Aus dem Institut für Klinische Diabetologie am
Deutschen Diabetes-Zentrum,
Leibniz-Zentrum für Diabetesforschung an der
Heinrich-Heine-Universität Düsseldorf

Direktor: Univ.-Prof. Dr. Michael Roden

Nerve fibre loss and regeneration in type 2 diabetes patients with painful and painless polyneuropathy

Dissertation

zur Erlangung des Grades eines Doktors der Medizin
der Medizinischen Fakultät der Heinrich-Heine-Universität Düsseldorf

vorgelegt von
Gidon Josia Bönhof
2020

Als Inauguraldissertation gedruckt mit Genehmigung der Medizinischen Fakultät der Heinrich-Heine-Universität Düsseldorf
gez.:
Dekan: Prof. Dr. Nikolaj Klöcker
Erstgutachter: Prof. Dr. Dan Ziegler
Zweitgutachter: PD Dr. Michael Gliem
Drittgutachter: Prof. Dr. Alexander Grimm

„In einem Augenblick gewährt die Liebe,
Was Mühe kaum in langer Zeit erreicht.“

Johann Wolfgang von Goethe

Teile dieser Arbeit wurden veröffentlicht:

Bönhof, G.J., Strom, A., Püttgen, S., Ringel, B., Brüggemann, J., Bódis, K., Müssig, K., Szendroedi, J., Roden, M., Ziegler, D. (2017). Patterns of cutaneous nerve fibre loss and regeneration in type 2 diabetes with painful and painless polyneuropathy. *Diabetologia* 60(12): 2495-2503

Abstract

Diabetic distal symmetric sensorimotor polyneuropathy (DSPN) affects approximately 30% of people with diabetes and its sequelae, such as neuropathic pain and foot ulcers, contribute to substantial morbidity, high socioeconomic burden, reduced quality of life, and increased risk of mortality. The determinants and mechanisms of the development of DSPN remain unclear and the conundrum of why some individuals with DSPN develop neuropathic pain, while others do not, has not been solved. Current options to prevent and treat painful and painless DSPN are limited.

DSPN is characterized by progressive nerve fibre loss and impaired nerve function. The present study aimed to examine the degree of cutaneous nerve fibre loss and regeneration in individuals with type 2 diabetes with painful or painless DSPN compared with individuals with recent-onset type 2 diabetes and corresponding healthy volunteers with normal glucose tolerance.

Skin biopsy is a reliable, minimally invasive tool for the assessment of epidermal and dermal nerve fibres in peripheral nerve disorders. The pan-neuronal marker protein gene product 9.5 (PGP9.5), a member of the ubiquitin hydroxylase system, is widely used to detect and quantify cutaneous nerve fibres. Growth-associated protein 43 (GAP-43) is a membrane protein that is involved in the process of peripheral nerve regeneration. As it is a major constituent in axonal growth cones after nerve injury, GAP-43 is expressed in peripheral nerve fibre areas with high neural plasticity such as the epidermis and dermis. Hence, GAP-43 is widely used in immunohistology as a marker for regenerative nerve fibres.

Skin biopsies were taken from the distal lateral calf and were analysed using double immunofluorescence staining for PGP9.5 and GAP-43. For both markers, intraepidermal nerve fibre density (IENFD) and length (IENFL) and dermal nerve fibre length (DNFL) were determined. For each parameter, the corresponding ratios between both markers were calculated to assess the regenerative capacity.

The present study demonstrated an enhanced regenerative capacity of dermal nerve fibres in both DSPN entities compared with corresponding control individuals, but to a higher extent in individuals with painful DSPN compared to those with painless DSPN. A higher dermal nerve fibre regenerative capacity was associated with more advanced intraepidermal nerve fibre loss, lower dermal nerve fibre length, and peripheral nerve dysfunction.

These findings suggest that nerve repair mechanisms are preserved in DSPN, but are ultimately not sufficient to adequately counteract epidermal neurodegenerative processes due to type 2 diabetes, leading to a loss of epidermal nerve fibres. The higher degree of dermal regenerative capacity found in painful compared with painless DSPN indicates that regenerative processes may contribute to the painful phenotype.

Zusammenfassung (deutsch)

Ungefähr 30% aller Menschen mit Diabetes mellitus leiden an diabetischer distal-symmetrischer sensomotorischer Polyneuropathie (DSPN). Die Folgen dieser Erkrankung, zu denen unter anderem neuropathische Schmerzen und chronische Fußulzera zählen, tragen zu deutlich erhöhter Morbidität und Mortalität und eingeschränkter Lebensqualität bei und stellen eine erhebliche sozioökonomische Belastung dar. Die maßgeblichen Faktoren, die zur Entwicklung der DSPN beitragen, bleiben weiterhin ungeklärt. Außerdem ist nicht bekannt, warum die DSPN bei bestimmten Menschen mit neuropathischen Schmerzen einhergeht, während sie bei anderen schmerzlos verläuft. Sowohl bei der schmerzhaften als auch der schmerzlosen Entität sind die aktuell verfügbaren Optionen hinsichtlich Therapie und Prävention limitiert.

Die DSPN zeichnet sich durch einen fortschreitenden Verlust an Nervenfasern und Einschränkung der Nervenfunktion aus. Die vorliegende Studie hatte zum Ziel, sowohl den Verlust an Nervenfasern, als auch neuroregenerative Prozesse in der Haut von Patienten mit Typ-2-Diabetes und schmerzhafter oder schmerzloser DSPN sowie bei denjenigen mit kürzlich diagnostiziertem Typ-2-Diabetes im Vergleich mit stoffwechselgesunden Probanden ohne Nervenschäden zu quantifizieren.

Hautbiopsien stellen eine zuverlässige, minimal-invasive Methode dar, um epidermale und dermale Nervenfasern bei Erkrankungen des peripheren Nervensystems beurteilen zu können. Um diese Nervenfasern aufzufinden und zu quantifizieren, wird üblicherweise der pan-axonale Marker *protein gene product 9.5* (PGP9.5), ein Mitglied der Ubiquitin-C-terminalen Hydroxylase-Familie, verwendet. *Growth-associated protein 43* (GAP-43) ist ein Membranprotein, welches an neuroregenerativen Prozessen im peripheren Nervensystem beteiligt ist, indem es eine wichtige Rolle bei der Elongation des proximalen Stumpfs verletzter peripherer Nervenfasern spielt. Es wird somit auch in Nervenfasern exprimiert, die einem hohen Maß an physiologischem Remodeling unterliegen, zu denen auch diejenigen in den oberen Hautschichten gehören. Aufgrund dieser Eigenschaften eignet sich GAP-43 als Marker für regenerierende Nervenfasern. In der vorliegenden Studie wurden Hautproben aus dem distal-lateralen Unterschenkel mittels doppelter Immunfluoreszenzmikroskopie für PGP9.5 und GAP-43 analysiert. Intraepidermale Nervenfaserdichte und -länge sowie dermale Nervenfaserlänge wurden für beide Marker quantifiziert. Für alle drei Parameter wurde auch das Verhältnis beider Marker zueinander bestimmt, um Rückschlüsse auf die regenerative Kapazität der Nervenfasern zu ziehen.

Bei beiden DSPN Gruppen wurde gegenüber der zugehörigen Kontrollgruppe eine erhöhte regenerative Kapazität der dermalen Nervenfasern festgestellt, die in der schmerzhaften im Vergleich mit der schmerzlosen DSPN-Gruppe deutlicher ausgeprägt war. Eine höhere regenerative Kapazität der dermalen Nervenfasern war mit stärkerem epidermalen Nervenfaserverlust, geringerer dermaler Nervenfaserlänge, sowie mit peripherer Nervendysfunktion assoziiert.

Diese Ergebnisse weisen darauf hin, dass die Reparaturmechanismen in kutanen Nervenfasern bei DSPN grundsätzlich weiterhin nachweisbar sind, aber letztlich nicht in der Lage sind, die durch Typ-2-Diabetes bedingten Vorgänge, die zu epidermalem Nervenverlust führen, adäquat zu kompensieren. Eine höhere dermale regenerative Kapazität bei der schmerzhaften gegenüber der schmerzlosen DSPN könnte auf eine Beteiligung neuroregenerativer Prozesse bei der Entstehung neuropathischer Schmerzen bei DSPN hinweisen.

Abbreviations

ADA.....*American Diabetes Association*
AGEs.....*advanced glycation end-products*
C fibres.....*group C nerve fibres*
CAN.....*cardiovascular autonomic neuropathy*
CCM.....*corneal confocal microscopy*
CIPD ..... *chronic inflammatorory demyelinating polyneuropathy*
CVD..... *cardiovascular disease*
DFS.....*diabetic foot syndrome*
DNFL..... *dermal nerve fibre length*
DRGs .....*dorsal root ganglia*
DSPN ....*diabetic distal symmetric sensorimotor polyneuropathy*
ECs.....*endothelial cells*
FFA ..... *free fatty acids*
GAPDH.....*glyceraldehyde 3-phosphate dehydrogenase*
HRV .....*heart rate variability*
IENFD..... *intraepidermal nerve fibre density*
IENFL .....*intraepidermal nerve fibre length*
IFG.....*impaired fasting glucose*
IGT..... *impaired glucose tolerance*
MNSI .....*Michigan Neuropathy Screening Instrument*
mTCNS..*modified Toronto Clinical Neuropathy Scale*
NAD^+ .....*oxidized nicotinamide adenine dinucleotide*
NCS .....*nerve conduction studies*
NCV..... *nerve conduction velocity*
NDS .....*Neuropathy Disability Score*
NF-κB .....*nuclear factor-κB*
NGT ..... normal glucose tolerance
NRS .....*Numerical Rating Scale*
NSS.....*Neuropathy Symptom Score*
OGTT.....*oral glucose tolerance test*
PARP1 .....*poly(ADP-ribose) polymerase-1*
PGP9.5..... protein gene product 9.5
PKC .....*protein kinase C*
PNS.....*peripheral nervous system*
QoL.....*quality of life*
QST..... *quantitative sensory testing*
RAGE .....*surface receptor for AGEs*
ROS ..... *reactive oxidative species*
SNAP.....*sensory nerve action potential*
SOD2 .....*superoxide dismutase 2*
TDT .....*thermal detection threshold*
UENS.....*Utah Early Neuropathy Scale*
VPT.....*vibration perception threshold*

Contents

List of figures

1 Introduction

Diabetic neuropathies represent the most prevalent chronic complications of diabetes, of which diabetic distal symmetric sensorimotor polyneuropathy (DSPN) is the most relevant clinical manifestation. DSPN affects approximately 30% of people with diabetes (1, 2). DSPN contributes substantially to reduced quality of life, increased morbidity, and high socioeconomic burden (3, 4). The underlying pathophysiology contributing to the development of DSPN and its sequelae such as neuropathic pain is not well understood and the current options to prevent and treat are limited (5).

1.1 Type 2 diabetes

Diabetes mellitus is a group of heterogeneous metabolic diseases characterised by hyperglycaemia due to deficits in pancreatic insulin secretion and/or insulin sensitivity of insulin-sensitive target tissues (6). According to the American Diabetes Association (ADA), diabetes is diagnosed based on plasma glucose criteria, by (1) the fasting plasma glucose (defined as no caloric intake for at least 8 h) or (2) the 2-h plasma glucose during a 75 g oral glucose tolerance test using a glucose load containing the equivalent of 75 g anhydrous glucose dissolved in water (OGTT), or (3) haemoglobin A1c (HbA1c) criteria: Fasting plasma glucose ≥ 126 mg/dl (7.0 mmol/l) or 2-h plasma glucose ≥ 200 mg/dl (11.1 mmol/l) during OGTT or HbA1c ≥ 6.5% (48 mmol/mol) or, in a patient with classic symptoms of hyperglycaemia or hyperglycaemic crisis, a random plasma glucose ≥ 200 mg/dL (11.1 mmol/l). In the absence of unequivocal hyperglycaemia, results should be confirmed by repeated testing (7).

Type 2 diabetes represents the most prevalent category of diabetes, accounting for around 90-95% of cases with diabetes, corresponding to over 400 million people worldwide in the age group of 20 to 79 years as of 2019 (8). Data from 65 million people insured by the German statutory health insurance suggest a number of 6.9 million people with type 2 diabetes in Germany as of 2015. Taking trends in incidence and demographic factors into account, a considerable increase of cases with type 2 diabetes between 54% and 77% can be expected until 2040, representing a substantial growth in socioeconomic burden (9).

In contrast to type 1 diabetes, which is characterized by pancreatic beta cell destruction, usually leading to absolute insulin deficiency, type 2 diabetes is a form of diabetes mellitus that encompasses individuals with chronically reduced insulin sensitivity and

usually, to a certain degree, preserved insulin secretion. As hyperglycaemia often develops gradually and not severely enough for the individual to notice symptoms in the early course of type 2 diabetes, it frequently goes undiagnosed for many years along with an increasing risk of developing complications. The risk of developing type 2 diabetes increases with higher age, obesity, and low physical activity (6).

Current therapeutic concepts in type 2 diabetes care primarily target controlling hyperglycaemia in order to prevent complications (10), as type 2 diabetes has generally been considered an incurable chronic disease. There is growing evidence that remission of type 2 diabetes is possible in selected patients with carbohydrate or calorie restricted diets or bariatric surgery leading to weight loss. However, long-term data concerning sustainability is limited. The number of non-responders in the published studies is high, and it remains unclear whether remission of type 2 diabetes is an achievable goal for individuals with longer lasting diabetes (11-13).

Type 2 diabetes is associated with several potentially severe chronic complications of hyperglycaemia involving dysfunction or failure of various organ systems (6). Vascular complications are of particular importance in type 2 diabetes. Arterial circulation is frequently affected, spanning from large elastic arteries to minute capillaries. Cardiovascular disease (CVD) is the leading cause of mortality in individuals with type 2 diabetes (14). Large vessel disorders associated with type 2 diabetes include atherosclerotic cardiovascular, cerebrovascular, and peripheral arterial disease (6). Impaired microcirculation culminates in characteristic diabetic microvascular complications. These include diabetic retinopathy, a frequent cause of newly-developed blindness among adults in developed countries (15), diabetic nephropathy, a major cause of chronic kidney disease and end-stage renal failure (16), and diabetic neuropathy, the most prevalent chronic complication of diabetes (17). Diabetic neuropathy leads to debilitating sequelae such as impaired sensation, neuropathic pain, foot ulcers, orthostatic hypotension as well as gastrointestinal and genitourinary complications (1-3).

1.2 Diabetic Neuropathy

Diabetic neuropathies are defined by impairments of the peripheral nervous system (PNS) due to diabetes after exclusion of other causes. Both somatic and autonomic parts of the PNS can be affected, leading to diabetic somatosensory and autonomic neuropathies, respectively, which either can remain subclinical or manifest clinically. Evidence has

accumulated suggesting that diabetic neuropathies, rather than representing "late" complications of diabetes, can develop early in the course of diabetes and even in prediabetes (1).

Peripheral neuropathies are characterised by sensory and/or motor nerve dysfunction. They include comparably rare mononeuropathies or radiculopathies which are considered atypical forms of diabetic neuropathies, and diffuse neuropathies, the most frequent and clinically relevant of which is diabetic distal symmetric sensorimotor polyneuropathy (DSPN) accounting for about 75% of diabetic neuropathies (1, 3, 17, 18).

Diabetic autonomic neuropathy, characterized by dysfunction of parasympathetic and/or sympathetic neurons, can affect various organs of human body. The most studied manifestation, cardiovascular autonomic neuropathy (CAN), is detectable at an early subclinical stage by reduced heart rate variability (HRV), later followed by clinical signs such as orthostatic hypotension and resting tachycardia (17, 19). Other clinical signs of CAN include exercise intolerance due to a blunted increase in cardiac output in response to physical activity and an increased intra- or perioperative cardiovascular instability, when autonomic response does not completely compensate for vasodilating effects of anaesthesia (20). Impaired sudomotor (distal hypohydrosis/anhidrosis, gustatory sweating), gastrointestinal (gastroparesis, diarrhoea, or constipation), genitourinary (neurogenic bladder, sexual dysfunction), and pupillary function as well as decreased hypoglycaemia awareness are further clinically relevant manifestations of diabetic autonomic neuropathy (17).

1.2.1 Diabetic distal symmetric sensorimotor polyneuropathy (DSPN)

1.2.1.1 Definition of DSPN

According to the Toronto Diabetic Neuropathy Expert Group consensus statement published in 2010, DSPN is '*a symmetrical, length-dependent sensorimotor polyneuropathy attributable to metabolic and microvessel alterations as a result of chronic hyperglycaemia exposure (diabetes) and cardiovascular risk covariates*' (21). In brief, the following definitions were proposed:

1. Possible DSPN (presence of symptoms or signs of DSPN)
2. Probable DSPN (presence of symptoms and signs of DSPN)
3. Confirmed DSPN (symptoms or signs of DSPN and either the presence of an abnormal nerve conduction test or a validated measure of small fibre neuropathy)

4. Subclinical DSPN (no symptoms or signs, but abnormal nerve conduction test or a validated measure of small fibre neuropathy)

Definitions 1, 2, or 3 (possible, probable, confirmed DSPN) were recommended for use in clinical practice, while definitions 3 or 4 (confirmed, subclinical DSPN) should serve primarily in research studies (21).

1.2.1.1.1 Definition of painful DSPN

In clinical practice, painful DSPN is defined as DSPN with painful neuropathic symptoms, based on the patient's description of pain. For research settings, a definition of DSPN associated with chronic neuropathic pain for >6 months and a weekly pain score ≥4 on an 11-point Numerical Rating Scale (NRS) after exclusion of pain not associated with DSPN was suggested (21).

1.2.1.2 Epidemiology of DSPN

DSPN affects approximately one third of patients with diabetes. The incidence of DSPN is approximately 2% per year. In hospital-based patients with type 2 diabetes, the prevalence ranges between approximately 18% and 75% (type 1 diabetes: 13-23%), while in general populations or primary care, the prevalence ranges between around 13% and 51% in type 2 diabetes (type 1 diabetes: 8-63%). DSPN may start very early during the course of diabetes. In prediabetes, DSPN may be found in around 11% to 25% of cases (1). In a large population based study, the prevalence of DSPN was 13.0% among individuals with impaired glucose tolerance (IGT) and 11.3% with impaired fasting glucose (IFG) (3, 22). In a cohort of recent-onset type 2 diabetes individuals with a known diabetes duration up to one year and very good glycaemic control, signs or symptoms of possible DSPN were reported in 23.2%, while confirmed DSPN was found in 6.6% of participants (23).

Despite its high prevalence, DSPN remains a frequently underdiagnosed and undertreated condition. Thus, the clinical impact of DSPN is still being underestimated (24, 25). Data obtained from a recent study as part of a German nationwide educational initiative suggested an alarming prevalence of previously undiagnosed peripheral neuropathy of 69.9% in participants with type 2 diabetes. Even 57.0% of participants with type 2 diabetes and peripheral neuropathy with neuropathic pain were not aware of having the condition (26).

Risk factors and comorbidities associated with DSPN include higher age, longer duration of diabetes, poor glycaemic control, height, cigarette smoking, alcohol intake, hyperlipidaemia, hypertension, the presence of other diabetic complications, and depression. Associations with higher body weight and hypoinsulinaemia have been reported specifically for type 2 diabetes (3).

1.2.1.3 Diagnosis of DSPN

The identification and characterization of DSPN requires a careful neurologic examination. It is a diagnosis of exclusion, eliminating other possible causes of neuropathy. Important differential diagnoses include nutrient deficiencies (e.g. B vitamins), drugs and toxins (e.g. ethanol, antineoplastic agents), infectious and inflammatory diseases (e.g. HIV, Guillain-Barré syndrome, chronic inflammatory demyelinating polyneuropathy (CIPD), monoclonal gammopathies), metabolic conditions (e.g. renal failure, critical illness), inherited diseases (e.g. Charcot-Marie-Tooth, hereditary neuropathies), paraneoplastic syndrome, vascular diseases (e.g. peripheral vascular disease), endocrinopathies (e.g. untreated hypothyroidism), and nerve compression syndromes (e.g. spinal disc herniation) (27, 28).

