Peripheral polyneuropathy in type 2 diabetes mellitus and impaired glucose tolerance. Correlations between morphology, neurophysiology, and clinical findings

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Doctoral thesis

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To my family

CONTENTS

ABSTRACT 9

THESIS AT A GLANCE 10

ABBREVIATIONS 12

LIST OF PAPERS 13

INTRODUCTION 15

1. BACKGROUND 17

1.1 Definition of diabetes and impaired glucose tolerance 17

1.2 Classification and characteristics of diabetic neuropathy 18
1.2.1 Chronic distal symmetrical sensory-motor polyneuropathy 18
1.2.2 Autonomic neuropathy 19
1.2.3 Focal/asymmetrical neuropathies 20

1.3 Prevalence of diabetic peripheral polyneuropathy 21

1.4 Pathogenesis and etiology of diabetic peripheral polyneuropathy 21
1.4.1. Metabolic nerve injury 21
1.4.2. Vascular/ischemic nerve injury 22
1.4.3. Autoimmune nerve injury 22

1.5 Diagnosis of peripheral polyneuropathy 23
1.5.1 Clinical examination 23
1.5.2 Neurophysiological examination 24
1.5.3 Sural nerve biopsy 26
1.5.4 Skin biopsy 27

1.6 Treatment of diabetic neuropathy 29
1.6.1 Pathogenetic treatment 29
1.6.2 Preventive treatment 30
1.6.3 Symptomatic treatment 30

1.7 Neuropathy associated with impaired glucose tolerance 30

2. AIMS 32

3. SUBJECTS AND STUDY DESIGN 33

4. METHODS 35

4.1. Oral glucose tolerance test (OGTT) 35

4.2 Clinical examination 35
4.2.1 Papers I and II 35
4.2.2 Paper III 35

4.3 Neurophysiological examination 36
4.3.1 Nerve conduction studies (NCS) 36
4.3.2 Quantitative sensory testing (QST) 36
4.3.3 Tactilometry 36

4.4 Sural nerve biopsy 37
4.4.1 Biopsy sampling and processing 37
4.4.2 Quantification of myelinated nerve fibers 37
4.4.3 Endoneurial capillary morphometry 38
4.5 Skin biopsy and quantification of intraepidermal nerve fibers 39
4.6. Statistics 39
5. RESULTS 40
5.1 Endoneurial capillary abnormalities in relation to glucose tolerance (Paper I) 40
5.2 Endoneurial capillary abnormalities in relation to peripheral polyneuropathy (Paper I) 40
5.3 Sural nerve biopsy and future progression of nerve dysfunction (Paper II) 40
5.4 Intraepidermal nerve fibers in relation to clinical scoring of diabetic polyneuropathy (Paper III) 41
5.5 Intraepidermal nerve fibers in relation to neurophysiological scoring of diabetic polyneuropathy (Paper III) 41
5.6 Vibrotactile sense in median and ulnar nerve innervated fingers in relation to glucose intolerance (Paper IV) 41
6. GENERAL DISCUSSION 43
6.1 Microangiopathy, glucose intolerance, and neuropathy (Paper I) 44
6.2 Myelinated nerve fiber density (Paper II) 45
6.3 Intraepidermal nerve fibers (Paper III) 47
6.4 Vibrotactile sense (Paper IV) 50
6.5 IGT and polyneuropathy 51
7. CONCLUSIONS 53
8. SWEDISH SUMMARY (Sammanfattning på svenska) 54
9. ICELANDIC SUMMARY (Samantekt á íslensku) 56
10. ACKNOWLEDGEMENTS 59
11. APPENDIX A - G 61
12. REFERENCES 68
13. ORIGINAL PUBLICATIONS 81

ABSTRACT

Diabetic peripheral polyneuropathy (PN) is a common and serious complication of diabetes. The prevalence of PN is rising with the global burden of type 2 diabetes. The causal mechanisms of PN are not fully understood, but both vascular and metabolic factors play a role. New methods of investigating PN need to be correlated with standard methods in well-defined, population-based cohorts.

Objectives: The overall aim was to investigate endoneurial microvascular abnormalities of the sural nerve and intraepidermal nerve fiber loss in type 2 diabetes and impaired glucose tolerance (IGT), in relation to glucose dysmetabolism, and clinical and neurophysiological measures of PN.

Methods: Subjects were recruited from a prospective, population-based study of males from Malmö, Sweden (6956 responders). From this cohort, 182 individuals in three groups were identified, [69 with type 2 diabetes, 51 IGT, and 62 normal glucose tolerance (NGT)], matched for age, height, and body mass index (BMI). Endoneurial microangiopathy and myelinated nerve fiber density (MNFD) were assessed in 30 sural nerve biopsies (from 10 men with type 2 diabetes, 10 IGT, and 10 NGT). Intraepidermal nerve fibers (IENF) were assessed in skin biopsies from the distal leg in 86 subjects (50 men with type 2 diabetes, 15 IGT, and 21 NGT) and graded as absent IENF, low (1–3 IENF/section), or high ($\geq$ 4 IENF/section) counts of IENF. The subjects underwent oral glucose tolerance test, clinical examination (Total Neuropathy Score; combined Neuropathy Symptom Score and Neuropathy Disability Score), and neurophysiological tests (nerve conduction and quantitative sensory testing) at baseline and at follow-up (6–10 years later). Vibrotactile sense of the index (median nerve) and little fingers (ulnar nerve) was assessed in 58 subjects (23 type 2 diabetes, 7 IGT, 28 NGT) with persistent glucose tolerance for 15 years.

Results: Increased endoneurial capillary density was linked to current diabetes and future progression from IGT to diabetes. Decreased capillary luminal area was associated with deterioration of glucose tolerance. Increased basement membrane area was related to clinical PN. A low baseline sural nerve MNFD ($\leq$ 4700 fibers/mm^2) was associated with future progression of neurophysiological dysfunction in the peroneal and median nerves. MNFD correlated negatively with BMI. Absence of IENF was related to low sural nerve amplitude and conduction velocity, and high cold perception threshold. Vibrotactile sense was impaired in the index and particularly the little finger of diabetic subjects, mainly at high frequencies (250–500 Hz). IGT did not affect vibrotactile sense.

Conclusions: Sural nerve endoneurial microangiopathy is related to glucose dysmetabolism and clinical PN. MNFD may predict future nerve dysfunction. Obesity may be a risk factor for PN. IENF count correlates with neurophysiological measures of PN. Vibrotactile sense is impaired in the fingers, particularly innervated by the ulnar nerve at high frequencies, in patients with type 2 diabetes but not those with IGT.

THESIS AT A GLANCE

Overall aim: To investigate endoneurial microvascular abnormalities of the sural nerve and intraepidermal nerve fiber loss in type 2 diabetes and impaired glucose tolerance (IGT), in relation to glucose dysmetabolism and clinical and neurophysiological measures of diabetic polyneuropathy. Subjects were recruited from a prospective, population-based study of males from Malmö. From this cohort, 182 individuals in three groups [69 with type 2 diabetes, 51 IGT, and 62 normal glucose tolerance (NGT)] were identified and matched for age, height, and body mass index (BMI).

Paper I:
Aim: To investigate baseline endoneurial microangiopathy of the sural nerve in relation to (1) glucose intolerance, (2) clinical peripheral polyneuropathy, (3) nerve dysfunction of the sural nerve, in subjects with type 2 diabetes, IGT, or NGT.

Method: Baseline assessment: oral glucose tolerance test, clinical examination, nerve conduction test of the sural nerve, endoneurial capillary morphology (capillary density, luminal area, basement membrane area), myelinated nerve fiber density in sural nerve biopsies from 30 subjects (10 with type 2 diabetes, 10 IGT, 10 NGT). Follow-up (6 years later): assessment of oral glucose tolerance, clinical examination, and nerve conduction of the contralateral sural nerve.

Results: Increased capillary density is associated with current and future development of diabetes, and mild dysfunction of the sural nerve. Reduced luminal area is linked to deterioration of glucose tolerance, from IGT to diabetes, or from NGT to IGT. Thickened basement membrane is related to clinical peripheral neuropathy and morphological loss of myelinated nerve fibers in subjects with type 2 diabetes or IGT.

Conclusion: Endoneurial microangiopathy may: (1) presage deterioration of glucose tolerance, (2) presage and accompany peripheral neuropathy. Higher density of endoneurial capillaries may suggest compensation for hypoperfusion and hypoxia in early diabetic polyneuropathy.

Paper II
Aim: To investigate the progression of nerve dysfunction over time in relation to baseline nerve biopsy.

Method: Baseline assessment: myelinated nerve fiber density (MNFD) in sural nerve biopsies from 30 subjects [10 with type 2 diabetes, 10 impaired (IGT), and 10 normal glucose tolerance (NGT)], nerve conduction test, and quantitative perception thresholds. Follow-up (7–10 years later): assessment of nerve conduction and quantitative perception thresholds.

Results: Subjects with low baseline MNFD ($\leq$ 4700 fibers/mm^2) show decline of peroneal amplitude and conduction velocity, as well as median sensory amplitude and motor conduction velocity from baseline to follow-up. Low MNFD correlates negatively with BMI.

Conclusion: A low baseline sural nerve MNFD, in subjects with type 2 diabetes, IGT, or NGT, may predict later progression of neurophysiological dysfunction in the peroneal and median nerves. Obesity may be a risk factor for peripheral neuropathy.

Paper III
Aim: To investigate the potential correlation between intraepidermal nerve fiber (IENF) count and clinical and neurophysiological measures of diabetic peripheral polyneuropathy.

Method: Quantification of IENF (with immunohistochemistry and light microscopy) in skin biopsies (type 2 diabetes, IGT, or NGT) and assessment of clinical polyneuropathy (Total Neuropathy Score) and neurophysiological examination (nerve conduction test and quantitative perception thresholds).

Results: Subjects with absent IENF have lower sural nerve amplitude and conduction velocity, and higher cold perception threshold compared to subjects with low (1–3 IENF/section) and high ($\geq$ 4 IENF/section) IENF counts. Total Neuropathy Score does not differ between the three groups with different IENF counts.

Conclusion: IENF count in subjects with type 2 diabetes, IGT or NGT, quantified with a simple technique, correlates with neurophysiological measures of both large and small fiber neuropathy.

Paper IV
Aim: To investigate vibrotactile sense (large fiber neuropathy) at different frequencies in index and little fingers (median and ulnar nerves, respectively).

Method: Assessment of vibrotactile sense at different frequencies in 58 subjects with stable glucose tolerance for 15 years (type 2 diabetes, IGT or NGT).

Results: Vibrotactile sense is impaired in index (median nerve) and little fingers (ulnar nerve) in diabetes but not in IGT. Vibration thresholds are particularly increased at 16, 250, and 500 Hz in the little finger.

Conclusion: Tactilometry, with a multi-frequency approach, is a sensitive technique to screen for large fiber neuropathy in type 2 diabetes. Frequency-related changes may mirror dysfunction of various receptors.

ABBREVIATIONS

AAN	American Academy of Neurology
AANEM	American Academy of Neuromuscular and Electrodiagnostic Medicine
AAPM&R	American Academy of Physical Medicine and Rehabilitation
ADA	The American Diabetes Association
AN	autonomic neuropathy
ARI	aldose reductase inhibitor
BMA	basement membrane area
BMI	body mass index
CIDP	chronic inflammatory demyelinating polyneuropathy
DCCT	The Diabetes Control and Complications Trial
DM	diabetes mellitus
EFNS	European Federation of Neurological Societies
IENF	intraepidermal nerve fibers
IGT	impaired glucose tolerance
IQR	interquartile range
MNF	myelinated nerve fiber
NDS	neuropathy disability score
NGT	normal glucose tolerance
NDS	neuropathy disability score
NSS	neuropathy symptom score
OGTT	oral glucose tolerance test
PN	distal symmetrical sensory-motor polyneuropathy
QPT	quantitative perception thresholds
QST	quantitative sensory testing
SFN	small-fiber neuropathy
SNAP	sural nerve action potential amplitude
SNCV	sural nerve conduction velocity
TNS	total neuropathy score
VPT	vibration perception threshold
WHO	World Health Organization

LIST OF PAPERS

I **Endoneurial capillary abnormalities presage deterioration of glucose tolerance and accompany peripheral neuropathy in men.**
Thrainsdottir S, Malik RA, Dahlin LB, Wiksell P, Eriksson K-F, Rosén I, Peterson J, Greene DA, Sundkvist G. *Diabetes* 52:2615–2622, 2003.

II **Sural nerve biopsy may predict future nerve dysfunction.**
Thrainsdottir S, Malik RA, Rosén I, Jakobsson F, Bakhtadze E, Petersson J, Sundkvist G, Dahlin LB. *Acta Neurol Scand* 120:38-46, 2009.

III **Intraepidermal nerve fiber loss correlates with neurophysiological severity of diabetic neuropathy.**
Thrainsdottir S, Englund E, Rosén I, Petersson J, Sundkvist G, Dahlin LB. *Submitted.*

IV **Vibrotactile sense in median and ulnar nerve innervated fingers of men with type 2 diabetes, normal or impaired glucose tolerance.**
Dahlin LB, Thrainsdottir S, Cederlund R, Thomsen NO, Eriksson KF, Rosén I, Speidel T, Sundkvist G. *Diabet Med*, 25:543–549, 2008 Epub 2008 Mar 13.

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INTRODUCTION

The prevalence of diabetes is increasing dramatically in both developed and developing countries. The prevalence was estimated to be 2.8%, affecting 171 million people worldwide, in the year 2000 (1). The increase in prevalence is chiefly attributed to type 2 diabetes, resulting in significantly rising health care costs. There are several factors at work bringing about the increase: a growing and aging population, sedentary lifestyle, lack of physical activity, high-fat diet, and obesity (2-6). With current trends, the prevalence worldwide is estimated to reach 4.4%, affecting 366 million people by the year 2030 (1). Diabetes and its complications are major causes of mortality, morbidity, and decreased quality of life. The complications include both microvascular (neuropathy, nephropathy, and retinopathy) and macrovascular (atherosclerotic) disease. Consequently, the burden of diabetes is enormous (3; 7-10).

Neuropathies are among the most common long-term complications of diabetes, affecting up to 50% of patients (11). The prevalence of diabetic neuropathies is rising with the global burden of type 2 diabetes (12). Given that diabetes affects approximately 246 million people worldwide, it is estimated that 20–30 million people are affected by symptomatic diabetic neuropathy (13). There are a wide variety of neuropathies in diabetes, affecting single (mononeuropathy), several (mononeuropathy multiplex), or many nerves (polyneuropathy). The most common form is the distal symmetrical polyneuropathy, affecting sensory more than motor nerves. Small and/or large nerve fibers may be affected, usually both. This neuropathy is a leading and independent risk factor for mortality (14) and morbidity as a result of foot ulceration and amputation (15). It affects up to 50% of type 1 or type 2 diabetic patients (16). The patients often suffer from neuropathic pain that is difficult to treat. In this thesis, the term “peripheral polyneuropathy” (PN) is used for this form of diabetic neuropathy. Other symmetrical neuropathies include autonomic neuropathy, acute painful neuropathy associated with weight loss, insulin neuritis, polyneuropathy after ketoacidosis, and the recently described neuropathy associated with “prediabetes” or impaired glucose tolerance (IGT) (11; 17-22). In order to prevent the development of PN it is essential to improve glycemic control, encourage good foot care, and screen for symptoms and signs of PN with a neurological examination.

