IMPROVING ASSESSMENTS in SMALL FIBER NEUROPATHY

PROEFSCHRIFT

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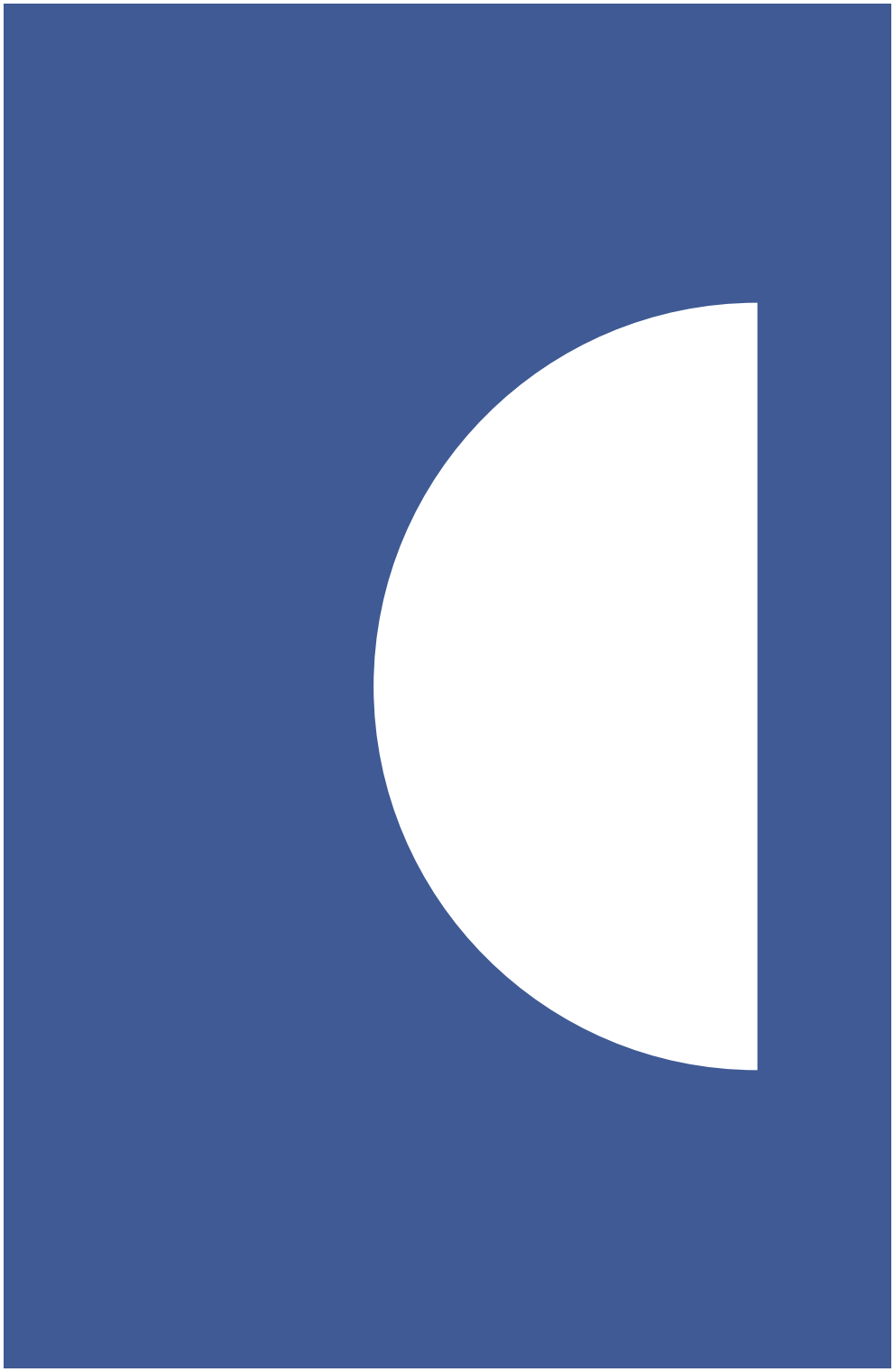
Table of contents

PART 1

INTRODUCTION



CHAPTER 1

GENERAL INTRODUCTION AND OUTLINE OF THE THESIS

Introduction

The International Association for the Study of Pain (IASP) defines neuropathic pain as pain caused by a lesion or disease of the somatosensory nervous system. Small fiber neuropathy (SFN) refers to a subtype of polyneuropathies that is dominated by neuropathic pain, caused by affected small nerve fibers, namely the myelinated A-delta fibers and unmyelinated C fibers. Additionally, the thinly myelinated preganglionic and unmyelinated postganglionic autonomic fibers are damaged.[1-3]

The clinical picture of SFN is characterized by positive and negative symptoms and/or signs related to pain, temperature and autonomic functions.[4,5] Patients usually describe their symptoms as burning, stabbing and/or shooting pain and they may have a decreased pain-, temperature- and/or pinprick sensation. Autonomic symptoms may also occur, such as dry eyes or mouth, palpitations, orthostatic hypotension, bowel and micturition disturbances, diminished ejaculation or vaginal lubrication, sweat changes and hot flushes.

The clinical presentation differs between patients, from mild neuropathic symptoms with some autonomic complaints to severe pain with a variety of positive autonomic symptoms which have a high impact on patients' Quality of Life (QoL).[6] SFN generally presents as a length dependent pain syndrome although non-length dependent patterns do occur.[7,8] SFN patients may have autonomic complaints exclusively, or an overlap of somatic and autonomic symptoms, which (partly) can be explained by the presence of specific underlying conditions. Some accompanying symptoms are at times unacknowledged due to their non-specific nature such as fatigue, daily performance decline, anxiety, and depression.[9]

SFN is associated with various underlying conditions including diabetes mellitus, autoimmune diseases (e.g., celiac disease, sarcoidosis, Sjögren's syndrome), infections (e.g., human immunodeficiency virus, hepatitis C), alcohol dependence and exposure to toxins, nutritional deficiencies (e.g., vitamin B12 deficiency), amyloidosis, paraneoplastic syndromes, and hereditary causes (e.g., Fabry's disease, sodium channelopathies). However, comprehensive testing fails to identify an underlying condition up to 50% of patients with SFN.[10]

Diagnostic assessments

Based on diagnostic criteria, SFN can be graded into possible, probable or definite SFN.[1] Small fiber dysfunction cannot be captured by diagnostic tests performed for diagnosing large fiber neuropathy (i.e nerve conduction studies). The diagnostic landscape to confirm the presence of SFN has a great variability. A recent systematic review showed that substantial heterogeneity exists in the diagnostic criteria used for identifying patients with SFN in clinical research studies.[11] In clinical practice, no broadly accepted guideline exists as to the appropriate combination of signs and symptoms or specific investigations necessary to confirm an accurate diagnosis of SFN. However, skin biopsies for determining the intra-epidermal nerve fiber density (IENFD) and quantitative sensory testing (QST) for testing small nerve fiber function are the universally recommended tests for SFN.[12,13] Typical symptoms and signs of small nerve fiber damage, and absence of large nerve fiber involvement are required for the diagnosis SFN. In other words, the diagnosis SFN is set by the combination of typical symptoms and signs at examination (neuropathic pain, allodynia, hyperalgesia), an abnormal IENFD in skin biopsy and/or an abnormal QST[5,14], in the absence of large fiber involvement.

A skin biopsy permits to demonstrate the predominant and sometimes exclusive degeneration of small nerve fibers. It is an easy, but time-consuming and therefore expensive, procedure that can be performed by a physician or physician's assistant. Although it is an invasive technique, it causes minimal pain and discomfort. The European Federation of Neurological Communities recommended a skin biopsy with staining of protein gene product 9.5 (PGP 9.5) as a grade A recommendation.[12] It is a reliable tool to confirm the diagnosis SFN, by using age- and gender specific normative values.[15] Diagnosis of SFN is made when the nerve fiber density is below the 5th percentile. Guidelines for the use of skin biopsy in clinical practice have been published as well, providing standardization of the technical procedures, including the counting procedure.[12,16] It should be emphasized that skin biopsy is a tool to diagnose small nerve fiber damage rather than small nerve fiber dysfunction *per se*. Recently, it was showed that up to 60% of large SFN cohorts showed a normal IENFD.[5,10,17,18] The difference in sensitivity could be explained by methodological differences, since in one of the studies signs and symptoms as criteria for SFN were not included, leading more likely to observe a normal IENFD.

QST is used to determine the functional impairment of small nerve fibers by measuring warm, cool and pain thresholds.[13] QST is a psychophysical test in nature, requiring cooperation from the patient. QST seems to have a relatively poor power in terms of localization of the injury in the somatosensory system.[19,20] If abnormal, the result may signal dysfunction between the receptor apparatus, the primary sensory cortex, and the association cortex. Furthermore, psychological factors figure prominently in sensory function perception. Other factors may influence the results, such as electrode size, site of stimulation, and frequency and rate of change of the stimuli. Because the environment of the test laboratory and instructions to the patients may differ between laboratories, local normative values were suggested, but are not always feasible.[21]

Sensitivity of skin biopsy and specificity of the QST are moderate. Nevertheless, the diagnostic criteria for SFN have been defined[5,14], but a ´gold standard´ for clinical practice and research is not available. Therefore, new diagnostics with a higher sensitivity and higher specificity might be of additional value to confirm an accurate diagnosis of SFN. Also, tests that have lower costs and are non-invasive are preferable to use in a diagnostic logarithm. In this thesis, two existing diagnostic tools, with the potential of being of additional value in the diagnosis of SFN, will be presented.

Corneal confocal microscopy

Corneal confocal microscopy (CCM) is a non-invasive technique for detecting small nerve fiber loss in the cornea. The cornea is innervated by small nerve fibers of trigeminal origin that enter through the middle third of the stroma. These fibers are then visualized by means of the CCM and quantification can take place, based on the pictures that are obtained with this technique. The use of this tool is limited in that it requires specific equipment and trained technicians and examiners. Nowadays, it is mostly used for research purposes. The function of the confocal microscope is to produce a point source of light and reject out-of-focus light, which provides the ability to image deep into tissues with high resolution. The basic principle of confocal microscopy is that illumination and detection optics are focused on the same diffraction-limited spot, which is moved over the sample to build the complete image on the detector. The microscopy provides optical sectioning while lessening the haze observed in standard light microscopy. To date, CCM has predominantly been used to assess patients with diabetes mellitus.[22-26]

In diabetic neuropathy, CCM showed a progressive reduction in corneal nerve fiber density and branch density in patients with an abnormal IENFD. However, the CCM quantifies small fiber damage rapidly and non-invasively and detects earlier stages of nerve damage compared with IENF pathology.[27]

CCM is also a surrogate endpoint for evaluating therapeutic efficacy in clinical trials of patients with diabetic neuropathy[28], even to detect regeneration of small nerve fiber, after pancreas transplantation.[29]

In SFN, CCM was also reported to be sensitive and to correlate with a variety of parameters in a small group of SFN patients.[30] International normative reference values has been documented for these CCM parameters.[31] In this thesis, the clinical application of the CCM, as a possible additional tool in the diagnostic algorithm in SFN, will be addressed.

Stimulated Skin Wrinkling

Skin wrinkling upon water immersion has been used as an indicator of autonomic limb nerve function for more than 80 years. Until recently, routine use of the test has been hampered by a poor understanding of the physiology and lack of standardization. The process underlying stimulated skin wrinkling has been identified as dependent on digital vasoconstriction mediated via sympathetic nerve fibers.[32] Vasoconstriction is postulated to drive wrinkling through loss of digit volume, which induces a negative pressure in the digit pulp and exerts a downward pull on the underlying skin and ultimately results in wrinkles. Substituting water with EMLA for inducing wrinkles have made the test easier to use it on bedside.[33]

In current clinical practice, skin wrinkling for assessing autonomic dysfunction in patients with SFN-like symptoms is performed in the hands and/or feet, using a published 5-point visual scale. This method of grading is subjective and the degree of natural wrinkles due to age and/or gender has not been taken into consideration.[34] For example, skin elasticity, extensibility and echogenicity all decrease with age.[35] Considering the mentioned limitations in current SFN diagnostics, a more sensitive, specific and preferably non-invasive screening tool to detect small nerve fiber dysfunction would be of great value. In this thesis, normative values by means of the grading method are presented and its

applicability for clinical use is investigated. Also, a quantitative technique for evaluating stimulated skin wrinkling will be introduced.

Genetic assessment of peripheral neuropathies

A genetic substrate for neuropathic pain is an accepted hypothesis in the scientific community. In the previous decade, novel pathogenic mutations have been identified in *SCN9A, SCN10A*, and *SCN11A* genes encoding for three sodium channels ($Na_v1.7$, $Na_v1.8$ and $Na_v1.9$ respectively)[36-40], known to play a critical role in the generation and conduction of action potentials in nociceptors and their terminal axons. Previous findings suggest that sodium channel mutations can change the physiological properties of nociceptors and impair also the integrity of their neurites, eventually leading to the degeneration of small nerve fibers.[41] The identification of mechanisms underlying these changes in key neuronal structures would allow to bridge functional abnormalities and structural changes in nociceptors. Evidence suggested that $Na_v1.7$ acts within the dorsal horn to facilitate synaptic transmission of pain signals to second-order pain-signaling neurons.[42] Based on its distribution and physiological features, $Na_v1.7$ is poised as a molecular gatekeeper of pain detection at peripheral nociceptors and is considered a 'threshold channel' for pain sensation. Gain-of-function missense mutation in the *SCN9A* gene, encoding for $Na_v1.7$, was first found in two rare human painful conditions: inherited erythromelalgia (IEM) and paroxysmal extreme pain disorder (PEPD).[43,44] These disorders are dominated by severe changes in the sensitivity to pain and typical alternations of the electrophysiological properties of DRG nociceptors, which may explain the occurrence of symptoms in patients. IEM, also termed as 'primary erythromelalgia', is characterized by burning pain and redness in the extremities in response to mild warmth. PEPD, previously named familial rectal pain syndrome, is characterized by attacks of excruciating deep burning pain often in de rectal, ocular, or jaw areas, with a start typically in infancy. The rectal pain is accompanied with skin flushing of the lower or upper body or face and can present in a harlequin pattern[45], which can alternate between the left and right sides of the body during different pain episodes.[46] Attacks can be triggered by factors such as defecation, cold wind, eating, and emotion. Furthermore, gain-of-function mutations of voltage-gated sodium channels $Na_v1.7$, $Na_v1.8$ and $Na_v1.9$ have been identified in SFN and may play a key role in the pathogenesis of SFN and axonal degeneration.[38,47-50] Increased Na^+ influx into axons expressing

mutant $Na_v1.7$ channels might trigger calcium influx into IENF and their small-diameter parent axons via reverse (Ca^{2+}-importing) Na_+-Ca^{2+} exchange leading to axonal degeneration.[47]

Moreover, in vitro studies showed a decreased length of DRG neurites expressing the $Na_v1.7$ gain-of-function variant I228M.[51] Estacion et al. found neurite degeneration after several weeks in vitro in $Na_v1.7$ mutant G856D challenged with two stressors, including extended depolarization and inhibition of glycolysis.[47] No degeneration was observed in wildtype $Na_v1.7$–expressing neurons. This observation suggests that the expression of mutated sodium channels, in combination with additional stressor(s) might lead to loss of IENFs. As with all in vitro studies of neurodegenerative disorders, cultured neurons do not fully recapitulate the in vivo situation. The time- and length-dependent patterns of axonal damage cannot fully mimic the situation that occurs in patients with SFN.

To expand the unraveling of the genetic architecture of neuropathic pain conditions, the PROPANE study ('Probing the role of sodium channels in painful neuropathies') aimed to identify novel variants in the *SCN3A, SCN7A-SCN11A, SCN1B-4B* genes in painful and painless (diabetic) neuropathies and aimed to provide a list of new candidate pain-related genes. The stratification of patients based on pain-inducing and pain-protective sodium channel gene mutations and novel gene variants, will identify biomarkers for targeted treatment strategies.

General aim of the thesis

The general aim of this thesis is to improve the clinical and genetic assessments in the diagnosis of SFN. In this thesis, studies are reported that investigate (1) the applicability of existing tests in the diagnosis of SFN, in order to facilitate the diagnostic process, and (2) the presence and the clinical characteristics of variants in genes encoding sodium channels in a large groups of SFN patients and painful and painless diabetic neuropathy patients.

Outline of the thesis

After this short introduction, **Chapter 2** reviews the diagnosis of SFN, diagnostic tests that assess quantification and function of the small nerve fibers. The review also gives an overview of sodium channelopathies related to clinical phenotypes. SFN can be positioned in a continuum of genetic pain disorders.

In **Chapter 3**, a study is presented assessing the applicability of the CCM in patients with suspected SFN. A total of 183 patients were examined to find out whether the CCM is an accurate technique for clinical use and if a correlation is found between the CCM parameters, other diagnostic tools and possible underlying conditions.

Chapter 4 presents age-dependent normative values for the use of stimulated skin wrinkling (SSW), based on the 5-point scale method. The Digit Wrinkle Scan© (DWS©) is introduced as a new method to quantitatively examine the wrinkling. The inter-observer reliability is also assessed for both methods.

In **Chapter 5,** the development is described of the Molecular Inversion Probes-Next generation sequencing technique (MIPs-NGS), a next generation genetic testing technique for the identification of variants in the sodium channel genes within the PROPANE study. The method was compared with the TruSeq Custom Amplicon-Next generation sequencing (TSCA-NGS).

Chapter 6 describes the clinical features of *SCN9A SCN10A* and *SCN11A* variants in 1139 patients with pure SFN, and provides a rationale for genetic screening. Pathogenicity of variants was classified according to established guidelines by the Association for Clinical Genetic Science and frequencies were determined.

In **Chapter 7**, the genetic differences between painful/painless diabetic neuropathy and painful idiopathic SFN were explored. Diabetic neuropathy is the most important cause of neuropathic pain. We report genetic variants of ten sodium channel genes (*SCN3A, SCN7A-SCN11A*, and *SCN1B-4B*), expressed in the nociceptive pathway by means of molecular Inversion Probes targeted Next Generation Sequencing approach (MIP-targeted NGS).

In **chapter 8**, we present a patient with an *SCN9A*-variant and clinical features of IEM, PEPD and SFN, as an example of an *SCN9A*-pain triangle phenomenon.

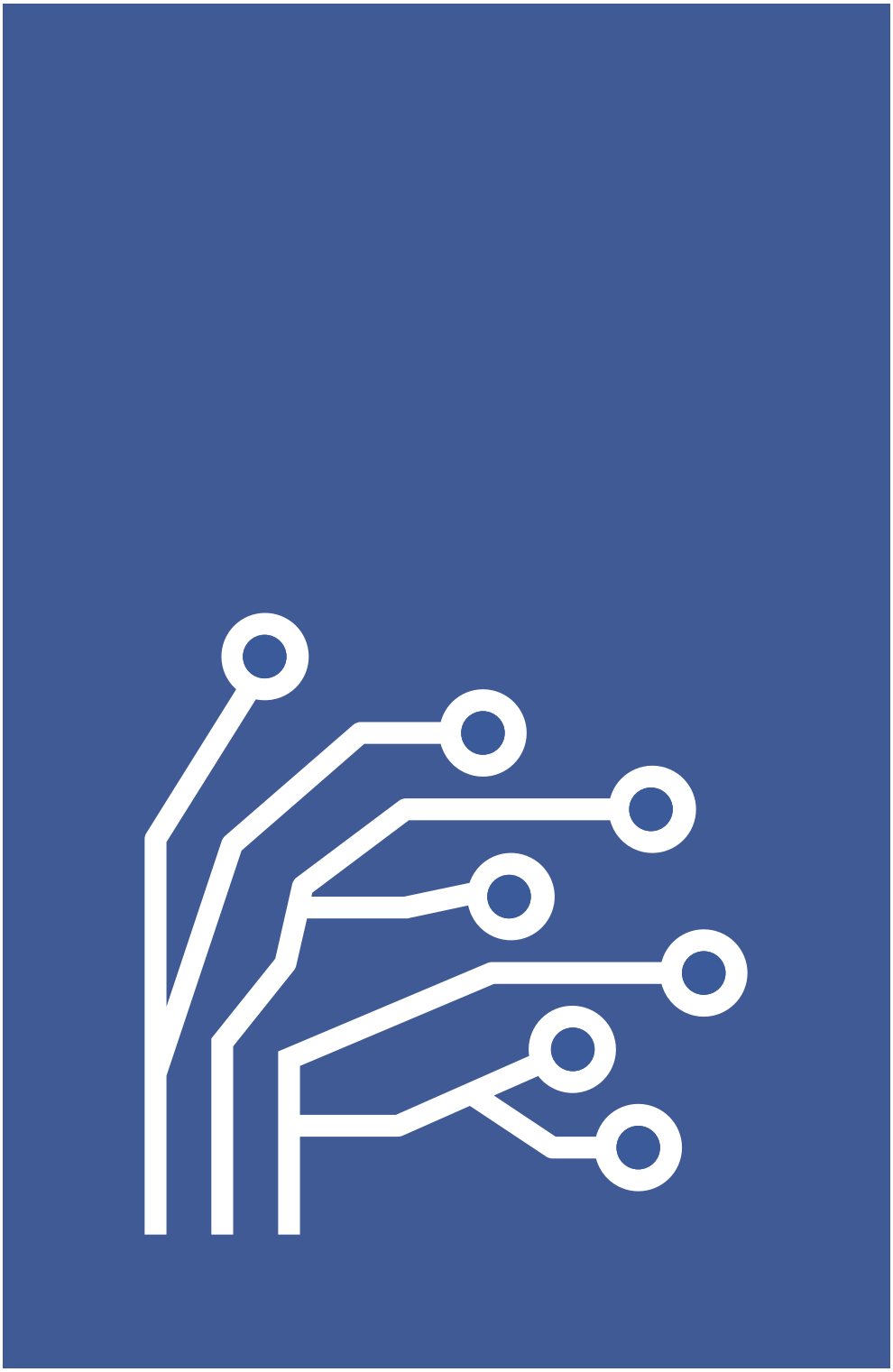
In **Chapter 9**, the main findings and conclusions from this thesis are discussed. Also, future perspectives are outlined. Lastly, **Chapter 10** presents the impact paragraph, which gives a reflection on the scientific and social impact of the results.

References

1. Sopacua M, et al. Small-fiber neuropathy: Expanding the clinical pain universe. J Peripher Nerv Syst. 2019;24(1):19-33.
2. Lauria G, Merkies IS, Faber CG. Small fibre neuropathy. Curr Opin Neurol. 2012;25(5):542-9.
3. Devigili G, Cazzato D, Lauria G. Clinical diagnosis and management of small fiber neuropathy: an update on best practice. Expert Rev Neurother. 2020;20(9):967-80.
4. Terkelsen AJ, et al. The diagnostic challenge of small fibre neuropathy: clinical presentations, evaluations, and causes. Lancet Neurol. 2017;16(11):934-44.
5. Devigili G, et al. Diagnostic criteria for small fibre neuropathy in clinical practice and research. Brain. 2019;142(12):3728-36.
6. Bakkers M, et al. Small fibers, large impact: quality of life in small-fiber neuropathy. Muscle Nerve. 2014;49(3):329-36.
7. Khan S, Zhou L. Characterization of non-length-dependent small-fiber sensory neuropathy. Muscle Nerve. 2012;45(1):86-91.
8. Khoshnoodi MA, et al. Longitudinal Assessment of Small Fiber Neuropathy: Evidence of a Non-Length-Dependent Distal Axonopathy. JAMA Neurol. 2016;73(6):684-90.
9. Basantsova NY, et al. Small-fiber neuropathy definition, diagnosis, and treatment. Neurol Sci. 2019;40(7):1343-50.
10. de Greef BTA, et al. Associated conditions in small fiber neuropathy - a large cohort study and review of the literature. Eur J Neurol. 2018;25(2):348-55.
11. Haroutounian S, et al. Diagnostic criteria for idiopathic small fiber neuropathy: A systematic review. Muscle Nerve. 2021;63(2):170-7.
12. Lauria G, et al. European Federation of Neurological Societies/Peripheral Nerve Society Guideline on the use of skin biopsy in the diagnosis of small fiber neuropathy. Report of a joint task force of the European Federation of Neurological Societies and the Peripheral Nerve Society. Eur J Neurol. 2010;17(7):903-12, e44-9.
13. Bakkers M, et al. Optimizing temperature threshold testing in small-fiber neuropathy. Muscle Nerve. 2015;51(6):870-6.
14. Freeman R, et al. Idiopathic distal sensory polyneuropathy: ACTTION diagnostic criteria. Neurology. 2020;95(22):1005-14.
15. Lauria 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.
16. Gasparotti R, et al. New technologies for the assessment of neuropathies. Nat Rev Neurol. 2017;13(4):203-16.
17. Devigili G, et al. The diagnostic criteria for small fibre neuropathy: from symptoms to neuropathology. Brain. 2008;131(Pt 7):1912-25.
18. Fabry V, et al. Which Method for Diagnosing Small Fiber Neuropathy? Front Neurol. 2020; 11:342.
19. Hansson P, Backonja M, Bouhassira D. Usefulness and limitations of quantitative sensory testing: clinical and research application in neuropathic pain states. Pain. 2007;129(3):256-9.
20. Bakkers M, et al. Temperature threshold testing: a systematic review. J Peripher Nerv Syst. 2013;18(1):7-18.
21. Shy ME, et al. Quantitative sensory testing: report of the Therapeutics and Technology Assessment Subcommittee of the American Academy of Neurology. Neurology. 2003;60(6): 898-904.
22. Azmi S, et al. Corneal Confocal Microscopy Identifies Small-Fiber Neuropathy in Subjects With Impaired Glucose Tolerance Who Develop Type 2 Diabetes. Diabetes Care. 2015;38(8): 1502-8.
23. Ferdousi M, et al. Diagnosis of Neuropathy and Risk Factors for Corneal Nerve Loss in Type 1 and Type 2 Diabetes: A Corneal Confocal Microscopy Study. Diabetes Care. 2021;44(1): 150-6.
24. Tavakoli M, Malik RA. Corneal confocal microscopy: a novel non-invasive technique to quantify small fibre pathology in peripheral neuropathies. J Vis Exp. 2011;(47):2194.

25. Alam U, et al. Diagnostic utility of corneal confocal microscopy and intra-epidermal nerve fibre density in diabetic neuropathy. PLoS One. 2017;12(7):e0180175.
26. Kalteniece A, et al. Corneal confocal microscopy detects small nerve fibre damage in patients with painful diabetic neuropathy. Sci Rep. 2020;10(1):3371.
27. Quattrini C, et al. Surrogate markers of small fiber damage in human diabetic neuropathy. Diabetes. 2007;56(8):2148-54.
28. Azmi S, et al. Corneal confocal microscopy shows an improvement in small-fiber neuropathy in subjects with type 1 diabetes on continuous subcutaneous insulin infusion compared with multiple daily injection. Diabetes Care. 2015;38(1):e3-4.
29. Mehra S, et al. Corneal confocal microscopy detects early nerve regeneration after pancreas transplantation in patients with type 1 diabetes. Diabetes Care. 2007;30(10):2608-12.
30. Tavakoli M, et al. Corneal confocal microscopy: a novel means to detect nerve fibre damage in idiopathic small fibre neuropathy. Exp Neurol. 2010;223(1):245-50.
31. Tavakoli M, et al. Normative values for corneal nerve morphology assessed using corneal confocal microscopy: a multinational normative data set. Diabetes Care. 2015;38(5):838-43.
32. Wilder-Smith EP. Water immersion wrinkling--physiology and use as an indicator of sympathetic function. Clin Auton Res. 2004;14(2):125-31.
33. Ping Ng KW, et al. EMLA-Induced Skin Wrinkling for the Detection of Diabetic Neuropathy. Front Neurol. 2013;4:126.
34. Vasudevan TM, et al. Skin wrinkling for the assessment of sympathetic function in the limbs. Aust N Z J Surg. 2000;70(1):57-9.
35. Batisse D, et al. Influence of age on the wrinkling capacities of skin. Skin Res Technol. 2002; 8(3):148-54.
36. Estacion M, et al. Intra- and interfamily phenotypic diversity in pain syndromes associated with a gain-of-function variant of NaV1.7. Mol Pain. 2011;7:92.
37. Han C, et al. The G1662S NaV1.8 mutation in small fibre neuropathy: impaired inactivation underlying DRG neuron hyperexcitability. J Neurol Neurosurg Psychiatry. 2014;85(5):499-505.
38. Faber CG, et al. Gain of function Nanu1.7 mutations in idiopathic small fiber neuropathy. Ann Neurol. 2012;71(1):26-39.
39. Huang J, et al. Gain-of-function mutations in sodium channel Na(v)1.9 in painful neuropathy. Brain. 2014;137(Pt 6):1627-42.
40. Faber CG, et al. Gain-of-function Nav1.8 mutations in painful neuropathy. Proc Natl Acad Sci U S A. 2012;109(47):19444-9.
41. Persson AK, et al. Neuropathy-associated Nav1.7 variant I228M impairs integrity of dorsal root ganglion neuron axons. Ann Neurol. 2013;73(1):140-5.
42. Minett MS, et al. Distinct Nav1.7-dependent pain sensations require different sets of sensory and sympathetic neurons. Nat Commun. 2012;3:791.
43. Dib-Hajj SD, et al. Paroxysmal extreme pain disorder M1627K mutation in human Nav1.7 renders DRG neurons hyperexcitable. Mol Pain. 2008;4:37.
44. Estacion M, et al. NaV1.7 gain-of-function mutations as a continuum: A1632E displays physiological changes associated with erythromelalgia and paroxysmal extreme pain disorder mutations and produces symptoms of both disorders. J Neurosci. 2008;28(43):11079-88.
45. Fertleman CR, et al. Paroxysmal extreme pain disorder (previously familial rectal pain syndrome). Neurology. 2007;69(6):586-95.
46. Choi JS, et al. Paroxysmal extreme pain disorder: a molecular lesion of peripheral neurons. Nat Rev Neurol. 2011;7(1):51-5.
47. Estacion M, et al. Ca^{2+} toxicity due to reverse Na^{+}/Ca^{2+} exchange contributes to degeneration of neurites of DRG neurons induced by a neuropathy-associated Nav1.7 mutation. J Neurophysiol. 2015;114(3):1554-64.
48. Han C, et al. Nav1.7-related small fiber neuropathy: impaired slow-inactivation and DRG neuron hyperexcitability. Neurology. 2012;78(21):1635-43.

49. Huang J, et al. Small-fiber neuropathy Nav1.8 mutation shifts activation to hyperpolarized potentials and increases excitability of dorsal root ganglion neurons. J Neurosci. 2013;33(35): 14087-97.
50. Han C, et al. The Domain II S4-S5 Linker in Nav1.9: A Missense Mutation Enhances Activation, Impairs Fast Inactivation, and Produces Human Painful Neuropathy. Neuromolecular Med. 2015;17(2):158-69.
51. Lee SI, et al. The small fiber neuropathy NaV1.7 I228M mutation: impaired neurite integrity via bioenergetic and mitotoxic mechanisms, and protection by dexpramipexole. J Neurophysiol. 2020;123(2):645-57.



CHAPTER 2

SMALL-FIBER NEUROPATHY: EXPANDING THE CLINICAL PAIN UNIVERSE

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J Peripher Nerv Syst, 2019; 24(1): 19 – 33.

Abstract

Small fiber neuropathy (SFN) is a disorder of thinly myelinated Aδ and unmyelinated C fibers. SFN is clinically dominated by neuropathic pain and autonomic complaints, leading to a significant reduction in quality of life. According to international criteria, the diagnosis is established by the assessment of intra-epidermal nerve fiber density and/or quantitative sensory testing. SFN is mainly associated with autoimmune diseases, sodium channel gene mutations, diabetes mellitus, and vitamin B12 deficiencies, although in more than one-half of patients a non-genetic aetiology cannot be identified. Recently, gain-of-function mutations in the genes encoding for the $Na_v1.7$, $Na_v1.8$ and $Na_v1.9$ sodium channel subunits have been discovered in SFN patients, enlarging the spectrum of underlying conditions. Sodium channel gene mutations associated with SFN can lead to a diversity of phenotypes, including different pain distributions and presence or absence of autonomic symptoms. This suggests that SFN is part of a clinical continuum. New assessments might contribute to a better understanding of the cellular and molecular substrates of SFN, and might provide improved diagnostic methods and trial designs in the future. Identification of the underlying mechanisms may inform the development of drugs that more effectively address neuropathic pain and autonomic symptoms of SFN.

Introduction

Knowledge of small fiber neuropathy (SFN) has advanced substantially over the last two decades, both at pathophysiological and clinical level. SFN is a condition that selectively involves thinly myelinated Aδ-fibers and unmyelinated C-fibers. It is clinically characterized by neuropathic pain, most frequently described as burning, shooting and/or prickling. Most cases present with a length-dependent or stocking-glove distribution[1], although a non-length-dependent pattern of symptoms may also occur.[2-5] Dysautonomic features may include dry eyes or mouth, orthostatic dizziness, bowel and micturition disturbances, a change of the perspiration pattern, accommodation problems, impotence, diminished ejaculation or vaginal lubrication, hot flushes, and/or cardiac palpitations.[6,7]

In general, pure SFN does not show abnormalities in motor and large sensory nerve fiber function at neurological examination, while hyperalgesia and allodynia frequently accompany nociceptive and temperature sensation loss.[8] In patients with pure SFN, nerve conduction studies reveal no signs of large nerve fiber involvement. In addition, over the last years, there has been increased awareness of chronic itch as a symptom of SFN.[9-12] Muscle cramps have also been described to be a symptom of SFN, possibly reflecting the location of small nerve fibers as thermoreceptor and nociceptor muscle afferents.[13,14]

Epidemiology

To date, the only epidemiological study in SFN has been performed in the Netherlands. It showed an overall minimum incidence of 12 cases per 100,000 inhabitants per year with long-term persistent complaints.[15]

Children can also suffer from SFN. Although SFN in children is difficult to diagnose because of the absence of criteria and validated questionnaires, several case reports have been published.[16-19]

Quality of Life

SFN leads to a significant reduction in the overall QoL[20], at least in part due to pain and autonomic symptoms. Thermal thresholds and reduced IENFD have been reported to correlate with the deterioration of QoL.[21] A significant association was found between pain severity and burden, with an association between the severity of pain and subject-reported health status and function.[22]

Furthermore, greater adjusted direct and indirect costs were reported at increasing levels of pain in idiopathic SFN.[22]

Definition and diagnosis

The diagnosis of SFN is made according to a clinically-based definition, including symptoms and signs suggestive of SFN and their distribution.[23] The diagnosis can be graded as follows:

- *Possible*: presence of length-dependent symptoms and/or clinical signs of small fiber damage;
- *Probable*: presence of length-dependent symptoms, clinical signs of small fiber damage, and normal sural NCS;
- *Definite*: presence of length-dependent symptoms, clinical signs of small fiber damage, normal sural nerve conduction study (NCS), and reduced intraepidermal nerve fiber density (IENFD) at the ankle and/or abnormal thermal thresholds.[1,24,25]

However, this definition only includes length-dependent symptoms, whereas the spectrum of clinical signs has widened from the classical length- dependent SFN to include non-length-dependent patterns.[2-5] Furthermore, according to this definition, the diagnosis SFN should be considered only in patients with pure or isolated impairment of the Aδ and C-fibers. Patients with predominant features of small fiber neuropathy and clinical and NCS findings of large sensory fiber dysfunction should be considered to have a mixed (small and large fiber) sensory neuropathy. A correct classification of SFN is of importance, as it impacts the work-up for an underlying condition and the design of clinical trials.[25]

Diagnostic tests

In the past years, new technologies for the assessment of peripheral neuropathies, including SFN, have become available.[26,27] A number of diagnostic tools is available for the detection of SFN (Table 2.1). A distinction can be made in methods that quantify small nerve fibers and methods that test small nerve fiber function. New imaging techniques are likely to impact the diagnostic field in SFN as well.

Table 2.1 Diagnostic tests in SFN.

***Quantification of small nerve fibers**
Skin biopsy
intra-epidermal nerve fiber density (IENFD)
dermal nerve fiber length
sweat gland and pilomotor muscle innervation
Cornea confocal microscopy
corneal nerve fiber density (CNFD)
corneal nerve branche density (CNBD)
corneal nerve fiber length (CNFL)
corneal nerve fiber turtuosity (CNFT)
***Functionality of small nerve fibers**
Quantitative sensory testing
assessment of large and small sensory nerve fiber function
Microneurography
assessment of activity of C-nociceptors
Nociceptive Evoked Potentials
generation by laser (LEPs), contact heat (CHEPs) or pain-related (PREPs)
intra-epidermal electrical stimulation (IES)
***Imaging**
Peripheral Nerve Ultrasound
(Functional) Magnetic Resonance Imaging
***Autonomic Testing**
Thermoregulatory sweat testing
Quantitative sudomotor axon reflex testing (QSART)
Silicone impression method
Quantitative direct and indirect axon reflex testing
Sympathetic skin response (SSR)
Electrochemical skin conductance
Neuropad
Stimulated skin wrinkling (SSW)

Abbrevations: CHEP: contact heat-evoked potential; LEP, laser-evoked potential; PREP, pain-related-evoked potential.

Quantification of small nerve fibers

** Skin biopsy*

The diagnostic value of skin biopsy with IENFD in patients with clinically suspected SFN has been established and the method is generally considered the 'gold standard' for the diagnosis.[26,28] IENF are unmyelinated sensory endings with exclusive somatic function that arise from nerve bundles of the sub-papillary dermis.[29] They lose the Schwann cell ensheathment as they cross the dermal–epidermal junction[30-32] and widely express the capsaicin receptor, making them the most distal nociceptors. Skin biopsy is commonly taken with a 3-mm disposable punch, from the lower leg, 10 centimeters proximal from the lateral malleolus, within the territory of the sural nerve. By means of immuno-

histochemistry, IENF are visualised using antibodies against the protein gene product (PGP9.5), a cytoplasmic ubiquitin carboxyl-terminal hydrolase. The number of fibers crossing the dermal-epidermal junction is quantified, the length of the section is measured and the linear density of IENF per millimetre is obtained and compared with age- and gender-matched normative values.[28] Recent studies have shown that right and left-side IENFD overlap in healthy subjects and in patients with length-dependent SFN, and that IENFD is stable when re-assessed within a 3-week period that is the time of epidermal renewal, through a follow-up biopsy in the same sensory territory.[33]

Disadvantages of skin biopsy are that the analysis is time-consuming and relatively costly, and that sensitivity is moderate. Indeed, some patients with symptoms of SFN may have normal IENFD and possibly represent pre-degenerative functional impairment of the nerve fibers.[34]

IENFD decreases with ageing[28] and values in upper arm and proximal thigh are significantly higher than in wrist and distal leg, respectively.[35] One study performed to monitor IENFD during disease course in idiopathic SFN found similar rates of decrease in proximal and distal sites of the lower limb.[3] The rates of IENFD decrease over time do not differ between idiopathic SFN, diabetic SFN and impaired glucose tolerance SFN.[3]

IENFD has been reported to be reduced also in other painful conditions, such as Guillain-Barré syndrome[36], meralgia paraesthestica[37], notalgia[38], Ehlers-Danlos syndrome[39], and fibromyalgia[40], and non-painful disorders, such as Parkinson's disease and related disorders[41-43], amyotrophic lateral sclerosis[44-46], critical illness[47], and peripheral arterial diseases.[48]

New techniques to determine the IENFD with indirect immunofluorescence[49], automated PGP9.5 immunofluorescence staining (laboratory developed test)[50] and 3D-analysis[51] have been reported. One study investigated the global spatial sampling in order to determine the epidermal nerve fiber length density (ENFLD) taking into account its biologic complexity.[52] Results showed that ENFLD is comparable with IENFD in differentiating between SFN and healthy individuals.[52]

In hairy skin, dermal nerve fibers are organized in small bundles. The bundles located just below the dermal– epidermal junction constitute the subepidermal neural plexus, from which fibers arise to reach the epidermis. Other bundles can

be found in the deeper dermis. Most fibers are unmyelinated, and the minority of myelinated fibers are detectable in the upper dermis, usually close to hair follicles or vascular structures.[32] A method for the assessment of dermal nerves by measuring the overall length of the fibers was shown to be reliable in terms of diagnostic yield in patients with pure SFN.[53]

The skin is also rich with autonomic nerve fibers, innervating different autonomic structures such as sweat glands and pilomotor muscles. The innervation of dermal autonomic structures can be investigated using markers for adrenergic, noradrenergic, and cholinergic sympathetic fibers and vasodilatory peptidergic fibers.[32] Indeed, several methods have been described to obtain a morphometry of sweat gland and pilomotor muscle innervation.[54,55]

** Corneal Confocal Microscopy*

Corneal Confocal Microscopy (CCM) is a method that visualizes the unmyelinated C-nerve fibers that originate from the trigeminal nerve and travel to the Bowman's membrane of the cornea.[56] It allows an *in vivo* evaluation of disease or surgery-induced alterations of corneal nerves.[57,58]

Four established parameters - corneal nerve fiber density (CNFD), corneal nerve branch density (CNBD), corneal nerve fiber length (CNFL) and corneal nerve fiber tortuosity (CNFT) – can be quantified by means of the software program CCMetrics. An international normative dataset of these corneal nerve fiber parameters has been published.[59]

CCM is a non-invasive tool with high repeatability.[60] Studies in patients with non-length dependent SFN[61] and length-dependent SFN[62] demonstrated a decrease in CNFD. However, these studies included small patient groups (6 and 25, respectively). Regeneration of the small fibers in the cornea was found in diabetic patients after kidney and pancreas transplantation[63] and after continuous subcutaneous insulin therapy in comparison with injections without improvement in the IENFD and QST.[64]

CCM has been used to detect small fiber damage in other neurological diseases, such as Fabry's disease, chronic inflammatory demyelinating polyneuropathy, Charcot-Marie Tooth type 1A, and multiple sclerosis.[65-68] Conversely, research on patients with Parkinson's showed an increase in CNBD and CNFL.[41]

Assessment of the function of small nerve fibers

** Quantitative sensory testing*

Quantitative sensory testing (QST) is a non-invasive psychophysical method that quantifies the thresholds of sensory perception carried by large and small nerve fibers.[69] QST is considered a diagnostic tool in SFN.[34,70] A recent consensus meeting has provided recommendations for clinical use of QST[71], emphasizing the need of a standardized protocol, adequate equipment, trained staff and use of normative values. The method of levels (i.e. a reaction time–independent method; the subject answers per stimulus whether a warmer or cooler temperature is sensed) has several advantages: there is no effect of stimulus temperature change rate, applicability is possible even in subjects with cognitive impairment and children, and repeatability is comparable or better compared to the method of limits (reaction time-dependent; pushing a button when a change in temperature or pain is sensed).[72-75] The combination of bilateral warm and cold thresholds of the hands and feet by the levels method probably provides the most optimal sensitivity and specificity.[76]

Thermal threshold deterioration was associated with intensity of pain in peripheral neuropathy.[77] In diabetes without sensory large nerve involvement, a significantly lower IENFD and higher cold perception threshold were found in comparison with controls, irrespective of whether they had symptoms of polyneuropathy or not. However, a reduction of IENFD was the most frequent abnormal finding in the subgroup of patients with neuropathic symptoms, and therefore seemed more sensitive as a diagnostic tool.[78] Furthermore, QST requires the patient to be alert and cooperative, the test cannot discriminate between central and peripheral nervous system diseases[79] and may be influenced by malingering or other nonorganic factors.[80-83] For all these reasons, QST should be used in relation to the clinical context and in conjunction with other tests, and not alone for the diagnosis of a neurological lesion.[84]

** Microneurography*

Microneurography is used to record the activity of C-nociceptors and sympathetic fibers and to test the efficacy of different compounds in blocking abnormal on-going activity in both animal models and in patients.[85] The use of microneurography is increasing in disorders affecting the peripheral nervous system.[86-89] However, its application in clinical practice remains limited due to the

technical challenges, the amount of time needed to perform the examination, the small number of nerve fibers that can be studied in any given patient, and the test awaits validation of diagnostic value.[90]

** Nociceptive Evoked Potentials*

Nociceptive evoked potentials can be used to investigate the conduction properties of small nerve fibers in a fashion not dependent on patients' cooperation and attention.[91] These nociceptive evoked potentials can be generated by either radiant heat (laser-evoked potentials, LEPs) or contact heat (contact heat-evoked potentials, CHEPs). Both LEPs and CHEPs are based on selective of Aδ- and C-fiber activation, whereas induction of pain-related evoked potentials (PREPs) involves the preferential stimulation of Aδ-fibers.[92] Skin denervation induced by topical capsaicin causes the decrease of LEP amplitude.[93] LEPs are a validated technique to investigate the neural bases of nociception.[94,95] LEP amplitudes correlate with the reported intensity of perceived pain[96], and negatively with age.[97] Moreover, it is modulated by opioids[98] and pain expectation.[99,100]

Comparable to QST, LEPs cannot discriminate the site of pathology (peripheral nerves, plexus, roots, spinal cord or brainstem)[101], and should therefore also be considered a supportive tool for diagnosing SFN.

