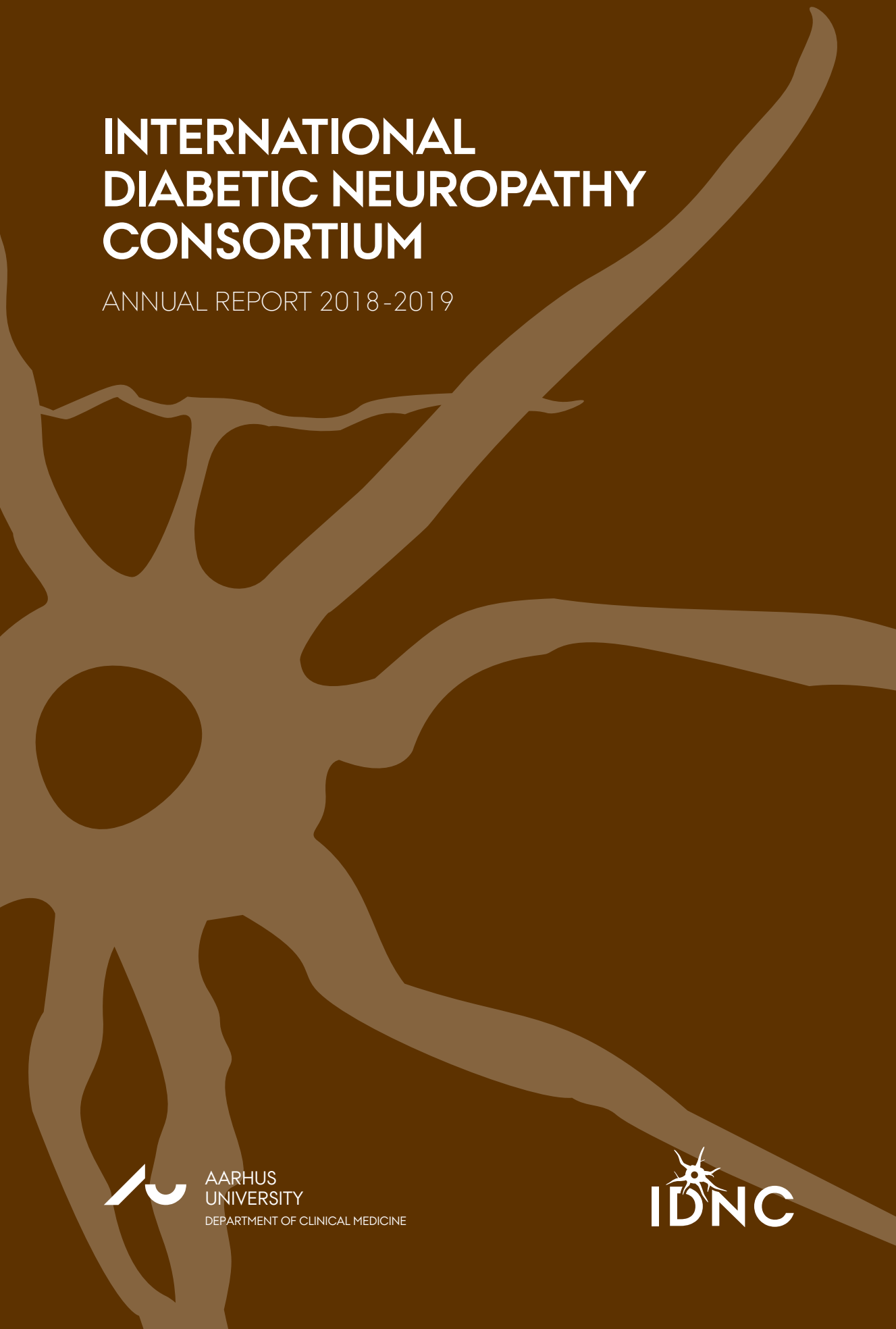
INTERNATIONAL DIABETIC NEUROPATHY CONSORTIUM

ANNUAL REPORT 2018-2019



AARHUS
UNIVERSITY
DEPARTMENT OF CLINICAL MEDICINE

IDNC

novo nordisk fonden

The International Diabetic Neuropathy Consortium was awarded a grant of 60 million Danish Kroner for a 6-year period from the Novo Nordisk Foundation in December 2014, with project start-up in May 2015.

Novo Nordisk Foundation: Grant number NNF14OC0011633

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> The pandemic of diabetic neuropathy calls for new therapeutic and preventive initiatives.

PREFACE

I am pleased to present the fourth annual report of the International Diabetic Neuropathy Consortium (IDNC).

The IDNC is supported by a Challenge grant from the Novo Nordic Foundation (NNF) to study diabetic neuropathy in a consolidated collaboration between Aarhus University, the University of Southern Denmark, the University of Michigan, USA and the University of Oxford, UK.

Several important reasons exist to study diabetic neuropathy: it represent one of the most common complications of diabetes; it is associated with increased morbidity and mortality; it has severe consequences for the quality of life of affected people and it carries a major economic burden to the society.

The mechanisms underlying diabetic neuropathy is still an enigma. IDNC has taken up the challenge to study diabetic neuropathy from different angles: assess the possible role of hypoxia of nerve fibers for their degeneration, develop new techniques to document early changes of nerve damage in humans, study the effect of neuropathy with and without pain on the quality of life and identify risk factors for this condition.

Collaboration between Danish universities, the new Steno Diabetes centers in Denmark and prestigious universities abroad represent a unique opportunity for IDNC to continue our research.

During the last year, members of the IDNC have been active in their research and at different congresses. I would like to extend my sincere thanks to all collaborators of the IDNC for their enthusiasm, commitment and hard work. Also my gratitude to our international scientific advisory board and to Aarhus University for their help and support.

Troels Staehelin Jensen
Director of the IDNC

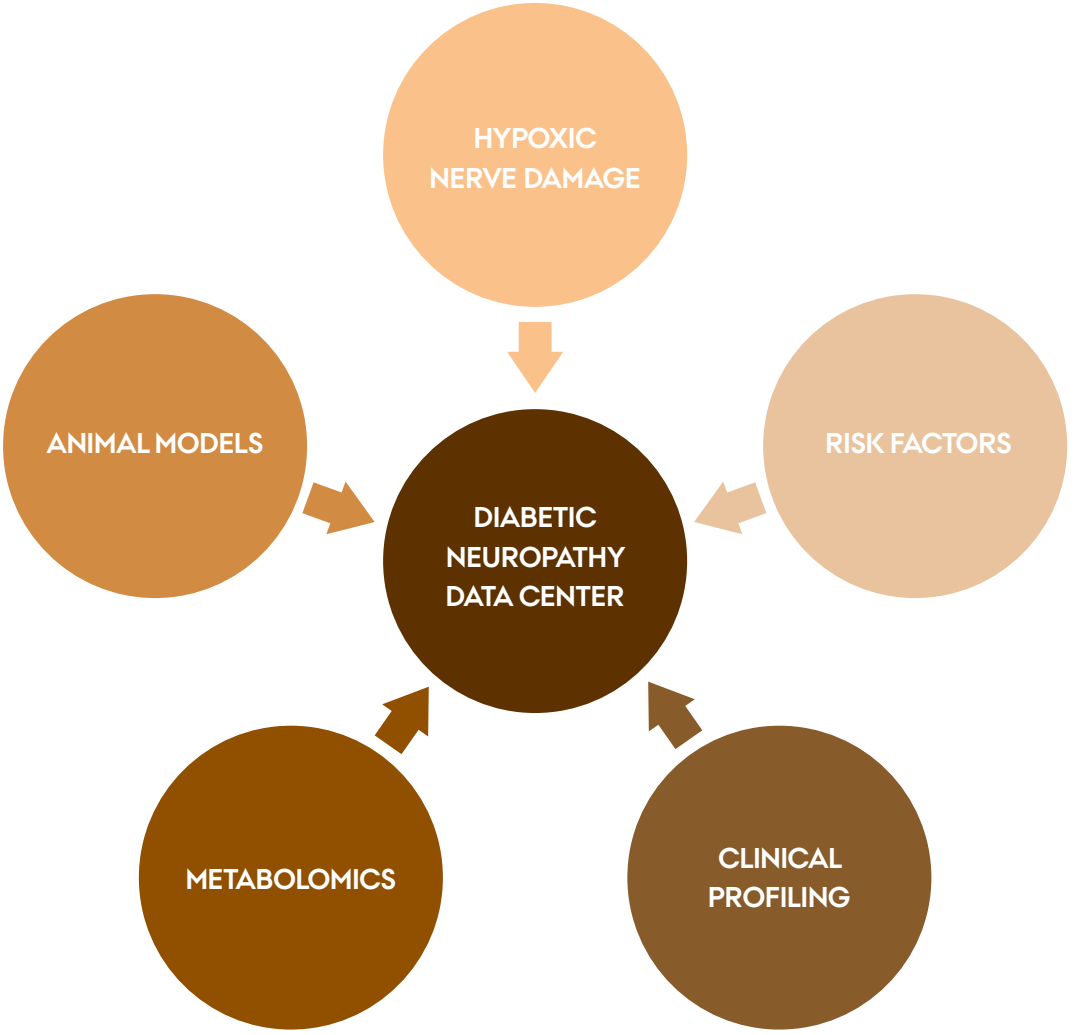
IDNC AT A GLANCE

Vision: To be a leading research group on diabetic neuropathy in Denmark with an outreach to the world.

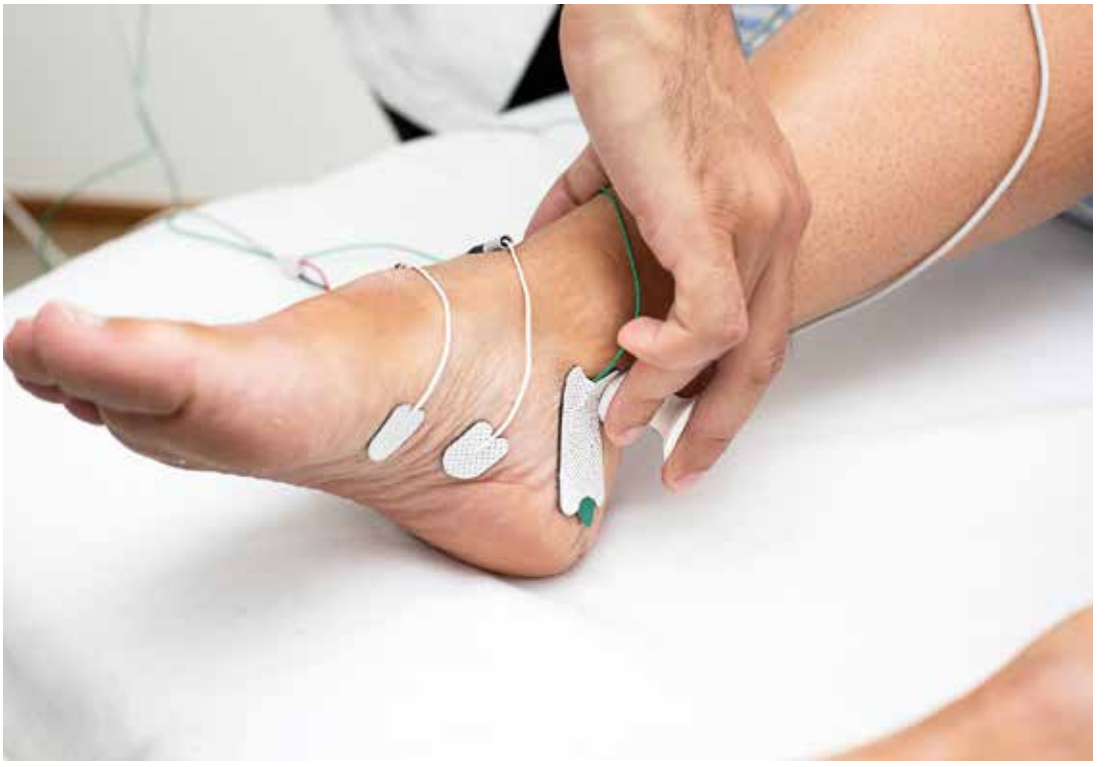
Mission: To study and unravel pathophysiological mechanisms of diabetic neuropathy, contribute to early identification, improve and develop a uniform international classification in order to better treat and prevent the detrimental consequences of diabetic neuropathy. The IDNC does so by bringing researchers and clinicians together in a stimulating and multidisciplinary environment in order to integrate and facilitate translational aspects of diabetic neuropathy.

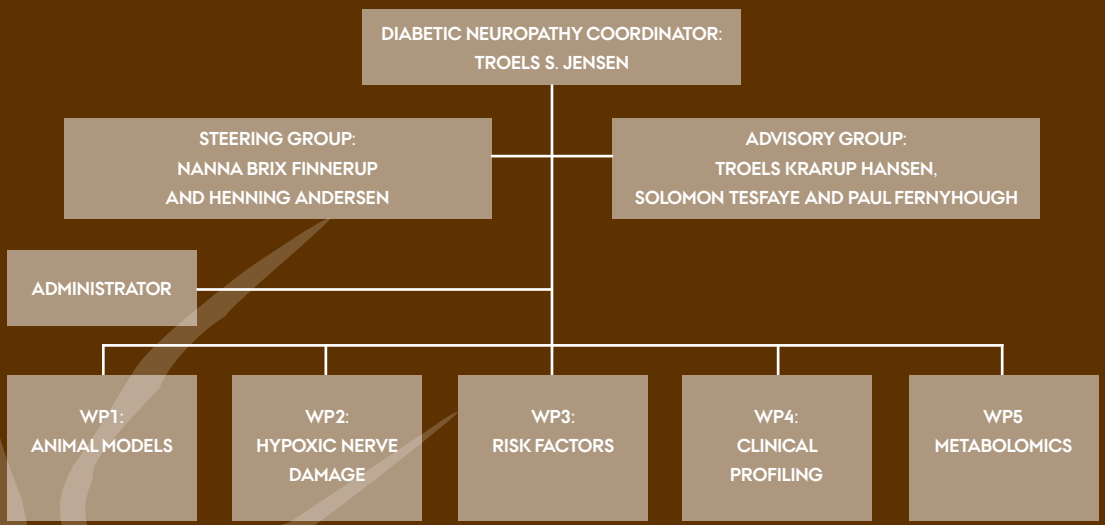
Structure: A series of work packages in which four universities: University of Michigan, University of Oxford, South Danish University and Aarhus University work together in an effort to understand mechanisms of diabetic neuropathy, risk factors for neuropathy and pain and the clinical and metabolic profile of diabetic neuropathy.

Funding: A 6-year Novo Nordisk Foundation Challenge Program grant (Grant number NNF14OC0011633).


HYPOXIC
NERVE DAMAGE
ANIMAL MODELS
RISK FACTORS
DIABETIC
NEUROPATHY
DATA CENTER
METABOLOMICS
CLINICAL
PROFILING

Assessments of neuropathy





ORGANIZATION

The management structure of the IDNC consists of the director, the steering group and the scientific advisory board. The steering group helps to identify important research initiatives and implement them in the IDNC. The internationally renowned scientific advisory board helps identifying research questions critical to improving our understanding of diabetic neuropathy.

Aarhus University, Health hosts and supports the administration of the IDNC. The Danish Pain Research Center at Aarhus University Hospital provides housing facility for IDNC management.

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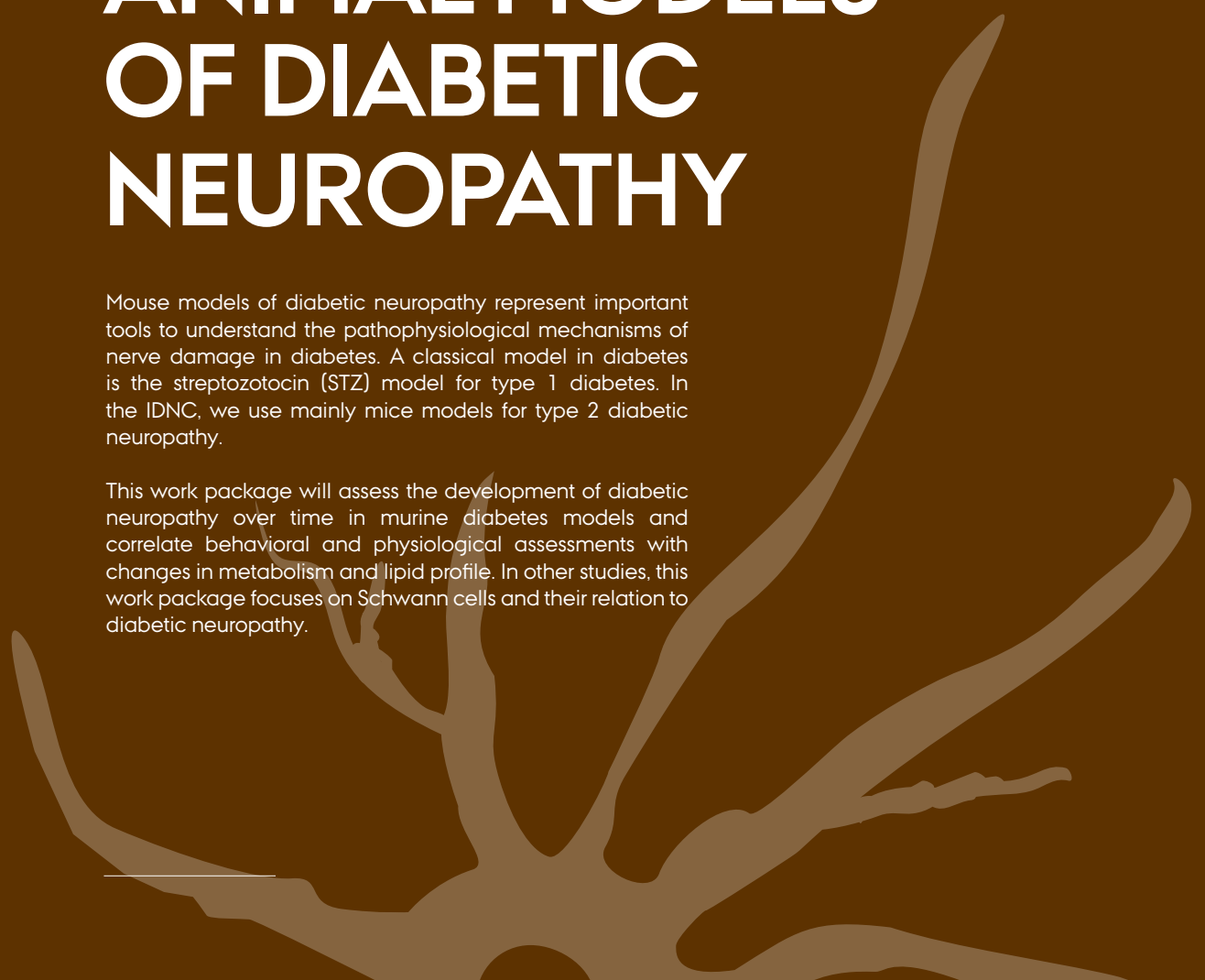
WP1: ANIMAL MODELS OF DIABETIC NEUROPATHY

Mouse models of diabetic neuropathy represent important tools to understand the pathophysiological mechanisms of nerve damage in diabetes. A classical model in diabetes is the streptozotocin (STZ) model for type 1 diabetes. In the IDNC, we use mainly mice models for type 2 diabetic neuropathy.

