4.12 Corneal Confocal microscopy

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

i. CNFD: the number of main nerve fibres / mm².

ii. CNBD: the number of main nerve branches / mm².

iii. CNFL: the sum of the length (mm./mm²) of all nerve structures.

iv. CNFT: the tortuosity or non-linearity of the main nerve fibres.

Patients underwent examination with a corneal confocal microscope. Images are obtained using the HRT III-RCM (Heidelberg Engineering GmbH, Heidelberg, Germany) (Figure 2.9). The laser is a 670 nm red wavelength Helium Neon diode laser and does not pose any danger to ocular structures. The corneal sub-basal
nerve plexus is determined by continuous imaging of the cornea through use of an OCD camera.

**Figure 2-9 Corneal confocal microscopy**

Patient demographics were entered in the Heidelberg Eye Explorer software (Heidelberg Engineering GmbH, Heidelberg, Germany) and the confocal microscope is prepared for examination. One eye was selected and anesthetized with one drop of 0.4% benoxinate hydrochloride (oxybuprocaine hydrochloride) (Chauvin Pharmaceuticals Ltd., Essex, UK). The objective lens of the confocal microscope was disinfected (isopropyl alcohol 70% vol/vol, swabs), and 1 drop of Viscotears liquid gel (Carbomer 980, 0.2%; Novartis, UK) was applied onto the tip of the lens and advanced forward until the gel touched the cornea thus allowing visual but not physical contact between the lens and corneal epithelium (Figure 2.10). The cornea was scanned in 2 min and en face two-dimensional images were acquired. Approximately three to five high-quality images of Bowman’s layer were examined as it contains the main nerve plexus (Figure 2.11 and 2.12). When enough images had been captured the procedure was repeated for the other eye.
Morphometric measurements of the images were undertaken and the assessors of the images were masked with respect to the identity of the patient.

Figure 2-10 Positioning of the TomoCap® on the cornea

Figure 2-11 Image of the corneal subbasal nerves using corneal confocal microscopy
Figure 2-12 A preview of an image from the corneal subbasal nerves on the Heidelberg eye explorer with a simultaneous view of the location of the image through the CCD camera.

2.4.13 Manual Image Analysis: Chapter 4,5 & 8

Images are analysed manually by the assessor using purpose built software called “CCM image analysis tools v0p6” (CCMIATv0p6) (M.A. Dabbah, Imaging Science, The University of Manchester). The following parameters were quantified to define corneal nerve fibre damage and repair: 1) nerve fibre density (CNFD), the total number of major nerves per square millimetre of corneal tissue; 2) nerve fibre length (CNFL), the total length of all nerve fibres and branches per square millimetre of corneal tissue; 3) nerve branch density (CNBD), the number of branches emanating from each nerve trunk per square millimetre of corneal tissue; and 4) nerve fibre tortuosity (CNFT), a parameter that is mathematically derived from the images (4).
Figure 2-13 An CCM image of a control subject analysed to quantify corneal subbasal nerve morphology in DSPN. The red colour corresponds to CNFD and the CNFT, the green dots highlight CNBD at the points of junction with main nerve fibres and CNFL, the length of nerve structures in the entire CCM image is highlighted under the red and blue colour.

2.4.14 Automated Image Analysis: Chapter 3 only

Automated corneal nerve fibre quantification consists of two steps: (1) CCM image enhancement and nerve fibre detection and (2) quantification of CNFD, CNBD and CNFL. The detection of nerve fibre is a challenging task, as the nerve fibres often show poor contrast in the relatively noisy images. As described in our earlier work (5-6), a dual-model feature descriptor combined with a neural network classifier was used to train the computer to distinguish nerve fibres from the background (noise and underlying connective tissue). In the nerve fibre quantification process, all the end points and branch points of the detected nerve fibres are extracted and used to construct a connectivity map. Each segment in the connectivity map can then be connected and classified as main nerve fibres or branches according to the nerve intensity, orientation and length.
2.5 METHODS: Vitamin D Studies – Chapters 6, 7 & 9

Studies were undertaken from a population-based cohort at the Central Manchester University Hospitals NHS Foundation Trust, Manchester, UK of those with Type 1 and 2 DM attending a tertiary diabetes centre. Ethical approval was not required for this specific analysis as the data were extracted retrospectively from clinical databases and did not extend beyond standard clinical practice.

Retrospective vitamin D studies undertaken are as follows:


b. Chapter VII: Differential effects of different vitamin D replacement strategies in patients with Diabetes

c. Chapter IX: Vitamin D Deficiency is not associated with Diabetic Retinopathy or Maculopathy in Diabetes Mellitus

2.5.1 Participant Exclusion Criteria for Chapters 6, 7 & 9

Those with renal impairment (eGFR <30 mL/min/1.73 m² (CKD stage 4 and below), granulomatous diseases (tuberculosis, sarcoidosis etc), malabsorption syndromes (Coeliac disease, bacterial overgrowth, concomitant orlistat treatment), pregnant and lactating women.

2.5.2 Blood pressure and Anthropometric measurements

Body mass index (BMI) was measured as per the standard equation (mass (kg)/(height(m))². Weight was measured with a digital scale (Seca 701, Seca,
Hamburg, Germany) to the nearest 0.1kg and height to the nearest 0.1cm. 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.

2.5.3 Assessment of Demographics, Cardiovascular disease and Medications

An assessment of patient demographics, previous cardiovascular events and medications were made through analysis of medical records and an in-hospital medical record database (Diamond database, Hicom, Surrey, UK). The subject demographics extracted were age, sex, ethnicity (Caucasian, South Asian, Far East Asian and Afro-Caribbean descent), smoking status (never, previous and current) and type (Type 1 and 2 diabetes) and duration of diabetes. Previous cardiovascular events extracted were ischaemic heart disease (IHD), myocardial infarction (MI), cerebrovascular accidents (CVA) and peripheral vascular disease (PVD).

2.5.4 Laboratory Measurements

Standard assessment of 25(OH)D was instituted in the Manchester Diabetes Centre, Manchester, UK in August 2009 as part of routine haematological and biochemical laboratory measurements which included HbA1c, (Complete Blood count (CBC)), Urea and Electrolytes (UE), Liver function tests (LFT), bone profile (Corrected Calcium (CCa2+), Alkaline Phosphatase (ALP), Albumin (Alb)), and lipid profile (Total Cholesterol (T-CHL), High Density lipoprotein Cholesterol (HDL), Triglycerides (TRIG)). Low Density lipoprotein (LDL) analyses were not
undertaken and patients were routinely advised that bloods were to be collected under fasting conditions.

2.5.5 Diabetic Retinopathy Assessment

The retinopathy data were collected according to the grading criteria set out to a national standard by the National Screening Committee (NSC)(7-8). Previous studies have shown acceptable levels of quality and accuracy of grading compared to expert graders within the English NSC (9). The national guidelines do not contain R1.5 or M0.5 grades and are categorised as Pre-Proliferative Diabetic and Diabetic Maculopathy being present respectively. These sub-gradings are used locally in screening centres have thus been included. The grading of retinopathy are as follows:

R0 - No Diabetic retinopathy (NDR)

R1 - Background Diabetic Retinopathy (NDR): microaneurysms, retinal haemorrhages, exudates

R1.5 - Moderate numbers of intra-retinal haemorrhages, hard exudates >1 disc diameter (DD) from fovea, 3-6 cotton wool spots visible

R2 - Pre-Proliferative Diabetic Retinopathy (PPDR): Venous beading or looping, deep haemorrhages visible, other microvascular anomaly visible.

R3 - Proliferative Diabetic Retinopathy (PDR): New vessel formation, vitreous haemorrhage, pre- retinal haemorrhage or fibrosis and/or retinal detachment.

The outlines for maculopathy grading are as follows:

M0 - No maculopathy.

M0.5 - Hard exudates within the arcades >1DD from the centre of the fovea.
M1 - Exudates <1DD within the centre of the fovea, retinal thickening <1DD of the centre of the fovea.

The outlines for photocoagulation grading are as follows:

P0 – No photocoagulation scarring

P1 – Photocoagulation scarring present

2.5.6 25(OH) Vitamin D Assay

The specific laboratory used for the biochemical assay measurements (Vitamin D Research Group Manchester Royal Infirmary, UK) was accredited to ISO 9001:2008 and ISO 13485:2003 by Lloyd’s Register Quality Assurance, certificate number LRQ 4001542 and participated successfully in the Vitamin D quality assurance scheme (DEQAS). Serum was separated from whole blood and stored at -20°C until assay. The assay used was an automated platform assay (ImmunoDiagnostic Systems Ltd, Boldon, Tyne and Wear, UK) and is based on chemiluminescence technology. Briefly, samples were subjected to a pre-treatment step to denature the vitamin D binding protein. The treated samples were then neutralised in assay buffer and a specific anti-25(OH)D antibody labelled with acridinium was added. Following an incubation step, magnetic particles linked to 25(OH)D were added. Following a further incubation step, the magnetic particles were “captured” using a magnet. After a washing step and addition of trigger reagents, the light emitted by the acridinium label was inversely proportional to the concentration of 25(OH)D in the original sample. The concentration of 25(OH)D was calculated automatically using a 4-point logistic curve. The cross reactivity for vitamin D₂ (of the assay) as per manufacturers assertion was 100% (relative to vitamin D₃) and the assay has excellent correlation to existing globally recognised
assays, in combination with good sensitivity and precision (10). The reportable range of the assay was 5-140 ng/mL. Inter- and intra-assay variation of the in-house control was 5.6% and 9.7% respectively. Vitamin D deficiency (<20ng/ml) and insufficiency (<30ng/ml) were defined according to the Institute of Medicine (IOM) of the National Academies (11).

2.6 REFERENCES


3 Chapter III - Rapid Nerve Fibre Decline in patients with Type 1 diabetes can be readily detected using Corneal Confocal Microscopy.

**Contribution:** Uazman Alam contributed to the conception and design of the study and made a major contribution to the recruitment of subjects, clinical neuropathy assessments, all statistical analyses and writing of this chapter.

**To be submitted for publication**

3.1 ABSTRACT

Diabetic neuropathy is associated with increased morbidity and is an independent risk factor for mortality. Defining a population which has progressive rather than stable neuropathy would allow risk stratification and also perhaps identify those who may respond optimally to therapeutic intervention. We have assessed whether corneal confocal microscopy (CCM) can detect progression of neuropathy by establishing a decline in corneal nerve morphology.

Fifty subjects with Type 1 diabetes (T1DM) and sixteen non-diabetic healthy control subjects (C) underwent assessment of neurologic deficits: quantitative sensory testing (QST), electrophysiology and CCM at baseline and two year follow up.

Neuropathic symptom profile (NSP) (P<0.0006), McGill pain index (P=0.008), neuropathy disability score (NDS) (P=0.008), HbA1c (P<0.0001), and vibration perception threshold (VPT) (P<0.0001) were significantly increased and sural nerve conduction velocity and amplitude (P<0.0001 and P=0.0003 respectively), peroneal nerve conduction velocity and amplitude (P<0.0001 and P=0.0003 respectively) were significantly reduced in T1DM compared to controls. Corneal nerve fibre density (CNFD) (P<0.0001), corneal nerve branch density (CNBD) (P=0.007) and corneal nerve fibre length (CNFL) (P<0.0001) were significantly reduced at baseline in T1DM compared with control subjects. There was no significant change for: NDS, vibration perception threshold (VPT), warm sensation threshold (WST), electrophysiology and CCM measures in both T1DM and controls at 2 year follow up. However, we identified 11/50 T1DM patients who had a greater than 14.4% decline in CNFL over 2 years based on a 2 standard deviation cut off for intra-individual variation in the control group. In this cohort: sural sensory
(P=0.04) and peroneal motor (P=0.05) nerve conduction velocities, CNFD (18.8±10.2 vs 11.5±9.4 no./mm², P=0.0006), CNBD (24.1±15.6 vs 10.7±12.8 no./mm², P=0.0002) and CNFL (12.1±5.0 vs 8.5±4.5 mm/mm², P=0.0002) were significantly reduced from baseline to follow up after 2 years, which was not related to any change in HbA1c, lipids or BP.

A rapid small fibre decline is readily detectable in a subgroup of subjects with Type 1 diabetes using CCM and this novel modality provides an ideal surrogate endpoint in defining those who are at risk of progressive DPN.
3.2 INTRODUCTION

Diabetic peripheral neuropathy (DPN) is a distressing condition which affects around one half of patients during the natural history of the disease (1) although prevalence data varies (2-3). DPN is a confirmed risk factor for debilitating lower limb amputation (4) and large nerve fibre dysfunction in diabetes is itself predicted by cardiovascular disease (5) and is one of only three independent and significant risk factors for mortality as confirmed through the Action to Control Cardiovascular Risk in Diabetes (ACCORD) study (6). Risk factors for DPN include duration of diabetes, glycaemic control and elevated triglycerides (7) and improvement of glycaemic control as shown through the Diabetes Control and Complications Trial (DCCT) and the Epidemiology of Diabetes Interventions and Complications (EDIC) to improve neuropathy outcomes both in the short and long term respectively (8-9). An analysis of a placebo control arm in a multi-centre, double-blind study showed that short term metabolic improvements in glycaemic control and serum triglyceride levels have an independent, additive and durable effect on restoration of nerve function as evaluated by neurophysiology (10). We have previously shown that improvements in corneal nerve fibre density (CNFD) occur with an improvement in HbA1c (11) and corneal nerve fibre density (CNFD) and branch density (CNBD) improves with simultaneous pancreas and kidney transplantation (12). Smith et al (13) also showed that intra-epidermal nerve fibre density (IENFD) improved with diet and exercise in a pre-diabetic group. Deterioration in small nerve fibres may progress overtime and indeed a postulated longitudinal decline in IENFD (14) and small fibre function with diminished axon reflex-mediated vasodilator response is thought to occur (15). Longitudinal studies of neuropathy are limited; however, they do show deterioration of neuropathy overtime in both Type 1 diabetes (16) and
Type 2 diabetes using electrophysiology (17). The progression of DPN is thought to occur much slower than previously thought and is likely in part due to the positive impact on the peripheral nerve from commonly prescribed agents such as ACE inhibitors (18) and statins (19). This lack of placebo decline is of course one of the postulated reasons for the dramatic failure of pathogenetic treatments for DPN and Dyck et al concluded that future clinical trials should include subjects who are developing neuropathy rather than those with established neuropathy (20).

In the current study, we evaluated a cohort of subjects with Type 1 Diabetes at baseline and 2 year follow up in relation to their neuropathy status with the aim of delineating subjects who have rapidly progressive DPN through the novel modality of CCM.
3.3 RESEARCH DESIGN AND METHODS

3.3.1 Selection of patients

Fifty subjects with Type 1 diabetes (T1DM) (n=50) and sixteen age and sex matched non-diabetic healthy control subjects (C) (n=16) were assessed at baseline and two year follow up. Control subjects were selected through the University of Manchester staff network or friends and family of diabetic participants. Both diabetic patients and control subjects underwent full neurologic assessment and were included in final analyses on completing the protocol of baseline and 2 year follow up visit. Retinopathy status evaluation through retinal photography was not a part of the experimental protocol. All causes of peripheral neuropathy (except diabetes in T1DM) and those with a history of ocular trauma or previous ocular surgery were excluded. This study was of an observational nature only with no active study intervention although alterations to anti-glycaemic, anti-hypertensive and lipid modifying medication may have been instituted either in primary or secondary care. The study was approved by the North West Research Ethics committee, and written informed consent was obtained according to the Declaration of Helsinki.

3.3.2 Assessment of neuropathy

All patients and control subjects underwent a detailed evaluation of neurologic symptoms according to the neuropathy symptom profile (NSP), and the McGill visual analogue score (VAS) was used to assess the severity of painful neuropathy. Clinical neurologic deficits were assessed using the modified neuropathy disability score (NDS), which includes evaluation of vibration, pin prick, and temperature perception as well as the presence or absence of ankle reflexes.
to establish the severity of neuropathy (21). Quantitative sensory testing included an assessment of vibration perception threshold (VPT), measured using a Neurothesiometer (Horwell, Scientific Laboratory Supplies, Wilford, Nottingham, U.K.), cold sensation threshold (CST) (Aδ fibres) and warm sensation threshold (WST) (22) (c fibres) using the method of limits with the MEDOC TSA II (Medoc, Ramat Yishai, Israel) on the dorsum of the left foot.

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

### 3.3.3 Corneal Confocal Microscopy

Patients underwent examination with the Heidelberg retina tomography III in vivo corneal confocal microscope employing our established methodology for image acquisition (23). Several scans of the entire depth of the cornea were recorded by turning the fine focus of the objective lens backward and forward for ~2 min using the section mode, which enables manual acquisition and storage of single images of all corneal layers. This provides en face two-dimensional images with a lateral resolution of ~2 mm/pixel and final image size of 400 x 400 pixels of the subbasal nerve plexus of the cornea from each patient and control subject. Each sub-basal nerve fibre bundle contains unmyelinated fibres, which run parallel to Bowman's
layer before dividing and terminating as individual axons underneath the surface epithelium. Five images per patient from the centre of the cornea were selected and examined in a masked and randomised fashion (24). Three corneal nerve parameters were quantified: 1) CNFD, the total number of major nerves per square millimetre of corneal tissue (no.mm\(^2\)); 2) corneal nerve branch density (CNBD), the number of branches emanating from all major nerve trunks per square millimetre of corneal tissue (no.mm\(^2\)); and 3) corneal nerve fibre length (CNFL), the total length of all nerve fibres and branches (mm/mm\(^2\)) within the area of corneal tissue. Automated corneal nerve fibre quantification (ACCMetrics software, University of Manchester, Manchester, UK) was undertaken and consists of two steps: (1) CCM image enhancement and nerve fibre detection and (2) quantification of three morphometric parameters i.e. CNFD, CNBD and CNFL. In the nerve fibre quantification process, all the end points and branch points of the detected nerve fibres are extracted and used to construct a connectivity map. Each segment in the connectivity map can then be connected and classified as main nerve fibres or branches according to the nerve properties such as intensity, orientation and length.

3.3.4 Statistical analysis and Power calculation

Previous natural history studies are lacking in assessing CCM parameters in the progression of diabetic neuropathy and thus power calculations are based on unpublished data. For the diabetic cohort, an assumption of paired groups was used to calculate the sample size considering a change in CNFD over 2 years. The standard deviation between groups is likely to be 9 nerves/mm\(^2\). Therefore, recruiting a minimum of 28 patients for the diabetic group will provide 80% chance
to detect a clinically meaningful change in CNFD of 5 nerves/mm$^2$ and an assumption of a type 1 error ($\alpha$-level) of 0.05. Considering a greater change of 7.5 nerves/mm$^2$ at 80% power to detect a clinically meaningful change, 14 patients would be required with an assumption of type 1 error of 0.05. Statistical analyses were undertaken on Statsdirect (Statsdirect, Cheshire, UK). The data are expressed as Mean $\pm$ standard deviation (SD). Paired t-test or a non-parametric counterpart, Mann-Whitney-U were used to assess differences between baseline and follow up data on normality of the data. Unpaired t-test or Mann-Whitney-U test were used to assess differences between baseline control and T1DM data, depending on normality of the data. Chi squared analyses were used to assess frequencies of gender and ethnicity. A significant P value was considered to be $\leq$0.05 corrected for multiple comparison tests. A post hoc analysis was undertaken of those showing a worsening neuropathy through assessment of decline in CNFL defined as rapid nerve fibre decline based on a two standard deviation cut off of CNFL variation over 2 years in the control group.
3.4 RESULTS

3.4.1 Demographics (Table 3.1)

The participant demographics and metabolic and anthropometric measurements in diabetic subjects and age-matched control subjects are summarized in Table 3.1. There were no significant differences in age (Control baseline: 41.4±11.4 vs T1DM baseline: 48.2±15.5 years, P=NS) and duration of diabetes was 33.1±16.7 years at baseline in the T1DM group. There were no differences in gender between controls and T1DM but there were a higher proportion of white Europeans in T1DM (Chi² P<0.0001).
Table 3-1 Participant demographics and clinical neuropathy parameters in control subjects and patients with T1DM at baseline and 2 year follow up, with statistically significant differences between groups

<table>
<thead>
<tr>
<th></th>
<th>Control BL (n=16)</th>
<th>Control FU (n=16)</th>
<th>P</th>
<th>T1DM BL (n=50)</th>
<th>T1DM FU (n=50)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>41.4±11.4</td>
<td></td>
<td></td>
<td>48.2±15.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gender (Male) (%)</td>
<td>63</td>
<td></td>
<td></td>
<td>54</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethnicity (White European) (%)</td>
<td>75</td>
<td></td>
<td></td>
<td>96</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duration of Diabetes (years)</td>
<td>N/a</td>
<td></td>
<td></td>
<td>33.1±16.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NDS (-/10) Median(IQR)</td>
<td>0.5±1.1*</td>
<td>0.4±1.1</td>
<td>NS</td>
<td>3.4±3.5*</td>
<td>3.3±3.6</td>
<td>NS</td>
</tr>
<tr>
<td>NSP (-/38) Median(IQR)</td>
<td>0.1±0.25**</td>
<td>0</td>
<td>NS</td>
<td>3.8±5.3**</td>
<td>4.1±6.7</td>
<td>NS</td>
</tr>
<tr>
<td>McGill VAS (~/10cm) Median(IQR)</td>
<td>0.3±1.25†</td>
<td>0</td>
<td>NS</td>
<td>2.3±3.3†</td>
<td>1.5±3.0</td>
<td>NS</td>
</tr>
</tbody>
</table>

Post hoc analyses (Control Baseline vs T1DM Baseline)

* P=0.0006; **P= <0.0001; † P=0.008.

Table key
BL – Baseline; FU – Follow Up; McGill VAS – McGill Visual Analogue Score; NDS – Neuropathy Disability Score; NSP – Neuropathy Symptom Profile.
3.4.2 Metabolic and Anthropometric measurements (Table 3.2)

BMI did not differ between patients with T1DM and control subjects at baseline. HbA1c was significantly higher in T1DM (P<0.0001) compared to controls with no significant change at 2 year follow up. Although, there was a significant reduction in HbA1c in controls at follow up, both baseline and follow up values were in the normal non-diabetic range. The total cholesterol was significantly lower (P<0.0001) in diabetic patients due to greater statin use, whilst triglycerides and HDL were comparable between T1DM and control subjects with no changes at follow up. Systolic BP was no different in controls compared to T1DM at baseline. T1DM (P=0.04) exhibited a mild drop in systolic BP at 2 year follow up with no change in controls at baseline and follow up. There were no differences in the estimated glomerular filtration rate between T1DM and controls at baseline, however, there was a decline in eGFR in T1DM at 2 year follow up (P=0.02).
Table 3-2 Metabolic parameters in control subjects and T1DM patients at baseline and 2 year follow up, with statistically significant differences between groups

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Control FU</th>
<th>P</th>
<th>T1DM</th>
<th>T1DM FU</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BL (n=16)</td>
<td>(n=16)</td>
<td>P</td>
<td>BL (n=50)</td>
<td>(n=50)</td>
<td></td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.6±0.3</td>
<td>5.3±0.3</td>
<td></td>
<td>8.2±1.3*</td>
<td>8.1±1.6</td>
<td></td>
</tr>
<tr>
<td>HbA1c (mmol/mol)</td>
<td>38.0±3.3</td>
<td>34.8±3.4</td>
<td>0.003</td>
<td>66.2±14.3*</td>
<td>65.2±17.1</td>
<td>NS</td>
</tr>
<tr>
<td>BMI (kg/m2)</td>
<td>25.7±4.2</td>
<td>24.7±3.8</td>
<td>NS</td>
<td>26.9±4.4</td>
<td>27.1±4.7</td>
<td>NS</td>
</tr>
<tr>
<td>T-CHL (mmol/l)</td>
<td>5.1±0.9</td>
<td>4.8±0.7</td>
<td>NS</td>
<td>4.3±0.9**</td>
<td>4.3±0.9</td>
<td>NS</td>
</tr>
<tr>
<td>HDL (mmol/l)</td>
<td>1.5±0.3</td>
<td>1.5±0.3</td>
<td>NS</td>
<td>1.7±0.5</td>
<td>1.7±0.5</td>
<td>NS</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>1.4±0.7</td>
<td>1.2±0.5</td>
<td>NS</td>
<td>1.1±0.7</td>
<td>1.1±0.5</td>
<td>NS</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>125±21</td>
<td>120±18</td>
<td>NS</td>
<td>132±18</td>
<td>127±21</td>
<td>0.04</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>75±12</td>
<td>74±11</td>
<td>NS</td>
<td>73±8</td>
<td>66±9</td>
<td>0.004</td>
</tr>
<tr>
<td>eGFR (ml/min/1.73)</td>
<td>86±7</td>
<td>83±7</td>
<td>NS</td>
<td>81±19</td>
<td>75±17</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Post hoc analyses (Control Baseline vs T1DM Baseline)

*P<0.0001; ** P=0.003.

Table key

BL – Baseline; BMI – Body Mass Index; BP – Blood Pressure; eGFR – estimated Glomerular Filtration Rate; FU – Follow Up; HbA1c – Glycated Haemoglobin A1c; HDL – High Density Lipoprotein Cholesterol; T-CHL – Total Cholesterol.
3.4.3 Neuropathy evaluation (Table 3.3)

3.4.3.1 Symptoms and Deficits

Neuropathic symptoms as assessed with the NSP were significantly greater in diabetic patients than in control subjects at baseline ($P<0.0006$), but there was no significant change at 2 year follow up for either controls or T1DM. The McGill pain index was significantly ($P=0.008$) greater at baseline compared with control subjects and did not show a significant change at 2 years follow up in T1DM. The modified NDS was significantly ($P=0.008$) greater at baseline compared with control subjects, indicating a mild to moderate neuropathy, and did not change significantly at 2 year follow up in T1DM.

3.4.3.2 Vibration Perception and Thermal Thresholds

VPT was significantly greater in T1DM ($P<0.0001$) compared with control subjects at baseline with no significant change from baseline to 2 year follow up in either controls or T1DM. CST was significantly greater in T1DM ($P=0.002$) compared with control subjects at baseline and further increased at 2 year follow up in T1DM ($P=0.02$) although the latter isolated change may be due to chance. WST did not differ between T1DM and control subjects at baseline and did not change at follow up.

3.4.3.3 Electrophysiology

Sural nerve conduction velocity and amplitude were significantly lower ($P<0.0001$ and $P=0.0003$ respectively) at baseline in T1DM compared with control subjects. Peroneal nerve conduction velocity and amplitude were significantly lower ($P<0.0001$ and $P=0.0003$ respectively) at baseline in T1DM compared with control subjects.
subjects. There were no significant changes in electrophysiology from baseline to 2 year follow up in either controls or T1DM.

3.4.3.4 Corneal sensation and structure

NCCA was significantly greater in T1DM compared to control subjects and did not progress over 2 years of follow up in either T1DM or controls. CNFD (P<0.0001), CNBD (P=0.007) and CNFL (P<0.0001) were reduced in T1DM compared to controls at baseline with no significant changes at 2 year follow up in either controls or T1DM.
Table 3-3 Small and large fibre tests of nerve structure and function in control subjects and diabetic patients at baseline and 2 year follow up, with statistically significant differences between groups.

<table>
<thead>
<tr>
<th></th>
<th>Control BL (n=16)</th>
<th>Control FU (n=16)</th>
<th>T1DM BL (n=50)</th>
<th>T1DM FU (n=50)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCCA (mBar)</td>
<td>0.6±0.3</td>
<td>0.6±0.4</td>
<td>1.4±2.3†</td>
<td>1.5±2.3</td>
<td>NS</td>
</tr>
<tr>
<td>CNFD (no/mm²)</td>
<td>30.1±4.9</td>
<td>28.3±5.5</td>
<td>19.5±9.1*</td>
<td>18.7±9.9</td>
<td>NS</td>
</tr>
<tr>
<td>CNBD (no/mm²)</td>
<td>36.6±15.5</td>
<td>39.3±18.0</td>
<td>23.9±15.0**</td>
<td>22.6±15.8</td>
<td>NS</td>
</tr>
<tr>
<td>CNFL (mm/mm²)</td>
<td>16.9±2.8</td>
<td>16.6±3.0</td>
<td>12.0±4.6***</td>
<td>12.1±5.0</td>
<td>NS</td>
</tr>
<tr>
<td>CST (°C)</td>
<td>28.6±2.1</td>
<td>26.8±4.8</td>
<td>24.8±6.9</td>
<td>22.5±8.4</td>
<td>0.02</td>
</tr>
<tr>
<td>WST (°C)</td>
<td>37.6±3.5</td>
<td>38.9±3.8</td>
<td>39.6±4.6</td>
<td>40.2±5.7</td>
<td>NS</td>
</tr>
<tr>
<td>VPT (volts)</td>
<td>5.3±4.8</td>
<td>6.0±5.2</td>
<td>15.5±13.6ª</td>
<td>15.9±12.6</td>
<td>NS</td>
</tr>
<tr>
<td>SSNCV (m/s)</td>
<td>49.4±3.9</td>
<td>48.1±4.7</td>
<td>43.6±6.3³</td>
<td>41.3±6.4</td>
<td>NS</td>
</tr>
<tr>
<td>SSNAmp (µV)</td>
<td>21.0±10.8</td>
<td>18.6±8.7</td>
<td>10.1±6.7³³</td>
<td>8.7±6.8</td>
<td>NS</td>
</tr>
<tr>
<td>PMNCV (m/s)</td>
<td>47.8±3.6</td>
<td>47.3±4.2</td>
<td>40.6±7.0⁵</td>
<td>40.0±6.6</td>
<td>NS</td>
</tr>
<tr>
<td>PMNAmp (mV)</td>
<td>6.0±1.6</td>
<td>5.8±1.2</td>
<td>3.4±2.4⁶</td>
<td>3.2±2.0</td>
<td>NS</td>
</tr>
</tbody>
</table>

Post hoc analyses (Control Baseline vs T1DM Baseline)

†P=0.02; *P<0.0001; **P=0.007; ***P<0.0001; ³P=0.002; ⁴P<0.0001; ⁵P<0.0001; ⁶P=0.0003; ⁷P<0.0001; ⁸P=0.0003.

Table key

3.4.3.5 Rapid Nerve Fibre Decline (RNFD) in T1DM

(Table 3.4, Figures 3.1 & 3.2)

The mean intra-individual change in CNFL over the 2 year follow up in the control group was 7.9±7.2%. A 2 standard deviation intra-individual change is therefore 14.4%. We thus used this parameter as a cut off for RNFD in the T1DM group. We identified eleven subjects (n=11) out of fifty in the T1DM group who had a greater than 14.4% decline in CNFL over 2 years. This analysis is based on the unpublished data presented by Professor Bruce Perkins (at the American Diabetes Association conference 2013) in which rapid nerve fibre decline was defined as a change in CNFL of -14% (25). The mean intra-individual change in CNFL over 2 year follow up in the T1DM group was 27.8±32.4%.

There were no significant differences for age HbA1c, BMI, T-CHL and systolic BP in those with RNFD. There were no significant changes in VPT (Baseline: 17.5±13.1 vs 2 yr FU: 19.7±14.2 volts, P=NS), CST (Baseline: 25.5±4.0 vs 2 year FU: 23.3±4.4 (˚C), P=NS) and WST (Baseline: 39.2±4.2 vs 2 year FU: 40.1±4.3 (˚C), P=NS).

There were concomitant reductions at 2 year follow up in sural sensory and peroneal motor nerve conduction velocities (P=0.04 and P=0.05 respectively) with no change in sural and peroneal amplitudes over the same period.