Age- and gender adjusted normative values have been reported for the clinical use of CHEPs.[102] More recently, a strong correlation between CHEP amplitudes with the degree of skin innervation was found in a large SFN cohort.[103] Patients with sensory neuropathy and an IENF loss have lower-amplitude CHEPs.[104-106] However, CHEPs cannot be recorded in all healthy participants, which makes the clinical interpretation of absent CHEPs difficult.[102]
Intraepidermal electrical stimulation (IES) has also been described as a potential additional tool in detecting functional changes in Aδ-fibers and C-fibers in SFN[107,108], and in patients with neuropathic pain.[109]

Imaging

** Peripheral Nerve Ultrasound*

Enlargement in cross-sectional area (CSA) of the sural nerve was found with ultrasound (US) in SFN patients with reduced IENFD, compared with body mass

index matched healthy controls[110], indicating changes in structure or morphology of larger nerve fibers. Possible explanations for this large nerve fiber enlargement include impaired axoplasmic flow in proximal (larger) nerve segments due to loss or injury of distal small nerve fibers, or sodium channel dysfunction, leading to axonal degeneration with axonal swelling.[111,112] Alternatively, changes in the extracellular space within peripheral nerves and/or change in non-neuronal connective tissue surrounding the axons may contribute. At present, more data are needed to establish the value of ultrasound as a diagnostic tool in SFN.

** Magnetic Resonance Imaging*

Non-invasive imaging techniques, such as functional magnetic resonance imaging (fMRI), are used to measure neuronal activity in humans in order to study regional activation in various parts of the brain in chronic pain states. The advantage of fMRI is the ability to ascribe function to specific brain regions. The resolution of fMRI images has become more detailed with increasing magnet strength.

Skin denervation has been associated with abnormal recruitment of pain-related regions in the brain[113], especially in diabetic neuropathic pain[114,115], suggesting altered patterns of activation of the brain in painful neuropathy. Volume reduction was most notable in pain-processing regions, particularly the bilateral anterior cingulate cortices, which was associated with greater depletion of IENF.[116] However, whether a specific activation pattern can be seen depends on many factors, such as type of brain imaging modality.[117] It is conceivable that a particular type of pain (stimulus) may enhance a specific pain brain pattern, but patient-specific factors (i.e. gender, genetic and epigenetic factors) may influence the pain activation network.[118-120] Psychological modulation as well as chronicity of pain may influence the activation network, and should therefore be taken into account.[121-126]

Autonomic testing

Changes in peripheral autonomic nervous system function may be an early manifestation in SFN.[127] Dysfunction of the sudomotor system may result in an increase or decrease in sweat production, resulting in disturbances of thermoregulation. Traditional measurements of sudomotor function include thermoregulatory sweat testing, quantitative sudomotor axon reflex testing

(QSART), silicone impressions, quantitative direct and indirect axon reflex testing, and the sympathetic skin response (SSR).[128]

** Thermoregulatory sweat testing*

Thermoregulatory sweat testing is performed by increasing the ambient room temperature which in turn raises blood and skin temperature. The degree and extent of sweat production is then visualized with an indicator dye.[128] The test is time-consuming, requires special equipment, and special preparation and treatment of the patient, and is therefore only performed in highly specialized centres.

** Quantitative sudomotor axon reflex testing (QSART)*

QSART is used to evaluate postganglionic sympathetic cholinergic sudomotor function by measuring the axon-reflex mediated sweat response over time. QSART can be of value in the diagnosis of SFN.[129,130] It has been suggested to add QSART as one of the core diagnostic tests, requiring abnormality on two measures for the diagnosis (clinical findings, QST, QSART, and skin biopsy). Although QSART can be of value in the diagnosis of SFN, normative data are needed to determine its usefulness for clinical practice.[25]

** Silicone impression method*

The silicone impression method is used to evaluate the postganglionic sympathetic cholinergic sudomotor function by measuring the direct and axon-reflex mediated sweat response at specific time points.[131] Although the silicone impression method is probably the easiest method to perform in the clinical realm, artifacts may influence the test results.

** Quantitative direct and indirect axon reflex testing*

Quantitative direct and indirect axon reflex testing is a method to evaluate the postganglionic sympathetic cholinergic sudomotor function by measuring the direct and axon-reflex mediated sweat response in a dynamic fashion. The test is simple, but further studies are required to determine its diagnostic value in SFN.[128]

** Sympathetic Skin Response*

Sympathetic Skin Response (SSR) is a measure of electrodermal activity and provides a surrogate measure of sympathetic cholinergic sudomotor function.

Although easy to perform, there is high variability within and between subjects, and sensitivity and specificity of the method are low.[70,132]

** Electrochemical skin conductance*

More recently, the Sudoscan was developed. It is a simple, quick, painless and non-invasive quantitative test measuring C-fiber postganglionic sympathetic nerve function in sweat glands of the palms and soles of the feet, areas that contain a high density of these glands.[1,133-135] The Sudoscan measures the electrochemical skin conductance.[135-137] Most studies on the Sudoscan have been performed in patients with diabetic neuropathy, demonstrating a decrease of electrochemical skin conductance and a correlation with small fiber dysfunction and neuropathic symptoms.[135,138,139] A recent review concluded that normative values are inconsistent across publications, and large combined data sets do not support a high sensitivity and specificity.[140] Therefore, the value of Sudoscan as a diagnostic tool for SFN still needs to be determined.

** Neuropad*

Another recently developed test, the Neuropad, was introduced to measure sweat production based on the colour change of a cobalt II compound.[141] Moderate sensitivity and specificity (68% and 49%, respectively) were found using the warm perception threshold as a reference method, and these were enhanced when the corneal nerve fiber length (CNFL) was used as a reference method (83% and 80% respectively). The contribution of this test to the diagnosis SFN needs to be established.

** Stimulated Skin Wrinkling*

Stimulated Skin Wrinkling (SSW) is a test for sympathetic function based on changes of dermal arteriovenous tissue vasoconstriction of the digits. A negative digit pulp pressure will occur after a warm water bath for 30 minutes. Wrinkling would occur when epidermal skin is drawn down unevenly, because of its varying tautness.[142] The eutectic mixture of local anaesthetics (EMLA©) cream can also be used as a vasoconstrictive factor with similar results.[143-145] In clinical practice, SSW is usually performed in the hands and graded using a published 5-point-scale.[146-148] Foot skin wrinkling is hardly ever performed, as wrinkling is poor because of higher sympathetic nerve activity to the lower limbs.[149] Reduced SSW was found in patients with diabetic neuropathy[150-152] and in idiopathic SFN.[146-148] However, the value of SSW as a diagnostic tool is currently limited.

Outcome measures

Surveys might help clinicians to diagnose and assess treatment responses. The 13-item SFN-Symptom Inventory Questionnaire© (SFN-SIQ), an ordinal based multi-item composite measuring 13 SFN-related symptoms, was transformed through Rasch to an interval measure which can be used as a diagnostic screening tool enabling parametric analyses.[153,154]

Furthermore, a disease-specific 32-item SFN-Rasch-built Overall Disability Scale (SFN-RODS©) questionnaire was developed via Rasch analyses, suitable for detecting activity limitations and participation restrictions in patients with SFN.[153]

A small-fiber symptom survey has also been developed with satisfactory psychometric properties, indicating potential future utility for surveying patient-reported symptoms; however, this is an ordinal scale, hampering meaningful calculations.[155] Finally, the Utah Early Neuropathy Scale was developed to evaluate the sensory signs and symptoms in sensory and small-fiber nerve neuropathy, and may be a useful tool for clinical use and in trials.[156]

Underlying conditions and pathophysiology

SFN is associated with multiple diseases which can be categorized as metabolic, immune-mediated and infectious diseases, exposure to drugs and toxins, and genetic causes.[24,25] In a large cohort of 921 patients, 75% of them did not have a known preselected comorbidity before the diagnostic workup. Immunological conditions were found in 175 patients (19%); other associated conditions were sodium channel gene mutations (16.7%), diabetes mellitus (7.7%), vitamin B12 deficiency (4.7%), alcohol abuse (3.0%), chemotherapy (2.2%), monoclonal gammopathy of undetermined significance (MGUS) (1.4%), and haemo-chromatosis (0.3%) (Figure 2.1). Systemic dysimmunity was more prevalent in idiopathic SFN patients than in the general population, though the pathogenic role of isolated autoantibodies remains uncertain.[157] Another smaller study confirmed the presence of immunological abnormalities (e.g. antinuclear antibodies, extractable nuclear antigens and celiac autoantibodies), whereas diabetes, prediabetes, and hypertriglyceridemia were not associated with SFN.[158] A large study demonstrated that the prevalence of Fabry´s disease is irrelevant in adult SFN patients, a finding that allows excluding this genetic screening in patients with confirmed diagnosis of SFN.[159]

Early degeneration of small nerve fibers can occur in the pre-symptomatic stage of patients carrying transthyretin mutations[160], whereas patients with a symptomatic stage of familial amyloid neuropathy more like present with a mixed neuropathy.[161] Even in patients with a known possible aetiology, additional underlying causes can be found in 27% of patients.[159] It is therefore recommended to screen patients with pure SFN at least for autoimmune diseases, diabetes mellitus including glucose intolerance, vitamin B12 deficiency and sodium channel gene variants, even when they already have a potential underlying condition at referral.

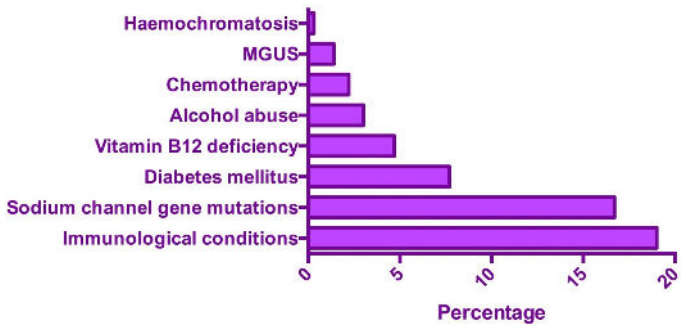


Figure 2.1 Prevalence of underlying causes in patients with SFN.[157]
Immunological causes: Sarcoidosis, Sjogren's disease, coeliac disease, other autoimmune diseases, and abnormal immunological laboratory findings (antinuclear antibodies, anti-neutrophil cytoplasmic antibodies, monoclonal gammopathy, soluble interleukin-2 receptor, anti-tissue transglutaminase, and antiextractable nuclear antigen antibodies); MGUS: monoclonal gammopathy of undetermined significance; SFN, small fiber neuropathy

** Voltage-gated sodium channelopathies in SFN*

Voltage-gated sodium channels play an essential role in regulating the excitability of nociceptive primary afferent neurons. Three voltage-gated sodium channels, $Na_V1.7$, $Na_V1.8$ and $Na_V1.9$, encoded by genes *SCN9A, SCN10A* and *SCN11A*, are preferentially expressed in peripheral neurons and are known to play a role in human pain disorders.[162]

Gain-of-function *SCN9A* variants have been described in three painful human pain conditions: inherited erythermyalgia (IEM), paroxysmal extreme pain disorder (PEPD) and SFN. By contrast, congenital insensitivity to pain (CIP) is associated with autosomal recessive loss-of-function *SCN9A* variants. Increased

understanding of the pathophysiological mechanisms underlying sodium channelopathies[163,164] paved the way for the development of isoform-selective blockers as a targeted treatment modality.[165-167]

$Na_v1.7$ in IEM

IEM (or erythermalgia; OMIM 133020) is characterized by attacks of bilateral symmetrical burning pain together with redness and warmth in the feet or hands. Moderate exercise and heat provoke and aggravate the attacks, whereas cold, rest and raising the affected limbs may provide relief.[168-170] In most patients with IEM, symptoms start in early childhood (prior to 5-6 years of age); occasional families show an older age at onset.[171] Except for reddening of the skin of affected body parts due to vasomotor dysregulation during attacks[172], autonomic symptoms, such as in SFN, has been rarely reported in IEM.[169]

IEM is an autosomal dominant painful neuropathy, caused by variants in *SCN9A*.[169,171,173] Gain-of-function variants that shift activation of $Na_v1.7$ in a hyperpolarizing direction, slow deactivation, and enhance ramp currents cause IEM. Over 20 different IEM mutations have been discovered in $Na_v1.7$, and almost all mutations investigated so far result in a hyperpolarizing shift of activation, allowing $Na_v1.7$ to open at lower potentials compared with the wild type[174-187], in familial cases[179,188-190] and children.[181,191] This left shift of activation enhances excitability, intuitively explaining the pain phenotype.[174,192,193] The phenotype, however, can be complex and variable[189,194-199], even within families carrying the same variant.[169] The I234T-mutation, which causes IEM-like pain phenotype, exhibits a complex phenotype that includes automutilation[198,200] and bilateral congenital corneal anesthesia.[201] These findings, which suggest both gain of function and loss of function at the clinical level for patients carrying this variant, are explained by the unusually large hyperpolarization of activation of the mutant channel, which produces a massive depolarization in the resting potential of some dorsal root ganglion (DRG) neurons, thus silencing them.[202] Several variants have been reported without functional testing.[203,204] Recently, a $Na_v1.8$ variant has been linked to a syndrome with clinical characteristics similar to IEM.[205]

$Na_v1.7$ in PEPD

PEPD, previously known as familial rectal pain (OMIM 167400) is an inherited condition characterized by paroxysms of rectal, ocular, or submandibular pain

with flushing. Patients with PEPD can also suffer from autonomic dysfunction leading to poor feeding and reflux, vomiting, tonic attacks, breath holding spells, and bradycardia that sometimes requires insertion of a pacemaker. PEPD is caused by gain-of-function $Na_v1.7$ variants that mostly result in impaired fast-inactivation. So far, 10 variants in $Na_v1.7$ are known that cause PEPD.[206-212] It is thought that the variant induces a depolarizing shift of steady-state fast inactivation, hampering channel closure during action potential electrogenesis.

$Na_v1.7$ in channelopathy-associated insensitivity to pain

Patients with CIP do not perceive physical pain (OMIM 243000). The difference between sharp and dull and hot and cold is felt, but the pain awareness is absent. Young children with CIP may accrue mouth or finger wounds due to repeated self-biting may also experience multiple burn-related injuries, and may injure bones and joints without experiencing pain. They also have a complete loss of the sense of smell (anosmia). *SCN9A* homozygous missense and deletion variants have been described in these patients, who do not produce functional Nav1.7 channels, and has been linked to the absence of pain perception.[213-223] Partial deletion of pain perception was also described.[224] The clinical phenotype of patients with reduced pain sensibility due to $Na_v1.9$-variants is different than $Na_v1.7$-associated CIP.[225-227]

Large hyperpolarizing shifts in the voltage dependence of activation in the mutated $Na_v1.9$ channels in these cases are associated with insensitivity to pain. This evokes a massive degree of membrane depolarization that renders DRG neurons hypoexcitable.[228]

$Na_V1.7$ in SFN

The first gain-of-function variants in Nav1.7 that change the properties of the channel and the excitability of DRG neurons were described in 2012 in skin biopsy- and QST-confirmed idiopathic SFN.[229] Unexpectedly, while there is a strong correlation between genotype and phenotype for many variants, some patients carrying Nav1.7 mutations show a remarkable degree of genotype-phenotype variability. Thus a single $Na_v1.7$ variant can be associated with a range of clinical phenotypes, and the same clinical phenotype may be associated with multiple different variants.[12,229-236] The I228M variant, for example, may present with facial pain, or with a distal SFN.[231] Most SFN variants in Nav1.7 are associated with distal pain, but the G856D variant was linked to a more complex

phenotype of very severe pain, together with erythema, dysautonomia and small hand and small feet (acromesomelia).[234] The IEM-associated G856R variant was recently also shown to be associated with impaired distal limb development, suggesting that some gain-of-function mutations of $Na_V1.7$ may adversely affect limb morphogenesis during development.[191] Furthermore, some variants present with severe autonomic symptoms, while others do not. This differential effect of certain $Na_V1.7$ variants, rendering DRG neurons hyperexcitable and sympathetic ganglion neurons hypoexcitable, can be explained by the presence or absence of $Na_V1.8$ in dorsal root ganglion versus sympathetic ganglion neurons, respectively.[172,232] In addition to providing a mechanistic basis for pain and autonomic symptoms in SFN, the presence of gain-of-function mutations in Na_V channels may provide insights about the mechanisms that lead to degeneration of axons in SFN. In vitro studies have demonstrated that reverse-mode (Ca^{2+} -importing) Na/Ca exchange can be triggered by a small but sustained influx of Na^+ ions due to pathogenic sodium channel mutations found in SFN patients, thereby impairing neurite outgrowth, suggesting a molecular mechanism of axon degeneration in SFN.[111]

Multiple modulatory factors can shape the pain experience of patients carrying $Na_V1.7$ gain-of-function variants; for example, a recent study of two patients with IEM both carrying the same $Na_V1.7$ variant but with different pain profiles, demonstrated that a variant of a second gene, in a potassium channel, can introduce a degree of resilience to pain.[237] Some patients with painful SFN can develop diabetes years after SFN becomes clinically manifest. It has been speculated that $Na_v1.7$ mutations, present in pancreatic ß-cells as well as DRG neurons, may increase susceptibility for development of diabetes via ß-cell injury and produce painful neuropathy via a distinct effect on DRG neurons.[238] This hypothesis remains to be experimentally tested.

$Na_v1.8$ in SFN

The $Na_v1.8$ sodium channel, expressed in DRG neurons and peripheral nerve axons, contributes most of the sodium current underlying the action potential upstroke and supports repetitive firing in response to sustained depolarization.[163,239-241] Gain-of-function variants in $Na_v1.8$ have been found in patients with painful neuropathy[242], which had an enhanced channel response to depolarization and produced hyperexcitability in DRG neurons, including reduced current threshold, increased firing frequency and spontaneous activity. Other

$Na_v1.8$ variants have also been linked to SFN[243,244], some with a clinical phenotype that includes a clinical picture that suggests severe dysautonomia.[245] Variants in $Na_V1.8$ were found in almost 5% of a group of 921 consecutive patients with SFN.[157]

$Na_v1.9$ in SFN

$Na_V1.9$ is preferentially expressed in small-diameter DRG neurons, trigeminal ganglion neurons, and intrinsic myenteric neurons.[246] Several human pain disorders have been linked to dominant gain-of-function $Na_V1.9$ variants, including early-onset pain in distal extremities[247-249], cold-aggravated pain[250], and SFN.[251,252] The expression of $Na_V1.9$ in myenteric neurons can explain the gastrointestinal symptoms reported by patients harbouring *SCN11A* variants.[248] Finally, gain-of-function variants in $Na_V1.9$ have been reported in patients with a complex clinical syndrome that includes insensitivity to pain.[226,227,253] The loss of pain sensibility in these cases arises from a massive depolarization of DRG neurons that inactivates the sodium channels in these cells and reduces their excitability.[228]

Overlap between pain disorders

With the description of painful SFN caused by $Na_V1.7$ variants, it has become clear that the phenotype of $Na_V1.7$ variants expands, and that the boundaries between these phenotypes are not always distinct (Figure 2.2). Clinically, burning pain with a stocking-glove distribution is a common characteristic in SFN but is also seen in IEM.[188,229] Facial and diffuse or widespread pain can be seen in SFN and PEPD[206,229,231], and also in IEM.[170] Although this suggests that the function of small nerve fibers is equally impaired, IEM is usually not characterized by a loss of epidermal nerve fiber density.[254]

Reddening of the skin can occur in both IEM and PEPD and, to a lesser extent, in SFN. One study suggested that the activity of mutant $Na_v1.7$ channels in smooth muscle cells and sympathetic fibers innervating skin vessels may contribute to this phenomenon.[255] Mixed phenotypes of IEM and SFN, IEM and PEPD, or SFN and PEPD associated with one variant have been described. Among SCN9A variants, the R185H has been found in patients diagnosed with either PEPD[256] or SFN.[229] The A1632E variant has been found in a patient with a mixed phenotype of IEM and PEPD, and causes a mixed physiological change in channel function, of hyperpolarized activation and impaired fast inactivation of the channel, which are

typically associated with IEM and PEPD, respectively.[257] The heterozygous L245V variant that was found in a large family with IEM did not affect channel activation, but instead resulted in incomplete fast inactivation and a small hyperpolarizing shift in steady-state slow inactivation, which is more characteristic for PEPD.[258] Overall at the structural level, most IEM variants tend to be located within the domains I and II of the protein, while PEPD variants are commonly located in the domains III and IV. The structural dichotomy parallels the biophysical effects of the two types of variants.[259]

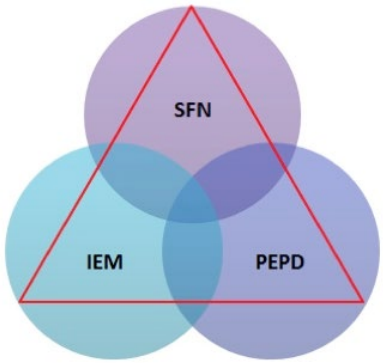


Figure 2.2 The triangle of *SCN9A*-related pain disorders. Modified from Hoeijmakers, thesis: SFN and sodium channels: a paradigm shift, 2014, chapter 9, Figure 2.1.[260] SFN=small fiber neuropathy, IEM=inherited erythromelalgia, PEPD=paroxysmal extreme pain disorder.

Electrophysiology and pathogenicity of SCN variants

Although we know that some variants in sodium channels $Na_v1.7$, $Na_v1.8$, and $Na_v1.9$ can cause pain disorders, it is important to discriminate disease-causing variants, from disease-contributing variants and variants of uncertain significance.[236] IEM and PEPD are due to rare, high impact, fully penetrant variants in $Na_v1.7$. The frequency of specific variants is still low in the SFN population, and one could argue whether these variants can be considered risk factors or variants contributing to the disease, but not causing the disease. The clinical utility of *in silico* mutation-prediction programs is at best moderate, since these algorithms do not always accurately predict changes in channel function.[236] Consensus has been reached that newly described gene variants of *SCN9A, SCN10A,* and *SCN11A* should be assessed in the context of phenotype, family history, *in-silico* analysis, and functional profiling of the variant channel, and urge

that gene variants be interpreted cautiously within clinical practice in the absence of segregation with symptoms in a large kindred and/or a pathogenic functional signature showing clear pro-excitatory changes in channel physiology.[236]

Management

Primary goals of the management of neuropathic pain in SFN are to detect (potentially treatable) underlying causes, to eliminate risk factors, and to manage the pain. Patients with SFN typically suffer from severe neuropathic pain that may be difficult to treat. At present, therapeutic strategies are largely symptomatic. Three main categories of drugs are most commonly used for treating neuropathic pain: antidepressants, anti-epileptics, and opioids.[261] Using the Grading of Recommendations Assessment, Development, and Evaluation (GRADE), recommendations were made for the pharmacotherapy of neuropathic pain based on the results of a systematic review and meta-analysis. There is a strong recommendation for use and proposal as first-line treatment for tricyclic antidepressants, serotonin-noradrenaline reuptake inhibitors, pregabalin, and gabapentin; a weak recommendation for use and proposal as second line for lidocaine patches, capsaicin high-concentration patches, and tramadol; and a weak recommendation for use and proposal as third line for strong opioids and botulinum toxin A. Topical agents and botulinum toxin A were recommended for peripheral neuropathic pain only. A substantial subset of patients with SFN is aged 65 years or older, and comorbidities and polypharmacy make neuropathic pain treatment more challenging.[262] At present, treatment of neuropathic pain is often disappointing, leading overall to pain relief of about 50% in only one-half of the patients, and the drug often has to be discontinued due to unpredictable side-effects. The recent genetic and functional findings in SFN may pave the way for the development of new analgesics, through both pharmacogenomic targeting of existing medication[263,264], and the development of a new generation of specific sodium channel blockers.[165-167]

As pain is a complex symptom, in which not only physical factors but also psychological, neurophysiological, socioeconomic, and cultural aspects may influence the experience and continuation of pain, a multidisciplinary approach in line with the biopsychosocial model is required in optimizing treatment for the individual patient.[265] Physical therapy modalities and rehabilitation techniques are important options.[266] Moreover, supervised exercise in patients with metabolic

syndrome showed an increased cutaneous regenerative capacity, suggesting potential benefits of peripheral nerve function.[267]

Conclusions

The universe of causes of SFN is expanding. Sodium channel gene mutations associated with SFN have been linked to a spectrum of clinical presentations, including different pain distributions together with the presence or absence of autonomic symptoms. The observation of mixed or overlap phenotypes suggests that multiple different pain disorders, currently considered as clinically distinct, may be part of a physiological continuum or spectrum. The number of diagnostic tests for SFN is increasing, although the clinical relevance of many is still not established. With the discovery of sodium channel variants underlying SFN, the understanding of the pathophysiology of the disorder has increased. Variants in sodium channel genes have been found in a relatively small percentage of SFN patients, and while their number is likely to increase, other genetic aetiologies are likely to emerge. Recent progress is likely to inform the development of new treatments and provide a mechanism-based precision medicine approach to neuropathic pain.

Review criteria

A literature search was performed to find studies and reviews published on SFN. If appropriate, historical papers were also included. PubMed search was performed using the keywords "small fiber (fiber) neuropathy", in combination with any of the following keywords: "aetiology/etiology", "pathogenesis", "diagnosis", "prognosis", "treatment", "skin biopsy", "quantitative sensory testing", "nerve conduction study (studies)". Furthermore, the bibliographies of all articles published between 1997 and 2017 regarding SFN were checked. Only articles in published in English were included.

References

1. Tesfaye S, Boulton AJ, Dyck PJ, Freeman R, Horowitz M, Kempler P, et al. Diabetic neuropathies: update on definitions, diagnostic criteria, estimation of severity, and treatments. Diabetes Care. 2010;33(10):2285-93.
2. Gorson KC, Herrmann DN, Thiagarajan R, Brannagan TH, Chin RL, Kinsella LJ, et al. Non-length dependent small fibre neuropathy/ganglionopathy. J Neurol Neurosurg Psychiatry. 2008;79(2):163-9.
3. Khoshnoodi MA, Truelove S, Burakgazi A, Hoke A, Mammen AL, Polydefkis M. Longitudinal Assessment of Small Fiber Neuropathy: Evidence of a Non-Length-Dependent Distal Axonopathy. JAMA Neurol. 2016;73(6):684-90.
4. Khan S, Zhou L. Characterization of non-length-dependent small-fiber sensory neuropathy. Muscle Nerve. 2012;45(1):86-91.
5. Gemignani F, Giovanelli M, Vitetta F, Santilli D, Bellanova MF, Brindani F, et al. Non-length dependent small fiber neuropathy. a prospective case series. J Peripher Nerv Syst. 2010;15(1): 57-62.
6. Lauria G. Small fibre neuropathies. Curr Opin Neurol. 2005;18(5):591-7.
7. Stewart JD, Low PA, Fealey RD. Distal small fiber neuropathy: results of tests of sweating and autonomic cardiovascular reflexes. Muscle Nerve. 1992;15(6):661-5.
8. Blackmore D, Siddiqi ZA. Pinprick Testing in Small Fiber Neuropathy: Accuracy and Pitfalls. J Clin Neuromuscul Dis. 2016;17(4):181-6.
9. Brenaut E, Marcorelles P, Genestet S, Menard D, Misery L. Pruritus: an underrecognized symptom of small-fiber neuropathies. J Am Acad Dermatol. 2015;72(2):328-32.
10. Misery L, Brenaut E, Le Garrec R, Abasq C, Genestet S, Marcorelles P, et al. Neuropathic pruritus. Nat Rev Neurol. 2014;10(7):408-16.
11. Martinelli-Boneschi F, Colombi M, Castori M, Devigili G, Eleopra R, Malik RA, et al. COL6A5 variants in familial neuropathic chronic itch. Brain. 2017;140(3):555-67.
12. Devigili G, Eleopra R, Pierro T, Lombardi R, Rinaldo S, Lettieri C, et al. Paroxysmal itch caused by gain-of-function Nav1.7 mutation. Pain. 2014;155(9):1702-7.
13. Mense S. Group III and IV receptors in skeletal muscle: are they specific or polymodal? Prog Brain Res. 1996;113:83-100.
14. Lopate G, Streif E, Harms M, Weihl C, Pestronk A. Cramps and small-fiber neuropathy. Muscle Nerve. 2013;48(2):252-5.
15. Peters MJ, Bakkers M, Merkies IS, Hoeijmakers JG, van Raak EP, Faber CG. Incidence and prevalence of small-fiber neuropathy: a survey in the Netherlands. Neurology. 2013;81(15): 1356-60.
16. Wakamoto H, Hirai A, Manabe K, Hayashi M. Idiopathic small-fiber sensory neuropathy in childhood: A diagnosis based on objective findings on punch skin biopsy specimens. J Pediatr. 1999;135(2 Pt 1):257-60.
17. Hoeijmakers JG, Faber CG, Miedema CJ, Merkies IS, Vles JS. Small Fiber Neuropathy in Children: Two Case Reports Illustrating the Importance of Recognition. Pediatrics. 2016;138(4).
18. Kafaie J, Kim M, Krause E. Small Fiber Neuropathy Following Vaccination. J Clin Neuromuscul Dis. 2016;18(1):37-40.
19. Oaklander AL, Klein MM. Evidence of small-fiber polyneuropathy in unexplained, juvenile-onset, widespread pain syndromes. Pediatrics. 2013;131(4):e1091-100.
20. Bakkers M, Faber CG, Hoeijmakers JG, Lauria G, Merkies IS. Small fibers, large impact: quality of life in small-fiber neuropathy. Muscle Nerve. 2014;49(3):329-36.
21. Lin MT, Lee LJ, Chao CC, Hsieh ST. Quality of life in polyneuropathy: association with biomarkers of small fiber impairment. Health Qual Life Outcomes. 2015;13:169.

22. Schaefer C, Mann R, Sadosky A, Daniel S, Parsons B, Nalamachu S, et al. Health status, function, productivity, and costs among individuals with idiopathic painful peripheral neuropathy with small fiber involvement in the United States: results from a retrospective chart review and cross-sectional survey. J Med Econ. 2014;17(6):394-407.
23. Hoeijmakers JG, Faber CG, Lauria G, Merkies IS, Waxman SG. Small-fibre neuropathies-advances in diagnosis, pathophysiology and management. Nat Rev Neurol. 2012;8(7):369-79.
24. Lauria G, Merkies IS, Faber CG. Small fibre neuropathy. Curr Opin Neurol. 2012;25(5):542-9.
25. Cazzato D, Lauria G. Small fibre neuropathy. Curr Opin Neurol. 2017;30(5):490-9.
26. Gasparotti R, Padua L, Briani C, Lauria G. New technologies for the assessment of neuropathies. Nat Rev Neurol. 2017;13(4):203-16.
27. Lauria G, Hsieh ST, Johansson O, Kennedy WR, Leger JM, Mellgren SI, et al. European Federation of Neurological Societies/Peripheral Nerve Society Guideline on the use of skin biopsy in the diagnosis of small fiber neuropathy. Report of a joint task force of the European Federation of Neurological Societies and the Peripheral Nerve Society. Eur J Neurol. 2010;17(7):903-12, e44-9.
28. 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.
29. Lauria G, Lombardi R, Camozzi F, Devigili G. Skin biopsy for the diagnosis of peripheral neuropathy. Histopathology. 2009;54(3):273-85.
30. Boulais N, Misery L. The epidermis: a sensory tissue. Eur J Dermatol. 2008;18(2):119-27.
31. Lauria G, Borgna M, Morbin M, Lombardi R, Mazzoleni G, Sghirlanzoni A, et al. Tubule and neurofilament immunoreactivity in human hairy skin: markers for intraepidermal nerve fibers. Muscle Nerve. 2004;30(3):310-6.
32. Lauria G, Merkies ISJ, Waxman SG, Faber CG. Epidermal Nerve Fibers. In: Aminoff MJ, Daroff RB, editors. Encyclopedia of the Neurological Sciences. 2nd ed. ed. Oxford: Academic Press; 2014:76-9.
33. Lauria G, Dacci P, Lombardi R, Cazzato D, Porretta-Serapiglia C, Taiana M, et al. Side and time variability of intraepidermal nerve fiber density. Neurology. 2015;84(23):2368-71.
34. Devigili G, Tugnoli V, Penza P, Camozzi F, Lombardi R, Melli G, et al. The diagnostic criteria for small fibre neuropathy: from symptoms to neuropathology. Brain. 2008;131(Pt 7):1912-25.
35. Liu Y, Fan X, Wei Y, Piao Z, Jiang X. Intraepidermal nerve fiber density of healthy human. Neurol Res. 2014;36(10):911-4.
36. Ruts L, van Doorn PA, Lombardi R, Haasdijk ED, Penza P, Tulen JH, et al. Unmyelinated and myelinated skin nerve damage in Guillain-Barre syndrome: correlation with pain and recovery. Pain. 2012;153(2):399-409.
37. Wongmek A, Shin S, Zhou L. Skin biopsy in assessing meralgia paresthetica. Muscle Nerve. 2016;53(4):641-3.
38. Lauria G, Lombardi R. Skin biopsy: a new tool for diagnosing peripheral neuropathy. BMJ. 2007;334(7604):1159-62.
39. Cazzato D, Castori M, Lombardi R, Caravello F, Bella ED, Petrucci A, et al. Small fiber neuropathy is a common feature of Ehlers-Danlos syndromes. Neurology. 2016;87(2):155-9.
40. Kosmidis ML, Koutsogeorgopoulou L, Alexopoulos H, Mamali I, Vlachoyiannopoulos PG, Voulgarelis M, et al. Reduction of Intraepidermal Nerve Fiber Density (IENFD) in the skin biopsies of patients with fibromyalgia: a controlled study. J Neurol Sci. 2014;347(1-2):143-7.
41. Kass-Iliyya L, Javed S, Gosal D, Kobylecki C, Marshall A, Petropoulos IN, et al. Small fiber neuropathy in Parkinson's disease: A clinical, pathological and corneal confocal microscopy study. Parkinsonism Relat Disord. 2015;21(12):1454-60.
42. Schrempf W, Katona I, Dogan I, Felbert W, Wienecke M, Heller J, et al. Reduced intraepidermal nerve fiber density in patients with REM sleep behavior disorder. Parkinsonism Relat Disord. 2016;29:10-6.
43. Podgorny PJ, Suchowersky O, Romanchuk KG, Feasby TE. Evidence for small fiber neuropathy in early Parkinson's disease. Parkinsonism Relat Disord. 2016;28:94-9.

44. Dalla Bella E, Lombardi R, Porretta-Serapiglia C, Ciano C, Gellera C, Pensato V, et al. Amyotrophic lateral sclerosis causes small fiber pathology. Eur J Neurol. 2016;23(2):416-20.
45. Nolano M, Provitera V, Manganelli F, Iodice R, Caporaso G, Stancanelli A, et al. Non-motor involvement in amyotrophic lateral sclerosis: new insight from nerve and vessel analysis in skin biopsy. Neuropathol Appl Neurobiol. 2016;43(2):119-32.
46. Truini A, Biasiotta A, Onesti E, Di Stefano G, Ceccanti M, La Cesa S, et al. Small-fibre neuropathy related to bulbar and spinal-onset in patients with ALS. J Neurol. 2015; 262(4):1014-8.
47. Skorna M, Kopacik R, Vlckova E, Adamova B, Kostalova M, Bednarik J. Small-nerve-fiber pathology in critical illness documented by serial skin biopsies. Muscle Nerve. 2015;52(1): 28-33.
48. Grone E, Uceyler N, Abahji T, Fleckenstein J, Irnich D, Mussack T, et al. Reduced intraepidermal nerve fiber density in patients with chronic ischemic pain in peripheral arterial disease. Pain. 2014;155(9):1784-92.
49. Provitera V, Gibbons CH, Wendelschafer-Crabb G, Donadio V, Vitale DF, Stancanelli A, et al. A multi-center, multinational age- and gender-adjusted normative dataset for immunofluorescent intraepidermal nerve fiber density at the distal leg. Eur J Neurol. 2016;23(2):333-8.
50. Van Acker N, Rage M, Sluydts E, Knaapen MW, De Bie M, Timmers M, et al. Automated PGP9.5 immunofluorescence staining: a valuable tool in the assessment of small fiber neuropathy? BMC Res Notes. 2016;9:280.
51. Dauch JR, Lindblad CN, Hayes JM, Lentz SI, Cheng HT. Three-dimensional imaging of nociceptive intraepidermal nerve fibers in human skin biopsies. J Vis Exp. 2013(74):e50331.
52. Karlsson P, Moller AT, Jensen TS, Nyengaard JR. Epidermal nerve fiber length density estimation using global spatial sampling in healthy subjects and neuropathy patients. J Neuropathol Exp Neurol. 2013;72(3):186-93.
53. Lauria G, Cazzato D, Porretta-Serapiglia C, Casanova-Molla J, Taiana M, Penza P, et al. Morphometry of dermal nerve fibers in human skin. Neurology. 2011;77(3):242-9.
54. Gibbons CH, Illigens BM, Wang N, Freeman R. Quantification of sweat gland innervation: a clinical-pathologic correlation. Neurology. 2009;72(17):1479-86.
55. Nolano M, Provitera V, Caporaso G, Stancanelli A, Vitale DF, Santoro L. Quantification of pilomotor nerves: a new tool to evaluate autonomic involvement in diabetes. Neurology. 2010;75(12):1089-97.
56. Tavakoli M, Hossain P, Malik RA. Clinical applications of corneal confocal microscopy. Clin Ophthalmol. 2008;2(2):435-45.
57. Patel DV, Tavakoli M, Craig JP, Efron N, McGhee CN. Corneal sensitivity and slit scanning in vivo confocal microscopy of the subbasal nerve plexus of the normal central and peripheral human cornea. Cornea. 2009;28(7):735-40.
58. Oliveira-Soto L, Efron N. Morphology of corneal nerves using confocal microscopy. Cornea. 2001;20(4):374-84.
59. Tavakoli M, Ferdousi M, Petropoulos IN, Morris J, Pritchard N, Zhivov A, et al. Normative values for corneal nerve morphology assessed using corneal confocal microscopy: a multinational normative data set. Diabetes Care. 2015;38(5):838-43.
60. Petropoulos IN, Manzoor T, Morgan P, Fadavi H, Asghar O, Alam U, et al. Repeatability of in vivo corneal confocal microscopy to quantify corneal nerve morphology. Cornea. 2013; 32(5):e83-9.
61. Gemignani F, Ferrari G, Vitetta F, Giovanelli M, Macaluso C, Marbini A. Non-length-dependent small fibre neuropathy. Confocal microscopy study of the corneal innervation. J Neurol Neurosurg Psychiatry. 2010;81(7):731-3.
62. Tavakoli M, Marshall A, Pitceathly R, Fadavi H, Gow D, Roberts ME, et al. Corneal confocal microscopy: a novel means to detect nerve fibre damage in idiopathic small fibre neuropathy. Exp Neurol. 2010;223(1):245-50.