This work package will assess the development of diabetic neuropathy over time in murine diabetes models and correlate behavioral and physiological assessments with changes in metabolism and lipid profile. In other studies, this work package focuses on Schwann cells and their relation to diabetic neuropathy.



WP1: SCHWANN CELLS AND THEIR ROLE IN DIABETIC NEUROPATHY

Nadia Gonçalves *addresses the role of Schwann cells in diabetic neuropathy in her postdoc project. Associate Professor Christian Bjerregaard Vægter leads the research (Department of Biomedicine, Aarhus University, Denmark).*

Diabetic neuropathy is characterized by damage to neurons, Schwann cells and blood vessels within the nerve. The concept of Schwannopathy in diabetic neuropathy is evolving and posits that Schwann cell stress triggered by the high glucose levels and hyperlipidemia leads to loss of axonal support, degeneration and Schwann cell dedifferentiation. A central player for all of these physiological paradigms is the p75 neurotrophin receptor ($p75^{NTR}$), with an *in vitro* ability to regulate Schwann cell myelination and proliferation. Therefore, in this study, we further tested how disruption of $p75^{NTR}$ signaling in Schwann cells modulates diabetic neuropathy progression.

Electron microscopy data from sciatic nerves from mice with high fat diet induced diabetes, where $p75^{NTR}$ was conditionally knockdown in Schwann cells – SC-$p75^{NTR}$-KO, denoted prominent segmental demyelination (Fig. 1A), axonal atrophy (Fig. 1B) and significant loss of C-fiber density (Fig. 1C and 1D), as compared with diabetic counterparts. RNA sequencing examination disclosed several pre-clinical signaling alterations in the diabetic peripheral nerves, dependent on Schwann cell $p75^{NTR}$ signaling. Apart from the similarly activated pathways, such as the PPAR signaling pathway, glycerolipid metabolism or defense response, our results highlight that Schwann cell $p75^{NTR}$ depleted nerves exhibit changes in gene expression levels in a higher extent (Fig. 2A), particularly relating to actin cytoskeleton, immune system, peroxisomes, lysosomes and phagosomes (Fig. 2B).

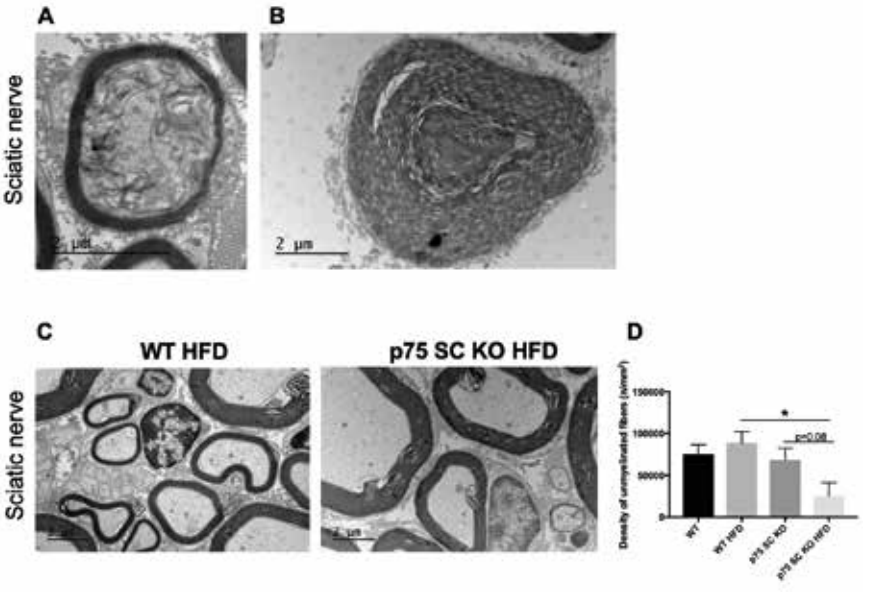


Fig. 1*. Morphological abnormalities found in nerve samples from p75 SC KO mice under the high fat diet (HFD) include: segmental demyelination* ***(A)****, axonal atrophy* ***(B)*** *and decreased number of C-fibers* ***(C)****, confirmed by morphometric quantification* ***(D)****.*

The phagolysosomal pathway is particularly relevant since electron microscopy analysis showed several lysosomes and autophagosomes in the axoplasm of C-fibers from the diabetic SC-p75^{NTR}-KO nerves. Furthermore, over-activation of Cathepsins at transcriptional and translational levels suggest increased cell stress and death, probably accounting for the C-fiber loss in diabetic SC-p75^{NTR}-KO nerves (Fig. 2C).

This study unravels a valuable rodent model, closely resembling nerve pathological hallmarks observed in patients with diabetic neuropathy, and points towards an important role of p75^{NTR} expressed by the Schwann cells for regulation of nerve transcriptomics in the context of diabetic neuropathy.

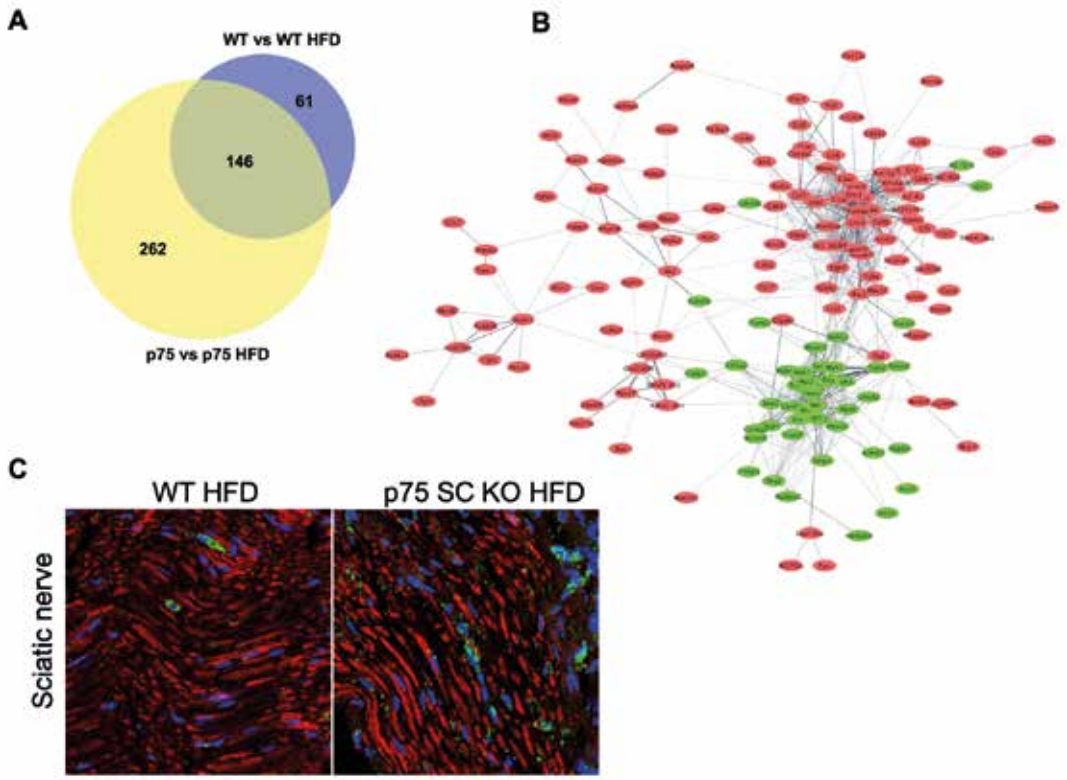


***Fig. 2**. In mice lacking Schwann cell p75NTR, feeding with a high fat diet (HFD) induced a significant stronger gene remodeling, with 146 shared genes among the two genotypes, as illustrated in the Venn diagram **(A)**. A STRING network analysis in Cytoscape for the 262 genes only differentially regulated in the p75 SC KO mice fed with HFD (as compared with HFD fed WT mice) revealed that most of the upregulated genes (represented in red) are related with the immune system and autophagosomal pathways and the downregulated genes (represented in green) are associated with actin cytoskeleton **(B)**. In **(C)**, pictures show Cathepsin B protein levels in the sciatic nerve, obtained by confocal microscopy. Axons are labeled in red with Illtubulin, Cathepsin B is immunolabeled in green and nuclei were stained blue with Hoechst. Magnification 20x.*

WP1: BASIC NEUROPHYSIOLOGICAL STUDY

Martin Nors Skov *is PhD student at the Comparative Medicine Lab at Aarhus University (DK). Professor Michael Pedersen leads the research with Ass. Professor, Hatice Tankisi and Ass. Professor Vladimir Matchkov. as co-supervisors*

Study aim: The development of a telemetric implant to investigate peripheral neuropathy.

Status: With a dissertation deadline January 5, 2020, the project has reached its final phase. As of now, the focus has changed to the Qtrac threshold tracking setup implant. A front-end amplifier has been developed and shows conformity with the clinical Digitimer Qtrac setup. The diabetic neuropathy study in rats has revealed no significant differences in nerve function between rats undergoing hyberbaric oxygen treatment and normobaric-treated rats.

We have been looking at two telemetric implant designs for autonomic nerve recording and an implant that works with the Qtrac setup. For the autonomic nerve recordings, we found in proof-of-concept studies that acquisition of data was challenged due to damaged nerves (the nerves are very small and fragile).

The primary focus shifted to the Qtrac setup. We created a working pre-amp and filter prototype with a size of 2x2x0.5 cm for the front-end of the telemetric implant. In tests, the front-end device showed to equally match the results obtained with the clinical Digitimer Qtrac setup (Fig.1, Show compassion in human testing). Aarhus University's Transfer and Technology Office is assessing the patent suitability with respect to the novelty of pre-amp front-end device. In 2019, we have completed our STZ diabetic neuropathy and hyberbaric oxygen treatment studies using a 3-weeks intervention period. We interestingly found no statistically significances of differences of electrophysiological parameters (Qtrac) between control rats and STZ rats with and without hyberbaric oxygen treatment. In parallel, we found that hyperbaric treatment stimulated a larger light evoke response compared to those subjected to normobaric treatment.

Martin had a 14-days stay at Prof. Christian Krarup and Ass. Prof. Mihai Moldovan research laboratory at Department of Clinical Medicine, University of Copenhagen. We tested the pre-amp on some mice with an impressive results (Fig. 2, Show electrophysiogical measures in mice). The newly developed device was presented at the conferences "NeuPSIG 2019, London" and "ECCN 2019, Warsaw", and there was a great interest in the device from both scientists and industry. Martin is planning a research visit to the working lab of Eli Lilly, UK, to exploit industrial partnership.

With the positive results of the front-end device, a final study is planned autumn 2019. The study is planned to run for 8 weeks. In week one we will implant electrodes, making it possible to run a Qtrac TROND protocol at the same anatomic spot each week. The rats will have the electrodes connected to a headplug, which is connected to our device during examinations. In the second week, the rat undergoes a STZ protocol to induce diabetes. For the following 7 weeks (compared to the 3-weeks period in the previous study), the rats will develop diabetic neuropathy, and each rat will 1-2 times a week undergo an electrophysiological test. With this study, we hope to find new insight about the dynamic relationship between development of diabetic neuropathy and the appearance of the compound action potential.

Martin testing mice at Panum institute, Copenhagen University

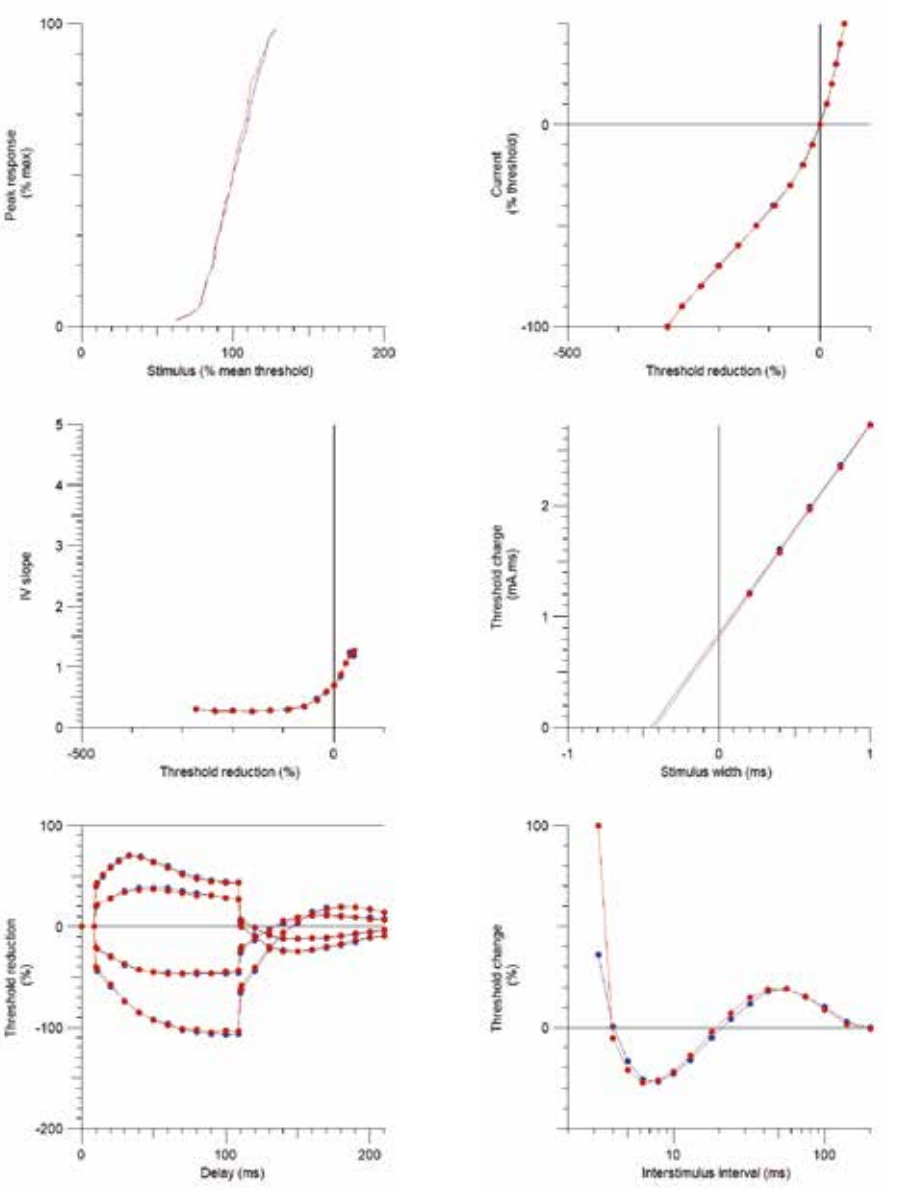


***Fig. 1**. Human testing. Compassion between Digitimer setup (Red) and Pre-amp device (Blue).*

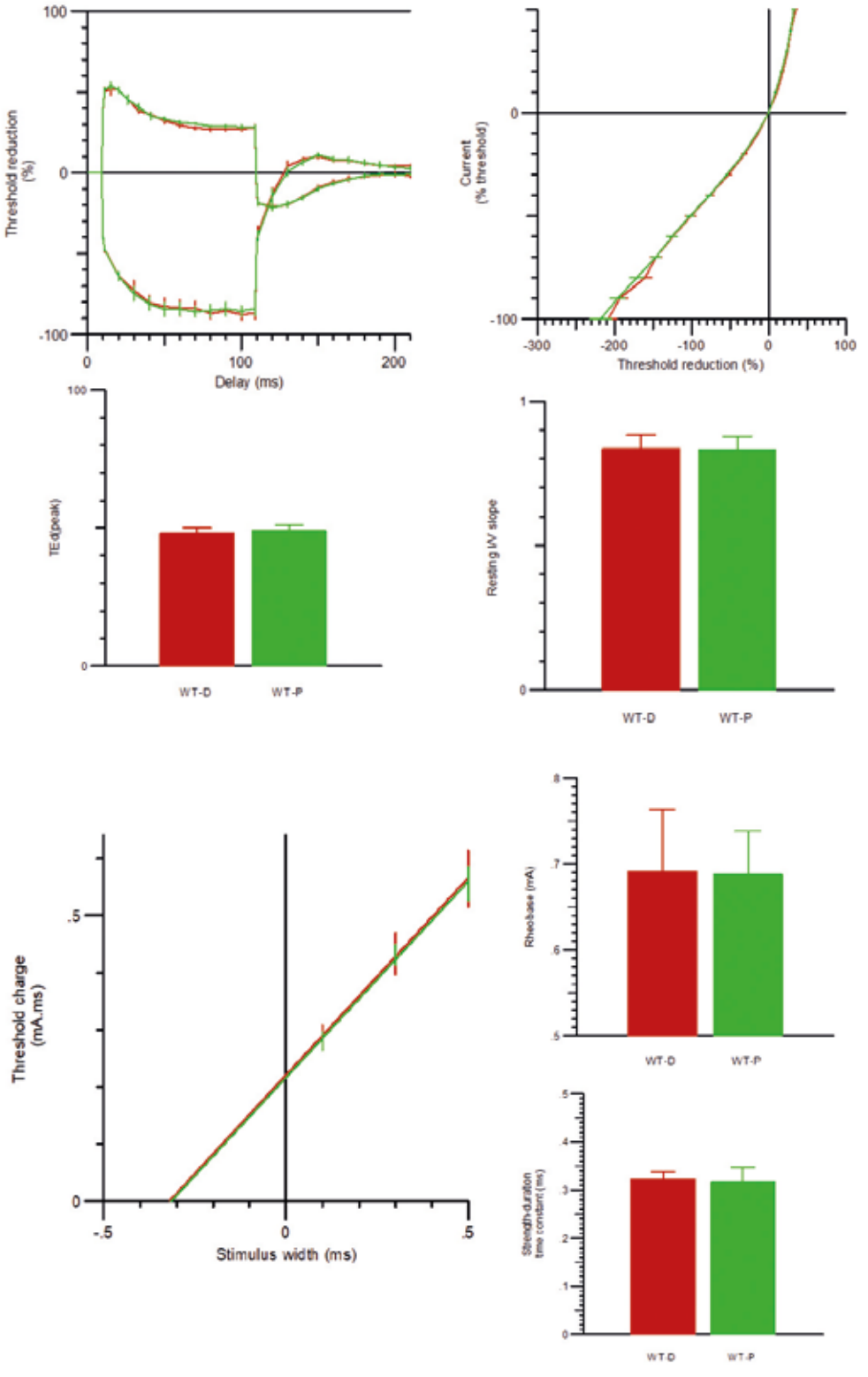


***Fig. 2**. Mice test. Comparison between Digitimer setup (Red) and Pre-amp device (Green). A. Threshold electrotonus, B. Current-threshold relationship, C. TEd (peak response), D. IV slope, E. Strength- duration time constant, F. Rheobase.*

WP2: HYPOXIC NERVE DAMAGE

In this work package, the idea is that capillary flow is lost in diabetes due to endothelial glycocalyx damage, loss of pericytes, thickening of capillary basement membranes and elevated blood viscosity. Capillary flow in sural nerves of both type 1 and type 2 models for diabetic neuropathy are studied using two-photon microscopy combined with optical coherence tomography (OCT). With these methods, we test the hypothesis that elevated capillary transit time heterogeneity and reduced oxygen tension are involved in diabetic neuropathy in mice.