Whilst PN has received considerable attention over the last century, however, the pathophysiological mechanisms are not fully understood. Furthermore, study-cohorts of patients with PN are not well defined and there is marked variability between studies in diagnostic methods and study selection criteria. Additional confounding factors include referral bias, the severity of PN, duration of diabetes, glycemic control, and sex and age of the patients. Early identification and treatment of patients at risk is essential to minimize and delay further progression and disability. Therefore, studies with well-defined cohorts are needed to clarify the causative mechanisms and to develop new methods to identify patients at risk for PN.

In this thesis, a well-defined cohort of men with type 2 diabetes, IGT, or normal (NGT) glucose tolerance (controls) has been studied. The subjects were matched for age, height, body mass index (BMI), and gender. The subjects were prospectively followed-up during a period of around 15 years, from when they were mean 61 years to mean 75 years. In this cohort, the occurrence of PN was investigated, with clinical,

neurophysiological, and histopathological methods. Microangiopathic abnormalities of the vasa nervorum were studied in sural nerve biopsies. Microangiopathy may develop early and play an important role in the development of diabetic PN. To find out how early these abnormalities develop, individuals with IGT, a form of prediabetes, (23) and control subjects were investigated. A follow-up was performed 7–10 years later, and possible deterioration of glucose tolerance and development of PN related to microangiopathy and loss of sural nerve myelinated nerve fibers. Sural nerve biopsies are difficult to obtain for research for ethical reasons as the procedure may cause persistent postoperative complaints (24; 25). Thus the data generated from this material is unique. To our knowledge, no previous studies are available that prospectively evaluate the relation between baseline sural nerve pathology and future glucose dysmetabolism and nerve dysfunction.

Small fiber neuropathy (SFN) may be difficult to diagnose as quantitative sensory testing methods are limited (26), and more objective measures are needed. In recent years, different techniques for quantification of the sensory cutaneous nerves in a skin biopsy have been used to investigate SFN. In this thesis the aim was to develop a simple and quick method to count the intraepidermal nerve fibers using light microscopy to allow quantification in any standard pathology laboratory and hence increase its use in clinical practice.

1. BACKGROUND

1.1 Definition of diabetes and impaired glucose tolerance

Diabetes mellitus is a metabolic syndrome characterized by chronically high levels of blood glucose caused by a defect in insulin secretion and/or insulin resistance. There are two main types of diabetes, type 1 and type 2 diabetes. Type 1 diabetes, previously called insulin-dependent diabetes, or juvenile-onset diabetes is an autoimmune disorder resulting in destruction of the insulin-producing beta cells in the pancreas (27). Type 1 diabetes occurs at all ages, but usually presents at young age, often with ketoacidosis. Patients with type 1 diabetes develop absolute insulin deficiency and usually need insulin early in the disease. The occurrence of type 1 diabetes shows clear geographical variations and is most common in Caucasians in Finland, Scandinavia, and Sardinia (28-30). Type 2 diabetes, previously called non-insulin-dependent diabetes, or adult-onset diabetes, results from insulin resistance, usually with relative (rather than absolute) insulin deficiency or insulin secretory defect with insulin resistance. Hence, there is insufficient insulin to compensate for the insulin resistance. Most patients with this form of diabetes are obese, and the obesity may cause some degree of insulin resistance. Type 2 diabetes is the most common form of diabetes, accounting for over 95% of all diagnosed cases of diabetes. The prevalence varies greatly between countries and ethnic groups, being 50% in Pima/Papago Indians, 5–8% in Europe (31), and 3–4% in Sweden (32; 33). Type 2 diabetes typically has a later age of onset, but an alarming increase in the prevalence of type 2 diabetes among adolescents and children has been reported (34). In this thesis, type 2, and not type 1, diabetic patients were studied.

An oral glucose tolerance test (OGTT) may be performed to determine the glucose tolerance, whether it is normal (NGT), impaired (IGT), or diabetic. Glucose concentration in whole blood or plasma is measured fasting (0 min) and 120 min after ingestion of 75g of glucose. In accordance with the WHO criteria from 1998 (35), a fasting capillary blood-glucose concentration $\geq$ 6.1 mmol/L and/or a 120 min blood-glucose $\geq$ 11.1 mmol/L is defined as diabetes. In plasma, the glucose levels are higher, therefore, capillary plasma-glucose concentration $\geq$ 7.0 mmol/L and/or a 120 min glucose $\geq$ 12.2 mmol/L is defined as diabetes (Table 1). These criteria are used in this thesis. In papers I and II, capillary blood-glucose was measured, but from 2004, our laboratory predominantly measures plasma-glucose levels. Hence, in papers III and IV, capillary plasma-glucose is measured.

Table 1

	Capillary blood	Capillary plasma	Venous plasma
Diabetes mellitus			
Fasting value	$\geq$ 6.1	$\geq$ 7.0	$\geq$ 7.0
120 min value	$\geq$ 11.1	$\geq$ 12.2	$\geq$ 11.1
IGT			
Fasting value	< 6.1	< 7.0	< 7.0
120 min value	7.8–11.0	8.9–12.1	7.8–11.0

Diagnostic criteria for diabetes mellitus and IGT (35). All values are mmol/L.

In 2003 (27), the American Diabetes Association (ADA) introduced new criteria based on the following premises: 1) symptoms of diabetes (polyuria, polydipsia, and unexplained weight loss) together with a random venous plasma-glucose concentration ≥ 11.1 mmol/L or 2) fasting venous plasma-glucose concentration ≥ 7.0 mmol/L on two different days, or 3) a 120 min venous plasma-glucose ≥ 11.1 mmol/L. In the latest reviews by WHO in 2006 (36), and ADA in 2009 (37), no changes in the classification of diabetes were made. A majority of patients with type 2 diabetes have features of the metabolic syndrome, defined as central obesity together with at least two of the following components: dyslipidemia, hypertonia, and type 2 diabetes or IGT and/or raised fasting plasma-glucose (≥ 5.6 mmol/L)(38).

IGT was first introduced in 1979 by the National Diabetes Data Group and the WHO as a risk factor for type 2 diabetes, replacing the term "borderline" diabetes, but did not confer a risk of microvascular complications (39). IGT is not uncommon, having prevalence of 2–25% in adults (40). There is growing evidence that IGT is associated with peripheral neuropathy, especially in patients with small nerve fiber neuropathy (22). IGT is defined as fasting capillary blood-glucose concentration of < 6.1 mmol/L combined with a 120 min value of 7.8–11.0 mmol/L (Papers I and II)(Table 1), or a fasting capillary plasma-glucose concentration of < 7.0 mmol/L combined with a 120 min value of 8.9–12.1 mmol/L (Papers III and IV). Hence, there is a spectrum of severity in hyperglycemia, ranging from "prediabetic state" to overt diabetes. Individuals with IGT are considered at increased risk of developing type 2 diabetes, cardiovascular disease, and death (41). However, proper lifestyle intervention with routine exercise and modest weight loss is thought to prevent or delay the onset of overt diabetes (23; 42).

1.2 Classification and characteristics of diabetic neuropathy

Neurological complications in diabetes are heterogeneous, affecting different parts of the nervous system, and may present with diverse clinical manifestations. There are numerous classifications of syndromes affecting the peripheral nervous system in diabetes (11). The classification shown in Table 2 is based on that originally proposed by Thomas (11; 43; 44). In this thesis, the most common form of diabetic neuropathies – the chronic distal symmetrical sensory-motor polyneuropathy (PN), representing 70% of diabetic neuropathies (12) – was studied. Other forms of diabetic neuropathies are described in this chapter, but are not discussed further.

1.2.1 Chronic distal symmetrical sensory-motor polyneuropathy

Chronic distal symmetrical sensory-motor polyneuropathy (PN) is a "dying back" neuropathy with predominantly sensory deficits and a variable degree of autonomic involvement. It is mainly axonal with a mixed large and small fiber involvement. The presentation is frequently insidious with burning or lancinating pain, numbness, paresthesia, and hyperalgesia in both feet and lower limbs (symmetrical). Typically, at a more severe stage, the hands become affected. The neuropathic pain is typically worse at night and is often relieved by walking around. Another common feature is the intolerance of the blanket to touch the feet. Up to 50% of diabetic patients may be

Table 2

Neuropathy class	Neuropathy type
Generalized/symmetrical polyneuropathies	Sensory-motor (chronic) Acute sensory Autonomic
Focal and multifocal neuropathies	Proximal motor (amyotrophy) Focal limb Cranial Thoracolumbar radiculoneuropathy
Rapidly reversible neuropathy	Hyperglycemic
Superimposed chronic inflammatory demyelinating polyneuropathy (CIDP)	

Classification of diabetic neuropathies (11; 43).

asymptomatic and the PN is only detected by neurological examination (45) or by a nerve conduction study (46). Approximately 10–20% may experience severe sensory symptoms that require treatment (11). Some patients are diagnosed when presenting with a painless, often infected, foot ulcer (45). Sensory neuropathy with numbness in feet is considered a major predisposing factor to diabetic foot ulcer and amputation (45). PN is related to the duration of diabetes and glycemic control (47), although it may be the presenting symptom of diabetes (48; 49). Independent of age or duration of diabetes, there are no major clinical differences of PN between patients with type 1 or type 2 diabetes (12).

Different neuropathies may cause preferential injury to different types of nerve fibers, predominantly affecting small or large diameter nerve fibers, or both. Accordingly, neuropathies may be distinguished in small fiber neuropathy, large fiber neuropathy, or mixed (small and large) fiber neuropathy. Each fiber type is responsible for different functions and when injured, causes different symptoms (Table 3)[adapted from Kiernan (50)].

1.2.2 Autonomic neuropathy

Autonomic neuropathy (AN) is a common and serious complication in both type 1 and type 2 diabetes. AN can affect one, several, or all innervated organs. Generally it is considered a disorder of the sympathetic and/or parasympathetic nervous system, affecting small nerve fibers. Common symptoms are dizziness (orthostatic hypotension), resting tachycardia, edema, bladder dysfunction, and erectile dysfunction. Diabetic patients with AN have significantly increased mortality risk compared to diabetic patients without AN, mainly due to renal failure, sudden death, and cardiovascular events (51). The prevalence of AN varies widely depending on the cohort studied and the method of assessment with prevalence rates ranging from 7.7% in newly diagnosed patients with type 1 diabetes (52) to 90% in potential pancreas

Table 3

Fiber class	Fiber type /size		Functional class	Fiber dysfunction
Myelinated nerve fibers	**Large fibers**	Aα motor neurons 12-20 µm	Motor	Weakness, atrophy, cramps, fasciculations
		Aβ fibers 5-15 µm	Sensory	Abnormal proprioception, vibration and touch sensation
	Small fibers	Aδ fibers 3-8 µm	Sensory	Deep and lancinating pain, abnormal cold and pressure sensation
Unmyelinated nerve fibers	**Small fibers**	C fibers 0.2-1.5 µm	Sensory	Burning pain and abnormal heat sensation
		C fibers 0.2-1.5 µm	Autonomic	Abnormal sweating, bowel, bladder, and sexual function, abnormal blood pressure control

Classification of nerve fibers in peripheral nerves (50).

transplant recipients (53). In a large study of 1171 diabetic patients (647 type 1 and 524 type 2), cardiovascular AN was diagnosed (at least 3 of 7 tests abnormal) in 16.8% of type 1 and in 22.1% of type 2 diabetic patients (52). Cardiovascular AN (at least 3 of 7 tests abnormal) is seen in 13% of diabetic patients without PN, 49% of those with symptomatic PN, and in 100% of those with the latter and concomitant autonomic symptoms (54). In addition to the risk factors for PN, autoimmunity is thought to play an important role in the pathogenesis of AN (51; 55; 56). Autoantibodies against adrenal medulla, sympathetic ganglia, and the vagal nerve have been detected and related to future development of cardiac and peripheral AN (56). Treatment is mainly symptomatic, but improvement in glycemic control seems to improve AN (57).

1.2.3 Focal/asymmetrical neuropathies

Focal/asymmetrical diabetic neuropathies may involve a single nerve (mononeuropathy), or several different nerves (mononeuropathy multiplex). Some focal neuropathies are caused by nerve entrapment, commonly involving the ulnar, median, and peroneal nerves (11; 45). An increased susceptibility to nerve compression in diabetes has been described (58) and about one third of diabetic patients have nerve entrapment (45). The most common nerve entrapment is carpal tunnel syndrome, i.e. compression of the median nerve at the wrist (11). Other focal/multifocal neuropathies in diabetes may have an ischemic basis and often present with sudden onset of severe pain. Cranial neuropathies are considered to result from microvascular nerve infarct, typically involving the third, fourth, sixth, and seventh cranial nerves (11; 45). Cranial neuropathies are rare and occur in older individuals

with a long duration of diabetes (11). The majority of the cranial neuropathies resolve spontaneously over several months but can recur in 25% of patients (11). Nerve infarcts have also been demonstrated in the proximal lower limb motor neuropathy (amyotrophy) but there is evidence that focal inflammatory lesions (including vasculitic) may be involved (11; 43).

1.3 Prevalence of diabetic peripheral polyneuropathy

Diabetic PN is the most common peripheral polyneuropathy in the developed world (18; 59). Since the prevalence of diabetes is increasing, the prevalence of diabetic PN is also expected to rise (13; 59). Diabetic PN affects approximately 50% of diabetic patients in the US (54% in type 1 and 45% in type 2 diabetes) (16). The prevalence of PN among diabetic patients varies between studies depending on duration of diabetes, glycemic control, different diagnostic criteria, and tests used. Among 8757 Italian diabetic patients attending outpatient clinics, clinical PN was diagnosed in 32.3% and increased dramatically to 83.5% when quantitative neurological examination and nerve conduction studies were employed (46). At the time of diabetes diagnosis, a Finnish study showed that 8% of diabetic patients had PN (diagnosed with clinical examination and nerve conduction tests) compared to 2% of non-diabetic controls (49). After 10 years of follow-up, the prevalence had increased to 42% in the diabetic patients and 6% in the controls. Pirart's classical study, published in 1978, is the most extensive epidemiological study so far (47). Between 1947 and 1973, 4400 patients were studied and the prevalence of neuropathy rose from 7.5% at the time of diagnosis of diabetes to approximately 50% after 25 years.

1.4 Pathogenesis and etiology of diabetic peripheral polyneuropathy

Risk factors for the development of PN include diabetes duration, degree of hyperglycemia, hyperlipidemia, hypertension, and height (11; 45). Retinopathy and nephropathy are highly associated with PN, occurring in 55% and 32% of type 2 diabetic patients, respectively (16). The pathophysiological mechanisms for PN, however, are not yet fully understood. Several different factors are thought to be involved, both metabolic and vascular (60-63). In addition, immune factors are thought to play an important role in the pathogenesis of AN in type 1 diabetes (51; 56) and perhaps also of PN in both type 1 and type 2 diabetes (64) (Table 4).

1.4.1. Metabolic nerve injury

Among the metabolic factors, hyperglycemia is probably the most important common initiator. In hyperglycemia, excess of glucose is converted to sorbitol via the enzyme aldose reductase (through the polyol pathway) leading to accumulation of sorbitol and depletion of myo-inositol in nerve tissue (65). Hyperglycemic activation of polyol pathway may lead to changes in the NAD/NADH ratio, causing direct neuronal damage and/or decreased nerve blood flow (51). In experimental studies, these metabolic changes induce osmotic swelling and alteration in sodium-potassium ATPase activity in diabetic nerves (65). A fall in sodium-potassium ATPase activity has been linked to slowing of conduction velocity in diabetic rats (66). In addition, hyperglycemia may induce the production of advanced glycation end products and free radicals, which are neurotoxic. Oxidative stress, in turn, may affect mitochondrial

permeability leading to activation of programmed cell death caspase pathways, promoting apoptosis of neurons and Schwann cells (67). A number of neuronal growth factors and insulin itself promote survival and outgrowth of neurons. Hence failed signaling from these factors and impaired nerve regeneration may induce PN (12).