63. Tavakoli M, Mitu-Pretorian M, Petropoulos IN, Fadavi H, Asghar O, Alam U, et al. Corneal confocal microscopy detects early nerve regeneration in diabetic neuropathy after simultaneous pancreas and kidney transplantation. Diabetes. 2013;62(1):254-60.
64. Azmi S, Ferdousi M, Petropoulos IN, Ponirakis G, Fadavi H, Tavakoli M, et al. Corneal confocal microscopy shows an improvement in small-fiber neuropathy in subjects with type 1 diabetes on continuous subcutaneous insulin infusion compared with multiple daily injection. Diabetes Care. 2015;38(1):e3-4.
65. Tavakoli M, Marshall A, Thompson L, Kenny M, Waldek S, Efron N, et al. Corneal confocal microscopy: a novel noninvasive means to diagnose neuropathy in patients with Fabry disease. Muscle Nerve. 2009;40(6):976-84.
66. Tavakoli M, Marshall A, Banka S, Petropoulos IN, Fadavi H, Kingston H, et al. Corneal confocal microscopy detects small-fiber neuropathy in Charcot-Marie-Tooth disease type 1A patients. Muscle Nerve. 2012;46(5):698-704.
67. Stettner M, Hinrichs L, Guthoff R, Bairov S, Petropoulos IN, Warnke C, et al. Corneal confocal microscopy in chronic inflammatory demyelinating polyneuropathy. Ann Clin Transl Neurol. 2016;3(2):88-100.
68. Bitirgen G, Akpinar Z, Malik RA, Ozkagnici A. Use of Corneal Confocal Microscopy to Detect Corneal Nerve Loss and Increased Dendritic Cells in Patients With Multiple Sclerosis. JAMA Ophthalmol. 2017;135(7):777-82.
69. Dyck PJ, Zimmerman I, Gillen DA, Johnson D, Karnes JL, O'Brien PC. Cool, warm, and heat-pain detection thresholds: testing methods and inferences about anatomic distribution of receptors. Neurology. 1993;43(8):1500-8.
70. Hoitsma E, Drent M, Verstraete E, Faber CG, Troost J, Spaans F, et al. Abnormal warm and cold sensation thresholds suggestive of small-fibre neuropathy in sarcoidosis. Clin Neurophysiol. 2003;114(12):2326-33.
71. 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.
72. Pertovaara A, Kauppila T, Hamalainen MM. Influence of skin temperature on heat pain threshold in humans. Exp Brain Res. 1996;107(3):497-503.
73. Yarnitsky D, Ochoa JL. Warm and cold specific somatosensory systems. Psychophysical thresholds, reaction times and peripheral conduction velocities. Brain. 1991;114 (Pt 4): 1819-26.
74. Bakkers M, Faber CG, Peters MJ, Reulen JP, Franssen H, Fischer TZ, et al. Temperature threshold testing: a systematic review. J Peripher Nerv Syst. 2013;18(1):7-18.
75. Kemler MA, Reulen JP, van Kleef M, Barendse GA, van den Wildenberg FA, Spaans F. Thermal thresholds in complex regional pain syndrome type I: sensitivity and repeatability of the methods of limits and levels. Clin Neurophysiol. 2000;111(9):1561-8.
76. Bakkers M, Faber CG, Reulen JP, Hoeijmakers JG, Vanhoutte EK, Merkies IS. Optimizing temperature threshold testing in small-fiber neuropathy. Muscle Nerve. 2015;51(6):870-6.
77. Ng Wing Tin S, Ciampi de Andrade D, Goujon C, Plante-Bordeneuve V, Creange A, Lefaucheur JP. Sensory correlates of pain in peripheral neuropathies. Clin Neurophysiol. 2014; 125(5):1048-58.
78. Loseth S, Stalberg E, Jorde R, Mellgren SI. Early diabetic neuropathy: thermal thresholds and intraepidermal nerve fibre density in patients with normal nerve conduction studies. J Neurol. 2008;255(8):1197-202.
79. 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.
80. Yarnitsky D, Sprecher E, Tamir A, Zaslansky R, Hemli JA. Variance of sensory threshold measurements: discrimination of feigners from trustworthy performers. J Neurol Sci. 1994;125(2):186-9.

81. Verdugo RJ, Ochoa JL. Use and misuse of conventional electrodiagnosis, quantitative sensory testing, thermography, and nerve blocks in the evaluation of painful neuropathic syndromes. Muscle Nerve. 1993;16(10):1056-62.
82. Shy ME, Frohman EM, So YT, Arezzo JC, Cornblath DR, Giuliani MJ, et al. Quantitative sensory testing: report of the Therapeutics and Technology Assessment Subcommittee of the American Academy of Neurology. Neurology. 2003;60(6):898-904.
83. Dyck PJ, Dyck PJ, Kennedy WR, Kesserwani H, Melanson M, Ochoa J, et al. Limitations of quantitative sensory testing when patients are biased toward a bad outcome. Neurology. 1998;50(5):1213.
84. Hansson P, Backonja M, Bouhassira D. Usefulness and limitations of quantitative sensory testing: clinical and research application in neuropathic pain states. Pain. 2007;129(3):256-9.
85. Serra J. Microneurography: an opportunity for translational drug development in neuropathic pain. Neurosci Lett. 2010;470(3):155-7.
86. Donadio V, Liguori R. Microneurographic recording from unmyelinated nerve fibers in neurological disorders: an update. Clin Neurophysiol. 2015;126(3):437-45.
87. Liguori R, Giannoccaro MP, Di Stasi V, Pizza F, Cortelli P, Baruzzi A, et al. Microneurographic evaluation of sympathetic activity in small fiber neuropathy. Clin Neurophysiol. 2011;122(9):1854-9.
88. Ochoa JL, Campero M, Serra J, Bostock H. Hyperexcitable polymodal and insensitive nociceptors in painful human neuropathy. Muscle Nerve. 2005;32(4):459-72.
89. Kleggetveit IP, Schmidt R, Namer B, Salter H, Helas T, Schmelz M, et al. Pathological nociceptors in two patients with erythromelalgia-like symptoms and rare genetic Nav 1.9 variants. Brain Behav. 2016;6(10):e00528.
90. Mainka T, Maier C, Enax-Krumova EK. Neuropathic pain assessment: update on laboratory diagnostic tools. Curr Opin Anaesthesiol. 2015;28(5):537-45.
91. Le Pera D, Valeriani M, Niddam D, Chen AC, Arendt-Nielsen L. Contact heat evoked potentials to painful and non-painful stimuli: effect of attention towards stimulus properties. Brain Topogr. 2002;15(2):115-23.
92. Merkies IS, Faber CG, Lauria G. Advances in diagnostics and outcome measures in peripheral neuropathies. Neurosci Lett. 2015;596:3-13.
93. Rage M, Van Acker N, Facer P, Shenoy R, Knaapen MW, 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.
94. Garcia-Larrea L, Frot M, Valeriani M. Brain generators of laser-evoked potentials: from dipoles to functional significance. Neurophysiol Clin. 2003;33(6):279-92.
95. Mobascher A, Brinkmeyer J, Warbrick T, Musso F, Wittsack HJ, Saleh A, et al. Laser-evoked potential P2 single-trial amplitudes covary with the fMRI BOLD response in the medial pain system and interconnected subcortical structures. Neuroimage. 2009;45(3):917-26.
96. Garcia-Larrea L, Peyron R, Laurent B, Mauguiere F. Association and dissociation between laser-evoked potentials and pain perception. Neuroreport. 1997;8(17):3785-9.
97. Truini A, Galeotti F, Romaniello A, Virtuoso M, Iannetti GD, Cruccu G. Laser-evoked potentials: normative values. Clin Neurophysiol. 2005;116(4):821-6.
98. Hoeben E, Smit JW, Upmalis D, Rusch S, Schaffler K, Reitmeir P, et al. Dose-response relationship after single oral dose administrations of morphine and oxycodone using laser-evoked potentials on UVB- and capsaicin-irritated skin in healthy male subjects. Pain. 2012; 153(8):1648-56.
99. Colloca L, Sigaudo M, Benedetti F. The role of learning in nocebo and placebo effects. Pain. 2008;136(1-2):211-8.
100. Hird EJ, Jones AKP, Talmi D, El-Deredy W. A comparison between the neural correlates of laser and electric pain stimulation and their modulation by expectation. J Neurosci Methods. 2017;293:117-27.
101. Cruccu G, Aminoff MJ, Curio G, Guerit JM, Kakigi R, Mauguiere F, et al. Recommendations for the clinical use of somatosensory-evoked potentials. Clin Neurophysiol. 2008;119(8):1705-19.

102. Lagerburg V, Bakkers M, Bouwhuis A, Hoeijmakers JG, Smit AM, Van Den Berg SJ, et al. Contact heat evoked potentials: normal values and use in small-fiber neuropathy. Muscle Nerve. 2015;51(5):743-9.
103. Wu SW, Wang YC, Hsieh PC, Tseng MT, Chiang MC, Chu CP, et al. Biomarkers of neuropathic pain in skin nerve degeneration neuropathy: contact heat-evoked potentials as a physiological signature. Pain. 2017;158(3):516-25.
104. Atherton DD, Facer P, Roberts KM, Misra VP, Chizh BA, Bountra C, et al. Use of the novel Contact Heat Evoked Potential Stimulator (CHEPS) for the assessment of small fibre neuropathy: correlations with skin flare responses and intra-epidermal nerve fibre counts. BMC Neurol. 2007;7:21.
105. Chao CC, Hsieh SC, Tseng MT, Chang YC, Hsieh ST. Patterns of contact heat evoked potentials (CHEP) in neuropathy with skin denervation: correlation of CHEP amplitude with intraepidermal nerve fiber density. Clin Neurophysiol. 2008;119(3):653-61.
106. Casanova-Molla J, Grau-Junyent JM, Morales M, Valls-Sole J. On the relationship between nociceptive evoked potentials and intraepidermal nerve fiber density in painful sensory polyneuropathies. Pain. 2011;152(2):410-8.
107. Inui K, Kakigi R. Pain perception in humans: use of intraepidermal electrical stimulation. J Neurol Neurosurg Psychiatry. 2012;83(5):551-6.
108. Kodaira M, Inui K, Kakigi R. Evaluation of nociceptive Adelta- and C-fiber dysfunction with lidocaine using intraepidermal electrical stimulation. Clin Neurophysiol. 2014;125(9):1870-7.
109. Omori S, Isose S, Misawa S, Watanabe K, Sekiguchi Y, Shibuya K, et al. Pain-related evoked potentials after intraepidermal electrical stimulation to Adelta and C fibers in patients with neuropathic pain. Neurosci Res. 2017.
110. Ebadi H, Siddiqui H, Ebadi S, Ngo M, Breiner A, Bril V. Peripheral Nerve Ultrasound in Small Fiber Polyneuropathy. Ultrasound Med Biol. 2015;41(11):2820-6.
111. Persson AK, Liu S, Faber CG, Merkies IS, Black JA, Waxman SG. Neuropathy-associated Nav1.7 variant I228M impairs integrity of dorsal root ganglion neuron axons. Ann Neurol. 2013; 73(1):140-5.
112. Persson AK, Hoeijmakers JG, Estacion M, Black JA, Waxman SG. Sodium Channels, Mitochondria, and Axonal Degeneration in Peripheral Neuropathy. Trends Mol Med. 2016;22(5):377-90.
113. Tseng MT, Chiang MC, Chao CC, Tseng WY, Hsieh ST. fMRI evidence of degeneration-induced neuropathic pain in diabetes: enhanced limbic and striatal activations. Hum Brain Mapp. 2013;34(10):2733-46.
114. Cauda F, Sacco K, Duca S, Cocito D, D'Agata F, Geminiani GC, et al. Altered resting state in diabetic neuropathic pain. PloS One. 2009;4(2):e4542.
115. Cauda F, D'Agata F, Sacco K, Duca S, Cocito D, Paolasso I, et al. Altered resting state attentional networks in diabetic neuropathic pain. J Neurol Neurosurg Psychiatry. 2010;81(7): 806-11.
116. Hsieh PC, Tseng MT, Chao CC, Lin YH, Tseng WY, Liu KH, et al. Imaging signatures of altered brain responses in small-fiber neuropathy: reduced functional connectivity of the limbic system after peripheral nerve degeneration. Pain. 2015;156(5):904-16.
117. Apkarian AV, Bushnell MC, Treede RD, Zubieta JK. Human brain mechanisms of pain perception and regulation in health and disease. Eur J Pain. 2005;9(4):463-84.
118. Cole LJ, Farrell MJ, Gibson SJ, Egan GF. Age-related differences in pain sensitivity and regional brain activity evoked by noxious pressure. Neurobiol Aging. 2010;31(3):494-503.
119. Quiton RL, Greenspan JD. Sex differences in endogenous pain modulation by distracting and painful conditioning stimulation. Pain. 2007;132 Suppl 1:S134-49.
120. Paulson PE, Minoshima S, Morrow TJ, Casey KL. Gender differences in pain perception and patterns of cerebral activation during noxious heat stimulation in humans. Pain. 1998;76(1-2):223-9.
121. Rainville P, Duncan GH, Price DD, Carrier B, Bushnell MC. Pain affect encoded in human anterior cingulate but not somatosensory cortex. Science. 1997;277(5328):968-71.

122. Ploghaus A, Tracey I, Gati JS, Clare S, Menon RS, Matthews PM, et al. Dissociating pain from its anticipation in the human brain. Science. 1999;284(5422):1979-81.
123. Ploghaus A, Tracey I, Clare S, Gati JS, Rawlins JN, Matthews PM. Learning about pain: the neural substrate of the prediction error for aversive events. Proc Natl Acad Sci U S A. 2000; 97(16):9281-6.
124. Phillips ML, Gregory LJ, Cullen S, Coen S, Ng V, Andrew C, et al. The effect of negative emotional context on neural and behavioural responses to oesophageal stimulation. Brain. 2003;126(Pt 3):669-84.
125. Gracely RH, Petzke F, Wolf JM, Clauw DJ. Functional magnetic resonance imaging evidence of augmented pain processing in fibromyalgia. Arthritis Rheum. 2002;46(5):1333-43.
126. Grachev ID, Fredrickson BE, Apkarian AV. Abnormal brain chemistry in chronic back pain: an in vivo proton magnetic resonance spectroscopy study. Pain. 2000;89(1):7-18.
127. Low VA, Sandroni P, Fealey RD, Low PA. Detection of small-fiber neuropathy by sudomotor testing. Muscle Nerve. 2006;34(1):57-61.
128. Illigens BM, Gibbons CH. Sweat testing to evaluate autonomic function. Clin Auton Res. 2009;19(2):79-87.
129. Thaisetthawatkul P, Fernandes Filho JA, Herrmann DN. Contribution of QSART to the diagnosis of small fiber neuropathy. Muscle Nerve. 2013;48(6):883-8.
130. Namer B, Pfeffer S, Handwerker HO, Schmelz M, Bickel A. Axon reflex flare and quantitative sudomotor axon reflex contribute in the diagnosis of small fiber neuropathy. Muscle Nerve. 2013;47(3):357-63.
131. Stewart JD, Nguyen DM, Abrahamowicz M. Quantitative sweat testing using acetylcholine for direct and axon reflex mediated stimulation with silicone mold recording; controls versus neuropathic diabetics. Muscle Nerve. 1994;17(12):1370-7.
132. Lacomis D. Small-fiber neuropathy. Muscle Nerve. 2002;26(2):173-88.
133. Grandinetti A, Chow DC, Sletten DM, Oyama JK, Theriault AG, Schatz IJ, et al. Impaired glucose tolerance is associated with postganglionic sudomotor impairment. Clin Auton Res. 2007;17(4):231-3.
134. Mao F, Liu S, Qiao X, Zheng H, Xiong Q, Wen J, et al. SUDOSCAN, an effective tool for screening chronic kidney disease in patients with type 2 diabetes. Exp Ther Med. 2017;14(2): 1343-50.
135. Nevoret ML, Vinik AI. CIDP variants in diabetes: measuring treatment response with a small nerve fiber test. J Diabetes Complications. 2015;29(2):313-7.
136. Bordier L, Dolz M, Monteiro L, Nevoret ML, Calvet JH, Bauduceau B. Accuracy of a Rapid and Non-Invasive Method for the Assessment of Small Fiber Neuropathy Based on Measurement of Electrochemical Skin Conductances. Front Endocrinol (Lausanne). 2016;7:18.
137. Sato K, Kang WH, Saga K, Sato KT. Biology of sweat glands and their disorders. II. Disorders of sweat gland function. J Am Acad Dermatol. 1989;20(5 Pt 1):713-26.
138. Casellini CM, Parson HK, Richardson MS, Nevoret ML, Vinik AI. Sudoscan, a noninvasive tool for detecting diabetic small fiber neuropathy and autonomic dysfunction. Diabetes Technol Ther. 2013;15(11):948-53.
139. Parson HK, Nguyen VT, Orciga MA, Boyd AL, Casellini CM, Vinik AI. Contact heat-evoked potential stimulation for the evaluation of small nerve fiber function. Diabetes Technol Ther. 2013;15(2):150-7.
140. Rajan S, Campagnolo M, Callaghan B, Gibbons CH. Sudomotor function testing by electrochemical skin conductance: does it really measure sudomotor function? Clin Auton Res. 2019;29(1):31-9.
141. Ponirakis G, Petropoulos IN, Fadavi H, Alam U, Asghar O, Marshall A, et al. The diagnostic accuracy of Neuropad for assessing large and small fibre diabetic neuropathy. Diabet Med. 2014;31(12):1673-80.
142. Wilder-Smith EP. Water immersion wrinkling--physiology and use as an indicator of sympathetic function. Clin Auton Res. 2004;14(2):125-31.

143. Willatts DG, Reynolds F. Comparison of the vasoactivity of amide and ester local anaesthetics. An intradermal study. Br J Anaesth. 1985;57(10):1006-11.
144. Hsieh CH, Huang KF, Liliang PC, Huang PC, Shih HM, Rau CS. EMLA and water immersion cause similar vasodilatation in replanted fingers. J Surg Res. 2007;143(2):265-9.
145. Wilder-Smith E, Chow A. Water immersion and EMLA cause similar digit skin wrinkling and vasoconstriction. Microvasc Res. 2003;66(1):68-72.
146. Teoh HL, Chow A, Wilder-Smith EP. Skin wrinkling for diagnosing small fibre neuropathy: comparison with epidermal nerve density and sympathetic skin response. J Neurol Neurosurg Psychiatry. 2008;79(7):835-7.
147. Wilder-Smith EP. Stimulated skin wrinkling as an indicator of limb sympathetic function. Clin Neurophysiol. 2015;126(1):10-6.
148. Wilder-Smith EP, Guo Y, Chow A. Stimulated skin wrinkling for predicting intraepidermal nerve fibre density. Clin Neurophysiol. 2009;120(5):953-8.
149. Anderson EA, Wallin BG, Mark AL. Dissociation of sympathetic nerve activity in arm and leg muscle during mental stress. Hypertension. 1987;9(6 Pt 2):III114-9.
150. Vasudevan TM, van Rij AM, Nukada H, Taylor PK. Skin wrinkling for the assessment of sympathetic function in the limbs. Aust N Z J Surg. 2000;70(1):57-9.
151. Clark CV, Pentland B, Ewing DJ, Clarke BF. Decreased skin wrinkling in diabetes mellitus. Diabetes Care. 1984;7(3):224-7.
152. Ping Ng KW, Ong JJ, Nyein Nyein TD, Liang S, Chan YC, Lee KO, et al. EMLA-Induced Skin Wrinkling for the Detection of Diabetic Neuropathy. Front Neurol. 2013;4:126.
153. Brouwer BA, Bakkers M, Hoeijmakers JG, Faber CG, Merkies IS. Improving assessment in small fiber neuropathy. J Peripher Nerv Syst. 2015;20(3):333-40.
154. Bakkers M, Faber CG, Drent M, Hermans MC, van Nes SI, Lauria G, et al. Pain and autonomic dysfunction in patients with sarcoidosis and small fibre neuropathy. J Neurol. 2010; 257(12):2086-90.
155. Treister R, Lodahl M, Lang M, Tworoger SS, Sawilowsky S, Oaklander AL. Initial Development and Validation of a Patient-Reported Symptom Survey for Small-Fiber Polyneuropathy. J Pain. 2017.
156. 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.
157. de Greef BT H, J.G.J, Merkies, I.S.J., Faber, C.G. Associated conditions in small fiber neuropathy – A large cohort study and review of the literature. Eur J Neurol. 2018;25(2):348-55.
158. Lang M, Treister R, Oaklander AL. Diagnostic value of blood tests for occult causes of initially idiopathic small-fiber polyneuropathy. J Neurol. 2016;263(12):2515-27.
159. de Greef BT, Hoeijmakers JG, Wolters EE, Smeets HJ, van den Wijngaard A, Merkies IS, et al. No Fabry Disease in Patients Presenting with Isolated Small Fiber Neuropathy. PLoS One. 2016;11(2):e0148316.
160. Masuda T, Ueda M, Suenaga G, Misumi Y, Tasaki M, Izaki A, et al. Early skin denervation in hereditary and iatrogenic transthyretin amyloid neuropathy. Neurology. 2017;88(23):2192-7.
161. Adams D, Suhr OB, Hund E, Obici L, Tournev I, Campistol JM, et al. First European consensus for diagnosis, management, and treatment of transthyretin familial amyloid polyneuropathy. Curr Opin Neurol. 2016;29 Suppl 1:S14-26.
162. Dib-Hajj SD, Yang Y, Black JA, Waxman SG. The Na(V)1.7 sodium channel: from molecule to man. Nat Rev Neurosci. 2013;14(1):49-62.
163. Dib-Hajj SD, Geha P, Waxman SG. Sodium channels in pain disorders: pathophysiology and prospects for treatment. Pain. 2017;158 Suppl 1:S97-S107.
164. Cummins TR, Rush AM, Estacion M, Dib-Hajj SD, Waxman SG. Voltage-clamp and current-clamp recordings from mammalian DRG neurons. Nat Protoc. 2009;4(8):1103-12.
165. Cao L, McDonnell A, Nitzsche A, Alexandrou A, Saintot PP, Loucif AJ, et al. Pharmacological reversal of a pain phenotype in iPSC-derived sensory neurons and patients with inherited erythromelalgia. Sci Transl Med. 2016;8(335):335ra56.

166. Alexandrou AJ, Brown AR, Chapman ML, Estacion M, Turner J, Mis MA, et al. Subtype-Selective Small Molecule Inhibitors Reveal a Fundamental Role for Nav1.7 in Nociceptor Electrogenesis, Axonal Conduction and Presynaptic Release. PLoS One. 2016;11(4):e0152405.
167. Zakrzewska JM, Palmer J, Morisset V, Giblin GM, Obermann M, Ettlin DA, et al. Safety and efficacy of a Nav1.7 selective sodium channel blocker in patients with trigeminal neuralgia: a double-blind, placebo-controlled, randomised withdrawal phase 2a trial. Lancet Neurol. 2017;16(4):291-300.
168. Drenth JP, Michiels JJ. Three types of erythromelalgia. BMJ. 1990;301(6750):454-5.
169. McDonnell A, Schulman B, Ali Z, Dib-Hajj SD, Brock F, Cobain S, et al. Inherited erythromelalgia due to mutations in SCN9A: natural history, clinical phenotype and somatosensory profile. Brain. 2016;139(Pt 4):1052-65.
170. Drenth JP, Waxman SG. Mutations in sodium-channel gene SCN9A cause a spectrum of human genetic pain disorders. J Clin Invest. 2007;117(12):3603-9.
171. Burns TM, Te Morsche RH, Jansen JB, Drenth JP. Genetic heterogeneity and exclusion of a modifying locus at 2q in a family with autosomal dominant primary erythermalgia. Br J Dermatol. 2005;153(1):174-7.
172. Rush AM, Dib-Hajj SD, Liu S, Cummins TR, Black JA, Waxman SG. A single sodium channel mutation produces hyper- or hypoexcitability in different types of neurons. Proc Natl Acad Sci U S A. 2006;103(21):8245-50.
173. Michiels JJ, te Morsche RH, Jansen JB, Drenth JP. Autosomal dominant erythermalgia associated with a novel mutation in the voltage-gated sodium channel alpha subunit Nav1.7. Arch Neurol. 2005;62(10):1587-90.
174. Dib-Hajj SD, Rush AM, Cummins TR, Hisama FM, Novella S, Tyrrell L, et al. Gain-of-function mutation in Nav1.7 in familial erythromelalgia induces bursting of sensory neurons. Brain. 2005;128(Pt 8):1847-54.
175. Lampert A, Dib-Hajj SD, Tyrrell L, Waxman SG. Size matters: Erythromelalgia mutation S241T in Nav1.7 alters channel gating. J Biol Chem. 2006;281(47):36029-35.
176. Lampert A, O'Reilly AO, Dib-Hajj SD, Tyrrell L, Wallace BA, Waxman SG. A pore-blocking hydrophobic motif at the cytoplasmic aperture of the closed-state Nav1.7 channel is disrupted by the erythromelalgia-associated F1449V mutation. J Biol Chem. 2008;283(35):24118-27.
177. Lampert A, Dib-Hajj SD, Eastman EM, Tyrrell L, Lin Z, Yang Y, et al. Erythromelalgia mutation L823R shifts activation and inactivation of threshold sodium channel Nav1.7 to hyperpolarized potentials. Biochem Biophys Res Commun. 2009;390(2):319-24.
178. Stadler T, O'Reilly AO, Lampert A. Erythromelalgia mutation Q875E Stabilizes the activated state of sodium channel Nav1.7. J Biol Chem. 2015;290(10):6316-25.
179. Choi JS, Cheng X, Foster E, Leffler A, Tyrrell L, Te Morsche RH, et al. Alternative splicing may contribute to time-dependent manifestation of inherited erythromelalgia. Brain. 2010;133(Pt 6):1823-35.
180. Cregg R, Laguda B, Werdehausen R, Cox JJ, Linley JE, Ramirez JD, et al. Novel mutations mapping to the fourth sodium channel domain of Nav1.7 result in variable clinical manifestations of primary erythromelalgia. Neuromolecular Med. 2013;15(2):265-78.
181. Estacion M, Yang Y, Dib-Hajj SD, Tyrrell L, Lin Z, Yang Y, et al. A new Nav1.7 mutation in an erythromelalgia patient. Biochem Biophys Res Commun. 2013;432(1):99-104.
182. Novella SP, Hisama FM, Dib-Hajj SD, Waxman SG. A case of inherited erythromelalgia. Nat Clin Pract Neurol. 2007;3(4):229-34.
183. Harty TP, Dib-Hajj SD, Tyrrell L, Blackman R, Hisama FM, Rose JB, et al. Na(V)1.7 mutant A863P in erythromelalgia: effects of altered activation and steady-state inactivation on excitability of nociceptive dorsal root ganglion neurons. J Neurosci. 2006;26(48):12566-75.
184. Ahn HS, Vasylyev DV, Estacion M, Macala LJ, Shah P, Faber CG, et al. Differential effect of D623N variant and wild-type Na(v)1.7 sodium channels on resting potential and interspike membrane potential of dorsal root ganglion neurons. Brain Res. 2013;1529:165-77.
185. Kim MK, Yuk JW, Kim HS, Park KJ, Kim DS. Autonomic dysfunction in SCN9A-associated primary erythromelalgia. Clin Auton Res. 2013;23(2):105-7.

186. Namer B, Orstavik K, Schmidt R, Kleggetveit IP, Weidner C, Mork C, et al. Specific changes in conduction velocity recovery cycles of single nociceptors in a patient with erythromelalgia with the I848T gain-of-function mutation of Nav1.7. Pain. 2015;156(9):1637-46.
187. Sheets PL, Jackson JO, 2nd, Waxman SG, Dib-Hajj SD, Cummins TR. A Nav1.7 channel mutation associated with hereditary erythromelalgia contributes to neuronal hyperexcitability and displays reduced lidocaine sensitivity. J Physiol. 2007;581(Pt 3):1019-31.
188. Yang Y, Wang Y, Li S, Xu Z, Li H, Ma L, et al. Mutations in SCN9A, encoding a sodium channel alpha subunit, in patients with primary erythermalgia. J Med Genet. 2004;41(3):171-4.
189. Cheng X, Dib-Hajj SD, Tyrrell L, Waxman SG. Mutation I136V alters electrophysiological properties of the Na(v)1.7 channel in a family with onset of erythromelalgia in the second decade. Mol Pain. 2008;4:1.
190. Yang Y, Huang J, Mis MA, Estacion M, Macala L, Shah P, et al. Nav1.7-A1632G Mutation from a Family with Inherited Erythromelalgia: Enhanced Firing of Dorsal Root Ganglia Neurons Evoked by Thermal Stimuli. J Neurosci. 2016;36(28):7511-22.
191. Tanaka BS, Nguyen PT, Zhou EY, Yang Y, Yarov-Yarovoy V, Dib-Hajj SD, et al. Gain-of-function mutation of a voltage-gated sodium channel NaV1.7 associated with peripheral pain and impaired limb development. J Biol Chem. 2017;292(22):9262-72.
192. Cummins TR, Dib-Hajj SD, Waxman SG. Electrophysiological properties of mutant Nav1.7 sodium channels in a painful inherited neuropathy. J Neurosci. 2004;24(38):8232-6.
193. Choi JS, Dib-Hajj SD, Waxman SG. Inherited erythermalgia: limb pain from an S4 charge-neutral Na channelopathy. Neurology. 2006;67(9):1563-7.
194. Drenth JP, te Morsche RH, Guillet G, Taieb A, Kirby RL, Jansen JB. SCN9A mutations define primary erythermalgia as a neuropathic disorder of voltage gated sodium channels. J Invest Dermatol. 2005;124(6):1333-8.
195. Han C, Rush AM, Dib-Hajj SD, Li S, Xu Z, Wang Y, et al. Sporadic onset of erythermalgia: a gain-of-function mutation in Nav1.7. Ann Neurol. 2006;59(3):553-8.
196. Han C, Lampert A, Rush AM, Dib-Hajj SD, Wang X, Yang Y, et al. Temperature dependence of erythromelalgia mutation L858F in sodium channel Nav1.7. Mol Pain. 2007;3:3.
197. Gurkiewicz M, Korngreen A, Waxman SG, Lampert A. Kinetic modeling of Nav1.7 provides insight into erythromelalgia-associated F1449V mutation. J Neurophysiol. 2011;105(4): 1546-57.
198. Meijer IA, Vanasse M, Nizard S, Robitaille Y, Rossignol E. An atypical case of SCN9A mutation presenting with global motor delay and a severe pain disorder. Muscle Nerve. 2014;49(1): 134-8.
199. Han C, Dib-Hajj SD, Lin Z, Li Y, Eastman EM, Tyrrell L, et al. Early- and late-onset inherited erythromelalgia: genotype-phenotype correlation. Brain. 2009;132(Pt 7):1711-22.
200. Ahn HS, Dib-Hajj SD, Cox JJ, Tyrrell L, Elmslie FV, Clarke AA, et al. A new Nav1.7 sodium channel mutation I234T in a child with severe pain. Eur J Pain. 2010;14(9):944-50.
201. Kim DT, Rossignol E, Najem K, Ospina LH. Bilateral congenital corneal anesthesia in a patient with SCN9A mutation, confirmed primary erythromelalgia, and paroxysmal extreme pain disorder. J AAPOS. 2015;19(5):478-9.
202. Huang J, Mis MA, Tanaka B, Adi T, Estacion M, Liu S, et al. Atypical changes in DRG neuron excitability and complex pain phenotype associated with a Nav1.7 mutation that massively hyperpolarizes activation. Sci Rep. 2018;8(1):1811.
203. Skeik N, Rooke TW, Davis MD, Davis DM, Kalsi H, Kurth I, et al. Severe case and literature review of primary erythromelalgia: novel SCN9A gene mutation. Vasc Med. 2012;17(1):44-9.
204. Dabby R, Sadeh M, Gilad R, Lampl Y, Cohen S, Inbar S, et al. Chronic non-paroxysmal neuropathic pain - Novel phenotype of mutation in the sodium channel SCN9A gene. J Neurol Sci. 2011;301(1-2):90-2.
205. Kist AM, Sagafos D, Rush AM, Neacsu C, Eberhardt E, Schmidt R, et al. SCN10A Mutation in a Patient with Erythromelalgia Enhances C-Fiber Activity Dependent Slowing. PloS One. 2016; 11(9):e0161789.

206. Fertleman CR, Baker MD, Parker KA, Moffatt S, Elmslie FV, Abrahamsen B, et al. SCN9A mutations in paroxysmal extreme pain disorder: allelic variants underlie distinct channel defects and phenotypes. Neuron. 2006;52(5):767-74.
207. Jarecki BW, Sheets PL, Xiao Y, Jackson JO, 2nd, Cummins TR. Alternative splicing of Na(V)1.7 exon 5 increases the impact of the painful PEPD mutant channel I1461T. Channels (Austin). 2009;3(4):259-67.
208. Theile JW, Jarecki BW, Piekarz AD, Cummins TR. Nav1.7 mutations associated with paroxysmal extreme pain disorder, but not erythromelalgia, enhance Navbeta4 peptide-mediated resurgent sodium currents. J Physiol. 2011;589(Pt 3):597-608.
209. Imai N, Miyake N, Saito Y, Kobayashi E, Ikawa M, Manaka S, et al. Short-lasting unilateral neuralgiform headache attacks with ispilateral facial flushing is a new variant of paroxysmal extreme pain disorder. J Headache Pain. 2015;16:519.
210. Suter MR, Bhuiyan ZA, Laedermann CJ, Kuntzer T, Schaller M, Stauffacher MW, et al. p.L1612P, a novel voltage-gated sodium channel Nav1.7 mutation inducing a cold sensitive paroxysmal extreme pain disorder. Anesthesiology. 2015;122(2):414-23.
211. Dib-Hajj SD, Estacion M, Jarecki BW, Tyrrell L, Fischer TZ, Lawden M, et al. Paroxysmal extreme pain disorder M1627K mutation in human Nav1.7 renders DRG neurons hyperexcitable. Mol Pain. 2008;4:37.
212. Choi JS, Boralevi F, Brissaud O, Sanchez-Martin J, Te Morsche RH, Dib-Hajj SD, et al. Paroxysmal extreme pain disorder: a molecular lesion of peripheral neurons. Nat Rev Neurol. 2011;7(1):51-5.
213. Shorer Z, Moses SW, Hershkovitz E, Pinsk V, Levy J. Neurophysiologic studies in congenital insensitivity to pain with anhidrosis. Pediatr Neurol. 2001;25(5):397-400.
214. Shorer Z, Wajsbrot E, Liran TH, Levy J, Parvari R. A novel mutation in SCN9A in a child with congenital insensitivity to pain. Pediatr Neurol. 2014;50(1):73-6.
215. Cox JJ, Reimann F, Nicholas AK, Thornton G, Roberts E, Springell K, et al. An SCN9A channelopathy causes congenital inability to experience pain. Nature. 2006;444(7121):894-8.
216. Cox JJ, Sheynin J, Shorer Z, Reimann F, Nicholas AK, Zubovic L, et al. Congenital insensitivity to pain: novel SCN9A missense and in-frame deletion mutations. Hum Mutat. 2010;31(9): E1670-86.
217. Goldberg YP, MacFarlane J, MacDonald ML, Thompson J, Dube MP, Mattice M, et al. Loss-of-function mutations in the Nav1.7 gene underlie congenital indifference to pain in multiple human populations. Clin Genet. 2007;71(4):311-9.
218. Bartholomew F, Lazar J, Marqueling A, Lee-Messer C, Jaradeh S, Teng JM. Channelopathy: a novel mutation in the SCN9A gene causes insensitivity to pain and autonomic dysregulation. Br J Dermatol. 2014;171(5):1268-70.
219. Staud R, Price DD, Janicke D, Andrade E, Hadjipanayis AG, Eaton WT, et al. Two novel mutations of SCN9A (Nav1.7) are associated with partial congenital insensitivity to pain. Eur J Pain. 2011;15(3):223-30.
220. Nilsen KB, Nicholas AK, Woods CG, Mellgren SI, Nebuchennykh M, Aasly J. Two novel SCN9A mutations causing insensitivity to pain. Pain. 2009;143(1-2):155-8.
221. Bogdanova-Mihaylova P, Alexander MD, Murphy RP, Murphy SM. SCN9A-associated congenital insensitivity to pain and anosmia in an Irish patient. J Peripher Nerv Syst. 2015;20(2):86-7.
222. Mansouri M, Chafai Elalaoui S, Ouled Amar Bencheikh B, El Alloussi M, Dion PA, Sefiani A, et al. A novel nonsense mutation in SCN9A in a Moroccan child with congenital insensitivity to pain. Pediatr Neurol. 2014;51(5):741-4.
223. Kurban M, Wajid M, Shimomura Y, Christiano AM. A nonsense mutation in the SCN9A gene in congenital insensitivity to pain. Dermatology. 2010;221(2):179-83.
224. Yuan R, Zhang X, Deng Q, Si D, Wu Y, Gao F, et al. Two novel SCN9A gene heterozygous mutations may cause partial deletion of pain perception. Pain Med. 2011;12(10):1510-4.
225. King MK, Leipold E, Goehringer JM, Kurth I, Challman TD. Pain insensitivity: distal S6-segment mutations in NaV1.9 emerge as critical hotspot. Neurogenetics. 2017;18(3):179-81.

226. Phatarakijnirund V, Mumm S, McAlister WH, Novack DV, Wenkert D, Clements KL, et al. Congenital insensitivity to pain: Fracturing without apparent skeletal pathobiology caused by an autosomal dominant, second mutation in SCN11A encoding voltage-gated sodium channel 1.9. Bone. 2016;84:289-98.
227. Woods CG, Babiker MO, Horrocks I, Tolmie J, Kurth I. The phenotype of congenital insensitivity to pain due to the NaV1.9 variant p.L811P. Eur J Hum Genet. 2015;23(5):561-3.
228. Huang J, Vanoye CG, Cutts A, Goldberg YP, Dib-Hajj SD, Cohen CJ, et al. Sodium channel NaV1.9 mutations associated with insensitivity to pain dampen neuronal excitability. J Clin Invest. 2017;127(7):2805-14.
229. Faber CG, Hoeijmakers JG, Ahn HS, Cheng X, Han C, Choi JS, et al. Gain of function Nanu1.7 mutations in idiopathic small fiber neuropathy. Ann Neurol. 2012;71(1):26-39.
230. Brouwer BA, Merkies IS, Gerrits MM, Waxman SG, Hoeijmakers JG, Faber CG. Painful neuropathies: the emerging role of sodium channelopathies. J Peripher Nerv Syst. 2014;19(2): 53-65.
231. Estacion M, Han C, Choi JS, Hoeijmakers JG, Lauria G, Drenth JP, et al. Intra- and interfamily phenotypic diversity in pain syndromes associated with a gain-of-function variant of NaV1.7. Mol Pain. 2011;7:92.
232. Han C, Hoeijmakers JG, Liu S, Gerrits MM, te Morsche RH, Lauria G, et al. Functional profiles of SCN9A variants in dorsal root ganglion neurons and superior cervical ganglion neurons correlate with autonomic symptoms in small fibre neuropathy. Brain. 2012;135(Pt 9):2613-28.
233. Hoeijmakers JG, Faber CG, Merkies IS, Waxman SG. Painful peripheral neuropathy and sodium channel mutations. Neurosci Lett. 2015;596:51-9.
234. Hoeijmakers JG, Han C, Merkies IS, Macala LJ, Lauria G, Gerrits MM, et al. Small nerve fibres, small hands and small feet: a new syndrome of pain, dysautonomia and acromesomelia in a kindred with a novel NaV1.7 mutation. Brain. 2012;135(Pt 2):345-58.
235. Hoeijmakers JG, Merkies IS, Gerrits MM, Waxman SG, Faber CG. Genetic aspects of sodium channelopathy in small fiber neuropathy. Clin Genet. 2012;82(4):351-8.
236. Waxman SG, Merkies IS, Gerrits MM, Dib-Hajj SD, Lauria G, Cox JJ, et al. Sodium channel genes in pain-related disorders: phenotype-genotype associations and recommendations for clinical use. Lancet Neurol. 2014;13(11):1152-60.
237. Mis MA, Yang Y, Tanaka BS, Gomis-Perez C, Liu S, Dib-Hajj F, et al. Resilience to Pain: A Peripheral Component Identified Using Induced Pluripotent Stem Cells and Dynamic Clamp. J Neurosci. 2019;39(3):382-92.
238. Hoeijmakers JG, Faber CG, Merkies IS, Waxman SG. Channelopathies, painful neuropathy, and diabetes: which way does the causal arrow point? Trends Mol Med. 2014;20(10):544-50.
239. Blair NT, Bean BP. Roles of tetrodotoxin (TTX)-sensitive Na+ current, TTX-resistant Na+ current, and Ca2+ current in the action potentials of nociceptive sensory neurons. J Neurosci. 2002; 22(23):10277-90.
240. Renganathan M, Cummins TR, Waxman SG. Contribution of Na(v)1.8 sodium channels to action potential electrogenesis in DRG neurons. J Neurophysiol. 2001;86(2):629-40.
241. Garrison SR, Weyer AD, Barabas ME, Beutler BA, Stucky CL. A gain-of-function voltage-gated sodium channel 1.8 mutation drives intense hyperexcitability of A- and C-fiber neurons. Pain. 2014;155(5):896-905.
242. Faber CG, Lauria G, Merkies IS, Cheng X, Han C, Ahn HS, et al. Gain-of-function Nav1.8 mutations in painful neuropathy. Proc Natl Acad Sci U S A. 2012;109(47):19444-9.
243. Huang J, Yang Y, Zhao P, Gerrits MM, Hoeijmakers JG, Bekelaar K, et al. Small-fiber neuropathy Nav1.8 mutation shifts activation to hyperpolarized potentials and increases excitability of dorsal root ganglion neurons. J Neurosci. 2013;33(35):14087-97.
244. Han C, Vasylyev D, Macala LJ, Gerrits MM, Hoeijmakers JG, Bekelaar KJ, et al. The G1662S NaV1.8 mutation in small fibre neuropathy: impaired inactivation underlying DRG neuron hyperexcitability. J Neurol Neurosurg Psychiatry. 2014;85(5):499-505.