All measures of CCM were significantly reduced at follow up: CNFD (Baseline: 18.8±10.2 vs 2 yr FU: 11.5±9.4 no./mm², P=0.0006), CNBD (Baseline: 24.1±15.6 vs 2 yr FU: 10.7±12.8 no./mm², P=0.0002) and CNFL (Baseline: 12.1±5.0 vs 2 yr FU: 8.5±4.5 mm/mm², P=0.0002) (Figure 3.1a, b & c). Figure 3.2a & b shows images from a subject with RNFD at baseline and 2 year follow up. In assessing
subjects with reductions in peroneal motor and sural sensory nerve conduction velocity, concomitant reductions in CNFD, CNBD and CNFL were also noted, however data are analysed based on those with a reduction of 14.4% in CNFL or greater.
Table 3-4 T1DM subjects with Rapid Nerve Fibre Decline at baseline and 2 year follow up with statistically significant differences

<table>
<thead>
<tr>
<th></th>
<th>T1DM BL (n=11)</th>
<th>T1DM FU (n=11)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>54.8±9.2</td>
<td>N/a</td>
<td>-</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>7.9±0.6</td>
<td>7.7±1.0</td>
<td>NS</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27.9±3.3</td>
<td>28.8±4.3</td>
<td>NS</td>
</tr>
<tr>
<td>T-CHL (mmol/l)</td>
<td>4.6±1.2</td>
<td>4.6±1.3</td>
<td>NS</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>141±20</td>
<td>132±24</td>
<td>NS</td>
</tr>
<tr>
<td>SSNCV (m/s)</td>
<td>42.7±6.1</td>
<td>38.1±7.0</td>
<td>0.04</td>
</tr>
<tr>
<td>SSNAmp (µV)</td>
<td>9.3±8.7</td>
<td>6.3±5.8</td>
<td>NS</td>
</tr>
<tr>
<td>PMNCV (m/s)</td>
<td>42.4±3.3</td>
<td>41.1±4.2</td>
<td>0.05</td>
</tr>
<tr>
<td>PMNAmp (mV)</td>
<td>2.8±2.1</td>
<td>3.0±1.5</td>
<td>NS</td>
</tr>
</tbody>
</table>

Table key

Figure 3-1a, b & c. CNFD, CNBD and CNFL and baseline and 2 year follow up.
Figure 3-2 a & b. CCM images of a subject with RNFD at baseline and 2 year follow up

CCM progression
Baseline (A) vs. 2 Years (B)

- Nerve Branches
- Nerve Fibres
3.5 DISCUSSION

The major findings of this study are: 1) Type 1 diabetes patients show no significant progression of neuropathy over 2 years; 2) A subgroup of patients with Type 1 diabetes shows rapidly progressive and readily detectable decline in corneal nerve fibre structure using CCM 2) and 3) CCM provides an ideal surrogate endpoint for the assessment and progression of diabetic neuropathy. These findings are both of importance in identifying those who have progression of DPN with subsequent risk of ensuing complications such as foot ulceration and lower limb amputation but also mortality. Furthermore, it may also be important in identifying patients who should be recruited into clinical trials of DPN as Dyck et al (20) concluded that future trials of DPN should include those with developing neuropathy and also select endpoints which show a discernible worsening. CCM provides a seemingly suitable surrogate endpoint for the assessment of DPN and has also shown an excellent correlation with the current gold standard IENFD (26).

At 2 years there were no significant changes in the majority of our diabetic subjects and this is in keeping with the notion that DPN progresses much slower than previously thought (27). This may provide a partial explanation for the failure of pathogenetic treatments in clinical trials of DPN. Nerve conduction velocity is only gradually diminished by DPN, with estimates of a loss of ~0.5m/s/ year (28). In the 10 year natural history study by Partanen et al (17), nerve conduction velocity deteriorated in all nerve segments evaluated with the largest deficit of 3.9 m/s for the sural nerve. The DCCT showed a similar slow progression of change in nerve conduction velocity over a 5 year period (29). Previously, a placebo controlled study has shown improvements in signs and VPT in the placebo-treated arm with a small reduction in electrophysiological measures (30) and this has also been
demonstrated in another placebo controlled study (31). Both the study by Partanen et al (17) and the DCCT (16) were undertaken prior to the widespread use of ACE inhibitors and statins, both of which are likely to impact positively on the peripheral nerve (18-19). Electrophysiology, of course focuses on large fibre function wherein the primary pathology lies in small fibres and predates abnormalities in nerve conduction studies (32). Therefore a measure of small fibre damage may be the most sensitive to detect an early abnormality and progression. Small fibre neuropathy in particular that of sudomotor dysfunction may result in dryness of foot skin and has been associated with foot ulceration (33) and CCM provides an ideal surrogate marker for small fibre neuropathy.

The Toronto criteria for DPN (32) advocates electrophysiology for epidemiologic surveys or controlled clinical trials of DPN as an early and reliable indicator of the occurrence of neuropathy. However, our study clearly shows a lack of concomitant reductions in nerve amplitudes particularly sural amplitude which is a considered a particularly valid and sensitive marker of nerve damage (34). Furthermore, variability in measurements (intra-observer variability) can be a significant concern when interpreting electrophysiological results, particularly in multi centre trials (35) and short term improvements in metabolic control can cause significant increases in nerve conduction parameters (10) although whether this correlates to actual repair or enhancement of large nerve fibre structure is unknown. Our data shows globally reduced measures of corneal nerve structure at baseline confirming previous studies which have shown that corneal small nerve fibre damage can be detected prior to abnormalities in electrophysiology and quantitative sensory testing (26). CCM also has the ability to detect nerve fibre repair and regeneration.
as evidenced by our recently published data on subjects undergoing simultaneous pancreas and kidney transplantation with amelioration of Type 1 diabetes (12). We have no obvious metabolic explanation for the basis of rapid nerve fibre decline observed in the subset of patients with T1DM as there were no significant changes in HbA1c, lipids and blood pressure in the overall T1DM cohort. This is clearly different from the results of the Eurodiab study which showed that the development of neuropathy was related to a range of cardiovascular risk factors (7). However, the control group was relatively small and the cut offs used for RNFD may be different if derived from a larger cohort and the duration of follow up was relatively short.

In conclusion, we have shown that CCM can identify a subcohort of patients with T1DM with a rapid degree of small fibre decline. Furthermore, given that we have recently shown that CCM can detect early nerve fibre repair after SPK (12) this adds to the evidence that CCM may be an ideal surrogate endpoint for clinical trials of new therapies in diabetic neuropathy.
3.6 REFERENCES


points assessed longitudinally for change and monotonicity. Diabetes Care 2007;30:2619-2625


Chapter IV - Diagnostic Utility of Corneal Confocal Microscopy and Intra-Epidermal Nerve Fibre Density in Diabetic Neuropathy

**Contribution:** Uazman Alam contributed to the conception and design of the study and made a major contribution to the recruitment of subjects, clinical tests including neuropathy assessments and skin biopsies, skin biopsy analyses, all statistical analyses and writing of this chapter.

*To be submitted for publication*

**Uazman Alam**, Maria Jeziorska, Ioannis N Petropoulos, Omar Asghar, Hassan Fadavi, Georgios Ponirakis, Andrew Marshall, Mitra Tavakoli, Andrew JM Boulton, Nathan Efron, Rayaz A Malik
4.1 ABSTRACT

Corneal confocal microscopy (CCM) is a rapid, non-invasive, reproducible technique that quantifies small nerve fibres. We have compared the diagnostic capability of CCM against a range of established measures of nerve damage in patients with diabetic neuropathy.

Thirty subjects with Type 1 diabetes without neuropathy (T1DM), thirty one subjects with Type 1 diabetes and neuropathy (DSPN) and twenty seven non-diabetic healthy control subjects underwent detailed assessment of neuropathic symptoms and neurologic deficits, quantitative sensory testing (QST), electrophysiology, skin biopsy and CCM.

Comparing subjects with DSPN and T1DM, those with DSPN they were older (P=0.0002), had a longer duration of diabetes (P<0.0001), lower eGFR (P=0.006) and higher albumin-creatinine ratio (ACR) (P=0.03) with no significant difference for HbA1c, BMI, lipids and blood pressure. Patients with DSPN had more symptoms (neuropathy symptom profile (NSP) (P<0.0001), McGill visual analogue score (McGill VAS) (P=0.01) and McGill pain score (P=0.02), higher neuropathy disability score (NDS) (P<0.0001), CST (P=0.007), WST (P=0.0004), vibration perception threshold (VPT) (P<0.0001) and lower peroneal motor nerve conduction velocity (PMNCV) and amplitude (PMNAmp) (P<0.0001 and P<0.0001), sural sensory nerve conduction velocity (SSNCV) and amplitude (SSNAmp) (P<0.0001), intra-epidermal nerve fibre density (IENFD) (P=0.001), corneal nerve fibre density (CNFD) (P<0.0001), corneal nerve branch density (CNBD) (P=0.02) and corneal nerve fibre length (CNFL) (P=0.001). CNFD correlated better with PMNCV (Spearman’s Rho= 0.60 P<0.0001) than IENFD with PMNCV (Spearman’s Rho= 0.56 P<0.0001). For the diagnosis of diabetic neuropathy the sensitivity for CNFD
was 0.77 and specificity was 0.79 with Wilcoxon estimate of area under the ROC
curve of 0.81. IENFD had a diagnostic sensitivity of 0.61, specificity of 0.80 and
area under the ROC curve of 0.73.
CCM is a valid accurate non-invasive method which is superior to skin biopsy in
diagnosing DPN.
4.2 INTRODUCTION

Diabetic peripheral neuropathy is a debilitating condition which may lead to pain, foot ulceration and eventual amputation (1). Therefore it is important to accurately diagnose diabetic neuropathy at the earliest stage of damage. Clinical neurological assessment is poorly reproducible (2), and whilst QST can measure both large and small nerve fibre function it is subjective and requires an alert and motivated subject. Nerve conduction studies remain the mainstay for the diagnosis and progression of diabetic neuropathy (3). However, the earliest nerve fibres to degenerate (4-5) and regenerate (6) are the small unmyelinated fibres and indeed they are central to the genesis of pain and the development of foot ulceration (7).

In 2005 the European Federation of Neurological Societies published guidelines on the use of skin biopsy in the diagnosis of peripheral neuropathies (8) and more recently the value of the technique has been further emphasized (9). Currently, skin biopsy with an assessment of intra-epidermal nerve fibres (IENF) is considered the gold standard for the evaluation of small fibre neuropathy and has been advocated as measure of treatment response in clinical trials (10). Previous studies have demonstrated good diagnostic ability of skin biopsy for small fibre neuropathy (11-14), however, these studies have assessed mixed populations of small fibre neuropathy and the diagnostic utility of CCM versus IENF have not been evaluated. Hence reproducible and reliable processing and accurate quantification methods have been established for assessing IENF pathology against normative ranges (15). However, despite being advocated as an endpoint in clinical trials of diabetic neuropathy there is surprisingly scarce data which have established the diagnostic ability of skin biopsy for diabetic neuropathy (7).
Furthermore skin biopsy is invasive with a small but significant risk for bleeding and infection and requires expertise in laboratory assessment (16). CCM is a non-invasive ophthalmic application, which is rapid, non-invasive and readily reproducible for quantifying small nerve fibres and thereby has been shown to diagnose and track the progression of diabetic neuropathy (17-19). CCM has been shown to have reasonable diagnostic utility in detecting DPN diagnosed using NDS (20) with good reproducibility (21). More recently CCM has been shown to correlate with functional measures of small nerve fibre injury (22). However, the differential ability of CCM against currently accepted gold standard FDA approved methods such as skin biopsy, QST and nerve conduction studies for the diagnosis of diabetic neuropathy has not been evaluated to date. We have therefore compared the ability of CCM, QST and skin biopsy in the diagnosis of diabetic neuropathy using the Toronto criteria (3).
4.3 RESEARCH DESIGN AND METHODS

4.3.1 Selection of patients

Sixty one consecutive subjects with Type 1 diabetes were assessed and subsequently divided into two groups based their neuropathy status. Patients were recruited from general diabetes clinics in the Manchester Diabetes Centre, Central Manchester Foundation Trust. Thirty subjects with Type 1 diabetes without neuropathy (T1DM) (n=30), thirty one T1DM subjects with neuropathy (DSPN) (n=31) and twenty seven non-diabetic healthy control subjects were studied. Subjects with a history of neurologic conditions, ocular trauma or previous ocular surgery were excluded. The study was approved by the North Manchester Research Ethics committee, and written informed consent was obtained according to the Declaration of Helsinki.

4.3.2 Definition of Neuropathy

Diabetic neuropathy was defined according to the Toronto criteria by the presence of an abnormality of electrophysiology and a symptom or symptoms or a sign or signs of neuropathy (3).

4.3.3 Assessment of neuropathy

All patients and control subjects underwent a detailed evaluation of neurologic symptoms according to the NSP, and the McGill VAS was used to assess the severity of painful neuropathy. Clinical neurologic deficits were assessed using the modified neuropathy disability score, which includes an evaluation of vibration, pin prick, and temperature perception as well as the presence or absence of ankle reflexes (23). Quantitative sensory testing included an assessment of the VPT,
measured using a neurothesiometer (Horwell, Scientific Laboratory Supplies, Wilford, Nottingham, U.K.), CST (Aδ fibres) and WST (24) (c fibres) thresholds using the method of limits with the MEDOC TSA II (Medoc, Ramat Vishay, Israel) on the dorsum of the left foot. Electro-diagnostic studies were undertaken using a Dante “Key point” system (Dante Dynamics, Bristol, U.K.) equipped with a DISA temperature regulator to keep limb temperature constantly between 32°C and 35°C. Peroneal motor and sural sensory nerves were assessed in the right lower limb by a consultant neurophysiologist. The motor study was performed using silver-silver chloride surface electrodes at standardized sites defined by anatomical landmarks, and recordings for the sural nerve were taken using antidromic stimulation over a distance of 100 mm.

4.3.4 Corneal Confocal Microscopy

Patients underwent examination with the Heidelberg retina tomography III in vivo corneal confocal microscope employing our established methodology for image acquisition (25). Several scans of the entire depth of the cornea were recorded by turning the fine focus of the objective lens backward and forward for ~2 min using the section mode, which enables manual acquisition and storage of single images of all corneal layers. This provides en face two-dimensional images with a lateral resolution of ~2 mm/pixel and final image size of 400 x 400 pixels of the subbasal nerve plexus of the cornea from each patient and control subject. Each sub-basal nerve fibre bundle contains unmyelinated fibres, which run parallel to Bowman’s layer before dividing and terminating as individual axons underneath the surface epithelium. Five images per patient from the centre of the cornea were selected and examined in a masked and randomized fashion (26). Three corneal nerve
parameters were quantified: 1) CNFD, the total number of major nerves per square millimetre of corneal tissue (no.mm$^2$); 2) CNBD, the number of branches emanating from all major nerve trunks per square millimetre of corneal tissue (no.mm$^2$); and 3) CNFL, the total length of all nerve fibres and branches (mm/mm$^2$) within the area of corneal tissue (27).

4.3.5 Skin biopsy and immunohistochemistry

Participants underwent a 3-mm punch skin biopsy from the dorsum of the foot; 2 cm above the second metatarsal head after local anaesthesia (1% lidocaine). The biopsy site was closed using Steristrips, and the specimen was immediately fixed in PBS-buffered 4% paraformaldehyde for 18-24 hours, washed in Tris-buffered saline, cryoprotected in sucrose, frozen in liquid nitrogen and stored at -80°C and subsequently cut into 50-μm sections on a cryostat microtome. Five floating sections per subject were immunostained for PGP9.5 neuronal marker. Non-specific protein binding and endogenous peroxidase activity were blocked by incubation in 5% goat serum and 0.3% hydrogen peroxide, respectively. The anti-PGP9.5 antibody (Milipore 1:1000; Billerica, MA) was followed first by goat anti-rabbit IgG and then by HRP-Streptavidin (both diluted 1:1000, both from Vector Laboratories, Peterborough, UK). Nerve fibres were visualised by SG chromogen (Vector Laboratories). IENFD was calculated as the number of nerve fibres crossing the basement membrane of the epidermis and expressed per millimetre length of epidermis (28).

4.3.6 Statistical analysis

Statistical analyses were undertaken on Statsdirect (Statsdirect, Cheshire, UK). The data are expressed as Mean ± standard deviation (SD). ANOVA method or a
non-parametric counterpart, Kruskal-Wallis were used to assess differences between groups depending on normality of the data. The Mann-Whitney U test was used to compare T1DM with DSPN for the duration of diabetes. Chi squared analyses were used to assess frequencies of gender, and ethnicity. Overall the P value was maintained at 0.05 for multiple comparison tests (Bonferoni adjustment or Conover-Inmann pairwise comparison). Spearman’s rank correlation was undertaken for CNFD, CNBD, CNFL and IENFD versus NDS, McGill VAS, NSP, IENFD, thermal thresholds, VPT and nerve conduction studies. ROC curve analyses were used to define the Wilcoxon estimate of area under ROC curve, optimal cut offs with associated sensitivity and specificity for CCM parameters, IENFD, VPT and thermal thresholds. Positive predictive value (PPV) and negative predictive value (NPV) were calculated for the three diagnostic measures, which had the greatest Wilcoxon estimate of area under the ROC curve.
4.4 RESULTS

4.4.1 Demographics, Metabolic and Anthropometric Assessment (Table 4.1)

The participant demographics and metabolic and anthropometric measurements in diabetic patients and control subjects are summarized in Table 4.1. Those with DSPN (53.3±11.9) were significantly older compared to controls (41.0±14.9 years, P=0.0008) and T1DM (38.8±12.5, P<0.0001) and the duration of diabetes was greater in DSPN compared to T1DM (P<0.0001). The non white European ethnicities in table 4.1 were Asian with one participant of African origin in the control and T1DM group. HbA1c (P<0.0001) was significantly higher in diabetic patients compared with control subjects with no difference between patients with T1DM and DSPN. Total cholesterol was significantly lower in diabetic patients with T1DM (P=0.006) and DSPN (P=0.002) compared to control subjects. BMI, HDL, triglycerides, systolic and diastolic blood pressure were comparable between diabetic patients and control subjects. The estimated glomerular filtration rate (eGFR) was lower in DSPN compared to T1DM (P=0.006), the Albumin-Creatinine Ratio (ACR) was higher in DSPN compared to T1DM (P=0.03) and controls (P=0.004), although median values for controls, T1DM and DSPN were all within the normal range for ACR.
Table 4-1 Participant demographics and metabolic parameters in control subjects and diabetic patients without (T1DM) and with neuropathy (DSPN), with statistically significant differences between groups

<table>
<thead>
<tr>
<th></th>
<th>C (n=27)</th>
<th>T1DM (n=30)</th>
<th>DSPN (n=31)</th>
<th>T1DM v DSPN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>41.0±14.9</td>
<td>38.8±12.5</td>
<td>53.3±11.9</td>
<td>0.0002</td>
</tr>
<tr>
<td>Gender (Male) (%)</td>
<td>59</td>
<td>43</td>
<td>61</td>
<td>-</td>
</tr>
<tr>
<td>Ethnicity (White European) (%)</td>
<td>77</td>
<td>90</td>
<td>97</td>
<td>-</td>
</tr>
<tr>
<td>Aetiology of Diabetes (Type 1 DM) (%)</td>
<td>-</td>
<td>100</td>
<td>100</td>
<td>NS</td>
</tr>
<tr>
<td>Duration of Diabetes (years)</td>
<td>-</td>
<td>17.2±12.0</td>
<td>37.2±13.1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.5±0.3</td>
<td>8.0±1.3</td>
<td>8.5±1.5</td>
<td>NS</td>
</tr>
<tr>
<td>HbA1c (mmol/mol)</td>
<td>36.9±3.4</td>
<td>61.0±21.0</td>
<td>70.0±17.0</td>
<td>NS</td>
</tr>
<tr>
<td>BMI (kg/m2)</td>
<td>26.9±4.0</td>
<td>26.3±4.4</td>
<td>27.2±4.2</td>
<td>NS</td>
</tr>
<tr>
<td>T-CHL (mmol/l)</td>
<td>5.0±0.8</td>
<td>4.4±0.9</td>
<td>4.3±0.9</td>
<td>NS</td>
</tr>
<tr>
<td>HDL (mmol/l)</td>
<td>1.6±0.4</td>
<td>1.6±0.4</td>
<td>1.6±0.5</td>
<td>NS</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>1.3±0.6</td>
<td>1.2±0.8</td>
<td>1.3±0.7</td>
<td>NS</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>128±18</td>
<td>126±17</td>
<td>132±22</td>
<td>NS</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>70±10</td>
<td>71±10</td>
<td>72±9</td>
<td>NS</td>
</tr>
<tr>
<td>ACR (mg/mmol)</td>
<td>0.4±0.4</td>
<td>0.7±0.9</td>
<td>2.8±4.8</td>
<td>0.03</td>
</tr>
<tr>
<td>(Median)IQR</td>
<td>0.3(0.2-0.4)</td>
<td>0.3(0.2-0.5)</td>
<td>0.7(0.2-2.7)</td>
<td></td>
</tr>
<tr>
<td>eGFR (ml/min/1.73)</td>
<td>85±7</td>
<td>90±3</td>
<td>80±18f</td>
<td>0.006</td>
</tr>
</tbody>
</table>
Post Hoc Analyses

Age: C vs T1DM (NS); C vs DSPN (P=0.0008); T1DM vs DSPN (P<0.0001).

Gender: Chi$^2$ – P=0.02.

Ethnicity: Chi$^2$ – P<0.0001.

HbA1c: C vs T1DM (P<0.0001); C vs DSPN (P<0.0001); T1DM vs DSPN (NS).

T-CHL: C vs T1DM (P=0.006); C vs DSPN (P=0.002); T1DM vs DSPN (NS).

ACR: C vs T1DM (NS); C vs DSPN (P=0.004); T1DM vs DSPN (P=0.03).

eGFR: C vs T1DM (NS); C vs DSPN (NS); T1DM vs DSPN (P=0.006).

Table key

ACR – Albumin Creatinine Ratio; BMI – Body Mass Index; BP – Blood Pressure; C – Controls, estimated Glomerular Filtration Rate; HbA1c – Glycated Haemoglobin A1c; T-CHL – Total Cholesterol; HDL – High Density Lipoprotein Cholesterol.

4.4.2 Symptoms and deficits (Table 4.2)

The NDS was significantly increased in patients with DSPN compared with control subjects (P<0.0001) and T1DM (P<0.0001), with no significant difference between controls and T1DM. The NSP was significantly higher in patients with DSPN (P<0.0001) compared to control subjects and T1DM (P<0.0001). The McGill pain score and McGill VAS were significantly increased in diabetic patients with DSPN compared with control subjects (P=0.001 and P=0.0007 respectively) and patients with T1DM (P=0.01 and P=0.02 respectively). There were no differences between in NDS, NSP, McGill pain score and VAS between controls and T1DM.
Table 4-2 Neuropathy symptoms and deficits in control subjects and diabetic patients without (T1DM) and with neuropathy (DSPN), with statistically significant differences between groups

<table>
<thead>
<tr>
<th></th>
<th>C (n=27)</th>
<th>T1DM (n=30)</th>
<th>DSPN (n=31)</th>
<th>T1DM v DSPN</th>
</tr>
</thead>
<tbody>
<tr>
<td>NDS (-/10)</td>
<td>0.4±0.8</td>
<td>1.2±2.0</td>
<td>4.6±3.3</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>(Median (IQR))</td>
<td>0(0-1)</td>
<td>0(0-2)</td>
<td>5(2-7)</td>
<td></td>
</tr>
<tr>
<td>NSP (-/38)</td>
<td>0.1±0.4</td>
<td>1.3±2.0</td>
<td>5.0±6.2</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>(Median (IQR))</td>
<td>0(0-0)</td>
<td>0(0-2)</td>
<td>2.5(0-6)</td>
<td></td>
</tr>
<tr>
<td>McGill VAS (/-10cm)</td>
<td>0.2±1.0</td>
<td>1.0±2.3</td>
<td>3.3±3.8</td>
<td>0.01</td>
</tr>
<tr>
<td>(Median (IQR))</td>
<td>0(0-0)</td>
<td>0(0-0)</td>
<td>0(0-0)</td>
<td></td>
</tr>
<tr>
<td>McGill Pain score</td>
<td>0.1±0.4</td>
<td>1.9±6.5</td>
<td>4.2±6.5</td>
<td>0.02</td>
</tr>
<tr>
<td>(Median (IQR))</td>
<td>0(0-0)</td>
<td>0(0-0)</td>
<td>2(0-5)</td>
<td></td>
</tr>
</tbody>
</table>

Post Hoc Analyses

NDS: C vs T1DM (NS); C vs DSPN (P<0.0001); T1DM vs DSPN (P<0.0001).

NSP: C vs T1DM (NS); C vs DSPN (P<0.0001); T1DM vs DSPN (P<0.0001).

McGill VAS: C vs T1DM (NS), C vs DSPN (P=0.001); T1DM vs DSPN (P=0.01).

McGill Pain score: C vs T1DM (NS); C vs DSPN (P=0.0007); T1DM vs DSPN (P=0.02).

Table key

C – Controls; McGill VAS – McGill Visual Analogue Score; NDS – Neuropathy Disability Score, NSP – Neuropathy Symptom Profile.
4.4.3 Neuropathy evaluation (Table 4.3)

4.4.3.1 Electrophysiology

Peroneal nerve conduction velocity was significantly lower in DSPN compared to controls (P<0.0001) and T1DM (P<0.0001) and between T1DM compared to controls (P<0.0001). Peroneal nerve amplitude was significantly lower in DSPN compared with T1DM (P<0.0001) and controls (P<0.0001). Sural nerve conduction velocity and amplitude were significantly lower in DSPN (P<0.0001 and P<0.0001 respectively) compared with control subjects and T1DM (P<0.0001 and P<0.0001 respectively). Sural nerve conduction velocity was lower in T1DM compared to controls (P=0.0008). However, values for sural and peroneal nerve conduction velocities and amplitudes were within the normal reference range in T1DM suggesting that there was minimal large fibre deficit in this group.

4.4.3.2 Vibration Perception and Thermal Thresholds

VPT was significantly greater in DSPN compared to T1DM (P<0.0001) and control subjects (P<0.0001). CST was significantly greater in DSPN compared to T1DM (P=0.0007) and control subjects (P<0.0001). WST was significantly greater in DSPN compared to T1DM (P=0.0004) and control (P<0.0001). There were no differences in VPT, CST and WST between controls and T1DM.

4.4.3.3 IENFD and CCM

Figure 4.1 shows skin biopsy specimens with highlighted IENF in controls, T1DM and DSPN. IENFD was significantly reduced in subjects with DSPN compared to T1DM (P=0.001) and control subjects (P<0.0001) and in T1DM compared to controls (P=0.02).
Figure 4.2 shows CCM images with highlighted corneal nerves in controls, T1DM and DSPN from the same subjects as in Figure 4.1. CNFD, CNBD and CNFL were significantly lower in DSPN compared with both T1DM (CNFD: P<0.0001, CNBD: P=0.02 and CNFL: P=0.001) and controls (CNFD: P<0.0001, CNBD: P<0.0001 and CNFL: P<0.0001). These parameters were also significantly reduced in patients with T1DM compared to controls (CNFD: P<0.0001, CNBD: P=0.0008 and CNFL: P<0.0001) suggesting early small fibre damage.

Excluding non-white European subjects from the control group (6 participants) (n=21) made no significant difference to any of the demographic, metabolic or neuropathy parameters. Therefore the control group in this study had no significant confounding effect through ethnicity (Appendix 2, Table 13.2). There were no significant differences when analysing an older control subgroup (Age: 54.4±9.9 years, n=13) which was more closely matched in age to the DSPN group (Age: 53.3±11.9) compared to the overall complete control group. There were no significant differences in demographic, metabolic and neuropathy parameters between the older control subgroup (n=13) and complete control group (n=27). The control group in this study is therefore representative sample which may be appropriately used for comparison to both T1DM and DSPN (see appendix 2, Table 13.3).
Figure 4-1a, b & c. Skin biopsy images of IENF in Controls (A), T1DM (B) and DSPN (C)

Red arrows show intra-epidermal nerve fibres

Figure 4-2a, b & c. CCM images in Controls (A), T1DM (B) and DSPN (C)

Subjects from right to left: Control, T1DM & DSPN

- ➡ Nerve Branches
- ➤ Nerve Fibres
Table 4-3 Small and large fibre tests of nerve structure and function in control subjects and diabetic patients without (T1DM) and with neuropathy (DSPN), with statistically significant differences between groups

<table>
<thead>
<tr>
<th></th>
<th>C (n=27)</th>
<th>T1DM (n=30)</th>
<th>DSPN (n=31)</th>
<th>T1DM v DSPN</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCCA (mBar)</td>
<td>0.5±0.3</td>
<td>0.8±0.7</td>
<td>1.3±2.6</td>
<td>NS</td>
</tr>
<tr>
<td>CNFD (no/mm$^2$)</td>
<td>37.2±5.1</td>
<td>30.1±6.7</td>
<td>19.8±9.2</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>CNBD (no/mm$^2$)</td>
<td>92.0±36.2</td>
<td>60.7±27.9</td>
<td>45.4±32.0</td>
<td>0.02</td>
</tr>
<tr>
<td>CNFL (mm/mm$^2$)</td>
<td>26.6±3.8</td>
<td>21.5±4.8</td>
<td>15.8±7.0</td>
<td>0.001</td>
</tr>
<tr>
<td>IENFD (no/mm)</td>
<td>10.2±3.3</td>
<td>8.3±5.5</td>
<td>4.7±4.3</td>
<td>0.001</td>
</tr>
<tr>
<td>CST (°C)</td>
<td>28.6±2.0</td>
<td>27.5±2.0</td>
<td>21.4±9.1</td>
<td>0.0007</td>
</tr>
<tr>
<td>WST(°C)</td>
<td>36.4±2.0</td>
<td>38.1±3.4</td>
<td>41.8±4.5</td>
<td>0.0004</td>
</tr>
<tr>
<td>VPT (volts)</td>
<td>5.3±4.1</td>
<td>5.6±2.5</td>
<td>18.4±12.2</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>SSNCV (m/s)</td>
<td>50.6±4.2</td>
<td>47.1±4.1</td>
<td>39.4±6.1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>SSN Amp (µV)</td>
<td>20.2±8.8</td>
<td>15.1±6.1</td>
<td>5.5±4.2</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>PMNCV (m/s)</td>
<td>49.2±3.7</td>
<td>45.5±2.2</td>
<td>35.4±8.6</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>PMN Amp (mV)</td>
<td>6.1±2.4</td>
<td>7.3±9.7</td>
<td>2.4±2.1</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>
Post hoc analyses

CNFD: C vs T1DM (P<0.0001); C vs DSPN (P<0.0001); T1DM vs DSPN (P<0.0001).
CNBD: C vs T1DM (P=0.0008); C vs DSPN (P<0.0001); T1DM vs DSPN (P=0.02).
CNFL: C vs T1DM (P<0.0001); C vs DSPN (<0.0001); T1DM vs DSPN (P=0.001).
IENFD: C vs T1DM (P=0.02); C vs DSPN (P<0.0001); T1DM vs DSPN (P=0.001).
CST: C vs T1DM (NS); C vs DSPN (P<0.0001); T1DM vs DSPN (P=0.0007).
WST: C vs T1DM (NS); C vs DSPN (P<0.0001); T1DM vs DSPN (P=0.0004).
VPT: C vs T1DM (NS); C vs DSPN (P<0.0001); T1DM vs DSPN (P<0.0001).
SSNCV: C vs T1DM (P=0.0008); C vs DSPN (P<0.0001); T1DM vs DSPN (P<0.0001).
SNAnmp: C vs T1DM (NS); C vs DSPN (P<0.0001); T1DM vs DSPN (P<0.0001).
PMNCV: C vs T1DM (P<0.0001); C vs DSPN (P<0.0001); T1DM vs DSPN (P<0.0001).
PMNAnmp: C vs T1DM (NS); C vs DSPN (P<0.0001); T1DM vs DSPN (P<0.0001).

