# Assessment of peripheral neuropathy - Focusing on multi-frequency vibrometry and intraepidermal nerve fiber density

Ekman, Linnéa

2024

*Document Version:*
Förlagets slutgiltiga version

# Assessment of peripheral neuropathy

## Focusing on multi-frequency vibrometry and intraepidermal nerve fiber density

LINNÉA EKMAN
DEPARTMENT OF TRANSLATIONAL MEDICINE | FACULTY OF MEDICINE | LUND UNIVERSITY

Assessment of peripheral neuropathy

# Assessment of peripheral neuropathy

## Focusing on multi-frequency vibrometry and intraepidermal nerve fiber density

Linnéa Ekman


LUND
UNIVERSITY

DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Medicine at Lund University to be publicly defended on December 11th, 2024, at 09.00 in Belfragesalen, Klinikgatan 32, Lund

*Faculty opponent*
Associate Professor Rayomand Press
Department of Neurology at Karolinska University Hospital, Stockholm

**Organization** LUND UNIVERSITY

**Document name** DOCTORAL THESIS **Date of issue** 11th December 2024

**Author** Linnéa Ekman

**Title and subtitle**

Assessment of peripheral neuropathy – Focusing on multi-frequency vibrometry and intraepidermal nerve fiber density

**Abstract**

In the field of peripheral neuropathy, there is a need for diagnostic methods that can assess early impairments in nerve function, thus enhancing the efficiency of diagnosis and enabling preventive treatment. This thesis focuses on two novel techniques that have been suggested to detect peripheral neuropathy: examination of vibration perception thresholds (VPTs) through multi-frequency vibrometry (MFV) and assessment of the intraepidermal nerve fiber density (IENFD) in thin 5-µm skin samples. These methodologies hold the potential for numerous advantages over current gold standards, offering increased accessibility, reduced costs, and shorter examination time requirements. However, there is a substantial knowledge gap concerning reference values, the possibility of differentiating between healthy and diseased states, and the optimal application of the methods within a clinical context. Thus, the doctoral work aimed to establish normative values of VPTs and IENFDs and investigate the clinical implementation in patients with type 1 (T1DM) or type 2 diabetes (T2DM).

The aim of Paper I was to explore the distribution of VPTs and its affecting factors within a cohort of healthy adults. The key outcome of the study was the normative values for men and women of various ages, where VPTs were found to deteriorate with increasing age. In Paper II, VPT assessment through MFV was compared to nerve conduction studies in patients with T1DM. The study revealed a strong correlation between sural nerve amplitude and VPTs at the 125 Hz frequency. Paper III aimed to explore the distribution and the potentially affecting factors for IENFD assessment in thin 5-µm skin sections obtained from healthy adults. Outcomes beyond the normative values suggested differences in IENFD across ages, sex, and excision sites, with the most notable finding being significantly lower IENFD among individuals >65 years. Lastly, in Paper IV, the temporal trend of IENFD was studied in people with T2DM and healthy controls. Although levels were lower within T2DM at baseline, aging resulted in similar IENFD levels for controls at follow-up.

By exploring the normative values and the clinical implementations in patients with T1DM and T2DM, a foundation has been established for further research and future employment of the methods. The studies have also added to the understanding and knowledge of how diabetic neuropathy affects both small and large nerve fibers.

**Keywords** Polyneuropathy, Small Fiber Neuropathy, Large Fiber Neuropathy, Diabetes Complications, Nerve Conduction Studies, Skin Biopsy, Intraepidermal Nerve Fiber Density, Immunohistochemistry, Vibration Perception Threshold, Multi-Frequency Vibrometry

**Language** English **ISSN** 1652-8220

**ISBN** 978-91-8021-647-0 **Number of pages** 91



**Signature** **Date** 29th October 2024

# Assessment of peripheral neuropathy

## Focusing on multi-frequency vibrometry and intraepidermal nerve fiber density

Linnéa Ekman


LUND
UNIVERSITY

Coverphoto *Soil punch biopsy*



Faculty of Medicine
Department of Translational Medicine

ISBN 978-91-8021-647-0
ISSN 1652-8220

Printed in Sweden by Media-Tryck, Lund University
Lund 2024

*The great art of life is sensation, to feel that we exist*
*– even though in pain*

Lord Byron

# Table of Contents

# List of papers

This thesis is based on the following publications which are hereafter referred to by their Roman numerals:

*Paper I*

**Ekman L**, Lindholm E, Brogren E, Dahlin LB. Normative values of the vibration perception thresholds at finger pulps and metatarsal heads in healthy adults. *PLoS One.* 2021 Apr 6;16(4):e0249461. doi: 10.1371/journal.pone.0249461.

*Paper II*

**Ekman L**, Dahlin LB, Andersson GS, Lindholm E. Diagnostic contribution of multi-frequency vibrometry to detection of peripheral neuropathy in type 1 diabetes mellitus compared with nerve conduction studies. *PLoS One.* 2024 Jan 10;19(1):e0296661. doi:10.1371/journal.pone.0296661.

*Paper III*

**Ekman L**, Dahlin LB, Englund E. Assessment of intraepidermal nerve fiber densities in 5 μm sections from arm and leg – a search for normative age-related values. *Free Neuropathology*. 2024 Oct 11; 5:24. doi: 10.17879/freeneuropathology-2024-5815.

*Paper IV*

**Ekman L**, Pourhamidi K, Englund E, Lagali N, Rolandsson O, Dahlin LB. Temporal trend of small fibre degeneration in people with and without type 2 diabetes mellitus. *Diabetic Medicine.* 2022 Mar;39(3):e14691. doi: 10.1111/dme.14691. Epub 2021 Sep 22.

*List of publications not included in the thesis*

Badian RA, **Ekman L**, Pripp AH, Utheim TP, Englund E, Dahlin LB, Rolandsson O, Lagali N. Comparison of Novel Wide-Field In Vivo Corneal Confocal Microscopy with Skin Biopsy for Assessing Peripheral Neuropathy in Type 2 Diabetes. *Diabetes*. 2023 Jul;72(7):908-917.

Lindholm E, **Ekman L**, Elgzyri T, Lindholm B, Löndahl M, Dahlin L. Diabetic Neuropathy Assessed with Multifrequency Vibrometry Develops Earlier than Nephropathy but Later than Retinopathy. *Exp Clin Endocrinol Diabetes*. 2023 Apr;131(4):187-193.

Frostadottir D, **Ekman L**, Zimmerman M, Dahlin LB. Cold sensitivity and its association to functional disability following a major nerve trunk injury in the upper extremity – A national registry-based study. *PLoS One*. 2022 Jul;17(7):e0270059.

Frostadottir D, **Ekman L**, Zimmerman M, Andersson S, Arner M, Brogren E, Dahlin LB. Cold sensitivity, functional disability and predicting factors after a repaired digital nerve injury. *Sci Rep*. 2022 Mar;12(1):4847.

Ising E, **Ekman L**, Elding Larsson H, Dahlin LB. Vibrotactile sense might improve over time in paediatric subjects with type 1 diabetes-A mid-term follow-up using multifrequency vibrometry. *Acta Paediatr*. 2022 Feb;111(2):411-417. Epub Oct 2021.

**Ekman L**, Persson Löfgren J, Dahlin LB. Examining practice effects in repeated measurements of vibration perception thresholds on finger pulps of healthy individuals – Is it possible to improve your results over a clinically relevant test interval? *PLoS One*. 2019 Dec;14(12):e0226371.

Dahlin LB, Elgzyri T, Löndahl M, **Ekman L**, Lindholm E. Improved metabolic control using glucose monitoring systems leads to improvement in vibration perception thresholds in type 1 diabetes patients. *Acta Diabetol*. 2020 Apr;57(4):433-438. Epub Nov 2019.

**Ekman L**, Thrainsdottir S, Englund E, Thomsen N, Rosén I, Hazer Rosberg DB, Petersson J, Eriksson KF, Dahlin LB. Evaluation of small nerve fiber dysfunction in type 2 diabetes. *Acta Neurol Scand*. 2020 Jan;141(1):38-46. Epub Oct 2019.

# Thesis at a glance

The overall aim of the thesis was to study two novel techniques for assessment of neuropathy: multi-frequency vibrometry (MFV) and measurements of intraepidermal nerve fiber density (IENFD). Specific aims for each paper are described below.

*Paper I*

**Normative values of the vibration perception thresholds at finger pulps and metatarsal heads in healthy adults**

*Aim:* To establish normative values of vibration perception thresholds (VPTs) using MFV, and to investigate potential impact of age, sex, height, weight, foot- or handedness, and skin temperature.

*Method:* VPTs were examined in 924 healthy and randomly selected subjects in southern Sweden (mean 46 years; 628 women and 296 men). VPTs were measured at the finger pulps of the index and little finger, as well as the first and fifth metatarsal heads of the foot.

*Results and conclusions:* Normative VPTs were presented for men and women on age decades. VPTs deteriorated as age increased (0.1-0.6 dB per year; $p<0.001$) and improved with higher temperatures in finger pulps. Height was negatively associated with VPTs at metatarsal heads, whereas sex, weight, and handedness showed no impact.

*Paper II*

**Diagnostic contribution of multi-frequency vibrometry to detection of peripheral neuropathy in type 1 diabetes mellitus compared with nerve conduction studies**

*Aim:* To assess the use of MFV in detecting diabetic peripheral neuropathy (DPN) in type 1 diabetes in comparison to nerve conduction studies (NCS) and neurothesiometer (NT).

*Method:* Adults with type 1 diabetes were examined regarding nerve function in the lower limbs through MFV, NT, and NCS.

*Results and conclusions:* Through NCS assessment, 33 participants (50%) were diagnosed with DPN. VPTs correlated negatively with all NCS parameters, where the strongest correlation was found between sural nerve amplitude and the 125 Hz frequency. A combination of two low (4 and 8 Hz) and two high (125 and 250 Hz) frequencies showed the highest classification efficacy (AUC 0.83). VPT testing for DPN in the lower limb could thus be shortened in time.

*Paper III*

**Assessment of intraepidermal nerve fiber densities in 5 µm sections from arm and leg – a search for normative age-related values**

*Aim:* To assess intraepidermal nerve fiber density (IENFD) in thin 5 µm skin sections from healthy adults, and to investigate the potential impact of age, sex, and excision site.

*Methods:* IENFD was assessed in 602 skin tissue samples from individuals between 18 and 97 years, previously examined and stored at the Pathology Department. Samples were re-sectioned and stained with the protein gene product 9.5-antibody and assessed for IENFD (assessment criteria: nerve fiber length >15 µm).

*Results and conclusions:* Normative IENFD values for thin sections were presented. Individuals >65 years presented with significantly lower levels of IENFD in comparison to all younger adults, and levels were generally higher in the arm compared to the leg.

*Paper IV*

**Temporal trend of small nerve fiber degeneration in people with and without type 2 diabetes mellitus**

*Aim:* To investigate the temporal trend of IENFD and the association between changes in IENFD and metabolic factors in individuals with and without type 2 diabetes (T2DM).

*Method:* Participants underwent clinical and electrophysiological examinations, as well as a skin biopsy both at baseline and at the follow-up visit (mean 8.1 ± 0.5 years).

*Results and conclusions:* IENFD decreased in both groups, with a greater decline in the group without diabetes than in T2DM (-2.3 and -0.6 fibers/mm respectively; $p<0.001$). Despite lower IENFD levels at baseline in T2DM, IENFD was equal between the groups at follow-up. A decrease in IENFD is to a limited extent affected by body weight, and HbA1c, but age seems to be the long-term determinant of IENFD in an elderly population.

# Populärvetenskaplig sammanfattning

Polyneuropati (från grekiskans *poly* – flera, *neuro* – nerver, och *pati* – lidande) är en sjukdom som drabbar flertalet av kroppens nervtrådar och medför nedsatt funktion i dessa. Det finns många olika varianter av sjukdomen och symtomen är beroende på vilka nervtrådar som har drabbats. Symtomen sträcker sig från milt obehag med känselstörningar så som stickningar och domningar, till ihållande och skärande smärta samt bortfall av viljestyrda muskelrörelser. Polyneuropati kan utvecklas utan känd orsak (s.k. idiopatisk polyneuropati), på grund av ärftliga faktorer, eller som följd av och ett symptom på en bakomliggande sjukdom. Den vanligaste utlösande faktorn för polyneuropati är diabetes, där ungefär varannan person förväntas drabbas någon gång under sin livstid. Bland den vuxna befolkningen i världen lever idag 537 miljoner personer med diabetes, en siffra som år 2045 beräknas vara 783 miljoner. I takt med att prevalensen av diabetes konstant ökar innebär det också att antalet patienter med polyneuropati blir fler. Det finns än idag ingen specifik behandling för polyneuropati, utan man förlitar sig på symptomlindring och, i de fall det går, preventiva åtgärder. För patienter med diabetes handlar detta främst om att upprätta en god blodsockerkontroll.

För att kunna arbeta preventivt är det också viktigt att vi kan upptäcka tidiga förändringar associerade med neuropati genom så kallade screeningmetoder. Dessa metoder behöver vara lättillgängliga, användarvänliga och tillförlitliga. I denna avhandling har två sådana nya metoder undersökts med målet att se hur metoderna fungerar i klinisk praxis, hur de står sig mot redan befintliga metoder, samt att upprätta referensmaterial så att resultaten av metoderna kan tolkas. De undersökta metoderna är *multifrekvens-vibrametri* samt *bestämning av nervtäthet i huden*. Avhandlingen är uppdelad i fyra delarbeten, varav varje metod har ett referensmaterial samt en klinisk tillämpning.

Under det första delarbetet undersöktes över 900 vuxna personer med multifrekvens-vibrametri i syfte att framställa referensvärden samt att undersöka vilka faktorer som påverkar förmågan att upptäcka små finjusterade vibrationer i händer och fötter. Genom att placera antingen ett finger eller fotens trampdyna på en apparat som avger vibrationer i olika frekvenser – därav namnet – kan patientens känsel undersökas. Testproceduren påminner mycket om hörseltestet du förmodligen genomgick som barn. En viktig upptäckt som gjordes i studien är att vibrationskänsligheten minskar med ökad ålder. I det andra delarbetet jämfördes användningen av multifrekvens-vibrametri med en redan etablerad metod för att undersöka nervfunktion – *neurografi*

– hos patienter med diabetes typ 1. Neurografi är en objektiv metod där man mäter nervernas elektriska signaleringsförmåga. Resultaten från studien visade bland annat att en förkortad testprocedur av multifrekvens-vibrametri, bestående av fyra frekvenser, var jämförbar med neurografi när det gäller att bedöma om patienten har polyneuropati.

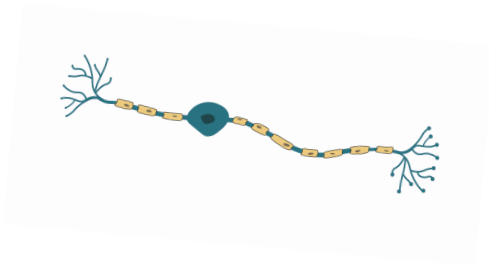
I de tredje och fjärde delarbetena undersöktes möjligheten att bestämma nervtätheten i tunna snitt från hudprover. Huden innehåller de yttersta ändarna av våra nerver, som likt små, tunna trådar breder ut sig och bidrar till att vi exempelvis kan avgöra temperaturen på vattnet i kranen. Dessa nervtrådar är ofta de första att ta skada vid en polyneuropati, vilket gör att undersökning av nervtätheten kan vara en bra indikator för tidiga förändringar. För att räkna nervtrådarna behöver man ta prov från en bit hud – en *biopsi* – som sedan behandlas i laboratorium med en specifik antikropp för att göra nervtrådarna synliga under mikroskop. I det tredje arbetet insamlades över 600 hudprover för mikroskopisk undersökning. I stället för att ta biopsier från friska frivilliga individer, användes redan insamlat material som finns arkiverat på Patologen i Lund. Skillnader i nervtäthet observerades mellan olika åldersgrupper, där individer över 65 år framför allt uppvisade lägre nivåer. Dessutom visade resultaten att nervtätheten var något högre i armarna än i benen. I det fjärde projektet, som syftade till att jämföra nervtätheten mellan friska personer och de med diabetes typ 2, konstaterades att åldern har en betydande inverkan, även om diabetes påskyndar förloppet.

Sammanfattningsvis utgör denna avhandling en utvärdering av två diagnostiska metoder för polyneuropati, tillämpning på patientmaterial, samt ett försöka att etablera normalvärden för respektive metod. Avhandlingen har därmed lagt en grund för vidare forskning kring de undersökta metoderna och framtida studier bör undersöka möjligheten att integrera dessa i den dagliga rutinvården. Dessutom har arbetet bidragit till en ökad förståelse av hur diabetisk polyneuropati påverkar olika nervtyper i kroppen.

# Abbreviations and units

| | |
|---|---|
| AGE | Advanced glycation end-product |
| AI | Artificial intelligence |
| ALS | Amyotrophic lateral sclerosis |
| AUC | Area under the curve |
| BMI | Body mass index |
| CCM | Corneal confocal microscopy |
| CNS | Central nervous system |
| DAG | Diacylglycerol |
| dB | Decibels, logarithmic unit of measurement |
| DCCT/EDIC | Diabetes Control and Complications Trial/Epidemiology of Diabetes Interventions and Complications |
| DPN | Diabetic polyneuropathy |
| DSPN | Distal symmetrical polyneuropathy |
| EFNS | European Federation of Neurological Societies |
| eIENFD | Estimated intraepidermal nerve fiber density |
| EMG | Electromyography |
| HAVS | Hand-arm vibration syndrome |
| HbA1c | Hemoglobin A1c |
| Hz | Hertz, unit of frequency (event per second) |
| IENF | Intraepidermal nerve fiber |
| IENFD | Intraepidermal nerve fiber density |
| IGT | Impaired glucose tolerance |
| MetS | Metabolic syndrome |

| | |
|---|---|
| MFV | Multi-frequency vibrometry |
| MNSI | Michigan Neuropathy Screening Instrument |
| MTI | First metatarsal head |
| MTV | Fifth metatarsal head |
| N | Newton, unit of force (mass times acceleration) |
| NCS | Nerve conduction study |
| NDS | Neuropathy Disability Score |
| NGT | Normal glucose tolerance |
| NSAID | Non-steroidal anti-inflammatory drug |
| NSS | Neuropathy Screening Instrument |
| NT | Neurothesiometer |
| PGP 9.5 | Protein gene product 9.5 |
| PKC | Protein kinase C |
| PNS | Peripheral nervous system |
| POTS | Postural orthostatic tachycardia syndrome |
| QST | Quantitative sensory testing |
| ROC | Receiver operating characteristic |
| ROS | Reactive oxygen species |
| SFN | Small fiber neuropathy |
| SI | Sensibility index |
| SNRIs | Serotonin-norepinephrine reuptake inhibitors |
| SSRIs | Selective serotonin reuptake inhibitors |
| TCNS | Toronto Clinical Neuropathy Scale |
| T1DM | Type 1 diabetes mellitus |
| T2DM | Type 2 diabetes mellitus |
| VPT | Vibration perception threshold |
| VSM | VibroSense Meter® |
| µm | Micrometer, 1/1000 mm |



# Introduction

*Prevention is better than cure*
– Desiderius Erasmus

Peripheral neuropathy refers to disease or damage affecting the nerves of the peripheral nervous system. It can manifest idiopathically or result from various underlying conditions. The pattern of nerve dysfunction mirrors the structural and functional impairment of the affected nerves, with one common presentation being the involvement of multiple nerves, known as polyneuropathy. Among the prevalent polyneuropathies, diabetic polyneuropathy (DPN) is the most frequent, accounting for around 35% of the cases.[1,2] DPN is also one of the most common diabetes-related complications, although it often receives less attention than others, such as nephropathy and retinopathy. Like polyneuropathies in general, DPN currently lacks curative treatments, despite considerable efforts to find them.

The potentially devastating consequences of DPN, including foot ulcers and amputations, make it a formidable challenge in diabetes management. Beyond the personal suffering it causes to individuals, DPN also imposes substantial economic burdens, with annual costs in the U.S. exceeding 10 billion dollars as early as 2003.[3]

Given the lack of disease-modifying therapies, early detection followed by prompt and effective interventions is crucial for preventing further disease progression. Polyneuropathies can involve damage to both myelinated and unmyelinated nerve fibers, as well as both small and large nerve fibers, meaning several methods may be used to evaluate nerve dysfunction. While current screening methods are generally quick and easy to administer, they often lack diagnostic accuracy in the form of sensitivity, making early-stage DPN detection challenging. Thus, searching for more reliable diagnostic tools, alongside the continuing research on effective interventions and treatments, could improve diagnostics and patient care. By exploring the clinical applicability of two novel methods – multi-frequency vibrometry (MFV) and intraepidermal nerve fiber density (IENFD) – this thesis hopes to contribute to improved screening and diagnosis of DPN, ultimately leading to better patient outcomes and quality of life.

# 1. Background

## 1.1. The peripheral nervous system

Our nervous system is an incredibly complex network, encompassing fast-traveling nerve signals protruding from the brain throughout the body and back again. The brain together with the spinal cord constitutes the central nervous system (CNS).[4] The nerve fibers branching out from the cord and throughout the body are jointly called the peripheral nervous system (PNS). The PNS can be further subdivided into the autonomic and somatic parts, where the autonomic nervous system is responsible for regulating the function of our internal organs and comprises the sympathetic, parasympathetic, and enteric nervous systems. The somatic nervous system includes afferent sensory nerves that register external stimuli and transmit this information to the CNS, as well as efferent motor nerves that carry signals back to the body and its muscles.

### 1.1.1. The peripheral nerve

A nerve cell, or *neuron*, comprises a cell body and projections in the form of dendrites and axons.[5] In afferent neurons, the dendrites establish connections with upper neurons via synapses in the spinal cord, while the cell body is situated outside the spinal cord within the dorsal root ganglia. The length of the axon can vary significantly, depending on the location of the associated sensory receptor. For efferent neurons, both the dendrites and the cell body are located in the ventral horn of the spinal cord, and the axon continues outward to a motor end plate.