Current clinical practice guidelines recommend a basic diagnostic evaluation including a standard battery of laboratory tests, i.e. complete blood count, creatinine, inflammation markers, TSH, vitamin B12, folic acid, liver enzymes, and immunoelectrophoresis (28).

Signs and symptoms of DSPN should be individually assessed using standardised clinical instruments, such as the Michigan Neuropathy Screening Instrument (MNSI) (29), the modified Toronto Clinical Neuropathy Scale (mTCNS) (30), the Utah Early Neuropathy Scale (UENS) (31), the Neuropathy Disability Score (NDS) (32), the Neuropathy Symptoms Score (NSS), and the 11-point Numerical Rating Scale (NRS) (17, 28).

1.2.1.3.1 Clinical symptoms of DSPN

Around 50% of patients with DSPN may experience symptoms of DSPN, while others remain asymptomatic (17). Symptoms may affect both motor and sensory nerve functions. Sensory symptoms typically appear first symmetrical in toes and feet, than gradually spreading to more distal parts of the limbs, resembling a stocking- or glove-like pattern of manifestation (27). Both motor and sensory symptoms can be divided into positive or negative categories.

Positive sensory symptoms include sensations without adequate stimuli representing spontaneous or exaggerated actions within the sensory system. Positive sensory neuropathic symptoms, typically present at rest and with a tendency to worsen during the night, include paraesthesiae (prickling, tingling), dysaesthesiae (unpleasant paraesthesiae), pain (spontaneous burning, stabbing, aching, constricting, electric, lancinating pain), or numbness sensations (Figure 1) (27, 33). Typical positive motor symptoms include muscle cramps.

Negative sensory neuropathic symptoms result from decreased function of sensory receptors, fibres, or central systems. Patients report decreased sensation to tactile stimuli including touch, vibration, temperature, noxious stimuli with the risk to develop ataxia, and imbalance (27, 33, 34). Compared to the sensory deficits, motor involvement is usually less prominent and restricted to the distal lower limbs resulting in muscle atrophy and weakness at the toes and foot. The lack of coordination due to neuropathic deficits fosters an increased propensity to stumble (27).

As a side note, there seems to be ambiguity about the definition of numbness as a positive or negative sensory symptom in the present literature as well as in the author's own experience with patients. While some refer to numbness as an active sensation like gone to sleep limbs (asleep-numbness), others use it to describe the perceived loss of sensation (hypaesthesia) (18, 27, 33).

Figure 1: Typical distribution of clinical neuropathic symptoms of DSPN.
Numbness, stabbing pain, burning pain, tingling. Symptoms usually develop symmetrically in both feet following a length-dependent distal to proximal gradient (dying-back pattern).

1.2.1.3.1.1 Neuropathic pain

Up to 50% of patients with DSPN may experience neuropathic pain symptoms. Chronic painful DSPN is encountered in approximately 13-26% of individuals with diabetes (1). Neuropathic pain in diabetes can be defined as '*pain arising as a direct consequence of abnormalities in the somatosensory system in people with diabetes*' (21, 35). It is to be distinguished from both nociceptive pain ('*pain through activation of nociceptors in non-*

neural tissues by actual or threatened tissue injury') and nociplastic pain (*'pain that arises from altered nociception despite no clear evidence of actual or threatened tissue damage causing the activation of peripheral nociceptors or evidence for disease or lesion of the somatosensory system causing the pain*') (36).

In diabetic neuropathy, pain typically occurs spontaneously at rest (worse at night) (17) or less frequently as an evoked exaggerated response to otherwise less painful or non-painful stimuli (hyperalgesia or allodynia) (1, 37). Biomarkers to predict the development of neuropathic pain are still lacking and the conundrum of why some individuals with DSPN develop neuropathic pain while others do not has not been solved (38, 39). Extensive efforts have been undertaken to determine whether there are specific patterns of sensory nerve function loss, hyperalgesia, and allodynia in peripheral neuropathies in general (1, 40). While neuropathic pain usually is associated with sensory loss, the concept of an 'irritable nociceptor' phenotype has been developed, which is characterized by preserved sensation together with hyperalgesia (41). However, evidence to support this concept in painful diabetic neuropathy is limited. In a large cross-sectional study, only a minority of participants with painful diabetic neuropathy had allodynia (41). Only a very small proportion of participants with neuropathic pain could be allocated to the irritable nociceptor phenotype (6.3%), similar to another study in which it was attributed to 14.6% of patients with painful diabetic neuropathy (1, 42).

1.2.1.3.2 Clinical neuropathic signs (deficits) in DSPN

Clinical signs (impairments, deficits) of peripheral neuropathies are assessed using quantitative or semi-quantitative bedside tests. Signs must be distinguished from clinical symptoms that represent subjective patients' reports. Instruments to assess sensory clinical signs include the pinprick test to assess hypoalgesia, tests to assess impaired touch, temperature, vibration sense, and testing joint position sense. Knee and ankle reflexes and muscle strength represent clinical signs of motor nerve function (30, 43).

1.2.1.3.2.1 Quantitative sensory testing

Quantitative sensory testing (QST), is a powerful tool to non-invasively assess sensory nerve function in response to controlled stimuli (e.g. cold, warmth, vibration). Research studies confirmed the usefulness of QST in the assessment and monitoring of sensory function in DSPN and other neuropathies (44). In particular, QST can be used to discriminate between large and small nerve fibre involvement. However, the patient is

required to be alert and cooperative due to the psychophysical nature of the tests. To date, QST has not yet been widely adapted in clinical practice (45).

1.2.1.3.3 Electrophysiological testing

Nerve conduction studies (NCS) are an objective, sensitive, repeatable, and hence well established and useful tool in the diagnosis of DSPN (21, 46) (Figure 2). NCS have been performed for more than half of a century to investigate patients with DSPN (47). NCS can help to confirm or refute a diagnosis of DSPN and to estimate its severity (21, 48). Routine NCS assess only large myelinated somatic nerve fibres in peripheral nerves by activating the entire nerve with an electric stimulus and recording the response which can indicate conditions of normal conduction, axonal injury, or demyelination. The sum of single nerve fibre action potentials responses is represented by the sensory nerve action potential (SNAP) amplitude which is roughly proportional to the number of sensory axons between stimulating and recording electrodes in the corresponding nerve. Hence, SNAP is reduced when axonal loss has occurred. In conditions with axonal injury, nerve conduction velocity (NCV) is primarily reduced, when the fastest axons within the nerve are affected. In demyelinating nerves, NCV is remarkedly reduced due to the impaired saltatory conduction (49). While length-dependent polyneuropathies are typically characterized by axonal loss, reduced NCV in DSPN can be attributed to either axonal loss or demyelination, while axon loss predominates distally (50).

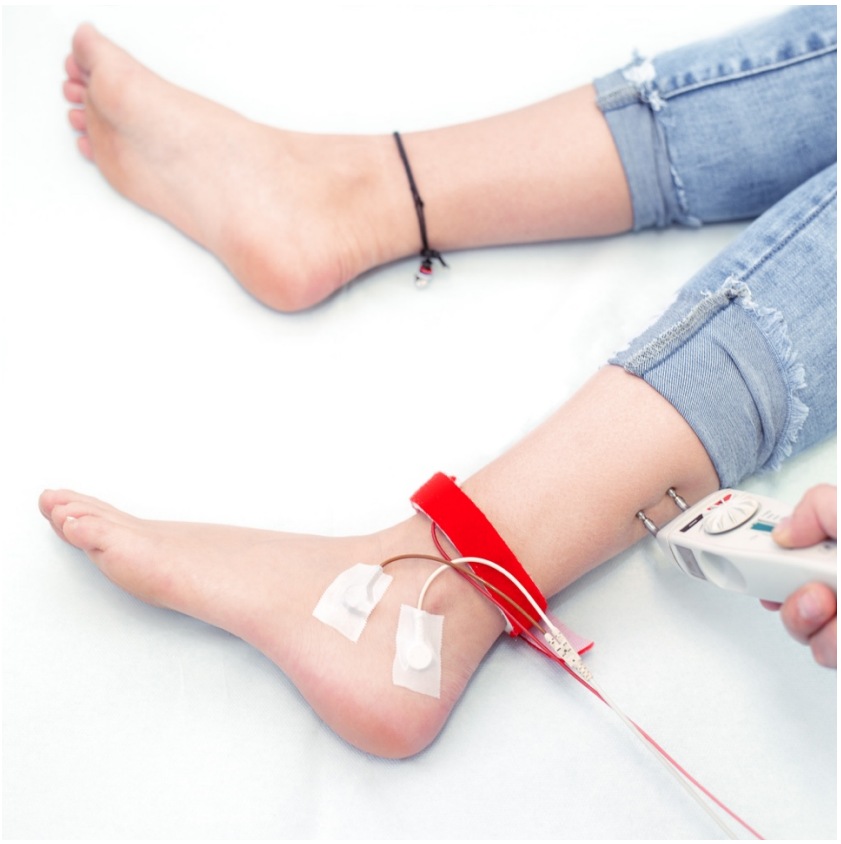

Figure 2: Recording of sural sensory nerve conduction velocity.
Nerve conduction studies are the current gold-standard to assess large nerve fibre dysfunction in peripheral neuropathies and can be used to estimate the severity of DSPN.

1.2.1.4 Clinical complications of DSPN

Numerous studies have consistently reported an impaired quality of life (QoL) in patients with painful DSPN. QoL in these patients is related to reduced physical activity, lack of sleep, and higher stress levels, which can all worsen the perception of pain, leading to a vicious cycle (51). It can be reasonably assumed that painless DSPN, independent from complications, may affect QoL due to unpleasant paraesthesia, hypaesthesia, and impaired balance. However, there is not enough evidence available to corroborate that claim. Nonetheless, neuropathic deficits were found to be positively associated with depressive symptoms (52), and diabetic neuropathy is a major factor in the pathogenesis of diabetic foot syndrome (DFS) (53), both regardless of the presence of neuropathic pain. Diabetic neuropathy is accountable for approximately 85% of cases of DFS. In about 50% of cases it is considered to be the sole main cause in absence of peripheral arterial occlusive disease, the other main pathogenetic factor contributing in 50% of cases, and in 15% without involving diabetic neuropathy (53).

DFS, primarily characterised by the occurrence of foot ulcers, is known to have a profound impact on health-related QoL (54). The lifetime incidence to develop at least one foot ulceration among individuals with diabetes has been estimated at approximately 19-34% according to most recent calculations. If a foot ulcer has occurred at least once in a person with diabetes, the risk of death at 10 years is doubled. Typically, a diabetic foot ulcer occurs, when protective sensation at the foot is impaired due to the sensory component of DSPN. Foot deformities may be present due to the motor component of DSPN, and sweat gland function may be affected by autonomic neuropathy, leading to dry skin. Each factor contributes to increased stress culminating in skin breakdown. Decreased perfusion due to peripheral artery occlusive disease affects wound healing (55). Diabetes remains the most frequent cause of nontraumatic amputations in many countries (56). In people with diabetes, amputations in the lower extremity are 10-20 times more common compared to individuals without diabetes (8). There is ample evidence that DSPN is an independent risk factor associated with amputations (57).

Charcot neuroarthropathy (‘Charcot foot’) holds a special position within the DFS. It is a noninfective arthropathy, typically presenting initially with a warm, swollen foot which may or may not be accompanied by pain or discomfort. It affects bones, joints, and soft tissues in the foot and ankle. Untreated, it leads to severe fractures and deformities with a collapse of the midfoot joint (‘rocker bottom foot’) as a hallmark (58). Today, diabetic neuropathy is by far the leading cause of Charcot neuroarthropathy. While the exact pathomechanisms are not well understood, a combined aetiology of DSPN and diabetic autonomic neuropathy is most likely. Sensorimotor deficits may lead to repeated microtrauma due to loss of protective sensation and increased muscle atrophy resulting in intracapsular effusions, ligamentous laxity, and joint instability. Autonomic dysfunction may result in bone atrophy due to impaired vasoconstriction leading to dysregulated, increased blood flow increasing local bone resorption (56, 59).

Comorbidities are very frequent among patients with DSPN. A large German multicentre study identified the presence of at least one comorbidity apart from diabetes and neuropathy in 90% of patients with diabetic peripheral neuropathy. The most frequent comorbidities were hypertension, hyperlipidaemia, other chronic pain, macrovascular diabetic complications, and depression (60).

1.2.1.5 Pathophysiology of painful and painless DSPN

Similarities and differences between painful and painless DSPN have been described, but the mechanisms contributing to the development of the respective entities are poorly understood (18, 38). Hyperglycaemia and dyslipidaemia initiate pathways implicated in the pathophysiology of neuronal damage (Figure 3). Hyperglycaemia leads to higher intracellular levels of glucose-6-phosphate which is the initial substrate in glycolysis that converts it to pyruvate. Dyslipidaemia results in higher intracellular levels of free fatty acids (FFA) (1). The substrate excess of pyruvate and FFAs in mitochondria increases the formation of reactive oxidative species (ROS) due to an increased electron leakage to oxygen in the mitochondrial electron transport chain and as a by-product of several enzymatic reactions (61). The accumulation of ROS such as superoxide (O_2^-) is accompanied by decreased antioxidant protection in diabetes, leading to increased oxidative stress and cell death (1). In recent-onset type 2 diabetes, a dermal overexpression of mitochondrial superoxide dismutase 2 (SOD2), an important antioxidant enzyme responsible for superoxide detoxification, was observed, suggesting a compensatory antioxidative defence against increased oxidative stress (1, 62). Oxidative stress and mitochondrial dysfunction are considered to play a pivotal role not only in the pathogenesis of diabetic complications, but also in the development of insulin resistance and pancreatic beta cell dysfunction, the characteristic features of type 2 diabetes. They are among the earliest detectable pathophysiological findings in the natural history of type 2 diabetes and its complications (63).

Mitochondrial dysfunction leads to increased levels of glucose-derived reactive carbonyls such as glyoxal or methylglyoxal, formed by autoxidation, which are precursors of advanced glycation end products (AGEs) (64, 65). DNA modifications as a consequence of reactive carbonyls and ROS activate the nuclear enzyme poly(ADP-ribose) polymerase-1 (PARP1), an important downstream effector of DNA damage (1, 66). PARP1 activation triggers altered gene expression and a depletion of oxidized nicotinamide adenine dinucleotide (NAD^+) and ATP levels resulting in reduced cytoplasmatic levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (1, 67).

Ultimately, inhibited GAPDH may reinforce four major pathways of hyperglycaemic damage according to experimental studies using endothelial cells (65). GAPDH is a glycolytic enzyme as it catalyses the reaction of glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate. When this reaction is inhibited by lower GAPDH, the upstream

glycolytic metabolites accumulate and are shunted into the four pathways: polyol pathway, hexosamine pathway, protein kinase C pathway, and AGE pathway (68). At the first step along glycolysis, glucose flux through the polyol pathway increases. Excessive glucose gets converted to sorbitol by aldose reductase with the cofactor NADPH and sorbitol to fructose by sorbitol dehydrogenase under consumption of NAD^+, causing a compensatory depletion of osmolytes like myo-inositol and taurine. The efflux of myo-inositol impairs the physiological function of neural Na^+/K^+-ATPase, leading to acute axonal dysfunction. Furthermore, the increased polyol flux promotes oxidative stress by the depletion of NADPH inhibiting the regeneration of the important antioxidant glutathione as well as by increased production of fructose enhancing the formation of AGEs. (69, 70).

Increased flux through the hexosamine pathway leads to conversion of fructose-6-phosphate, a glycolysis intermediate, to UDP-N-acetylglucosamine which modifies common transcription factors promoting injury of complication prone tissues and inflammation (70).

Increased levels of glyceraldehyde-3-phosphate, the next intermediate among the glycolysis lead to the initiation of the protein kinase C pathway and the AGE pathway. Glyceraldehyde-3-phosphate is metabolized to diacylglycerol which activates neuronal protein kinase C (PKC). Activated PKC alters several metabolic processes leading to increased insulin resistance, impaired function of neuronal Na^+/K^+-ATPase, and altered gene expression of growth factors. In experimental diabetes models, increased activation of PKC resulted in vasoconstriction, hypoxia, and neuronal damage (70). Methylglyoxal, a major intracellular precursor of AGEs, is formed from glyceraldehyde-3-phosphate. Hence, increased glyceraldehyde-3-phosphate contributes to the accumulation of AGEs. Intracellular AGEs modify proteins and thereby alter cytoplasmic and nuclear factors which regulate gene transcription (71). However, the role of intracellular AGEs in peripheral nerves remains poorly understood and more studies have focused on effects resulting from the interaction between extracellular AGEs with the cell surface receptor for AGEs (RAGE). RAGE activation initiates a series of pathways leading to increased expression of extracellular cytokines, formation of ROS, and the activation of the transcription factor nuclear factor-κB (NF-κB) (72). NF-κβ triggers the expression of various cytokines (e.g. TNF-α, TGFβ, IL1β, IL6), chemokines, cell adhesion molecules, growth factors, and pro-apoptotic genes. NF-κβ activation increases vascular calcification and, hence, may impair endothelial function of the vasa nervorum leading to ischaemic

damage (73). In a large prospective study, higher systemic levels of methylglyoxal were associated with the risk of incident peripheral neuropathy in type 2 diabetes patients (1, 74).

However, it is a matter of debated whether oxidative stress is in fact an upstream or downstream effect of the aforementioned four major pathways implicated in the pathogenesis of diabetic neuropathy. Experimental data supporting the upstream theory were not derived from neuronal or Schwann cells but rather from endothelial cells. Nonetheless, the latter are located in the vasa nervorum representing a target tissue for microvascular damage (65).

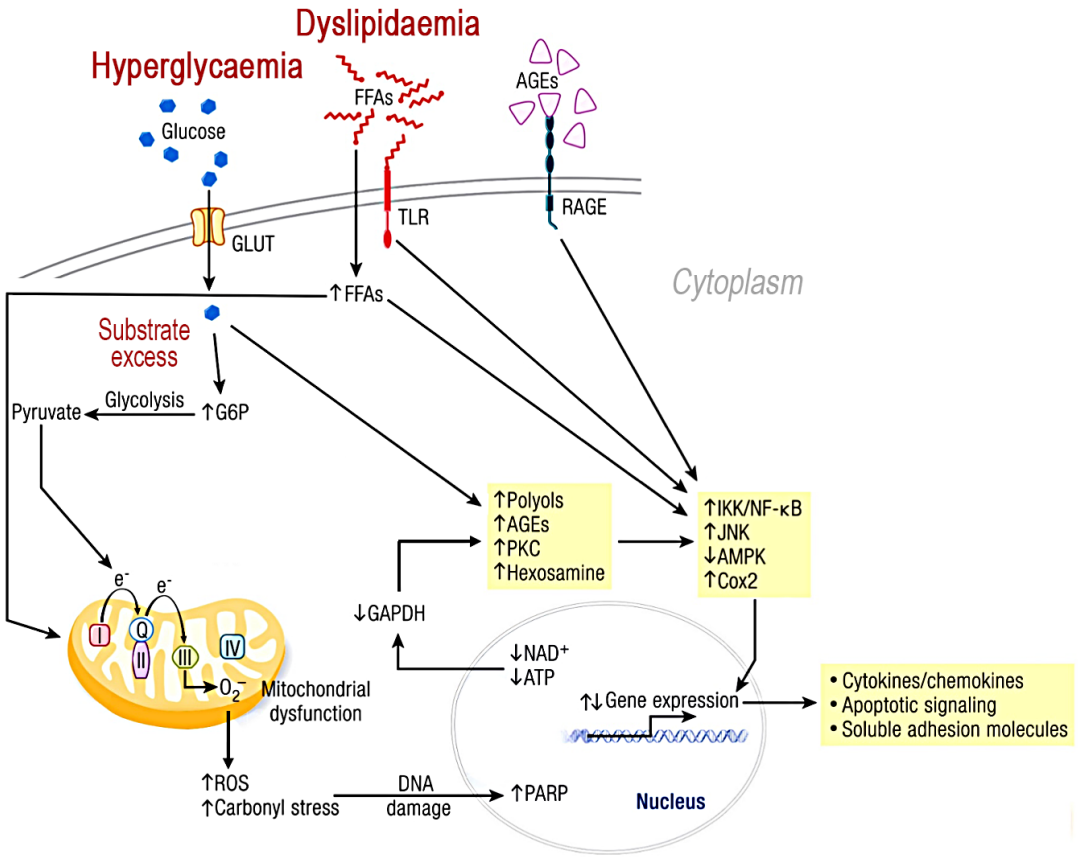

Figure 3: Cellular pathways implicated in the pathophysiology of DSPN.
AGEs, advanced glycation end-products; AMPK, 59 adenosine monophosphate–activated protein kinase; FFAs, free fatty acids; G6P, glucose-6-phosphate; GLUT, glucose transporter; IKK, IkB kinase; JNK, c-Jun N-terminal kinase; NAD^+, oxidized nicotinamide adenine dinucleotide; RAGE, receptor of AGE; TLR, toll-like receptor.
Modified from Bönhof et al. Endocr Rev 2019; 40: 153-92, used with permission.