Table key

4.4.3.4 Correlates of CCM & IENFD (Table 4)

To explore the relationship of CCM parameters and IENFD with other diagnostic modalities, Spearman’s rank correlations of these measures were undertaken and are highlighted in table 4.4. The strongest correlation was between CNFD and peroneal motor nerve conduction velocity (Rho= 0.60, P<0.0001). Other neurophysiology measures correlated well with CNFD (peroneal motor nerve amplitude: Rho= 0.52 P<0.0001, sural sensory nerve conduction velocity: Rho= 0.52 P<0.0001, sural sensory nerve amplitude: Rho= 0.48 P<0.0001). IENFD correlated less well (peroneal motor nerve conduction velocity Rho= 0.56 P<0.0001, peroneal motor nerve amplitude: Rho= 0.45 P<0.0001, sural sensory nerve conduction velocity: Rho= 0.45 P<0.0001, sural sensory nerve amplitude: Rho= 0.50 P<0.0001). Only a low to moderate correlation (Rho=0.3-0.49) was found between CCM parameters and IENFD (CNFD: Rho= 0.33 P=0.001, CNBD: Rho= 0.33 P=0.003, CNFD: Rho= 0.32 P=0.002). However, both signs (NDS) and in particular symptoms (NSP and McGill VAS) correlated better with CNFD and IENFD.
The strongest correlations are for CCM parameters and IENFD are highlighted in bold.

Table 4-4 Spearman’s rank correlation of CNFD, CNBD, CNFL and IENFD versus NDS, McGill VAS, NSP, IENFD, thermal thresholds, VPT and nerve conduction studies

<table>
<thead>
<tr>
<th></th>
<th>CNFD</th>
<th>CNBD</th>
<th>CNFL</th>
<th>IENFD</th>
</tr>
</thead>
<tbody>
<tr>
<td>NDS (-/10)</td>
<td>Rho = -0.45</td>
<td>Rho = -0.27</td>
<td>Rho = -0.34</td>
<td>Rho = -0.47</td>
</tr>
<tr>
<td></td>
<td>P &lt; 0.0001</td>
<td>P = 0.01</td>
<td>P = 0.001</td>
<td>P &lt; 0.0001</td>
</tr>
<tr>
<td>McGill VAS (-/10cm)</td>
<td>Rho = -0.43</td>
<td>Rho = -0.34</td>
<td>Rho = -0.43</td>
<td>Rho = -0.45</td>
</tr>
<tr>
<td></td>
<td>P &lt; 0.0001</td>
<td>P = 0.002</td>
<td>P &lt; 0.0001</td>
<td>P &lt; 0.0001</td>
</tr>
<tr>
<td>NSP (-/38)</td>
<td>Rho = -0.51</td>
<td>Rho = -0.28</td>
<td>Rho = -0.39</td>
<td>Rho = -0.51</td>
</tr>
<tr>
<td></td>
<td>P &lt; 0.0001</td>
<td>P = 0.009</td>
<td>P = 0.0002</td>
<td>P &lt; 0.0001</td>
</tr>
<tr>
<td>IENFD (no/mm)</td>
<td>Rho = 0.33</td>
<td>Rho = 0.31</td>
<td>Rho = 0.32</td>
<td>Rho = 0.32</td>
</tr>
<tr>
<td></td>
<td>P = 0.001</td>
<td>P = 0.003</td>
<td>P = 0.002</td>
<td>P = 0.002</td>
</tr>
<tr>
<td>CST (°C)</td>
<td>Rho = 0.37</td>
<td>Rho = 0.23</td>
<td>Rho = 0.26</td>
<td>Rho = 0.33</td>
</tr>
<tr>
<td></td>
<td>P = 0.005</td>
<td>P = 0.04</td>
<td>P = 0.02</td>
<td>P = 0.002</td>
</tr>
<tr>
<td>WST(°C)</td>
<td>Rho = -0.39</td>
<td>Rho = -0.35</td>
<td>Rho = -0.33</td>
<td>Rho = -0.52</td>
</tr>
<tr>
<td></td>
<td>P = 0.003</td>
<td>P = 0.009</td>
<td>P = 0.002</td>
<td>P &lt; 0.0001</td>
</tr>
<tr>
<td>VPT (volts)</td>
<td>Rho = -0.49</td>
<td>Rho = -0.31</td>
<td>Rho = -0.37</td>
<td>Rho = -0.47</td>
</tr>
<tr>
<td></td>
<td>P &lt; 0.0001</td>
<td>P = 0.004</td>
<td>P = 0.0004</td>
<td>P &lt; 0.0001</td>
</tr>
<tr>
<td>SSNCV (m/s)</td>
<td>Rho = 0.52</td>
<td>Rho = 0.40</td>
<td>Rho = 0.43</td>
<td>Rho = 0.45</td>
</tr>
<tr>
<td></td>
<td>P &lt; 0.0001</td>
<td>P = 0.0002</td>
<td>P = 0.0001</td>
<td>P &lt; 0.0001</td>
</tr>
<tr>
<td>SSNAmplitude (µV)</td>
<td>Rho = 0.48</td>
<td>Rho = 0.28</td>
<td>Rho = 0.34</td>
<td>Rho = 0.50</td>
</tr>
<tr>
<td></td>
<td>P &lt; 0.0001</td>
<td>P = 0.01</td>
<td>P = 0.002</td>
<td>P &lt; 0.0001</td>
</tr>
<tr>
<td>PMNCV (m/s)</td>
<td>Rho = 0.60</td>
<td>Rho = 0.46</td>
<td>Rho = 0.54</td>
<td>Rho = 0.46</td>
</tr>
<tr>
<td></td>
<td>P &lt; 0.0001</td>
<td>P &lt; 0.0001</td>
<td>P &lt; 0.0001</td>
<td>P &lt; 0.0001</td>
</tr>
<tr>
<td>PMNAmplitude (mV)</td>
<td>Rho = 0.52</td>
<td>Rho = 0.40</td>
<td>Rho = 0.52</td>
<td>Rho = 0.45</td>
</tr>
<tr>
<td></td>
<td>P &lt; 0.0001</td>
<td>P = 0.0002</td>
<td>P &lt; 0.0001</td>
<td>P &lt; 0.0001</td>
</tr>
</tbody>
</table>
4.4.3.5 Receiver-Operating Characteristic (ROC) analysis (Table 4.5, Figure 4.3)

To assess the diagnostic ability of small and large fibre tests including optimal cut-offs, sensitivity and specificity ROC analysis was undertaken for all measures of neuropathy. As the delineation of T1DM from DSPN was based on the nerve conduction studies (Toronto criteria for definite diabetic neuropathy (3)) we have used the ROC analysis for peroneal motor nerve conduction velocity as the referent value for Wilcoxon estimate of area under ROC curve = 0.98 (95% CI: 0.49 – 1), sensitivity = 0.94 (95% CI: 0.79 - 0.99) and specificity = 1 (95% CI: 0.88 – 1). The Wilcoxon estimate of area under the ROC curve was greatest for VPT at 0.85 with an optimal cut off of 13 volts, sensitivity of 0.67, specificity of 1, PPV of 1.0 and NPV 0.75. The small fibre test with the greatest Wilcoxon estimate of area under the ROC curve was CNFD at 0.81 (Figure 4.3), which was superior to IENFD (Figure 4.3). The PPV and NPV for CNFD were 0.8 and 0.77 respectively for a cut off of 25 fibres/mm. IENFD had a lower Wilcoxon estimate of area under the ROC curve at 0.73, which was similar to CNFL (0.74), CST (0.76) and WST (0.74). The PPV and NPV were also lower for IENFD at 0.76 and 0.67 respectively for a cut off of 4.5 fibres/mm.
Table 4-5 ROC analysis with area under the curve, optimal cut off and respective sensitivity and specificity with 95% confidence interval in T1DM versus DSPN for CNFD, CNBD, CNFL, IENFD, VPT, CST and WST

<table>
<thead>
<tr>
<th></th>
<th>Optimal Cut off</th>
<th>AUC (95% CI)</th>
<th>Sensitivity (95% CI)</th>
<th>Specificity (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNFD (no/mm²)</td>
<td>25.0</td>
<td>0.81 (0.47 - 1.0)</td>
<td>0.77 (0.59 - 0.90)</td>
<td>0.79 (0.60 - 0.92)</td>
</tr>
<tr>
<td>CNBD (no/mm²)</td>
<td>36.5</td>
<td>0.67 (0.45 - 0.90)</td>
<td>0.58 (0.39 - 0.75)</td>
<td>0.79 (0.60 - 0.92)</td>
</tr>
<tr>
<td>CNFL (mm/mm²)</td>
<td>16.8</td>
<td>0.74 (0.46 - 1.0)</td>
<td>0.61 (0.42 - 0.78)</td>
<td>0.86 (0.68 - 0.96)</td>
</tr>
<tr>
<td>IENFD (no/mm)</td>
<td>4.5</td>
<td>0.73 (0.46 - 1.0)</td>
<td>0.61 (0.42 - 0.78)</td>
<td>0.80 (0.61 - 0.92)</td>
</tr>
<tr>
<td>VPT (Volts)</td>
<td>13</td>
<td>0.85 (0.74 - 0.95)</td>
<td>0.67 (0.47 - 0.83)</td>
<td>1.0 (0.88 - 1.0)</td>
</tr>
<tr>
<td>CST (°C)</td>
<td>24.7</td>
<td>0.76 (0.46 - 1.0)</td>
<td>0.57 (0.37 - 0.76)</td>
<td>0.89 (0.72 - 0.98)</td>
</tr>
<tr>
<td>WST (°C)</td>
<td>38</td>
<td>0.74 (0.61 - 0.88)</td>
<td>0.86 (0.67 - 0.96)</td>
<td>0.64 (0.44 - 0.81)</td>
</tr>
</tbody>
</table>

Table key
CNFD – Corneal Nerve Fibre Density; CNBD – Corneal Nerve Branch Density; CNFL – Corneal Nerve Fibre Length; CST – Cold Sensation Threshold; IENFD – Intra Epidermal Nerve Fibre Density; VPT – Vibration Perception Threshold; WST – Warm Sensation Threshold.
Figure 4-3 Receiver-operated characteristic (ROC) curves, based on the analysis of CNFD and IENFD in T1DM versus DSPN

Black line – CNFD
Red line – IENFD
4.5 DISCUSSION

There is a need for surrogate end points of diabetic neuropathy, which accurately detect early disease, quantify disease progression and measure therapeutic response (29). The current ‘gold’ standard for the diagnosis of neuropathy is neurophysiology, a robust measure that also predicts foot ulceration and mortality in diabetes (30). Other measures of neuropathy such as neurological assessment are poorly reproducible (2), QST is subjective and more accurate measures such as skin and nerve biopsy are invasive and require specialist analysis (3). Small fibre neuropathy has direct pathophysiological relevance to the main outcomes of pain and foot ulceration (7) and therefore skin biopsy assessment of IENF has been proposed as a valid measure of diabetic neuropathy (8). Whilst skin biopsy detects early small nerve fibre damage even when electrophysiology and QST are still normal (4-5), the use of this test in clinical trials is limited by its invasive nature. CCM is a novel, rapid and readily reiterative technique, which quantifies small nerve fibres non-invasively and shows promise (17-18; 31-34). The major findings of this study which address major questions in relation to the diagnostic ability of different small fibre tests in diabetic neuropathy are: 1) early subclinical small nerve fibre decline can be detected by CCM and IENFD; 2) CNFD has higher diagnostic utility than IENFD in the diagnosis of DPN; 3) CNFD correlates better with nerve conduction studies than IENFD.

Early intervention with improved glycaemic control in type 1 DM can lead to a durable reduction in DPN (35-36). Furthermore, the need for early evaluation of subclinical small fibre neuropathy has been demonstrated by Smith et al (6) where lifestyle intervention with diet and exercise in a pre-diabetic neuropathy group lead to cutaneous re-innervation and improved pain. Previous studies have employed
ROC curve analysis of IENFD at the distal leg and shown a specificity of 95%-97% and sensitivity of 45%-80% (15; 37) for small fibre neuropathy but these studies were not specifically in patients with DPN. Recently Nebuchennykh et al (13) in a study of patients with polyneuropathy from varying causes showed a sensitivity of 35% and specificity of 95% using a cut off point of 6.7 fibres/mm for IENFD. In another study by Vlckova-Moravcova et al (12), the diagnostic sensitivity for detecting neuropathy was 80% and the specificity was 82% with an optimal IENFD cut off point of ≤8.8 fibres/mm. Although, ROC curve analysis is a standard and appropriate method for establishing diagnostic validity these studies were flawed as they assessed a disease group against a healthy control population and thus sensitivities and specificities will be inappropriately high and the delineation of the optimal cut off points inaccurate. The need to identify DPN in diabetic subjects should mean that optimal cut off points for neuropathy should be based on data from a population with diabetes with and without neuropathy rather than a healthy control population versus DPN. Therefore we have performed the present study in a population of diabetic patients with and without neuropathy using the robust Toronto criteria and utilised a best fit ROC curve analysis (38-39) to derive optimal cut off points, sensitivities, and specificities to assess the diagnostic validity of CCM measures and IENFD. ROC curve analysis in this study showed that IENFD had a sensitivity of 61% and specificity of 80% at an optimal cut off point of 4.5 fibres/mm. These diagnostic validity measures are clearly lower than in the current published literature (12-14), but we believe are more representative. Using exactly the same population and methods we show that CNFD has a better sensitivity of 77% and an almost identical specificity of 79% at an optimal cut off point of 25.0 fibres/mm.
Previous studies of IENFD have found either absent or weak correlations with nerve conduction studies (12) and sural nerve action potentials (15; 40-41). In the present study CNFD showed a superior correlation than IENFD with peroneal motor nerve conduction velocity and amplitude and sural sensory nerve conduction velocity. These data support the study of Shun et al (14) which showed a significant correlation between IENFD with sural nerve action potential and warm sensation threshold. Thermal thresholds continue to an important psychophysical test in evaluating small nerve fibres and our data show that the strongest correlation was indeed between IENFD and WST. For DPN, thermal thresholds have previously shown a sensitivity which ranges from 36%-85% (6; 42-43). Sensitivities reported in our study are 57% and 86% respectively for CST and WST. Furthermore, we have shown negative correlations of WST with CCM measures and IENFD confirming a previous study (42). Interestingly CNBD, CNFD and CNFL correlated with IENFD but the association was a low one (Spearman’s Rho 0.33, 0.31 and 0.32 respectively). Although both CCM and skin biopsy measure small nerve fibres, the sites of assessment are anatomically distinct. There are no reported significant variations of IENFD calculated in adjacent sections from the same biopsy or in adjacent biopsies from the same site (15). However, even with IENFD differences in mean values exist between differing sites on the lower limb (15; 44) although no direct correlations were assessed between sites. Although DPN is considered a length dependent neuropathy, recent studies suggest that lesions may occur in a proximal (45) multifocal fascicular pattern (46). IENFD correlates somewhat better with NDS, McGill VAS and NSP than CNFD. Clinical measures of neuropathy in part directly assess distal symptoms which would be in keeping with distal measures such as IENFD. We suggest that CCM
measures are similar to IENFD despite assessing a distinct clinical site than typically assessed through clinical measures of diabetic neuropathy. VPT had the greatest AUC and specificity but this reflects the method of delineation of neuropathy through the Toronto criteria which uses electrophysiology with signs and/or symptoms. Both VPT and electrophysiology are large fibre measures and therefore the ability of VPT as a diagnostic measure for diabetic neuropathy to be high particularly as the groups were divided based on electrophysiology. However, CNFD was still superior to IENFD despite the delineation of the groups with a large fibre test.

The current study provides the first direct comparison between IENFD and CCM in the assessment DPN. Both CNFD and IENFD correlated with clinical signs (NDS), and symptoms (McGill VAS and NSP). Furthermore, CNFD had better diagnostic utility for DPN through ROC curve analyses and correlated better with electrophysiology than the current ‘gold’ standard of IENFD.
4.6 REFERENCES


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Curr Diab Rep 2010;10:276-282


5 Chapter V - Enhanced Small Fibre Neuropathy in Patients with Latent Autoimmune Diabetes in Adults

**Contribution:** Uazman Alam contributed to the conception and design of the study and made a major contribution to the recruitment of subjects, clinical tests including neuropathy assessments and skin biopsies, skin biopsy analyses, all statistical analyses and writing of this chapter.

**To be submitted for publication**

**Uazman Alam**, Maria Jeziorska, Ioannis N Petropoulos, Omar Asghar, Hassan Fadavi, Georgios Ponirakis, Andrew Marshall, Mitra Tavakoli, Andrew JM Boulton, Nathan Efron, Rayaz A Malik
5.1 ABSTRACT

Latent Autoimmune Diabetes in Adults (LADA) is often misdiagnosed and patients have relatively poorer glycaemic control with a potential for an increased risk of neuropathy. Surprisingly, few studies have been undertaken in these patients in relation to the prevalence of neuropathy.

Subjects with LADA (LADA) (n=19), type 2 DM (T2DM) (n=22) and non-diabetic healthy control subjects (C) (n=33) underwent detailed assessment of neurologic deficits, quantitative sensory testing (QST), electrophysiology, skin biopsy and corneal confocal microscopy (CCM).

There was no significant difference for age, duration of diabetes, lipids and blood pressure, but HbA1c was higher (P<0.0001) and the BMI was lower (P=0.008) in patients with LADA compared with T2DM. There was no significant difference in the neuropathy disability score (NDS), vibration perception threshold (VPT) or electrophysiology between LADA and T2DM. However, cold sensation threshold (CST) (P=0.008), and warm sensation threshold (WST) (P=0.008), were significantly higher and intra-epidermal nerve fibre density (IENFD) (P=0.02), corneal nerve fibre density (CNFD) (P=0.03), corneal nerve fibre length (CNFL), (P=0.02), and corneal nerve branch density (CNBD) (P=0.003) were significantly lower in LADA compared to T2DM.

Despite, comparable age, duration of diabetes and cardiovascular risk factors, subjects with LADA have a significant small fibre neuropathy compared to matched patients with T2DM.
5.2 INTRODUCTION

LADA is often misdiagnosed as type 2 diabetes mellitus (DM), but pathophysiologically is more closely related to type 1 DM. (1). Hence, these patients present at a younger age due to a slow autoimmune destruction of pancreatic β-cells (2-5). The prevalence of LADA in patients with type 2 DM (T2DM) ranges from a minimum of 4% (6-7) to 10% as shown in the UKPDS (3). Thus patients with LADA represent a significant proportion of patients with a faster decline in C-peptide (6) and delayed treatment with insulin resulting in a significant period of poor glycaemic control (8). Given, that early and optimal treatment of glycaemia reduces the incidence of neuropathy in Type 1 DM as shown by the DCCT and EDIC studies (9-10) then patients with LADA with poorer glycaemic control may well have an increased risk of developing the long term complications (11-12).

However, the long term complications in patients with LADA, in particular diabetic somatic polyneuropathy (DSPN) have been poorly studied. Hence, surprisingly individuals with LADA had a lower prevalence of diabetic neuropathy assessed using electrophysiology, quantitative sensory testing and autonomic function, despite having a HbA1c which was 1.1% (~12mmol/mol) higher when compared to patients with T2DM (13). Yet in another study the prevalence of neuropathy defined using the Michigan Neuropathy screening instrument was higher in the LADA group (~38%) when compared to T2DM (~30%) (14). Whilst there was no difference for neuropathy in LADA compared to Type 2 DM when assessed using the Neuropathy Disability score (NDS) and Vibration Perception Threshold (VPT) (15). These data reflect no clear consensus on the prevalence of DSPN in patients with LADA and raise the important issue of the sensitivity of different tests to
diagnose diabetic neuropathy. Intra-epidermal nerve fibre density (IENFD) is significantly reduced in patients with normal electrophysiology suggesting early damage to small nerve fibres (16). Similarly, corneal confocal microscopy (CCM) is a non-invasive technique that can detect early small sensory nerve fibre loss (17) and repair on normalisation of glycaemia after pancreas transplantation in Type 1 diabetes (18). Therefore the assessment of small fibres may be the most sensitive means to detect early neuropathy in diabetes (19).

We have undertaken a comprehensive study phenotyping diabetic neuropathy in subjects with LADA to identify large and small fibre neuropathy compared to matched patients with Type 2 DM.
5.3  RSEARCH DESIGN AND METHODS

5.3.1  Selection of patients

19 subjects with LADA, 22 subjects with T2DM and 33 age and sex matched non-diabetic control subjects were studied. Subjects with a history of neurologic conditions, ocular trauma or previous ocular surgery were excluded. The study was approved by the North West and Salford and Trafford Research Ethics committee, and written informed consent was obtained according to the Declaration of Helsinki.

5.3.2  LADA definition

LADA was defined as follows: age of onset less than 50 years, anti-glutamic decarboxylase (GAD) antibodies (GAD-Ab) positivity, exclusion of patients with ketonuria/diabetic ketoacidosis at diagnosis and a lack of insulin treatment within the first six months after diagnosis of diabetes mellitus, suggesting type 1 DM (1). All subjects with LADA had elevated positive GAD-Ab titres which were consistent with autoimmune diabetes. All subjects with T2DM had a clinical LADA screening score of 0 or 1 and thus low risk of autoimmune diabetes as defined in the study by Fourlanos et al (20).

5.3.3  Assessment of neuropathy

All patients and control subjects underwent a detailed evaluation of neurologic symptoms according to the neuropathy symptom profile (NSP), and the McGill visual analogue score (McGill VAS) VAS was used to assess the severity of painful neuropathy. Clinical neurologic deficits were assessed using the modified neuropathy disability score, which includes evaluation of vibration, pin prick, and
temperature perception as well as the presence or absence of ankle reflexes to establish the severity of neuropathy (21). Quantitative sensory testing (QST) included an assessment of vibration perception threshold (VPT), measured using a Neurothesiometer (Horwell, Scientific Laboratory Supplies, Wilford, Nottingham, U.K.), cold sensation threshold (CST) (Aδ fibres) and warm sensation threshold (WST) (22) (c fibres) thresholds using the method of limits with the MEDOC TSA II (Medoc, Ramat Yishai, Israel) on the dorsum of the left foot. Electro-diagnostic studies were undertaken using a Dantec “Keypoint” system (Dantec Dynamics, Bristol, U.K.) equipped with a DISA temperature regulator to keep limb temperature constantly between 32°C and 35°C. Peroneal motor and sural sensory nerves were assessed in the right lower limb by a consultant neurophysiologist. The motor study was performed using silver-silver chloride surface electrodes at standardized sites defined by anatomical landmarks, and recordings for the sural nerve were taken using antidromic stimulation over a distance of 100 mm.

5.3.4 Corneal Confocal Microscopy

Patients underwent examination with the Heidelberg retina tomography III in vivo corneal confocal microscope employing our established methodology for image acquisition (23). Several scans of the entire depth of the cornea were recorded by turning the fine focus of the objective lens backward and forward for ~2 min using the section mode, which enables manual acquisition and storage of single images of all corneal layers. This provides en face two-dimensional images with a lateral resolution of ~2 mm/pixel and final image size of 400 x 400 pixels of the subbasal nerve plexus of the cornea from each patient and control subject. Each subbasal nerve fibre bundle contains unmyelinated fibres, which run parallel to Bowman's
layer before dividing and terminating as individual axons underneath the surface epithelium. Five images per patient from the centre of the cornea were selected and examined in a masked and randomized fashion (24). Four corneal nerve parameters were quantified: 1) CNFD, the total number of major nerves per square millimetre of corneal tissue (no. mm$^2$); 2) CNBD, the number of branches emanating from all major nerve trunks per square millimetre of corneal tissue (no. mm$^2$); 3) CNFL, the total length of all nerve fibres and branches (mm/mm$^2$) within the area of corneal tissue and 4) corneal nerve fibre tortuosity (CNFT), a parameter mathematically derived from the images (25).

5.3.5 Skin biopsy and immunohistochemistry

A sub-cohort of participants (Controls n=19, T2DM n=12, and LADA n=9) underwent a 3-mm punch skin biopsy from the dorsum of the foot; 2 cm above the second metatarsal head after local anaesthesia (1% lidocaine). The biopsy site was closed using Steristrips, and the specimen was immediately fixed in PBS-buffered 4% paraformaldehyde. After 18–24 hours, it was rinsed in Tris-buffered saline and soaked in 33% sucrose (2–4 hours) for cryoprotection. It was then embedded in optimal cutting temperature–embedding compound, rapidly frozen in liquid nitrogen, and cut into 50-μm sections using a cryostat (model OTF; Bright Instruments, Huntington, U.K.). Four floating sections per subject underwent melanin bleaching (0.25% KMnO$_4$ for 15 min followed by 5% oxalic acid for 3 min), a 4-h protein block with a Tris-buffered saline solution of 5% normal swine serum, 0.5% powdered milk, and 1% Triton X-100, and overnight incubation with 1:1,200 Biogenesis polyclonal rabbit anti-human PGP9.5 antibody (Serotec, Oxford, U.K.). Biotinylated swine anti-rabbit secondary antibody (1:300; DakoCytomation, Ely,
U.K.) was then applied for 1 hour; sections were quenched with 1% H$_2$O$_2$ in 30% MeOH-PBS (30 min) prior to a 1 hour incubation with 1:500 horseradish peroxidase–streptavidin (Vector Laboratories, Peterborough, U.K.). Nerve fibres were demonstrated using 3, 3-diaminobenzidine chromogen (Sigma-Aldrich, Manchester, U.K.). Sections were mildly counterstained with eosin to better localize the basement membrane to identify nerve fibres passing through it. Negative control subjects consisted of replacing the anti- PGP9.5 antibody with rabbit immunoglobulin (DakoCytomation) at a concentration matching that of the primary antibody, which showed no immunostaining. IENFD, i.e., the number of fibres passing through the dermo-epidermal junction per millimetre of basement membrane was quantified in accord with established criteria and techniques and expressed as number per millimetre (26).

5.3.6 Statistical analysis

Statistical analyses were undertaken on Statsdirect (Statsdirect, Cheshire, UK). The data are expressed as Mean ± standard deviation (SD). ANOVA method or a non-parametric counterpart, Kruskal-Wallis were used to assess differences between groups depending on normality of the data. The Mann-Whitney U test was used to compare T2DM with LADA for the duration of diabetes. Chi squared analyses were used to assess frequencies of gender, ethnicity and aetiology of diabetes. Overall the P value was maintained at 0.05 for multiple comparison tests (Bonferoni adjustment or Conover-Inmann pairwise comparison).
5.4 RESULTS

5.4.1 Demographics and Clinical Neuropathy (Table 5.1)

The participant demographics and clinical neuropathy measurements in subjects with diabetes and age-matched control subjects are summarized in Table 5.1. There were no significant differences in age (C: 53.3±9.8 vs T2DM: 54.9±6.8 vs LADA: 50.6±11.9 years, P=NS) and duration of diabetes (T2DM: 9.8±5.4 vs LADA: 11.4±10.2 years, P=NS). There were no differences between the groups in their ethnic mix. The NDS was significantly greater in patients with T2DM (P=0.02) and LADA (P=0.0003) compared to control subjects, consistent with a mild neuropathy, but did not differ between T2DM and LADA. The NSP was significantly higher in T2DM and LADA (P<0.0001 and P=0.0002 respectively) with no differences in the McGill VAS compared to controls. There were no significant difference in the degree of neuropathic pain in T2DM (Median (IQR) (0.5(0-4)) compared to LADA (0(0-0) (P=NS)).
Table 5-1 Participant demographics in Controls, T2DM and LADA

<table>
<thead>
<tr>
<th></th>
<th>C (n=20)</th>
<th>T2DM (n=17)</th>
<th>LADA (n=19)</th>
<th>T2DM v LADA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (years)</strong></td>
<td>53.3±9.8</td>
<td>54.9±6.8</td>
<td>50.6±11.9</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Gender (Male) (%)</strong></td>
<td>65</td>
<td>76</td>
<td>58</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Ethnicity (White European) (%)</strong></td>
<td>60</td>
<td>59</td>
<td>58</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Duration of Diabetes (years)</strong></td>
<td>-</td>
<td>9.8±5.4</td>
<td>11.4±10.2</td>
<td>NS</td>
</tr>
<tr>
<td><strong>NDS (~/10)</strong></td>
<td>0.5±1.0</td>
<td>2.1±1.9*</td>
<td>3.9±3.6**</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Median (IQR)</strong></td>
<td>0(0-1)</td>
<td>1(0-4)</td>
<td>3(1-6)</td>
<td></td>
</tr>
<tr>
<td><strong>NSP (~/38)</strong></td>
<td>0.1±0.3</td>
<td>4.5±5.6^</td>
<td>4.4±6.0^^</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Median (IQR)</strong></td>
<td>0(0-0)</td>
<td>2(1-4)</td>
<td>2.5(0-6)</td>
<td></td>
</tr>
<tr>
<td><strong>McGill VAS (~/10cm)</strong></td>
<td>0.6±1.8</td>
<td>1.9±2.5</td>
<td>0.5±1.2</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Median (IQR)</strong></td>
<td>0(0-0)</td>
<td>0.5(0-4)</td>
<td>0(0-0)</td>
<td></td>
</tr>
</tbody>
</table>

**Post hoc analyses**

NDS  *C vs T2DM – P=0.02;  **C vs LADA– P=0.0003.

NSP  ^C vs T2DM – P<0.0001;  ^^C vs LADA – P=0.0002.

McGill VAS  C vs T2DM – P=NS;  C vs LADA – P=NS.

**Table Key**

IQR – Inter quartile Range; McGill VAS – McGill Visual Analogue Score; NDS – Neuropathy Disability Score; NSP – Neuropathy Symptom Profile.
5.4.2 Metabolic and Anthropometric measurements (Table 5.2)

Metabolic and anthropometric measurements are detailed in Table 5.2. HbA1c was significantly higher in subjects with LADA compared to T2DM and controls (P<0.0001). BMI was significantly higher in T2DM subjects (P=0.001 and P=0.0005 respectively) compared to subjects with LADA and control subjects. The total cholesterol was significantly lower (P<0.0001) in diabetic patients due to greater statin use with no difference between the patients with T2DM and LADA. HDL was significantly lower in T2DM compared to controls (P=0.002) and subjects with LADA (P=0.03). There were no differences between blood pressure either systolic or diastolic in any group. There were no differences in the estimated glomerular filtration rate (eGFR) between diabetic subjects and controls.
Table 5-2 Clinical and metabolic parameters in control subjects and patients with T2DM and LADA

<table>
<thead>
<tr>
<th></th>
<th>C (n=20)</th>
<th>T2DM (n=17)</th>
<th>LADA (n=19)</th>
<th>T2DM v LADA</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HbA1c (%)</td>
<td>5.7±0.4</td>
<td>7.0±0.7*</td>
<td>9.8±2.3**</td>
<td></td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>HbA1c (mmol/mol)</td>
<td>38.4±4.2</td>
<td>52.8±7.4*</td>
<td>83.4±24.7**</td>
<td></td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>BMI (kg/m2)</td>
<td>27.5±4.5</td>
<td>32.8±4.6†</td>
<td>27.1±4.0</td>
<td></td>
<td>0.001</td>
</tr>
<tr>
<td>T-CHL (mmol/l)</td>
<td>5.5±0.7</td>
<td>4.1±1.3††</td>
<td>4.5±1.3††</td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>HDL (mmol/l)</td>
<td>1.6±0.4</td>
<td>1.1±0.4˚</td>
<td>1.4±0.4</td>
<td></td>
<td>0.03</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>1.5±0.6</td>
<td>2.0±1.3</td>
<td>1.6±1.2</td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>133±16</td>
<td>137±25</td>
<td>137±22</td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>76±10</td>
<td>79±11</td>
<td>76±11</td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>eGFR (ml/min/1.73)</td>
<td>80±9</td>
<td>79±16</td>
<td>82±13</td>
<td></td>
<td>NS</td>
</tr>
</tbody>
</table>

Post hoc analyses

HbA1c: *C vs T2DM – P<0.0001; **C vs LADA – P<0.0001.
BMI: †C vs T2DM – P=0.0005; C vs LADA – P=NS.
T-CHL: ††C vs T2DM – P<0.0001; †††C vs LADA – P=0.003.

