Laser Doppler Imager Flare (LDIflare) small fibre function

by

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Submission Declaration

I hereby declare that I am the sole author of this thesis. The published works which this thesis is based are presented alongside and I can confirm that I have been closely involved in authoring them.

I declare that the submitted material as a whole is not substantially the same as published or unpublished material that I have previously submitted, or am currently submitting, for a degree, diploma, or similar qualification at any university or similar institution. No parts of the works submitted have been submitted previously for any aforementioned qualification.

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Paper 3 The rate of decline in small fibre function assessed using axon reflex-mediated neurogenic vasodilatation and the importance of age related centile values to improve the detection of clinical neuropathy (2013). **PLoS ONE** **8**(7): e69920. Prash Vas jointly concieved the idea of the paper and jointly designed the study with Gerry Rayman . He undertook the study assessments, interpreted the data and analysed the final cohort. He took the lead role in writing the manuscript in liasion with the co-author. Gerry Rayman responded to the reviewers as the corresponding author but recieved significant contribution from Prash Vas.	Vas, PRJ Rayman, G
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Copies of these statements of contribution, signed by all co-authors can be found in Appendix A.	

Abbreviations used:

CBNFD	Corneal branch nerve fibre density
CHEPs	Contact evoked potentials
CIPN	Chemotherapy induced peripheral neuropathy
CNFD	Corneal nerve fibre density
CNFL	Corneal nerve fibre length
CPT	Current perception thresholds
DPN	Diabetic Peripheral Neuropathy
DSPN	Diabetic Sensorimotor Polyneuropathy
EMG	Electromyography
HBA1c	Glycated haemoglobin
HiTG	Triglycerides > 2.25 mmol/L
HTG	Hyperriglyceridaemia
IENFD	Intra epidermal nerve fibre density
IVCCM	In-vivo Corneal Confocal Microscopy
LDIflare	Laser Doppler Imager flare, area expressed in cm^2
LDL	Low density lipoprotein
LEPs	Laser evoked potentials
MiTG	Triglycerides between 1-7 and 2.25 mmol/L
MV-	Microvascular disease Negative
MV+	Microvascular disease Positive
NCS	Nerve conduction studies
NDS	Neuropathy disability score (scored out of 10)
NIS-LL	Neuropathy Impairment Score in the Lower Limbs
QSART	Quantitative Sudomotor Axon Reflex
QST	Quantitative Sensory Tests
SFF	Small fibre function
SFN	Small fibre neuropathy
SNAP	Sural nerve action potential
SNCV	Sural nerve conduction velocity
SNMFD	Sural nerve myelinated fibre density
TG	Serum triglycerides

Abstract:

Distal sensorimotor peripheral neuropathy (DSPN), the classical length dependent symmetrical neuropathy of diabetes, can affect up to 50% of those with diabetes leading to significant morbidity, mortality and healthcare costs. There is increasing recognition that small nerve fibres, mediating pain, temperature and autonomic function, are involved early in the course of diabetic neuropathy, preceding large fibre involvement. However, assessment small fibre neuropathy (SFN), continues to be a significant challenge. The currently available options are either invasive, subjective with poor reproducibility, may not directly assess the region of interest or are still in a research phase. Thus, there is an ongoing need for simple, non-invasive and reproducible techniques for the evaluation of SFN.

The laser Doppler imager flare (LDIflare) is one such novel, non-invasive technique of assessing small fibre function. It has been shown to be a reliable indicator of small fibre neuropathy, even when other SFN markers are either inconclusive or normal. However, the original methodology, took over an hour to complete, which limited its use as a clinical tool. The LDIflare methodology was therefore modified to overcome this limitation by incorporating an accelerated acclimatisation phase and a shorter duration of skin heating but at a higher final temperature reducing the total procedure time to under 30 minutes. The size of the resultant flares was nearly twice as large compared to the older method while demonstrating similar group differences in those with and without clinical neuropathy. Assessment of the LDIflare in healthy volunteers (n=94) demonstrated significant inverse relationship of LDIflare size to age ($r=-0.42$, $p<0.0001$) but not with other anthropometric or metabolic factors except for fasting triglycerides ($r=-0.36$, $p<0.0001$). Furthermore, the LDIflare possessed a

sensitivity of 77%, specificity of 90%, positive predictive value (PPV) of 82% and negative predictive value of (NPV) 87% for the detection of clinical neuropathy.

Recent observations, exploring into the aetiopathogenesis of diabetic neuropathy, have suggested that triggers for neuropathy development in the two main forms of diabetes may be different. Small fibre function (SFF) in individuals with type 1 diabetes with (MV+, n=24) and without (MV-, n=24) renal and retinal microvascular disease, but all without clinical neuropathy was assessed using the LDIflare. The finding of abnormal SFF only in the MV- group suggests that direct microvascular damage is an early aetiopathogenic factor in type 1 diabetes. Furthermore, in another study of normal glucose tolerant individuals not meeting the criteria for metabolic syndrome, SFF was observed to be abnormal with increasing fasting triglyceride concentrations. This is suggestive that hypertriglyceridaemia may play a pathogenic role in the development of neural dysfunction, and may partly explain the presence of neuropathy early in the course of type 2 diabetes, when significant hyperglycaemia is not a factor.

The LDIflare in its current modification is a novel, reliable, non-invasive measure and objective method of detecting small fibre neuropathy. It has good reproducibility and offers excellent accuracy for the detection of clinical neuropathy. The age based normative values allow for a clear distinction of abnormal results. While further comparative studies between the LDIflare and modern markers of SFN are desired, the studies included in this submission support the use of the LDIflare technique to investigate abnormalities in the peripheral nervous system, in particular small nerve fibres, in research but also in clinical domains.

Laser Doppler Imager Flare (LDIflare) small fibre function

1.0 Introduction

Diabetes Mellitus is a complex metabolic disorder characterised by defects in insulin action, insulin secretion or both, resulting in disturbances of carbohydrate, fat and protein metabolism [1]. The burden of diabetes has been rapidly increasing, affecting up to 347 million individuals worldwide, with prevalence rates reaching 9.8% in men and 9.3% in women [2] . In addition, a significant number live with the condition undiagnosed and it is projected that with an aging population and the continued rise in obesity, the global prevalence of diabetes will reach 530 million individuals by the year 2035. At the same time, an increasing number of individuals are being diagnosed with prediabetes, a biochemical state where the glycaemic variables that are higher than normal, but lower than the thresholds for diabetes, many of whom will eventually develop diabetes [3]. The prevalence of prediabetes is projected to reach 470 million by year 2030, a number not dissimilar to projected diabetes figures for same time period [3-4]. Given the enormity of the numbers affected, diabetes is perceived as a major public health challenge with substantial healthcare costs associated with managing the condition and its complications [5-6]. A recent study from the United Kingdom (UK) put the total cost of managing diabetes in the year 2010/2011 at around £24 billion while estimating that by the year 2036/2037, these costs will rise to nearly £40 billion [5]. Figures emerging from other countries also demonstrate a similar or higher degree of economic burden and the overall picture is one of high cost with continued individual and societal suffering [6-7].

The chronic complications accompanying diabetes can be divided into macrovascular – those affecting the larger blood vessels such as myocardial

infarction, stroke or peripheral vascular disease and, microvascular – those affecting the small blood vessels, leading to the development of neuropathy, nephropathy and retinopathy. Neuropathy is perhaps the most common microvascular complication, ultimately affecting more than 50% of those with diabetes [8]. Diabetic Neuropathy (DN) is a collective term for a heterogeneous group of conditions that affect different parts of the nervous system and present with diverse clinical manifestations [9]. Although any part of the nervous system can be affected, the most common presentation is the length dependent distal sensory predominant sensorimotor neuropathy (DSPN) which accounts for over 80% of the cases.

The consequences of DN are significant – it can lead to considerable morbidity [10] and is increasingly recognised to confer an increased mortality risk [11-12]. Development of DSPN in particular, may lead to neuropathic pain, foot deformities, loss of protective pain sensation and foot ulceration. In the latter group, development of infection may increase the risk of a future lower extremity amputation [13]. The life expectancy of patients with neuropathic foot ulceration is approximately 50% at 5 years, an outcome worse than many of the major cancers including breast, colon and prostate [14]. Like diabetes in general, the healthcare costs associated with DN and its associated complications are significant. In the year 2003, it was estimated that the direct cost of managing DSPN in the UK was £252 million[15], but a more recent estimate for the year 2010/2011, commissioned by the charity Diabetes UK, which included the cost of all diabetes related neurological conditions and foot disease, has put the figure closer to £1.2 billion [6]. At the moment, there are no pathogenesis-based treatments available for diabetic neuropathy, apart from good glycaemic control.

Although many putative agents with disease modifying potential have been investigated, none are currently licensed for the treatment of DN/DSPN [16].

Therefore, early recognition is vital. Findings from the Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications (DCCT/EDIC) studies have demonstrated that the clinical course of diabetic complications in type 1 diabetes, DSPN in particular, may be influenced by improved glycaemic control [17]. It is thought that the development of DSPN has a temporal trend resulting in a clinical spectrum ranging from early, almost undetectable, asymptomatic changes to an advanced stage with loss of protective sensation [18-20]. The early neuropathic changes by virtue of being asymptomatic are unlikely to be recognised by patients and clinicians alike. Unlike retinal imaging for diabetic retinopathy and microalbuminuria for nephropathy, no simple marker indicative of early neural damage exists for DSPN. Commonly used neuropathy screening devices such as the 10gm monofilament or the 128Hz tuning fork only detect advanced neuropathy and lack sensitivity for early neuropathy [21]. It has been suggested by experts that more advanced neuropathy is less likely to be amenable to therapy [16, 22]. Recognising neuropathy earlier may thus increase the 'window of opportunity' - allowing for intensification of diabetes therapy, management of associated vascular risk factors and for institution of interventions aimed at arresting the progression to more advanced neuropathy. Therefore, research aimed at recognition of early diabetic neuropathy and amelioration of its progression is considered an urgent clinical need.

2.0 Small Fibre Neuropathy in Diabetic Neuropathy : Significance and Emerging focus

2.1 The nerve fibres

The Nobel laureates Gasser and his mentor Erlanger in studies of mammalian neural tissue, observed neurologic structural deficits by cautious sectioning of nerve fibres and subsequently classified nerve fibres into A, B and C groups. Subtypes of the A-fibres - A -alpha (afferent or efferent fibres), A -beta (afferent or efferent fibres), A -gamma (efferent fibres) along with B- fibres are considered as large fibres on account of their generous myelination. These are fast conducting and mediate sensory modalities such as proprioception, touch, pressure and vibration alongside mediating afferent and efferent motor function. Small nerve fibres are slow conducting and comprise of the thinly myelinated A -delta and the non-myelinated C-fibres (Table 1). Overall, the small fibres make up between 55% - 70% of the peripheral nerves; the A-delta fibres constitute ~80% of primary sensory nerves sprouting from dorsal root ganglia, whereas the C -fibres make up ~20% of the primary afferents [23]. Together, these nerve fibres mediate pain, temperature perception and autonomic function. Thus, small fibre neuropathy (SFN) may lead to generation of nociceptive pain (neuropathic pain), abnormalities of temperature perception, impairment of vasodilatory responses and also impaired sweating, heart rate variability, abnormal blood pressure response to stimuli and symptoms of visceral dysautonomia.

Table 1**.** Classification of Nerve Fibres

Type	Size	Conduction	Innervation	Myelination
Large Nerve fibres				
A-alpha	13-20 micrometers	70-120 m/s	Muscular spindles and tendon organs , Limb proprioception	Yes
A-beta	6-12 micrometers	35-75 m/s	Limb proprioception, vibration, pressure, capture touch receptors	Yes
A-gamma	4-8 micrometers	15-40 m/s	touch, pressure, motor neurons to muscle spindles	Yes
Small nerve fibres				
A-delta	1-5 micrometers	4-30 m/s	Mechanical sharp pain Cold sensation	Yes, thinly
C-Fibres	0.2-1.5 micrometers	0.5-2 m/s	Warm sensation , Thermal pain, mechanical/ burning pain Autonomic fibres	No

m/s= metres/second

2.2 Diabetic Neuropathy and Small Nerve Fibres

There is increasing recognition that small nerve fibres are involved early in the course of neuropathy development in both forms of diabetes [24-27] and also in prediabetic states [28-29] . Although the precise sequence of nerve fibre damage remains unclear, mainly because of the lack of longitudinal studies, assessment of small fibre parameters in crossectional cohorts has suggested that small fibre change *may* precede large fibre involvement [27, 30-32]; however these findings are not consistent [18]. Therefore, small fibre neuropathy (SFN) assessment techniques have an important role in not only in characterising the presence of diabetic neuropathy but also in the recognition of early diabetic neuropathy.

In addition to the potential role in the diagnosis of early diabetic neuropathy, understanding small fibre neuropathy may be important in unravelling the aetiopathogenesis of diabetic neuropathy. There is emerging evidence that the pathogenesis of neuropathy differs between type 1 and type 2 diabetes. Ultrastructural studies have suggested that paranodal degeneration seen in neuropathy of type 1 diabetes is not seen in type 2 diabetes, with axonal loss and atrophy expressed to a larger extent in the former [33]. While improvement in glycaemic control has shown to significantly reduce the risk of diabetic neuropathy in type 1 diabetes [17, 34], there is only a modest improvement in neuropathy risk reduction in type 2 diabetes [9, 34]. Therefore, factors in addition to hyperglycaemia are thought to potentially contribute diabetic neuropathy, especially in type 2 diabetes, and may also be responsible for driving the earliest damage. This is supported by a growing body of literature linking prediabetes and neuropathy, which is typically of the small fibre type [35]. The metabolic, immunologic an clinical correlates of SFN may provide with insights into how

neuropathy develops and may also in time allow for the development of interventions designed to alter the course of neuropathy.

At the same time, it is felt by experts that small fibre assessments may be better suited as future surrogate endpoints in neuropathy clinical trials than the current large fibre based measures [9, 22]. Longitudinal follow up of diabetic subjects has suggested the progression of large fibre neuropathy is minimal during a 1-5 year follow up period, typical of most studies, but that the progression of small fibres may be more rapid [36-38]. However, at what stage such a progression in a particular small fibre marker constitutes a meaningful, clinically relevant change is also unclear. This particular area needs attention as until this occurs, their relevance as true 'clinical endpoints' for research will remain challenged.

