Assessing Small Nerve Fibre Function in Diabetes

Towards Early Detection and a Deeper Pathopsysiological Understanding

Røikjer, Johan

DOI (link to publication from Publisher):
10.54337/aau528830728

Publication date:
2022

Document Version
Publisher's PDF, also known as Version of record

ASSESSING SMALL NERVE FIBRE FUCNTION IN DIABETES

TOWARDS EARLY DETECTION AND A DEEPER PATHOPHYSIOLOGICAL UNDERSTANDING

BY
JOHAN MØLLER RØIKJER

DISSERTATION SUBMITTED 2022

AALBORG UNIVERSITY
DENMARK

ASSESSING SMALL NERVE FIBRE FUCNTION IN DIABETES

TOWARDS EARLY DETECTION AND A DEEPER PATHOPHYSIOLOGICAL UNDERSTANDING

by

Johan Møller Røikjer

AALBORG UNIVERSITY
DENMARK

Dissertation submitted 2022

Dissertation submitted: December 2022

PhD supervisor: Professor Niels Ejskjær,
Steno Diabetes Center North Denmark,
Aalborg University Hospital
Department of Clinical Medicine, Aalborg University

Assistant PhD supervisors: Associate Prof. Carsten Dahl Mørch,
Faculty of Health Science and Technology,
Center for Neuroplasticity and Pain (CNAP),
Integrative Neuroscience, Aalborg University

Professor Peter Vestergaard,
Steno Diabetes Center North Denmark,
Aalborg University Hospital
Department of Clinical Medicine, Aalborg University

PhD committee: Clinical Professor Marianne Tang Severinsen, (chair)
Aalborg University, Denmark

Senior Lecturer, Dr. D. Selvarajah,
Sheffield Teaching Hospitals, United Kingdom

Senior Lecturer, Dr. Prash Vas
King's College Hospital, United Kingdom

PhD Series: Faculty of Medicine, Aalborg University

Department: Department of Health Science and Technology

ISSN (online): 2246-1302
ISBN (online): 978-87-7573-774-1

Published by:
Aalborg University Press
Kroghstræde 3
DK – 9220 Aalborg Ø
Phone: +45 99407140
aauf@forlag.aau.dk
forlag.aau.dk

Printed in Denmark by Stibo Complete, 2023

CURRICULUM VITAE

Johan Røikjer, MD

Mail: j.roeikjaer@rn.dk
Phone: +45 53349610

Short biography

Educated MD from Aarhus University, Denmark in June 2017. Since August 2018 I have been employed at Steno Diabetes Center North Denmark and Integrative Neuroscience at Aalborg University. I have been affiliated with the Department of Endocrinology, Aalborg University Hospital since November 2014. My primary focus has been on diabetes both clinically and in research, where my special interests are diabetes neuropathy, the diabetic foot and neuropathic pain.

Clinical experience

January 2015 - June 2015: Department of Endocrinology, Aalborg University Hospital, Medical Doctor (substitute)

June 2016 – September 2016: Department of Geriatric Medicine, Aalborg University Hospital, Medical Doctor (substitute)

August 2017 – February 2018: Department of Urology, Aarhus University Hospital, Medical Doctor

February 2018 – August 2018: Department of Infectious Diseases, Aarhus University Hospital, Medical Doctor

August 2018 – August 2020: Department of Endocrinology, Aalborg University Hospital

January 2023 – : Specialized training (endocrinology)

Publications

[1] **Røikjer J**, Lindmark I, Knudsen T. Cancer in ectopic breast tissue. Ugeskr Laeger 2015;177.

[2] **Røikjer J**, Jensen MH, Vestergaard P, Sørensen AM, Laursen HVB, Ejskjær N. Twenty years with diabetes and amputations: a retrospective population-based cohort study. Diabet Med 2020:dme.14251. https://doi.org/10.1111/dme.14251.

[3] **Røikjer J**, Mørch CD, Ejskjaer N. Diabetic Peripheral Neuropathy: Diagnosis and Treatment. Curr Drug Saf 2020;15. https://doi.org/10.2174/1574886315666200731173113.

[4] Laursen HVB, **Røikjer J**, Dal J, Jensen MH. Sodium Glucose Cotransporter-2 Inhibitor Treatment and the Risk of Diabetic Ketoacidosis in Denmark: A Retrospective Cohort Study of Five Years of Use. Curr Drug Saf 2020;16:73–81. https://doi.org/10.2174/1574886315666200819114629.

[5] Werkman NCC, Nielen JTH, van den Bergh JPW, Ejskjaer N, **Røikjer J**, Schaper NC, et al. Use of Sodium-Glucose Co-Transporter-2-Inhibitors (SGLT2-Is) and Risk of Lower Limb Amputation. Curr Drug Saf 2020;16:62–72. https://doi.org/10.2174/1574886315666200805103053.

[6] **Røikjer J**, Werkman NCC, Ejskjaer N, van den Bergh JPW, Vestergaard P, Schaper NC, et al. Incidence, hospitalization and mortality and their changes over time in people with a first ever diabetic foot ulcer. Diabet Med 2021. https://doi.org/10.1111/DME.14725.

[7] **Røikjer J**, Ejskjaer N. The conundrum and enigma of painful and painless neuropathy. Diabet Neuropathy 2022:123–33. https://doi.org/10.1016/b978-0-12-820669-0.00012-8.

[8] **Røikjer J**, Croosu SS, Hansen TM, Frøkjær JB, Andersen HH, Arendt-Nielsen L, et al. The histamine-induced axon-reflex response in people with type 1 diabetes with and without peripheral neuropathy: A clinical, observational study. J Pain 2022;0. https://doi.org/10.1016/J.JPAIN.2022.01.002.

[9] Croosu SS, Hansen TM, **Røikjer J**, Mørch CD, Ejskjaer N, Frokjær JB. Gray Matter Brain Alterations in Type 1 Diabetes - Findings Based on Detailed Phenotyping of Neuropathy Status. Exp Clin Endocrinol Diabetes 2022. doi:10.1055/A-1835-1877.

[10] **Røikjer J**, Ejskjaer N. Diabetic Peripheral Neuropathy. Handb Exp Pharmacol 2022;274:309–28. https://doi.org/10.1007/164_2022_585.

[11] **Røikjer J**, Croosu SS, Hansen TM, Frøkjær JB, Arendt-Nielsen L, Ejskjaer N, Mørch CD. Perception Threshold Tracking: Validating a novel method for assessing function of large and small sensory nerve fibers in diabetic peripheral neuropathy with and without pain. PAIN 2022. doi:10.1097/j.pain.0000000000002780.

[12] Croosu SS, **Røikjer J**, Mørch CD, Ejskjaer N, Frøkjær JB, Hansen TM. Alterations in Functional Connectivity of Thalamus and Primary Somatosensory Cortex in Painful and Painless Diabetic Peripheral Neuropathy. Diabetes Care 2023;46:1–10. doi:10.2337/DC22-0587.

[13] **Røikjer J**, Croosu SS, Hansen TM, Frøkjær JB, Brock C, Mørch CD, Ejskjaer N. The co-existence of peripheral and autonomic neuropathy in type 1 diabetes with and without pain. **Under Review.**

[14] **Røikjer J**, Croosu SS, Sejergaard BF, Hansen TM, Frøkjær JB, Søndergaard CB, Petropoulos IN, Malik RA, Nielsen E, Mørch CD, Ejskjaer N. Diagnostic accuracy of perception threshold tracking in detection of small fiber damage in type 1 diabetes. **Under Review.**

[15] **Røikjer J**, Croosu SS, Borbjerg MK, Hansen TM, Frøkjær JB, Arendt-Nielsen L, Ejskjaer N, Mørch CD. Optimizing the diagnostic time and accuracy of the histamine-induced axon-reflex flare response. **Under Review.**

Conference abstracts (as presenting author)

- 2019: 20 years with diabetes and amputation, Danish Endocrine Society Annual Meeting

- 2019: 20 years with diabetes and amputation, International Symposium for the Diabetic Foot

- 2019: 20 years with diabetes and amputation, European Association for the Study of Diabetes Annual Meeting

- 2020: Incidence, hospitalization and mortality and their changes over time in people with a first ever diabetic foot ulcer, Danish Endocrine Society Annual Meeting

- 2020: Incidence, hospitalization and mortality and their changes over time in people with a first ever diabetic foot ulcer, European Association for the Study of Diabetes Annual Meeting

- 2020: Prevention of the first diabetic foot ulcer initiative (invited talk), Diabetic Foot Study Group Annual Meeting

- 2021: Histamine-induced axon flair-mediated response and diabetic peripheral neuropathy, European Association for the Study of Diabetes Annual Meeting

- 2021: Prevention of the first diabetic foot ulcer initiative (invited talk), Diabetic Foot Study Group Annual Meeting

- 2022: Perception Threshold Tracking: A novel method for early detection of functional damage to small nerve fibres, Danish Endocrine Society Annual Meeting

- 2022: Perception Threshold Tracking: A novel method for early detection of functional damage to small nerve fibres, European Association for the Study of Diabetes Annual Meeting

- 2022: Perception Threshold Tracking: A novel method for early detection of functional damage to small nerve fibres, NeuroDiab Annual Meeting

Formal teaching

- Medical students, Aarhus University: Case-based, clinical education: 30 hours
- Medical students, Aalborg University: Formalized case-based education: 105 hours
- Medical students, Aalborg University: Project supervisor (bachelor, master, semester projects): 200 hours
- Medical doctors, Aalborg University hospital (research training, diabetes, neuropathy, diabetic foot): 35 hours
- PhD students, Danish Diabetes Academy (diabetes, neuropathy, and diabetic foot): 20 hours
- People with diabetes, Podcast: "Diabetesforskerne" (diabetes neuropathy), Two times invited speaker "Diabetesforeningen" (diabetic foot + neuropathy): 10 hours

Other research activities

- Investigator in 7 multicenter trials
- Member of the Diabetic Foot Study Group-initiated "*prevention of the first ulcer initiative*" (https://dfsg.org/about-dfsg/dfsg-research-group)
- Member of Danish Society of Endocrinology, Danish Society for Internal Medicine, and "Young Endocrinologists in Denmark".
- Award: "*Best Abstract*" at Annual meeting of the Danish Endocrine Society 2022
- Award: "*Young Investigators Award for Best Oral Presentation*" at NeuroDiab 2022.

LIST OF PAPERS

Røikjer J, Croosu SS, Hansen TM, Frøkjær JB, Andersen HH, Arendt-Nielsen L, Mørch CD, Ejskjaer N. The histamine-induced axon-reflex response in people with type 1 diabetes with and without peripheral neuropathy: A clinical, observational study. J Pain 2022;0. https://doi.org/10.1016/J.JPAIN.2022.01.002.

Røikjer J, Croosu SS, Frøkjær JB, Hansen TM, Arendt-Nielsen L, Ejskjaer N, Mørch CD. Perception threshold tracking: validating a novel method for assessing function of large and small sensory nerve fibers in diabetic peripheral neuropathy with and without pain. Pain 2022. doi:10.1097/j.pain.0000000000002780.

Røikjer J, Croosu SS, Sejergaard BF, Hansen TM, Frøkjær JB, Søndergaard CB, Petropoulos IN, Malik RA, Nielsen E, Mørch CD, Ejskjaer N. Diagnostic accuracy of perception threshold tracking in the detection of small fiber damage in type 1 diabetes. **Under review**.

ABBREVIATIONS

AGE: Advanced glycation end-products

CCM: Corneal confocal microscopy

CNBD: Corneal nerve branch density

CNFD: Corneal nerve fibre density

CNFL: Corneal nerve fibre length

CNFT: Corneal nerve fibre tortuosity

CDT: Cold detection threshold

CHEPs: Contact heat-evoked potentials

CPT: Cold pain threshold

DFNS: German Research Network on Neuropathic Pain

DN4: Douleur Neuropathique en 4

DFU: Diabetic foot ulcer

DPN: Diabetic peripheral neuropathy

DSPN: Distal symmetrical polyneuropathy

FDA: The U.S. Food and Drug Administration

FLPI: Full-field laser perfusion imaging

HbA1c: Haemoglobin A1c

HDT: Heat detection threshold

HPT: Heat pain threshold

IENFD: Intra-epidermal nerve fibre density

(LDI_{FLARE}): Laser Doppler imaging flare

LEP: Laser-evoked potential

LOPS: Loss of protective sensation

MEDON: Methods for Early Detection Of diabetic peripheral Neuropathy

mm: Millimetre

ms: Milliseconds

mN: Millinewton

no.: Number

PDPN: Painful diabetic peripheral neuropathy

PGP 9.5: Protein gene product 9.5

PU: Perfusion units

SFN: Small fibre neuropathy

T1DM: Type 1 diabetes mellitus

T2DM: Type 2 diabetes mellitus

QSART: Sudomotor axon reflex testing

QST: Quantitative sensory testing

TCA: Tricyclic antidepressants

VPT: Vibration perception threshold

ENGLISH SUMMARY

Adequate assessment of the small nerve fibres is pivotal when striving for early detection of diabetic peripheral neuropathy. Current screening methods are either insufficient, time-consuming, or only assess structural damage to the nerve fibres without providing information on their remaining function. These issues justify the development of new rapid measurements, that could be applied in a clinical setting.

The overall aim of this PhD thesis was to evaluate novel methods for rapid and adequate assessment of small nerve fibre function in people with type 1 diabetes. In addition, the thesis explores the nebulous concepts of diabetic peripheral neuropathy and painful diabetic peripheral neuropathy and tries to assess whether these can be successfully distinguished from each other using novel methodology.

The thesis is based on two published, peer-reviewed, original papers and one original paper submitted for publication. The three papers focus on two novel methods for functional assessment of small nerve fibres: a novel perception threshold tracking technique utilizing transcutaneous electrical stimulation for activation of Aδ-fibres, and a novel method utilizing histamine to evoke a C-fibre-mediated axon-reflex flare response.

The thesis presents clinical data from both examinations and compares perception threshold tracking to one of the established standards for structural assessment of small fibre neuropathy (corneal confocal microscopy). The study population used for all papers are from the “MEDON-cohort”, which was created as a part of the present PhD-project. The cohort consists of a total of 80 participants equally divided into four distinct groups. A group with type 1 diabetes and painful diabetic peripheral neuropathy (n=20), a group with type 1 diabetes and painless diabetic peripheral neuropathy (n=20), a group with type 1 diabetes without diabetic peripheral neuropathy or pain (n=20), and healthy controls (n=20). The participants were individually matched on age and sex between the groups.

The thesis concludes, that both perception threshold tracking and the histamine-induced axon-reflex flare response are promising methods for assessment of small nerve fibre function in diabetes, and that further research in prospective studies are warranted.

DANSK RESUME

En hurtig og præcis evaluering af små nervefibre er altafgørende hvis man ønsker tidlig detektion af diabetes neuropati. Eksisterende screeningsmetoder er enten upræcise, tidsforbrugende, eller formår kun at evaluere nervefibrenes strukturelle forandringer uden at bidrage med information om deres resterende funktion. Disse mangler betyder, at der er behov for nye og bedre metoder, der kan anvendes til screening i både forskning og klinik.

Det overordnede mål for denne Ph.d.-afhandling var at afprøve nye metoder til hurtig og præcis evaluering af de små nervefibres funktion i mennesker med type 1 diabetes. Derudover forsøger afhandlingen ligeledes at give indsigt i forskellene mellem de personer, som har ledsagende nervesmerter, og de personer som ikke har.

Afhandlingen er baseret på to publicerede, fagfællebedømte, originale videnskabelige artikler, samt en original artikel sendt til vurdering hos et videnskabeligt tidsskrift. De tre artikler fokuserer på to nye metoder til funktionel evaluering af diabetes neuropati: en nyudviklet teknik til elektrofysiologisk vurdering af perceptionstærsklen for små Aδ-fibre (”perception threshold tracking”), og en ny, indirekte, vurdering af små C-fibre via et histamin-induceret flare-respons (FLPI).

Afhandlingen præsenterer klinisk data fra begge metoder, og sammenligner den elektrofysiologiske evaluering af perceptionstærsklen med en etableret guldstandard i form af konfokalmikroskopi af hornhinden. Studiepopulationen i alle artikler stammer fra ”MEDON-kohorten”, som blev lavet som en del af aktuelle Ph.d.-afhandling, og som består af 80 personer fordelt i fire karakteristiske grupper. En gruppe med type 1 diabetes og smertefuld diabetes neuropati (n=20), en gruppe med type 1 diabetes og smerteløs diabetes neuropati (n=20), en gruppe med type 1 diabetes og hverken diabetes neuropati eller smerter (n=20) og en gruppe uden diabetes og smerter (n=20). Alle deltagere er matchet på alder og køn i et 1:1:1:1 forhold.

Afhandlingen konkluderer, at både ”perception threshold tracking” og FLPI er lovende kandidater til funktionel evaluering af små nervefibre i diabetes, og at de inkluderede studier danner grobund for fremtidige studier i prospektive kohorter.

ACKNOWLEDGEMENTS

The submission of this thesis is the culmination of a fruitful collaboration between Aalborg University and Aalborg University Hospital and marks the end of an amazing journey into the conundrum and enigma of diabetes neuropathy.

I am deeply grateful to everyone who helped making this dissertation possible, and to everyone who have contributed along the way.

I would like to express my most profound gratitude to my main supervisor, Niels Ejskjær, who took me under his wings and introduced me to the dazzling universe of diabetes neuropathy. I thank you for your endless enthusiasm, support, and commitment, and for always encouraging me to reach for the sky. Your philosophy on research is inspiring, and your ability to involve and encourage your students is unparalleled. It has been an honour to work under your leadership and guidance, and I look forward to continuing our relationship in future projects.