All axons receive metabolic support from glial cells, which are called Schwann cells in the PNS. Schwann cells may be myelinating, meaning they produce a supporting fatty sheath that wraps around the individual axons, improving the conduction velocity of nerve impulses. Regarding the unmyelinated nerve fibers, a single Schwann cell organizes and encapsulates multiple axons together in a structure called Remak bundles, as demonstrated in Figure 1.1. Further, all axons are organized into larger bundles through four different connective tissue structures: the endoneurium, the perineurium, the epineurium, and the mesoneurium. The endoneurium surrounds the individual nerve fibers, while the perineurium forms fascicles within the nerve by encapsulating groups of axons. The epineurium encompasses all the fascicles together, contains

extrinsic blood vessels, and thus provides both metabolic and mechanical support to the fascicles.[5] Finally, the whole nerve structure is covered in the mesoneurium, a connective tissue sheath important for friction-free movement of the nerve within other structures.

All nerves receive blood supply from nerve-specific blood vessels – called the *vasa nervorum*. Extrinsic blood vessels run along the nerve trunk, with branches segmentally supplying the intrinsic parts of the vascular system – a longitudinal plexus of blood vessels within the epineurium, perineurium, and, finally, the endoneurium with its endoneurial capillaries.[6]

Nerve fibers are classified based on their diameter and amount of myelination, as illustrated in Figure 1.2. The largest heavily myelinated Aα motor fibers reach conduction velocities around 100 m/s and are responsible for muscle control and proprioception.[4] Among the sensory nerve fibers, we find the moderately myelinated Aβ fibers and the thinly myelinated Aδ fibers. Lastly, we have the thinnest and unmyelinated C-fibers where signals transmit at around 1 m/s. Most peripheral nerves include both motor and sensory functions and thus contain axons of different diameters and degrees of myelination.

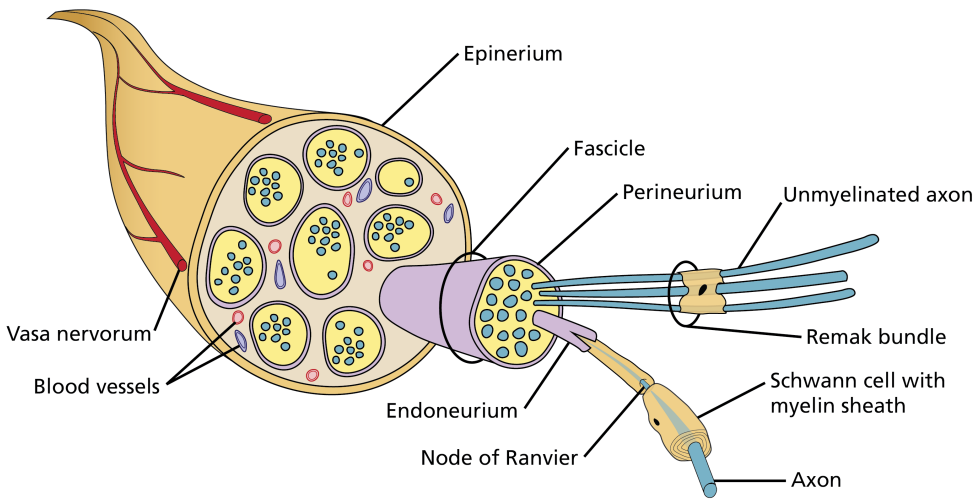



Figure 1.1. Anatomy of the peripheral nerve

Illustration of the anatomy of a peripheral nerve. Axons can either be individually myelinated by Schwann cells or grouped together with multiple unmyelinated fibers in Remak bundles. The axons are further supported by the endoneurium, forming a fascicle with other axons and then surrounded by the perineurium. Multiple fascicles are bundled together to form the complete nerve, which is then enclosed by the epineurium and supplied by the vasa nervorum. The nodes of Ranvier, gaps between the myelin sheaths, enable saltatory conduction of nerve signals, allowing for rapid signal transmission.

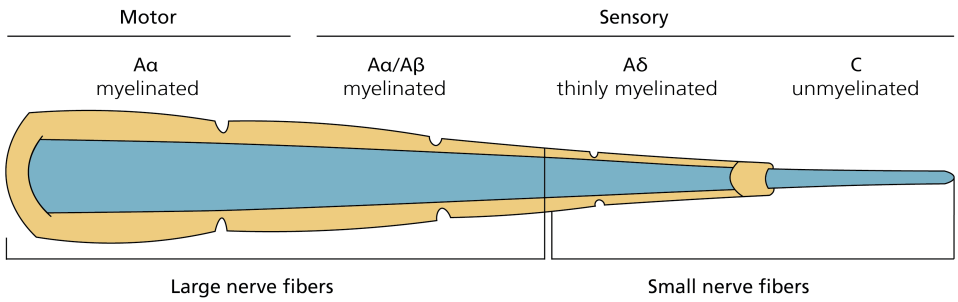



Figure 1.2. Classification of nerve fibers

Schematic illustration depicting the spectrum of nerve fiber types, classified by their diameter and degree of myelination. Motor nerve fibers are consistently large, myelinated Aα fibers. In contrast, sensory fibers vary in form, ranging from large, myelinated Aα or Aβ fibers to smaller Aδ fibers, which are thinly myelinated, and C fibers, which are unmyelinated.

### 1.1.2. Innervation of the skin and mechanoreceptors

With an area of approximately two square meters, the skin constitutes the largest organ of the human body. The skin plays many important roles, being both a physical, chemical, and immunological barrier against the environment and its external threats. It is also important for our temperature regulation, vitamin production, and finally sensation. The skin is densely innervated with different kinds of nerve endings and sensory receptors that register external stimuli and transmit signals from the skin, via the PNS, to the CNS and the primary somatosensory cortex where the signals are interpreted.[5] The cutaneous sensory receptors can be found in the subcutis, dermis, and epidermis, the three layers of the skin, and include thermoreceptors, nociceptors, and mechanoreceptors, see Figure 1.3. Thermoreceptors register cold and heat, whereas nociceptors transmit signals regarding pain caused by e.g. toxic agents or hot temperatures. Both these receptors are free nerve endings of either Aδ or C fibers.[7] Mechanoreceptors respond, as the name implies, to mechanical stimuli such as pressure and vibration.

There are four types of complex mechanoreceptors: Pacinian, Meissner's, and Ruffini corpuscles, as well as Merkel discs, all innervated by Aβ fibers. Meissner corpuscles are located beneath the epidermis in the apex of the dermal papillae in glabrous skin.[8] They are supplied by several axons which terminate between lamellar Schwann cells and form an encapsulated structure. Meissner corpuscles are sensitive to light touch as well as low-frequency vibrations, particularly around 50 Hz.[4] Merkel discs, or more accurately, the Merkel cell-neurite complexes are found at the basal layer of the epidermis. The complex involves a specialized tactile cell, the Merkel cell, and a flattened nerve ending that together forms a synapse-like membrane contact. Merkel discs detect stimuli of

pressure, static touch, and vibration of low frequencies (optimally 5 Hz). Pacinian corpuscles are large, onion bulb-like structures with a central axon that is surrounded by several layers of connective tissue. Pacinian corpuscles detect surface textures, gross pressure changes, and vibrations of high frequencies (200 Hz). They have a large receptive field but are fewer in count than the other receptor types. Last, we have the Ruffini corpuscles, which are fusiform bulbous nerve endings intertwined among collagen bundles. These corpuscles are sensitive primarily for skin stretch and finger positioning and movement. Both Pacinian and Ruffini corpuscles are found either deeply situated in the dermis, or the subcutis.

Mechanical stimuli can also be detected by hair root plexuses as well as free nerve endings of Aδ fibers. Whereas the myelinated Aβ fibers only reach the subcutaneous and dermal layers, free unmyelinated nerve endings can protrude to *stratum basale* and *stratum spinosum,* i.e. the two deeper layers of the epidermis.[5] In order to be receptive to the signals provided by surrounding cells, such as melanocytes and dendritic cells, the nerve fiber sheds its Schwann cell layer as it crosses the dermal-epidermal junction. As a result, the nerve fibers within the epidermis are "naked" axons.[9] Beyond the sensory nerve fibers mentioned above, there are also autonomous nerve endings to be found in the skin, innervating e.g. the sweat glands and arrector pili muscles.

The distribution of mechanoreceptors and their afferent fibers varies across the body and exhibits differences also among hairy and glabrous skin. For example, the palms of the hands, and particularly the fingers, are more densely innervated with receptors, which also have smaller receptive fields in these regions.[10] Consequently, discriminative touch is more pronounced in certain areas, such as the finger pulp compared to the arm.

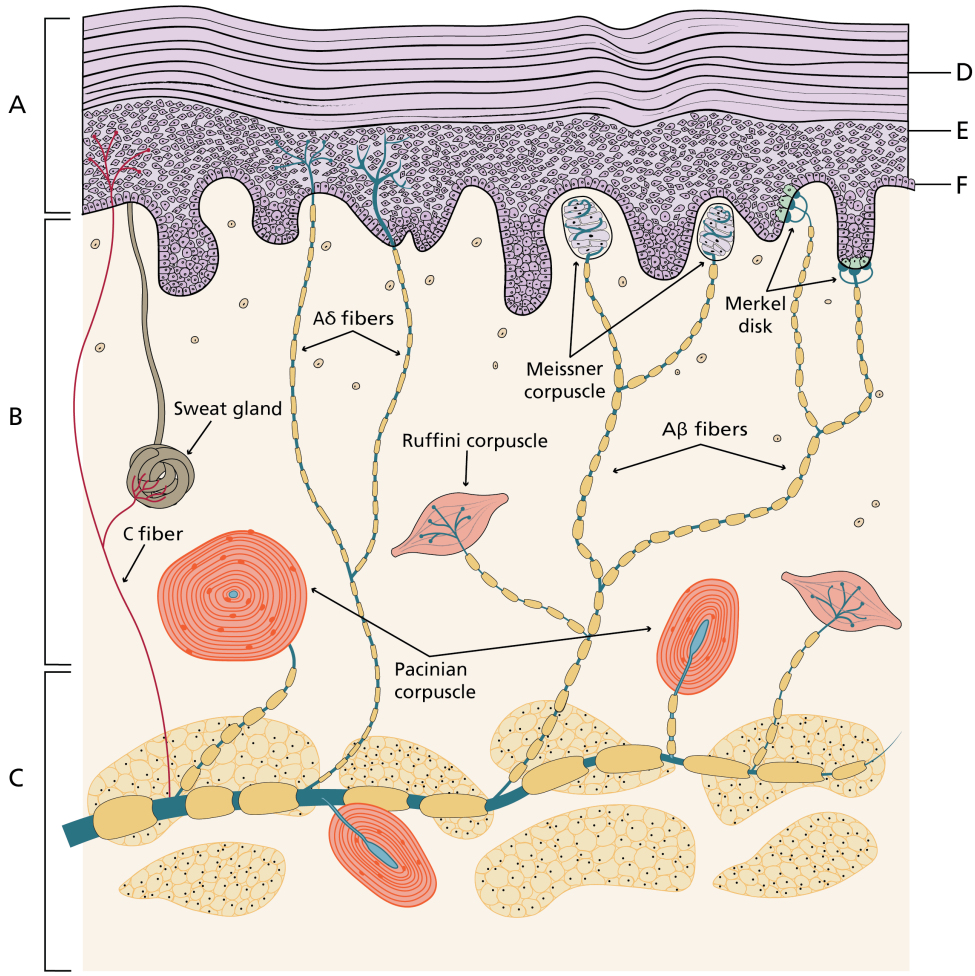



Figure 1.3. Innervation of the skin

Schematic illustration of the nerve fiber types and mechanoreceptors present in the different layers of the skin. The layers include A: epidermis (with D: stratum corneum, E: stratum spinosum, and F: stratum basale), B: dermis, and C: subcutis. Meissner corpuscles are located at the apex of the dermal papillae, while Merkel disks are part of complexes situated in the basal layer of the epidermis (stratum basale). Pacinian and Ruffini corpuscles are found deeper in the dermis. All the mechanoreceptors are innervated by Aβ fibers. Unmyelinated C-fibers innervate sweat glands or extend as free nerve endings into the epidermis, while Aδ fibers can also reach epidermis after first shedding their myelin.

## 1.2. Peripheral neuropathy

Damage or disease to either the axon or the myelin sheath, the primary components of peripheral nerves, leads to peripheral neuropathy. This impairment disrupts normal signal transmission, resulting in a spectrum of symptoms and complications. The manifestation varies depending on the cause, its severity, and the specific nerves affected. The disease pattern reflects the structure and function of the damaged nerves, with clinical presentation influenced by whether motor, sensory, or autonomic nerves are involved, as well as the number of nerves affected.

Affected Aα fibers, i.e. motor neuropathy, result in loss of strength and function of the skeletal muscles. The lower extremities are most commonly affected, leading to impaired postural stability, balance, and gait.[11,12] These challenges in walking and maintaining balance significantly increase the risk of falls. Motor neuropathy can also cause imbalances in the muscle tonus between the flexor and extensor muscles, resulting in foot deformities. Damage to the Aβ fibers results in sensory large fiber neuropathy, characterized by symptoms such as reduced sensibility, paresthesia, and numbness. Instead, when Aδ and/or C-fibers are affected, it is called *small fiber neuropathy* (SFN). Symptoms and dysfunctions of SFN include tingling, burning, and loss of temperature sensation. Although symptoms are very specific for the axon type and its location, it is common for both sensory and motor fibers, as well as for small and large nerve fibers, to be involved, resulting in a *mixed neuropathy*. This complexity further complicates the clinical landscape, contributing to the varied symptoms experienced by affected individuals.

In cases where a single nerve trunk is affected, termed *mononeuropathy*, we find conditions with local deficits, such as carpal tunnel syndrome, i.e. entrapment of the median nerve, and Bell's palsy, an idiopathic facial paralysis. Multiple nerve involvement, termed *polyneuropathy*, will be thoroughly described below.

### 1.2.1 Polyneuropathy

Peripheral neuropathies frequently manifest as distal symmetrical polyneuropathy (DSPN), presenting in a stocking-and-glove pattern where the distal regions of the arms and legs are the first to be affected.[13] There are multiple etiologies of polyneuropathy. Inflammatory conditions, like Guillain-Barré syndrome, infectious diseases such as leprosy, and hereditary disorders like familial amyloid polyneuropathy, all contribute to the diverse etiology of polyneuropathy.

Additionally, acquired metabolic or toxic factors, such as vitamin B12 deficiencies and chronic alcoholism, further underscore the complexity of this condition. Further examples can be found in Table 1.1. In approximately 30% of the patients, signs and symptoms of polyneuropathy are present without any known cause, a condition called

idiopathic polyneuropathy. However, the most common cause of polyneuropathy is diabetes, accounting for around 40% of the cases.[2]

Table 1.1. Examples of causes and underlying diseases associated with polyneuropathy

| Type | | Examples |
|---|---|---|
| Hereditary | | Charcot-Marie-Tooth's disease |
| | | Familial amyloid polyneuropathy |
| Metabolic | | Diabetes mellitus |
| | | Hypothyroidism |
| | | Chronic kidney disease |
| | | Nutritional deficiencies (e.g. vitamin $B_{12}$, $B_1$, folate) |
| Toxic | | Alcohol |
| | | Chemotherapy (vinca alkaloids, taxanes) |
| | | Heavy metals (e.g. lead, mercury, arsenic) |
| Inflammatory | Infectious | Lyme disease |
| | | HIV |
| | | Diphtheria |
| | Non-infectious | Guillan-Barré syndrome |
| | | Amyloidosis |
| | | Connective tissue diseases (e.g. Sjögren's syndrome) |

HIV: human immunodeficiency virus

Patients can suffer from a range of symptoms, including abnormal sensations, such as tingling and numbness, weakness in the extremities, and debilitating pain. The pain, experienced by nearly half of the patients, is associated with burning or stabbing sensations, significantly impacting their quality of life by disrupting sleep, reducing mobility, and limiting daily life activities.[14] Management is mainly focused on the underlying cause along with pain treatment in cases where needed. Pharmacological strategies include selective serotonin reuptake inhibitors (SSRIs), serotonin-norepinephrine reuptake inhibitors (SNRIs), tricyclic depressants, and anticonvulsants with the intention to handle pain and allodynia.

## 1.4. Diabetic polyneuropathy

Diabetic polyneuropathy (DPN) is not only the most common type of polyneuropathy but also one of the most common microvascular complications of diabetes. The prevalence numbers vary depending on diabetes duration, but it is believed to affect up to 50% of all individuals with diabetes mellitus over their lifetime.[15] DPN affects both individuals with type 1 diabetes mellitus (T1DM) as well as type 2 diabetes mellitus (T2DM), and the incidence numbers are approximately three times higher in T2DM compared to T1DM. As the prevalence of diabetes is steadily increasing, from 537 million adults in 2021 to the predicted 783 million by 2045, the related complications are also likely to increase.[16]

Like peripheral neuropathies in general, DPN is mainly manifested as a DSPN, accounting for approximately 75% of all cases.[17] Nonetheless, it is a heterogenous disease with various clinicopathological patterns, such as focal or entrapment neuropathies, and autonomic, as well as motor neuropathies.

### 1.4.1. Definition

The term DPN is employed when the underlying cause of the peripheral neuropathy cannot be attributed to factors other than diabetes mellitus, as stated in the often-used definition "presence of signs and/or symptoms of peripheral nerve dysfunction in people with diabetes after the exclusion of other causes" by Boulton et al.[18] Another more specific definition was proposed by Tesfaye et al. in 2010 on behalf of the Toronto Diabetic Neuropathy Expert Group.[19] This definition is subdivided into possible, probable, and confirmed DPN, and relies on certain criteria for each step. *Possible DPN* requires at least one sign or symptom of DPN, e.g. tingling or burning sensations and impaired ankle reflexes. For *probable DPN*, a minimum of two signs and/or symptoms of the following needs to be present: "neuropathic symptoms, decreased distal sensation, or unequivocally decreased or absent ankle reflexes". To be diagnosed with a *confirmed DPN* however, Tesfaye et al. suggest that the signs and/or symptoms must be confirmed by an abnormal result obtained within nerve conduction studies (NCS; further described later in this chapter).[19]

### 1.4.2. Pathogenesis

A principal risk factor for DPN is, of course, the hyperglycemia brought on by either the underlying insulin deficiency or insulin resistance.[20] The DCCT/EDIC trial showed that improved glycemic control in T1DM can reduce the risk of developing DPN.[21] However, this impact seems less important among patients with T2DM, wherein other metabolic factors lately have gained more focus.[22] The pathogenesis of

DPN is thus complex and multifactorial, involving both microvascular changes and metabolic disturbances. Thereto, the development of DPN can be both dependent on, as well as accelerated by factors such as genetic vulnerability, disease duration, comorbidities, and lifestyle. The role of hyperglycemia, metabolic syndrome, and vascular changes will be briefly described below.

*Hyperglycemia-induced nerve damage*

Since neurons and Schwann cells have free access to glucose, relying on osmosis rather than insulin-dependent receptors, their intracellular glucose concentrations rise parallel with blood glucose levels.[23] Although the complete role of glucose in nerve damage is not fully understood, studies have shown that hyperglycemia disturbs the mitochondrial redox state, which results in the formation of reactive oxygen species (ROSs). These are highly reactive and directly harmful to the cell when abundant.[24] Besides the damage caused by ROSs, hyperglycemia induces several additional metabolic pathways that contribute to the neural damage. In *the polyol pathway*, the enzyme aldose reductase is triggered to metabolize glucose into sorbitol. Increased levels of sorbitol, along with its metabolite fructose, induce osmotic stress within the cell, subsequently prompting efflux of myoinositol.[25] Myoinositol is essential for the $Na^+/K^+$-ATPase, i.e. the sodium-potassium pump, and its depletion within the cell results in impaired axonal transport. The increased aldose reductase activity also induces the formation of additional ROSs, causing oxidative stress and eventually cellular dysfunction.[25,26]

Hyperglycemia also yields an increased glycolysis, resulting in the production of diacylglycerol (DAG). Accumulation of DAGs activates the protein kinase C (PKC), which in turn can cause further damage to the $Na^+/K^+$-ATPase. This is referred to as *the PKC pathway*. The PKC can also contribute to hypoxia and vasoconstriction, by altering gene expression of important growth factors.[25] Moreover, glycolysis triggers a third pathway, wherein the intermediate fructose-6-phosphate accumulates and activates *the hexosamine pathway*.[26] The final metabolite of the pathway, uridine 5-diphosphate-N-acetylglycosamine, can be transferred to specific sites on proteins, thereby modifying their function. Key proteins affected by this modification include the glucose transporter 4 and the insulin receptor substrates 1 and 2.[27] Yet another result of hyperglycemia is increased glycation of proteins, i.e. formation of advanced glycation end products (AGEs), through *the AGE pathway*. Glycation can e.g. affect lipids crucial for myelin production, and proteins involved in cytoskeletal structures, and thus yield both demyelination and axonal degeneration. Additionally, the presence of AGEs induces inflammatory responses, and the glycation of extracellular matrix proteins hinders regenerative activity in the cells.[25]

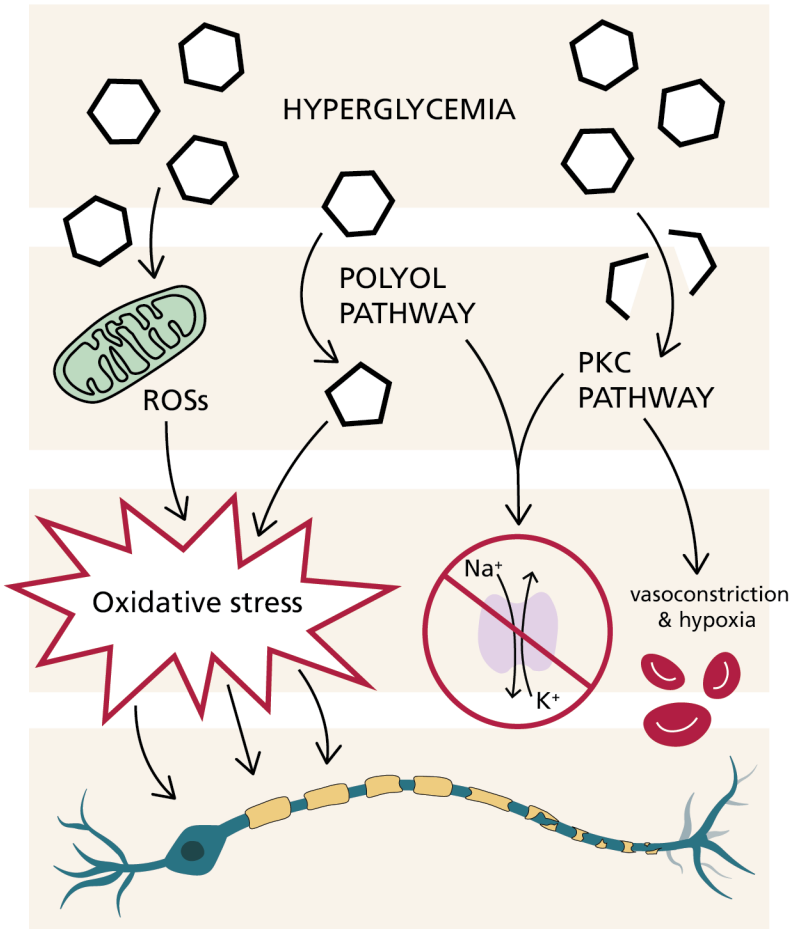



Figure 1.4. Hyperglycemia-induced nerve damage

Illustration depicting a selection of the proposed pathways and pathological processes associated with hyperglycemia and the development of diabetic polyneuropathy (DPN). Excess glucose levels, i.e. hyperglycemia, disrupt the mitochondrial redox state, leading to increased production of reactive oxygen species (ROSs), which in turn cause oxidative stress within the cells. Through the polyol pathway, oxidative stress and damage to the $Na^+/K^+$-ATPase occur. Additionally, the protein kinase C (PKC) pathway exacerbates $Na^+/K^+$-ATPase damage and induces hypoxia and vasoconstriction within the nerve. The combined impact of these processes leads to demyelination and axonal degeneration.