Despite significant research and many molecules with putative disease modification potential entering into phase III trials, there are currently no licensed treatments for diabetic neuropathy. The most significant reason for failure in clinical trials has been the lack of demonstrable treatment efficacy. While the reasons for this have been greatly debated including poor subject selection, short duration on intervention, and lack of homogeneity between study groups [16, 22, 39-40], a recent line of enquiry has been questioning the appropriateness of study endpoints recommended by regulatory authorities, which are predominantly clinical and large fibre based by which time the neuropathy may not be reversible [41-42] . It has been advocated by some that small fibre based endpoints may be superior, as these fibres are damaged early, and may be therefore, potentially more amenable to regenerative or corrective therapy [22, 43]. However, there is no current consensus on which of the small fibre techniques are best suited. As a consequence of the above factors, a significant clinical and research focus is

emerging into the understanding of small fibre neuropathy and into its detection techniques.

2.3 Diagnostic Criteria for Small Fibre Neuropathy

The diagnostic criteria of SFN in diabetes mellitus were reviewed by the Toronto Consensus on Diabetic Neuropathy panel, who proposed a grading of SFN into : i) Possible SFN - length dependent symptoms and/or clinical signs ii) Probable SFN - symptoms and signs as above and normal sural nerve electrophysiology, iii) Definite SFN - symptoms and signs as above, normal sural nerve electrophysiology and altered intra-epidermal nerve fibre density at the ankle and/or abnormal quantitative sensory testing of thermal thresholds at the foot [31]. 'Subclinical SFN' was considered if there were abnormalities on a validated SFN marker but no symptoms and signs.

2.4 Techniques for Small Nerve Fibre Assessment

Detection of small fibre neuropathy, however, continues to be a significant challenge. DSPN in particular, is typically suspected when symptoms such as numbness, burning/throbbing pain, hyperaesthesia, hyperalgesia or parasthaesia are reported. However, early diabetic neuropathy can be asymptomatic and typical symptoms are often not recognised until the abnormalities are well advanced. Clinical examination for reflexes, proprioception, light touch, vibratory perception and motor strength is normal in *pure* SFN. Therefore in the absence of clinical signs and symptoms to guide the diagnosis, there is a need for precise and reliable testing methodologies for detecting and confirming SFN. Nerve conduction studies are widely used to assess and quantitate nerve function but they detect only large fibre function, and are therefore, not suitable for the initial assessment

of SFN. Their role in SFN is complementary - to detect or exclude any associated large fibre component [31, 44]. Recognising the importance of detecting such early neuropathy, the Toronto Diabetic Neuropathy Consensus document has recommended the use of small fibre testing, using a validated marker, when nerve conduction assessment is normal [31].

Until recently, most diabetic neuropathy guidelines and position statements paid limited cognizance to the importance of small fibre neuropathy. This was primarily because most small fibre techniques were still in a research phase with limited clinical utility. The 2017 position statement on diabetic neuropathy by the American Diabetes Association has recently recommended using pinprick and temperature sensation as bedside screening tests for small fibre function [9]. However, over the last three decades, significant progress has been made in the development of techniques which allow for more detailed, clinically valuable, small fibre characterisation and measurement [45]. They are divided into two main groups:

I) Structural methods - allowing for morphometric analysis or quantitative measurement of the small nerves. Examples include skin biopsy and in-vivo corneal confocal microscopy (IVCCM).

and

II) Functional markers - these assess function of the small fibres, mainly indirectly. Many functional techniques are qualitative but quantitative methodologies are available and beginning to be more widely utilised. Examples include quantitative sensory testing (QST) for thermal and pain thresholds,

contact heat evoked potentials, sudomotor assessment techniques and the laser Doppler imager flare (LDIflare) [45].

Table 2 summarises the characteristics, principles and limitations of some of the main small fibre assessment tools.

2.4.1 The case for newer small fibre techniques

Skin biopsy with measurement of the intra-epidermal nerve fibre density (IENFD) has been recently proposed as a 'gold standard' technique for the assessment of SFN [46-47]. It allows for precise structural quantification and has well established worldwide normative data [48]. However, its invasive nature is a limitation, especially for application in large longitudinal studies requiring repetitive assessments. Quantitative sensory testing (QST) of temperature and pain thresholds is a non-invasive technique widely used in clinical practice as well as research studies [46, 49-51]. Well recognised limitations include the subjective, psychophysical nature of testing and its inability to distinguish between a peripheral and a central lesion. Additionally and specifically in relevance to diabetic neuropathy, it has been shown that there is poor relationship between quantitative sensory tests and morphometric indicators of small nerve fibre damage and repair [52]. In-vivo corneal confocal microscopy (IVCCM) with visualisation small nerve fibres in the subbasal nerve layer of the Bowman layer of the cornea has received significant recent attention [53-54]. It is easy to acquire the images, non-invasive, has been validated against IENFD [55-56] and possesses good sensitivity and specificity for the detection of DSPN [56-57] . In addition, multi-centre reference values have been recently established [58]. A major limitation is that IVCCM detects changes that could be considered 'far

away' from the distal leg. Moreover, morphometric analysis of the corneal epithelial nerve terminals is not possible and variation in data has been reported, especially in cloudy and fibrotic corneas [59]. Furthermore, it is unclear which among the three parameters measured using IVCCM are best suited for neuropathy assessment. While other SFN techniques are available and summarised in Table 2, many are still in a research validation phase with limited clinical experience.

Another challenge for SFN techniques is their ability to detect meaningful improvement with treatment. Although IENFD has been shown to improve in subjects receiving intensive lifestyle treatment for impaired glucose tolerance [60] and IVCCM has shown improvement in treated type 2 diabetes [61] and after pancreatic transplantation [62-63], the cohorts studied were small or the within group changes not significant. Thus, there is an overall need for newer SFN measures that are simple, non-invasive, reliable and with an ability to detect a response to treatment.

Table 2. Overview of the main tests used in small fibre assessment currently.

Structural tests for SFN							
Test	**Type**	**Technique**	**Equipment Needed**	**Time to acquire results**	**Normative Data**	**Operating Characteristics for DSPN**	**Limitations**
Skin Biopsy	Invasive (minimally), quantitative	Measurement of intra-epidermal nerve fibre density	Sterile equipment for biopsy, access to trained personnel and laboratory	Procedure takes 5-10 minutes but takes a few days to get the results back.	Worldwide normative Data present	Published sensitivity to detect DSPN is between 60% and 95% and specificity between 90% and 95%	Challenging to use in prospective studies of very large cohorts, infection risk at site of biopsy Needs operator training and access to high quality pathology services
Sural nerve biopsy	Invasive, quantitative	Ultrastructure and morphometric analysis of sural nerve biopsy specimens	Experienced operator who can perform biopsy and access to pathologist and at times, electron or confocal microscope	Procedure may take up to 45 minutes. Results usually take a few days.	None available	None available	Infection, pain and hypoesthesia at biopsy site
Corneal Confocal Microscopy	Non-invasive, quantitative	Measurement of nerve parameters of the corneal sub-basal layer	Corneal scanning confocal microscope, trained technician	Image acquisition takes 5-10 minutes. Results available immediately if automated counting used	Worldwide normative Data present	Reported sensitivity of 85% and specificity of 84%	Surrogate marker of DSPN rather than a direct indicator. Previously reliant on manual counting but newer automated methods emerging. Unclear which of the three- CNFB, CNFL or CBNFD best representative/predictive of DSPN Cost of equipment is significant

Functional tests for SFN							
Quantitative sensory testing (QST)	Non-invasive, quantitative	Computerised measurement of thermal thresholds and heat pain thresholds	Computerised assessment device, temperature controlled laboratory and a trained technician	Takes about half an hour but could take longer, depending on subject concentration	Commercial normative data present from the bigger manufacturers.		Psychophysical test- results are dependent on subject compliance and attention. Complex testing protocols present . Varying reproducibility depending on experience of the unit undertaking testing.
Laser Doppler imager Flare (LDIflare)	Non-invasive, quantitative	Measurement of the axon-reflex mediated flare response as a marker of small fibre function	Laser Doppler imager, temperature controlled room, operator with experience	Image acquisition took ~1 hour with the older method. Newer method takes approximately 25 minutes (discussed within the thesis). Results available immediately	One site normative values determined at a single centre. Larger data set of normative valves desired	For the newer technique: Sensitivity of 70-75%, specificity of 66-85% , positive predictive value of 74%, and negative predictive value of 86%	Dependent on the microcirculation. Patients need to have no significant macrovascular distal circulatory impairment.
Current perception threshold (CPT)	Non-invasive, quantitative	Low current intensity stimulation of the small nerve fibres at frequency of 250 Hz for A-delta fibres and the 5 Hz for C-fibres.	Neurometer device temperature controlled room and a trained technician	Takes about half an hour but could take longer, depending on subject concentration	None available. Most studies have included age matched controls for comparison.	None available	Requires active patient co-operation. Like QST, therefore reproducibility has been a challenge and other methodological challenges persist (such as what frequency to use. Not widely available.

Contact Heat Evoked Potentials (CHEPs)	Non-invasive, quantitative	Measure cerebral responses to thermal stimuli mediated by A-delta fibres	Needs thermal threshold testing first. Then small discs are placed on the head to record signals received to the brain from application of 10 to 20 short (a fraction of a second) heat or cold stimuli at a particular point of interest (face, arm or leg)	Takes about half an hour but could take longer, depending on subject concentration	Multicentre normative data on 226 adult subjects are available	The AUC for DSPN detection in a small sample has been estimated at 0.778.	Requires active patient co-operation. Like QST and CPT, therefore reproducibility has been a challenge. Not widely available. Also unclear if both A-delta and C-fibres are assessed. Participant discomfort is another major limitation of use.
Microneuro-graphy	Minimally invasive, semi quantitative	Measurement of Single fibre recordings from peripheral axons	Skilled operator and extensive equipment list. Preserve of a large neurophysiology lab rather than clinic based procedure.	May take up to 3 hours to get a satisfactory recording.	None available	None available. Considered by EFNS to possess grade A evidence for assessing function of the A-delta fibre pathways in patients with neuropathic pain	Still primarily a research tool. May have a role in assessment of neuropathic pain rather than early neuropathy. Expensive and needs skill to elicit responses. Patient cooperation is also extensively required.
Laser Evoked Potentials (LEPs)	Non-invasive, quantitative	Radiant heat generate by laser selectively excites free nerve endings in the superficial skin layers activating nearby A-delta and C -fibre nociceptors	CO2-laser stimulator, technician with experience and a temperature controlled room ideally.	May take up to 1 hour to complete the procedure and ensure no artefacts presents in readings gained	Single centre normative values available on 100 subjects. No decade specific data reported.	None available. Studies have used age matched control data.	Limited availability. May be useful in demonstrating reduced function but unable to detect enhanced transmission as found in hyperalgesia. Small changes in pain sensitivity are not easily detectable with LEP

Quantitative sudomotor axon reflex test (QSART)	Sudomotor Non-invasive, quantitative	Information on skin autonomic function and evaluation of postganglionic sudomotor function using acetylcholine iontophoresis	Purpose built lab, iontophoresis and sudomotor quantification equipment.	45-60 minutes to complete.	Normative data available from specific centres for QSART. A commercially available device QSWEAT is also available	No specific data available for DSPN but has been widely used, especially in the Rochester Diabetic Neuropathy study	Requires precautions for electrical safety and small risk of minor local injury to the skin
Thermo-regulatory sweat test (TST)	Sudomotor Non-invasive, semi-quantitative	When core temperature rises beyond a hypothalamic thermoregulatory set point (>38°C), sweating occurs	Needs a laboratory and a digital camera	90-120 minutes to perform correctly. Maximal sweating is achieved within 30–65 minutes.	Unclear	Helpful data on the TST available in DSPN mainly from the autonomic lab at the Mayo Clinic, Rochester USA.	Patients may not be able to tolerate 60 minutes of warming up
Sympathetic Skin Response	Sudomotor Non-invasive, quantitative	Information on skin autonomic function and evaluation of postganglionic sudomotor function using electrodermal activity	Purpose built lab, SSR equipment includes electrodes.	45-60 minutes to complete.	Normative data available from specific centres but usually has been derived from a small normative group	Minimal data only available in DSPN. Some helpful data in diabetic autonomic neuropathy and bladder dysfunction.	Limited availability, needs expertise and experience to test correctly. Popular in Japan.
Sudoscan®	Sudomotor Non-invasive, quantitative	Testing is based on stimulation of sweat glands by a low-voltage current (<4volts) representing a electrochemical reaction between electrodes and chloride ions,	Just the Sudoscan® device	Takes less than 5 minutes	Comes with inbuilt normative data. Limited experience at the moment	Increasing literature now available of its use in DSPN. Similar AUC as IENFD (0.761) in one study. For Cardiac autonomic neuropathy sensitivity was 65%, specificity 80%.	Still limited availability . Needs more detailed validation work for different ethnicities. Reproducibility is yet to be established. Longitudinal data is lacking.

Neuropad®	Simple qualitative indicator of sudomotor dysfunction	Simple sticker which changes colour in the presence of sweating.	Relatively cheap and easy to avail. Cost is approximately £8/pad.	Takes less than 10 minutes	Qualitative, does not need normative data.	Lots of available literature and has been validated against IENFD. In one study, Neuropad had a sensitivity 85% and specificity of 45% for detection of clinical DSPN.	Difficult to interpret when there is partial change in colour though. One centre has published data on semi-quantification using digital imaging of the Neuropad®.
Cardiovascular Autonomic tests	Quantitative and assess cardiac autonomic neuropathy	Complex laboratory based testing protocols	Special labs, equipment and expertise in testing. Usually undertaken by Neurophysiologists	Takes up to 90-120 minutes. Simpler algorithms may take less time (30-45 minutes)	No worldwide reference values, each laboratory tends to have its own values		Not easily available to frontline clinicians. Mainly a tool for research in DSPN or helpful in extremely atypical cases.

3.0 The LDIflare technique

The laser Doppler imager flare (LDIflare) is a novel non-invasive method for the detection of small fibre neuropathy, based on measurement of the axon-reflex mediated vasodilatory flare response, using skin heating as the nociceptive stimulus. This neurogenic vasodilatory response has been shown to be directly related to small nerve fibre and in particular, c-fibre function [64-66]. The axon reflex is part of the Lewis triple response which was first described by Sir Thomas Lewis in 1924 [67]. This response can be elicited by drawing a sharp object (a key) across the skin and comprises of three phases:

1) Red reaction or the Flush: This occurs in the first few seconds post injury and visually appears as a red line at the site of injury. It is due to capillary dilatation secondary to histamine release.

2) Wheal: This is localized (to around the region of the redline) and is due to increased capillary permeability and exudation of fluid from dilated arterioles, capillaries and venules driven by the local histamine release and appears approximately 30 seconds to 1 minute after the initial injury.