Table 4

Metabolic	Polyol pathway (aldose reductase) Glycosylation of important proteins (advanced glycation end products) Free radicals and oxidative stress Neuronal growth factors and insulin deficiency
Vascular/ischemic	Basement membrane reduplication in vasa nervorum Reduced endoneurial blood flow Reduced endoneurial oxygen tension
Autoimmune	Autoantibodies against adrenal medulla, sympathetic ganglia and vagal nerve Autonomic ganglia infiltrated by immune cells Calcium-dependent apoptosis of neuronal cells

Pathogenesis of diabetic polyneuropathy

1.4.2. Vascular/ischemic nerve injury

Microangiopathy with endothelial dysfunction of the vasa nervorum is considered the vascular factor causing ischemia and hypoxia in nerves of diabetic patients (12). Pathological studies of sural nerves of diabetic patients show a number of alterations, including thickening of the endoneurial capillary basement membrane, capillary closure, endothelial cell hypertrophy and hyperplasia, and pericyte degeneration (68-70). Impaired vasodilatation of the vasa nervorum (12; 71; 72) may develop early and presage microangiopathic changes, subsequently leading to narrowing of the capillary lumen, resulting in reduced capillary blood flow, hypoxia, and progression of neuropathy (73-75). Rheological alterations of the red blood cells may additionally contribute to microcirculatory disturbances (76-78). Degeneration of myelinated and unmyelinated nerve fibers may develop later and accompany the PN (75; 79; 80). The relationship between the severity of hyperglycemia and microangiopathy is not yet established and few studies on neural microangiopathy in IGT have been conducted (80).

1.4.3. Autoimmune nerve injury

Several studies suggest different mechanisms of autoimmune activation as a pathogenetic factor in the development of AN in both type 1 and type 2 diabetes (51). Circulating autoantibodies in sera of type 1 diabetic patients react with autonomic tissue, most notably sympathetic ganglia and the vagus nerve, and are associated with future development of AN (56). Postmortem studies of type 1 diabetic patients with severe AN have shown lymphocytic infiltration and small nerve fiber damage in

autonomic ganglia indicating vigorous immune response (81). Regarding PN, it is not clear whether autoimmunity plays a primary role in the pathogenesis; more likely, it accelerates PN initiated by metabolic or vascular injury. A significant greater activation of complement-independent calcium influx leading to apoptosis in cultured neuronal cells was shown in serum from type 2 diabetic patients with PN compared with serum from type 2 diabetic patients without PN (82). Autoimmune immunoglobulin was thought to induce apoptosis of the neuronal cells, and has been related to the severity of PN (83). This suggests that autoimmune mechanisms may act in concert with hyperglycemia to damage sensory/autonomic neurons (11; 51).

1.5 Diagnosis of peripheral polyneuropathy

Although experienced clinicians usually can diagnose PN with a clinical examination, a great inconsistency in diagnostic criteria exists in the literature. The American Academy of Neurology in conjunction with the American Association of Electrodiagnostic Medicine and the American Academy of Physical Medicine and Rehabilitation reported a case definition of distal symmetric polyneuropathy to standardize and facilitate clinical research and epidemiologic studies (84). Accordingly, the combination of neuropathic symptoms, signs, and electrodiagnostic findings provides the most accurate diagnosis of distal symmetric polyneuropathy.

1.5.1 Clinical examination

The clinical examination consists of an examination of peripheral sensation, tendon reflexes, and muscle strength. Neuropathic symptoms with distal sensory loss, absent tendon reflexes, and abnormal nerve conduction studies are highly suggestive of PN (84). Diabetic patients should be screened on a regular basis for PN as a simple clinical examination is a good predictor of future foot ulceration (15). The feet should be examined for ulcers, calluses, and deformities, and footwear should be inspected (85). The diagnosis of diabetic neuropathy also involves the exclusion of non-diabetic causes (i.e. metabolic, hereditary, nutritional deficiency, immunological, toxic) (86). Some metabolic conditions known to cause neuropathy (B12 deficiency, hypothyroidism, and uremia), and chronic inflammatory demyelinating polyneuropathy (CIDP) occur more frequently in diabetes. Excessive alcohol consumption should also be ruled out.

Neuropathy Disability Score

Scoring of signs of suspected neurological disease with different scales was used already in the 19th century (1886) (87). The use of composite scores to assess clinical signs was pioneered by Dyck and colleagues, who first described the Neuropathy Disability Score (NDS) (88) and later the Neuropathy Impairment Score (NIS) (89). They developed a scheme to detect and grade polyneuropathy. In the NDS, impairment of sensation, muscle strength, and tendon reflexes are individually scored, and all scores are summed to give a composite score of neurological deficits. The NDS evaluates cranial nerves, and upper and lower limbs bilaterally. This grading system may be used in epidemiological studies and clinical trials, as well as in clinical practice. Each item is graded 0 = no deficit, 1 = mild deficit, 2 = moderate deficit, 3 = severe deficit, and 4 = complete absence of function or the most severe deficit. A

modified NDS has been used in several large studies and is shown to predict future foot ulceration (15). Simple composite examination scores are as accurate as more complex examinations (84). In this thesis, a modified and simplified version of Dyck's original NDS was used to detect and define the severity of PN (80; 90, Paper III) (See methods).

Neuropathy Symptom Score

Neuropathy Symptom Score (NSS) was also developed by Dyck and his colleagues to define neuropathic symptoms (88). NSS comprises proximal and distal symptoms of muscle weakness, sensory disturbances (e.g. pain, numbness, unsteadiness in walking), and autonomic neuropathy (e.g. impotence, orthostatic hypotension, nocturnal diarrhea). Multiple neuropathic symptoms are more accurate than single symptoms in diagnosing PN (84). Altogether 17 symptoms are graded in the NSS; 1 point for the presence of a symptom, and 0 if absent. In this thesis, the NSS was modified from Dyck's description, with grading of seven different sensory symptoms.

1.5.2 Neurophysiological examination

Nerve conduction studies (NCS), quantitative sensory testing (QST), and vibrotactilometry are methods used to investigate peripheral nerve function. The former two are used in both routine clinical practice and in clinical trials, and tactilometry has been used primarily in trials. In the diagnosis of PN, NCS are the most informative part of the neurophysiological examination (84).

Nerve conduction studies (NCS)

NCS are electrophysiological studies commonly used in clinical practice to evaluate and follow up suspected PN and other neuromuscular disorders. NCS are generally not required for the diagnosis of diabetic PN, but the inclusion of NCS adds a higher level of specificity to the diagnosis (84). NCS should be requested in atypical cases of PN in diabetic patients, which might suggest other forms of neuropathy. NCS should be performed in rapidly progressing limb weakness (suspected CIDP), asymmetric/multifocal signs (suspected vasculitis), or a family history of neuropathy/feet deformities (suspected Charcot-Marie Tooth neuropathy). NCS are non-invasive, objective, and standardized, and provide a sensitive measure of the functional status of sensory and motor nerves. An electrical impulse, an action potential, is elicited by stimulating the nerve and conducted along a motor or sensory axon. In the normal nerve, the impulse propagates with a nerve conduction velocity of approximately 50–70 m/s. This can be measured with great accuracy and objectively used to determine the presence, severity, and level of peripheral nerve dysfunction. NCS can determine the distribution of abnormality (focal, multifocal, or diffuse) and whether the pathophysiology is predominantly segmental demyelination or axonal degeneration. Axonal loss causes reduction of the sensory nerve action potential and compound motor action potential. Demyelination causes reduction of the nerve conduction velocity and increased dispersion. Both afferent and efferent nerves can be tested, thus, motor and sensory neuropathies can be diagnosed. The ulnar and median nerves in the arm and the peroneal, posterior tibial, and sural nerves in the leg are commonly tested when PN is suspected. Guidelines and recommended protocol for

NCS in diagnosing PN in clinical research have been published (84; 91). The protocol includes unilateral studies of sural, ulnar, and median sensory nerves, and peroneal, tibial, median, and ulnar motor nerves with F waves. The minimum criterion for electrodiagnostic confirmation of distal symmetric polyneuropathy (PN) is an abnormality of any attribute of nerve conduction in two separate nerves, one of which must be the sural nerve (84).

Subcutaneous needle electrode magnifies the amplitude response as the needle comes in more proximity to the nerve, facilitating the measurement of the nerve conduction velocity. In isolated small fiber neuropathy NCS are normal but quantitative sensory testing is typically abnormal (see below).

Quantitative sensory testing (QST)

QST is a method used to investigate peripheral nerve function both in large myelinated Aβ (vibration) and in small thinly myelinated Aδ (cold), and in small unmyelinated C (heat) afferent nerve fibers (see Table 3). Vibration perception thresholds (VPT, μm) are generally measured at bony eminences in both upper and lower limbs. Heat (HPT, Δ°C) and cold (CPT, Δ°C) perception thresholds are measured on the lateral side of the foot and the thenar eminence of the hand. The two most commonly used methods of determining sensory thresholds are the "method of limits" and the "method of level". In this thesis the method of limits was used. In this method, the stimulus strength is increased from zero to the point where the sensation is first perceived and the subject presses a button. The stimulus strength is then decreased to the point where the sensation disappears, and the subject presses the button again. The threshold is determined by calculating the average of the perception and disappearance thresholds of three consecutive determinations on each test site.

QST examines the whole sensory pathway, from the receptor along afferent nerves and central pathways, to the sensory cortex. However, QST also measures the efferent motor pathways when the subject presses the button. Thus, the "method of limits" is limited by the subject's motor skills, reaction time, attention, and cooperation, and is influenced by the subject's sex, age, and height (26).

Tactilometry

The ability to feel vibrations is reflected by function in large nerve fibers and delicate receptors located in the finger pulps (92). Fast adapting receptors, such as Meissner's end organs and Pacini end organs, in contrast to slowly adapting receptors, are sensitive to fast and repeated deformation of skin. Threshold tests, such as tactilometry, are used to detect disturbances of vibrotactile sense, and assess large fiber neuropathy in diabetic patients (93-99). The ulnar and median nerves may be affected in diabetes, both due to PN and due to compression of these nerves at the elbow and the wrist, respectively. It is therefore important to investigate the occurrence and extent of neuropathy in the hands. However, little is known about vibratory perception in finger pulps of subjects with diabetes or IGT. In this doctoral thesis, age-matched subjects with persistent diabetes or IGT, and subjects with NGT were investigated. Tactilometry is influenced by age (26; 100) and former exposure to vibration (101).

In order to cover a larger spectrum of receptors, vibration thresholds may be analyzed at different frequencies (tactilometry). Pacinian corpuscle receptors are

responsible for vibrotactile sense at frequencies more than approximately 80 Hz (probably a maximum of 250 Hz) and are located deep in glabrous skin (92; 102; 103). Intracutaneous receptors of FA I type (Meissner's) are probably more important at lower frequencies (5–50 Hz) (92; 102). Slowly adapting afferent fibers in glabrous skin are also sensitive to vibration, particularly to low frequencies, but are considered not to be of crucial importance for the vibrotactile sense (103; 104). Detection of vibrotactile sense is poorer in hairy than in glabrous skin (105), but the discriminative performance shows striking similarities in the two types of skin with a possible exception at 50 Hz (103). A multifrequency approach (8–500 Hz) may fully evaluate the vibrotactile sense in finger pulps, which mediate high acuity tactile information to the brain.

A normal tactilogram has the form of a slope interrupted by peaks at 125 and 250 Hz. In patients with impaired vibrotactile sense, there is a change in the shape of the curve, and usually the highest frequencies are first affected. Few former studies have focused on investigating diabetic neuropathy in the hands. Tactilometry is a non-invasive method and may be sensitive to detect large fiber neuropathy (101). Disturbance in vibrotactile sense in the legs, previously investigated ideally at a single (125 Hz) frequency, is an indicator of neuropathy (99). Investigation of vibrotactile sense in finger pulps (sensibility index) is important to detect any large fiber neuropathy in the hands in diabetes, since such patients may have neurological symptoms that may be missed in clinical practice.

1.5.3 Sural nerve biopsy

Sural nerve biopsies are not routinely used for diagnosis of diabetic PN as the procedure may cause persistent postoperative complaints with sensory deficits (24). Nerve biopsy is generally accepted as useful in the diagnosis of inflammatory nerve diseases such as vasculitis, sarcoidosis, CIDP, infiltrative disorders such as tumor or amyloidosisis or infectious diseases such as leprosy (106). In 2009, the American Academy of Neurology (AAN), the American Academy of Neuromuscular and Electrodiagnostic Medicine (AANEM), and the American Academy of Physical Medicine and Rehabilitation (AAPM&R) published a report on the role of a nerve biopsy for the evaluation of peripheral neuropathy (106). Nerve biopsy was found most valuable in mononeuropathy multiplex or suspected vasculitic neuropathy (106), but there are no studies regarding the role of nerve biopsy in the evaluation of PN. Hence, no recommendations could be made regarding this issue (106).

The earliest reports on pathological degenerative changes in diabetic PN are from the 19th century. In the 20th century, Fagerberg (1959), demonstrated the relationship between microangiopathy and diabetic PN, and upheld the view that the damage was vascular in origin (107). He drew attention to the thickening and hyalinization of the walls of the small neural blood vessels, later shown to correspond to basement membrane thickening.

In diabetic PN, the sural nerve demonstrates loss of myelinated and unmyelinated nerve fibers and segmental demyelination. It also shows endoneurial microangiopathy characterized by basement membrane thickening, pericyte degeneration, and endothelial cell hyperplasia and luminal narrowing (68; 69). These pathological changes are associated with both clinical and neurophysiological measures of PN (68;

80; 108). However, diabetic patients without evidence of PN also demonstrate microangiopathy (69). Hence, microangiopathy develops early in diabetes and correlates with neuropathic severity, supporting a role in the pathogenesis of diabetic PN.

In 2008, the European Neuromuscular Centre published guidelines on the processing and evaluation of sural nerve biopsies in different peripheral neuropathies, but concluded that little high-quality evidence is available for that purpose (109). However, consensus about clinical indications for nerve biopsy and requirements for the person evaluating it were provided. Sural nerve tissue is obtained either by a fascicle biopsy or a whole nerve biopsy. The three most common methods of sampling nerve tissue from the sural nerve are: "fascicle sampling", "random sampling of whole nerve", and "systematic sampling of an area of every fascicle in the whole nerve" (110). Fascicular sampling was developed to minimize sensory deficits in sural nerve innervated skin area on the lateral side of the foot (24). The latter two methods are performed when the whole nerve is available, and systematic sampling is considered more accurate. In this thesis, a whole sural nerve biopsy was performed, and systematic sampling was applied for the quantification of myelinated nerve fibers (111). Sampling was not used for the assessment of microangiopathy as all endoneurial capillaries were counted in all fascicles in the whole nerve.

1.5.4 Skin biopsy

Skin biopsy has been used in recent years for investigating peripheral neuropathies, and has become a new diagnostic tool in small fiber neuropathy (SFN)(112-116). Different techniques for both processing the biopsy and nerve fiber assessment have been used. Intraepidermal nerve fiber (IENF) density is a sensitive measure and normative values for different body sites, age, and gender have been published (117-122). Reduced IENF density has been observed in both type 1 and type 2 diabetes (119), as well as in IGT (120). In studies examining skin biopsies at multiple sites, a decrease in IENF density from proximal to more distal sites has been observed (123; 124), suggesting that loss of small nerve fibers occurs in a length-dependent manner. Assessment of IENF density can reveal loss of small-diameter nerve fibers in patients with burning pain in their feet, whilst nerve conduction studies and clinical examination are normal. Hence, skin biopsy can give additional information for the diagnosis of SFN. The procedure is minimally invasive and can be repeated close to the site of the former biopsy to monitor disease progression in follow-up of patients or to investigate nerve regeneration in trials of neuroprotective treatments (125). Additionally, as a measure of SFN, skin biopsy is less invasive and more sensitive than sural nerve biopsy (126).