WP2: ARE DIABETIC NERVES SUFFOCATING?

Anete Dudele *is a postdoc at the Center of Functionally Integrative neuroscience (CFIN), Aarhus University (DK) Professor Leif Østergaard leads the research.*

Anete hypothesizes in her studies that diabetic peripheral neuropathy (DPN) develops due to dysfunctional microvascular blood flow in the nerves, leading to limited oxygen delivery to the nervous tissue, which consequently causes tissue damage.

Using state of the art in vivo two-photon microscopy, we have established a method to investigate this in peripheral nerves of mice. This method enables us to visualize and quantify nerve perfusion in real time during rest and electric stimulation, at the level of individual endoneureal capillaries, and capillary networks (Fig.1.).

Utilizing electrophysiological methods learned during a visit to prof. Eva Feldman's lab at the University of Michigan in 2016, we have validated a mouse model of DPN that we have used for further investigation of the microvascular endoneureal blood flow. This mouse model resembles DPN characteristic to human type 2 diabetes, as the mice have developed obesity (Fig. 2A.) after consumption of a diet with high fat content, which has led to development type 2 diabetes (Fig. 2B.) and DPN (Fig. 2C and D).

In addition, we are developing a new method of data analysis acquired from in vivo microscopy. With this new approach, we will be able to extract information on which mechanisms responsible for regulation of local blood flow (endothelial, neurological or myogenic) have been affected by progression of diabetes.

These studies will enable us to understand exactly which microvascular events occur as type 2 diabetes leads to development of DPN, and in what order. This information will allow us to work towards development of highly targeted interventions aimed at the key mechanisms during the right windows of opportunity.

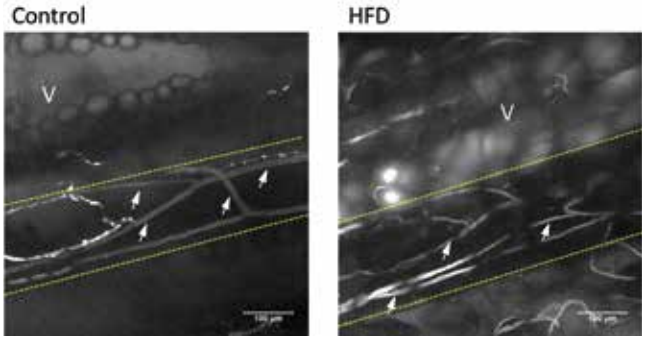


Fig. 1*. Maximal projection image from two-photon in vivo microscopy scan, depicting imaging location of murine sural nerve in 25 weeks old, C57 black male mice fed either control or high fat diet (HFD) for 20 weeks. Sural nerve highlighted by yellow dotted lines, all plasma appears in white. Arrows point to sural nerve vessels that have been scanned to obtain red blood cell velocity and sural nerve vessel diameter measurements. V –vein adjacent to the sural nerve.*

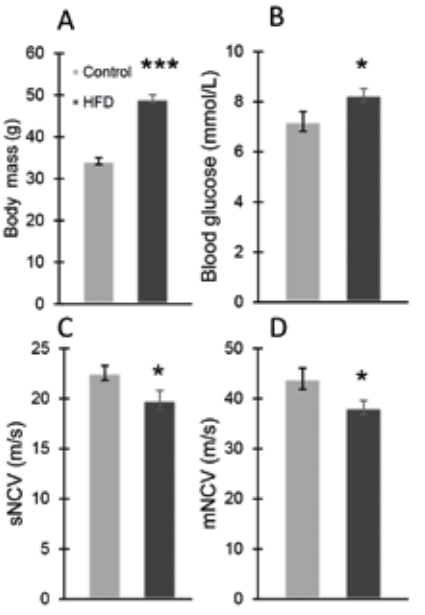
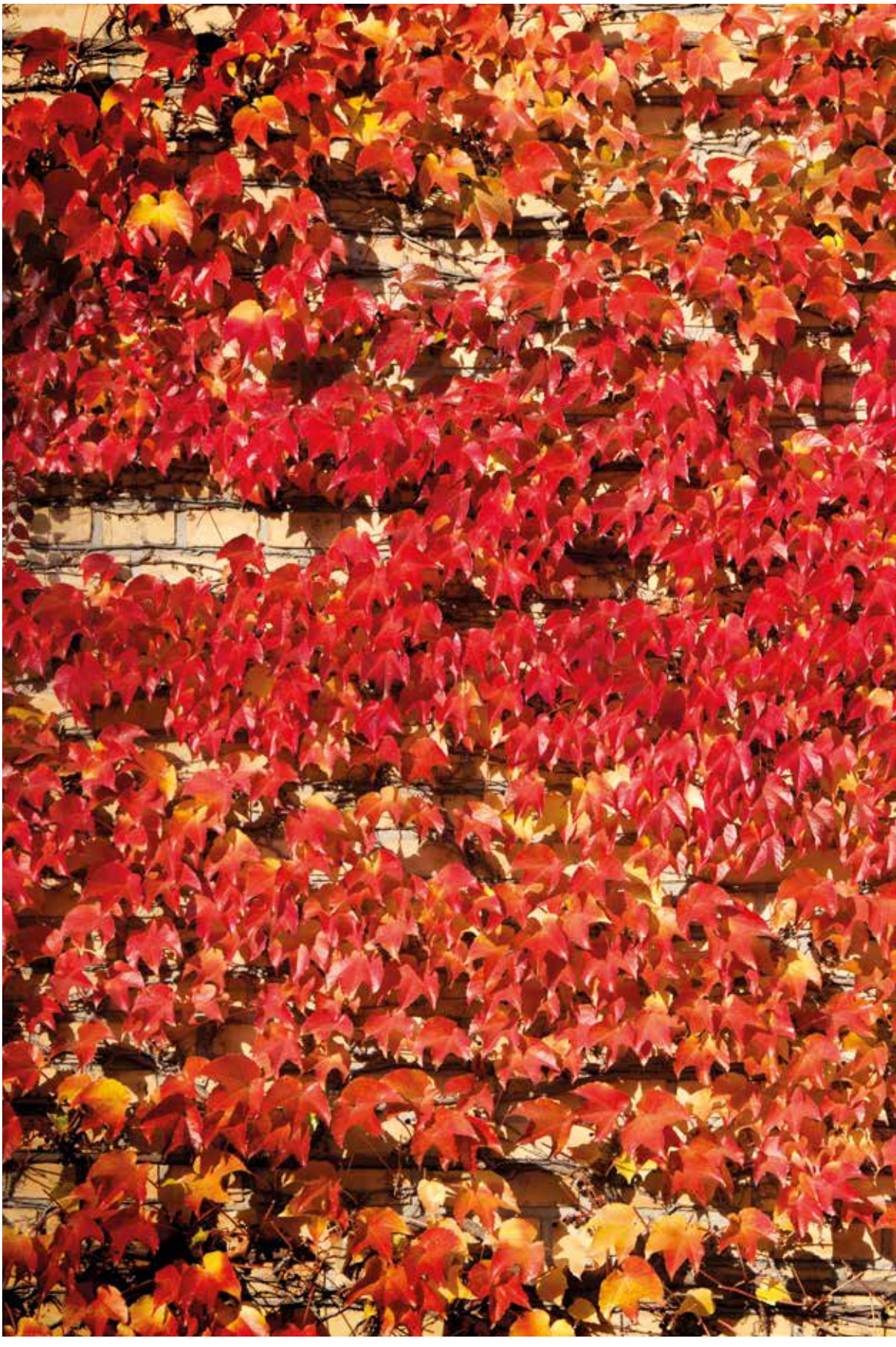
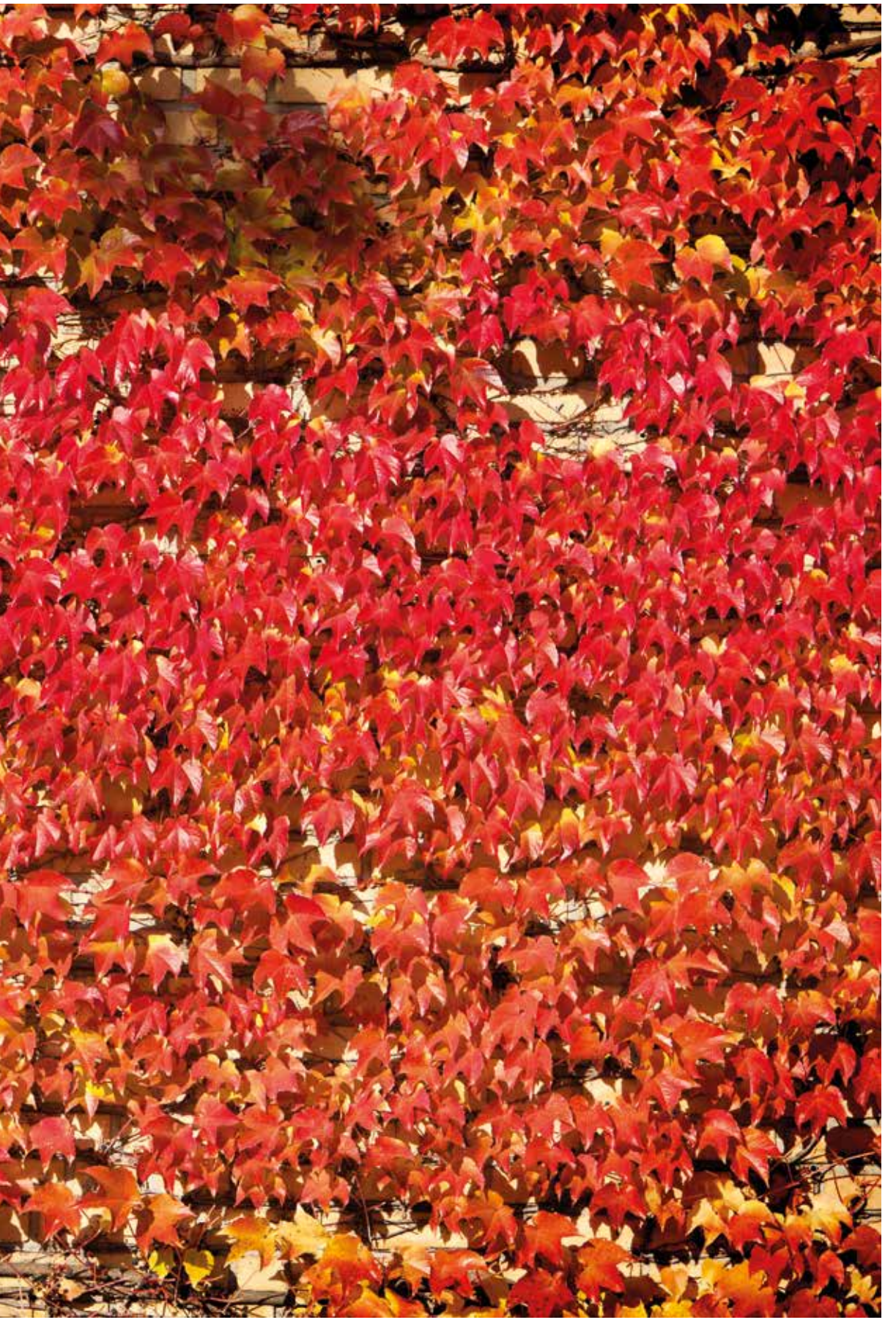


Fig. 2*. Body weight(A),non-fasting blood glucose(B), sensory(C) and motor(D) nerve conduction velocity (NCV) of 25 weeks old, C57 black male mice fed either control (n=8) or high fat diet (n=9;HFD) for 20 weeks. Data presented as mean +/- standard error. Data compared by one-way ANOVA, followed by a t-test where *p<0.05, ***p<0.0001.*





WP3: RISK FACTORS FOR DIABETIC NEUROPATHY

The ADDITION cohort and the DD2 cohort are the basis of this work package where we are studying:
1) the metabolic risk factors for diabetic neuropathy,
2) the effect of therapy on diabetic neuropathy and
3) the determinants for the clinical course of diabetic neuropathy and its prognosis.

The ADDITION Study (Anglo-Danish-Dutch Study of Intensive Treatment in People with Screen Detected Diabetes in Primary Care) is a study on early detection and intensive treatment of type 2 diabetes in primary care, where patients have been followed since their screen-detected diagnosis of type 2 diabetes 15 years ago.

The prospective Danish Center for Strategic Research in Type 2 Diabetes (DD2) cohort and biobank continuously enroll newly diagnosed type 2 diabetes patients throughout Denmark. The DD2 database was started in 2010 and currently holds approx. 10,000 individuals.

WP3: DIABETIC NEUROPATHY IN A COHORT WITH SCREEN-DETECTED TYPE 2 DIABETES: ADDITION-DENMARK

***Signe Toft Andersen** has continued in the IDNC as a part-time researcher and project manage after she successfully defend her PhD thesis "Diabetic neuropathy and type 2 diabetes" in December 2018 based on data from the ADDITION-Denmark study.*

Signe is involved in a number of ongoing studies based on data from the ADDITION Denmark and with the planning of a research project in general practice in collaboration with Steno Diabetes Center Aarhus. Some of these studies are listed below:

With medical student Laura Linnea Määta as first author *"A Prospective Study of Neuropathic Symptoms Preceding Clinically Diagnosed Diabetic Polyneuropathy: ADDITION-Denmark"*; is published online ahead-of-print in Diabetes Care. This study assess the course of nerve fiber specific symptoms during 13 years and their association with clinically diagnosed diabetic polyneuropathy (DPN) from the diagnosis of type 2 diabetes by screening. This study provides no support for the proposed hypothesis for the course of nerve fiber damage reflected by nerve fiber specific symptoms.

In collaboration with diabetes epidemiologists, Signe is working on the project *"Heterogeneity in neuropathy measures"*. This is a data-driven factor analysis using all available neuropathy measures in the ADDITION-Denmark study to identify clusters of neuropathy measures with the aim of identifying a more simple or a more accurate definition of diabetic neuropathy.

"Subclinical cardiovascular autonomic neuropathy associates with increased all-cause mortality and incident cardiovascular disease in screen-detected type 2 diabetes". Based on data from ADDITION-Denmark a follow-up period of seven years shows a hazard ratio of 2 for the risk of all-cause mortality and cardiovascular disease (CVD) events in participants with cardiovascular autonomic neuropathy (CAN) compared to those without CAN. This effect is above the effect of conventional CVD risk factors. A manuscript is under preparation (Fig.1.).