3) Flare: Spreading redness, extending beyond the redline, secondary to the axon flare reflex.

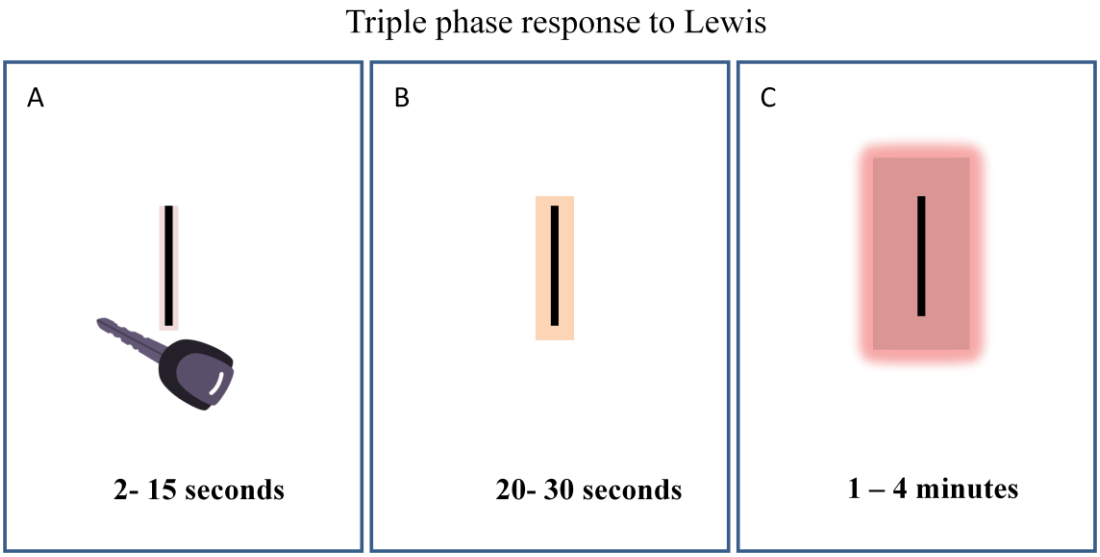


Figure 1: The Lewis Triple Response.

In panel: A) the skin is injured with a key resulting in a pink flush; B) Wheal developing immediately as a direct response and C) Flare response secondary to activation of the local small fibre network.

3.1 Principle of LDIflare technique

Stimulation of nociceptive C-fibres leads to the simultaneous conduction of impulses orthodromically to the spinal cord and antidromically to the nerve endings abutting arterioles, where the release of neurovascular transmitters causes vasodilatation and increased blood flow to injured tissues [68]. Substance P and Calcitonin Gene Related Peptide (CGRP) are the two main neurovascular transmitters that are released from C-fibres and cause vasodilatation of the arteriolar blood vessels innervated by the neural network [69]. Substance P acts on its preferred receptor, the NK1 receptor and on endothelial cells to cause plasma leakage [70]. Substance P also degranulates mast cells, causing the release of mast cell amines. These together

with the stimulatory activity of CGRP acting on its receptor contribute to arteriolar dilatation [71-72]. The neural course of the axon- reflex is illustrated in Figure 2. The resultant flare response may be detected visually, and objectively quantified using the laser Doppler imager (LDI).

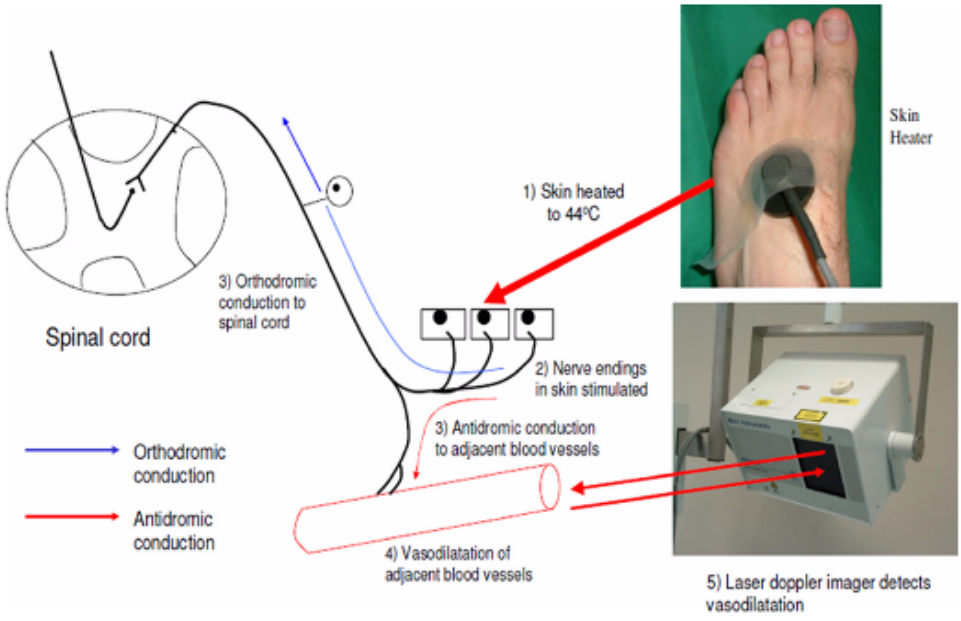


Figure 2**:** Principle of the LDIflare technique.

The course of the nerve axon-reflex and the antidromic conduction into the adjacent blood vessels is picked up by the laser Doppler imager.

3.2 Interpreting the results

Baseline perfusion

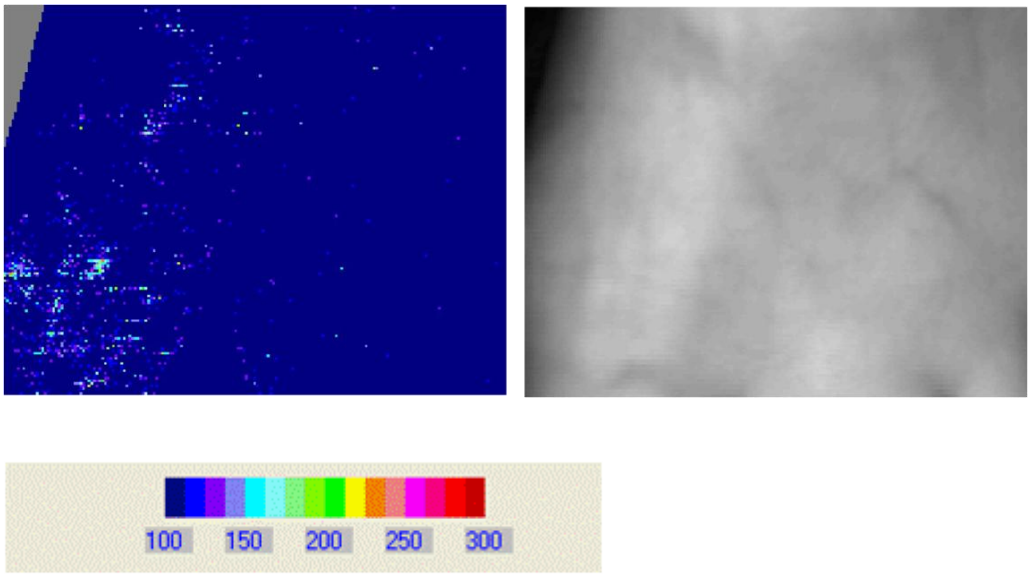


Figure 3: Baseline perfusion in the foot skin prior to a skin stimulus being applied. On the right, is a representative B&W image of the scanned area. Using the palette provided one can see that the perfusion in this image is <100PU in most of the scanned area.

Post Stimulation change in perfusion and flare response

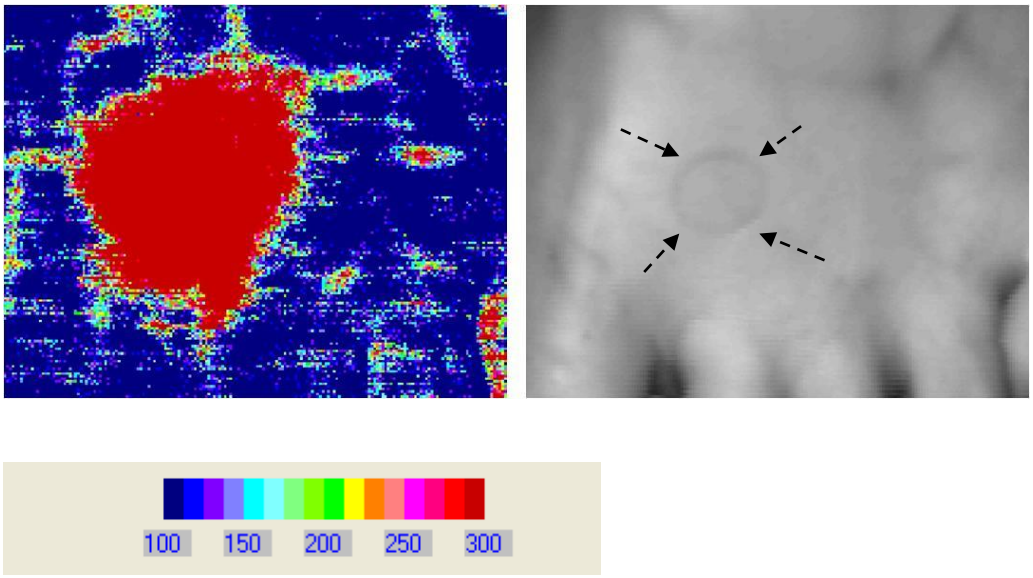
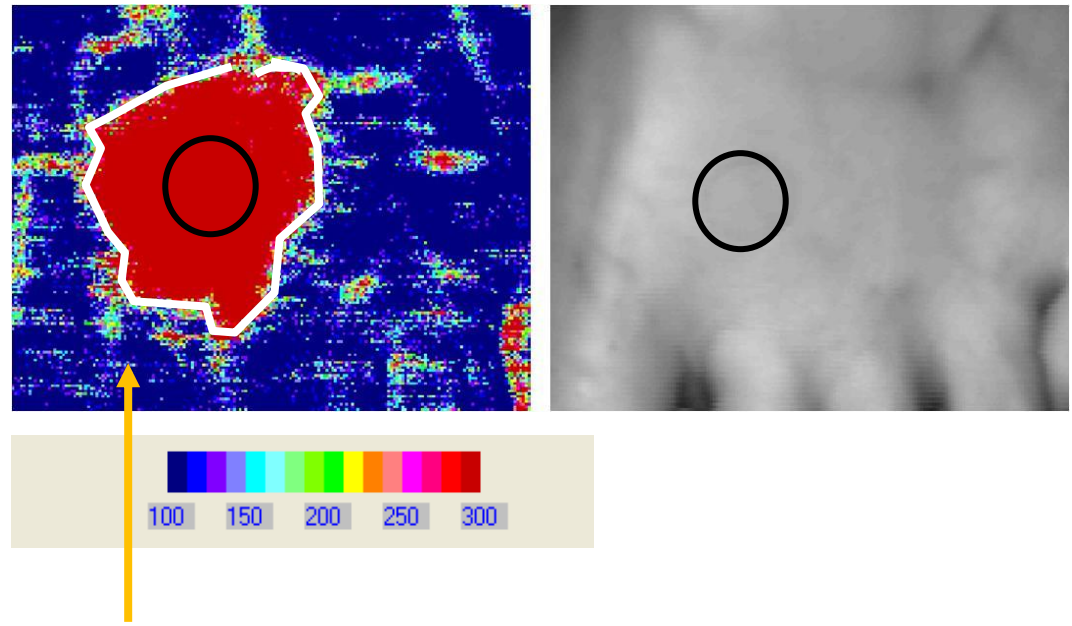


Figure 4: The post stimulation (skin heating) laser Doppler image acquired using a LDI.

The image analysis palate has been set such that flux < 100PU appears as dark blue (representing standard blood flow) and anything >300PU appears as bright red. The arrows point to the position where the skin heater was applied.

Measurement of the LDIflare area.



White Freeform= LDIflare area, cm^2

Black circle= skin heating area, 1 cm^2

Figure 5: Calculation of the LDIflare area.

The white freeform constitutes the area of the post skin heating resultant flare (LDIflare) and is expressed in cm^2. The area under the heater (black circle) represents the area directly under the heater probe and the change in perfusion is non-neurogenic and endothelium dependent. It is termed LDImax and is a measure of endothelial function. The area outside the skin heating is the true axon-reflex mediated flare (LDIflare).

3.3 LDIflare and Small Fibre Function

The original methodology of the LDIflare (oLDIflare) was described in 2004 [73] . Initial validation demonstrated good reproducibility along with an ability to detect early neuropathic abnormalities, even when QST's were normal [73-74]. In a select cohort of impaired glucose tolerant individuals without clinical neuropathy, the oLDIflare demonstrated evidence of small nerve fibre dysfunction [75]. At the same time, a group of patients with longstanding type 1 diabetes, specifically selected for the absence of any micro/macrovascular complications demonstrated normal small fibre function. Discussing these findings in an editorial, Prof. Boulton and Prof. Malik, highlighted the oLDIflare as a emerging marker of SFN with potential for application in longitudinal cohorts and neuropathy drug trials [76]. The Toronto Consensus on Diabetic Neuropathy also provided further recognition but suggested that further studies were required to validate the technique and develop its diagnostic potential [31]. In order to achieve this, the methodology needed addressing to make the oLDIflare more practical and accessible - testing took 90 minutes to complete - making examination of large patient numbers difficult. It was unclear if the original heating temperature of 44^{o}C fully stimulated the small nerve fibres and there were additional validity concerns regarding its specificity as a marker of small fibre function. Furthermore, it lacked normative data and precise operating characteristics for the detection of diabetic neuropathy - important attributes required for clinical application.

In order to overcome these challenges, modifications were made to the methodology to allow the testing procedure to be completed faster and further validation studies undertaken using the modified LDIflare technique (mLDIflare).

4.0 Synopsis of Studies included in this submission

In this section, I have provided a brief synopsis of the studies included in this submission. They include experiments conducted to validate the LDIflare methodology, determination of normative values and operating characteristics such as sensitivity and specificity for the detection of clinical diabetic neuropathy. A further study examining the relationship of fasting triglycerides in normal glucose tolerant on small fibre function is described. In addition, in a group of individuals with type 1 diabetes, normal small fibre function is demonstrated in the absence of clinical microvascular disease elsewhere.