I would also like to offer my most sincere gratitude and appreciation to my co-supervisor Carsten Dahl Mørch, who has introduced me to the fields of neuroscience and electrophysiology. I thank you for your guidance, support, and patience, and for enduring the initial shortcomings in my understanding of the biophysical aspects of the field. Thank you for your everlasting involvement and positive attitude. I am confident that Integrative Neuroscience will thrive under your leadership, and I sincerely hope our collaboration will continue in the future.

I also want to extend a special thanks to my co-supervisor Peter Vestergaard, who has introduced me to research and welcomed me as a part of the amazing research team at Steno Diabetes Center North Denmark. Your door has always been open when I needed support or guidance, and for that I am eternally grateful. I admire your leadership and feel privileged to have you as my Head of Research.

A special thanks to Jens Brøndum Frøkjær, Tine Maria Hansen, and Suganthiya Santhiapillai Croosu for their participation in the MEDON-project, and for the fruitful discussions at our research meetings.

A special thanks to my colleagues and friends at Steno Diabetes Center North Denmark and the Department of Endocrinology, Aalborg University Hospital, and at Integrative Neuroscience and Center for Neuroplasticity and Pain, Aalborg University. I would also like to express my gratitude to my collaborators at the Department of Ophthalmology and the Department of Neurophysiology, who have been essential for the completion of the studies.

A special thanks to Mette Pilegaard, who has been an unparalleled support in the administration and daily conduction of the project.

I would also like to thank all the participants for volunteering to participate. Without your support, this study was not possible.

Finally, I would like to express my most sincere gratitude to my family and friends for their continuous love and support. To my wife Kirstine for your endless support and for enduring periods with lots of conferences and late nights at the office, and to my daughter Iben, for being an endless source of joy. Without your support, this thesis would never have existed.

Johan Møller Røikjer, December 2022

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

1.1. FIGURES

1.2. TABLES

CHAPTER 2. INTRODUCTION

Diabetes mellitus is a disease caused by a mismatch between the insulin excretion from the β-cells and the insulin resistance in the peripheral tissues. The condition is characterized by the presence of hyperglycaemia and consists of a heterogeneous group of metabolic diseases. The two most common types of diabetes mellitus are type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM). The two conditions are pathophysiologically vastly different, but ultimately share similar challenges including the development of late complications.

Diabetic peripheral neuropathy (DPN) is the most common late complication and affects as much as 50% of all individuals with diabetes mellitus[1,2]. The condition can present as many different phenotypes, but most often presents as a symmetrical, length-dependent, slowly progressing polyneuropathy. The condition is mainly sensory in the early stages and is therefore often seen as a progressive loss of sensation in the feet[3].

While DPN is most often asymptomatic in the early stages, 20-30% of the population with DPN experience (often severe) neuropathic pain[4,5]. Severe painful diabetic peripheral neuropathy (PDPN) is associated with markedly reduced quality of life, higher health care costs, and increased morbidity and mortality[6]. Treating PDPN is often difficult, and due to a lacking understanding of the underlying pathophysiological mechanisms, the current treatment is often insufficient and accompanied by severe side effects[7]. It is currently unknown why some develop PDPN while others do not, although some studies have suggested that neuropathic pain often accompany more severe damage to the pain-sensing Aδ and C-fibres[8,9].

In the clinic, early signs of DPN are often overlooked due to insufficient screening methods, but as severity increases, so does the risk of further complications. This means that DPN is often not diagnosed before an individual with diabetes has reached a stage with loss of protective sensation (LOPS), which can ultimately lead to the development of a diabetic foot ulcer (DFU) with subsequent risk of major amputation and early death[10–12].

Once DPN has progressed to a stage with LOPS it appears impossible to revert the changes and thus restore peripheral sensation. As of now, no nerve-specific, disease-modifying treatment exists for treating, preventing or even halting the progression of DPN, which means individuals who has gotten their first ever DFU more often than not experience rapid recurrence[7,8,13].

To truly combat the socio-economic burden of diabetic foot disease, and to truly prevent the first ever DFU, one must therefore focus on preventing early onset of DPN. Unfortunately, preventive measures are currently limited to optimization of

external factors such as treating hypertension and hypercholesterolemia, or by enforcing early, strict glycaemic control, which (at least in T1DM) has been shown to halt the progression of DPN[14–16]. The vast limitations in treatment-options for DPN are caused by insufficient knowledge of the underlying pathophysiology, and by insufficiently sensitive measurements of nerve fibre integrity[7,17]. The latter issue is somewhat present for most types of nerve fibres, although the problem primarily resides with the diagnostics of damage to the small (Aδ and C) nerve fibres. Unfortunately, the small nerve fibres appear to have a temporal relationship with damage preceding that of the large (Aα and Aβ) fibres, which makes assessing them adequately pivotal when striving for early detection[18–20].

For years, quantitative sensory testing (QST) was considered the gold standard for assessing small nerve fibres, but although being a resourceful method to assess neuropathy in large cohorts, the method has consistently failed as a clinical endpoint in individual people[21,22].

Over the latest decades, researchers have provided several alternatives to QST for assessing the integrity of small nerve fibres. The two most well-known and internationally acknowledged are corneal confocal microscopy (CCM) and quantification of intra-epidermal nerve fibre density (IENFD) using skin biopsies [23–27]. Both methods have different pros and cons, with skin biopsies being invasive, time-consuming, and unfit for large scale screening, while critiques of CCM claim that the method has too much variation even amongst healthy subjects, and that the method is limited by its lacking specificity to diabetes mellitus, as other systemic conditions like rheumatoid arthritis has shown similar reductions in the measured parameters[7,28]. Finally, the two methods also share the common limitation of only measuring the extend of structural damage without providing any information regarding the function of the remaining nerve fibres.

The above-mentioned limitations in the current methods for assessing the integrity of small nerve fibres justify a look into novel methods for adequate, precise, and rapid assessment of small nerve fibre function, to be used as a supplement or replacement for the existing methods.

CHAPTER 3. BACKGROUND

3.1. TYPE 1 DIABETES MELLITUS

T1DM is a chronic, autoimmune disease affecting the islet cells of the pancreas. The condition is a vast topic of which most are out of the scope of the present thesis. T1DM accounts for approximately 5-10% of the total number of cases with diabetes worldwide and both incidence and prevalence appear to be increasing[29,30]. Although T1DM is not as common as T2DM, it accounts for a rather large proportion of the population seen in most outpatient clinics. T1DM often debut early in life and require life-long treatment with exogeneous insulin. Polyuria, polydipsia, and weight loss are some of the most common symptoms leading to diagnosis in children, although up to a third debut with more rapid onsets and mild to severe ketoacidosis[31]. In adults, the onset can be a bit more varied and sometimes lack the classic symptoms seen in children, leading to an initial misclassification as T2DM [31].

The diagnosis of diabetes mellitus is nowadays usually given based on a haemoglobin A1c (HbA1c) > 48 mmol/mol, but the detection of any plasma-glucose ≥ 11.1 mmol/mol alongside classic symptoms, a fasting plasma-glucose ≥ 7.0 mmol/mol, or a plasma-glucose ≥ 11.1 mmol/mol following an oral glucose tolerance test are still relevant tools, especially in people with rapid onset of T1DM or with chronic kidney disease, recent blood transfusion, haematological diseases, or any other conditions affecting the lifespan of the erythrocytes[29,32,33].

The cornerstone in the treatment of T1DM is administration of exogenous insulin, which can be delivered using an insulin pen or using a pump with varying degrees of automatization[34]. Optimal glycaemic control often requires multiple-dose therapy to mimic physiological insulin release, which means a combination of long- and rapid-acting insulin is required if using pen. Over the last decades, the use of continuous glucose monitoring has gradually increased, and although fully automated closed-loop systems are still not available, the overall usage of smart technologies have now become a mainstay in the treatment of T1DM [35].

T1DM is associated with several acute and chronic complications. Ketoacidosis and hypoglycaemia are the most common of the acute complications and are associated with life-threatening outcomes if not treated timely and adequately[36]. The chronic complications to diabetes mellitus are often divided into micro- (retinopathy, nephropathy, neuropathy) and macrovascular (i.e., stroke, acute myocardial infarction, limb ischemia) complications[37]. Although the different types of diabetes mellitus historically have been associated with different types of complications, the more modern view is that all complications can happen to all people with all forms of diabetes mellitus[38].

Research-wise, studying most types of diabetes have different pros and cons, but as T1DM often have less co-morbidities outside of those related to diabetes, and thus poses less uncertainties concerning the origin of their symptoms, this phenotype will be the focus of this thesis.

3.2. DIABETIC PERIPHERAL NEUROPATHY

According to the Toronto Diabetic Neuropathy Expert Group, typical DPN is defined as [1]:

> "*A symmetrical, length-dependent sensorimotor polyneuropathy attributable to metabolic and microvessel alterations as a result of chronic hyperglycaemia exposure (diabetes) and cardiovascular risk covariates*"

This definition underlines the fact that DPN is indeed considered a microvascular complication, with atherosclerosis of the small blood vessels leading to nerve ischemia. Despite DPNs common occurrence (affecting up to half the population with diabetes mellitus), the exact underlying pathophysiological mechanisms remain unknown[7,39]. Most agree that the toxic effects of hyperglycaemia play an important role in the underlying pathogenesis of DPN besides the mentioned microvascular changes. For years, the shift in glucose metabolism caused by saturation of the hexamine pathway and increased activity in the polyol pathway with subsequent intra-cellular accommodation of sorbitol was thought to be the primary mechanism behind the complication[40]. However, this mechanism is probably only a small part of a larger picture, and might even be driven by an increased formation of advanced glycation end products (AGEs) and decreased levels of glutathione leading to oxidative stress rather than the accumulation of sorbitol itself, as several studies have since reported insignificant concentrations of sorbitol accumulated in the nerves of people with diabetes[41]. Factors such as dyslipidaemia, hypertension, mitochondrial dysfunction, and an overload of reactive oxygen species have been associated with the development of DPN, and recently also the role of systemic low-grade inflammation has gained increased interested as a potential contributing factor[42–44]. Risk factors such as age, alcohol abuse, high body mass index, ethnicity, sex, diabetes duration, diabetes type, glycaemic control, other microangiopathic complications, waist circumference, hypertension, vitamin deficiency, and smoking have all been associated with the development of DPN, but far from everyone with these risk factors develop the condition, while some develop it despite having only a few of the risk factors[43,45]. Overall, as researchers continuously uncover new potential mechanisms of action (also besides the direct effects of hyperglycaemia), it has become increasingly apparent, that we are yet to uncover the full picture of the natural history of DPN and unravel the mysteries of the human nerves and their inevitable demise[43].

DPN is a condition affecting all sensory nerves in a length-dependant manner. The large (Aα and Aβ) nerve fibres are myelinated with periodic gaps with a high density of ion-channels known as nodes of Ranvier, which helps them achieve a high conduction velocity and excitability[43,46]. The large nerve fibres are responsible for the sensation of touch, vibration, and proprioception, and are usually assessed using a 10g-monofilamnent (touch), a biothesiometer (vibration) or conventional nerve conduction studies. The small (Aδ- and C) nerve fibres are either thinly myelinated or unmyelinated, which result in a slower conduction velocity (especially for the unmyelinated C-fibres) and a lower excitability than that displayed by the large fibres, and can cause the sensation evoked by the nerves to be more diffuse and long-lasting[43,46].

The temporal relationship between damage to the different types of nerves remains a bit nebulous. Most recent studies support the hypothesis that DPN is characterized by predominant and progressive injury to the small nerve fibres followed by subsequent large fibre impairment, with the small nerve fibres often displaying damage years in advance of the large fibres[3,18–20]. This natural history is accepted by many, and appear plausible from a theoretical point of view, as small unmyelinated/thinly myelinated nerve fibres should be more susceptible to external damage than the large, myelinated fibres. Unfortunately, this topic is again limited by the lack of longitudinal studies and unprecise and insufficient measurements of nerve fibre damage, and while some newer studies does exist with opposite findings, the issues of insufficient and unprecise measurements remain[47,48].

Returning to the initial definition of DPN by the Toronto Diabetic Neuropathy Expert Group, it is also noteworthy that the condition is defined as a *sensorimotor polyneuropathy*. This means that not only is DPN a universal neuropathy, but it also might affect both sensory and motor nerves[1]. Involvement of both sensory and motor nerves are however rare in the initial stages, where the degeneration is predominantly of the sensory nerves. Although DPN is most commonly seen as a symmetrical, length-dependant, polyneuropathy affecting the most distal parts of the body first with a slow proximal progression, it can present in many different shapes and form ranging from transient mononeuropathies to rapidly progressing autonomic neuropathies with severe symptoms[3]. In its usual from, symptoms are rare in the early stages, where an asymptomatic loss of sensation is the usual clinical picture. As the DPN worsens, symptoms like numbness, tingling, prickling, or the sensation of “walking on cotton wool” become increasingly common, and some (up to 20-30%) might develop accompanying neuropathic pain[7,49,50]. The most common presentations of DPN are depicted in *figure 3-1*.

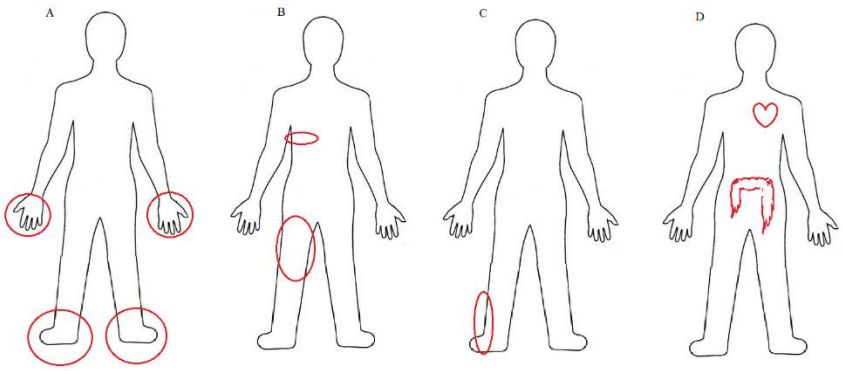

Figure 3-1 Schematic overview of the most common types of diabetes neuropathy: *Distal symmetrical polyneuropathy (A), radiculopathy (B), mononeuropathy (C), and autonomic neuropathy (D)*

3.2.1. PAINFUL DIABETIC PERIPHERAL NEUROPATHY

PDPN is a heterogeneous condition that remains an enigma for both clinicians and scientists alike. PDPN sometimes presents as a transient phenomenon after only a few years living with diabetes, but is often linked to longer diabetes duration and more severe stages of neuropathy[8,9]. The underlying pathophysiological differences between those with painless and those with painful DPN remain unknown, although many agree, that the pain probably arise from a combination of damage to the peripheral nerve endings and changes within the central nervous system including altered signal processing and reduced inhibition of descending pathways within the central nervous system[6].

The lacking understanding of the pathophysiological changes leading to pain is the main reason why no disease-modifying treatment for PDPN is currently approved by any major regulatory authority[7,51]. However, clinicians do have several therapies available for relieving the often-severe symptoms. Unfortunately, these options are usually ineffective in lower dosages and associated with side-effects in high dosages, which is why combination-therapy is often required to achieve an acceptable ratio between pain relief and side-effects[7,52]. The U.S. Food and Drug Administration (FDA) approved the calcium-channel modulator, pregabalin, and the serotonin-noradrenaline reuptake-inhibitor, duloxetine, more than 25 years ago, and more recently added tapentadol, a dual-action opioid and norepinephrine reuptake-inhibitor with extended-release to the list[7]. Other commonly used drugs include tricyclic antidepressants (TCAs) like amitriptyline, or drugs with similar actions as the ones already approved, like gabapentin (a calcium-channel modulator) or venlafaxine (a

serotonin-noradrenaline reuptake-inhibitor)[7]. Recently, a pioneering head-to-head, crossover trial investigating the effect of amitriptyline with the addition of pregabalin, pregabalin with the addition of amitriptyline, and duloxetine with the addition of pregabalin found similar analgesic effect for all monotherapies, but enhanced effect in all combination regimes[53]. Sodium channel blockers (carbamazepine and its successor oxcarbazepine) are also worth mentioning, as their unique mechanism of action provide a potential pathogenic target in subgroups with PDPN. Carbamazepine was previously tested in PDPN but was later withdrawn due to significant side effects[54]. Oxcarbazepine has received limited testing and mediocre results, but a sub-group analysis based on deep phenotyping by QST revealed promising effects in subgroups with PDPN, pushing us towards much needed personalized treatment regimens[55]. Similarly, interesting results have also been found utilizing functional magnetic resonance imaging, where deep learning has been used to successfully predict treatment response in small cohorts of people with PDPN[56]. Other pharmacological treatment targeting the proposed mechanisms leading to pain include α-lipoic acid, C-peptide, benfotiamine and aldose-reductase inhibitors, although the effect of neither of these drugs are currently well-documented due to insufficient study designs and lack of subgroupings[7,57]. Local treatment options are also available, with capsaicin being the most well-documented and widely used[58,59]. Although several options are available, some people might not experience adequate pain-relief using traditional pharmacological interventions[7]. Therefore, several non-pharmacological alternatives have been made available. These include transcutaneous, percutaneous, or more direct frequency-modulated electromagnetic nerve stimulations, alongside initiatives focused on different coping-strategies[60,61].