Peripheral nerves are vascularized by endoneurial, perineurial, and epineurial vessels. In diabetes, proliferation of endothelial cells (ECs), basement membrane thickening, and altered secretion of vasoactive, proinflammatory, and prothrombotic substances may

contribute to impaired nerve function. Furthermore, a dysregulation of the capillary flow may alter the extraction of diffusible molecules from the blood stream into neurons and Schwann cells and hence may contribute to nerve ischemia. Sensory neurons in the dorsal root ganglia (DRGs) are characterised by a higher independently regulated blood flow with lower oxygen tension compared with peripheral nerve trunks and might be particularly susceptible to microvascular alterations (1, 75).

Many of the risk factors associated with DSPN are triggers of subclinical inflammation and there is increasing evidence that subclinical inflammation might play a role in the pathogenesis of DSPN. Higher systemic levels of IL1β, IL6, and TNF-α have been linked with the presence of neuropathic pain and reduced NCV in animal models (1). In a recent prospective population-based study, systemic levels of six out of 71 biomarkers reflecting multiple aspects of immune activation were associated with incident peripheral neuropathy (76). Using the same method in a cross-sectional study, deficits in systemic cytokines, chemokines, and growth factors promoting nerve regeneration in patients with type 2 diabetes were associated with polyneuropathy in general but not specifically with the painful or painless entity. Thus, biomarkers of systemic neuroinflammation and nerve regeneration do not appear useful to differentiate between the two DSPN entities (77).

Sensory nerve fibres innervating the skin arise from the DRGs. They are bundled into peripheral nerves and ultimately reach into the different layers of the skin. There are unmyelinated and myelinated sensory nerve fibres. Unmyelinated group C nerve fibres (C fibres) measuring approximately 1 µm or less in diameter are afferent fibres slowly transmitting tactile (warmth, sensual touch) and nociceptive (pain, itch) sensations (78). C fibre axons are grouped together into so called Remak bundles. These occur when a non-myelinating Schwann cell bundles the axons close together by surrounding them. Myelinated fibres are wrapped individually by a myelinating Schwann cell forming myelin shafts separated only by unmyelinated nodes of Ranvier for fast saltatory nerve conduction (1). Thick myelinated Aβ fibres have a diameter of approximately 10 µm and rapidly transmit stimuli by mechanoreceptors localized in various layers of the skin. Thinly myelinated Aδ fibres respond to mechanical and chemical stimuli and mediate acute first pain provoked by noxious triggers such as intense pressure, heat, or chemical substances (78). Small fibre neuropathies (SFNs) are characterized by an impairment of the thinner Aδ and C fibres, whereas large-fibre neuropathies refer to predominant involvement of thicker nerve fibres such as Aβ fibres or motor nerve fibres (79). DSPN is mostly characterized by mixed small- and large-fibre damage (17).

In the development of diabetic neuropathies, multiple degenerative, remodelling, and regenerative processes in axons, glia cells, and the axon-surrounding microenvironment culminate in impaired nerve function (1). Axonal atrophy, demyelination, nerve fibre loss, and blunted nerve fibre regeneration coexist in DSPN (80). Peripheral nerve damage is followed by a series of morphological and molecular changes in the perikarya of injured nerves into a regenerative state, priming neurons for regrowth (81). Further understanding of neural plasticity, regenerative capacity, and nerve fibre degeneration is required to identify novel therapeutic and preventative approaches specific for painful and painless DSPN. Whether the small or large fibre components are affected earlier in the cause of DSPN is subject of ongoing debate. While some studies suggested that small fibre damage may precede large fibre impairment (82) and that small fibre damage may be considered a prerequisite for the presence of neuropathic pain in DSPN, there is no definitive evidence of an exclusive or predominant involvement of small fibres in either painful or painless DSPN, and biomarkers to predict the development of neuropathic pain are still lacking (38). Early changes in DSPN include signs of both degenerative and compensatory regenerative processes in unmyelinated nerve fibres resulting in reduced axon diameters. In myelinated nerve fibres, segmental demyelination and remyelination as well as abnormalities around the nodes of Ranvier can precede axonal defects, suggesting an early occurrence of Schwann cell impairment (1).

Following axonal injury, the axon stump distal to the injury site undergoes Wallerian degeneration to allow for regrowth. This process involves the activation of Schwann cells and macrophages and was shown to be delayed in experimental diabetes. The transected axon proximal to the injury sprouts growth cones aiming to reach the distal stump. This elongation process is dependent on supporting factors such as growth factors and neuronal proteins associated with regeneration such as α-tubulin and growth-associated-protein-43 (GAP-43) (1, 80).

1.3 Skin biopsy: A window into the peripheral nervous system

Skin biopsy is a reliable, minimally invasive tool for the assessment of epidermal and dermal nerve fibres in peripheral nerve disorders such as SFNs or DSPN (83). It is usually performed under local anaesthesia using a sterile 3 mm biopsy punch (Figure 4). The risk of complications such as bleeding or local infection is very low and healing is usually complete within 1 week. The collected specimen can be analysed using specific

antibodies to stain different structures present in the epidermal and dermal layers of the skin. The epidermis is composed of four layers of keratinocytes that differentiate while progressing from the stratum basale to the outermost stratum corneum. The upper dermal layer is organized as papillae that include blood vessels, hair follicles, erector pili muscles, sebaceous glands, and sweat glands. Bundles of unmyelinated as well as myelinated somatic and autonomic nerve fibres can be found in the dermis. Nerve fibres which cross the dermal-epidermal junction lose their Schwann cell ensheathment and are mostly nociceptive (83, 84) .

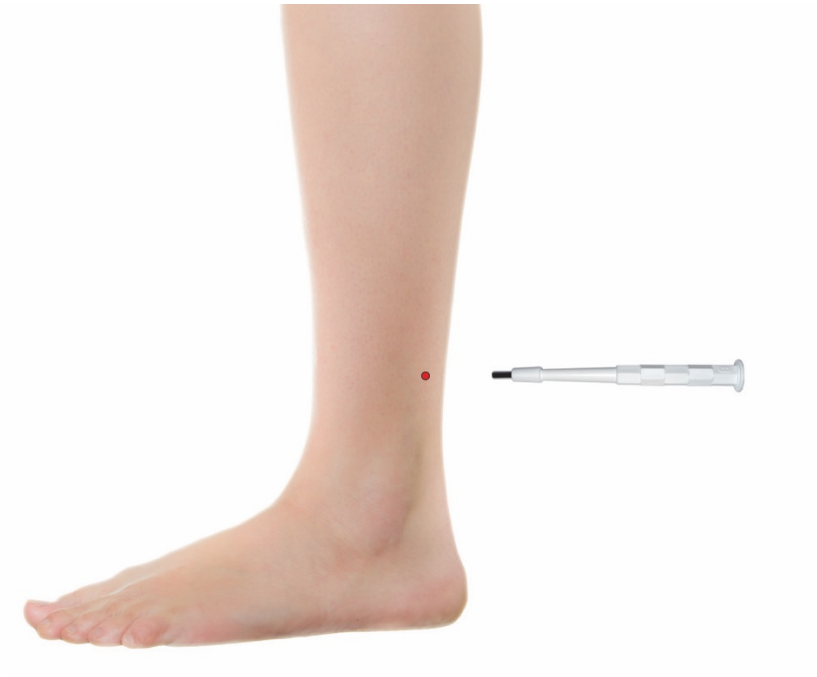

Figure 4: Schematic image for the standardised biopsy site at the distal-lateral calf and biopsy punch tool.
Worldwide normative reference values are available for intraepidermal nerve fibre density (IENFD) at this site using protein gene product 9.5 (PGP9.5) immunohistochemistry. IENFD represents the gold standard measurement to diagnose small fibre damage in peripheral neuropathies.

1.3.1 PGP9.5: pan-axonal marker

The assessment of intraepidermal nerve fibres using the pan-axonal marker protein gene product 9.5 (PGP9.5, a member of the ubiquitin hydroxylase system, is the current gold standard for the diagnosis of small fibre damage in peripheral neuropathies including DSPN (1, 83). Using this technique, studies have shown that PGP9.5-positive intraepidermal nerve fibres are already reduced at an early stage of type 2 diabetes (85) and are also diminished in small fibre neuropathies (SFNs) of different origins, including

diabetes (83, 86). PGP9.5 can be used for both bright-field and immunofluorescence techniques which show high levels of agreement and comparable results. Manual counting of fibres crossing the dermal-epidermal junction, manual morphometric analyses, or semiautomatic assessment can be used to evaluate cutaneous nerve fibre counts, lengths, and densities (1). Worldwide normative reference is available for intraepidermal nerve fibre density (IENFD) at the distal-lateral calf approximately 10 cm above the lateral malleolus using immunohistochemistry staining for bright-field microscopy of 50 µm thick sections. IENFD gradually declines with increasing age and normative values were found to be higher in healthy women compared to men (87).

1.3.2 GAP-43: marker of regenerative axons

GAP-43 is a membrane protein that is involved in the process of peripheral nerve regeneration (88, 89). It is a major constituent of the axonal growth cones after nerve injury, where it is localised in the membrane skeleton (80). GAP-43 is expressed in peripheral nerve fibre areas with high neural plasticity such as the epidermis and dermis. Hence, it can be used as a marker to stain regenerating nerve fibres (90, 91). Using double-staining for PGP9.5 and GAP-43 in combination with immunofluorescence microscopy and morphometric assessment, densities and lengths of nerve fibres positive for GAP-43 can be directly compared with those positive for the established pan-axonal marker PGP9.5 in the epidermal and dermal layers, to gain insights into neural plasticity of cutaneous nerve fibres. The ratio between GAP-43- and PGP9.5-positive IENFD has been suggested to reflect the degree of axonal regeneration (92, 93).

1.4 Approval of the Ethics Committee

- Probing the Role of Sodium Channels in Painful Neuropathies (ROPANE) study:
  - Ethics committee of Heinrich Heine University; approval No. 4369R (13.08.2013)
- German Diabetes Study (GDS):
  - Ethics committee of Heinrich Heine University; approval No. 4508 (16.12.2013)

1.5 Aims of the study

Several studies reported that nerve regeneration may be altered in diabetes with and without DSPN (92-95). However, the varying results due to small population sizes, heterogeneous study populations, and methodological differences do not give a conclusive answer as to whether enhanced nerve regeneration may contribute to the phenotype of painful as opposed to painless DSPN.

In the present study, we aimed to determine the degree of cutaneous nerve fibre loss and regeneration in type 2 diabetes patients with painful and painless DSPN as well as in recent-onset type 2 diabetes patients with a known diabetes duration not longer than one year compared to corresponding control participants with normal glucose tolerance (NGT).

We hypothesised that the patterns of cutaneous nerve fibre loss and repair may differ between painful and painless DSPN in type 2 diabetes, contributing to different phenotypes as well as between individuals with recent-onset type 2 diabetes and NGT reflecting early changes due to diabetes. Furthermore, we hypothesised that the degree of nerve fibre loss and regenerative capacity may be associated with clinical measures of DSPN and neuropathic pain.

2 Original publication: Patterns of cutaneous nerve fibre loss and regeneration in type 2 diabetes with painful and painless polyneuropathy, Bönhof, G.J., Strom, A., Püttgen, S., Ringel, B., Brüggemann, J., Bódis, K., Müssig, K., Szendroedi, J., Roden, M., Ziegler, D., *Diabetologia*, 60: 2495-2503, (2017)

Diabetologia (2017) 60:2495–2503
DOI 10.1007/s00125-017-4438-5

ARTICLE

Patterns of cutaneous nerve fibre loss and regeneration in type 2 diabetes with painful and painless polyneuropathy

Gidon J. Bönhof[1] · Alexander Strom[1,2] · Sonja Püttgen[1] · Bernd Ringel[1] · Jutta Brüggemann[1] · Kálmán Bódis[1] · Karsten Müssig[1,2,3] · Julia Szendroedi[1,2,3] · Michael Roden[1,2,3] · Dan Ziegler[1,2,3]

Received: 13 June 2017 / Accepted: 2 August 2017 / Published online: 15 September 2017

Abstract

Aims/hypothesis The determinants and mechanisms of the development of diabetic sensorimotor polyneuropathy as a painful (DSPN+p) or painless (DSPN-p) entity remain unclear. We examined the degree of cutaneous nerve fibre loss and regeneration in individuals with type 2 diabetes with DSPN+p or DSPN-p compared with individuals with recent-onset type 2 diabetes and corresponding healthy volunteers.

Methods In this cross-sectional study, skin biopsies taken from the distal lateral calf were obtained from individuals with recent-onset type 2 diabetes (n = 32) from the German Diabetes Study, with DSPN+p (n = 34) and DSPN-p (n = 32) from the PROPANE study, and volunteers with normal glucose tolerance (n = 50). Double immunofluorescence staining for protein gene product 9.5 (PGP9.5) (pan-neuronal marker) and growth-associated protein 43 (GAP-43) (nerve regeneration marker) was applied to assess intraepidermal nerve fibre density (IENFD) and length (IENFL) and dermal nerve fibre length (DNFL). DSPN was diagnosed using the modified Toronto Consensus (2011) criteria, while neuropathic pain was assessed using an 11-point Numerical Rating Scale.

Results After adjustment for age, sex, BMI and HbA_{1c}, IENFD and IENFL were reduced for both markers in individuals with recent-onset diabetes and both DSPN groups compared with control participants (all $p < 0.05$), but did not differ between the DSPN groups. The DNFL GAP-43/PGP9.5 ratio was higher in the DSPN+p and DSPN-p groups compared with control participants (1.18 ± 0.28 and 1.07 ± 0.10 vs 1.02 ± 0.10; $p \leq 0.05$) and in the DSPN + p group compared with DSPN-p ($p < 0.05$). Correlation analyses showed distinct inverse associations between the DNFL GAP-43/PGP9.5 ratio and PGP9.5 positive IENFD as well as DNFL (IENFD: $\beta = -0.569$, DNFL: $\beta = -0.639$; both $p < 0.0001$) in individuals with type 2 diabetes, but not in the control group. A similar pattern was found for correlations between the DNFL GAP-43/PGP9.5 ratio and peripheral nerve function tests.

Conclusions/interpretation Dermal nerve fibre regeneration is enhanced in DSPN, particularly in DSPN+p, and increases with advancing intraepidermal nerve fibre loss. These data suggest that, despite progressive epidermal fibre loss, dermal nerve repair is preserved, particularly in DSPN+p, but fails to adequately counteract epidermal neurodegenerative processes.

Keywords Nerve regeneration · Neuropathic pain · Neuropathy · Skin biopsy · Type 2 diabetes

✉ Dan Ziegler
dan.ziegler@ddz.uni-duesseldorf.de

[1] Institute for Clinical Diabetology, German Diabetes Center (DDZ), Leibniz Center for Diabetes Research at Heinrich Heine University, Auf'm Hennekamp 65, 40225 Düsseldorf, Germany

[2] German Center for Diabetes Research (DZD), Munich, Neuherberg, Germany

[3] Division of Endocrinology and Diabetology, Medical Faculty, Heinrich Heine University, Düsseldorf, Germany

Abbreviations

DNFL	Dermal nerve fibre length
DSPN	Diabetic sensorimotor polyneuropathy
DSPN+p	Painful diabetic sensorimotor polyneuropathy
DSPN-p	Painless diabetic sensorimotor polyneuropathy
GAP-43	Growth-associated protein-43
GDS	German Diabetes Study
IENFD	Intraepidermal nerve fibre density
IENFL	Intraepidermal nerve fibre length

NCV	Nerve conduction velocity
NDS	Neuropathy Disability Score
NRS	Numerical Rating Scale
NSS	Neuropathy Symptom Score
PGP9.5	Protein gene product 9.5
PROPANE	Probing the Role of Sodium Channels in Painful Neuropathies
SFN	Small fibre neuropathy
SNAP	Sensory nerve action potential
TDT	Thermal detection threshold
VPT	Vibration perception threshold

Introduction

Diabetic sensorimotor polyneuropathy (DSPN) affects approximately 30% of people with diabetes and its sequelae, such as neuropathic pain and foot ulcers, contribute to substantial morbidity, high socioeconomic burden, reduced quality of life and increased mortality rates [1, 2]. Since the mechanisms contributing to the development of DSPN as a painful (DSPN+p) and painless (DSPN-p) entity are not well understood, the current options to prevent and treat either are limited [3, 4].

In diabetic neuropathies, axonal atrophy, demyelination, loss of nerve fibres and blunted regeneration of nerve fibres are present, and both nerve regeneration as well as degeneration contribute to nerve pathology [4]. Nerve damage is followed by a series of morphological and molecular changes in the perikarya of injured nerves into a regenerative state, priming neurons for regrowth [5]. Therefore, further understanding of neural plasticity, regenerative capacity and nerve fibre degeneration is required to identify novel therapeutic and preventive approaches specific for the two types of DSPN.

Although small nerve fibre damage is considered a prerequisite for the presence of neuropathic pain in diabetes, there is no definite evidence of an exclusive or prominent involvement of small fibres in DSPN+p, and biomarkers to predict the development of neuropathic pain are still lacking [6]. Similarities and differences between DSPN+p and DSPN-p have been described, but the conundrum of why some individuals with DSPN develop neuropathic pain while others do not has not been solved [6, 7].

Skin biopsy is a reliable, minimally invasive tool for the assessment of epidermal and dermal nerve fibres in peripheral nerve disorders [8]. The pan-neuronal marker protein gene product 9.5 (PGP9.5), a member of the ubiquitin hydroxylase system, is widely used to detect and quantify cutaneous nerve fibres [9]. Using this technique, studies have shown that small-calibre nerve fibres penetrating the epidermis, which are mostly nociceptive [8], are already reduced in the early stages of type 2 diabetes [10] and are diminished in small fibre neuropathies (SFNs) of different origins, including diabetes [8, 11].

Growth-associated protein 43 (GAP-43) is a membrane protein that is involved in the process of nerve regeneration [12, 13]. As it is a major constituent in axonal growth cones after nerve injury [4], GAP-43 is expressed in peripheral nerve fibre areas with high neural plasticity such as the epidermis and dermis. Hence, it can be used as a marker for regenerating nerve fibres [14, 15].

Several studies have reported that nerve regeneration may be altered in diabetes mellitus with and without DSPN, but the varying results due to small population sizes, heterogeneous study populations and methodological differences do not give a conclusive answer about regenerative capacity and the variable by which it is reflected best [16–19]. Here we determined the degree of cutaneous nerve fibre loss and regeneration in individuals with type 2 diabetes with DSPN+p and DSPN-p, compared with individuals with recent-onset diabetes and corresponding control participants. We hypothesised that the patterns of cutaneous nerve fibre loss and repair may differ between the two types of DSPN, as well as between individuals with recent-onset diabetes and normal glucose tolerance.