*The metabolic syndrome*

As previously stated, managing hyperglycemia does not prevent the development of DPN in T2DM to the same extent as it does in T1DM. This suggests that other factors are also contributing to the development of DPN. While hyperglycemia remains a likely contributing factor, it operates alongside a range of other comorbidities and risk factors. One of these factors is the so-called metabolic syndrome (MetS), which is a cluster of individual conditions known to increase the risk of stroke and heart disease: abdominal obesity, dyslipidemia, insulin resistance, and hypertension. Both MetS and some of its components have also been shown to be significant risk factors for the development of both T2DM and subsequent DPN.[28] MetS has also been associated with neuropathy in cases with prediabetes, i.e. impaired glucose tolerance (IGT).[29]

Dyslipidemia has been found to trigger altered lipid synthesis, leading to mitochondrial dysfunction such as impaired motility.[30] This, in turn, can result in an insufficient number of mitochondria in the distal parts of the nerve, causing a failure to produce adequate energy for cellular requirements. Eventually, this results in distal axonal injury.[31] In a study on the association between body composition and DPN, abdominal obesity was pointed out as an important risk factor.[32] In addition to metabolic factors, genetics are also believed to contribute to the complexity by increasing susceptibility to developing DPN.[20]

*The microvascular component*

Given that DPN is a microvascular complication of diabetes, the vascular component is of course significant in addition to the pivotal metabolic disturbances. Nerve cells are unique in their structure; being extremely long and thin, stretching e.g. from the spine to the toes. In addition to their distinctive anatomy, neurons also have high metabolic demands and thus depend heavily on energy supply. To meet this requirement, nerve trunks are highly vascularized via the segmentally approaching blood vessels through the epineurial intrinsic blood vessels and the endoneurial capillaries. However, long-lasting hyperglycemia can result in structural changes to the endothelial cells and cause microvascular damage, eventually leading to severe ischemia in neurons.[33]

Vascular changes have been reported to be associated with neuropathy since the 1950s when basal membrane thickening was found to occur in concurrence of DPN. Subsequent research has identified additional microvascular changes, such as endothelial hyperplasia and micro-occlusions, all contributing to reduced endoneurial blood flow.[34,35] Microvascular damage is also fundamental in other diabetic complications, including retinopathy, nephropathy, and impaired wound healing associated with foot ulceration. Moreover, these changes play a crucial role in the development of nerve entrapment disorders, such as carpal tunnel syndrome and nerve entrapment at the elbow, which are relevant also in diabetes.[36,37]

### 1.4.3. The debilitating consequences

The consequences of DPN extend beyond the sensory impairments and include reduced mobility and neuropathic pain. The impaired sensation and proprioception, together with muscle weakness and loss of reflexes, increases the risk of falling and thus, fall-related injuries. A significant fear of falling can arise, causing the patient to decline in mobility even further.[38] The neuropathic pain, in turn, is a distressing symptom of DPN associated with poor responsiveness to treatment.[39] Painful DPN has been reported to affect up to half of all patients living with DPN.[40] Living with chronic pain can yield further concerns, such as reduced ability to perform daily tasks, compromised sleep, and anxiety or depression.[41] Collectively, these issues contribute to a significant reduction in patients' quality of life.

Moreover, DPN is associated with a severe complication that can increase mortality rates, i.e. the development of diabetic foot ulcers.[42] These ulcers arise from trauma or irritation and loss of protective sensation inherent in DPN, often combined with poor circulation. Consequently, patients with DPN are predisposed to acquiring wounds and experiencing impaired wound healing, leading to chronic ulcers. In cases of necrotizing tissues, lower limb amputation may be necessary. In high-income countries, DPN is a leading cause of non-traumatic amputations performed within the general health care system.[43] Thus, the impact of DPN is multifaceted, affecting not only the sensory system or the emotional well-being of patients, but also resulting in serious medical complications that necessitate significant medical interventions.

### 1.4.4. Treatment strategies

There is no cure or disease-modifying treatment for DPN per se. However, managing the underlying disease and associated factors can have preventive effects. This includes not only monitoring and regulating the hyperglycemia but also addressing factors associated with the MetS. The growing use of insulin pump therapy in patients with T1DM is a promising step toward reducing the incidence of multiple diabetes-related complications.[44] Progression of neuropathy has also been shown to reduce or even halt with adherence to lifestyle changes, such as dietary weight loss and supervised exercise.[45,46] Additionally, neuropathy symptom relief has been associated with changes in lipid levels due to lifestyle interventions.[47]

For painful DPN, as well as for painful neuropathy in general, simple analgesics such as paracetamol and NSAIDs have little to no effect.[48,49] Instead, pain can be treated with SNRIs (e.g. duloxetine), tricyclic antidepressants (e.g. amitriptyline), gabapentin, or pregabalin.

## 1.5. Assessment of polyneuropathy

The assessment of polyneuropathy, or the diagnostic methods and routes to diagnosis, can look different based on the type of neuropathy, its potential underlying causes, and whether the onset and course are acute or chronic. For instance, an acute onset of neurological deficits in a previously healthy individual will be managed differently than a slow progression of dysfunction, such as sensory loss and numbness in the toes in a patient with a long history of diabetes. However, there are some general techniques and methods to assess nerve function of both large and small nerve fibers, and these will be described below.

In addition to these technical approaches, it is essential to investigate the patients' clinical history and somatic status. A sample of blood and/or cerebrospinal fluid can be valuable for diagnosing potential vitamin deficiencies or infections. Hence, diagnosis of polyneuropathy can be based on either solely or a combination of clinical findings, symptom scales, and objective measures by different instruments and chemical analyses.

### 1.5.1. Electrophysiology

Electrophysiological studies of the peripheral nerves involve nerve conduction studies (NCS) and electromyography (EMG), two important diagnostic tools for the evaluation of nerve and muscle function. Electrophysiology parameters are both objective and quantitative, and thus often considered the gold standard for assessment of large nerve fiber dysfunction. EMG measures muscle activity in response to an electrical stimulation of the associated nerve.[50] This is done by inserting small needle electrodes through the skin, into the muscle, and upon muscle contraction, the amplitude and pattern of the muscle response can be recorded. By EMG, abnormalities associated with denervation and neuromuscular disorders can be found.

NCS evaluates the function of specific nerves and is performed by applying small electrical pulses against the skin covering the specific nerve you want to evaluate.[51] By exciting and recording the nerves' reaction to the stimuli, neurophysiologists are enabled to evaluate different parameters, such as nerve amplitudes, conduction velocities, latencies, and F-waves. Amplitude, or properly the action potential amplitude, is the magnitude of a nerve signal, and a reduced amplitude indicates that axonal degeneration has taken place. Latency refers to the time for a nerve impulse to travel along the nerve to a specific point and is used to calculate the conduction velocity, i.e. the speed of the nerve impulse. The conduction velocity reflects the level of myelination to the nerve. Thus, a low velocity indicates demyelination of the nerve fibers. Another NCS parameter is the F-wave, a late and low-amplitude response following the main motor response. It is measured in terms of latency and persistence, providing an estimate of the conduction velocity along the full length of the nerve. This

response can indicate the presence of an obstruction along the nerve, even distant from the specific region being tested.

Electrophysiological studies can be helpful in pretty much all PNS disorders since valuable information about potential damage to the nerve and its location can be found. Concerning polyneuropathy assessment of the lower limb, NCS of the tibial, peroneal, and sural nerves can be performed. Motor conduction velocities and response amplitudes are then recorded upon stimulation of the tibial and peroneal nerves, whereas sensory conduction velocity and response amplitude are recorded for the sural nerve.

### 1.5.2. Nerve biopsy

A nerve biopsy assessment involves the removal of the entire nerve, such as a whole sural nerve biopsy, or a small portion of a nerve, like a fascicular nerve biopsy. This is followed by tissue preparation and examination under a microscope. Due to its invasive nature, nerve biopsy is only used when a nerve injury cannot be adequately assessed through non-invasive methods, i.e. exhaustion has been made, and the results are believed to be useful in terms of initiating treatment. Indications for nerve biopsy can e.g. be a suspected peripheral nerve vasculitis or leprosy-induced neuropathy.[52,53] Commonly, a distal part, branch, or fascicle of the sural nerve is used, due to it being a pure sensory nerve that is easily accessible. Again, nerve biopsy is an invasive method with a significant risk for complications, such as chronic pain.[54] Therefore, obtaining well-informed consent from the patient is essential before performing a nerve biopsy. However, the risk should also be related to the severity of the neuropathy, i.e. if the sensory impairment is extensive, the risk may be considered low.

### 1.5.3. Testing the sense of vibration

*Tuning fork*

Vibratory sensation can be evaluated using a tuning fork, mostly with a fixed frequency of 128 Hz. The procedure involves striking the tuning fork against a surface to initiate vibrations, then placing it against a bony prominent on the patient, such as the first metatarsophalangeal joint or the malleolus. The patient is instructed to signal when the vibration has ceased. Sensation is thus evaluated based on duration and compared between the right and left side across the body.[55]

*Neurothesiometer*

Vibration perception thresholds (VPTs) can be measured with a biothesiometer or a neurothesiometer (NT). It is a hand-held device, and its probe is applied against similar locations as the tuning fork, e.g. the bony prominent of the metatarsophalangeal joint

or the medial malleolus. The vibratory output, i.e. the amplitude, is gradually increased by the assessor until the patient reports that they can feel the vibration. The assessor notes the vibratory output, which is measured in volts (electric tension or difference in potential), and then repeats the test.

In Paper II, VPTs were measured with the Horwell Neurothesiometer (Scientific Laboratory Supplies, Nottingham, U.K). This specific NT vibrates within a frequency of 100 Hz.

### 1.5.4. Monofilaments

The Semmes-Weinstein's monofilaments are employed for assessing the cutaneous sensory perception threshold. This tool consists of a variety of nylon treads that are applied perpendicular to the skin for a couple of seconds, repeated three times, with sufficient pressure causing the filament to bend. The test procedure should be performed in a calm and quiet room to minimize potential distractions, and the patients are instructed to indicate when they first perceive the pressure. Semmes-Weinstein's monofilaments are available in a range of pressure forces required for perception, ranging from 0.008 g to 300 g. In clinical practice, a set of five filaments (called 2.83, 3.61, 4.31, 4.56, 6.65) are typically utilized and applied in ascending order of pressure from low to high until the patient detects the sensation. For DPN screening, the sole use of the 10 g monofilament (referred to as 5.07) is recommended and used to identify potential loss of protective sensation.[56,57] The use of monofilaments in the assessment of DPN was validated against VPT testing in 1997, demonstrating reproducibility as well as reasonable sensitivity and specificity for diagnosing DPN.[58] However, a study from 2023 indicated that the diagnostic accuracy of the 10 g monofilament is, contrary to previous findings, poor.[59]

### 1.5.5. Quantitative sensory testing of temperature thresholds

The term quantitative sensory testing (QST) refers to a group of tests that measure sensory thresholds of different modalities, such as discriminative touch, temperature, pain, and vibration. These tests are considered semi-objective because, while the test procedure itself can be standardized, they rely on active cooperation from the patient. The QST battery may include tools like the monofilaments, tuning fork, or neurothesiometer, which have been described above. This section will now focus on the QST of temperature thresholds. Temperature thresholds, such as the detection of cooling or painful heat, can be measured using several devices. In Paper IV, thermal testing was conducted with a device called Thermotest® (Somedic AB, Hörby, Sweden). Testing of cold and heat detection was performed according to the 'method of limits', which means that the stimulus intensity is gradually increased from an undetectable

level up until the patient signals that they can perceive the cold or heat. One of the main advantages of QST is its ability to assess the function of different nerve fiber types; vibration testing evaluates large nerve fibers (Aβ), while cold and heat stimuli target the small nerve fibers (Aδ- and C-fibers, respectively). However, the primary limitation of QST is the patient-dependency.

### 1.5.6. Scoring systems and symptom scales

Different kinds of scales, scores, or questionnaires can be useful for assessing the severity of neuropathy as well as its impact on daily life. Among the common screening instruments, we find e.g. the Michigan Neuropathy Screening Instrument (MNSI) and the Toronto Clinical Neuropathy Scale (TCNS). The MNSI involves a patient-administered part, with simple yes and no questions, as well as a part of physical assessment. The TCNS also consists of both physical assessment as well as questions on present symptoms, although asked by the examiner. In Paper IV, a modified version of the Neuropathy Disability Score (NDS) and Neuropathy Symptom Score (NSS) were applied.

*Neuropathy Symptom Score*

The Neuropathy Symptom Score (NSS) was introduced by Dyck in 1988 and is a tool used to assess the symptoms of sensory disturbances, autonomic symptoms, and muscle weakness, experienced by the patient.[60] It comprises 17 items regarding peripheral motor, sensory, and autonomic neuropathy symptoms. Each item is dealt a score of either 1 point if the symptom is present, or 0 if absent.

The modified version used in Paper IV consists of seven items regarding numbness, abnormal sensation to heat or cold, pins and needles, different kinds of pain (burning, lancinating, and dull), and contact dysesthesia to bedclothes. This modified NSS, which has been previously described, was applied to evaluate incident neuropathy symptoms in the study population.[35,61] Symptoms are graded and scored based on whether they are absent (0 points) or occur occasionally (1 point), regularly (2 points), or frequently at night (3 points). These questions are asked related to both the upper and lower extremities and can thus yield a maximum score of 42 points.

*Neuropathy Disability Score*

The Neuropathy Disability Score (NDS), also introduced by Dyck in 1988, is a tool used to quantify and grade the severity of neuropathy.[60] The NDS involves examinations of the upper and lower limbs, as well as cranial nerves, assessing sensory perception, muscle strength, and tendon reflexes. The individual tests are scored on a scale from 0 to 4, where 0 indicates no deficit. A score of 1 is given for a mild deficit,

2 for a moderate deficit, 3 for a severe deficit, and 4 when there is a complete absence of function. These scores are then summed to create a composite score.

In Paper IV, a modified version of the NDS was applied to assess the severity of neuropathy in the cohort. This modified version of the NDS has been previously described in detail.[61] In summary, the sensory perception tests included light touch (cotton wool), pinprick (needle), vibration (tuning fork), and cold (metal item). These modalities were assessed at arm, leg, knee, and lateral malleolus. Reflexes were tested at the biceps, ankle, and patella, while muscle strength was evaluated for the toe, foot, knee, finger, wrist, and elbow.

## 1.6. Screening for diabetic polyneuropathy

When it comes to DPN, and other neuropathies with clear associations to their underlying disease, objective evidence provided by e.g. NCS is not required in routine clinical practice. Instead, simple and rapid methods are applied, such as the abovementioned 10 g monofilament and tuning fork. With regards to the high risk for patients with diabetes of developing foot ulcers, testing for DPN shall be performed regularly. According to the American Diabetes Association, patients with diabetes should be tested/screened for DPN annually, with the first assessment performed directly after being diagnosed with T2DM, or five years after the onset of diabetes for those with T1DM.[62]

It is however important to note that the 10 g monofilament, and probably also the tuning fork, only detects advanced neuropathy, where the risk of developing foot ulcers is already relatively high. Thus, methods that could detect changes at an earlier stage, but still be useful in a simple screening procedure, are warranted.

## 1.7. The methods in focus for the thesis

In the upcoming two chapters, I will present the two methods that were the focus of my thesis: multi-frequency vibrometry (MFV) and assessment of intraepidermal nerve fiber density (IENFD). Although these methods are not brand new, they are not yet established for nerve fiber testing. Instead, they are under investigation to determine their efficacy and applicability. Both methods hold the potential to provide a comprehensive picture of peripheral nerve health in a relatively simple manner compared to current methods.

The methods differ quite significantly in their approach: MFV investigates large nerve fiber function and requires patient participation, whereas IENFD offers an objective morphological assessment of the small nerve fibers, relying more on the

examiner than on the patient. The goal of my thesis was to establish a reference material for each method and test their applicability in a clinical context to facilitate future use of these techniques in a clinical setting.

# 2. Multi-frequency vibrometry

This chapter will be focused on the first of the two novel methods investigated in this thesis, namely the multi-frequency vibrometry (MFV). I will present the medical background and technical properties of the method, as well as shortly describe the aims, methods, and results for Papers I and II. Lastly, the outcome of the two papers will be discussed and put into a clinical context.

## 2.1. Medical background and motivation

The ability to perceive vibration, known as *pallesthesia*, is mediated through the mechanoreceptors in the skin, as mentioned in the first chapter. The receptors detect rapid, repetitive stimuli and generate action potentials that travel through the afferent nerves to the brain where they are interpreted as a sensation of vibration. Thus, the vibrational sensation can be studied and provide valuable information about the integrity of the pathway, where impaired pallesthesia suggests damage to either the peripheral nerves and their receptors, or the central areas.

### 2.1.1. Vibration perception thresholds

A vibration perception threshold (VPT) refers to the lowest vibrational intensity required to detect a stimulus. Determination of VPTs is a form of quantitative sensory testing that can be used to assess the loss of protective sensation as well as to evaluate large nerve fiber dysfunction. Traditionally, VPTs are measured using a tuning fork or a neurothesiometer. The application of tuning forks for assessing pallesthesia and thus nerve function can be traced back to the late 1880s.[63] A study from 1899 showed that VPTs measured with the 128 Hz tuning fork decreased with increasing age. Five years later, it was found that vibration sensation diminished when peripheral nerves were injured. Both studies are referenced in a study by Gordon from 1936.[63]

Several factors have been shown to impact VPTs, such as age, gender, and anatomical location of VPT testing.[64] Additional factors significantly influencing the thresholds include the frequency of vibration, the size and contact force of the vibrating probe, as well as skin properties, such as temperature.[65-69] To ensure representative measures of

VPTs, it is therefore crucial to adhere to the international standards for mechanical vibrations as outlined in ISO 13091-1.[70]

In contrast to the tuning fork or neurothesiometer, which vibrates at a fixed frequency, MFV is a method applied for VPT measures at multiple frequencies.

### 2.1.2. The history of multi-frequency vibrometry

Multi-frequency vibrometry was invented by the hand surgeon and Professor Emeritus Göran Lundborg and his colleagues. The concept for MFV was founded on the premise that testing across a spectrum of frequencies would provide information about different receptor types and that using additional test sites would offer insights into multiple nerve innervation areas. The examination procedure and tool were adapted from a Békésy audiometer, traditionally used for hearing tests.[71] For this adaption, the headphones were replaced with a probe and a vibration exciter.[72] The probe protruded through a small testing table, against which a finger pulp was placed. Thus, instead of presenting tones for the patient to hear and register, the ability to feel vibrations of different frequencies was tested. The VPTs were measured by gradually increasing the vibration amplitude until the patient detected it and pressed a hand switch, prompting a decrease in amplitude until the vibration was no longer felt, at which point the patient released the switch. This data was continuously plotted on a chart, resulting in a diagram of VPTs measured at different frequencies – i.e. a *vibrogram*.[72]

The device was first utilized in a study comparing VPTs between healthy controls and patients with carpal tunnel syndrome. Vibrograms demonstrated a correlation with symptom severity: mild symptoms, such as periodic paresthesia, were associated with slight impairments at higher frequencies, while more severe symptoms, including abnormal two-point discrimination, correlated with significantly higher thresholds across all frequencies.[72] This study marked the starting point for the tool's application in research, leading to several subsequent studies, with an important focus being patients with hand-arm vibration syndrome (HAVS). One study revealed strong correlations between pathological vibrograms and both subjective symptoms and exposure time to vibration, suggesting that the use of MFV could be suitable for screening purposes.[73] Another study indicated that impaired vibrotactile sense among vibration-exposed workers was linked to decreased grip force and increased cold sensitivity. Clear relationships were also observed between the vibrotactile sense and the results of the Stockholm Workshop Scale, which is today's standard for assessing HAVS symptoms. These relationships further supported the utility of MFV in assessing and evaluating the severity of vibration-induced work.[74] In 1992, a reference population of 171 males of various ages was collected and assessed.[75] The study showed that VPTs increased with both frequency and age, with the most significant age effect observed at the highest frequencies. Based on these reference values, a more objective method to quantify the results was developed.[75,76] Until then, test results had been based on a visual

inspection of vibrogram patterns. The introduction of the sensibility index (SI), which is the ratio between the examined patient's results and age-matched reference values, provided a more objective measure.[76]

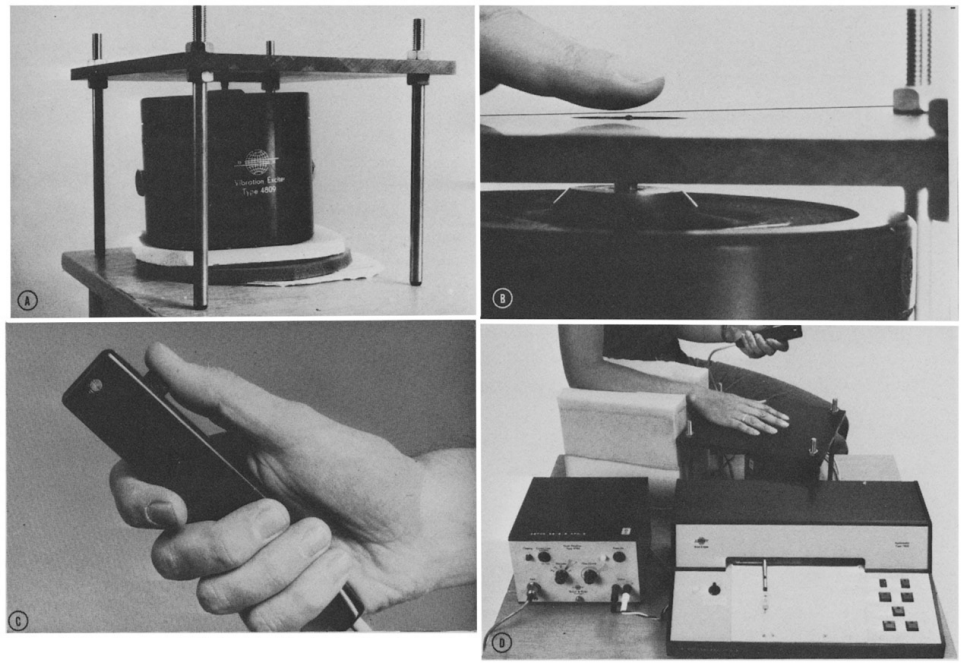


Figure 2.1. The original multi-frequency vibrometer device from 1986
Pictures of the first device built based on a modified Békésy audiometer. A. Vibration exciter. B. Finger pulp placed on the probe protruding through the test table. C. Hand switch used for registering perceived vibrations. D. Full set-up of the device with the recorder in the foreground, plotting the vibrogram in real time. Reprinted from Lundborg et al.[72] with permission from Elsevier.