Table key

ACR – Albumin Creatinine Ratio; BMI – Body Mass Index; BP – Blood Pressure; estimated Glomerular Filtration Rate; HbA1c – Glycated Haemoglobin A1c; HDL – High Density Lipoprotein Cholesterol; T-CHL – Total Cholesterol.
5.4.3 Neuropathy evaluation (Table 5.3)

5.4.3.1 Electrophysiology

Peroneal motor nerve conduction velocity was significantly lower in T2DM (P=0.004) and LADA (P=0.0001) compared with control subjects with no difference between the T2DM and LADA groups. Peroneal motor nerve amplitude was significantly lower in T2DM (P=0.01) and LADA (P=0.002) compared with control subjects. Sural sensory nerve conduction velocity and amplitude were significantly lower in LADA (P=0.01 and P=0.03 respectively) with no difference in T2DM compared with control subjects with no difference between T2DM and LADA groups. However, values for sural sensory and peroneal motor nerve conduction velocities and amplitudes were within the normal reference range for T2DM and LADA groups suggesting that there was minimal large fibre deficit.

5.4.3.2 Vibration Perception, Thermal Thresholds & Neuropad

VPT was significantly greater in T2DM (P=0.009) and LADA (P=0.004) compared with control subjects with no difference between T2DM and LADA. CST was significantly lower in T2DM (P=0.002) and LADA (P<0.0001) compared with control subjects and was significantly lower in LADA (P=0.03) compared to T2DM. WST was significantly greater in T2DM (P<0.0001) and LADA (P<0.0001) compared with control subjects and was significantly greater in LADA (P=0.02) compared to T2DM. There were no differences between controls, T2DM and LADA in the neuropad assessment.
5.4.3.3 IENFD and CCM

Figure 5.1 shows skin biopsy specimens with highlighted IENF in controls, LADA and T2DM (A, B and C respectively). IENFD was significantly reduced in subjects with LADA compared to control subjects and T2DM (P=0.001 and P=0.03 respectively) with no difference between controls and T2DM. Those undergoing skin biopsy were representative of the overall cohorts in terms of age, ethnicity, gender and duration of diabetes. There were no significant differences in age and duration of diabetes between those subjects who had skin biopsy (controls vs T2DM vs LADA).

Figure 5-1 Skin biopsies stained for PGP9.5. Healthy control (A) shows numerous long intra-epidermal nerve fibres and a well-developed sub-epidermal nerve plexus, compared with short intra-epidermal nerves and less pronounced sub-epidermal plexus in a LADA patient (B) and comparable sub-epidermal plexus but more numerous intra-epidermal nerves in a T2DM patient with neuropathy (C). (Bar = 100 µm).
Figure 5.2 shows CCM images with highlighted corneal nerves in controls, LADA and T2DM (A, B and C respectively). CNFD was significantly reduced in both T2DM (P=0.003) and LADA (P=0.001) compared with control subjects. CNBD (P=0.0008) and CNFL (P=0.0005) were significantly lower in LADA with no significant difference in T2DM compared to controls. CCM parameters were further significantly reduced in patients with LADA compared to T2DM (CNFD (P=0.03), and CNFL (P=0.01)).

Figure 5-2 Healthy control (A) shows numerous corneal nerve fibres and branches compared with much fewer and less pronounced subbasal nerve plexus in a LADA patient (B) and comparable subepidermal plexus but greater numbers of corneal nerves and branches in a T2DM patient with neuropathy (C).

Control (A) vs LADA (B) vs Mild Neuropathy (C)
Table 5-3 Small and large fibre neuropathy tests in control subjects, T2DM and LADA

<table>
<thead>
<tr>
<th></th>
<th>C (n=20)</th>
<th>T2DM (n=17)</th>
<th>LADA (n=19)</th>
<th>T2DM v LADA</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCCA (mBar)</td>
<td>0.7±0.8</td>
<td>1.0±0.8</td>
<td>1.0±1.2</td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td>CNFD (no/mm²)</td>
<td>36.7±5.1</td>
<td>30.5±11.25*</td>
<td>24.2±5.3**</td>
<td>P=0.03</td>
<td></td>
</tr>
<tr>
<td>CNBD (no/mm²)</td>
<td>93.4±37.1</td>
<td>70.1±29.4</td>
<td>60.4±29.3††</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>CNFL (mm/mm²)</td>
<td>26.1±5.3</td>
<td>24.7±6.9</td>
<td>19.9±4.8¥¥</td>
<td>P=0.01</td>
<td></td>
</tr>
<tr>
<td>CNFT</td>
<td>16.0±3.7</td>
<td>19.7±4.9*</td>
<td>19.5±5.3**</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>IENFD (no/mm)</td>
<td>9.9±3.2</td>
<td>7.4±4.4</td>
<td>3.6±3.0††</td>
<td>P=0.03</td>
<td></td>
</tr>
<tr>
<td>CST (°C)</td>
<td>28.6±2.0</td>
<td>26.0±3.1*</td>
<td>22.6±6.9***</td>
<td>P=0.03</td>
<td></td>
</tr>
<tr>
<td>WST(°C)</td>
<td>36.8±2.6</td>
<td>41.3±3.4*</td>
<td>43.9±3.6†††</td>
<td>P=0.02</td>
<td></td>
</tr>
<tr>
<td>Neuropad (%)</td>
<td>87±20</td>
<td>79±33</td>
<td>68±35</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>VPT (volts)</td>
<td>6.0±3.5</td>
<td>10.5±6.9††</td>
<td>13.5±11.8***</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>SSNCV (m/s)</td>
<td>49.6±4.0</td>
<td>46.6±5.7</td>
<td>45.2±6.4*:</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>SSNAmp (µV)</td>
<td>17.8±8.2</td>
<td>12.6±8.2</td>
<td>10.8±6.0€</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>PMNCV (m/s)</td>
<td>47.8±3.6</td>
<td>42.5±7.4*</td>
<td>40.9±7.2**:</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>PMNAmp (mV)</td>
<td>6.1±1.9</td>
<td>4.2±2.1§</td>
<td>3.8±2.8§§</td>
<td>NS</td>
<td></td>
</tr>
</tbody>
</table>

Post hoc analyses

CNFD: °C vs T2DM – P=0.003; °°C vs LADA – P=0.001.
CNBD: C vs T2DM – P=NS; ††C vs LADA – P=0.0008.
CNFL: C vs T2DM – P=NS; ¥¥C vs LADA – P=0.0005.
CNFT: °C vs T2DM – P=NS; °°C vs LADA – P=NS.
IENFD: C vs T2DM – P=NS; ‡‡C vs LADA – P=0.001.
CST: °°C vs T2DM – P=0.002; °°°C vs LADA – P<0.0001.
WST: °°C vs T2DM – P<0.0001; °°°C vs LADA – P<0.0001.
VPT: °°C vs T2DM – P=0.009; °°°C vs LADA – P=0.004.
SSNCV: C vs T2DM – P=NS; °C vs LADA – P=0.01.
SSNAmp: C vs T2DM – P=NS; °C vs LADA – P=0.03.
PMNCV: °C vs T2DM – P=0.004; °°C vs LADA – P=0.0001.
PMNamp: §C vs T2DM – P=0.01, §§C vs LADA – P=0.002.

**Table Key**

5.5 DISCUSSION

LADA is a slowly progressive form of autoimmune diabetes which is often misclassified as type 2 diabetes and therefore results in a protracted period of relatively poor glycaemic control due to a delay in the introduction of insulin (1). Longitudinal studies in patients with LADA have found a relatively rapid decline in insulin secretion in the first few years after diagnosis of diabetes (27-28). In a recent study, individuals with LADA had worse glycaemic control than patients with type 2 diabetes despite a longer time on insulin therapy (29). Two small studies have shown beneficial effects on β-cell reserve of early insulin therapy in patients with LADA (27; 30). Our LADA cohort also had a greater degree of hyperglycaemia reflecting this late recognition and late introduction of insulin.

Given that poorer glycaemic control is associated with an increased risk for the development of neuropathy in both type 1 (31-32) and type 2 diabetes (19; 33), patients with LADA may therefore have a greater propensity for the development of neuropathy (29). However, in subjects with LADA there are limited studies (13-15) which have shown a varying severity of neuropathy (6; 13; 34). In a recent study current perception thresholds and electrophysiology were found to be similar in patients with LADA and type 1 and 2 diabetes (6). In the few other studies which have been undertaken, the assessment of neuropathy was crude as symptoms and clinical neurological deficits or vibration perception were assessed (14-15), groups were not age matched. Given that small nerve fibre damage precedes large fibre deficits (17; 35-37), and no direct assessment of small fibre function or structure was undertaken (13; 15), neuropathy may not have been detected.

Thus in the present study we show that subjects with both T2DM and LADA have an abnormal NDS, VPT and electrophysiology. However, patients with LADA have
comparable measures of large fibre neuropathy to subjects with T2DM. Cold and warm thermal thresholds were increased, and both IENFD and corneal nerve parameters were reduced in patients with T2DM and LADA. However, these small fibre parameters were further significantly reduced in patients with LADA compared to patients with T2DM. This relative more advanced pathology of small fibres may explain the discrepant findings in previous studies where patients with LADA have been shown to have either no abnormality or indeed mild comparable neuropathy to patients with T2DM. However, despite less small fibre neuropathy in T2DM compared to LADA there was no difference in levels of neuropathic pain as demonstrated by the McGill VAS. In a previous community based study an excess of pain was South Asian subjects with diabetes (38) although in our study, groups were accurately matched for ethnicity.

Of potential pathogenetic relevance, GAD-Ab, which are used to diagnose LADA have been linked to other neurological conditions such as stiff man syndrome (39), however there has been no direct correlation of GAD-Ab status with peripheral/autonomic neuropathy in diabetes mellitus (40-41).

In conclusion the present study shows a clear adverse consequence of LADA with a greater degree of small fibre neuropathy compared to matched patients with T2DM. This likely consequence is due to the poorer glycaemic control, as the other major risk factors for neuropathy such as lipid profile were comparable and BMI was in fact lower (32). Thus patients with LADA require assessment for small fibre neuropathy, perhaps to enable risk stratification and earlier commencement of insulin, limiting the progression of neuropathy. Our study also highlights the need to undertake detailed assessment of small fibres using either skin biopsy or corneal confocal microscopy to identify small fibre neuropathy.
5.6 REFERENCES


Chapter VI - Marked vitamin D deficiency in patients with diabetes from secondary care: Ethnic and seasonal differences and an association with dyslipidaemia

Contribution: Uazman Alam conceived the study and subsequently designed it, extracted data, performed statistical analyses and wrote the manuscript which constitutes the basis for this chapter and was submitted and accepted for publication in the journal Diabetic Medicine 2012;29:1343-1345

Uazman Alam, Osman Najam, Sarah Al-Himdani, Sophie Benoliel, Pushpa Jinadev, Jacqueline Berry, Michelle Kew, Omar Asghar, Ioannis Petropolous, Rayaz A Malik
6.1 ABSTRACT

Vitamin D deficiency has been implicated in cardiovascular disease in subjects with diabetes. We aimed to assess the 25-hydroxyvitamin D (25(OH)D) status in patients from a secondary care hospital. This cross-sectional population-based cohort study assessed 25(OH)D levels in 563 unselected subjects with diabetes and 44 age and ethnicity matched subjects without diabetes, in relation to metabolic, anthropometric and demographic factors. The mean 25(OH)D was lower in subjects with diabetes (15.7 ± 9.1 ng/ml) compared to age and ethnicity subjects without diabetes (21.3 ± 14.6 ng/ml) (p=0.009) with vitamin D deficiency (<30 ng/ml) in 91% compared to 78% in the respective groups. The severest (<10ng/ml) vitamin D deficiency occurred in South Asian subjects (54%) compared to white Europeans (22%). There were no significant differences in BMI, systolic and diastolic blood pressure, ALP, corrected calcium or HbA1c between the different quartiles of 25(OH)D (<10ng/ml, 10-19.9ng/ml, 20-29.9ng/ml, >30ng/ml). However, HDL (1.3±0.7, 1.3±0.4, 1.4±0.5, 1.6±0.6mmol/l, p=0.003) was higher and triglycerides (2.2±2.8, 1.7±1.5, 1.5±0.9, 1.6±1.3mmol/l, p=0.04) were significantly lower in subjects with diabetes with 25(OH)D >30ng/ml. There was a statistically but clinically non-significant seasonal variation in the 25(OH)D status in the white European cohort in summer (20.1±8.8ng/ml) versus winter (16.0±6.1ng/ml) (p=0.007), with no change in the South Asian/Middle Eastern cohort.

There is profound vitamin D deficiency in the North West of England, particularly in South Asians which is associated with lower HDL levels, possibly mediating elevated cardiovascular risk. Further research is warranted to define whether replacement of vitamin D may alter cardiometabolic risk.
6.2 INTRODUCTION

Over one billion people worldwide are vitamin D deficient (1). Vitamin D deficiency is highly prevalent in the UK (2) and in a nationwide cohort study, 87.1% and 60.9% were deficient in vitamin D in the winter/spring and summer/autumn months respectively (2) using ≥30ng/ml as a cut off for adequate levels (1; 3). At risk groups for deficiency include those with pigmented skin (4) and severe deficiency is highly prevalent in South Asians living in northern latitudes (5).

The role of Vitamin D is well established in the regulation of bone metabolism and calcium homeostasis. Hence concentrations of ≥30ng/ml of vitamin D have been identified as necessary for optimal bone health (3) by maximal suppression of parathyroid hormone, greatest calcium absorption, and highest bone mineral density (6). Concentrations ≥10ng/ml are sufficient to prevent the severe skeletal manifestations leading to rickets in children and osteomalacia in adults (7). Thus a serum concentration of ≥30 ng/ml is considered sufficient, 20 to <30ng/ml insufficient, 10 to <20ng/ml deficient and <10ng/ml as severe deficiency (1).

Vitamin D may play a pivotal role in several chronic diseases such as diabetes and cardiovascular disease (1; 4). The vitamin D receptor (VDR) is expressed ubiquitously including in tissues such as cardiomyocytes, vascular endothelium and lymphopoeitic cells (1). With regard to diabetes, the binding of the activated hormonal form of vitamin D (1,25-hydroxyvitamin D [1,25(OH)₂D]) to VDR in pancreatic β-cells leads to the production of insulin (8). The development of both type 1 and 2 diabetes has been causally associated with vitamin D deficiency and has been validated in both experimental and human interventional studies (9-11). In the Finnish birth cohort study, more than 10,000 individuals were followed over 30 years and children who were supplemented with 2000IU daily of vitamin D for
the first year of life had a relative risk of 0.22 (95% CI 0.05-0.89) for the development of type 1 diabetes when compared to those who were not supplemented (9). There is also a propensity for vitamin D deficiency, particularly in Type 2 diabetes (12). VDR activation may be beneficial in terms of proliferation of vascular smooth muscle and cardiomyocytes (1). Indeed two large prospective studies have demonstrated increased cardiovascular mortality in subjects with severe deficiency (≤10ng/ml) (13-14). The Framingham cohort study has also shown similar results with a cut off of 15ng/ml (15) and a recent meta-analysis showed a significant association between high levels of vitamin D and a reduction of cardiovascular disease risk (33% reduction compared to low levels of vitamin D), and metabolic syndrome (51% reduction) (16). The relationship of vitamin D to cardiovascular disease is further supported considering the shared metabolic pathway with cholesterol production, via 7-dehydrocholesterol (17). In a recent study an approximate increase of 4.2mg/dl in HDL was associated with every 10ng/ml increase of 25(OH)D (18). Indeed, activated vitamin D inhibits foam cell formation and an impairment of VDR signalling results in acceleration of this process in patients with diabetes (19). This postulated link to cholesterol metabolism and cardiovascular outcomes is further strengthened by the demonstration that statins increase vitamin D levels (20). However, large prospective randomised trials of vitamin D intervention assessing cardiovascular outcomes as the primary endpoint are absent, thus the relationship may be regarded by some as post hoc ergo propter hoc (correlation not causation).

This cross-sectional study was undertaken in a group of unselected subjects with diabetes attending a hospital based diabetes clinic and a control group without diabetes from a general medical clinic. We defined the prevalence of vitamin D
deficiency and its association with the constituents of metabolic syndrome and cardiovascular disease risk.
6.3 RESEARCH DESIGN AND METHODS

Data from a population-based cohort of 563 subjects with diabetes and 44 age and ethnicity matched subjects without diabetes from Central Manchester Foundation Trust, Manchester, UK were analyzed. All patients attending the clinics were assessed irrespective of a history suggestive of vitamin D deficiency. Ethical approval was not required as the data were extracted retrospectively and did not extend beyond standard clinical practice. All those found to be deficient in 25(OH)D, were provided with appropriate replacement therapy in accordance with standard clinical practice.

6.3.1 Subjects

This cross sectional study was conducted in an unselected group of subjects with and without diabetes in a general medical population. All participants were aged ≥ 18 years old attending clinics at the Central Manchester Foundation Trust, Manchester from August 2009 to July 2010. Those with renal impairment (eGFR <30 mL/min/1.73m² (CKD stage 4 and below), granulomatous diseases (tuberculosis, sarcoidosis etc), malabsorption syndromes (Coeliac disease, bacterial overgrowth, concomitant orlistat treatment), pregnant and lactating women, and those currently on vitamin D supplementation were excluded from the analysis. From previous prevalence studies in the UK we anticipated the proportion of individuals with vitamin D deficiency to be greater than 50% (4).

6.3.2 Blood pressure and Anthropometric measurements

Body mass index (BMI) was measured as per the standard equation (mass (kg)/(height(m)))². Weight was measured with a digital scale (Seca 701, Seca,
Hamburg, Germany) to the nearest 0.1kg and height to the nearest 0.1cm. 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.

6.3.3 Assessment of Demographics, Cardiovascular disease and Medications

An assessment of patient demographics, previous cardiovascular events and medications were made through analysis of medical records and an in-hospital medical record database (Diamond database, Hicom, Surrey, UK). Subject demographics extracted were age, sex, ethnicity (White European, South Asian, Far East Asian and Afro-Caribbean descent), smoking status (never, previous and current) and type (Type 1 and 2 diabetes) and duration of diabetes. Previous cardiovascular events extracted were ischaemic heart disease (IHD), myocardial infarction (MI), cerebrovascular accidents (CVA) and peripheral vascular disease (PVD).

6.3.4 Laboratory Measurements

Standard assessment of 25(OH)D was instituted in August 2009 as part of routine haematological and biochemical laboratory measurements which included HbA1c, Complete Blood count (CBC), Urea and Electrolytes (UE), Liver function tests (LFT), bone profile (Corrected Calcium (CCa2+), Alkaline Phosphatase (ALP), Albumin (Alb), and Lipid profile (Total Cholesterol (T-CHL), High Density lipoprotein Cholesterol (HDL), Triglycerides (TRIG)). Low Density lipoprotein (LDL)
analyses were not undertaken and patients were routinely advised that bloods were to be collected under fasting conditions.

For 25(OH)D measurement, serum was separated from whole blood and stored at -20°C until assay. The laboratory used for the biochemical assay measurements (Vitamin D Research Group Manchester Royal Infirmary, UK) was accredited to ISO 9001:2008 and ISO 13485:2003 by Lloyd’s Register Quality Assurance certificate number LRQ 4001542 and participated successfully in the Vitamin D quality assurance scheme (DEQAS). Serum 25(OH)D was measured using the IDS-iSYS multi-discipline automated analyzer (Immunodiagnostic Systems Ltd, Boldon, Tyne and Wear, UK). The assay is based on chemiluminescent technology and was performed exactly as per the manufacturer’s instructions. Briefly, samples were subjected to a pre-treatment step to denature the vitamin D binding protein. The treated samples were then neutralised in assay buffer and a specific anti-25(OH)D antibody labelled with acridinium was added. Following an incubation step, magnetic particles linked to 25(OH)D were added. Following a further incubation step, the magnetic particles were “captured” using a magnet. After a washing step and addition of trigger reagents, the light emitted by the acridinium label was inversely proportional to the concentration of 25(OH)D in the original sample. Concentration of 25(OH)D was calculated automatically using a 4-point logistic curve. The reportable range of the assay was 5-140 ng/mL. Inter- and intra-assay variation of the in-house control was 5.6% and 9.7% respectively.

6.3.5 Statistical analysis

Statistical analyses were undertaken on Statsdirect (Statsdirect, Cheshire, UK). Mean 25(OH)D ± standard deviation (SD) are presented as a whole and per
season and subdivided for ethnic background. ANOVA method or a non-parametric counterpart, Kruskal-Wallis were used to assess differences between groups depending on normality of the data. Overall the P value was maintained at 0.05 for multiple comparison tests (Bonferoni adjustment or Dwass-Steel-Chritchlow-Fligner pairwise comparison). Further descriptive statistics are presented for characteristics of 25(OH)D (≥30ng/ml, 20 to <30ng/ml, 10 to <20ng/ml and <10ng/ml). Multiple linear regression models, after assessment of normality, were used to examine relationships between vitamin D level and lipids, adjusting for BMI/type of diabetes and ethnicity. A P value of <0.05 was considered to be statistically significant. All values are presented as mean ± SD.
6.4 RESULTS

The mean serum 25(OH)D concentration was 15.7 ± 9.1 ng/ml in an unselected consecutive sample of 563 subjects with diabetes. The smaller cohort of 44 subjects without diabetes from a general medical clinic had a significantly higher 25(OH) vitamin D of 21.3 ± 14.6 ng/ml (Table 6.1). The two cohorts did not differ in either age or ethnicity.

Table 6-1 Baseline characteristics of subjects with and without diabetes

<table>
<thead>
<tr>
<th></th>
<th>Subjects with Diabetes</th>
<th>Control subjects (without Diabetes)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>563</td>
<td>44</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>59.7±13.3</td>
<td>58.1±15.6</td>
<td>NS</td>
</tr>
<tr>
<td>White European, n= (%)</td>
<td>334 (59%)</td>
<td>28 (63%)</td>
<td>NS</td>
</tr>
<tr>
<td>25(OH)D (ng/ml)</td>
<td>15.7±9.1</td>
<td>21.3±14.6</td>
<td>0.009</td>
</tr>
</tbody>
</table>

Subjects with diabetes were divided into categories based on their 25(OH)D status and their respective characteristics are detailed in table 3.2. Using the clinically recognised cut off of 30 ng/ml, 511 (91%) subjects had vitamin D deficiency and 179 (32%) had severe (<10 ng/ml) vitamin D deficiency (Figure 6.1). The severest vitamin D deficiency occurred in those of South Asian/Middle Eastern descent with 54% of subjects falling into this group, although 22% of white Europeans were also severely deficient. Despite marked deficiency in vitamin D there were no significant differences in either ALP or CCa and these were well within the normal range in each category. There was no difference in the percentage free of cardiovascular events in the categories although this was marginally higher in category 4. Although chi² analyses did not reach significance (P=0.06), there seemed to be a greater medication load in the form of multidrug therapy for
hypertension in the lower echelons (category 1 and 2) of vitamin D status compared to category 3 and 4. A significant confounding factor is possibly the longer duration of diabetes in category 4 and the presence of asymptomatic undeclared CVD, thus obscuring the true cardiovascular event profile. Nevertheless, we demonstrate a significant difference in HDL ($P=0.003$) and triglycerides ($P=0.04$) between the categories. On post hoc analysis, HDL in category 3 and 4 were significantly higher than category 1 (1.4±0.5mmol/l vs 1.3±0.7mmol/l, $P=0.02$ and 1.6±0.6mmol/l vs 1.3±0.7mmol/l, $P=0.002$ respectively) and a similar relationship was found with triglycerides, with triglycerides being significantly lower in categories 2, 3 and 4 compared to category 1 (1.7±1.5mmol/l vs 2.2±2.8mmol/l, $p=0.002$, 1.5±0.9mmol/l vs 2.2±2.8mmol/l, $P=0.02$ and 1.6±1.3mmol/l vs 2.2±2.8mmol/l, $P=0.05$ respectively). However, after adjusting for BMI, season, ethnicity and lipid medication, regression analyses between 25(OH)D and triglycerides was no longer significant (Table 6.3 ($r=-0.06$, $P=NS$), particularly as the lowest 25(OH)D (category 1) had a higher proportion of Asian subjects with type 2 diabetes and features of metabolic syndrome. Table 6.3 shows a regression analyses of lipid fractions with 25(OH)D levels and demonstrates a significant relationship with HDL ($P=0.006$) after adjusting for BMI, ethnicity, season and lipid medication. Figure 6.2 represents a scatter plot showing a positive linear trend of 25(OH)D versus HDL.
Figure 6-1 Histogram of 25(OH)D Status in subjects with diabetes*

*Minimal reported 25(OH)D was 5ng/ml
Table 6-2 Characteristics by categories of 25(OH)D in 563 unselected subjects with diabetes.

<table>
<thead>
<tr>
<th>Serum 25(OH)D Category 1</th>
<th>Category 2</th>
<th>Category 3</th>
<th>Category 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>179</td>
<td>233</td>
<td>99</td>
</tr>
<tr>
<td>Men (%)</td>
<td>53</td>
<td>57</td>
<td>48</td>
</tr>
<tr>
<td>Type 2 DM (%)</td>
<td>85</td>
<td>75</td>
<td>69</td>
</tr>
</tbody>
</table>

| White European, n (%)     | 71 (40)    | 146 (63)   | 73 (74)    | 44 (84)    |
| South Asian/Middle East, n (%) | 96 (54)    | 61 (26)    | 16 (16)    | 6 (12)     |
| Afro-Caribbean, n (%)     | 8 (4)      | 14 (6)     | 8 (8)      | 2 (4)      |
| Far-East Asian, n (%)     | 4 (2)      | 12 (5)     | 2 (2)      | 0 (0)      |

| % free of Cardiovascular events | 79 | 80 | 77 | 84 |
| % on CHL meds                  | 88 | 85 | 82 | 81 |

| % on BP meds (No therapy/single therapy/multidrug therapy) | 76 (25/31/45) | 76 (24/23/53) | 69 (31/30/39) | 76 (23/39/38) |

<table>
<thead>
<tr>
<th>Mean±SD</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>58.8±13.7</td>
</tr>
<tr>
<td>Duration of Diabetes (years)</td>
<td>15.4±9.4</td>
</tr>
<tr>
<td>BMI (kg/m2)</td>
<td>31.2±6.5</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>129±17</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>69±10</td>
</tr>
<tr>
<td>HbA1c (%) (IFCC (mmol/mol))</td>
<td>8.6±1.9, 69±24</td>
</tr>
<tr>
<td>T-CHL (mmol/l)</td>
<td>4.2±1.3</td>
</tr>
<tr>
<td>HDL (mmol/l)</td>
<td>1.3±0.7</td>
</tr>
<tr>
<td>TRIG (mmol/l)</td>
<td>2.2±2.8</td>
</tr>
<tr>
<td>ALP (U/L)</td>
<td>94±43</td>
</tr>
<tr>
<td>CCa (mmol/l)</td>
<td>2.3±0.1</td>
</tr>
<tr>
<td>eGFR (ml/min/l)</td>
<td>73±20</td>
</tr>
</tbody>
</table>

Table key
ALP – Alkaline Phosphatase; BMI – Body Mass Index; BP – Blood Pressure; CCa – Corrected Calcium; eGFR – estimated Glomerular Filtration Rate; HDL – High-density Lipoprotein Cholesterol; T-CHL – Total Cholesterol; TRIG – Triglycerides;
Table 6-3 Regression analyses of 25(OH)D and T-CHL, HDL and triglycerides in subjects with diabetes

<table>
<thead>
<tr>
<th></th>
<th>r</th>
<th>P</th>
<th>r*</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(unadjusted)</td>
<td>(unadjusted)</td>
<td>(adjusted)</td>
<td>(adjusted)</td>
</tr>
<tr>
<td>T-CHL</td>
<td>0.014</td>
<td>NS</td>
<td>-0.007</td>
<td>NS</td>
</tr>
<tr>
<td>HDL</td>
<td>0.158</td>
<td>0.0002</td>
<td>0.12</td>
<td>0.006</td>
</tr>
<tr>
<td>TRIG</td>
<td>-0.112</td>
<td>0.009</td>
<td>-0.06</td>
<td>NS</td>
</tr>
</tbody>
</table>

*adjusted for BMI, ethnicity, season and lipid medication.

Table key

HDL – High-density Lipoprotein Cholesterol; T-CHL – Total Cholesterol; TRIG – Triglycerides.

Figure 6-2 Relationship between serum 25(OH)D and high-density lipoprotein cholesterol (HDL) in subjects with diabetes

*adjusted for BMI, ethnicity, season and lipid medication.
Table 6.4 shows ethnic differences in 25(OH)D with a further sub-categorisation for South Asian/Middle Eastern and white European subjects. There were significant differences between the South Asian/Middle Eastern group and white Europeans (11.3±9.6ng/ml vs 18.0±7.0ng/ml, P<0.0001), African-Caribbean (11.3±9.6ng/ml vs 16.4±7.8ng/ml, P=0.001) and Far-East Asian groups (11.3±9.6ng/ml vs 14.5±4.5ng/ml, P=0.02). There were no differences in BMI between the ethnic subcategories. There was a statistically significant but clinically non-significant seasonal variation in the 25(OH)D status in the white European group in summer (20.1±8.8ng/ml) versus winter (16.0±6.1ng/ml) (P=0.007) but there was no seasonal variation in the South Asian/Middle Eastern group. There were insufficient data to assess seasonal variation in either the Afro-Caribbean or the Far-East Asian group.