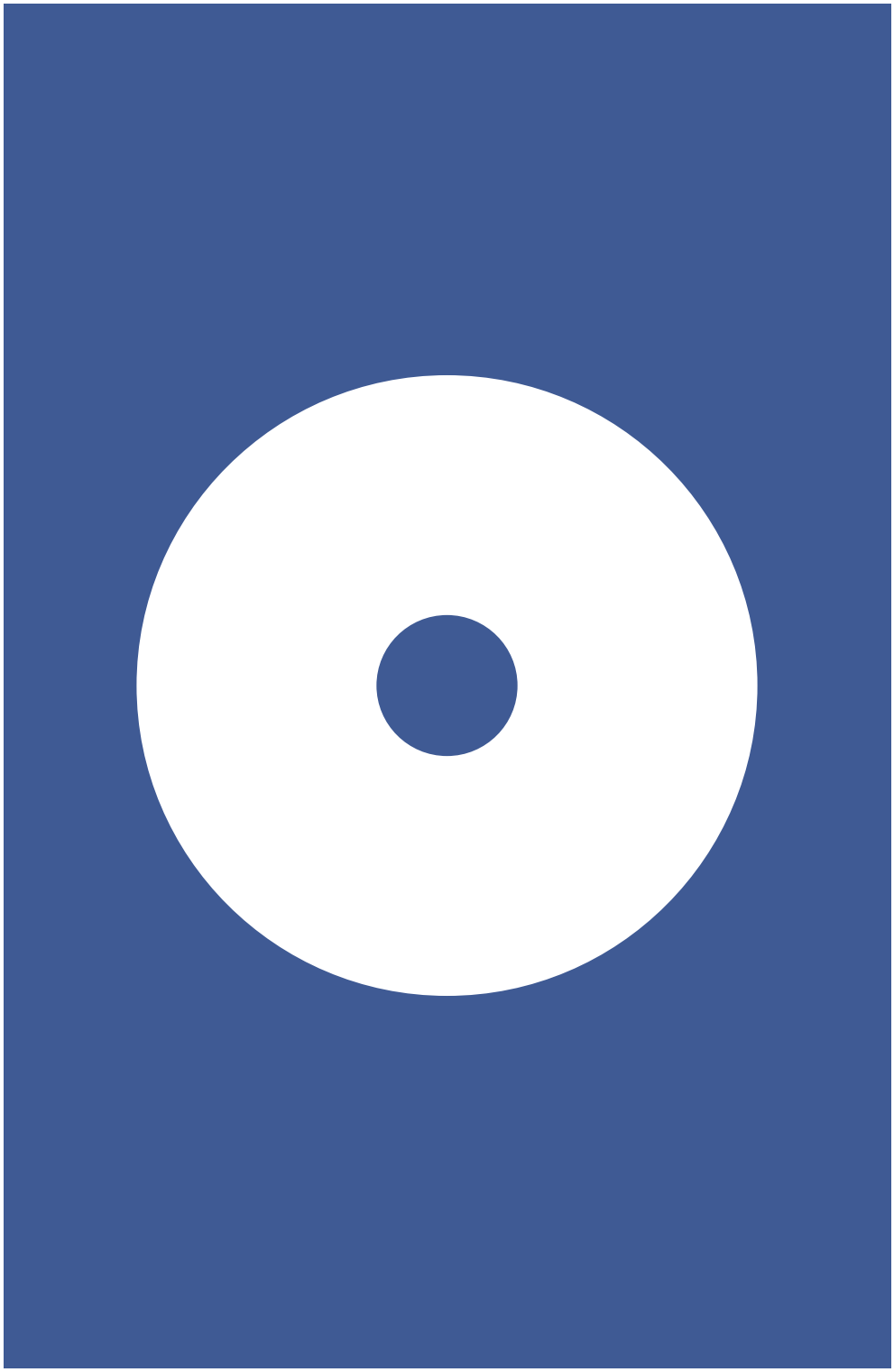
245. Dabby R, Sadeh M, Broitman Y, Yosovich K, Dickman R, Leshinsky-Silver E. Painful small fiber neuropathy with gastroparesis: A new phenotype with a novel mutation in the SCN10A gene. J Clin Neurosci. 2016;26:84-8.
246. Dib-Hajj SD, Black JA, Waxman SG. NaV1.9: a sodium channel linked to human pain. Nat Rev Neurosci. 2015;16(9):511-9.
247. Okuda H, Noguchi A, Kobayashi H, Kondo D, Harada KH, Youssefian S, et al. Infantile Pain Episodes Associated with Novel Nav1.9 Mutations in Familial Episodic Pain Syndrome in Japanese Families. PloS One. 2016;11(5):e0154827.
248. Han C, Yang Y, Te Morsche RH, Drenth JP, Politei JM, Waxman SG, et al. Familial gain-of-function Nav1.9 mutation in a painful channelopathy. J Neurol Neurosurg Psychiatry. 2017;88(3):233-40.
249. Zhang XY, Wen J, Yang W, Wang C, Gao L, Zheng LH, et al. Gain-of-function mutations in SCN11A cause familial episodic pain. Am J Hum Genet. 2013;93(5):957-66.
250. Leipold E, Hanson-Kahn A, Frick M, Gong P, Bernstein JA, Voigt M, et al. Cold-aggravated pain in humans caused by a hyperactive NaV1.9 channel mutant. Nat Commun. 2015;6:10049.
251. Han C, Yang Y, de Greef BT, Hoeijmakers JG, Gerrits MM, Verhamme C, et al. The Domain II S4-S5 Linker in Nav1.9: A Missense Mutation Enhances Activation, Impairs Fast Inactivation, and Produces Human Painful Neuropathy. Neuromolecular Med. 2015;17(2):158-69.
252. Huang J, Han C, Estacion M, Vasylyev D, Hoeijmakers JG, Gerrits MM, et al. Gain-of-function mutations in sodium channel Na(v)1.9 in painful neuropathy. Brain. 2014;137(Pt 6):1627-42.
253. Leipold E, Liebmann L, Korenke GC, Heinrich T, Giesselmann S, Baets J, et al. A de novo gain-of-function mutation in SCN11A causes loss of pain perception. Nat Genet. 2013;45(11):1399-404.
254. Mantyh WG, Dyck PJ, Dyck PJ, Engelstad JK, Litchy WJ, Sandroni P, et al. Epidermal Nerve Fiber Quantification in Patients With Erythromelalgia. JAMA Dermatol. 2016.
255. Rice FL, Albrecht PJ, Wymer JP, Black JA, Merkies IS, Faber CG, et al. Sodium channel Nav1.7 in vascular myocytes, endothelium, and innervating axons in human skin. Mol Pain. 2015;11:26.
256. Meglic A, Perkovic-Benedik M, Trebusak Podkrajsek K, Bertok S. Painful micturition in a small child: an unusual clinical picture of paroxysmal extreme pain disorder. Pediatr Nephrol. 2014;29(9):1643-6.
257. Estacion M, Dib-Hajj SD, Benke PJ, Te Morsche RH, Eastman EM, Macala LJ, et al. NaV1.7 gain-of-function mutations as a continuum: A1632E displays physiological changes associated with erythromelalgia and paroxysmal extreme pain disorder mutations and produces symptoms of both disorders. J Neurosci. 2008;28(43):11079-88.
258. Emery EC, Habib AM, Cox JJ, Nicholas AK, Gribble FM, Woods CG, et al. Novel SCN9A mutations underlying extreme pain phenotypes: unexpected electrophysiological and clinical phenotype correlations. J Neurosci. 2015;35(20):7674-81.
259. Cheng X, Dib-Hajj SD, Tyrrell L, Wright DA, Fischer TZ, Waxman SG. Mutations at opposite ends of the DIII/S4-S5 linker of sodium channel Na V 1.7 produce distinct pain disorders. Mol Pain. 2010;6:24.
260. Hoeijmakers JG. Small fiber neuropathy and sodium channels. A paradigm shift. Maastricht: Maastricht University; 2014.
261. Finnerup NB, Attal N, Haroutounian S, McNicol E, Baron R, Dworkin RH, et al. Pharmacotherapy for neuropathic pain in adults: a systematic review and meta-analysis. Lancet Neurol. 2015;14(2):162-73.
262. Brouwer BA, de Greef BT, Hoeijmakers JG, Geerts M, van Kleef M, Merkies IS, et al. Neuropathic Pain due to Small Fiber Neuropathy in Aging: Current Management and Future Prospects. Drugs Aging. 2015;32(8):611-21.
263. Geha P, Yang Y, Estacion M, Schulman BR, Tokuno H, Apkarian AV, et al. Pharmacotherapy for Pain in a Family With Inherited Erythromelalgia Guided by Genomic Analysis and Functional Profiling. JAMA Neurol. 2016;73(6):659-67.

264. Yang Y, Adi T, Effraim PR, Chen L, Dib-Hajj SD, Waxman SG. Reverse pharmacogenomics: carbamazepine normalizes activation and attenuates thermal hyperexcitability of sensory neurons due to Nav 1.7 mutation I234T. Br J Pharmacol. 2018;175(12):2261-71.
265. McCarberg BH, Stanos S, Williams DA. Comprehensive chronic pain management: improving physical and psychological function (CME multimedia activity). Am J Med. 2012;125(6):S1.
266. Akyuz G, Kenis O. Physical therapy modalities and rehabilitation techniques in the management of neuropathic pain. Am J Phys Med Rehabil. 2014;93(3):253-9.
267. Singleton JR, Marcus RL, Lessard MK, Jackson JE, Smith AG. Supervised exercise improves cutaneous reinnervation capacity in metabolic syndrome patients. Ann Neurol. 2015;77(1): 146-53.

II

PART 2

DIAGNOSTIC ASSESSMENTS IN SMALL FIBER NEUROPATHY



CHAPTER 3

NO ADDITIONAL VALUE FOR CORNEAL CONFOCAL MICROSCOPY IN THE DIAGNOSIS OF SMALL FIBER NEUROPATHY

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Submitted.

Abstract

Background

According to the international criteria, the diagnosis small fiber neuropathy (SFN) is based on clinical symptoms and signs in combination with a reduced intra-epidermal nerve fiber density (IENFD) in skin biopsy. Temperature threshold testing (TTT) can be supportive for the diagnosis. The sensitivity of skin biopsy is moderate to good; however, the test is invasive, time-consuming and not widely available. The specificity of the TTT is moderate and may be influenced by psychosocial factors. Corneal confocal microscopy (CCM) is a non-invasive tool that visualizes the small nerve fibers in the cornea. Degeneration and regeneration of small nerve fibers has been shown in various conditions. The aim of this study was to examine the applicability of CCM in patients with the suspicion of SFN, and the value of CCM as an additional diagnostic tool in SFN.

Methods

From January 2016 to June 2017, 183 patients with a suspicion of SFN and 25 healthy subjects were included in this study. All patients received a standardized procedure of investigations for SFN. Thereafter, The CCM parameters corneal nerve fiber density (CNFD), branch density (CNBD), fiber length (CNFL), and fiber tortuosity (CNFT) were determined. The sensitivity and specificity of the CCM was determined. Furthermore, we investigated whether any differences were found between SFN-associated conditions, and the CCM parameters.

Results

In this study, 140 patients were diagnosed with SFN (based on 7.7% abnormal IEFND, 44.8% abnormal TTT, 26.2% both abnormal), while in 43 patients the diagnosis could not be confirmed (non-SFN). In the SFN and non-SFN group hardly any CCM parameter was affected (the highest affected parameter was seen for the CNFL (14.3% in SFN / 18.6% in non-SFN). From the associated conditions to the diagnosis of SFN that were tested, 32.8% of the patients in the SFN-group was idiopathic. Overall, the CCM had a moderate specificity (60.5%) and a low sensitivity (39.3%) in comparison to the actual reference tests. However, specifically at least one CCM abnormality was detected in 57.7% (15/26) of the SFN-patients with an impaired glucose tolerance.

Conclusion

CCM as a diagnostic tool does not increase the diagnostic yield in SFN. However, specifying subgroups according to associated conditions may be important for future studies to determine the position of the CCM in diagnosing SFN.

Introduction

Small fiber neuropathy (SFN) is a form of peripheral neuropathy, which selectively affects the small Aδ nerve fibers and C fibers. SFN is characterized by neuropathic pain and autonomic dysfunction.[1,2] The minimal prevalence of SFN is estimated to be 53/100.000.[3] The diagnosis is made if typical symptoms and signs are present, in combination with a reduced intra-epidermal nerve fiber density (IENFD) in skin biopsy.[4] In addition, abnormal temperature threshold testing (TTT) can be supportive for the diagnosis.[1,5-7] The diagnostic value of skin biopsy in patients with SFN has been established.[4] However, the process of staining and counting the nerve fibers is time consuming, and the test is not widely available. Moreover, up to 60% of patients with SFN-symptoms IENFD is normal, possibly representing pre-degenerative functional impairment of the nerve fibers.[8-11] TTT is a widely available diagnostic tool, but this test lacks specificity.[12], and may be influenced by malingering or other non-organic factors.[13] To optimize the diagnostic process, a sensitive, preferably non-invasive, screening tool to detect damage of the small nerve fibers would be of value.

In vivo corneal confocal microscopy (CCM) has evolved rapidly from a predominantly research application to a diagnostic tool with a variety of clinical applications in ocular and neurological diseases, and with a high repeatability.[14] CCM can be used to examine small nerve fibers in the sub-basal nerve plexus (SNP) of the cornea.[15] Normative corneal nerve fiber density (CNFD), corneal nerve fiber branch density (CNBD), corneal nerve fiber length (CNFL), and corneal nerve fiber tortuosity (CNFT) reference values have been published.[16] Previous studies showed the detection of nerve fiber loss/damage in patients with diseases that may also affect the small nerve fibers, like Charcot-Marie-Tooth type 1A (CMT1A), impaired glucose intolerance, sarcoidosis-related neuropathy, Fabry disease, Parkinson's disease, peripheral auto-immune neuropathy, human immunodeficiency virus (HIV), and diabetic neuropathy.[17-26] CCM may also detect regeneration of small nerve fibers after treatment, for example after pancreas transplantation in patients with diabetes neuropathy.[27] Furthermore, CCM was used as a surrogate endpoint for evaluating therapeutic efficacy in clinical trials of patients with diabetic neuropathy and sarcoidosis.[28,29]

Small nerve fiber pathology was found in a small group of patients with idiopathic SFN (n=25), using CCM.[19] The primary aim of this study was to determine the value of the CCM in the diagnostic work-up of SFN in a large group of patients

with suspicion having SFN. To ensure that the international published normative values could be used in this study, a small number of healthy subjects was included.[16] Furthermore, the inter- and intra-observer reliability of the manual analysis method was investigated, and also compared with the automatic analysis method.

Materials and methods

The study took place between January 2016 and June 2017 at the Maastricht University Medical Center+ (Maastricht UMC+). The study was approved by the local Medical Ethics Committee, and written informed consent was obtained from each participant according to the declaration of Helsinki.

Study population

A total of 25 healthy participants were included in this study to confirm the applicability of the age- and gender dependent normative values which have previously been published.[16] Healthy controls were eligible to participate when they had a minimal age of 18 years, without a history of corneal diseases and/or neuropathic pain and underlying conditions that could influence nerve fiber functions.

Patients with possible SFN were eligible for inclusion when they were 18 years or older, and were referred to the Maastricht UMC+ for suspected SFN. Patients were excluded from the study if they had a history of diseases of the eye such atopic keratoconjunctivitis[30], epithelial membrane basement dystrophies[31] or cystic corneal disorders.[31] Also, patients with signs of an axonal or demyelinating polyneuropathy on nerve conduction studies (NCS) were excluded. Furthermore, patients were excluded when they did not want to be informed about defects or accidental findings, which could be found during the pre-screening by the Ophthalmology department.

Patients underwent a standardized procedure of clinical investigations for SFN. This contained a medical assessment, TTT, skin biopsy 10 cm above the lateral malleolus to determine IENFD, NCS to exclude involvement of large nerve fibers, neurological examination (including Medical Research Council (MRC) grading, tendon reflexes and sensory testing) and completion of the Visual Analogue Scale

(VAS) for pain and the SFN-Symptom Inventory Questionnaire (SFN-SIQ). The SFN-SIQ evaluates several symptoms as well as autonomic symptoms in a simple manner. The questionnaire consists of 13 questions and was derived from the original SIQ, and from the composite autonomic symptoms scale (COMPASS).[32] The diagnosis of SFN was set by the clinical presentation in combination with an abnormal TTT and/or abnormal IENFD.[1,33] For further investigation of underlying conditions, blood analysis was performed to determine possible causes of SFN such as diabetes, thyroid dysfunction, vitamin B6-intoxication, vitamin B12 insufficiency, Borrelia infection, immunological deficiencies, hepatitis C and *SCN9A*, *SCN10A* and *SCN11A*-variants.[34,35]

CCM-procedure

If there were no abnormalities during the pre-screening of the ophthalmologist, the participant was scanned with a laser IVCCM (Heidelberg Retinal Tomography III Rostock Cornea Module) to examine the nerve plexus present in the sub-basal membrane in the (peri-)center of the cornea. Participants' eyes were anaesthetized using a drop of 0.4% Benoxinate hydrochloride after which Viscotears were applied for lubrication of the eye. The anaestheticum works for approximately 30 minutes. A drop of viscotears was placed on the tip of the objective lens and a sterile disposable perplex cap was placed over the lens. The participant was instructed to fixate on a light with the eye that was not examined. The lens was placed against the cornea and focused on the basal layer of the cornea by turning the fine focus of the objective lens backwards and forwards for approximately two minutes per eye. When focused on the (peri-)center of the neuroplexus, we shot approximately 50 images of the unmyelinated nerve fibers which run parallel to Bowman's membrane and supply the overlying corneal epithelium. The images were two-dimensional with a lateral resolution of approximately 2 μm/pixel and a final image size of 400 x 400 pixels. Of all images that were shot, 4 high-quality images were selected with a (sub)optimal visualization of the small nerve fibers of each eye. Pictures with the highest quality for assessing the small fibers around the center of the cornea were selected.

Analysis with CCMetrics Image Analysis Tools v. 1.1.

The selected images were manually analysed using the CCMetrics image analysis tool version 1.1 (provided by the University of Manchester, United Kingdom). The images were uploaded into the program, and the visible nerve fibers were overdrawn by the analyser (MS).[36] Four corneal nerve parameters were

quantified: 1) CNFD, the total number of major nerves per square millimetre (no. mm^2); 2) CNBD, the number of branches emanating from all major nerve trunks per square millimetre of corneal tissue; 3) CNFL, the total length of all nerve fibers and branches (mm/mm^2) within the area of corneal tissue; 4) CNFT, expressed as the tortuosity coefficient (TC).[36] The CCM was considered abnormal if one or more of these variables were abnormal when compared to the normative data set[16], using the mean value of the four selected pictures of the right and left eye per CCM variable. Quantification of corneal nerve parameters was undertaken in a blinded fashion.

Afterwards, pictures were divided in two categories: 1) picture with an optimal resolution where the whole sub-basal plexus could be examined (Category 1; Figure 3.1A); 2) picture with a suboptimal resolution of the sub-basal plexus where at least 75% of the picture could be examined (Category 2; Figure 3.1B). Due to technical and/or anatomical variation, in some cases, optimal pictures could not be taken.

To determine the intraclass correlation coefficient (ICC) of the CNFL, CNFD, CNBD and CNFT between observers, 240 randomly picked images were examined. Both examiners were blind for the diagnostic results. In order to determine the intra-observer reliability, one observer assessed the same 240 pictures with an interval of, at least two weeks without having access to the previous findings. To compare the manual technique with the automated manner, all images were selected.[37] For the automated analyses, CNFT was not included.

Statistical analysis

For the storage and analysis of the data we used SPSS 23.0 (IBM Corp. Released 2015. IBM SPSS Statistics for Windows, Version 23.0. Armonk, NY: IBM Corp). Inter-observer reliability and the correlation between the manual and automated analysis were measured using ICCs for each corneal parameter by means of the Two-Way Random-Effects Model.[38] The ICC values less than 0.5 were indicated as poor, values between 0.5 and 0.75 as moderate, values between 0.75 and 0.9 as good, and values greater than 0.90 as excellent reliability.

To apply the international normative values for our Dutch sample, a heterogeneous group of healthy subjects was examined to determine if their values were within the range of the international normative dataset.

Demographics were shown as means with the standard deviation (SD). The associated conditions which cause SFN-like symptoms in the total patient group were visualised. The Mann-Whitney-U test was executed in order to determine whether analysis of one eye (left or right) was sufficient for further analysis. Two tables are presented with the number of patients with/without CCM abnormalities and how many per subject, divided in (1) SFN versus non-SFN, and (2) Category 1 versus Category 2 pictures. The relationship between associated conditions of SFN and CCM outcome measures was presented by a bar chart.

Lastly, we showed a cross-tabulation to determine the sensitivity, specificity, likelihood ratios and predictive values of the CCM to the actual golden standard.

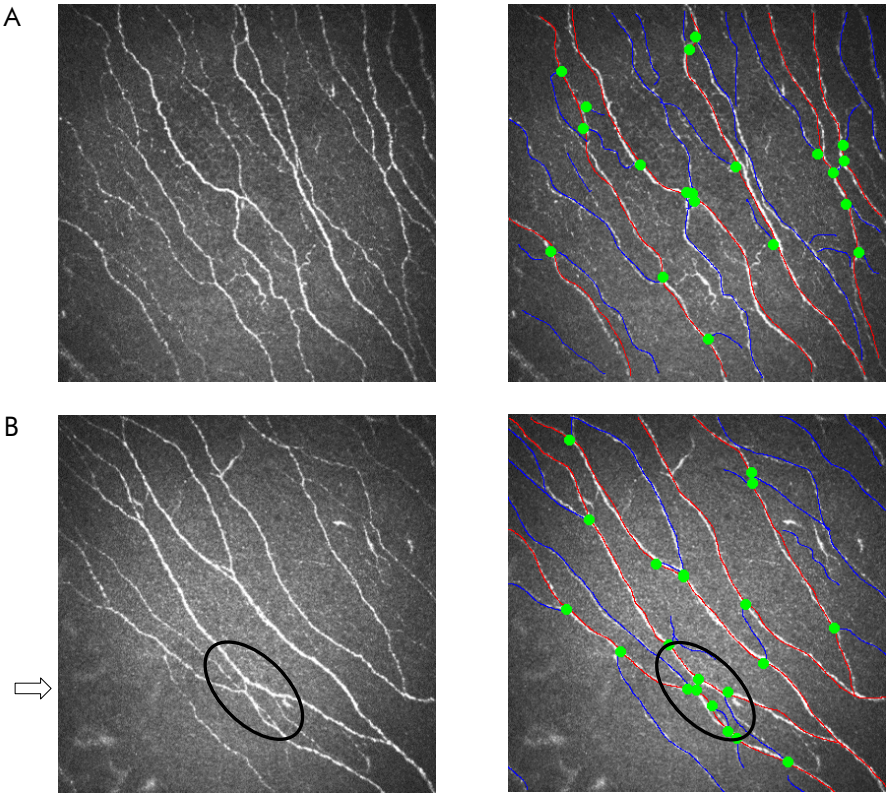

Figure 3.1 **A**: optimal resolution picture (Category 1). Field of view is 0.4 mm by 0.4 mm. **B**: suboptimal resolution pictures (Category 2). The circle indicate the stroma of the cornea. Field of view is 0.4 mm by 0.4 mm.

RESULTS

Group of participants

Findings from our healthy group (*n*=25) in all age groups (18-25, 26-35, 36-45, 46-55, 56-65, and older than 65 years) confirmed the applicability of age- and gender adjusted normative values.[16] None of the healthy participants had a CNFD, CNFB or CNFL lower than the cut-off points. Furthermore, the CNFT was within the normal range without reaching the cut-off point.

A total of 359 patients underwent screening for SFN between January 2016 - June 2017. Eventually, 183 participants were included (Figure 3.2). For 140 patients, the diagnosis of SFN was obtained according to the international criteria. The clinical characteristics are summarized in Table 3.1.

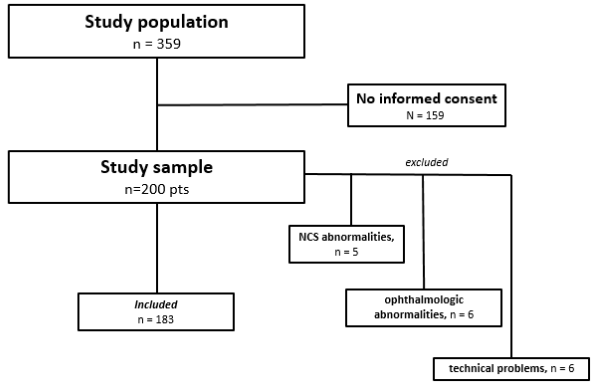

Figure 3.2 Flow-chart inclusion participants.

Table 3.1 Patient group demographic and clinical characteristics.

	SFN-group (*n* = 140)	non-SFN group (*n* = 43)
Gender, M/F	51/89	6/35
Mean age ‡, years	51 ± 12	45 ± 13
Age of onset ‡, years	44 ± 14	41 ± 16
Only abnormal IENFD	14	0
Only abnormal TTT	78	4*
Both abnormal IEFND and TTT	48	0
Idiopathic, Y/N	47/93	18/25
VAS, min-max	64, 38-82	53, 24-73

‡ Data are shown as mean (SD); # data are shown as mean, min-max; * diagnosis was not set because the clinical picture was not typical for SFN. SFN, small fiber neuropathy; VAS, visual analogue scale; IENFD, intra-epidermal nerve fiber density; TTT, temperature threshold testing. The diagnosis SFN was made based on clinical symptoms in combination with a reduced intra-epidermal nerve fiber density (IENFD) in skin biopsy and/or abnormal temperature threshold testing (TTT).[1,4,5,59]

The total group of included patients (n=183) contained 124 females (67.8%). The age group 46-55 years was the greatest group with 56 patients (30.6%). From the patient group diagnosed having SFN (n=140), 48 patients (26.2%) were diagnosed with definite SFN based on an abnormal TTT and abnormal IENFD, whereas 14 patients (7.7%) only had an abnormal IENFD and 78 patients (44.8%) only an abnormal TTT. The IENFD and TTT were both normal in 39 patients (20.2%). Four patients (2.2%) had an abnormal TTT, but the diagnosis could not be made, because the clinical picture was considered not typical for SFN. The last two mentioned groups together were defined as the non-SFN group (n=43).

Figure 3.3 shows the associated conditions for the SFN-group (n=140). A percentage of 32.8% was diagnosed with idiopathic SFN. The distribution of associated conditions in our sample is comparable to a large patient cohort.[34]

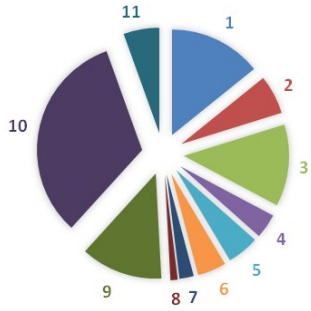

Figure 3.3 The associated conditions of in the SFN-group (n=140); 1: glucose intolerance (14.2%); 2: hypercholesterolemia (6.0%); 3: Immunological abnormalities (12.6%); 4: Liver- and kidney abnormalities (3.8%); 5: Borrelia (4.9%); 6: Thyroid dysfunction (4.4%); 7: Vitamin B12 deficiency (2.2%); 8: Vitamin B6 intoxication (1.1%); 9: Genetic variant (12.6%); 10: idiopathic (32.8%); 11: Other (5.5%).

Inter- and intra-observer reliability

The manual CCM examinations were adequate to highly reproducible, indicated by ICCs ranging between 0.790 and 0.987 for the intra-rater reliability. The ICC was performed best in CNFT. Measurements in CNFD, CNBD and CNFT were highly reproducible between observers, indicated by an ICC of 0.836 (CNFD), 0.914 (CNBD), and 0.948 (CNFT). The reliability of CNFL was intermediate with 0.691. When automated analysis was performed, an excellent ICC was found on

CNFL, namely 0.959 (CI: 0.945-0.969), and good reliability score on CNBD (0.796; CI: 0.727-0.848). However, the ICC for the CNFD was poor (0.325; CI: 0.096-0.496).

Comparison left and right eye

In Table 3.2, a comparison was made between the left and right eye. No significant differences were found in CNFD, CNBD, CNFL and CNFT between both eyes. Therefore, it was decided to continue the calculations, based on the right eye.

Table 3.2 Comparison left and right eye.

	Median R	Median L	*p*
CNFD	23.44	23.44	0.963
CNBD	50.00	51.56	0.287
CNFL	18.82	19.10	0.862
CNFT	15.72	16.04	0.342

CCM abnormalities

In the SFN group, 76.4% (107/140) did not show any CCM abnormalities, whereas 74.4% (32/43) in the non SFN group showed normal CCM parameters. A percentage of 25.0% (18/72) showed only CCM abnormalities in the right eye.

In 23.6% of the SFN-group, and in 25.6% of the non-SFN-group, abnormalities in CCM were found (Table 3.3). The CNFL had the highest frequencies of abnormalities, in comparison to the other CCM parameters (SFN-group: 14.3% (20/140); non-SFN-group: 18.6% (8/43)). Furthermore, patients were divided in the amount of abnormalities (0 to 4). No significant differences between the SFN- and non-SFN-group were found over all CCM parameters. Table 3.4 presented the pictures of the SFN-group in two subgroups, namely the Category 1 and Category 2 group. The percentages of abnormalities in the Category 2-group were higher, but not significant, than in Category 1, indicating that a higher number of scores in CNFD, CNFL and CNBD exceeded the cut-off values.[16]

Table 3.3 Frequencies of CCM-abnormalities in the SFN-group (n=140) and non-SFN-group (n=43).

	CCM parameters				Total CCM abnormalities* per subject				
	CNFD	CNBD	CNFL	CNFT	0	1	2	3	4
SFN-group	12.1%	10.0%	14.3%	10.0%	76.4%	12.1%	3.6%	6.4%	1.4%
	17/140	14/140	20/140	14/140	107/140	17/140	5/140	9/140	2/140
Non-SFN-group	11.6%	11.6C%	18.6%	9.3%	74.4%	9.3%	11.6%	2.3%	2.3%
	5/43	5/43	8/43	4/43	32/43	4/43	5/43	1/43	1/43

* an abnormality was defined as under/above the cut-off values, in comparison with the international normative values.[16] CNFD=cornea nerve fiber density; CNBD=cornea nerve fiber branch density; CNFL=cornea nerve fiber length; CNFT=cornea nerve fiber tortuosity; n/a=not applicable. CNFD, CNBD and CNFL in no/mm^2 ; CNFT in tortuosity coefficient (TC).

Table 3.4 Frequencies of CCM-abnormalities in the Category 1-group and Category 2-group in SFN-group (n=140).

	CCM parameters				Total CCM abnormalities* per subject				
	CNFD	CNBD	CNFL	CNFT	0	1	2	3	4
Category 1 group	6.1%	5.1%	8.2%	8.2	82.7%	12.2%	1.0%	3.1%	1.0%
	6/98	5/98	8/98	8/98	81/98	12/98	1/98	3/98	1/98
Category 2 group	26/2%	19.0	28.6%	14.3%	61,9%	11.9%	9.5%	14.3%	2/4%
	11/42	9/42	12/42	6/42	26/42	5/42	4/42	6/42	1/42

* an abnormality was defined as under/above the cut-off values, in comparison with the international normative values [16]. CNFD = cornea nerve fiber density; CNBD = cornea nerve fiber branch density; CNFL = cornea nerve fiber length; CNFT = cornea nerve fiber tortuosity; n/a = not applicable. CNFD, CNBD and CNFL in no/mm^2 ; CNFT in tortuosity coefficient (TC).

CCM and associated conditions

The highest frequencies of normal and abnormal CCM were found in the idiopathic group. In the SFN-group, 29.3% (41/140) was idiopathic and showed a normal CCM, while 13.6% (19/140) had at least one abnormal CCM variable. Of all possible associated conditions that were found in the SFN-group, 15 of the 26 patients (57.7%) with a glucose intolerance had at least one abnormal CCM variable. Furthermore, 71.4% (5/7) of the patients with an abnormal liver and/or kidney-function, according to the local laboratory normative values, showed CCM abnormalities (Figure 3.4).

Additional value of CCM in SFN diagnostics

In Table 3.5, a cross-tabulation was shown that indicate CCM test results against the reference tests (IENFD and TTT). For this calculation, abnormalities in the left and/or right eye were included. The specificity of this test was 39.3% (CI: 31.1-47.9), while the specificity was 60.5% (CI: 44.4-75.0).

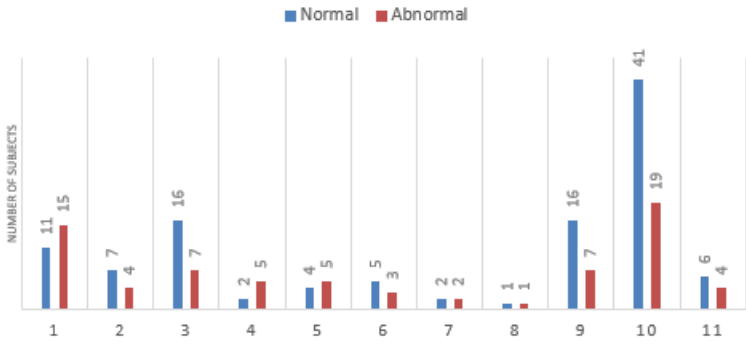

Figure 3.4 The associated conditions of SFN in relation to CCM variables (n=140). 1: glucose intolerance; 2: hypercholesterolemia; 3: Immunological abnormalities; 4: Liver- and kidney abnormalities; 5: Borrelia infection; 6: Thyroid dysfunction; 7: Vitamin B12 deficiency; 8: Vitamin B6 intoxication; 9: Genetic variant; 10: idiopathic; 11: Other.

Table 3.5 Cross-tabulation of the CCM versus SFN.

	SFN group	Non-SFN group	
Abnormal CCM	55	17	PPV: 76.4%; PLR 0.99
Normal CCM	85	26	NPV: 23.4%; NLR 1.00
Sensitivity: 39.3%;	Specificity: 60.5%		Accuracy: 44.3%

Note: an abnormal CCM was defined as at least one CCM abnormality, observed in the right and/or left eye.

Discussion

In this study, we found a moderate to high reproducibility of the manual CCM analysis method. It was confirmed that the international normative values were applicable for our center. We concluded that the CCM has no additional value in the diagnostic process to detect SFN, according to its moderate specificity (60.5%) and low sensitivity (39.3%) in comparison to the actual reference tests.

Intra- and inter reliability of CCM

In our study, we used the section mode for manual analysis, because the normative values of CCM parameters were based on manual measurements.[16] We found a high reproducibility for CNFD, CNBD, CNFT and moderate repeatability for CNFL. The repeatability of CCM in a previous study for manual analysis in healthy subjects was reported good, except for CNBD[39], whereas the highest ICC was found for CNFL in early diabetic sensorimotor polyneuropathy.[40]

We calculated the CCM parameters by selecting pictures around the peri-center of the plexus and calculated the mean per CCM variable per subject. Although we demonstrated an intermediate to high reproducibility, we noticed challenges in the manual CCM analysis. An important disadvantage of manual image analysis is that it is time-consuming and needs qualified personal. For the future, to conduct the CCM procedure, automated image analysis could be an advantage, as it allows rapid analysis without specialized personal. Alternative modes are the sequence mode, which permits capture of a movie of up to 100 images[36], and the volume scan which is able to capture subsequent incremental stepwise images. Nowadays, software can used for a wide field mosaicking of the sub-basal plexus nerves which would give a better overview of the nerve plexus in the cornea.[41] Previous research found that the clockwise whorl which lies inferiorly detects abnormalities in both diabetic neuropathy patients and non-diabetic neuropathy patients.[42]

Previously, the clinical use of the CCM in the diagnostic process of neuropathic pain syndromes was reported for underlying causes such as diabetes mellitus type I and II[20,43,45], or an abnormal glucose intolerance.[46] In this study, the subgroup with an abnormal glucose intolerance showed a percentage of 57.7% abnormalities on the cornea. It is assumed that both prediabetes and diabetes lead to microstructural alternations in affected nerves, which seem to begin in small unmyelinated C fibers.[47] Here, patients with evident large fiber involvement (i.e. abnormalities in NCS) were excluded. In diabetic polyneuropathy, the sensitivity and specificity of the CCM is moderate to high. It is indicated that corneal nerve pathology is more pronounced in patients with diabetic polyneuropathy and is associated with its clinical severity.[14,48] Moreover, it is indicated that the CNFD is a more valid test to identify small nerve fiber pathology than the IENFD in diagnosing diabetic neuropathy.[20] Corneal aspects such as cornea nerve fiber size and fiber loss in the inferior whorl have also been suggested as biomarker in this subgroup.[49-51] Furthermore, increased Langerhans cells and corneal keratocyte density were correlated with corneal nerve damage in diabetic peripheral neuropathy.[52,53] We did not include this analysis in our study. Another biomarker that not was included in this study, is the dendritic cell density. In a recent study, it was reported that an increase in dendritic cell density was found in persons with chronic inflammatory demyelinating polyneuropathy (CIDP), in comparison to diabetic neuropathy and controls.[54] In our sample, immunological deficiencies are the third group of underlying conditions in

patients with SFN-like symptoms. Therefore, future studies that will investigate an immunological disorder, should take this biomarker into account for its presence.

The non-length-dependent nature of neuropathy was defined based on the distribution of sensory symptoms and signs restricted to, or predominant in, body areas different from the expected "stocking and glove" topography, indicating a non-length-dependent pattern. As CCM investigates the sensory terminals of trigeminal nerve, it would be interesting to find out whether non-length dependent SFN-patients would have more CCM abnormalities than patients with a length-dependent pattern. In this study, only a few patients had a non-length dependent pattern. Previous study showed that CNFD was decreased in a small SFN group (n=6).[55] This could not be confirmed in our patient population.

Future diagnostics in SFN

The CCM was reported as comparable utility with quantitative sensory testing and nerve conduction in the diagnostics of patients with diabetic neuropathy.[56] Although our study showed that CCM is not valuable in the diagnostic logarithm of SFN, CCM could potentially be a tool to evaluate treatment strategies in patients with neuropathic pain, which have previously been showed after simultaneous pancreas and kidney transplantation in diabetes mellitus type I[27,57], and in medication trials.[28,29] Also, in case of longitudinal studies, corneal nerve regeneration occurred after the improvement in risk factors in diabetic neuropathy.[58]

A follow-up study in SFN patients may provide more information on pain intensity and treatment(s) of the underlying condition(s) and its relationship to possible regeneration of corneal nerve fibers, even more when symptoms show a non-length distribution.

In conclusion, CCM is not of additional value in the diagnosis of SFN. However, it seems that CCM may serve as a reliable diagnostic tool in SFN subgroups with an underlying condition, like glucose intolerance. The reliability of the CCM analysis method is high for both manual and automatic manner.

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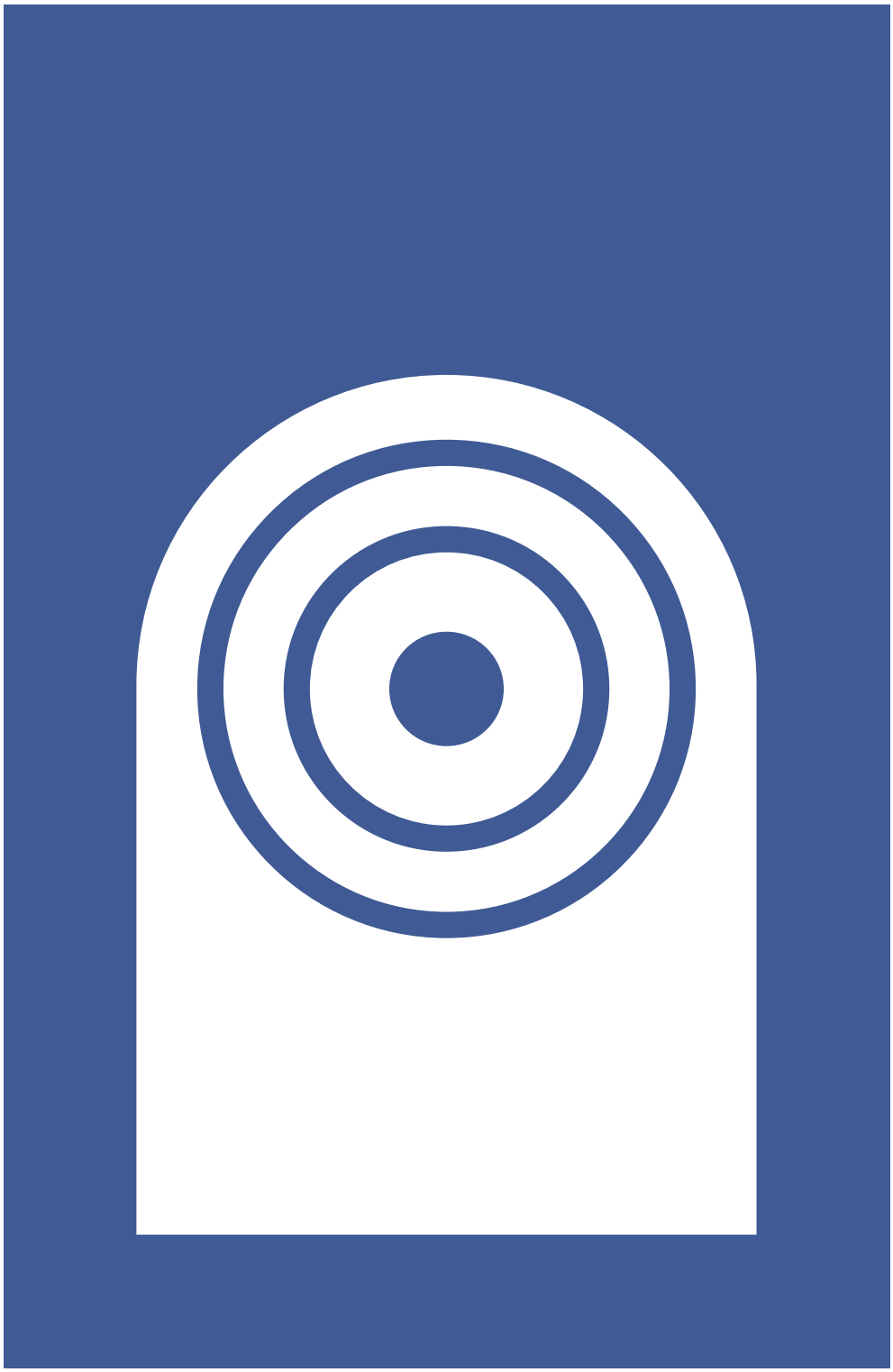
References

1. Cazzato D, Lauria G. Small fibre neuropathy. Curr Opin Neurol. 2017;30(5):490-9.
2. Devigili G, Cazzato D, Lauria G. Clinical diagnosis and management of small fiber neuropathy: an update on best practice. Expert Rev Neurother. 2020;20(9):967-80.
3. Peters MJ, et al. Incidence and prevalence of small-fiber neuropathy: a survey in the Netherlands. Neurology. 2013;81(15):1356-60.
4. Lauria 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.
5. Bakkers M, et al. Optimizing temperature threshold testing in small-fiber neuropathy. Muscle Nerve. 2015;51(6):870-6.
6. Devigili G, et al. Diagnostic criteria for small fibre neuropathy in clinical practice and research. Brain. 2019;142(12):3728-36.
7. Freeman R, et al. Idiopathic distal sensory polyneuropathy: ACTTION diagnostic criteria. Neurology. 2020;95(22):1005-14.
8. Devigili G, et al. The diagnostic criteria for small fibre neuropathy: from symptoms to neuropathology. Brain. 2008;131(Pt 7):1912-25.
9. De Greef BT, Hoeijmakers JGJ, Merkies ISJ, Faber CG. Associated conditions in small fiber neuropathy – A large cohort study and review of the literature. Eur J Neurol. 2018;25(2): 348-55.
10. Eijkenboom I, et al. Yield of peripheral sodium channels gene screening in pure small fibre neuropathy. J Neurol Neurosurg Psychiatry. 2019;90(3):342-52.
11. Fabry V, et al. Which Method for Diagnosing Small Fiber Neuropathy? Front Neurol. 2020;11:. 342.
12. Maier C, 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.
13. Shy ME, et al. Quantitative sensory testing: report of the Therapeutics and Technology Assessment Subcommittee of the American Academy of Neurology. Neurology. 2003;60(6): 898-904.
14. Petropoulos IN, et al. Corneal nerve loss detected with corneal confocal microscopy is symmetrical and related to the severity of diabetic polyneuropathy. Diabetes Care. 2013;36(11): 3646-51.
15. Patel DV, McGhee CN. Mapping of the normal human corneal sub-Basal nerve plexus by in vivo laser scanning confocal microscopy. Invest Ophthalmol Vis Sci. 2005;46(12):4485-8.
16. Tavakoli M, et al. Normative values for corneal nerve morphology assessed using corneal confocal microscopy: a multinational normative data set. Diabetes Care. 2015;38(5):838-43.
17. Tavakoli M, et al. Corneal confocal microscopy: a novel noninvasive means to diagnose neuropathy in patients with Fabry disease. Muscle Nerve. 2009;40(6):976-84.
18. Tavakoli M, et al. Corneal confocal microscopy detects small-fiber neuropathy in Charcot-Marie-Tooth disease type 1A patients. Muscle Nerve. 2012;46(5):698-704.
19. Tavakoli M, et al. Corneal confocal microscopy: a novel means to detect nerve fibre damage in idiopathic small fibre neuropathy. Exp Neurol. 2010;223(1):245-50.
20. Alam U, et al. Diagnostic utility of corneal confocal microscopy and intra-epidermal nerve fibre density in diabetic neuropathy. PLoS One. 2017;12(7):e0180175.
21. Bitirgen G, et al. Use of Corneal Confocal Microscopy to Detect Corneal Nerve Loss and Increased Dendritic Cells in Patients With Multiple Sclerosis. JAMA Ophthalmol. 2017;135(7): 777-82.
22. Kemp HI, et al. Use of Corneal Confocal Microscopy to Evaluate Small Nerve Fibers in Patients With Human Immunodeficiency Virus. JAMA Ophthalmol. 2017;135(7):795-800.