"Early indication of diabetic neuropathy is associated with higher risk of subsequent cardiovascular disease in screen-detected type 2 diabetes" is primarily carried out by MD, PhD Lasse B. Hansen. This study shows a higher risk of incident CVD (incidence rate ratio of 1.5 in the fully adjusted model) for those with DPN at the diagnosis of diabe-

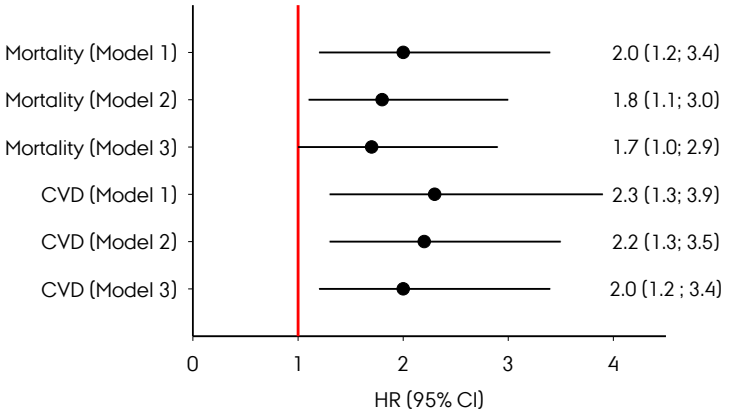
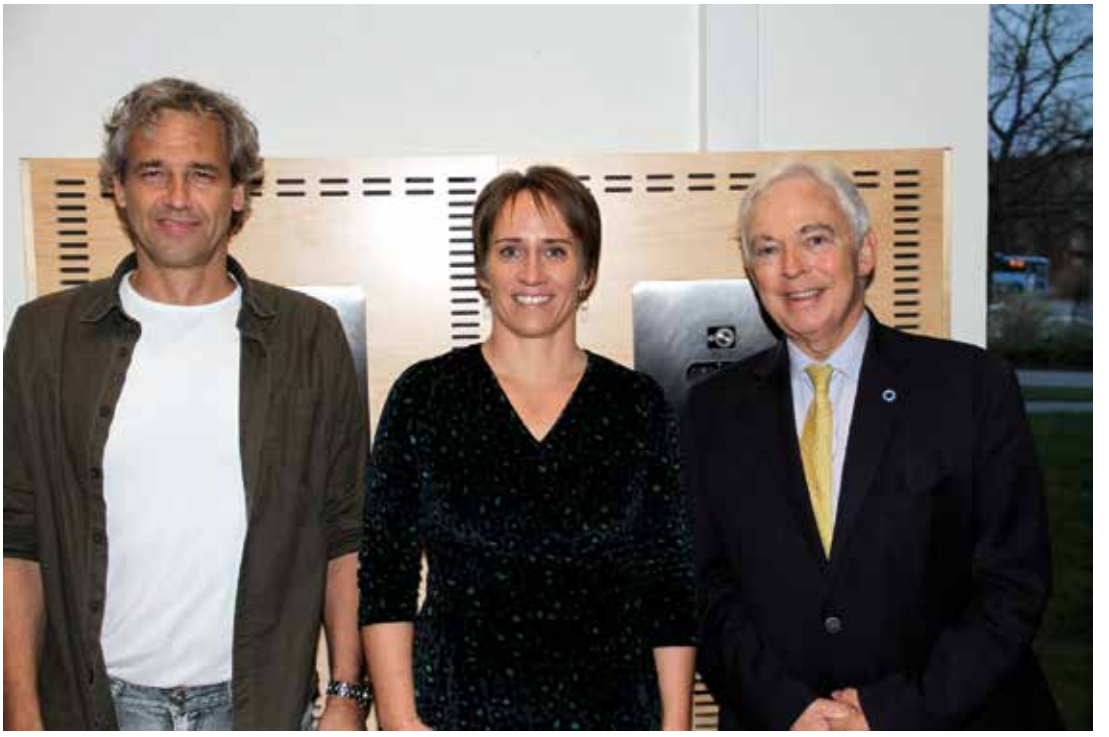


***Fig. 1**. Risk of mortality and CVD in presence of CAN. Risk is expressed by hazard ratios (95% CI) for all-cause mortality and time-to first CVD event during 7 years of follow-up by presence of CAN 6 years after a diagnosis of screen-detected type 2 diabetes: ADDITION-Denmark*
***Model 1**: Adjusted for sex, age, randomization-group*
***Model 2**: Adding adjustments for systolic blood pressure and LDL cholesterol*
***Model 3**: Adding adjustment for history of CVD*

tes as defined by the Michigan Neuropathy Screening Instrument questionnaire. These analyses will be extended to include data from the DD2.

"Screening for subclinical cardiovascular autonomic neuropathy in people with type 2 diabetes in the Central Denmark Region: to improve the care and the prognosis of people with type 2 diabetes". This project is under preparation in collaboration with researchers based in the Steno Diabetes Center Aarhus. Around 500 people with type 2 diabetes will be screened for cardiovascular autonomic neuropathy (CAN) in general practice with further clinical profiling focusing on continuous glucose measures and signs and markers of heart failure. The overarching aims are to optimize the detection and treatment of people with type 2 diabetes at high risk of cardiovascular disease and sudden death followed in general practice.



A happy PhD candidate after the defend of her thesis "Diabetic Neuropathy and type 2 diabetes" on December 3rd 2018 at Aarhus University, DK. Associate professor Peter Gæde, DK (to the left) and Professor Andrew J.M. Boulton, UK (to the right) assessed the thesis together with Professor Bjørn Richelsen, DK as chair

WP3: DD2 COHORT AND REGISTRIES

Diana Hedevang Christensen *is a PhD student at the Department of Clinical Epidemiology, Aarhus University Hospital (DK). Associate Professor Reimar W. Thomsen supervises the research.*

Diana Hedevang Christensen has provided a detailed cohort profile paper describing the DD2 cohort that forms the basis for both epidemiological and clinical studies in the IDNC.

In an ongoing study based on data from the IDNC-DD2 questionnaire survey on neuropathy (N = 6,276, total response rate 86%), Sandra Sif Gylfadottir and Diana found a prevalence of diabetic polyneuropathy (DPN) and painful DPN of 18% and 10%, respectively, among patients with early type 2 diabetes. Both DPN and painful DPN was associated with lower quality-of-life and more symptoms of depression, anxiety, and sleep disturbance. Interestingly, DPN itself had larger impact on mental health than neuropathic pain.

Adding detailed primary collected DD2 data and linked register-data to the IDNC-DD2 questionnaire data, has led to an ongoing broad investigation of the association between DPN and the metabolic profile at time of diabetes diagnosis (Fig. 1.). We found that DPN was related to modifiable risk factors including general and central obesity (Fig. 2.), low-grade inflammation, glycemic control, insulin resistance (c-peptide), dyslipidemia, and unhealthy lifestyle, i.e. tobacco smoking, low physical activity level, and high alcohol consumption. Neuropathic pain occurrence in DPN may share some (e.g. smoking) but not all of these risk factors.

During her PhD-project, Diana and colleagues have established a large nationwide register-based dataset on the Danish diabetes population from 1977 onwards including up to 5 controls for each diabetes patient. These data are now being used in ongoing pharmaco-epidemiological studies investigating the impact of lipid-lowering drugs and newer glucose-lowering drugs on DPN risk.

Furthermore, Diana and colleagues got permission to access nationwide longitudinal data on diabetic foot examinations from all Danish podiatrists, which will be linked to the DD2 cohort.

Currently, Diana finishes her PhD-dissertation, which she will hand in primo October 2019.

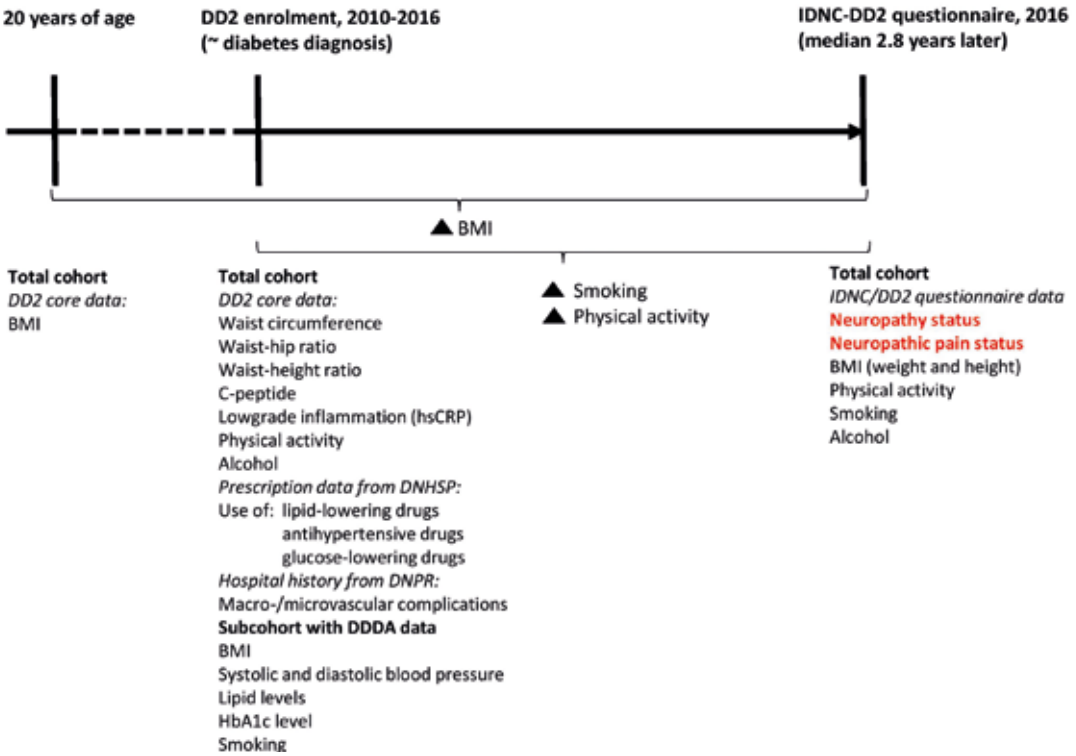


Fig. 1*. Timeline of assessment of obesity measures, other metabolic and lifestyle factors, and DPN-status in the study based on IDNC-DD2 questionnaire data, DD2 data, and linked register-data. The DNHSP is the Danish National Health Service Prescription Registry, the DNPR is the Danish National Patient Register, and the DDDA is the Danish Diabetes Database for Adults; the latter can be linked to ~70% of the DD2 cohort.*

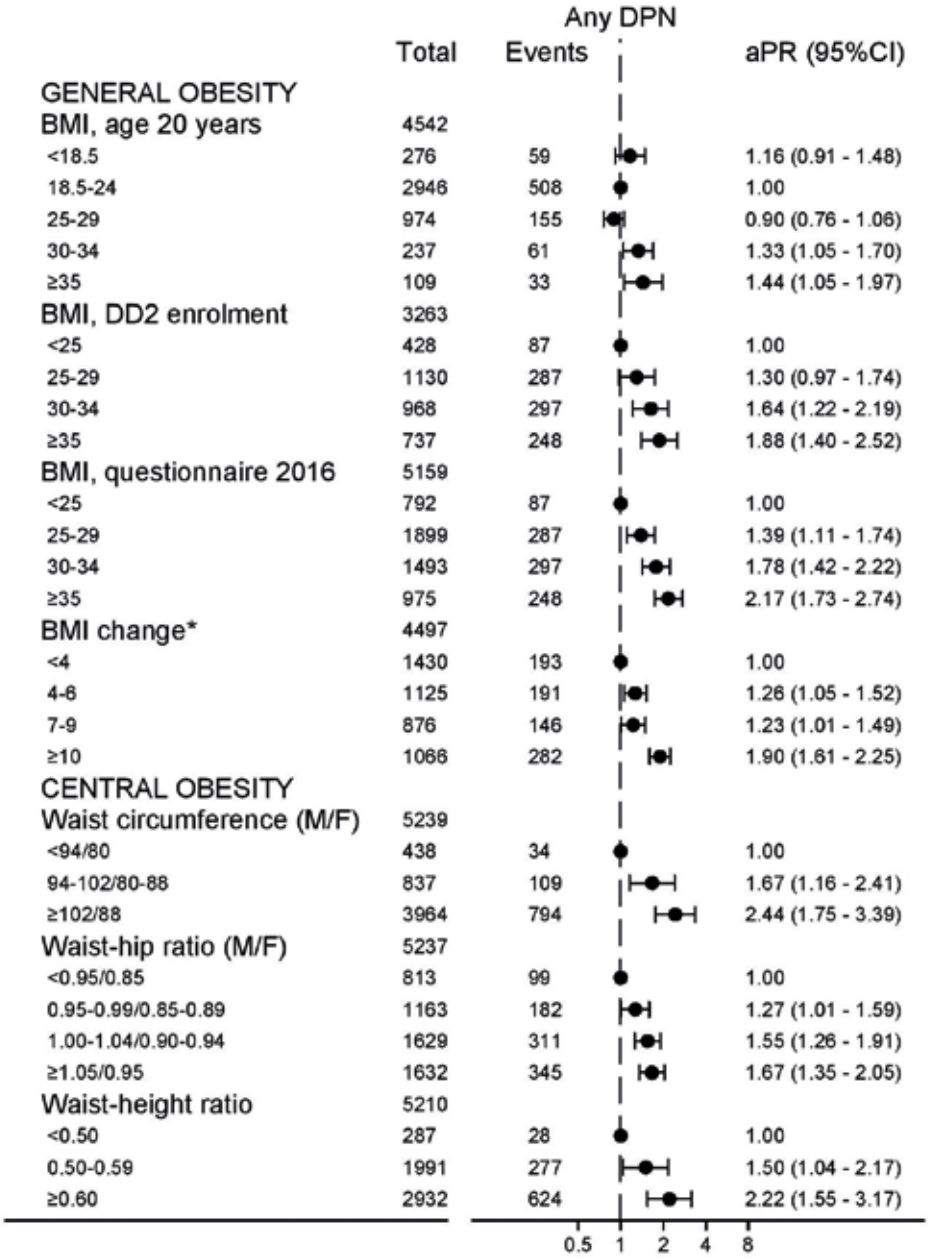


Fig. 2. *Prevalence ratios of DPN with general and central obesity.*

WP3: DD2 COHORT AND REGISTRIES

Frederik Pagh Kristensen *is a medical student and research year student at the Department of Clinical Epidemiology, Aarhus University Hospital (DK). Associate Professor Reimar W. Thomsen supervises the research.*

Frederik is working on the project; *Statin therapy and risk of polyneuropathy in type 2 diabetes: A nationwide cohort study.*

Dyslipidemia and central obesity are associated with increased risk of diabetic polyneuropathy (DPN), possibly through increased levels of reactive oxygen species and local nerve inflammation. The HMG-CoA reductase inhibitors (statins) are widely used to lower cholesterol levels in type 2 diabetes patients. Besides the cholesterol-lowering effect, statins exert pleiotropic effects including endothelial activation, anti-inflammatory, and anti-oxidative effects. Thus, statin therapy initiated before or at time of type 2 diabetes diagnosis may potentially reduce the risk of future DPN. This ongoing research year project investigates the effect of statin therapy on the risk of subsequent DPN.

Frederik used the nationwide population-based registries to conduct a cohort study of all incident type 2 diabetes patients in Denmark from January 2, 2002 – July 5, 2016 (n=282,292). Risk of developing DPN was assessed in new users- and prevalent users of statins compared with never user of statins. Patients were followed from 6 months after diagnosed type 2 diabetes. DPN was defined using an algorithm of G- and E-chapter ICD-10 diagnosis codes previously validated by the IDNC.

Preliminary results showed that over a mean follow-up of 6.7 years, neither new use nor prevalent use of statins were associated with increased DPN risk (incidence rate 4.0 vs 3.7 events per 1000 person-years, adjusted hazard ratio (aHR) 1.04 (95% CI, 0.98-1.11) and a HR 0.95 (95% CI, 0.89-1.01), respectively) (Fig. 1.).

Our study proposes that previous studies investigating the association of statin therapy and diabetic polyneuropathy have been falsely inflated by a mix-up of statin-exposure groups and by non-validated diagnosis codes. This study will, therefore, improve our understanding of the possible role of statin therapy in the development of DPN.

Further analysis will be initiated in fall 2019 with use of the clinical laboratory information system (LABKA) to investigate the impact of lipid levels before the diagnosis of type 2 diabetes and the association between statin treatmet and risk of DPN.

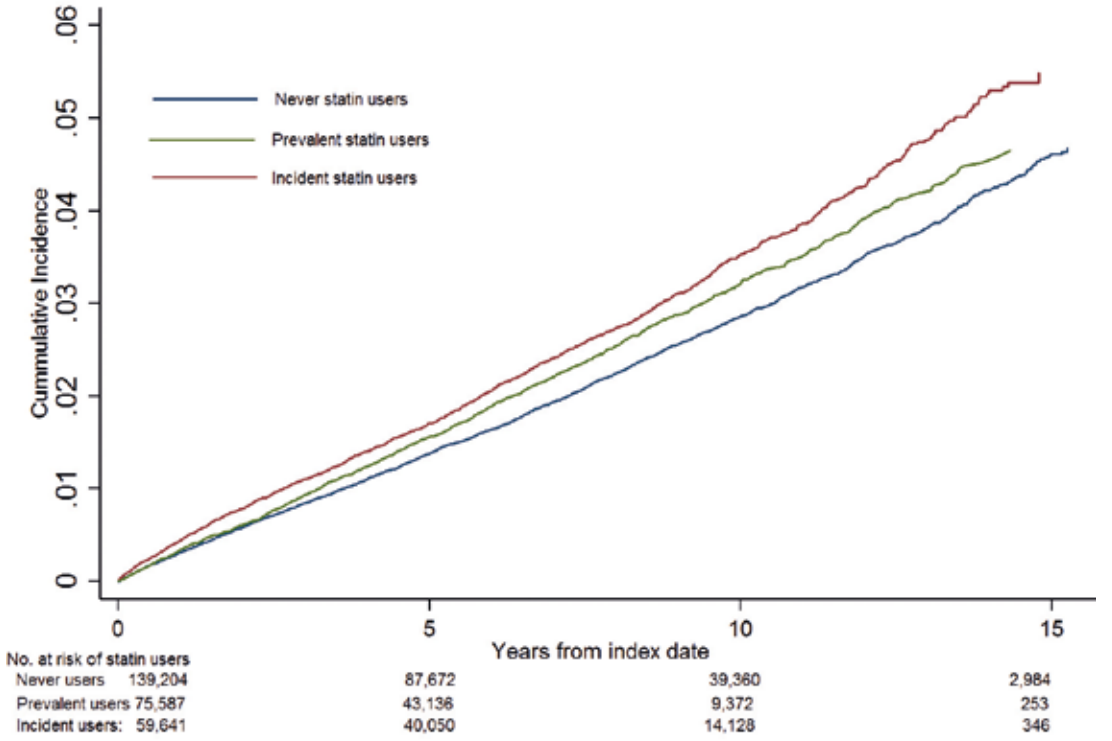


***Fig. 1**. Preliminary results: Crude cumulative incidence of DPN stratified according to statin use treating death as competing risk.*

WP4: CLINICAL PROFILING

In this large work package, we will determine the presence of pain and sensory abnormalities in type 2 diabetes. The hypothesis is that based on a neurological history, and in particular clinical examination and detailed sensory profiling, it will be possible to find distinguishing characteristics in patients with type 2 diabetes, diabetic neuropathy and painful diabetic neuropathy.