In 2005 the European Federation of Neurological Societies (EFNS) published a guideline on the use of skin biopsy in peripheral neuropathy (127). The guideline included recommendations on methods for tissue processing and quantification of IENF (127), as well as normative data and correlations with other clinical, electrophysiological, and morphologic tests. The EFNS recommends performing 3 mm punch skin biopsy at the distal leg, and quantifying the linear density of IENF in at least three 50 µm thick sections per biopsy. In addition, the EFNS concluded that morphologic nerve fiber changes, such as axonal swellings, may be predictive of

progression of polyneuropathy, but further studies are needed to determine their diagnostic accuracy.

Another method of obtaining skin samples involves applying a suction capsule to the skin (128). This creates a blister in which the epidermis is separated from the dermis and can be removed with the innervation intact. This blister is less invasive, bloodless and painless, but dermal myelinated and autonomic nerve fibers cannot be studied (129; 130), as in the skin biopsy. The blister technique was recently compared with the punch skin biopsy and demonstrated a good correlation with no statistical differences in IENF density between the two methods (131).

The use of bright-field immunohistochemistry or immunofluorescence with antibodies against PGP 9.5, a non-specific panaxonal marker widely distributed in the peripheral nervous system, is also recommended by the EFNS. Laser scanning confocal microscopy with computerized image analysis may also be used for quantification of IENF density. However, these methods are expensive, time-consuming, and require extensive training of the investigator to achieve adequate experience and reliability. Therefore, the aim of this thesis was to develop a simple and quick method that could be applied in any pathology laboratory as routine histopathological practice where the skin biopsies are fixed in formalin, embedded in paraffin, and cut in 5 μm thick sections. By ordinary light microscopy, IENF are easily identified and counted. Thin biopsy-sections have been used by others, Koskinen et al. used similar technique with 5–10 μm thick sections (132), and Dabby et al. quantified dermal autonomic nerve fibers in 5 μm thick sections, but found thin sections of limited value for the evaluation of IENF density (133).

Recent studies have focused on correlating IENF density with other measures of diabetic PN. Such studies are important to answer the question whether the quantification of IENF may be an alternative or complementary method in diagnosing SFN. There is a significant correlation between reduced nerve length in the dermis and slowing of sural nerve conduction velocity (134). In addition, IENF density in the distal leg has been shown to correlate with heat (119; 124) and cold perception threshold, heat pain, pressure sense, and total neurological disability score (124). Furthermore, IENF density showed negative correlation with vibration perception threshold and Michigan Neuropathy Screening Instrument (135). More recently, IENF density and corneal nerve fiber density were shown to correlate with heat pain and cold perception threshold (113). However, some studies have not shown statistically significant correlation between IENF density and thermal thresholds (136) or only among patients with abnormal nerve conduction studies, indicating more severe neuropathy involving both small and large diameter nerve fibers (112). Hence, in paper III, the aim was to investigate the relationship between IENF count, NCS, QST, and clinical symptoms and signs of PN.

The validity of IENF density quantification in skin biopsies for the detection of SFN is high, with a diagnostic efficiency of 88%, a positive predictive value of 75%, and a negative predictive value of 90% (117). In 2009, the AAN, in conjunction with the AANEM and the AAPM&R, published an evidence-based review of the role of a skin biopsy for the evaluation of distal symmetric polyneuropathy (PN) and small fiber neuropathy (SFN) (106). Studies assessing IENF density in patients with and without PN or SFN were included. The clinical impression of PN or SFN was used as the

diagnostic reference standard. Accordingly, the presence of symptoms and/or signs with abnormal NCS was diagnosed as PN but SFN if the NCS was normal. Based on this, the sensitivity of reduced IENF density for the diagnosis of PN was 45%–90%, and the specificity of normal IENF density for the absence of PN was 95%–97%. The sensitivity of IENF density assessment for SFN was 24%–90%, and the specificity was 95%–98%. Thus, normal IENF density would not rule out PN/SFN, but reduced IENF density would raise the likelihood of PN/SFN (106). However, none of the studies addressed the question whether a skin biopsy would accurately distinguish patients with symptomatic PN from patients with other conditions causing painful feet, and such studies were recommended for future research. The review provided recommendations for the use of skin biopsy in clinical practice, and concluded that the IENF density assessment using PGP 9.5 immunohistochemistry is a validated and reproducible marker of SFN. For symptomatic patients with suspected PN, skin biopsy may be considered to diagnose polyneuropathy, particularly with small fiber involvement (106).

In this thesis, a well-defined group of patients with diabetes or IGT is investigated. The patients are elderly men, matched for age, gender, height and BMI. Other studies of skin biopsies often include a non-homogenous group of patients at different ages, both sexes, and with peripheral neuropathies of different etiology, making comparisons between groups difficult. IENF density is higher at more proximal body sites, in younger patients, and in women. Therefore, studies need to match for these parameters when comparing groups (118; 122; 137).

1.6 Treatment of diabetic neuropathy

Apart from improved glycemic control, there is no treatment that genuinely modifies progression of PN, despite several decades of research (138; 139). Epidemiologic and experimental data link the metabolic syndrome to PN, even without hyperglycemia, supporting aggressive treatment of high blood pressure and lipids, weight reduction in obesity, and smoking cessation (22). However, even with control of these risk factors, many patients with diabetes still develop PN through time.

The management can be divided into three different approaches: pathogenetic, preventive, and symptomatic.

1.6.1 Pathogenetic treatment

Targeted therapies of the underlying mechanisms of diabetic PN are essential to slow the progression of the disease. However, many attempts have failed, as the etiology is not clear. Targeted therapies have shown some effect in animals with improvement in nerve conduction velocity, but modest benefit in diabetic patients with PN (139; 140). Aldose reductase inhibitors (ARIs) may reduce the accumulation of sorbitol and increase myo-inositol in nerve tissue, but are ineffective in clinical trials. Many of these substances have been withdrawn due to severe adverse events (140). In addition, α-lipoic-acid may lower oxygen free radicals, γ-linolenic acid may affect essential fatty acid metabolism, and growth factors may induce nerve regeneration and growth (45). However none of these agents have convincingly been shown to reverse or slow the clinical progression of diabetic PN (141; 142), perhaps because patients with relatively advanced PN were included in these trials (143).

1.6.2 Preventive treatment

The improvement of blood glucose control is the only proven preventive measure in diabetic neuropathy. The Diabetes Control and Complications Trial (DCCT) followed 1441 patients with type 1 diabetes prospectively for 3.5–9 years and showed that patients receiving intensive insulin therapy aimed at achieving normoglycemia were 64% less likely to develop clinical neuropathy over a 5-year follow-up compared to patients receiving routine care (144; 145). Foot care education and foot treatment, if needed, are essential as all patients with diabetic neuropathy are at increased risk of foot ulceration and amputation (85; 146; 147). Pancreas transplantation may stabilize or improve PN in type 1 diabetes, but is not yet routinely performed (139; 148; 149).

1.6.3 Symptomatic treatment

Painful diabetic neuropathy is one of most common causes of neuropathic pain, causing substantial morbidity. Treatment of pain in diabetic neuropathy includes both centrally and peripherally acting agents, but many patients do not respond well to traditional pain therapies. Four main classes of drugs are used: tricyclic antidepressants, selective serotonin reuptake inhibitors, antiepileptics, and opioids. The tricyclics amitriptyline and imipramine are efficacious and show the lowest number needed to treat (NNT = 2.6) (150). However, they have troublesome anticholinergic and central side effects, which limit their use. The antiepileptics gabapentin and pregabalin (151) are the most commonly prescribed drugs with milder central side effects if titrated slowly. Duloxetine, a new serotonin and norepinephrine reuptake inhibitor, has shown efficacy (152), but it takes up to a month to achieve full therapeutic effect. In 2004, EFNS published guidelines to be used in the management of patients with neuropathic pain (153).

1.7 Neuropathy associated with impaired glucose tolerance

IGT neuropathy and early diabetic PN are clinically similar, characterized by preferential injury to small nerve fibers which is usually accompanied by sensory symptoms and disabling pain (41; 154-157). The nature of the relationship between IGT and neuropathy is not clear, although microangiopathy (68; 69; 107), episodic postprandial hyperglycemia (22; 63), and endothelial dysfunction (158) may be causal factors. No large well-designed studies of the prevalence and long-term prognosis of IGT neuropathy have been conducted. However, several studies have demonstrated a high prevalence of IGT in individuals with idiopathic peripheral neuropathy (45%) (154; 156), suggesting that the disease may represent the earliest stage of diabetic nerve injury.

A few smaller studies have demonstrated loss of skin innervation in IGT neuropathy (155; 157; 159), but larger studies of the relationship between IENF density, clinical neuropathy, and neurophysiological tests are missing. The most comprehensive study of IGT neuropathy is a prospective study that followed up 71 subjects with IGT related neuropathy for up to 3 years (160). The most frequently abnormal tests were measures of SFN. Cold detection threshold was abnormal in 64% and IENF density was reduced in 83%.

There have been no therapeutic trials for IGT neuropathy. However, lifestyle intervention with diet and exercise counseling may increase IENF density and improve neuropathic pain (160). Lifestyle intervention may also reduce the risk of progression from IGT to diabetes (23; 42). Approximately 30% of untreated IGT individuals develop diabetes (23), and lifestyle intervention may lower this risk by 58% (23; 42). The metabolic syndrome has also been linked to PN, independent of hyperglycemia (138; 161). Hence, in addition to lifestyle intervention, each element of the metabolic syndrome should be aggressively treated in IGT subjects (162).

2. AIMS

The overall aim of the present thesis was to investigate the relationship between histopathology of nerves (the sural nerve and small nerve fibers in epidermis) and different clinical and neurophysiological methods of diagnosing peripheral polyneuropathy in diabetes and IGT. We also aimed to define endoneurial microangiopathy in the sural nerve and its association with glucose intolerance and peripheral polyneuropathy.

More specifically we aimed to:

1a. Define the relationship between endoneurial microangiopathy and glucose intolerance (Paper I).

1b. Define the relationship between endoneurial microangiopathy and the presence/development of peripheral polyneuropathy (Paper I).

2. Determine whether baseline sural nerve myelinated nerve fiber density may predict later development or progression of neurophysiological dysfunction. (Paper II)

3. Investigate the potential correlation between histopathologically quantified intraepidermal nerve fibers and neurophysiological and clinical scoring of diabetic peripheral polyneuropathy (Paper III).

4. Investigate vibrotactile sense (large fiber neuropathy) at different frequencies in index and little fingers (median and ulnar nerves, respectively) of subjects with diabetes, impaired and normal glucose tolerance (Paper IV).

3. SUBJECTS AND STUDY DESIGN

The study subjects are men from representative subgroups of a large original cohort. A population-based health-screening program (The Malmö Preventive Project) was initiated between 1975 and 1979 in Malmö, Sweden. All 47–49-year-old men, born in 1926–1931, initially residents of Malmö, Sweden, were invited to participate. Of a total population of 9033, 6956 men (77% attendance rate) agreed to participate (163). There were 101 subjects known to have diabetes, and 6811 subjects underwent an oral glucose tolerance test (OGTT). There were 423 subjects with an abnormal OGTT on two consecutive occasions. These subjects were invited to participate in a long-term intervention. Between 1989 and 1991, the same individuals, now with a mean age of 61 ± 1.3 years, were screened again and reclassified according to the WHO criteria (diabetes, IGT, NGT) (35). Of the 182 individuals three groups were identified ;

1. **Subjects with type 2 diabetes** (n=69; 35 had IGT and 34 had diabetes [persistent diabetes] in 1975–79).
2. **Subjects with persistent IGT** (n=51) twice in the initial survey (1975–79) and still in 1989–91 (163).
3. **Control subjects with persistent NGT** on three consecutive occasions (n=62) were randomly selected from the original cohort of 6956, and matched according to gender (men), age, height, and BMI.

All 182 individuals underwent a neurophysiological evaluation of peripheral nerve function (93). Between 1992 and 1993, 10 subjects from each group (10 with diabetes, 10 with IGT, and 10 with NGT, mean age 63 ± 1.3 years) were randomly invited to perform a whole sural nerve biopsy, clinical, and neurophysiological examination (111).

Between 1998 and 1999, 5–6 years after the biopsy, the subjects who underwent sural nerve biopsy were invited to a follow-up OGTT, clinical and neurophysiological examination (Papers I and II) (80; 90). Twenty-four subjects accepted the complete follow-up investigation; 6 with diabetes, 10 with IGT, and 8 with NGT.

Between 2003 and 2004, the subjects from the cohort of 182 individuals, now with a mean age of 74.8 ± 1.3 years, were invited to a follow-up study comprising a repeated OGTT, clinical and neurophysiological examination, and a skin biopsy from the distal leg (Paper III). By clinical examination, 107 subjects were included. Seven subjects were excluded from the follow-up study because of neurological disease or neuropathy associated with disorders other than diabetes. Accordingly, 100 subjects were included in the follow-up study; 33 of 69 subjects originally classified as having diabetes, 30 of 51 originally classified as having IGT, and 37 of 62 originally classified as having NGT at baseline. Skin biopsy was performed in 86 subjects (50 with diabetes, 15 with IGT and 21 with NGT), and 80 subjects accepted the complete follow-up investigation.

In 2003–2005, subjects from the cohort of paper III, who had stable glucose tolerance between 1989–1991 and 2003–2005 (i.e. around 15 years), were invited for an evaluation of vibrotactile sense with a tactilometry (Paper IV) (164). There were 58

subjects with stable glucose tolerance (mean age 73.4 ± 0.12 years), 23 with persistent diabetes, seven with persistent IGT, and 28 with persistent NGT.

4. METHODS

4.1. Oral glucose tolerance test (OGTT)

An OGTT was performed on four different occasions from 1975–1979 to 2003–2004. The first OGTT was performed in 1975–1979 (initial health screening study), the second OGTT in 1989–1991 (Papers I and II), the third OGTT in 1998–1999 (follow-up after sural nerve biopsy, Paper I), and the fourth OGTT in 2003–2004 (Papers III and IV).

After ingestion of 75g of glucose, blood-glucose was measured fasting (0 min) and 120 min later.

4.2 Clinical examination

4.2.1 Papers I and II

At baseline, the subjects were examined by a neurologist, and patients with absent ankle reflexes and/or altered/reduced sensory perception and/or neuropathic symptoms in toes or feet were considered to have clinical PN (111). At follow-up, a modified version of Dyck's original Neuropathy Symptom Score (NSS) and Neuropathy Disability Score (NDS)(88) were employed to define the incidence and severity of PN (80). Sensory function at different levels in lower limbs (from big toe to knee) was examined. The severity and frequency of seven different symptoms in the feet were scored (Appendix A). Each of the seven symptoms was scored; lack of symptoms was scored as 0, sometimes as 1, often as 2, and during most nights as 3. The scores were added into the NSS, giving a range of 0–21. Sensory perception was assessed with regard to light touch (cotton wool), pin prick (needle), vibration (vibration fork), and cold (cold metal item) (Appendix C). Lack of sensation for the individual modality was given a score of 1. The sensory modalities were added into a NDS sub-score A (NDS A), and the sum divided by 2, giving a range of 0–20. Ankle and knee reflexes were assessed in the legs (Appendix E). Reflex findings were added into a NDS sub-score B (NDS B), in which normal reflex was given a score of 0, reduced reflex a score of 1, and absent reflex a score of 2, giving a range of 0–8. NDS A and NDS B were also added together to give a composite NDS, ranging from 0–28.