Methods

Participant selection This cross-sectional study was conducted in accordance with the Declaration of Helsinki and was approved by the ethics committee of Heinrich Heine University, Düsseldorf, Germany. After obtaining written informed consent, 98 participants with type 2 diabetes, defined according to the ADA criteria [20], were allocated to three groups: recent-onset type 2 diabetes (n = 32) DPSN-p (n = 32), and DSPN+p (n = 34). The presence of pain in the distal lower extremities lasting ≥1 year with a pain intensity ≥4 (24 h average or maximum) on the 11-point Numerical Rating Scale (NRS) in the absence of analgesic treatment, or according to the medical history (recall and/or records) prior to analgesic treatment, was used to define DSPN+p. Individuals with DSPN-p reported a pain intensity on the 24 h average NRS of 0, except for two who had an NRS of 1 without analgesic treatment. Pain not associated with DSPN due to conditions such as low back pain or osteoarthritis was not considered in the pain assessment [21, 22]. There was a difference in age between the recent-onset group and the DSPN groups; therefore, two glucose-tolerant control groups of similar age and sex were formed from 50 individuals with normal glucose tolerance. The Control 1 group which included all 50 individuals was used for comparison with the recent-onset group. The Control 2 group included the 25 oldest individuals from the Control 1 group for comparison with the DSPN groups. Individuals with recent-onset type 2 diabetes and

control participants were recruited from the German Diabetes Study (GDS), which is a prospective longitudinal cohort study investigating the impact of subphenotypes on the course of the disease (ClinicalTrials.gov Identifier: NCT01055093). The study design and cohort profile of the GDS have been published elsewhere [23]. Individuals with type 2 diabetes with DSPN were recruited from the Probing the Role of Sodium Channels in Painful Neuropathies (PROPANE) study, an observational study investigating the factors that contribute to the development of neuropathic pain (ClinicalTrials.gov Identifier: NCT02243475) [24]. Inclusion criteria for entry into the PROPANE study for the present cohort were age ≥18 years, type 1 or type 2 diabetes [20], and a diagnosis of sensory, sensorimotor and/or small fibre DSPN as possible, probable or confirmed according to the Toronto Consensus criteria [21]. Exclusion criteria were other causes of neuropathy (hypothyroidism, renal failure, vitamin B_{12} deficiency, monoclonal gammopathy, alcohol abuse [>5 U/day], malignancies, drugs known to cause neuropathy) and concomitant diseases that might interfere with the participant's ability to fill in questionnaires. No biopsies were taken from individuals on anticoagulant therapy or with peripheral artery disease, chronic infections of the lower extremities or presenting other contraindications. All assessments in individuals from the PROPANE study were performed specifically for the present study, while data from some individuals from the GDS related to neurological examination and peripheral nerve function had been published previously [10].

Peripheral nerve function Electrophysiological testing, quantitative sensory testing and neuropathy score surveys were performed as previously described [10]. Motor nerve conduction velocity (NCV) was measured in the peroneal nerve, while sensory NCV and sensory nerve action potentials (SNAPs) were determined in the sural nerve at a skin temperature of 33–34°C using surface electrodes (Nicolet VikingQuest, Natus Medical, San Carlos, CA, USA). Vibration perception threshold (VPT) was measured at the medial malleolus using the method of limits (Vibrameter, Somedic, Stockholm, Sweden). Thermal detection thresholds (TDTs) to warm and cold stimuli were determined on the dorsum of the foot using the method of limits (TSA-II NeuroSensory Analyzer, Medoc, Ramat Yishai, Israel). Neurological examination was performed using the Neuropathy Disability Score (NDS) [25]. Neuropathic symptoms were assessed by the Neuropathy Symptom Score (NSS) [25] and the NRS for neuropathic pain average over 24 h and maximum scores.

Skin biopsy Skin punch biopsy specimens (3 mm) were taken under local anaesthesia (Meaverin-Actavis 1%, 1–1.8 ml, Actavis, Langenfeld, Germany) from the left distal lateral calf, about 10 cm proximal to the lateral malleolus. Details of sampling and processing of the biopsy have been published previously [26, 27]. In brief, after fixation with 2% periodate-lysine-paraformaldehyde at 4°C for 24 h tissue was rinsed twice for 10 min with 0.1 mol/l Sorensen buffer. Following incubation in 33% sucrose for 3 h the specimens were incubated with 0.02 mol/l Sorensen buffer containing 20% glycerol at 4°C overnight, embedded and stored at −80°C.

Immunofluorescence staining The double immunofluorescence staining for PGP9.5 and GAP-43 was performed using three 50 μm sections for each individual using the free-floating method. After blocking with 5% BSA and 5% normal goat serum for 30 min, sections were incubated with the rabbit polyclonal anti-PGP9.5 (AB1761, 1:3000, Merck Millipore, Billerica, MA, USA) and the mouse anti-GAP-43 (MAB347, 1:1000, Merck Millipore) antibodies overnight. Thereafter, sections were incubated with Alexa488 conjugated goat anti-rabbit IgG (A-11034, 1:500, Thermo Fisher Scientific, Waltham, MA, USA) and Alexa555 conjugated goat anti-mouse IgG (A-21424, 1:500, Thermo Fisher Scientific) secondary antibodies for 1 h. Nuclei were stained with Hoechst 33,342 (1 mg/ml) for 5 min. Image stacks were acquired using a Nikon Ti-E inverted microscope (Nikon, Minato, Japan) equipped with a DS-Qi2 digital monochrome camera, corresponding filters and a x20/0.75 CFI P-Apo DM objective. Each antibody was validated separately prior to use in double immunofluorescence.

Image analysis After focus stacking and general brightness and contrast adjustment, images were analysed using the NIS-Elements AR v4.50.00 (Nikon) or ImageJ 1.49 (National Institutes of Health, Bethesda, MD, USA) software. For each individual and marker, three sections were analysed. Intraepidermal nerve fibre density (IENFD) was determined as described previously [18]. To determine intraepidermal nerve fibre length (IENFL) and dermal nerve fibre length (DNFL), the epidermal and dermal areas of interest were defined. The epidermal area of interest was defined using nucleus staining. The dermal area of interest was limited by the epidermal–dermal junction and a line 200 μm below the dermal–epidermal junction using the 25% method [28]. Then, the lengths of the fibres within these areas were measured and the total fibre length (μm) was divided by the total corresponding area (mm^2). Thicker nerve fibre bundles were considered as fibres and measured likewise. IENFD, IENFL and DNFL for each individual were expressed as the means of three analysed sections. GAP-43/PGP9.5 ratios for each of these three measures were obtained by calculating the proportion of GAP-43 to PGP9.5 positive fibres. Representative images of a double-immunofluorescent stained section with indication of intraepidermal and dermal nerve fibres in an individual with normal glucose tolerance are shown in Fig. 1. Microscopy and image analysis were performed with anonymised samples and the examiners were blinded to group allocation.

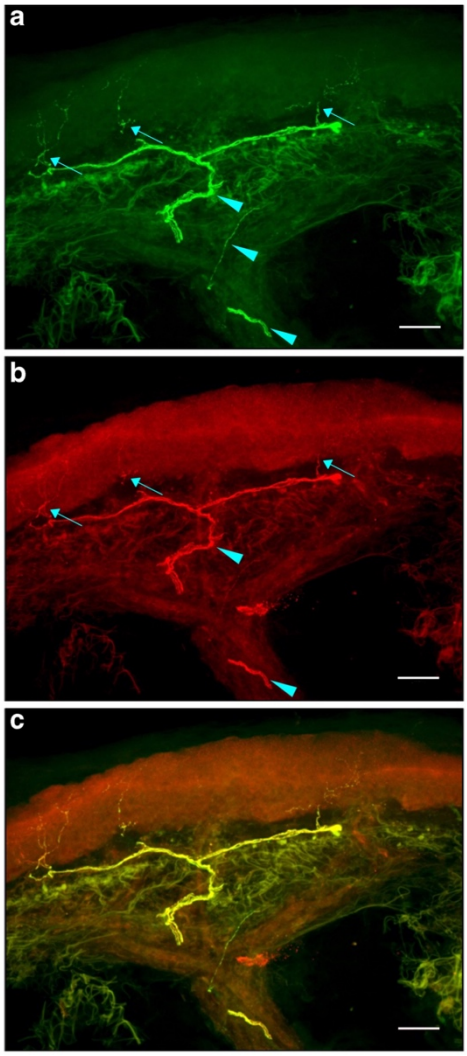

Fig. 1 Double immunofluorescence with PGP9.5 and GAP-43. Detection of (**a**) PGP9.5 (green), (**b**) GAP-43 (red) and (**c**) positive nerve fibres in skin biopsies (yellow; PGP9.5 and GAP-43 merged). Arrows, intraepidermal nerve fibre; arrow heads, dermal nerve fibre. Scale bars, 50 μm

Statistical analysis Categorical data are expressed as percentages of participants. Continuous data are expressed as mean ± SD. Categorical variables were compared using the χ^2 test. For normally distributed data, parametric tests were used (Student's t test or Pearson correlation), otherwise non-parametric tests (Mann–Whitney U test or Spearman rank correlation) were applied. All group comparisons were adjusted for sex, age and BMI, except for the skin biopsy measures which were additionally adjusted for HbA_{1c}. To determine possible associations between two variables, multiple linear regression analyses with adjustments for sex, age and BMI were performed. Variables that were not normally distributed were $\log_e$-transformed before being entered into the regression models. The level of significance was set at $\alpha = 0.05$. All analyses were performed with SPSS Statistics for Windows, Version 22.0 (IBM, Armonk, NY, USA). All graphs were generated using GraphPad Prism, Version 6.04 (GraphPad Software, La Jolla, CA, USA).

Results

The demographic, neurophysiological and clinical characteristics of the groups studied are shown in Table 1. All three diabetes groups showed a higher BMI compared with the corresponding control participants. The DSPN-p group were older compared with the Control 2 group, while those with DSPN+p were younger than those with DSPN-p. Both DSPN groups had higher total cholesterol and HbA_{1c} levels compared with the Control 2 group (all $p < 0.05$). Among the individuals with recent-onset type 2 diabetes, 21.9% had DSPN-p, while none had DSPN+p. In the DSPN+p/DSPN-p groups, 47.1%/6.3% were using alpha-2-delta ligands (gabapentin or pregabalin), 8.8%/0% were using serotonin noradrenaline (norepinephrine) reuptake inhibitors (duloxetine or venlafaxine), 8.8%/3.1% were using opiates and 8.8%/0% were using other analgesics for neuropathic pain.

After adjustment for age, sex and BMI, peroneal motor, sural sensory NCV were reduced in both DSPN groups compared with the Control 2 group. Sural SNAPs were reduced in the recent-onset and DSPN groups compared with the corresponding control participants. The VPTs were reduced in the DSPN groups compared with the Control 2 group. Warm TDTs were reduced in the DSPN+p and cold TDTs were reduced in both DSPN compared with Control 2 groups. NSS and NDS were higher in the DSPN groups compared with the Control 2 group. As expected, NSS was higher in the DSPN+p than in the DSPN-p groups, and the average and maximum pain scores were higher in individuals with DSPN+p compared with DSPN-p and the Control 2 group (all $p < 0.05$).

Table 2 shows the cutaneous nerve fibre density and length in the five groups studied. After adjustment for age, sex, BMI and HbA_{1c}, both DSPN groups showed reduced IENFD and IENFL compared with the Control 2 group for both markers ($p < 0.05$), while no differences were found between the DSPN+p and DSPN-p groups. IENFD and IENFL were also reduced in the recent-onset group compared with the Control 1 group ($p < 0.05$). Differences in DNFL were not found between the recent-onset and Control 1 groups or between the DSPN-p and the Control 2 groups or between the DSPN groups. No between-group differences in the GAP-43/PGP9.5 ratio for IENFD or IENFL were noted. The DNFL GAP-43/

Table 1 Demographic, neurophysiological and clinical characteristics of the study participants

	Control 1	Recent-onset type 2 diabetes	Control 2	DSPN-p	DSPN+p
n	50	32	25	32	34
Sex (% male)	84.0	75.0	92.0	90.6	76.5
Age (years)	53.0 ± 16.0	51.0 ± 10.1	64.9 ± 4.9	71.3 ± 8.1†	67 ± 8.6‡
Height (cm)	178 ± 8	177 ± 7	179 ± 9	177 ± 8	175 ± 7
BMI (kg/m^2)	27.4 ± 5.8	32.5 ± 5.5*	26.3 ± 3.0	28.9 ± 4.1†	31.0 ± 4.4†
Diabetes duration (years)	–	0.2 ± 0.4	–	15.2 ± 10.7	15.7 ± 9.9
Current smoker (%)	16.0	16.1	12.0	6.3	20.6
Heart rate (bpm)	66 ± 9	72 ± 11	67 ± 10	70 ± 11	71 ± 10
Systolic BP (mmHg)	127 ± 17	132 ± 15	134 ± 17	136 ± 18	138 ± 16
Diastolic BP (mmHg)	72 ± 10	74 ± 11	76 ± 8	69 ± 9	72 ± 9
Total cholesterol (mmol/l)	5.25 ± 0.85	5.43 ± 1.06	5.95 ± 0.44	4.91 ± 1.27	4.94 ± 1.01
HDL-cholesterol (mmol/l)	1.50 ± 0.51	1.31 ± 0.36	1.41 ± 0.28	1.35 ± 0.38	1.36 ± 0.39
LDL-cholesterol (mmol/l)	3.34 ± 0.85	3.52 ± 0.93	3.90 ± 0.41	3.00 ± 1.14	3.08 ± 0.88
C-reactive protein (nmol/l)[a]	14.3 ± 13.3	39.0 ± 28.6*	–	22.4 ± 24.5	32.4 ± 30.5
HbA_{1c} (%)	5.24 ± 0.30	6.42 ± 1.00*	5.36 ± 0.24	7.31 ± 0.88†	7.40 ± 1.32†
HbA_{1c} (mmol/mol)	33.78 ± 3.29	46.65 ± 10.96*	35.05 ± 2.67	56.39 ± 9.57†	57.34 ± 14.40†
Peroneal motor NCV (m/s)[a]	45.4 ± 4.5	44.0 ± 4.8	44.3 ± 3.4	38.2 ± 6.42†	38.3 ± 6.42†
Sural sensory NCV (m/s)[a]	45.0 ± 4.6	44.9 ± 5.8	44.2 ± 4.0	33.6 ± 7.22†	32.5 ± 8.12†
Sural SNAP (μV)[a]	11.0 ± 5.8	7.2 ± 3.81	10.3 ± 6.3	4.6 ± 3.82†	3.1 ± 3.42†
Malleolar VPT (μm)[a]	1.7 ± 1.8	2.11 ± 2.74	2.1 ± 2.1	11.2 ± 8.32†	10.1 ± 8.42†
Foot warm TDT (°C)[a]	39.1 ± 3.8	40.5 ± 4.0	40.0 ± 3.9	44.3 ± 5.5	45.5 ± 5.32†
Foot cold TDT (°C)[a]	27.8 ± 4.1	26.3 ± 6.6	27.1 ± 3.7	19.0 ± 10.32†	15.9 ± 12.52†
NSS[a]	0.12 ± 0.85	0.78 ± 2.18	0.24 ± 1.20	5.84 ± 2.08†	7.11 ± 1.41†,‡
NDS[a]	0.78 ± 1.05	1.41 ± 1.64	1.27 ± 1.10	5.65 ± 2.88†	6.83 ± 2.64†
Average NRS pain score (points)[a]	0	0	0	0.06 ± 0.25	3.32 ± 2.61†,‡ (4.34 ± 3.02[b])
Maximum NRS pain score (points)[a]	0	0	0	0.27 ± 0.89	5.43 ± 2.67†,‡ (6.44 ± 2.80[b])

*$p < 0.05$ vs Control 1, †$p < 0.05$ vs Control 2, ‡$p < 0.05$ vs DSPN-p

[a] Adjusted for sex, age and BMI

[b] From medical history prior to analgesic treatment

PGP9.5 ratio was higher in both DSPN groups compared with the Control 2 group ($p < 0.05$). Moreover, the DNFL GAP-43/PGP9.5 ratio was higher in the DSPN+p compared with the DSPN-p group ($p < 0.05$). The number of participants showing a DNFL GAP-43/PGP9.5 ratio >95th percentile of the control individuals was 1/25 (4.0%) in the Control 2 group and 9/34 (26.5%) in the DSPN+p group ($p < 0.05$).

Inverse correlations between PGP9.5 positive IENFD and DNFL GAP-43/PGP9.5 ratio ($\beta = -0.569$, $p < 0.0001$), as well as PGP9.5 positive DNFL and DNFL GAP-43/PGP9.5 ratio ($\beta = -0.639$, $p < 0.0001$), were found for all individuals with type 2 diabetes. In contrast, no such correlations were observed in the control individuals ($\beta = 0.052$, $p = 0.697$; $\beta = -0.127$, $p = 0.344$, respectively).

The correlations of cutaneous nerve fibre density and length with measures of large and small fibre function are shown in Table 3. After adjustment for sex, age and BMI, GAP-43 positive IENFD was positively associated with sural sensory NCV and sural SNAPs in both DSPN groups, and also positively associated with peroneal motor NCV and cold TDT and inversely associated with warm TDT in the DSPN+p group ($p < 0.05$). GAP-43 positive DNFL was positively associated with peroneal motor NCV in the DSPN+p group, and with sural sensory NCV and sural SNAPs in both DSPN

Table 2 IENFD, IENFL and DNFL immunostained with PGP9.5 and GAP-43

	Control 1	Recent-onset type 2 diabetes	Control 2	DSPN-p	DSPN+p
PGP9.5					
IENFD (fibres/mm)	7.76 ± 3.28	5.11 ± 2.96*	7.01 ± 3.07	3.81 ± 3.85†	2.43 ± 2.86†
IENFL (μm/mm²)	6.21 ± 3.57	2.65 ± 1.69*	5.09 ± 2.73	2.89 ± 3.34†	2.09 ± 3.09†
DNFL (μm/mm²)	6.29 ± 2.09	5.84 ± 1.59	5.22 ± 1.31	4.72 ± 2.28	3.85 ± 2.67
GAP-43					
IENFD (fibres/mm)	7.20 ± 3.23	4.86 ± 2.67*	6.65 ± 3.37	3.48 ± 3.28†	2.29 ± 2.61†
IENFL (μm/mm²)	4.93 ± 3.29	1.98 ± 1.09*	4.06 ± 2.71	2.07 ± 2.62†	1.44 ± 2.07†
DNFL (μm/mm²)	6.16 ± 1.96	5.60 ± 1.46	5.30 ± 1.23	5.00 ± 2.19	4.15 ± 2.60
GAP-43/PGP9.5 ratio					
IENFD	0.92 ± 0.12	1.00 ± 0.23	0.93 ± 0.13	0.96 ± 0.15	1.08 ± 0.44
IENFL	0.80 ± 0.30	0.83 ± 0.23	0.82 ± 0.35	0.87 ± 0.40	1.25 ± 1.99
DNFL	0.99 ± 0.09	0.97 ± 0.11	1.02 ± 0.10	1.07 ± 0.10†	1.18 ± 0.28‡,§

*$p < 0.05$ vs Control 1, † $p < 0.05$ vs Control 2, ‡ $p = 0.05$ vs Control 2, § $p < 0.05$ vs DSPN-p (all adjusted for sex, age, BMI and HbA_{1c})

groups ($p < 0.05$). The correlations of PGP9.5 positive fibres with measures of large and small fibre function corresponded to those found for GAP-43 (data not shown). The DNFL GAP-43/PGP ratio was inversely associated with sural SNAPs in the DSPN-p group ($p < 0.05$). In the entire type 2 diabetes group, all variables of cutaneous nerve fibre density and length were associated with each measure of large and small fibre function ($p < 0.05$). No such associations were noted for the control participants (Table 3) or in the recent-onset group (data not shown).