The clinical profiling involves work done at the University of Oxford (UK), University of Southern Denmark and Aarhus University (DK). Clinical profiling in Denmark is carried out on the basis of the DD2 cohort. The examinations and profiling carried out at these three study sites are similar to those done in the major multicenter project DOLORisk, which aims to understand risk factors and determinants for neuropathic pain (dolorisk.eu). DOLORisk is funded by the European Commission Horizon 2020-PHC-2014 and is coordinated by IDNC affiliated researcher Professor David Bennett, Oxford University with Professor Nanna Brix Finnerup, Aarhus University as deputy project coordinator.

WP4: SOMATOSENSORY PHENOTYPING, THRESHOLD TRACKING AND GENETICS IN DIABETIC NEUROPATHY

The Oxford group consist of Professor David LH Bennett, BSc Andreas Themistocleous and clinical research nurse Jishi John who aims to answer the following question;
"What are the drivers of neuropathic pain in diabetic neuropathy and can we better stratify those patients with neuropathic pain?"

The priority this year has been to investigate genetic risk as a determinant of neuropathic pain (NeuP) in the context of diabetic neuropathy. This is being studied in the context of a highly phenotyped cohort (which have undergone the NeupSIG grading system for neuropathic pain) of patients with diabetic neuropathy recruited in Oxford.

We have initially examined the relationship between variants in the voltage-gated sodium channel Nav1.7 and NeuP. The rationale for studying Nav1.7 is that this voltage gated sodium channel is expressed in nociceptors and amplifies subthreshold stimuli a property recently shown in our study of patients with loss of function mutations in Nav1.7 (McDermott et al., 2019). It is therefore a key determinant of nociceptor excitability and selective blockers of Nav1.7 are under active development as novel analgesics. Nav1.7 has been shown to be important in pathological pains states in humans.

Although no rare variants were found in participants with painless DPN, we identified twelve rare Nav1.7 variants in ten study participants with painful DPN. Five of these variants had previously been described in the context of other NeuP disorders and seven have not previously been linked to NeuP. Those patients with rare variants reported more severe pain and greater sensitivity to pressure stimuli on quantitative sensory testing.

In vitro electrophysiological characterisation of two of the novel variants demonstrated gain of function changes as a consequence of markedly impaired channel fast inactivation. Our observations suggest that rare Nav1.7 variants contribute to the development of NeuP in patients with DPN. We are now extending these observations in a number of ways.

Firstly we are now extending the examination of rare variants to other sodium channels such as Nav1.8 and Nav1.9. This may be relevant to future treatment opportunities given that a number of non-selective sodium channel blockers are available and that more selective sodium channel blockers are being developed.

We are also extending this genetic approach as a collaboration between the DOLORisk consortium (See Pascal et al., 2019) and IDNC.

The IDNC has undertaken questionnaire based sensory phenotyping of the DD2 cohort and 800 of these subjects have evidence of diabetic neuropathy on the basis of the Michigan Neuropathy Screenig Instrument and neuropathic pain status is being determined by the DN4 questionnaire. These will be combined with 2000 highly phenotyped diabetic neuropathy patients as part of DOLORisk. A genome wide association study will examine common variants which act as risk factors for neuropathic pain and exome sequencing will be used to investigate rare variants.

As future plans we are also continuing our work on using electro-physiological tools to discriminate between patients with painful and painless DPN. Threshold tracking is a neurophysiological tool that assesses large nerve fibre axonal excitability. It is an indirect measure of the ion channel excitability within myelinated nerve fibres. We have recorded from 151 participants in Oxford. Preliminary data analysis of four outcomes does not show differences between participants with painful and painless DPN. We will continue to explore the data to determine whether symptoms or other clinical variables are related to our four key outcomes. We also hope to combine this data with that collected in Aarhus which will generate a very large cohort in which to examine the relationship of nerve excitability variables to sensory phenotype.

WP4: CLINICAL PROFILING OF DIABETIC NEUROPATHY

Mustapha Itani *is a neurologist at the department of neurology at Odense University Hospital (OUH) and a PhD student at the University of Southern Denmark (SDU). Professor Søren Sindrup leads the research*

Mustapha has 3 distinct projects based on the detailed clinical profiling of a sample of type 2 diabetes patients from the national DD2 cohort:

1) In the first project he is in collaboration with Sandra Sif Gylfadottir, looking at the prevalence of diabetic polyneuropathy (DPN) and risk factors of DPN. Furthermore, he and Sandra will be validating 2 well known neuropathy scores, the Michigan Neuropathy Screening Instrument (MNSI) and the Dolour Neuropatique en 4 Question (DN4). These scores have not yet been validated in a population of people with type 2 diabetes.

2) In the second project, he will determine the pattern of DPN, subdividing DPN into its 3 main groups: Large fiber neuropathy (LFN), small fiber neuropathy (SFN) and mixed fiber neuropathy (MFN). The primary purposes of this subdivision are to examine whether there exist neuropathy subgroup specific risk factors and to determine if SFN is a precursor of DPN or rather a separate entity (Fig. 1.) These aspects are lacking in the literature.

3) In the third and last project, he will compare the pattern of polyneuropathy in type 2 diabetes with the pattern of polyneuropathy seen in idiopathic polyneuropathy based on a local database of idiopathic polyneuropathy patients at the department of neurology at Odense University Hospital.

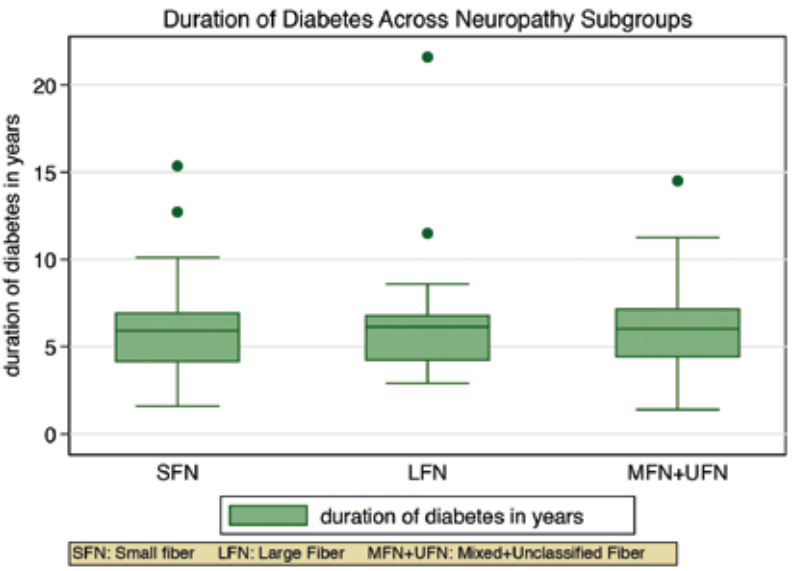


Fig. 1*. Duration of diabetes across neuropathy sub-groups*

WP4: CLINICAL PROFILING OF PAINFUL DIABETIC NEUROPATHY

Sandra Sif Gylfadottir
is a PhD student. Her main supervisor is professor Nanna Brix Finnerup, Danish Pain Research Center, Aarhus University (DK)

The main focus of this PhD study is to estimate the prevalence of painful diabetic polyneuropathy (DPN) and to compare patients with and without pain. We wish to identify abnormalities specific to pain and to examine morphological chances in intraepidermal nerve fibers and differentiate between patients with and without pain. I will present some preliminary results.

We invited participants from the nationwide Danish Centre for Strategic Research in type 2 Diabetes (DD2) cohort to participate in a clinical examination at two centers in Denmark, i.e. Aarhus and Odense based on responses from a questionnaire study conducted in 2016, where patients answered questions on neuropathy and pain. The inclusion of patients lasted from October 2016 to October 2018. The examination consisted of a bedside neurological examination and history taking, nerve conduction studies, skin biopsy, quantitative sensory testing, confocal corneal microscopy and blood samples. We included 389 diabetes patients (Fig. 1.).

Of a sub-cohort of 330 patients (no DPN (63), possible DPN (50), probable DPN (88) and definite DPN (126)), 41.5% were females and median (IQR) age was 66.6 (58.6; 71.8) years. Median BMI was 31.2 (27.5; 35.6) and the patients had suffered from diabetes for 5.9 (4.2; 7.1) years. Of the 126 patients with definite DPN, 73 had DPN without pain and 53 painful DPN. We found that a subgroup of patients with non-painful DPN had unpleasant abnormal sensation (dysesthesia) in their feet.

The bedside sensory examination revealed a gradient of increased spatial distribution (distal to proximal) of sensory loss from non-painful DPN to dysesthetic DPN to painful DPN for light brush stroking, pinprick and cold and warm sensations (Fig. 2.).

In conclusion, patients with painful DPN presented with more somatosensory loss than patients with painless DPN. The intermediate group of patients with dysesthesia could represent a milder form of painful DPN and is important to consider in the clinic and in studies comparing patients with and without pain.

Hopefully, the identification of specific sensory profiles of painful DPN will provide further information about the underlying pathophysiology of DPN and eventually lead to a better diagnostic approach and treatment strategy for this condition.

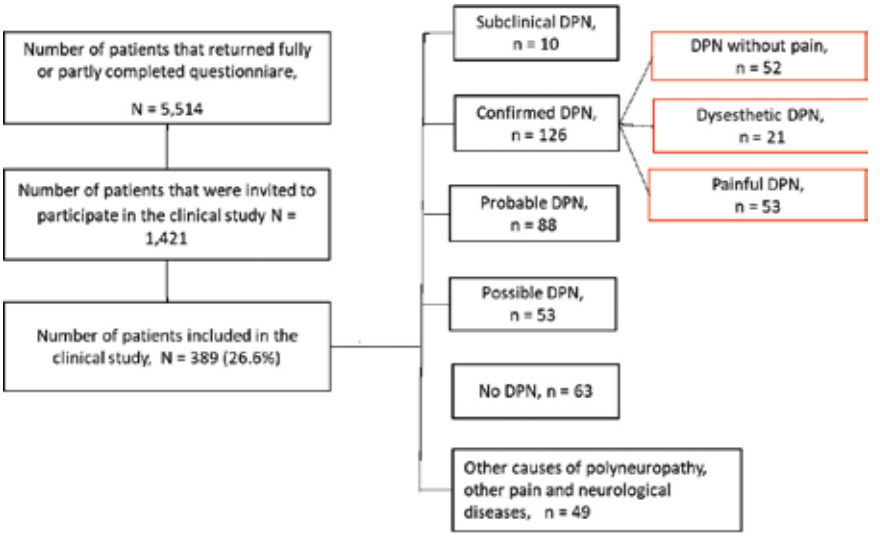


***Fig. 1**. Flow diagram of patient inclusion and diagnosis of diabetic polyneuropathy according to the Toronto criteria (Tesfaye et al, 2010) and the neuPSIG definition of neuropathic pain (Finnerup et al, 2016).*

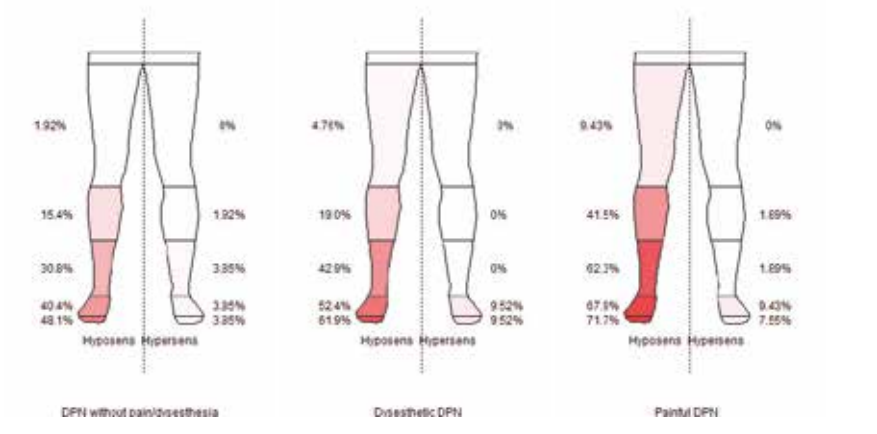


***Fig. 2**. There was an increased spatial dissemination of sensory loss (displayed in percentages from toes to thighs) from DPN without pain and dysesthesia to dysesthetic DPN and to painful DPN. (Bedside examination of pinprick with Semmes-Weinstein monofilament no 5.88).*

WP4: MOTOR DYSFUNCTION IN DIABETIC NEUROPATHY

Karolina Snopek *carries out the PhD project entitled: Falls, motor dysfunction and the effects of resistance training in diabetic polyneuropathy and is supervised by Professor Henning Andersen, Department of Neurology, Aarhus University Hospital (DK).*

Motor dysfunction is a debilitating complication of diabetic polyneuropathy (DPN) that can impair postural stability and increase the risk of falling. The underlying causation of falling and the relation between motor dysfunction and postural instability in DPN remain ambiguous. Physical exercise has shown benefits in preventing and delaying diabetes disease progression. The role of exercise in DPN has however been sparsely studied and the effect of resistance training remains to be explored.

In a cross-sectional nationwide questionnaire study, we examined the frequency of self-reported falls in relation to symptoms of DPN in patients with newly diagnosed type 2 diabetes (Fig.1.). We found that falls were 2.32 times more prevalent when DPN was present, after adjusting for possible confounders (prevalence ratio (PR): 2.32 (2.06-2.63)). Patients with DPN did not have a higher prevalence of fractures when compared to those without DPN (PR:1.26(0.64-2.47)).

In the second part of this study, 92 type 2 diabetes patients and 39 controls were evaluated to describe the characteristics of diabetic fallers with the association of motor dysfunction, postural instability and DPN. Falls were more common in diabetes patients (36% (n= 33)) when compared to healthy controls (15%, n=6), this finding was irrespective of DPN. Fallers had a slower six-minute walk test (6MWT) (86±29%), five-time sit to stand test (FTSST) (120±42%) and a higher instability index (ST) (131±74%) when compared to non-fallers (p<0.02 for all).

Lastly, we examined the effects of a 12-week high-intensity resistance training (HIRT) protocol in a randomized single blinded controlled trial in type 2 diabetes patients with and without DPN.

A total of 90 participants completed the study with 30 participants in each of the three groups (DPN versus non-DPN and healthy controls). HIRT resulted in gains of strength of the knee extensors/flexors in all three groups (13%±4.5 in the DPN group, 7% ±2.2 in the non-DPN group and 6%±0.8 in the control group (p<0.05), whereas no increase in strength was observed at the ankle. The 6MWT, FTSST and Toronto Clinical Neuropathy Score (TCNS) only improved in the DPN group (p<0.02). Combining all diabetes patients the gain in muscle strength correlated to the improvement in the function tests (6MWT (r=0.53, p=0.0001), FTSST (r=0.34, P=0.001)).

These findings provide evidence that patients with DPN have a higher prevalence of falls. Furthermore, the study shows that fallers with diabetes have a higher degree of motor dysfunction. In patients with DPN, HIRT resulted in improved motor function and neuropathy scores. This suggests that HIRT may prevent falls, however, this needs to be studied in prospective large-scaled controlled studies.

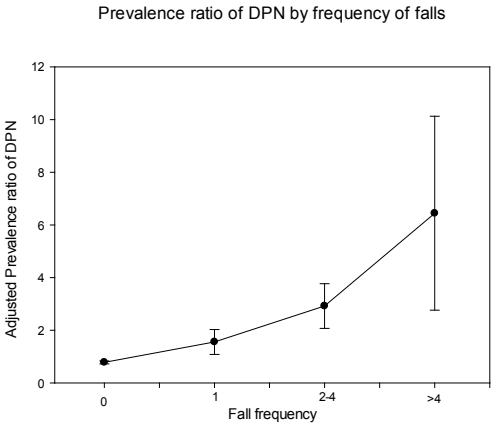


Fig. 1*. Adjusted prevalence ratio (PR) of DPN by the frequency of falls within 1 year prior to the questionnaire response. The PR was adjusted for BMI, smoking, alcohol, gender, age, diabetes duration, Charlson comorbidity index and eye disease.*

WP4: CLINICAL NEUROPHYSIOLOGICAL MEASURES IN DIABETIC NEUROPATHY

Alexander Gramm Kristensen *is a PhD student at Danish Pain Research Center, Clinical Institute, Aarhus University and Department of Clinical Neurophysiology, Aarhus University Hospital. Associate Professor Hatice Tankisi, Department of Neurophysiology, Aarhus University Hospital (DK) supervises the research.*

Alexander attempts to obtain the optimal strategy for diagnosing diabetic neuropathy using neurophysiology and to examine the mechanisms behind diabetic neuropathy.

Alexander has finished inclusion into his studies after which he spent a month in Japan with professor Kuwabara. There he received valuable input for the interpretation of his nerve excitability data, which was presented at the 17th European Congress for Clinical Neurophysiology in Warsaw. Since then, Alexander has been focused on completing his dissertation while also finishing the papers for publication. We are happy to report, that the first article "Detection of early motor involvement in diabetic polyneuropathy using a novel MUNE method – MScanFit MUNE" has been published (Kristensen et al, Clin Neurophysiol2019. doi.org/10.1016/j.clinph.2019.08.003).

The next paper on nerve excitability in type 2 diabetics is soon to be finished. The results of this study are summarized in Fig. 1. and 2.

From these figures, it is evident that both motor and sensory nerve excitability did not differ much in diabetics with increasing likelihood of neuropathy. Compared to previous studies, the patients in this study had a lower duration of disease, but some of the parameters that differed significantly between groups matched prior excitability studies. The significance of this study is, that while there are changes in excitability with increasing probability of diabetic neuropathy, they are overshadowed by changes in nerve conduction study parameters that better correlate to clinical measures.

The final study of Alexanders PhD will be based on results of motor unit number estimation and muscle excitability in the lower limb. Preliminary results of this study can be seen in Fig. 3.

This study ties in with the first study on motor unit number in the upper extremity, and shows that these findings also translate to the lower limb where diabetic neuropathy symptoms are more prevalent in early stages of this disease.