<table>
<thead>
<tr>
<th></th>
<th>White European</th>
<th>South Asian/Middle East</th>
<th>Afro-Caribbean*</th>
<th>Far East Asian*</th>
<th>P for comparison of means</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean 25(OH)D</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(ng/ml)</td>
<td>18.0±7.0</td>
<td>11.3±9.6</td>
<td>16.4±7.8</td>
<td>14.5±4.5</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Season</td>
<td>Seasonal variation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spring (ng/ml)</td>
<td>17.2±9.9</td>
<td>10.6±6.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Summer (ng/ml)</td>
<td>20.1±8.8</td>
<td>11.0±5.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Autumn (ng/ml)</td>
<td>17.8±9.9</td>
<td>10.9±6.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Winter (ng/ml)</td>
<td>16.0±6.1</td>
<td>12.5±8.9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P for comparison</td>
<td>0.009</td>
<td>NS</td>
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<tr>
<td>of means</td>
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*Insufficient number to undertake seasonal analysis.
6.5 DISCUSSION

Vitamin D deficiency is endemic in the United Kingdom and our data confirm this showing a pronounced severe (<10ng/ml) vitamin D deficiency in 32% of individuals, with more than half of South Asian and Middle Eastern subjects demonstrating severe deficiency. Whilst this highlights the need for routine testing in patients with diabetes, the general patient population are also deficient as shown in this study and others (2; 21). However, we have clearly shown a higher 25(OH)D in the age and ethnicity matched subjects without diabetes in this study. Vitamin D deficiency has not only been implicated in the causation of both type 1 and 2 diabetes, but those with type 2 diabetes are particularly prone to vitamin D deficiency, in part due to obesity with the proposed sequestration of 25(OH)D in adipose tissue (22). Although, a previous study has shown a propensity for vitamin D deficiency even when adjusting for BMI (23). The reasons for this may be multifactorial including genetic polymorphisms and variants of vitamin D binding protein (24). The VDR is expressed on the pancreatic β-cell and activated vitamin D affects insulin production (8). The predisposition for the development of vitamin D deficiency may contribute to worsening glycaemic control, leading to the development of impaired glucose tolerance and eventually diabetes. Unfortunately most interventional studies of vitamin D have either been secondary analyses of osteoporosis interventions or have generally had small numbers with low doses of vitamin D replacement. This has significant implications on the guidelines for supplementation of vitamin D particularly as the current recommended daily allowance (RDA) of 400IU, which is less than the RDAs elsewhere, appears woefully inadequate in the absence of adequate sunlight exposure (25).
Seasonal variation in levels of vitamin D are often cited as plausible reasons for overestimation of vitamin D deficiency, particularly in South Asians, but this is a controversial issue and previous studies have had conflicting results (26-27). In the present study in white European subjects, the difference of 4.1 ng/ml from winter to summer is not considered to be clinically significant. Furthermore, a lack of seasonal variation in South Asian and Middle Eastern subjects, which may in-part be related to lack of sunlight exposure through body attire, particularly in women of South Asian origin, further blunts this minimal seasonal effect. Current data on the association of vitamin D deficiency with glucose intolerance seems to be population specific (24). Reduction in serum 25(OH)D concentration has been noted in a UK South Asian population at risk for Type 2 diabetes compared with subjects not at risk (28). In the same South Asian population, short-term vitamin D supplementation increased insulin secretion (28). The data from the Third National Health and Nutrition Examination study (NHANES) also showed an inverse association between vitamin D status and diabetes in non-Hispanic white and Mexican American people but not in non-Hispanic black people (29). However, serum 25(OH)D levels have not been related to glucose status in a white European population from the UK (30). Assessing vitamin D status in population specific targets may an important facet in addressing poorer glycaemic control particularly in ethnic minorities at risk of vitamin D deficiency living in the UK.

Cholesterol and vitamin D share a common metabolic pathway through 7-dehydrocholesterol which is a precursor for both. Our study has shown a positive but albeit weak correlation between 25(OH)D and HDL, confirming the results of other studies (18). Our subjects are largely (approximately 80%) on statin therapy which, of course, impacts on HDL and probably also on 25(OH)D. Atorvastatin and
Rosuvastatin have been shown to increase levels of 25(OH)D, and statins may require adequate levels of vitamin D to function optimally (20). However, statins may worsen diabetes and predispose to dysglycaemia which would go against the notion of statins increasing vitamin D levels may protect against diabetes mellitus. However, the mechanism by which statins may cause dysglycaemia is distinct from the vitamin D pathway and may be via inhibition of β-cell glucose transporters, delayed ATP production, pro-inflammatory and oxidative β-cell effects of plasma-derived cholesterol, inhibition of calcium channel-dependent insulin secretion and β-cell apoptosis (31). In a recent study culturing macrophages from obese, diabetic, hypertensive patients with 1,25(OH)₂D suppressed foam cell formation by reducing LDL uptake, whilst deletion of the VDR in these macrophages accelerated foam cell formation through increased LDL uptake (19). Thus vitamin D may play an important, perhaps even permissive role in atherosclerosis.

Whilst this cross-sectional study has limitations as it provides association and not cause and effect a major strength is that all participants were from a single centre and from a small geographical locality. The north west of England is known to have lower levels of sunlight when compared to other areas of the UK (2) and this may potentially impact on transferability of results. However, despite these shortcomings, we are mindful that the results of this study are not dissimilar to those of other studies in the UK (2).

In conclusion, we provide further confirmation of the epidemic of vitamin D deficiency in diabetic patients (85% in Type 1 diabetes and 93% in Type 2 diabetes) in England. Furthermore, we provide an association with lipid abnormalities providing a potential mechanistic link observed in the landmark longitudinal studies such as the Framingham cohort study (15) which showed
approximately twice the cardiovascular risk in those with low levels of 25(OH)D compared to higher levels. We also confirm the lower levels in those from South Asian, Far Eastern and Afro-Caribbean ethnicity. Moreover we show that seasonal variation contributes minimally to differences in vitamin D status, particularly in South Asians. These data therefore highlight the need to assess 25(OH)D levels in all patients with diabetes, not only those with symptoms of osteomalacia and also confirms the inability of corrected calcium or alkaline phosphatase levels to target individuals for assessment. Whether supplementation will lower cardiovascular risk is not known and the results of the VITAL study (a randomised placebo controlled trial of 25(OH)D and omega-3) are awaited (clinicaltrials.gov - NCT01169259).
6.6 REFERENCES


4. Pearce SH, Cheetham TD: Diagnosis and management of vitamin D deficiency. BMJ 2010;340:b5664


Chapter VII - Differential effects of different vitamin D replacement strategies in patients with diabetes

Contribution: Uazman Alam conceived the study and subsequently designed it, acquired data, performed statistical analysis and wrote the manuscript which constitutes the basis for this chapter and was submitted and accepted for publication in the Journal of Diabetes and its Complications 2013 (Epub ahead of print).

Uazman Alam, Agnes WS Chan, April Buazon, Cristiano Van Zeller, Jacqueline L Berry, Ravinder S Jugdey, Omar Asghar, John Kennedy Cruickshank, Ioannis N Petropoulos, Rayaz A Malik
7.1 ABSTRACT

The optimal treatment regimen for correcting vitamin D insufficiency in diabetic patients has not been established.

Two hundred and forty four adult diabetic patients with vitamin D insufficiency were enrolled to receive: Ergocalciferol (D$_2$) 50,000 IU daily over 10 days (500,000 IU) followed by Calcichew D$_3$ (calcium carbonate/Cholecalciferol) BID (~24,000IU cholecalciferol/month) (ECC) (n=53); Cholecalciferol (D$_3$) 40,000 IU daily over 10 days (400,000 IU) followed by Calcichew D$_3$ BID (~24,000IU cholecalciferol/month) (CCC) (n=94) or Cholecalciferol 40,000 IU daily over 10 days (400,000 IU) followed by Cholecalciferol 40,000 IU monthly (CC) (n=97). The 25(OH)D, HbA1c, lipids, blood pressure and eGFR were assessed at baseline and after a mean follow up of 8.0±4.0 months.

Treatment increased 25(OH)D concentrations significantly in ECC (17.4±13.8 vs 29.9±9.6ng/ml, P<0.0001), CCC (14.2±6.6 vs 30.9±13.1ng/ml, p<0.0001) and CC (13.5±8.4 vs 33.9±14.4ng/ml, P<0.0001). The relative increase in 25(OH)D was significantly lower with ECC compared to CC (+14.6±12.2 vs +20.6±15.0, P=0.01) and the majority of subjects in the ECC group (63%) remained vitamin D deficient (25(OH)D<30ng/ml) compared to CCC (46%) and CC (36%)(P=0.0005).

This study demonstrates that relatively aggressive treatment regimens of both vitamin D$_2$ and D$_3$ increase 25(OH)D concentrations in diabetic patients, but the ability to raise 25(OH)D status to ‘sufficient’ levels is inadequate in a large proportion of individuals.
7.2 INTRODUCTION

There is an epidemic of vitamin D deficiency worldwide (1). Vitamin D has been implicated not only in bone health but also a host of cardiovascular diseases including diabetes, hypertension and metabolic syndrome (2). Vitamin D deficiency poses a significant health problem in many countries irrespective of economic, cultural or geographic diversity (3).

There is currently consensus that levels below 10ng/ml (25nmol/L) qualify as ‘deficient’ and require treatment, but beyond this there is currently no standard definition of ‘optimal’ 25(OH)D with some sources suggesting that levels above 20ng/ml (50nmol/L) are ‘sufficient’, while 28-32ng/ml (70–80nmol/L) are ‘optimal’ (4). The Department of Health in the UK has set dietary recommendations for children aged 6 months to 5 years, those aged 65 years and over (5), and pregnant or lactating women and also recommends that people at risk of low sun exposure should get 10 µg of vitamin D a day, through supplementation (6). However, the recommended daily allowance (RDA) has not changed despite its inability to prevent severe vitamin D deficiency and ensuing bone or metabolic complications in the absence of adequate sunlight exposure (7-9).

Recent data confirm the high prevalence of vitamin D deficiency and no impact of dietary vitamin D intake in the UK due to low amounts of fortification (10). Furthermore, proposed treatment strategies recommending ‘over the counter’ vitamin D preparations have been found to be largely ineffective in correcting vitamin D deficiency (2; 7; 11). Currently there are limited data to guide policy on replacement strategies (2; 7). Previous studies have suggested that vitamin D₂ is 2-3 times less effective at increasing serum 25-hydroxyvitamin D (25(OH)D) than
vitamin D$_3$, but that vitamin D$_2$ taken on a daily basis in high enough doses may be as effective in maintaining 25(OH)D as vitamin D$_3$ (12-14).

We assessed the effects of 3 different vitamin D replacement regimens (using combinations of ergocalciferol, cholecalciferol and calcium carbonate/cholecalciferol) dependent on the clinician’s preference for treatment of vitamin D deficiency.
7.3 RESEARCH DESIGN AND METHODS

The study was undertaken at the Central Manchester University Hospitals NHS Foundation Trust, Manchester, UK. Data from a population-based cohort of 244 patients with Diabetes (Type 1 and 2 Diabetes Mellitus) attending a tertiary diabetes centre were analyzed. Ethical approval was not required for this analysis as all the data were extracted retrospectively. This therefore did not extend beyond standard clinical practice. All patients with a 25(OH)D <30ng/ml were allocated to 3 different treatment regimens based on the prescribing choice of three different clinicians: Ergocalciferol (D$_2$) 50,000 IU daily over 10 days (500,000 IU) followed by Calcichew D$_3$ (calcium carbonate/Cholecalciferol) BID (~24,000IU cholecalciferol/month) (ECC) (n=53); Cholecalciferol (D$_3$) 40,000 IU daily over 10 days (400,000 IU) followed by Calcichew D$_3$ BID (~24,000IU cholecalciferol/month) (CCC) (n=94) or Cholecalciferol 40,000 IU daily over 10 days (400,000 IU) followed by Cholecalciferol 40,000 IU monthly (1$^{st}$ day of each month (CC (n=97). All subjects were provided with vitamin D regimens as soon as vitamin D results were made available, however, data on medication compliance were unavailable.

7.3.1 Blood pressure and Anthropometric measurements

Body mass index (BMI) was measured as per the standard equation (mass (kg)/(height(m))$^2$. Weight was measured with a digital scale (Seca 701, Seca, Hamburg, Germany) to the nearest 0.1kg and height to the nearest 0.1cm with a calibrated stadiometer. Both height and weight were taken at comparable times at baseline and follow up. Blood pressure (BP) was assessed using an automated BP device (Dinamap pro 100v2, GE Medical Systems, Freiburg, Germany) with an
appropriate cuff size. Two measurements of systolic and diastolic BP were made five minutes apart with the lowest reading recorded.

7.3.2 Assessment of Demographics, Cardiovascular disease and Medications

An assessment of patient demographics was made through analysis of medical records and an in-hospital medical record database (Diamond database, Hicom, Surrey, UK). Subject demographics extracted were age, sex, ethnicity (White European, South Asian, Far East Asian and Afro-Caribbean descent), type (T1DM/T2DM), duration of diabetes and current anti-diabetic therapy with any alterations to treatment during the intervention period.

7.3.3 Laboratory Measurements

Assessment of 25(OH)D was instituted in August 2009 as part of routine haematological and biochemical laboratory measurements which included HbA1c, Complete Blood count (CBC), Urea and Electrolytes (UE), Liver function tests (LFT), bone profile (Corrected Calcium (CCa2+), Alkaline Phosphatase (ALP), Albumin (Alb), and Lipid profile (Total Cholesterol (T-CHL), High Density lipoprotein Cholesterol (HDL), Triglycerides (TRIG)). For 25(OH)D measurement, serum was separated from whole blood and stored at -20° C until assay. The laboratory used for the biochemical assay measurements (Vitamin D Research Group Manchester Royal Infirmary, UK) was accredited to ISO 9001:2008 and ISO 13485:2003 by Lloyd’s Register Quality Assurance certificate number LRQ 4001542 and participated successfully in the Vitamin D quality assurance scheme (DEQAS). Serum 25(OH)D was measured using the IDS-iSYS multi-discipline automated
analyzer (Immunodiagnostic Systems Ltd, Boldon, Tyne and Wear, UK). The assay is based on chemiluminescent technology and was performed exactly as per the manufacturer’s instructions. The cross reactivity for vitamin D$_2$ (of the assay) as per manufacturers assertion was 100% (relative to vitamin D$_3$) and the assay has excellent correlation to existing globally recognised assays, in combination with good sensitivity and precision (15). The reportable range of the assay is 5-140 ng/ml Inter- and intra-assay variation of the in-house control was 5.6% and 9.7% respectively.

7.3.4 Statistical analysis

Statistical analyses were undertaken using Statsdirect (Statsdirect, Cheshire, UK). ANOVA method or a non-parametric counterpart, Kruskal-Wallis were used to assess differences between groups depending on normality of the data. Overall the P value was maintained at 0.05 for multiple comparison tests (after Bonferoni adjustment or Conover-Inman pairwise comparison). Paired t-tests were used for intra-individual observations. A P value of ≤0.05 was considered to be statistically significant. All values are presented as mean ± standard deviation (SD).
7.4 RESULTS

Demographics of the three cohorts are shown in Table 7.1. They did not differ in for age, type of diabetes, sex and ethnicity and there were no differences in the period of follow up. A significant proportion of subjects still remained deficient on ECC regimen of supplementation (P=0.0005) compared to the other regimens (CCC and CC). Anti-diabetic therapies used in each cohort are detailed in Table 7.1. There were significant differences in the types of anti-diabetic regimens used (chi$^2$ P=0.01) across the 3 groups, however, there were no significant differences in the numbers of individuals who had intensification of their diabetes treatment in each cohort from baseline to follow up. In those individuals requiring insulin administration, mean doses of insulin units required per day did not differ.
Table 7-1 Clinical and demographic data and Characteristics of Anti-Diabetic therapy in all three cohorts of all three intervention cohorts

<table>
<thead>
<tr>
<th></th>
<th>ECC (n=53)</th>
<th>CCC (n=94)</th>
<th>CC (n=97)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (years)</strong></td>
<td>60.7±12.1</td>
<td>58.2±13.3</td>
<td>57.0±12.2</td>
</tr>
<tr>
<td><strong>Sex (% male)</strong></td>
<td>55</td>
<td>57</td>
<td>52</td>
</tr>
<tr>
<td><strong>Type 2 DM (%)</strong></td>
<td>81</td>
<td>68</td>
<td>82</td>
</tr>
<tr>
<td><strong>Duration of diabetes (years)</strong></td>
<td>16.7±11.2</td>
<td>21.1±12.8</td>
<td>17.1±10.3</td>
</tr>
<tr>
<td><strong>Ethnicity (% White European)</strong></td>
<td>55</td>
<td>57</td>
<td>52</td>
</tr>
<tr>
<td><strong>% on lipid lowering therapy</strong></td>
<td>80</td>
<td>90</td>
<td>87</td>
</tr>
<tr>
<td><strong>Period of follow up (months)</strong></td>
<td>8.0±3.7</td>
<td>7.8±3.9</td>
<td>8.1±4.3</td>
</tr>
<tr>
<td><strong>Insufficient after supplementation with 25(OH)D (&lt;30ng/ml) n (%)</strong></td>
<td>32 (63)*</td>
<td>43 (46)*</td>
<td>35 (36)*</td>
</tr>
<tr>
<td><strong>Treatment regimens</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diet controlled n (%)</td>
<td>0 (0)</td>
<td>3 (3)</td>
<td>1 (1)</td>
</tr>
<tr>
<td>Single oral therapy n (%)</td>
<td>8 (15)</td>
<td>7 (7)</td>
<td>4 (4)</td>
</tr>
<tr>
<td>Oral combination therapy n (%)</td>
<td>20 (38)</td>
<td>22 (23)</td>
<td>31 (32)</td>
</tr>
<tr>
<td>Oral and/or Insulin + GLP-1 mimetic n (%)</td>
<td>1 (2)</td>
<td>8 (9)</td>
<td>10 (10)</td>
</tr>
<tr>
<td>Oral + Insulin therapy n (%)</td>
<td>15 (28)</td>
<td>21 (22)</td>
<td>30 (31)</td>
</tr>
<tr>
<td>Insulin alone n (%)</td>
<td>12 (23)</td>
<td>31 (33)</td>
<td>20 (21)</td>
</tr>
<tr>
<td>CSII therapy n (%)</td>
<td>2 (4)</td>
<td>2 (2)</td>
<td>1 (1)</td>
</tr>
<tr>
<td><strong>Mean Insulin dose/day</strong></td>
<td>86±81**</td>
<td>92±149**</td>
<td>84±63**</td>
</tr>
<tr>
<td><strong>(mean±SD) Median(IQR)</strong></td>
<td>64(44-76)</td>
<td>68.5(48-88)</td>
<td>65(52-104)</td>
</tr>
<tr>
<td><strong>Escalation in Anti diabetic</strong></td>
<td>11 (19)***</td>
<td>20 (21)***</td>
<td>17 (18)***</td>
</tr>
<tr>
<td><strong>Therapy n (%)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>No. of subjects with increase in insulin n (%)</strong></td>
<td>1 (2)</td>
<td>10 (11)</td>
<td>2 (2)</td>
</tr>
<tr>
<td><strong>Average dose increase in insulin/day (units)</strong></td>
<td>6±0</td>
<td>4±1.3</td>
<td>2±0</td>
</tr>
</tbody>
</table>

Post hoc analyses

*P=0.0005 (Chi² test); **Chi² for treatment regimens – P=0.01; **KrusKal-Wallis – P=NS.

***Chi² for intensification of anti-diabetic therapy – P=NS.

Type 2 DM – Type 2 Diabetes Mellitus.

Table key

CSII – Continuous Subcutaneous Insulin Infusion, DM – Diabetes Mellitus, IQR – Interquartile range, SD – Standard deviation.
25(OH)D status and metabolic data are presented in Table 7.2. The 25(OH)D concentrations at baseline were higher in the ECC cohort when compared to CCC and CC (17.4±13.8 vs 14.2±6.6 vs 13.5±8.4ng/ml), respectively, although this did not reach statistical significance. After treatment 25(OH)D concentrations increased significantly in the ECC (17.4±13.8 vs 29.9±9.6, P<0.0001), CCC (14.2±6.6 vs 30.9±13.1, P<0.0001) and CC (13.5±8.4 vs 33.9±14.4ng/ml, P<0.0001) cohorts, respectively. There was no difference in the increase in 25(OH)D comparing ECC to CCC (+14.6±12.2 vs +16.7±13.4ng/ml). However, there was a significantly greater increase in CC compared to ECC (+20.6±15.0 vs +14.6±12.2, P=0.01) with no difference compared to CCC. However, 63% of patients remained deficient (25(OH)D <30ng/ml) after treatment in the ECC cohort compared to 46% and 36% in the CCC and CC cohorts, respectively (chi^2 P=0.0005). When further subdividing the three cohorts into those with an initial 25(OH)D <15ng/ml and >15ng/ml (based on the study by Wang et al (16)) there were significant differences between pre- and post-intervention, with a more pronounced increase in 25(OH)D in those with a lower baseline 25(OH)D (ECC: +21.2±8.4 vs +6.4±11.4ng/ml, p<0.0001, CCC: +20.1±13.8 vs +12.3±11.6ng/ml, P=0.005, CC: +24.4±14.5 vs +12.1±12.9ng/ml, P=0.01)(figures 7.1a, b and c).

There were no significant changes in BMI, systolic and diastolic blood pressure, lipid profile (T-CHL, HDL and TRIG) and eGFR after treatment in any of the cohorts. However, there was a significant reduction in HbA1c after treatment in ECC (-0.5% or 5.2mmol/mol, P=0.02), with no change in the CCC (8.3±1.7 vs 8.3±1.7%) or CC (8.2±1.6 vs 8.1±1.6%) cohorts.
Table 7-2 Change in 25(OH)D status, metabolic data and anthropometric measurements following intervention in all three cohorts

<table>
<thead>
<tr>
<th></th>
<th>ECC (n=53)</th>
<th>CCC (n=94)</th>
<th>CC (n=97)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Follow up</td>
<td>Baseline</td>
</tr>
<tr>
<td>25(OH)D (ng/ml)</td>
<td>17.4±13.8*</td>
<td>29.9±6.6^/*</td>
<td>14.2±6.6^^</td>
</tr>
<tr>
<td>BMI (kg/m2)</td>
<td>29.6±8.6</td>
<td>30.0±7.3</td>
<td>31.4±7.3</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>126±16</td>
<td>132±16</td>
<td>128±15</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>68±10</td>
<td>67±13</td>
<td>68±10</td>
</tr>
<tr>
<td>HbA1c (%) (IFCC (mmol/mol))</td>
<td>8.5±1.7*** (69.8±18.6)</td>
<td>8.0±1.5*** (64.6±16.8)</td>
<td>8.3±1.7 (66.8±18.7)</td>
</tr>
<tr>
<td>T-CHL (mmol/l)</td>
<td>4.1±1.3</td>
<td>4.3±1.5</td>
<td>4.0±1.0</td>
</tr>
<tr>
<td>HDL (mmol/l)</td>
<td>1.4±0.5</td>
<td>1.3±0.5</td>
<td>1.4±0.5</td>
</tr>
<tr>
<td>TRIG (mmol/l)</td>
<td>1.7±1.3</td>
<td>2.0±2.4</td>
<td>1.6±0.9</td>
</tr>
<tr>
<td>eGFR (ml/min/l)</td>
<td>75±18</td>
<td>77±16</td>
<td>74±17</td>
</tr>
</tbody>
</table>

Post hoc analyses

25(OH)D - *p<0.0001; ^ ECC vs CC (p=0.01); **P<0.0001, ^^P<0.0001.
HbA1c - ***P=0.02.

Table key

BMI – Body Mass Index; BP – Blood Pressure; eGFR – estimated Glomerular Filtration Rate; HDL – High-density Lipoprotein Cholesterol; T-CHL – Total Cholesterol; TRIG – Triglycerides.
Figure 7-1a Box and Whisker plots for 25(OH)D pre- and post-intervention in ECC subdividing into <15ng/ml and >15ng/ml

25 (OH)D pre- and post-intervention dependent on baseline Vitamin D status

25 (OH)D ng/ml

P<0.0001

+6.4 ± 11.4

+21.2 ± 8.4

1st VIT D ECC <15ng/ml 2nd Vit D ECC <15ng/ml 1st VIT D ECC >15ng/ml 2nd Vit D ECC >15ng/ml
Figure 7-2b Box and Whisker plots for 25(OH)D pre- and post-intervention in CCC subdividing into <15ng/ml and >15ng/ml

25 (OH)D pre- and post-intervention dependent on baseline Vitamin D status

1st VIT D CCC <15 ng/ml 2nd Vit D CCC <15 ng/ml 1st VIT D CCC >15 ng/ml 2nd Vit D CCC >15 ng/ml

+20.1 ± 13.8

P=0.005

+12.3 ± 11.6
Figure 7.1c Box and Whisker plots for 25(OH)D pre- and post-intervention in CC subdividing into <15ng/ml and >15ng/ml

25 (OH)D pre- and post-intervention dependent on baseline Vitamin D status

P = 0.01

+24.4 ± 14.5

+12.1 ± 12.9
7.5 DISCUSSION

Vitamin D deficiency is highly prevalent and current guidelines recommending replacement regimes appear inadequate (17). In 2011, the US Institute of Medicine (IOM) Recommended Dietary Allowance (RDA) of vitamin D was 800 IU per day for older adults. A serum 25(OH)D of 20 ng/ml is considered to be sufficient by the IOM. However, recently the US Endocrine Society's Clinical Practice Guideline suggest 1500-2000 IU per day for adults aged 19 years or more to maintain 25(OH)D above the optimal level of 30 ng/ml (18). Earlier data suggested that at least 55 µg (2200IU)/day is necessary to increase 25(OH)D concentrations from 20-40 nmol/L (8-16 ng/ml) to 80 nmol/L (32 ng/ml) (19). Yet both the UK Department of Health and NICE continue to endorse dietary supplementation of 10 µg (400IU)/day and UK recommendations, particularly for primary care advocate ‘over the counter’ supplements or dual calcium carbonate and cholecalciferol supplementation which only contain 100-400IU of cholecalciferol/per tablet (7; 20).

Recent guidelines by Holick et al (21) have suggested in adults that a dose of 400,000IU vitamin D₂ over 8 weeks followed by a maintenance therapy of 1500-2000IU daily is adequate in the treatment of deficiency. In a retrospective cohort study from the US, a dosing schedule of D₂ (50,000IU on alternate weeks) has recently been shown to be effective, with a baseline 25(OH)D of 31.0 ± 10.6 ng/mL increasing to 48.3 ± 13.4 ng/mL (22). Our treatment protocols at the Manchester Diabetes centre consisted of a loading dose of vitamin D₂ (ergocalciferol) or D₃ (cholecalciferol) at non-bioequivalent doses of 500,000IU and 400,000IU respectively, followed by a maintenance dose of cholecalciferol with or without calcium carbonate. In our study the baseline 25(OH)D concentration was lower,
than the previous study, and therefore despite a higher dose replacement the increase in 25(OH)D was not as marked (D$_2$ ~12-13ng/ml, D$_3$ ~20ng/ml). Most treatment protocols do not differentiate between the therapeutic effect of vitamin D$_2$ and D$_3$ (7; 23), although, clearly this has implications on therapeutic effects and monitoring of treatment. The difference in increasing the concentrations of 25(OH)D is both physiologically and pharmacologically meaningful with studies showing that increasing 25(OH)D improves calcium absorption (2), reduces fall frequency (24) and osteoporotic fracture risk (25). Previous studies have suggested that 50,000IU of vitamin D$_2$ may be biologically equivalent to ~15,000IU of vitamin D$_3$ (13). Of course, vitamin D$_2$ is effective in elevating 25(OH)D concentrations, but has been considered not to be bio-equivalent with a suggestion for dose adjustment in treatment protocols (26). However, conclusions regarding the biological consequence and functional effects of the D$_2$ and D$_3$ cannot clearly be drawn. Additional studies are needed to elucidate the pharmacokinetic parameters and the varying vitamin D$_2$ and vitamin D$_3$ 25(OH)D metabolites produced which may have differing biological affinity and thus differing physiological responses (19; 27).

Despite these differences a considerable proportion of individuals still remain vitamin D deficient on these regimens. Thus even with doses of cholecalciferol which were approximately equivalent to the current recommended maintenance therapy in addition to an ‘adequate’ loading dose still resulted in around 46% of individuals being below the target 25(OH)D of 30ng/ml. This cannot be due to inadequate time of exposure to treatment as the time to equilibrium of vitamin D$_3$ is approximately 5 months (28). Our data confirm a significant disparity of effect between those supplemented at lower compared to higher baseline levels (29). It
has been previously suggested that there is essentially a linear relationship up to 10000IU (4), however there are minimal data directly assessing baseline 25(OH)D status and response to a standardised vitamin D intervention. Variations in circulating concentrations can also be attributed to differences in the transport of vitamin D in the circulation following exposure to the sun and oral routes (30) with an initial rapid hepatic delivery of vitamin D via the oral route but the increase in concentrations is not as well sustained. However, in the present study all three interventions were oral and of sufficiently long duration to ensure a steady state concentration. Therefore the differences can be primarily attributed to pharmacological differences in the replacement regimens, although genetic polymorphisms may affect the vitamin D metabolic pathway (31). Furthermore, we show that patients with a lower baseline show the most marked rise in concentrations of 25 (OH)D, despite equivalent doses. This may be important for dosing considerations when establishing replacement protocols and the need for less conservative treatment (32). In fact vitamin D shares a similar intake-response curve as other nutrients and in persons whose baseline values differ, an identical nutrient intake may or may not produce a measurable response (33). Unfortunately, most of the randomized, controlled trials of vitamin D that have been published to date have paid little attention to baseline status (33).

A range of potentially beneficial metabolic effects of vitamin D have been proposed (34-36). Vitamin D receptors are of course expressed ubiquitously along with vitamin D activating enzymes (2) and are also present on pancreatic β-cells (2). Circulating 25(OH)D is inversely associated with insulin resistance and possibly insulin secretion (37), and there is a inverse relationship between 25(OH)D concentrations and the risk of Type 2 DM (38). Moreover, a recent study has
shown an improvement in HbA1c when diabetic patients were given vitamin D (39). A recent meta-analysis, stated ‘there was a insufficient evidence of beneficial effect to recommend vitamin D supplementation as a means of improving glycaemia or insulin resistance in patients with diabetes, normal fasting glucose or impaired glucose tolerance’, although the doses used for replacement were not adequate (40). In the present study, whilst there was an overall intensification of anti-diabetic treatment in all groups there was no significant difference between the cohorts. Importantly the administration of D$_2$ did not achieve adequate plasma concentrations of 25(OH)D which was lower than CCC and CC cohorts which did not produce significant drops in glycaemia. Therefore the improvement in blood glucose control cannot be entirely accredited to ergocalciferol administration. This study provides hypothesis generating data to assess any differential effect of vitamin D$_2$ and D$_3$ on glycaemic control.

A major limitation of the present retrospective analysis was that it was not randomised (to treatment groups) and there was no placebo arm and was therefore subject to a greater degree of confounding. However, it provides interesting hypotheses generating ‘real world’ data on the ‘correct’ dosing regimens in vitamin D deficiency and the need for more intensive dosing regimens for patients with vitamin D deficiency. This highlights the significant need for high quality randomised controlled trials assessing the effectiveness of cholecalciferol and ergocalciferol in the treatment of vitamin D deficiency and metabolic outcomes in diabetes.
7.6 REFERENCES


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8 Chapter VIII - Vitamin D Deficiency Contributes to Painful Diabetic Neuropathy

Contribution: Uazman Alam contributed to the conception and design of the study and made a major contribution to the recruitment of subjects, clinical tests including neuropathy assessments and skin biopsies, skin biopsy analyses, all statistical analyses and the writing of this chapter.