Table 3 Correlations of cutaneous nerve fibre density and length with measures of large and small fibre function

	Control		DSPN-p		DSPN+p		All individuals with diabetes	
	r	*p*	*r*	*p*	*r*	*p*	*r*	*p*
Log_e(IENFD-GAP-43)								
Peroneal motor NCV	0.263	0.071	0.324	0.099	0.579	0.001*	0.351	0.002*
Sural sensory NCV	0.366	0.011	0.574	0.005*	0.554	0.005*	0.473	<0.0001*
Log_e (sural SNAP)	0.376	0.008	0.587	0.004*	0.455	0.025*	0.456	<0.0001*
Log_e (VPT)	−0.421	0.003	0.026	0.890	−0.222	0.222	−0.281	0.010*
Log_e (warm TDT)	−0.194	0.187	−0.129	0.481	−0.523	0.001*	−0.257	0.016*
Log_e (cold TDT)	0.330	0.025	0.094	0.610	0.621	<0.0001*	0.257	0.016*
DNFL GAP-43								
Peroneal motor NCV	0.175	0.234	0.365	0.059	0.569	0.001*	0.489	<0.0001*
Sural sensory NCV	0.181	0.224	0.579	0.005*	0.516	0.01*	0.517	<0.0001*
Log_e (sural SNAP)	0.180	0.217	0.692	0.0004*	0.534	0.007*	0.588	<0.0001*
DNFL ratio								
Log_e (sural SNAP)	−0.163	0.262	−0.548	0.008*	−0.037	0.862	−0.359	0.001*
Log_e (cold TDT)	−0.100	0.507	−0.199	0.276	−0.446	0.008	−0.433	<0.0001*

*$p < 0.05$ after adjustment for sex, age and BMI

Discussion

In this comprehensive study using PGP9.5 and GAP-43 double staining, we demonstrate differential patterns of cutaneous nerve fibre loss and regeneration in various groups of individuals with type 2 diabetes with and without painful DSPN. First, dermal nerve fibre regeneration assessed by the DNFL GAP-43/PGP9.5 ratio was enhanced in both types of DSPN, to a higher extent in individuals with DSPN+p compared with those with DSPN-p. Second, the degree of dermal nerve fibre regeneration was associated with more advanced intraepidermal nerve fibre loss and peripheral nerve dysfunction. Third, the IENFD-GAP-43/PGP9.5 ratio did not differ between the groups, questioning the usefulness of this ratio as a marker of nerve regeneration in type 2 diabetes. Thus, we suggest that, despite progressive epidermal nerve fibre loss, dermal nerve repair remains stimulated in DSPN, particularly in DSPN+p, but fails to adequately counteract epidermal neurodegenerative processes.

A major finding presented herein is the increased DNFL GAP-43/PGP9.5 ratio in DSPN, suggesting an increased attempt at regeneration by dermal nerve fibres, particularly in DSPN+p. Although apparently contradictory to the common assumption that regenerative capacity is reduced in individuals with diabetes [4, 19], these results are in line with basic principles of neural plasticity in the peripheral nervous system. Physiologically, nerve remodelling and regrowth are part of an intact peripheral neural plasticity [14], representing processes in which numerous extrinsic and intrinsic factors, such as GAP-43, are involved. These factors are fairly robust against degenerative events in cutaneous sensory nerve fibres [29]. Following nerve injury, GAP-43 is strongly upregulated in the axonal growth cone at the distal end of the proximal axonal stump [4], to compensate for axonal loss. While GAP-43 may not exclusively label regenerating nerve fibres, its preponderance in the regenerating growth cones compared with degenerating distal axonal stumps or uninjured axons justifies its use as a post-injury regenerative marker [4]. Therefore, the increase in GAP-43 DNFL relative to PGP9.5 DNFL in individuals with DSPN and the inverse association with IENFD could mirror a compensatory upregulation against the neurodegenerative effects of diabetes which ultimately may be insufficient to increase IENFD in a diabetes-induced microenvironment inhospitable for axonal regrowth [30]. Interestingly, an enhanced DNFL GAP-43/PGP9.5 ratio was associated with more advanced intraepidermal nerve fibre loss and peripheral nerve dysfunction in individuals with type 2 diabetes, whereas no such association was found in control participants. Together, these skin biopsy measures correlated with large fibre function (NCV) for both types of DSPN, while they were only associated with small fibre function (TDT) in DSPN+p. These findings support the concept of a higher rate of maladaptive small fibre regeneration processes in DSPN+p [31–33], which may result in enhanced pain perception due to peripheral sensitisation. Supporting this rationale, a recent study by Xie et al [34] reported that nerve regeneration in a spinal nerve injury model resulted in tangled GAP-43 positive neuromas and that blocking these regenerative processes reduced neuropathic pain.

To the best of our knowledge, this is the first study in which both IENFL and DNFL were quantified manually in adequately large groups of individuals with type 2 diabetes and control participants using double immunofluorescence for PGP9.5 and GAP-43. Direct comparisons of the various aspects of this study with previous reports are difficult because of differences in methodology and aetiology of neuropathy. For example, previous studies have not specified the proportion of individuals with DSPN [18, 28, 35, 36], have used bright-field microscopy [8, 37, 38] or relied on automatic measurement of nerve fibre density [16]. Focusing on IENFD, Cheng et al [17] found no difference in IENFD for PGP9.5 positive fibres between DSPN+p and DSPN-p but, in contrast to our findings, they reported more GAP-43 positive fibres in DSPN+p and reduced intraepidermal GAP-43/PGP9.5 ratios, particularly in DSPN-p, compared with individuals without DSPN. A previous study by Sorensen et al [39] showed reduced PGP9.5 positive IENFD in people with DSPN+p compared with a group of individuals without pain, but it is unclear how many individuals in the latter group had DSPN. Compatible with our data, Themistocleous et al [38] reported similarly reduced PGP9.5 positive IENFD in both DSPN entities using bright-field microscopy. However, 30% of the DSPN-p group were receiving analgesic pharmacotherapy [38], casting doubt on the validity of the DSPN-p group.

In contrast to previous studies, we found no evidence of dermal nerve fibre loss in the three type 2 diabetes groups studied. In a small study, Krishnan et al [37] reported reduced dermal nerve fibre density in DSPN-p but not DSPN+p using only 5 μm thin sections. Lauria et al [28] found reduced DNFL in individuals with SFN, but they neither reported the proportions of participants with diabetic vs idiopathic SFN nor did they discriminate between painful and painless SFN.

The higher proportion of GAP-43 compared with PGP9.5 positive cutaneous nerve fibres found in the DSPN groups is challenging given the recognised role of PGP9.5 as a pan-neuronal marker. A possible explanation could be that GAP-43 is superior to PGP9.5 in detecting emerging nerve fibre endings because of different axonal transport mechanisms. PGP9.5 is an enzyme that relies on the slow axonal transport component B (2–8 mm/day), while GAP-43 uses fast vesicular axonal transport (50–400 mm/day) [12, 40]. Hence, GAP-43 could be more susceptible than PGP9.5 to recently grown nerve fibres. This could be an advantage in areas of high neural plasticity and needs to be evaluated in further studies.

Major strengths of this work are the manual assessment of both epidermal and dermal nerve fibres using double immunofluorescence microscopy including sophisticated morphometry and the extensive functional phenotyping performed in relatively large samples of individuals with type 2 diabetes and control participants. One limitation is the cross-sectional nature of this study which does not give information on the temporal sequence of the findings presented. Another limitation is the restriction to largely descriptive morphological and functional assessment which may not provide direct insights into the pathological mechanisms. Moreover, topical capsaicin application and excision axotomy models have been introduced to study nerve regeneration [19, 30], but these require sampling of multiple serial skin biopsies usually feasible only in a limited number of individuals.

In conclusion, the regenerative capacity of dermal nerve fibres assessed by the DNFL GAP-43/PGP9.5 ratio was enhanced in both types of DSPN, particularly in DSPN+p, but not in individuals with recent-onset type 2 diabetes. The extent of the dermal regenerative capacity was associated with more advanced intraepidermal nerve fibre loss and peripheral nerve dysfunction. We propose that the DNFL GAP-43/PGP9.5 ratio should be considered as a marker to assess the regenerative capacity of cutaneous nerve fibres. Whether this biomarker has the potential to predict the progression or regression of DSPN or the susceptibility to regenerative pharmacotherapy remains to be established in future prospective observational studies and clinical trials.

Acknowledgements The authors wish to thank the staff of the Research Group Neuropathy, Institute for Clinical Diabetology at the German Diabetes Center (DDZ), Düsseldorf, Germany, especially F. Battiato, N. Reuß and M. Schroers-Teuber, for their excellent work. The GDS Group consists of A.E. Buyken (Department of Sports and Health, Paderborn University, Paderborn, Germany), G. Geerling (Department of Ophthalmology, Medical Faculty, Heinrich Heine University, Düsseldorf, Germany), J. Eckel, H. Al-Hasani, C. Herder, A. Icks, J. Kotzka, O. Kuss, E. Lammert, D. Markgraf, K. Müssig, W. Rathmann, J. Szendroedi, D. Ziegler and M. Roden (speaker) (all DDZ). Some of the data were presented as an abstract at the 52nd EASD Annual Meeting in Munich in 2016.

Data availability The data sets generated during and/or analysed during the current study are not publicly available, since they are subject to national data protection laws and restrictions imposed by the ethics committee to ensure data privacy of the study participants. However, they can be applied for through an individual project agreement with PROPANE and/or GDS.

Funding The GDS was initiated and financed by the German Diabetes Center, which is funded by the German Federal Ministry of Health (Berlin, Germany), the Ministry of Innovation, Science, Research and Technology of the state North Rhine-Westphalia (Düsseldorf, Germany), grants from the German Federal Ministry of Education and Research (BMBF) to the German Center for Diabetes Research (DZD), and partly funded through an EFSD award supported by Novartis to DZ and AS. The PROPANE study was initiated by the PROPANE consortium and received funding from the European Union Seventh Framework Programme FP7/2007-2013 (grant no. 602273).

Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

Contribution statement All authors were involved in revising the manuscript critically for important intellectual content and gave final approval of the version to be published. GJB contributed to acquisition, analysis and interpretation of data and wrote the manuscript. AS and SP contributed to the acquisition, analysis and interpretation of data, BR and JB contributed to the acquisition of data. KB, KM, JS and MR contributed to the analysis and interpretation of data. DZ contributed to conception and design of the study and to the analysis and interpretation of data. DZ is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

References

1. Happich M, John J, Stamenitis S, Clouth J, Polnau D (2008) The quality of life and economic burden of neuropathy in diabetic patients in Germany in 2002—results from the Diabetic Microvascular Complications (DIMICO) study. Diabetes Res Clin Pract 81:223–230
2. Ziegler D, Papanas N, Vinik AI, Shaw JE (2014) Epidemiology of polyneuropathy in diabetes and prediabetes. Handb Clin Neurol 126:3–22
3. Landowski LM, Dyck PJ, Engelstad J, Taylor BV (2016) Axonopathy in peripheral neuropathies: mechanisms and therapeutic approaches for regeneration. J Chem Neuroanat 76:19–27
4. Yasuda H, Terada M, Maeda K et al (2003) Diabetic neuropathy and nerve regeneration. Prog Neurobiol 69:229–285
5. Zochodne DW (2012) The challenges and beauty of peripheral nerve regrowth. J Peripher Nerv Syst 17:1–18
6. Spallone V, Greco C (2013) Painful and painless diabetic neuropathy: one disease or two? Curr Diab Rep 13:533–549
7. Tesfaye S, Vileikyte L, Rayman G et al (2011) Painful diabetic peripheral neuropathy: consensus recommendations on diagnosis, assessment and management. Diabetes Metab Res Rev 27:629–638
8. Lauria G, Lombardi R, Camozzi F, Devigili G (2009) Skin biopsy for the diagnosis of peripheral neuropathy. Histopathology 54:273–285
9. Day IN, Thompson RJ (2010) UCHL1 (PGP 9.5): neuronal biomarker and ubiquitin system protein. Prog Neurobiol 90:327–362
10. Ziegler D, Papanas N, Zhivov A et al (2014) Early detection of nerve fiber loss by corneal confocal microscopy and skin biopsy in recently diagnosed type 2 diabetes. Diabetes 63:2454–2463
11. Kennedy WR, Wendelschafer-Crabb G, Johnson T (1996) Quantitation of epidermal nerves in diabetic neuropathy. Neurology 47:1042–1048
12. Denny JB (2006) Molecular mechanisms, biological actions, and neuropharmacology of the growth-associated protein GAP-43. Curr Neuropharmacol 4:293–304
13. Benowitz LI, Routtenberg A (1997) GAP-43: an intrinsic determinant of neuronal development and plasticity. Trends Neurosci 20:84–91
14. Verze L, Viglietti-Panzica C, Maurizo S, Sica M, Panzica G (2003) Distribution of GAP-43 nerve fibers in the skin of the adult human hand. Anat Rec A Discov Mol Cell Evol Biol 272:467–473
15. Fantini F, Johansson O (1992) Expression of growth-associated protein 43 and nerve growth factor receptor in human skin: a

comparative immunohistochemical investigation. J Invest Dermatol 99:734–742
16. Narayanaswamy H, Facer P, Misra VP et al (2012) A longitudinal study of sensory biomarkers of progression in patients with diabetic peripheral neuropathy using skin biopsies. J Clin Neurosci 19: 1490–1496
17. Cheng HT, Dauch JR, Porzio MT et al (2013) Increased axonal regeneration and swellings in intraepidermal nerve fibers characterize painful phenotypes of diabetic neuropathy. J Pain 14:941–947
18. Bursova S, Dubovy P, Vlckova-Moravcova E et al (2012) Expression of growth-associated protein 43 in the skin nerve fibers of patients with type 2 diabetes mellitus. J Neurol Sci 315:60–63
19. Polydefkis M, Hauer P, Sheth S, Sirdofsky M, Griffin JW, McArthur JC (2004) The time course of epidermal nerve fibre regeneration: studies in normal controls and in people with diabetes, with and without neuropathy. Brain 127:1606–1615
20. ADA (2012) Diagnosis and classification of diabetes mellitus. Diabetes Care 35(Suppl 1):S64–S71
21. Tesfaye S, Boulton AJ, Dyck PJ et al (2010) Diabetic neuropathies: update on definitions, diagnostic criteria, estimation of severity, and treatments. Diabetes Care 33:2285–2293
22. Dworkin RH, Turk DC, Peirce-Sandner S et al (2010) Research design considerations for confirmatory chronic pain clinical trials: IMMPACT recommendations. Pain 149:177–193
23. Szendroedi J, Saxena A, Weber KS et al (2016) Cohort profile: the German Diabetes Study (GDS). Cardiovasc Diabetol 15:59
24. Lauria G, Ziegler D, Malik R et al (2014) The role of sodium channels in painful diabetic and idiopathic neuropathy. Curr Diab Rep 14:538
25. Young MJ, Boulton AJ, MacLeod AF, Williams DR, Sonksen PH (1993) A multicentre study of the prevalence of diabetic peripheral neuropathy in the United Kingdom hospital clinic population. Diabetologia 36:150–154
26. Lauria G, Hsieh ST, Johansson O et al (2010) European Federation of Neurological Societies/Peripheral Nerve Society guideline on the use of skin biopsy in the diagnosis of small fiber neuropathy. Report of a joint task force of the European Federation of Neurological Societies and the Peripheral Nerve Society. Eur J Neurol 17(903–912):e944–e909
27. McCarthy BG, Hsieh ST, Stocks A et al (1995) Cutaneous innervation in sensory neuropathies: evaluation by skin biopsy. Neurology 45:1848–1855
28. Lauria G, Cazzato D, Porretta-Serapiglia C et al (2011) Morphometry of dermal nerve fibers in human skin. Neurology 77:242–249
29. Cheng C, Guo GF, Martinez JA, Singh V, Zochodne DW (2010) Dynamic plasticity of axons within a cutaneous milieu. J Neurosci 30:14735–14744
30. Ebenezer GJ, O'Donnell R, Hauer P, Cimino NP, McArthur JC, Polydefkis M (2011) Impaired neurovascular repair in subjects with diabetes following experimental intracutaneous axotomy. Brain 134:1853–1863
31. Costigan M, Scholz J, Woolf CJ (2009) Neuropathic pain: a maladaptive response of the nervous system to damage. Annu Rev Neurosci 32:1–32
32. Orstavik K, Namer B, Schmidt R et al (2006) Abnormal function of C-fibers in patients with diabetic neuropathy. J Neurosci 26:11287–11294
33. Navarro X, Vivo M, Valero-Cabre A (2007) Neural plasticity after peripheral nerve injury and regeneration. Prog Neurobiol 82:163–201
34. Xie W, Strong JA, Zhang JM (2017) Active nerve regeneration with failed target reinnervation drives persistent neuropathic pain. eNeuro. https://doi.org/10.1523/ENEURO.0008-17.2017
35. Scheytt S, Riediger N, Braunsdorf S, Sommer C, Uceyler N (2015) Increased gene expression of growth associated protein-43 in skin of patients with early-stage peripheral neuropathies. J Neurol Sci 355:131–137
36. Vlckova-Moravcova E, Bednarik J, Dusek L, Toyka KV, Sommer C (2008) Diagnostic validity of epidermal nerve fiber densities in painful sensory neuropathies. Muscle Nerve 37:50–60
37. Krishnan ST, Quattrini C, Jeziorska M, Malik RA, Rayman G (2009) Abnormal LDIflare but normal quantitative sensory testing and dermal nerve fiber density in patients with painful diabetic neuropathy. Diabetes Care 32:451–455
38. Themistocleous AC, Ramirez JD, Shillo PR et al (2016) The Pain in Neuropathy Study (PiNS): a cross-sectional observational study determining the somatosensory phenotype of painful and painless diabetic neuropathy. Pain 157:1132–1145
39. Sorensen L, Molyneaux L, Yue DK (2006) The relationship among pain, sensory loss, and small nerve fibers in diabetes. Diabetes Care 29:883–887
40. Rage M, Van Acker N, Facer P et al (2010) The time course of CO_2 laser-evoked responses and of skin nerve fibre markers after topical capsaicin in human volunteers. Clin Neurophysiol 121:1256–1266

3 Discussion

The factors and mechanisms contributing to the phenotype of painful and painless DSPN are not well understood, so that there is currently no conclusive answer to the clinically important question why some patients with DSPN develop neuropathic pain, while others do not. In this cross-sectional study, we investigated the patterns of cutaneous nerve fibre loss and regeneration in individuals with type 2 diabetes with painful or painless DSPN and in recent-onset type 2 diabetes patients without DSPN compared to corresponding glucose-tolerant controls.

Skin biopsy sections at a standardised site at the distal-lateral calf were obtained and immunofluorescent markers of all axons and regenerative axons were used to determine the densities and lengths of intraepidermal and dermal nerve fibres reflecting the degree of nerve fibre damage and repair.

Using PGP9.5 and GAP-43 double staining, immunofluorescence microscopy, and manual morphometric assessment of skin biopsy sections (Figure 5), differential patterns of nerve fibre loss and regeneration were observed in the various groups studied. To the best of our knowledge, ours is the first study in which intraepidermal nerve fibre density (IENFD) and both intraepidermal and dermal nerve fibre lengths (IENFL, DNFL) were quantified manually in adequately large groups of individuals with type 2 diabetes and control participants using double immunofluorescence. Comprehensive phenotyping of DSPN was performed including electrophysiological testing, QST, and clinical scores and questionnaires.