3.3. DIAGNOSING DIABETIC PERIPHERAL NEUROPATHY

In the clinic, DPN is a diagnosis of exclusion. Signs and symptoms of peripheral nerve dysfunction are combined with the exclusion of other causes of neuropathy (i.e., vitamin-deficiencies, alcohol abuse, paraneoplasia, or rheumatologic- or neurological diseases) to give the final diagnosis. Often, this diagnosis can be given be a specialist in diabetes without the need for further examinations, but in some cases, the diagnosis needs to be confirmed by conventional nerve conduction study or by a consulting neurologist[62].

The Toronto Diabetic Neuropathy Expert Group suggested a distinction between typical (DSPN) and atypical DPN. The group also listed four definitions of DSPN to be used in both clinical practice and research, with 1-3 being recommended as a clinical standard, and 3-4 being recommended for research[1]:

(1) *Possible DSPN: Symptoms-decreased sensation, positive neuropathic sensory symptoms (e.g., "asleep numbness," prickling or stabbing, burning or aching pain) predominantly in the toes, feet, or legs; or signs–symmetric*

decrease of distal sensation or unequivocally decreased or absent ankle reflexes.

(2) Probable DSPN: The presence of a combination of symptoms and signs of neuropathy include any two or more of the following: neuropathic symptoms, decreased distal sensation, or unequivocally decreased or absent ankle reflexes.

(3) Confirmed DSPN: The presence of an abnormality of NC and a symptom or symptoms or a sign or signs of neuropathy confirm DSPN. If NC is normal, a validated measure of small fibre neuropathy (SFN) (with class 1 evidence) may be used.

(4) Subclinical DSPN: The presence of no signs or symptoms of neuropathy are confirmed with abnormal NC or a validated measure of SFN (with class 1 evidence).

Notably, the diagnosis of confirmed typical DPN is reliant on a validated measure of small fibre neuropathy (SFN) in cases with a normal conventional nerve conduction. However, the method of choice is not clear-cut, which becomes apparent the groups' definition of SFN, where both thermal thresholds and skin biopsies are mentioned[1]:

(1) Possible SFN: the presence of length-dependent symptoms and/or clinical signs of small fibre damage

(2) Probable SFN: the presence of length-dependent symptoms, clinical signs of small fibre damage, and normal sural NC study

(3) Definite SFN: the presence of length-dependent symptoms, clinical signs of small fibre damage, normal sural NC study, and altered IENF density at the ankle and/or abnormal quantitative sensory testing thermal thresholds at the foot.

Since the publication of the mentioned guidelines, CCM has become increasingly popular as a marker for small fibre damage, although its usage as a confirmatory test is still being heavily debated.

3.3.1. SKIN BIOPSIES

Skin biopsies with quantification of intra-epidermal nerve fibre density (IENFD) are considered the gold standard for assessing small nerve fibres in the skin in most neuropathic conditions including diabetes[27,63]. The method relies on a 3 mm skin

biopsy taken from the calf, in an area located approximately 10 cm proximal of the lateral malleolus[64]. After being taken, the biopsy requires fixation followed by cryoprotection and subsequent staining (usually by the pan-axonal marker protein gene product 9.5 (PGP 9.5)), before it can be studied using bright-field microscopy or immunofluorescence[65]. The method excels due to it being objective with high inter-observer reproducibility, while also not being confounded by height and weight like most other electrophysiological methods[66]. Normative datasets (adjusted for age and sex) have been developed for both bright-field microscopy and immunofluorescence, which allow for easy comparison between different populations and studies[67]. While skin biopsies are historically used primarily for quantification of the IENFD, modern technology has allowed for more advanced research with specialized staining targeting specific structures (like small peptides or subsets of ion-channels) within the nerve fibres[68,69]. In addition to specialised staining, the role of axonal swellings as a potential marker for early damage has also been gaining increased interest[70,71]. Despite its many pros, skin biopsies do suffer several inherent weaknesses. Firstly, it is invasive and require specialized laboratories with experience in preparation and analysis. Secondly, the intra-person reproducibility remains unclear, as only a limited number of studies have assessed this outside of healthy subjects[72]. Thirdly, the method is limited to only assessing the morphological changes of the nerves without providing any information of the function of the remaining nerve fibres[7]. Due to the mentioned limitations, the method is not suitable for large scale screening, but might serve as a confirmatory test or as a clinical endpoint for research studies. Representative images from skin biopsies of people with different severities of DPN and axonal swellings are depicted in *figure 3-2*.

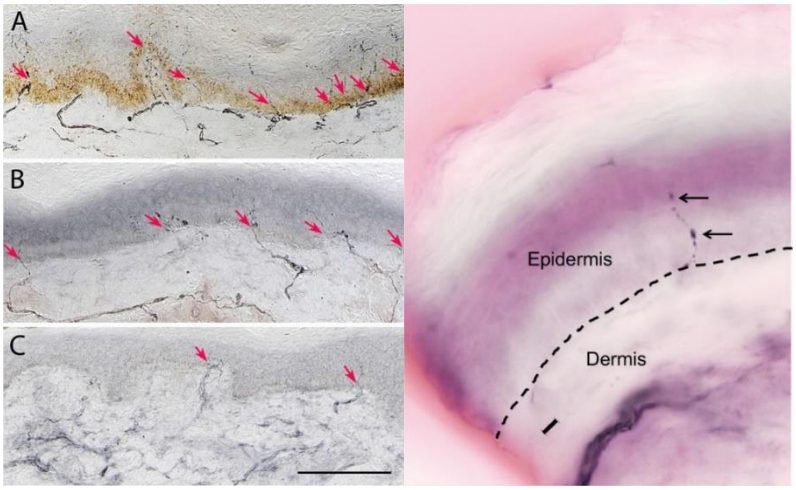

Figure 3-2 Representative images of skin biopsies: *Left: a healthy person (A), a person with diabetes of similar age (B) and that same person 6.5 years later. Right: a person with diabetes and axonal swellings. Adapted from* [70] and [73].

3.3.2. QUANTITATIVE SENSORY TESTING

QST has been a cornerstone in neuropathy and (especially) pain research for decades, although the procedure was not properly standardized until the German Research Network on Neuropathic Pain (DFNS) established their procedure in the early 2000's[74]. The method consists of multiple tests, involving thermal perception and pain thresholds, vibration perception, touch and pinprick sensations, and deep musculoskeletal perception, ultimately assessing the function of both large and small nerve fibres[74]. The method is non-invasive and diagnose both gain and loss of sensory function. Furthermore, a large database containing normative values have been developed, and were recently validated in multiple centres across Europe[75]. Even more recently, the method has also been used as a tool for subgrouping people with PDPN, potentially improving performance and giving additional insight into the heterogeneity of the condition[76]. Unfortunately, issues like time-consumption, labour intensity, lacklustre sensitivity, low reproducibility, and disease-specific sensitivity remain important concerns, limiting the use of QST as a reliable endpoint in everyday clinical work and in clinical trials[7,17,22].

3.3.3. CORNEAL CONFOCAL MICROSCOPY

CCM is an emerging method for indirect assessment of SFN[23,24]. The method has been gaining interest as a surrogate marker for SFN due to its rapidness and high diagnostic agreement with skin biopsies[77,78]. The method utilizes the fact that the sub-basal nerve plexus located near Bowman's layer in the cornea has the highest density of nerve fibre endings in the human body, and the fact that these can be visualized without the need for invasive procedures. The method is rapid and is claimed to be reproducible with a high inter-observer correlation[79–81]. In some studies, CCM has also been able to distinguish between people with and without PDPN, although an equal number of studies have failed to show any differences, once again hinting that PDPN is a heterogeneous condition[73,82,83]. CCM has also been utilized in more exploratory studies, including a recent study in people with T1DM undergoing pancreatic transplantation. Here, the authors found nerve fibre regeneration in the months following the transplantation, hinting a high sensitivity to change[84].

The measurements usually derived from CCM are corneal nerve fibre density (CNFD), corneal nerve fibre length (CNFL), corneal nerve branch density (CNBD) and corneal nerve fibre tortuosity (CNFT). Of these, CNFL appear to be the most robust parameter, while CNFT has fallen out of favour due to inconsistency in its results[7]. Normative values have also been published, making comparison between different sites easier, although some variation still exists as a standardized method for image selection is yet to be published[85]. Recently, studies using CCM in diabetes

have reported an increased numbers of corneal and epidermal Langerhans cells, with some studies even reporting high correlations between this and the other markers of corneal nerve loss[86].

Although CCM has some unique advantages over many other methods for assessing small nerve fibres it does suffers from some equally unique disadvantages. Firstly, the method appears to be rather unspecific to diabetes or even neuropathy, as several conditions have been associated with reductions in the measured corneal parameters, including common conditions like rheumatoid arthritis[28,87]. Secondly, while the method itself is rapid, the subsequent analysis is not, discouraging its use in large scale screening. This issue has led to the development of software for automatic image analysis, which (despite some initial good results) tends to underestimate several of the measured parameters compared to the manual analysis[83,88]. Thirdly, the lack of a standardized image selection process hurts the diagnostic capabilities, which were already challenged by the large variations in the normative values[85]. Representative images of the cornea from different stages of DPN assessed by CCM are depicted in *figure 3-3*.

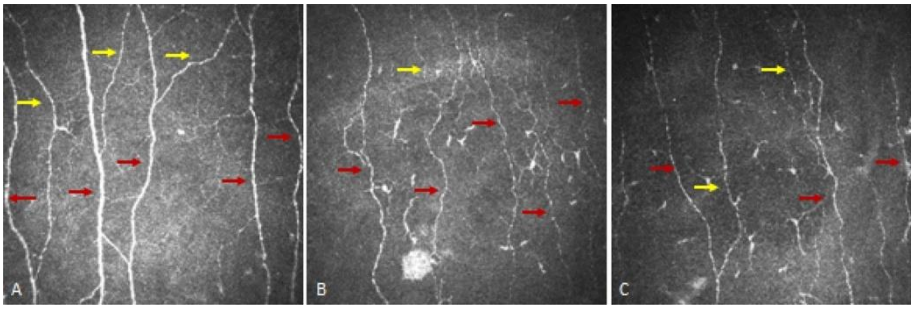

Figure 3-3 Representative images of the sub-basal nerve plexus in the cornea: *a healthy person (A), a person with diabetes of similar age (B) and that same person 6.5 years later. Red arrows mark main nerves and yellow arrows mark nerve branches. Adapted from* [73].

OTHER METHODS FOR SMALL FIBRE ASSESSMENT

While the above-mentioned methods are the most widely used for detection of small nerve fibre pathology in diabetes, several alternatives do exist. Some of the most mentionable include full-field laser perfusion imaging (FLPI), laser- (LEP) or contact heat-evoked potentials (CHEPs), and microneurography[89–92].

The FLPI is a technique utilizing Doppler to assess the C-fibre-mediated flare response (LDI_{FLARE}) in the skin as the result of a local stimulus[93]. The most widely used and studied stimuli in diabetes are local heating followed by iontophoresis of acetylcholine, but other stimuli like histamine have also been applied especially outside of diabetes research[89,93–95]. The LDI_{FLARE} response has been validated against QST, CCM and skin biopsies, and has been shown to be progressively reduced with worsening neuropathy[89,95–97]. Longitudinal data from the method are currently limited, although preliminary data from longitudinal studies has been presented at conferences but is yet to be published. A meta-analysis from 2017 likewise concluded that there are evidence for an attenuated axon-reflex flare response in people with diabetes compared to people without diabetes, but failed to establish a relationship between the degree of neuropathy and the size and intensity of the axon-reflex flare response due to an insufficient number of studies with varying design and methodology[98]. Furthermore, the method is limited in its clinical applicability by the requirement of specialized equipment and time-consumption (30+ minutes per evaluation) in addition to a lack of normative data[95,99].

The LEP or CHEPs are similar electrophysiological methods to detect functional changes in small nerve fibres[90].The methods record the central response to a peripheral stimuli, and excel due to their ability to measure direct and objectively. The methods are widely used in several neurological conditions (with available normative values), but their role in diabetes research is yet to be fully established, while their potential for clinical application are extremely low due to the requirement of specialized equipment and complex procedural algorithms[99–102]. Recently, pain-related evoked potentials have also been proposed as a method for studying neuropathic pain, although more studies are needed to confirm their usability[103].

Microneurography is a very time-consuming method with unique abilities for studying underlying pathophysiological mechanisms of neuropathy. The method utilizes tungsten needles to penetrate peripheral nerve fascicles, but has been associated with nerve damage and is probably not a pivotal part of future DPN research[92,104].

Additional methods for assessing peripheral autonomic C-fibre (sudomotor) function also exist and include methods like qualitative sudomotor axon reflex testing (QSART), SUDOSCAN, or even the Neuropad, although these are only mentioned here to complete the list and will not described any further[105–107].

CONVENTIONAL THRESHOLD TRACKING

Conventional threshold tracking is an electrophysiological method that provides information on motor or sensory axonal nerve fibre excitability. Conventional threshold tracking is therefore sensitive to changes in membrane properties, giving it an unique ability to indirectly study ion-channels and electrogenic ion-pumps[46]. This contrasts with the more widely used electrophysiological tests that mainly focus on conduction velocities caused by damages to the myelin sheet and compound action potential amplitude reduction caused by axonal degeneration[46]. While conventional threshold tracking does indeed provide some unique insight into the pathophysiological changes happening within the nerve membrane, its usefulness is limited by the sheer complexity and specialized equipment needed to perform the examination. This means that the method is rarely known or seen outside of highly specialized neurophysiological clinics and is by no means a candidate for large scale clinical use in diabetes. Consensus guidelines were recently published on how to best perform the examinations and how to interpret the results underlining the difficulties associated with the methods[108]. The reason the method is mentioned here, is the fact that the perception threshold tracking technique presented in this thesis is built on the foundation of this method, utilizing the discoveries related to impulse shapes, nerve fibre accommodation properties, and strength-duration behaviour[46].

Accommodation is the ability of a nerve to adapt to a slowly rising (ramped) stimulus by increasing the its activation threshold[109,110]. Different nerve fibres possess different accommodation properties based on their ion-channel composition and resulting ability to maintain a steady membrane potential[111,112]. Large nerve fibres have a good ability to accommodate, while small nerve fibres do not. This is caused by small nerve fibres expressing a large number of persistent voltage-gated sodium channels, while the large nerve fibres predominantly express transient voltage-gated sodium channels[110,113].

The strength-duration behaviour of different nerve fibres is depicted schematically in *figure 3-4*. The minimal current of infinite duration needed to activate a nerve fibre is known as the rheobase, while the minimal time required to double the strength of the rheobase is known as the chronaxie[46,114,115]. The differences in excitability between the different nerve fibres are caused by several factors including size, myelination, ion-channel composition and the presence of nodes of Ranvier, which ultimately results in large, myelinated, Aβ-fibres being markedly more excitable than small, thinly myelinated or unmyelinated, Aδ- and C-fibres[43,114].

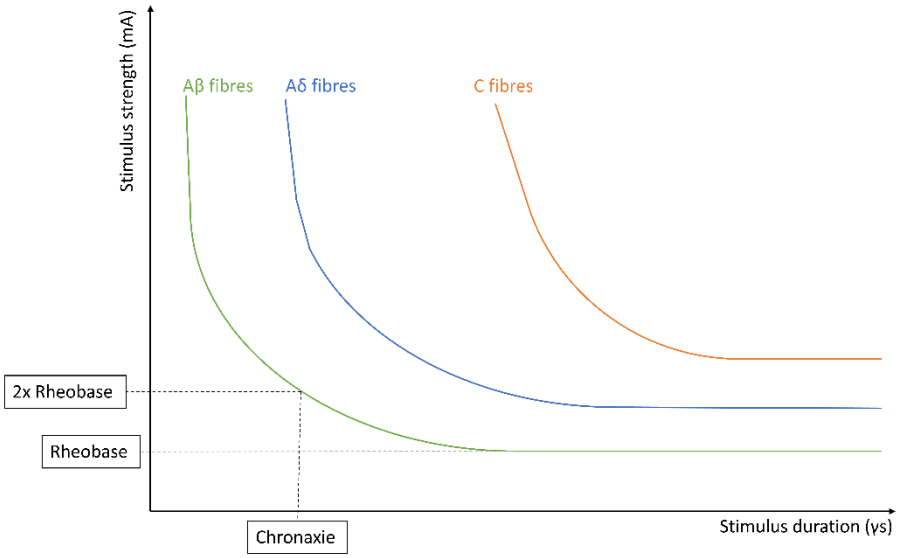

Figure 3-4 Schematic overview of strength-duration curves for different nerve fibres: *Aβ-fibres (green) have a lower activation threshold than Aδ-fibres (blue) and both have lower activation thresholds than C-fibres (orange). The rheobase (here shown for Aβ-fibres as a dotted grey line) is the minimal current of infinite duration needed to activate the nerve fibres. The chronaxie (here shown for Aβ-fibres as a dotted black line) is the minimal time required to double the strength of the rheobase. Figure created based on* [46,114].

3.3.4. LARGE FIBRE ASSESSMENT

Functional assessment of large nerve fibres using conventional nerve conduction studies is considered the gold standard for confirming large fibre nerve damage in most neuropathies including that caused by diabetes[1]. The examination usually consists of an evaluation of both motor and sensory nerves, usually including the median, the ulnar, the radial, the tibial, the peroneal and the sural nerves. While the method is considered a clinical standard in most modern health care systems, the method does still require specialized equipment and training, is somewhat time-consuming, and has limited potential in several settings including most large clinical trials. The NC-stat DPNCheck is a rapid alternative to the full conventional test and provides a bedside option for usage in clinical trials or large-scale screening[116]. The method excels due to its speed and handheld form, but although the method has shown excellent agreement with conventional nerve conductions, and has been validated against methods like the LDI_{FLARE}, the NC-stat DPNCheck only provides information

on the sural nerve and often require multiple tries to achieve a correct assessment[117,118]. Alternative bedside methods include assessment of the vibration perception threshold (VPT) using a biothesiometer or a tuning fork, or assessment of touch sensation using the 10g-monofilament. Both methods have shown to be decent predictors for the development of a DFU but are all test for LOPS rather than early neuropathy[119].