4.1 Modification and validation of the LDIflare technique.

The primary modifications to the LDIflare methodology were: 1) the shortening of the acclimatisation period, 2) reducing the duration of skin heating required to elicit a nociceptive response, thus allowing the process to be completed faster, and 3) utilising a higher final skin heating temperature. The methodology is detailed in the papers submitted. The neurogenic nature of the mLDIflare was confirmed in a study in which a local anaesthetic cream (EMLA; lidocaine 2.5% and prilocaine 2.5%; AstraZeneca, Luton, UK) was applied over the heating area prior to eliciting the mLDIflare. The axon-mediated flare response was near completely obliterated (9.3 ± 3.0 cm^2 before and 1.7 ± 0.3 cm^2 after; $p < 0.0001$), confirming the neurogenic nature of the LDIflare. In another study it was shown that there was near perfect correlation of flare sizes between the right and left feet (Pearson's coefficient $r= 0.95$, $p<0.0001$). Finally, and in agreement with the original technique, the mLDIflare was significantly lower in the group with diabetic neuropathy (DN+) compared to healthy controls (HC) (1.99 ± 1.1 cm^2 v 9.9 ± 3.4 cm^2; $p<0.001$) and also

when the DN+ group was compared to those without diabetic neuropathy (DN-) (1.99±1.1 cm^2 v 6.78±2.78 cm^2; p<0.001) [73].

4.2 Estimating the rate of decline in LDIflare small fibre function assessed and developing age-related centile values in the detection of clinical neuropathy.

The peripheral nervous system, both somatic and autonomic, changes with age [48, 58, 77-78] . Determination of accurate normative values underpins the diagnostic validity and overall performance of a test [79]. Given the potential role of the LDIflare in future testing of early DSPN, it was essential to understand the variables, both clinical and metabolic, that influence it. It was also important to establish operating characteristics, such as sensitivity and specificity to determine what values can be confidently defined as 'abnormal'. Therefore, studies were undertaken to establish determinants of small fibre function as assessed by the LDIflare method and derive age-related centile values. An additional aim was to determine which of the two analytical techniques, receiver -operator derived cut-off values or centile based values, were superior in assessing the operating characteristics of the LDIflare. Therefore, across-sectional cohort of 94 healthy volunteers was recruited to understand the variables that influence the LDIflare and develop age-specific normative values. Glucose dysregulation was excluded using a composite of fasting glucose <6.0 mmol/L *and* HbA1c <6.0% (42 mmol/mol).

4.3 Additional validation of the LDIflare

The two studies described below were not specifically designed to validate the LDIflare. They provide crucial supportive literature evidence for the LDIflare as a useful SFN marker, but are not part of the thesis submission.

4.3.1 LDIflare and point-of-care nerve conduction device (NC-stat®|DPNCheck™) for the measurement of early diabetic Neuropathy

More recently, a simple point-of-care device (POCD) has been developed and validated for the detection of sensory changes in the sural nerve and to serve as an acceptable proxy to NCS [80]. The hand-held device: NC-stat®|DPNCheck™ system (developed by Neurometrix, Waltham, MA) allows the quantitative measurement of sural nerve conduction velocity (SNCV) and amplitude of the sensory nerve action potential (SNAP). Validation studies have suggested that it has excellent correlation with conventional NCS ($r=0.95$, $p<0.001$) and is reliable (inter-rater reproducibility values of 0.83 for SNAP and 0.79 for SNCV) [80-81]. In addition, the sensitivity and specificity for identification of DSPN defined by electrophysiological criteria was 95% and 71% respectively [81].

The accuracy of the NC-Stat®|DPNCheck™ was compared with the modified LDIflare technique for the detection of DSPN categorised into no (0-2), mild (3-5), moderate (6-8) and severe (9-10) DSPN using the neuropathy disability score (NDS) [82]. A total of 80 healthy controls (HC) alongside 162 diabetic individuals (50% type 1 diabetes) were recruited. All groups were age ($p=0.22$) and gender ($p=0.57$) matched. Among HC, the LDIflare correlated strongly with both SNAP ($r=0.88$, $p<0.0001$) and SNCV ($r=0.90$, $p<0.0001$). Similar significant correlation between

the LDIflare and POCD measures were noted within the no, mild, moderate and severe DSPN groups [82]. The LDIflare was significantly smaller among those with DM when compared to HC (5.81±2.09 vs. 9.11± 2.17 cm^2, p<0.0001). The results with the POCD were similarly significantly lower in the DM cohort - SNCV (42.04±9.11 vs. 50.24±5.69 m/s; p=<0.0001) and SNAP (10.13±3.12 vs. 18.49±4.13 µV; p=<0.0001). In addition, the LDIflare and POCD both demonstrated individual reduction in mean values across the DSPN categories [82]. However, while there was a highly significant difference in the LDIflare sizes between the HC and the diabetic control group (those with NDS 0-2) (9.11± 2.17 vs 7.52 ± 2.59 cm^2, p < 0.0001) there was no such difference between the same groups for SNAP (18.49 ± 4.13 vs 16.61± 8.45, p=0.15) and SNCV (50.22 ± 5.69 vs 48.95 ± 12.70, p=0.11) [82]. The AUC for the LDIflare and POCD were as follows (Table 3, data extracted from Sharma et al [82]).

AUC (Area under the curve)	No DSPN	Mild DSPN	Moderate DSPN	Severe DSPN
LDIflare	0.901	0.768	0.767	0.964
SNAP	0.868	0.703	0.804	0.869
SNCV	0.896	0.743	0.814	0.907

Table 3: The area under the curve for LDIflare for detection of DSPN categories.

4.3.2 The LDIflare in chemotherapy-induced peripheral neuropathy

A common, unpredictable and at times dose limiting complication of cancer drug treatment regimes is the chemotherapy-induced peripheral neuropathy (CIPN). The

symptoms of CIPN such as paraesthesia, dysaesthesia, hyperalgesia, hypoalgesia and patients are similar to those described in DSPN. Patients may also experience at the same burning, shooting or electric-shock type discomfort described in diabetic neuropathic pain [83]. Furthermore, like DSPN, the distribution of symptoms is in a "stocking and glove" manner, due to the vulnerability of the long nerves [84]. The diagnosis of CIPN is clinical as there is no specific test. However, it is understood that IENFD depletion occurs early despite normal peripheral nerve axon counts and preserved nerve conduction results and may at times be the only hallmark of CIPN [83, 85].

The LDIflare was evaluated 24 patients with established CIPN and distal sensory symptoms to determine its utility in assessing the diagnosis [86]. Of these, 12 were CIPN secondary to platinum analogues and 12 with damage from taxanes. An additional 24 age, gender and BMI matched healthy controls were also studied. Apart from the LDIflare, additional neurological assessments included determination of vibration perception thresholds (VPT), sural nerve amplitude (SNAP) and conduction velocity (SNCV) [86]. The LDIflare was significantly reduced in CIPN group compared to HC (3.75 ±1.68 vs 6.53 ±0.75 cm^2 , $p<0.001$) while SNAP ($p= 0.06$ and SNCV ($P = 0.09$) were not [86]. Additionally, the LDIflare was the only neurological marker to correlate with European Organisation for Research and Treatment of Cancer - QLQ-CIPN20 disease severity questionnaire [86].

4.4 LDIflare small fibre function in normal glucose tolerant subjects with and without hypertriglyceridaemia

The relationship between early diabetic neuropathy and dyslipidemia, and hypertriglyceridaemia in particular, is receiving increasing attention. However, the relationship between small nerve fibres and triglycerides (TG) in neurologically

asymptomatic glucose tolerant individuals remains unclear. Neuropathy studies in non-diabetic individuals have either focussed on small cohorts with marked hypertriglyceridaemia (HTG) [87-89], or on those referred to hospital with overt positive sensory neurological symptoms [88, 90-91]. Furthermore, such studies have typically utilised large fibre assessments. Therefore, we explored the relationship of TG's on small fibre function, measured using the LDIflare, in groups with normotriglyceridaemia (HC), mild hypertriglyceridemia (MiTG, TG=1.7 and 2.25 mmol/L) and significant hypertriglyceridemia (HiTG , TG=>2.25 mmol/L) [92]. A total of 79 age-matched subjects (HC=43, MitG=17 and HiTG=19) and were selected for normal glucose tolerance and absence of metabolic syndrome (Met-S) as defined by International Diabetes Federation (IDF) [92].

Analysis of variance (ANOVA) between the 3 groups showed that total cholesterol, Log_{10} converted triglycerides and TC/HDL ratio differed significantly (F-statistic - 8.6, 110.0, 15.9 respectively, $p<0.0001$). LDL-C also differed between the groups (F-statistic 4.6, p=0.01). Post-hoc analysis showed no significant difference in total cholesterol, LDL-C, HDL-C or TC/HDL ratio between MiTG and HiTG groups. However, the difference in Log_{10} converted triglycerides remained significant [92]. For all 3 groups combined, there was a significant inverse association between LDIflare and age (r=-0.42, $p<0.0001$), BMI (-0.51, $p<0.0001$), log_{10} triglycerides (r=-0.66,$p<0.0001$,), total cholesterol (r=-0.26, p=0.02) and TC/HDL ratio (r=-0.37, p=0.002). However, on multivariate regression analysis, only log_{10} triglycerides (B coefficient -5.8, 95% CI -7.5 to -4.2, $p<0.0001$) and age (-0.08, 95% CI -0.13 to -0.03, p=0.003) were independently associated . A similar association for age (B-coefficient -0.10, 95% CI -0.18 to -0.02, p=0.02) and Log_{10} triglycerides (B-

coefficient -5.0, 95% CI -7.9 to -2.1, p=0.01) was noted when the MiTG and HiTG groups were combined.

4.5 Small fibre dysfunction, microvascular complications and glycaemic control in type 1 diabetes: a case-control study

The relationship of microvascular disease and small fibre function was investigated using the LDIflare in a group of type 1 diabetic individuals of moderate duration of disease with (MV+) and without (MV-) microvascular complications and compared to healthy controls (HC) [93]. Each group consisted of 24 individuals without overt clinical neuropathy (neuropathy disability score, NDS <3 and Toronto clinical neuropathy score, TCNS <5). Subjects with type 1 diabetes were considered to have microvascular disease if they had either confirmed diabetic retinopathy for at least 2 years' duration as per the English National Screening Programme for Diabetic Retinopathy [94] and/or microalbuminuria (urinary albumin to creatinine ratio >2.5 mg/mmol for males and >3.5 mg/mmol for females). In addition, duration-averaged HbA1C was calculated in all participants by calculating the mean of their HbA1C recorded at annual review assessments over the period they had diabetes [74].

The 3 groups (HC, MV- and MV+) did not differ for sex, age, height, weight or BMI. The duration of diabetes was not significantly different between the two type 1 groups (MV- 17.7±5.7 years versus MV+ 20.1±5.2 years, p=0.21). The HbA1C values at the point of LDIflare estimation were also similar (MV- 8.0±1.2% [64±10 mmol/mol] versus MV+ 8.0±0.9% [64±9 mmol/mol], p=0.53). Neither of the above HbA1C correlated with LDIflare. However, the duration-averaged HbA1C was significantly higher in the MV+ group (MV+ 8.6±0.9% [70±9 mmol/mol] versus MV− 7.6±0.6% [60±7 mmol/mol], p<0.001). Combining the two diabetic groups,

there was a significant inverse correlation between the LDIflare size and duration-averaged HbA1C, ($r= -0.50$, $p<0.001$) which persisted after adjustment for age, BMI, and lipids [93].

The LDIflare size did not differ between the HC and MV- groups (HC 10.0±3.09 cm^2 versus MV- 9.9±2.9 cm^2, $p=0.55$). However, the LDIflare size was significantly lower in the MV+ group (5.1±1.8 cm^2) when compared to HC ($p<0.0001$) and MV- ($p<0.001$) groups. The LDImax, which relates to the area directly under the heater probe and a measure of non-neurogenic vasodilatation, was not significantly different between the MV- and MV+ groups (MV- 685±141 PU versus 632±156 PU, $p=0.10$) [74]. The MV+ demonstrated a higher fasting triglyceride concentration compared to the MV- group (MV+ 1.23±0.12 mmol/l versus MV− 0.92±0.07, $p=0.04$). There was no intra-group correlation between LDIflare size and any of the lipid parameters including triglycerides. However, when the MV- and MV+ groups were combined, after adjusting for age, gender and BMI, the LDIflare inversely correlated with triglycerides ($r= -0.304$, $p=0.036$).

5.0 Discussion

Measurement of small fibre nerve impairment is a major challenge. While studies in diabetic retinopathy and nephropathy have had access to clinically relevant indicators of early abnormalities such as digital retinal images and urine albumin excretion rate estimation, no such reliable marker of early damage exists in neuropathy. Although, small fibre abnormalities are increasingly recognised as the earliest to be acquired and may potentially present a higher regenerative ability, the lack of accessible tools to quantitate such damage has been a limitation. Hence, studies looking into fundamental clinical questions such as - does good glycaemic control impact on neural function - have had to rely on indicators of more established peripheral nerve involvement such as vibratory sensation, light touch or ankle reflexes [95]. This may be a major reason for the failure of putative pharmacological interventions to demonstrate a reduction in relative risk for nerve events, especially in type 2 diabetes [34, 95]. It also has meant that the natural course and history of diabetic neuropathy continues to remain poorly understood.

The data presented in this thesis validates the modifications to the LDIflare and confirms that it is a novel, non-invasive, reproducible and importantly, an objective marker of small fibre function thereby fulfilling an important niche in small fibre testing. The coefficient of variation (CoV) was 11% and compared well with the original methodology which was between 12 and 15% [75]. The testing process can be completed in less than 30 minutes, thus allowing subjects to be assessed relatively quickly and is similar to the testing time required to perform a nerve conduction study. This specification, in particular, will allow for its application in studies with larger cohorts. Additionally, the testing site is directly representative of the most clinically relevant pathological site in DSPN. The analysis of the results obtained is

straightforward and unlike IVCCM or CHEPS, there is only one neurogenic variable to analyse. The non-invasive nature allows for the test to be repeated as many times as required. Finally, the simplicity of the testing process, allows for the procedure to be carried out in a clinic environment without the need for complex, purpose built laboratory space. It is of note that after a short period of training all 5 researchers in the Ipswich Diabetes Research unit have achieved CoV of less than 15%. A potential disadvantage of the method is the cost of the LDI scanning device, however this is on par with the cost of a IVCCM or a CASE-IV™ QST device.