4.2.2 Paper III

The author of this thesis performed the clinical examination. The same protocol was used as for papers I and II, except that muscle strength, sensory function, and tendon reflexes were examined in both upper and lower limbs: 1) the subject was asked for neuropathic symptoms in hands and feet, giving an NSS range of 0–42 (Appendix B); 2) sensory perception was assessed in the hands and feet, giving an NDS A range of 0–32 (Appendix D); 3) reflexes were evaluated in arms and legs, giving an NDS B range of 0–12 (Appendix F); 4) muscle strength was estimated in the hands and feet (Appendix G). Muscle strength was graded 0 to 3, with normal strength given a score of 0 and paralyses a score of 3, added into NDS sub-score C (NDS C), giving a range of 0–60. NDS A (sensory function), NDS B (reflexes), and NDS C (muscle strength) were added together, giving a composite NDS, ranging 0–104. NSS and NDS scores were added together to give a composite total neuropathy score (TNS), ranging 0–146.

Accordingly, subjects with high scores had more symptoms and signs of PN than subjects with low scores.

4.3 Neurophysiological examination

Nerve conduction studies (NCS), quantitative sensory testing (QST), and vibrotactilometry were performed (see below). In papers III and IV, neurophysiological results were converted to Z scores (the number of SDs from the normal mean dependent on the subject's gender, age, and height) (165; 166). Abnormal neurophysiological results were defined as values more than 2.00 SD below (amplitudes and conduction velocities) or above (sensory thresholds) the reference value (one-sided 95% confidence interval). The neurophysiological studies were performed at the Department of Clinical Sciences and Neurophysiology, Lund, and reference values for amplitudes, CVs, and sensory thresholds were taken from a large sample, routinely used in our laboratory in Lund and Malmö for many years (165; 166).

4.3.1 Nerve conduction studies (NCS)

Surface electrodes (Papers II and III) were used for stimulation and recording in motor and sensory nerves. Needle electrodes were used for the sural nerve in paper I (all subjects) and in paper III (in two subjects). NCS of the sural (Papers I, II and III), peroneal (Papers II and III), and the median nerve (Papers II and IV) was performed. Motor NCS included the peroneal nerve in the foot and median nerve in the arm. Sensory NCS included the sural nerve in the foot and the median nerve in the arm. For both motor and sensory nerves, amplitude and conduction velocity (CV) were measured (166).

4.3.2 Quantitative sensory testing (QST)

Vibration perception thresholds (VPT) were measured at the big toe with the Goldberg-Lindblom type of vibrameter (99), and sensory thresholds for heat and cold perception were measured on the lateral side of the foot with the Termotest equipment (Somedic, Eslöv, Sweden) (165). The sensory thresholds were determined by the "method of limits", described in chapter 1.5.2.

4.3.3 Tactilometry

Vibrotactile thresholds at seven different frequencies (8, 16, 32, 64, 125, 250 and 500 Hz; ascending order) were measured in the upper extremities by a tactilometer (101) (PID AB and Vibrosense AB, Malmö, Sweden) in a room at stable temperature. Sinusoidal vibrations were delivered by the vibrating probe to the pulps of index and little fingers, reflecting function of median and ulnar nerves, respectively.

For each investigated frequency, the patient controlled the vibration amplitude by pressing a hand switch. At the beginning, the vibration amplitude was increased until the subject perceived the vibration. The subject pressed and held the switch while the vibration amplitude was decreased until the subject no longer perceived a vibration stimulus. This sequence was repeated four times for each frequency while the vibration amplitude was increased or decreased at a constant rate of 3 dB/s until the subject responded. The vibration perception threshold value was calculated as the

arithmetic mean of the peak (ascending) and bottom (descending) thresholds for each frequency. The values of the levels were expressed in dB. A tactilogram was obtained from each finger. The tactilogram was graphically displayed on an inverted scale with low intensity (amplitude) at the top and high intensity at the bottom. Vibrotactile thresholds in the pulps of the index and little fingers of both hands were recorded. A sensibility index (SI) was calculated (101), where the area beneath the curve was divided by the area of the curve from a healthy reference population (index = 1 normal function; index < 0.8 abnormal) (101). The SI reflects the overall dysfunction of the tactilogram.

4.4 Sural nerve biopsy

4.4.1 Biopsy sampling and processing

The biopsies were performed by a co-author (L. B. D.) at the Department of Clinical Sciences, Hand Surgery in Malmö. After infiltration local anesthesia (1% mepivacaine without adrenaline), a 7–8 cm length longitudinal incision was made between the lateral malleolus and the Achilles tendon (24). The sural nerve was identified and blocked at least 1.5 cm proximal to the proximal incision. The nerve was then freed from the surrounding connective tissue and the proximal end was cut. Thereafter the nerve was dissected for 6 cm and the distal end excised by a scalpel. The wound was closed with interrupted 4.0 monofilament proline sutures. A normal dressing was applied. The patient was asked to keep his foot elevated for at least 3 hours. The dressing was changed on day 3 postoperatively and the sutures were removed between 10 and 14 days later.

The 5–6 cm length whole (full-thickness) sural nerve was divided into five 1-cm segments (111). The first, fourth, and fifth (proximal to distal) segments were immediately frozen in liquid nitrogen and stored at –70°C until shipped overnight frozen to the Nerve Biopsy Laboratory where they were recorded with random identification numbers to conceal their identity from the analysts. The second and third segments were fixed in 0.1 mol/L cacodylate buffered 2.5% gluteraldehyde. The fixed 1-cm segments were rinsed and further divided into three equal segments, postfixed in 1% osmium, dehydrated in ascending concentrations of ethanol, and placed in propylene oxide prior to embedding in Epon 812 (111).

4.4.2 Quantification of myelinated nerve fibers

In papers I and II, a whole sural nerve biopsy was performed, and systematic sampling was applied. The largest fascicle meeting criteria for cross-sectional area ($\geq$ 100,000 μm^2), fixation, and mechanical distortion ($\leq$ 6% endoneurial area) was selected for morphometric analysis (111). Preliminary studies comparing myelinated nerve fiber density (MNFD) in 20 large readable fascicles to that of the whole nerve specimen revealed a relative difference of 0.2 ± 2.6% (111). When the ratio of MNFD of each fascicle to the MNFD of the whole specimen was plotted for all fascicles, there was a systematic deviation toward lower MNFD in fascicles < 50,000 μm^2; therefore, 100 000 μm^2 was used as a lower threshold for an acceptable fascicle (111). MNFD was analyzed by both light and electron microscope. Following digital imaging at 400 X, a trained reader examined the selected fascicle (light microscope). Following image

capture, montage formation, and axon cueing, MNFD was analyzed (electron microscope)(Figure 1). The identity of the biopsy was concealed from the reader. Because of inadequate tissue fixation, MNFD could not be assessed in four subjects, one with diabetes, one with IGT, and two with NGT at baseline.

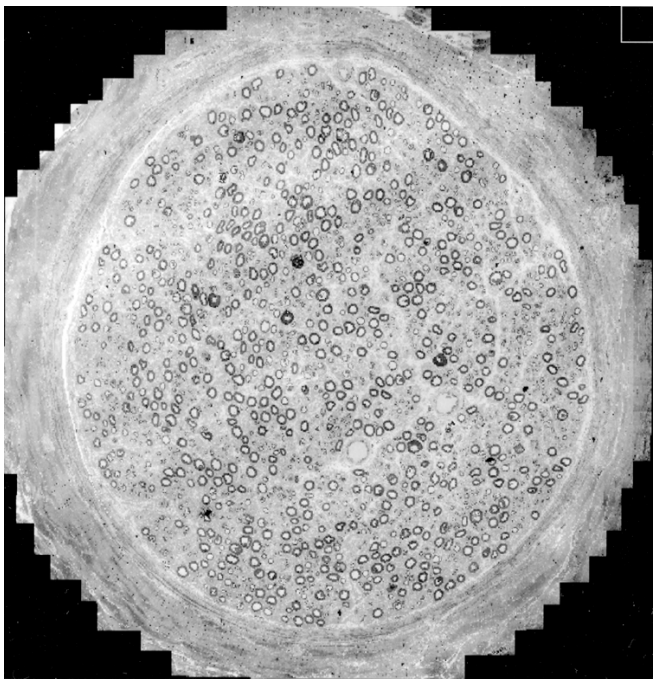

Fig 1. A digitized and montaged electron micrograph of an entire sural nerve fascicle (111).

Permission to reprint the figure has been granted by the publisher.

4.4.3 Endoneurial capillary morphometry

The endoneurial capillary density was determined directly by light microscopy from semithin sections stained with Thionin and Acridine Orange. Sampling was not used because all endoneurial capillaries were counted directly in all fascicles of the whole nerve and related to the fascicular area to calculate a density. Electron micrographs (magnification x 6000) were prepared on an average of 10 randomly selected endoneurial capillaries per biopsy (range 8–12; no difference between the three groups of different glucose tolerances), and the luminal area, endothelial cellular area, and basement membrane area were derived by tracing the image analysis cursor around each capillary profile. The endothelial cell profile nuclear number and pericyte cell nuclear number per capillary were counted directly from each micrograph. Capillary density (number/mm^2), luminal area (μm^2), basement membrane area (μm^2), endothelial/pericyte nuclear ratio were analyzed. The detailed methodology for the quantification of capillary morphology has been described (167; 168).

Because of inadequate tissue fixation, capillary density could not be assessed in three subjects, one with diabetes, one with IGT, and one with NGT at baseline. Similarly, endothelial/pericyte ratio, luminal area, and basement membrane area could not be assessed in three subjects, two with IGT and one with NGT at baseline.

4.5 Skin biopsy and quantification of intraepidermal nerve fibers

The skin biopsies were performed by the author of this thesis. During local anesthesia (10% carbocain with adrenaline) a punch skin biopsy (3 mm) was performed at the lateral distal leg, approximately 10 cm proximal to the lateral malleolus. The procedure was well tolerated, although, one subject with diabetes developed a local infection. One subject with IGT developed a minor neuroma at the biopsy site.

Biopsies were immediately fixed in 4% paraformaldehyde lysine phosphate for at least 24 h, dehydrated, and embedded in paraffin. Five-µm thick sections were obtained from the paraffin-embedded blocks, mounted on glass slides and dried one hour at 60 ºC. The sections were dewaxed, rehydrated and microwave pre-treated in 10 mM citrate buffer to achieve antigen retrieval. An automated immunostainer was used for the staining procedure. The rabbit polyclonal antibody Protein Gen Product (PGP) 9.5 was used as primary antibody (169).

Intraepidermal nerve fibers (IENF) were assessed in two sections from each skin biopsy using light microscopy (magnification × 40). Two observers (the author of this thesis and a co-author, E. E.), blinded to the identities, manually counted the nerve fibers in the epidermis. The higher IENF count in one of the two sections was recorded. The subjects were divided into three groups: 1) subjects with absent IENF (total loss); 2) low IENF (1–3 IENF/section); and 3) high IENF counts (4 or more IENF/section).

4.6. Statistics

Differences between groups were compared using the Kruskal-Wallis and Mann-Whitney U tests for numeric variables whereas Fisher's Exact or Chi square tests were used for nominal variables. Wilcoxon's paired test was used to evaluate differences within groups. Correlations were analyzed by Spearman Rank Correlation test.
$P < 0.05$ was considered significant. Data are presented as median (interquartile range) unless otherwise stated.

5. RESULTS

The results presented demonstrate an association between endoneurial microangiopathy in the sural nerve and glucose intolerance. In addition, the results show the relationship between endoneurial microangiopathy and loss of intraepidermal nerve fibers and different measures of peripheral neuropathy (clinical examination, nerve conduction studies, and quantitative sensory testing). For details the reader is referred to the individual papers.

5.1 Endoneurial capillary abnormalities in relation to glucose tolerance (Paper I)

Baseline sural nerve capillary density, analyzed in whole sural nerve biopsies, was higher in subjects with diabetes at follow-up 6 years later compared with individuals with persistent NGT. In addition, capillary density was higher in subjects progressing from IGT to diabetes compared to subjects with persistent IGT. Hence, higher density of endoneurial capillaries was associated with current diabetes or future development of diabetes, suggesting compensation for hypoperfusion and hypoxia in early diabetic neuropathy. Capillary luminal area was reduced in subjects deteriorating in glucose tolerance, (from IGT to diabetes or from NGT to IGT), compared to subjects whose glucose tolerance was stable (constant NGT or constant IGT). Hence, capillary luminal narrowing may presage deterioration of glucose tolerance.

5.2 Endoneurial capillary abnormalities in relation to peripheral polyneuropathy (Paper I)

Increased capillary density, observed in sural nerve biopsies, was associated with reduced sural nerve conduction velocity at follow-up, in subjects with diabetes or IGT at baseline. Hence, higher density of sural nerve capillaries was associated with mild dysfunction of the sural nerve. Among subjects with diabetes or IGT, those with clinical PN had increased basement membrane area compared to those without PN. In addition, among subjects with diabetes or IGT, basement membrane area correlated negatively with myelinated nerve fiber density, and sural nerve amplitudes. Hence, thickening of the basement membrane was associated with clinical PN and both morphological and neurophysiological loss of large nerve fibers.

5.3 Sural nerve biopsy and future progression of nerve dysfunction (Paper II)

Subjects with low baseline sural nerve myelinated nerve fiber density (MNFD) ($\leq$ 4700 fibers/mm^2) showed a decline of peroneal amplitude and conduction velocity, as well as median sensory amplitude and motor conduction velocity from baseline to follow-up 7–10 years later. Subjects with low MNFD had higher vibration perception threshold at the big toe at baseline and at follow-up compared to subjects with normal MNFD (> 4700 fibers/mm^2). MNFD showed a negative correlation with vibration perception threshold at the big toe and with body mass index at baseline. In linear regression analyses, diabetes influenced the decline of nerve conduction over time.

5.4 Intraepidermal nerve fiber loss in relation to clinical scoring of diabetic polyneuropathy (Paper III)

Intraepidermal nerve fiber count (IENF) did not differ between groups with different glucose tolerance (diabetes, IGT, or NGT). Total Neuropathy Score (combined Neuropathy Symptom Score and Neuropathy Disability Score) did not differ between the three groups of different IENF counts.

5.5 Intraepidermal nerve fiber loss in relation to neurophysiological scoring of diabetic polyneuropathy (Paper III)

Subjects with absent IENF had lower sural nerve amplitude and conduction velocity Z scores compared to subjects with low (1–3 IENF/section) and high ($\geq$ 4 IENF/section) IENF. Subjects with absent IENF also exhibited higher cold perception threshold Z score compared to subjects with low and high IENF. Hence, IENF count was related to neurophysiological measures of peripheral polyneuropathy.

5.6 Vibrotactile sense in median and ulnar nerve innervated fingers in relation to glucose intolerance (Paper IV)

Vibrotactile sense (sensibility index) was impaired in index (median nerve) and little fingers (ulnar nerve) bilaterally in diabetic subjects. Vibration thresholds were particularly increased at 16, 250, and 500 Hz in the little finger. Diabetic subjects showed electrophysiological signs of neuropathy in the median nerve, both in the forearm and at the wrist. Subjects with persistent IGT or NGT showed no impairment of vibrotactile sense or nerve conduction in the upper extremity. Hence, tactilometry, with a multi-frequency approach, was impaired in type 2 diabetes, and is a sensitive technique to screen for large fiber neuropathy in the upper extremity.