CHAPTER 4. RATIONALE AND AIMS

Adequate assessment of the small nerve fibres is pivotal when striving for early detection of DPN. Current screening methods are either insufficient, time-consuming, or only assess structural damage to the nerve fibres without providing information on their remaining function. These issues justify the development of new rapid measurements, that could be applied in a clinical setting.

The overall aim of this PhD thesis was to evaluate novel methods for rapid and adequate assessment of small nerve fibre function in people with T1DM, with the ultimate end-goal of providing robust clinical endpoints for early detection, prevention, and hopefully future pharmacological interventions to either halt, prevent or even cure DPN, PDPN and ultimately DFUs. In addition, the thesis explores the nebulous concepts of DPN and PDPN and tries to assess whether these can be successfully distinguished from each other using novel methods.

The thesis is based on two published, peer-reviewed, original papers and one original paper submitted for publication. All three papers derive from the MEDON-study (*Methods for Early Detection Of diabetic peripheral Neuropathy, Clinicaltrials.gov: NCT04078516)*, but have different hypotheses and aims. An overview of the different papers with corresponding aims and utilized methods are depicted in *figure 4-1*.

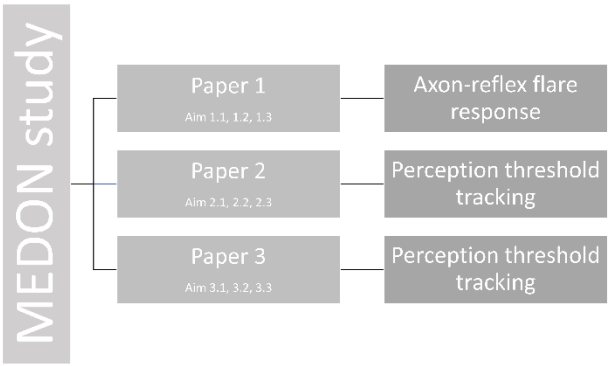

Figure 4-1 Overview of papers and aims: *paper 1 investigates the axon-reflex flare response, while paper 2 and 3 investigate perception threshold tracking (see 4.1 Papers below).*

4.1. PAPERS

4.1.1. PAPER 1

Aim 1.1: To test if the axon-reflex flare response can be evoked by histamine applied by a simple handheld skin-prick device.

Aim 1.2: To assess whether the presence of T1DM attenuates the intensity of the axon-reflex flare response compared to the response in people without diabetes.

Aim 1.3: To assess whether the presence of DPN attenuates the intensity of the axon-reflex flare response compared to the response in people with T1DM without DPN.

Aim 1.4: To assess if the presence of PDPN attenuates the intensity of the axon-reflex flare response compared to the response in people with T1DM and painless DPN.

4.1.2. PAPER 2

Aim 2.1: To validate the novel perception threshold tracking technique in people with diabetes and ensure preserved selective stimulation of Aβ and Aδ-fibres, respectively.

Aim 2.2: To assess if perception threshold tracking can distinguish different groups of people with and without T1DM, DPN and PDPN.

Aim 2.3: To compare the diagnostic performance of perception threshold tracking to established parameters of temperature sensation obtained from QST

4.1.3. PAPER 3

Aim 3.1: To compare the outcomes of perception threshold tracking and CCM.

Aim 3.2: Investigate the diagnostic performance of perception threshold tracking for detecting small fibre damage using CCM as a reference.

Aim 3.3: Investigate the relationship between structural and functional measures of small nerve fibre damage.

CHAPTER 5. METHODS

All data provided in this thesis were obtained from the MEDON-study, which was created as a part of the present PhD-study. The study was conducted in accordance with the Declaration of Helsinki and was approved by the North Denmark Region Committee on Health Research Ethics (ethics: *N-20190003*) and the North Denmark Region (data protection: *2019-32*). The study was prospectively registered on ClinicalTrials.gov (ID: *NCT04078516)*. The MEDON-study also included an MRI-session, which is not relevant for the present thesis. The MEDON-study will be described below, but only methods relevant for the actual thesis will be presented in detail.

5.1. THE MEDON-STUDY

The MEDON-study was set up as a collaboration between Steno Diabetes Center North Denmark, the department of Radiology at Aalborg University Hospital, and Integrative Neuroscience at Aalborg University. Furthermore, the department of Endocrinology, the department of Neurophysiology, and the department of Ophthalmology participated as collaborators regarding specific tasks (participant recruitment, conventional nerve conduction studies and CCM, respectively). The overall aim of the MEDON-study was to evaluate methods for assessment and deep-phenotyping of neuropathic complication in T1DM. The study was designed as an observational, cross-sectional, case-control study including 80 participants matched individually (1:1:1:1) on age (+/- 2 years) and sex between four groups: T1DM+PDPN (n=20), T1DM+DPN (n=20), T1DM-DPN (n=20), and healthy controls (n=20). To be in the group with T1DM+PDPN the participants needed to have T1DM and clinical signs and symptoms of PDPN supported by a Douleur Neuropathique en 4 (DN4)-score of ≥ 4. To be in the group with T1DM+DPN the participants needed to have signs and symptoms of DPN, no signs and symptoms of PDPN, and a VPT $\geq$ 25V. To be in the group with T1DM-DPN the participants needed to have no signs or symptoms of DPN or PDPN, and a VPT < 25V. To be in the group with health controls the participants needed to not have diabetes (defined by HbA1c), while also not having any signs and symptoms of neuropathy and a VPT < 25V (*see figure 5-1*). The reasoning behind this grouping was to depict the clinical standard and then later re-group the participants based on whether they had large fibre involvement based on conventional nerve conduction and whether they had small fibre involvement based on QST.

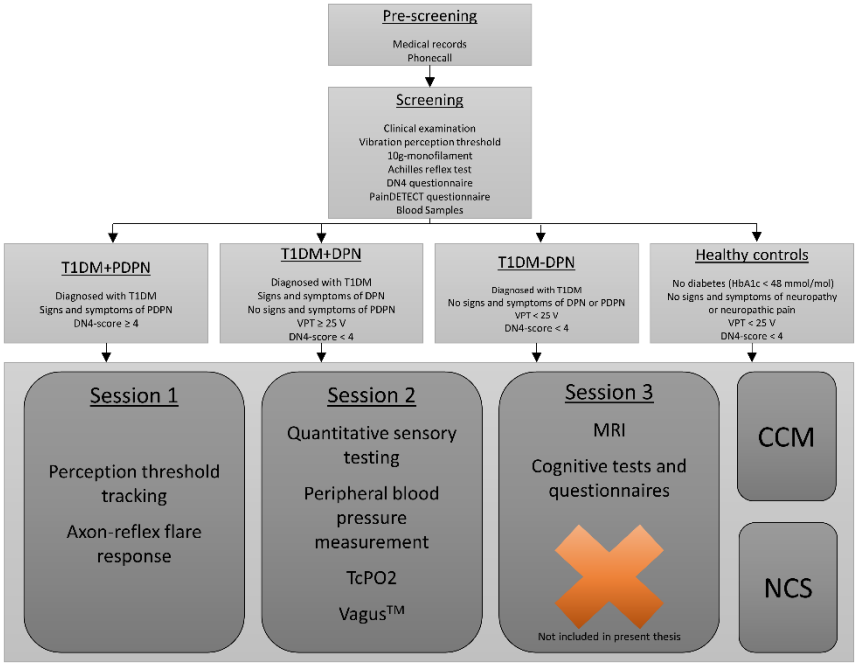

Figure 5-1 Overview of the MEDON-study: *Data from screening, session 1, session 2, and the sessions containing corneal confocal microscopy and conventional nerve conduction studies are included in the present thesis, while data from session 3 is published as a separate thesis with original publications.*

Abbreviations: ***CCM:*** *Corneal Confocal Microscopy,* ***DN4:*** *Douleur Neuropathique en 4,* ***DPN:*** *Diabetic Peripheral Neuropathy,* ***HbA1c:*** *Haemoglobin A1c,* ***MRI:*** *Magnet Resonance Imaging,* ***NCS:*** *Nerve Conduction Studies,* ***PDPN:*** *Painful Diabetic Peripheral Neuropathy,* ***T1DM:*** *Type 1 Diabetes Mellitus,* ***TcPO2:*** *Transcutaneous Oximetry,* ***VPT:*** *Vibration Perception Threshold*

To ensure the thorough matching across the groups, each participant with T1DM+PDPN were always recruited first, and matching participants in the other groups were subsequently recruited based on age and sex. Participants with T1DM were recruited from the outpatient clinic at Steno Diabetes Center North Denmark/department of Endocrinology, while healthy controls were recruited from an existing database of volunteers. Medical records of potential participants were pre-screened prior to contact and only participants still eligible after pre-screening were contacted by phone and invited to a screening visit. At the screening visit all causes of neuropathy other than that caused by diabetes were excluded (medical history and broad screening blood samples). The full set of exclusion criteria are available in *table 5-1*. Prior to all sessions, a blood glucose between 4.0-14.0 mmol/L was ensured.

Exclusion criteria
Current or previous alcohol- or drug abuse
Abnormal screening blood samples
Not being able to understand Danish written and/or verbally
Not being able to corporate to examination (e.g., not being able to speak, suffering from senile dementia etc)
Previous chemotherapy or intake of experimental medicine
Active herpes- or varicella zoster infection or known HIV
Severe skin disease
Known neural damage or disease in the neural system (e.g., Guillain-Barre etc)
Critical limb ischemia
Allery or intolerance to histamine or inability to make do without antihistamine for one day
Pregnancy
Active cancer-disease

Table 5-1 Exclusion criteria for MEDON: *Any drug or current alcohol abuse or previous alcohol abuse for more than 1 year, abnormal screening blood samples including vitamins (mild vitamin-D insufficiency were allowed by corrected prior to enrolment), not being able to understand Danish or corporate to the examinations, any previous chemotherapy or experimental medicine, severe viral infections, skin damage or known neuronal disease, critical limb ischemia (as per clinical guidelines), allergy to histamine or inability to do without antihistamine for a day, pregnancy and active cancer.*

87 participants were invited to screening. Seven of those were screen failures and were not included in the study. The reasons for screen failures are depicted in *table 5-2*.

Reason	Excluded
Previous alcohol abuse	3
Non-neuropathic pain	1
Drug abuse (marijuana)	1
Occult cancer	1
Porphyria	1

Table 5-2 Reasons for screen failure in MEDON: *Three had previous alcohol abuse, one had non-neuropathic (ischaemic) pain, one had active marijuana abuse, one was diagnosed with multiple myeloma based on screen blood samples (positive M-component), and one had unregistered porphyria.*

5.2. LARGE FIBRE ASSESSMENT

Large fibre dysfunction was initially identified using biothesiometry (VPT) as per the Danish clinical guidelines for the management of diabetes[33]. The VPT was determined by placing the probe on the participants finger and then slowly increasing the intensity of the vibrations to make the participant aware of the sensation. The probe was then placed on the first toe and the participants were instructed to close their eyes and indicate when the vibrations were felt. The intensity of the vibrations was then slowly increased until the participants indicated the sensation was felt. The intensity of the vibrations was then increased approximately 20% and subsequently lowered until the participants indicated the feeling was no longer present. This was repeated three times for each foot, and the vibration perception for each foot was determined as the average value of all tests on that foot.

Touch sensation was assessed using the 10g-monofilament. The monofilament was initially applied on the finger of the participant to indicate the correct sensation. The participants were then instructed to close their eyes and indicate when a similar touch was felt on their foot. The monofilament was applied 4 times (bending to approximately 45°) on each foot in 4 pre-defined locations following established guidelines[120]. All 4 applications of the monofilament should be registered by the participant for the examination to be normal. Otherwise, the test would be scored as diminished or absent as per the Michigan Neuropathy Screening Instrument[121].

The Achilles reflex was evaluated following established guidelines and were graded into present, reinforced and absent as per the Michigan Neuropathy Screening Instrument[121,122].

Large fibre dysfunction was confirmed by conventional nerve conductions studies of the median, the ulnar, the radial, the tibial, the peroneal and the sural nerves performed at the department of Neurophysiology at Aalborg University Hospital following established clinical guidelines (including standardized 32° Celsius skin temperature). Established local normative values were used to determine cut-offs. If a nerve was not detectable the valued was denoted with zero.

5.3. SMALL FIBRE ASSESSMENT

5.3.1. QUANTITATIVE SENSORY TESTING

The function of both large and small nerve fibres was assessed by QST following the established protocol by the DFNS[74]. The protocol is well-known and quite extensive, but in short, the cold detection threshold (CDT), heat detection threshold (HDT), cold pain threshold (CPT), and heat pain threshold (HPT) were obtained by slowly changing the temperature from the starting value of 32° Celsius upwards towards 50° Celsius or downwards towards 0° Celsius (Advanced Thermosensory Stimulator, Advanced Medical Systems, Israel). The participant was asked to press a button whenever they felt the neutral temperature change to either cold (CDT), warm (HDT), painfully cold (CPT), or painfully warm (HPT). Each threshold was obtained three times in a row and the final threshold was determined as the mean values of the three tests. Paradoxical temperature sensations were assessed by alternating between increasing and decreasing the temperature. The participants were asked to indicate whether the felt sensations were “cold”, “warm”, “burning” or “painfully burning” each time they indicated a change in temperature. The mechanical detection threshold was obtained by using von Frey hair (Aestiesiometer III, Somedic SenseLab, Sweden) with varying force[74]. The lower mechanical detection threshold was determined as the lowest force the participant was not able to feel, while the upper mechanical detection threshold was determined as the lowest force the participant was able to feel. Each threshold was determined five times and the final threshold reported as the mean of the five values. Upper and lower mechanical pain thresholds were determined in a similar fashion using pinpricks (custom-made, Aalborg University, Denmark). The stimulus-response functions (mechanical pain sensitivity and dynamic mechanical allodynia) were obtained by asking the participant to indicate the pain induced on a scale from 0-10 using pre-defined combinations of above-mentioned pinprick set in combination with cotton wool, a cotton swap, and a brush (Somedic SenseLab, Sweden)[74]. The wind-up ratio (increasing pain intensity by repeatable stimulations) was determined by applying a 256 mN pinprick (custom-made, Aalborg University, Denmark) once and subsequently ten times in a row. The pain intensity after one

application and after 10 was rated on a scale from 0-10. This was repeated five times and reported as the mean of the five examinations. The pressure pain threshold was obtained using a pain pressure device on the foot (Algometer, Somedic SenseLab, Sweden) three times. The pain pressure threshold was determined as the mean of the three examinations. The VPT was determined using a Rydel−Seiffer tuning fork (64 Hz, 8/8 scale) on the lateral malleolus, asking the participants to indicate when the vibrating sensation stopped. This examination was performed three times and the VPT was determined as the mean value of the three tests. All examinations were conducted in an area located 2-3 cm proximally to the area between the second and third toe.

5.3.2. QUESTIONNAIRES

The participants were asked to fill out two questionnaires as part of the study: the DN4- and the PainDETECT-questionnaires[123,124]. DN4 served to ensure those with PDPN indeed had neuropathic pain and helped identify which type of pain each participant experienced. PainDETECT helped quantify the pain intensity, as it provided information on average and peak pain over the last four weeks on a scale from 0-10. In addition to that, PainDETECT also helped identify and subtype neuropathic pain, although the questionnaire has not been validated for this purpose in diabetes.

5.3.3. CORNEAL CONFOCAL MICROSCOPY

All participants underwent in vivo CCM (Heidelberg Retinal Tomograph III Rostock Cornea Module, Heidelberg Engineering GmbH, Germany) at the department of Ophthalmology at Aalborg University Hospital. The participants received local anaesthesia using eye drops, and 100 images with a resolution of 400 x 400 μm were obtained using a volume scan through the central cornea. Afterwards, a total of 6-8 representative images were selected for each participant, and analysed manually using CCMetrics (M.A. Dabbah, Imaging Science and Biomedical Engineering, University of Manchester, United Kingdom). CNFL, CNBD, CNFD, and CNFT were determined as the mean value of the 6-8 images. CNFL was defined as the total length of all nerve fibres per frame (mm/mm^2). CNBD was defined as the number of branches on the main nerves ($no./mm^2$). CNFD was defined as the number of main nerve fibres defined as fibres taking up more than 50% of the total frame length per frame ($no./mm^2$). The tortuosity coefficient was calculated using CCMetrics[125].

5.3.4. AXON-REFLEX FLARE RESPONSE

All participants underwent an examination of the axon-reflex flare response provoked by an epidermal application of one drop of 1% histamine (Lofarma, Italy). The histamine was applied using a skin-prick lancet with a standardized 85g pressure in an area located 2-3 cm proximally to the area between the second and third toe[126]. Images of the dermal blood flow were captured before the application of histamine and each subsequent minute for 15 minutes using an FLPI (moorFLPI, Moor Instruments, United Kingdom). The mean flux ("blood flow" measured in perfusion units (PU)) of the region of interest (a circular area with an area of 450 mm^2 located around the application site) was determined for each image using moorFLPI-2 Review V5.0 (Moor Instruments, United Kingdom). The examinations were conducted in a room with standardized temperature and lighting. Pictures with poor quality were identified and removed (*see figure 5-2*). The mean flux of each image was analysed as a change from baseline by subtracting the mean flux of the baseline image (before application of histamine) from each subsequent image. The maximum flux and the time-constant (time before reaching approximately 63.2% of the maximal intensity) for each participant were derived from individual fits to inverse exponential decay:

$$(Y = A * (1 - e^{(-b*t)})$$

Where A is the maximum flux and b is 1 divided by the time constant (please see thesis paper 1*: The histamine-induced axon-reflex response in people with type 1 diabetes with and without peripheral neuropathy: A clinical, observational study*, section "*Regrouping and Statistical Analysis*" and *figure 1* for further explanation and visualization).