Succeeding Professor Lars B. Dahlin initiated research on the vibrotactile sense in patients with diabetes, starting with comparisons of VPTs between healthy controls and patients with T2DM and IGT.[77] Given that DPN typically manifests in the feet, a specialized foot device was soon developed. The following studies demonstrated increased VPTs in the feet of patients with diabetes, both T1DM and T2DM, and that impaired low-frequency VPTs correlated with the risk of foot ulcers.[78,79] Impaired vibrotactile sense has also been observed in children and adolescents with T1DM.[80] The follow-up study demonstrated improved VPTs.[81] Monitoring VPT changes over time also led to a test-retest study, aiming to determine whether repeated measurements would yield any practice effects that could impact the interpretation of results. During intense periods of examinations, with tests each month, small VPT improvements were

observed. However, in a clinically relevant test interval of once or twice a year, no or only minor effects were found.[82]

In 2005, the tool and method were commercialized as the VibroSense Meter® when Professor Lundborg and engineer Toni Speidel founded VibroSense Dynamics AB. Throughout this thesis, the first version of the marketed device, VibroSense Meter® I (VSM I), has been employed. The methodology for assessing VPTs using the VSM I will be detailed below. For disclosure, neither I nor my supervisors are affiliated with or have financial interests in the company.

Figure 2.2. Setup of the VSM I device for examination of the hand
The finger pulp is placed on the probe (shown in the center of the right picture) and the forearm is resting against the wooden box. The red button is placed in the contralateral hand of the patient and used for registering the perceived vibrations. Light-emitting diodes indicate the pressure exerted by the finger on the probe, ranging from low to high (lower right-hand corner of the right picture). Pictures adapted from VibroSense Dynamics AB with permission.

## 2.2. Assessment of vibration perception thresholds

Assessment of VPTs with the VSM I can be performed in the pulp of the index and little fingers, as well as at the first (MTI) and fifth metatarsal heads (MTV) in the sole of the foot. In Paper I, both finger pulps and metatarsal heads in the left and right hands and feet, respectively, were investigated. In Paper II, however, only the feet were examined. The designated area is stimulated with a vibrating probe, administering vibrations at seven different frequencies (8, 16, 32, 64, 125, 250, and 500 Hz for finger pulps; 4, 8, 16, 32, 64, 125, and 250 Hz for metatarsal heads).

The subject for examination is instructed to be seated comfortably on a chair in front of the VSM device with either the finger pulp or metatarsal head placed on the probe. The temperature of the examined area is measured either by an internal temperature probe or a hand-held thermometer. Surrounding area temperature is also controlled to be within limit according to the ISO 13091-1 requirements (maintained between 20-22°C).[70] Subjects are briefed on the procedure and instructed to press and hold the

response button upon perceiving vibrations and release it when vibration perception ceases. They are also encouraged to maintain a high level of concentration throughout the test which lasts approximately 4 minutes per test location. The examination is conducted in a secluded and noise-controlled environment to eliminate external disturbances. The subjects are thereto provided with hearing protection and encouraged to close their eyes if needed for further focus.

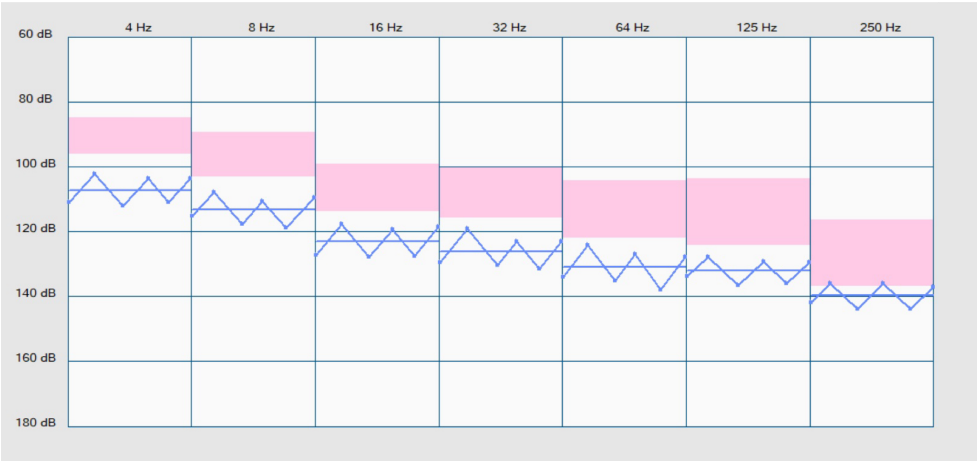



Figure 2.3. Vibrogram showing impaired VPTs in a young female with probable neuropathy
A vibrogram presented in the VSM software after examination of the right MTI using the VSM II device. The VPTs (blue line, with points indicating each press and release of the response button) are increased (note that the dB scale is inverted) compared to the reference values (pink areas).

Vibrations are applied through the probe, measuring 4 mm in diameter, following a von Békésy up-and-down psychophysical algorithm. The acceleration of the probe is expressed in decibels (dB; relative to $10^{-6}$ m/s$^2$). The test procedure starts at a level of 100 dB and then increases with an amplitude ramp rate of 3 dB/s until the subject of examination presses the response button. Pressing the button causes the intensity to decrease at a corresponding speed of 3 dB/s until the subject no longer can perceive any vibrations and thus releases the button again. For examinations of finger pulps, the protocol of measuring VPTs adheres to the ISO standard for mechanical vibration, i.e. ISO13091-1, Method A, conducted without a surround and with a contact force of $0.15 \pm 0.09$ N between the finger pulp and the probe.[70] This contact force is equivalent to a static skin indentation of approximately 1.5 mm. For examination of the feet, no such standardization exists, but a modified version of the method is employed, ensuring consistency in contact force. Visual contact with the probe is precluded as the hand device was shielded, and during metatarsal examination, the foot itself covers the probe.

Before exhibiting the first frequency, a trial recording at the second frequency (16 Hz for fingers and 8 Hz for feet) is provided to familiarize the subject with the

procedure. The examination procedure is then fully automated, with frequencies progressing from low to high. Each frequency is repeated four times, whereas the last three recordings are used to calculate the mean VPT for each frequency. The tests are supervised and recorded using the VSM software on a computer connected to the device. Continuous monitoring is carried out by the operator throughout the test to maintain the force within the required limits, as well as to observe potentially deviant patterns and/or measurements that require a retry. Test results are presented in the VSM software as a vibrogram and SI-values for each frequency measured. Actual VPT values are visible upon data withdrawal from the program.

## 2.3. Aims of the projects

The overall aim for the projects behind Papers I and II was to further lay the foundation for the potential future use of MFV in diagnosing neuropathies. The identified knowledge gaps that were targeted were as follows:

a. Identifying which factors affect VPTs measured with MFV and establishing normative values for VPTs in both hands and feet.
b. Investigating how the technique performs in comparison to a gold standard method, i.e. nerve conduction studies, and if the test procedure could be shortened.

## 2.4. Paper I: Establishing normative values

To establish a reference material for VPTs in hands and feet measured with MFV, recruitment of healthy adults aged 18 years and older began in 2014. After four years and extensive VPT assessment, involving recruitment and travels across southern Sweden, data from 924 individuals were collected. The cohort comprises 628 women and 296 men between the ages of 18 and 90 years. Prior to the examination, participants had completed a questionnaire regarding their medical status and potential symptoms of neuropathy to confirm their health. Direct exclusion criteria included diabetes mellitus or other potential nerve-affecting disorders or injuries. Following data collection, the questionnaires and vibrograms were manually reviewed jointly by the examiner (myself or my predecessors) and the principal investigator of the study (Lars B. Dahlin). Further exclusions of either single frequencies or full vibrograms were made if not reaching the criteria of adequate curve patterns.

As part of establishing normative values, the identification of potentially affecting factors was also pursued. Based on this previous research, the factors of focus in this study were age, sex, height, weight, skin temperature, and hand/foot dominance. The

dependence of age on vibration sensation has been known since the 1920s, as stated and referenced in a study by Wiles et al.[83] Several studies, including the one by Wiles et al. have demonstrated this age-dependency for VPTs assessed with a biothesiometer.[83-85] Additional factors influencing VPTs measured with the biothesiometer involve female sex and taller height, which are both associated with lower VPTs at the ankle.[83,86] A study on the sensitivity to monofilaments observed differences between the dominant and non-dominant hand, prompting the inclusion of foot- and handedness in the statistical models.[87] Lastly, in a study that incorporated a portion of the data from this present study, the VPTs – now measured with the VSM – were shown to increase with increasing age. Differences were also observed between sexes, with men exhibiting higher VPTs than women.[79]

*Ethical statement and statistical analyses*

The study received ethical approval from Lund University (386/2007), and written informed consent was obtained from all participants before the examination. To ensure data privacy and confidentiality, all collected information was decoded, stored securely, and presented at a group level.

Reference ranges for VPT values were presented as mean values with 95% confidence intervals, aiming to ensure with 95% certainty that the population mean was included. Simple and multiple linear regression models were employed to examine potential influencing factors on VPTs. The factors considered were age, sex, height, weight, dominant hand/foot, and skin temperature. Due to a large number of comparisons being made, an alpha level of 0.001 was chosen to minimize the risk of type I errors.

*Results*

The complete results of the study are presented in Paper I. In brief, normative values for VPTs were presented for a large cohort of 913 participants, with data stratified by sex and age decades. These reference ranges can be found in Tables 1, 2, 5, and 6 of the paper, but are also visually illustrated for the male population in Figure 2.4. The regression analyses demonstrated that VPTs deteriorated in association with increasing age, with a decline of 0.1-0.6 dB per year. Additionally, skin temperature showed a negative linear relationship with VPTs, primarily at the finger pulp of the index finger, indicating that warmer skin temperatures are associated with lower thresholds.

*Note*

An erratum for the second table in Paper I is required, as data was omitted during publication. The mean VPT value for the 500 Hz frequency in male subjects between 60 and 69 years of age should be 132.5 dB.

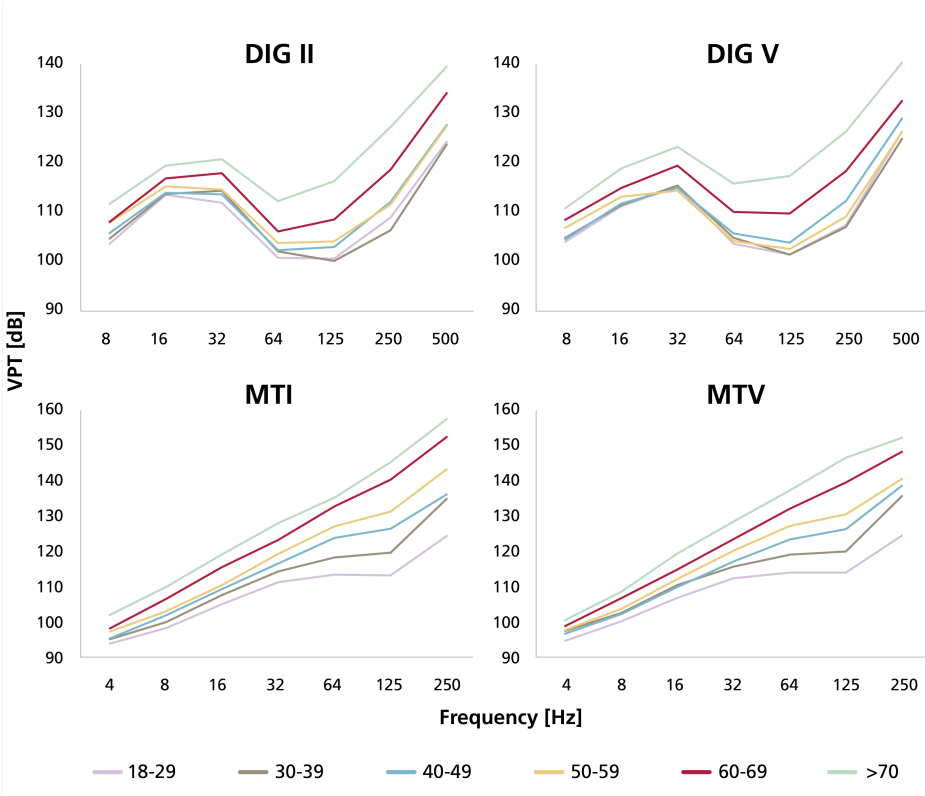



Figure 2.4. Vibration perception thresholds assessed with multi-frequency vibrometry
Mean vibration perception thresholds (VPTs; measured in decibels, dB) of the male subjects from Paper I, stratified by age (years) in categories according to the color scheme at the bottom. Data are presented for seven different frequencies (indicated at the X-axis) in each of the four test sites: index (DIG II) and little (DIG V) fingers, as well as first (MTI) and fifth (MTV) metatarsal heads.

## 2.5. Paper II: Comparing the method with a gold standard

When introducing a new method, especially when considering its use in a clinical setting, it should demonstrate that it is at least comparable to, if not better than, existing methods already available on the market. As mentioned in the first chapter, the gold standard diagnostic test for peripheral nerve injuries and disorders is the NCS. Compared to NCS, the assessment of VPTs offers potential advantages in terms of accessibility and cost-effectiveness. The ease of performing VPT assessment with the MFV makes the method feasible in various clinical contexts, as the device is relatively small, portable, and simple to use. Although not directly comparable to the NCS, since it is not a fully objective method, VPT assessment could potentially serve as an effective

screening tool. Regardless of whether diagnoses are based on VSM results or followed up with NCS examinations, the number of referrals to neurophysiologists could be drastically reduced. However, both NCS and VPT assessments through MFV are time-consuming and, consequently, more costly. As part of the project, efforts were made to explore whether the test procedure could be shortened by reducing the number of frequencies tested. A previous study suggested a minimum of four frequencies and preferably a combination of both low and high frequencies to be studied.[79]

In addition to NCS, VPT measurements using MFV were compared to those obtained with the NT. Although not considered a gold standard, NT is commonly used for assessing DPN. Given that the study was based on individuals with T1DM, including the NT in the battery of tests was a logical choice. Compared to NCS, the NT offers even greater advantages over the MFV in terms of accessibility, ease of use, and cost. However, it also has limitations, such as measuring VPTs only at one frequency and being used at bony prominences of the feet, like the medial malleolus, which are not sensory areas. Additionally, the use of NT introduces subjectivity not only based on the examined person but also from the examiner, as the fact that the device is handheld can result in varying applied pressures by the probe against the skin.

*Ethical statement and statistical analyses*

Ethical permission was obtained from Lund University (386/2007, 2015/683, 2017/386). Study participants were verbally informed about the study and provided written consent.

Correlations between VPTs and NCS parameters were analyzed using the Spearman Rank Correlations test due to non-parametric data. To test how the test procedure could be shortened, and which frequencies should be used, MFV values were converted to z-scores and then combined in four different models. A z-score is a measure of how many standard deviations a value deviates from the mean. It is calculated by subtracting the population mean ($\mu$) from your value ($x$) and dividing it by the standard deviation ($\sigma$), as follows: $z=(x-\mu)/\sigma$. The population means and standard deviations were collected from age- and gender-matched data (i.e. data from Paper I). The models consisted of either all frequencies (Model A), the two highest frequencies (125 and 250 Hz; Model B), the two lowest frequencies (4 and 8 Hz; Model C), or a combination of the two lowest and the two highest (Model D).

*Results*

A total of 66 individuals with T1DM were examined through VPT assessment in the feet, using both NT and MFV, along with NCS of the tibial, peroneal, and sural nerves. The results are detailed in Paper II, but a summary follows. Based on a blinded review of NCS results, 50% of the cohort was diagnosed with DPN. All VPTs were significantly higher ($p<0.0001$) within the DPN group, as demonstrated in Table 2.1, and moderate to strong negative correlations were found between all VPTs and NCS

parameters. The strongest correlation was found between the 125 Hz frequency at MTV and the amplitude of the sural nerve action potential.

Different combinations of z-values were applied and compared with the NCS outcome. Regarding the test procedure and frequency selection, a combination of two low frequencies (4 and 8 Hz) and two high frequencies (125 and 250 Hz) demonstrated the highest efficacy in classifying subjects as healthy or having DPN. In the search for a cut-off value, a z-score of 0.5 yielded the highest Youden index, with sensitivity and specificity values of 79% and 76%, respectively.

Table 2.1. Vibration perception thresholds at the feet in individuals with and without DPN

| | Frequency | No DPN | DPN |
|---|---|---|---|
| MTI | 4 Hz | 93.9 (90.2-101.7) | 100.9 (96.8-109.9) |
| | 8 Hz | 101.7 (95.9-108.7) | 110.4 (105.2-118.0) |
| | 16 Hz | 109.4 (104.7-117.2) | 118.5 (112.7-127.2) |
| | 32 Hz | 116.6 (111.5-122.9) | 127.1 (121.0-132.7) |
| | 64 Hz | 123.2 (112.8-130.5) | 135.0 (130.0-141.8) |
| | 125 Hz | 129.2 (117.8-139.3) | 145.2 (137.9-152.5) |
| | 250 Hz | 138.7 (126.8-148-7) | 158.3 (149.2-165.3) |
| MTV | 4 Hz | 91.6 (87.2-99.0) | 100.4 (96.6-106.5) |
| | 8 Hz | 99.9 (95.2-107.2) | 110.1 (104.9-117.1) |
| | 16 Hz | 108.3 (104.4-116.2) | 119.7 (114.2-125.6) |
| | 32 Hz | 117.0 (110.4-122.3) | 125.8 (121.8-133.4) |
| | 64 Hz | 124.7 (114.8-130.0) | 134.0 (130.6-140.5) |
| | 125 Hz | 126.4 (117.1-139.4) | 142.8 (138.2-147.3) |
| | 250 Hz | 141.8 (128.5-153.6) | 156.2 (150.2-163.3) |

Median ($25^{th}$-$75^{th}$ percentile) vibration perception thresholds (VPTs; expressed in dB) assessed with multi-frequency vibrometry at the first (MTI) and fifth (MTV) metatarsal heads of the feet in individuals with type 1 diabetes, either with or without diabetic polyneuropathy (DPN). Comparisons with the Mann-Whitney U test showed that VPTs were significantly higher in the DPN group compared to the group of no DPN for all frequencies (all $p<0.0001$).

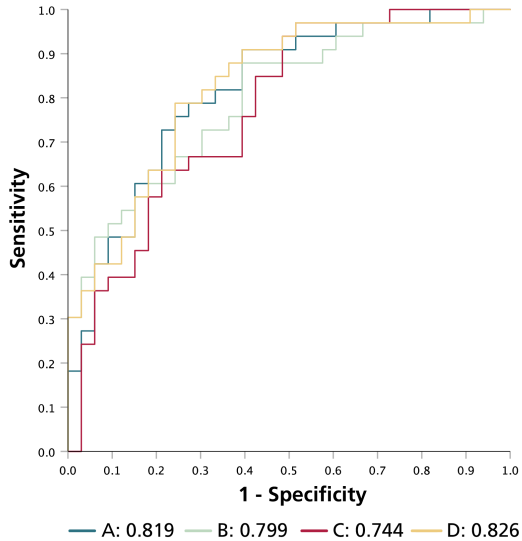



Figure 2.5. ROC curves for DPN classification of four different MFV models
Receiver operating characteristics (ROC) analyses using diabetic peripheral neuropathy (DPN) status (determined by nerve conduction studies) as the reference variable, and combinations of frequencies tested with multi-frequency vibrometry (MFV) in four different models as the predictor variables. Model A includes all frequencies, Model B focuses on the two highest frequencies (125 and 250 Hz), Model C includes the two lowest frequencies (4 and 8 Hz), and Model D combines the two highest and two lowest. Each model is presented with a specific color in the ROC plot, see the color scheme at the bottom where also area under the curve (AUC) values are displayed.

# 2.6. Discussion

In the present section, the potential use of MFV will be discussed alongside the results of the two papers presented in this chapter. A broader discussion on the implementation of new methods, strengths and limitations of all the studies included in the thesis, as well as future directions, is provided in Chapter 4.

## 2.6.1. Assessing VPTs at multiple frequencies

As illustrated in Figure 2.4, the pattern of VPTs across frequencies differs markedly between the finger pulps and the metatarsal heads. First and foremost, VPTs are also measured over slightly different frequency ranges – 8 to 500 Hz for the hand, and 4 to 250 Hz for the foot. This variation is based on the distinct dynamics of sensation in the hands and feet, likely influenced by the distribution and density of mechanoreceptors, such as Pacinian and Meissner corpuscles.[66] The choice of frequencies for testing is thus driven by the sensory properties of human skin, but also

by the technical limitations of the instrument. To be able to perceive the 500 Hz frequency in the feet, the instrument would need to generate much greater forces with consequences being e.g. a high-pitched sound. In fact, similar sounds occasionally arise within the VSM I device when patients are unable to detect the highest frequencies until very large amplitudes are applied. The sound causes distraction, and therefore it is of great importance to consider the use of hearing protection during the test procedure for more accurate test results.