Submitted to Diabetes awaiting revision and resubmission

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8.1 ABSTRACT

The aetiology of painful diabetic neuropathy (PDN) is unclear. Low vitamin D levels have been associated with a number of painful conditions. We have evaluated vitamin D levels in diabetic patients with and without painful neuropathy. Forty three type 1 diabetic patients subdivided into painless neuropathy (DPN) (n=20) and painful neuropathy (PDN) (n=23) and 14 non-diabetic healthy control subjects (C) underwent detailed assessment of neurologic deficits, quantitative sensory testing (QST), electrophysiology, skin biopsy and corneal confocal microscopy (CCM) together with measurement of serum 25(OH)D. There were no significant differences for age, BMI, HbA1c, lipids, neurological evaluation, QST, electrophysiology, intra-epidermal nerve fibre density (IENFD) and corneal nerve morphology when comparing DPN with PDN. Both positive (hyperalgesia and allodynia) and negative symptoms (paraesthesia and numbness) of diabetic neuropathy were greater in PDN compared to DPN (P=0.009 and P=0.02 respectively). Serum 25(OH)D level were significantly lower in PDN (24.0±14.1ng/ml) compared to DPN (34.6±15.0ng/ml, P=0.01) and controls (34.1±8.6ng/ml, P=0.03). The odds ratio in favour of painful diabetic neuropathy was 9.8 (P=0.003 (95% CI 2.2-76.4) for vitamin D deficiency (<20ng/ml) and 4.4 (P=0.03 (95% CI 1.1-19.8)) for vitamin D insufficiency (<30ng/ml).

This study provides a novel association between vitamin D deficiency and painful diabetic neuropathy.
8.2 INTRODUCTION

A large population based study has recently shown that the prevalence of painful diabetic neuropathy is ~21% and painful symptoms are more prevalent in patients with type 2 diabetes, females and South Asians (1). It is characterised by symmetrical lower limb paraesthesiae, dysaesthesiae, lancinating pains and allodynia with nocturnal exacerbation (2) which are associated with significant sleep disturbance and reduced quality of life (3). National and international guidelines advocate a range of therapies providing symptomatic relief (4-5). However, the therapeutic efficacy for all recommended medications is at best ~50% pain relief and is limited due to unwanted side effects (2; 6). Apart from peripheral and central alterations (7), metabolic alterations such as increased glycaemic flux (8) and elevated plasma methylglyoxal levels have been implicated in patients with painful diabetic neuropathy (9).

Two recent observational studies in diabetic patients have demonstrated a significant association between vitamin D deficiency and both paraesthesiae and numbness (10) and diabetic neuropathy per se, defined using the neuropathy symptom score, neurological deficits and electrophysiology (11). However, neither of these studies delineated negative from positive symptoms and there was no definitive assessment of pain. There is of course an association between vitamin D deficiency and painful symptoms in the general population (12). In relation to a mechanistic link between vitamin D and pain, a recent study has shown that nociceptive calcitonin gene-related peptide (CGRP)-positive neurones have a distinct vitamin D phenotype with hormonally-regulated ligand and receptor levels (13). Vitamin D deficiency results in increased numbers of axons containing CGRP, and in culture vitamin D receptor (VDR) expression is increased in growth cones.
and sprouting appears to be regulated by VDR-mediated rapid response signalling pathways (14). Nerve Growth Factor (NGF) is known to be depleted in experimental diabetes (15) and in a study of patients with diabetic neuropathy NGF immunostaining on skin keratinocyte correlated with skin axon-reflex vasodilation (16). In an experimental study a preservation of NGF expression was shown in sciatic nerves of diabetic animals treated with a vitamin D analogue (CB1093). Similarly Tacalcitol, an active vitamin D3 induces NGF production in human epidermal keratinocytes (17). Given that demyelination and axonal degeneration are the hallmarks of diabetic neuropathy (18-20), of potential relevance, treatment with vitamin D3 has been shown to reduce demyelination in a cuprizone experimental model of demyelination (21) and in a separate spinal cord compression model it has been shown to induce axonal regeneration (22).

In relation to the potential therapeutic benefits of vitamin D therapy, a prospective study by Lee et al (23) of 51 patients with Type 2 diabetes and painful neuropathy given ~2000 IU of cholecalciferol daily for 3 months showed a ~50% decrease in pain scores as measured by Visual Analogue Score (VAS). Similarly a dramatic improvement in neuropathic symptoms has been described recently after treatment with 50000U of D2 weekly in a type 1 diabetic patient who had been refractory to treatment with tricyclics, gabapentin, pregabalin and oxycodone (24).

In the present study we have evaluated the association between the levels of 25(OH)D and painful versus painless diabetic neuropathy matched for all other measures of neuropathy.


8.3 RESEARCH DESIGN AND METHODS

8.3.1 Selection of patients

Forty three type 1 diabetic patients were categorized into two groups: painless neuropathy (DPN) (n=20) and painful neuropathy (PDN) (n=23) using the McGill Visual Analogue Score (McGill VAS) and McGill pain score, and compared with 14 age, sex and ethnicity matched non-diabetic healthy control subjects (C). Subjects with any history of neurologic conditions, ocular trauma or ocular surgery were excluded. The study was approved by the North West and Salford and Trafford Research Ethics committee, and written informed consent was obtained according to the Declaration of Helsinki.

8.3.2 Assessment of neuropathy

All patients and control subjects underwent a detailed evaluation of neurologic symptoms according to the neuropathy symptom profile (NSP), and the McGill VAS was used to assess the severity of painful neuropathy. Neurologic deficits were assessed using the modified neuropathy disability score (NDS), which includes evaluation of vibration, pin prick, and temperature perception as well as the presence or absence of ankle reflexes (25). Quantitative sensory testing included an assessment of vibration perception threshold (VPT), measured using a Neurothesiometer (Horwell, Scientific Laboratory Supplies, Wilford, Nottingham, U.K.), cold sensation threshold (CST) (Aδ fibres) and warm sensation threshold (WST) (26) (C fibres) thresholds using the method of limits with the MEDOC TSA II (Medoc, Ramat Yishai, Israel) on the dorsum of the left foot. Computer-Aided Sensory Evaluator (CASE IV) (WR Medical Electronic Ltd, Maplewood, Maine, USA) was used to measure the heart rate response to deep breathing (HRV-DB)
over two 8-cycle breathing series interspersed by a 5-min period of normal breathing. Electro-diagnostic studies were undertaken using a Dantec “Keypoint” system (Dantec Dynamics, Bristol, U.K.) equipped with a DISA temperature regulator to keep limb temperature constantly between 32°C and 35°C. Peroneal motor and sural sensory nerves were assessed in the right lower limb by a consultant neurophysiologist. The motor study was performed using silver-silver chloride surface electrodes at standardized sites defined by anatomical landmarks, and recordings for the sural nerve were taken using antidromic stimulation over a distance of 100 mm.

8.3.3 Corneal Confocal Microscopy

Patients underwent examination with the Heidelberg retina tomography III in vivo corneal confocal microscope employing our established methodology for image acquisition (27). Several scans of the entire depth of the central cornea were recorded by turning the fine focus of the objective lens backward and forward for ~2 min using the section mode, which enables manual acquisition and storage of single images of all corneal layers. This provides en face two-dimensional images with a lateral resolution of ~2 mm/pixel and final image size of 400 x 400 pixels of the subbasal nerve plexus of the cornea. Each nerve fibre bundle contains unmyelinated fibres, which run parallel to Bowman’s layer before dividing and terminating as individual axons underneath the surface epithelium. Five images per patient from the centre of the cornea were selected and examined in a masked and randomized fashion (28). Three corneal nerve parameters were quantified: 1) corneal nerve fibre density (CNFD), the total number of major nerves per square millimetre of corneal tissue (no.mm²); 2) corneal nerve branch density (CNBD), the
number of branches emanating from all major nerve trunks per square millimetre of corneal tissue (no. mm²); and 3) corneal nerve fibre length (CNFL), the total length of all nerve fibres and branches (mm/mm²) within the area of corneal tissue.

8.3.4 Skin biopsy and immunohistochemistry

Intra-epidermal nerve fibre density (IENFD) was assessed in a sub-cohort of participants (Controls n=10, DPN n=11, and PDN n=9) who agreed to undergo a 3-mm punch skin biopsy from the dorsum of the foot; 2 cm proximal to the second metatarsal head, after local anaesthesia (1% lidocaine). The biopsy specimen was immediately fixed in PBS-buffered 4% paraformaldehyde and after 18–24 hours rinsed in Tris-buffered saline and soaked in 33% sucrose (2–4 hours) for cryoprotection. It was then embedded in optimal cutting temperature-embedding compound, rapidly frozen in liquid nitrogen, and cut into 50-μm sections using a cryostat (model OTF; Bright Instruments, Huntington, U.K.). Four floating sections per subject were subjected to melanin bleaching (0.25% KMnO₄ for 15 min followed by 5% oxalic acid for 3 min), a 4-h protein block with a Tris-buffered saline solution of 5% normal swine serum, 0.5% powdered milk, and 1% Triton X-100, and overnight incubation with 1:1,200 Biogenesis polyclonal rabbit anti-human PGP9.5 antibody (Serotec, Oxford, U.K.). Biotinylated swine anti-rabbit secondary antibody (1:300; DakoCytomation, Ely, U.K.) was then applied for 1 hour; sections were quenched with 1% H₂O₂ in 30% MeOH-PBS (30 min) before a 1 hour incubation with 1:500 horseradish peroxidase–streptavidin (Vector Laboratories, Peterborough, U.K.). Nerve fibres were demonstrated using 3, 3-diaminobenzidine chromogen (Sigma-Aldrich, Manchester, U.K.). Sections were mildly counterstained with eosin to better localize the basement membrane to identify
nerve fibres passing through it. Negative control subjects consisted of replacing the anti-PGP9.5 antibody with rabbit immunoglobulin (DakoCytomation) at a concentration matching that of the primary antibody and showed no immunostaining. IENFD, i.e., the number of fibres per millimetre of basement membrane, was quantified in accord with established criteria and techniques and expressed as number per millimetre (29).

8.3.5 25(OH) Vitamin D Assay

The laboratory used for the biochemical assay measurements (Vitamin D Research Group Manchester Royal Infirmary, UK) was accredited to ISO 9001:2008 and ISO 13485:2003 by Lloyd’s Register Quality Assurance certificate number LRQ 4001542 and participated successfully in the Vitamin D quality assurance scheme (DEQAS). Serum was separated from whole blood and stored at -20°C until assay. The assay used was an automated platform assay (ImmunoDiagnostic Systems Ltd, Bolden, Tyne and Wear, UK) and is based on chemiluminescence technology. Briefly, samples were subjected to a pre-treatment step to denature the vitamin D binding protein. The treated samples were then neutralised in assay buffer and a specific anti-25(OH)D antibody labelled with acridinium was added. Following an incubation step, magnetic particles linked to 25(OH)D were added. Following a further incubation step, the magnetic particles were “captured” using a magnet. After a washing step and addition of trigger reagents, the light emitted by the acridinium label was inversely proportional to the concentration of 25(OH)D in the original sample. The concentration of 25(OH)D was calculated automatically using a 4-point logistic curve. The cross reactivity for vitamin D₂ (of the assay) as per manufacturers assertion was 100% (relative to
vitamin D₃) and the assay has excellent correlation to existing globally recognised assays, in combination with good sensitivity and precision (30). The reportable range of the assay was 5-140 ng/mL. Inter- and intra-assay variation of the in-house control was 5.6% and 9.7% respectively. Vitamin D deficiency (<20ng/ml) and insufficiency (<30ng/ml) were defined according to the Institute of Medicine (IOM) of the National Academies (31).

8.3.6 Statistical analysis

Statistical analyses were undertaken on Statsdirect (Statsdirect, Cheshire, UK). All values are presented as mean ± SD. ANOVA method or a non-parametric counterpart, Kruskal-Wallis were used to assess differences between groups depending on normality of the data. Overall the p value was maintained at 0.05 for multiple comparison tests (Bonferoni adjustment or Dwass-Steel-Chritchlow-Fligner pairwise comparison). Unpaired t-test or Mann-Whitney U test were used for analysis for DPN versus PDN for the duration of diabetes and IENFD. Chi² analyses were used to assess frequencies of gender, ethnicity and aetiology of diabetes. Odds Ratios for painful symptoms were calculated by further delineating DPN and PDN groups based on the cut offs for vitamin D deficiency (<20ng/ml) and insufficiency (<30ng/ml).
8.4 RESULTS

8.4.1 Demographics, Metabolic and Anthropometric Assessment (Table 8.1)

The participant demographics and metabolic and anthropometric measurements in diabetic patients and control subjects are summarized in Table 8.1. There were no significant differences in age (C: 59.3±7.8 vs DPN: 57.1±13.5 vs PDN: 59.8±11.7 years, P=NS), duration of diabetes (DPN: 36.0±17.5 vs PDN: 35.5±14.9 years, P=NS) gender, type of diabetes and all subjects were of white European origin. BMI was lower in diabetic patients but this was not significant. HbA1c (P<0.0001) was significantly higher in diabetic patients compared with control subjects with no difference between patients with PDN and DPN. The total cholesterol was significantly lower in diabetic patients with DPN (P=0.003) and PDN (P=0.02) compared to control subjects, due to greater statin use. HDL, triglycerides, systolic and diastolic blood pressure were comparable between diabetic patients and control subjects. The estimated glomerular filtration rate was comparable between diabetic patients and control subjects, but the Albumin-Creatinine Ratio (ACR) was higher in the DPN (P=0.009) and PDN (P=0.002) groups versus control subjects with no difference between DPN and PDN. Serum vitamin B12 levels were comparable between groups.
Table 8-1 Participant demographics and metabolic parameters in control subjects and diabetic patients with DPN and PDN, with statistically significant differences between groups

<table>
<thead>
<tr>
<th></th>
<th>C (n=14)</th>
<th>DPN (n=20)</th>
<th>PDN (n=23)</th>
<th>DPN v PDN</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (years)</strong></td>
<td>59.3±7.8</td>
<td>57.1±13.5</td>
<td>59.8±11.7</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Gender (Male) (%)</strong></td>
<td>57</td>
<td>60</td>
<td>53</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Ethnicity (White European) (%)</strong></td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Aetiology of Diabetes (Type 1 DM) (%)</strong></td>
<td>-</td>
<td>85</td>
<td>91</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Duration of Diabetes (years)</strong></td>
<td>-</td>
<td>36.0±17.5</td>
<td>35.5±14.9</td>
<td>NS</td>
</tr>
<tr>
<td><strong>HbA1c (%)</strong></td>
<td>5.7±0.2</td>
<td>8.1±1.1*</td>
<td>8.0±1.5**</td>
<td>NS</td>
</tr>
<tr>
<td><strong>HbA1c (mmol/mol)</strong></td>
<td>38.3±2.5</td>
<td>65.2±12.0*</td>
<td>63.7±16.3**</td>
<td>NS</td>
</tr>
<tr>
<td><strong>BMI (kg/m2)</strong></td>
<td>31.0±4.2</td>
<td>28.1±4.1</td>
<td>26.6±4.9</td>
<td>NS</td>
</tr>
<tr>
<td><strong>T-CHL (mmol/l)</strong></td>
<td>5.1±1.2</td>
<td>4.2±1.0†</td>
<td>4.4±0.8††</td>
<td>NS</td>
</tr>
<tr>
<td><strong>HDL (mmol/l)</strong></td>
<td>1.7±0.5</td>
<td>1.7±0.4</td>
<td>1.7±0.6</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Triglycerides</strong></td>
<td>1.6±0.7</td>
<td>1.2±0.5</td>
<td>1.3±0.8</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Systolic BP (mmHg)</strong></td>
<td>138±15</td>
<td>141±26</td>
<td>141±23</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Diastolic BP (mmHg)</strong></td>
<td>77±8</td>
<td>71±7</td>
<td>71±11</td>
<td>NS</td>
</tr>
<tr>
<td><strong>ACR (mg/mmol)</strong></td>
<td>0.4±0.3</td>
<td>7.1±16.7¥</td>
<td>5.3±6.7¥¥</td>
<td>NS</td>
</tr>
<tr>
<td>(Median)IQR)</td>
<td>(0.2(0.2-0.7))</td>
<td>(0.2(0.2-0.7))</td>
<td>(2.2(0.5-10.2))</td>
<td></td>
</tr>
<tr>
<td><strong>eGRF (ml/min/1.73)</strong></td>
<td>85±7</td>
<td>77±16</td>
<td>78±15</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Serum Vitamin B12 (ng/l)</strong></td>
<td>247±69</td>
<td>342±121</td>
<td>317±111</td>
<td>NS</td>
</tr>
</tbody>
</table>
**Post hoc analyses**

HbA1c: *C vs DPN (P<0.0001); **C vs PDN (P<0.0001); DPN vs PDN (NS).

T-CHL: †C vs DPN (P=0.003); ††C vs PDN (P=0.02); DPN vs PDN (NS).

ACR: ¥C vs DPN (P=0.009); ¥¥C vs PDN (P=0.002); DPN vs PDN (NS).

**Table key**

ACR – Albumin Creatinine Ratio; BMI – Body Mass Index; C – Controls; DPN – Diabetic Peripheral Neuropathy; estimated Glomerular Filtration Rate; HDL – High Density Lipoprotein Cholesterol; PDN – Painful Diabetic Neuropathy; T-CHL – Total Cholesterol.
8.4.2 Symptoms and deficits (Table 8.2)

The NSP was significantly higher in patients with PDN (P<0.0001) compared to control subjects and between patients with PDN compared to DPN (P<0.0005). Positive symptoms (out of 6) were significantly greater in PDN compared to control subjects (P=0.05) and DPN (P=0.009). Negative symptoms (out of 4) were significantly greater in PDN compared to control subjects (P=0.009) and DPN (P=0.02). The McGill pain score and McGill VAS were significantly increased in diabetic patients with PDN compared with control subjects (P<0.0001) and patients with DPN (P<0.0001). The NDS was significantly increased in patients with DPN (P=0.01) and PDN (P=0.002) compared with control subjects, but there were no significant difference between DPN and PDN.
Table 8-2 Clinical neuropathy symptoms and deficits in control subjects and diabetic patients with DPN and PDN, with statistically significant differences between groups

<table>
<thead>
<tr>
<th></th>
<th>C (n=14)</th>
<th>DPN (n=20)</th>
<th>PDN (n=23)</th>
<th>DPN v PDN</th>
</tr>
</thead>
<tbody>
<tr>
<td>NDS (-/10) (Median(IQR)</td>
<td>1.4±1.5</td>
<td>3.9±3.2*</td>
<td>4.5±3.2**</td>
<td>NS</td>
</tr>
<tr>
<td>NSP (-/38) (Median(IQR)</td>
<td>1±1.5</td>
<td>2.2±2.6</td>
<td>6.3±5.5^</td>
<td>&lt;0.0005</td>
</tr>
<tr>
<td>+ve Symptoms on NSP (-/6)(Median(IQR))</td>
<td>0.5±0.9 (0±0)</td>
<td>0.25±0.6 (0±0)</td>
<td>1.6±1.8σ (1±0-3)σ</td>
<td>0.009</td>
</tr>
<tr>
<td>-ve symptoms on NSP (-/4)(Median(IQR))</td>
<td>0±0 (0±0)</td>
<td>0.3±0.4 (0.5±0-1)</td>
<td>1.1±1.2¥ (1±0-2)¥</td>
<td>0.02</td>
</tr>
<tr>
<td>McGill VAS (-/10cm)</td>
<td>0.5±1.4</td>
<td>0±0</td>
<td>5.7±2.3†</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>McGill Pain score</td>
<td>0.4±0.9</td>
<td>0±0</td>
<td>6.1±6.5††</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Post hoc analyses

NDS: *C vs DPN (P=0.01); **C vs PDN (P=0.002); DPN vs PDN (NS).

NSP: C vs DPN (NS); ^C vs PDN (P<0.0001); DPN vs PDN (P=0.0005).

+ve Symptoms: C vs DPN (NS); σC vs PDN (P=0.05); DPN vs PDN (P=0.009).

-ve Symptoms: C vs DPN (NS); ¥C vs PDN (P=0.009); DPN vs PDN (P=0.02).

McGill VAS: C vs DPN (NS); †C vs PDN (P<0.0001); DPN vs PDN (P<0.0001).

McGill Pain score: C vs DPN (NS); ††C vs PDN (P<0.0001); DPN vs PDN (P<0.0001).

Table key

C – Controls; DPN – Diabetic Peripheral Neuropathy; IQR – Inter Quartile Range; McGill VAS – McGill Visual Analogue Score; NDS – Neuropathy Disability Score; NSP – Neuropathy Symptom Profile; PDN – Painful Diabetic Peripheral Neuropathy.
8.4.3 Quantitative sensory tests (Table 8.3)

VPT, CST and WST did not differ between diabetic patients and control subjects or between patients with DPN and PDN.

8.4.4 Electrophysiology (Table 8.3)

Peroneal motor nerve conduction velocity and amplitude were significantly lower in DPN (P=0.004 and P=0.003, respectively) and PDN (P=0.0008 and P=0.001, respectively) compared with control subjects, but there was no difference between patients with DPN and PDN. Sural sensory nerve conduction velocity and amplitude were significantly lower in DPN (P=0.02 and P=0.007, respectively) and PDN (P=0.005 and P=0.04, respectively) compared with control subjects, but there was no significant difference between patients with DPN and PDN.

8.4.5 Autonomic function, IENFD and CCM (Table 8.3)

HRV-DB did not differ between diabetic patients and control subjects or between patients with DPN and PDN. IENFD did not differ between patients with DPN and control subjects but was significantly reduced in PDN compared to control subjects (P=0.05) with no difference between DPN and PDN. CNFD was significantly reduced in patients with DPN (P=0.0008) and PDN (P<0.0001) compared with control subjects with no difference between DPN and PDN. CNBD was significantly reduced only in patients with PDN (P<0.03) compared with control subjects with no difference between DPN and PDN. CNFL was significantly reduced in patients with DPN (P=0.03) and PDN (P<0.0009) compared with control subjects with no difference between DPN and PDN.
Table 8-3 Small and large fibre tests of nerve structure and function in control subjects and diabetic patients with DPN and PDN, with statistically significant differences between groups

<table>
<thead>
<tr>
<th></th>
<th>C (n=14)</th>
<th>DPN (n=20)</th>
<th>PDN (n=23)</th>
<th>DPN v PDN</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNFD (no/mm(^2))</td>
<td>34.6±5.4</td>
<td>24.5±8.4*</td>
<td>20.4±10.0**</td>
<td>NS</td>
</tr>
<tr>
<td>CNBD (no/mm(^2))</td>
<td>75.9±24.2</td>
<td>56.1±31.8</td>
<td>45.5±29.3†</td>
<td>NS</td>
</tr>
<tr>
<td>CNFL (mm/mm(^2))</td>
<td>24.3±3.6</td>
<td>19.8±5.7¥</td>
<td>15.8±7.2¥¥</td>
<td>NS</td>
</tr>
<tr>
<td>IENFD (no/mm)</td>
<td>7.6±3.4</td>
<td>5.2±3.7</td>
<td>3.9±2.9a</td>
<td>NS</td>
</tr>
<tr>
<td>(n=10)</td>
<td>(n=11)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DB-HRV (beats/min)</td>
<td>23±10</td>
<td>18±9</td>
<td>18±14</td>
<td>NS</td>
</tr>
<tr>
<td>CST (˚C)</td>
<td>27.2±1.9</td>
<td>24.0±6.6</td>
<td>23.5±6.8</td>
<td>NS</td>
</tr>
<tr>
<td>WST (˚C)</td>
<td>39.8±3.8</td>
<td>40.7±4.7</td>
<td>42.0±4.9</td>
<td>NS</td>
</tr>
<tr>
<td>VPT (volts)</td>
<td>10.7±6.6</td>
<td>17.0±14.0</td>
<td>19.5±13.1</td>
<td>NS</td>
</tr>
<tr>
<td>SSNCV (m/s)</td>
<td>47.7±5.1</td>
<td>41.9±6.2†</td>
<td>42.2±4.6**</td>
<td>NS</td>
</tr>
<tr>
<td>SSNAm (µV)</td>
<td>9.6±2.7</td>
<td>6.9±4.8€</td>
<td>5.3±5.3€€</td>
<td>NS</td>
</tr>
<tr>
<td>PMNCV (m/s)</td>
<td>45.9±3.3</td>
<td>38.3±9.3ª</td>
<td>38.4±8.7ªª</td>
<td>NS</td>
</tr>
<tr>
<td>PMNAm (mV)</td>
<td>4.9±1.0</td>
<td>2.6±1.9§</td>
<td>2.6±2.5§§</td>
<td>NS</td>
</tr>
</tbody>
</table>

Post hoc analyses

CNFD: C vs DPN (P=0.0008); **C vs PDN (P<0.0001); DPN vs PDN (NS).
CNBD: C vs DPN (NS); †C vs PDN (P=0.03); DPN vs PDN (NS).
CNFL: ¥C vs DPN (P=0.03); ¥¥C vs PDN (P=0.0009); DPN vs PDN (NS).
IENFD: C vs DPN (NS); aC vs PDN (P=0.05); DPN vs PDN (NS).
SSNCV: *C vs DPN (P=0.02); **C vs PDN (P=0.005); DPN vs PDN (P=NS).
SSNAm: 6C vs DPN (P=0.007); 66C vs PDN (P=0.04); DPN vs PDN (NS).
PMNCV: 8C vs DPN (P=0.004); 88C vs PDN (P=0.0008); DPN vs PDN (P=NS).
PMNAm: 6C vs DPN (P=0.003); 66C vs PDN (P=0.001); DPN vs PDN (P=NS).
Table Key

8.4.6 25(OH)D status (Figure 8.1)

The serum 25(OH)D level was significantly lower in PDN (24.0±14.1 ng/ml) compared to DPN (34.6±15.0 ng/ml, P=0.01) and control subjects (34.1±8.6 ng/ml, P=0.03). The odds ratio in favour of painful diabetic neuropathy was 9.8 (P=0.003 (95% CI 2.2-76.4) for vitamin D deficiency (<20ng/ml) and 4.4, (P=0.03 (95% CI 1.1-19.8)) for vitamin D insufficiency (<30ng/ml).

Figure 8-1 Graph showing 25(OH)D levels in ng/ml in C, DPN and PDN

Post hoc analyses

C vs DPN – P=NS; *C vs DPN – P=0.03; **DPN vs PDN – P=0.01.

(Overall P for Kruskal Wallis = 0.02).
8.5 DISCUSSION

Painful neuropathy is an extremely disabling condition which may be present in at least one fifth of diabetic patients (1). The treatment of this disabling condition has remained unsatisfactory with a ‘good’ response to conventional medication rated at between 30-50% pain relief (5). Available drugs are often moderately effective and their use is limited by side effects.

The aetiology of painful diabetic neuropathy is not clear. Central changes comprising of increased thalamic vascularity (32), Aβ fibre sprouting into lamina II of the dorsal horn, and reduced inhibition via descending pathways (2) together with axonal atrophy in peripheral nerves have been demonstrated in patients with painful diabetic neuropathy (33). In the present study we carefully phenotyped diabetic patients into those with painful and painless diabetic neuropathy. Painful diabetic neuropathy has also been associated with significantly greater autonomic dysfunction than painless DPN (34). Previously we have shown that the LDIflare, a measure of small fibre function, is abnormal in patients with painful diabetic neuropathy, whilst conventional quantitative sensory testing and dermal nerve fibre density did not differ from those with painless neuropathy (35). Given the results of these studies suggesting greater small fibre, particularly autonomic dysfunction we undertook detailed assessment of large and small fibre neuropathy. There was no difference for electrophysiology, quantitative sensory testing and autonomic function between diabetic patients with painful and painless neuropathy. We have also shown a greater reduction in both intraepidermal nerve and corneal nerve fibre length in diabetic patients with painful neuropathy (36). A recent detailed immunophenotyping study has shown increased axonal growth through growth associated proteins (higher GAP43/PGP) and axonal swellings, positive for
tropomyosin-receptor-kinase A and substance P in patients with painful compared to painless neuropathy (37). In the present study, although patients with painful diabetic neuropathy had greater deficits for IENFD and corneal nerve morphology compared to diabetic patients with painless neuropathy, this was not significantly different between diabetic patients with painful and painless neuropathy. Although IENFD was lower in PDN, however due to a fewer number of subjects consenting for skin biopsy compared to the overall group this did not reach significance.

Painful neuropathic symptoms tend to vary in their severity over time and therefore it is difficult to reconcile these ‘hard wired’ changes with the fluctuating symptoms. Changes in sodium channel distribution and expression, altered peripheral blood flow and glycaemic flux have also been implicated (2). Given the potential link between vitamin D and pain, together with the high prevalence of vitamin D deficiency in diabetic populations (38) we have explored the link with painful diabetic neuropathy. Two recent studies have shown a relationship between vitamin D deficiency and diabetic neuropathy (10-11), but did not assess the link between positive and negative neuropathic symptoms in these cohorts. In the present study we have shown a markedly increased risk of diabetic painful neuropathy in diabetic patients with vitamin D deficiency and insufficiency. We observed no relationship for painful diabetic neuropathy with any other risk factor for neuropathy such as glycaemic control or severity of neuropathy, which challenges the two recent studies showing a link between vitamin D deficiency and diabetic neuropathy (10-11). Previous epidemiological studies have shown a higher prevalence of painful diabetic neuropathy in South Asians despite a lower prevalence of neuropathy (1) compared to other ethnic groups and of course South Asian are at the highest risk of vitamin D deficiency (38). In our previous study
(38), 55% of our South Asian patients were severely deficient with a 25(OH)D <10ng/ml. The cohort of subjects evaluated in this study was exclusively of white European origin thus minimising ethnicity as a confounding factor.

Whilst vitamin D supplementation has been used to treat pain particularly in rheumatological conditions (39) a Cochrane review (40) concluded that there was a poor evidence base for the use of vitamin D in chronic pain. Intervention studies in diabetic neuropathy are limited. Whilst Valensi et al (41) showed benefit for painful symptoms with a topical compound (QR-333) containing quercetin (aldose reductase inhibitor effects), ascorbyl palmitate and vitamin D3, with three different compounds that may benefit painful neuropathy it is difficult to define the relative effectiveness of vitamin D3. Lee et al (23) showed benefit with oral cholecalciferol in painful diabetic neuropathy, however, this study had neither a placebo group nor was it randomised. Most recently a dramatic improvement was observed in a single type 1 patient with painful neuropathy, refractory to a range of standard therapies (24). However given that recent studies of novel drugs in the treatment of painful diabetic neuropathy have dramatically failed (42-43) with active treatment being barely superior to placebo there is an urgent need to explore new mechanisms and hence treatments for diabetic painful neuropathy.

Whilst this is a small study a major strength is the detailed phenotyping undertaken to ensure that diabetic patients with and without painful neuropathy were absolutely matched for all other measures of neuropathy and demographic characteristics, except painful symptoms. The results appear unequivocally in favour of a relationship between vitamin D deficiency and painful neuropathic symptoms. A well constructed controlled trial of vitamin D supplementation in painful diabetic
neuropathy is required in order to assess the effectiveness of a potentially simple treatment with no obvious side effects.
8.6 REFERENCES


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42. Selvarajah D, Gandhi R, Emery CJ, Tesfaye S: Randomized Placebo-Controlled Double-Blind Clinical Trial of Cannabis-Based Medicinal Product (Sativex) in Painful Diabetic Neuropathy: Depression is a major confounding factor. Diabetes Care 2010;33:128-130

9 Chapter IX - Vitamin D Deficiency is not associated with Diabetic Retinopathy or Maculopathy: A Cross Sectional study

Contribution: Uazman Alam conceived the study and subsequently designed it, extracted data, performed statistical analyses and wrote the manuscript which constitutes the basis for this chapter.

To be submitted for publication

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9.1 ABSTRACT

Experimental data and several small clinical studies suggest a possible association between vitamin D deficiency and both diabetic retinopathy and maculopathy.

We have performed a cross-sectional study in adults aged 18-80 years with Type 1 and 2 Diabetes Mellitus. The relationship between the presence and severity of diabetic retinopathy and maculopathy with serum 25-hydroxyvitamin D concentration was evaluated using logistic regression analyses in the presence of demographic and clinical covariates, such as age, race, gender, duration of diabetes, anthropometric measures (Body Mass Index (BMI) and Blood Pressure (BP)) and metabolic parameters (HbA1c and lipid fractions).