The clinical translation of any technique is dependent on the definition of clear normative values, allowing for the determination of whether a condition is present or absent. It is also important to understand the various factors that influence a particular technique, as statistical adjustments may need to be undertaken, especially in longitudinal studies, to evaluate potential epoch and cohort effects [79]. If age, as a dependent variable plays a significant role, then it is important that the test results are interpreted accordingly. For example, if there is a significant inverse relationship with age, what may be abnormal value for a young individual, may be well within the normal range in the elderly. Age was a major determinant of small fibre function as assessed by the LDIflare [78] (Figure 6). In addition, fasting triglycerides but not total cholesterol or LDL-Cholesterol was an independent predictor.

The relationship of small fibre indices with age are in keeping with findings reported by the worldwide normative reference study on IENFD [48] as well as the recently published multinational normative data on IVCCM values [58]. Normative values of small fibre function markers such as contact heat evoked potential (CHEPS) [96] as well as laser evoked potentials (LEPs) [97] have also demonstrated a similar association with age. The lack of influence of height, weight, body mass index

(BMI), HbA1C (within the normal range) on SFN indices has also been reported previously [48, 58]. Other normative datasets have noticed a gender association [48, 96, 98], however, we did not find such an association. Although the LDIflare elicited larger flare sizes in females, the difference did not reach statistical significance [78]. This may be partly explained by the relatively moderate size of our normative sample and similar sized cohorts in IVCCM and laser evoked potential studies have not demonstrated a gender effect [99-100]. Importantly, the consistent influence of age in almost every published study underscores the need for all small fibre markers to have clear age-determined normative values [101].

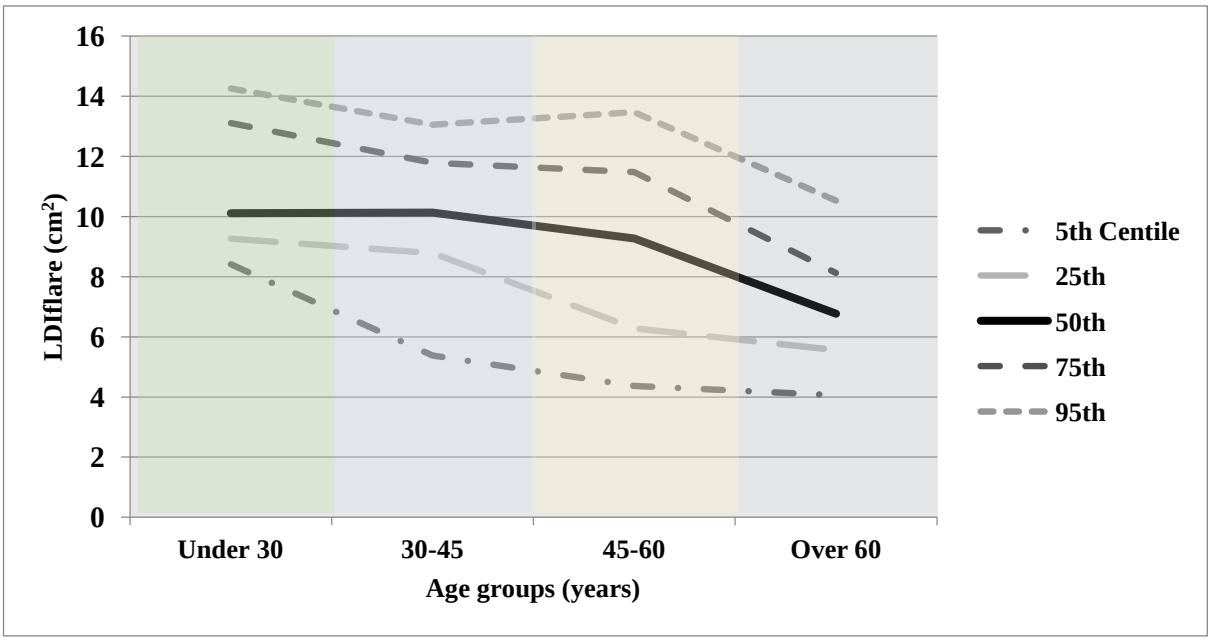


Figure 6: Normogram of the LDIflare.

The LDIflare demonstrated excellent sensitivity and specificity for the detection of DSPN and compares favourably with other established small fibre markers. Using NDS>3 to stratify the presence of DSPN, IVCCM has a reported sensitivity of 82%

and specificity of 52% [57]. The sensitivity and specificity for the original LDIflare has been reported by other groups. Nouri et al, measured the LDIflare in a group with type 1 diabetes and in healthy controls. Using the subclinical sural nerve impairment based criteria as reference standard, and employing the original LDIflare (heating to 44°C for 20 minutes) they found a sensitivity of 79% and a specificity of 60% with an area under the curve (AUC) of 0.75 for a cut off value of 1.90 cm^2 [102]. In the same study using the 'England criteria' [103] as reference standard, the sensitivity and specificity were 66% and 70% respectively, with AUC 0.72 for a cut off value of 1.90 cm^2 [102]. In a cohort of 74 subjects with idiopathic small fibre painful neuropathy (with normal nerve conduction studies), Ebadi et al, using IENFD cut-off of <5.4 fibres/mm as the reference standard for the presence of SFN, estimated the sensitivity and specificity of the LDIflare at 54% and 54% respectively, giving an AUC of 0.54 at a diagnostic threshold of 1.96 cm^2 [104]. However, there are important differences between the methodology used by Nouri et al and Ebadi et al and those of the studies undertaken in this thesis. A major limitation of these studies, both of which originated from the same research unit, was the lack of clarity in the LDIflare methodology and the absence of published data on the reproducibility values for the LDIflare technique in their hands. In addition, a smaller size heating probe was used. The mean flare sizes of 2.1 ± 1.1 cm^2 and 2.3 ± 1.2 cm^2 their studies were much smaller than those obtained in the original studies in the Ipswich unit (5.2 cm^2 (IQR 3.9 –5.9 cm^2) possibly reflecting the different in probe size and/or lack of appreciation in the importance of scrupulous attention to acclimatisation and precision of technique which is required to achieve reproducibility. This may have contributed to the inconsistent operating characteristics reported. Furthermore, and importantly, their flare sizes are far from

those observed with the modified LDIflare technique. More recently, the same team has investigated the utility of LDIflare testing in individuals presenting to a neuromuscular clinic with typical positive neuropathic symptoms in the lower limbs, of which 26% had diabetes. [105]. They reported that while the LDIflare performed moderately in all-cause SFN (combined sensitivity 64%), it had excellent performance in diabetic mixed fibre neuropathy (sensitivity 86%) with demonstrable superiority over quantitative thermal thresholds (sensitivity 36% for cooling and 0% for heat thresholds for all cause SFN and 79% and 29% for diabetes respectively) [105]. All individuals with reduced IENFD also had reduced LDIflare [105]

The studies using the NC-Stat®|DPNCheck™ and in subjects with CIPN provide *additional* validation of the LDIflare technique. Indeed, the strong AUC (0.901) for those without clinical DSPN suggests that the LDIflare also possesses a strong negative predictive value for DSPN. Furthermore, they demonstrate a strong correlation with sensory nerve conduction parameters, which are themselves the earliest perceptible large fibre changes [31]. The finding of normal sural sensory nerve conduction among those without clinical DSPN but abnormal LDIflare adds further to the suggestion that small fibre dysfunction may precede changes in large fibre markers. In another context, the ability of the LDIflare to detect early small fibre change, when traditional neurophysiology is non-contributory or even normal, underlines the importance of using tests of small fibre function in future SFN research studies.

The relationship between small fibre function and triglyceride levels is novel and intriguing, especially the hitheto unrecognised findings noted in healthy subjects and/or in the context of mildly abnormal triglyceride levels. Although the inverse correlation in one cross-sectional study cannot determine causality, it does suggest

the possibility that triglyceride levels above the normal may have an incremental, direct neurotoxic effect on small nerve fibres. The exact mechanism of damage is unclear. One hypothesis is that generation of long chain free fatty acids may damage the Schwann cells [106-108] and oxidised or glycated LDL may bind to surface receptors and trigger intracellular oxidative stress through a process not dissimilar to direct effects of hyperglycaemia [109]. Another possibility is that dyslipidemia may drive direct axonal damage by way of mitochondrial dysfunction [110-111]. The findings, in part, may explain the presence of neuropathy in early type 2 diabetes (and in prediabetic states) and account for the persistent risk of diabetic neuropathy in specific, but microvascular complications in general, despite good glycaemic control.

The relationship between SFN and microvascular disease in type 1 diabetes has remained inconclusive. The LDIflare observed abnormal small fibre function in those with microvascular disease but preserved function in those *without* microvascular disease. The findings support previous observations in the DCCT [17] and EUROBIAB [112] studies that glycaemic control plays a significant role in the development diabetic neuropathy of type 1 diabetes and provides specific evidence that this also pertains to early small fibre change. However, those studies predominantly used history, focussed neurological examination, nerve conduction studies and autonomic testing to assess DSPN and did not use any validated SFN measure.

The important role of hyperglycaemia in the aetiopathogenesis of diabetic neuropathy is undisputed. Studies have clearly established a link between diabetes and glycaemic control and neuropathy development [10, 109, 113]. Whether the effect of hyperglycaemia as the pathogenic driver of early neuropathy is directly

mediated (through the polyol pathway, oxidative-nitrosative stress or lack of c-peptide stimulation) or indirect through the development of neuronal microvascular damage remains unclear [109]. Green et al, in an earlier study using the older LDIflare technique noted that individuals with type 1 diabetes, carefully selected for absence of retinopathy and nephropathy had evidently preserved small fibre function, while a group with prediabetes simultaneously studied, demonstrated significant small fibre dysfunction [75]. Studies in type 2 diabetes have noted the presence of neuropathy early into the diagnosis of diabetes [18, 25, 114-115] and similar findings have also been noted in prediabetes [29, 116-118]. Such individuals have relatively mild hyperglycaemia and significantly less exposure to glycaemic perturbations than the type 1 groups studied. Taken together, the findings with the LDIflare suggest that aetiopathogensis the neuropathies of type 1 and type 2 diabetes may be different - that in those with type 2 diabetes, metabolic factors may play a significant early role while in those with type 1 diabetes, microangiopathy may be key [33, 119] . If these observations are proven, they may have a important implications on how patients are recruited for future therapeutic trials of early diabetic neuropathy. Putative agents having a significant impact on microangiopathy and the microvasculature may need to recruit more subjects with type 1 diabetes ; while those with corrective effect on metabolic parameters may be better off with recruiting predominantly type 2 diabetes subjects.

6.0 Limitations of the papers presented:

Although the experiments and studies presented validate the LDIflare technique and highlight the important role of small fibre function in DSPN, they do have limitations.

The stratification of neuropathy was based on the Neuropathy Disability Score (NDS). The points accrued in the NDS are based on clinical examination which may be subjective with a wide inter-rater variability [120]. Formal nerve conduction studies were not undertaken; hence our study subjects did not fulfil the Toronto consensus case-definition of confirmed/absent neuropathy. The primary reason is that the studies were conceived and ethical approval gained prior to the publication of the Toronto consensus. Limited funding for the studies was an additional factor for the reliance on NDS. Nonetheless, the use of NDS is in keeping with other previously published studies on small fibre neuropathy assessment techniques [57, 121-123]. Importantly, all the studies presented had a single operator which would have significantly reduced any variation in the subsequent stratification. In addition, there are only two diabetes groups, those with clinical neuropathy (NDS≥3) and those without (NDS<3), in the validation work undertaken. A more detailed assessment, allowing the assessment and validation of the modified LDIflare technique against neuropathy stratified as mild, moderate and severe using the NDS or NIS-LL would have been desirable.

Another potential limitation is the reliance of a single small fibre measure. However, the LDIflare has been previously validated against intra-epidermal nerve fibre density and has been shown to have a strong correlation [75]. In the extension of the validation studies, a strong correlation with early sensory sural nerve conduction

parameters has also been demonstrated [82]. Furthermore, in our experience, the utility of quantitative tests of thermal thresholds is limited as they are subjective and time consuming [49, 78]. Future studies with the LDIflare should ideally include an additional objective SFN marker, such as IENFD or IVCCM to provide additional supportive evidence.

All subjects included in the studies did not have any obvious macrovascular disease. Thus, the LDIflare has not been validated for use in this group. Further studies would be required to assess suitability of the LDIflare to measure small fibre function in this specific group. However, we expect most individuals being assessed for early neuropathy not to have significant symptomatic or major structural vascular disease, which frequently coexists with advanced stage neuropathy and loss of protective sensation. Therefore, this limitation is unlikely to impact on the broad applicability of the technique. Future work should evaluate the LDIflare in this particular group, to evaluate reliability, validity and importantly, safety.

Finally, given the complex interplay of microvascular response to nociceptive stimuli, some experts have suggested that the LDIflare may be a reflection of the underlying microvascular status and endothelial function. The LDIflare measures changes in dermal perfusion, but is also dependent on the ability of the underlying microvasculature to dilate to the efferent neurogenic stimuli. Vascular tone is dependent on extrinsic factors such sympathetic activity (neurogenic tone) [124], circulating angiotensin II [125] and intrinsic factors such as smooth muscle mechanics, nitric oxide (vasodilator) [126-127], endothelin (vasoconstrictor) [125, 128], local humoro-chemicals such as histamine, bradykinins [129] and local oxygenation [130]. The nerve–axon reflex related vasodilatation has been validated as an objective method to evaluate C-nociceptive fibre function [64, 122, 131-132].

In support of the neurogenic nature of the LDIflare, both thermal and pharmacological stimuli have been shown to induce the nerve–axon reflex [65, 133]. Application of a local anaesthetic agent has been shown to reduce the acetylcholine mediated response (endothelium dependent) by 71% ± 12% [64, 134] but not the response to sodium nitroprusside (endothelium-independent and a smooth muscle relaxant) [134]. In many of the above studies, laser Doppler Flowmetry (LDF) with single-point measurement rather than laser Doppler imaging (with scanning of the entire response) was undertaken and may have impacted on the conclusions derived. Inherently LDF has a much higher coefficient of variation of up to 38%, while we have demonstrated a CoV of~11% using the LDIflare. Recently, Emanuel and colleagues using the LDF technique alongside nerve conduction studies and IENFD measurement have provided additional support for the neurogenic nature of the LDIflare by demonstrating that impaired microvascular endothelium-dependent vasodilation does not contribute to neuropathy in type 2 diabetes and vice versa [135]. Thus, from a mechanistic perspective, the LDIflare results represent the significant (but in certain situations, not exclusive) role of small fibres in the nerve-axon mediated vasodilatation.