Secondly, the pattern of the vibrogram presents with notable differences between hands and feet (Figure 2.4). The VPTs for metatarsal heads exhibit a consistent linear increase in VPTs as the frequency rises. In contrast, the vibrogram for finger pulps shows a more intricate pattern, where VPTs increase from 8 to 32 Hz, then decline at 64 and 125 Hz, before rising again at the higher frequencies. This distinct non-linear pattern for the finger pulps was documented already in the work presented by Lundborg et al. and has also been confirmed in subsequent studies.[72,88] The different patterns of the vibrograms are probably, and again, the result of the distinct distributions and densities of mechanoreceptors in hands compared to feet.[66,89,90]

### 2.6.2. Potential impact of age and skin temperature on VPTs

In Paper I, a significant association was found between increasing age and impaired VPTs across all seven frequencies measured at each site. Hence, the results were in alignment with the previous research, although many of these studies were conducted with fewer frequencies and on different instruments, such as the biothesiometer.[79,83-85] Aging is characterized by several biological hallmarks, including cellular senescence, mitochondrial dysfunction, altered intercellular communication, loss of proteostasis, and chronic inflammation.[91] These processes also affect the PNS, where nerves may suffer from e.g. neuroinflammation, disrupted axonal transport, and reduced expression of essential proteins, leading to demyelination and axonal degeneration.[92] Additionally, loss of Meissner corpuscle and Merkel disks has been observed with aging.[93] These degenerative changes result in functional declines, such as slower nerve conduction velocities and reduced sensory discrimination. Thereto, the ability for axonal regeneration declines with age, further exacerbating nerve degeneration.[92,94-96]

Another notable observation from Paper I was the association between lower skin temperatures and higher VPTs, i.e. decreased sensitivity. The impact of skin temperatures on VPTs has been well-documented in previous studies.[97,98] For instance, Verrillo et al. demonstrated a gradual increase in vibrotactile sensation with rising temperatures from 15°C to 30°C, but no further changes were observed beyond 30°C.[98] In Paper I, participants' skin temperatures were ensured to be maintained within a range of 20°C to 37°C.[70] Thus, quite some space was allowed for variation within the temperature range, and it is therefore reasonable that the association was observed. The effect of temperature on nerve function is attributed to altered ion

channel function, as described by Kiernan et al.[99] Higher core temperatures accelerate temperature-dependent sodium channel activation, ultimately resulting in increased conduction velocity. Although skin temperatures are likely to be lower than the core temperatures, Burke et al. suggest that measures at the skin can serve as a qualitative indicator of the temperature in the nerve when examining nerve function through the skin surface.[100] This relationship further supports the notion that skin temperature is an important factor to consider in sensory assessments.

Despite the associated factors being found in Paper I, a lot is left to explore regarding differences in VPTs. Results of the statistical analyses yielded a maximum explanation rate of 29% in the finger pulps and 42% at the metatarsals. These numbers suggest that explanatory factors are missing. In the paper, we suggested that anatomical variations and within-subjects variability could be potential factors.

### 2.6.3. Diagnostic utility of MFV

The MFV has been employed to study VPTs across various conditions and diseases, some of which have been discussed earlier in this chapter, including carpal tunnel syndrome and diabetic neuropathy.[72,78,80,101] Additionally, MFV has been used to assess VPTs in conditions not previously mentioned in this thesis, such as chemotherapy-induced neuropathy.[102-104] However, the use of MFV for assessing HAVS has perhaps gained the most traction. According to VibroSense Dynamics, nearly all Occupational and Environmental Medicine departments at the university hospitals across Sweden and Norway are now incorporating the VSM into their diagnostic protocols for HAVS investigations. Beyond the work with the VSM, other studies have demonstrated beneficial results when applying the assessment of VPTs at multiple frequencies. For instance, Rolke et al. demonstrated that VPT assessment, across a range of frequencies from 2 to 600 Hz, was a far more sensitive parameter for the detection of HAVS in comparison to measures of both temperature thresholds and NCS.[105]

Regarding the potential use of MFV for diagnosing DPN, the thesis by E. Ising suggests that MFV could constitute a valuable tool for screening of impaired vibrotactile sense – which could serve as an indicator of DPN – in children and adolescents with T1DM.[106] Ising's work also aligned with the findings in Lindholm et al. where VPTs of the lower frequencies showed to be the best predictors for the risk of DPN and subsequent foot ulcers.[79,106] In the study by Lindholm et al., the use of two lower and two higher frequencies was suggested to be included in an examination of DPN. The results from Paper II further confirmed this, as a combination of the two lowest and two highest frequencies yielded the highest agreement with the diagnosis of DPN, as assessed by NCS. However, the AUC levels were not convincingly high, and not all individuals identified as having DPN through NCS presented with VPT values above the suggested cut-off, i.e. a z-score of 0.5. This cut-off value was thereto

unexpectedly low, which may indicate that a further refinement of the testing method or comparisons are needed to improve a future diagnostic accuracy.

Moreover in Paper II, the 125 Hz frequency demonstrated the strongest correlation with NCS parameters, and particularly with the sural nerve amplitude. This further aligns with previous studies, where VPTs of the higher frequencies (125, 250, and 500 Hz) were found to be elevated in patients with diabetes, even those with relatively short disease durations of less than ten years.[79,107] One of these studies also showed that VPTs at the higher frequencies correlated well with clinical scores but not with NCS. These findings could collectively suggest that both the low and the high frequencies are valuable indicators for DPN, but also that these might reflect different aspects of the condition – something that potentially is not detectable through NCS.

*Advantages*

The possible benefits of using MFV as a diagnostic tool are several, starting with the fact that the test procedure is entirely non-invasive and harmless. Compared to more invasive procedures like skin or nerve biopsies, which carries certain risks and discomfort, as well as the NCS that some patients find unpleasant – VPT assessment using MFV is gentle and well-tolerated. At worst, patients might find the sensation slightly ticklish, which can be amusing rather than uncomfortable.

Another important advantage of MFV is the ability to test VPTs at multiple frequencies, which provides a more comprehensive assessment than traditional methods for VPT assessment such as the tuning fork and biothesiometer. This broader approach allows for the evaluation of different mechanoreceptors and innervation areas, which may be relevant in different situations. Additionally, MFV offers the capability to compare a patient's current results with their previous data. Although this is a feature available also for other diagnostic methods, the straightforwardness of comparing VPTs across different examinations could be useful for monitoring disease progression and treatment effectiveness in the future. Perhaps the most significant advantage is that MFV is applicable in most clinical contexts. It is easy to maneuver, simple to use, practically automated, and relatively time-efficient, making it a practical choice for busy healthcare settings.

*Disadvantages*

There are also some limiting properties and weaknesses with MFV. One notable drawback is the lack of randomization in the frequency presentation. Unfortunately, the VSM device presents the frequencies in a fixed order, from low to high, which raises the potential for a practice effect. This occurs when the patient develops a familiarity with the test procedure and alters the response based on prior stimuli. All test frequencies begin at the same baseline level of 100 dB, and higher frequencies generally demand higher amplitudes to be detected. Detection is thus expected to be delayed when frequencies are sequentially increased. However, after the first two or three

frequencies, which are often detected immediately, it is sometimes observed that the patient registers detection before any perceivable vibration has been presented. A randomized task presentation would perhaps mitigate this pattern of false registrations. On the other hand, practice effects were investigated in a previous study and considered to be non-existent when testing is performed over clinically relevant time intervals.[82] It is also important to highlight that the potential issue of premature registrations often is compensated by the fact that the first registered VPT point for each frequency is automatically deleted and not part of the test result. Additionally, the frequency can be rerun if necessary.

Another limitation is the subjectivity of the test procedure, where the patient needs to be actively participating for the test to be performed. Test results can be easily affected by disturbing factors, such as noises, discomfort, or lack of concentration. Additionally, the communication between the observer and patient can be limited by other factors such as language barriers.

# 3. Intraepidermal nerve fiber density

In this chapter, I will delve into the second method investigated throughout this thesis, i.e. assessment of intraepidermal nerve fiber density (IENFD) in skin biopsies. As in the previous chapter, the medical background and technicalities will be discussed, followed by a brief description of the aims, methods, and results from Papers III and IV. At last, the results of the papers and other aspects of the method will be discussed.

## 3.1. Medical background and methodology

### 3.1.1. An evolving method

The technique for evaluation of nerve fiber density within the epidermis, referred to as IENFD, has undergone significant evolution in recent decades. The first protocol for assessing IENF in the skin was published in 1959 by Arthur and Shelley.[108] It was a rather complicated technique where the skin was studied from above, by scrolling up and down through the depth of a thick section. A new protocol of quantification was introduced in 1995 which overcame the issues with the prior technique. This new protocol became feasible due to the findings of two research groups at the Karolinska Institute in Stockholm. There, antibodies against the Protein Gene Product (PGP) 9.5 showed to be a superior agent for visualizing nerve fibers in the skin, compared to previously used antibodies and neuropeptides, when using bright-field immunohistochemistry.[109,110] The cytoplasmic protein PGP 9.5 is part of the ubiquitin hydrolase family and is highly expressed in neurons and their projections. It currently offers the most reliable and selective staining for cutaneous innervation.[9]

The developing methodology caught the attention of neurologists, leading to the first studies investigating skin biopsies to find an explanation for why patients with normal NCS and nerve biopsy findings could still suffer from neuropathic pain in their lower limbs.[111,112] These studies suggested that the symptoms could be linked to a reduced amount of small nerve fibers in the epidermal layer of the skin, i.e. SFN. These fibers are crucial for thermal sensation and nociception, and thus when impaired, can yield symptoms such as allodynia and hyperalgesia.

Shortly after, guidelines detailing the procedure for conducting skin biopsies with subsequent assessment of IENFD were published by a task force under the European Federation of Neurological Societies (EFNS).[113] The recommended methodologies for obtaining skin biopsies and preparing the tissue for IENFD assessment – i.e. immunohistochemistry or immunofluorescence – will be thoroughly described in the section below. Normative reference values obtained with the two different methods were published in 2010 as well as in 2016. These studies revealed that IENFD declines with age and that it also tends to be slightly higher among women.[114,115]

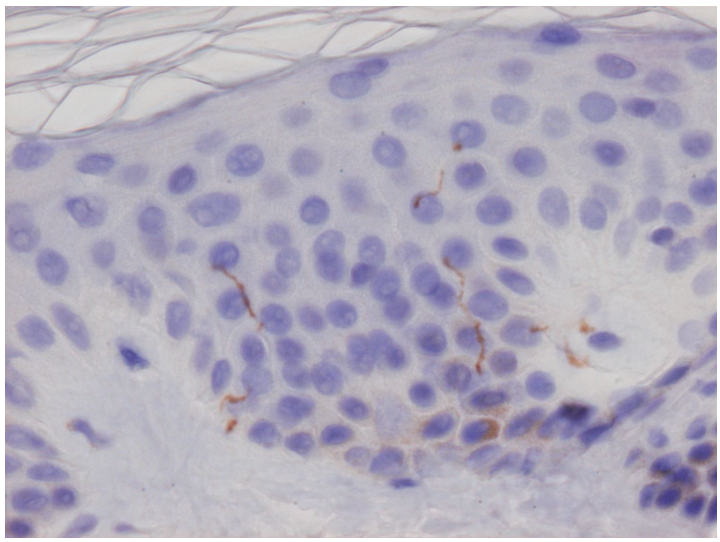


Figure 3.1. Photography of a 5 µm skin section immunostained with PGP 9.5
Cross-section of a skin biopsy stained with hematoxylin-eosin, showing the epidermal layer (with the stratum corneum visible in the upper left corner). The application of immunostaining with an antibody against protein gene product 9.5 (PGP 9.5) enables visualization of nerve fiber material, which appears in brown in the photograph.

### 3.1.2. The EFNS guidelines

For diagnostic purposes in polyneuropathy, as per the recommendations of the EFNS guidelines, a 3 mm punch skin biopsy is obtained from the distal leg.[113] In the case of investigating a length-dependent loss, which is typical for axonal polyneuropathies, a biopsy can also be obtained from the proximal thigh for comparison. The guidelines do not provide information about anesthesia, wound closure, and aftercare, but indicate that the procedure is safe if performed using processes that ensure sterility and hemostasis. Typically, the biopsy is taken under local anesthesia, such as by lidocaine, and the wound is closed using either sutures or surgical tape. The biopsy should be immediately fixed in either 2% paraformaldehyde-lysine-periodate (PLP) or Zamboni's

fixative (2% paraformaldehyde, picric acid). Skin sample preparations and IENFD assessment can then be performed through either bright-field immunohistochemistry or immunofluorescence with or without confocal microscopy.

According to the EFNS guidelines, an assessment of IENFD should be performed in at least three sections of 50 µm thickness, stained with the PGP 9.5-antibody. Fibers are counted at high magnification (40×) as single fibers if they cross the dermal-epidermal junction. Nerve fibers that branch below the junction and project into the epidermis individually are counted as two (or more) fibers. Branching above the junction is not taken into consideration, with the nerve fiber still counted as one. Fragments in the epidermis without visible crossing of the junction are not included in the counting. To obtain the nerve fiber density, the number of fibers detected is divided by the length of the section measured in millimeters, i.e. IENF/mm.[113]

### 3.1.3. From 50 to 5 micrometers

When the technique for assessment of IENFD was introduced in our research group, adjustments were an inevitable necessity. In Sweden, and possibly also in many other countries, clinical pathology laboratories do not have protocols for tissue handling that align with the guidelines from EFNS for diagnostic testing of neuropathies.[114] Implementing these protocols would be impractical for policy reasons due to the complexity, cost, and high technical expertise required. This issue, along with other challenges associated with handling thick cryosections – such as the tendency of 50 µm specimens to fall off the objective glass during preparation due to their weight – highlighted the need for a more feasible approach.

Using paraffin-embedded tissue blocks and performing immunohistochemical staining on 5 µm sections is a technique that likely exists in, or can be readily adopted by, all clinical laboratories. This method offers several advantages, such as improved time stability of paraffin-embedded samples, allowing for long-term storage and re-assessment, which is more challenging with cryosections. The immunohistochemical approach and assessment criteria for this method are detailed below.

*Immunohistochemical skin tissue processing*

Skin biopsies, or excisions, are immediately fixed in a 4% buffered formaldehyde solution for at least 24 hours. At the clinical pathology laboratory, samples are dehydrated and embedded in paraffin. These paraffin-embedded tissue blocks are sectioned using a microtome into 5 µm slices, which are then mounted on positively charged glass slides for immunohistochemical staining. The sections are dried for one hour at 60° C, followed by de-waxing and rehydration. Microwave pre-treating in 10 mM citrate buffer (pH 6.0) for 19 minutes at 750 W is performed to achieve antigen retrieval. Immunohistochemical staining is performed using an automated

immunostainer (TechMate 500 Plus; Dako). The rabbit polyclonal PGP 9.5 antibody (Cell Marque, Rocklin, USA) is used as the primary antibody at a dilution of 1:3000.

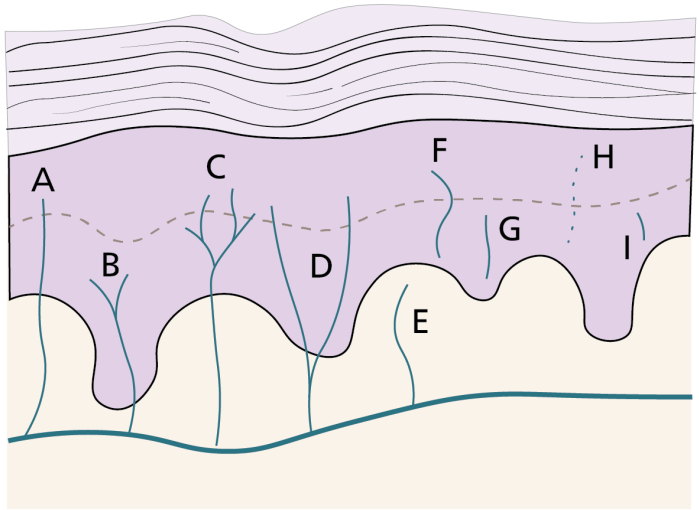



Figure 3.2. Assessment criteria for IENFD

Fibers are counted as one if they are at least as long as half the width of the epidermal layer (stratum corneum excluded; indicated by the dashed grey line), regardless of whether they visibly cross the dermal-epidermal junction (A) or not (F). If a nerve fiber branches within the epidermis, it is counted as one (C), while fibers branching before crossing the junction are counted as separate fibers (D; two fibers). Fiber fragments are counted if they form a pattern consistent with a coiling or winding fiber (e.g. in a dotted line, H) suggesting that the fiber is twisting through the section. Individual fibers or small fragments that do not reach a substantial length are not counted (B, G, and I). The IENFD is calculated by dividing the number of nerve fibers detected by the length of the epidermis in millimeters, resulting in a unit of fibers/mm.

*Criteria for assessment of intraepidermal nerve fibers*

The shift to thinner sections required a revision of the assessment criteria. In the 50-µm sections, the three-dimensional shape of the nerve fibers, with branches extending in multiple directions, can be easily visible. The assessment criteria for these thicker sections emphasize counting each "tree" structure as a single nerve fiber and require a clear visual crossing of the dermal-epidermal junction for fibers to be counted. With thinner sections, however, it is not possible to achieve a complete visualization of the nerve fibers and their arborized pattern. Instead, the three-dimensional structure is likely to be fragmented. Consequently, the assessment criteria had to be revised and rewritten to accommodate these limitations and were initially as follows: individual epidermal fibers are counted if their length is at least half the width of the epidermal layer in the examined area. Fibers that branch within the epidermis are counted as a single nerve fiber, while those that branch in the dermis are counted for each fiber that crosses the dermal-epidermal junction. Even well-defined fibers within the epidermis

that do not visibly cross the junction are still counted, provided that all other criteria are satisfactorily met. The assessment criteria are further detailed in Figure 3.2.

Within the Lund/Malmö research group, six papers have been published on this method for IENFD assessment using 5-µm sections, two of which are included in this thesis. The early studies from 2009 focused on comparing IENFD between individuals with diabetes and healthy controls.[116,117] Since then, IENFD has also been correlated with the advanced imaging techniques of nanotomography and corneal confocal microscopy (CCM).[118,119] However, a crucial missing piece in advancing this work has been the ability to compare samples against a reference material, which was one of the objectives of this present thesis. During the process of establishing that reference material, issues with the existing assessment criteria were identified, prompting the initiation of further development of the assessment method. These developments are detailed in Paper III and will be further discussed below.

## 3.2. Aims of the projects

The overarching aim of the projects outlined in Paper III and Paper IV was to establish a foundation for the potential future application of IENFD for diagnostic purposes. The identified knowledge gaps and specific aims of the studies were:

a. Establishing normative reference values of IENFD assessed in thin sections of 5 µm, and exploring the impact of age, sex, and site of excision.
b. Investigating the temporal trend of IENFD and associations between IENFD changes and metabolic factors in individuals with and without T2DM.

## 3.3. Paper III: Establishing normative values

It is challenging and time-consuming to enroll healthy individuals to establish a reference material. Consequently, prior normative reference values on IENFD have been developed through multi-center collaborations. However, we employed a different approach to overcome these difficulties. By utilizing material that had already been obtained and stored according to the Swedish Biobank Act, unnecessary harm and/or discomfort to study participants could be avoided. Specifically, we searched among archival samples at the Department of Pathology in Lund and Malmö. These skin samples, originally collected for investigating skin lesions and tumors such as squamous cell carcinoma and malignant melanoma, are often taken with wide margins to ensure radical removal. As a result, these samples include end snippets of benign tissue. The careful selection of samples to include in the study was based on clinical as well as morphological data: the cause of referral, established diagnosis, and the availability of

an adequate amount of benign material. Morphological findings (confirmed by pathology reports) of inflammatory lesions, e.g. eczema psoriatic lesions, and infectious foci, prevented inclusion. Medical records for all the individuals included in the study were reviewed to identify potential neurotoxic conditions, disorders, or treatments. Exclusion from the study based on medical records was primarily due to diabetes (T1DM and T2DM) or prior cytostatic treatment.

*Sample site*

The previously published reference materials for IENFD in 50-µm sections primarily focus on the distal leg. However, multiple studies have indicated the presence of a proximal-distal gradient of IENFD in the legs.[120-122] Furthermore, a previous study within my research group highlighted interesting findings regarding sex and skin type differences in IENFD at the wrist level.[116] These discoveries fueled the interest in establishing normative values for IENFD at various sites in both the arms and legs. Initially, the plan was to comprehensively map IENFD along the limbs, but due to referral texts being vague or lacking in detail, this approach had to be reconsidered. Instead, samples were categorized into upper and lower arm, and upper and lower leg, as illustrated in Figure 3.3.

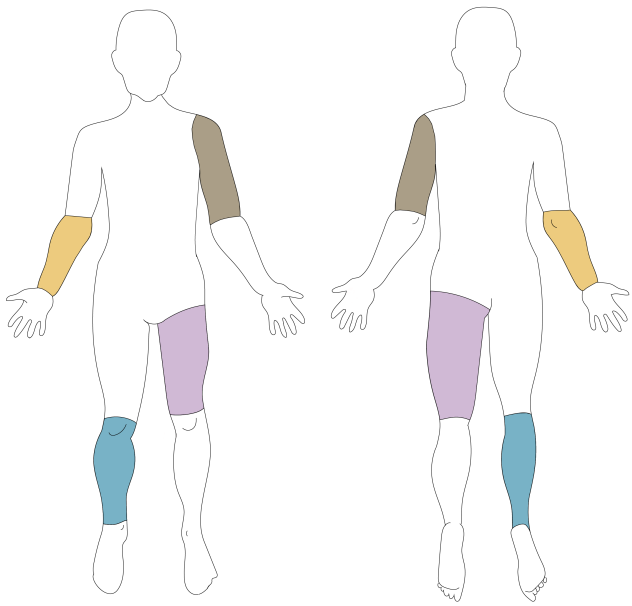


Figure 3.3. Illustration representing the four anatomical sites of excision used in Paper III
Proximal (brown) and distal (yellow) arms, as well as proximal (purple) and distal (blue) legs.