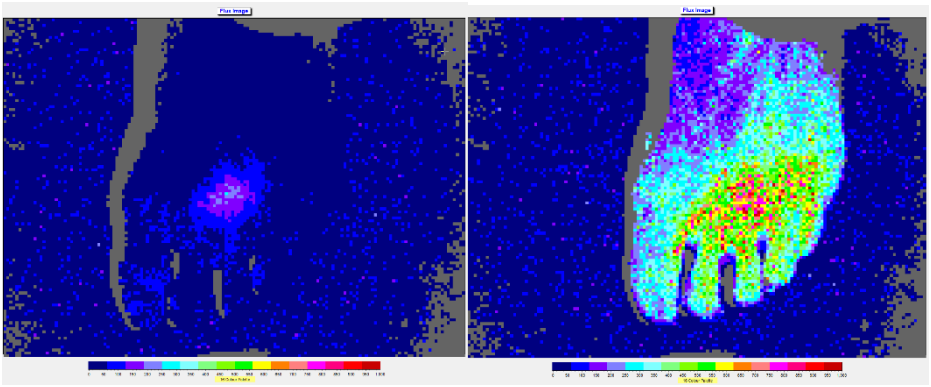

Figure 5-2 Example of poor image quality from FLPI: *Representative image of a flux image obtained from FLPI (left) and an image of poor quality (right). Unpublished data from paper 1.*

Abbreviations: ***FLPI:*** *Full-field Laser Perfusion Imaging*

5.3.5. PERCEPTION THRESHOLD TRACKING

All participants underwent perception threshold tracking on the right foot in an area located 2-3 cm proximally to the area between the second and third toe. Perception threshold tracking is a novel method to selectively stimulate large Aβ- and small Aδ-fibres in the skin using weak electrical currents applied through two different transcutaneous electrodes: a patch electrode (Neuroline 700, Ambu A/S, Denmark as cathode and Pals Neurostimulation Electrode, Axelgaard, CO., United States as anode) for stimulation of the large nerve fibres and a pin electrode (custom-made, Aalborg University, Denmark) for stimulation of small nerve fibres. The custom-made pin-electrode was designed as a concentric steel ring (serving as the anode) surrounding a printed circuit board with 16 blunted steel pins placed in a circle (serving as cathodes) (*see figure 5-3*). The selective stimulation of small nerve fibres was achieved by applying a current that was predominantly present near the termination of the free nerve endings in the epidermis without reaching the dermis and the termination of the large nerve fibres (*see figure 5-3*).

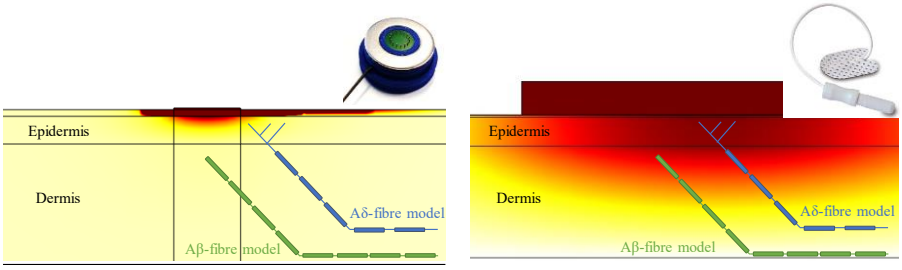

Figure 5-3 Electrodes used for perception threshold tracking and their current intensity in different skin layers: *the pin electrode for stimulation of small nerve fibres (left) produces a current predominantly present in the epidermis near the termination of the small nerve fibres, while the patch electrode for stimulation of the large nerve fibres (right) produces an unspecific current present in both dermis and epidermis. Due to the lower activation threshold of the large Aβ-fibres these are activated before the small nerve fibres. Adapted and modified from* [127].

The electrical current for both electrodes was delivered by an electrical stimulator (DS5 electrical stimulator, Digitimer Ltd, UK) controlled by a computer utilizing a protocol implemented in a custom-made program (LabBench Io, Inventors Way, Denmark). The protocol controlled both shape, duration, and intensity of the applied current. The perception threshold was estimated using square-shaped electrical impulses with varying duration (0.1 millisecond (ms), 1ms and 50ms) by increasing the current intensity until the stimulus was perceived. To ensure precision, a perceived stimulus should be confirmed by three successfully perceived stimuli in a row at the same current intensity. The current intensity was then subsequently lowered until the stimulus was no longer felt three times in a row. A perceived stimulus was registered by the participants pressing a handheld button (custom-made, Aalborg University,

Denmark). This procedure was repeated three to five times (depending on pulse-type) for each pulse. The variation between the number of times each pulse was tested was due to the 1ms pulse serving as normalization for some of the other pulse types (to ensure subthreshold stimulations of the pulses containing pre-pulses). Accommodation (*see 3.5.5 conventional threshold tracking*) was determined by applying a 100ms ramp-shaped pulse repeated three times. The setup is depicted in *figure 5-4*.

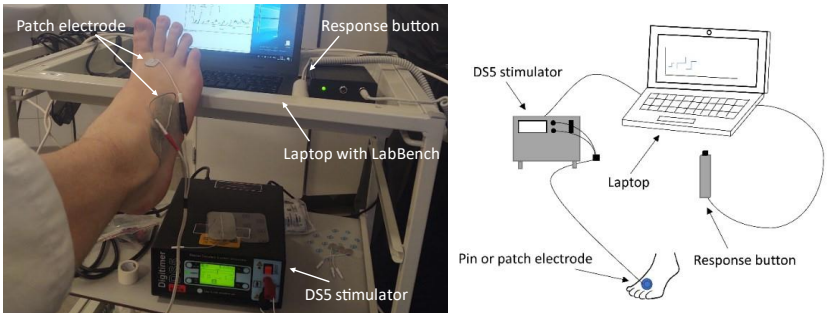

Figure 5-4 Setup used for perception threshold tracking: *(left): The setup depicted in the lab. The small electrode is the cathode, and the large electrode is the anode. The electrodes are connected to a DS5 electrical stimulator, which is connected to a laptop receiving information on perception through a response button. (right): The same setup depicted schematically. Adapted from thesis paper 2.*

Based on the thresholds obtained by the square pulses of varying duration, a strength-duration curve was plotted for each participant and the rheobase and chronaxie were derived (*see 3.3.5 conventional threshold tracking* and *figure 3-4)*.

5.3.6. OTHER EXAMINATIONS

The participants in the MEDON-study also underwent several other examinations including a test for cardiac autonomic neuropathy (Vagus™, Medicus Engineering, Denmark) using the classic cardiac autonomic reflex tests (Expiration:inspiration ratio/deep breathing), Valsalva ratio (breathing through resistance), and 30:15 supine to standing ratio (postural change), an assessment of peripheral blood flow by measurements of transcutaneous O_2-pressure, ankle-brachial index, and toe-brachial index (Periflux 6000, Perimed, Sweden), and several MRI-scans of both brain and leg utilizing methods like blood-oxygen-level dependent sequences for assessment of functional brain connectivity, diffusion tensor imaging and spectroscopy, although the MRI assessments are not considered a part of the present thesis[128,129].

5.4. STATISTICAL ANALYSIS

The statistical analysis used in the present thesis are different between the three thesis papers. Relevant information about the different statistical analyses is described in detail in each paper. In short, normality was assessed visually by quantile-quantile plots and histograms supported by Shapiro-Wilk or Kolmogorov-Smirnov tests. Non-normally distributed data were log-transformed to achieve normality, but in cases where it was impossible the data were analysed by non-parametric tests (Spearman's rank correlation or Kruskal-Wallis H tests followed by Bonferroni-corrected pairwise Mann-Whitney U tests and displayed as medians with corresponding interquartile ranges). Normally distributed data were analysed with parametric tests (t-tests, one-way analysis of variance (ANOVA) and displayed as means with a corresponding standard deviation). Categorical data were analysed with Chi-squared or Fisher's exact test and expressed as percentages. Logistic regressions were used to generate receiver operating characteristic (ROC) curves.

Most analyses were conducted following the initial MEDON-grouping (*figure 5-1*) and were then repeated following a re-grouping of the participants with T1DM based on them having small- (defined by abnormal CDT and HDT from QST) or large fibre involvement (defined by abnormal sural nerve conduction velocity and amplitude). This re-grouping was done, as the initial MEDON-grouping relied heavily on a test for large fibre function (vibration, *figure 5-1*), which means people in the "T1DM-DPN"-group could have subclinical SFN without concomitant large fibre involvement, and thus influence the results when assessing the function of small fibres. This re-grouping was decided prior to study initiation and was pre-registered at clinicaltrials.gov (*NCT04078516*).

CHAPTER 6. KEY RESULTS

As stated earlier, the overall goal of the present PhD thesis was to evaluate novel methods for rapid and adequate assessment of small nerve fibre function in people with T1DM, with the sub-goal of exploring peripheral differences between people with T1DM with and without neuropathic pain. To address these goals, the overall key findings from the three thesis papers are presented in the following chapter, while the details of each key finding are found in the respective paper (thesis paper 1-3).

6.1. DEMOGRAPHICS OF THE MEDON-COHORT

The cohort used for all papers is derived from the MEDON-cohort. Demographical data from this cohort are available in *table 6-1*.

	T1DM+PDPN (n=20)	**T1DM+DPN** (n=20)	**T1DM-DPN** (n=20)	**HC** (n=20)	*p*
Age	50.5 [43.5;57.0][a]	51.5 [45.5;58.5][a]	50.5 [44.5;58.5][a]	50.5 [44.0;58.5][a]	ns
Sex (% male)	50.0%[a]	50.0%[a]	50.0%[a]	50.0%[a]	ns
BMI	27.2 [25.1;30.4][a]	27.8 [24.2;30.8][a]	27.1 [24.6;30.2][a]	24.3 [23.1;27.9][a]	ns
HbA1c (mmol/mol)	70.0 [59.0;78.5][a]	73.0 [65.5;78.0][a]	64.5 [58.0;72.3][a]	34.0 [31.8;35.0][b]	< 0.01
Diabetes Duration (years)	33.0 [22.5;40.5][a]	34.5 [30.0;29.0][a]	25.5 [15.5;31.0][b]	-	< 0.05
NCV (m/sec)	13.5 [0.0;39.0][a]	15.5 [0.0;39.5][a]	47.5 [45.0;48.5][b]	54.5 [48.0;57.0][c]	< 0.01
NCA (µV)	0.4 [0.0;2.7][a]	1.1 [0.0;3.6][a]	5.0 [2.7;7.8][b]	10.3 [6.7;13.3][c]	< 0.01
CDT (°Celsius)	20.3 [7.3;25.1][a]	14.6 [7.3;20.9][a]	28.1 [26.7;30.3][b]	30.1 [25.6;30.7][b]	< 0.01
HDT (°Celsius)	45.3 [43.2;47.2][a]	44.3 [40.6;49.3][a]	40.0 [37.3;42.0][b]	37.5 [35.5;41.1][b]	< 0.01
Pain Score (Peak Intensity)	8.0 [6.0;9.0][a]	0.0 [0.0;0.0][b]	0.0 [0.0;0.0][b]	0.0 [0.0;0.0][b]	< 0.01
Pain Score (Average Intensity)	5.0 [4.0;7.5][a]	0.0 [0.0;0.0][b]	0.0 [0.0;0.0][b]	0.0 [0.0;0.0][b]	< 0.01

MNSI	4.5 [3.0;5.0][a]	4.0 [3.0;5.0][a]	0.0 [0.0;0.0][b]	0.0 [0.0;0.0][b]	< 0.01
DN4 Score	5.0 [4.0;6.0][a]	0.0 [0.0;1.3][b]	0.0 [0.0;0.0][b]	0.0 [0.0;0.0][b]	< 0.01
ABI	1.12 [0.90;1.25][a]	1.15 [1.04;1.25][a]	1.25 [1.17;1.31][a]	1.28 [1.18;1.31][a]	ns
TBI ǂ	0.81 ± 0.27[a]	0.80 ± 0.25[a]	0.87 ± 0.19[a]	0.87 ± 0.23[a]	ns

Table 6-1 Demographical data of the MEDON-cohort: *The table provides data on baseline characteristics and key results from some of the tests used for re-grouping (nerve conduction studies and quantitative sensory testing). Overall difference between the groups is calculated using Kruskal-Wallis H Test. Individual differences between the groups are calculated by Bonferroni-corrected pairwise Mann-Whitney U tests and denoted with letters. Groups with the same denoted letter (e.g., "a") is not statistically different from other groups denoted with the same letter but is statistically different from groups denoted with a different letter (e.g., "b"). ǂ indicates normal distribution, and such data is analysed with analysis of variance (ANOVA) and subsequent Bonferroni-corrected pairwise student's t-tests. Average and peak pain intensity is derived from the PainDETECT-questionnaire*[123]*. In cases where it was not possible to assess sural nerve conduction velocity or amplitude the value was set to 0.0.*

Table adapted and modified from thesis papers 1 and 2.

Abbreviations: ***ABI:*** *Ankle-brachial index,* ***BMI:*** *Body Mass Index,* ***CDT:*** *Cold detection threshold,* ***DN4****: Douleur Neuropathique en 4,* ***DPN****: Diabetic peripheral neuropathy,* ***HbA1c:*** *Glycated haemoglobin A1c,* ***HDT:*** *Heat detection threshold,* ***MNSI:*** *Michigan Neuropathy Screening Instrument,* ***NCA****: Nerve conduction amplitude (sural nerve),* ***NCV****: Nerve conduction velocity (sural nerve)* ***NCV****: Nerve conduction velocity (sural nerve),* ***PDPN****: Painful diabetic peripheral neuropathy,* ***T1DM****: Type 1 diabetes,* ***TBI:*** *Toe-brachial index*

6.2. PAPERS

6.2.1. KEY RESULTS FROM PAPER 1

- The axon-reflex flare response could be reliably evoked by an epidermal application of histamine applied with a simple handheld skin-prick device
- The intensity of the axon-reflex flare response was significantly attenuated in people with diabetes per se irrespective or their neuropathy status.
- The intensity of the axon-reflex flare response was significantly attenuated in people with T1DM and peripheral neuropathy compared to people with T1DM without peripheral neuropathy
- The presence of PDPN did not influence the intensity of the axon-reflex flare response

Comment: The results indicate that T1DM might cause early changes in the interaction between small cutaneous C-fibres and small blood vessels, and that these changes happen prior to the development of clinically distinct DPN. This could indicate that the method is a relevant option for future research into early detection of DPN. Furthermore, it seems that the axon-reflex flare response can reliably be evoked by a simple and rapid application of epidermal histamine, which in the future should be validated against the established method using local heating to reduce examination-time and move the method one step closer towards a potential clinical application.

6.2.2. KEY RESULTS FROM PAPER 2

- Perception threshold tracking can be applied in people with T1DM
- Perception threshold tracking can selectively stimulate large and small nerve fibres in people with T1DM-DPN, T1DM+DPN, and in healthy controls, while the selectivity in people with T1DM+PDPN is most likely deattenuated, although the possibility that the small nerve fibres in those with T1DM+PDPN have alternated accommodation properties compared to the small nerves of those without pain cannot be ruled out.
- Perception threshold tracking can (with the patch electrode) distinguish between people with T1DM with and without DPN based on large fibre grouping (initial MEDON-grouping).
- Perception threshold tracking can (with the pin electrode) distinguish between people with T1DM with and without DPN based on small fibre

assessment (per protocol regrouping), but not in the initial MEDON-grouping.

- Perception threshold tracking can possibly distinguish subtypes of PDPN from non-painful DPN.

- Perception threshold tracking has a good agreement with thermal sensation evaluated by QST in identifying small fibre involvement in DPN (AUC 0.82).

Comment: Perception threshold tracking appears to be a good, selective, and rapid method for functional assessment of large and small nerve fibres in diabetes, with the latter being of particular interest. The results from the present study suffer from the relatively small sample size, the fact that participants are included based on large fibre involvement, and the harsh correction for multiple testing. However, the absolute values indicate that the method warrants further studies in both painful and painless DPN.

6.2.3. KEY RESULTS FROM PAPER 3

- Perception threshold tracking (of small fibres) and all parameters from CCM can successfully distinguish people with T1DM and neuropathic complications from healthy controls

- Perception threshold tracking (of small fibres) correlates significantly with all parameters from CCM and total score of the Michigan Neuropathy Screening Instrument.

- Perception threshold tracking (of small fibres) has a high diagnostic agreement with all CCM parameters in detecting SFN with the highest performance against CNFL (94% sensitivity, 94% specificity, positive predictive value 97%, negative predictive value 89%)

- The optimal cut-off value for the rheobase for detecting SFN appears to be between 0.25 mA and 0.36 mA.

Comment: Perception threshold tracking (of small fibres) have a very high diagnostic agreement with CCM, which means the method shows great potential as a rapid screening method for SFN, although the results should be interpreted in the context of the highly selected study population. The positive results warrant future research comparing perception threshold tracking to both CCM and skin biopsies in larger, unselected cohorts. Based on the findings of the present study both functional and structural changes occur in DPN, although the magnitude of the changes might not be similar.