In our normative studies, and indeed in the two groups of type 1 diabetes, there was no correlation between LDIflare and LDImax, the latter which is the direct response to skin heating. This is further supportive that the LDIflare indeed quantifies neurogenic function. Additionally, application of the EMLA, virtually abolished the LDIflare but did not change the LDImax [136]. LDIflare has also been shown to correlate with IENFD as well as dermal nerve fibre density [137]. The work by Perkins and colleagues using the original LDIflare methodology demonstrated a moderate but significant correlation between corneal confocal microscopy markers

and the LDIflare (r= 0.25-0.41, p<0.01) [138]. Comparison with sural sensory conduction parameters using the NC-Stat®|DPNCheck™ demonstrating a strong correlation has provided further evidence for the LDIflare as a surrogate marker of diabetic neuropathy [82].

7.0 Conclusions and future outlook

The detection of the early forms of DSPN, especially small fibre neuropathy, remains a significant challenge. Symptoms, signs and standard neurophysiology cannot be relied upon as they primarily reflect large fibre deficits. While a number of small fibre techniques are currently available, their widespread application is limited either by their invasive nature, poor reproducibility, poor sensitivity, need specialist equipment/personnel or a clear lack of normative data.

7.1 LDIflare as a validated marker of small fibre function

In the series of papers presented, the LDIflare, in its current modification, is supported as a novel, relatively rapid, non-invasive, reproducible, reliable and accurate marker for small fibre function. This has been achieved by:

a) Shortening the acclimatisation and heating period and by using a higher skin heating temperature,
b) Determining normative values for the LDIflare by applying the technique in healthy, non-neuropathic adult volunteers categorised into 4 age-groups,
c) Assessment of operating characteristics such as sensitivity, specificity, positive and negative predictive values.

The current technique is able to detect the same group difference as the original method but with greater clarity, given the larger flare sizes in those without or mild small fibre dysfunction. Additional validation for the technique has been provided by studies comparing the LDIflare with the NC-stat®|DPNCheck™ device and through studies undertaken by other research groups.

Emerging work utilising the capsaicin nerve injury model has shown that the axon-mediated flare area measured with the laser Doppler imager technique, is able to consistently demonstrate differences between capsaicin denervated and placebo treated skin in addition showing ability to detect reinnervation by demonstrating an improvement in flare area sizes 2 weeks post denervation [139]. These findings, while preliminary, can be considered encouraging. as an important value of any small fibre test in an interventional study would be its ability to detect a response to treatment [22].

The validation findings in this submission and those available in literature (direct and surrogate) demonstrate the potential of LDIflare as a SFN marker which could be reliably utilised in translational studies of future disease modifying therapies in diabetic neuropathy. It could also be used in larger cohort studies required to understand the epidemiology of diabetic neuropathy and monitor progression. In addition, the technique could arguably be also considered ready for clinical use in specialist units dealing in small fibre neuropathy and its diagnosis, as an important *additional* methodology alongside a battery of other tests such as IENFD, IVCCM and autonomic function assessments.

7.2 Small fibre nerves and pathogenesis of diabetic neuropathy

The relationship between small fibre function and microvascular complications in neurologically asymptomatic individuals with type 1 diabetes is extremely intriguing and is in contrast to findings reported in prediabetes and type 2 diabetes. The reporting of preserved small fibre function in those without retinal or renal microvascular disease is novel. This supports the hypothesis that the aetiopathogenesis of neuropathy may be different in the two forms of diabetes: that

in type 2 the initial effects are mediated through metabolic defects while in type 1 diabetes, vascular injury may be the primary trigger.

A recent study has noted abnormalities of small fibre structure in type 1 diabetes, measured using IVCCM, in those without retinopathy or microalbuminuria [123]. These findings are contrary to our findings of preserved LDIflare in type 1 diabetes without early renal or retinal abnormalities. The participants in that study had a higher NDS score (1.8 ± 0.7 v 0.16±0.5 in our cohort) which may have contributed to the findings. However, another view may be that small fibre nerve damage perhaps starts *prior* to demonstrable abnormalities of retinopathy and nephropathy. Within the specialism of small fibre assessment, apart from underscoring the need for larger cohort studies of well characterised subjects, the disparity in currently published LDIflare and IVCCM findings also lead to the question of which occurs first - Small fibre structural or functional damage? To answer this, prospective studies simultaneously assessing both small fibre and function in well characterised subjects are required.

The findings of an inverse correlation between small fibre function and triglycerides are novel and not been previously reported. The present findings could be considered early work and larger cohorts of both normal glucose-tolerant and diabetic subjects are required to understand the relationship further. In addition, the LDIflare small fibre dysfunction model may have a potential role in understanding the effect of anti-hyperlipidaemic therapy on progression/reversal of neuropathy.

7.3 Future Outlook for the LDIflare

The reliability and operating characteristics of a test are likely to be enhanced with increasing numbers of subjects tested allowing apparent any inconsistencies - methodological and analytical - to be further improved upon. This is also applicable to the LDIflare. Although age-specific normative values have been derived, they will be further fortified by the assessment of a larger number of normal glucose tolerant (NGT) individuals. This larger sample size may confirm if the LDIflare has an association with gender like QST and IENFD and IVCCM and further refine the normative data. Ideally, this should be through a worldwide, multicentre collaborative effort with other centres using the LDIflare similar to initiatives in IENFD [48] and IVCCM [58]. A prospective follow-up study may clarify the annual rate of change in the LDIflare in healthy controls and those with diabetes in addition to delineating the clinical and biological factors associated with such change. The prospective study may also clarify if the LDIflare is able to demonstrate improvement in SFF when there is sustained improvement in glycaemic control. There is already evidence that IENFD [60, 140] and IVCCM [62, 141] are able to detect such a response to treatment.

Another validation wish list for the LDIflare is to confirm its correlation with formal nerve conduction studies. In addition, a direct comparison with all of the IVCCM variables, which have emerged over the past decade as simple, reliable and non-invasive marker of small fibre structure is desirable. This is may further strengthen the place of LDIflare as a suitable supplementary endpoint for early neuropathy case definition alongside invasive morphometric measure such as IENFD or sural nerve biopsy.

Currently, there is no single small fibre marker that can be considered as the ‘gold standard’. It remains to be seen if the operating characteristics for early DSPN detection and for SFN assessment can be enhanced if LDIflare and IVCCM findings are combined compared to their individual sensitivities and specificities. It is also unclear which of the two - small fibre structure or function - is the first to be impaired. The lack of a simple and objective functional marker of SFN has been a limiting factor. The LDIflare fulfils, and in conjunction with the IVCCM or IENFD, may be suitable for the investigation of this fundamental conundrum.

One future challenge is that there are now two validated protocols for the LDIflare. There is a need for those involved in small fibre research and utilising the LDIflare to utilise the best validated method and promote it, for example as done by the German Pain Network for NCS and QST testing. This will, again, require collaborative discussions between various research units that utilise the LDIflare technique [142]. The current iteration on account of its simple investigational algorithm and short heating duration should be encouraged. With diabetic neuropathy research being a domain of relatively select research units, the need for multicentre cooperation and standardisation of the technique is important.

The findings of SFN in those with type 1 diabetes without renal and retinal disease is opening up newer paradigms of thought and investigation. These, alongside well characterised SFN studies in early type 2 diabetes suggesting that neuropathy may occur prior to the development of retinopathy challenge how glucose thresholds are used to derive the definition of diabetes, especially type 2 diabetes (143). The current diagnostic glucose thresholds have been derived from retinopathy prevalence studies, which have rarely included neuropathy measures. If neuropathy precedes retinopathy, or indeed simply the presence of neuropathy may change or add an

additional dimension to our perception of glucose thresholds. The LDIflare on account of its sensitivity may be an useful neuromarker in future studies investigating the glycaemic cut-off targets.

Finally, there is a need to reassign the current definition of DSPN. Many studies, including those in this submission, have supported the notion that small fibre neuropathy may be asymptomatic. The current Toronto Consensus would classify such individuals as subclinical neuropathy, until they developed symptoms or signs, even if there was quantitative change in the interim. Furthermore, there is no current severity staging system in place for SFN. The LDIflare is objective, quantitative and reliable -and will be valuable tool in future studies of severity stratification.

References:

[1] World Health Organization (1999) Definition, Diagnosis and Classification of Diabetes Mellitus and its Complications: Report of a WHO Consultation. Part 1: Diagnosis and Classification of Diabetes Mellitus. Geneva, World Health Org. URL http://www.who.int/iris/handle/10665/66040. Accessed 27/10/2017.

[2] Danaei G, Finucane MM, Lu Y, et al. (2011) National, regional, and global trends in fasting plasma glucose and diabetes prevalence since 1980: systematic analysis of health examination surveys and epidemiological studies with 370 country-years and 2.7million participants. The Lancet 378: 31-40

[3] Tabák AG, Herder C, Rathmann W, Brunner EJ, Kivimäki M (2012) Prediabetes: a high-risk state for diabetes development. The Lancet 379: 2279-2290

[4] Perreault L, Pan Q, Mather KJ, Watson KE, Hamman RF, Kahn SE (2012) Effect of regression from prediabetes to normal glucose regulation on long-term reduction in diabetes risk: results from the Diabetes Prevention Program Outcomes Study. Lancet 379: 2243-2251

[5] Hex N, Bartlett C, Wright D, Taylor M, Varley D (2012) Estimating the current and future costs of Type 1 and Type 2 diabetes in the UK, including direct health costs and indirect societal and productivity costs. Diabet Med 29: 855-862

[6] Kerr, M. (2016) Improving footcare for people with diabetes and saving money:an economic study in England. Diabetes UK.
URL https://diabetes-resources-production.s3-eu-west-1.amazonaws.com/diabetes-storage/migration/pdf/Improving%2520footcare%2520economic%2520study%2520%28January%25202017%29.pdf. Accessed 27/10/2017

[7] American Diabetes Association (2013) Economic Costs of Diabetes in the U.S. in 2012. Diabetes Care 36: 1033-1046

[8] Partanen J, Niskanen L, Lehtinen J, Mervaala E, Siitonen O, Uusitupa M (1995) Natural history of peripheral neuropathy in patients with non-insulin-dependent diabetes mellitus. N Engl J Med 333: 89-94

[9] Pop-Busui R, Boulton AJM, Feldman EL, et al. (2017) Diabetic Neuropathy: A Position Statement by the American Diabetes Association. Diabetes Care 40: 136-154

[10] Boulton AJ, Vinik AI, Arezzo JC, et al. (2005) Diabetic neuropathies: a statement by the American Diabetes Association. Diabetes Care 28: 956-962

[11] Maser RE, Mitchell BD, Vinik AI, Freeman R (2003) The Association Between Cardiovascular Autonomic Neuropathy and Mortality in Individuals With Diabetes: A meta-analysis. Diabetes Care 26: 1895-1901

[12] Ziegler D, Zentai CP, Perz S, et al. (2008) Prediction of mortality using measures of cardiac autonomic dysfunction in the diabetic and nondiabetic population: the MONICA/KORA Augsburg Cohort Study. Diabetes Care 31: 556-561

[13] Vas PR, Edmonds ME (2016) Early recognition of diabetic peripheral neuropathy and the need for one-stop microvascular assessment. Lancet Diabetes Endocrinology 4(9): 723-725.

[14] Armstrong DG, Wrobel J, Robbins JM (2007) Guest Editorial: are diabetes-related wounds and amputations worse than cancer? International Wound Journal 4: 286-287

[15] Gordois A, Scuffham P, Shearer A, Oglesby A (2003) The healthcare costs of diabetic peripheral neuropathy in the UK. The Diabetic Foot Journal 6: 62-73

[16] Malik RA (2016) Wherefore Art Thou, O Treatment for Diabetic Neuropathy? Int Rev Neurobiol 127: 287-317

[17] Martin CL, Albers JW, Pop-Busui R (2014) Neuropathy and Related Findings in the Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications Study. Diabetes Care 37: 31-38

[18] Ziegler D, Papanas N, Zhivov A, et al. (2014) Early detection of nerve fiber loss by corneal confocal microscopy and skin biopsy in recently diagnosed type 2 diabetes. Diabetes 63: 2454-2463

[19] Sumner CJ, Sheth S, Griffin JW, Cornblath DR, Polydefkis M (2003) The spectrum of neuropathy in diabetes and impaired glucose tolerance. Neurology 60: 108-111

[20] Azmi S, Ferdousi M, Petropoulos IN, et al. (2015) Corneal Confocal Microscopy Identifies Small-Fiber Neuropathy in Subjects With Impaired Glucose Tolerance Who Develop Type 2 Diabetes. Diabetes Care 38(8): 1502-1508.