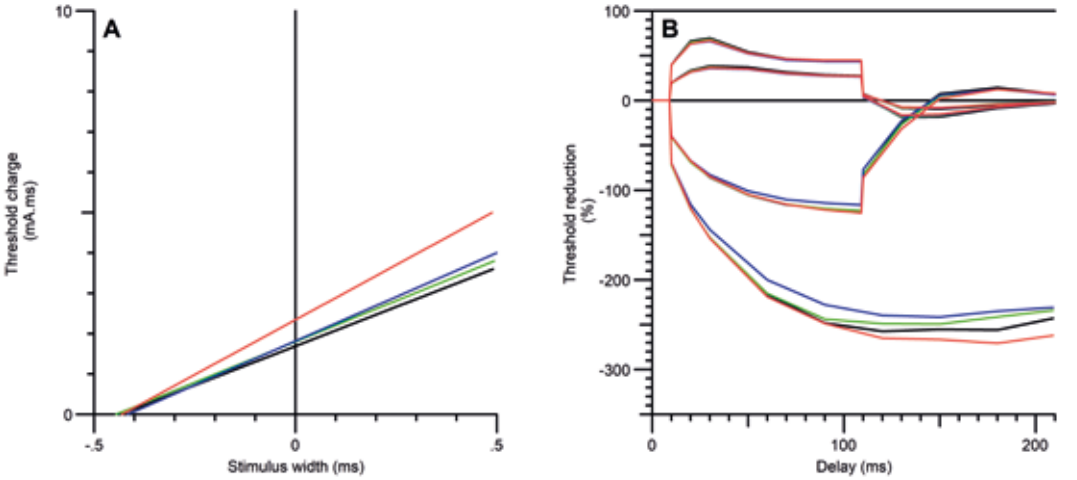


***Fig. 1**. Motor nerve excitability results from the median nerve. A: Strength duration relationship. B: Threshold Electrotonus. Black – Healthy controls, Green – Diabetic patients without neuropathy, Blue – Diabetic patients with possible and probable neuropathy, Red – Diabetic patients with nerve conduction study confirmed neuropathy.*

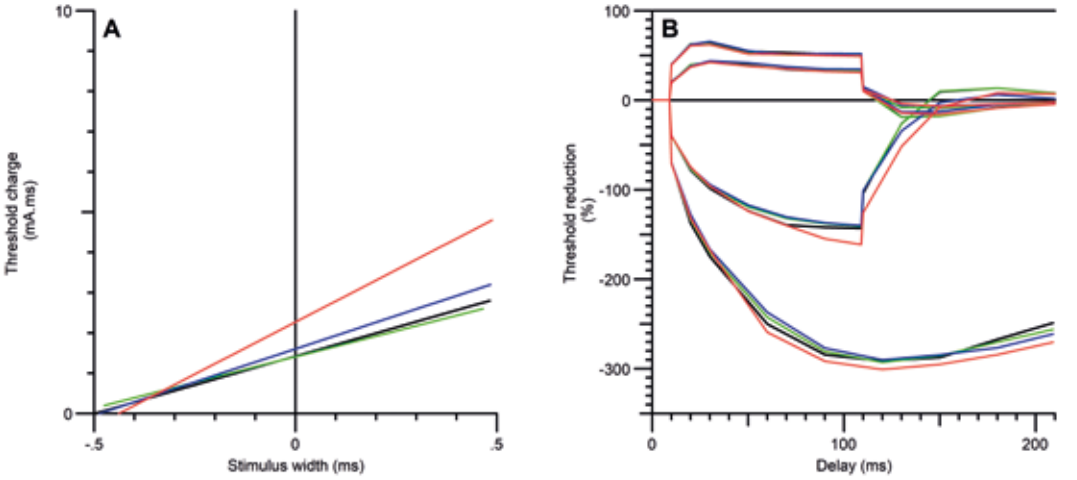


***Fig. 2**. Sensory nerve excitability results from the median nerve. **A**: Strength duration relationship. **B**: Threshold Electrotonus. Black – Healthy controls, Green – Diabetic patients without neuropathy, Blue – Diabetic patients with possible and probable neuropathy, Red – Diabetic patients with nerve conduction study confirmed neuropathy.*

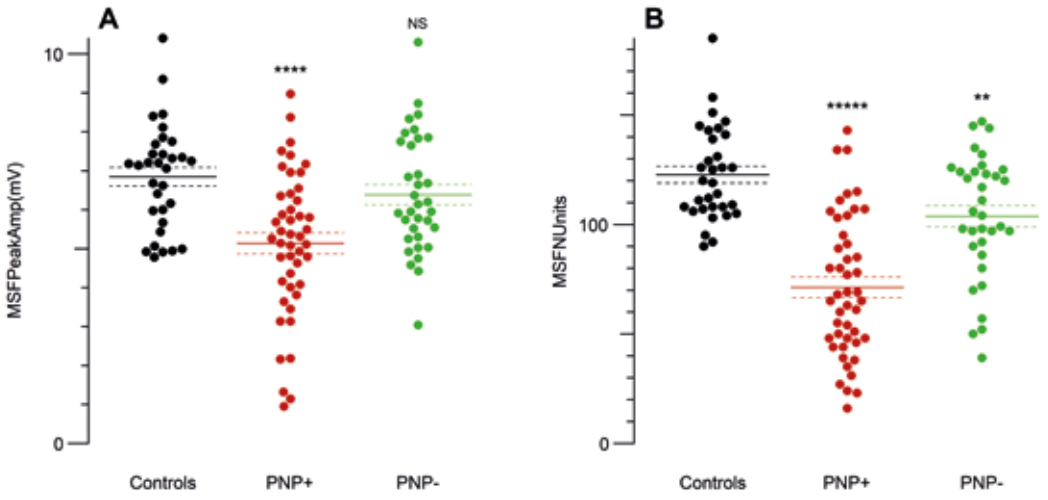


***Fig. 3**. Preliminary results from MScanFit MUNE of the anterior tibial muscle. HC: Healthy Controls, PNP+: Diabetic patients with nerve conduction study defined neuropathy, PNP-: Diabetic patients without neuropathy. A: Peak amplitude of each group. B: Estimated number of motor units for each group. Solid lines denote the mean, dashed lines are standard error.*

WP4: AUTONOMIC NEUROPATHY IN DIABETES

Thorsten Kamlarczyk Rasmussen *is a PhD student with Associate professor Astrid Juhl Terkelsen, Department of Neurology, Aarhus University Hospital being main supervisor of the project*

Thorsten carries out a project entitled; *Small fiber neuropathy: Clinical and physiological characteristics with focus on adrenergic dysfunction.*

Small nerve fibers of Aδ and C type are essential for the perception of heat, cold and pain and the transmission of autonomic regulation of heart and endo- and exocrine gland functions such as sweat and smooth muscles. These autonomic small fibers ensure homeostasis through strict regulation of cardiovascular system, renal, bowel, bladder and sexual functions. Following injury to the autonomic system nerves, such as in diabetes, severe changes may occur.

Small nerve fiber damage resulting in neurogenic autonomic dysfunction and small fiber polyneuropathy is common but overseen in diabetes and associated with reduced quality of life, increased morbidity and sudden death. Patients with autonomic involvement may experience symptoms such as orthostatic hypotension or syncope, enteric dysfunction, sexual impotence, anhidrosis and urinary retention or incontinence.

This project is a two-part study with a joint focus on neurogenic adrenergic autonomic dysfunction, primarily in type 2 diabetics.

Study 1 will assess the neuropathic impact on the adrenergic autonomic nerves in type 2 diabetics through assessment of a validated autonomic nervous system test-battery comprised of tilt table testing, Valsalva maneuver (Fig. 1.) deep respiration (Fig. 2.), and the cardiovascular autonomic reflex test battery. These patients will furthermore be examined through 24-hour blood pressure profiling and 123-MIBG scintigraphy to estimate the association between these different clinical adrenergic markers.

In study 2 we aim to combine the utilization of clinical autonomic testing and skin biopsy findings for the evaluation of neuropathy in diabetics and patients with known small fiber neuropathy. We aim to quantify the innervation of cutaneous autonomic elements (sweat glands, microvascular vasomotor nerve fibers, and arrector pili) in patients with known small fiber neuropathy, compared to healthy controls. We furthermore aim to estimate the association between stereologically quantified autonomic skin biopsy markers, and their clinically measured functions. More specifically, we want to assess the association between biopsy quantified adrenergic microvascular blood vessel innervation and the microvascular blood flow rate dynamics, and the association between sweat gland innervation, and the clinical sudomotor function assessed by quantitative sudomotor axon reflex testing and skin conductance dynamics.

Collection of data in both studies are currently ongoing. We expect the tools and methods investigated in these studies will further promote and encourage the utilization of objectively quantifiable autonomic markers in the diagnostic assessment of diabetic neuropathy and small fiber neuropathy.

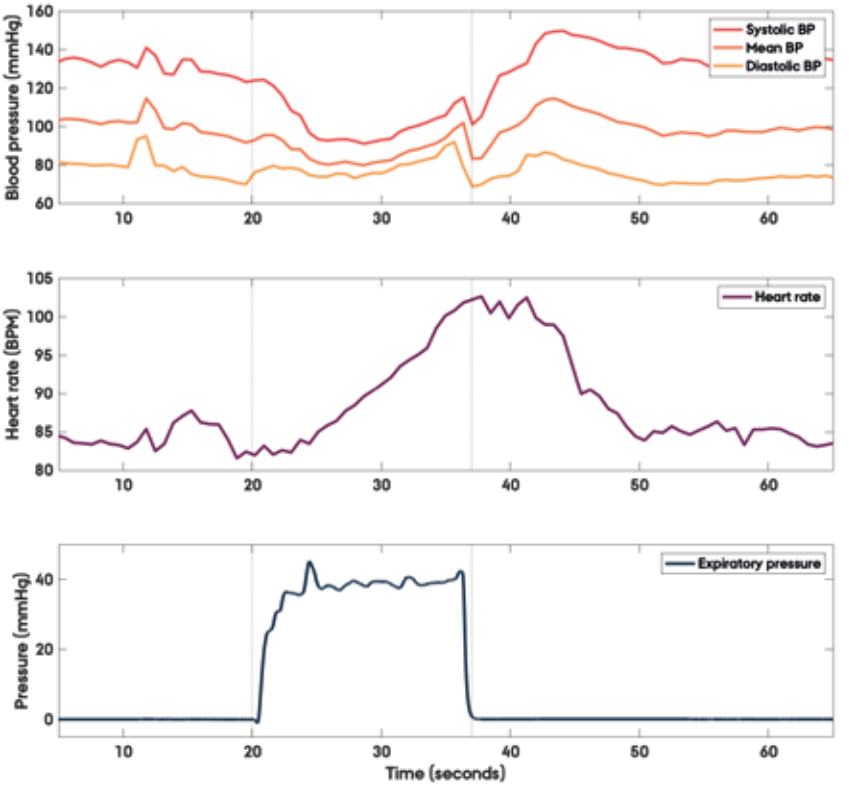


***Fig. 1**. Blood pressure, heart rate and expiratory pressure during the Valsalva maneuver. The presence of a rising blood pressure during expiration indicates a normal cardiovascular adrenergic function. The heart rate response with a significant Valsalva ratio indicates a normal cardiovagal and cardiovascular adrenergic function.*

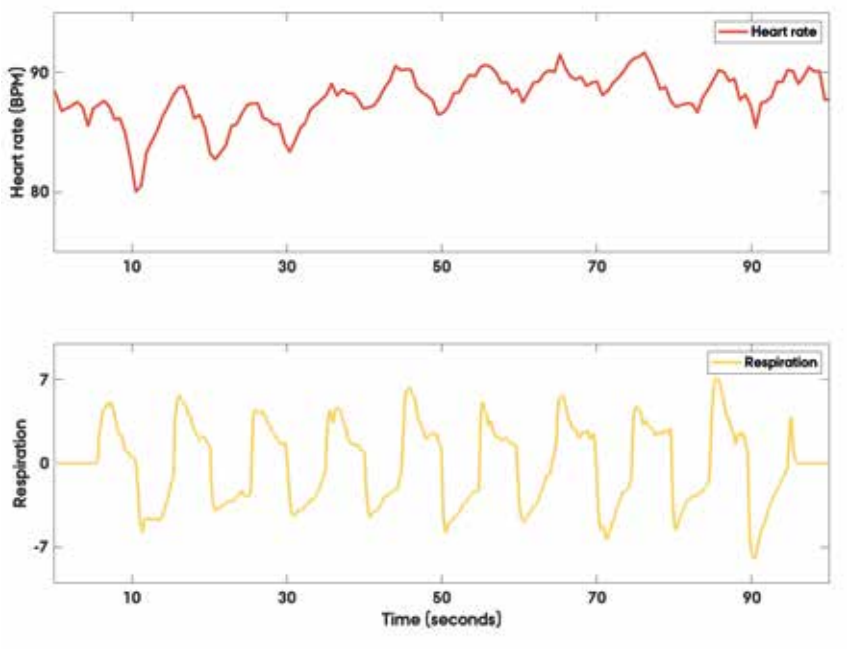


***Fig. 2**. Heart rate variability to deep respiration. The significant variation in heart rate indicates a preserved cardiovagal activity.*

WP4: DIABETIC NEUROPATHY; PATIENT PERSPECTIVES

Signe Vogel
Anthropologist Signe Vogel works on a project at the Danish Pain Research Center, Aarhus University (DK) to examine how life with diabetic neuropathy is experienced by patients

Signe is studying the effects of diabetic polyneuropathy without pain (DPN) and with pain (DPNP) in patients from the WP4 studies.

Pain and other sensory disturbances correlated with DPN and DPNP can be approached as neurological findings but these sensations can also be studied as experiences in a patient's life. A qualitative approach can explore and examine important aspects of DPN and DPNP which are not accessible through quantitative research. Accordingly, the main aim of this study is to get a better understanding of the impact DPN and DPNP can have on the patient's lifeworld.

We invited participants from the WP4 studies to participate in an exploratory interview at the Danish Pain Research Center, Aarhus University (DK). The inclusion started in December 2018 and ended May 2019. During this time-period 27 patients were interviewed. Ten were diagnosed with diabetes, seven were diagnosed with DPN, and ten were diagnosed with DPNP.

Findings demonstrate that the burden of illness for DPN and DPNP is considerable and that it can have an impact on both physical, psychological, and social health-related quality of life. The areas of impact include fundamental aspects of everyday life such as work, domestic work, exercising, leisure activities, mood, social relationships, and identity construction. Furthermore, clinical encounters related to DNP and DPNP symptoms are often experienced as contributing to the burden of illness instead of alleviating it.

In conclusion, these findings demonstrate that it is important to understand the full impact DPN and DPNP can have on life, not just the physical sensations and limitations but also the psychological and social consequences. It is pivotal that clinicians and researchers direct their attention to the highly complex, interactive, relationship between context and experience of symptoms when trying to measure, assess, and compare DPN and DPNP symptoms and their impact on quality of life.

WP4: DETAILED SKIN INNERVATION ANALYSIS IN PAINLESS AND PAINFUL DIABETIC POLYNEUROPATHY

Pall Karlsson, *Assistant Professor Pall Karlsson, Danish Pain Research Center and Core Center for Molecular Morphology, Section for Stereology and Microscopy, Department of Clinical Medicine, Aarhus University (DK) has active collaboration with leading experts on skin biopsies, e.g. Guiseppe Lauria in Milan (Italy), Michael Polydefkis in Baltimore (US), and more recently with Maria Nolano in Naples (Italy).*

Trigeminal nociceptive function and oral somatosensory functional and structural assessment in patients with DPN

Early 2019 we published in Scientific Reports the findings of a study where we aimed to compare the trigeminal nociceptive function, the intraoral somatosensory profile and structural nerve changes between DPN patients and healthy participants. We did so by taking skin biopsies from the distal leg and at the mucosa side of the cheek, performing nociceptive blink reflex (nBR) at the cheek and quantitative sensory testing (QST) at the distal leg and cheek. The nBR is an electrophysiological test that can be used to evaluate the trigeminal nociceptive function using surface electrodes. The nBR and QST is standard battery of psychophysical tests that provides a comprehensive phenotyping of the somatosensory function.

This work was important to undertake as no systematic combined investigation of possible orofacial neurophysiological, somatosensory and structural consequences of DPN has been reported so far.

The main findings were that DPN patients presented trigeminal hyperexcitability in combination with decreased nerve fiber length density from skin biopsies, and that loss of intraoral somatosensory function occurred more often in DPN patients compared to healthy participants.

We therefore concluded that early signs of trigeminal nociceptive facilitation, intraoral somatosensory abnormalities and loss of intraoral neuronal tissue could be detected in DPN patients. Indeed, the orofacial somatosensory and neurophysiological consequences of DPN appear to present heterogeneous characteristics, considering that not only signs of enhanced trigeminal nociceptive function, but also loss of intraoral nerve fibre length density can be identified along with minor somatosensory alterations (Fig. 1.)

Axonal swellings are frequently present in skin biopsies from patients with diabetes

It is well known that patients with diabetes and especially those with DPN have decreased nerve fiber density from skin biopsies – a validated and sensitive diagnostic tool for the assessment of small fibre neuropathies and DPN. It is, however, unclear if the nerve fiber density differentiates between patients with painless or painful DPN. Another structural difference sometimes seen on the remaining nerve fibers are axonal swellings. The question is if axonal swellings are related to neuropathy- or pain status in patients with diabetes or with other clinical variables often associated with diabetes. We therefore recently finished a study that raised the question if axonal swellings are related to neuropathy, or, HbA1C, BMI, diabetes duration, age, and gender in well-defined diabetic patients that have undergone detailed and rigorous DPN assessment.