23. Khan A, et al. Corneal Confocal Microscopy Detects Corneal Nerve Damage in Patients Admitted With Acute Ischemic Stroke. Stroke. 2017;48(11):3012-8.
24. Brines M, et al. Corneal nerve quantification predicts the severity of symptoms in sarcoidosis patients with painful neuropathy. Technology. 2013;01(01):20-6.
25. Ferdousi M, et al. Greater small nerve fibre damage in the skin and cornea of type 1 diabetic patients with painful compared to painless diabetic neuropathy. Eur J Neurol. 2021;28(5): 1745-51.
26. Lim SH, et al. Corneal confocal microscopy detects small fibre neurodegeneration in Parkinson's disease using automated analysis. Sci Rep. 2020;10(1):20147.
27. Mehra S, et al. Corneal confocal microscopy detects early nerve regeneration after pancreas transplantation in patients with type 1 diabetes. Diabetes Care. 2007;30(10):2608-12.
28. Azmi S, et al. Corneal confocal microscopy shows an improvement in small-fiber neuropathy in subjects with type 1 diabetes on continuous subcutaneous insulin infusion compared with multiple daily injection. Diabetes Care. 2015;38(1):e3-4.
29. Dahan A, et al. ARA 290 improves symptoms in patients with sarcoidosis-associated small nerve fiber loss and increases corneal nerve fiber density. Mol Med. 2013;19:334-45.
30. Hu Y, et al. Corneal in vivo confocal scanning laser microscopy in patients with atopic keratoconjunctivitis. Ophthalmology. 2008;115(11):2004-12.
31. Rosenberg ME, et al. Corneal structure and sensitivity in type 1 diabetes mellitus. Invest Ophthalmol Vis Sci. 2000;41(10):2915-21.
32. Greco C, et al. Validation of the Composite Autonomic Symptom Score 31 (COMPASS 31) for the assessment of symptoms of autonomic neuropathy in people with diabetes. Diabet Med. 2017;34(6):834-8.
33. Tesfaye S, et al. Diabetic neuropathies: update on definitions, diagnostic criteria, estimation of severity, and treatments. Diabetes Care. 2010;33(10):2285-93.
34. de Greef BTA, et al. Associated conditions in small fiber neuropathy - A large cohort study and review of the literature. Eur J Neurol. 2018;25(2):348-55.
35. Lang M, Treister R, Oaklander AL. Diagnostic value of blood tests for occult causes of initially idiopathic small-fiber polyneuropathy. J Neurol. 2016;263(12):2515-27.
36. Tavakoli M, Malik TA. Corneal confocal microscopy: a novel non-invasive technique to quantify small fibre pathology in peripheral neuropathies. J Vis Exp. 2011;(47):2194.
37. Scarpa F, Colonna A, Ruggeri A. Multiple-Image Deep Learning Analysis for Neuropathy Detection in Corneal Nerve Images. Cornea. 2020;39(3):342-7.
38. Koo TK, Li MY. A Guideline of Selecting and Reporting Intraclass Correlation Coefficients for Reliability Research. J Chiropr Med. 2016;15(2):155-63.
39. Petropoulos IN, et al. Repeatability of in vivo corneal confocal microscopy to quantify corneal nerve morphology. Cornea. 2013;32(5):e83-9.
40. Hertz P, et al. Reproducibility of in vivo corneal confocal microscopy as a novel screening test for early diabetic sensorimotor polyneuropathy. Diabet Med. 2011;28(10):1253-60.
41. Vaishnav YJ, et al. Rapid, automated mosaicking of the human corneal subbasal nerve plexus. Biomed Tech (Berl). 2017;62(6):609-13.
42. Petropoulos IN, et al. The Inferior Whorl For Detecting Diabetic Peripheral Neuropathy Using Corneal Confocal Microscopy. Invest Ophthalmol Vis Sci. 2015;56(4):2498-504.
43. Petropoulos IN, et al. Rapid automated diagnosis of diabetic peripheral neuropathy with in vivo corneal confocal microscopy. Invest Ophthalmol Vis Sci. 2014;55(4):2071-8.
44. Li Q, et al. Quantitative analysis of corneal nerve fibers in type 2 diabetics with and without diabetic peripheral neuropathy: Comparison of manual and automated assessments. Diabetes Res Clin Pract. 2019;151:33-8.
45. Kalteniece A, et al. Corneal confocal microscopy detects small nerve fibre damage in patients with painful diabetic neuropathy. Sci Rep. 2020;10(1):3371.
46. Asghar O, et al. Corneal confocal microscopy detects neuropathy in subjects with impaired glucose tolerance. Diabetes Care. 2014;37(9):2643-6.

47. Malik RA, et al. Sural nerve pathology in diabetic patients with minimal but progressive neuropathy. Diabetologia. 2005;48(3):578-85.
48. Papanas N, Ziegler D. Corneal confocal microscopy: a new technique for early detection of diabetic neuropathy. Curr Diab Rep. 2013;13(4):488-99.
49. Brines M, et al. Corneal nerve fiber size adds utility to the diagnosis and assessment of therapeutic response in patients with small fiber neuropathy. Sci Rep. 2018;8(1):4734.
50. Kalteniece A, et al. Greater corneal nerve loss at the inferior whorl is related to the presence of diabetic neuropathy and painful diabetic neuropathy. Sci Rep. 2018;8(1):3283.
51. Ferdousi M, et al. Diabetic Neuropathy Is Characterized by Progressive Corneal Nerve Fiber Loss in the Central and Inferior Whorl Regions. Invest Ophthalmol Vis Sci. 2020;61(3):48.
52. D'Onofrio L, et al. Small Nerve Fiber Damage and Langerhans Cells in Type 1 and Type 2 Diabetes and LADA Measured by Corneal Confocal Microscopy. Invest Ophthalmol Vis Sci. 2021;62(6):5.
53. Kalteniece A, et al. Keratocyte Density Is Reduced and Related to Corneal Nerve Damage in Diabetic Neuropathy. Invest Ophthalmol Vis Sci. 2018;59(8):3584-90.
54. Fleischer M, et al. Corneal confocal microscopy differentiates inflammatory from diabetic neuropathy. J Neuroinflammation. 2021;18(1):89.
55. Gemignani F, et al. Non-length-dependent small fibre neuropathy. Confocal microscopy study of the corneal innervation. J Neurol Neurosurg Psychiatry. 2010;81(7):731-3.
56. Ferdousi M, et al. Corneal confocal microscopy compared with quantitative sensory testing and nerve conduction for diagnosing and stratifying the severity of diabetic peripheral neuropathy. BMJ Open Diabetes Res Care. 2020;8(2):e001801.
57. Tavakoli M, et al. Corneal confocal microscopy detects early nerve regeneration in diabetic neuropathy after simultaneous pancreas and kidney transplantation. Diabetes. 2013;62(1): 254-60.
58. Tavakoli M, et al. Corneal confocal microscopy detects improvement in corneal nerve morphology with an improvement in risk factors for diabetic neuropathy. Diabet Med. 2011; 28(10):1261-7.
59. Yarnitsky D, Sprecher E. Thermal testing: normative data and repeatability for various test algorithms. J Neurol Sci. 1994;125(1):39-45.



CHAPTER 4

THE APPLICABILITY OF THE DIGIT WRINKLE SCAN TO QUANTIFY SYMPATHETIC NERVE FUNCTION

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Abstract

Objective

Stimulated skin wrinkling test (SSW) has been launched as a non-invasive diagnostic procedure. However, no normative age dependent values have been reported that can be applied in clinical practice. The objectives of the study were to (1) collect age-dependent normative values according to the 5-point scale assessment for the SSW, to (2) determine reliability scores for the obtained norm values, and to (3) introduce a new digital method for SSW assessment, the Digit Wrinkle Scan© (DWS©) for detection of wrinkles in a more quantitative manner.

Methods

Firstly, 82 healthy participants were included, divided in 5 age groups. The participants underwent SSW using lidocaine and prilocaine topical cream. Secondly, 35 healthy participants were included to test whether the DWS© could be a novel manner to assess the grade of wrinkling quantitatively. We determined the inter-observer reliability of both methods. Also, the intra-observer reliability was calculated for the DWS©.

Results

We found a decrease in normative values over age. The inter-observer reliability of assessment by the 5-point scale method was moderate after SSW (Cohen's k: 0.53). Results of the DWS© indicate that total wrinkle length per mm^2 showed moderate to good agreement for the 4^{th} and 5^{th} digits after SSW, and a low agreement for the other digits.

Conclusion

Age-dependent normative values were obtained according to the 5-point scale, but its clinical application is doubtful since we found a moderate inter-observer reliability. We introduced the DWS© as a possible new method in order to quantify the grade of wrinkling.

Significance

We found unsatisfactory reliability scores, which hampers its usefulness for clinical practice.

Introduction

Small fiber neuropathy (SFN) is a form of peripheral neuropathy in which thinly myelinated Aδ-fibers and unmyelinated C-fibers are selectively affected without involvement of the large nerve fibers. The condition is clinically characterized by neuropathic pain and autonomic dysfunction.[1-3] Diagnosis of SFN can be challenging since small nerve fibers are not always well investigated by electrophysiological testing and clinical presentation is difficult to interpret.[4]
Currently, SFN diagnosis is based on the following international criteria: (i) clinical signs of neuropathic pain and dysautonomia, (ii) a decreased intraepidermal never fiber density (IENFD) in skin biopsy and/or abnormal temperature threshold testing (TTT) and (iii) normal nerve conduction studies (NCS).[5-7] In other words, a combination of a clinical, functional and structural approach to the diagnosis of SFN is reliable and relevant both for clinical practice and clinical trial design.

Although the diagnostic value of skin biopsy has been established[8], it is an invasive method and assessment of obtained tissue is expensive and time consuming. Although the specificity of the IENFD is high, its sensitivity is moderate.[9-11] While TTT is more accessible and non-invasive, the main disadvantage of this test is its moderate specificity.[12,13] Also, test outcomes may be influenced by malingering or other nonorganic factors.[14]

Previous studies have introduced Stimulated Skin Wrinkling (SSW) as a reliable and convenient tool to examine the sympathetic nerve function in hands and feet[15,16], in diabetic neuropathy[17], and in HIV neuropathy.[18] Skin-wrinkling was originally induced by water immersion, which leads to diffusion and subsequent vasoconstrictive wrinkling of overlying skin.[19] Besides water, also EMLA (eutectic mixture of local anaesthetics) cream© was suggested as a usable vasoconstrictive factor. It is thought that EMLA causes vasoconstriction through direct effect on smooth muscle cells and postganglionic neuron Ca^{2+} channels.[19] Although SSW induced by water immersion and EMLA cream show nearly identical wrinkling, EMLA produces a more linear response curve than water and wrinkling persists for over 90 minutes allowing sufficient time for grading.[20]

In current clinical practice, skin wrinkling for assessing autonomic dysfunction in patients with SFN-like symptoms is performed in the hands and/or feet, using a published 5-point-scale.[21] This method of grading is subjective and the degree of

natural wrinkles due to age and/or gender has been disregarded.[21] For example, skin elasticity, extensibility and echogenicity all decrease with age.[22] Considering the mentioned limitations in current SFN diagnostics, a more sensitive, specific and preferably non-invasive screening tool to detect small nerve fiber dysfunction would be of great value.

In the current study, we firstly aimed to obtain normative values for the SSW-test with EMLA, in a cohort of healthy individuals stratified for age under pre-defined standardized (room temperature, duration, assessed digits) assessment conditions. Assessment was done by the 5-point scale method. In addition, the inter-observer reliability was also examined as a minimum requirement for using the test in daily practice. Secondly, we have investigated whether the SSW assessment, using a new software program, the Digit Wrinkle Scan (DWS©), could improve the assessment of skin wrinkling in healthy individuals. The DWS© program digitally quantified the wrinkles in the fingertips, before and after EMLA application. For this analysis, the inter- and intra-observer reliability were determined.

Methods

We conducted a prospective cross-sectional diagnostic study in which we performed SSW with the 5-point scale method (part I) and the DWS© (part II) in healthy subjects.

Part I of the study took place between December 2012 and April 2013, while part II was performed between May 2017 and May 2018 at Maastricht University Medical Center+ (Maastricht UMC+), Maastricht, the Netherlands after having finished the development of the DWS© software. The study was approved by the local Medical Ethics Committee and written informed consent was obtained from each subject according to the declaration of Helsinki.

Subjects

Healthy subjects were recruited through advertisements in Maastricht UMC+. The following inclusion criteria applied: no SFN-related complaints as assessed using the SFN-Symptom Inventory Questionnaire (SFN-SIQ) and normal neurological testing (including Medical Research Council (MRC) grading, tendon reflexes and

sensory testing). The SFN-SIQ evaluates several symptoms as well as autonomic symptoms in a simple manner. The questionnaire consists of 13 questions and was derived from the original SIQ, and from the composite autonomic symptoms scale (COMPASS).[23] Healthy subjects were excluded when they (i) had complaints of burning/tingling feet or hands; (ii) are known with previous neurological disorders, such as (poly)neuropathy, carpal tunnel syndrome, spinal cord and root disease, or significant limb trauma; (iii) have known conditions that may cause neuropathy: diabetes mellitus, hypothyroidism, renal failure, vitamin B12 deficiency, monoclonal gammopathy, alcohol abuse (more than five IU/day), malignancies, drugs that cause neuropathy (e.g. chemotherapy, amiodarone, propafenone); (iv) use skin cream at day of testing and (v) use antihypertensive drugs with effect on the sympathetic nervous system at peripheral effect (α1 adrenergic antagonists, β-blockers, calcium channel blockers, ACE inhibitors, AT1 receptor antagonists).

Study design

In order to create normative values for the SSW, participants were stratified for age, forming five age groups (20-29, 30-39, 40-49, 50-59, ≥60 years), each group consisting at least 5 males plus 5 females.

To determine the applicability of the DWS©, we randomly selected 35 new healthy participants.

Study procedure

All SSW examinations took place in a standardized, temperature controlled (21-24°C) room, before lunch time. Subjects were instructed not to drink coffee or tea at least two hours before testing, since caffeine might influence autonomic function. In the first part, baseline photographs of the distal tip of the, 4th and 5th digit of both hands were taken, because it was reported that these fingers showed the clearest and most pronounced SSW with EMLA.[20] In the second part of the study, we took pictures of the distal tip of the 2nd, 3rd, 4th and 5th digit of both hands, since it is unknown if the DWS© would show other results during analyses. A review study has advised to use the average score of the 2nd, 3rd, 4th and 5th digit.[15] However, in other studies the average of digit 3, 4 and 5[24], or digit 2, 3, and 4[17] have been used. A fixed setup was used: the hand was held in front of a dark background with the palm of the hand towards the digital camera (CANON

Eos 10D, macro lens). The camera was positioned at a standardized distance of 30 cm above the background with diffuse ring lighting around the lens.

In all subjects, skin wrinkling was induced by EMLA cream© (lidocaine 2.5% and prilocaine 2.5% AstraZeneca). Approximately 1 g (the amount needed to thickly and completely cover the distal digit pulp) of EMLA cream© was applied to the distal tip of the mentioned fingers of both hands and then left to soak into the skin for 30 minutes after covering with a Tegaderm© plaster. Any residual EMLA was removed afterwards. Subsequently, new photographs of EMLA-treated digits were taken in the same way as baseline pictures were made. All taken photographs were uploaded in a web-based program (MACRO), after they had been assigned a study number.

For the first part of the study, two trained observers graded the wrinkling pattern after SSW according to the previously published 5–point-scale[21], in order to create normative values and to determine the inter-observer reliability of this assessment.

In the second part of the study, two independent observers carried out DWS© analyses in a blinded fashion. To determine the length and width of each distal digit tip, two straight lines were drawn after which the program automatically calculated the tip surface in millimeters^2 (mm^2) (Figure 4.1). Subsequently, the researcher drew lines over all wrinkles (Figure 4.2). Determination of surface area and total wrinkle length was done according to a protocol. The total length of wrinkles in millimeters (mm) and the total wrinkle length in mm/mm^2 were calculated by the software. In order to determine the intra-observer reliability, one observer assessed each picture with an interval of, at least, two weeks without having access to the previous records.

Data analysis

Statistical analysis was carried out on a PC using Microsoft Excel and the IBM SPSS statistical package version 24.

The inter-observer variability for wrinkle scores according to the 5-point-scale was assessed using Cohen's Kappa's score. Furthermore, the inter-observer reliability was measured using intraclass correlation coefficient (ICC) for each digit by means of the Two-Way Random-Effects Model.[25] Lastly, the ICCs for the intra-

observer reliability for the DWS© was calculated. The confidence interval was calculated with 95%.

The ICC values less than 0.5 were indicated as poor, values between 0.5 and 0.75 as moderate, values between 0.75 and 0.9 as good, and values greater than 0.90 as excellent reliability.

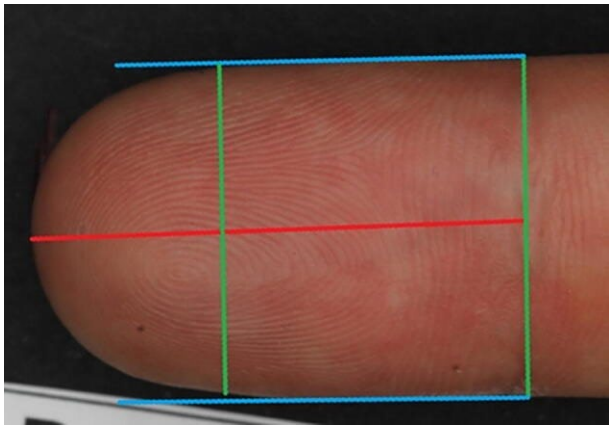


Figure 4.1 The length of the digit is determined by drawing the red straight line (in mm). The width is the mean of both green lines, which are drawn perpendicular to the blue lines (in mm).

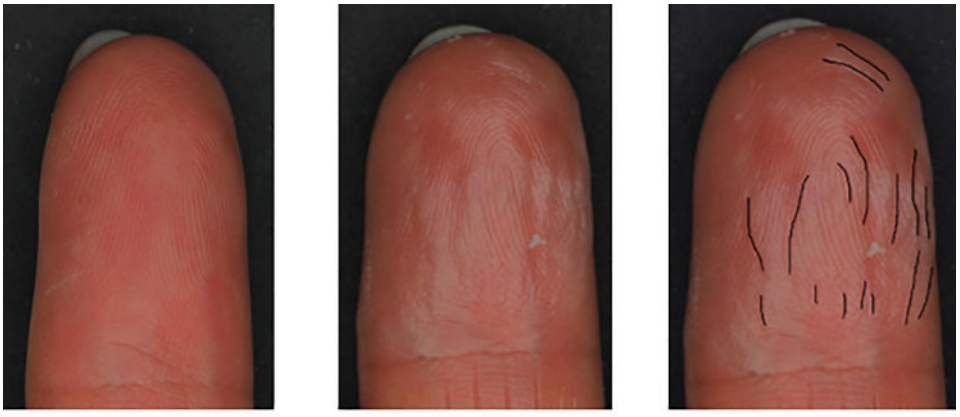


Figure 4.2 This is an example of the assessment process of one digit: a picture before EMLA application (left), after EMLA-removal (middle) and after uploading in the software and drawing the wrinkle lines (in mm) (right).

RESULTS

5-point scale outcome measures

In part I, SSW was conducted in 82 healthy volunteers (women: n=47, men: n=35). Mean age for both gender was 49.8 (SD 16.9, range 21-77 years). Normative data per age group were shown for digit 4 and 5 in Table 4.1. With increasing age, the difference between wrinkling before and after SSW decreases. There was a minimal difference between digit 4 and 5.

Table 4.1 Normative values for SSW before and after EMLA application (range).

Age category (in years)	t=0		t=30	
	digit 4	digit 5	digit 4	digit 5
20-29	0	0	3.9 (3-4)	3.9 (3-4)
30-39	0	0	3.7 (3-4)	3.7 (1-4)
40-49	0.2 (0-1)	0.2 (0-1)	3.5 (2-4)	3.5 (1-4)
50-59	0	0	3.5 (1-4)	3.5 (1-4)
≥60	0.2 (0-3)	0.2 (2-3)	3.3 (1-4)	3.2 (1-4)

The inter-observer reliability scores at t=0 were good for all examined digits (Table 4.2). The scores were moderate at t=30 for digit 4 and 5; scores for digit 4 were slightly better than for digit 5 at t=30.

Table 4.2 Inter-observer reliability (ICC) on t=0 and t=30.

t=0	ICC	CI 95%
Digit 4, right	1.0	n/a
Digit 5, right	0.96	0.94 – 1.0
Digit 4, left	0.96	0.94 – 1.0
Digit 5, left	1.0	n/a
t=30		
Digit 4, left	0.53	0.47 – 0.55
Digit 5, left	0.50	n/a

DWS outcome measures

In the second part of the study, we included a total of 35 healthy subjects (40% male; mean age 35.3 years; range 20-80 years). We calculated the ICC's, based on the wrinkle length and the wrinkle length per mm^2, at t=0 and t=30.

At t=30 after EMLA-application, the inter-observer reliability was higher in the 4^{th} and 5^{th} digit, in comparison to the 2^{nd} and 3^{rd} digit (Table 4.3), but moderate for

both. A good to excellent reliability (ICC≥0.75) was only found on t=30 in digit IV of the left hand (ICC: 0.788, CI: 0.570-0.895) for the wrinkle length/mm^2 (Table 4.3).

Table 4.3 The inter-observer reliability of the line length and line length/mm^2 before, and after EMLA-immersion.

	ICC (CI 95%)	ICC (CI 95%)	ICC (CI 95%)	ICC (CI 95%)
Digit, side	**line length, t= 0**	**line length, t=30**	**line length/mm², t=0**	**line length/mm², t=30**
2nd, right	0.656 (0.319-0.827)	0.608 (0.215-0.804)	0.464 (-0.062-0.279)	0.459 (-0.082-0.730)
3rd, right	0.520 (0.049-0.758)	0.368 (-0.265-0.684)	0.637 (0.280-0.817)	0.749 (0.498-0.875)
4th, right	0.658 (0.322-0.827)	0.674 (0.348-0.837)	0.535 (0.078-0.765)	0.686 (0.372-0.843)
5th, right	0.247 (-0.492-0.620)	0.765 (0.530-0.883)	0.560 (0.129-0.778)	0.711 (0.421-0.856)
2nd, left	0.548 (0.104-0.772)	0.370 (-0.262-0.685)	0.508 (0.026-0.752)	0.337 (-0.328-0.669)
3rd, left	0.642 (0.290-0.819)	0.632 (0.264-0.816)	0.650 (0.306-0.823)	0.226 (-0.550-0.613)
4th, left	0.333 (-0.332-0.663)	0.660 (0.311-0.832)	0.442 (-0.105-0.718)	0.788 (0.570-0.895)
5th, left	0.584 (0.177-0.190)	0.645 (0.281-0.825)	0.637 (0.281-0.817)	0.734 (0.462-0.869)

The intra-observer reliability (ICC) for the total group of 35 participants with the DWS showed a good reliability in two digits, namely 2nd digit (right) and 4th digit (left), 0.838 [CI: 0.578-0.937) and 0.827 [CI: 0.563-0.932] respectively after EMLA-application (Table 4.4). The remaining correlations were moderate.

Table 4.4 The intra-observer reliability of the line length and line length/mm^2 before, and after EMLA-immersion.

	ICC (interval)	ICC (interval)
Digit, side	**line length/mm², t=0**	**line length/mm², t=30**
2nd, right	0.374 (-0.530-0.760)	0.838 (0.578-0.937)
3rd, right	0.577 (-0.069-0.833)	0.585 (-0.078-0.804)
4th, right	0.469 (-0.341-0.798)	0.532 (-0.184-0.815)
5th, right	0.576 (-0.071-0.832)	0.698 (0.216--0.884)
2nd, left	0.416 (-0.475-0.769)	0.687 (0.209-0.876)
3rd, left	-0.118 (-1.826-0.557)	0.703 (0.248-0.882)
4th, left	0.600 (-0.011-0.842)	0.827 (0.563-0.932)
5th, left	0.692 (0.221-0.878)	0.628 (0.059-0.853)

Discussion

This study presents the normative values of SSW according to the 5-point scale assessment. Changes were found in SSW after EMLA application, namely a decrease with increasing age, suggesting a decrease of autonomic function. No effect was found for gender (results not published). Furthermore, reliability scores

show moderate agreement. This suggests that the use of the categorical assessment, as intuitively simple as it might look and consequently the application of the normative values, is controversial and should be discouraged for clinical practice.
The age-dependent normative values that we obtained, showed a decrease in wrinkles with age. Various factors may contribute to age-related changes in the sensory system; a change in properties of the dermis, demyelination and fiber loss in peripheral nerves, and degenerative changes in the central nervous system.[26] Although the extreme scores (i.e. score 0 or 4) can be well distinguished, the other categories are more doubtful. That could be the reason why relatively high ICCs were found before SSW and moderate agreement after EMLA-application. The scale used thus far is an ordinal one, based on classical test theory (CTT).[27] A major limitation of CTT is that scores create measurement at an ordinal level with unequal intervals that hamper accurate measurement of differences in scores and changes over time among individuals. Therefore, a more accurate and linear scale would be needed to properly assess skin wrinkling, both in terms of normative values as in clinical application, particularly when examining medical interventions.

For the second part of the study, we developed the DWS© in order to quantify the presence of natural wrinkling and wrinkling after EMLA application, on a ratio scale. DWS© provides a seemingly more specific, detailed and objective image of the presence of wrinkles on the digits as compared to the grading method. We found that the 4th and 5th finger of each hand after EMLA application had the highest ICC. These results may indicate that these fingers are best judged, which was previously reported.[20] However, the reliability scores cannot be marked as good/excellent and therefore hamper applicability in clinical practice.

The DWS© software program has several main advantages over the 5-point scale visual assessment. The DWS© takes physiological wrinkles into account, which are naturally present on the digit surface. In this way, the increase or decrease of wrinkles after EMLA-application can be determined. However, the DWS© protocol requires further improvement. Future studies should focus on improving the screening conditions (camera resolution, environmental light, picture angle), analysis techniques (3D analysis, Doppler imaging[28]) and final outcome measures.

In conclusion, our results show that the SSW 5-point scale does not fulfill clinimetric requirements, which hampers its use in the clinical setting. Moreover, the DWS© is a more specific method to examine alterations in wrinkle formation after SSW, however, inter-observer reliability showed also moderate to good agreement at best. Therefore, more reliable techniques, in larger study samples, should be investigated (i.e. 3D-techniques, or subdermal visualization), to determine whether these methods could improve the assessments of autonomic dysfunction. If a reliable technique is available, international normative values, corrected for age, should be determined before it could serve as a tool in the diagnostics of autonomic dysfunction in neuropathies, like SFN.

References

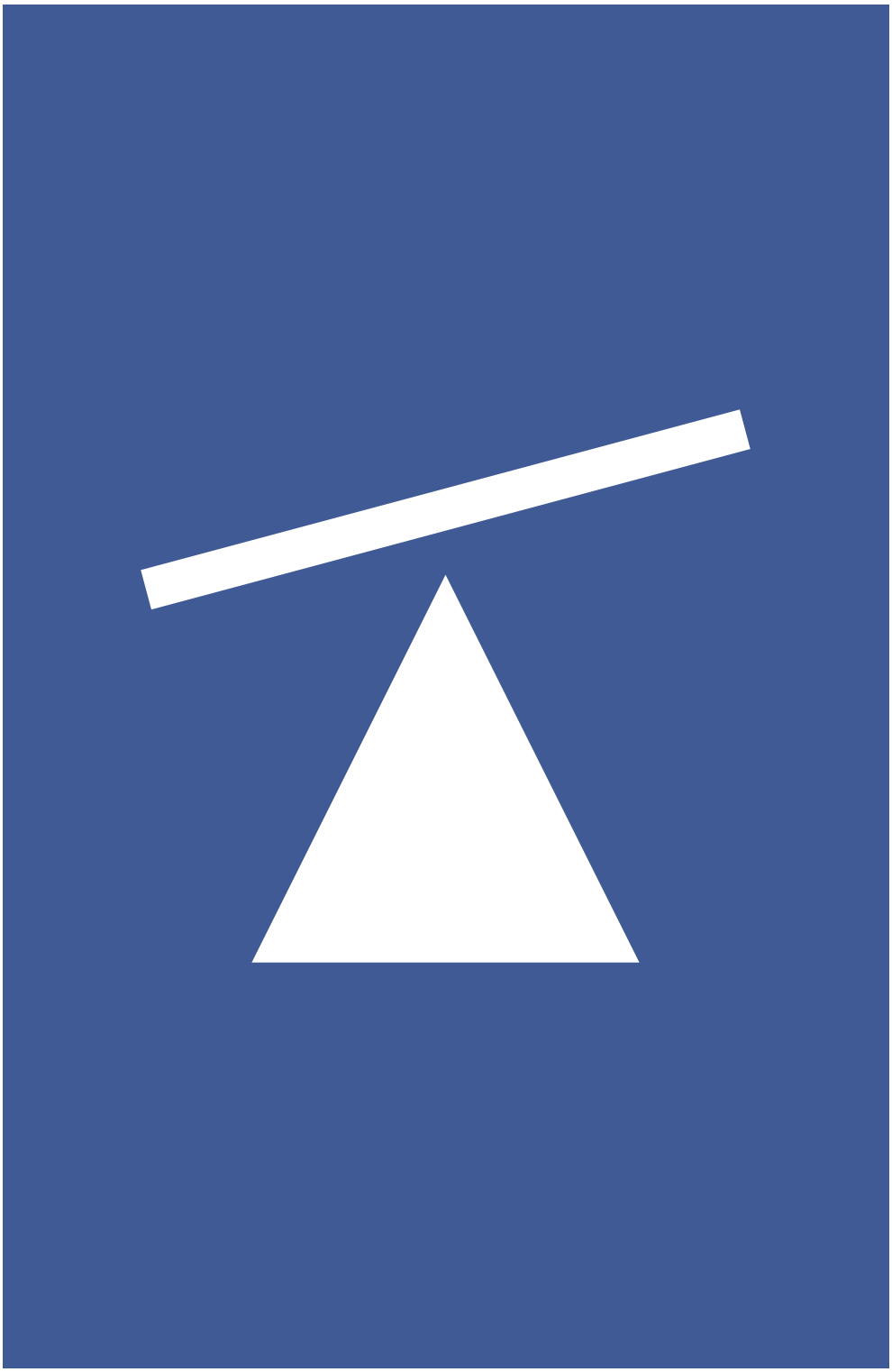
1. Lauria G, Merkies IS, Faber CG. Small fibre neuropathy. Curr Opin Neurol. 2012;25(5):542-9.
2. Devigili G, Cazzato D, Lauria G. Clinical diagnosis and management of small fiber neuropathy: an update on best practice. Expert Rev Neurother. 2020;20(9):967-80.
3. Sopacua M, et al. Small-fiber neuropathy: Expanding the clinical pain universe. J Peripher Nerv Syst. 2019;24(1):19-33.
4. Hoeijmakers JG, et al. Small-fibre neuropathies--advances in diagnosis, pathophysiology and management. Nat Rev Neurol. 2012;8(7):369-79.
5. Tesfaye S, et al. Diabetic neuropathies: update on definitions, diagnostic criteria, estimation of severity, and treatments. Diabetes Care. 2010;33(10):2285-93.
6. Freeman R, et al. Idiopathic distal sensory polyneuropathy: ACTTION diagnostic criteria. Neurology. 2020;95(22):1005-14.
7. Devigili G, et al. Diagnostic criteria for small fibre neuropathy in clinical practice and research. Brain. 2019;142(12):3728-36.
8. Lauria 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.
9. De Greef BT, Hoeijmakers JGJ, Merkies ISJ, Faber CG. Associated conditions in small fiber neuropathy – A large cohort study and review of the literature. Eur J Neurol. 2018;25(2): 348-55.
10. Fabry V, et al. Which Method for Diagnosing Small Fiber Neuropathy? Front Neurol. 2020;11: 342.
11. Jin P, et al. Low Sensitivity of Skin Biopsy in Diagnosing Small Fiber Neuropathy in Chinese Americans. J Clin Neuromuscul Dis. 2018;20(1):1-6.
12. Maier C, 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.
13. Shy ME, et al. Quantitative sensory testing: report of the Therapeutics and Technology Assessment Subcommittee of the American Academy of Neurology. Neurology. 2003;60(6): 898-904.
14. Yarnitsky D, et al. Variance of sensory threshold measurements: discrimination of feigners from trustworthy performers. J Neurol Sci. 1994;125(2):186-9.
15. Wilder-Smith EP. Stimulated skin wrinkling as an indicator of limb sympathetic function. Clin Neurophysiol. 2015;126(1):10-6.
16. Falanga V. The "wrinkle test": clinical use for detecting early epidermal resurfacing. J Dermatol Surg Oncol. 1993;19(2):172-3.
17. Ping Ng KW, et al. EMLA-Induced Skin Wrinkling for the Detection of Diabetic Neuropathy. Front Neurol. 2013;4:126.
18. Mawuntu AHP, et al. Early detection of peripheral neuropathy using stimulated skin wrinkling test in human immunodeficiency virus infected patients: A cross-sectional study. Medicine (Baltimore). 2018;97(30):e11526.
19. Wilder-Smith EP. Water immersion wrinkling--physiology and use as an indicator of sympathetic function. Clin Auton Res. 2004;14(2):125-31.
20. Wilder-Smith E, Chow A. Water immersion and EMLA cause similar digit skin wrinkling and vasoconstriction. Microvasc Res. 2003;66(1):68-72.
21. Vasudevan TM, et al. Skin wrinkling for the assessment of sympathetic function in the limbs. Aust N Z J Surg. 2000;70(1):57-9.
22. Batisse D, et al. Influence of age on the wrinkling capacities of skin. Skin Res Technol. 2002; 8(3):148-54.
23. Greco C, et al. Validation of the Composite Autonomic Symptom Score 31 (COMPASS 31) for the assessment of symptoms of autonomic neuropathy in people with diabetes. Diabet Med. 2017;34(6):834-8.

24. Teoh HL, Chow A, Wilder-Smith EP. Skin wrinkling for diagnosing small fibre neuropathy: comparison with epidermal nerve density and sympathetic skin response. J Neurol Neurosurg Psychiatry. 2008;79(7):835-7.
25. Koo TK, Li MY. A Guideline of Selecting and Reporting Intraclass Correlation Coefficients for Reliability Research. J Chiropr Med. 2016;15(2):155-63.
26. Wickremaratchi MM, Llewelyn JG. Effects of ageing on touch. Postgrad Med J. 2006;82(967): 301-4.
27. DeVellis RF. Classical test theory. Med Care. 2006;44(11 Suppl 3):S50-9.
28. Gechev A. Fluid Containing Structures in the tips of the fingers and toes delineated by Ultrasound Imaging before and after Induced Skin Wrinkling. Sci Rep. 2019;9(1):1640.

III

PART 3

EPIDEMIOLOGY OF GENETIC MUTATIONS IN SMALL FIBER NEUROPATHY



CHAPTER 5

EVALUATION OF MOLECULAR INVERSION PROBE VERSUS TRUSEQ® CUSTOM METHODS FOR TARGETED NEXT-GENERATION SEQUENCING

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Abstract

Resolving the genetic architecture of painful neuropathy will lead to better disease management strategies, risk stratification, and counselling. We aimed to develop a reliable technique re-sequence multiple genes in a large cohort of painful neuropathy patients at low cost. In this study, we compared the sensitivity, specificity, targeting efficiency, performance and cost effectiveness of Molecular Inversion Probes-Next generation sequencing (MIPs-NGS) and TruSeq® Custom Amplicon-Next generation sequencing (TSCA-NGS). Capture probes were designed to target nine sodium channel genes (*SCN3A, SCN8A-SCN11A*, and *SCN1B-SCN4B*). One hundred sixty six patients with diabetic and idiopathic neuropathy were tested by both methods, 70 patients were also validated by Sanger sequencing. The average targeted regions coverage for MIPs-NGS was 97.3% versus and 93.9% for TSCA-NGS. MIPs-NGS has a more versatile assay design and is more flexible than TSCA-NGS. The cost of MIPs-NGS is >5 times cheaper than TSCA-NGS when 500 or more samples are tested.

In conclusion, MIPs-NGS is a reliable, flexible, and relatively inexpensive method to detect genetic variations in a large cohort of patients. In our centers, MIPs-NGS is currently implemented as a routine diagnostic tool for screening of sodium channel genes in painful neuropathy patients.

Introduction

Over the last decade, the field of molecular genetic diagnostic has undergone tremendous changes. Introduction of next generation sequencing (NGS) enabled replacement of single gene tests with comprehensive gene panels, whole exome sequencing (WES) and whole genome sequencing (WGS) to increase the likelihood of identifying causal variants while decreasing the number of tests.[1-4]

Currently, there is a growing clinical use of WES or WGS to identify causal variants and to discover new disease related genes in patients. However, routine sequencing of large numbers of WES or WGS remains expensive for clinical use. Several issues such as incidental findings that pose significant ethical problems, cost effectiveness and technical challenges of clinical interpretation of enormous numbers of genetic variants remain challenging.[5,6] Moreover, WGS generates a huge amount of data that require complex bioinformatic analysis tools for data handling and storage.[1]

To fully leverage the power of NGS in a large number of samples in a cost and time-effective manner, several targeted enrichment approaches are available.[7,8] Therefore, many diagnostic laboratories are implementing targeted enrichment NGS methods to focus on specific gene panels, or genomic regions for genetically heterogeneous diseases.[9-12] The most commonly used custom-enrichment approaches are based on capture by hybridization, PCR-based methods (highly multiplexed PCR) and capture by circularization.[7,13-17]

Neuropathic pain is a common feature of peripheral neuropathy causing a significant impact on patients' quality of life and health care costs. Millions of individuals (7-10% of the general population) worldwide suffer from neuropathic pain.[18] However, not all individuals with peripheral neuropathy develop pain, and it is not possible to predict who is more or less susceptible among those with similar risk exposure.[19] Current inability to identify high-risk individuals hinders development and application of therapies to counteract neuropathic pain and to address targeted prevention strategies. Pathogenic variants in voltage-gated sodium channel (VGSC) genes expressed in the peripheral nociceptive pathway such as *SCN9A, SCN10A*, and *SCN11A* have been reported to play a key role in neuropathic pain.[20-23]

In order to provide a genetic diagnosis in patients with neuropathic pain, identification of causal variants in genes encoding for the sodium channel subunits as well as other genes involved in painful neuropathy pathway is needed. Resolving the genetic architecture of painful neuropathy will lead to better disease management strategies, risk stratification, and counselling. Therefore, the aim of this study was to develop, validate and implement a reliable technique to rapidly and accurately re-sequence multiple genes in a large cohort of neuropathic pain patients at low cost. We present here the assessment of Molecular Inversion Probes-Next generation sequencing (MIPs-NGS) and TruSeq® Custom Amplicon-Next generation sequencing (TSCA-NGS, Illumina, Inc., San Diego, CA, USA) methods, using a custom gene panel, to identify genetic variants in patients with neuropathic pain. For both methods, we constructed a targeted enrichment kit to capture the coding and exon-flanking intron sequences of nine sodium channel genes (*SCN3A, SCN8A-SCN11A*, and *SCN1B-4B*). We applied the two methods to test 166 different patients and systematically compared the sensitivity, specificity, flexibility, targeting efficiency, reproducibility of performance and cost effectiveness of MIPs-NGS and TSCA-NGS approaches.

Materials and methods

Patient samples

In total, 166 samples from patients diagnosed with diabetic and idiopathic neuropathy were tested by MIPs-NGS and TSCA-NGS. For 70 of these patients, exons and exon-flanking intron sequences of *SCN9A, SCN10A,* and *SCN11A* genes were analyzed by Sanger sequencing to validate MIPs-NGS and TSCA-NGS methods.[23] Local Medical Ethical Committees of Fondazione IRCCS Istituto Neurologico "Carlo Besta" (Italy), Maastricht University Medical Center (the Netherlands), University of Manchester (United Kingdom) and the Deutsche Diabetes Forschungsgesellschaft EV (Germany) approved this study. Informed consent for genetic testing was given by patients to participate in this study.

Genomic DNA was extracted from peripheral blood by using QIAamp DNA Blood Maxi Kit, Puregene® Blood Core Kit (Qiagen, Hilden, Germany) or NucleoSpin®8 Blood Isolation kit (Macherey-Nagel, Düren, Germany). Quality and concentration of the DNA was determined by NanoDrop (Thermo Scientific, Wilmington, USA), and Qubit® 2.0 Fluorometer using the Qubit® dsDNA BR assay

kit (Life technologies, Bleiswijk, The Netherlands). Isolated DNA was stored with a unique numeric code in the central DNA bank at Maastricht University Medical Centre and IRCCS Foundation "Carlo Besta" Neurological Institute.

Targeted Enrichment, paired-end sequencing and data processing

Targeted enrichment kits were constructed for this study to capture the coding and exon-flanking intron sequences (±20 base pairs [bp]) of nine VGSC genes; *SCN3A, SCN8A-SCN11A*, and *SCN1B-4B*. The probes were designed for the two methods using their respective informatics pipelines and methods are provided in the subsections below. The workflow summary of MIPs-NGS and TSCA-NGS are presented in Figure 5.1. Probe features and sequencing characteristics are given in Table 5.1.