657 adults with diabetes were stratified based on retinopathy grading: No Diabetic retinopathy (NDR) (39%), Background Diabetic Retinopathy (BDR) (37%), Pre-Proliferative Diabetic Retinopathy (PPDR) (21%) and Proliferative Diabetic Retinopathy (PDR) (3%), respectively. There were no differences in serum 25-hydroxyvitamin D concentrations (25(OH)D) between the groups (15.3±9.0 vs 16.4±10.5 vs 15.9±10.4 vs 15.7±8.5ng/ml, P=NS). Logistic regression analysis demonstrated no statistically significant relationship between the severity of retinopathy and serum 25(OH)D. Furthermore, there was no difference in serum 25(OH)D between those with (n=94, 14%) and without (n=563, 86%) diabetic maculopathy (16.2±10.0 vs 15.8±9.8, p=NS) and no relationship was demonstrated by logistic regression analyses between the two variables.

This study has found no association between serum 25(OH)D concentrations and the presence and severity of diabetic retinopathy or maculopathy.
9.2 INTRODUCTION

The prevalence of diabetic retinopathy (DR) approaches 93 million people worldwide (1) and is one the leading causes of premature visual loss in the UK and worldwide (2-3). Indeed, the World Health Organization estimates that whilst DR accounts for approximately 5% of the global prevalence of blindness, the prevalence rises sharply to 15-17% in developed countries (4). Several risk factors are implicated in the aetiology of DR with hyperglycemia and hypertension showing the strongest association (5), yet interventions aimed at correcting these risk factors have demonstrated moderate success (6-7). Therefore the interactions between neural and retinal vascular dysfunction and the mechanisms resulting in retinal pathology including neovascularisation have been questioned recently (8).

Furthermore, micronutrients including Vitamin C, Vitamin E and magnesium have been postulated to play a role in DR (9).

Vitamin D deficiency has been linked to a host of cardiovascular diseases including diabetes and hypertension (10-11). Vitamin D receptor (VDR) genotypes have been associated with the cumulative prevalence of diabetic retinopathy (12). In two separate studies of the VDR gene in the French population, FokI and TaqI single nucleotide polymorphisms have been associated with DR (13-14). In a study of Caucasians with C-peptide-negative type 1 diabetes, there was a novel association between the functional FokI VDR polymorphism and severe DR (13). VDR dependent calcium binding proteins have been isolated in the human retina, particularly in the photoreceptor layer of the cones (15) and immunostaining in animal models has shown that VDR is expressed in the ganglion cells, the inner and outer plexiform layer and the photoreceptor layer (16). In an in-vitro study of
retinoblastoma tissue expressing VDR, supplementation with vitamin D resulted in a reduction of growth and apoptosis of the retinoblastoma cells (17). 1,25-dihydroxyvitamin D$_3$ (1,25(OH)$_2$D$_3$) closely regulates Vascular Endothelial Growth Factor in experimental models (18) and there is an inverse correlation of 25(OH)D with Vascular Endothelial Growth Factor, postulated to be related to tissue hypoxia (19). In a mouse model of ischaemic retinopathy, 1,25(OH)$_2$D$_3$ was shown to inhibit neovascularisation in retinal tissue (20). Vitamin D may also have a direct effect on the renin-angiotensin-aldosterone-system (RAS) and the RAS is known to be overexpressed in type 1 diabetic patients with retinopathy (21) and blockade of this system reduces DR progression (22). A vitamin D analogue (paricalcitol) has shown an improvement in microalbuminuria through a mechanism related to inhibition of RAS (23). Aksoy H et al, demonstrated an inverse correlation in a Turkish cohort between worsening diabetic retinopathy and lower 1,25-hydroxyvitamin D$_3$ (active vitamin D) in a population of 66 subjects (24). Furthermore, severe vitamin D deficiency has been shown to predict not only mortality but the development of nephropathy and retinopathy in type 1 diabetes (5). In a recent cross-sectional study of children and adolescents with type 1 diabetes, retinopathy prevalence was higher in children and adolescents with lower levels of vitamin D (25). Other cross-sectional studies which have assessed vitamin D status in relation to DR in adults have either had small numbers (26) or have been based on retrospective analysis of data collected from the National Health and Nutrition Examination Survey between 1988 and 1994 (27). However, since then the targets for glycaemia, blood pressure and lipids have changed and also this study made no assessment of diabetic maculopathy (27). Therefore we have undertaken a study to establish the
relationship between vitamin D status and the severity of DR and maculopathy in a large adult population with type 1 and type 2 diabetes.
9.3 RESEARCH DESIGN AND METHODS

All patients attending clinics were assessed for the level of 25(OH)D, irrespective of a history suggestive of vitamin D deficiency. Ethical approval was not required as the data were extracted retrospectively and did not extend beyond standard clinical practice. 25(OH)D was added as a standard routine test from June 2009 due to the high levels of deficiency noted.

9.3.1 Subjects

All participants were aged ≥ 18 years old attending clinics at the Central Manchester Foundation Trust, Manchester from August 2009 to May 2011. Those with renal impairment (eGFR <30 mL/min/1.73m² (CKD stage 4 and below), granulomatous diseases (tuberculosis, sarcoidosis etc), malabsorption syndromes (Coeliac disease, bacterial overgrowth, concomitant Orlistat treatment), pregnant and lactating women, and those currently on vitamin D supplementation were excluded from the analysis.

9.3.2 Blood pressure and Anthropometric measurements

BMI was measured as per the standard equation (mass (kg)/(height(m)))². Weight was measured with a digital scale (Seca 701, Seca, Hamburg, Germany) to the nearest 0.1kg and height to the nearest 0.1cm. Blood pressure measurements were obtained with the use of an automated device (Dinamap pro 100v2, GE Medical Systems, Freiburg, Germany) with an appropriate cuff size. A minimum of two measurements of systolic and diastolic blood pressures were made five minutes apart with the lowest reading recorded and the mean of the preceding 2 years blood pressure results were used. Metabolic variables were also recorded.
with a mean of 2 year retrospective readings for glycosylated haemoglobin A1c (HbA1c) and components of the lipid profile (total cholesterol (T-CHL), high density lipoprotein cholesterol (HDL) and triglycerides). The following measurements were taken as ‘spot readings’ at the same date as baseline 25(OH)D measurements: BMI; bone profile markers such as corrected calcium (CCa), alkaline phosphatase (ALP) and estimated glomerular filtration rate (eGFR).

9.3.3 Assessment of Demographics, Cardiovascular disease and Medications

An assessment of patient demographics, previous cardiovascular events and medications were made through analysis of medical records and an in-hospital medical record database (Diamond database, Hicom, Surrey, UK). Subject demographics extracted were age, sex, ethnicity (Caucasian, South Asian, Far East Asian and Afro-Caribbean descent), smoking status (never, previous and current) and type (type 1 and 2 diabetes) and duration of diabetes. Dates of baseline 25(OH)D were used to obtain respective retinopathy screening data. Only retinopathy screening data within 1 year of the baseline 25(OH)D and prior to vitamin D supplementation were included.

Baseline 25(OH)D status and retinopathy data were collected for 657 patients who had attended their retinopathy screening appointments. The retinopathy data were collected according to the grading criteria of the National Screening Committee (28-29). Previous studies have shown acceptable level of quality and accuracy of grading compared to expert graders within the English National Screening Committee (30). The national guidelines do not contain R1.5 or M0.5 grades and are categorised as Pre-Proliferative Diabetic Retinopathy and Diabetic
Maculopathy, respectively. These sub-gradings were used locally in screening centres and have been included. Retinopathy was graded as follows:

R0 - No Diabetic retinopathy (NDR).

R1 - Background Diabetic Retinopathy (BDR): microaneurysms, retinal haemorrhages, exudates.

R1.5 - Moderate numbers of intra-retinal haemorrhages, hard exudates >1 disc diameter (DD) from fovea, 3-6 cotton wool spots visible.

R2 - Pre-Proliferative Diabetic Retinopathy (PPDR): Venous beading or looping, deep haemorrhages visible, other microvascular anomaly visible.

R3 - Proliferative Diabetic Retinopathy (PDR): New vessel formation, vitreous haemorrhage, pre-retinal haemorrhage or fibrosis and/or retinal detachment.

M0 - No maculopathy.

M0.5 - Hard exudates within the arcades >1 disc diameter from the centre of the fovea.

M1 - Exudates <1 DD from the centre of the fovea, retinal thickening <1 disc diameter from the centre of the fovea.

P0 – No photocoagulation scarring.

P1 – Photocoagulation scarring.

9.3.4 25(OH) vitamin D Assay

Assessment of 25(OH)D was instituted in August 2009 as part of routine haematological and biochemical laboratory measurements which included HbA1c, (Complete Blood count (CBC)), Urea and Electrolytes (UE), Liver function tests (LFT), bone profile (Corrected Calcium (CCa2+), Alkaline Phosphatase (ALP), Albumin (Alb), Phosphate (phos)), and Lipid profile (Total Cholesterol (T-CHL),
High Density lipoprotein Cholesterol (HDL), Triglycerides (TRIG)). Serum was separated from whole blood and stored at -20°C until assay. The laboratory used for the biochemical assay measurements (Vitamin D Research Group Manchester Royal Infirmary, UK) was accredited to ISO 9001:2008 and ISO 13485:2003 by Lloyd’s Register Quality Assurance certificate number LRQ 4001542 and participated successfully in the Vitamin D quality assurance scheme (DEQAS). Serum 25(OH)D was measured using the IDS-iSYS multi-discipline automated analyzer (Immunodiagnostic Systems Ltd, Boldon, Tyne and Wear, UK). The assay is based on chemiluminescent technology and was performed exactly as per the manufacturer’s instructions. The cross reactivity for vitamin D₂ (of the assay) as per manufacturer’s assertion was 100% (relative to vitamin D₃) and the assay has excellent correlation to existing globally recognised assays, in combination with good sensitivity and precision (31). The reportable range of the assay is 5-140 ng/ml Inter- and intra-assay variation of the in-house control was 5.6% and 9.7% respectively.

9.3.5 Statistical analysis

Data were analysed using StatsDirect (StatsDirect, Altringham, Cheshire, UK). The data were stratified according to retinopathy (NDR, BDR, PPDR and PDR) and maculopathy (No maculopathy and maculopathy) status and a comparison of means was undertaken using either ANOVA or Krus-Kal Wallis for DR data and Unpaired t-test or Mann-Whitney U for maculopathy data. Chi Squared test were used for aetiology of diabetes, ethnicity, gender and smoking status. Logistic regression analyses were undertaken to assess the association between serum 25(OH)D levels and retinopathy and maculopathy status (either present (1) or not
present (0)), adjusting for mean values of duration of diabetes, smoking status, HbA1c, total cholesterol, HDL, triglycerides, systolic and diastolic blood pressure. Further assessment of the results comparing vitamin D categories (Severely deficient (<10ng/ml), Deficient (10-<20ng/ml), Insufficient (20-<30ng/ml) and Sufficient (>30ng/ml)) and retinopathy (NDR, BDR, PPDR and PDR), maculopathy (No maculopathy and maculopathy) and photocoagulation status (No photocoagulation and photocoagulation) were performed using chi² testing. Appropriate statistical analyses were employed depending on the normality of the data. Overall the P value was maintained at 0.05 for multiple comparison tests (Bonferoni adjustment or Dwass-Steel-Chritchlow-Fligner pairwise comparison).
9.4 RESULTS

657 patients were stratified according to their retinopathy status: NDR (n=257, 39%), BDR (n= 243, 37%), PPDR (n=135, 21%) and PDR (n=22, 3%); No Diabetic Maculopathy (n=563, 86%) and Diabetic Maculopathy (n=94, 14%). 206 (31%) of the patients had severe vitamin D deficiency with 25(OH)D levels below 10ng/ml, 284 (43%) were deficient with 25(OH)D of 10<-20ng/ml, 101(14%) were insufficient with 25(OH)D of 20-<30ng/ml. Only 65 (10%) individuals had ‘adequate’ levels of 25 (OH)D at >30ng/ml. The mean 25(OH)D for the population was 15.8 ± 9.4 ng/ml.

Table 9.1 shows demographic and metabolic data based on retinopathy grading; NDR, BDR, PPDR and PDR respectively. There were no differences in 25(OH)D status between the groups (15.3±9.0 vs 16.4±10.5 vs 15.9±10.4 vs 15.7±8.5, P=NS). Subjects were matched for age (59.8±13.8 vs 58.8±13.3 vs 60.8±10.9 vs 55.1±13.6 years), however, the duration of diabetes was significantly lower in NDR (11.3±8.7 vs 18.7±11.7 vs 21.0±9.8 vs 19.7±10.0 years, P<0.0001). The median number of metabolic and anthropometric measurements over the preceding two year period from the baseline 25(OH)D result was 4 (Interquartile range 3-5). Two year mean HbA1c (%) (8.2±1.6 vs 8.6±1.7 vs 8.9±1.6 vs 8.9±1.5, P<0.0006) showed a significantly lower HbA1c, lower systolic Blood pressure (129±13 vs 131±15 vs 134±15 vs 134±11mmHg, P=0.007) and higher eGFR (76.3±16.9 vs 75.9±17.5 vs 70.9±16.9 vs 69.0±21.6, P=0.02) in NDR (Table 9.1). There was no difference for aetiology of diabetes, ethnicity, sex, smoking status, BMI, lipid and bone parameters and diastolic blood pressure between the grades of DR. Table 9.2 shows logistic regression analyses for DR status with Odds Ratios (OR) and 95% CI. There was no correlation of DR with 25(OH)D (OR 1.00 (95% CI 0.98-
1.02), P=NS), gender or ethnicity. However, lower age (OR 0.97 (95% CI 0.96-0.99), P=0.01), longer duration of diabetes (OR 1.09 (95% CI 1.06-1.13), P<0.0001), higher HbA1c (OR 1.22 (95% CI 1.07-1.39), P=0.003) and systolic blood pressure (OR 1.02 (95% CI 1.00-1.04), P=0.02) were all associated with DR.
Table 9-1 Demographic and metabolic parameters in subgroups based on severity of retinopathy

<table>
<thead>
<tr>
<th></th>
<th>No Diabetic Retinopathy (NDR) (n=257)</th>
<th>Background Diabetic Retinopathy (BDR) (n=243)</th>
<th>Pre-Proliferative Diabetic Retinopathy (PPDR) (n=135)</th>
<th>Proliferative Diabetic Retinopathy (PDR) (n=22)</th>
<th>P value</th>
</tr>
</thead>
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<tr>
<td>Age (years)</td>
<td>59.8±13.8</td>
<td>58.8±13.3</td>
<td>60.8±10.9</td>
<td>55.1±13.6</td>
<td>NS</td>
</tr>
<tr>
<td>Duration of Diabetes (years)</td>
<td>11.3±8.7†</td>
<td>18.7±11.7†</td>
<td>21.0±9.8†</td>
<td>19.7±10.0†</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Type 2 DM (%)</td>
<td>88</td>
<td>75</td>
<td>80</td>
<td>77</td>
<td>NS</td>
</tr>
<tr>
<td>Ethnicity (White European/South Asian (%))</td>
<td>48/45</td>
<td>55/38</td>
<td>50/45</td>
<td>52/48</td>
<td>NS</td>
</tr>
<tr>
<td>Sex (male (%))</td>
<td>51</td>
<td>50</td>
<td>48</td>
<td>53</td>
<td>NS</td>
</tr>
<tr>
<td>Current Smokers/Past smokers/Never smokers (%)</td>
<td>14/25/61</td>
<td>14/33/53</td>
<td>14/26/60</td>
<td>4/23/71</td>
<td>NS</td>
</tr>
<tr>
<td>BMI (kg/m2)</td>
<td>31.6±10.6</td>
<td>31.1±7.1</td>
<td>31.9±6.4</td>
<td>31.1±6.9</td>
<td>NS</td>
</tr>
<tr>
<td>25(OH)D (ng/ml)</td>
<td>15.3±9.0</td>
<td>16.4±10.5</td>
<td>15.9±10.4</td>
<td>15.7±8.5</td>
<td>NS</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>8.2±1.6*</td>
<td>8.6±1.7*</td>
<td>8.9±1.6*</td>
<td>8.9±1.5</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>T-CHL (mmol/l)</td>
<td>4.1±1.0</td>
<td>4.2±1.1</td>
<td>4.2±1.3</td>
<td>4.3±1.1</td>
<td>NS</td>
</tr>
<tr>
<td>HDL (mmol/l)</td>
<td>1.3±0.5</td>
<td>1.4±0.5</td>
<td>1.4±0.7</td>
<td>1.3±0.4</td>
<td>NS</td>
</tr>
<tr>
<td>TRIG (mmol/l)</td>
<td>1.8±0.9</td>
<td>1.8±1.5</td>
<td>1.8±0.9</td>
<td>2.5±2.9</td>
<td>NS</td>
</tr>
<tr>
<td>eGFR (ml/min/l)</td>
<td>76.3±16.9**</td>
<td>75.9±17.5</td>
<td>70.9±16.9**</td>
<td>69±21.6</td>
<td>0.02</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>129±13***</td>
<td>131±14.6</td>
<td>134±15***</td>
<td>134±11</td>
<td>0.007</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>70±7</td>
<td>69±7</td>
<td>70±8</td>
<td>71±8</td>
<td>NS</td>
</tr>
<tr>
<td>ALP (u/L)</td>
<td>87±39</td>
<td>83±34</td>
<td>84±32</td>
<td>93±43</td>
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</tr>
<tr>
<td>CCa (mmol/l)</td>
<td>2.4±0.1</td>
<td>2.4±0.1</td>
<td>2.3±0.1</td>
<td>2.4±0.2</td>
<td>NS</td>
</tr>
</tbody>
</table>
Post hoc analyses
Duration of Diabetes – †NDR vs BDR (P<0.0001); NDR vs PPDR (P<0.0001);
                 NDR vs PDR (P=0.001).
HbA1c –            *NDR vs BDR (P=0.01); NDR vs PPDR (P<0.0001).
eGFR –             **NDR vs PPDR (P=0.04).
Systolic BP –      ***NDR vs PPDR (P=0.008).

Table key
ALP – Alkaline Phosphatase; BMI – Body Mass Index; BP – Blood Pressure; CCa – Corrected Calcium; eGFR – estimated Glomerular Filtration Rate; HDL – High-density Lipoprotein Cholesterol; T-CHL – Total Cholesterol; TRIG – Triglycerides.
Table 9-2 Logistic regression analyses for the relationship between retinopathy, 25(OH)D status and other confounding variables

<table>
<thead>
<tr>
<th></th>
<th>Odds Ratio</th>
<th>95% CI</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>25(OH)D (ng/ml)</td>
<td>1.00</td>
<td>0.98-1.02</td>
<td>NS</td>
</tr>
<tr>
<td>Age (years)</td>
<td>0.97</td>
<td>0.96-0.99</td>
<td>0.01</td>
</tr>
<tr>
<td>Duration of Diabetes (years)</td>
<td>1.09</td>
<td>1.06-1.13</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Never Smoker</td>
<td>0.48</td>
<td>0.21-1.09</td>
<td>NS</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>1.22</td>
<td>1.07-1.39</td>
<td>0.003</td>
</tr>
<tr>
<td>T-CHL (mmol/l)</td>
<td>1.09</td>
<td>0.88-1.36</td>
<td>NS</td>
</tr>
<tr>
<td>HDL (mmol/l)</td>
<td>0.88</td>
<td>0.55-1.41</td>
<td>NS</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>0.98</td>
<td>0.77-1.25</td>
<td>NS</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>1.02</td>
<td>1.00-1.04</td>
<td>0.02</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>0.98</td>
<td>0.94-1.01</td>
<td>NS</td>
</tr>
<tr>
<td>eGFR (ml/min/l)</td>
<td>0.99</td>
<td>0.98-1.00</td>
<td>NS</td>
</tr>
</tbody>
</table>

**Table key**

BMI – Body Mass Index, BP – Blood Pressure, eGFR – estimated Glomerular Filtration Rate, HDL – High-density Lipoprotein Cholesterol, T-CHL – Total Cholesterol.
Table 9.3 shows demographic and metabolic data in diabetic patients with (n=94, 14%) and without (n=563, 86%) maculopathy. There were no differences in 25(OH)D status between patients with and without maculopathy (16.2±10.0 vs 15.8±9.8 ng/ml, P=NS). Subjects were matched for age (59.1±11.5 vs 59.5±13.3 years), however, the duration of diabetes was significantly longer in patients with maculopathy (15.9±11.1 v 19.2±9.7 years, P=0.0003). Two year mean HbA1c (%) (8.4±1.6 vs 9.1±1.5, P<0.0001) and systolic blood pressure (130±14 vs 134±14mmHg, P=0.01) were significantly higher in diabetic patients with maculopathy. There were no differences for type of diabetes, ethnicity, sex, smoking status, BMI, lipid and bone parameters and diastolic blood pressure between patients with and without maculopathy. Table 9.4 shows logistic regression analyses for diabetic maculopathy status with Odds Ratios and 95% CI. There was no relationship of maculopathy status with 25(OH)D (OR 1.00 (95% CI 0.98-1.03), P=NS), age, gender, ethnicity, systolic blood pressure or lipid fractions. However, a longer duration of diabetes (OR 1.03 (95% CI 1.00-1.05), P=0.01) and higher HbA1c (OR 1.22 (95% CI 1.05-1.43), P=0.009) were associated with maculopathy status.
Table 9-3 Demographic and metabolic parameters in subgroups based on maculopathy

<table>
<thead>
<tr>
<th></th>
<th>No Diabetic Maculopathy (n=563)</th>
<th>Diabetic Maculopathy (n=94)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>59.5±13.3</td>
<td>59.1±11.5</td>
<td>NS</td>
</tr>
<tr>
<td>Duration of Diabetes (years)</td>
<td>15.9±11.1</td>
<td>19.2±9.7</td>
<td>0.0003</td>
</tr>
<tr>
<td>Type 2 DM (%)</td>
<td>82</td>
<td>80</td>
<td>NS</td>
</tr>
<tr>
<td>Ethnicity (White European/South Asian (%))</td>
<td>40/50</td>
<td>49/48</td>
<td>NS</td>
</tr>
<tr>
<td>Sex (male (%))</td>
<td>51</td>
<td>47</td>
<td>NS</td>
</tr>
<tr>
<td>Current/Past/ Never smokers (%)</td>
<td>15/28/57</td>
<td>16/27/57</td>
<td>NS</td>
</tr>
<tr>
<td>BMI</td>
<td>31.4±8.8</td>
<td>31.3±6.2</td>
<td>NS</td>
</tr>
<tr>
<td>25(OH)D (ng/ml)</td>
<td>15.8±9.8</td>
<td>16.2±10.0</td>
<td>NS</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>8.4±1.6</td>
<td>9.1±1.5</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>T-CHL (mmol/l)</td>
<td>4.1±1.1</td>
<td>4.4±1.4</td>
<td>NS</td>
</tr>
<tr>
<td>HDL (mmol/l)</td>
<td>1.3±0.5</td>
<td>1.4±0.8</td>
<td>NS</td>
</tr>
<tr>
<td>TRIG (mmol/l)</td>
<td>1.8±1.2</td>
<td>1.9±1.6</td>
<td>NS</td>
</tr>
<tr>
<td>eGFR (ml/min/l)</td>
<td>75±18</td>
<td>73±20</td>
<td>NS</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>130±14</td>
<td>134±14</td>
<td>0.01</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>71±7</td>
<td>70±7</td>
<td>NS</td>
</tr>
<tr>
<td>ALP (u/L)</td>
<td>87±53</td>
<td>100±129</td>
<td>NS</td>
</tr>
<tr>
<td>CCa (mmol/l)</td>
<td>2.3±0.1</td>
<td>2.4±0.1</td>
<td>NS</td>
</tr>
</tbody>
</table>

Table key

ALP – Alkaline Phosphatase; BMI – Body Mass Index; BP – Blood Pressure; CCa – Corrected Calcium; eGFR – estimated Glomerular Filtration Rate; HDL – High-density Lipoprotein Cholesterol; T-CHL – Total Cholesterol; TRIG – Triglycerides.
Table 9-4 Logistic regression analyses for the relationship between maculopathy, 25(OH)D status and other confounding variables

<table>
<thead>
<tr>
<th></th>
<th>OR</th>
<th>95% CI</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>25(OH)D</td>
<td>1.00</td>
<td>0.98-1.03</td>
<td>NS</td>
</tr>
<tr>
<td>Age (years)</td>
<td>0.99</td>
<td>0.97-1.02</td>
<td>NS</td>
</tr>
<tr>
<td>Duration of Diabetes (years)</td>
<td>1.03</td>
<td>1.00-1.05</td>
<td>0.01</td>
</tr>
<tr>
<td>Never Smoker</td>
<td>1.24</td>
<td>0.73-2.12</td>
<td>NS</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>1.22</td>
<td>1.05-1.43</td>
<td>0.009</td>
</tr>
<tr>
<td>T-CHL (mmol/l)</td>
<td>1.05</td>
<td>0.84-1.33</td>
<td>NS</td>
</tr>
<tr>
<td>HDL (mmol/l)</td>
<td>1.12</td>
<td>0.73-1.73</td>
<td>NS</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.19</td>
<td>0.77-1.25</td>
<td>NS</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>1.01</td>
<td>0.99-1.04</td>
<td>NS</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>1.03</td>
<td>0.98-1.07</td>
<td>NS</td>
</tr>
<tr>
<td>eGFR (ml/min/l)</td>
<td>0.99</td>
<td>0.97-1.00</td>
<td>NS</td>
</tr>
</tbody>
</table>

Table key
ALP – Alkaline Phosphatase; BMI – Body Mass Index; BP – Blood Pressure; CCa – Corrected Calcium; eGFR – estimated Glomerular Filtration Rate; HDL– High-density Lipoprotein Cholesterol; T-CHL – Total Cholesterol.
The frequencies for both severity of retinopathy, maculopathy and photocoagulation were similar between the four vitamin D categories (severely deficient (<10ng/ml), deficient (10–<20ng/ml), insufficient (20–<30ng/ml) and sufficient (>30ng/ml) (Figure 9.1, Table 9.5).

Figure 9-1 Frequencies of retinopathy, maculopathy and photocoagulation scarring categorised by 25(OH)D status
Table 9-5 Frequencies of retinopathy, maculopathy and photocoagulation scarring categorised by 25(OH)D status

<table>
<thead>
<tr>
<th></th>
<th>&lt;10ng/ml</th>
<th>10-&lt;20ng/ml</th>
<th>20-&lt;30ng/ml</th>
<th>&gt;30ng/ml</th>
<th>p</th>
</tr>
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<tbody>
<tr>
<td>No Diabetic Retinopathy</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>43</td>
<td>39</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Background Diabetic Retinopathy</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>36</td>
<td>35</td>
<td>35</td>
<td>45</td>
<td></td>
</tr>
<tr>
<td>Pre-Proliferative Diabetic Retinopathy</td>
<td>25</td>
<td>19</td>
<td>21</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>Proliferative Diabetic Retinopathy</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>3</td>
<td>5</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>No Maculopathy</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>85</td>
<td>84</td>
<td>87</td>
<td>83</td>
<td></td>
</tr>
<tr>
<td>Maculopathy</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>16</td>
<td>13</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>No Photocoagulation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>87</td>
<td>80</td>
<td>83</td>
<td></td>
</tr>
<tr>
<td>Photocoagulation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>13</td>
<td>20</td>
<td>17</td>
<td></td>
</tr>
</tbody>
</table>
9.5 DISCUSSION

Vitamin D deficiency has wide ranging implications for insulin resistance, beta cell dysfunction and hypertension and therefore provides a potential link with diabetic complications (32). Experimental studies have postulated an important link between vitamin D deficiency and retinopathy (33) and an increased risk of diabetic retinopathy has been demonstrated in the presence of VDR polymorphisms (13). However, our study has shown no relationship between the vitamin D status and the severity of diabetic retinopathy or maculopathy in a large cohort of patients with predominantly type 2 diabetes, after correcting for glycaemic control, blood pressure and lipids. We confirm that the ‘usual culprits’ of longer duration of diabetes, higher HbA1c and systolic blood pressure are directly related to retinopathy and maculopathy (1), thereby providing confidence in the validity of our data. Furthermore, the metabolic and anthropometric measurements used in the regression analysis were taken over an extended period of time as opposed to ‘spot’ readings taken in other studies (24; 26). A possible explanation for the lack of relationship between vitamin D deficiency and retinopathy could be the striking extent of vitamin D deficiency in this population, although this is consistent with our previous data (34). Thus the majority of patients demonstrated deficiency (~90%) and indeed severe deficiency (~31%). Therefore any relationship between retinopathy and adequacy of vitamin D could not be explored adequately. Only a limited number of clinical studies have investigated the role of vitamin D deficiency in DR. In one of the earliest studies Aksoy et al showed an inverse relationship between 1,25(OH)₂D₃ and worsening retinopathy, although the short half life of 1,25(OH)₂D₃ may limit the interpretation of any such relationship (24). Another smaller North American study has shown that subjects with DR, in particular PDR,
have lower levels of 25(OH)D (26). Whilst in a recent study the percentage of individuals with vitamin D deficiency increased with the severity of retinopathy, regression analysis did not demonstrate a statistically significant relationship between retinopathy severity and serum 25(OH)D concentration (27). In a prospective observational follow-up study of a cohort of type 1 diabetic patients, although severe vitamin D deficiency independently predicted all-cause mortality, it was not related to the development of either retinopathy or nephropathy (5).

In conclusion, this large cross-sectional study found no association of vitamin D status with diabetic retinopathy or maculopathy. A population with a larger spread of vitamin D levels may provide further insight into a possible association, but this may not be possible due to the high prevalence of vitamin D deficiency.
9.6 REFERENCES


30. Patra S, Gomm EMW, Macipe M, Bailey C: Interobserver agreement between primary graders and an expert grader in the Bristol and Weston diabetic


10 Chapter X - Discussion
The chronic hyperglycaemia of DM is associated with end-organ damage, dysfunction and failure, including the retina, kidney, nerves, heart and blood vessels (1). The IDF estimates an overall prevalence of DM to be 366 million in 2011, and this is expected to rise to 552 million by 2030 (2). The prevalence of DSPN is thought to be around 30% (3-4) and increases with a longer duration of the disease. When diabetes has been present for greater than 25 years the prevalence rises to approximately 50% (4).