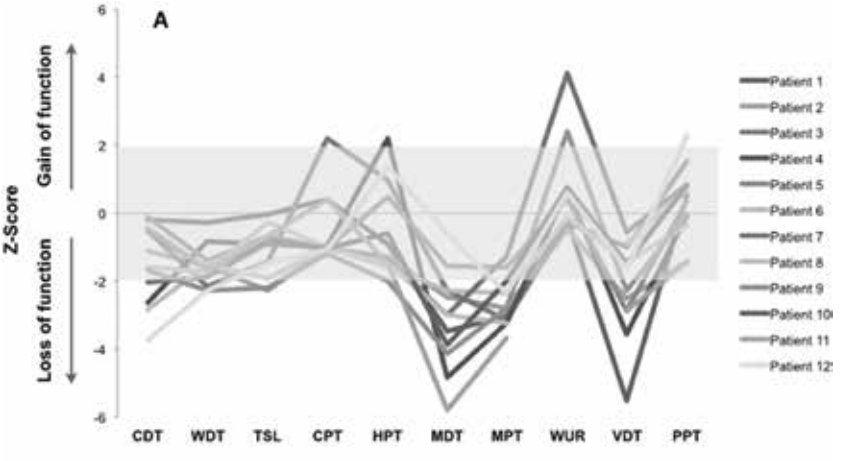


***Fig. 1**. Somatosensory profiles from the buccal mucosa of diabetic peripheral neuropathy patients. The grey zone represents normal range level of the reference group while a Z-score above the zone indicates a gain in somatosensory function and a score below loss of somatosensory function. The figure is modified from Costa et al., 2019 (www.ncbi.nlm.nih.gov/pmc/articles/PMC6336810/).*

Our key findings were, contrary to our initial expectation, that diabetic participants with nerve fiber density ≥1.0 fibres/mm and regardless of DPN presence, had increased swelling ratio compared with non-diabetic controls, and that there was no difference in swelling ratios between diabetic patients with painless DPN and those with painful DPN (Fig. 2.). Finally, there was no statistically significant correlation between axonal swellings and the clinical variables assessed in this study. These findings indicate that diabetic participants with some nerve fibers remaining have higher axonal swelling ratio compared with non-diabetics and that axonal swellings are not directly related to DPN. These findings are important as they indicate that axonal swellings are associated with diabetes and not specifically DPN. A manuscript based on these findings is under preparation.

New study: Novel risk markers and potential treatment targets for neuropathic pain in type 1 diabetes

Researchers from the IDNC recently received a DKK 4.378.100 grant from the Novo Nordisk Foundation to identify biomarkers of neuropathic pain and autonomic dysfunction in diabetes patients. This three-year-long, national multicentre collaborative project will be conducted at three Steno Diabetes Centres in Denmark (in Aarhus, Odense and Copenhagen) in collaboration with research-groups from Heidelberg University and University of Naples.

There is an urgent need to develop methods to understand why and how some patients develop neuropathic pain and to widen the scope of potential targets for more efficient

treatment. Detailed skin biopsy analysis is important for a better subgrouping of neuropathic pain patients and elucidate the different mechanisms underlying pain and autonomic dysfunction, both of which are important steps towards the development of treatment strategies.

This project will identify biomarkers of neuropathic pain and autonomic dysfunction, thereby identifying novel treatment targets by using a large panel of validated antibodies on skin biopsies in well-characterized cohorts of type 1 diabetes patients with DPN with and without pain, and relate the changes to painful symptoms and autonomic dysfunction.

This project is important because the results are likely to:

i. Increase our knowledge of why some, but not all, neuropathic patients develop neuropathic pain

ii. Relate autonomic dysfunction to autonomic structural changes

iii. Enable us to improve classification of patients based on the morphological changes seen in the skin, and

iv. Ultimately, aid in the development of novel treatment strategies for neuropathic pain.

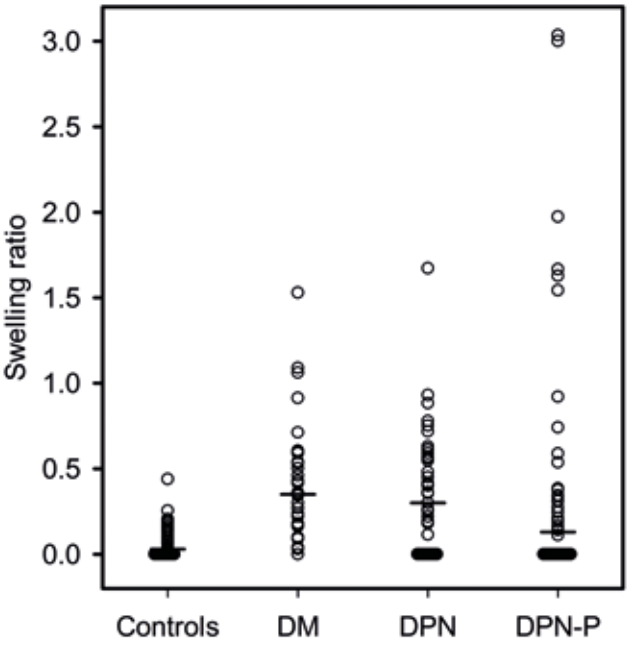


***Fig. 2**. Swelling ratio for participants with IENFD ≥1.0 fibres/mm*

WP5: METABOLOMICS AND LIPIDOMICS

In this work package, the focus is on oxidative stress pathways and lipid metabolites for the identification and quantitation of the proteins and metabolites that are predictive of diabetic neuropathy. This work package is led by Professor Eva Feldman, Department of Neurology, University of Michigan, Ann Arbor (US).

WP5: METABOLOMICS AND LIPIDOMICS

Eva Feldman's Laboratory at the University of Michigan, Ann Arbor, USA, uses a systems approach employing transcriptomics, metabolomics, and metabolic flux analysis to identify nerve-specific differences in type 1 (T1D) and 2 diabetic (T2D) mice and man.

Our goal is to identify highly conserved pathways across human and murine models that are likely to play a role in diabetic peripheral neuropathy (DPN) pathogenesis and provide new possible mechanism-based targets for DPN therapy.

Progress during the last years
In the last 4 years, we have summarized our approach and sentinel findings in 33 published papers. These papers include primary articles on our research findings along with a series of reviews focused on the clinical problem, our approach to diagnosis, and the implications our work has on both an understanding of neuropathy in patients with T1D and T2D and the development of novel treatments for these patients.

Our recent clinical studies in man indicate that drivers of neuropathy include not only glucose, but obesity and associated components of the metabolic syndrome. These data serve as the cornerstone of our work in vitro and in murine models of T1D and T2D, where our goal is to better understand the intersection of hyperglycemia and the components of the metabolic syndrome, particularly obesity, in driving the onset and progression of DPN.

Mitochodrial function in T2D
To better understand mitochondrial function in T2D, we treated primary adult mouse dorsal root ganglion (DRG) neurons with physiologically relevant concentrations of the saturated fatty acid (SFA) palmitate and glucose to evaluate the impact of hyperlipidemia and hyperglycemia on mitochondrial transport, depolarization, and bioenergetics.

Treatment with palmitate significantly reduced the number of motile mitochondria in DRG axons, while glucose did not impair mitochondrial trafficking dynamics. Palmitate-treated DRG neurons exhibiting a decrease in motile mitochondria also showed a reduction in mitochondrial velocity, decreased ATP production, and mitochondrial depolarization.

These results suggest that SFAs induce DRG neuron mitochondrial depolarization, inhibiting axonal mitochondrial trafficking and decreasing ATP production.

Further studies then examined how chain length of SFAs regulates mitochondrial trafficking and function. While DRG neurons treated with C16:0 and C18:0 SFAs exhibited impaired motility, mitochondrial depolar-

Professor Eva Feldman presenting at the IDNC Annual Meeting 2018

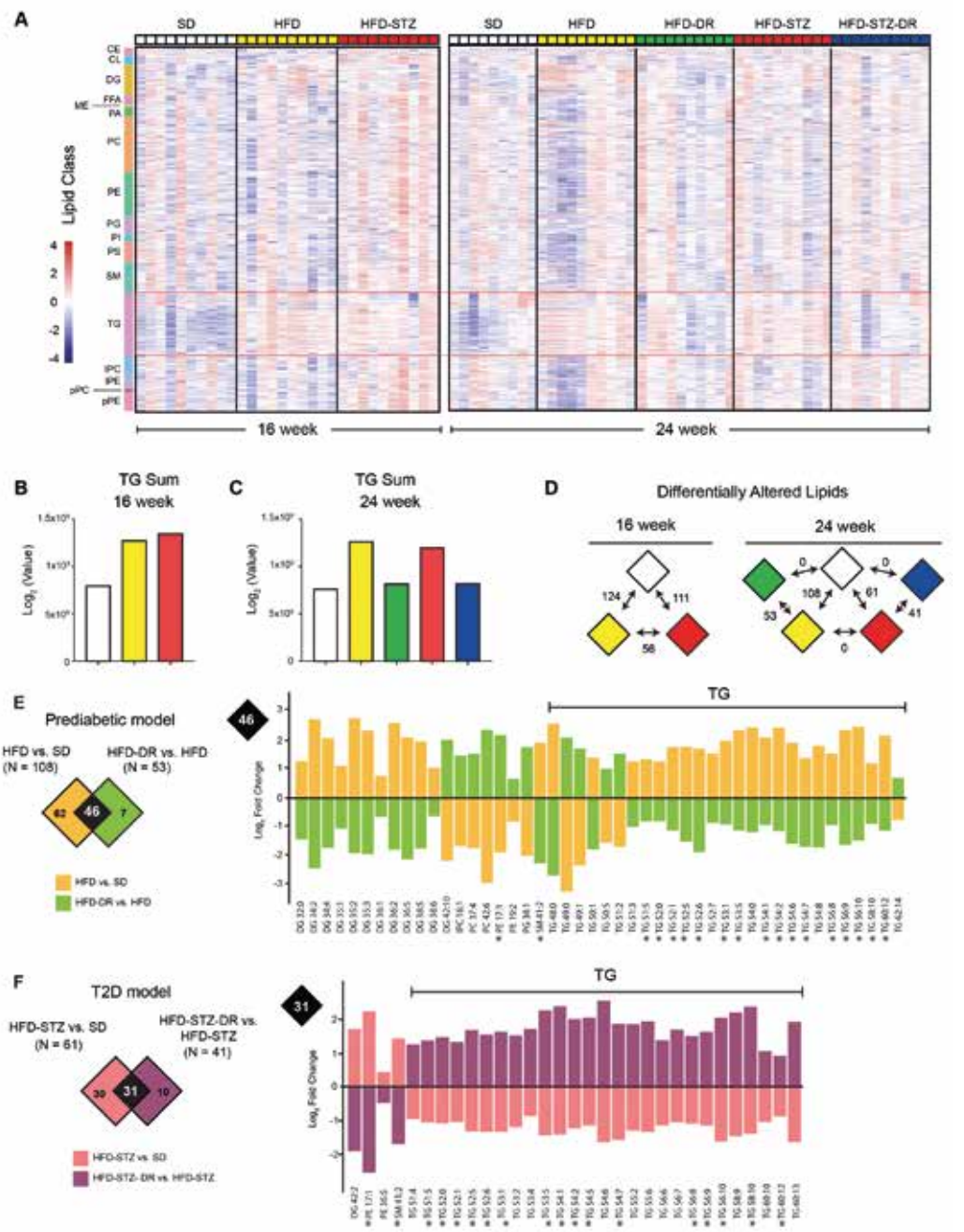


Fig. 1. *Nerve TG levels correlated with neuropathy phenotype. (A) Untargeted shotgun lipidomics was performed on SCN tissue isolated at 16 (left) and 24 weeks (right). Differences in TG were observed when comparing groups at either time-point (red box) (n = 9-10). The scale bar represents lipid levels that were z-score transformed at each lipid species. The sum of the TG was determined at the (B) 16 or (C) 24-week time-point. (D) The number of significant DALs was determined between groups at the 16 or 24-week time-point (adjusted p-value = 0.05). (E) To determine significant DALs in the prediabetic model, the HFD vs. SD diabetic dataset (orange) was compared to the HFD-DR vs. HFD reversal dataset (lime) (left panel). The overlapping DALs were plotted according to log2-485 fold change (right panel). (F) To determine significant DALs in the type 2 diabetic model, the HFD-STZ vs. SD diabetic dataset (salmon) was compared to the HFD-STZ-DR vs. HFD-STZ reversal dataset (purple) (left panel). The overlapping DALs were plotted according to log2-fold change (right panel). Lipids that were common in both prediabetic and type 2 diabetic models are denoted by an asterisk (*). CE, cholesteryl esters; CL, cardolipins; DAL, differentially altered lipid; DG, diglycerides; FFA, free fatty acids; ME, methyl esters; PA, phosphatidic acids; PC, phosphatidylcholines; PE, phosphatidylethanolamines; PG, phosphatidylglycerols; PI, phosphatidylinositols; PS, phosphatidylserines; SM, sphingomyelins; TG, triglycerides, lPC, lysophosphatidylcholines; lPE, lysophosphatidylethanolamines; pPC, plasmenyl-phosphatidylcholines; pPE, plasmenyl495 phosphatidylethanolamines.*
From: O'Brien, P.D., et al., Integrated lipidomic and transcriptomic analyses identify altered nerve triglycerides in mouse models of prediabetes and type 2 diabetes. Disease Models and Mechanisms, Submitted.

ization, and apoptosis, treatment with C12:0 and C14:0 SFAs had no impact on motility, the mitochondria retained polarization (Fig. 1), and apoptosis was blocked.

These data thus indicate that SFA chain length has important implications on mitochondrial trafficking and function in DRG neurons and provide new mechanistic insight into the association of dyslipidemia and DPN.

Finally, we examined the differential effect of SFAs and monounsaturated fatty acids (MUFAs) on the development of neuropathy in mice. We found that mice fed a high fat diet rich in SFAs developed a robust peripheral neuropathy, while switching the mice from the SFA-rich diet to a MUFA-rich high fat diet completely reversed neuropathy. Further mechanistic studies determined that the MUFA oleate prevented the impairment of mitochondrial transport and protected mitochondrial membrane potential in cultured sensory neurons treated with mixtures of oleate and the SFA palmitate, and it also preserved intracellular ATP levels, prevented apoptosis induced by palmitate treatment, and promoted lipid droplet formation in sensory neurons.

These results suggest that MUFAs reverse the progression of neuropathy by protecting mitochondrial function and transport through the formation of intracellular lipid droplets in sensory neurons that may protect sensory neurons from lipotoxicity.

Transcriptomics in DPN

Additional mechanistic insight into DPN was also acquired through transcriptomic analyses. In one study, we reanalyzed previously published DN-related microarray datasets from human and multiple murine models to identify a possible disease mechanism conserved between various models and humans with DPN. Using eight microarray datasets on peripheral nerve samples from murine models of DPN and human participants with non-progressive and progressive DPN, we identified DEGs between non-diabetic and diabetic samples in murine models, and non-progressive and progressive human samples, using a unified analysis pipeline. Pathway and centrality analyses revealed highly connected genes and pathways, including LXR/RXR activation, adipogenesis, glucocorticoid receptor signaling, and multiple cytokine and chemokine pathways. The major clusters involving the highest number of pathways influencing the network of the disease state include inflammatory response, degradation pathways involved with lipid utilization, apoptosis, and immune and kinase related signaling.

These highly conserved pathways across human and murine models provide even further support for our hypothesis that dyslipidemia underlies DPN.

Integrated lipidomics analyses

In another study, we performed an integrated lipidomic and transcriptomic analysis in sciatic nerve to comprehensively characterize changes that occur during neuropathy progression in prediabetic and T2D mice (Fig. 2.). Dietary reversal from a high-fat diet to a standard diet rescues neuropathy, so we also characterized the key lipidomic and transcriptomic alterations that occur upon reversal. We found an increase in triglycerides (TGs) containing saturated fatty acids.

In parallel, transcriptomic analysis confirmed the dysregulation of lipid pathways. Integration of lipidomic and transcriptomic analyses identified an increase in diacylglycerol acyltransferase 2 (DGAT2), the enzyme required for the last and committed step in TG synthesis. Increased DGAT2 expression was present not only in the murine models but also in sural nerve biopsies from hyperlipidemic diabetic patients with PN.

Collectively, these findings support the hypothesis that abnormal nerve-lipid signaling is an important factor in peripheral nerve dysfunction in both prediabetes and T2D. Notably, this contention extends throughout life. Given the increasing neuropathy prevalence in children and adolescents, we established juvenile murine models of diet induced obesity (prediabetes) and T2D that both exhibited similar neuropathy severity, again emphasizing the idea that hyperglycemia alone does not drive early neuropathy.

In an additional study, we also assessed the role of DNA methylation, an epigenetic mechanism important for the regulation of gene expression and the interaction between genetic and environmental factors, in

the progression of DPN in type 2 diabetes. We compared genome-wide DNA methylation profiles of human sural nerve biopsies from subjects with significant nerve regeneration (regenerators) and subjects with significant nerve degeneration (degenerators). Using reduced representation bisulfite sequencing, we identified 3,460 differentially methylated CpG dinucleotides between the two groups. The genes associated with differentially methylated CpGs were highly enriched in biological processes that have previously been implicated in DPN, such as nervous system development, neuron development, and axon guidance, as well as glycerophospholipid metabolism and mitogen-activated protein kinase (MAPK) signaling.

These findings are the first to provide a comprehensive analysis of DNA methylation profiling in human sural nerves of subjects with DPN and suggest that epigenetic regulation has an important role in the progression of this prevalent diabetic complication.

Intervention studies

Intervention studies were also pursued to determine the efficacy of mechanism-based therapies. First, based on our recently identified fundamental differences in mitochondrial responses of complication-prone tissues to T2D in BKS-db/db mice, we treated BKS-db/db mice with the mitochondrial uncoupler, niclosamide ethanolamine (NEN), to determine the effects of mitochondrial uncoupling therapy on T2D, and the pathogenesis of DPN, diabetic kidney disease (DKD), and diabetic retinopathy (DR).

Our data indicated that NEN treatment from 6-24 wk of age had little effect on the development of T2D and diabetic complications, suggesting that globally targeting mitochondria with an uncoupling agent is unlikely to provide therapeutic benefit for DPN, DKD, or DR in T2D.

These data also highlight the need for further insights into the role of tissue-specific metabolic reprogramming in the pathogenesis of diabetic complications.

In parallel, we performed a side-by-side comparison in T1D and T2D mouse models for all prevalent microvascular complications to systematically evaluate the potential efficacy of minocycline, a tetracycline antibiotic with anti-inflammatory and anti-apoptotic properties, which we compared to insulin and pioglitazone. Minocycline ameliorated DR and DKD in T1D and T2D animals, respectively, but was ineffective in improving DPN in either model.