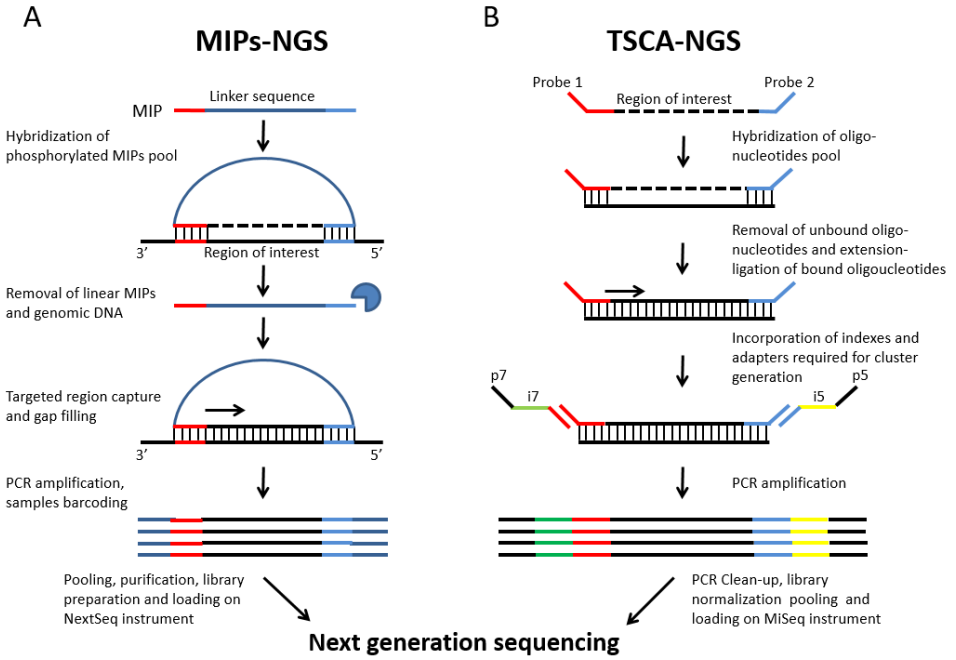

Figure 5.1 MIPs-NGS and SCA-NGS workflow summary. A and B: Graphical depiction of MIPs-NGS and TSCA-NGS methods for library construction and capture protocol for targeted next-generation sequencing.

Table 5.1 Comparison of recommended DNA input, probe features, sequencing kits, costs and sample processing time for MIPs-NGS and TSCA-NGS.

	MIPs-NGS	TSCA-NGS
Recommended DNA input (ng)	50-100	250
Probe type	DNA; molecular inversion probe	DNA; oligonucleotides
Probe strategy	Multiple amplicons	Multiple amplicons
Length of probes (bp)	77-80 mers	50 mers*
Gap fill length	220-230	425
Number of probes	276	186
Sequencing kit	2x150/2x250	2x250
Hands on time per library (min)	45	130
Total duration per library (days)	2	1-2

*probes+adapter

MIPs-NGS

Two hundred and seventy six molecular inversion probes (MIPs) were designed to capture 37.467 bp that represent all exons and exon-flanking intron sequences of nine VGSC genes. A modified version of MIPgen tool (http://shendurelab.github.io/MIPGEN/) was used to design MIPs.
All probes were fixed in to 77-80-mer in length and each MIP contains two targeting arms; an extension arm ranging in length from 16 to 20 nucleotides (nt) and a ligation arm ranging in length from 20 to 24 nt. These arms were joined by a 30 nt common linker sequence which contains two universal PCR primer sites (complimentary probe arms are available upon request). MIPs were designed to have an overlapping of 20 bps and every base in the targeted region should be covered and captured at least by one probe. All MIPs with high arm copy count (>5x) were excluded. Furthermore, 3′ and 5′untranslated regions (3′ and 5′ UTRs) were not included in the design. Common single nucleotide polymorphisms (SNPs) (>1%) in the extension and ligation arms of the MIPs were excluded whenever possible. In some cases where SNPs were not avoidable, two MIPs were designed to match both wildtype and variant genotype for the same locus (n=12). Constructed BED file of the targeted regions and MIPs corresponding to these nine candidate genes were uploaded to UCSC genome browser (http://genome.ucsc.edu/) in order to validate each designed MIP.

To reduce costs, the standard gap-fill length of 112 nt between the extension and ligation arm (region of interest) of the MIPs[24] was adapted to 220-230 nt. Each MIP contains a single molecule tag of 5 nt to remove duplicates introduced by PCR amplification and sequencing. Probes were synthesized by Integrated DNA

Technologies (IDT, Iowa, USA) and delivered individually. Subsequently, probes were equimolarly pooled and phosphorylated at 5' end of the probe.

Experimental workflow was done by following standard protocols.[16,24,25] In brief, 50-100 ng of high quality, non-fragmented genomic DNA was used for hybridization. After gap filling and ligation, circularized DNA molecules were used as template in PCR with universal primers complementary to the linker sequence. Then, sample-specific barcode sequences and Illumina adaptors were introduced during the PCR amplification step. Fragment size, quality, and quantity of the amplified captured material were determined by Qubit® 2.0 Fluorometer using the Qubit® dsDNA BR assay kit (Life technologies, Bleiswijk, The Netherlands) and Agilent 2100 bioanalyzer using the Agilent DNA 1000 Kit (Agilent Technologies, Santa Clara, CA, USA) following manufacturer's instructions. Next, samples were pooled and purified using Ampure XP beads (Beckman Coulter, Inc, Brea, California) according to manufacturer's instructions. Pooled samples were then paired-end sequenced (2 x 150 bp or 2 x 250 bp) using the MiSeq or NextSeq 500 Instrument (Illumina, Inc., San Diego, CA, USA) to achieve >30x coverage per bp.

After a pilot experiment with 10 DNA samples, MIPs with a poor performance (15-30 reads) were rebalanced by adding a 5 fold concentration of early poorly performing probe to the original MIPs pool, and MIPs with no sequence reads were replaced by adding new MIPs to the original MIPs pool. Then, the pilot experiment was repeated as described above.

Sequenced data was analyzed by using our in-house MIPs-targeted NGS data analysis pipeline. This pipeline aligns sequenced data to the human reference sequence GRCh37, trims probe arm sequences, de-duplicates the data on the basis of UMIs, and annotates variants according with information and frequencies form ExAC, dbSNP, cadd, Gencode, and calculates coverages per sample, number of bases, mean and median coverage per MIP target. Due to the coverage depth used for this approach, no copy number variant (CNV) analysis has been performed.

TSCA-NGS

One hundred and eighty-five oligonucleotides were designed to capture 38.258 bp that represent all exons and exon-flanking intron sequences of nine

VGSC genes. The design of oligonucleotides was assessed by the free tool Design Studio, which is available on Illumina website (https://designstudio.illumina.com/ (Illumina Inc., San Diego, CA, USA)). Targeted regions were selected by entering the genes name to the tool and only coding exons were selected. All probes were fixed in to 50-mer in length (including the adapters) and the gap-fill length was set to 425 bp (amplicons size). In case that the tool failed to locate automatically the probes hybridization sites, due to the high CG content or high SNPs rates, we manually added the genomic regions by entering the chromosome coordinates. The *a priori* calculation of the target regions coverage using this tool provided better results when adapting 425 bp amplicon size, instead of the 250 bp option. Increasing the amplicon size resulted in costs reduction in terms of number of oligonucleotides probes spanning the entire target region. One pair of oligonucleotides was designed for each amplicon and provided as a pool in a single tube.

TruSeq Custom Amplicon libraries preparation was performed according to manufacturer's protocol (TruSeq Custom Amplicon Library Preparation Guide, Part # 15027983 Rev. C). In brief, 250 ng genomic DNA was hybridized to the custom made pool of oligonucleotides specific to the targeted regions of interest. Then, unbound oligonucleotides were removed by washing the samples on the provided filtering plate. Subsequently, DNA polymerase was added to extend from the upstream oligonucleotide through the targeted region, followed by ligation to the 5' end of the downstream oligonucleotide using DNA ligase. The extension-ligation resulted in the formation of products containing the targeted regions of interest flanked by sequences required for PCR amplification. During amplification, the extension-ligation products were amplified using indexed-primers that added sample multiplexing index sequences (i5 and i7) as well as common adapters required for cluster generation (P5 and P7). After purification with PCR Clean-Up AMPure XP beads (Beckman Coulter, Inc.), the library was normalized with Library Normalization Beads. Finally, equal volumes of normalized library were combined, diluted in hybridization buffer, and heat-denatured for sequencing on the MiSeq instrument (paired-end sequenced 2 x 250 bp) (Illumina, Inc., San Diego, CA, USA). No probe dosage adjustment was required for TSCA-NGS.

To identify sequence variations in *SCN3A, SCN8A-SCN11A, SCN1B-4B* by TSCA-NGS, Raw Fastq files were aligned to reference sequence GRCh37 by using CLC Genomics Workbench software (CLCbio, Qiagen, Hilden, Germany). Variant

calling, annotation and coverage analysis was performed CLC Genomics Workbench (CLCbio, Qiagen, Hilden, Germany). No CNV analysis was carried out for this approach.

Sanger sequening

Coding exons and exon-flanking intronic regions of *SCN9A*, *SCN10A* and *SCN11A* of 70 patients were amplified by PCR and sequenced by Sanger sequencing as previously described[23], to assess the sensitivity and specificity both targeted-NGS methods.

Cross-comparative analysis of genetic variations detection between MIPs-NGS and TSCA-NGS

A cross-comparative analysis was performed in order to compare the sensitivity, specificity and reproducibility of genetic variations detection between MIPs-NGS and TSCA-NGS. Regions with high variation rates in exons or mapping at the ends of the reads were excluded to avoid mismatches due to the low quality of reads (Table 5.2). Variants covered less than 30x, with reads supporting the variant call less than 20%, or that were present in more than 90% of the tested samples were filtered-out to exclude potential artifacts. For the cross-comparative analysis, the overlap between each approach was determined. Disconcordant calls were qualitatively and quantitavely assessed.

Table 5.2 Performance comparison between MIPs-NGS and TSCA-NGS.

Gene name	Targeted region (bp)		Number of probes (n)		Average coverage (%)		Number of probes with no reads (n)	
	MIPs-NGS	TSCA-NGS	MIPs-NGS	TSCA-NGS	MIPs-NGS	TSCA-NGS	MIPs-NGS	TSCA-NGS
SCN3A	7043	7175	55	33	99.6	99.3	0	0
SCN8A	6943	6983	49	33	97.6	97.1	0	2 (ex 12*, 21*)
SCN9A	6934	6974	49	32	98.7	95.8	0	1 (ex 27*)
SCN10A	6871	6951	43	34	99.9	98.6	0	0
SCN11A	6379	6416	46	32	99.9	91.6	1 (ex 1)	1 (ex 1)
SCN1B	967	1216	13	6	93.6	91.3	1 (ex 1)	1 (ex 1)
SCN2B	768	808	6	5	100.0	93.2	0	0
SCN3B	808	848	6	5	100.0	98.7	0	0
SCN4B	754	887	9	5	86.5	79.7	1 (ex 1)	1 (ex 1)
Total	**37467**	**38258**	**276**	**185**	**97.3**	**93.9**	**3**	**6**

*exon partially uncovered, bp: base pair, ex: exon.

RESULTS

Performance of MIPs-NGS and TSCA-NGS

We constructed two targeted enrichment kits, the MIPs-NGS kit which contains 276 probes and TSCA-NGS which has 185 probes to capture all exons and exon-flanking intron sequences (±20 bp) of the nine VGSC genes *SCN3A, SCN8A-SCN11A*, and *SCN1B-4B.* To assess the performance, capture efficiency and sequencing coverage of the on-target regions for these nine VGSC genes, 166 samples were captured and enriched by both methods. Data from this study showed a performance and capture efficiency for the SCN (*SCN3A, SCN8A-SCN11A*, and *SCN1B-4B)* MIPs-NGS of 98.9% (n=273/276 MIPs), compared to 96.7% for the TSCA-NGS (n=179/185 probes). To increase the overall performance for MIPs-NGS (>30x, number of unique sequencing reads needed to obtain accurate genotype call at certain position), 28 SCN MIPs were rebalanced by adding a 5 fold concentration of each poorly performing probe to the original MIPs pool, and seven MIPs with no sequence reads were replaced by added new MIPs to the original MIPs pool, prior to the testing of the 166 samples. Probes dosage adjustment for TSCA –NGS was unnecessary.

The average targeted regions coverage (coverage >30x/bp) was 97.3% in MIPs-NGS, and 93.9% in TSCA-NGS. Capture efficiencies of individual probes was highly reproducible per region and between different samples for both methods. No sequence reads were obtained for three MIPs (1.1%) (n=3/276) and for six TSCA probes (3.3%, n=6/185). The first coding exon of *SCN1B, SCN4B* and *SCN11A* genes failed completely to be captured and enriched by both approaches. The last coding region (exon 27) of *SCN9A* was partially covered by TSCA-NGS, while it was fully captured by MIP-NGS (Table 5.2).

Seven exons of *SCN3B* (exon 2), *SCN8A* (exons 13, 16, 21 and 26), *SCN10A* (exon 13) and *SCN11A* (exon 14) tested by MIPs-NGS and three exons of *SCN4B* (exon 2), *SCN10A* (exon 13) and *SCN11A* (exon 14) tested by TSCA-NGS showed high variation in sequencing reads.

Specificity and sensitivity of MIPs-NGS and TSCA-NGS

To determine the specificity and sensitivity of both targeted NGS approaches, 70 patients were analyzed for sequence variations in *SCN9A, SCN10A* and *SCN11A* by Sanger sequencing. Sixty-eight unique variants were identified in

these *SCN9A, SCN10A* and *SCN11A*, including 65 nucleotide substitutions and three indels, located either in the exonic regions or in the exon-flanking intron sequences (±20 bp). Sixty-four of the 68 variants are present in both MIPs-NGS and TSCA-NGS sets, including their status being homozygous or heterozygous. Four variants were missed by both methods, as these variants are located within regions with low quality or no coverage (one variant in exon 13 in *SCN10A* and three variants in *SCN11A;* one variant in exon 1 and two variants in exon 14). Based on these results, both capture panels demonstrated a 94.1% sensitivity for variant detection (n=64/68 variants). When we excluded Sanger sequencing variants located in regions with low quality or no coverage (4 variants) by MIPs-NGS and TSCA-NGS (n=4 variants), we observed a perfect agreement (100%; no differences in number of variants and zygosity status of variants) between Sanger sequencing data and those obtained using MIPs-NGS and TSCA-NGS.

By excluding regions with low quality (exons with high variation in reads), the overall false-positive rate in *SCN9A*, *SCN10A*, and *SCN11A* was 0% for MIPs-NGS and TSCA-NGS.

Cross comparative analysis of genetic variations detection

To compare variants calls from MIPs-NGS versus TSCA-NGS, sequence data of the same 166 diabetic and idiopathic neuropathy patients was analyzed with our in-house data analysis pipeline. The overlap in on-target regions from all 166 tested subjects was 3642 (123 unique variants) by MIPs-NGS and 3658 (122 unique variants) by TSCA-NGS. We found that 3642 variants were correctly called by both methods and a true positive call of 99.6% was observed. The 16 dissimilar variants (all the same unique variant present in 16 samples) (0.4%) were due to coverage differences between the two methods (low coverage or the coverage was just below the threshold).

MIPs-NGS vs. TSCA-NGS workflow and costs

MIPs-NGS and TSCA-NGS showed a high multiplexing level and require low sample DNA input (between 50-250 ng). Both protocols are straightforward, but hands-on time and the methodological complexity should be taken into consideration. MIPs-NGS is easier and less time-consuming than TSCA-NGS. The workflow for TSCA-NGS library preparation pass through 86 steps, combining enzymatic and PCR reactions, purification and normalization steps with filer plates and magnetic beads, while MIPs-NGS only required 7 steps to obtain the final

library pool.[16,24,25] The hands-on time to create a library was 45 minutes for MIPs-NGS versus 130 minutes for TSCA-NGS, regardless of sample size, DNA purity, and concentration of starting material (Table 5.1).

In this study, costs per sample, including probes, chemical reagents and efforts was lower for MIPs-NGS compared to TSCA-NGS. However, when a large number of patients, e.g. 500 samples were included, the costs per sample for MIPs-NGS could drop to >5 times as cheap as TSCA-NGS (Table 5.3).

Table 5.3 Price comparison MIPs-NGS and TSCA-NGS on different sequencing platforms.

	MiSeq		NextSeq 500/550	NovaSeq600	
	MiSeq Reagent Kit v2 (300 cycles)	MiSeq Reagent Kit v2 (500 cycles)	NextSeq 500/550 High Output Kit v2.5 (300 cycles)	NovaSeq 6000 SP Reagent Kit (300 cycles)	NovaSeq 6000 SP Reagent Kit (500 cycles)
MIPs-NGS					
Sample price* for 100 samples (€)	44.6	45.9	45.5	42.8	46.8
Sample price* for 300 samples (€)	23.5	24.9	24.5	21.7	25.8
Sample price* for 500 samples (€)	19.3	20.7	20.3	17.5	21.6
Sample price* for 1000 samples (€)	16.1	17.5	17.1	14.4	18.4
TSCA-NGS					
Sample price‡ for 100 samples (€)	-[1]	163.9	-[1]	-[1]	192.5
Sample price‡ for 300 samples (€)	-[1]	125.0	-[1]	-[1]	-[2]
Sample price‡ for 500 samples (€)	-[1]	113.4	-[1]	-[1]	-[2]
Sample price‡ for 1000 samples (€)	-[1]	103.3	-[1]	-[1]	-[2]

* price per samle based on probes (€5.6/probe), reagents (€1.8), rebalancing, optimization and validation costs (€1575) and sequencing costs (varies from €1073 to €6246). Prices are without VAT, company discounts, labor and equipment costs. ‡ price per sample based on TruSeq® Custom Amplicon Kit v1.5 (€6142-€12488), TruSeq® Custom Amplicon Index Kit (€870) and sequencing costs (€1204-€5162). Prices are without VAT, company discounts, labor and equipment costs. [1] not calculated, read length 300 cycles (2 x 150 bp) too short for TSCA-NGS. [2] not calculated, maximum number of available sample indexes for TSCA-NGS was n=96.

Discussion

Selection of an approach for screening a panel of genes depends for most laboratories depends on a width range of criteria, including clinical use of the test, panel size, sensitivity and specificity for the genetic regions of interest, expected number of patients to be tested, turnaround time, approach flexibility and scalability, available equipment, work flow, costs, technical expertise, and availability of bioinformatics support.

In this study, we developed a MIPs-NGS and TSCA-NGS targeted re-sequencing panels for nine sodium channel genes (*SCN3A, SCN8A-SCN11A*, and *SCN1B-4B)* known to be associated with neuropathic pain[19,26], and compared their sensitivity, specificity, targeting efficiency, performance and cost effectiveness. The average targeted regions coverage was 97.3% in MIPs-NGS, and 93.3% in TSCA-NGS. Sensitivity, specificity and performance of both methods were comparable (Table 5.2). However, MIPs-NGS has a more versatile assay design, and is flexible and cheaper than TSCA-NGS (Table 5.1 and Table 5.3).

Molecular Inversion Probes (MIPs), also known as padlock probes, belong to the category of molecular techniques that capture sequences by circularization. This technology first described in 1994, was initially developed for multiplex target discovery and SNP genotyping[27,28], and has recently been combined with next generation sequencing. TSCA-NGS is an amplicon-based approach for targeted re-sequencing. This approach is based on the design of synthetic oligonucleotides (probes), with complementary sequence to the flanking regions of the target DNA to be sequenced. Amplicon-based sequencing approaches are cheaper than Sanger sequencing, WES, and WGS, and characterized by high specificity and deep coverage. Moreover, MIPs-NGS and TSCA-NGS have been successfully employed with good-quality DNA sources such as blood or frozen tissues, and with more challenging samples extracted from formalin-fixed and paraffin-embedded tissues.[29]

Development of a sensitive diagnostic custom targeted NGS enrichment kit requires proper design of specific primers or probes for candidate genes. For MIPs-NGS and TSCA-NGS probe design, we used the freely available tools, MIPgen software (http://shendurelab.github.io/MIPGEN/) and Design Studio, which is available on Illumina website (https://designstudio.illumina.com/), respectively. Both software tools were user-friendly, and simplify the probe designing process based on the criteria defined by the user.

MIPs-NGS and TSCA-NGS showed high multiplexing level, low DNA input requirements (50-250ng) and no need for DNA shearing compared to other targeted enrichment methods.[15,30]

MIPs-NGS and TSCA-NGS have, in general, straightforward laboratory workflow, however TSCA-NGS pass through many steps compared to MIPs-NGS. The hands-on time per library were 2.9 times shorter for MIPs-NGS compared to

TSCA-NGS (Table 5.1). In addition, development automated reaction setups is feasible for MIPs-NGS approach since small number of enzymatic reactions and processing steps are required to achieve targeted region capture and sample barcoding. Such an automated laboratory workflow was recently established for smMIP enrichment to detect genetic variations in BRCA1 and BRCA2.[31] Furthermore, this study concluded that smMIPs-NGS has a superior accuracy and turnaround time compared to other genetic testing methods for gene panels or targeted regions.[31]

The performance and capture efficiency of MIPs-NGS and TSCA-NGS targeted approaches for *SCN3A, SCN8A-SCN11A*, and *SCN1B-4B* was high (98.9% for MIPs-NGS versus 96.7% for TSCA-NGS) and variation per read low; both required for a reliable variant detection. No sequence reads were obtained by MIPs-NGS and TSCA-NGS approaches for the first coding exon of *SCN1B, SCN4B* and *SCN11A*. These regions have a high GC content (>50%) which influence the capture efficiency. To avoid false positive variant calling, data of seven MIPs (1678 bp) versus three TSCA probes (794 bp) should be excluded from the analysis. To provide a full coverage for the *SCN3A, SCN8A-SCN11A*, and *SCN1B-4B,* 2321 bp (6.1%) for the MIPs-NGS approach versus 2467 bp (6.4%) for TSCA-NGS should subsequently been analysed by Sanger sequencing (costs per sample excluding VAT are €29.70 for MIPs-NGS versus €24.75 for TSCA-NGS).

The utility of custom targeted enrichment NGS panels may be limited in some contexts because the kits are inflexible in terms of adding or excluding targeted regions and usually expensive.[32] In many diagnostic laboratories, adjusting existing diagnostic panels have gained popularity specially in investigating genetically heterogeneous disease.[33] MIPs-NGS approach offered a higher degree in flexibility and in optimizing the probe performance compared to TSCA-NGS and other targeted enrichment NGS methods.[2,33] All MIPs were ordered individually, so each individual MIP can be combined into various panels and can be add to an existing pool. This means that changing the MIP kit content as the regions of interest change over time and to keep up with the ever-increasing numbers of diagnostics requests is possible. Moreover, the performance of poorly performing probes can be improved by individual MIP rebalancing or by redesigning new probes. In our study the performance of MIPs-NGS was improved by adding a 5 fold concentration of each poorly performing probe (n=28 MIPs) to existing probes pool without the need to order new probes. TSCA–

NGS approch is less flexible because changing the kit content or optimizing specific probe(s) performance is dependent on manipulations by the supplying company. However, in this study probes dosage adjustment was not needed.

Applying WGS as a comprehensive clinical testing approach is at this time still too expensive. Sequencing the entire human genome is associated with the generation of a massive amount of data and the need of complex downstream data analyses and variants interpretation. The majority of known disease- causing variants are located in exons; thus WES and targeted gene panels are often used to identify clinically relevant variants in known disease genes.[34] WES -nearly- include all the exonic regions of the human genes, so using WES eliminates the need to select the gene panel content and exonic variants are more comprehensively assessed. However, the amount of data generated by WES is significantly higher than targeted gene panels which increase the computational requirements and data storage costs. In contrast to WES[34], targeted NGS enrichment gene panels are often used for re-sequencing a selective group of phenotype-specific disease genes in common genetic disorders. This results in more manageable and easier data interpretation, usually with higher quality and depth at lower cost.[35]

MIPs-NGS has a higher performance and capture efficiency for *SCN3A, SCN8A-SCN11A*, and *SCN1B-4B* compared to the commonly used Agilent SureSelectXT Human All Exon v5 (Illumina) WES enrichment kit. Similar to our data (Table 5.2 and Table 5.3), several studies demonstrated that MIPs-NGS is an efficient and inexpensive method to detect genetic variations in different diseases.[31,36,37] For example, Zhang *et al.* and Pérez Millán *et al.* presented in their studies the use of MIPs based NGS panels to detect pathogenic mutations in early-onset colorectal cancer patients and in patients with hypopituitarism, respectively.[36,37] Another potential application of MIPs-NGS is to target noncoding and low covered regions in WES.

High throughput NGS platforms such as Nextseq 500/550 and NovaSeq6000 (Illumina, Inc., San Diego, CA, USA) have enabled reductions in sequencing costs (see Table 5.3 for comparison costs of sequencing *SCN3A*, SCN8A-SCN11A, and SCN1B-SCN4B on MiSeq versus NextSeq 500/550 and NovaSeq) and time that have resulted in wide use in diagnostic laboratories. Nevertheless, full capacity high-throughput sequencing runs must be achieved to get the best cost-efficiency (based on maximum number of available sample indexes, 384 samples/run for

MIPs-NGS versus 96 samples/run for TSCA-NGS). MIPs-NGS and TSCA-NGS can be easily customized to individual needs. Multiple different targeted MIPs-NGS can be combined in one Mid-(48-96 samples) or High output run (96-384 samples) without affecting data quality and coverage for each individual sample by which in diagnostics a turn-around time of 2-4 weeks/sample from arrival to reporting can be achieved.

When designing specific targeted enrichment NGS experiments, total number of samples involved, and cost of probes and reagents must be considered. Our data show that the costs of MIPs-NGS are lower than TSCA-NGS. Using MIPs with a gap-fill length of 220-230 nt,[24] and TSCA probes with a region of interest of 425 nt, the cost of MIPs-NGS compared to TSCA-NGS is around 5 times lower per sample (€20.70 for MIPs-NGS versus €113.40 for TSCA-NGS) when 500 samples and more are planned to be tested with MIPs-NGS (Table 5.3).

In conclusion, our results provide a validation and performance assessment of MIPs-NGS and TSCA-NGS as reliable methods for variant detection in a disease-specific subset of genes. Our results suggests that MIPs-NGS is a more flexible and less expensive method for detection of genetic variations and is a reliable screening approach for laboratories involved in diagnostic service for patients with pain-related disorders.

References

1. Gullapalli RR, Desai KV, Santana-Santos L, Kant JA, Becich MJ. Next generation sequencing in clinical medicine: Challenges and lessons for pathology and biomedical informatics. J Pathol Inform. 2012;3:40.
2. Sikkema-Raddatz B, Johansson LF, de Boer EN, Almomani R, Boven LG, van den Berg MP, et al. Targeted Next-Generation Sequencing can Replace Sanger Sequencing in Clinical Diagnostics. Hum Mutat. 2013;34(7):1035-42.
3. Saunders CJ, Miller NA, Soden SE, Dinwiddie DL, Noll A, Alnadi NA, et al. Rapid whole-genome sequencing for genetic disease diagnosis in neonatal intensive care units. Sci Transl Med. 2012; 4(154):154ra135
4. Alazami AM, Patel N, Shamseldin HE, Anazi S, Al-Dosari MS, Alzahrani F, et al. Accelerating novel candidate gene discovery in neurogenetic disorders via whole-exome sequencing of prescreened multiplex consanguineous families. Cell Rep. 2015;10(2):148-61.
5. Roberts JS, Dolinoy DC, Tarini BA. Emerging Issues in Public Health Genomics. Annu Rev Genomics Hum Genet. 2014;15:461-80.
6. Hehir-Kwa JY, Claustres M, Hastings RJ, Van Ravenswaaij-Arts C, Christenhusz G, Genuardi M, et al. Towards a European consensus for reporting incidental findings during clinical NGS testing. Eur J Hum Genet. 2015;23(12):1601-6.
7. Turner EH, Ng SB, Nickerson DA, Shendure J. Methods for Genomic Partitioning. Annu Rev Genomics Hum Genet. 2009;10:263-84.
8. Rizzo JM, Buck MJ. Key principles and clinical applications of "next-generation" DNA sequencing. Cancer Prevention Research. 2012;5(7):887-900.
9. Rehm HL. Disease-targeted sequencing: A cornerstone in the clinic. Nature Reviews Genetics. 2013;14(4):295-300.
10. Wooderchak-Donahue WL, O'Fallon B, Furtado L V., Durtschi JD, Plant P, Ridge PG, et al. A direct comparison of next generation sequencing enrichment methods using an aortopathy gene panel- clinical diagnostics perspective. BMC Med Genomics. 2012;5:50.
11. Lu Y, Zhou X, Jin Z, Cheng J, Shen W, Ji F, et al. Resolving the genetic heterogeneity of prelingual hearing loss within one family: Performance comparison and application of two targeted next generation sequencing approaches. J Hum Genet. 2014;59(11):599-607.
12. Consugar MB, Navarro-Gomez D, Place EM, Bujakowska KM, Sousa ME, Fonseca-Kelly ZD, et al. Panel-based genetic diagnostic testing for inherited eye diseases is highly accurate and reproducible, and more sensitive for variant detection, than exome sequencing. Genet Med. 2015;17(4):253-61.
13. Albert TJ, Molla MN, Muzny DM, Nazareth L, Wheeler D, Song X, et al. Direct selection of human genomic loci by microarray hybridization. Nat Methods. 2007;4(11):903-5.
14. Mertes F, ElSharawy A, Sauer S, van Helvoort JMLM, van der Zaag PJ, Franke A, et al. Targeted enrichment of genomic DNA regions for next-generation sequencing. Brief Funct Genomics. 2011;10(6):374-86.
15. Almomani R, Van Der Heijden J, Ariyurek Y, Lai Y, Bakker E, Van Galen M, et al. Experiences with array-based sequence capture; Toward clinical applications. Eur J Hum Genet. 2011; 19(1):50-5.
16. Porreca GJ, Zhang K, Li JB, Xie B, Austin D, Vassallo SL, et al. Multiplex amplification of large sets of human exons. Nat Methods. 2007;4(11):931-6.
17. Li JB, Gao Y, Aach J, Zhang K, Kryukov G V., Xie B, et al. Multiplex padlock targeted sequencing reveals human hypermutable CpG variations. Genome Res. 2009;19(9):1606-15.
18. Colloca L, Ludman T, Bouhassira D, Baron R, Dickenson AH, Yarnitsky D, et al. Neuropathic pain [Review]. Nat Rev Dis Prim. 2017;3:17002.
19. Waxman SG, Merkies ISJ, Gerrits MM, Dib-Hajj SD, Lauria G, Cox JJ, et al. Sodium channel genes in pain-related disorders: Phenotype-genotype associations and recommendations for clinical use. Lancet Neurol. 2014;13(11):1152-60.

20. Faber CG, Lauria G, Merkies ISJ, Cheng X, Han C, Ahn H-S, et al. Gain-of-function Nav1.8 mutations in painful neuropathy. Proc Natl Acad Sci. 2012;109(47):19444-9.
21. Han C, Yang Y, de Greef BTA, Hoeijmakers JGJ, Gerrits MM, Verhamme C, et al. The Domain II S4-S5 Linker in Nav1.9: A Missense Mutation Enhances Activation, Impairs Fast Inactivation, and Produces Human Painful Neuropathy. NeuroMolecular Med. 2015;17(2):158-69.
22. Devigili G, Eleopra R, Pierro T, Lombardi R, Rinaldo S, Lettieri C, et al. Paroxysmal itch caused by gain-of-function Nav1.7 mutation. Pain. 2014;155(9):1702-7.
23. Eijkenboom I, Sopacua M, Hoeijmakers JGJ, De Greef BTA, Lindsey P, Almomani R, et al. Yield of peripheral sodium channels gene screening in pure small fibre neuropathy. J Neurol Neurosurg Psychiatry. 2019;90(3):342-52.
24. Hiatt JB, Pritchard CC, Salipante SJ, O'Roak BJ, Shendure J. Single molecule molecular inversion probes for targeted, high-accuracy detection of low-frequency variation. Genome Res. 2013; 23(5):843-54.
25. O'Roak BJ, Vives L, Fu W, Egertson JD, Stanaway IB, Phelps IG, et al. Multiplex targeted sequencing identifies recurrently mutated genes in autism spectrum disorders. Science. 2012; 338(6114):1619-22.
26. Alsaloum M, Estacion M, Almomani R, Gerrits M, Bönhof G, Ziegler D, et al. [EXPRESS] A gain-of-function sodium channel β2 subunit mutation in painful diabetic neuropathy. Mol Pain. 2019;15:1744806919849802.
27. Nilsson M, Malmgren H, Samiotaki M, Kwiatkowski M, Chowdhary BP, Landegren U. Padlock probes: Circularizing oligonucleotides for localized DNA detection. Science. 1994; 265(5181):2085-8.
28. Hardenbol P, Banér J, Jain M, Nilsson M, Namsaraev EA, Karlin-Neumann GA, et al. Multiplexed genotyping with sequence-tagged molecular inversion probes. Nat Biotechnol. 2003;21(6):673-8.
29. Zucca S, Villaraggia M, Gagliardi S, Grieco GS, Valente M, Cereda C, et al. Analysis of amplicon-based NGS data from neurological disease gene panels : a new method for allele drop-out management. BMC Bioinformatics. 2016;17(Suppl 12):339.
30. Bodi K, Perera AG, Adams PS, Bintzler D, Dewar K, Grove DS, et al. Comparison of commercially available target enrichment methods for next-generation sequencing. J Biomol Tech. 2013;24(2):73-86.
31. Neveling K, Mensenkamp AR, Derks R, Kwint M, Ouchene H, Steehouwer M, et al. BRCA testing by single-molecule molecular inversion probes. Clin Chem. 2017;63(2):503-12.
32. Wilson KD, Shen P, Fung E, Karakikes I, Zhang A, InanlooRahatloo K, et al. A Rapid, High-Quality, Cost-Effective, Comprehensive and Expandable Targeted Next-Generation Sequencing Assay for Inherited Heart Diseases. Circ Res. 2015;117(7):603-11.
33. Schenk D, Song G, Ke Y, Wang Z. Amplification of overlapping DNA amplicons in a single-tube multiplex PCR for targeted next-generation sequencing of BRCA1 and BRCA2. PLoS One. 2017;12(7):e0181062.
34. Rehm HL, Bale SJ, Bayrak-Toydemir P, Berg JS, Brown KK, Deignan JL, et al. ACMG clinical laboratory standards for next-generation sequencing. Genet Med. 2013;15(9):733-47.
35. Sikkema-Raddatz B, Johansson LF, de Boer EN, Almomani R, Boven LG, van den Berg MP, et al. Targeted Next-Generation Sequencing can Replace Sanger Sequencing in Clinical Diagnostics. Hum Mutat. 2013;34(7):1035-42.
36. Pérez Millán MI, Vishnopolska SA, Daly AZ, Bustamante JP, Seilicovich A, Bergadá I, et al. Next generation sequencing panel based on single molecule molecular inversion probes for detecting genetic variants in children with hypopituitarism. Mol Genet Genomic Med. 2018;6(4):514-25.
37. Zhang J, Wang X, de Voer RM, Hehir-Kwa JY, Kamping EJ, Weren RDA, et al. A molecular inversion probe-based next-generation sequencing panel to detect germline mutations in Chinese early-onset colorectal cancer patients. Oncotarget. 2017;8(15):24533-24547.

CHAPTER 6

YIELD OF PERIPHERAL SODIUM CHANNELS GENE SCREENING IN PURE SMALL FIBER NEUROPATHY

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Abstract

Background

Neuropathic pain is common in peripheral neuropathy. Recent genetic studies have linked pathogenic voltage-gated sodium channel (VGSC) variants to human pain disorders. Our aims are to determine the frequency of *SCN9A*, *SCN10A* and *SCN11A* variants in patients with pure small-fiber neuropathy (SFN), analyze their clinical features, and provide a rationale for genetic screening.

Methods

Between September 2009 and January 2017, 1139 patients diagnosed with pure SFN at our reference center were screened for *SCN9A*, *SCN10A* and *SCN11A* variants. Pathogenicity of variants was classified according to established guidelines of the Association for Clinical Genetic Science and frequencies were determined. SFN patients were grouped according to the VGSC variants detected, and clinical features were compared.

Results

Among 1139 SFN patients, 132 (11.6%) patients harbored 73 different (potentially) pathogenic VGSC variants, of which 50 were novel and 22 were found in ≥1 patient. The frequency of (potentially) pathogenic variants was 5.1% (n=58/1139) for *SCN9A,* 3.7% (n=42/1139) for *SCN10A*, and 2.9% (n=33/1139) for *SCN11A*. Only erythromelalgia-like symptoms and warmth-induced pain were significantly more common in patients harboring VGSC variants.

Conclusion

(Potentially) pathogenic VGSC variants are present in 11.6% of pure SFN patients. Therefore, genetic screening of *SCN9A, SCN10A* and *SCN11A* should be considered in pure SFN patients, independently of clinical features or underlying conditions.

Introduction

According to the International Association for the Study of Pain (IASP), pain is defined as an unpleasant sensory and emotional experience associated with actual or potential tissue damage. As in neuropathic pain the somatosensory nervous system is affected[1], it is not surprising that pain is frequently reported in peripheral neuropathy, especially when the small diameter sensory nerve fibers are involved.[2] Pure small-fiber neuropathy (SFN) is a peripheral neuropathy in which the thinly myelinated Aδ-fibers and unmyelinated C-fibers are selectively affected, leading to sensory symptoms and autonomic dysfunction.[2] In general, neuropathic pain is the main symptom, reflected by allodynia and hyperalgesia, but also thermal sensory loss, pinprick loss, restless legs syndrome, sicca syndrome, accommodation problems, hyperhidrosis or hypohydrosis, micturation disturbances, impotence and/or diminished ejaculation or lubrication, bowel disturbances (constipation, diarrhea, irritability, gastroparesis, cramps), hot flushes, orthostatic dizziness, and cardiac palpitations may be present.[2] Pharmacotherapy for neuropathic pain in SFN is challenging since the efficacy of currently available medications is moderate and side-effects are often dose-limiting.[3,4] A better understanding of genetic causes and pathophysiology of peripheral neuropathy may provide a basis for development of more effective, personalized treatment.

Voltage-gated sodium channels (VGSCs) are integral membrane polypeptides that are mainly present in excitable cells. The large α-subunit is constructed of four homologous domains (DI-DIV) with six transmembrane segments (S1-S6), forming an ion-selective pore. Associated smaller auxiliary β-subunits contribute to targeting and anchoring of the channel at specific sites in the plasma membrane, modulating gating properties of the α-subunit.[5] VGSCs $Na_V1.7$, $Na_V1.8$, and $Na_V1.9$, respectively encoded by *SCN9A, SCN10A* and *SCN11A,* are preferentially expressed in the small diameter dorsal root ganglion neurons (DRGs) and their peripheral axons. They play important roles in generation and conduction of action potentials in the physiological pain pathway.[5,6] In addition, $Na_V1.7$ is present in sympathetic ganglion neurons of the peripheral autonomic nervous system.[5]

Gain-of-function VGSC variants or dysregulated VGSC expression can cause pathological pain states characterized by spontaneous and prolonged pain.[6] A causal link between pathogenic *SCN9A* variants and multiple human pain syndromes has been reported. *SCN9A* variants that increase the excitability of DRG were initially found in inherited erythromelalgia (IEM), an autosomal dominant disorder characterized by episodic painful red discolored extremities

due to warm temperatures and exercise.[7,8] Shortly thereafter, pathogenic *SCN9A* variants were shown to be responsible for the episodic pain and autonomic features in the ocular, mandibular and sacral regions in paroxysmal extreme pain disorder (PEPD).[9,10] Several years later, *SCN9A* gain-of-function variants were demonstrated in 28.6% (n=8/28) of a cohort of patients with skin biopsy-confirmed idiopathic pure SFN.[11] Subsequently, pathogenic *SCN10A* and *SCN11A* variants were found in in SFN patients.[12,13]

A follow-up study of 393 patients diagnosed with SFN, based on typical clinical features (neuropathic pain and autonomic complaints) in combination with an abnormal intraepidermal nerve fiber density (IENFD) in skin biopsy and/or abnormal temperature threshold testing (TTT), showed that 9.1% (n=34/393) harbored an *SCN9A* variant, 4.2% (n=15/359) an *SCN10A* variant, and 3.5% (n=12/345) an *SCN11A* variant.[13,14]

Over the past years our SFN-cohort has expanded to 1502 patients. Aims of the current study were to provide more precise data on *SCN9A, SCN10A* and *SCN11A* variant frequencies and a rationale for the genetic screening of patients with pure SFN and to compare the clinical features of SFN patients with and without VGSC variants.

Methods

Study population and clinical characterization

The retrospective study was conducted at the Departments of Neurology and Clinical Genetics of the Maastricht University Medical Centre+ (Maastricht UMC+), Maastricht, The Netherlands, a tertiary national referral center for patients with clinical symptoms of SFN. Between September 2009 and January 2017, 1502 adult patients (age ≥18 years old) with SFN symptoms were examined in a structured day case setting. The following records were taken:

i. Demographic data; age of onset of complaints; duration of symptoms; altered pain sensation; presence of erythromelalgia symptoms, itch or cramps; influence of temperature, exercise or rest on pain; medical history and family history; neuropathic pain medication used at moment of presentation;
ii. Neurological examination (muscle strength, pinprick sensation, vibration- and position sense, tendon reflexes);
iii. Nerve conduction studies (motor nerves: peroneal and tibial nerves to determine the compound muscle action potential amplitude, distal latency,

and conduction velocities; sensory nerves: ulnar and/or median and sural nerve to determine sensory nerve action potential amplitudes, distal latencies and conduction velocities);

iv. Thermal threshold testing (TTT)[15];
v. Skin biopsy for determination of IENFD[16];
vi. Multiple questionnaires, including the Visual Analogue Scale (VAS) to assess pain intensity[17]; Neuropathic Pain Scale (NPS) to evaluate ten qualities of neuropathic pain[18]; and the SFN Symptom Inventory Questionnaire (SFN-SIQ) which includes 13 SFN specific symptoms.[19]

According to the international criteria, the diagnosis pure SFN was established when typical clinical symptoms were present in combination with a decreased IENFD in skin biopsy and/or abnormal TTT, without signs of large nerve fiber damage on neurological examination and/or NCS.[2,20] Other underlying diseases or use of medication were excluded as possible causes. To search for associated conditions, patients diagnosed with pure SFN underwent extensive blood analyses as described previously.[21]

Variant screening

Genomic DNA was extracted from whole blood using NucleoSpin®8 Blood Isolation kit (Macherey-Nagel, Düren, Germany) according to manufacturer's instructions. Coding exons and exon-flanking intronic regions of *SCN9A*, *SCN10A* and *SCN11A* were amplified by PCR and sequenced by Sanger sequencing. Sequences were compared with reference sequence GRCh37. Variants detected were annotated according to guidelines of the Human Genome Variation Society (http://www.hgvs.org/mutnomen/). Variants which were located in functional domain of the protein and/or at a highly conserved amino acid in mammalian paralogues/human VGSC orthologues were classified and reported according the Practice Guidelines of the Association for Clinical Genetic Science (ACGS) and recommendations of Waxman et al.[22,23] Co-segregation of (potentially) pathogenic variants with the disease was tested, if possible, in cases with a positive family history.