*New assessment criteria*

As mentioned above, the assessment criteria for nerve fiber counting were reconsidered during this project. After evaluating approximately one hundred sections, a pause was taken. At this point, it had become evident that many nerve fibers were being missed due to the requirement that fibers must reach a length of at least half the width of the epidermal layer (excluding the stratum corneum). Discussions about potential modifications to the criteria were initiated, and various approaches were tested. In the method for 50-µm sections, there is no length requirement for nerve fibers to be counted. The length criterion was implemented in the early studies on thin sections to account for their reduced thickness. Eliminating this criterion would likely result in the inclusion of numerous fragmented fibers and background debris, which may not represent actual nerve fibers. Instead, it was decided to maintain a minimum length requirement. At the Department of Pathology in Lund and Malmö, histological samples are scanned and reviewed digitally using the medical imaging software Sectra IDS7. This program enables precise digital measurements, allowing for accurate assessment of both the length of the epidermis and the nerve fibers. Through testing various lengths, we settled on a minimum length of 15 µm. A 20-µm minimum was deemed too long, with the risk of excluding many fibers, while a shorter length proved impractical due to the difficulty in distinguishing fibers from debris. Moreover, 15 µm is approximately the combined length of two adjacent keratinocytes, making it a suitable and easy-to-use benchmark also for assessments through conventional light microscopy. The new assessment criteria, further detailed in Figure 3.4, were tested on a portion of the included samples and evaluated through comparisons between observers. Intra-observer reliability was considered high (no statistical testing was performed), and the consensus was that these criteria were easy to implement without any further adjustments. Consequently, the approximately hundred samples already assessed were reassessed using the new criteria. Once the reassessment was completed, the inclusion of additional samples could resume.

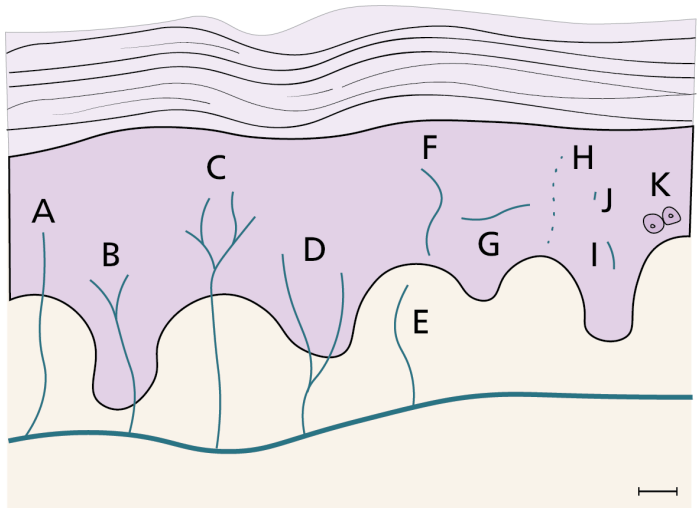



Figure 3.4. The new criteria for IENFD assessment developed during the work with Paper III
Fibers are counted as one if >15 µm, regardless of visibly crossing the baseline (A) or not (F and G), and if branching within the epidermis (B and C), or if in a dotted line (H). If a nerve fiber has branched within the dermis and crosses the junction as separate fibers, the fibers are counted individually (D; two fibers). The bar represents 15 µm, which is the approximate length of two neighboring keratinocytes (K). The IENFD is calculated by dividing the number of fibers counted by the epidermal length, i.e. fiber/mm.

*Ethical statement and statistical analyses*

The Swedish Ethical Review Authority (2020-03597) approved the study. Data was collected from archived material stored in accordance with the Swedish Biobank Act. All samples sent to the Department of Pathology are stored and available for further diagnostic, educational, quality control, and research purposes. The clinician performing the biopsy and/or excision is responsible for informing patients about biobank storage and is also required to register whether the patient wishes to opt-out. Patients can also submit a "no-ticket" and thus request retroactively withdrawal of data from the biobank. Therefore, no additional consent for participation in this study was obtained.

Data on IENFD were presented stratified by sex and age decades, with results shown as median values along with the 25$^{th}$ and 75$^{th}$ percentiles. Comparisons between groups were performed using the Kruskal-Wallis test, followed by Dunn's post hoc test, with adjustments for multiple comparisons using Bonferroni correction. Factors associated with IENFD were investigated through quantile regression analysis.

*Results*

A total of 602 samples from 591 individuals, aged between 18 and 97 years, were selected, re-sectioned, stained, and manually assessed for IENFD. Median IENFD

values, stratified on age decades, sex, and site of excision, are presented in Table 3.1. The overall distribution of IENFD across the entire cohort was significantly more variable and irregular than anticipated. An overall difference was found for IENFD across all age decades and sexes, but no linear relationship between them could be established. Nor did quantile regression analyses show any conclusive results regarding affecting factors, as both age and sex exhibited varying patterns across different quantiles. Significant differences were, however, found when comparing individuals over 65 years with either young or middle-aged participants, although no differences were observed between the two younger groups. Additional results can be found in Paper III.

Table 3.1. Intraepidermal nerve fiber densities (IENFD) in 5 µm skin sections from healthy adults

| | | Proximal arm | | Distal arm | | Proximal leg | | Distal leg | |
|---|---|---|---|---|---|---|---|---|---|
| | Age | n | IENFD | n | IENFD | n | IENFD | n | IENFD |
| M | 18-29 | *11* | 2.75 (1.59-3.60) | *11* | 1.62 (1.16-3.69) | *11* | 1.39 (0.82-2.53) | *11* | 0.73 (0.56-2.55) |
| | 30-39 | *10* | 2.12 (1.02-3.73) | *13* | 1.58 (1.40-2.61) | *10* | 1.56 (0.96-2.61) | *12* | 0.85 (0.31-1.11) |
| | 40-49 | *10* | 2.40 (1.24-3.27) | *10* | 2.18 (1.06-3.04) | *10* | 1.53 (0.87-3.01) | *11* | 0.45 (0.20-1.06) |
| | 50-59 | *10* | 1.85 (0.96-2.35) | *10* | 0.89 (0.53-2.33) | *10* | 1.40 (1.04-3.08) | *10* | 1.10 (0.60-1.55) |
| | 60-69 | *10* | 0.90 (0.55-2.20) | *10* | 0.89 (0.50-1.26) | *10* | 0.82 (0.58-2.26) | *10* | 0.55 (0.23-0-86) |
| | 70-79 | *10* | 0.93 (0.48-1.76) | *10* | 0.83 (0.53-1.49) | *10* | 0.57 (0.22-1.30) | *11* | 0.15 (0.00-0.82) |
| | >80 | *14* | 1.03 (0.32-1.77) | *11* | 0.39 (0.27-0.65) | *12* | 0.48 (0.20-0.75) | *14* | 0.22 (0.00-0.54) |
| W | 18-29 | *11* | 2.31 (1.81-5.37) | *10* | 2.21 (1.34-3.08) | *10* | 1.60 (1.27-2.09) | *11* | 0.98 (0.63-1.32) |
| | 30-39 | *10* | 2.36 (1.81-5.21) | *11* | 2.12 (1.13-2.56) | *10* | 2.46 (2.01-3.76) | *11* | 2.28 (0.61-2.97) |
| | 40-49 | *10* | 2.19 (1.53-5.27) | *10* | 2.38 (1.79-4.19) | *10* | 2.32 (1.82-3.60) | *10* | 1.72 (1.15-3.08) |
| | 50-59 | *10* | 1.34 (0.82-2.70) | *10* | 2.39 (1.52-2.94) | *12* | 2.10 (1.57-2.81) | *11* | 0.82 (0.42-1.11) |
| | 60-69 | *11* | 1.98 (0.89-3.09) | *10* | 1.69 (0.63-2.17) | *10* | 0.80 (0.21-1.98) | *10* | 0.78 (0.18-1.15) |
| | 70-79 | *10* | 1.17 (0.37-2.14) | *10* | 1.49 (0.73-2.24) | *10* | 1.06 (0.63-1.61) | *10* | 0.40 (0.08-1.50) |
| | >80 | *13* | 0.59 (0.33-1.70) | *13* | 0.70 (0.43-1.32) | *11* | 0.90 (0.28-2.07) | *15* | 0.13 (0.00-0.48) |

Median ($25^{th}$-$75^{th}$ percentiles) IENFD values presented for men (M) and women (W), respectively, in age groups based on decades. *n:* number of included samples.

## 3.4. Paper IV: Investigating temporal and metabolic effects

The project for Paper IV was conducted in collaboration with research colleagues in Umeå, Stockholm, and Linköping, Sweden. The study focused on investigating differences in IENFD over time in individuals with and without T2DM, as longitudinal studies are particularly useful in terms of investigating relationships between risk factors and outcomes. At the time of initiation of this project, all previous longitudinal studies had been performed on 50 µm sections and over shorter time frames of a maximum of five years.[123-125]

The study participants were part of a cohort recruited in Skellefteå, Sweden, during 2003 and 2004, as detailed in K. Pourhamidi's thesis.[126] This cohort originally consisted of 119 individuals aged 60 years, categorized into three groups: 39 with normal glucose tolerance (NGT), 29 with impaired glucose tolerance (IGT), and 51 with T2DM. Individuals with NGT and IGT had been recruited from the Västerbotten Intervention Programme, while participants with T2DM were recruited from the primary health care. Several studies have been published based on data from this baseline study.[127-129] For the follow-up study, the participants were reinvited to a second assessment approximately eight years later. A final total of 80 participants were investigated through clinical and electrophysiological examinations, blood sampling, and skin biopsies at both baseline and follow-up. Group assignment was confirmed through an oral glucose tolerance test. Skin biopsies were obtained, processed, and immunohistochemical treated according to the procedures detailed in section 3.1.3. Since the work was conducted prior to Paper III, the assessment was also carried out using the previous criteria for IENF counting, as outlined on page 40. The IENFD was assessed in all sections through bright-field microscopy by two observers – me and Elisabet Englund. Due to a small number of IGT at follow-up (n=9), the group was removed from statistical analyses regarding temporal changes.

*Ethical statement and statistical analyses*

The study was approved by the ethical review board of Umeå University (2013-21-31 M), and written informed consent was obtained from all participants.

Changes between follow-up and baseline were tested using the Wilcoxon signed-rank test. To explore potential associations between biometric or metabolic factors and changes in IENFD over time, linear mixed models were employed. Three models were performed: one including all participants, and two separate models for the NGT and T2DM groups. Factors included in the models were age, sex, height, weight, blood pressure, statin treatment, and HbA1c.

*Results*

The overall finding of the study was that IENFD was significantly lowered at follow-up in both the NGT and T2DM groups. In contrast to what was anticipated, however,

a larger decrease in IENFD was observed for the individuals with NGT compared to T2DM. In fact, at follow-up, the groups had reached similarly low levels of IENFD. In the search for metabolic factors, HbA1c appeared to be associated with IENFD levels in the entire group. However, this impact diminished when analyzing the T2DM and NGT groups separately. No association between blood pressure or statin treatment and IENFD was found. Aside from age, weight emerged as the only factor with a significant negative relationship to IENFD, both for the cohort as a whole and within the separate groups, although this relationship was relatively weak. For more detailed descriptions of the methods and results, see Paper IV.

## 3.5. Discussion

Following below, the modalities and applicability of the IENFD assessment in thin 5 µm sections will be discussed, along with the results of the two papers presented in this chapter. Further discussion regarding the implementation of new methods, as well as strengths and limitations of the studies, and potential future directions, are covered in Chapter 4.

### 3.5.1. Assessment in thinner sections

Although a thorough review of the research area has been published together with methodological guidelines, there are also several other things to take into consideration when employing a new method.[113] Besides the guidelines presented to the scientific community, healthcare system regulations specific to each area or country must also be considered, as they often dictate the feasibility of implementing new methods. The use of thinner sections for IENFD assessment has also been adopted by other research groups. While Koskinen et al. studied IENFD in sections with a thickness of 10 µm, Dabby et al. focused on dermal autonomic fibers in 5 µm sections.[130,131] At the Department of Pathology, Skåne University Hospital, Lund, Sweden, the need for a method that was easily applicable, and frankly, cost-effective, drove the development of a new IENFD assessment approach tailored to the specific clinical context.

The 5-µm method developed in Lund for IENFD assessment has been applied in both previous projects and the papers of this thesis. It offers both advantages and disadvantages in comparison to the recommended methodologies. A significant benefit is the long-term stability and reliability provided by the formaldehyde fixation and paraffin embedding. This allows for long-term storage and assures reassessment of both the glass-mounted section as well as the remaining tissue block. For instance, in a previous study, numerous archived sections from 2014 were readily interpretable when reassessed in 2019, demonstrating the robustness of these samples over time.[117] In

contrast, cryosections require storage in a -80°C freezer and are typically viable for only up to 1 year, making them less suitable for long-term storage and retrospective analysis.

Alongside our search for the normative values of IENFD assessed in thin sections, we were informed by our former project collaborator K. Pourhamidi that he, together with his associate O. Aspegren, had been working on a similar project. Like us, they had identified limitations with the previous assessment method and initiated their search for a more accurate strategy. While we opted to maintain a minimum length requirement for nerve fibers to be counted, they chose a more inclusive approach by counting almost all the positively immunostained material in samples obtained from 68 individuals. Our decision to exclude shorter fragments was driven by the concern that such positively stained fragments might not truly represent nerve fibers but rather debris or artifacts. However, Aspegren and Pourhamidi suggested that the broader inclusion yielded an estimation of the real IENFD (eIENFD), and the study, which was published in April 2024, showed promising results.[132] By assessing eIENFD, their results showed more comparable numbers with studies performed on 50 µm sections, in comparison to the results of our Paper III.

Despite the encouraging outcome of the eIENFD assessment, reservations regarding its practical use in clinical settings are warranted. Counting small nerve fiber fragments presents challenges in both accurate identification and also the amount of time required for assessment. Thereto, discerning these kinds of short fragments is very difficult, if not using imaging software where annotations and measurements can be applied. Even then, it can be challenging since every small fragment might need to be measured or noted, which takes time. Thus, when transitioning from the "half-the-width" to the 15-µm method in Paper III, the importance of establishing an easily applicable length requirement was discussed. The length requirement was designed to be practical even "at a glance" – i.e. without always measuring the nerve fibers directly. It was also intended to be useful in conventional light microscopy by providing a reference that could be applied based on surrounding structures, such as the size of neighboring keratinocytes. In my view, using conventional microscopes for assessing eIENFD is highly impractical and although digital imaging software simplifies this task, the process of examination still requires considerable time. However, with the rapid advancement of digital tools, particularly artificial intelligence (AI), and their growing implementation in clinical settings, there is potential to further streamline or even fully automate the process – which could ultimately yield a method of great potential. In fact, a recent pilot study on AI-assisted IENFD assessment using 50 µm immunofluorescent slides demonstrated high levels of agreement when compared with evaluations conducted by a pathologist. This work was performed by C. Gibbons and colleagues from Harvard Medical School, Boston, USA, and presented as a poster at the 2024 Peripheral Nerve Society Annual Meeting in Montréal, Canada.[133]

Going back to comparing results with previous studies, it is also important to note the differences in the size of our cohorts. While we included 602 samples from

individuals between 18 and 97 years, Aspegren and Pourhamidi assessed eIENFD in samples from 68 individuals aged 20 to 59 years. In fact, all prior studies presenting reference values on IENFD are based on either smaller populations or on multi-center collections of data, as demonstrated in Table 3.2 below. This discrepancy likely reflects the logistical challenges associated with gathering data from larger populations. However, it may also suggest that the methods employed in these previous studies are more time-consuming and therefore less suitable for large-scale assessment compared to the method developed by our group.

Table 3.2. Reference studies on IENFD

| Article | No. of study centers | No. of samples |
|---|---|---|
| McArthur et al. 1998[134] | 1 | 98 |
| Umapathi et al. 2006[122] | 1 | 84 |
| Lauria et al. 2010[114] | 8 | 550 |
| Provitera et al. 2016[115] | 4 | 528 |
| Collongues et al. 2018[135] | 1 | 298 |
| Aspegren et al. 2024[132] | 1 | 68 |
| Ekman et al. 2024 (Paper III) | 1 | 602 |

Examples of studies presenting reference ranges or normative data on IENFD with the number of study centers collaborating in the study as well as the number of samples, i.e. the size of the population.

### 3.5.2. Potential impact of age, weight, and excision site on IENFDs

As part of laying a foundation for IENFD assessment in thin sections, one focus of interest has been to identify factors potentially impacting the IENFD. As highlighted in Paper IV, age emerged as the most significant factor associated with IENFD, while weight also showed a potential relationship. These findings are consistent with previous studies on IENFD assessment in 50 µm sections, including the reference materials, which have found an age-related decline.[114,115,122] On the other hand, the reference values presented by McArthur et al. showed no overall effect of age, except for the significantly higher IENFDs found among the youngest group of their study, including individuals in ages between 10-19 years, and thus representing a pediatric population.[134] As described in Chapter 2, nerve-affecting processes of normal aging include reduced cellular communication, neuroinflammation, and atrophy.[91,92] Evidence of axonal loss due to normal aging can e.g. be demonstrated by reduced sural nerve amplitudes.[136] There are also structural changes in the skin associated with aging,

such as decreasing epidermal thickness and flattening of the dermal-epidermal junction.[137,138] It is imaginable that these alterations in the surrounding tissue create microenvironments that are less favorable for the nerve fibers, thereby further negatively impacting skin innervation.

The impact of age was also supported by the findings of Paper III, although with a cut-off around the age of 65 years. This could suggest that the impact of aging on IENFD may become more evident later in life, with nerve fiber densities remaining relatively stable during younger and middle adulthood, followed by a later life decline. Considering the environmental changes, dermal-epidermal junction changes have been shown to appear from the ages of 60 years and upwards.[139] These changes result in less robust and thus, more vulnerable skin tissue. Another consequence is the impaired connection between the dermal and epidermal areas, ultimately resulting in a loss of nutrient supply to the epidermis.[140] Hence, it is reasonable to believe that epidermal innervation is reduced after this age.

Weight was another factor found to be associated with IENFD in Paper IV. Although more prominently observed in individuals with T2DM, where obesity is a well-known risk factor for DPN, a significant negative association was also observed between weight and IENFD in healthy individuals.[141] A study examining individuals at various stages of metabolic syndrome demonstrated signs of SFN among those with obesity, even in the absence of hyperglycemia or hyperinsulinemia.[142] Increased body mass index (BMI) has also been shown to be associated with reduced action potential amplitudes, though not with impaired nerve conduction velocities.[143,144] This indicates that weight, or particularly obesity and the presence of fat tissue, may contribute more significantly to axonal loss than to demyelination. A higher adipocyte content within the endoneurium could play a role in this relationship. It would have been interesting to explore this relationship further in Paper III, but this was not possible due to the lack of data on participants' weight.

Another finding of Paper III was the differences in IENFD among excision sites, where higher densities were observed in the arm compared to the leg. However, no statistically significant differences could be found between the proximal and distal parts of each limb. This is in contrast with previous studies, which have proposed factors such as the proximal-distal gradient as well as the "dying-back" phenomenon of nerve damage.[122,123,134] Besides this intra-site variability, variations could also stem from an intra-punch variability, as suggested by McArthur et al.[134] This is something I dwelled upon during the assessment process. Along with the potential differences between, for instance, a lateral and medial calf, there could also be significant variability even between sites located adjacent to one another. This topographical variability became more apparent when examining excisions that could reach lengths of 10-20 millimeters, compared to the smaller biopsies of approximately 3 mm. In certain samples, it was almost evident that nerve fibers appeared clustered together in small groves or gatherings rather than being evenly distributed across the skin surface. This observation

could be attributed to the thinly cut sections, which might result in specific branches of a nerve fiber "tree" being visible only in proximity to each other. Alternatively, it could suggest that nerve fibers are more abundant in certain areas, thereby forming a patchy distribution pattern within the skin. For instance, Merkel cells are located in the skin within so-called touch domes, i.e. the Merkel disks. These structures are typically found in areas of high tactile sensitivity, such as the lips and fingertips, but can also occur in hairy skin.[145] Although Merkel disks per se are not counted during IENFD assessment, their distribution could influence the nerve fiber pattern in the epidermis. If this is the case, the inter-punch variability may be significant, which would limit the diagnostic utility of IENFD assessment. However, a potential solution could also be to exclude epidermal sections where Merkel disks are present as suggested by Collongues et al.[135]

### 3.5.3. Assessment of IENFD as a diagnostic tool

Unlike for large nerve fiber assessment, there is currently no universally accepted gold standard for evaluating small fiber neuropathy. The most suggested diagnostic approaches, both in clinical and research contexts, are the QST and skin biopsy with IENFD assessment. As mentioned in the first chapter, QST assesses the perception thresholds for vibration and temperature. However, these measurements depend not only on the actual sensory thresholds but also on the patient's capacity to cooperate during the test, potentially introducing variability. In contrast, skin biopsy offers a more objective assessment by directly quantifying IENFD in the sampled skin area.

In recent years, the use of CCM has gained increasing interest as a diagnostic tool for SFN and other types of neuropathies, particularly DPN.[146-150] The use of CCM allows for a noninvasive, *in vivo* examination of the corneal nerve fiber plexus, offering a potential alternative to the more invasive diagnostic methods. However, in a recent study that I was involved in, we applied an automated wide-field imaging protocol to analyze a larger area of the cornea. Thereafter, automated fiber detection and quantification were applied to minimize human selection bias. Following these advancements in the methodology, no significant associations were found between CCM measures and IENFDs or clinical measures of DPN.[119] The advantages of IENFD assessment in comparison to other methods for investigation of small nerve fibers lie thus in the direct morphological view of the nerves, but also in the higher correlation with clinical findings.

When comparing the IENFD assessment in 5-µm sections to the suggested 50-µm method, the 5-µm approach offers distinct advantages as a diagnostic tool. The advantages already mentioned, such as the simpler immunohistochemical process making it more affordable and thus more feasible in a clinical laboratory, could therefore enhance the practicality of IENFD assessment as a diagnostic tool.

Despite its utility, IENFD is currently not a stand-alone diagnostic tool, and its results must be interpreted in conjunction with clinical findings. For instance, an international consensus on diagnostic criteria for SFN was established in 2018, requiring at least one symptom and at least one sign of SFN to confirm the diagnosis.[151] However, multiple commercial laboratories in the U.S., which can be found online, offer SFN diagnosis based solely on skin biopsy and IENFD assessment, i.e. without clinically confirming the diagnosis. Furthermore, conditions like Parkinson's disease or amyotrophic lateral sclerosis (ALS) can present with reduced IENFD.[152] However, this may not be evidence enough for a neuropathy diagnosis, unless specific antibody staining with e.g. alpha-synuclein is applied.