CHAPTER 7. DISCUSSION

The present thesis has utilized two novel methods for functional assessment of small nerve fibre pathology in T1DM: the intensity of the histamine-induced axon-reflex flare response and an assessment of the perception threshold through transcutaneous electrical stimulation. Furthermore, the thesis has also compared the diagnostic performance of perception threshold tracking to CCM. In the following, both methods will be discussed individually and put into context.

7.1. THE HISTAMINE-INDUCED AXON-REFLEX FLARE RESPONSE

Thesis paper 1 found an attenuated axon-reflex flare response intensity in people with T1DM irrespective of severity of DPN compared to healthy controls, a further attenuated response as neuropathy worsens, no influence of the presence of PDPN on the response, and that the response could be reliably evoked by an epidermal application of histamine. These findings are in line with most of the available studies using the axon-reflex flare response as a marker for C-fibre dysfunction in diabetes, although most studies either use local heating or iontophoresis of acetylcholine, with only few studies using (iontophoresis of) histamine[95,130]. In addition to this, most studies tend to use flare area size rather than flare intensity when using local heating as stimulus, as studies using this method has previously established that the size of the flare response (rather than the intensity) is what correlates to nerve fibre function, while the intensity is primarily driven by vascular function[131]. Although this is not the case when using the epidermal application of histamine, an evaluation of flare area size in addition to the provided measures of flare intensity could be useful for comparison between the methods. In fact, analyses made for a coming paper comparing the histamine-induced axon-reflex flare response intensity and flare area size to each other and to CCM indicates that flare area size might also be superior to flare intensity when evoking the response with histamine, as flare area size yields better results when compared to both QST (*AUC 0.91 versus 0.82, $p<0.01$, submitted, unpublished data*) and CCM (*AUC 0.88 versus 0.79, $p<0.01$, submitted, unpublished data*). A direct comparison to other existing literature can be found in the discussion section of thesis paper 1.

Critiques of the skin-prick method for delivery of active substances argue that the dissemination cannot be regulated, and that glycosylated skin could interfere with the delivery and response[131,132]. While these concerns are indeed valid, the main goal of the present thesis was to evaluate methods for measuring small fibre function that could be applied in a clinical setting. By applying histamine through a simple skin-prick device rather than applying prolonged local heating, it is possible to reduce the

preparation time from approximately 30 minutes to less than 30 seconds. This makes clinical application much more feasible (although still not obvious due to the requirement of specialized equipment), but it might compromise reproducibility. In thesis paper 1, the data are presented as the response measured over 15 minutes, which would again not be feasible for clinical screening. However, it could appear that the intensity of the axon-reflex flare response could be different between different groups of people with T1DM after only a few minutes, in which case a clinical application would move closer (*see figure 1, Thesis paper 1*). In fact, analyses made for a coming paper comparing the histamine-induced axon-reflex flare response intensity and flare area size to each other and to CCM reveals that people with T1DM+DPN and T1DM-DPN can be distinguished after six minutes using axon-reflex flare response intensity ($p<0.01$, *submitted, unpublished data*), and after only four minutes ($p<0.01$, *submitted, unpublished data*) using flare area size.

Thesis paper 1 contributes to the existing literature by confirming previous results found by the utilization of the axon-reflex flare area size obtained through local heating, while also confirming the applicability of a simple epidermal application of histamine to evoke the response in diabetes. In the future, the flare area size should be analysed in the present cohort and compared to the measurements reported in thesis paper 1. Beyond that, a direct comparison between local heating and epidermal application of histamine should be made, and the reproducibility should be further investigated. Finally, it should also be mentioned, that the study likewise investigated the impact of PDPN, which appears to not impact the intensity (or the size, *submitted, unpublished data*) of the axon-reflex flare response. Although being a negative finding it is still of interest, as recent studies utilizing skin biopsies have found increased levels of peptides related to the histamine-pathway (substance P and calcitonin gene-related peptide) in people with PDPN, hinting of a potential mechanistic pathway[68,69].

7.2. PERCEPTION THRESHOLD TRACKING

Thesis paper 2 found that perception threshold tracking is applicable in diabetes, can indeed preferentially stimulate large and small nerve fibres selectively, can distinguish some groups of people with and without T1DM and neuropathic complications, and has a good agreement with measurements of peripheral thermal sensation. Thesis paper 3 build on top of these findings, displaying correlations with the Michigan Neuropathy Screening Instrument and a very high diagnostic agreement with all CCM parameters at an optimal cut-off value between 0.25 mA and 0.36 mA. As perception threshold tracking is unique in its application, comparing it directly to existing literature is not possible. The discussion sections of thesis paper 2 and 3 draw parallels to conventional threshold tracking although the methods are not directly comparable. As these parallels are available in thesis paper 2 and 3, they will not be discussed any further in the present section.

Due to its relatively rapid examination-time and high diagnostic agreement with an established marker for SFN (CCM), perception threshold tracking could be a potential candidate for future large-scale screening. Before the method is ready for large scale application, there is however several issues that need to be addressed. Firstly, the pin electrode (for stimulation of the small fibres) needs to be available as a cheap single-use electrode, as the current pin electrode is custom-made, require constant cleaning, and is not easily distributed to other centres to confirm the applicability in multicentre trials. Secondly, the method needs to be validated against the structural gold standard in skin biopsies, and preferably also CCM, in larger, unselected cohorts. Thirdly, the reproducibility and variance of the thresholds needs to be assessed to ensure that the method is indeed sensitive enough to detect worsening or even small improvements. Fourthly, the predictive capability of the method needs to be addressed in longitudinal studies. Finally, the speed of the examination could be further increased with a protocol utilizing the PSI-method for a rapid estimation of the perception threshold[133]. The method is algorithm-based, and functions by optimizing the information that is gained from each stimulus by adjusting the intensity based on the information received from previous stimuli. Using this method, it is possible to establish a psychometric function based on a fixed number between 30-50 stimulations (see *figure 7-1*). We recently examined the applicability of the method in a small cohort of people with diabetes (n=43) (*unpublished data*). In that study, we found the method to have a similar performance with less stimulations than the methods of limits (applied in thesis papers 2 and 3), although it appeared unfit in people with severe neuropathy, as they would often respond randomly, thus destroying the functionality of the algorithm. This issue should therefore also be solved before applying the method to randomly selected cohorts.

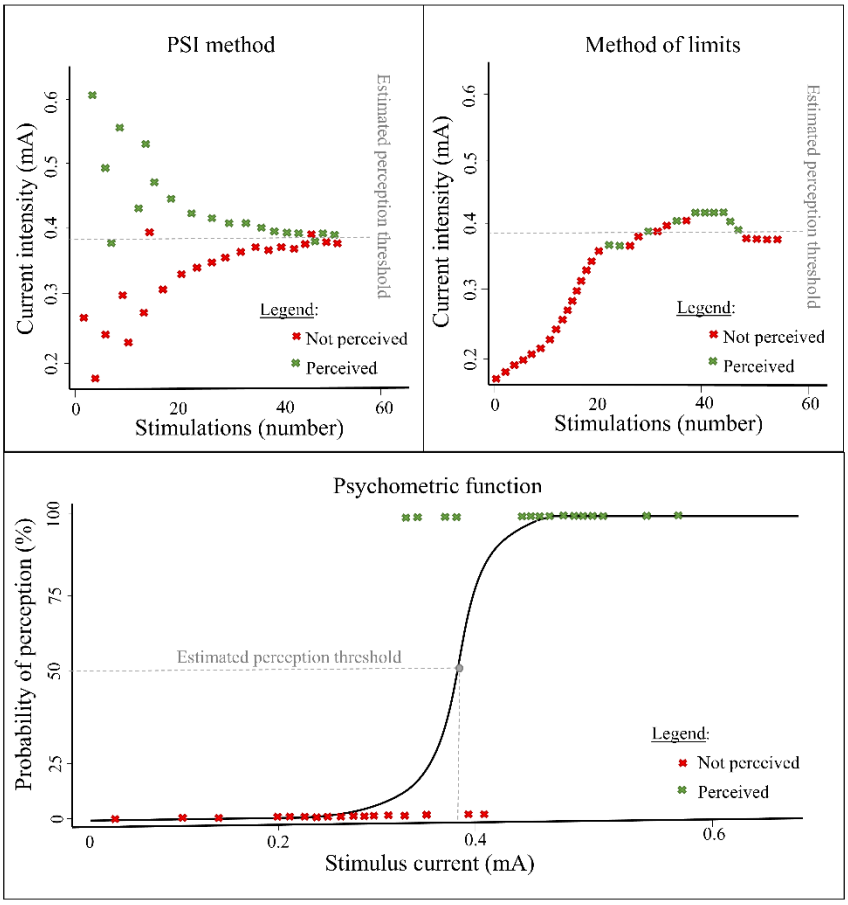

Figure 7-1 Schematic overview of the PSI-method, the method of limits and the psychometric function: *perceived stimuli are marked with green and unperceived stimuli are marked with red. The PSI-method uses a computer-generated algorithm to maximize the information gained from each impulse based on previous inputs, while the method of limits slowly climbs until a stimulus-intensity is confirmed to be perceived four repetitive times in a row. It then declines until the stimulus is no longer felt four repetitive times in a row. The psychometric function is the sigmoid curve that the PSI-method is built upon. The estimated threshold is defined as the stimulus current where the probability of the participant perceiving a stimulus is 50%.*

Abbreviations: ***mA:*** *Milliampere*

7.3. LIMITATIONS

Despite the many positive findings, the present thesis does suffer several inherent issues, which will be discussed in the following sections.

7.3.1. LIMITATIONS IN THE MEDON-STUDY

As the first, larger, investigator-initiated, clinical neuropathy-trial at Steno Diabetes Center North Denmark, the MEDON-study is a landmark for what is to come in future projects. However, the design of the MEDON-study has several flaws with the most important one being the procedure/requirements for inclusion in each of the original groups. The philosophy when designing the study was an initial, clinical, grouping followed by a subsequent regrouping based on large- and small nerve fibre involvement, respectively. The idea was formed based on a vision of clinical research directly on top of daily clinical activities, but ultimately the inclusion of four thoroughly matched groups based solely on signs and symptoms of (mainly large fibre) neuropathy supported by a VPT (and ankle-reflexes) alongside a validated questionnaire for assessment of neuropathic pain proved insufficient in a study focusing on SFN. For large fibre assessment, the clinical distinction proved acceptable, with only one participant from the original group with T1DM+DPN having near-normal sural nerve conduction velocity and amplitude, and one participant from the original group with T1DM-DPN having borderline abnormal sural nerve conduction velocity and amplitude. However, when re-grouping participants based on their thermal sensation assessed by QST, four participants were drastically re-grouped (i.e., from the original group with T1DM-DPN to the re-grouped participants with "definite" [both abnormal cold- and heat-perception threshold red.] SFN). This means that 4/60 participants in the original cohorts were probably wrongly grouped in the clinical groupings, which limits the strength of the initial comparisons as well as limiting the impact of the otherwise thorough matching in the original groups. No study design is perfect, but in similar future projects the initial grouping should at least be based on the Toronto criteria for "definite neuropathy", which means an abnormal nerve conduction study should be a requirement for enrolment into a group with T1DM+DPN[1]. Unfortunately, this would not solve the issue of wrongly classifying participants based on small fibre involvement, which could only be solved by performing either skin biopsies with quantification of the IENFD or QST at the screening visit, which is a quite comprehensive protocol, and even then, still limited by the questionable performance on an individual level[21,22]. Fortunately, the procedure/requirements for inclusion did ultimately not impact the results too much but is still something to be improved in future studies, which is also the case for our upcoming "NeuroPredict"-project (*Clinical trials: NCT05546138*) focusing on early identification and prediction of early onset neuropathy.

7.3.2. LIMITATION IN THE AXON-REFLEX FLARE RESPONSE

As mentioned in thesis paper 1, the methodology used for assessment of the C-fibre mediated axon-reflex flare response has several weaknesses, which are explained in detail in the paper and in the *section 7.1* of the present thesis. In addition to the limitations mentioned there, the lack of standardized skin temperature (only standardized room temperature), the lack of a comparison to the established method using local heating, and the lack of an analysis of flare size (again for comparison to established methods) does limit the comparison to existing literature[95]. These shortcomings are due to practical and technical limitations during the period of performing the study and should be solved before future trials utilizing the method.

7.3.3. LIMITATIONS IN PERCEPTION THRESHOLD TRACKING

As described in thesis papers 2 and 3, perception threshold tracking does have several weaknesses. These limitations are described in detail in thesis paper 2, and include considerations on sample size, prolonged use of electrical stimulation, risk of signal shunting through hairy skin, questionable selectivity at very high current intensities, and the impact of age and sex. Paper 3 likewise mentions the reduction in sample size due to several participants being excluded from undergoing CCM, which might have limited the strength of the associations provided in that study. To elaborate on the unique issues related to perception threshold tracking, the questions regarding the impact of prolonged stimulations are of particular interest. Thesis paper 2 describes how prolonged stimulation might incidentally activate slowly conducting C-fibres, which is felt as an unspecific pain/heat/tingling sensation, making the sensation of activated Aδ-fibres harder to perceive. In addition, prolonged stimulation likewise causes “a drift” in the perception threshold, where the perception threshold would slowly increase due to the concept of habituation[134,135]. This concept is important when performing prolonged sessions (and were considered in the present protocol by normalizing the pulses containing pre-pulses prior to running them) but is not important when performing rapid estimations of the perception threshold (what is presented in the present thesis and suggested as a potential candidate for clinical screening).

CHAPTER 8. CONCLUSION AND FUTURE PERSPECTIVES

The present thesis provides promising results for both the histamine-induced axon-reflex flare response and perception threshold tracking and their ability to perform a functional assessment of small nerve fibres in diabetes. The histamine-induced axon-reflex flare response provides a rapid alternative to the established standard of local heating for assessment of small cutaneous C-fibres, but reproducibility and diagnostic agreement needs to be examined further. Likewise, perception threshold tracking provides a very rapid evaluation of small cutaneous Aδ-fibres, with great diagnostic agreement with CCM. Perception threshold tracking also provides an option for further pathophysiological studies into both painful and painless neuropathy, although this utilization of the technique requires further examination and subgrouping of those with a painful condition.

The present thesis has described an alternative way of evoking an axon-reflex flare response in diabetes, the first ever clinical application of perception threshold tracking, and the first ever use of the method in diabetes. The results are promising, but much work is needed before any of the two methods are serious contenders for large scale screening. Perception threshold tracking is the most promising of the two regarding its applicability in a clinical setting due to its rapid nature. The initial requirements and studies needed beforehand are already described in the discussion, and several of the projects have already been initiated.

All in all, the work that has been done in the present thesis has laid the foundation for many future studies and PhD-projects, which should further test and develop the methods described, with the ultimate end-goal of providing robust clinical endpoints for both researchers and clinicians, and ultimately reduce the burden of disease in people with diabetes.

REFERENCES

[1] Tesfaye S, Boulton AJM, Dyck PJ, Freeman R, Horowitz M, Kempler P, et al. Diabetic neuropathies: Update on definitions, diagnostic criteria, estimation of severity, and treatments. Diabetes Care 2010;33:2285–93. https://doi.org/10.2337/dc10-1303.

[2] Hicks CW, Selvin E. Epidemiology of Peripheral Neuropathy and Lower Extremity Disease in Diabetes. Curr Diab Rep 2019;19. https://doi.org/10.1007/s11892-019-1212-8.

[3] Feldman EL, Callaghan BC, Pop-Busui R, Zochodne DW, Wright DE, Bennett DL, et al. Diabetic neuropathy. Nat Rev Dis Prim 2019;5. https://doi.org/10.1038/s41572-019-0092-1.

[4] Abbott CA, Malik RA, Van Ross ERE, Kulkarni J, Boulton AJM. Prevalence and characteristics of painful diabetic neuropathy in a large community-based diabetic population in the U.K. Diabetes Care 2011;34:2220–4. https://doi.org/10.2337/dc11-1108.

[5] Shillo P, Sloan G, Greig M, Hunt L, Selvarajah D, Elliott J, et al. Painful and Painless Diabetic Neuropathies: What Is the Difference? Curr Diab Rep 2019;19. https://doi.org/10.1007/s11892-019-1150-5.

[6] Tesfaye S, Boulton AJM, Dickenson AH. Mechanisms and management of diabetic painful distal symmetrical polyneuropathy. Diabetes Care 2013;36:2456–65. https://doi.org/10.2337/dc12-1964.

[7] Røikjer J, Mørch CD, Ejskjaer N. Diabetic Peripheral Neuropathy: Diagnosis and Treatment. Curr Drug Saf 2020;15. https://doi.org/10.2174/1574886315666200731173113.

[8] Røikjer J, Ejskjaer N. The conundrum and enigma of painful and painless neuropathy. Diabet Neuropathy 2022:123–33. https://doi.org/10.1016/b978-0-12-820669-0.00012-8.

[9] Kalteniece A, Ferdousi M, Azmi S, Mubita WM, Marshall A, Lauria G, et al. Corneal confocal microscopy detects small nerve fibre damage in patients with painful diabetic neuropathy. Sci Rep 2020;10:3371. https://doi.org/10.1038/s41598-020-60422-7.

[10] Røikjer J, Jensen MH, Vestergaard P, Sørensen AM, Laursen HVB, Ejskjær N. Twenty years with diabetes and amputations: a retrospective population-based cohort study. Diabet Med 2020:dme.14251. https://doi.org/10.1111/dme.14251.