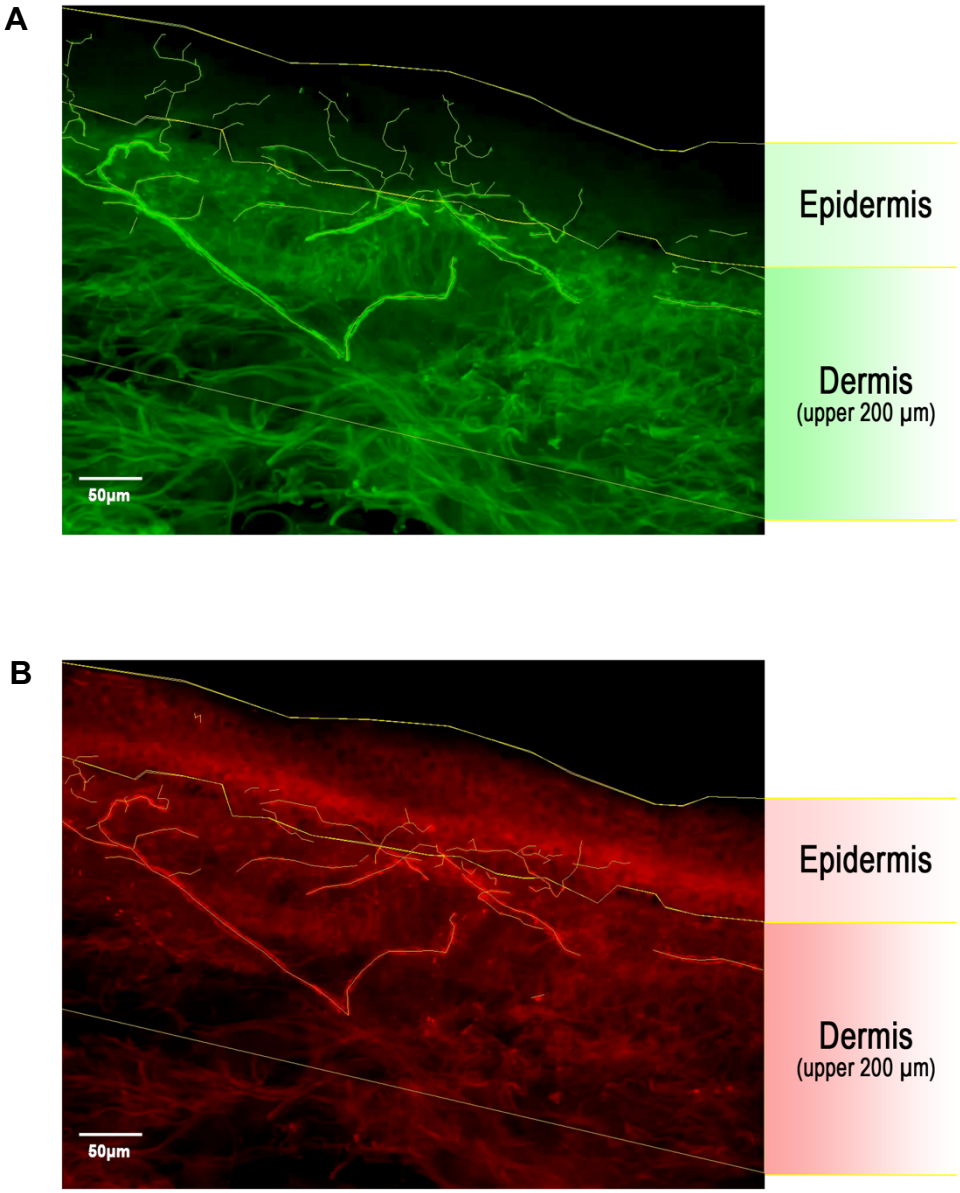

Figure 5: Manual morphometric assessment of epidermal and dermal nerve fibres using double immunofluorescence microscopy.
Images show epidermal and dermal areas and corresponding nerve fibres (yellow overlay) in a skin biopsy segment stained with **(A)** protein gene product 9.5 (PGP9.5) and **(B)** growth-associated-protein-43 (GAP-43). Typical nerve fibre distribution in a healthy elderly individual.

Three main results were obtained. First, the regenerative capacity of dermal nerve fibres assessed by the ratio of GAP-43 and PGP9.5 positive dermal nerve fibres (DNFL GAP-43/PGP9.5 ratio) was higher in both DSPN entities than in the corresponding control individuals, but to a higher extent in patients with painful DSPN compared to those with painless DSPN.

Second, the extent of dermal nerve fibre regenerative capacity expressed by the DNFL GAP-43/PGP9.5 ratio was associated with more advanced intraepidermal nerve fibre loss, lower dermal nerve fibre length, and peripheral nerve dysfunction assessed by electrophysiological testing and QST (sural nerve SNAP, cold thermal detection threshold (TDT)).

Third, the intraepidermal ratio between GAP-43 and PGP9.5 positive nerve fibres (IENFD GAP-43/PGP9.5 ratio) did not differ between any of the groups, casting doubt on the usefulness of this ratio as a marker of nerve regeneration in type 2 diabetes.

These findings suggest that nerve repair mechanisms remain active in DSPN, but are obviously not sufficient to adequately counteract epidermal neurodegenerative processes due to type 2 diabetes, leading to a loss of epidermal nerve fibres. It is tempting to speculate that the higher degree of dermal regenerative capacity found in painful compared with painless DSPN indicates that regenerative processes, albeit maladaptive, to the painful phenotype.

3.1 Clinical characteristics of painful and painless DSPN

The allocation of individuals to the painful and painless DSPN groups in the present study was not actively controlled for demographic and clinical characteristics to maintain a preferably real world representation of patients. Nonetheless, no differences were observed between the groups with respect to their demographic and clinical characteristics, except for a slightly lower mean age in the painful DSPN group. No differences were observed in measures reflecting large nerve fibre dysfunction (nerve conduction studies, vibration perception threshold (VPT)) or small nerve fibre impairment (IENFD, thermal thresholds). Moreover, the NDS including simple clinical tests to assess both large and small fibre dysfunction did not differ between the DPSN groups, indicating a similar severity of neuropathic deficits in the painful and painless DSPN groups.

Several factors have recently been linked to the painful DSPN entity. Large cross-sectional studies suggested that neuropathic pain appears to be associated with female gender, increasing age, obesity, and higher neuropathy severity (36, 38, 42). Raputova and colleagues (42) reported that neuropathic pain in DSPN correlated with the severity of neuropathic signs and symptoms and thermal hyposensitivity assessed by QST but not with nerve conduction studies. In the present study, no differences between the groups with painful and painless DSPN were noted for age, sex, and neuropathy severity, but this may be due to inadequate statistical power. Likewise, other studies did not find associations between neuropathic pain and neuropathy severity (96-99), and it is worth noting that neuropathic pain can occur at any stage of DSPN (38).

There is an ongoing debate as to whether painful DSPN is associated with predominant small fibre damage (82, 85). However, most of the existing data supporting preferential small fibre involvement in painful DSPN is based on smaller studies (100-102) and the definition, diagnosis, and severity of DSPN varies considerably. In the present study, measures of small and large fibre involvement were reduced in painful and painless DSPN to a similar extent. Thus, our results do not support the notion that painful DSPN is linked to predominant small fibre dysfunction. Overall, the DSPN groups were comparable without being confounded by different neuropathy severity, diabetes duration, or HbA1c levels. Furthermore, group comparisons were adjusted for sex, age, and BMI. Skin biopsy results were additionally adjusted for HbA1c.

3.2 Intraepidermal nerve fibres

The number of fibres crossing the epidermal-dermal junction (IENFD) and the overall length of intraepidermal nerve fibres (IENFL) were lower in recent-onset type 2 diabetes and in both DSPN groups compared with the corresponding control groups for both PGP9.5 and GAP-43 markers. In the recent-onset type 2 diabetes group, reduced IENFD and IENFL were the measures indicating early nerve damage. In an earlier study including more participants with recent-onset type 2 diabetes from the same cohort, IENFD was reduced to a similar degree, but nerve conduction studies and QST indicated an early parallel involvement of small and large nerve fibres in type 2 diabetes (85).

No difference in epidermal innervation assessed by IENFD and IENFL was found between painful und painless DSPN regardless of using PGP9.5 or GAP-43 immunostaining. In all groups studied, IENFD and IENFL were similar with both

markers, and no difference was observed between the corresponding GAP-43/PGP9.5 ratios. Both markers showed comparable IENFD and IENFL. In all groups studied, the IENFD GAP-43/PGP9.5 ratio was close to 1, indicating similar proportions of PGP9.5- and GAP-43-positive nerve fibres crossing the epidermal-dermal junction. The IENFL GAP-43/PGP9.5 ratio was numerically highest in the group with painful DSPN compared to the other groups, but this difference did not achieve statistical significance. These data suggest that painful and painless DSPN show a similar degree of intraepidermal nerve fibre loss and that the regenerative capacity of the remaining nerve fibres sprouting into the epidermis remains normal.

Direct comparisons of this study with previous reports are difficult due to differences in the definition of painful and painless neuropathy or in the methodology used to assess cutaneous nerve fibres. Focusing on IENFD, a previous study by Cheng and colleagues (93) found no difference in IENFD for PGP9.5 positive fibres between type 2 diabetes patients with painful and painless DSPN using a similar double immunofluorescence technique with PGP9.5 and GAP-43. In contrast to our study, more GAP-43 positive intraepidermal nerve fibres were reported in patients with painful DSPN compared to those with painless DSPN and those with type 2 diabetes without DSPN. IENFD GAP-43/PGP9.5 ratio was higher in type 2 diabetes patients with painful compared to painless DSPN or those without DSPN. In painless DSPN, it was lower compared with painful DSPN or with type 2 diabetes or normal glucose tolerance without DSPN. IENFD GAP-43/PGP9.5 ratio did not exceed 0.5, indicating generally more than twice as many PGP9.5- than GAP-43-positive fibres. However, while parameters of DSPN were similarly reduced in both DSPN groups, age and diabetes duration were higher in the painful DSPN group, and group comparisons were not adjusted for those potential confounders.

A previous study by Sorensen et al. (103) compared patients with diabetes, 84% of whom had type 2 diabetes, with and without painful DSPN and reported reduced PGP9.5 positive IENFD in the painful DSPN group using bright-field microscopy. However, it is unclear how many participants in the group without painful DSPN actually had DSPN as the median neuropathy score was in the normal range in this group, and no adjustment was performed when comparing the two groups. Therefore, the reduced IENFD in the painful DSPN group was possibly related to DSPN itself rather than specifically to the painful phenotype. Comparable with our findings, a cross-sectional study reported a similar reduction in PGP9.5 positive IENFD in DSPN with and without neuropathic pain

using bright-field microscopy. However, the validity of the painless DSPN group is questionable, as 30% of the individuals in that group were receiving analgesic pharmacotherapy (41). Another previous study examined IENFD using separate immunofluorescence staining with PGP9.5 and GAP-43 in participants with peripheral neuropathies of different aetiologies, 4% of which were attributed to diabetes, but not analysed separately. Within this heterogeneous peripheral neuropathy group, higher IENFDs were observed in GAP-43-stained compared with PGP.5-stained sections with a median GAP-43/PGP9.5 ratio of 1.6 (104). In a recent study by Galosi and colleagues (105), both markers were studied using bright-field microscopy in reasonably matched painful or painless DSPN groups and in subgroups of burning pain and mechanical allodynia. The highest mean IENFD, either PGP9.5-positive or GAP-43-positive, was observed in the group with allodynia, indicating a functional impairment rather than a loss of innervation of the skin. A higher GAP-43-positive IENFD and higher GAP-43/PGP9.5 ratio, but not PGP9.5-positive IENFD, were found in the group with ongoing burning pain compared with the painless DSPN group, suggesting a role for regenerative sprouting in the development of burning pain in DSPN.

3.3 Dermal nerve fibres

The was no evidence of a reduced overall length of dermal nerve fibres (DNFL) in each of the three type 2 diabetes groups studied, regardless of the marker used. In contrast, Krishnan and colleagues (106) reported reduced dermal nerve fibre density in individuals with type 2 diabetes and painless but not painful DSPN compared with healthy individuals. However, the groups were small, the control group was neither matched for age nor were the results adjusted accordingly, and only thin 5 μm sections were used. Reduced DNFL was reported by Lauria and colleagues (82) in individuals with SFN, but that study neither specified how many participants had SFN due to diabetes nor did it discriminate between painful and painless SFN. In a previous study by Vlčková-Moravcová and colleagues (107), lower subepidermal nerve fibre densities were reported in patients with peripheral neuropathy of heterogeneous aetiology compared with control individuals as well as in patients with mixed small and large fibre neuropathies compared to those with pure SFN. However, less than a third of the patients with neuropathy had diabetes. A recent study by Pál and colleagues (108) assessed subepidermal nerve fibre density only semiquantitatively comparing individuals with idiopathic and secondary SFN, but only 7% of cases with secondary SFN had diabetes and no control group was

included. No difference in subepidermal nerve fibre density was reported between patients with idiopathic and secondary SFN. A recent study assessed dermal nerve fibres in drug-induced neuropathy and reported a lower nerve fibre density in the subepidermal layer subjacent to the dermal-epidermal junction, but instead more nerve fibres in the dermis below the subepidermal area compared with healthy control individuals (109). In our study, no distinction was made between different layers of the dermis. Future studies could assess whether innervation in different layers of the dermis is affected differently in DSPN.

3.3.1 DNFL GAP-43/PGP9.5 ratio

In the present study, the DNFL GAP-43/PGP9.5 ratio was higher in both DSPN groups compared with the corresponding control group in contrast to the intraepidermal GAP-43/PGP9.5 ratios, for which no differences were observed between the groups. Moreover, the DNFL GAP-43/PGP9.5 ratio was higher in painful DSPN compared with painless DSPN. More than a quarter of individuals with painful DSPN showed a DNFL GAP-43/PGP9.5 ratio higher than the 95th percentile of the control group. This major finding, not reported previously, suggests an increased regenerative activity in dermal nerve fibres, particularly in painful DSPN. No difference in DNFL GAP-43/PGP9.5 ratio was found in the recent-onset type 2 diabetes group compared with the corresponding control group. The higher dermal as opposed to epidermal GAP-43/PGP9.5 ratio in DSPN indicates ongoing regenerative nerve fibre sprouting in the dermal but not epidermal layer compared with individuals without diabetes. As a consequence this pattern suggests that dermal nerve fibre length remains normal despite the presence of DSPN, while epidermal nerve fibres are diminished.

3.4 Associations of skin biopsy markers with nerve function tests

The correlations of PGP9.5 positive fibres with measures of small and large fibre function corresponded to those found for GAP-43. Lower epidermal innervation measured by IENFD was associated with increasing large fibre dysfunction in both DSPN groups, while associations with measures of small fibre dysfunction were observed in painful, but not in painless DSPN. In the entire group of participants with type 2 diabetes, epidermal nerve fibre loss was associated with both small and large fibre dysfunction confirming the results of a recent large cross-sectional study (41). A new finding is the association of lower levels of dermal innervation with large fibre dysfunction in both DSPN groups as

well as in a group of all participants with type 2 diabetes combined (including those with recent-onset type 2 diabetes, painful DSPN, and painless DSPN). Increased regenerative processes in dermal nerve fibres, reflected by an enhanced DNFL GAP-43/PGP9.5 ratio, were associated with both impaired small and large fibre function in patients with type 2 diabetes. In DSPN, only an association with one measure of large fibre dysfunction was observed in the painless entity. No such associations were observed in individuals with normal glucose tolerance or recent-onset type 2 diabetes. Interestingly, enhanced dermal regenerative processes were associated with more advanced intraepidermal nerve fibre loss and lower dermal nerve fibre length in individuals with type 2 diabetes, suggesting that regenerative processes in the dermis, where nerve fibre length remains in the normal range, are not successful to maintain epidermal innervation by regenerative sprouting of nerve fibres crossing the dermal-epidermal junction.

3.5 Neural plasticity in DSPN

The findings of the present study provide information about both the degenerative and regenerative processes relevant to DSPN. Although the analysis of skin biopsy markers primarily allows conclusions about structural changes and not about functional dynamics, the associations between skin biopsy parameters and those of peripheral nerve function support the concept of maladaptive small fibre regeneration processes, particularly in painful DSPN.

Injuries to peripheral nerves can initiate either favourable regenerative processes to compensate for the functional deficits or contrarily maladaptive regenerative attempts resulting in the formation of errant nerve sprouts that are hypofunctional, not adequately restoring the functional deficit, or by contrast hyperfunctional, resulting in positive symptoms such as paraesthesiae or neuropathic pain (110, 111). Reduced functionality of C fibres in DSPN was demonstrated in a study by Ørstavik and colleagues (112) using microneurography. Individuals with DSPN had a substantially lower distribution of normal mechanoreceptive to mechano-insensitive nociceptors that had lost mechanical and heat responsiveness.

The higher dermal as opposed to epidermal GAP-43/PGP9.5 ratio reported in this study indicates an enhanced dermal regenerative sprouting which may represent a compensatory attempt to repair intraepidermal nerve fiber loss, while dermal nerve fibre

length remains normal. We assume that regenerative sprouting is sufficient to counteract neural degenerative processes due to diabetes in the dermal but not epidermal layer.

As expected, DSPN was characterised by epidermal denervation in the present study. Epidermal denervation was associated with enhanced dermal regeneration reflected by increased DNFL GAP-43/PGP9.5-ratio. This finding suggests that dermal regeneration attempts are triggered under conditions leading to nerve fibre loss, but are insufficient to successfully reinnervate the epidermis. The defining characteristic of the epidermal layer is the abundance of keratinocytes. Keratinocytes modulate epidermal innervation by producing nerve repellent, but also by providing elongation factors to guide epidermal nerve fibres which they surround. In diabetes, the structure and function of keratinocytes may be altered. A recent translational study demonstrated an upregulation of semaphorin 3A, an inhibitor of nerve regeneration produced by keratinocytes, in the skin under diabetic conditions accompanied by lower IENFD, suggesting a role of impaired keratinocyte function in the reduction of epidermal nerve fibres in diabetes (1, 113). Keratinocytes in the epidermis underlie a regeneration cycle of 28 days which forces the epidermal nerve endings to constantly extend. Moreover, Schwann cells that foster axonal regrowth up to the level of the dermis are not present in the epidermis (114). These factors indicate that epidermal innervation may be more difficult to sustain compared to the innervation in deeper skin layers (112), which could explain why intraepidermal, but not dermal nerve fibres were reduced in the type 2 diabetes groups in our study. These conclusions are further strengthened by our recent study assessing small corneal nerve fibres in participants from the same cohort using corneal confocal microscopy (CCM). Despite a similar degree of corneal nerve fibre loss and peripheral nerve dysfunction in individuals with painful and painless DSPN, corneal nerve branching was enhanced in those with painful DSPN, pointing to some susceptibility of corneal nerve fibres toward regeneration in the painful entity, albeit not resulting in normal corneal nerve fibre density (115).

Although apparently contradictory to the common assumption that regenerative capacity is reduced in individuals with diabetes (80, 95), these results are compatible with basic principles of neural plasticity in the peripheral nervous system. Physiologically, nerve remodelling and regrowth are part of an intact peripheral neural plasticity (91), representing processes in which numerous extrinsic and intrinsic factors, such as GAP-43, are involved. These factors are fairly robust against degenerative events in cutaneous sensory nerve fibres (116). Assuming that diabetes leads to a microenvironment

inhospitable to nerve fibres, this could either culminate in damage to existing nerve fibres or in a hampered growth of new nerve fibres or in a combination of both. Furthermore, the physiological degeneration and regeneration cycle defining neural plasticity may be altered leading to faster or slower regeneration rates. The functional quality of existing or regrown nerve fibres could be affected leading to an impaired sensory function. The results of the present study suggest that regenerative processes are physiologically and sufficiently initiated in the dermis as a response to degenerative processes. In the epidermis however, degenerative processes result in actual nerve fibre loss, because the number of dermal nerve fibre regenerative sprouts crossing the epidermal-dermal junction remains inadequate. Thus, regenerative processes within the epidermis are not adequately stimulated to maintain normal levels of epidermal innervation. Consequently, a relative rather than absolute deficit in nerve regeneration can be assumed in type 2 diabetes, at least when DSPN has reached a clinical stage.

3.6 Factors contributing to neuropathic pain

Regenerative sprouting can contribute to enhanced pain perception in DSPN due to improper functional abilities, such as abnormal stimulus thresholds, ectopic impulse generation, slower conduction, and reduced inhibition. This maladaptive plasticity has been described as a substantial mechanism in the development of neuropathic pain which neither protects against noxious stimuli nor supports tissue repair. Once a painful sensation is generated by an injured nerve fibre, the hypersensitivity usually persists, especially when the underlying cause such as diabetes continues to affect nerve fibres. Spontaneous pain arises from ectopic action potential generation along nociceptive pathways including the neuroma at the site of the injury, the axons, and even in neighbouring afferents (110). In experimental neuropathic pain models, Xie and colleagues (117) reported that regenerating nerve fibres constitute the primary source of abnormal spontaneous activity after injury. Regeneration attempts after injury resulted in GAP-43-positive neuromas without target reinnervation. Blocking these regenerative processes with semaphorin 3A reversed spontaneous activity and neuropathic pain underscoring the role of regeneration in the development of neuropathic pain.