6. GENERAL DISCUSSION

This thesis has shown that PN in diabetes and IGT is associated with myelinated nerve fiber loss and microangiopathy in sural nerve biopsies. In addition, myelinated nerve fiber loss in the sural nerve predicts future nerve dysfunction of both peroneal and median nerves. Microangiopathy of the sural nerve is related to existing PN and future deterioration of glucose tolerance. Loss of small intraepidermal nerve fibers in skin biopsies is associated with measures of both large (sural nerve function) and small nerve fiber (cold perception threshold) dysfunction. Vibrotactile sense of the median and ulnar nerve innervated finger pulps is impaired in diabetes.

Taken together, the morphology of nerve fibers and capillaries in the sensory sural nerve and the morphology of cutaneous nerve fibers correlate with neurophysiology and clinical findings. Quantification of intraepidermal nerve fibers is a simple and safe method and may be used in clinical studies of peripheral polyneuropathy. Tactilometry, with multifrequency analysis of vibration thresholds, is a simple method for screening large fiber neuropathy. A comprehensive discussion of the results of the thesis follows below. However, there are a number of limitations in this thesis:

1. Risk of selection bias

Of the total cohort of 9033 men, 6811 subjects underwent a glucose tolerance test (OGTT) and 101 had known diabetes (see chapter 3). There may have been a selection bias as the prevalence of known diabetes was higher in the non-responder group (3.8%) than in the cohort as a whole (2.0%).

Of the subjects who underwent OGTT, 423 subjects showed an abnormal test on two consecutive occasions (93). Subjects under medical treatment elsewhere or with complicating disorder such as liver disease or severe hypertension were excluded from the study, which may have caused a selection of healthier IGT subjects.

2. Number of subjects

The Ethics Committee approved 30 sural nerve biopsies (10 diabetes, 10 IGT, and 10 NGT) as the biopsy procedure may cause persistent postoperative complains (24; 25). Hence, 24 subjects participated in the follow-up after the sural nerve biopsy (Paper II). In clinical studies a high follow-up rate is often difficult to obtain, especially in older individuals, as we experienced, but 24/30 subjects must be considered satisfactory in this type of clinical study.

During the long follow-up, a significant number of subjects deteriorated from IGT to diabetes between 1989–91 and 2003–05. Hence, of a group of 51 IGT individuals, only 7 subjects had persistent IGT over 15 years. The number of dropouts may also induce selection bias.

6.1 Microangiopathy, glucose intolerance, and neuropathy (Paper I)

Key alterations in the sural nerve endoneurial vasculature were quantified in relation to the development and progression of glucose intolerance. This thesis demonstrates that sural nerve endoneurial capillary density is increased in subjects with IGT who develop diabetes and in patients with manifest diabetes compared to subjects with NGT. This thesis also demonstrates a reduction in capillary luminal area in subjects progressing from NGT to IGT and from IGT to diabetes.

These findings supports the concept that reduced nerve blood flow is associated with deterioration of glucose tolerance and suggests that increased endoneurial capillary density may be compensatory for endoneurial hypoperfusion and hypoxia. Sural nerve oxygen tension is reduced in diabetic patients with established PN (170), but without a significant alteration in either luminal area or endoneurial capillary density (167; 168; 171-173). Increase in epineurial capillaries is seen in a variety of advanced neuropathies, including diabetic PN (174). Also in subclinical PN, increased epineurial nerve blood flow may reflect arterio-venous shunting, rendering the endoneurium ischemic (175). Increased capillary density is also seen in skeletal muscle and correlates with poor aerobic capacity (176).

The microangiopathic changes described are of relevance to the pathogenesis of diabetic/IGT PN. The endoneurial microenvironment is maintained by endoneurial vessels which maintain nerve fiber function. One of the key alterations regulating endoneurial perfusion is the endoneurial capillary density and hence the intercapillary distance (177). This thesis demonstrates a link between increased endoneurial capillary density and disturbed nerve function, which is suggestive of a compensatory increase in vascular density in early neuropathy. As no alteration in endoneurial capillary density is seen in established neuropathy (167; 168), endoneurial capillary density seems to decrease with progression of PN.

This thesis demonstrates that subjects with PN (both diabetic and IGT) have significantly thickened basement membrane compared to those without PN. In addition, thickened basement membrane is related to loss of myelinated nerve fibers, also supported by low sural nerve amplitude. Hence, basement membrane thickening is related to clinical, neurophysiological, and morphologic measures of neuropathy in subjects with diabetes and IGT.

Basement membrane thickening is seen in patients with both mild (168) and more severe (167; 171-173) diabetic PN, and may even precede the development of PN (69) (16). Many studies (167; 168; 171-173) have shown a strong correlation between basement membrane thickening and neuropathic severity in diabetic and nondiabetic neuropathies.

No correlation was found between the basement membrane area and the degree of glucose tolerance. Hence, basement membrane thickening is specifically related to the development and progression of PN, not simply to hyperglycemia. On the other hand, thickened basement membrane is associated with hypoinsulinemia in our type 2 diabetic subjects. Hypoinsulinemia, relative or absolute, may promote the development of PN, regardless of the degree of hyperglycemia (49). Hypoinsulinemia shows no association with loss of myelinated nerve fibers, supporting the hypothesis that hypoinsulinemia primarily affects the basement membrane, thereby promoting the development of PN.

In this thesis capillary luminal area is reduced in subjects deteriorating in glucose tolerance, (from IGT to diabetes or from NGT to IGT), compared to subjects whose glucose tolerance was stable (persistent NGT or persistent IGT). Hence, capillary luminal narrowing may presage deterioration of glucose tolerance. Luminal occlusion has been demonstrated in some (73), but not all (167; 168; 171-173), studies. The luminal area is not different between subjects with or without PN, and there is no relation between luminal area, nerve function, or axonal loss, which supports the majority of these studies (167; 168; 171-173). This thesis also demonstrates that endothelial cell hyperplasia and/or pericyte degeneration in the sural nerve occurs in IGT and diabetes, and may be present before the development of PN (69).

Insulin resistance is associated with IGT and early type 2 diabetes, and is one component feature of the metabolic syndrome. There is an established relationship between insulin resistance and alterations in the function and structure of blood vessels in classically insulin-responsive tissues, such as muscle and fat (178). Insulin is a vasoactive hormone, which at physiological concentrations increases skeletal muscle tissue perfusion by recruiting microvascular beds (179), whereas insulin resistance displays impaired insulin-mediated vasodilatation (72). Increased capillary density in both nerve and muscle (176) precedes the development of diabetes in subjects with IGT. Insulin concentrations are increased in our subjects with diabetes or IGT (80), but do not correlate with capillary density. Hence, early endoneurial capillary microangiopathy may be attributed to insulin resistance, rather than hyperinsulinemia per se.

Accordingly, the data are consistent with the hypothesis that endoneurial capillary microangiopathy presages deterioration in glucose tolerance and is an early and persistent feature in the processes underlying diabetic PN. Basement membrane thickening may be related to insulin resistance and the accompanying development of PN when hypoinsulinemia occurs (49).

6.2 Myelinated nerve fiber density (Paper II)

This thesis demonstrates an association between myelinated nerve fiber density (MNFD) of the sural nerve and the clinical and neurophysiological assessment of PN 7–10 years later. MNFD in the sural nerve not only reflects the concomitant function of the sural nerve but also predicts future progression of neuropathic severity in other peripheral nerves. Subjects with low MNFD show future progression of axonal loss and mild demyelination of both peroneal and median nerves, as indicated by decreases in amplitudes and conduction velocities from baseline to follow-up. In contrast, such changes are not apparent among subjects with a normal MNFD.

Subjects with low MNFD demonstrate loss of large, fast conducting nerve fibers in the legs from baseline to follow-up, indicated by a decline in both peroneal nerve motor amplitude and conduction velocity, although demyelination with increased temporal dispersion may also cause a decline of amplitudes. Similarly, subjects with low MNFD demonstrate an increase in vibration perception threshold in the foot from baseline to follow-up, and a negative correlation between MNFD and vibration perception threshold in agreement with some (108) but not all studies (180). This discrepancy between studies may reflect differences in the severity of PN. In the arms,

subjects with low MNFD demonstrate a mild PN, signified by a decrease in median nerve sensory amplitude and motor conduction velocity.

The length-dependent nature of PN is demonstrated in subjects with low MNFD by a predominantly sensory affection in the legs, as indicated by decreased sural nerve sensory amplitude, preserved peroneal nerve motor amplitude, and lack of median nerve dysfunction. The longer neurons in the legs are involved earlier and more severely than the shorter neurons in the arms.

Clinical PN is only detected in the group with low MNFD and not in the group with normal MNFD. In subjects with PN, MNFD is lower compared to those without PN (111). This suggests that simply preexisting PN causes decline in nerve function in subjects with low MNFD. However, regression analysis shows that glucose dysmetabolism (diabetes, high HbA1c, and high fasting-insulin level), but not the presence of PN, affects decline in nerve function. Hence, both low MNFD and diabetes are associated with decline in nerve function. Although subjects with diabetes have comparable baseline MNFD and nerve function with NGT and IGT subjects (90), only subjects with low MNFD deteriorate in nerve function. This suggests that subjects with early loss of myelinated nerve fibers (low baseline MNFD) are more liable to factors affecting nerve function, and therefore show faster progression of nerve dysfunction. In the diabetes group this factor is probably glucose dysmetabolism, but an obvious factor cannot be inferred in the IGT and NGT groups. Thus, if sural nerve biopsy is performed in diabetic patients, a finding of low MNFD may predict greater risk of progression of neuropathic changes and may identify individuals with the highest risk of developing PN.

In the group with low MNFD, 4 had NGT. Three of them had developed clinical and nerve conduction deficits at follow-up, however, only 1 had progressed to IGT and none to diabetes. The interpretation is that this may be an effect of normal aging. These subjects may be unable to compensate for the normal effects of aging and could develop clinically evident deficits over time. Reduced distal sensation and decreased reflexes are part of normal aging and definition of PN in elderly is not clear (181). Reduction of myelinated nerve fibers with aging has been demonstrated in peripheral nerves from autopsy specimens (182), and neurophysiological function decline with increasing age (183-185).

Regarding the IGT group, aging in conjunction with features of the metabolic syndrome may affect nerve function (161) (see below). In the whole study group, the sural nerve amplitudes fall significantly from baseline to follow-up, with no relationship to baseline MNFD or glucose tolerance. This is also most likely a manifestation of aging. Sural nerve amplitudes correlate negatively with age and an absent sural nerve response is found in more than 20% of subjects older than 70 years (183).

This study also suggests that obesity may have an important role in the development of PN. It shows that low MNFD correlates with increased body mass index (BMI), even in subjects with NGT. The mechanism behind this finding is complex, and to our knowledge the literature lacks previous reports demonstrating this relationship. In support of this notion, obesity has been proposed to influence nerve conduction in both diabetic (186) and non-diabetic subjects (187). Obesity is one of the component features of the metabolic syndrome, and constitutes an independent risk factor for PN

in diabetic patients (138). Patients with type 1 diabetes who develop PN have higher BMI than those without PN, and markers of insulin resistance are associated with an increased risk of PN (138). In insulin-resistant states, increased vascular tone, predisposing to endothelial dysfunction (188-190), may therefore promote basement membrane thickening and later development of peripheral PN (see discussion Paper I). Again, microangiopathy and insulin as a vasoactive hormone (72; 178) may be a link between obesity and PN.

6.3 Intraepidermal nerve fibers (Paper III)

The technique of quantifying intraepidermal nerve fibers (IENF) in thin biopsy sections by light microscopy used in this thesis correlates with neurophysiological function of both small and large nerve fibers. IENF loss is related to both abnormal cold perception threshold and sural nerve dysfunction. Intra- and inter-observer agreement is 84% and 83%, respectively (Tables 5 and 6). However, IENF loss does not statistically differ between subjects with diabetes, IGT, or NGT, nor does the Total Neuropathy Score differ between groups with different IENF counts. Still, these results support the notion that our technique can provide useful additional information in the assessment of diabetic PN and may be used in research of cohorts of patients for pathological correlate with neurophysiological measures.

The skin biopsy is performed at the distal leg as recommended by the EFNS guidelines (127). More loss of IENF is seen at distal sites, reflecting the length-dependent degenerative process of axonal PN in diabetes (22; 41; 137; 155; 191). A gradient is also seen in normal individuals (122; 137). In this thesis, the IENF are semi-quantitatively graded, based on the counting of nerve fibers, and three groups are identified. Low IENF count (1–3 IENF/section) is probably pathological, indicating mild small fiber neuropathy (SFN). Axonal swellings and fragmentation or sprouting of cutaneous/subepithelial nerves are observed but not quantitatively assessed. Such changes may be an early sign of SFN (137; 192; 193) and a consequence of nerve remodeling and regeneration (194) in healing.

In contrast with most previous studies, thin section procedure is employed in this thesis, which has the advantage of being simple to perform and easy to assess and reproduce**.** Koskinen et al. quantified IENF density using a similar method with 5–10 μm thick sections (132), and Dabby et al. quantified dermal autonomic nerve fibers using 5 μm thick sections, but found thin sections of limited value for the evaluation of IENF density (133). A disadvantage of our technique is the relatively low nerve fiber quantities, which are of a range in which possible group mean differences (diabetic vs. IGT and IGT vs. NGT) may become indiscernible even when present. Instead of calculating the mean IENF count of the two biopsy-sections quantified, which would lower the quantitative value ranges further, we record the higher IENF count of the two sections.

Quantification of IENF is difficult to standardize, with large variations even between normal subjects (118). In this study the IENF count is compared with neurophysiological tests, but this method should also be compared with other methods of quantifying IENF. EFNS has recommended (127) bright-field immunohistochemistry (195) or immunofluorescence, with or without (124; 196; 197) confocal microscopy. However, no systematic study has compared these techniques,

which are more complicated, time-consuming, and expensive than standard immunohistochemistry on thin sections, as presently used. Bright-field immunohistochemistry (112; 117; 118; 122) or immunofluorescence techniques (124; 197) both show a higher IENF density compared with the technique in this study. A use of thicker biopsy sections allowing more IENF to be recognized and counted is the most likely explanation for this difference.

The intra- and inter-observer agreement is 84% and 83%, respectively (Tables 5 and 6), which is similar to other investigators (120), although a recent study showed a significant difference in IENF density between three observers (198). This difference was explained by difficulties in counting IENF exactly, especially with low as well as high IENF density values, and may be a relevant problem in clinical diagnostics. With the method used in this thesis, correlation coefficients (ranging 0.86–0.96) (117; 132) or the relative inter-trial variability (RIV) (120) cannot be calculated as the IENF values are normative. Others have presented interobserver reliability as the mean difference in IENF density (0.4 ± 1.5 fibers/mm) between two observers (118). As demonstrated in Table 5, the two observers grade 71 biopsies (12+34+25=71) of total 86 biopsies into the same IENF group (absent, low or high IENF). This accounts for an inter-observer agreement of 83% (71/86). Similarly, as shown in Table 6, observer 1 graded 72 biopsies (18+33+21=72) of a total of 86 biopsies in the same IENF group, accounting for an intra-observer agreement of 84% (72/86).

Table 5 **Observer 2**

Observer 1	Absent IENF	Low IENFD	Normal IENFD	Total
Absent IENFD	12	6	1	19
Low IENFD	1	34	3	38
Normal IENFD	1	3	25	29
Total	14	43	29	86

Inter-observer agreement

Table 6 **Observer 1**

Observer 1	Absent IENF	Low IENFD	Normal IENFD	Total
Absent IENFD	18	4	1	23
Low IENFD	0	33	7	40
Normal IENFD	1	1	21	23
Total	19	38	29	86

Intra-observer agreement

We found a relationship between a low IENF count and reduced sural nerve amplitudes and conduction velocities, although both parameters measure function of large nerve fibers. Typically, diabetic PN is a mixed, small and large fiber (predominately sensory) polyneuropathy (12). Hence, a correlation between small and

large fiber measures is expected. Abnormal IENF density is seen in 81% of patients with mixed small and large fiber neuropathy, and in 88% of patients with pure SFN (199). In this thesis, 87% of subjects with low sural nerve amplitudes had absent or low IENF, but only 25% of the subjects with absent or low IENF had abnormally low sural nerve amplitudes. Accordingly, a SFN is not necessarily associated with large nerve fiber neuropathy. On the other hand, a subject with involvement of large nerve fibers is likely to have developed a previous SFN, a chronology, which for the lower extremities is supported by several authors (22; 112; 136), and reflects a more severe neuropathic process involving both fiber types.