Perhaps the most obvious disadvantage of IENFD assessment as a diagnostic tool is its invasiveness. Although it is a minor surgical procedure with generally good healing prospects and a very small risk of complications, any invasive procedure carries inherent risks. Non-invasive methods are always preferable when applicable. Potential complications following a skin biopsy include poor wound healing and infections. To mitigate these risks, the harvesting process should be performed with extra safety measures. In addition to sterile conditions, aftercare of the wound can also be enhanced. As an example, within the research group, we have implemented various techniques, including suturing, suture tape, and bandaging, to facilitate healing and minimize the risk of infection. Nevertheless, in Paper IV, biopsies were taken and left to heal without sutures, and the results were positive with no complications reported. This suggests that while there are some limited risks associated with skin biopsies, IENFD assessment can yield favorable outcomes as long as the entire procedure is carefully managed.

# 4. General discussion

During my PhD, I have had the opportunity to attend the NeuroDiab conference twice – first in Bergen in 2022 and then in Rome in 2024. NeuroDiab is a diabetic neuropathy study group based in Europe that has held annual meetings since its start in 1991. The most significant take-home message I gathered from both conferences was this: there are numerous assessment methods used, and everyone advocates for their own. Initially, this left me feeling somewhat disillusioned, especially after the first meeting, where I questioned the necessity of my work. Why, I wondered, introduce two more methods into an already crowded field?

However, by the time of the second meeting, I began to see things differently. Instead of feeling discouraged, I was struck by the thought that perhaps we are at a point where we need to carefully assess whether the best method already exists – and, if so, how we should adopt and standardize it. As I sit here now, though, I am plagued by yet another question. What if this diversity of methods and tests is symptomatic? What if, there is no "one-size-fits-all" solution or diagnostic tool? That it is simply impossible to fully grasp peripheral neuropathies, such as DPN, with one type of test? Perhaps all the researchers in this field are engaged in an inevitable struggle, but hopefully, it is simply part of the journey that drives us to continuously refine our methods in pursuit of perfection.

## 4.1. Evaluation of new methods

As emphasized throughout this thesis, there are multiple ways to study the peripheral nerves and the diseases that affect them. They range from simple sensory tests to more advanced electrophysiological techniques and morphological assessments. While nerve biopsies can provide detailed insights into pathological processes and are sometimes essential for diagnosis, they are invasive and inevitably impair nerve function. Instead, we typically rely on less invasive methods that estimate the condition of the specific nerve or evaluate function from the external. Regardless of the method employed, factors such as accuracy, accessibility, ease of use, and minimal patient harm are essential attributes of a good diagnostic tool.

In the context of diabetic neuropathy, methods that enable early detection are particularly crucial to prevent irreversible damage. When a method is used for

screening, the goal is not to diagnose a disease but rather to distinguish between individuals likely to have the condition and those with a low likelihood of having it. In contrast, pure diagnostic tests should provide a higher accuracy but are generally more resource-intensive. Thus, finding a balance between utilizing precise diagnostic techniques and implementing those that may facilitate early detection becomes essential.

The aim of this doctoral work was to establish normative values for VPTs and IENFDs and to explore the clinical implementation of the methods in individuals with T1DM and T2DM. Progress has been made regarding both determining reference data and assessing how these methods can be integrated into clinical practice. However, for IENFD assessment in thin sections, a definite conclusion regarding its ability to differentiate between healthy and diseased states is not yet reached. As for the VPTs, the findings are more promising. A test procedure involving a combination of two low and two high frequencies was suggested and demonstrated good agreement with the diagnosis of DPN. A cut-off value was also proposed, although measures of sensitivity and specificity were not optimal. Thus, while strides have been made in developing a test procedure, further refinement is needed to enhance its diagnostic accuracy and differentiation between healthy and diseased individuals.

## 4.2. Strengths and limitations

There are limitations to my studies that need to be addressed. One key limitation is that in Paper III, participants were neither asked nor screened for signs or symptoms of neuropathy. I did not meet these individuals personally and was therefore unable to make a clinical judgment. This contrasts with Paper I, where either I or one of my predecessors met each participant in person, which is one of the main strengths of that study. The decision to base the study of Paper III on archived material was, however, due to ethical considerations, as conducting skin biopsies on over 600 individuals without first validating the method would have raised other ethical concerns. While distributing questionnaires to gather additional data could have been an alternative, we opted to review the medical records of all participants. As a result, all participants included in the study were ensured to not have any documented history of neuropathy or other nerve-related disorders. Still, the risk of missing any underreported conditions remains. Interestingly, while not meeting the participants for Paper III is a limitation in that manner, it also eliminates the potential for hierarchal dependency or bias that might arise between researchers and their patients/participants.

Another potential limitation – although a positive one – is that the participants with T2DM in Paper IV were closely monitored and considered relatively healthy despite their diagnosis. Moreover, all participants who dropped out of the study between the

baseline and follow-up were from the T2DM group, which could have contributed to an even healthier cohort in the end.

In terms of strengths, the studies presented in this thesis benefit from comprising two large data sets – Paper I and Paper III. Additionally, all skin sections analyzed in both Paper III and IV were observed by a single observer, which enhances the comparability of the two works and adds robustness to Paper III. Furthermore, when examining the use of MFV and IENFD assessment in the two diabetes cohorts, neuropathy status could be confirmed with either NCS or QST, both of which are considered gold standard methods for assessing the function of large and small nerve fibers. This significantly enhances the impact of Papers II and III, as the proposed methods could be validated against established techniques. Importantly, this also means that the thesis as a whole has explored multiple nerve fiber types – ranging from thin unmyelinated fibers to large myelinated fibers – thereby capturing various modalities of neuropathy.

## 4.3. Future directions

The potential use of IENFD assessment in thin 5 µm sections warrants further exploration, and there is also room for incorporating more qualitative evaluations into the assessment of SFN. This includes assessing the number of retracted and enlarged axonal remains in the basal lamina or beneath it, as well as evaluating the presence of very short nerve fiber remnants in the epidermal layer. This is a work currently in progress.

Another interesting avenue for future work would be to examine how the two methods relate to one another. Although they represent measures of two distinct types of nerve fibers, some conditions may benefit from being assessed through multiple approaches. The original plan for this thesis included such a comparative study; however, this was canceled due to the Covid-19 pandemic and its impact on research activities. Notably, both MFV and IENFD assessments have recently been utilized in a project evaluating autonomic and peripheral nerve dysfunction in patients with postural orthostatic tachycardia syndrome (POTS). To my knowledge, the neuropathic component of POTS is not yet fully understood or established, but a comprehensive battery of tests for neuropathies was applied in the study. Preliminary results suggest that there are no significant differences in either VPTs or IENFDs between patients with POTS and healthy controls. As a result, it is currently challenging to draw any meaningful comparisons between the two methods.

# 5. Conclusions

This thesis involves findings on new approaches for diagnosing peripheral neuropathy, intending to establish simpler assessment methods with less time and cost requirements for early detection of conditions such as diabetic neuropathy. The thesis aimed to establish normative values for VPTs and IENFDs and to explore the clinical applicability of these methods by studying diabetic neuropathy. The work covers both physiological insights and practical considerations for further integrating these methods into clinical practice. Specific conclusions for the individual papers are as follows:

- **Paper I** presents normative VPTs for men and women of various ages, providing reference data for future comparisons. The study concludes that age and skin temperature are important associated factors, further enhancing the need for reference data and a standardized test procedure.
- In **Paper II**, it is concluded that VPTs at the 125 Hz frequency of MFV strongly correlate with measures of the sural nerve amplitude in patients with T1DM. It is also concluded that VPT testing with MFV could advantageously be performed based on four frequencies, instead of the original seven, and thus be more time efficient.
- **Paper III** presents normative IENFD values for thin-sectioned skin samples for men and women of various ages and different sites of excision. Age is concluded to be associated with decreased nerve densities in the skin for individuals over 65 years of age.
- In **Paper IV**, age is proposed to be the long-term determinant of IENFD, with the process being further accelerated by weight and HbA1c, leading to an earlier onset of decline in patients with T2DM.

The clinical implications I hope will result from my thesis involve facilitating screening and diagnosis of nerve-affecting disorders, such as DPN, by employing simple yet reliable methods. These tools could enable more accessible and efficient follow-ups in both primary and diabetic care settings, ultimately improving patient outcomes through earlier detection and intervention.

# Acknowledgments

*Over my troubled mind, under the moon. Rope walking.*
– Edda Magnason

I would like to thank all the people who have made this journey possible. Not just in the cheesy way, but sincerely – for giving me the opportunity to take a PhD in clinical medicine although I'm not a clinician at all. It is a one-time opportunity and even though it has been utterly challenging and heavy on my mind, I'm so proud of myself for not letting go, but continuing and eventually getting here – to the end.

**Lars B. Dahlin** my main supervisor, professor, and boss. From the moment we first met, we instantly connected over our shared hometown and just a few hours later, you offered me a job. What started as a six-month project turned into another six months, and now here we are, seven years later. You gave me the opportunity to earn my bachelor's degree and begin my journey toward a Ph.D. I am deeply grateful for all the chances you've given me and for always believing in me, no matter what.

**Elisabet Englund** my co-supervisor and idol in both medicine and research. Do you remember the first e-mail I sent you? I was thrilled when Lasse told me about the project with you and that I could be a part of it. Since then, the Pathology Department has felt like a second home to me. Forever grateful for your encouragement, commitment, and optimism – it's infectious.

**Eero Lindholm** my co-supervisor and personal expert in diabetes. Thank you for your knowledge and critical mind. I also want to thank you for encouraging and challenging me, which has motivated me to be better and braver.

**Elisabeth Brogren** my co-supervisor and steady rock. I am truly grateful that you accepted to be part of my team of supervisors, although you weren't much involved in the projects per se. You have been a significant support for me on this journey. Thank you for being present, for listening, and for always cheering me up with tons of compliments (although I suck at receiving them, I know).

**Gert Andersson, Kaveh Pourhamidi, Neil Lagali,** and **Olov Rolandsson** my co-authors for Papers II and IV. Thank you for your invaluable cooperation, support, and input during these projects.

Thank you, **Helena & Tina,** for taking care of me since the very first day on this job. Together with **Sharon,** who joined later, you have been my fellow non-doctors on the third floor. Thank you for letting me be a part of your gang and all our fika sessions. I will miss you!

I also want to thank my dear research colleagues and friends **Jin Persson Löfgren** and **Linnéa Tyrberg** for being a part of our troika since the start of med school as well as for introducing me to Lasse and his research. I truly wouldn't be here without you. **Marie Taylor**, for sharing experiences along this Ph.D. journey and making Zoom courses a bit less lonely. **Signe Säwén**, for always helping and supporting me during the toughest moments of preparation. **Lena Stenberg**, for all the walks and never-ending conversations. Your perspectives and attitude towards life inspire me, and I appreciate our bond so much. And **Drífa Frostadóttir**, for being my biggest inspiration and support. I don't have enough words to thank you properly. The best days of my Ph.D. were those spent in your office, deeply sunken into the sofa, listening to jazz, and writing side by side with you.

**Annica, Josefine & Amanda** the women of my family – my mother and sisters – and the most important people in my life. I don't think that any of you have read my articles (let alone this book) or fully understood what I've been working on. Like that time when my mum surprised me with prosecco after hearing that one of my articles got published (it wasn't even the first). She raised a toast, took a sip, and then asked "So, what does this really mean...?". Nevertheless, you've all been the best supporters ever on this journey, always just a phone call away. I love you bunches!

**Alex** my love. This has been a tough journey not only for me, but also for you. I can't thank you enough for your patience (listening to my rants, day in and day out), love (serving me wonderful weekend breakfasts and ridiculously good cocktails), and incredible mind (suggesting we should buy the cottage when I needed it the most). For all the things you've done to keep me going – thank you & I love you.

*Linnéa Ekman*

*October 2024 Malmö*

# References


1. Visser NA, Notermans NC, Linssen RS, van den Berg LH, Vrancken AF. Incidence of polyneuropathy in Utrecht, the Netherlands. Neurology. 2015;84(3):259-64.
2. Hoffman EM, Staff NP, Robb JM, St Sauver JL, Dyck PJ, Klein CJ. Impairments and comorbidities of polyneuropathy revealed by population-based analyses. Neurology. 2015;84(16):1644-51.
3. Gordois A, Scuffham P, Shearer A, Oglesby A, Tobian JA. The health care costs of diabetic peripheral neuropathy in the US. Diabetes Care. 2003;26(6):1790-5.
4. Purves D, Augustine GJ, Fitzpatrick D, Hall W, LaMantia AS, McNamara JO, et al. Neuroscience. 4th ed. Sunderland, MA: Sinauer Associates, Inc; 2008.
5. Kahle W, Frotscher M. Color Atlas of Human Anatomy: Vol. 3 Nervous System and Sensory Organs. 7th ed. Stuttgart, Germany: Thieme; 2015.
6. Boissaud-Cooke M, Pidgeon TE, Tunstall R. Chapter 37 - The Microcirculation of Peripheral Nerves: The Vasa Nervorum. In: Tubbs RS, Rizk E, Shoja MM, Loukas M, Barbaro N, Spinner RJ, editors. Nerves and Nerve Injuries. San Diego: Academic Press; 2015. p. 507-23.
7. Laverdet B, Danigo A, Girard D, Magy L, Demiot C, Desmoulière A. Skin innervation: important roles during normal and pathological cutaneous repair. Histol Histopathol. 2015;30(8):875-92.
8. Kim SH, Lee YH. Re-evaluation of the distribution of Meissner's corpuscles in human skin. Anat Cell Biol. 2020;53(3):325-9.
9. Lauria G, Borgna M, Morbin M, Lombardi R, Mazzoleni G, Sghirlanzoni A, Pareyson D. Tubule and neurofilament immunoreactivity in human hairy skin: markers for intraepidermal nerve fibers. Muscle Nerve. 2004;30(3):310-6.
10. Corniani G, Saal HP. Tactile innervation densities across the whole body. J Neurophysiol. 2020;124(4):1229-40.
11. Parasoglou P, Rao S, Slade JM. Declining Skeletal Muscle Function in Diabetic Peripheral Neuropathy. Clin Ther. 2017;39(6):1085-103.
12. Li L, Zhang S, Dobson J. The contribution of small and large sensory afferents to postural control in patients with peripheral neuropathy. J Sport Health Sci. 2019;8(3):218-27.
13. Kumar V, Abbas AK, Aster JC. Robins & Cotran Pathologic Basis of Disease. Philadelphia, PA: Elsevier Saunders; 2015.
14. Sommer C, Geber C, Young P, Forst R, Birklein F, Schoser B. Polyneuropathies. Dtsch Arztebl Int. 2018;115(6):83-90.

15. Elafros MA, Andersen H, Bennett DL, Savelieff MG, Viswanathan V, Callaghan BC, Feldman EL. Towards prevention of diabetic peripheral neuropathy: clinical presentation, pathogenesis, and new treatments. Lancet Neurol. 2022;21(10):922-36.
16. International Diabetes Federation. IDF Diabetes Atlas [Internet]. Brussels, Belgium; 2021.10th ed. [cited 2024 Mar 21]. Available from: https://www.diabetesatlas.org.
17. Hicks CW, Selvin E. Epidemiology of Peripheral Neuropathy and Lower Extremity Disease in Diabetes. Curr Diab Rep. 2019;19(10):86.
18. Boulton AJ, Malik RA. Diabetic neuropathy. Med Clin North Am. 1998;82(4):909-29.
19. Tesfaye S, Boulton AJ, 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(10):2285-93.
20. Eid SA, Rumora AE, Beirowski B, Bennett DL, Hur J, Savelieff MG, Feldman EL. New perspectives in diabetic neuropathy. Neuron. 2023;111(17):2623-41.
21. Martin CL, Albers JW, Pop-Busui R. Neuropathy and related findings in the diabetes control and complications trial/epidemiology of diabetes interventions and complications study. Diabetes Care. 2014;37(1):31-8.
22. 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(10):2244-9.
23. Magnani P, Thomas TP, Tennekoon G, DeVries GH, Greene DA, Brosius FC, 3rd. Regulation of glucose transport in cultured Schwann cells. J Peripher Nerv Syst. 1998;3(1):28-36.
24. Brownlee M. The pathobiology of diabetic complications: a unifying mechanism. Diabetes. 2005;54(6):1615-25.
25. Feldman EL, Nave KA, Jensen TS, Bennett DLH. New Horizons in Diabetic Neuropathy: Mechanisms, Bioenergetics, and Pain. Neuron. 2017;93(6):1296-313.
26. Mizukami H, Osonoi S. Pathogenesis and Molecular Treatment Strategies of Diabetic Neuropathy Collateral Glucose-Utilizing Pathways in Diabetic Polyneuropathy. Int J Mol Sci. 2020;22(1).
27. Pang L, Lian X, Liu H, Zhang Y, Li Q, Cai Y, et al. Understanding Diabetic Neuropathy: Focus on Oxidative Stress. Oxid Med Cell Longev. 2020;2020:9524635.
28. Kazamel M, Stino AM, Smith AG. Metabolic syndrome and peripheral neuropathy. Muscle Nerve. 2021;63(3):285-93.
29. Stino AM, Smith AG. Peripheral neuropathy in prediabetes and the metabolic syndrome. J Diabetes Investig. 2017;8(5):646-55.
30. Stino AM, Rumora AE, Kim B, Feldman EL. Evolving concepts on the role of dyslipidemia, bioenergetics, and inflammation in the pathogenesis and treatment of diabetic peripheral neuropathy. J Peripher Nerv Syst. 2020;25(2):76-84.

31. Rumora AE, Lentz SI, Hinder LM, Jackson SW, Valesano A, Levinson GE, Feldman EL. Dyslipidemia impairs mitochondrial trafficking and function in sensory neurons. Faseb j. 2018;32(1):195-207.
32. Oh TJ, Lee JE, Choi SH, Jang HC. Association between Body Fat and Diabetic Peripheral Neuropathy in Middle-Aged Adults with Type 2 Diabetes Mellitus: A Preliminary Report. J Obes Metab Syndr. 2019;28(2):112-7.
33. Popov D. Endothelial cell dysfunction in hyperglycemia: Phenotypic change, intracellular signaling modification, ultrastructural alteration, and potential clinical outcomes. International Journal of Diabetes Mellitus. 2010;2(3):189-95.
34. Malik RA, Tesfaye S, Thompson SD, Veves A, Hunter A, Sharma AK, et al. Transperineurial capillary abnormalities in the sural nerve of patients with diabetic neuropathy. Microvasc Res. 1994;48(2):236-45.
35. Thrainsdottir S, Malik RA, Dahlin LB, Wiksell P, Eriksson KF, Rosén I, et al. Endoneurial capillary abnormalities presage deterioration of glucose tolerance and accompany peripheral neuropathy in man. Diabetes. 2003;52(10):2615-22.
36. Dahlin LB, Meiri KF, McLean WG, Rydevik B, Sjöstrand J. Effects of nerve compression on fast axonal transport in streptozotocin-induced diabetes mellitus. An experimental study in the sciatic nerve of rats. Diabetologia. 1986;29(3):181-5.
37. Dahlin LB, Zimmerman M, Calcagni M, Hundepool CA, van Alfen N, Chung KC. Carpal tunnel syndrome. Nat Rev Dis Primers. 2024;10(1):37.
38. Hewston P, Deshpande N. Falls and Balance Impairments in Older Adults with Type 2 Diabetes: Thinking Beyond Diabetic Peripheral Neuropathy. Can J Diabetes. 2016;40(1):6-9.
39. Finnerup NB, Sindrup SH, Jensen TS. The evidence for pharmacological treatment of neuropathic pain. Pain. 2010;150(3):573-81.
40. Abbott CA, Malik RA, van Ross ER, Kulkarni J, Boulton AJ. Prevalence and characteristics of painful diabetic neuropathy in a large community-based diabetic population in the U.K. Diabetes Care. 2011;34(10):2220-4.
41. Gore M, Brandenburg NA, Dukes E, Hoffman DL, Tai KS, Stacey B. Pain severity in diabetic peripheral neuropathy is associated with patient functioning, symptom levels of anxiety and depression, and sleep. J Pain Symptom Manage. 2005;30(4):374-85.
42. Chammas NK, Hill RL, Edmonds ME. Increased Mortality in Diabetic Foot Ulcer Patients: The Significance of Ulcer Type. J Diabetes Res. 2016;2016:2879809.
43. Boulton AJM, Armstrong DG, Kirsner RS, Attinger CE, Lavery LA, Lipsky BA, et al. Diagnosis and Management of Diabetic Foot Complications. Compendia. 2018;2018(2).
44. Zabeen B, Craig ME, Virk SA, Pryke A, Chan AK, Cho YH, et al. Insulin Pump Therapy Is Associated with Lower Rates of Retinopathy and Peripheral Nerve Abnormality. PLoS One. 2016;11(4):e0153033.
45. Callaghan BC, Reynolds EL, Banerjee M, Akinci G, Chant E, Villegas-Umana E, et al. Dietary weight loss in people with severe obesity stabilizes neuropathy and improves symptomatology. Obesity (Silver Spring). 2021;29(12):2108-18.

46. Singleton JR, Marcus RL, Lessard MK, Jackson JE, Smith AG. Supervised exercise improves cutaneous reinnervation capacity in metabolic syndrome patients. Ann Neurol. 2015;77(1):146-53.
47. Effects of a long-term lifestyle modification programme on peripheral neuropathy in overweight or obese adults with type 2 diabetes: the Look AHEAD study. Diabetologia. 2017;60(6):980-8.
48. Sloan G, Alam U, Selvarajah D, Tesfaye S. The Treatment of Painful Diabetic Neuropathy. Curr Diabetes Rev. 2022;18(5):e070721194556.
49. Feldman EL, Callaghan BC, Pop-Busui R, Zochodne DW, Wright DE, Bennett DL, et al. Diabetic neuropathy. Nature Reviews Disease Primers. 2019;5(1):41.
50. Larsson L-E. Neurofysiologi - En bok om hur hjärnan fungerar. Lund: Studentlitteratur; 2000.
51. Mallik A, Weir AI. Nerve conduction studies: essentials and pitfalls in practice. J Neurol Neurosurg Psychiatry. 2005;76 Suppl 2(Suppl 2):ii23-31.
52. Gisslander K, Dahlin LB, Smith R, Jayne D, O'Donovan DG, Mohammad AJ. The Role of Sural Nerve Biopsy in the Diagnosis of Vasculitis. J Rheumatol. 2022;49(9):1031-6.
53. Chimelli L, Freitas M, Nascimento O. Value of nerve biopsy in the diagnosis and follow-up of leprosy: the role of vascular lesions and usefulness of nerve studies in the detection of persistent bacilli. J Neurol. 1997;244(5):318-23.
54. Dahlin LB, Eriksson KF, Sundkvist G. Persistent postoperative complaints after whole sural nerve biopsies in diabetic and non-diabetic subjects. Diabet Med. 1997;14(5):353-6.
55. Reggars JW. VIBRATORY SENSATION TESTING: Practice Tip. COMSIG Rev. 1995;4(1):14-5.
56. American Diabetes Association. 11. Microvascular Complications and Foot Care: Standards of Medical Care in Diabetes—2019. Diabetes Care. 2018;42(Supplement_1):S124-S38.
57. Swedish Association of Local Authorities and Regions. Fotundersökning vid diabetes enligt nationellt vårdprogram för prevention av fotkomplikationer vid diabetes. Stockholm: Swedish Association of Local Authorities and Regions; 2018.
58. Valk GD, de Sonnaville JJ, van Houtum WH, Heine RJ, van Eijk JT, Bouter LM, Bertelsmann FW. The assessment of diabetic polyneuropathy in daily clinical practice: reproducibility and validity of Semmes Weinstein monofilaments examination and clinical neurological examination. Muscle Nerve. 1997;20(1):116-8.
59. Dunker Ø, Uglem M, Bu Kvaløy M, Løseth S, Hjelland IE, Allen SM, et al. Diagnostic accuracy of the 5.07 monofilament test for diabetes polyneuropathy: influence of age, sex, neuropathic pain and neuropathy severity. BMJ Open Diabetes Res Care. 2023;11(6).
60. Dyck PJ. Detection, characterization, and staging of polyneuropathy: assessed in diabetics. Muscle Nerve. 1988;11(1):21-32.
61. Thrainsdottir S, Malik RA, Rosén I, Jakobsson F, Bakhtadze E, Petersson J, et al. Sural nerve biopsy may predict future nerve dysfunction. Acta Neurol Scand. 2009;120(1):38-46.