Increased ectopic impulse generation could be intensified by mutations of voltage-gated sodium channels which are the backbone of an intact electric signalling cascade in the PNS, allowing cells to depolarise via influx of sodium channels. (1). In a large cohort,

several hyperfunctional variants in the α subunits $Na_v1.7$ of voltage-gated sodium channels were found in some participants with painful DSPN, but in none with painless DSPN (1, 118). A recent case-report including a participant with painful DSPN from the present study's cohort, another gain-of-function sodium channel mutation leading to hyper-excitability was revealed in a β2-subunit of voltage-gated sodium channels (119). However, current evidence is not sufficient to draw the conclusion, that voltage-gated sodium channel mutations are a predominant contributor to the development of the painful phenotype.

3.7 Reappraisal of GAP-43 as a marker of nerve regeneration

Following nerve injury, GAP-43 is strongly upregulated in the axonal growth cone at the distal end of the proximal axonal stump (80) to compensate for axonal loss. While GAP43 may not exclusively label regenerating nerve fibres, its preponderance in the regenerating growth cones compared with degenerating distal axonal stumps or uninjured axons has justified its use as a post-injury regenerative marker (80). However, the proportions of GAP-43-positive to PGP9.5-positive nerve fibres reported in the current literature vary considerably. A few studies (104, 105) including ours, but not the majority of published studies (92, 93, 114), provide evidence of higher nerve fibre density or length using GAP-43 than those obtained with PGP9.5 immunostaining, resulting in GAP-43/PGP9.5 ratios above 1 (Figure 6). This finding is challenging given the recognised role of PGP9.5 as a pan-axonal marker. A possible explanation could be that GAP-43 may detect similar proportions of nerve fibres as does PGP9.5 in areas of high neural plasticity such as the skin (figure 7), where remodelling is frequent, but is actually superior to PGP9.5 in detecting emerging nerve fibre endings because of different axonal transport mechanisms. PGP9.5 is an enzyme that relies on the slow axonal transport component B (up to 8 mm/day), while GAP-43 uses fast vesicular axonal transport (approximately 50-400 mm/day) (88, 120). Performing skin biopsies before and at several times after a chemical axotomy with topical cutaneous application of capsaicin, Ragé and colleagues (120) found faster regeneration rates in subepidermal nerve fibre density using GAP-43 compared with intraepidermal nerve fibre density using PGP9.5, which took approximately 25 days longer to reach about 40% of the initial density before application of capsaicin. Moreover, GAP-43-positive subepidermal nerve fibre density correlated well with recovery of laser-evoked potentials used to stimulate and functionally assess nociceptive fibres. In contrast, no GAP-43-positive intraepidermal nerve fibres were

detected. However, immunohistochemistry images from a recent study by Anand et al. (121) who counted very few GAP-43 positive intraepidermal nerve fibres even in healthy control individuals using the same GAP-43 antibody, suggest that the identification of intraepidermal nerve fibres may be challenging using this specific staining protocol.

The variability of the results of published studies may be attributed to methodological differences such as different staining techniques (antibody used, bright-field vs immunofluorescence, single vs double staining), section thickness (thin vs thick sections), or definition of the areas (e.g. depth of dermal area) (122). Moreover, no blinding in regard to antibody has been reported, making it difficult to rule out confirmation bias toward reduced GAP-43 against the pan-axonal marker PGP9.5 (1). Added together, GAP-43 may be more amenable than PGP9.5 to recently grown nerve fibres. This could offer an advantage in areas of high neural plasticity and needs to be evaluated in further studies.

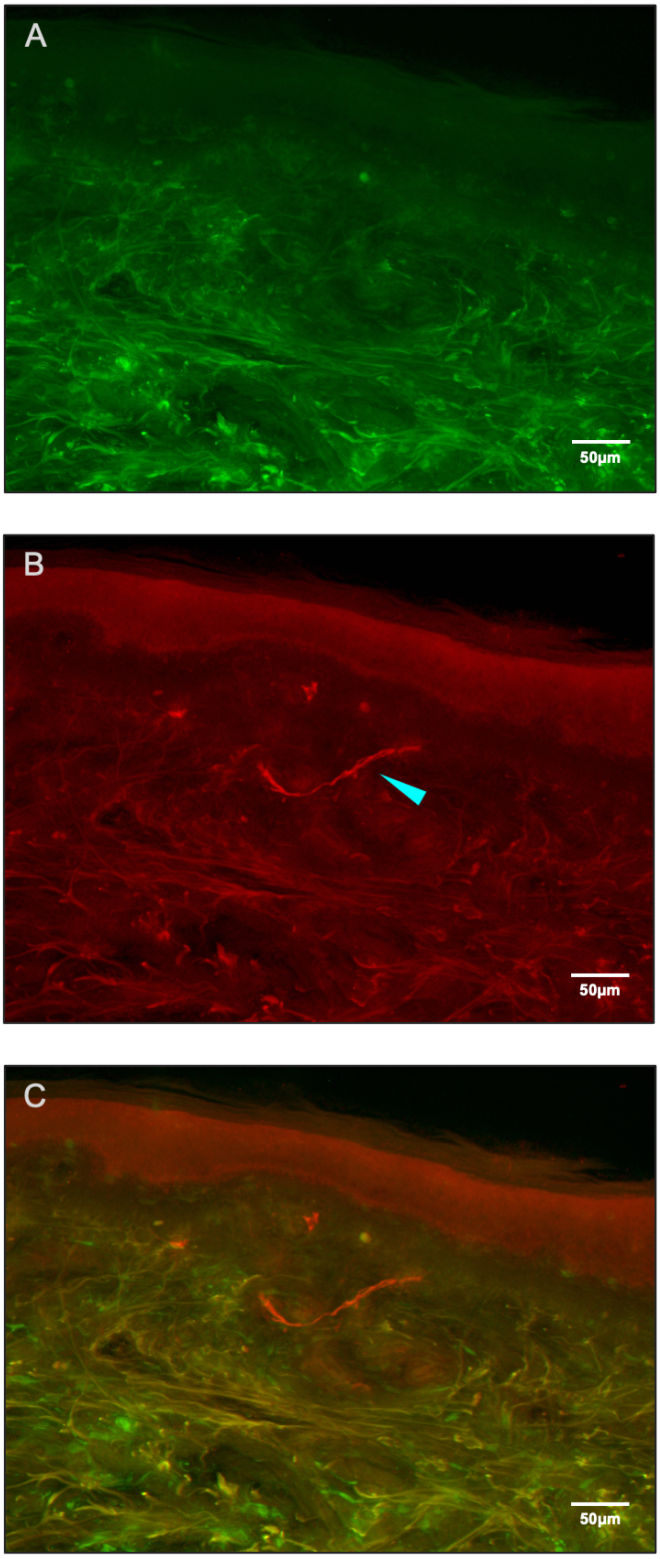

Figure 6: Double immunofluorescence staining of a skin biopsy section from a patient with type 2 diabetes and painful DSPN.
In this individual, no intraepidermal nerve fibres were detected (IENFD: zero fibres/mm) using either marker (**A**: protein gene product 9.5 (PGP9.5), **B**: growth-associated-protein-43 (GAP-43). Images show a segment in which dermal nerve fibres were detected using GAP-43 (arrow), but not with PGP9.5. **C** shows both markers combined in a merged image.

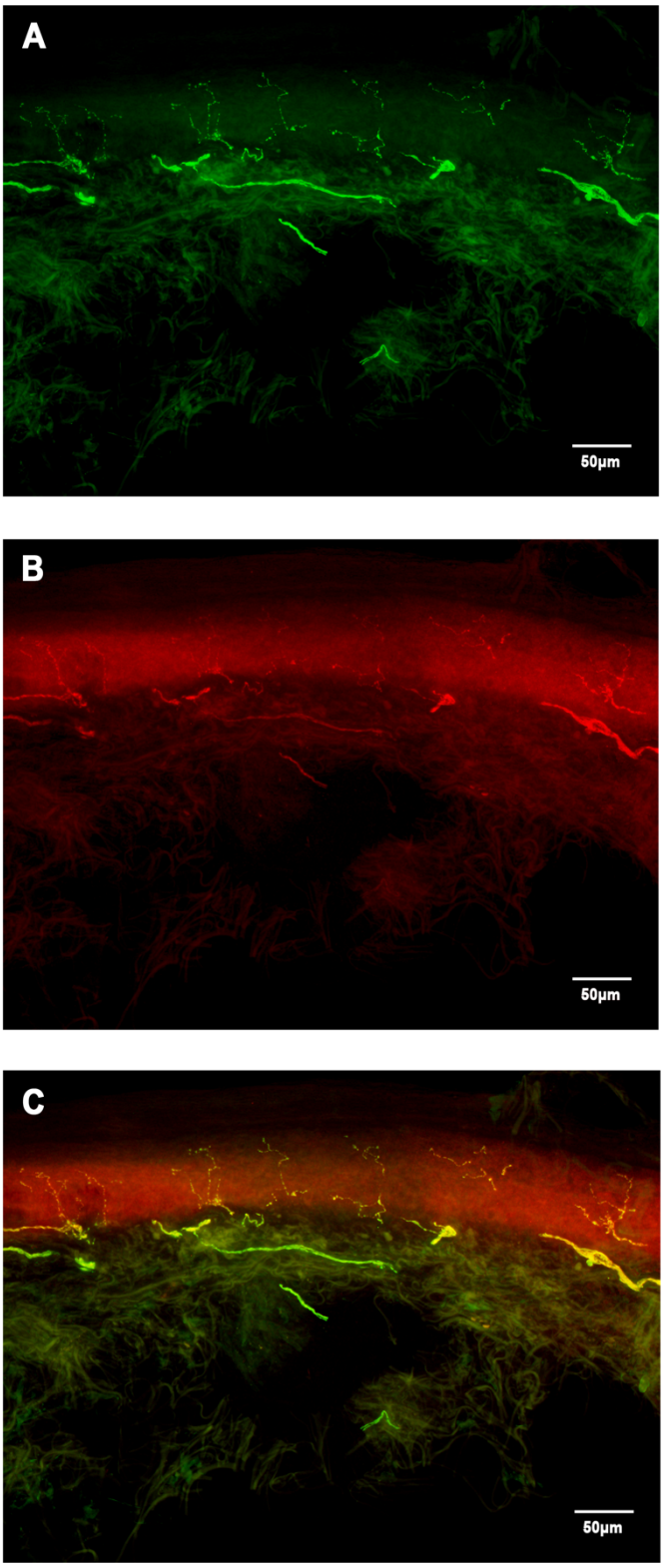

Figure 7: Double immunofluorescence images showing similar proportions of cutaneous nerve fibres.
A: protein gene product 9.5 (PGP9.5), **B:** growth-associated-protein-43 (GAP-43), **C:** both markers combined in a merged image.

3.8 Strengths and limitations

Major strengths of the present study are the relatively large groups of individuals with type 2 diabetes and healthy control persons with NGT, the extensive functional phenotyping using state-of-the-art methodology, including gold standard techniques to assess large fibre function (nerve conduction studies) and small fibre morphology (skin biopsy). Moreover, blinded assessment of both epidermal and dermal nerve fibres was performed using double immunofluorescence microscopy including sophisticated and time-consuming manual morphometric measurements. It should be emphasised that this study includes data from two different cohorts to examine both early type 2 diabetes and long-term type 2 diabetes with clinically manifest DSPN. However, prospective data is needed to explore the temporal sequence of our findings to explore the predictive value of skin biopsy biomarkers in predicting the course of DSPN as a painful or painless entity and their progression. Another limitation is the restriction to largely descriptive morphological and functional assessment which may not provide direct insights into pathogenetic mechanisms. Topical capsaicin application and excision axotomy models have been introduced to study nerve regeneration (95, 114), but these require sampling of multiple serial skin biopsies that are usually feasible in small cohorts only.

3.9 Conclusions

In conclusion, the present study demonstrates enhanced regenerative sprouting of dermal nerve fibres reflected by an increased DNFL GAP-43/PGP9.5 ratio in both DSPN phenotypes, particularly in painful DSPN, but not in recent-onset type 2 diabetes, accompanied by normal dermal, but reduced intraepidermal innervation. The extent of the dermal regenerative capacity is associated with more pronounced intraepidermal nerve fibre loss and peripheral nerve dysfunction in type 2 diabetes. Hence, a relative rather than absolute deficit in cutaneous nerve fibre regeneration can be assumed in type 2 diabetes patients with clinically manifest DSPN. We propose that the DNFL GAP-43/PGP9.5 ratio should be considered as a marker to assess the regenerative capacity of cutaneous nerve fibres. The potential of this biomarker to predict the progression or regression of DSPN or the susceptibility to regenerative pharmacotherapy should be established in future prospective observational studies and clinical trials.

4 References

1. Bönhof GJ, Herder C, Strom A, Papanas N, Roden M, Ziegler D. Emerging Biomarkers, Tools, and Treatments for Diabetic Polyneuropathy. Endocr Rev. 2019;40(1):153-92.

2. Herder C, Roden M, Ziegler D. Novel Insights into Sensorimotor and Cardiovascular Autonomic Neuropathy from Recent-Onset Diabetes and Population-Based Cohorts. Trends Endocrinol Metab. 2019;30(5):286-98.

3. Ziegler D, Papanas N, Vinik AI, Shaw JE. Epidemiology of polyneuropathy in diabetes and prediabetes. Handb Clin Neurol. 2014;126:3-22.

4. Happich M, John J, Stamenitis S, Clouth J, Polnau D. The quality of life and economic burden of neuropathy in diabetic patients in Germany in 2002—results from the Diabetic Microvascular Complications (DIMICO) study. Diabetes Res Clin Pract. 2008;81(2):223-30.

5. Landowski LM, Dyck PJ, Engelstad J, Taylor BV. Axonopathy in peripheral neuropathies: Mechanisms and therapeutic approaches for regeneration. J Chem Neuroanat. 2016;76(Pt A):19-27.

6. American Diabetes Association. Diagnosis and classification of diabetes mellitus. Diabetes Care. 2014;37 Suppl 1:S81-90.

7. American Diabetes Association. Classification and Diagnosis of Diabetes: Standards of Medical Care in Diabetes-2018. Diabetes Care. 2018;41(Suppl 1):S13-S27.

8. International Diabetes Federation. IDF Diabetes Atlas. 9th ed. Brussels, Belgium: International Diabetes Federation; 2019.

9. Tönnies T, Rockl S, Hoyer A, Heidemann C, Baumert J, Du Y, et al. Projected number of people with diagnosed Type 2 diabetes in Germany in 2040. Diabet Med. 2019;36(10):1217-25.

10. van Ommen B, Wopereis S, van Empelen P, van Keulen HM, Otten W, Kasteleyn M, et al. From Diabetes Care to Diabetes Cure-The Integration of Systems Biology, eHealth, and Behavioral Change. Front Endocrinol (Lausanne). 2018;8:381.

11. Al-Mrabeh A, Zhyzhneuskaya SV, Peters C, Barnes AC, Melhem S, Jesuthasan A, et al. Hepatic Lipoprotein Export and Remission of Human Type 2 Diabetes after Weight Loss. Cell Metab. 2020;31(2):233-49 e4.

12. Hallberg SJ, Gershuni VM, Hazbun TL, Athinarayanan SJ. Reversing Type 2 Diabetes: A Narrative Review of the Evidence. Nutrients. 2019;11(4).

13. Chowdhury TA. Diabetes remission: a realistic goal? Clin Med (Lond). 2018;18(2):116-7.

14. Strain WD, Paldanius PM. Diabetes, cardiovascular disease and the microcirculation. Cardiovasc Diabetol. 2018;17(1):57.

15. Solomon SD, Chew E, Duh EJ, Sobrin L, Sun JK, VanderBeek BL, et al. Diabetic Retinopathy: A Position Statement by the American Diabetes Association. Diabetes Care. 2017;40(3):412-8.

16. Lim A. Diabetic nephropathy - complications and treatment. Int J Nephrol Renovasc Dis. 2014;7:361-81.

17. Pop-Busui R, Boulton AJM, Feldman EL, Bril V, Freeman R, Malik RA, et al. Diabetic Neuropathy: A Position Statement by the American Diabetes Association. Diabetes Care. 2017;40(1):136-54.

18. Dyck PJ, Albers JW, Andersen H, Arezzo JC, Biessels GJ, Bril V, et al. Diabetic polyneuropathies: update on research definition, diagnostic criteria and estimation of severity. Diabetes Metab Res Rev. 2011;27(7):620-8.

19. Freeman R. Diabetic autonomic neuropathy. Handb Clin Neurol. 2014;126:63-79.

20. Vinik AI, Ziegler D. Diabetic cardiovascular autonomic neuropathy. Circulation. 2007;115(3):387-97.

21. Tesfaye S, Boulton AJM, Dyck PJ, Freeman R, Horowitz M, Kempler P, et al. Diabetic neuropathies: update on definitions, diagnostic criteria, estimation of severity, and treatments. Diabetes care. 2010;33(10):2285-93.

22. Ziegler D, Rathmann W, Dickhaus T, Meisinger C, Mielck A, Group KS. Prevalence of polyneuropathy in pre-diabetes and diabetes is associated with abdominal obesity and macroangiopathy: the MONICA/KORA Augsburg Surveys S2 and S3. Diabetes Care. 2008;31(3):464-9.

23. Szendroedi J, Saxena A, Weber KS, Strassburger K, Herder C, Burkart V, et al. Cohort profile: the German Diabetes Study (GDS). Cardiovasc Diabetol. 2016;15:59.

24. Pafili K, Papanas N, Maltezos E. Treatment of diabetic complications: how can we learn by seeking and blundering? Angiology. 2015;66(4):301-3.

25. Ziegler D, Strom A, Lobmann R, Reiners K, Rett K, Schnell O. High prevalence of diagnosed and undiagnosed polyneuropathy in subjects with and without diabetes participating in a nationwide educational initiative (PROTECT study). J Diabetes Complications. 2015;29(8):998-1002.

26. Ziegler D, Landgraf R, Lobmann R, Reiners K, Rett K, Schnell O, et al. Painful and painless neuropathies are distinct and largely undiagnosed entities in subjects participating in an educational initiative (PROTECT study). Diabetes Res Clin Pract. 2018;139:147-54.

27. Zochodne DW. Clinical features of diabetic polyneuropathy. Handb Clin Neurol. 2014;126:23-30.

28. Ziegler D. Diabetische Neuropathie. Internist (Berl). 2020;61(3):243-53.

29. Feldman EL, Stevens MJ, Thomas PK, Brown MB, Canal N, Greene DA. A practical two-step quantitative clinical and electrophysiological assessment for the diagnosis and staging of diabetic neuropathy. Diabetes care. 1994;17(11):1281-9.

30. Bril V, Tomioka S, Buchanan RA, Perkins BA, mTCNS Study Group. Reliability and validity of the modified Toronto Clinical Neuropathy Score in diabetic sensorimotor polyneuropathy. Diabet Med. 2009;26(3):240-6.

31. Singleton JR, Bixby B, Russell JW, Feldman EL, Peltier A, Goldstein J, et al. The Utah Early Neuropathy Scale: a sensitive clinical scale for early sensory predominant neuropathy. J Peripher Nerv Syst. 2008;13(3):218-27.

32. Young MJ, Boulton AJ, MacLeod AF, Williams DR, Sonksen PH. A multicentre study of the prevalence of diabetic peripheral neuropathy in the United Kingdom hospital clinic population. Diabetologia. 1993;36(2):150-4.

33. Apfel SC, Asbury AK, Bril V, Burns TM, Campbell JN, Chalk CH, et al. Positive neuropathic sensory symptoms as endpoints in diabetic neuropathy trials. J Neurol Sci. 2001;189(1-2):3-5.

34. Neundorfer B, Thomas P. Symmetric distal polyneuropathy. In: Gries FA, Low PA, Cameron NE, editors. Textbook of Diabetic Neuropathy. New York: Thieme; 2003. p. 199-202.

35. Treede RD, Jensen TS, Campbell JN, Cruccu G, Dostrovsky JO, Griffin JW, et al. Neuropathic pain: redefinition and a grading system for clinical and research purposes. Neurology. 2008;70(18):1630-5.