As expected, there is a relationship between abnormal cold perception, which is a function of small (thinly myelinated) Aδ fibers, and absent IENF. Surprisingly, there is no relationship between heat perception, which is a function of small (unmyelinated) C fibers, and IENF. Quantitative sensory testing examines the whole sensory pathway, from the receptor along afferent nerves and central pathways, to the sensory cortex, and the efferent motor pathways, when the subject presses the button. Perception thresholds measured by the method of limits are reaction-time-dependent, and are limited by the subject's height, sex, and age (26). In a sample of elderly men, motor skills, attention, and cooperation, may influence our results. The heat perception threshold become increasingly variable with age, and thermal perception thresholds are not examined in patients above 70 years of age in clinical practice (in the Department of Neurophysiology, University Hospital of Lund and Malmö, Sweden). Recent studies have also found cold perception threshold to be more sensitive than heat perception threshold, and suggest that patients with PN detect cold more precisely than warm stimuli, which may merge with a burning sensation (112; 136). Reproducibility of quantitative sensory testing varies from poor to excellent, and in fact, is not recommended as a part of the neurophysiological examination in clinical research (84).

SFN has been proposed to be an early manifestation of nerve damage in diabetes or IGT (157; 200), and often presents with pain, paresthesiae, and loss of IENF, without objective clinical signs or neurophysiological evidence of nerve damage. Previous studies have shown a relationship between low IENF density and neuropathic symptoms and signs (124; 136; 201), and a reduction of IENF density in painful compared with painless diabetic PN (135). Devigili et al. found both abnormal clinical and skin biopsy findings in 43% of patients with SFN (the diagnosis was based on at least 2 abnormal examinations of clinical findings, quantitative perception thresholds or skin biopsy) (199).

As stated above, there is no statistical association between IENF loss and clinical symptoms and signs, as Total Neuropathy Score did not differ between groups with different IENF counts. Similarly, Shun et al. (119) did not find a correlation between the presence of neuropathic pain and IENF density, although diabetic patients with paresthesiae and leg pain had much lower IENF density than those with symptoms limited to toes and feet. Approximately half of their patients with painful diabetic PN had reduced IENF density but not all patients with reduced IENF density had neuropathic pain (119).

In this thesis, 67% of patients with clinical symptoms of SFN have reduced IENF count. However, only 35% of those with reduced IENF count had neuropathic

symptoms. Other studies have shown a relationship between reduced IENF density and PN symptoms and signs (124; 136), and a reduction of IENF density in painful compared with painless diabetic PN (135). A likely explanation why we did not find a statistical difference in Total Neuropathy Score between groups with different IENF counts is that our patients with PN are not distinguished in small, large, or mixed (large and small) fiber neuropathy, and the Total Neuropathy Score may be more sensitive to detect mixed rather than SFN.

The material is homogenous in respect to type of diabetes (type 2), gender (men), age, height, and BMI, diminishing the influence of these parameters on the results. Others have included patients of different ages and few older patients (117; 118; 122). On the other hand, it is necessary to make a further evaluation of female and younger patients with the technique used in this thesis. The subjects had difficulties remembering their disease duration (diabetes or IGT), and the patients' medial records were not accessible. Therefore, IENF count was not correlated with disease duration, which might have demonstrated more clear-cut differences within the diabetes group. A further expansion with regard to the number of sections counted should also be tested, as should the assessment of granular positivities and short nerve fiber fragments, whether representing transverse and obliquely sectioned fibers or degraded nerve fiber debris. Hence, although the technique used in this thesis shows good correlation with neurophysiological tests and may provide an important tool for studies of diabetic PN, it needs further refinement to be used in the evaluation of an individual patient in clinical practice.

6.4 Vibrotactile sense (Paper IV)

Vibrotactile sense (sensibility index) was impaired in finger pulps of index (median nerve) and little fingers (ulnar nerve) bilaterally in subjects with diabetes for more than 15 years, but not in subjects with persistent IGT or NGT during at least 15 years. Particularly, the little finger was affected at three frequencies (16, 250, and 500 Hz) bilaterally when vibration thresholds were analyzed at individual frequencies, indicating a predilection for ulnar nerve dysfunction in diabetes. In the index fingers, only the vibration thresholds at 500 and 125 Hz, the latter the common frequency investigated clinically, were significantly altered.

In this thesis, electrophysiology of the median nerve was used as the gold standard to detect and verify large fiber neuropathy in upper extremity, showing differences between diabetes, IGT, or NGT (202). Motor and sensory conduction velocities in the forearm were converted to Z scores and added (compound Z score forearm), and sensory conduction velocity and distal latency at the wrist were converted to Z scores and added (compound Z score wrist). The low compound Z scores of the median nerve indicated large fiber neuropathy in the forearm in subjects with diabetes. At the wrist, the diabetic subjects also showed electrophysiological signs of nerve compression, although they did not have any symptoms (carpal tunnel syndrome) in accordance with previous studies (202). Impaired vibrotactile sense (sensibility index) was associated with low Z score in the forearm (in subjects with NGT) and at the wrist (both diabetes and NGT), as both parameters measure large fiber function.

The data show that the finger pulps innervated by the ulnar nerve have impaired vibrotactile sense in diabetes. The corresponding area innervated by the median nerve

was also affected as observed by analysis of sensibility index, but single frequencies were rarely affected. This may indicate a more pronounced large fiber neuropathy in the ulnar nerve than in the median nerve in diabetic patients, supporting findings in other studies with patient materials of different sex, age and treatments (203).

Trauma or ischemia has been proposed as a causative mechanism for ulnar nerve affection in diabetes (204). However, patients with diabetes did not have any clinical signs of ulnar nerve compression. Only 2 patients (1 with diabetes and 1 with NGT) had an operation for symptomatic median nerve compression at the wrist bilaterally (carpal tunnel syndrome), although Z score at the wrist was significantly impaired in the diabetes group, signifying an asymptomatic compression neuropathy (202). The possibility that the function of the ulnar nerve, and to a lesser extent the median nerve, is influenced by other factors than diabetes, is less likely even if diabetes may confer an increased susceptibility to trauma in the peripheral nerve (205).

Blood parameters gave no indication of other causes of nerve dysfunction. One possibility is that the tactile innervation of the little finger is sparser than that of the index finger, which may result in a more severe functional affection in the former, even with comparable nerve fiber degeneration of the two nerves caused by diabetes.

Impairment at low (16 Hz) and high (250 and 500 Hz) frequencies of the ulnar nerve indicates that several of the sensory receptors in the pulps, or their nerve fibers, may be affected by diabetes. Pacinian corpuscles are responsible for vibrotacile sense at higher frequencies and Meissner corpuscles at lower frequencies (92; 103; 206). Threshold changes in the index finger are affected by age, and the changes due to aging are most pronounced at the highest frequencies (100). Hence, in our sample of older men, age may influence our results as the vibrotactile sense in the little finger was particularly affected at 250 and 500 Hz. However, the three groups (diabetes, IGT or NGT) were matched for age, and therefore it cannot solely explain the affection at high frequencies in the diabetic group.

6.5 IGT and polyneuropathy

Whether IGT is associated with polyneuropathy has been debated (20; 207). Recent studies have indicated that 30–45% of patients with idiopathic polyneuropathy may have undiagnosed IGT, which require oral glucose tolerance test to diagnose (154; 156).

In this thesis, nerve function (sural, peroneal or median nerve) and quantitative perception thresholds were not affected in subjects with IGT, and did not differ from NGT subjects. This is surprising when 4 of 10 IGT subjects had PN at baseline (Papers I and II), but none of the NGT subjects. Endoneurial microangiopathy was associated with deterioration in glucose tolerance (from NGT to IGT and from IGT to diabetes) but capillary morphology did not differ between subjects with IGT and NGT (Paper I). In addition, myelinated nerve fiber and intraepidermal nerve fiber morphology did not differ between IGT and NGT. On the other hand, IGT subjects with clinical PN did have reduced myelinated nerve fiber density and thickened basement membranes of endoneurial capillaries. Hence, IGT induces large fiber neuropathy in the lower extremity, but not in the upper extremity, as vibrotactile sense in the fingers was not affected in subjects with persistent IGT for more than 15 years. This may be explained by the length-dependent presentation of PN, affecting the longer nerves in the feet

first, but at a more severe stage, the shorter nerves in the hands may also become affected. IGT preferentially affects small nerve fibers (41; 154-157), but this thesis also demonstrates large fiber involvement.

IGT is one component of the metabolic syndrome, which is also characterized by hyperlipidemia, hypertension, and central obesity (161). Additionally, insulin resistance and hyperinsulinemia are generally referred to the metabolic syndrome. Whether hyperglycemia or other features of the metabolic syndrome increase the risk of developing polyneuropathy has recently been studied in patients with idiopathic neuropathy (161). Polyneuropathy patients with IGT and NGT shared a similarly elevated prevalence of metabolic syndrome features other than hyperglycemia, and lipid abnormalities were particularly prevalent among polyneuropathy subjects.

The metabolic syndrome seems to be linked to polyneuropathy, independent of hyperglycemia, as supported by this thesis. An association between high BMI and reduced myelinated nerve fiber density of the sural nerve was found (Paper II), but no difference in BMI between subjects with diabetes, IGT, or NGT. However, the BMI did not differ between subjects with or without clinical PN (diabetes or IGT; Paper I). Surprisingly, diabetic subjects showed lower total cholesterol and LDL-cholesterol compared with IGT or NGT subjects, but this could be due to more use of statins and other lipid lowering agents in the diabetic group.

Compared with the NGT group, the IGT group (also those without PN) was suspected to demonstrate more affection of nerve function, morphological changes in sural nerve and skin biopsies, and clinical symptoms/signs of PN. One possible explanation for the preserved function of the IGT group is due to a selection bias (see below) and another possibility is that these individuals have been offered a life-long follow-up by a medical specialist doctor, who treated hypertension, hyperlipidemia, B12 deficiency, and other medical conditions that may induce neuropathy.

Both individuals with IGT or diabetes were discovered in a screening procedure when they were 47–49 years of age, and then followed-up from 1975–79 with an intervention program and annual checkups (IGT) and strict glycemic control (diabetes). This may have prevented the development of endoneurial microangiopathy, PN, and loss of IENF in some individuals. On the other hand, the control group was not included in a regular follow-up. Although this hypothesis is difficult to prove, other studies indicate that lifestyle intervention for individuals with IGT may improve neuropathy (160) and reduce the risk of developing diabetes (23; 42).

7. CONCLUSIONS

1a. Increased capillary density in the sural nerve, is associated with current type 2 diabetes or future progression from IGT to type 2 diabetes. Decreased capillary luminal area is associated with future deterioration of glucose tolerance, from IGT to diabetes, and from NGT to IGT. Microangiopathy is associated with current diabetes and may presage deterioration of glucose tolerance. Higher density of endoneurial capillaries indicates compensation for hypoperfusion and hypoxia in early diabetic peripheral polyneuropathy.

1b. Endoneurial basement membrane thickening in the sural nerve from subjects with type 2 diabetes and IGT is linked to clinically confirmed peripheral polyneuropathy, morphological loss of myelinated nerve fibers, and current and future nerve dysfunction, suggesting that microangiopathy may both presage and accompany peripheral polyneuropathy.

2. A low baseline sural nerve myelinated nerve fiber density (MNFD) in subjects with type 2 diabetes, IGT or NGT is related to future progression of neurophysiological dysfunction in the peroneal and median nerves. Thus, MNFD may predict later development or progression of neurophysiological dysfunction. Obesity may be a risk factor for peripheral polyneuropathy, as body mass index correlates with MNFD.

3. Intraepidermal nerve fiber count in subjects with type 2 diabetes, IGT, or NGT, quantified with a simple technique with immunohistochemical staining and light microscopy, correlated with neurophysiological measures of both large and small fiber neuropathy.

4. Vibrotactile sense in subjects with type 2 diabetes is impaired in the index and little fingers, reflecting median and ulnar nerve function respectively. Tactilometry, with a multi-frequency approach, is a sensitive technique to screen for large fiber neuropathy in type 2 diabetes. Frequency-related changes may mirror dysfunction of various receptors.

8. SWEDISH SUMMARY (Sammanfattning på svenska)

Perifer polyneuropati vid typ 2 diabetes mellitus och störd sockertolerans; Korrelationer mellan morfologi, neurofysiologi och kliniska undersökningsfynd.

Perifer polyneuropati (PN: perifer nervsjukdom) är en mycket vanlig komplikation till diabetes och kan drabba ungefär 50 % av diabetespatienter beroende på vilka diagnostiska metoder som används. PN vid diabetes är en oberoende riskfaktor för mortalitet och morbiditet och det finns egentligen ingen säkerställd medicinsk behandling, utöver förbättrad sockerkontroll. De senaste åren har studier visat att även individer med störd sockertolerans (impaired glucose tolerance, IGT) kan utveckla PN. Både metabola och vaskulära faktorer anses kunna bidra till utvecklingen av PN vid diabetes. Däremot är inte sambandet fastställt mellan graden av sockerintolerans och dessa faktorer. Patologi i nervernas små kapillärer (mikroangiopati) har framhållits som en viktig faktor och har visat sig föregå utvecklingen av PN.

I denna avhandling har förekomsten och utvecklingen av olika patofysiologiska aspekter av PN vid typ 2 diabetes studerats. Olika metoder att diagnostisera PN har även studerats. Dessa inkluderar såväl invasiva (nervbiopsier och hudbiopsier) som icke invasiva (klinisk neurologisk undersökning, neurografi, mätning av vibrationssinne och temperaturtrösklar) tekniker. Analyser av suralisnerv- och hudbiopsier för att se förekomsten av nervfiberförändringar och mikroangiopati har utförts. Dessa patologiska förändringar har sedan relaterats till icke invasiva tekniker både vid biopsitillfället och vid uppföljning upp till 10 år senare. Vibrationssinet i fingrar hos patienter med diabetes har även studerats med taktilometri.

Patientmaterialet baseras på äldre män med typ 2 diabetes. Patienterna rekryterades från en stor populationsbaserad, prospektiv hälsokontrollstudie i Malmö (Malmö Preventionstudien) mellan 1975 och 1979. En kohort av 6956 personer genomgick sockertoleranstester. Från denna kohort matchades 182 individer med typ 2 diabetes, IGT och normal (kontroller) sockertolerans (NGT) för ålder, längd och vikt (body mass index, BMI). Från denna subgrupp gjordes suralisnervbiopsier hos 30 individer (10 med diabetes, 10 med IGT och 10 med NGT) och senare hudbiopsier från underbenet hos 86 individer (50 med diabetes, 15 med IGT och 21 med NGT). Vibrationssinet i fingrar undersöktes sedan hos patienter med diabetes.