62. Pop-Busui R, Boulton AJ, Feldman EL, Bril V, Freeman R, Malik RA, et al. Diabetic Neuropathy: A Position Statement by the American Diabetes Association. Diabetes Care. 2017;40(1):136-54.
63. Gordon I. The Sensation of Vibration, with Special Reference to its Clinical Significance. J Neurol Psychopathol. 1936;17(66):107-34.
64. Wells C, Ward LM, Chua R, Inglis JT. Regional variation and changes with ageing in vibrotactile sensitivity in the human footsole. J Gerontol A Biol Sci Med Sci. 2003;58(8):680-6.
65. Johansson RS, Vallbo ÅB. Tactile sensory coding in the glabrous skin of the human hand. Trends in Neurosciences. 1983;6:27-32.
66. Johansson RS, Vallbo AB. Detection of tactile stimuli. Thresholds of afferent units related to psychophysical thresholds in the human hand. J Physiol. 1979;297(0):405-22.
67. Gu C, Griffin MJ. Spatial summation of vibrotactile sensations at the foot. Med Eng Phys. 2013;35(8):1221-7.
68. Zippenfennig C, Wynands B, Milani TL. Vibration Perception Thresholds of Skin Mechanoreceptors Are Influenced by Different Contact Forces. J Clin Med. 2021;10(14).
69. Schlee G, Sterzing T, Milani TL. Foot sole skin temperature affects plantar foot sensitivity. Clin Neurophysiol. 2009;120(8):1548-51.
70. ISO 13091–1. Mechanical vibration–Vibrotactile perception thresholds for the assessment of nerve dysfunction–Part 1: Methods of measurement at the fingertips. International Organization of Standardization: Geneva; 2011.
71. Békésy Gv. A New Audiometer. Acta Oto-Laryngologica. 1947;35(5-6):411-22.
72. Lundborg G, Lie-Stenström AK, Sollerman C, Strömberg T, Pyykkö I. Digital vibrogram: a new diagnostic tool for sensory testing in compression neuropathy. J Hand Surg Am. 1986;11(5):693-9.
73. Lundborg G, Sollerman C, Strömberg T, Pyykkö I, Rosén B. A new principle for assessing vibrotactile sense in vibration-induced neuropathy. Scand J Work Environ Health. 1987;13(4):375-9.
74. Flodmark BT, Lundborg G. Vibrotactile sense and hand symptoms in blue collar workers in a manufacturing industry. Occup Environ Med. 1997;54(12):880-7.
75. Lundström R, Strömberg T, Lundborg G. Vibrotactile perception threshold measurements for diagnosis of sensory neuropathy. Description of a reference population. Int Arch Occup Environ Health. 1992;64(3):201-7.
76. Lundborg G, Dahlin LB, Lundström R, Necking LE, Strömberg T. Vibrotactile function of the hand in compression and vibration-induced neuropathy. Sensibility index--a new measure. Scand J Plast Reconstr Surg Hand Surg. 1992;26(3):275-9.
77. Dahlin LB, Thrainsdottir S, Cederlund R, Thomsen NO, Eriksson KF, Rosén I, et al. Vibrotactile sense in median and ulnar nerve innervated fingers of men with Type 2 diabetes, normal or impaired glucose tolerance. Diabet Med. 2008;25(5):543-9.

78. Nelander J, Speidel T, Björkman A, Dahlin LB. Vibration thresholds are increased at low frequencies in the sole of the foot in diabetes-a novel multi-frequency approach. Diabet Med. 2012;29(12):e449-56.

79. Lindholm E, Löndahl M, Fagher K, Apelqvist J, Dahlin LB. Strong association between vibration perception thresholds at low frequencies (4 and 8 Hz), neuropathic symptoms and diabetic foot ulcers. PLoS One. 2019;14(2):e0212921.

80. Ising E, Dahlin LB, Elding Larsson H. Impaired vibrotactile sense in children and adolescents with type 1 diabetes - Signs of peripheral neuropathy. PLoS One. 2018;13(4):e0196243.

81. Ising E, Ekman L, Elding Larsson H, Dahlin LB. Vibrotactile sense might improve over time in paediatric subjects with type 1 diabetes-A mid-term follow-up using multifrequency vibrometry. Acta Paediatr. 2022;111(2):411-7.

82. Ekman L, Persson Löfgren J, Dahlin LB. Examining practice effects in repeated measurements of vibration perception thresholds on finger pulps of healthy individuals - Is it possible to improve your results over a clinically relevant test interval? PLoS One. 2019;14(12):e0226371.

83. Wiles PG, Pearce SM, Rice PJ, Mitchell JM. Vibration perception threshold: influence of age, height, sex, and smoking, and calculation of accurate centile values. Diabet Med. 1991;8(2):157-61.

84. Bloom S, Till S, Sönksen P, Smith S. Use of a biothesiometer to measure individual vibration thresholds and their variation in 519 non-diabetic subjects. Br Med J (Clin Res Ed). 1984;288(6433):1793-5.

85. Meh D, Denislic M. Influence of age, temperature, sex, height and diazepam on vibration perception. J Neurol Sci. 1995;134(1-2):136-42.

86. Gandhi MS, Sesek R, Tuckett R, Bamberg SJ. Progress in vibrotactile threshold evaluation techniques: a review. J Hand Ther. 2011;24(3):240-55; quiz 56.

87. Hage JJ, van der Steen LP, de Groot PJ. Difference in sensibility between the dominant and nondominant index finger as tested using the Semmes-Weinstein monofilaments pressure aesthesiometer. J Hand Surg Am. 1995;20(2):227-9.

88. Dahlin LB, Güner N, Elding Larsson H, Speidel T. Vibrotactile perception in finger pulps and in the sole of the foot in healthy subjects among children or adolescents. PLoS One. 2015;10(3):e0119753.

89. Strzalkowski NDJ, Ali RA, Bent LR. The firing characteristics of foot sole cutaneous mechanoreceptor afferents in response to vibration stimuli. J Neurophysiol. 2017;118(4):1931-42.

90. Mildren RL, Bent LR. Vibrotactile stimulation of fast-adapting cutaneous afferents from the foot modulates proprioception at the ankle joint. J Appl Physiol (1985). 2016;120(8):855-64.

91. López-Otín C, Blasco MA, Partridge L, Serrano M, Kroemer G. Hallmarks of aging: An expanding universe. Cell. 2023;186(2):243-78.

92. Verdú E, Ceballos D, Vilches JJ, Navarro X. Influence of aging on peripheral nerve function and regeneration. J Peripher Nerv Syst. 2000;5(4):191-208.

93. García-Piqueras J, García-Mesa Y, Cárcaba L, Feito J, Torres-Parejo I, Martín-Biedma B, et al. Ageing of the somatosensory system at the periphery: age-related changes in cutaneous mechanoreceptors. J Anat. 2019;234(6):839-52.
94. Maita KC, Garcia JP, Avila FR, Torres-Guzman RA, Ho O, Chini CCS, et al. Evaluation of the Aging Effect on Peripheral Nerve Regeneration: A Systematic Review. J Surg Res. 2023;288:329-40.
95. Wagstaff LJ, Gomez-Sanchez JA, Fazal SV, Otto GW, Kilpatrick AM, Michael K, et al. Failures of nerve regeneration caused by aging or chronic denervation are rescued by restoring Schwann cell c-Jun. Elife. 2021;10.
96. Kerezoudi E, Thomas PK. Influence of age on regeneration in the peripheral nervous system. Gerontology. 1999;45(6):301-6.
97. Green BG. The effect of skin temperature on vibrotactile sensitivity. Perception & Psychophysics. 1977;21(3):243-8.
98. Verrillo RT, Bolanowski SJ, Jr. The effects of skin temperature on the psychophysical responses to vibration on glabrous and hairy skin. J Acoust Soc Am. 1986;80(2):528-32.
99. Kiernan MC, Cikurel K, Bostock H. Effects of temperature on the excitability properties of human motor axons. Brain. 2001;124(Pt 4):816-25.
100. Burke D, Mogyoros I, Vagg R, Kiernan MC. Temperature dependence of excitability indices of human cutaneous afferents. Muscle Nerve. 1999;22(1):51-60.
101. Flondell M, Rosén B, Andersson G, Schyman T, Dahlin LB, Björkman A. Vibration thresholds in carpal tunnel syndrome assessed by multiple frequency vibrometry: a case-control study. J Occup Med Toxicol. 2017;12:34.
102. Marstrand SD, Buch-Larsen K, Andersson M, Jensen LT, Schwarz P. Vibration Perception Threshold and Heart Rate Variability as methods to assess chemotherapy-induced neuropathy in women with breast cancer – a pilot study. Cancer Treatment and Research Communications. 2021;28:100426.
103. Nielsen SW, Hasselsteen SD, Dominiak HSH, Labudovic D, Reiter L, Dalton SO, Herrstedt J. Oral cannabidiol for prevention of acute and transient chemotherapy-induced peripheral neuropathy. Support Care Cancer. 2022;30(11):9441-51.
104. Nielsen SW, Lindberg S, Ruhlmann CHB, Eckhoff L, Herrstedt J. Addressing Chemotherapy-Induced Peripheral Neuropathy Using Multi-Frequency Vibrometry and Patient-Reported Outcomes. J Clin Med. 2022;11(7).
105. Rolke R, Rolke S, Vogt T, Birklein F, Geber C, Treede RD, et al. Hand-arm vibration syndrome: clinical characteristics, conventional electrophysiology and quantitative sensory testing. Clin Neurophysiol. 2013;124(8):1680-8.
106. Ising E. Peripheral neuropathy in type 1 and type 2 diabetes - screening of vibrotactile sense and proteomics in human nerve biopsies. Lund, Sweden: Lund University; 2022.
107. Dahlin LB, Granberg V, Rolandsson O, Rosén I, Dahlin E, Sundkvist G. Disturbed vibrotactile sense in finger pulps in patients with Type 1 diabetes--correlations with glycaemic level, clinical examination and electrophysiology. Diabet Med. 2011;28(9):1045-52.

108. Arthur RP, Shelley WB. The innervation of human epidermis. J Invest Dermatol. 1959;32(3):397-411.

109. Dalsgaard CJ, Rydh M, Haegerstrand A. Cutaneous innervation in man visualized with protein gene product 9.5 (PGP 9.5) antibodies. Histochemistry. 1989;92(5):385-90.

110. Wang L, Hilliges M, Jernberg T, Wiegleb-Edström D, Johansson O. Protein gene product 9.5-immunoreactive nerve fibres and cells in human skin. Cell Tissue Res. 1990;261(1):25-33.

111. Holland NR, Crawford TO, Hauer P, Cornblath DR, Griffin JW, McArthur JC. Small-fiber sensory neuropathies: clinical course and neuropathology of idiopathic cases. Ann Neurol. 1998;44(1):47-59.

112. McCarthy BG, Hsieh ST, Stocks A, Hauer P, Macko C, Cornblath DR, et al. Cutaneous innervation in sensory neuropathies: evaluation by skin biopsy. Neurology. 1995;45(10):1848-55.

113. 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(10):747-58.

114. 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(3):202-7.

115. Provitera V, Gibbons CH, Wendelschafer-Crabb G, Donadio V, Vitale DF, Stancanelli A, et al. A multi-center, multinational age- and gender-adjusted normative dataset for immunofluorescent intraepidermal nerve fiber density at the distal leg. Eur J Neurol. 2016;23(2):333-8.

116. Thomsen NO, Englund E, Thrainsdottir S, Rosén I, Dahlin LB. Intraepidermal nerve fibre density at wrist level in diabetic and non-diabetic patients. Diabet Med. 2009;26(11):1120-6.

117. Ekman L, Thrainsdottir S, Englund E, Thomsen N, Rosén I, Hazer Rosberg DB, et al. Evaluation of small nerve fiber dysfunction in type 2 diabetes. Acta Neurol Scand. 2020;141(1):38-46.

118. Eckermann M, Peruzzi N, Frohn J, Bech M, Englund E, Veress B, et al. 3d phase-contrast nanotomography of unstained human skin biopsies may identify morphological differences in the dermis and epidermis between subjects. Skin Res Technol. 2021;27(3):316-23.

119. Badian RA, Ekman L, Pripp AH, Utheim TP, Englund E, Dahlin LB, et al. Comparison of Novel Wide-Field In Vivo Corneal Confocal Microscopy With Skin Biopsy for Assessing Peripheral Neuropathy in Type 2 Diabetes. Diabetes. 2023;72(7):908-17.

120. Holland NR, Stocks A, Hauer P, Cornblath DR, Griffin JW, McArthur JC. Intraepidermal nerve fiber density in patients with painful sensory neuropathy. Neurology. 1997;48(3):708-11.

121. Pittenger GL, Ray M, Burcus NI, McNulty P, Basta B, Vinik AI. Intraepidermal nerve fibers are indicators of small-fiber neuropathy in both diabetic and nondiabetic patients. Diabetes Care. 2004;27(8):1974-9.

122. Umapathi T, Tan WL, Tan NC, Chan YH. Determinants of epidermal nerve fiber density in normal individuals. Muscle Nerve. 2006;33(6):742-6.

123. Khoshnoodi MA, Truelove S, Burakgazi A, Hoke A, Mammen AL, Polydefkis M. Longitudinal Assessment of Small Fiber Neuropathy: Evidence of a Non-Length-Dependent Distal Axonopathy. JAMA Neurol. 2016;73(6):684-90.

124. Løseth S, Stålberg EV, Lindal S, Olsen E, Jorde R, Mellgren SI. Small and large fiber neuropathy in those with type 1 and type 2 diabetes: a 5-year follow-up study. J Peripher Nerv Syst. 2016;21(1):15-21.

125. Divisova S, Vlckova E, Srotova I, Kincova S, Skorna M, Dusek L, et al. Intraepidermal nerve-fibre density as a biomarker of the course of neuropathy in patients with Type 2 diabetes mellitus. Diabet Med. 2016;33(5):650-4.

126. Pourhamidi K. Peripheral nerve function - metabolic features, clinical assessment, and heat shock protein 27. Umeå, Sweden: Umeå University; 2013.

127. Pourhamidi K, Dahlin LB, Boman K, Rolandsson O. Heat shock protein 27 is associated with better nerve function and fewer signs of neuropathy. Diabetologia. 2011;54(12):3143-9.

128. Pourhamidi K, Dahlin LB, Englund E, Rolandsson O. No difference in small or large nerve fiber function between individuals with normal glucose tolerance and impaired glucose tolerance. Diabetes Care. 2013;36(4):962-4.

129. Pourhamidi K, Dahlin LB, Englund E, Rolandsson O. Evaluation of clinical tools and their diagnostic use in distal symmetric polyneuropathy. Prim Care Diabetes. 2014;8(1):77-84.

130. Koskinen M, Hietaharju A, Kyläniemi M, Peltola J, Rantala I, Udd B, Haapasalo H. A quantitative method for the assessment of intraepidermal nerve fibers in small-fiber neuropathy. J Neurol. 2005;252(7):789-94.

131. Dabby R, Vaknine H, Gilad R, Djaldetti R, Sadeh M. Evaluation of cutaneous autonomic innervation in idiopathic sensory small-fiber neuropathy. J Peripher Nerv Syst. 2007;12(2):98-101.

132. Aspegren O, Pourhamidi K. Reliable Method for Estimating Nerve Fiber Density in Epidermis Using Routine Histopathologic Tissue Preparation: A Promising Diagnostic Tool for Small Fiber Neuropathy. Appl Immunohistochem Mol Morphol. 2024;32(5):215-21.

133. Gibbons C, Bellaire B, Levine T, Freeman R. A novel quantitative artificial intelligence approach to measurement of intra-epidermal nerve fiber density. Proceeding of the Peripheral Nerve Society Annual Meeting. June 22-25, 2024; Montréal, Canada.

134. McArthur JC, Stocks EA, Hauer P, Cornblath DR, Griffin JW. Epidermal nerve fiber density: normative reference range and diagnostic efficiency. Arch Neurol. 1998;55(12):1513-20.

135. Collongues N, Samama B, Schmidt-Mutter C, Chamard-Witkowski L, Debouverie M, Chanson JB, et al. Quantitative and qualitative normative dataset for intraepidermal nerve fibers using skin biopsy. PLoS One. 2018;13(1):e0191614.

136. Taams NE, Drenthen J, Hanewinckel R, Ikram MA, van Doorn PA. Age-Related Changes in Neurologic Examination and Sensory Nerve Amplitude in the General Population: Aging of the Peripheral Nervous System. Neurology. 2023;101(13):e1351-e8.

137. Farage MA, Miller KW, Elsner P, Maibach HI. Characteristics of the Aging Skin. Adv Wound Care (New Rochelle). 2013;2(1):5-10.

138. Neerken S, Lucassen GW, Bisschop MA, Lenderink E, Nuijs TA. Characterization of age-related effects in human skin: A comparative study that applies confocal laser scanning microscopy and optical coherence tomography. J Biomed Opt. 2004;9(2):274-81.

139. Hull MT, Warfel KA. Age-related changes in the cutaneous basal lamina: scanning electron microscopic study. J Invest Dermatol. 1983;81(4):378-80.

140. Südel KM, Venzke K, Mielke H, Breitenbach U, Mundt C, Jaspers S, et al. Novel aspects of intrinsic and extrinsic aging of human skin: beneficial effects of soy extract. Photochem Photobiol. 2005;81(3):581-7.

141. Smith AG, Singleton JR. Obesity and hyperlipidemia are risk factors for early diabetic neuropathy. J Diabetes Complications. 2013;27(5):436-42.

142. Herman RM, Brower JB, Stoddard DG, Casano AR, Targovnik JH, Herman JH, Tearse P. Prevalence of somatic small fiber neuropathy in obesity. Int J Obes (Lond). 2007;31(2):226-35.

143. Buschbacher RM. Body mass index effect on common nerve conduction study measurements. Muscle Nerve. 1998;21(11):1398-404.

144. Miscio G, Guastamacchia G, Brunani A, Priano L, Baudo S, Mauro A. Obesity and peripheral neuropathy risk: a dangerous liaison. J Peripher Nerv Syst. 2005;10(4):354-8.

145. Moll I, Roessler M, Brandner JM, Eispert AC, Houdek P, Moll R. Human Merkel cells--aspects of cell biology, distribution and functions. Eur J Cell Biol. 2005;84(2-3):259-71.

146. Jiang MS, Yuan Y, Gu ZX, Zhuang SL. Corneal confocal microscopy for assessment of diabetic peripheral neuropathy: a meta-analysis. Br J Ophthalmol. 2016;100(1):9-14.

147. 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(7):e0180175.

148. Kalteniece A, Ferdousi M, Azmi S, Khan SU, Worthington A, Marshall A, et al. Corneal nerve loss is related to the severity of painful diabetic neuropathy. European Journal of Neurology. 2022;29(1):286-94.

149. 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):1859.

150. Quattrini C, Tavakoli M, Jeziorska M, Kallinikos P, Tesfaye S, Finnigan J, et al. Surrogate markers of small fiber damage in human diabetic neuropathy. Diabetes. 2007;56(8):2148-54.

151. Freeman R, Gewandter JS, Faber CG, Gibbons C, Haroutounian S, Lauria G, et al. Idiopathic distal sensory polyneuropathy: ACTTION diagnostic criteria. Neurology. 2020;95(22):1005-14.

152. Finnerup NB, Haroutounian S, Kamerman P, Baron R, Bennett DLH, Bouhassira D, et al. Neuropathic pain: an updated grading system for research and clinical practice. Pain. 2016;157(8):1599-606.

# Assessment of peripheral neuropathy

**IN THE FIELD** of peripheral neuropathy, there is a need for diagnostic methods that can assess early impairments in nerve function, thus enhancing the efficiency of diagnosis and enabling preventive treatment. This thesis focuses on two novel techniques that have been suggested to detect peripheral neuropathy: examination of vibration perception thresholds (VPTs) through multi-frequency vibrometry and assessment of the intraepidermal nerve fiber density (IENFD) in thin 5-µm skin samples. These methodologies hold the potential for numerous advantages over current gold standards, offering increased accessibility, reduced costs, and shorter time requirements. However, there is a substantial knowledge gap concerning reference values, the possibility of differentiating between healthy and diseased states, and the optimal application of the methods within a clinical context. Thus, the doctoral work aimed to establish normative values of VPTs and IENFDs, and to investigate the clinical implementation in patients with type 1 or type 2 diabetes.

**LINNÉA EKMAN** holds a Bachelor of Medical Sciences and began her clinical research journey at the Department of Hand Surgery in Malmö in 2017.

Printed by Media-Tryck, Lund 2024 NORDIC SWAN ECOLABEL 3041 0903

LUND UNIVERSITY

FACULTY OF MEDICINE

Department of Translational Medicine

Lund University, Faculty of Medicine
Doctoral Dissertation Series 2024:150
ISBN 978-91-8021-647-0
ISSN 1652-8220