In the same manner that identifying and treating microalbuminuria allows the prevention of diabetic nephropathy, identifying and treating abnormalities in small nerve fibre function may help to prevent diabetic neuropathy and its sequelae. Furthermore, earlier diagnosis of neuropathy would allow for the optimisation of conventional risk factors and consequently reduce the morbidity associated with neuropathy. The accurate detection and quantification of DSPN are important to identify at risk patients, anticipate deterioration of nerve function, and assess endpoints in clinical trials. Current methods lack sensitivity (QST), primarily focus on large fibres (NCS) or are invasive and require detailed laboratory processing (skin/nerve biopsy) and thus are not routinely performed within the health care system. CCM has been proposed as a surrogate endpoint of DSPN and allows for rapid, reiterative and non-invasive assessment of the corneal subbasal nerve plexus. The evaluation of corneal subbasal nerve morphology has shown significant diagnostic potential for a number of peripheral neuropathies and in particular DSPN (5-8). Corneal nerve pathology correlates well with IENF loss (9) and currently prospective natural history studies are underway will investigate the utility of CCM to assess the status and progression of DSPN in subjects with type 1
and type 2 DM (9). Furthermore, recent studies using CCM have also shown nerve fibre regeneration after simultaneous pancreatic and kidney transplant (10). The UKPDS demonstrated a significant risk reduction for cardiovascular disease and amputation for every 1% reduction in mean HbA1c (11). Intensive therapy reduced the development of confirmed clinical neuropathy by 64% in the DCCT after 5 years of follow-up (12). However, approximately 50% of patients with DSPN experience neuropathic symptoms (13) and these symptoms include; burning pain, electrical or stabbing sensations, paraesthesia, hyperaesthesia and a deep aching pain. The precise reasons for the development of neuropathic pain despite loss of sensation is not clearly elucidated but suggests that aberrations in pain signalling in the peripheral and central nervous system occur (13). Furthermore, there is an epidemic of vitamin D deficiency worldwide with over one billion people thought to be affected (14). Until recently, studies of vitamin D have largely focussed on bone health and calcium metabolism (14), however, there is an increasing body of evidence implicating the role of vitamin D in the nervous system (15-17). In experimental studies, vitamin D has been linked to the regulation of neurotrophins such as Nerve Growth Factor (NGF) and neuronal Ca2+ homeostasis, both may play a neuroprotective role in the peripheral nerve (18). Previous studies have shown improvements in diabetic and non-diabetic nephropathy with activated vitamin D administration through a mechanism linked to inhibition of the RAS system (19-21). Vitamin D is a potent RAS inhibitor (22). RAS over activity impacts on the prevalence of diabetes related complications and inhibition of this system may have a dramatic effect on the improvement of diabetic complications including retinopathy and neuropathy (23-24).
10.1 Rapid Nerve Fibre Decline in patients with Type 1 diabetes can be readily detected using Corneal Confocal Microscopy (To be submitted)

DSPN is a confirmed risk factor for debilitating lower limb amputation (25) and is one of only three independent and significant risk factors for mortality as confirmed through the ACCORD study (26). Longitudinal studies show deterioration in neuropathy over time in both type 1 diabetes (12) and type 2 diabetes by means of electrophysiology (27). However, the dramatic failure of pathogenetic treatments in DSPN (28) has been considered to be related to inappropriate surrogate endpoints and selection of patients for studies. The progression of DSPN has been shown to occur much slower than previously thought, likely due to overall improved diabetes care with better glycaemic control and the more prevalent use of ACE inhibitors (24) and statins (29), which are likely to have an impact on neuropathy. Therefore it has been proposed that subjects with deteriorating as opposed to stable nerve function should be recruited into clinical trials of DSPN (28). Delineating this cohort is essential in undertaking a well conducted clinical trial and also identifying the at risk patient for timely intervention. In this study, we have shown that CCM can be used to identify a subgroup of subjects with type 1 diabetes undergoing a more accelerated degree of small fibre decline. Therefore, in-vivo CCM provides an ideal surrogate endpoint in identifying those at risk of worsening DSPN and also for recruitment into future clinical trials of pathogenetic treatments for DSPN.

10.2 Diagnostic Utility of Corneal Confocal Microscopy and Intra-Epidermal Nerve Fibre Density in Diabetic Neuropathy (To be submitted)

DSPN is a debilitating condition which may lead to pain, foot ulceration and eventual amputation (30). Therefore it is important to accurately diagnose diabetic
neuropathy at the earliest stage of damage. However, the earliest nerve fibres to
degenerate (31-32) and regenerate (33) are the small unmyelinated fibres and
indeed they are central to the genesis of pain and the development of foot
ulceration (34). CCM is a non-invasive ophthalmic application, which is rapid, non-
invasive and readily reproducible for quantifying small nerve fibres and thereby has
been shown to diagnose and track the progression of diabetic neuropathy (5; 7;
35). CNFD has a superior sensitivity and an almost identical specificity compared
to IENFD. Subsequently, CNFD had better diagnostic utility for DSPN and
correlated better with electrophysiology than the current ‘gold’ standard of IENFD.
CCM is a valid accurate non-invasive method which is superior to skin biopsy in
diagnosing DSPN.

10.3 Enhanced Small Fibre Neuropathy in Patients with Latent Autoimmune
Diabetes in Adults (To be submitted)

Latent Autoimmune Diabetes in Adults is an under recognized (~10%) form of
diabetes which is often misdiagnosed as type 2 diabetes mellitus (36). The
pathophysiology is more closely related to autoimmune destruction of β-cells rather
than the insulin resistance found in type 2 diabetes. Patients with LADA are
autoantibody positive at a younger age of presentation, with a relatively low BMI
and have an earlier loss of glycaemic control which may influence the development
of complications (37-41). The complications of LADA have been poorly phenotyped
with only a handful of studies assessing neuropathy in this group (41-44). We
clearly demonstrate a greater degree of small fibre neuropathy in subjects with
LADA who have poorer glycaemic control. This was detected using conventional
small fibre tests such as thermal thresholds and IENFD but in particular the novel
non-invasive test of CCM. Hyperglycaemia predisposes to microvascular complications including DSPN (11; 45-46) and therefore poorer glycaemic control due to continued conventional oral hypoglycaemic treatment as opposed to earlier treatment with insulin in LADA (47) may result in the more prevalent small fibre damage. Furthermore, given the potential link between C-peptide and neuropathy, the faster decline in C-peptide levels may also predispose to more prevalent neuropathy (43). Thus patients with LADA require early and accurate assessment for neuropathy to identify those individuals who may be at greater risk for progression to early β-cell failure and loss of glycaemic control.


The purpose of this cross sectional study was to assess the prevalence of vitamin D deficiency in a diabetic population in relation to metabolic parameters. Using ≤30ng/ml as a cut off for deficiency (49) we found that 91% of our subjects with diabetes, compared to 78% age and ethnicity matched subjects without diabetes, were deficient (48). Despite particularly low vitamin D concentrations there was no association with an increase in ALP or low corrected calcium status, both of which are considered to be good metabolic indicators of vitamin D deficiency (50-51). This is an important observation as current local, national and international guidelines endorse a view that vitamin D should only be assessed if there are overt symptoms of osteomalacia or if the calcium is reduced and or ALP is elevated, in predefined high risk groups. An association was found, albeit weak with HDL cholesterol, suggesting an important relationship with lipids and a possible
explanation for the excess cardiovascular disease described in vitamin D deficiency states. Significant ethnic differences were seen with the lowest levels in South Asian/middle Eastern patients but the differences were not marked, again challenging the stereotype that vitamin D deficiency occurs predominantly in South Asians. Furthermore, there was a minimal seasonal variation in white European patients and there were no significant variation of note in South Asian/middle Eastern subjects. Seasonality is often cited as a reason for vitamin D insufficiency; however with a lack of sunlight exposure in most patients in all seasons this not likely to be a valid contributing factor. This study demonstrates an important deficiency of vitamin D which is not routinely assessed in diabetic patients and may play a role in cardiovascular disease states.

10.5 Differential effects of different vitamin D replacement strategies in patients with diabetes. (Alam U et al. J Diabetes Complications 2013. Accepted, [Epub ahead of print])

Sunlight exposure in the U.K. is insufficient in providing an adequate 25(OH)D status (52-53) and effective replacement strategies are required particularly in high risk groups. This study is an evaluation of vitamin D status at baseline and after intervention with vitamin D in over two hundred and forty participants assigned to three treatment strategies of Vitamin D_2 or D_3. Despite relatively aggressive regimens of vitamin D replacement there was significant vitamin D deficiency in a large subgroup, particularly in those treated with vitamin D_2. Furthermore, there was a significant disparity in regimens of vitamin D_2 and vitamin D_3 in relation to the relative effectiveness of the improvement in 25(OH)D concentrations which has also been suggested in previous literature (54). Current regimens do not adjust for
these differences (55) and the relative disparity of treatments may lead to inadequate replacement with consequent complications. Vitamin D shares a similar intake-response curve to other nutrients and in persons whose baseline values differ then an identical nutrient intake may or may not produce a measurable response (56). We have found reduced efficacy of vitamin D replacement in those with higher baseline vitamin D status. Differences in baseline concentrations and type of vitamin D (D$_2$/D$_3$) replacement need to be considered for future policies and guidelines. We believe our data provides considerable insights to enable more evidence based guidance to be developed.

10.6 Vitamin D Deficiency Contributes to Painful Diabetic Neuropathy (Alam U et al. Submitted Diabetes awaiting revision)

We have undertaken a detailed assessment in forty three subjects with mild diabetic neuropathy based on the neuropathy disability score with and without painful neuropathy compared to a healthy age matched control group. A previous study has shown a potential therapeutic benefit of modest intervention with vitamin D therapy (~2000 IU cholecalciferol daily) in patients with type 2 diabetes and painful neuropathy (57). Other than potential analgesic effects, active vitamin D$_3$ induces NGF (58) and NGF is known to be depleted in experimental diabetes (59). This suggests vitamin D may have a direct effect on the pathogenesis of diabetic neuropathy, particularly when considering that vitamin D$_3$ has been shown to reduce demyelination in a cuprizone experimental model of demyelination (60). The data provides a novel postulated link between vitamin D deficiency and painful diabetic neuropathy. Despite identical severity of neuropathy and metabolic control, the only abnormality noted was a lower level of vitamin D in a cohort of patients
with painful diabetic neuropathy. Furthermore, there was a significantly greater odds ratio for painful neuropathy in those with vitamin D insufficiency and in particular deficiency. This study provides a novel association between vitamin D deficiency and painful diabetic neuropathy. Recent studies of novel drugs in the treatment of painful diabetic neuropathy have dramatically failed (61-62) with active treatment being barely superior to placebo. There is an urgent need to explore new mechanisms and hence clinical trials assessing the efficacy of vitamin D replacement in painful diabetic neuropathy are urgently required.

10.7 Vitamin D Deficiency is not associated with Diabetic Retinopathy or Maculopathy: A Cross Sectional study (To be submitted).

A cross sectional evaluation of retinopathy and maculopathy in subjects with type 1 and type 2 diabetes in relation to vitamin D status, demographic, anthropometric measurement, blood pressure and blood metabolic chemistry was undertaken in six hundred and fifty seven participants. VDR genotypes have been associated with the cumulative prevalence of diabetic retinopathy (63) and the VDR is expressed in the ganglion cells, the inner and outer plexiform layer and the photoreceptor layer of the retina as demonstrated by immunostaining in animal models (64). Several risk factors are implicated in the aetiology of DR with hyperglycaemia and hypertension showing the strongest association (65). However, interventions aimed at correcting these risk factors have demonstrated moderate success (66-67). Recent studies have suggested a link with vitamin D status and DR in adolescents (68) and adults (69). Whilst we have shown there is a marked vitamin D deficiency in this population, we found no association between vitamin D status and the presence and severity of diabetic retinopathy or
maculopathy. However, well established risk factors such as HbA1c, age, duration of diabetes and systolic blood pressure were significantly associated with the severity of retinopathy. We conclude that further studies, in particular longitudinal evaluation is required to better understand the potential interplay between vitamin D and diabetic retinopathy and maculopathy.

10.8 Study limitations

There are several limitations to the current studies and should be acknowledged. A major limitation in all but the first study (chapter 3) is the cross-sectional design. Furthermore, the studies of vitamin D deficiency prevalence, differential vitamin D intervention and vitamin D status in relation to retinopathy were retrospective analyses of clinically derived data. Nevertheless, this work provides a real world analysis of the extent of this significant public health problem and detailed effectiveness of commonly used replacement regimens. This is particularly important as there is no consensus agreement on the optimal replacement strategies in vitamin D deficiency and many of the current proposed strategies lack an evidence base. The study of vitamin D status in relation to painful diabetic neuropathy provides a novel clinical association which demands further mechanistic studies and human clinical trials. A lack of association between vitamin D status and diabetic retinopathy and maculopathy in our study does not exclude causation, particularly as most of the subjects had a low level of vitamin D. Of course, epidemiological studies are liable to ecological fallacy. We also present a prospective study evaluating the ability to predict the development and progression of neuropathy through CCM. This builds on our previously published data showing that CCM can accurately stratify the severity of
neuropathy (70) and track an improvement in nerve structure (5). A large longitudinal study of CCM as a surrogate marker of DSPN is currently ongoing to establish the predictive value of this novel modality (9). There was an absence of skin biopsy data in the longitudinal study over 2 years. This therefore limits interpretation as IENFD is the current gold standard technique in assessing small nerve fibres and would allow direct comparison versus corneal nerve structure and peripheral nerve function thus allowing a more accurate prediction of the ability of CCM in the diagnosis and assessment of progression of DSPN. The restoration/regeneration of the peripheral nerve is of paramount importance and future studies of pathogenetic treatments that may show regression of neuropathy detected by CCM are of key significance. For recruitment into these studies the correct patient population and surrogate endpoints are required. CCM may indeed be a game changer if this current data are mirrored in longitudinal studies with accurate comparisons to IENFD and electrophysiology. It has been argued that the human cornea is avascular and immune privileged and therefore assessment of corneal nerves which are of course also much shorter has limited relevance to peripheral nerves and hence a dying back sensorimotor neuropathy which affects the most distal nerves first. However, the corneal subbasal nerve plexus is the most distal portion of the ophthalmic branch of the trigeminal nerve with its cell body in the trigeminal ganglion and therefore is directly comparable to the anatomy of any peripheral nerve. Furthermore, the observed alterations in corneal nerves appear to be the result of vascular-mediated effects which occur in the trigeminal ganglion and accompanying neurovascular bundle (71-72).
10.9 Future Work

In terms of CCM, the prognostic value of the corneal nerve morphometric parameters is currently being established through a number of longitudinal studies. A community based study, investigating the ability of CCM as a screening tool for DSPN would be invaluable and the rates of subsequent ‘hard’ endpoints such as foot ulceration would provide valuable evidence on the utility of the technique. Clearly subjects with LADA may have a significant predisposition to poorer glycaemic control and subsequent small fibre neuropathy and this population may require more intensive screening for diabetic complications, and CCM may provide a unique opportunity for non-invasive, reiterative screening of small nerve fibres.

Future epidemiological studies of vitamin D status relating to diabetic complications should be prospective and data derived, from multi-national sources consisting of a spread of vitamin D status including a significant proportion that have sufficient vitamin D concentrations. Of course, the definitive scientific evaluation should be through double blinded randomised placebo controlled trials of vitamin D supplementation for metabolic outcomes, diabetic complications and painful diabetic neuropathy. However, a number of obstacles must be considered; the likely effective dose as under dosing has been an issue in other studies, the type of vitamin D preparation and metabolites and the length of treatment required with the relevant vitamin D formulation. Vitamin D deficiency in relation to painful diabetic neuropathy is an important avenue which clearly needs urgent scientific and clinical trial evaluation as it represents an almost perfect treatment option with once weekly or even monthly dosing, minimal side effects and of course the potential for multiple other benefits.
10.10 Conclusion

In conclusion, this thesis provides evidence of marked vitamin D deficiency in a population with diabetes and a novel association with DSPN but not retinopathy. We also show that corneal nerve loss is readily detected with CCM and allows the identification of both progressive DSPN and early small fibre neuropathy in patients with LADA and type 1 diabetes.
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12 APPENDIX 1-STUDY RELATED DOCUMENTS
Patient information sheet - Ophthalmic markers of diabetic neuropathy

Central Manchester and Manchester Children’s University Hospitals

STUDY INFORMATION SHEET
Ophthalmic markers of diabetic neuropathy

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

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

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

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

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

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

Study Information Sheet Version 5, Date: 26/04/2011
warm and vibration on the foot. Another test that can reveal damage to the nerves is a standard ECG tracing of the heart during deep breathing and a change in blood pressure in standing up.

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

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

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

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

**DO I HAVE TO TAKE PART?**

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

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

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

**ARE THERE ANY POSSIBLE BENEFITS?**

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

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

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

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

Study Information Sheet Version 5, Date: 26/04/2011
COMPENSATION IN CASE OF INJURY

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

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

WHAT IF THERE IS A PROBLEM?

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

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.

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

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

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

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

I agree to take part in the above study.

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

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

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

Please initial box:

I for participant; 1 for researcher
12-3 Physical measurements and peripheral neuropathy assessment forms

**Check List:**
Surrogate markers of diabetic neuropathy (IGT- Diabetes- Transplant- JDRF)
Name of Patient:

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<thead>
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<th>Date</th>
<th>Notes</th>
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<td>Travel costs</td>
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<tr>
<td>Information sheet</td>
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<td>Skin biopsy</td>
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**Medical history**

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<th>Hypertension</th>
<th>Stroke</th>
<th>High cholesterol</th>
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<tr>
<td>Heart problems</td>
<td>Breathing problems</td>
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Other health issues:

**Medication**

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<th>Beta blockers</th>
<th>Warfarin</th>
<th>Synthrome</th>
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<tr>
<td>Aspirin</td>
<td>Clopidogrel</td>
<td>ACE inhibitor</td>
</tr>
<tr>
<td>A2RB</td>
<td>Statin</td>
<td>Fibrates</td>
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Other anti-hypertensive medication

**Neuropathy**

**Exclusion criteria**
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<th>Severe systemic diseases (e.g. congestive cardiac failure, rheumatoid disease, SLE)</th>
<th>Chronic renal impairment (serum creatinine &gt;250 umol/l)</th>
<th>Known peripheral vascular disease (e.g. previous bypass surgery, angioplasty or claudication)</th>
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</thead>
<tbody>
<tr>
<td>Alcohol intake &gt;21 units per week (males) or &gt;14 units/week (females)</td>
<td>Non-diabetic peripheral neuropathy</td>
<td>Aspirin and Clopidrogrel</td>
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### Ophthalmic examination sheet

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<th>Participant’s Full Name:</th>
<th>Date of Birth:</th>
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<tr>
<td>Date of Visit:</td>
<td>Investigator(s):</td>
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<td>Study &amp; Visit ID:</td>
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**IF NOT PART OF A TRIAL**

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<th>Patient referred from:</th>
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**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:

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<th>Date of Transplant:</th>
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<td>Duration on Renal Dialysis:</td>
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<td>Smoking:</td>
<td>per day</td>
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<tr>
<td>Drinking:</td>
<td>units per week</td>
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**Ophthalmic Examinations:**

Slit Lamp Biomicroscopy (draw findings):

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Corneal Aesthesiometry:

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<td>OS</td>
<td></td>
</tr>
</tbody>
</table>

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

Tear Tests:

BUT:
Schirmer Test:

Tear Sample collection:
- Schirmer strips:
- Microcappillary tubes:

Corneal Confocal Microscopy (HRT III-RCM)

<table>
<thead>
<tr>
<th>Epithelium</th>
<th>Bowman’s Layer/Nerve Plexus</th>
<th>Stroma</th>
<th>Endothelium</th>
</tr>
</thead>
<tbody>
<tr>
<td>OD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OS</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Corneal nerve parameters (values):

<table>
<thead>
<tr>
<th>NFD (no./mm²)</th>
<th>NBD (no./mm²)</th>
<th>NFL (mm/mm²)</th>
<th>NFT</th>
</tr>
</thead>
<tbody>
<tr>
<td>OD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OS</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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

Comments:

OD

OS

Comments:
13  APPENDIX 2 – Additional Analyses
Table 13-1 Baseline characteristics of the complete Control group (n=16) and the Control group with only white European participants (n=12) with significant differences.

<table>
<thead>
<tr>
<th></th>
<th>Complete Control BL (n=16)</th>
<th>White European Control BL (n=12)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>41.4±11.4</td>
<td>42.6±12.5</td>
<td>NS</td>
</tr>
<tr>
<td>Gender (Male) (%)</td>
<td>63</td>
<td>58</td>
<td>NS</td>
</tr>
<tr>
<td>Ethnicity (White European) (%)</td>
<td>75</td>
<td>100</td>
<td>NS</td>
</tr>
<tr>
<td>NDS (-/10)</td>
<td>0.5±1.1</td>
<td>0.6±1.3</td>
<td>NS</td>
</tr>
<tr>
<td>Median(IQR)</td>
<td>0(0-0)</td>
<td>0(0-1)</td>
<td></td>
</tr>
<tr>
<td>NSP (-/38)</td>
<td>0.1±0.25</td>
<td>0.1±0.29</td>
<td>NS</td>
</tr>
<tr>
<td>Median(IQR)</td>
<td>0(0-0)</td>
<td>0(0-0)</td>
<td></td>
</tr>
<tr>
<td>McGill VAS (-/10cm)</td>
<td>0.3±1.25</td>
<td>0.4±1.40</td>
<td>NS</td>
</tr>
<tr>
<td>Median (IQR)</td>
<td>0(0-0)</td>
<td>(0-0)</td>
<td></td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.6±0.3</td>
<td>5.6±0.3</td>
<td>NS</td>
</tr>
<tr>
<td>BMI (kg/m2)</td>
<td>25.7±4.2</td>
<td>24.9±4.2</td>
<td>NS</td>
</tr>
<tr>
<td>T-CHL (mmol/l)</td>
<td>5.1±0.9</td>
<td>5.1±1.0</td>
<td>NS</td>
</tr>
<tr>
<td>HDL (mmol/l)</td>
<td>1.5±0.3</td>
<td>1.5±0.3</td>
<td>NS</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>1.4±0.7</td>
<td>1.4±0.8</td>
<td>NS</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>125±21</td>
<td>127±24</td>
<td>NS</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>75±12</td>
<td>75±13</td>
<td>NS</td>
</tr>
<tr>
<td>eGFR (ml/min/1.73)</td>
<td>86±7</td>
<td>87±5</td>
<td>NS</td>
</tr>
<tr>
<td>NCCA (mBar)</td>
<td>0.6±0.3</td>
<td>0.7±0.3</td>
<td>NS</td>
</tr>
<tr>
<td>CNFD (no/mm2)</td>
<td>30.1±4.9</td>
<td>30.1±4.7</td>
<td>NS</td>
</tr>
<tr>
<td>CNBD (no/mm2)</td>
<td>36.6±15.5</td>
<td>34.1±16.1</td>
<td>NS</td>
</tr>
<tr>
<td>CNFL (mm/mm2)</td>
<td>16.9±2.8</td>
<td>16.6±2.9</td>
<td>NS</td>
</tr>
<tr>
<td>CST (`C)</td>
<td>28.6±2.1</td>
<td>28.5±2.2</td>
<td>NS</td>
</tr>
<tr>
<td>WST(˚C)</td>
<td>37.6±3.5</td>
<td>38.2±3.9</td>
<td>NS</td>
</tr>
<tr>
<td>VPT (volts)</td>
<td>5.3±4.8</td>
<td>6.0±5.5</td>
<td>NS</td>
</tr>
<tr>
<td>SSNCV (m/s)</td>
<td>49.4±3.9</td>
<td>49.2±3.9</td>
<td>NS</td>
</tr>
<tr>
<td>SSNAmp (µV)</td>
<td>21.0±10.8</td>
<td>21.6±12.1</td>
<td>NS</td>
</tr>
<tr>
<td>PMNCV (m/s)</td>
<td>47.8±3.6</td>
<td>47.2±4.1</td>
<td>NS</td>
</tr>
<tr>
<td>PMNAmp (mV)</td>
<td>6.0±1.6</td>
<td>5.4±1.0</td>
<td>NS</td>
</tr>
</tbody>
</table>
Table 13-2 Baseline characteristics of the complete Control group (n=27) and the Control group with only white European participants (n=21) with significant differences.

<table>
<thead>
<tr>
<th></th>
<th>Controls (n=27)</th>
<th>Controls White European only (n=21)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>41.0±14.9</td>
<td>42.9±16.0</td>
<td>NS</td>
</tr>
<tr>
<td>Gender (Male) (%)</td>
<td>59</td>
<td>57</td>
<td>NS</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.5±0.3</td>
<td>5.5±0.3</td>
<td>NS</td>
</tr>
<tr>
<td>HbA1c (mmol/mol)</td>
<td>36.9±3.4</td>
<td>36.9±3.4</td>
<td>NS</td>
</tr>
<tr>
<td>BMI (kg/m2)</td>
<td>26.9±4.0</td>
<td>26.9±4.3</td>
<td>NS</td>
</tr>
<tr>
<td>T-CHL (mmol/l)</td>
<td>5.0±0.8</td>
<td>5.0±0.9</td>
<td>NS</td>
</tr>
<tr>
<td>HDL (mmol/l)</td>
<td>1.6±0.4</td>
<td>1.6±0.4</td>
<td>NS</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>1.3±0.6</td>
<td>1.4±0.6</td>
<td>NS</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>128±18</td>
<td>129±20</td>
<td>NS</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>70±10</td>
<td>69±9</td>
<td>NS</td>
</tr>
<tr>
<td>eGFR (ml/min/1.73)</td>
<td>85±7</td>
<td>85±7</td>
<td>NS</td>
</tr>
<tr>
<td>NDS (-/10) Median(IQR)</td>
<td>0.4±0.8</td>
<td>0.5±0.9</td>
<td>NS</td>
</tr>
<tr>
<td>NSP (-/38) Median(IQR)</td>
<td>0.1±0.4</td>
<td>0.1±0.5</td>
<td>NS</td>
</tr>
<tr>
<td>McGill VAS (-/10cm) Median(IQR)</td>
<td>0.2±1.0</td>
<td>0.2±1.1</td>
<td>NS</td>
</tr>
<tr>
<td>NCCA (mBar)</td>
<td>0.5±0.3</td>
<td>0.6±0.3</td>
<td>NS</td>
</tr>
<tr>
<td>CNFD (no/mm²)</td>
<td>37.2±5.1</td>
<td>37.7±4.8</td>
<td>NS</td>
</tr>
<tr>
<td>CNBD (no/mm²)</td>
<td>92.0±36.2</td>
<td>92.2±34.1</td>
<td>NS</td>
</tr>
<tr>
<td>CNFL (mm/mm²)</td>
<td>26.6±3.8</td>
<td>27.0±2.8</td>
<td>NS</td>
</tr>
<tr>
<td>IENFD (no/mm)</td>
<td>10.2±3.3</td>
<td>10.2±3.3</td>
<td>NS</td>
</tr>
<tr>
<td>CST (°C)</td>
<td>28.6±2.0</td>
<td>28.5±2.1</td>
<td>NS</td>
</tr>
<tr>
<td>WST(°C)</td>
<td>36.4±2.0</td>
<td>36.6±2.2</td>
<td>NS</td>
</tr>
<tr>
<td>VPT (volts)</td>
<td>5.3±4.1</td>
<td>5.9±4.5</td>
<td>NS</td>
</tr>
<tr>
<td>SSNCV (m/s)</td>
<td>50.6±4.2</td>
<td>50.4±4.6</td>
<td>NS</td>
</tr>
<tr>
<td>SSNAmp (µV)</td>
<td>20.2±8.8</td>
<td>19.2±9.3</td>
<td>NS</td>
</tr>
<tr>
<td>PMNCV (m/s)</td>
<td>49.2±3.7</td>
<td>48.8±4.0</td>
<td>NS</td>
</tr>
<tr>
<td>PMNAmp (mV)</td>
<td>6.1±2.4</td>
<td>5.5±2.5</td>
<td>NS</td>
</tr>
</tbody>
</table>
Table 13-3 Baseline characteristics of the complete Control group (n=27) and the Control group with participants of an older age matched to the DSPN group (n=13) with significant differences.

<table>
<thead>
<tr>
<th></th>
<th>Controls (n=27)</th>
<th>Older Controls (n=13)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (years)</strong></td>
<td>41.0±14.9</td>
<td>54.4±9.9</td>
<td>0.007</td>
</tr>
<tr>
<td><strong>Gender (Male) (%)</strong></td>
<td>59</td>
<td>70</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Ethnicity (White European) (%)</strong></td>
<td>77</td>
<td>84</td>
<td>NS</td>
</tr>
<tr>
<td><strong>HbA1c (%)</strong></td>
<td>5.5±0.3</td>
<td>5.7±0.3</td>
<td>NS</td>
</tr>
<tr>
<td><strong>BMI (kg/m2)</strong></td>
<td>26.9±4.0</td>
<td>28.5±3.2</td>
<td>NS</td>
</tr>
<tr>
<td><strong>T-CHL (mmol/l)</strong></td>
<td>5.0±0.8</td>
<td>5.0±0.8</td>
<td>NS</td>
</tr>
<tr>
<td><strong>HDL (mmol/l)</strong></td>
<td>1.6±0.4</td>
<td>1.6±0.4</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Triglycerides</strong></td>
<td>1.5±0.5</td>
<td>1.4±0.6</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Systolic BP (mmHg)</strong></td>
<td>128±18</td>
<td>134±20</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Diastolic BP (mmHg)</strong></td>
<td>70±10</td>
<td>71±10</td>
<td>NS</td>
</tr>
<tr>
<td><strong>eGFR (ml/min/1.73)</strong></td>
<td>85±7</td>
<td>83±9</td>
<td>NS</td>
</tr>
<tr>
<td><strong>NDS (-/10)</strong></td>
<td>0.4±0.8</td>
<td>0.4±0.8</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Median(IQR)</strong></td>
<td>0(0-1)</td>
<td>0(0-1)</td>
<td></td>
</tr>
<tr>
<td><strong>NSP (-/38)</strong></td>
<td>0.1±0.4</td>
<td>0.2±0.6</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Median(IQR)</strong></td>
<td>0(0-0)</td>
<td>0(0-0)</td>
<td></td>
</tr>
<tr>
<td><strong>McGill VAS (-/10cm)</strong></td>
<td>0.2±1.0</td>
<td>0.4±1.4</td>
<td>NS</td>
</tr>
<tr>
<td><strong>Median(IQR)</strong></td>
<td>(0(0-0)</td>
<td>(0(0-0)</td>
<td></td>
</tr>
<tr>
<td><strong>NCCCA (mBar)</strong></td>
<td>0.5±0.3</td>
<td>0.6±0.3</td>
<td>NS</td>
</tr>
<tr>
<td><strong>CNFD (no/mm²)</strong></td>
<td>37.2±5.1</td>
<td>38.2±5.1</td>
<td>NS</td>
</tr>
<tr>
<td><strong>CNBD (no/mm²)</strong></td>
<td>92.0±36.2</td>
<td>99.7±40.5</td>
<td>NS</td>
</tr>
<tr>
<td><strong>CNFL (mm/mm²)</strong></td>
<td>26.6±3.8</td>
<td>27.8±3.8</td>
<td>NS</td>
</tr>
<tr>
<td><strong>IENFD (no/mm)</strong></td>
<td>10.2±3.3</td>
<td>9.1±2.3</td>
<td>NS</td>
</tr>
<tr>
<td><strong>CST (˚C)</strong></td>
<td>28.6±2.0</td>
<td>28.0±2.3</td>
<td>NS</td>
</tr>
<tr>
<td><strong>WST(˚C)</strong></td>
<td>36.4±2.0</td>
<td>37.2±2.0</td>
<td>NS</td>
</tr>
<tr>
<td><strong>VPT (volts)</strong></td>
<td>5.3±4.1</td>
<td>6.9±5.4</td>
<td>NS</td>
</tr>
<tr>
<td><strong>SSNCV (m/s)</strong></td>
<td>50.6±4.2</td>
<td>48.4±4.1</td>
<td>NS</td>
</tr>
<tr>
<td><strong>SSNAmp (µV)</strong></td>
<td>20.2±8.8</td>
<td>16.0±8.9</td>
<td>NS</td>
</tr>
<tr>
<td><strong>PMNCV (m/s)</strong></td>
<td>49.2±3.7</td>
<td>47.7±3.7</td>
<td>NS</td>
</tr>
<tr>
<td><strong>PMNAmp (mV)</strong></td>
<td>6.1±2.4</td>
<td>5.6±2.3</td>
<td>NS</td>
</tr>
</tbody>
</table>
**Key for Table 13.1, 13.2 & 13.3**