I suralisnervbiopsierna var mikroangiopati associerad med försämring av sockertoleransen och PN. Den kapillära densiteten (antal kapillärer/mm^2) var högre hos patienter som hade diabetes vid uppföljningen 6 år senare jämfört med individer som hade NGT. Kapillära densiteten var även högre hos individer med IGT vid biopsitagningen men som utvecklat diabetes vid uppföljningen jämfört med dem som hade konstant IGT. Därmed var ökad kapillär densitet associerad med diabetes och framtida utveckling av diabetes. Tjockleken av basalmembranen i kapillärerna var större hos diabetes/IGT patienter med PN jämfört med dem utan polyneuropati. Förtjockning av basalmembranen var således kopplad till förekomsten av PN. Arean av lumen i kapillärerna var dessutom mindre hos individer som försämrades i sockertoleransen (från IGT till diabetes eller från NGT till IGT) jämfört med individer med konstant sockertolerans (IGT eller NGT). Således var minskning av kapillärernas lumen associerat med framtida försämring av sockertoleransen. Förändringar i

nervkapillärer kan således föregå en försämrad sockertolerans och förekommer tillsammans med PN.

I ovan angivna suralisnervbiopsier har densiteten av myeliniserade nervfibrer kopplats till framtida elektrofysiologisk funktion. Personer med låg densitet av nervfibrer hade vid en klinisk och neurofysiologisk uppföljning upp till 10 år senare sämre peroneus- och medianusnerv-funktion. Densiteten av myeliniserade nervfibrer korrelerade negativt med BMI. I regressionsanalys noterades att diabetes påverkar försämringen av nervfunktionen. Dessa resultat indikerar att en låg densitet av myeliniserade nervfibrer kan förutspå försämring i nervfunktion och att det finns en förbindelse med fetma och förlust av myeliniserande nervfibrer.

I hudbiopsierna från 86 av de 100 individer som genomgick uppföljning undersöktes hudnervtrådar (intraepidermal nerve fibers, IENF) från underbenet. Förekomst av PN undersöktes med klinisk och neurofysiologisk undersökning och korrelerades till fynden i hudbiopsin (IENF). Hudbiopsierna kvantifierades i ljusmikroskop med en enkel metod med tunna hudsnitt, genom att mäta antalet nervtrådar i ytterhuden (epidermis). Nervtrådarna färgades med axonal markör (PGP9.5). Totalt bortfall av nervtrådar i huden var relaterat till försämring av elektrofysiologisk funktion i suralisnerven och till försämring av köldtrösklar i foten.

Förekomst av neuropati i den övre extremiteten har studerats hos 23 patienter med diabetes i över 15 år, 7 med konstant IGT och 28 med NGT. Med hjälp av ny metod att mäta vibrationssinnet vid olika frekvenser erhålls resultatet av vibrationströsklar i ett diagram vilket liknar ett audiogram där enskilda vibrationströsklar vid de sju frekvenserna (8, 16, 32, 64, 125, 250 och 500 Hz) mäts individuellt eller att hela arean under kurvan mäts. I studien mättes också elektrofysiologisk funktion i medianusnerven. Vibrationssinnet var försämrat både i pek- och långfinger hos patienter med diabetes och vibrationströsklarna var särskilt påverkade vid höga frekvenser (16, 250 och 500 Hz) i lillfingret, i ulnarisnerven. Patienterna med diabetes visade också tecken till neuropati i medianusnerven med elektrofysiologisk teknik. Vibrationssinnet och elektrofysiologisk funktion var inte påverkad hos individer med IGT eller NGT.

Sammanfattningsvis har avhandlingen visat att förekomst och utveckling av PN är relaterad till nervfiberförändringar i hudbiopsier och suralisnervbiopsier samt microangiopati i suralisnervbiopsier. Den har också visat att vibrationströsklar är förhöjda i fingrarna hos diabetespatienter, särskilt vid höga frekvenser i ulnarisinnerverade fingerpulpor. IGT förefaller inte vara en faktor som inducerar neuropati i övre extremiteten. Att mäta små nervtrådar (IENF) i hudbiopsier är en enkel och säker metod, korrelerar till neurofysiologisk funktion och bör kunna användas i kliniska studier på PN. Multifrekvensmätning av vibrationströsklar kan vara en enkel teknik för att screena neuropati i stora nervtrådar vid typ 2 diabetes.

9. ICELANDIC SUMMARY (Samantekt á íslensku)

Perifer polyneuropatia við týpu 2 sykursýki og skert sykurþol; tengsl milli vefjafræði, taugalífeðlisfræði og taugaeinkenna við líkamsskoðun.

Perifer polyneuropatia (PN: fjöltaugakvilli) er algengur fylgikvilli sykursýki. PN leggst aðallega á taugar í útlimum og veldur því að taugaboð um mismunandi skynjun og vöðvasamdrátt berast ekki eðlilega eftir taugunum. Algengustu einkennin eru óþægilegur nálardofi og verkir í fótum. U.þ.b. 50% af sykursjúkum fá PN en tíðnin ræðst m.a. af því hvaða aðferðum er beitt við að greina sjúkdóminn. PN við sykursýki er áhættuþáttur fyrir dauða (mortalitet) og sjúkleika (morbiditet) en það er í raun ekki til nein læknismeðferð við sjúkdómnum nema að stjórna blóðsykrinum. Undanfarin ár hafa rannsóknir sýnt að jafnvel einstaklingar með forstig sykursýki, þ.e. skert sykurþol (enska: impaired glucose tolerance, IGT) geta fengið PN. Bæði efnafræðilegir þættir og vefjabreytingar í háræðum tauganna eru talin orsaka PN við sykursýki. Sambandið milli gráðu sykuróþols og þessarra þátta er þó ekki þekkt. Vefjabreytingar í háræðum tauganna (þ.e. mikroangiopatia) eru taldar mikilvægur þáttur í sjúkdómsmynduninni og rannsóknir hafa sýnt að þær þróast jafnvel á undan PN.

Tilgangur þessa doktorsverkefnis var að með mismunandi aðferðum rannsaka PN og þróun hennar við týpu 2 sykursýki, en það er sú tegund af sykursýki sem eldra fólk fær. Taugasýni var tekið frá taug (sk súralistaug) og húð, líkamleg taugaskoðun gerð, neurografia (mæling á taugarafboðum) framkvæmd og titrings-, hita- og kuldaskyn metið.

Rannsóknarhópurinn voru eldri menn sem voru beðnir að taka þátt í heilsurannsókn í Malmö í Svíþjóð sem hófst milli 1975 og 1979. Af 6956 einstaklingum sem gengust undir sykurþolspróf voru 182 menn teknir inn í rannsóknina. Af þeim voru 69 með týpu 2 sykursýki, 51 með IGT og 62 með eðlilegt sykurþol (enska: normal glucose tolerance, NGT)(viðmiðunarhópur). Þeir voru allir sambærilegir að aldri, hæð og þyngd. Taugavefssýni frá súralistaug var tekið hjá 30 einstaklingum (10 með sykursýki, 10 með IGT og 10 með NGT) og þeim síðan fylgt eftir í 5-6 ár. Síðar var húðtaugasýni tekið frá 86 einstaklingum (50 með sykursýki, 15 með IGT og 21 með NGT). Titringsskynið í vísi- og litlafingri var metið hjá einstaklingum sem höfðu haft óbreytt sykurþol í 15 ár (23 með sykursýki, 7 með IGT og 28 með NGT).

Niðurstöðurnar benda til að vefjabreytingarnar í háræðum taugarinnar tengist sykuróþoli og PN. Þéttni háræða í tauginni (fjöldi háræða/mm^2) var meiri hjá þeim sjúklingum sem voru með sykursýki við eftirfylgni 5-6 árum síðar samanborið við viðmiðunarhópinn sem var með eðlilegt sykurþol. Þéttni háræða var einnig meiri hjá þeim einstaklingum sem voru með skert sykurþol við sýnatökuna úr súralistauginni en höfðu þróað sykursýki við eftirfylgnina samanborið við einstaklinga sem voru áfram með skert sykurþol. Niðurstaðan var því að hærri háræðaþéttni í tauginni tengdist sykursýki og að síðar þróa sykursýki. Þykktin á grunnhimnu háræðanna (enska: basement membrane area) var meiri meðal sjúklinga með sykursýki eða IGT og PN samanborið við þá sem voru ekki með PN. Þykknun á grunnhimnunni tengdist þannig PN. Þverskurðarflatarmál háræðaholrúmsins (enska: capillary luminal area) var þar að auki minna hjá þeim einstaklingum sem sykurþolið versnaði (frá IGT yfir í sykursýki eða frá NGT yfir í IGT) samanborið hjá einstaklingum með stöðugt sykurþol (IGT eða

NGT). Vefjabreytingarnar í háræðum taugarinnar gætu þar af leiðandi komið á undan versnun á sykurþoli en sjást einnig við tilvist PN.

Í súralistauginni eru stórir taugungar með þykkt taugaslíður (mýelínslíður) til einangrunar (sk mýldir taugungar, enska: myelinated nerve fibers). Tengls milli þéttni þessara stóru taugunga og seinni tíma taugavirkni mælt með neurógrafíu voru könnuð. Einstaklingar með lága þéttni mýldra taugunga reyndust hafa meira af einkennum PN við taugaskoðun og hafa verri taugavirkni í handlegg (í sk medianustaug) og fótlegg (í sk peroneustaug) allt að 10 árum seinna. Tengsl voru einnig milli færri mýldra taugunga og líkamsfitustuðuls (enska: body mass index), þ.e. eftir því sem einstaklingurinn er feitari þeim mun færri taugunga hefur hann. Einnig voru tengsl milli sykursýki og versnunar á taugavirkni. Niðurstöðurnar benda til að lág þéttni mýldra taugunga geti spáð fyrir um versnandi taugavirkni og að tengsl séu milli offitu og tapi á mýldum taugungum.

Fjöldi lítilla húðtaugaþráða (enska: intraepidermal nerve fibers, IENF) var kannaður í húðsýnum teknum frá neðanverðum sköflungnum hjá 86 af þeim 100 einstaklingum sem tóku þátt í eftirfylgni. Kannað var hvort einstaklingarnir væru með PN með líkamlegri taugaskoðun og neurografiu og niðurstöðurnar bornar saman við fjölda húðtaugaþráða. Algjört brottfall af taugaþráðum í húðinni tengdist skertri taugavirkni í súralistauginni og verra kuldaskyni í fæti.

Með nýrri tækni – taktilometriu- var kannað hvort einstaklingar með sykursýki, IGT eða NGT í meira en 15 ár, væru með PN í hendi. Þessi tækni metur titringsskynið við mismunandi tíðni og niðurstöðurnar eru birtar í línuriti (diagram) líkt og við heyrnarmælingu (audiogram). Titringsþröskuldur er mældur við hverja tíðni (8, 16, 32, 64, 125, 250 og 500 Hz) og flatarmálið undir bogalínunni er mælt. Niðurstöðurnar sýndu að titringsskynið er skert í bæði vísi- og litlafingri hjá sykursjúkum og sérstaklega við hærri tíðni (16, 250 och 500 Hz) í litlafingri sem ulnaristaugin ítaugar. Eins benti niðurstaðan úr neurografiunni til að sjúklingar með sykursýki væru með PN í medianustauginni. Titringsskynið var hins vegar eðlilegt hjá einstaklingum með IGT eða NGT.

Samantekið sýndu niðurstöður þessa doktorsverkefnis að PN og þróun hennar tengist tapi á húðtaugaþráðum og mýldum taugungum í súralistauginni sem og vefjabreytingum í háræðum súralistaugarinnar. Titringsskynið er skert í fingrum sykursýkissjúklinga, sérstaklega við hærri tíðni í fingurgómum sem ulnaristaugin ítaugar. IGT virðist ekki tengjast PN í hendi/handlegg. Að telja húðtaugaþræði (IENF) í húðsýnum er einföld og örugg aðferð til að meta litla taugaþræði í PN og tengist taugalífeðlisfræðilegri virkni og ætti því að vera nothæf í rannsóknum á PN. Mæling á titringsþröskuldum við mismunandi tíðni er einföld tækni til skimunar fyrir PN hjá sykursjúkum.

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11. APPENDIX A - G

Appendix A

Neuropathy Symptom Score (Anamnes perifer neuropati), Papers I and II

(0 = aldrig, 1 = ibland, 2 = ofta, 3 = nattligen)

	FÖTTER
1. DOMNINGSKÄNSLA	
2. VÄRME-KÖLD KÄNSLA	
3. STICKNINGAR	
4. BRÄNNANDE SMÄRTA	
5. UTSTRÅLANDE SMÄRTA	
6. MOLANDE VÄRK	
7. SÄNGKLÄDER IRRITATION	

NSS (FÖTTER)	

Appendix B

Neuropathy Symptom Score (Anamnes perifer neuropati), Paper III

(0 = aldrig, 1 = ibland, 2 = ofta, 3 = nattligen)

	FÖTTER	HÄNDER
1. DOMNINGSKÄNSLA		
2. VÄRME-KÖLD KÄNSLA		
3. STICKNINGAR		
4. BRÄNNANDE SMÄRTA		
5. UTSTRÅLANDE SMÄRTA		
6. MOLANDE VÄRK		
7. SÄNGKLÄDER IRRITATION		

NSS		
NSS (FÖTTER+HÄNDER)		

Appendix C

Neuropathy Disability Score (sensorik), Papers I and II

(0=normal, 1= nedsatt, 2=frånvaro)

	VÄ	HÖ		VÄ	HÖ
BOMULL			**STÄMGAFFEL**		
STORTÅ			**STORTÅ**		
MELLANFOT			**MELLANFOT**		
MALLEOL			**MALLEOL**		
UNDERBEN			**UNDERBEN**		
KNÄ			**KNÄ**		

	VÄ	HÖ		VÄ	HÖ
NÅL			**KYLA**		
STORTÅ			**STORTÅ**		
MELLANFOT			**MELLANFOT**		
MALLEOL			**MALLEOL**		
UNDERBEN			**UNDERBEN**		
KNÄ			**KNÄ**		

NDS-A (FÖTTER)	

Appendix D

Neuropathy Disability Score (sensorik), Paper III

(0=normal, 1= nedsatt, 2=frånvaro)

	VÄ	HÖ		VÄ	HÖ
BOMULL			**STÄMGAFFEL**		
BEN			***BEN***		
STORTÅ			**STORTÅ**		
MALLEOL			**MALLEOL**		
KNÄ			**KNÄ**		
ARM			***ARM***		
PEKFINGER			**PEKFINGER**		

	VÄ	HÖ		VÄ	HÖ
NÅL			**KYLA**		
BEN			***BEN***		
STORTÅ			**STORTÅ**		
MALLEOL			**MALLEOL**		
KNÄ			**KNÄ**		
ARM			***ARM***		
PEKFINGER			**PEKFINGER**		

NDS-A (FÖTTER+HÄNDER)	

Appendix E

Neuropathy Disbility Score B (reflexer), Papers I and II

(0=normal, 1= nedsatt, 2=frånvaro)

	VÄ	HÖ
PATELLAR		
ACHILLES		

NDS-B (FÖTTER)	

Appendix F

Neuropathy Disbility Score B (reflexer), Paper III

(0=normal, 1= nedsatt, 2=frånvaro)

	VÄ	HÖ
BEN		
PATELLAR		
ACHILLES		
ARM		
BICEPS		

NDS-B (FÖTTER+HÄNDER)	

Appendix G

Neuropathy Disability Score C (muskelstyrka), Paper III

**(0=normal, 1= nedsatt, 2= kraftigt nedsatt,
3= paralys)**

	VÄ	HÖ
BEN		
STORTÅ extension		
STORTÅ flexion		
FOTLED extension		
FOTLED flexion		
KNÄ extension		
ARM		
FINGER extension		
FINGER flexion		
HANDLED extension		
HANDLED flexion		
ARMBÅGE flexion		

NDS-C (FÖTTER+HÄNDER)	

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13. ORIGINAL PUBLICATIONS