36. Rosenberger DC, Blechschmidt V, Timmerman H, Wolff A, Treede RD. Challenges of neuropathic pain: focus on diabetic neuropathy. J Neural Transm (Vienna). 2020:doi: 10.1007/s00702-020-2145-7. [Epub ahead of print].

37. Tesfaye S, Boulton AJM, Dickenson AH. Mechanisms and management of diabetic painful distal symmetrical polyneuropathy. Diabetes care. 2013;36(9):2456-65.

38. Spallone V, Greco C. Painful and painless diabetic neuropathy: one disease or two? Curr Diab Rep. 2013;13(4):533-49.

39. Tesfaye S, Vileikyte L, Rayman G, Sindrup SH, Perkins BA, Baconja M, et al. Painful diabetic peripheral neuropathy: consensus recommendations on diagnosis, assessment and management. Diabetes Metab Res Rev. 2011;27(7):629-38.

40. Maier C, Baron R, Tolle TR, Binder A, Birbaumer N, Birklein F, et al. Quantitative sensory testing in the German Research Network on Neuropathic Pain (DFNS): somatosensory abnormalities in 1236 patients with different neuropathic pain syndromes. Pain. 2010;150(3):439-50.

41. Themistocleous AC, Ramirez JD, Shillo PR, Lees JG, Selvarajah D, Orengo C, et al. The Pain in Neuropathy Study (PiNS): a cross-sectional observational study determining the somatosensory phenotype of painful and painless diabetic neuropathy. Pain. 2016;157(5):1132-45.

42. Raputova J, Srotova I, Vlckova E, Sommer C, Uceyler N, Birklein F, et al. Sensory phenotype and risk factors for painful diabetic neuropathy: a cross-sectional observational study. Pain. 2017;158(12):2340-53.

43. Dyck PJ. Methodology for conduct of epidemiologic surveys and randomized controlled trials of diabetic polyneuropathy. Handb Clin Neurol. 2014;126:335-8.

44. Backonja MM, Attal N, Baron R, Bouhassira D, Drangholt M, Dyck PJ, et al. Value of quantitative sensory testing in neurological and pain disorders: NeuPSIG consensus. Pain. 2013;154(9):1807-19.

45. Javed S, Petropoulos IN, Tavakoli M, Malik RA. Clinical and diagnostic features of small fiber damage in diabetic polyneuropathy. Handb Clin Neurol. 2014;126:275-90.

46. Dyck PJ, Zimmerman BR, Vilen TH, Minnerath SR, Karnes JL, Yao JK, et al. Nerve glucose, fructose, sorbitol, myo-inositol, and fiber degeneration and regeneration in diabetic neuropathy. N Engl J Med. 1988;319(9):542-8.

47. Gilliatt RW, Willison RG. Peripheral nerve conduction in diabetic neuropathy. J Neurol Neurosurg Psychiatry. 1962;25:11-8.

48. Dupuis JE, Li J, Callaghan BC, Reynolds EL, London ZN. Bilateral nerve conduction studies in the evaluation of distal symmetric polyneuropathy. Muscle Nerve. 2019;60(3):305-7.

49. Bromberg MB. An electrodiagnostic approach to the evaluation of peripheral neuropathies. Phys Med Rehabil Clin N Am. 2013;24(1):153-68.

50. Said G. Diabetic neuropathy. Handb Clin Neurol. 2013;115:579-89.

51. Girach A, Julian TH, Varrassi G, Paladini A, Vadalouka A, Zis P. Quality of Life in Painful Peripheral Neuropathies: A Systematic Review. Pain Res Manag. 2019;2019:2091960.

52. Vileikyte L, Leventhal H, Gonzalez JS, Peyrot M, Rubin RR, Ulbrecht JS, et al. Diabetic peripheral neuropathy and depressive symptoms: the association revisited. Diabetes Care. 2005;28(10):2378-83.

53. Volmer-Thole M, Lobmann R. Neuropathy and Diabetic Foot Syndrome. Int J Mol Sci. 2016;17(6).

54. Navarro-Flores E, Cauli O. Quality of life in individuals with diabetic foot syndrome. Endocr Metab Immune Disord Drug Targets. 2020.

55. Armstrong DG, Boulton AJM, Bus SA. Diabetic Foot Ulcers and Their Recurrence. N Engl J Med. 2017;376(24):2367-75.

56. Boulton AJM. Diabetic neuropathy and foot complications. Handb Clin Neurol. 2014;126:97-107.

57. Reiber GE, Ledoux WR. Epidemiology of diabetic foot ulcers and amputations: Evidence for prevention. In: Williams R, Herman W, Kinmoth AL, Wareham NJ, editors. The evidence base for diabetes care. Chichester: John Wiley & Sons; 2002.

58. Rogers LC, Frykberg RG. The Charcot foot. Med Clin North Am. 2013;97(5):847-56.

59. Frykberg RG, Mendeszoon E. Management of the diabetic Charcot foot. Diabetes Metab Res Rev. 2000;16 Suppl 1:S59-65.

60. Ziegler D, Schneider E, Boess FG, Berggren L, Birklein F. Impact of comorbidities on pharmacotherapy of painful diabetic neuropathy in clinical practice. J Diabetes Complications. 2014;28(5):698-704.

61. Sifuentes-Franco S, Pacheco-Moises FP, Rodriguez-Carrizalez AD, Miranda-Diaz AG. The Role of Oxidative Stress, Mitochondrial Function, and Autophagy in Diabetic Polyneuropathy. J Diabetes Res. 2017;2017:1673081.

62. Ziegler D, Strom A, Bruggemann J, Ziegler I, Ringel B, Puttgen S, et al. Overexpression of cutaneous mitochondrial superoxide dismutase in recent-onset type 2 diabetes. Diabetologia. 2015;58(7):1621-5.

63. Fernyhough P, McGavock J. Mechanisms of disease: Mitochondrial dysfunction in sensory neuropathy and other complications in diabetes. Handb Clin Neurol. 2014;126:353-77.

64. Suzuki D, Miyata T. Carbonyl stress in the pathogenesis of diabetic nephropathy. Intern Med. 1999;38(4):309-14.

65. Mizisin AP. Mechanisms of diabetic neuropathy: Schwann cells. Handb Clin Neurol. 2014;126:401-28.

66. Obrosova IG, Li F, Abatan OI, Forsell MA, Komjati K, Pacher P, et al. Role of poly(ADP-ribose) polymerase activation in diabetic neuropathy. Diabetes. 2004;53(3):711-20.

67. Shakeel M. Recent advances in understanding the role of oxidative stress in diabetic neuropathy. Diabetes Metab Syndr. 2015;9(4):373-8.

68. Brownlee M. The pathobiology of diabetic complications: a unifying mechanism. Diabetes. 2005;54(6):1615-25.

69. Sima AA, Zhang W. Mechanisms of diabetic neuropathy: axon dysfunction. Handb Clin Neurol. 2014;126:429-42.

70. Feldman EL, Nave KA, Jensen TS, Bennett DL. New Horizons in Diabetic Neuropathy: Mechanisms, Bioenergetics, and Pain. Neuron. 2017;93(6):1296-313.

71. Chilelli NC, Burlina S, Lapolla A. AGEs, rather than hyperglycemia, are responsible for microvascular complications in diabetes: a "glycoxidation-centric" point of view. Nutr Metab Cardiovasc Dis. 2013;23(10):913-9.

72. Toth C. Diabetes and neurodegeneration in the brain. Handb Clin Neurol. 2014;126:489-511.

73. Suryavanshi SV, Kulkarni YA. NF-kappabeta: A Potential Target in the Management of Vascular Complications of Diabetes. Front Pharmacol. 2017;8:798.

74. Andersen ST, Witte DR, Dalsgaard EM, Andersen H, Nawroth P, Fleming T, et al. Risk Factors for Incident Diabetic Polyneuropathy in a Cohort With Screen-Detected Type 2 Diabetes Followed for 13 Years: ADDITION-Denmark. Diabetes Care. 2018;41(5):1068-75.

75. Østergaard L, Finnerup NB, Terkelsen AJ, Olesen RA, Drasbek KR, Knudsen L, et al. The effects of capillary dysfunction on oxygen and glucose extraction in diabetic neuropathy. Diabetologia. 2015;58(4):666-77.

76. Herder C, Kannenberg JM, Carstensen-Kirberg M, Strom A, Bönhof GJ, Rathmann W, et al. A Systemic Inflammatory Signature Reflecting Cross Talk Between Innate and Adaptive Immunity Is Associated With Incident Polyneuropathy: KORA F4/FF4 Study. Diabetes. 2018;67(11):2434-42.

77. Ziegler D, Strom A, Bönhof GJ, Kannenberg JM, Heier M, Rathmann W, et al. Deficits in systemic biomarkers of neuroinflammation and growth factors promoting nerve regeneration in patients with type 2 diabetes and polyneuropathy. BMJ Open Diabetes Res Care. 2019;7(1):e000752.

78. Ebenezer G, Polydefkis M. Epidermal innervation in diabetes. Handb Clin Neurol. 2014;126:261-74.

79. Gasparotti R, Padua L, Briani C, Lauria G. New technologies for the assessment of neuropathies. Nat Rev Neurol. 2017;13(4):203-16.

80. Yasuda H, Terada M, Maeda K, Kogawa S, Sanada M, Haneda M, et al. Diabetic neuropathy and nerve regeneration. Prog Neurobiol. 2003;69(4):229-85.

81. Zochodne DW. The challenges and beauty of peripheral nerve regrowth. J Peripher Nerv Syst. 2012;17(1):1-18.

82. Malik RA, Veves A, Tesfaye S, Smith G, Cameron N, Zochodne D, et al. Small fibre neuropathy: role in the diagnosis of diabetic sensorimotor polyneuropathy. Diabetes Metab Res Rev. 2011;27(7):678-84.

83. Lauria G, Lombardi R, Camozzi F, Devigili G. Skin biopsy for the diagnosis of peripheral neuropathy. Histopathology. 2009;54(3):273-85.

84. Lauria G, Devigili G. Skin biopsy as a diagnostic tool in peripheral neuropathy. Nat Clin Pract Neurol. 2007;3(10):546-57.

85. Ziegler D, Papanas N, Zhivov A, Allgeier S, Winter K, Ziegler I, et al. Early detection of nerve fiber loss by corneal confocal microscopy and skin biopsy in recently diagnosed type 2 diabetes. Diabetes. 2014;63(7):2454-63.

86. Kennedy WR, Wendelschafer-Crabb G, Johnson T. Quantitation of epidermal nerves in diabetic neuropathy. Neurology. 1996;47(4):1042-8.

87. Lauria G, Bakkers M, Schmitz C, Lombardi R, Penza P, Devigili G, et al. Intraepidermal nerve fiber density at the distal leg: a worldwide normative reference study. J Peripher Nerv Syst. 2010;15(3):202-7.

88. Denny JB. Molecular mechanisms, biological actions, and neuropharmacology of the growth-associated protein GAP-43. Curr Neuropharmacol. 2006;4(4):293-304.

89. Benowitz LI, Routtenberg A. GAP-43: an intrinsic determinant of neuronal development and plasticity. Trends Neurosci. 1997;20(2):84-91.

90. Verze L, Viglietti-Panzica C, Maurizo S, Sica M, Panzica G. Distribution of GAP-43 nerve fibers in the skin of the adult human hand. Anat Rec A Discov Mol Cell Evol Biol. 2003;272(1):467-73.

91. Fantini F, Johansson O. Expression of growth-associated protein 43 and nerve growth factor receptor in human skin: a comparative immunohistochemical investigation. J Invest Dermatol. 1992;99(6):734-42.

92. Bursova S, Dubovy P, Vlckova-Moravcova E, Nemec M, Klusakova I, Belobradkova J, et al. Expression of growth-associated protein 43 in the skin nerve fibers of patients with type 2 diabetes mellitus. J Neurol Sci. 2012;315(1-2):60-3.

93. Cheng HT, Dauch JR, Porzio MT, Yanik BM, Hsieh W, Smith AG, et al. Increased axonal regeneration and swellings in intraepidermal nerve fibers characterize painful phenotypes of diabetic neuropathy. The journal of pain : official journal of the American Pain Society. 2013;14(9):941-7.

94. Narayanaswamy H, Facer P, Misra VP, Timmers M, Byttebier G, Meert T, et al. A longitudinal study of sensory biomarkers of progression in patients with diabetic peripheral neuropathy using skin biopsies. J Clin Neurosci. 2012;19(11):1490-6.

95. Polydefkis M, Hauer P, Sheth S, Sirdofsky M, Griffin JW, McArthur JC. The time course of epidermal nerve fibre regeneration: studies in normal controls and in people with diabetes, with and without neuropathy. Brain. 2004;127(Pt 7):1606-15.

96. Jambart S, Ammache Z, Haddad F, Younes A, Hassoun A, Abdalla K, et al. Prevalence of painful diabetic peripheral neuropathy among patients with diabetes mellitus in the Middle East region. J Int Med Res. 2011;39(2):366-77.

97. Van Acker K, Bouhassira D, De Bacquer D, Weiss S, Matthys K, Raemen H, et al. Prevalence and impact on quality of life of peripheral neuropathy with or without neuropathic pain in type 1 and type 2 diabetic patients attending hospital outpatients clinics. Diabetes Metab. 2009;35(3):206-13.

98. Halawa MR, Karawagh A, Zeidan A, Mahmoud AE, Sakr M, Hegazy A. Prevalence of painful diabetic peripheral neuropathy among patients suffering from diabetes mellitus in Saudi Arabia. Curr Med Res Opin. 2010;26(2):337-43.

99. Truini A, Spallone V, Morganti R, Tamburin S, Zanette G, Schenone A, et al. A cross-sectional study investigating frequency and features of definitely diagnosed diabetic painful polyneuropathy. Pain. 2018;159(12):2658-66.

100. Umapathi T, Tan WL, Loke SC, Soon PC, Tavintharan S, Chan YH. Intraepidermal nerve fiber density as a marker of early diabetic neuropathy. Muscle Nerve. 2007;35(5):591-8.

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

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

103. Sorensen L, Molyneaux L, Yue DK. The relationship among pain, sensory loss, and small nerve fibers in diabetes. Diabetes Care. 2006;29(4):883-7.

104. Scheytt S. Quantifizierung von GAP 43 positiven intraepidermalen Nervenfasern bei Patienten mit Polyneuropathie und bei gesunden Kontrollen. Würzburg: University of Würzburg; 2013.

105. Galosi E, La Cesa S, Di Stefano G, Karlsson P, Fasolino A, Leone C, et al. A pain in the skin. Regenerating nerve sprouts are distinctly associated with ongoing burning pain in patients with diabetes. Eur J Pain. 2018;22(10):1727-34.

106. Krishnan ST, Quattrini C, Jeziorska M, Malik RA, Rayman G. Abnormal LDIflare but normal quantitative sensory testing and dermal nerve fiber density in patients with painful diabetic neuropathy. Diabetes Care. 2009;32(3):451-5.

107. Vlckova-Moravcova E, Bednarik J, Dusek L, Toyka KV, Sommer C. Diagnostic validity of epidermal nerve fiber densities in painful sensory neuropathies. Muscle Nerve. 2008;37(1):50-60.

108. Pál E, Fülöp K, Tóth P, Delli G, Pfund Z, Janzky J, et al. Small Fiber Neuropathy: Clinicopathological Correlations. Behav Neurol. 2020;2020(4):1-7.

109. Bechakra M, Nieuwenhoff MD, van Rosmalen J, Groeneveld GJ, Scheltens-de Boer M, Sonneveld P, et al. Clinical, electrophysiological, and cutaneous innervation changes in patients with bortezomib-induced peripheral neuropathy reveal insight into mechanisms of neuropathic pain. Mol Pain. 2018;14:1744806918797042.

110. Costigan M, Scholz J, Woolf CJ. Neuropathic pain: a maladaptive response of the nervous system to damage. Annu Rev Neurosci. 2009;32:1-32.

111. Navarro X, Vivo M, Valero-Cabre A. Neural plasticity after peripheral nerve injury and regeneration. Prog Neurobiol. 2007;82(4):163-201.

112. Orstavik K, Namer B, Schmidt R, Schmelz M, Hilliges M, Weidner C, et al. Abnormal function of C-fibers in patients with diabetic neuropathy. J Neurosci. 2006;26(44):11287-94.

113. Wu LY, Li M, Qu ML, Li X, Pi LH, Chen Z, et al. High glucose up-regulates Semaphorin 3A expression via the mTOR signaling pathway in keratinocytes: A potential mechanism and therapeutic target for diabetic small fiber neuropathy. Mol Cell Endocrinol. 2018;472:107-16.

114. Ebenezer GJ, O'Donnell R, Hauer P, Cimino NP, McArthur JC, Polydefkis M. Impaired neurovascular repair in subjects with diabetes following experimental intracutaneous axotomy. Brain. 2011;134(Pt 6):1853-63.

115. Püttgen S, Bönhof GJ, Strom A, Müssig K, Szendroedi J, Roden M, et al. Augmented Corneal Nerve Fiber Branching in Painful Compared With Painless Diabetic Neuropathy. J Clin Endocrinol Metab. 2019;104(12):6220-8.

116. Cheng C, Guo GF, Martinez JA, Singh V, Zochodne DW. Dynamic plasticity of axons within a cutaneous milieu. J Neurosci. 2010;30(44):14735-44.

117. Xie W, Strong JA, Zhang JM. Active Nerve Regeneration with Failed Target Reinnervation Drives Persistent Neuropathic Pain. eNeuro. 2017;4(1):ENEURO.0008-17.2017.

118. Blesneac I, Themistocleous AC, Fratter C, Conrad LJ, Ramirez JD, Cox JJ, et al. Rare NaV1.7 variants associated with painful diabetic peripheral neuropathy. Pain. 2018;159(3):469-80.

119. Alsaloum M, Estacion M, Almomani R, Gerrits MM, Bonhof GJ, Ziegler D, et al. A gain-of-function sodium channel beta2-subunit mutation in painful diabetic neuropathy. Mol Pain. 2019;15:1744806919849802.

120. Rage M, Van Acker N, Facer P, Shenoy R, Knaapen MWM, Timmers M, et al. The time course of CO2 laser-evoked responses and of skin nerve fibre markers after topical capsaicin in human volunteers. Clin Neurophysiol. 2010;121(8):1256-66.

121. Anand P, Elsafa E, Privitera R, Naidoo K, Yiangou Y, Donatien P, et al. Rational treatment of chemotherapy-induced peripheral neuropathy with capsaicin 8% patch: from pain relief towards disease modification. J Pain Res. 2019;12:2039-52.

122. Scheytt S, Riediger N, Braunsdorf S, Sommer C, Uceyler N. Increased gene expression of growth associated protein-43 in skin of patients with early-stage peripheral neuropathies. J Neurol Sci. 2015;355(1-2):131-7.

5 Acknowledgements

I would like to thank Prof. Dr. Dan Ziegler for being an outstanding mentor. Thank You for your excellent guidance, expertise, and kindness. Thank You for your role in designing and conducting the PROPANE study and your contribution to the German Diabetes Study.

I would also like to acknowledge:

Dr. Alexander Strom for introducing me to statistics and the methodological background, and for his endless patience and support.

Prof. Dr. Michael Roden as scientific director of the Institute for Clinical Diabetology and Principal Investigator of the German Diabetes Study for the opportunity to work at the German Diabetes Center.

Jutta Brüggemann (†) for introducing me to immunofluorescence microscopy. We miss her as a wonderful colleague and person.

Bernd Ringel, Marie-Ninon Krahnke-Schoelzel, and Iris Ziegler for their excellent laboratory work.

Sonja Püttgen for her valuable contribution to the PROPANE study.

Francesco Battiato, Nadine Reuß, Maria Schroers-Teuber (†), and Justyna Schubert for their excellent neurophysiological work.

Dr. Kálman B. Bódis, Dr. Oana-Patricia Zaharia, Prof. Dr. Julia Szendrödi, and Prof. Dr. Karsten Müssig for their valuable contribution to the German Diabetes Study.

Dr. Lina Persechini for language counselling.