[11] Røikjer J, Werkman NCC, Ejskjaer N, van den Bergh JPW, Vestergaard P, Schaper NC, et al. Incidence, hospitalization and mortality and their changes over time in people with a first ever diabetic foot ulcer. Diabet Med 2021.

https://doi.org/10.1111/dme.14725.

[12] Edmonds M, Manu C, Vas P. The current burden of diabetic foot disease. J Clin Orthop Trauma 2021;17:88–93. https://doi.org/10.1016/j.jcot.2021.01.017.

[13] Armstrong DG, Boulton AJM, Bus SA. Diabetic Foot Ulcers and Their Recurrence. N Engl J Med 2017;376:2367–75. https://doi.org/10.1056/NEJMra1615439.

[14] Charles M, Ejskjaer N, Witte DR, Borch-Johnsen K, Lauritzen T, Sandbaek A. Prevalence of neuropathy and peripheral arterial disease and the impact of treatment in people with screen-detected type 2 diabetes: The ADDITION-Denmark study. Diabetes Care 2011;34:2244–9. https://doi.org/10.2337/dc11-0903.

[15] Ismail-Beigi F, Craven T, Banerji MA, Basile J, Calles J, Cohen RM, et al. Effect of intensive treatment of hyperglycaemia on microvascular outcomes in type 2 diabetes: An analysis of the ACCORD randomised trial. Lancet 2010;376:419–30. https://doi.org/10.1016/S0140-6736(10)60576-4.

[16] Group TDC and CTR. The Effect of Intensive Treatment of Diabetes on the Development and Progression of Long-Term Complications in Insulin-Dependent Diabetes Mellitus. N Engl J Med 1993;329:977–86. https://doi.org/10.1056/NEJM199309303291401.

[17] Malik RA. Why are there no good treatments for diabetic neuropathy? Lancet Diabetes Endocrinol 2014;2:607–9. https://doi.org/10.1016/S2213-8587(14)70067-1.

[18] Breiner A, Lovblom LE, Perkins BA, Bril V. Does the prevailing hypothesis that small-fiber dysfunction precedes large-fiber dysfunction apply to type 1 diabetic patients? Diabetes Care 2014;37:1418–24. https://doi.org/10.2337/dc13-2005.

[19] Løseth S, Stålberg E, Jorde R, Mellgren SI. Early diabetic neuropathy: Thermal thresholds and intraepidermal nerve fibre density in patients with normal nerve conduction studies. J Neurol 2008;255:1197–202. https://doi.org/10.1007/s00415-008-0872-0.

[20] Divisova S, Vlckova E, Hnojcikova M, Skorna M, Nemec M, Dubovy P, et al. Prediabetes/early diabetes-associated neuropathy predominantly involves sensory small fibres. J Peripher Nerv Syst 2012;17:341–50. https://doi.org/10.1111/j.1529-8027.2012.00420.x.

[21] Azmi S, Petropoulos IN, Ferdousi M, Ponirakis G, Alam U, Malik RA. An update on the diagnosis and treatment of diabetic somatic and autonomic neuropathy. F1000Research 2019;8. https://doi.org/10.12688/f1000research.17118.1.

[22] Shy ME, Frohman EM, So YT, Arezzo JC, Cornblath DR, Giuliani MJ, et al. Quantitative sensory testing: Report of the therapeutics and technology assessment subcommittee of the American academy of neurology. Neurology 2003;60:898–904. https://doi.org/10.1212/01.WNL.0000058546.16985.11.

[23] Rosenberg ME, M. T, Tervo T, J. I, Immonen, J. L, et al. Corneal Structure and Sensitivity in Type 1 Diabetes Mellitus. Invest Ophthalmol Vis Sci 2000;41:2915–21. https://doi.org/10.1016/S0304-5412(12)70418-3.

[24] Malik RA, Kallinikos P, Abbott CA, Van Schie CHM, Morgan P, Efron N, et al. Corneal confocal microscopy: a non-invasive surrogate of nerve fibre damage and repair in diabetic patients. Diabetologia 2003;46:683–8. https://doi.org/10.1007/s00125-003-1086-8.

[25] Perkins BA, Lovblom LE, Bril V, Scarr D, Ostrovski I, Orszag A, et al. Corneal confocal microscopy for identification of diabetic sensorimotor polyneuropathy: a pooled multinational consortium study. Diabetologia 2018;61:1856–61. https://doi.org/10.1007/s00125-018-4653-8.

[26] Lauria G, Devigili G. Skin biopsy as a diagnostic tool in peripheral neuropathy. Nat Clin Pract Neurol 2007;3:546–57. https://doi.org/10.1038/ncpneuro0630.

[27] Lauria G, Cornblath DR, Johansson O, McArthur JC, Mellgren SI, Nolano M, et al. EFNS guidelines on the use of skin biopsy in the diagnosis of peripheral neuropathy. Eur J Neurol 2005;12:747–58. https://doi.org/10.1111/j.1468-1331.2005.01260.x.

[28] Villani E, Galimberti D, Viola F, Mapelli C, Papa N Del, Ratiglia R. Corneal involvement in rheumatoid arthritis: An in vivo confocal study. Investig Ophthalmol Vis Sci 2008;49:560–4. https://doi.org/10.1167/iovs.07-0893.

[29] Association AD. 2. Classification and diagnosis of diabetes: Standards of medical care in diabetes-2021. Diabetes Care 2021;44:S15–33. https://doi.org/10.2337/dc21-S002.

[30] Mobasseri M, Shirmohammadi M, Amiri T, Vahed N, Fard HH, Ghojazadeh M. Prevalence and incidence of type 1 diabetes in the world: A systematic review and meta-analysis. Heal Promot Perspect 2020;10:98–115. https://doi.org/10.34172/hpp.2020.18.

[31] DiMeglio LA, Evans-Molina C, Oram RA. Type 1 diabetes. Lancet 2018;391:2449–62. https://doi.org/10.1016/S0140-6736(18)31320-5.

[32] Kristensen JK, Balasubramaniam K, Bjerregaard-Andersen M, Breum L, Charles M, Hansen LJ, et al. Type 2 Diabetes. Https://EndocrinologyDk/Nbv/Diabetes-Melitus/Behandling-Og-Kontrol-Af-Type-2-Diabetes/ 2022.

[33] Søndergaard E, Danish Endocrine Society D. Type 1 Diabetes.

Https://EndocrinologyDk/Nbv/Diabetes-Melitus/Type-1-Diabetes-Mellitus/ 2022.

[34] Peters AL, Ahmann AJ, Battelino T, Evert A, Hirsch IB, Murad MH, et al. Diabetes technology-continuous subcutaneous insulin infusion therapy and continuous glucose monitoring in adults: An endocrine society clinical practice guideline. J Clin Endocrinol Metab 2016;101:3922–37. https://doi.org/10.1210/jc.2016-2534.

[35] Bailey TS, Chang A, Christiansen M. Clinical accuracy of a continuous glucose monitoring system with an advanced algorithm. J Diabetes Sci Technol 2015;9:209–14. https://doi.org/10.1177/1932296814559746.

[36] Realsen J, Goettle H, Chase HP. Morbidity and mortality of diabetic ketoacidosis with and without insulin pump care. Diabetes Technol Ther 2012;14:1149–54. https://doi.org/10.1089/dia.2012.0161.

[37] Viigimaa M, Sachinidis A, Toumpourleka M, Koutsampasopoulos K, Alliksoo S, Titma T. Macrovascular Complications of Type 2 Diabetes Mellitus. Curr Vasc Pharmacol 2019;18:110–6. https://doi.org/10.2174/1570161117666190405165151.

[38] Harding JL, Pavkov ME, Magliano DJ, Shaw JE, Gregg EW. Global trends in diabetes complications: a review of current evidence. Diabetologia 2019;62:3–16. https://doi.org/10.1007/s00125-018-4711-2.

[39] Sloan G, Selvarajah D, Tesfaye S. Pathogenesis, diagnosis and clinical management of diabetic sensorimotor peripheral neuropathy. Nat Rev Endocrinol 2021;17:400–20. https://doi.org/10.1038/s41574-021-00496-z.

[40] Sheetz MJ, King GL. Molecular understanding of hyperglycemia's adverse effects for diabetic complications. J Am Med Assoc 2002;288:2579–88. https://doi.org/10.1001/jama.288.20.2579.

[41] Oates PJ. Polyol pathway and diabetic peripheral neuropathy. Int Rev Neurobiol 2002;50. https://doi.org/10.1016/s0074-7742(02)50082-9.

[42] Fernyhough P. Mitochondrial Dysfunction in Diabetic Neuropathy: a Series of Unfortunate Metabolic Events. Curr Diab Rep 2015;15:1–10. https://doi.org/10.1007/s11892-015-0671-9.

[43] Røikjer J, Ejskjaer N. Diabetic Peripheral Neuropathy. Handb Exp Pharmacol 2022;274:309–28. https://doi.org/10.1007/164_2022_585.

[44] Pop-Busui R, Ang L, Holmes C, Gallagher K, Feldman EL. Inflammation as a Therapeutic Target for Diabetic Neuropathies. Curr Diab Rep 2016;16:1–10. https://doi.org/10.1007/s11892-016-0727-5.

[45] Papanas N, Ziegler D. Risk factors and comorbidities in diabetic neuropathy: An update 2015. Rev Diabet Stud 2015;12:48–62.

https://doi.org/10.1900/RDS.2015.12.48.

[46] Bostock H, Cikurel K, Burke D. Threshold tracking techniques in the study of human peripheral nerve. Muscle Nerve 1998;21:137–58. https://doi.org/10.1002/(SICI)1097-4598(199802)21:2<137::AID-MUS1>3.0.CO;2-C [pii].

[47] Ziegler D, Bönhof GJ, Strom A, Straßburger K, Karusheva Y, Szendroedi J, et al. Progression and regression of nerve fibre pathology and dysfunction early in diabetes over 5 years. Brain 2021;144:3251–63. https://doi.org/10.1093/brain/awab330.

[48] Määttä LL, Charles M, Witte DR, Bjerg L, Jørgensen ME, Jensen TS, et al. Prospective study of neuropathic symptoms preceding clinically diagnosed diabetic polyneuropathy: Addition-Denmark. Diabetes Care 2019;42:2282–9. https://doi.org/10.2337/dc19-0869.

[49] Pop-Busui R, Ang L, Boulton AJM, Feldman EL, Marcus RL, Mizokami-Stout K, et al. Diagnosis and Treatment of Painful Diabetic Peripheral Neuropathy. American Diabetes Association; 2022. https://doi.org/10.2337/DB2022-01.

[50] Røikjer J, Ejskjaer N. The conundrum and enigma of painful and painless neuropathy. Diabet Neuropathy 2022:123–33. https://doi.org/10.1016/b978-0-12-820669-0.00012-8.

[51] Azmi S, Alam U, Burgess J, Malik R a. State-of-the-art pharmacotherapy for diabetic neuropathy. Expert Opin Pharmacother 2021;22:55–68. https://doi.org/10.1080/14656566.2020.1812578.

[52] Tesfaye S, Wilhelm S, Lledo A, Schacht A, Tölle T, Bouhassira D, et al. Duloxetine and pregabalin: High-dose monotherapy or their combination? the "cOMBO-DN study" - A multinational, randomized, double-blind, parallel-group study in patients with diabetic peripheral neuropathic pain. Pain 2013;154:2616–25. https://doi.org/10.1016/j.pain.2013.05.043.

[53] Tesfaye S, Sloan G, Petrie J, White D, Bradburn M, Julious S, et al. Comparison of amitriptyline supplemented with pregabalin, pregabalin supplemented with amitriptyline, and duloxetine supplemented with pregabalin for the treatment of diabetic peripheral neuropathic pain (OPTION-DM): a multicentre, double-blind, randomise. Lancet 2022;400:680–90. https://doi.org/10.1016/S0140-6736(22)01472-6.

[54] Beydoun A, Shaibani A, Hopwood M, Wan Y. Oxcarbazepine in painful diabetic neuropathy: Results of a dose-ranging studya. Acta Neurol Scand 2006;113:395–404. https://doi.org/10.1111/j.1600-0404.2006.00631.x.

[55] Demant DT, Lund K, Vollert J, Maier C, Segerdahl M, Finnerup NB, et al. The effect of oxcarbazepine in peripheral neuropathic pain depends on pain

phenotype: A randomised, double-blind, placebo-controlled phenotype-stratified study. Pain 2014;155:2263–73. https://doi.org/10.1016/j.pain.2014.08.014.

[56] Teh K, Armitage P, Tesfaye S, Selvarajah D. Deep Learning Classification of Treatment Response in Diabetic Painful Neuropathy: A Combined Machine Learning and Magnetic Resonance Neuroimaging Methodological Study. Neuroinformatics 2022. https://doi.org/10.1007/s12021-022-09603-5.

[57] Javed S, Petropoulos IN, Alam U, Malik RA. Treatment of painful diabetic neuropathy. Ther Adv Chronic Dis 2015;6:15–28. https://doi.org/10.1177/2040622314552071.

[58] Simpson DM, Robinson-Papp J, Van J, Stoker M, Jacobs H, Snijder RJ, et al. Capsaicin 8% Patch in Painful Diabetic Peripheral Neuropathy: A Randomized, Double-Blind, Placebo-Controlled Study. J Pain 2017;18:42–53. https://doi.org/10.1016/j.jpain.2016.09.008.

[59] Tandan R, Lewis GA, Krusinski PB, Badger GB, Fries TJ. Topical capsaicin in painful diabetic neuropathy. Diabetes Care 1992;15:8–14. https://doi.org/10.2337/diacare.15.1.8.

[60] Bril V, England J, Franklin GM, Backonja M, Cohen J, Del Toro D, et al. Evidence-based guideline: Treatment of painful diabetic neuropathy: Report of the American Academy of Neurology, the American Association of Neuromuscular and Electrodiagnostic Medicine, and the American Academy of Physical Medicine and Rehabilitation. Neurology 2011;76:1758–65. https://doi.org/10.1212/WNL.0b013e3182166ebe.

[61] Yeung AM, Huang J, Nguyen KT, Xu NY, Hughes LT, Agrawal BK, et al. Spinal Cord Stimulation for Painful Diabetic Neuropathy. Https://DoiOrg/101177/19322968221133795 2022:193229682211337. https://doi.org/10.1177/19322968221133795.

[62] Pop-Busui R, Boulton AJM, Feldman EL, Bril V, Freeman R, Malik RA, et al. Diabetic neuropathy: A position statement by the American diabetes association. Diabetes Care 2017;40:136–54. https://doi.org/10.2337/dc16-2042.

[63] Lauria G, Hsieh ST, Johansson O, Kennedy WR, Leger JM, Mellgren SI, et al. 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 ne. Eur J Neurol 2010;17:903-e49. https://doi.org/10.1111/j.1468-1331.2010.03023.x.

[64] Lauria G, Lombardi R, Camozzi F, Devigili G. Skin biopsy for the diagnosis of peripheral neuropathy. Histopathology 2009;54:273–85. https://doi.org/10.1111/j.1365-2559.2008.03096.x.

[65] Kennedy WR, Wendelschafer-Crabb G, Johnson T. Quantitation of epidermal nerves in diabetic neuropathy. Neurology 1996;47:1042–8. https://doi.org/10.1212/WNL.47.4.1042.

[66] Bakkers M, Merkies ISJ, Lauria G, Devigili G, Penza P, Lombardi R, et al. Intraepidermal nerve fiber density and its application in sarcoidosis. Neurology 2009;73:1142–8. https://doi.org/10.1212/WNL.0b013e3181bacf05.

[67] Lauria G, Bakkers M, Schmitz C, Lombardi R, Penza P, Devigili G, et al. Intraepidermal nerve fiber density at the distal leg: A worldwide normative reference study. J Peripher Nerv Syst 2010;15:202–7. https://doi.org/10.1111/j.1529-8027.2010.00271.x.

[68] Albrecht PJ, Houk G, Ruggiero E, Dockum M, Czerwinski M, Betts J, et al. Keratinocyte Biomarkers Distinguish Painful Diabetic Peripheral Neuropathy Patients and Correlate With Topical Lidocaine Responsiveness. Front Pain Res 2021;2:102. https://doi.org/10.3389/fpain.2021.790524.

[69] Karlsson P, Provitera V, Caporaso G, Stancanelli A, Saltalamacchia AM, Borreca I, et al. Increased peptidergic fibers as a potential cutaneous marker of pain in diabetic small fiber neuropathy. Pain 2021;162:778–86. https://doi.org/10.1097/j.pain.0000000000002054.

[70] Karlsson P, Gylfadottir SS, Kristensen AG, Ramirez JD, Cruz P, Le N, et al. Axonal swellings are related to type 2 diabetes, but not to distal diabetic sensorimotor polyneuropathy. Diabetologia 2021;64:923–31. https://doi.org/10.1007/s00125-020-05352-9.

[71] Lauria G, Morbin M, Lombardi R, Borgna M, Mazzoleni G, Sghirlanzoni A, et al. Axonal swellings predict the degeneration of epidermal nerve fibers in painful neuropathies. Neurology 2003;61:631–6. https://doi.org/10.1212/01.WNL.0000070781.92512.A4.

[72] Lauria G, Dacci P, Lombardi R, Cazzato D, Porretta-Serapiglia C, Taiana M, et al. Side and time variability of intraepidermal nerve fiber density. Neurology 2015;84:2368–71. https://doi.org/10.1212/WNL.0000000000001666.

[73] Dhage S, Ferdousi M, Adam S, Ho JH, Kalteniece A, Azmi S, et al. Corneal confocal microscopy identifies small fibre damage and progression of diabetic neuropathy. Sci Rep 2021;11:1–9. https://doi.org/10.1038/s41598-021-81302-8.