Statistical analysis

The primary analysis was the comparison of clinical variables between pure SFN patients with and without VGSC variant. For categorical variables, the chi-square test was used or the Fisher exact test when necessary. For continuous variables, the independent student's t-test was chosen. Equal variances between two groups were tested with the Levene's test. For these analyses, a significance level of 0.05 was used.

Post-hoc analyses were performed to investigate whether differences between patients with specific VGSC variants and without variants were present. In total six post-hoc analyses per variable were executed. The analyses were performed in the same way as the primary analyses, however, the significance level was adjusted for multiple testing with the Bonferroni correction (0.05/6). Therefore, post-hoc analyses were compared with a significance level of 0.0083. Missing values where not imputed or estimated.

Results

Patient characteristics

The diagnosis of pure SFN was established in 1139 of 1502 patients (75.8%) referred to our center (Figure 6.1). More females (59.2%) were present than males. The mean age at presentation in our referral center was 52.1 years (SD 13.2 years), with an age of onset of symptoms at 46 years (SD 14.6 years). In 25.9% of patients the diagnosis of pure SFN was based on the clinical picture in combination with both an abnormal IENFD and TTT, in 6.9% of the patients only the IENFD was abnormal, while in 67.2% solely an abnormal TTT was found. In 61.1% (n=696) of pure SFN patients, additional workup revealed no associated conditions. Autoimmune diseases were found in 21.7% (n=247), glucose intolerance in 10.6% (n=121), vitamin B12 deficiency in 6.6% (n=75), diabetes mellitus in 5.2% (n=59), alcohol abuse in 2.7% (n=31), chemotherapy in 2.2% (n=25), monoclonal gammopathy of undetermined significance in 1.1% (n=13), and haemochromatosis in 0.6% (n=7) of patients, which is in line with our previous reports.[21] Multiple associated conditions can be found within one patient.

Genetic screening of *SCN9A*

Among 1139 pure SFN patients, 28 different (potentially) pathogenic heterozygous *SCN9A* variants were detected in 58 patients (5.1%, Figure 6.1 and Table 6.1). Six variants have already been published as pathogenic,[11,24-27] one variant as probably pathogenic[11], and two variants as risk factor.[23] Eighteen variants were novel and classified as possibly pathogenic (n=3) or of uncertain clinical significance (VUS, n=15; Table 6.1). Eleven *SCN9A* variants were found in >1 patient. Nine patients harbored more than one variant in *SCN9A* (Table 6.1). Finally, one patient was heterozygous for two *SCN9A* VUSs, c.1555G>A and c.2271G>A, and the pathogenic *SCN11A* c.3473T>C variant.

Table 6.1 Potentially pathogenic *SCN9A* variants identified in patients with pure small-fiber neuropathy at Maastricht UMC+ (n=1139 patients).

SCN9A variants, Chr 2, GRCh37, NM_002977.3		Number of patients	Class[#]	Location in *SCN9A*	MAF ExAC (%)	Cell electrophysiology	Co-segregation[§]	Classification (23)[€]	Ref.
c. position	p. position								
c.118A>G	p.(Lys40Glu)	1	3	N-terminus	0.003 (4/120754)	N/A	N/A	VUS	-
c.554G>A	p.(Arg185His)	3	3	Loop DI/S2-DI/S3	0.3 (398/120674)	Gain-of-function	Inconclusive (1 family)[§]	VUS (Risk factor)	[11,24]
c.684C>G	p.(Ile228Met)	7	4	DI/S4	0.1 (117/119144)	Gain-of-function	Segregation with disease (3 families)	Pathogenic variant	[11,25]
c.1007A>C	p.(Asn336Thr)	2	3	Loop DI/S5-DI/S6	-	N/A	N/A	VUS	-
c.1552G>T	p.(Val518Phe)	1	3	Linker DI/S6-DII/S1	-	N/A	N/A	VUS	-
c.1555G>A [$]	p.(Glu519Lys)	2	3	Linker DI/S6-DII/S1	0.04 (52/120678)	N/A	No segregation with disease (1 family)	VUS	-
c.1592A>C	p.(Thr531Asn)	1	3	Linker DI/S6-DII/S1	-	N/A	N/A	VUS	-
c.1867G>A	p.(Asp623Asn)	1	3	Linker DI/S6-DII/S1	-	Gain-of-function	Inconclusive (1 family)[§]	Pathogenic variant	[11,26]
c.2033A>C	p.(Ala678Glu)	1	3	Linker DI/S6-DII/S1	-	N/A	N/A	VUS	-
c.2128T>G	p.(Phe710Val)	1	3	Linker DI/S6-DII/S1	0.02 (10/45614)	N/A	N/A	VUS	-
c.2157G>C	p.(Trp719Cys)	1	3	Linker DI/S6-DII/S1	0.2 (79/43776)	N/A	N/A	VUS	-
c.2159T>A	p.(Ile720Lys)	2	3	Linker DI/S6-DII/S1	0.02 (8/43708)	Gain-of-function	Segregation with disease (1 family)	Pathogenic variant	[11]
c.2215A>G *	p.(Ile739Val)	14	4	DII/S1	0.3 (168/43128)	Gain-of-function	Segregation with disease (1 family), segregation inconclusive (2 families)[§]	VUS (Risk factor)	[11,24,27]
c.2266C>A	p.(Pro756Thr)	1	3	Loop DII/S1-DII/S2	0.005 (2/38560)	N/A	N/A	VUS	-
c.2271G>A [$]	p.(Met757Ile)	2	3	Loop DII/S1-DII/S2	0.008 (3/37456)	N/A	N/A	VUS	-
c.2567G>A	p.(Gly856Asp)	1	3	Loop DII/S4-DII/S5	-	Gain-of-function	Segregation with disease (1 family)	Pathogenic variant	[28]
c.2794A>C/ c.2971G>T *	p.(Met932Leu)/ p.(Val991Leu)	9	3 2	DII/S6 Linker DII/S6-DIII/S1	2.9 (3580/121398) 3.1 (3393/109198)	Gain-of-function	Segregation with disease (2 families)	Pathogenic variant	[11]
c.2969A>G	p.(Tyr990Cys)	1	3	Linker DII/S6-DIII/S1	0.06 (64/108972)	N/A	N/A	VUS	-
c.3689T>C	p.(Met1230Thr)	1	3	DIII/S2	-	N/A	No segregation with disease (1 family)	VUS	-

Table 6.1 (continued)

SCN9A variants, Chr 2, GRCh37, NM_002977.3		Number of patients	Class[#]	Location in *SCN9A*	MAF ExAC (%)	Cell electrophysiology	Co-segregation[§]	Classification (23)[€]	Ref.
c. position	c. position								
c.3836G>A	p.(Arg1279Gln)	1	3	DIII/S4	0.003 (3/103348)	N/A	N/A	VUS	-
c.4585_4590delAGTCAA	p.(Ser1529_Gln1530del)	1	3	Loop DIV/ S1-DIV/S2	-	N/A	N/A	VUS	-
c.4596G>A	p.(Met1532Ile)	1	3	Loop DIV/ S1-DIV/S2	0.0008 (1/119080)	Gain-of-function	N/A	Probably pathogenic variant	[11]
c.4612T>C *	p.(Trp1538Arg)	4	3	DIV/S2	0.2 (249/118528)	Gain-of-function	Segregation with disease (1 family)	Pathogenic variant	[29]
c.4859G>T	p.(Arg1620Leu)	1	4	DIV/S4	0.003 (4/121412)	N/A	N/A	Possibly pathogenic variant	-
c.4970A>C	p.(Tyr1657Ser)	1	4	DIV/S5	-	N/A	N/A	Possibly pathogenic variant	-
c.5260G>T	p.(Val1754Phe)	2	4	C-terminus	-	N/A	N/A	Possibly pathogenic variant	-
c.5912A>T	p.(Asp1971Val)	1	3	C-terminus	0.02 (16/83770)	N/A	N/A	VUS	-

c. position, location cDNA; p. position, location in protein; MAF ExAC, Minor Allele Frequency Exome Aggregation Consortium; N/A, not available; VUS, variants with uncertain clinical significance. [5]One patient was heterozygous for c.1555G>A, c.2271G>A and *SCN11A* c.3473T>C variant. *Three patients were heterozygous for c.2974A>C/c.2971G>T and c.4612T>C variant, and one patient was heterozygous for c.2215A>G, c.2974A>C/c.2971G>T and c.4612T>C variant. [#]Classification based on predictive algorithms[22]: 2, unlikely to be pathogenic; 3, uncertain clinical significance; 4, likely to be pathogenic. [§]Indicates results for families that were tested, Inconclusive, affected and unaffected family members within a family were heterozygous for the variant (c.2215A>G) or affected family members with minor complaints were negative for the variant (c.554G>A, c.1867G>A and c.2215A>G). [€]Classification according to Waxman recommendations.[23]

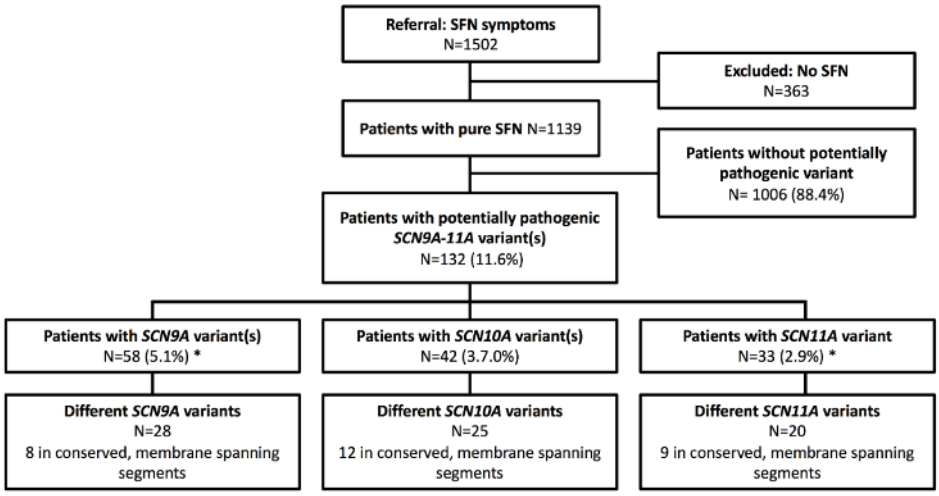

Figure 6.1 **Patients with SFN analyzed for potentially pathogenic *SCN9A*, *SCN10A* and *SCN11A* variants in the Maastricht University Medical Center+.** Diagnosis of pure SFN was made according to the international criteria by the clinical symptoms in combination with an abnormal intraepidermal nerve fiber density in skin biopsy and/or abnormal temperature threshold testing.[2,18] Genetic screening of *SCN9A*, *SCN10A* and *SCN11A* was performed with Sanger sequencing. Variants which were located in the functional domain of the protein and/or at a highly conserved amino acid in mammalian paralogues/human VGSC orthologues were classified according to the practice guidelines of the Association for Clinical Genetic Science and recommendations of Waxman.[22,23] Patients in this study were included between September 2009 and January 2017. *One patient was heterozygous for two *SCN9A* variants and one *SCN11A* variant. SFN, small-fiber neuropathy.

For 9 variants cell electrophysiology showed a gain-of-function of the $Na_V1.7$ channel[11,24-29], Table 6.1. Co-segregation with pain in the family was demonstrated for 6 variants, was inconclusive for 3 variants, and not supportive for 2 variants (Table 6.1).

Nine (potentially) pathogenic *SCN9A* variants detected in our cohort of pure SFN patients have been reported in patients with IEM, PEPD, paroxysmal itch, painful diabetic neuropathy and Dravet syndrome.[30-34] Two SFN patients, positive for one of these *SCN9A* variants, had a history consistent with erythromelalgia (variant c.554G>A), two SFN patients suffered from diabetes mellitus (one patient with variant c.1552G>T and one patient with variant c.2215A>G), and three SFN patients complained of itch (variant c.2215A>G). None of the other patients carrying ≥1 of these *SCN9A* variants were positive for erythromelalgia, PEPD, paroxysmal itch, PDN or Dravet syndrome.

The clinical features of each individual patient harboring (potentially) pathogenic *SCN9A* variants is shown in the Supplementary Table S6.1.

Genetic screening of *SCN10A*

In *SCN10A*, 25 different (potentially) pathogenic heterozygous variants were detected in 42 pure SFN patients (3.7%, n=45/1139, Figure 6.1 and Table 6.2). One variant was already published as pathogenic[35] and three variants as probably pathogenic.[12,36] Twenty-one variants were novel for SFN and classified as probably pathogenic (n=2), possibly pathogenic (n=2) or VUS (n=17; Table 6.2). Five *SCN10A* variants were present in >1 patient. Two patients harbored two *SCN10A* variants. For 3 variants cell electrophysiology showed a gain-of-function of the $Na_V1.8$ channel, and for one variant DRG neuron hyperexcitability was seen[12,35,36], Table 6.2. Co-segregation tested for 3 patients was only positive for one variant (Table 6.2).

Eight (potentially) pathogenic *SCN10A* variants detected in our cohort of pure SFN patients have been reported in patients with Brugada syndrome (BrS), atrial fibrillation (AF), sudden infant death syndrome (SIDS), Lennox-Gastaut syndrome (LGS), febrile infection-related epilepsy syndrome (FIRES) and autism.[37-40] Only the SFN patient with variant c.41G>T had arrhythmia. The other previously reported conditions were not seen in our cohort of patients with ≥1 *SCN10A* variants.

The individually clinical data of the 42 pure SFN patients with (potentially) pathogenic *SCN10A* variants is shown in the Supplementary Table S6.2.

Genetic screening of *SCN11A*

We found 20 different (potentially) pathogenic heterozygous variants in 33 pure SFN patients (2.9%, n=33/1139, Figure 6.1 and Table 6.3). Three variants were already published as probably pathogenic[13,41], and one variant as possibly pathogenic and 5 variants as VUS.[13] Eleven variants were novel and classified as possibly pathogenic (n=5) or VUS (n=6; Table 6.3). Six *SCN11A* variants were detected in >1 patient. Only one patient was heterozygous for two *SCN9A* VUSs (c.1555G>A and c.2271G>A) and the pathogenic *SCN11A* c.3473T>C variant. Cell electrophysiology showed a gain-of-function of 3 *SCN11A* variants[13,41], while a loss-of-function of the $Na_V1.9$ channel was seen for one variant.[41] Co-segregation was tested for 2 variants, and only supportive for one (Table 6.3). All 25 (potentially) pathogenic *SCN11A* variants detected in our cohort were specific for SFN.

Table 6.2 Potentially pathogenic *SCN10A* variants identified in patients with pure small-fiber neuropathy at Maastricht UMC+ (n=1139 patients).

SCN10A variants, Chr3, GRCh37,NM_006514.2		Number of patients	Class[#]	Location in *SCN10A*	MAF ExAC (%)	Cell electrophysiology	Co-segregation[§]	Classification (23)[€]	Ref.
c.position	p.position								
c.41G>T	p.(Arg14Leu)	4	3	N-Terminus	0.2 (234/121350)	N/A	Segregation inconclusive (1 family)[§]	VUS	-
c.626G>A	p.(Arg209His)	1	3	Loop DI/S3-DI/S4	0.003 (4/121360)	N/A	N/A	VUS	-
c.1141A>G	p.(Ile381Val)	1	4	DI/S6	0.05 (63/121384)	N/A	N/A	Possibly pathogenic variant	-
c.1667A>T	p.(Gln556Leu)	1	3	Linker DI/S6-DII/S1	-	N/A	N/A	VUS	-
c.1879T>C	p.(Ser627Pro)	1	3	Linker DI/S6-DII/S1	0.003 (3/120550)	N/A	N/A	VUS	-
c.2221C>G	p.(Leu741Val)	1	3	DII/S3	0.01 (18/120924)	N/A	N/A	VUS	-
c.2972C>T	p.(Pro991Leu)	4	3	Linker DII/S6-DIII/S1	0.09 (108/121082)	N/A	Segregation with disease (1 family), segregation inconclusive (1 family)[§]	VUS	-
c.3482T>C	p.(Met1161Thr)	1	4	DIII/S1	0.02 (27/121036)	N/A	N/A	Possibly pathogenic variant	-
c.3607C>T [$]	p.(Leu1203Phe)	1	3	DIII/S2	-	N/A	N/A	VUS	-
c.3674T>C	p.(Ile1225Thr)	1	3	DIII/S3	0.06 (70/121240)	N/A	Segregation with disease (1 family)	Probably pathogenic variant	-
c.3766C>T	p.(Arg1256Trp)	1	4	DIII/S4	0.003 (3/121100)	N/A	N/A	Probably pathogenic variant	-
c.3803G>A	p.(Arg1268Gln)	4	3	Loop DIII/S4-DIII/S5	0.2 (213/120708)	N/A	N/A	VUS	-
c.3910G>A	p.(Ala1304Thr)	1	4	DIII/S5	0.004 (5/121410)	Gain-of-function	N/A	Probably pathogenic variant	12
c.4139G>A	p.(Arg1380Gln)	1	3	Loop DIII/S5-DIII/S6	0.009 (10/110106)	N/A	N/A	VUS	-
c.4378C>T	p.(Arg1460Trp)	1	3	Linker DIII/S6-DIV/S1	0.05 (58/121296)	N/A	N/A	VUS	-
c.4562G>A	p.(Gly1521Asp)	1	3	DIV/S2	-	N/A	N/A	VUS	-
c.4568G>A [$*]	p.(Cys1523Tyr)	9	4	DIV/S2	0.1 (135/121358)	DRG neuron hyperexcitability	N/A	Probably pathogenic variant	12
c.4724T>C	p.(Ile1575Thr)	1	4	DIV/S4	-	N/A	N/A	VUS	-
c.4878G>A	p.(Met1626Ser)	1	3	DIV/S5	0.00008 (1/121408)	N/A	N/A	VUS	-
c.4984G>A	p.(Gly1662Ser)	3	4	Loop DIV/S5-DIV/S6	0.2 (190/121360)	Gain-of-function	N/A	Pathogenic variant	[12,35]
c.5116A>G	P.(Ile1706Val)	1	4	DIV/S6	-	Gain-of-function	N/A	Probably pathogenic variant	[36]
c.5200G>A*	p.(Glu1734Lys)	1	3	C-terminus	0.02 (20/121400)	N/A	N/A	VUS	-

Table 6.2 (continued)

SCN10A variants, Chr3, GRCh37,NM_006514.2 c.position	p.position	Number of patients	Class[#]	Location in *SCN10A*	MAF ExAC (%)	Cell electrophysiology	Co-segregation[§]	Classification (23)[€]	Ref.
c.5474T>C	p.(Met1825Thr)	1	3	C-terminus	-	N/A	N/A	VUS	-
c.5539C>T	p.(Arg1847Ter)	1	3	C-terminus	0.003 (3/121404)	N/A	N/A	VUS	-
c.5606G>A	p.(Arg1869His)	1	3	C-terminus	0.004 (5/121402)	N/A	N/A	VUS	-

c. position, location cDNA; p. position, location in protein; MAF ExAC, Minor Allele Frequency Exome Aggregation Consortium; N/A, not available; VUS, variants with uncertain clinical significance. [$]One patient was heterozygous for c.3607C>T and c.4568G>A variant. *One patient was heterozygous for c.4568G>A and c.5200G>A variant. [#]Classification based on predictive algorithms[22]: 2, unlikely to be pathogenic; 3, uncertain clinical significance; 4, likely to be pathogenic. [§]Indicates results for families that were tested, Inconclusive, affected family members with minor complaints were negative for the variant (c.41G>T and c.2972C>T). [€]Classification according to Waxman recommendations.[23]

Table 6.3 Potentially pathogenic *SCN11A* variants identified in patients with pure small-fiber neuropathy at Maastricht UMC+ (n=1139 patients).

SCN11A variants, Chr 3, GRCh37, NM_014139.2		Number of patients	Class[#]	Location in *SCN11A*	MAF ExAC (%)	Cell electrophysiology	Co-segregation[§]	Classification (23)[€]	Ref.
c.position	p.position								
c.95C>T	p.(Ala32Val)	2	3	N-terminus	-	N/A	N/A	VUS	-
c.151C>T	p.(Arg51Trp)	1	3	N-terminus	0.003 (3/121122)	N/A	N/A	VUS	-
c.1060C>T	p.(Arg354Trp)	1	4	Loop DI/S5-DI/S6	-	N/A	N/A	Possibly pathogenic	-
c.1142T>C	p.(Ile381Thr)	1	4	DI/S6	0.02 (20/120664)	Gain-of-function	N/A	Probably pathogenic	[13]
c.1257G>T	p.(Lys419Asn)	5	3	Linker DI/S6-DII/S1	0.01 (18/121332)	N/A	N/A	VUS	[13]
c.1744G>A	p.(Ala582Thr)	5	3	DII/S1	0.004 (5/121046)	N/A	N/A	Possibly pathogenic	[13]
c.1927A>G	p.(Ile643Val)	1	3	DII/S3	-	N/A	N/A	VUS	-
c.2042C>A	p.(Ala681Asp)	1	4	DII/S4	0.007 (8/121294)	Loss-of-function	N/A	VUS	[13]
c.2095G>A	p.(Gly699Arg)	1	4	Loop DII/S4-DII/S5	0.05 (64/121284)	Gain-of-function	N/A	Probably pathogenic	[41]
c.2419A>G	p.(Ile807Val)	2	3	DII/S6	-	N/A	Segregation with disease (1 family)	Possibly pathogenic	-
c.2513G>A	p.(Arg838Gln)	1	4	Linker DII/S6-DIII/S1	-	N/A	N/A	Possibly pathogenic	-
c.2524G>C	p.(Ala842Pro)	1	3	Linker DII/S6-DIII/S1	-	N/A	N/A	VUS	[13]
c.3222A>G	p.(Ile1074Met)	1	3	DIII/S1	-	N/A	N/A	VUS	-
c.3473T>C [$]	p.(Leu1158Pro)	3	4	DIII/S4	0.02 (21/121350)	Gain-of-function	N/A	Probably pathogenic	[13]
c.3745G>A	p.(Ala1249Thr)	1	3	Loop DIII/S5-DIII/S6	0.02 (22/121324)	N/A	N/A	VUS	-
c.4049G>A	p.(Arg1350Gln)	2	3	Linker DIII/S6-DIV/S1	-	N/A	N/A	VUS	-
c.4057-1G>A	p.?	1	3	Linker DIII/S6-DIV/S1	0.002 (2/121276)	N/A	Segregation inconclusive (1 family)[§]	VUS	[13]
c.4282G>A	p.(Gly1428Ser)	1	4	DIV/S3	0.006 (7/120924)	N/A	N/A	Possibly pathogenic	-
c.4309G>A	p.(Val1437Met)	1	4	DIV/S3	0.06 (77/121390)	N/A	N/A	Possibly pathogenic	-
c.5067C>G	p.(Phe1689Leu)	1	3	C-terminus	0.003 (3/121030)	N/A	N/A	VUS	[13]

c. position, location cDNA; p. position, location in protein; MAF ExAC, Minor Allele Frequency Exome Aggregation Consortium; N/A, not available; VUS, variants with uncertain clinical significance. [$]One patient was heterozygous for c.3473T>C, *SCN9A* c.1555G>A and *SCN9A* c.2271G>A. [#]Classification based on predictive algorithms[22]: 2, unlikely to be pathogenic; 3, uncertain clinical significance; 4, likely to be pathogenic. [§]Indicates results for families that were tested, Inconclusive, affected family members with minor complaints were negative for the variant (c.4057-1G>A). [€]Classification according to Waxman recommendations.[23]

The clinical features per patient harboring (potentially) pathogenic *SCN11A* variant is shown in the Supplementary Table S6.3.

Pure SFN patients with or without VGSC variant

The number of SFN patients with a VGSC variant and a decreased IENFD was not significantly higher than that of patients without a VGSC variant (37.9% vs. 32.6%; p=0.328). Furthermore, in both groups TTT was almost equally abnormal (92.4% with VGSC variant vs. 93.2% without VGSC variant; p=0.741). Patients that harbor an *SCN9A* variant reported significantly more often erythromelalgia-like symptoms compared to patients with an *SCN10A* variant or without VGSC variant (43.9% vs. 16.7%; p=0.004 and 43.9% vs. 26.4%; p=0.004). The proportion of SFN patients that experienced an aggravation of the pain by warm temperature was significantly higher in those with a VGSC variant compared to those without VGSC variant (45.2% vs. 30.9%; p=0.014). No differences were seen in other symptoms, obtained by history taking and various questionnaires (Figure 6.2-6.3 and Supplementary Table S6.4). In case patients were harboring multiple VGSC variants, in the same channel or in different channels, the type of complaints and severity was similar. In patients with a VGSC variant family history for SFN-related symptoms was more frequently positive than in the other pure SFN patients (33.9% vs. 24.6%; p=0.027; Supplementary Table S6.4). In 15.1% (n=66/436) of pure SFN patients with an underlying condition a (potentially) pathogenic VGSC variant was found. In the 132 SFN patients with a VGSC variant the following underlying conditions were demonstrated: in 22.0% (n=29) an immunological disease, in 6.8% (n=9) glucose intolerance, in 5.3% (n=7) vitamin B12 deficiency, in 3.0% (n=4) diabetes mellitus, in 2.3% (n=3) alcohol abuse, in 0.8% (n=1) a history of chemotherapy, and in 0.8% (n=1) a monoclonal gammopathy of undetermined significance. The number of patients per specific underlying condition was too small to study potential relationships between these conditions and particular VGSC variants.

As all of the patients in our cohort suffered from painful SFN and no patients with painless SFN were included, it was not possible to investigate if the presence of VGSC variants in patients with an associated condition is related to the development of pain. Since this study had a retrospective design, it was not possible to collect data about the use of pain medication in a standardized way to provide reliable information on the response to treatment. However, the data on pain features and intensity, indirectly suggest the poor efficacy of the drugs (Supplementary Tables S6.1-S6.3).

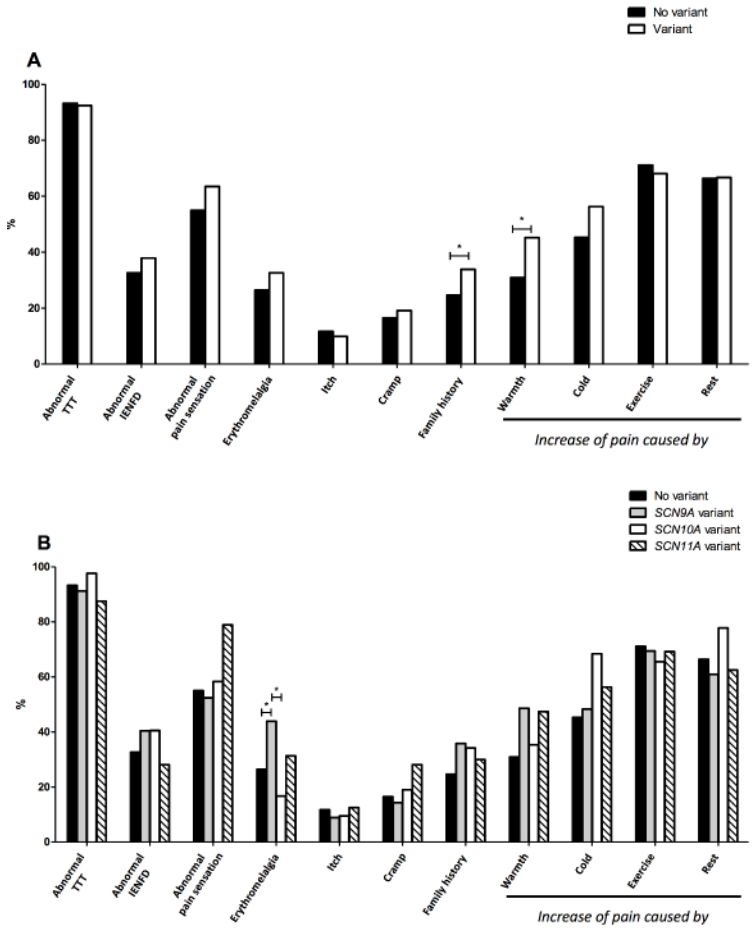

Figure 6.2 Clinical features of pure small-fiber neuropathy patients with or without (potentially) pathogenic voltage-gated sodium channel variant. (A) Patients with pure SFN without VGSC variant versus all patients with pure SFN with VGSC variant. (B) Patients with pure SFN without VGSC variant versus patients with pure SFN with SCN9A variant or SCN10A variant or SCN11A variant. *<0.0083. Aggravation of the pain by warm temperature was significantly higher in those with a VGSC variant compared to patients without variant (A). Erythromelalgia symptoms are significantly more frequently reported in pure SFN patients with a (potentially) pathogenic *SCN9A* variant compared to patients without VGSC variant or with an *SCN10A* variant (B). For exact data see Supplemental file. TTT = temperature threshold testing; IENFD = intraepidermal nerve fiber density.

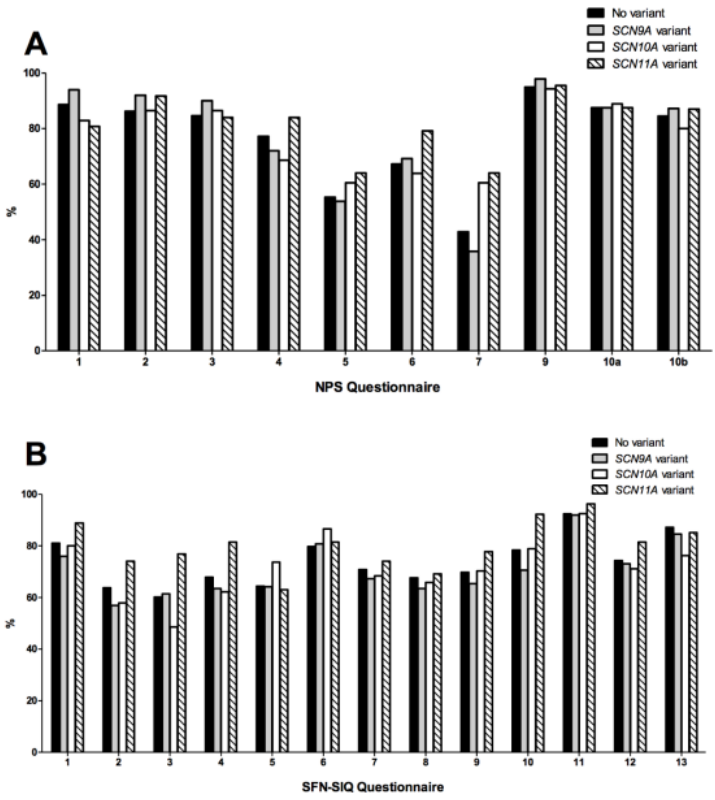

Figure 6.3 Questionnaires pure small-fiber neuropathy patients with or without (potentially) pathogenic voltage-gated sodium channel variant. A: NPS: neuropathic pain scale. NPS pain qualities: 1=severity; 2=sharpness; 3=hotness; 4=dullness; 5=coldness; 6=sensitivity; 7=itchiness; 9=unpleasantness; 10a=intensity deep pain; 10b=intensity surface pain. Each item is scored on an 11-point scale (0=not applicable to the experienced pain and 10=in the most severe form applicable to the experienced pain). An NPS score >3 is considered as a relevant pain quality. B: SFN-SIQ: small-fiber neuropathy inventory questionnaire. SFN-SIQ symptoms: 1=altered sweating pattern; 2=diarrhea; 3=constipation; 4=micturation problems; 5=dry eyes; 6=dry mouth; 7=orthostatic dizziness; 8=palpitations; 9=hot flashes; 10=sensitive skin legs; 11=burning feet; 12=intolerance for sheets; 13=restless legs. The answer options of the SFN-SIQ include 'never=1', 'sometimes=2', 'often=3' and 'always=4'. A symptom is considered to be present when the score is >1. No statistically significant differences in experienced pain qualities and small-fiber neuropathy symptoms were found between pure small fiber neuropathy patients with or without (potentially) pathogenic voltage-gated sodium channel variant. For exact data see Supplemental file.

Discussion

In our retrospective cohort, 132 of 1139 (11.6%) patients with pure SFN harbor potentially pathogenic heterozygous variants in *SCN9A*, *SCN10A* and/or *SCN11A*. *SCN9A* variants were found more frequently (5.1%, n=58/1139 patients) than *SCN10A* (3.7%, n=41/1139 patients) and *SCN11A* (2.9%, n=38/1139 patients) variants. Fifty variants were novel for SFN and classified as probably pathogenic (n=2), possibly pathogenic (n=10) or VUS (n=38). In this cohort, erythromelalgia showed a significant relationship with the presence of *SCN9A* variants. Furthermore, warmth-induced pain was significantly increased with the presence of potentially pathogenic VGSC variants. Other clinical features of pure SFN, such as abnormal TTT, abnormal IENFD, abnormal pain sensation, itch, cramp and cold-, exercise- and rest-induced pain, were not significant different for patients with and without VGSC variants (Figure 6.2). Also, the NPS and SFN-SIQ, revealed comparable results for patients with and without VGSC variants (Figure 6.3).

The frequencies of variants in the *SCN10A* and *SCN11A* genes in our pure SFN patients are slightly lower than those reported before; respectively 4.2-4.8% and 3.5-3.8%.[13,14,21] For *SCN9A*, however, the frequency has decreased over the years. At first, eight gain-of-function variants were identified in 28 subjects (28.6%) with biopsy-confirmed idiopathic pure SFN.[11] Subsequently, in the cohort of 393 consecutive patients diagnosed with SFN, 17 potentially pathogenic variants were found in 34 patients (9.1%).[13,14] Then, we reported in an extended cohort of 921 patients diagnosed with idiopathic pure SFN, 78 patients have ≥1 potentially pathogenic *SCN9A* variants (8.5%)[21], and here we report 58 subjects with ≥1 potentially pathogenic *SCN9A* variants in 1139 pure SFN patients (5.1%). The decline in frequency for *SCN9*A in the expanded cohort can be explained by less stringent inclusion criteria for the current cohort, more stringent variant classification criteria, extension of functional data obtained by cell electrophysiology and data of co-segregation analysis. For instance, variants with no change in channel function and/or co-segregation with the disease in affected family members, like c.3734A>G (p.Asn1245Ser) and c.3799C>G (p.Leu1267Val), were classified as unlikely to be pathogenic in the current study, while they were classified as VUS with frequencies respectively of 1.5% and 1.3% in the cohort of 393 SFN patients[13,14], and 1.1% and 0.9% in the cohort of 921 idiopathic pure SFN patients.

To date, only one other cohort of painful neuropathy patients (n=217) has been tested for *SCN9A*, *SCN10A* and *SCN11A* gene variations.[42] In this cohort, the number of patients with ≥1 low-frequency (minor allele frequency [MAF] <5% in

the NHLBI Exome Sequencing Project Exome Variant Server, European American [EVS-EA] population) missense variant in *SCN9A*, *SCN10A* and *SCN11A* was respectively 25%, 21% and 13%. From these low-frequency missense variants, 8.7% *SCN9A* (n=19/217), 0.9% *SCN10A* (n=2/217) and 0.9% *SCN11A* (n=2/17) variants have been previously reported in patients with IEM, SFN or PDN, including *SCN9A* variant c.3734A>G (n=7/217, 3.2%) and c.3799C>G (n=1/217, 0.5%).[42] Compared to our cohort, the incidence of potentially pathogenic variants in the 217 painful neuropathy patients was ~4-5-fold higher. The discrepancies in frequencies of reported variants between both cohorts are mainly caused by different variant filtering strategies (i.e. MAF <5% vs. <1%) and variant classification approaches (i.e. all missense mutations vs. highly conserved missense mutation). Different patient inclusion criteria and used sequencing platforms, may also have an effect the frequencies of variants detected.

Nine (potentially) pathogenic *SCN9A* variants detected in our cohort of pure SFN patients have been reported as disease-causing variants in other pain phenotypes and Dravet syndrome.[30-34] Multigenerational segregation with the disease in SFN families and/or functional testing in DRG neurons by cell electrophysiology support that these variants are causative for SFN (Table 6.1). As patients with SFN share clinical features with other pain-phenotypes, it is not surprising that these variants are reported for SFN, and IEM, paroxysmal itch and painful diabetic neuropathy. Features of Dravet syndrome have not been seen in our cohort, and no abnormal pain sensation have been reported for patients with Dravet syndrome and a (potentially) pathogenic *SCN9A* variant.[43] For *SCN10A* variants, eight (potentially) pathogenic variants have been report as disease-causing in BrS, AF, SIDS, LGS, FIRES and autism.[37-40] Multigenerational segregation with the disease in SFN families and/or functional testing in DRG neurons by cell electrophysiology indicate that the majority of the variants are causative for SFN (Table 6.2). Variant c.41G>T, p.(Arg14Leu), which has been described in patients with BrS and AF[37,38], was identified four times in our cohort of SFN patients. Only one patient had SFN and arrhythmia. SIDS, LGS, FIRES and autism have not been seen in our pure SFN patients. For *SCN11A*, none of potentially pathogenic variants identified in the current study have been associated with other inherited disorders. Taken together, these findings suggest that one variant can produce multiple different disease outcomes, depending on cell-type-specific expression[11,24,25] presence of additional disease-causing variants or modifiers[23], and/or additional underlying conditions (e.g. diabetes mellitus or vitamin B12 deficiency).[21]

For variants where segregation with disease in multi-generations was confirmed, this should be considered supporting, though not definite evidence for

pathogenicity. A definite conclusion would require at least 10 meiosis, which is generally not the case. In this study, co-segregation of the variant with disease was only tested for 15 potentially pathogenic variants in 20 families, because most families were too small to test segregation with disease properly or the proband was not in contact with their relatives.

About two-third of the potentially pathogenic *SCN9A* variants identified in our cohort of pure SFN patients have been localized to domain I (DI) and II (DII), and the intracellular linker between DI-DII and DII-domain III (DIII). This is in contrast with our findings for *SCN10A,* where approximately 70% of the *SCN10A* variants were localized to DIII and domain IV (DIV), the intracellular linker between DIII-DIV and C-terminus of the protein. For *SCN11A*, the distribution of potentially pathogenic variants was all across the gene. No possible hotspots were identified in this gene.

Patch clamp studies have shown that *SCN9A* variants associated with SFN produce gain-of-function channel changes, ranging from impaired slow-inactivation to depolarized slow- and fast-inactivation, and induce DRG neurons hyperexcitability.[11,24-29] Compared to PEPD, where most *SCN9A* variants were located in the intracellular linker between DIII-DIV and intracellular loop linking segments S4-S5 of DIII and DIV, effects on fast-inactivation of SFN *SCN9A* variants were relatively mild. IEM *SCN9A* variants exhibit hyperpolarized activation or enhanced ramp currents, and were mainly localized to transmembrane segment S4, S5 and S6, and the intracellular loop linking segments S4-S5.[44] Functional IEM characteristics were not demonstrated for SFN *SCN9A* variants. Although most potentially pathogenic *SCN9A* variants in this cohort were localized to DI and DII, and the intracellular linkers DI-DII and DII-III, it is still unclear how this type of $Na_V1.7$ channel dysfunction causes SFN. However, certain SFN *SCN9A* variants have been shown to impair regeneration and/or degeneration of sensory axons, suggesting that enhanced sodium channel activity and reverse Na-Ca exchange may contribute to a decrease in length of peripheral sensory axons.[45] Besides *SCN9A*, variants in *SCN10A* and *SCN11A* have been shown to participate in the pathophysiology of SFN. DRG neurons expressing *SCN10A* or *SCN11A* mutant channels exhibit increased excitability and abnormal spontaneous firing activity.[12,13,35,36,41] One *SCN10A* variant and nine *SCN11A* variants have been associated with other inherited pain disorders.[46-50] Although different cell electrophysiology properties were seen for several of these variants[12,13,35-37,41,46], functional data for *SCN10A* and *SCN11A* variants is too limited to correlate channel phenotype with clinical phenotype.
In conclusion, in this cohort of 1139 pure SFN patients the overall frequency of potentially pathogenic *SCN9A*, *SCN10A* and *SCN11A* variants is 11.6%.

Erythromelalgia and warmth-induced pain were the only SFN-related clinical features that showed a significant relationship with the presence of VGCS variants. As genetic screening for all pure SFN patients is debatable, we believe that in the future, as the number of well-characterized variants of Na_v channels increases, and an inter-expert concordance on variant classification is reached, the utility of genetic screening for clinical care will rise, and tailored treatments with specific sodium channel blockers can be developed. Furthermore, we have seen that certainty about the origin of symptoms, as well as, genetic counselling by which the patient and relatives are informed about the possibility of developing and transmitting the condition, is of great importance for patients with pure SFN. Therefore, genetic screening of *SCN9A, SCN10A* and *SCN11A* should be considered for patients with pure SFN, independently of clinical features or underlying conditions.