[21] Abbott CA, Carrington AL, Ashe H, et al. (2002) The North-West Diabetes Foot Care Study: incidence of, and risk factors for, new diabetic foot ulceration in a community-based patient cohort. Diabet Med 19: 377-384

[22] Malik RA (2014) Why are there no good treatments for diabetic neuropathy? The Lancet Diabetes & Endocrinology 2: 607-609

[23] Vallbo AB, Hagbarth K, Torebjork H, Wallin B (1979) Somatosensory, proprioceptive, and sympathetic activity in human peripheral nerves. Physiological Reviews 59: 919-957

[24] Ziegler D, Mayer P, Gries FA (1988) Evaluation of thermal, pain, and vibration sensation thresholds in newly diagnosed type 1 diabetic patients. J Neurol Neurosurg Psychiatry 51: 1420-1424

[25] Loseth S, Stalberg E, Jorde R, Mellgren SI (2008) Early diabetic neuropathy: thermal thresholds and intraepidermal nerve fibre density in patients with normal nerve conduction studies. J Neurol 255: 1197-1202

[26] Breiner A, Lovblom LE, Perkins BA, Bril V (2014) Does the prevailing hypothesis that small-fiber dysfunction precedes large-fiber dysfunction apply to type 1 diabetic patients? Diabetes Care 37: 1418-1424

[27] Jimenez-Cohl P, Grekin C, Leyton C, Vargas C, Villaseca R (2012) Thermal threshold: research study on small fiber dysfunction in distal diabetic polyneuropathy. J Diabetes Sci Technol 6: 177-183

[28] Singleton JR, Smith AG, Bromberg MB (2001) Painful sensory polyneuropathy associated with impaired glucose tolerance. Muscle Nerve 24: 1225-1228

[29] Nebuchennykh M, Loseth S, Jorde R, Mellgren SI (2008) Idiopathic polyneuropathy and impaired glucose metabolism in a Norwegian patient series. Eur J Neurol 15: 810-816

[30] Divisova S, Vlckova E, Hnojcikova M, et al. (2012) Prediabetes/early diabetes-associated neuropathy predominantly involves sensory small fibres. J Peripher Nerv Syst 17: 341-350

[31] Tesfaye S, Boulton AJ, Dyck PJ, et al. (2010) Diabetic neuropathies: update on definitions, diagnostic criteria, estimation of severity, and treatments. Diabetes Care 33: 2285-2293

[32] Umapathi T, Tan WL, Loke SC, Soon PC, Tavintharan S, Chan YH (2007) Intraepidermal nerve fiber density as a marker of early diabetic neuropathy. Muscle Nerve 35: 591-598

[33] Sima AAF, Kamiya H (2006) Diabetic Neuropathy Differs in Type 1 and Type 2 Diabetes. Annals of the New York Academy of Sciences 1084: 235-249

[34] Callaghan BC, Little AA, Feldman EL, Hughes RA (2012) Enhanced glucose control for preventing and treating diabetic neuropathy. Cochrane Database Syst Rev 6: CD007543. DOI 10.1002/14651858.CD007543.pub2

[35] Stino AM, Smith AG (2017) Peripheral Neuropathy in Prediabetes and the Metabolic Syndrome. Journal of Diabetes Investigation 8(5):646-655. doi: 10.1111/jdi.12650

[36] Løseth S, Stålberg EV, Lindal S, Olsen E, Jorde R, Mellgren SI (2016) Small and large fiber neuropathy in those with type 1 and type 2 diabetes: a 5-year follow-up study. Journal of the Peripheral Nervous System 21: 15-21

[37] Gibbons CH, Freeman R, Tecilazich F, et al. (2013) The evolving natural history of neurophysiologic function in patients with well-controlled diabetes. Journal of the Peripheral Nervous System 18: 153-161

[38] Sachedina S, Toth C (2013) Progression in idiopathic, diabetic, paraproteinemic, alcoholic, and B12 deficiency neuropathy. Journal of the Peripheral Nervous System 18: 247-255

[39] Polydefkis M, Arezzo J, Nash M, et al. (2015) Safety and efficacy of ranirestat in patients with mild-to-moderate diabetic sensorimotor polyneuropathy. J Peripher Nerv Syst 20: 363-371

[40] Bril V (2016) Chapter Three - The Perfect Clinical Trial. In: Nigel AC, Paul F (eds) International Review of Neurobiology. Academic Press, pp 27-41

[41] Calcutt N, Fernyhough P (2016) Chapter One - A Brief Introduction to the History and Controversies of Clinical Trials in Diabetic Neuropathy. International Review of Neurobiology. N. A. Calcutt and P. Fernyhough, Academic Press. 127: 3-8

[42] Apfel SC, Asbury AK, Bril V, et al. (2001) Positive neuropathic sensory symptoms as endpoints in diabetic neuropathy trials. Journal of the Neurological Sciences 189: 3-5

[43] Singleton JR, Marcus RL, Jackson JE, M KL, Graham TE, Smith AG (2014) Exercise increases cutaneous nerve density in diabetic patients without neuropathy. Ann Clin Transl Neurol 1: 844-849

[44] Themistocleous AC, Ramirez JD, Serra J, Bennett DLH (2014) The clinical approach to small fibre neuropathy and painful channelopathy. Practical Neurology

[45] Vas PR, Sharma S, Rayman G (2015) Distal Sensorimotor Neuropathy: Improvements in Diagnosis. Rev Diabet Stud 12: 29-47

[46] Haanpaa M, Attal N, Backonja M, et al. (2011) NeuPSIG guidelines on neuropathic pain assessment. Pain 152: 14-27

[47] Lauria G, Hsieh ST, Johansson O, et al. (2010) European Federation of Neurological Societies/Peripheral Nerve Society Guideline on the use of skin biopsy in the diagnosis of small fiber neuropathy. Report of a joint task force of the European Fe-deration of Neurological Societies and the Peripheral Nerve Society. European Journal of Neurology 17: 903-e949

[48] Lauria G, Bakkers M, Schmitz C, et al. (2010) Intraepidermal nerve fiber density at the distal leg: a worldwide normative reference study. J Peripher Nerv Syst 15: 202-207

[49] Shy ME, Frohman EM, So YT, et al. (2003) Quantitative sensory testing: Report of the Therapeutics and Technology Assessment Subcommittee of the American Academy of Neurology. Neurology 60: 898-904

[50] Quattrini C, Tavakoli M, Jeziorska M, et al. (2007) Surrogate markers of small fiber damage in human diabetic neuropathy. Diabetes 56: 2148-2154

[51] Cruccu G, Sommer C, Anand P, et al. (2010) EFNS guidelines on neuropathic pain assessment: revised 2009. Eur J Neurol 17: 1010-1018

[52] Malik RA, Veves A, Walker D, et al. (2001) Sural nerve fibre pathology in diabetic patients with mild neuropathy: relationship to pain, quantitative sensory testing and peripheral nerve electrophysiology. Acta Neuropathol 101: 367-374

[53] Malik RA, Kallinikos P, Abbott CA, et al. (2003) Corneal confocal microscopy: a non-invasive surrogate of nerve fibre damage and repair in diabetic patients. Diabetologia 46: 683-688

[54] Pritchard N, Edwards K, Vagenas D, et al. (2010) Corneal sensitivity as an ophthalmic marker of diabetic neuropathy. Optom Vis Sci 87: 1003-1008

[55] Petropoulos IN, Manzoor T, Morgan P, et al. (2013) Repeatability of in vivo corneal confocal microscopy to quantify corneal nerve morphology. Cornea 32: e83-89

[56] Chen X, Graham J, Dabbah MA, et al. (2015) Small nerve fiber quantification in the diagnosis of diabetic sensorimotor polyneuropathy: comparing corneal confocal microscopy with intraepidermal nerve fiber density. Diabetes Care 38: 1138-1144

[57] Tavakoli M, Quattrini C, Abbott C, et al. (2010) Corneal confocal microscopy: a novel noninvasive test to diagnose and stratify the severity of human diabetic neuropathy. Diabetes Care 33: 1792-1797

[58] Tavakoli M, Ferdousi M, Petropoulos IN, et al. (2015) Normative values for corneal nerve morphology assessed using corneal confocal microscopy: a multinational normative data set. Diabetes Care 38: 838-843

[59] Tervo T, Holopainen J, Belmonte C (2016) Confocal microscopy of corneal nerves—a limited but still useful technique to evaluate peripheral neuropathies. JAMA Ophthalmology 134: 990-991

[60] Smith AG, Russell J, Feldman EL, et al. (2006) Lifestyle intervention for pre-diabetic neuropathy. Diabetes Care 29: 1294-1299

[61] Tavakoli M, Kallinikos P, Iqbal A, et al. (2011) Corneal confocal microscopy detects improvement in corneal nerve morphology with an improvement in risk factors for diabetic neuropathy. Diabet Med 28: 1261-1267

[62] Mehra S, Tavakoli M, Kallinikos PA, et al. (2007) Corneal confocal microscopy detects early nerve regeneration after pancreas transplantation in patients with type 1 diabetes. Diabetes Care 30: 2608-2612

[63] Boucek P, Havrdova T, Voska L, et al. (2008) Epidermal Innervation in Type 1 Diabetic Patients. A 25-year prospective study after simultaneous pancreas/kidney transplantation 31: 1611-1612

[64] Caselli A, Rich J, Hanane T, Uccioli L, Veves A (2003) Role of C-nociceptive fibers in the nerve axon reflex-related vasodilation in diabetes. Neurology 60: 297-300

[65] Caselli A, Spallone V, Marfia GA, et al. (2006) Validation of the nerve axon reflex for the assessment of small nerve fibre dysfunction. J Neurol Neurosurg Psychiatry 77: 927-932

[66] Walmsley D, Wiles PG (1990) Assessment of the neurogenic flare response as a measure of nociceptor C fibre function. J Med Eng Technol 14: 194-196

[67] Haas LF (2005) Sir Thomas Lewis 1881–1945. Journal of Neurology, Neurosurgery & Psychiatry 76: 1157

[68] Roosterman D, Goerge T, Schneider SW, Bunnett NW, Steinhoff M (2006) Neuronal Control of Skin Function: The Skin as a Neuroimmunoendocrine Organ. Physiological Reviews 86: 1309-1379

[69] Hagains CE, Trevino LA, He J-W, Liu H, Peng YB (2010) Contributions of dorsal root reflex and axonal reflex to formalin-induced inflammation. Brain research 1359: 90-97

[70] Krause JE, Takeda Y, Hershey AD (1992) Structure, functions, and mechanisms of substance P receptor action. Journal of investigative dermatology 98: 2S-7S

[71] Van Rossum D, Hanisch U-K, Quirion R (1997) Neuroanatomical Localization, Pharmacological Characterization and Functions of CGRP, Related Peptides and Their Receptors. Neuroscience & Biobehavioral Reviews 21: 649-678

[72] Brain S, Williams T, Tippins J, Morris H, MacIntyre I (1985) Calcitonin gene-related peptide is a potent vasodilator. Nature 3-9;313(5997):54-6.

[73] Krishnan ST, Rayman G (2004) The LDIflare A novel test of C-fiber function demonstrates early neuropathy in type 2 diabetes. Diabetes Care 27: 2930-2935

[74] Krishnan ST, Quattrini C, Jeziorska M, Malik RA, Rayman G (2009) Abnormal LDIflare but normal quantitative sensory testing and dermal nerve fiber density in patients with painful diabetic neuropathy. Diabetes Care 32: 451-455

[75] Green AQ, Krishnan S, Finucane FM, Rayman G (2010) Altered C-fiber function as an indicator of early peripheral neuropathy in individuals with impaired glucose tolerance. Diabetes Care 33: 174-176

[76] Boulton AJ, Malik RA (2010) Neuropathy of impaired glucose tolerance and its measurement. Diabetes Care 33: 207-209

[77] Said G (2007) Diabetic neuropathy-a review. Nat Clin Pract Neuro 3: 331-340

[78] Vas PR, Rayman G (2013) The rate of decline in small fibre function assessed using axon reflex-mediated neurogenic vasodilatation and the importance of age related centile values to improve the detection of clinical neuropathy. PLoS ONE 8 (7): e69920. doi:10.1371/journal.pone.0069920

[79] O'Connor PJ (1990) Normative data: their definition, interpretation, and importance for primary care physicians. Fam Med 22: 307-311

[80] Perkins BA, Grewal J, Ng E, Ngo M, Bril V (2006) Validation of a novel point-of-care nerve conduction device for the detection of diabetic sensorimotor polyneuropathy. Diabetes Care 29: 2023-2027

[81] Lee JA, Halpern EM, Lovblom LE, Yeung E, Bril V, Perkins BA (2014) Reliability and validity of a point-of-care sural nerve conduction device for identification of diabetic neuropathy. PLoS ONE 9(1):e86515. doi: 10.1371/journal.pone.0086515

[82] Sharma S, Vas PR, Rayman G (2015) Assessment of diabetic neuropathy using a point-of-care nerve conduction device shows significant associations with the LDIFLARE method and clinical neuropathy scoring. J Diabetes Sci Technol 9: 123-131

[83] Han Y, Smith MT (2013) Pathobiology of cancer chemotherapy-induced peripheral neuropathy (CIPN). Front Pharmacol 4: 156

[84] Peltier AC, Russell JW (2002) Recent advances in drug-induced neuropathies. Curr Opin Neurol 15: 633-638

[85] Boyette-Davis J, Xin W, Zhang H, Dougherty PM (2011) Intraepidermal nerve fiber loss corresponds to the development of taxol-induced hyperalgesia and can be prevented by treatment with minocycline. Pain 152: 308-313

[86] Sharma S, Venkitaraman R, Vas PR, Rayman G (2015) Assessment of chemotherapy-induced peripheral neuropathy using the LDIFLARE technique: a novel technique to detect neural small fiber dysfunction. Brain Behaviour 5(7): e00354. doi: 10.1002/brb3.354

[87] Drory VE, Groozman GB, Rubinstein A, Korczyn AD (1999) Hypertriglyceridemia may cause a subclinical peripheral neuropathy. Electromyogr Clin Neurophysiol 39: 39-41

[88] McManis PG, Windebank AJ, Kiziltan M (1994) Neuropathy associated with hyperlipidemia. Neurology 44: 2185-2186

[89] Kassem HS, Azar ST, Zantout MS, Sawaya RA (2005) Hypertriglyceridemia and peripheral neuropathy in neurologically asymptomatic patients. Neuro Endocrinol Lett 26: 775-779

[90] Rajabally YA, Shah RS (2011) Dyslipidaemia in chronic acquired distal axonal polyneuropathy. J Neurol 258: 1431-1436

[91] Hughes RA, Umapathi T, Gray IA, et al. (2004) A controlled investigation of the cause of chronic idiopathic axonal polyneuropathy. Brain 127: 1723-1730

[92] Vas PR, Sharma S, Rayman G (2015) LDIflare small fiber function in normal glucose tolerant subjects with and without hypertriglyceridemia. Muscle Nerve 52: 113-119

[93] Vas PR, Green AQ, Rayman G (2012) Small fibre dysfunction, microvascular complications and glycaemic control in type 1 diabetes: a case-control study. Diabetologia 55: 795-800

[94] ENSPDR (2011) English National Screening Programme for Diabetic Retinopathy.