These data suggest that while minocycline alone is unlikely to improve outcomes in DR and DKD beyond that achieved with current available therapies in patients with T1D or T2D, it may serve a role as an adjunct therapy to current treatment protocols.

Likewise, empagliflozin (EMPA), an inhibitor of the sodium/glucose transporter 2 (SGLT2) that lowers blood glycemia in streptozotocin (STZ)-induced T1D and db/db T2D diabetes mouse models, did not ameliorate any microvascular complications in the T2D model despite robust control of blood glucose levels. EMPA did however favorably impact DPN in the T1D model, but did not significantly improve DR and DKD.

These results support the concept that glucose-centric treatments are more effective for neuropathy in T1D versus T2D.

Accomplisments and future plans

We continue to pursue the overarching hypothesis that fatty acid uptake, lipid oxidation, and lipid biosynthetic pathways are dysregulated by diabetes in the peripheral nervous system and that elucidation of these altered pathways will provide new mechanism-based therapeutic targets for neuropathy prevention and treatment.

During the first four years of IDNC funding, we completed clinical studies that support the idea that components of the metabolic syndrome, including dyslipidemia, converge with hyper-glycemia to mediate nerve injury and DPN. In vitro and in murine studies, we have used a combination of mitochondrial assessments, gene expression analysis, and steady-state and dynamic fluxomics and lipidomics and have discovered effects of lipids on mitochondrial function and a downregulation of energy metabolism in the peripheral nerve in mice with diabetes and DPN. This functional downregulation of glucose and lipid metabolism supports our contention that energy failure may likely underlie the pathogenesis of DPN.

LCSFAs palmitate and stearate induce mitochondrial depolarization in mouse DRG neurons

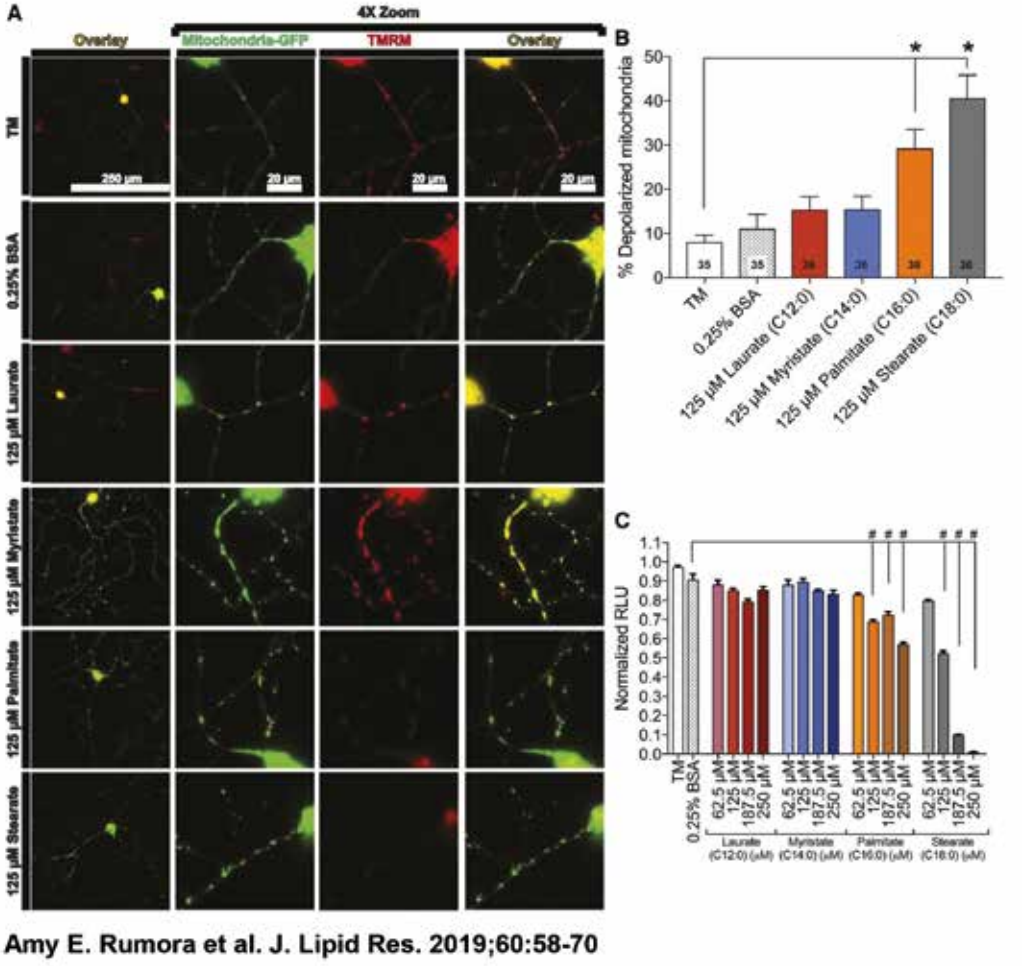
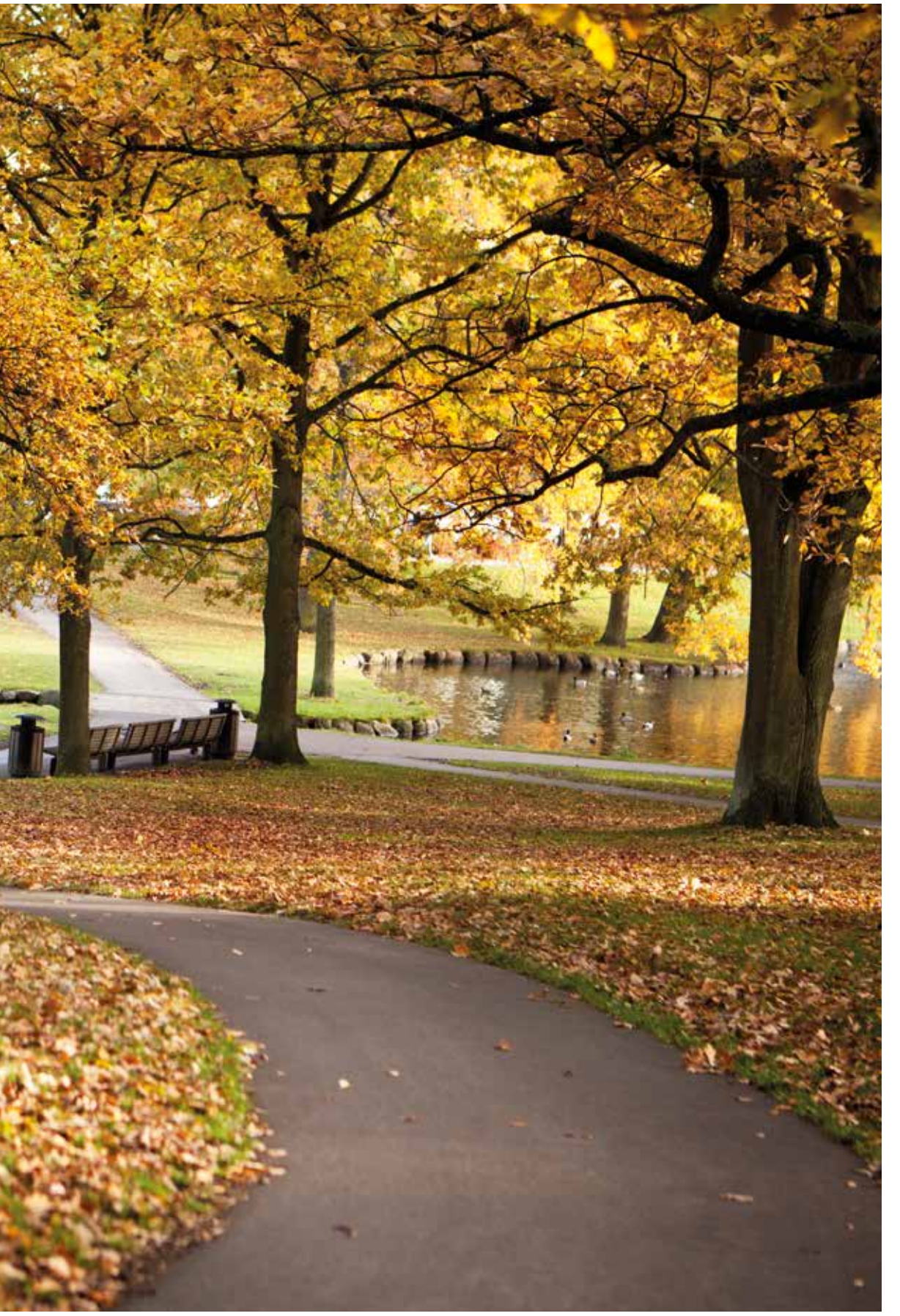


Fig. 2. *LCSFAs palmitate and stearate induce mitochondrial depolarization in mouse DRG neurons.*
*LCSFAs palmitate and stearate induce mitochondrial depolarization in mouse DRG neurons. A: DRG neurons expressing mito-GFP (green) were evaluated for changes in TMRM staining (red) using an overlay (yellow) of mito-GFP and TMRM signal that appears yellow in polarized mitochondria (left overlay column). Each neuron is displayed with a 4× zoom to depict the mitochondria-GFP signal, TMRM signal, and the merged image (right overlay column). Treatment conditions that retained DRG neuron mitochondrial membrane potential include TM, 0.25% BSA, 125 µM laurate, and 125 µM myristate. The 125 µM palmitate and stearate treatments exhibit diffuse TMRM staining (bottom two rows). B: Quantitation of TMRM signal in DRG neurons treated with 125 µM laurate (red bar) and myristate (blue bar) showed no significant effect on mitochondrial membrane potential relative to the TM and 0.25% BSA controls. Palmitate (orange bar) and stearate (black bar) at a concentration of 125 µM, however, exhibited a significant increase in the percentage of depolarized mitochondria relative to 125 µM laurate, 125 µM myristate, and the TM and 0.25% BSA control-treated neurons. The number displayed in the bar is the total number of DRG neurons that were evaluated for each treatment condition in three separate experimental trials. C: Palmitate- and stearate-treated 50B11 DRG neurons (from 125 to 250 µM) exhibit a reduction in intracellular ATP level compared with the 0.25% BSA control. Laurate and myristate treatments had no effect on ATP level. Values are expressed as mean ± SEM. * P < 0.01, ordinary one-way ANOVA with Tukey's multiple-comparisons test (B); #P < 0.0001, ordinary one-way ANOVA with Tukey's multiple-comparisons test (C).*
From: Rumora, A.E., et al., Chain length of saturated fatty acids regulates mitochondrial trafficking and function in sensory neurons. J Lipid Res, 2019. 60(1): p. 58-70



EDUCATIONAL ACTIVITIES AND NETWORKING

Educational activities and networking continue to constitute important elements of the IDNC. IDNC events are announced on the consortium's webpage (www.idnc.au.dk), university websites and mailing lists and are generally open to everyone interested – free of charge.

Reimar Thomsen at IDNC annual meeting 2018 (top) and Diana Hedevang Christensen presenting.

INTERNATIONAL SYMPOSIUM

Diabetes and CNS disorders
15 January 2019 Aarhus Denmark

IDNC organized a symposium about the possible role of central nerve cell damage in diabetes. Invited speakers from Holland, Germany and members of IDNC discussed during a one-day meeting the potential association between diabetes on one hand and stroke, depression, pain and cognitive decline on the other hand. This topic will be discussed further in the future.

RESEARCH STAYS ABROAD

Karolina Snopek had a one-month stay at Prof Rodica Pop-Busui's Department of Internal Medicine at University of Michigan in the Spring 2019.

Frederik Pagh Kristensen will have a 2-months stay in Prof Eva Feldman's and Brian Callaghan's group in Michigan Sep-Oct 2019.

AWARDS AND PRIZES

Hatice Tankisi received a grant on 650.000 DKK from Lundbeck foundation for contribution of Professor Hugh Bostock as a visiting professor in 2018-2019 at the Department of Clinical Neurophysiology, AUH. Prof. Hugh Bostock´s contribution has also included the IDNC projects and supervision of the PhD students in IDNC.

Troels Staehelin Jensen received the Benjamin Covino Lecture Award Harvard Medical School septmeber 2018. Troels S Jensen became honorary member of the International Association for the Study of Pain in September 2018 and honorary member of the Danish Neurological Society in March 2019 and in May 2019 he received the Mitchell Max Award from the American Academy of Neurology for his work on neuropathic pain.

Prof David Bennett was awarded a honorary Skou professorship (named after former Nobel Prize winner Jens Chr. Skou from Aarhus University) by Aarhus University. This professorship to Dr Bennett will facilitate collaboration between Aarhus and Oxford sites as part of the IDNC. Dr Bennett was also awarded the 2019 medal lecture of the British Pain Society.

Troels Staehelin receiving the Mitchell Max Award 2019 from the American Academy of Neurology

SCIENTIFIC MEETINGS AND TEACHING ACTIVITIES

Members of the IDNC gave lectures and poster presentations at numerous key national and international scientific meetings and courses on pain and diabetic neuropathy in 2018/2019, including the following:

31st International Congress of Clinical Neuro-physiology
Washington DC, USA, 1-6 May 2018

16th European Congress of Clinical Neurophysiology
Budapest, August 30 - September 02, 2018

NEURODIAB the 28th Annual Meeting of the Diabetic Neuropathy Study Group of the EASD
Rome, 4-7 September 2018

17th World Congress on Pain
Boston, MA, USA, 12-16 September 2018

World Congress for Microcirculation
Vancouver, Canada. September 2018

American Diabetes Association's 79th Scientific Sessions
San Francisco, California, USA,
7-11 June 2019

IASP Pain Camp
Kuching Malaysia April 6-11, 2019

ASEAPS congress,
Kuching Malaysia April 11-14, 2019

7th International Congress on Neuropathic Pain
London, UK, May 2019

American Academy of Neurology meeting
Philadelphia, USA May 6-11, 2019

17th European Congress of Clinical Neurophysiology (ECCN)
Warsaw, Poland, 5-8 June 2019

Active participant in the Danish "Folkemødet på Bornholm"
Svaneke, Denmark, June 13-16 2019

PNS 2019
Genoa, Italy, 22-25 June 2019

North American Pain School Montebello, Quebec
Canada 23-28 June 2019

55th EASD (European Association for the Study of Diabetes) Annual Meeting
Barcelona, Spain,16-20 September 2019

PUBLICATIONS

PEER-REVIEWED PUBLICATIONS

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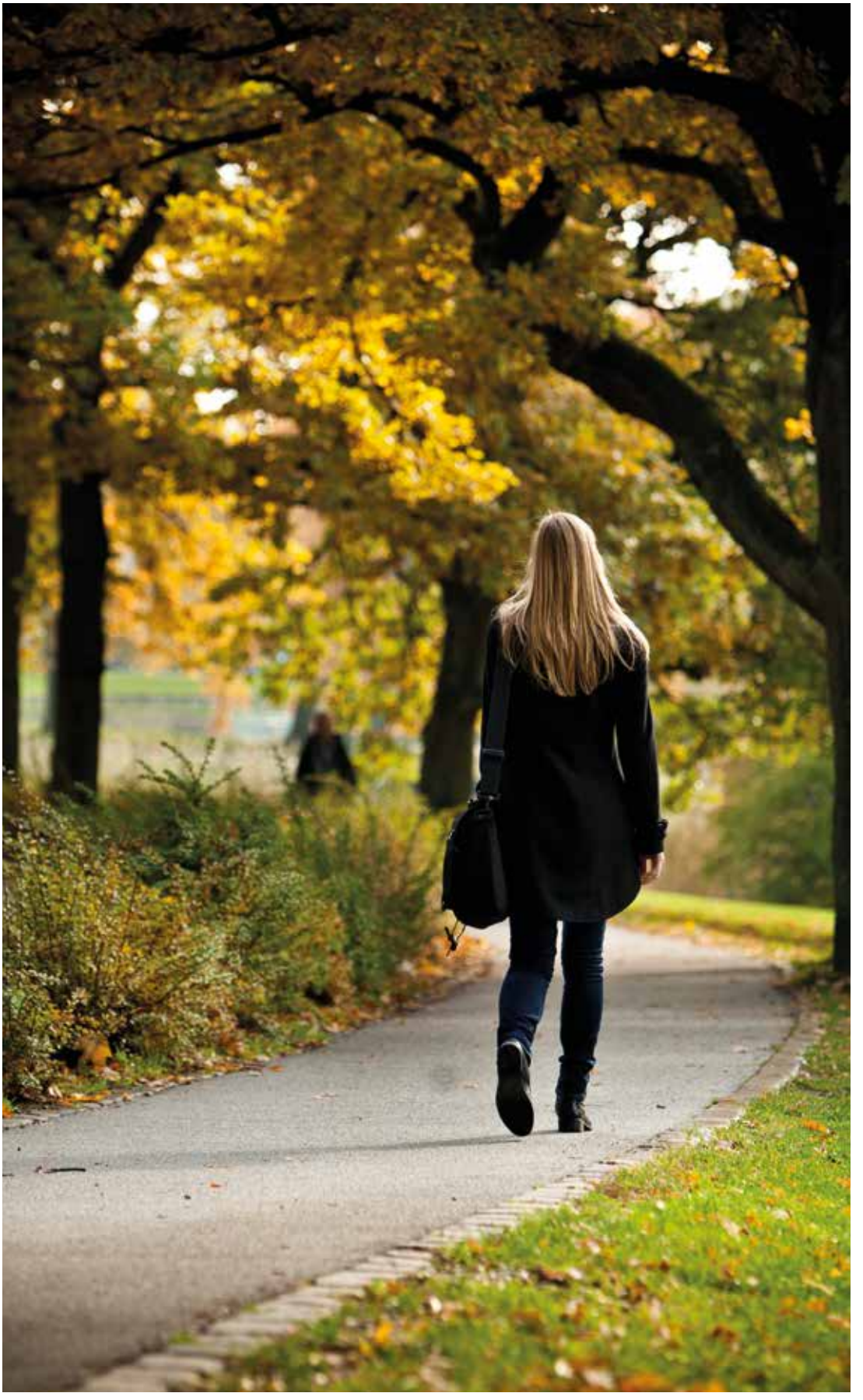

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