[74] Rolke R, Magerl W, Campbell KA, Schalber C, Caspari S, Birklein F, et al. Quantitative sensory testing: A comprehensive protocol for clinical trials. Eur J Pain 2006;10:77–88. https://doi.org/10.1016/j.ejpain.2005.02.003.

[75] Vollert J, Attal N, Baron R, Freynhagen R, Haanpää M, Hansson P, et al.

Quantitative sensory testing using DFNS protocol in Europe: An evaluation of heterogeneity across multiple centers in patients with peripheral neuropathic pain and healthy subjects. Pain 2016;157:750–8. https://doi.org/10.1097/j.pain.0000000000000433.

[76] Vollert J, Maier C, Attal N, Bennett DLH, Bouhassira D, Enax-Krumova EK, et al. Stratifying patients with peripheral neuropathic pain based on sensory profiles: Algorithm and sample size recommendations. Pain 2017;158:1446–55. https://doi.org/10.1097/j.pain.0000000000000935.

[77] Chen X, Graham J, Dabbah MA, Petropoulos IN, Ponirakis G, Asghar O, et al. Small nerve fiber quantification in the diagnosis of diabetic sensorimotor polyneuropathy: Comparing corneal confocal microscopy with intraepidermal nerve fiber density. Diabetes Care 2015;38:1138–44. https://doi.org/10.2337/dc14-2422.

[78] Alam U, Jeziorska M, Petropoulos IN, Asghar O, Fadavi H, Ponirakis G, et al. Diagnostic utility of corneal confocal microscopy and intra-epidermal nerve fibre density in diabetic neuropathy. PLoS One 2017;12. https://doi.org/10.1371/journal.pone.0180175.

[79] Hertz P, Bril V, Orszag A, Ahmed A, Ng E, Nwe P, et al. Reproducibility of in vivo corneal confocal microscopy as a novel screening test for early diabetic sensorimotor polyneuropathy. Diabet Med 2011;28:1253–60. https://doi.org/10.1111/j.1464-5491.2011.03299.x.

[80] Pacaud D, Romanchuk KG, Tavakoli M, Gougeon C, Virtanen H, Ferdousi M, et al. The reliability and reproducibility of corneal confocal microscopy in children. Investig Ophthalmol Vis Sci 2015;56:5636–40. https://doi.org/10.1167/iovs.15-16995.

[81] Petropoulos IN, Manzoor T, Morgan P, Fadavi H, Asghar O, Alam U, et al. Repeatability of in vivo corneal confocal microscopy to quantify corneal nerve morphology. Cornea 2013;32. https://doi.org/10.1097/ICO.0b013e3182749419.

[82] Andersen ST, Grosen K, Tankisi H, Charles M, Andersen NT, Andersen H, et al. Corneal confocal microscopy as a tool for detecting diabetic polyneuropathy in a cohort with screen-detected type 2 diabetes: ADDITION-Denmark. J Diabetes Complications 2018. https://doi.org/10.1016/j.jdiacomp.2018.09.016.

[83] Püttgen S, Bönhof GJ, Strom A, Müssig K, Szendroedi J, Roden M, et al. Augmented Corneal Nerve Fiber Branching in Painful Compared With Painless Diabetic Neuropathy. J Clin Endocrinol Metab 2019;104:6220–8. https://doi.org/10.1210/jc.2019-01072.

[84] Tavakoli M, Mitu-Pretorian M, Petropoulos IN, Fadavi H, Asghar O, Alam U, et al. Corneal confocal microscopy detects early nerve regeneration in

diabetic neuropathy after simultaneous pancreas and kidney transplantation. Diabetes 2013;62:254–60. https://doi.org/10.2337/db12-0574.

[85] Tavakoli M, Ferdousi M, Petropoulos IN, Morris J, Pritchard N, Zhivov A, et al. Normative values for corneal nerve morphology assessed using corneal confocal microscopy: A multinational normative data set. Diabetes Care 2015;38:838–43. https://doi.org/10.2337/dc14-2311.

[86] D'Onofrio L, Kalteniece A, Ferdousi M, Azmi S, Petropoulos IN, Ponirakis G, et al. Small nerve fiber damage and langerhans cells in type 1 and type 2 diabetes and LADA measured by corneal confocal microscopy. Investig Ophthalmol Vis Sci 2021;62:5–5. https://doi.org/10.1167/IOVS.62.6.5.

[87] Malik R. Clinical applications of corneal confocal microscopy. Clin Ophthalmol 2008;2:435. https://doi.org/10.2147/opth.s1490.

[88] Chen X, Graham J, Dabbah MA, Petropoulos IN, Tavakoli M, Malik RA. An automatic tool for quantification of nerve fibers in corneal confocal microscopy images. IEEE Trans Biomed Eng 2017;64:786–94. https://doi.org/10.1109/TBME.2016.2573642.

[89] Krishnan STM, Quattrini C, Jeziorska M, Malik RA, Rayman G. Abnormal LDIflare but normal quantitative sensory testing and dermal nerve fiber density in patients with painful diabetic neuropathy. Diabetes Care 2009;32:451–5. https://doi.org/10.2337/dc08-1453.

[90] Casanova-Molla J, Grau-Junyent JM, Morales M, Valls-Solé J. On the relationship between nociceptive evoked potentials and intraepidermal nerve fiber density in painful sensory polyneuropathies. Pain 2011;152:410–8. https://doi.org/10.1016/j.pain.2010.11.012.

[91] Valeriani M, Le Pera D, Niddam D, Chen ACN, Arendt-Nielsen L. Dipolar modelling of the scalp evoked potentials to painful contact heat stimulation of the human skin. Neurosci Lett 2002;318:44–8. https://doi.org/10.1016/S0304-3940(01)02466-1.

[92] Donadio V, Liguori R. Microneurographic recording from unmyelinated nerve fibers in neurological disorders: An update. Clin Neurophysiol 2015;126:437–45. https://doi.org/10.1016/j.clinph.2014.10.009.

[93] Parkhouse N, LeQuesne PM. Quantitative objective assessment of peripheral nociceptive C fibre function. J Neurol Neurosurg Psychiatry 1988;51:28–34. https://doi.org/10.1136/jnnp.51.1.28.

[94] Atherton DD, Facer P, Roberts KM, Peter Misra V, Chizh BA, Bountra C, et al. Use of the novel contact heat evoked potential stimulator (CHEPS) for the assessment of small fibre neuropathy: Correlations with skin flare responses and intra-epidermal nerve fibre counts. BMC Neurol 2007;7:21. https://doi.org/10.1186/1471-2377-7-21.

[95] Vas PRJ, Rayman G. Validation of the modified LDIFlare technique: A simple and quick method to assess C-fiber function. Muscle and Nerve 2013;47:351–6. https://doi.org/10.1002/mus.23532.

[96] Vas PRJ, Rayman G. 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 2013;8:e69920. https://doi.org/10.1371/journal.pone.0069920.

[97] Sharma S, Tobin V, Vas PRJ, Malik RA, Rayman G. The influence of age, anthropometric and metabolic variables on LDIFLARE and corneal confocal microscopy in healthy individuals. PLoS One 2018;13. https://doi.org/10.1371/journal.pone.0193452.

[98] Fuchs D, Dupon PP, Schaap LA, Draijer R. The association between diabetes and dermal microvascular dysfunction non-invasively assessed by laser Doppler with local thermal hyperemia: A systematic review with meta-analysis. Cardiovasc Diabetol 2017;16:11. https://doi.org/10.1186/s12933-016-0487-1.

[99] Sharma S, Vas P, Rayman G. Small Fiber Neuropathy in Diabetes Polyneuropathy: Is It Time to Change? J Diabetes Sci Technol 2022;16:321–31. https://doi.org/10.1177/1932296821996434.

[100] Ruscheweyh R, Emptmeyer K, Putzer D, Kropp P, Marziniak M. Reproducibility of contact heat evoked potentials (CHEPs) over a 6months interval. Clin Neurophysiol 2013;124:2242–7. https://doi.org/10.1016/j.clinph.2013.05.003.

[101] Treede RD, Lorenz J, Baumgärtner U. Clinical usefulness of laser-evoked potentials. Neurophysiol Clin 2003;33:303–14. https://doi.org/10.1016/j.neucli.2003.10.009.

[102] Lagerburg V, Bakkers M, Bouwhuis A, Hoeijmakers JGJ, Smit AM, Van Den Berg SJM, et al. Contact heat evoked potentials: Normal values and use in small-fiber neuropathy. Muscle and Nerve 2015;51:743–9. https://doi.org/10.1002/mus.24465.

[103] Mueller D, Obermann M, Koeppen S, Kavuk I, Yoon MS, Sack F, et al. Electrically evoked nociceptive potentials for early detection of diabetic small-fiber neuropathy. Eur J Neurol 2010;17:834–41. https://doi.org/10.1111/j.1468-1331.2009.02938.x.

[104] Vallbo ÅB. Microneurography: How it started and how it works. J Neurophysiol 2018;120:1415–27. https://doi.org/10.1152/jn.00933.2017.

[105] Yajnik CS, Kantikar V V., Pande AJ, Deslypere JP. Quick and Simple Evaluation of Sudomotor Function for Screening of Diabetic Neuropathy.

ISRN Endocrinol 2012;2012:1–7. https://doi.org/10.5402/2012/103714.

[106] Papanas N, Papatheodorou K, Christakidis D, Papazoglou D, Giassakis G, Piperidou H, et al. Evaluation of a New Indicator Test for Sudomotor Function (Neuropad®) in the Diagnosis of Peripheral Neuropathy in Type 2 Diabetic Patients. Exp Clin Endocrinol Diabetes 2005;113:195–8. https://doi.org/10.1055/s-2005-837735.

[107] Riedel A, Braune S, Kerum G, Schulte-Mönting J, Lücking CH. Quantitative sudomotor axon reflex test (QSART): A new approach for testing distal sites. Muscle and Nerve 1999;22:1257–64. https://doi.org/10.1002/(SICI)1097-4598(199909)22:9<1257::AID-MUS14>3.0.CO;2-J.

[108] Kiernan MC, Bostock H, Park SB, Kaji R, Krarup C, Krishnan A V., et al. Measurement of axonal excitability: Consensus guidelines. Clin Neurophysiol 2020;131:308–23. https://doi.org/10.1016/j.clinph.2019.07.023.

[109] Hugosdottir R, Mørch CD, Andersen OK, Helgason T, Arendt-Nielsen L. Preferential activation of small cutaneous fibers through small pin electrode also depends on the shape of a long duration electrical current. BMC Neurosci 2019;20:1–11. https://doi.org/10.1186/s12868-019-0530-8.

[110] Tigerholm J, Hoberg TN, Brønnum D, Vittinghus M, Frahm KS, Mørch CD. Small and large cutaneous fibers display different excitability properties to slowly increasing ramp pulses. J Neurophysiol 2020;124:883–94. https://doi.org/10.1152/JN.00629.2019/ASSET/IMAGES/LARGE/Z9K0092055870007.JPEG.

[111] Hodgkin AL, Huxley AF. A quantitative description of membrane current and its application to conduction and excitation in nerve. J Physiol 1952;117:500–44. https://doi.org/10.1113/jphysiol.1952.sp004764.

[112] Baker M, Bostock H. Depolarization changes the mechanism of accommodation in rat and human motor axons. J Physiol 1989;411:545–61. https://doi.org/10.1113/jphysiol.1989.sp017589.

[113] Djouhri L, Fang X, Okuse K, Wood JN, Berry CM, Lawson SN. The TTX-resistant sodium channel Nav 1.8 (SNS/PN3): Expression and correlation with membrane properties in rat nociceptive primary afferent neurons. J Physiol 2003;550:739–52. https://doi.org/10.1113/jphysiol.2003.042127.

[114] Weiss G. Sur la possibilite de rendre comparables entre eux les appareils servant a l'excitation electrique. Arch Ital Biol 1901;35:413–46.

[115] Geddes LA, Bourland JD. The Strength-Duration Curve. IEEE Trans Biomed Eng 1985;BME-32:458–9. https://doi.org/10.1109/TBME.1985.325456.

[116] Papanas N, Ziegler D. New vistas in the diagnosis of diabetic polyneuropathy. Endocrine 2014;47:690–8. https://doi.org/10.1007/s12020-014-0285-z.

[117] Shibata Y, Himeno T, Kamiya T, Tani H, Nakayama T, Kojima C, et al. Validity and reliability of a point-of-care nerve conduction device in diabetes patients. J Diabetes Investig 2019;10:1291–8. https://doi.org/10.1111/jdi.13007.

[118] Sharma S, Vas PR, Rayman G. 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 2015;9:123–31. https://doi.org/10.1177/1932296814551044.

[119] Crawford F, Inkster M, Kleijnen J, Fahey T. Predicting foot ulcers in patients with diabetes: a systematic review and meta-analysis. Qjm 2006;100:65–86. https://doi.org/10.1093/qjmed/hcl140.

[120] Hansen CS, Eldrup E, Yderstræde K, Vind BF, Andersen ST, Jakobsen PE, et al. Diabetisk neuropati. Https://EndocrinologyDk/Nbv/Diabetes-Melitus/Type-1-Diabetes-Mellitus/ 2022.

[121] Feldman EL, Stevens MJ, Thomas PK, Brown MB, Canal N, Greene DA. A practical two-step quantitative clinical and electrophysiological assessment for the diagnosis and staging of diabetic neuropathy. Diabetes Care 1994;17:1281–9. https://doi.org/10.2337/diacare.17.11.1281.

[122] Simpson GM, Blair JH. Achilles Reflex. Am J Psychiatry 1964;121:403–403. https://doi.org/10.1176/ajp.121.4.403.

[123] Jespersen A, Amris K, Bliddal H, Andersen S, Lavik B, Janssen H, et al. Is neuropathic pain underdiagnosed in musculoskeletal pain conditions? The Danish PainDETECTive study. Curr Med Res Opin 2010;26:2041–5. https://doi.org/10.1185/03007995.2010.502748.

[124] Greco C, Di Gennaro F, D'Amato C, Morganti R, Corradini D, Sun A, et al. Validation of the Composite Autonomic Symptom Score 31 (COMPASS 31) for the assessment of symptoms of autonomic neuropathy in people with diabetes. Diabet Med 2017;34:834–8. https://doi.org/10.1111/dme.13310.

[125] Dabbah MA, Graham J, Petropoulos IN, Tavakoli M, Malik RA. Automatic analysis of diabetic peripheral neuropathy using multi-scale quantitative morphology of nerve fibres in corneal confocal microscopy imaging. Med Image Anal 2011;15:738–47. https://doi.org/10.1016/j.media.2011.05.016.

[126] Andersen HH, Lundgaard AC, Petersen AS, Hauberg LE, Sharma N, Hansen SD, et al. The lancet weight determines wheal diameter in response to skin prick testing with histamine. PLoS One 2016;11:e0156211. https://doi.org/10.1371/journal.pone.0156211.

[127] Poulsen AH, Tigerholm J, Meijs S, Andersen OK, Mørch CD. Comparison of existing electrode designs for preferential activation of cutaneous nociceptors. J Neural Eng 2020;17. https://doi.org/10.1088/1741-2552/ab85b1.

[128] Croosu SS, Røikjer J, Mørch CD, Ejskjaer N, Frøkjær JB, Hansen TM. Alterations in Functional Connectivity of Thalamus and Primary Somatosensory Cortex in Painful and Painless Diabetic Peripheral Neuropathy. Diabetes Care 2023;46:1–10. https://doi.org/10.2337/DC22-0587.

[129] Croosu SS, Hansen TM, Røikjer J, Mørch CD, Ejskjaer N, Frøkjær JB. Gray Matter Brain Alterations in Type 1 Diabetes – Findings Based on Detailed Phenotyping of Neuropathy Status. Exp Clin Endocrinol Diabetes 2022. https://doi.org/10.1055/a-1835-1877.

[130] Aronin N, Leeman SE, Clements RS. Diminished flare response in neuropathic diabetic patients. Comparison of effects of substance P, histamine, and capsaicin. Diabetes 1987;36:1139–43. https://doi.org/10.2337/diab.36.10.1139.

[131] Krishnan STM, Rayman G. The LDIflare: A novel test of C-fiber function demonstrates early neuropathy in type 2 diabetes. vol. 27. 2004. https://doi.org/10.2337/diacare.27.12.2930.

[132] Durand S, Fromy B, Bouyé P, Saumet JL, Abraham P. Current-induced vasodilation during water iontophoresis (5 min, 0.10 mA) is delayed from current onset and involves aspirin sensitive mechanisms. J Vasc Res 2002;39:59–71. https://doi.org/10.1159/000048994.

[133] Mørch CD, Hennings K, Andersen OK. Estimating nerve excitation thresholds to cutaneous electrical stimulation by finite element modeling combined with a stochastic branching nerve fiber model. Med Biol Eng Comput 2011;49:385–95. https://doi.org/10.1007/s11517-010-0725-8.

[134] Hugosdottir R, Mørch CD, Andersen OK, Arendt-Nielsen L. Investigating stimulation parameters for preferential small-fiber activation using exponentially rising electrical currents. J Neurophysiol 2019;122:1745–52. https://doi.org/10.1152/jn.00390.2019.

[135] Hugosdottir R, Mørch CD, Jørgensen CK, Nielsen CW, Olsen MV, Pedersen MJ, et al. Altered excitability of small cutaneous nerve fibers during cooling assessed with the perception threshold tracking technique. BMC Neurosci 2019;20:1–13. https://doi.org/10.1186/s12868-019-0527-3.

ISSN (online): 2246-1302
ISBN (online): 978-87-7573-774-1

AALBORG UNIVERSITY PRESS