[95] Zoungas S, Arima H, Gerstein HC, et al. (2017) Effects of intensive glucose control on microvascular outcomes in patients with type 2 diabetes: a meta-analysis of individual participant data from randomised controlled trials. The Lancet Diabetes & Endocrinology 5(6):431-437. doi: 10.1016/S2213-8587(17)30104-3

[96] Lagerburg V, Bakkers M, Bouwhuis A, et al. (2015) Contact heat evoked potentials: normal values and use in small-fiber neuropathy. Muscle Nerve 51: 743-749

[97] Truini A, Galeotti F, Romaniello A, Virtuoso M, Iannetti G, Cruccu G (2005) Laser-evoked potentials: normative values. Clinical Neurophysiology 116: 821-826

[98] Yarnitsky D, Sprecher E (1994) Thermal testing: normative data and repeatability for various test algorithms. Journal of the Neurological Sciences 125: 39-45

[99] Wu T, Ahmed A, Bril V, et al. (2012) Variables associated with corneal confocal microscopy parameters in healthy volunteers: implications for diabetic neuropathy screening. Diabet Med 29: e297-303

[100] Truini A, Galeotti F, Romaniello A, Virtuoso M, Iannetti GD, Cruccu G (2005) Laser-evoked potentials: normative values. Clinical Neurophysiology 116: 821-826

[101] Vas PR, Sharma S, Rayman G (2014) Comment on Breiner et al. Does the prevailing hypothesis that small-fiber dysfunction precedes large-fiber dysfunction apply to type 1 diabetic patients? Diabetes care 2014;37:1418-1424. Diabetes Care 37: e240-241

[102] Nabavi Nouri M, Ahmed A, Bril V, et al. (2012) Diabetic neuropathy and axon reflex-mediated neurogenic vasodilatation in type 1 diabetes. PLoS ONE 7(4): e34807. doi: 10.1371/journal.pone.0034807

[103] England JD, Gronseth GS, Franklin G, et al. (2005) Distal symmetric polyneuropathy: a definition for clinical research: report of the American Academy of Neurology, the American Association of Electrodiagnostic Medicine, and the

American Academy of Physical Medicine and Rehabilitation. Neurology 64: 199-207

[104] Ebadi H, Perkins BA, Katzberg HD, Lovblom LE, Bril V (2012) Evaluation of proxy tests for SFSN: evidence for mixed small and large fiber dysfunction. PLoS ONE 7(8): e42208. doi: 10.1371/journal.pone.0042208

[105] Abraham A, Alabdali M, Alsulaiman A, et al. (2016) Laser Doppler Flare Imaging and Quantitative Thermal Thresholds Testing Performance in Small and Mixed Fiber Neuropathies. PLoS ONE 11 (11): e0165731
doi: 10.1371/journal.pone.0165731

[106] Hinder LM, Figueroa-Romero C, Pacut C, et al. (2014) Long-chain acyl coenzyme A synthetase 1 overexpression in primary cultured Schwann cells prevents long chain fatty acid-induced oxidative stress and mitochondrial dysfunction. Antioxid Redox Signal 21: 588-600

[107] Di Paolo G, Przedborski S (2013) When Schwann Cells Conspire with Mitochondria, Neighboring Axons Are under Attack by Glia-Derived Neurotoxic Lipids. Neuron 77: 801-803

[108] Viader A, Sasaki Y, Kim S, et al. (2013) Aberrant Schwann Cell Lipid Metabolism Linked to Mitochondrial Deficits Leads to Axon Degeneration and Neuropathy. Neuron 77: 886-898

[109] Callaghan BC, Cheng HT, Stables CL, Smith AL, Feldman EL (2012) Diabetic neuropathy: clinical manifestations and current treatments. Lancet Neurol 11: 521-534

[110] Vincent AM, Edwards JL, McLean LL, et al. (2010) Mitochondrial biogenesis and fission in axons in cell culture and animal models of diabetic neuropathy. Acta neuropathologica 120: 477-489

[111] Feldman EL, Nave K-A, Jensen TS, Bennett DL (2017) New Horizons in Diabetic Neuropathy: Mechanisms, Bioenergetics, and Pain. Neuron 93: 1296-1313

[112] Tesfaye S, Stevens LK, Stephenson JM, et al. (1996) Prevalence of diabetic peripheral neuropathy and its relation to glycaemic control and potential risk factors: the EURODIAB IDDM Complications Study. Diabetologia 39: 1377-1384

[113] Gubitosi-Klug RA (2014) The diabetes control and complications trial/epidemiology of diabetes interventions and complications study at 30 years: summary and future directions. Diabetes Care 37: 44-49

[114] I. M. Stratton, R. R. Holman, Boulton AJM (2004) Risk factors for neuropathy in UKPDS. Diabetologia 47 Suppl 1: A47

[115] Lee S-S, Han H-S, Kim H (2014) Peripheral Nerve Abnormalities in Patients with Newly Diagnosed Type I and II Diabetes Mellitus. Korean J Clin Neurophysiol 16: 8-14

[116] Rajabally YA (2011) Neuropathy and impaired glucose tolerance: an updated review of the evidence. Acta Neurol Scand 124: 1-8

[117] Singleton JR, Smith AG, Russell JW, Feldman EL (2003) Microvascular Complications of Impaired Glucose Tolerance. Diabetes 52: 2867-2873

[118] Nebuchennykh M, Loseth S, Lindal S, Mellgren SI (2009) The value of skin biopsy with recording of intraepidermal nerve fiber density and quantitative sensory testing in the assessment of small fiber involvement in patients with different causes of polyneuropathy. J Neurol 256: 1067-1075

[119] Callaghan BC, Hur J, Feldman EL (2012) Diabetic neuropathy: one disease or two? Curr Opin Neurol 25: 536-541

[120] Dyck PJ, Overland CJ, Low PA, et al. (2010) Signs and symptoms versus nerve conduction studies to diagnose diabetic sensorimotor polyneuropathy: Cl vs. NPhys trial. Muscle Nerve 42: 157-164

[121] Ponirakis G, Fadavi H, Petropoulos IN, et al. (2015) Automated Quantification of Neuropad Improves Its Diagnostic Ability in Patients with Diabetic Neuropathy. J Diabetes Res 2015: 847854. http://dx.doi.org/10.1155/2015/847854

[122] Gibbons CH, Freeman R, Veves A (2010) Diabetic neuropathy: a cross-sectional study of the relationships among tests of neurophysiology. Diabetes Care 33: 2629-2634

[123] Petropoulos IN, Green P, Chan AW, et al. (2015) Corneal confocal microscopy detects neuropathy in patients with type 1 diabetes without retinopathy or microalbuminuria. PLoS ONE 10: e0123517

[124] Clement DL (1976) Neurogenic influences on blood pressure and vascular tone from peripheral receptors during muscular contraction. Cardiology 61 suppl 1: 65-68

[125] Touyz RM, Schiffrin EL (2000) Signal Transduction Mechanisms Mediating the Physiological and Pathophysiological Actions of Angiotensin II in Vascular Smooth Muscle Cells. Pharmacological Reviews 52: 639-672

[126] Lincoln TM, Komalavilas P, Cornwell TL (1994) Pleiotropic regulation of vascular smooth muscle tone by cyclic GMP-dependent protein kinase. Hypertension 23: 1141-1147

[127] Lerman A, Zeiher AM (2005) Endothelial Function: Cardiac Events. Circulation 111: 363-368

[128] Westby CM, Weil BR, Greiner JJ, Stauffer BL, DeSouza CA (2011) Endothelin-1 vasoconstriction and the age-related decline in endothelium-dependent vasodilatation in men. Clinical Science 120: 485-491

[129] Hecker M, Dambacher T, Busse R (1992) Role of endothelium-derived bradykinin in the control of vascular tone. J Cardiovasc Pharmacol 20 Suppl 9: S55-61

[130] Leach RM, Hill HS, Snetkov VA, Ward J (2002) Hypoxia, energy state and pulmonary vasomotor tone. Respiratory physiology & neurobiology 132: 55-67

[131] Caselli A, Spallone V, Marfia G, et al. (2006) Validation of the nerve axon reflex for the assessment of small nerve fibre dysfunction. Journal of Neurology, Neurosurgery & Psychiatry 77: 927

[132] Rayman G, Williams SA, Spencer PD, Smaje LH, Wise PH, Tooke JE (1986) Impaired microvascular hyperaemic response to minor skin trauma in type I diabetes. Br Med J (Clin Res Ed) 292: 1295-1298

[133] Green AQ, Krishnan ST, Rayman G (2009) C-fiber function assessed by the laser doppler imager flare technique and acetylcholine iontophoresis. Muscle Nerve 40: 985-991

[134] Caselli A, Uccioli L, Khaodhiar L, Veves A (2003) Local anesthesia reduces the maximal skin vasodilation during iontophoresis of sodium nitroprusside and heating. Microvasc Res 66: 134-139

[135] Emanuel AL, Nieuwenhoff MD, Klaassen ES, et al. (2017) Relationships Between Type 2 Diabetes, Neuropathy, and Microvascular Dysfunction: Evidence From Patients With Cryptogenic Axonal Polyneuropathy. Diabetes Care 40: 583-590

[136] Vas PR, Rayman G (2012) Validation of the modified LDIFLARE technique: A simple and quick method to assess C-fiber function. Muscle Nerve 47(3):351-6.

[137] Krishnan ST, Quattrini C, Jeziorska M, Malik RA, Rayman G (2009) Abnormal LDIflare but normal quantitative sensory testing and dermal nerve fiber density in patients with painful diabetic neuropathy. Diabetes Care 32: 451-455

[138] Sivaskandarajah GA, Halpern EM, Lovblom LE, et al. (2013) Structure-function relationship between corneal nerves and conventional small-fiber tests in type 1 diabetes. Diabetes Care 36: 2748-2755

[139] Illigens BM, Siepmann T, Roofeh J, Gibbons CH (2013) Laser Doppler imaging in the detection of peripheral neuropathy. Auton Neurosci 177: 286-290

[140] Kluding PM, Pasnoor M, Singh R, et al. (2012) The effect of exercise on neuropathic symptoms, nerve function, and cutaneous innervation in people with diabetic peripheral neuropathy. Journal of Diabetes and Its Complications 26: 424-429

[141] Azmi S, Ferdousi M, Petropoulos IN, et al. (2015) Corneal confocal microscopy shows an improvement in small-fiber neuropathy in subjects with type 1 diabetes on continuous subcutaneous insulin infusion compared with multiple daily injection. Diabetes Care 38: e3-4

[142] Vas PRJ, Sharma S, Rayman G (2014) Comment on Breiner et al. Does the Prevailing Hypothesis That Small-Fiber Dysfunction Precedes Large-Fiber Dysfunction Apply to Type 1 Diabetic Patients? Diabetes Care 2014;37:1418–1424. Diabetes Care 37: e240-e241

[143] Vas PRJ, Alberti KG, Edmonds ME (2017) Prediabetes: moving away from a glucocentric definition. Lancet Diabetes Endocrinol 5: 848-849. doi 10.1016/S2213-8587(17)30234-6

Appendix A: Statements of contribution signed by all co-authors

Paper 1

Distal Sensorimotor Neuropathy: Improvements in Diagnosis (2015).

Vas, P. R., S. Sharma, Rayman,G

Rev Diabet Stud **12**(1-2): 29-47.

Prash Vas co-concieved the review, conducted the literature search and conducted the literature search. In addition, he wrote the manuscript which he edited witht he help of his coauthors and responded to the reviewers as the corresponding author

I agree that Prash Vas made the aforementioned contribution to the paper		
Name Gerry Rayman	Signature	Date 12 JULY 2017
Name Sanjeev Sharma	Signature	Date 12/07/2017.

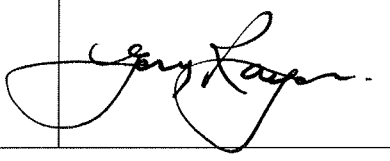
Paper 2

Validation of the modified LDIFlare technique: a simple and quick method to assess C-fiber function (2013)

Vas, P. R. and G. Rayman

Muscle Nerve **47**(3): 351-356.

Prash Vas jointly concieved the idea of the paper, and jointly designed the study with Gerry Rayman . He undertook the study assessments, interpreted the data and analysed the final results. He took the lead role in writing the manuscript in liasion with the co-author. Gerry Rayman responded to the reviewers as the corresponding author but recieved significant contribution from Prash Vas.

I agree that Prash Vas made the aforementioned contribution to the paper		
Name	Signature	Date
Gerry Rayman		12 JULY 2017

Paper 3

The rate of decline in small fibre function assessed using axon reflex-mediated neurogenic vasodilatation and the importance of age related centile values to improve the detection of clinical neuropathy (2013).

Vas, P. R. and G. Rayman

PLoS ONE 8(7): e69920.

Prash Vas jointly conceived the idea of the paper, and jointly designed the study with Gerry Rayman . He undertook the study assessments, interpreted the data and analysed the final results. He took the lead role in writing the manuscript in liaison with the co-author. Gerry Rayman responded to the reviewers as the corresponding author but received significant contribution from Prash Vas.

I agree that Prash Vas made the aforementioned contribution to the paper		
Name Gerry Rayman	Signature	Date 12 JULY 2017

Paper 4

Small fibre dysfunction, microvascular complications and glycaemic control in type 1 diabetes: a case-control study(2012).

Vas, P. R., A. Q. Green, Rayman, G

Diabetologia 55(3): 795-800.

Prash Vas contributed to the idea of the paper, and jointly designed the study with Gerry Rayman . He undertook the study assessments, interpreted the data and with the co-authors, led on writing the manuscript. Gerry Rayman responded to the reviewers as the corresponding author but received significant contribution from Prash Vas.

I agree that Prash Vas made the aforementioned contribution to the paper		
Name Gerry Rayman	Signature 	Date 12 July 2017
Name Al Q Green	Signature	Date

Paper 5

LDIflare small fiber function in normal glucose tolerant subjects with and without hypertriglyceridemia (2015).

Vas, P. R., S. Sharma, Rayman, G

Muscle Nerve 52(1): 113-119.

Prash Vas contributed to the idea of the paper, and jointly designed the study with Gerry Rayman . He undertook the study assessments, interpreted the data and with the co-authors, led on writing the manuscript. Gerry Rayman responded to the reviewers as the corresponding author but received significant contribution from Prash Vas

I agree that Prash Vas made the aforementioned contribution to the paper		
Name Gerry Rayman	Signature	Date 12 July 2017
Name Sanjeev Sharma	Signature	Date 12/07/2017.

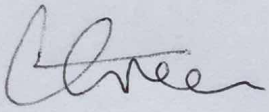
Paper 4

Small fibre dysfunction, microvascular complications and glycaemic control in type 1 diabetes: a case-control study (2012).

Vas, P. R., Green A.Q., Rayman, G

Diabetologia 55(3): 795-800.

Prash Vas contributed to the idea of the paper, and jointly designed the study with Gerry Rayman . He undertook the study assessments, interpreted the data and with the co-authors (AG, GR), led on writing the manuscript. Gerry Rayman responded to the reviewers as the corresponding author but received significant contribution from Prash Vas.

I agree that Prash Vas made the aforementioned contribution to the paper		
Name Gerry Rayman	Signature	Date
Name Al Q Green	Signature 	Date 13/7/17

Paper 6

Early recognition of diabetic peripheral neuropathy and the need for one-stop microvascular assessment (2016)

Vas, P. R. and M. E. Edmonds (2016).

Lancet Diabetes Endocrinol. doi: 10.1016/S2213-8587(16)30063-8.

Prash Vas concieved the idea of this commentary and wrote the manuscript woth contribution from Prof Edmonds. He responsed to the revieweres as the corresponding author.

I agree that Prash Vas made the aforementioned contribution to the paper		
Name Prof Michael Edmonds	Signature M Edmonds.	Date 18/7/17

Appendix B: Publications included in the thesis