References

1. 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.
2. Hoeijmakers JG, Faber CG, Lauria G, Merkies IS, Waxman SG. Small-fibre neuropathies--advances in diagnosis, pathophysiology and management. Nat Rev Neurol. 2012;8(7):369-79.
3. Finnerup NB, Attal N, Haroutounian S, McNicol E, Baron R, Dworkin RH, et al. Pharmacotherapy for neuropathic pain in adults: a systematic review and meta-analysis. Lancet Neurol. 2015;14(2):162-73.
4. Brouwer BA, de Greef BT, Hoeijmakers JG, Geerts M, van Kleef M, Merkies IS, et al. Neuropathic Pain due to Small Fiber Neuropathy in Aging: Current Management and Future Prospects. Drugs Aging. 2015;32(8):611-21.
5. Catterall WA, Goldin AL, Waxman SG. International Union of Pharmacology. XLVII. Nomenclature and structure-function relationships of voltage-gated sodium channels. Pharmacol Rev. 2005;57(4):397-409.
6. Dib-Hajj SD, Cummins TR, Black JA, Waxman SG. Sodium channels in normal and pathological pain. Annu Rev Neurosci. 2010;33:325-47.
7. Dib-Hajj SD, Rush AM, Cummins TR, Hisama FM, Novella S, Tyrrell L, et al. Gain-of-function mutation in Nav1.7 in familial erythromelalgia induces bursting of sensory neurons. Brain. 2005;128(Pt 8):1847-54.
8. Spillane J, Kullmann DM, Hanna MG. Genetic neurological channelopathies: molecular genetics and clinical phenotypes. J Neurol Neurosurg Psychiatry. 2016;87(1):37-48.
9. Fertleman CR, Ferrie CD. What's in a name--familial rectal pain syndrome becomes paroxysmal extreme pain disorder. J Neurol Neurosurg Psychiatry. 2006;77(11):1294-5.
10. Fertleman CR, Baker MD, Parker KA, Moffatt S, Elmslie FV, Abrahamsen B, et al. SCN9A mutations in paroxysmal extreme pain disorder: allelic variants underlie distinct channel defects and phenotypes. Neuron. 2006;52(5):767-74.
11. Faber CG, Hoeijmakers JG, Ahn HS, Cheng X, Han C, Choi JS, et al. Gain of function Nanu1.7 mutations in idiopathic small fiber neuropathy. Ann Neurol. 2012;71(1):26-39.
12. Faber CG, Lauria G, Merkies IS, Cheng X, Han C, Ahn HS, et al. Gain-of-function Nav1.8 mutations in painful neuropathy. Proc Natl Acad Sci U S A. 2012;109(47):19444-9.
13. Huang J, Han C, Estacion M, Vasylyev D, Hoeijmakers JG, Gerrits MM, et al. Gain-of-function mutations in sodium channel Na(v)1.9 in painful neuropathy. Brain. 2014;137(Pt 6):1627-42.
14. Brouwer BA, Merkies IS, Gerrits MM, Waxman SG, Hoeijmakers JG, Faber CG. Painful neuropathies: the emerging role of sodium channelopathies. J Peripher Nerv Syst. 2014; 19(2):53-65.
15. Yarnitsky D, Sprecher E. Thermal testing: normative data and repeatability for various test algorithms. J Neurol Sci. 1994;125(1):39-45.
16. 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.
17. Maxwell C. Sensitivity and accuracy of the visual analogue scale: a psycho-physical classroom experiment. Br J Clin Pharmacol. 1978;6(1):15-24.
18. Galer BS, Jensen MP. Development and preliminary validation of a pain measure specific to neuropathic pain: the Neuropathic Pain Scale. Neurology. 1997;48(2):332-8.
19. Brouwer BA, Bakkers M, Hoeijmakers JG, Faber CG, Merkies IS. Improving assessment in small fiber neuropathy. J Peripher Nerv Syst. 2015;20(3):333-40.
20. Tesfaye S, Boulton AJ, Dyck PJ, Freeman R, Horowitz M, Kempler P, et al. Diabetic neuropathies: update on definitions, diagnostic criteria, estimation of severity, and treatments. Diabetes Care. 2010;33(10):2285-93.

21. de Greef BTA, Hoeijmakers JGJ, Gorissen-Brouwers CML, Geerts M, Faber CG, Merkies ISJ. Associated conditions in small fiber neuropathy - a large cohort study and review of the literature. Eur J Neurol. 2018;25(2):348-55.
22. Wallis YP, S. McAnulty, C. et al. Practice Guidelines for the Evaluation of Pathogenicity and the Reporting of Sequence Variants in Clinical Molecular Genetics.2013. Available from: http://www.acgs.uk.com/media/774853/evaluation_and_reporting_of_sequence_variants_bpgs_june_2013_-_finalpdf.pdf.
23. Waxman SG, Merkies IS, Gerrits MM, Dib-Hajj SD, Lauria G, Cox JJ, et al. Sodium channel genes in pain-related disorders: phenotype-genotype associations and recommendations for clinical use. Lancet Neurol. 2014;13(11):1152-60.
24. Han C, Hoeijmakers JG, Liu S, Gerrits MM, te Morsche RH, Lauria G, et al. Functional profiles of SCN9A variants in dorsal root ganglion neurons and superior cervical ganglion neurons correlate with autonomic symptoms in small fibre neuropathy. Brain. 2012;135(Pt 9):2613-28.
25. Estacion M, Han C, Choi JS, Hoeijmakers JG, Lauria G, Drenth JP, et al. Intra- and interfamily phenotypic diversity in pain syndromes associated with a gain-of-function variant of NaV1.7. Mol Pain. 2011;7:92.
26. Ahn HS, Vasylyev DV, Estacion M, Macala LJ, Shah P, Faber CG, et al. Differential effect of D623N variant and wild-type Na(v)1.7 sodium channels on resting potential and interspike membrane potential of dorsal root ganglion neurons. Brain Res. 2013;1529:165-77.
27. Han C, Hoeijmakers JG, Ahn HS, Zhao P, Shah P, Lauria G, et al. Nav1.7-related small fiber neuropathy: impaired slow-inactivation and DRG neuron hyperexcitability. Neurology. 2012;78(21):1635-43.
28. Hoeijmakers JG, Han C, Merkies IS, Macala LJ, Lauria G, Gerrits MM, et al. Small nerve fibres, small hands and small feet: a new syndrome of pain, dysautonomia and acromesomelia in a kindred with a novel NaV1.7 mutation. Brain. 2012;135(Pt 2):345-58.
29. Cregg R, Laguda B, Werdehausen R, Cox JJ, Linley JE, Ramirez JD, et al. Novel mutations mapping to the fourth sodium channel domain of Nav1.7 result in variable clinical manifestations of primary erythromelalgia. Neuromolecular Med. 2013;15(2):265-78.
30. Goldberg YP, Price N, Namdari R, Cohen CJ, Lamers MH, Winters C, et al. Treatment of Na(v)1.7-mediated pain in inherited erythromelalgia using a novel sodium channel blocker. Pain. 2012;153(1):80-5.
31. Meglic A, Perkovic-Benedik M, Trebusak Podkrajsek K, Bertok S. Painful micturition in a small child: an unusual clinical picture of paroxysmal extreme pain disorder. Pediatr Nephrol. 2014;29(9):1643-6.
32. Devigili G, Eleopra R, Pierro T, Lombardi R, Rinaldo S, Lettieri C, et al. Paroxysmal itch caused by gain-of-function Nav1.7 mutation. Pain. 2014;155(9):1702-7.
33. 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.
34. Mulley JC, Hodgson B, McMahon JM, Iona X, Bellows S, Mullen SA, et al. Role of the sodium channel SCN9A in genetic epilepsy with febrile seizures plus and Dravet syndrome. Epilepsia. 2013;54(9):e122-6.
35. Han C, Vasylyev D, Macala LJ, Gerrits MM, Hoeijmakers JG, Bekelaar KJ, et al. The G1662S NaV1.8 mutation in small fibre neuropathy: impaired inactivation underlying DRG neuron hyperexcitability. J Neurol Neurosurg Psychiatry. 2014;85(5):499-505.
36. Huang J, Yang Y, Zhao P, Gerrits MM, Hoeijmakers JG, Bekelaar K, et al. Small-fiber neuropathy Nav1.8 mutation shifts activation to hyperpolarized potentials and increases excitability of dorsal root ganglion neurons. J Neurosci. 2013;33(35):14087-97.
37. Hu D, Barajas-Martinez H, Pfeiffer R, Dezi F, Pfeiffer J, Buch T, et al. Mutations in SCN10A are responsible for a large fraction of cases of Brugada syndrome. J Am Coll Cardiol. 2014;64(1):66-79.
38. Jabbari J, Olesen MS, Yuan L, Nielsen JB, Liang B, Macri V, et al. Common and rare variants in SCN10A modulate the risk of atrial fibrillation. Circ Cardiovasc Genet. 2015;8(1):64-73.

39. Neubauer J, Lecca MR, Russo G, Bartsch C, Medeiros-Domingo A, Berger W, et al. Post-mortem whole-exome analysis in a large sudden infant death syndrome cohort with a focus on cardiovascular and metabolic genetic diseases. Eur J Hum Genet. 2017;25(4):404-9.
40. Kambouris M, Thevenon J, Soldatos A, Cox A, Stephen J, Ben-Omran T, et al. Biallelic SCN10A mutations in neuromuscular disease and epileptic encephalopathy. Ann Clin Transl Neurol. 2017;4(1):26-35.
41. Han C, Yang Y, de Greef BT, Hoeijmakers JG, Gerrits MM, Verhamme C, et al. The Domain II S4-S5 Linker in Nav1.9: A Missense Mutation Enhances Activation, Impairs Fast Inactivation, and Produces Human Painful Neuropathy. Neuromolecular Med. 2015;17(2):158-69.
42. Wadhawan S, Pant S, Golhar R, Kirov S, Thompson J, Jacobsen L, et al. NaV channel variants in patients with painful and nonpainful peripheral neuropathy. Neurol Genet. 2017;3(6):e207.
43. Singh NA, Pappas C, Dahle EJ, Claes LR, Pruess TH, De Jonghe P, et al. A role of SCN9A in human epilepsies, as a cause of febrile seizures and as a potential modifier of Dravet syndrome. PLoS Genet. 2009;5(9):e1000649.
44. Tang Z, Chen Z, Tang B, Jiang H. Primary erythromelalgia: a review. Orphanet J Rare Dis. 2015;10:127.
45. Persson AK, Liu S, Faber CG, Merkies IS, Black JA, Waxman SG. Neuropathy-associated Nav1.7 variant I228M impairs integrity of dorsal root ganglion neuron axons. Ann Neurol. 2013;73(1):140-5.
46. Kist AM, Sagafos D, Rush AM, Neacsu C, Eberhardt E, Schmidt R, et al. SCN10A Mutation in a Patient with Erythromelalgia Enhances C-Fiber Activity Dependent Slowing. PloS One. 2016;11(9):e0161789.
47. Okuda H, Noguchi A, Kobayashi H, Kondo D, Harada KH, Youssefian S, et al. Infantile Pain Episodes Associated with Novel Nav1.9 Mutations in Familial Episodic Pain Syndrome in Japanese Families. PloS One. 2016;11(5):e0154827.
48. Zhang XY, Wen J, Yang W, Wang C, Gao L, Zheng LH, et al. Gain-of-function mutations in SCN11A cause familial episodic pain. Am J Hum Genet. 2013;93(5):957-66.
49. Kleggetveit IP, Schmidt R, Namer B, Salter H, Helas T, Schmelz M, et al. Pathological nociceptors in two patients with erythromelalgia-like symptoms and rare genetic Nav 1.9 variants. Brain Behav. 2016;6(10):e00528.
50. Han C, Yang Y, Te Morsche RH, Drenth JP, Politei JM, Waxman SG, et al. Familial gain-of-function Nav1.9 mutation in a painful channelopathy. J Neurol Neurosurg Psychiatry. 2017;88(3):233-40.

Supplementary data

Supplementary Table S1: Clinical characteristics of pure small fiber neuropathy patients with a (potentially) pathogenic *SCN9A* variant

c.position	Gender	Age	Age debute	IENFD (5th percentile)	IENFD	TTT	Associated condition(s)	Erythromelalgia	Itch	Cramps	Family history	Actual VAS	VASmin	VASmax	NPS: Pain Severity	Sharpness	Hotness	Dullness	Coldness	Sensitivity	Itchiness	Unpleasantness	Intensity deep pain	Intensity surface pain	SFN-SIQ No. autonomic	SFN-SIQ No. somatic	Neuropathic pain medication at moment hospital visit
c.118A>G	F	46	44	7,4 (5,7)	Normal	Abnormal	Idiopathic	No	No	No	Negative	70	59	83	7	7	6	8	9	6	2	8	8	7	8	2	None
c.554G>A	F	40	38	6,6 (5,7)	Normal	Abnormal	↓ vitamin B12	Yes	No	No	Negative	20	NA	59	3	5	NA	5	3	4	4	4	4	6	9	4	None
c.554G>A	M	52	22	1 (35)	Abnormal	Abnormal	Idiopathic	Yes	NA	NA	Positive	50	50	NA	6	7	8	0	0	7	0	7	0	8	0	4	Pregabalin 150mg BID
c.554G>A	F	25	23	4,9 (8,4)	Abnormal	Abnormal	Idiopathic	No	Yes	No	Negative	84	62	84	7	8	7	8	8	9	7	9	7	7	4	4	Phenytoin
c.684C>G*	M	28	20	7,5 (6,1)	Normal	Abnormal	Autoimmune disease	No	No	No	Positive	50	23	77	6	4	8	0	1	1	0	6	6	3	3	2	Lamotrigine 25mg BID
c.684C>G*	F	43	41	5,1 (5,7)	Abnormal	Normal	Idiopathic	No	No	No	Negative	52	49	49	4	8	2	NA	0	0	2	5	5	5	5	2	Gabapentin 300mg QID
c.684C>G*	F	37	36	11,1 (7,1)	Normal	Abnormal	Idiopathic	Yes	No	No	Positive	41	20	78	7	6	7	8	8	5	3	7	8	7	7	4	None
c.684C>G*	F	46	45	5,2 (5,7)	Abnormal	Abnormal	Idiopathic	Yes	No	No	Negative	75	33	83	8	7	8	3	0	8	0	8	3	9	4	2	None
c.684C>G*	F	54	51	8,3 (4,3)	Normal	Abnormal	↓ vitamin B12	No	No	No	Positive	49	46	94	8	9	9	9	6	10	5	9	9	6	9	4	None
c.684C>G*	F	41	13	11,1 (5,7)	Normal	Abnormal	↓ vitamin B12	Yes	No	No	Positive	NA	NA	NA	8	8	4	7	9	4	7	8	8	7	8	3	Gabapentin 900mg TID
c.684C>G*	M	51	17	1,6 (35)	Abnormal	Abnormal	Idiopathic	No	No	No	Negative	68	18	88	7	8	3	3	4	4	1	7	7	4	9	4	None
c.1007A>C	M	40	37	8,5 (4,4)	Normal	Abnormal	Idiopathic	No	No	Yes	Positive	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	None
c.1007A>C	M	50	47	10,1 (3,5)	Normal	Abnormal	Autoimmune disease	Yes	No	No	Negative	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	None
c.1552G>T	M	38	37	6,1 (5,2)	Normal	Abnormal	Autoimmune disease and DM	Yes	No	No	Negative	79	24	52	8	8	6	4	1	3	7	7	6	7	9	4	Tramadol and oxycodone on occasion
c.1555>nA	F	44	35	7,3 (5,7)	Normal	Abnormal	Idiopathic	Yes	No	No	Positive	67	NA	86	7	8	8	7	10	9	0	7	8	NA	9	4	None
c.1555>nA**	F	63	60	1,0 (3,2)	Abnormal	Abnormal	IGT	Yes	No	No	NA	36	21	65	5	6	7	5	6	5	3	5	3	3	6	4	Gabapentin 300mg daily
c.1592A>C	F	26	25	8 (8,4)	Abnormal	Normal	Idiopathic	Yes	No	No	Negative	75	7	74	8	7	10	0	0	0	0	NA	NA	NA	2	1	None
c.1867G>A*	F	64	59	2,8 (3,2)	Abnormal	Abnormal	Idiopathic	Yes	No	Yes	Positive	87	87	96	9	8	7	7	9	9	7	8	9	7	6	4	None
c.2033A>C	M	54	50	7,7 (3,5)	Normal	Abnormal	Idiopathic	Yes	No	No	Positive	70	53	88	8	8	8	0	0	8	0	NA	NA	NA	8	4	Duloxetine 120mg daily, oxycodone extended release 10mg TID, oxycodone 5mg QID
c.2128T>G	F	21	16	4,7 (8,5)	Abnormal	Normal	Idiopathic	Yes	No	No	Positive	64	0	85	7	5	8	7	0	5	0	6	7	5	5	3	None
c.2157G>C	F	46	39	6,9 (5,7)	Normal	Abnormal	Idiopathic	Yes	No	No	Positive	NA	NA	NA	9	9	3	9	9	9	1	9	9	9	1	1	Tramadol hydrochloride/paracetamol
c.2159T>A*	F	65	63	3,7 (3,2)	Normal	Abnormal	Idiopathic	No	No	No	Positive	NA	NA	NA	4	5	5	6	5	2	2	NA	2	2	4	3	None
c.2159T>A*	M	39	38	4,5 (5,2)	Abnormal	Abnormal	Idiopathic	No	No	No	Negative	56	34	74	7	6	7	7	8	3	0	7	8	7	6	4	NA
c.2215A>G	F	28	26	7,7 (8,4)	Abnormal	Abnormal	Idiopathic	No	No	No	Negative	77	56	89	8	8	NA	9	1	8	7	NA	NA	NA	8	4	Oxycodone 5mg 4 to 8 times a day, pregabalin 75mg 6 times a day
c.2215A>G	M	65	61	3,2 (2,8)	Normal	Abnormal	Idiopathic	Yes	Yes	No	Negative	45	7	46	6	0	9	0	6	10	0	10	2	10	2	1	None
c.2215A>G	F	56	53	1,3 (4,3)	Abnormal	Normal	Autoimmune disease	No	No	No	Negative	76	49	96	8	8	8	7	6	6	0	8	8	7	8	4	None
c.2215A>G	M	70	68	1 (2,2)	Abnormal	Abnormal	Idiopathic	No	No	No	Negative	22	21	NA	7	8	7	6	3	2	2	5	3	7	2	2	Pregabalin 75mg BID
c.2215A>G	F	48	41	NA	Normal	Abnormal	Autoimmune disease	Yes	No	No	Positive	46	22	86	7	8	7	7	4	8	4	9	7	8	0	4	None
c.2215A>G	M	59	39	2,2 (3,5)	Abnormal	Abnormal	Idiopathic	No	Yes	No	Positive	NA	NA	NA	9	9	7	3	1	1	1	8	8	8	7	4	Pregabalin, duloxetine
c.2215A>G	F	51	14	3,4 (4,3)	Abnormal	Abnormal	Idiopathic	Yes	Yes	Yes	Positive	53	46	NA	6	8	6	4	2	8	6	7	8	5	0	4	Duloxetine
c.2215A>G	M	67	61	0	Abnormal	Abnormal	Idiopathic	No	No	No	Negative	79	27	85	NA	NA	8	2	0	8	1	6	6	8	7	3	None
c.2215A>G	F	69	63	2,2 (3,2)	Abnormal	Abnormal	Idiopathic	Yes	No	No	Negative	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	Pregabalin 75mg TID
c.2215A>G	F	50	46	4,5 (4,3)	Normal	Abnormal	Idiopathic	Yes	No	No	Negative	NA	NA	NA	5	5	9	0	0	0	0	7	6	6	8	3	Pregabalin
c.2215A>G	F	75	70	6,7 (2,2)	Normal	Abnormal	DM	No	No	No	Negative	64	29	83	7	6	8	8	0	5	1	5	6	4	8	3	None
c.2215A>G	M	35	27	5,1 (5,2)	Abnormal	Abnormal	Alcohol abuse	No	Yes	No	Positive	56	28	87	6	7	8	0	5	6	0	9	9	6	5	4	Amitriptyline 10mg BID
c.2215A>G	F	66	64	2,3 (3,2)	Abnormal	Abnormal	Idiopathic	No	No	No	Negative	83	25	94	9	9	7	7	6	8	0	8	7	9	8	3	Gabapentin 800mg TID, tramadol 50mg TID
c.2215A>G +c.2794A>C;c.2971G>T* +c.4612T>C*	F	65	62	9,7 (3,2)	Normal	Abnormal	Idiopathic	No	No	No	Negative	91	71	95	9	9	7	8	8	9	8	9	9	8	7	4	Duloxetine 30mg QID, buprenorphine transdermal
c.2206G>A	F	71	63	6,4 (2,2)	Normal	Abnormal	Idiopathic	No	No	No	Negative	NA	NA	NA	7	10	10	2	0	8	0	10	10	7	3	1	Pregabaline 75mg daily
c.2271G>A	F	68	48	5 (3,2)	Normal	Abnormal	Autoimmune disease	Yes	No	Yes	Negative	NA	NA	NA	7	9	6	7	5	8	5	9	10	5	5	3	Duloxetine 60mg daily
c.2567G>A*	M	68	NA	3,7 (2,8)	Normal	Abnormal	NA	No	No	No	Positive	0	0	0	0	0	0	NA	0	0	0	0	0	0	0	1	None
c.2794A>C;c.2971G>T*	F	62	59	5,4 (3,2)	Normal	Abnormal	Idiopathic	No	No	No	Positive	60	28	81	6	6	7	5	1	8	2	NA	NA	NA	9	4	None
c.2794A>C;c.2971G>T*	M	21	16	4 (6,1)	Abnormal	Abnormal	Idiopathic	No	No	No	Positive	7	7	71	4	6	7	4	0	1	1	6	7	5	7	2	None
c.2794A>C;c.2971G>T*	F	36	34	6,4 (7,1)	Abnormal	Abnormal	Autoimmune disease	Yes	No	Yes	NA	87	80	100	9	9	8	9	10	7	6	10	10	8	9	4	Amitriptyline
c.2794A>C;c.2971G>T*	M	27	19	7,4 (6,1)	Normal	Abnormal	Autoimmune disease	No	No	Yes	Negative	88	39	100	9	9	8	9	7	10	0	9	9	9	7	4	None
c.2794A>C;c.2971G>T*	M	21	13	10,2 (6,1)	Normal	Abnormal	Idiopathic	No	No	No	NA	77	47	95	9	9	8	8	3	8	0	8	8	7	4	3	None
c.2794A>C;c.2971G>T* +c.4612T>C	M	50	49	0,6 (3,5)	Abnormal	Abnormal	Autoimmune disease	No	No	No	NA	29	13	59	4	5	3	1	1	0	1	5	4	3	5	2	None
c.2794A>C;c.2971G>T* +c.4612T>C	F	44	42	8,1 (5,7)	Normal	Abnormal	Idiopathic	No	No	Yes	Negative	68	27	78	6	7	6	7	6	9	5	5	8	7	9	4	Gabapentin 300mg TID, duloxetine 60mg daily
c.2794A>C;c.2971G>T* +c.4612T>C	M	58	56	9,6 (3,5)	Normal	Abnormal	Autoimmune disease	No	No	No	Negative	80	21	73	8	8	9	7	8	8	6	8	5	9	6	4	None
c.2969A>G	M	67	63	7,8 (2,8)	Normal	Abnormal	Autoimmune disease	No	No	No	Negative	71	19	87	8	8	9	3	8	9	0	8	8	4	3	1	Amitriptyline, lidocaine gel, oxycodone 5mg TID
c.3689T>C	F	27	26	4,5 (8,4)	Abnormal	Normal	Idiopathic	Yes	No	No	Positive	NA	NA	NA	4	0	6	7	8	1	6	5	1	7	5	3	None
c.3836>nA	M	64	59	7 (2,8)	Normal	Abnormal	Idiopathic	Yes	No	No	Negative	NA	NA	NA	NA	NA	NA	8	NA	NA	8	NA	9	NA	8	4	None
c.4585_4590delAGTCAA	F	42	40	9,1 (5,7)	Normal	Abnormal	Idiopathic	Yes	No	No	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	None
c.4596>nA	F	70	68	2,3 (2,2)	Normal	Abnormal	Idiopathic	Yes	No	Yes	Negative	35	35	75	8	8	8	8	9	7	8	7	8	5	3	4	None
c.4859G>T	M	30	25	3,6 (5,2)	Abnormal	Abnormal	Idiopathic	No	No	No	Positive	3	4	88	3	1	7	NA	0	0	0	5	7	1	3	1	None
c.4970A>C	F	47	45	8,2 (5,7)	Normal	Abnormal	Idiopathic	No	No	No	Negative	49	19	75	7	4	7	7	8	2	2	7	7	5	8	4	None
c.5260G>T	M	35	29	7,1 (5,2)	Normal	Abnormal	Idiopathic	No	No	No	Negative	NA	NA	NA	8	9	5	7	5	3	8	7	6	2	4	3	None
c.5260G>T	F	56	55	16,6 (4,3)	Normal	Abnormal	Idiopathic	No	No	No	Negative	NA	NA	NA	6	6	5	7	0	4	4	7	7	6	5	3	Amitriptyline 10mg daily
c.5912A>T	F	60	59	3,5 (3,2)	Normal	Abnormal	Idiopathic	Yes	No	No	Positive	NA	NA	NA	NA	NA	9	8	9	8	0	8	9	4	4	3	Gabapentin 400mg TID

F: female; M: male; IENFD: intraepidermal nerve fibre density; TTT: temperature threshold testing; VAS: visual analogue scale (VAS is ranging from 0-100 [0 = no pain and 100 = the worst possible pain]); min: minimum; max: maximum; NPS: neuropathic pain scale (0 = not applicable to the experienced pain and 10 = in the most severe form applicable to the experienced pain); SFN-SIQ: small fiber neuropathy inventory questionnaire; No. autonomic: number of positive autonomic symptoms; No. somatic: number of positive somatic symptoms; NA: not available; DM: diabetes mellitus; IGT: impaired glucose tolerance; ↓ vitamin B12: vitamin B12 deficiency; BID: twice daily; TID: three times daily; QID: four times daily.
*Variant classified as pathogenic according to the Waxman recommendations. **Also SCN11A variant c.3473T>C;p.(Leu1158Pro).

Supplementary Table 3: Clinical characteristics of pure small fiber neuropathy patients with a (potentially) pathogenic *SCN11A* variant

CHAPTER 7

PROPANE STUDY GROUP. GENETIC PROFILING OF SODIUM CHANNELS IN PAINFUL AND PAINLESS DIABETIC AND IDIOPATHIC SMALL FIBER NEUROPATHY

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Submitted.

Abstract

Background

Neuropathic pain is a frequent feature of diabetic peripheral neuropathy (DPN) and small fiber neuropathy (SFN). Resolving the genetic architecture of these painful neuropathies will lead to better disease management strategies, counselling and intervention. Our aims were to profile ten sodium channel genes (SCG) expressed in a nociceptive pathway in painful and painless DPN and painful and painless SFN patients, and to provide a perspective for clinicians who assess patients with a painful peripheral neuropathy.

Methods

Between June 2014 and September 2016, 1125 patients with painful-DPN (n=237), painless-DPN (n=309), painful-SFN (n=547) and painless-SFN (n=32), recruited in four different centres, were analysed for *SCN3A, SCN7A-SCN11A* and *SCN1B-SCN4B* variants by single molecule Molecular inversion probes-Next Generation Sequence. Patients were grouped based on phenotype and presence of SCG variants.

Results

Screening of *SCN3A, SCN7A-SCN11A, SCN1B-SCN4B* revealed 125 different (potential) pathogenic variants in 194 patients (17.2%, n=194/1125). A potential pathogenic variant was present in 18.1% (n=142/784) of painful neuropathy patients vs. 15.2% (n=52/341) of painless neuropathy patients (17.3% (n=41/237) for painful-DPN patients, 14.9% (n=46/309) for painless-DPN patients, 18.5% (n=101/547) for painful-SFN patients, and 18.8% (n=6/32) for painless-SFN patients). Of the variants detected, 70% were in *SCN7A, SCN9A, SCN10A* and *SCN11A*. The frequency of *SCN9A* and *SCN11A* variants was the highest in painful-SFN patients, *SCN7A* variants in painful-DPN patients, and *SCN10A* variants in painless-DPN patients.

Conclusion

Our findings suggest that rare SCG genetic variants may contribute to the development of painful neuropathy. Genetic profiling and SCG variant identification should aid a better understanding of the genetic variability in patients with painful and painless neuropathy and may lead to better risk stratification and the development of more targeted and personalized pain treatment.

Introduction

Neuropathic pain (NP) arises due to damage or a disease of the somatosensory nervous system, involving peripheral fibers (Aβ, Aδ, and C fibers) and central neurons.[1] NP is a frequent feature of peripheral neuropathy causing a significant impact on patients' quality of life and health care costs.[2] Not all individuals with neuropathy develop pain and it is not possible to predict who is more or less susceptible among those with similar risk exposure.

In small fiber neuropathy (SFN), thinly myelinated Aδ and the unmyelinated C fibers are affected resulting in sensory symptoms and autonomic dysfunction.[3] Pure SFN can be diagnosed on clinical symptoms, a reduction in intraepidermal nerve fiber density and/or an elevation in temperature threshold testing (TTT). Diabetes mellitus (DM) and sodium channel gene genetic variants are associated with SFN.[4] Establishing the underlying cause of SFN is important as some of these conditions can be treated.[4]

Diabetic peripheral neuropathy (DPN) is the most commonly reported complication of diabetes, affecting 50-90% of patients.[5] DPN is defined as a symmetrical, length-dependent sensorimotor polyneuropathy that develops as a consequence of longstanding hyperglycemia, associated metabolic derangements, and cardiovascular risk factors.[6] Clinically, painful-DPN is characterized by burning, tingling or shooting pain which interferes with daily activities of living and social functioning with a significant decrease in the quality of life.[7,8] The treatment of DPN relies on improved glycemic control and the use of medication to reduce pain. However, the currently available treatments for painful DPN are inadequate and are associated with side-effects which limits efficacy. A better understanding of the pathogenesis of painful DPN is urgently needed.

Pathogenic genetic variants of voltage-gated sodium channels genes have been reported in patients with a painful neuropathy.[9-13] These genes play an essential role in action potential generation in nociceptors and their propagation along the axons.[14] Given the importance of sodium channel genes in the activity of nociceptors, exploration of these genes may extend our knowledge of the pathophysiology of neuropathic pain in DPN and SFN, leading to the identification of new molecular druggable sites, with a possibility to develop more effective, personalized, treatment strategies.

Single-molecule Molecular Inversion Probes-Next Generation Sequencing (smMIPs-NGS) has recently been introduced for the re-sequencing of gene panels in large cohorts of patients. Using this relatively inexpensive and reliable

technique[15], ten genes encoding the alpha-subunits of Na_v 1.3 (*SCN3A*), Na_v 1.6 (*SCN8A*), Na_v 1.7 (*SCN9A*), Na_v 1.8 (*SCN10A*), Na_v 1.9 (*SCN11A*), Nax (*SCN7A*) and beta-subunits (*SCN1B, SCN2B, SCN3B and SCN4B*) were sequenced in patients with painful-DPN, painless-DPN and painful and painless SFN. The aim of the current study was to profile these genes according to the different phenotypes and to provide a perspective for clinicians who assess patients with peripheral neuropathy.

Methods

Study population

Patients (n=1325) were recruited in four different European centers from June 2014 until September 2016. Patients with painful-DPN and painless-DPN were recruited for this study at the University of Manchester (United Kingdom) and the Deutsche Diabetes Forschungsgesellschaft EV (Germany), and patients with SFN were recruited at the Fondazione IRCCS Instituto Neurologico "Carlo Besta" (Italy) and the Maastricht University Medical Center+ (The Netherlands). Local Medical Ethical Committees of each center approved this study. Informed consent was given by patients to participate in this study.

Inclusion- and exclusion criteria

Patients who met the following inclusion criteria were eligible to participate in this study: 1. the diagnosis of sensory neuropathy, based on established clinical, nerve conduction studies (NCS) and/or skin biopsy findings[6] with idiopathic or diabetic etiology, after ruling out other known causes of neuropathy including vitamin deficiencies, immune-mediated disorders like sarcoidosis, Sjögren syndrome, coeliac, leprosy, Epstein-Barr virus, toxins and drugs[4]; 2. Neuropathic pain was diagnosed based on the definition and grading system of Treede *et al.,* 2008.[16] Patients with neuropathic pain for more than one year and pain intensity equal or more than 4 on the pain intensity numerical rating scale (PI-NRS)[17] at the screening visit were defined as ´painful neuropathy´, whereas those with pain severity less than 4 were defined as ´non-painful neuropathy´; 3. Age of 18 years or older.

Clinical assessments

After written informed consent, all eligible patients underwent a screening visit and the following records and investigations were undertaken: medical and family history, neurological examination, quantitative sensory testing (QST) at the

feet to determine cold, warm, heat-pain and cold-pain thresholds (Mücke et al., 2016), nerve conduction studies of the tibial, peroneal and sural nerves, skin biopsy at the distal leg for determination of intra-epidermal nerve fibers density (IENFD), Visual Analogue Scale (VAS) to measure pain intensity[18], Neuropathy Pain Scale (NPS) to evaluate 10 qualities of neuropathic pain[19] and the SFN-symptom Inventory questionnaire (SFN-SIQ) to assess 13 SFN-related symptoms.[20] Blood withdrawal from patients was done (2x6 mL EDTA blood) for genetic analyses.

SCG mutation analysis by smMIPs-NGS

Genomic DNA was extracted from whole blood using QIAamp DNA Blood Maxi Kit /Puregene® Blood Core Kit (Qiagen, Hilden, Germany) or NucleoSpin ®8 Blood Isolation kit (Macherey-Nagel, Düren, Germany) according to manufacturer's instructions. Coding exons and exon-flanking intron sequences of *SCN3A, SCN7A-SCN11A*, and *SCN1B-SCN4B* were sequenced by smMIP-NGS. Three-hundred-twenty-four smMIPs were designed using a modified version of MIPgen (http://shendurelab.github.io/MIPGEN/). The gap-fill length between the extension - and ligation arm (region of interest) of the smMIPs was fixed to 220-230 nt. Probes were synthesized by Integrated DNA Technologies (IDT, Iowa, USA).

Targeted capture with smMIPs was performed according to standard protocols.[21] In brief, after hybridization, gap filling and ligation, circularized DNA molecules were used as template in a PCR with universal primers complementary to the linker sequence. Sample-specific barcode sequences and Illumina adaptors were introduced during the PCR amplification step. Next, samples were pooled and purified using Ampure XP beads according to manufacturer's instructions. Pooled samples were sequenced using an Illumina NextSeq500 system (Illumina, Inc., San Diego, CA, USA), with 2 × 150-bp paired-end reads (Illumina, Inc., San Diego, CA, USA). Sequenced data was analyzed by using an in-house smMIPs-NGS data analysis pipeline. Variants were included for analysis with >30x coverage and an alternative variant call of at least 20%. Regions with a poor performance (n=9; coverage <30x, high false positive variant calling) were analysed by Sanger sequencing.[22]

Patients' coding and immediate flanking regions of *SCN3A, SCN7A-SCN11A*, and *SCN1B-SCN4B* were compared with reference sequence GRCh37. Variants detected were annotated according to the guidelines of the Human Genome Variation Society (http://www.hgvs.org/mutnomen/), and classified according to current classification guidelines[23] using Alamut Mutation-Interpretation Software

(Interactive-Biosoftware, Rouen, France). Variants of interest were confirmed by Sanger sequencing.

Statistical analysis

The primary analysis was performed to compare patients with painful SFN, painless SFN, painful-DPN and painless-DPN with and without SCG variants and to measure the frequencies of the identified variants in the ten genes in the tested patients.

For categorical variables, the ÷2 test was used or the Fisher's exact test when necessary. For continuous variables, the independent student's t-test was chosen. Equal variances between two groups were tested with the Levene's test. For these analyses, a significance level of 0.05 was used.

Post hoc analyses were performed to investigate whether differences between patients with specific SCG variants and without variants were present. In total, six post hoc analyses per variable were executed. The analyses were performed in the same way as the primary analyses; however, the significance level was adjusted for multiple testing with the Bonferroni correction.

Results

Patient characteristics

A total of 1.325 patients were recruited for this study. Two-hundred patients were excluded based on incomplete clinical or diagnostic data and/or low quality smMIPs-NGS data (Figure 7.1). Eventually, 1.125 patients, including 237 painful-DPN, 309 painless-DPN, 547 painful-SFN and 32 painless-SFN patients, were profiled for gene variations in *SCN3A, SCN7A-SCN11A*, and *SCN1B-SCN4B*.

Among the 1125 patients, males (55.2%) were represented more than females. The mean age of the patient at recruitment for this study was 58.9 years (SD±14.0 years). The median age of onset of complaints was 53.0 years (SD±15.4 years) and the mean duration of neuropathy was 7.0 years (SD±8.1 years). One in five patients (20.2%) reported a family history of neuropathy.

The mean VAS at recruitment was 46.0 (SD±26.9) for the painful patients versus 21.8 (SD±23.38) for the painless patients, $p<0.001$. Of the DPN patients, 21.2% had type I diabetes, which was more prevalent in painless-DPN compared to

painful-DPN (25.6% vs. 15.6%, p=0.005). Patient characteristics per subgroup are given in Table 7.1.

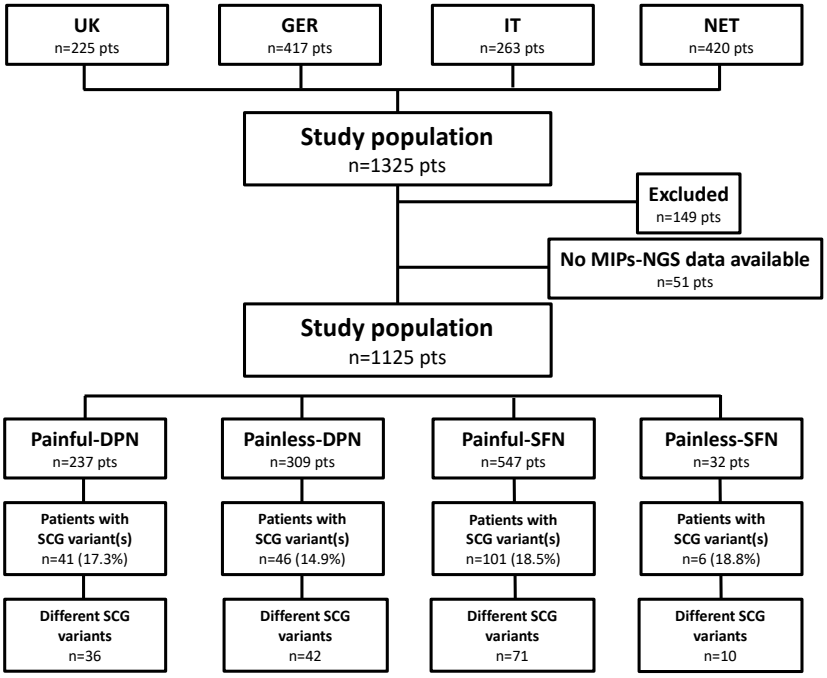


Figure 7.1 PROPANE study patients analysed for potentially pathogenic SCG variants by single molecule Molecular Inversion Probe-Next generation sequencing (smMIP-NGS). Between June 2014 and September 2016, patients with painful and painless diabetic peripheral neuropathy (DPN) and idiopathic small fiber neuropathy (SFN), recruited in four different centres, were screened for *SCN3A, SCN7A-SCN11A*, and *SCN1B-SCN4B* variants by smMIP-NGS. Variants' pathogenicity was classified according to to current classification guidelines.[23] UK, University of Manchester, United Kingdom; GER, Deutsche Diabetes Forschungsgesellschaft EV, Germany; IT, Fondazione IRCCS Instituto Neurologico "Carlo Besta", Italy; NET, Maastricht University Medical Centre+, the Netherlands; n, number; pts, patients; Painful-DPN, painful diabetic peripheral neuropathy; Painless-DPN, painless diabetic peripheral neuropathy; Painful-SFN, painful-idiopathic small fiber neuropathy; Painless-SFN, painless-idiopathic small fiber neuropathy; SCG, sodium channel genes.

The quality of neuropathic pain was assessed by NPS and peripheral neuropathy related symptoms were evaluated using the SFN-SIQ per subgroup. As expected for the 10 qualities of NPS, there was a clear difference between patients with painful neuropathy and painless neuropathy. Comparing patients with painful-DPN and painful-SFN, more patients experienced dullness, sensitivity, intensity deep and surface pain in the latter group, while the other qualities of the NPS scored similarly between both groups (Figure 7.2A).

Table 7.1 Patient characteristics of the study population.

	Painful-DPN (n=237)	Painless-DPN (n=309)	Painful-SFN (n=547)	Painless-SFN (n=32)	Painful-DPN vs. Painless DPN	Painful SFN vs. Painless SFN	Painful DPN vs. Painful SFN	Painful DPN/SFN vs. Painless DPN/SFN
Male (n)	150/237 (63.3%)	236/309 (76.4%)	220/547 (40.2%)	15/32 (46.9%)	<0.001	NS	<0.001	<0.001
Mean age at recruitment (in years +/- SD)	63.70 +/- 10.42 (0 missing)	64.92 +/- 11.88 (0 missing)	53.56 +/- 14.09 (0 missing)	54.69 +/- 17.11 (0 missing)	NS	NS	<0.001	<0.001
Mean age of onset neuropathy (in years +/- SD)	58.43 +/- 11.26 (69 missing)	62.29 +/- 11.82 (128 missing)	53.56 +/- 14.09 (0 missing)	45.79 +/- 14.16 (13 missing)	0.002	NS	<0.001	<0.001
Mean duration of neuropathy (in years +/- SD)	6.70 +/- 5.86 (70 missing)	5.62 +/- 6.60 (140 missing)	7.91 +/- 9.65 (218 missing)	6.42 +/- 6.30 (13 missing)	NS	NS	NS	0.010
Familial cases of neuropathy (n)	30/162 (18.5%) (75 missing)	24/185 (13.0%) (124 missing)	80/314 (25.5%) (233 missing)	0/4 (0%) (28 missing)	NS	NS	NS	0.003
Normal EMG (n)	0/237 (0%)	0/309 (0%)	547/547 (100%)	32/32 (100%)	ND	ND	ND	ND
Abnormal QST (n)					ND		ND	ND
Abnormal skin biopsy (n)	12/12 (100%) * (225 missing)	N/A*	137/364 (37.6%) (183 missing)	1/4 (25.0%) (28 missing)	ND	NS	ND	ND
VAS - current pain (mean +/- SD)	46.44 +/- 26.46 (86 missing)	31.94 +/- 23.53 (137 missing)	45.78 +/- 27.25 (283 missing)	14.25 +/- 16.46 (28 missing)	<0.001	0.022	NS	<0.001
VAS - maximum pain (mean +/- SD)	73.65 +/- 31.30 (86 missing)	36.01 +/- 30.48 (137 missing)	73.37 +/- 25.88 (283 missing)	63.25 +/- 39.18 (28 missing)	<0.001	NS	NS	<0.001
Diabetes mellitus type 1 (n)	37/237 (15.6%)	79/309 (25.6%)	3/547 (0.5%)	0/32 (0%)	0.005	NS	<0.001	ND
Diabetes mellitus type 2 (n)	198/237 (83.5%)	228/309 (73.8%)	25/547 (4.6%)	0/32 (0%)	0.006	NS	<0.001	ND
Hypertension (n)	137/237 (57.8%)	155/309 (50.2%)	78/547 (14.3%)	1/32 (3.1%)	NS	NS	<0.001	ND
Hypercholesterolemia (n)	113/237 (47.7%)	131/309 (42.4%)	50/547 (9.1%)	0/32 (0%)	NS	NS	<0.001	ND
Cardiovascular diseases (n)	84/237 (35.4%)	105/309 (34.0%)	33/547 (6.0%)	0/32 (0%)	NS	NS	<0.001	ND

* Not required for diagnosis. Painful-DPN, painful diabetic peripheral neuropathy; Painless-DPN, painless diabetic peripheral neuropathy; Painful-SFN, painful-idiopathic small fiber neuropathy; Painless-SFN, painless-idiopathic small fiber neuropathy; n, number; SD, standard deviation; N/A, not applicable; NS, not significant; ND, not determined.

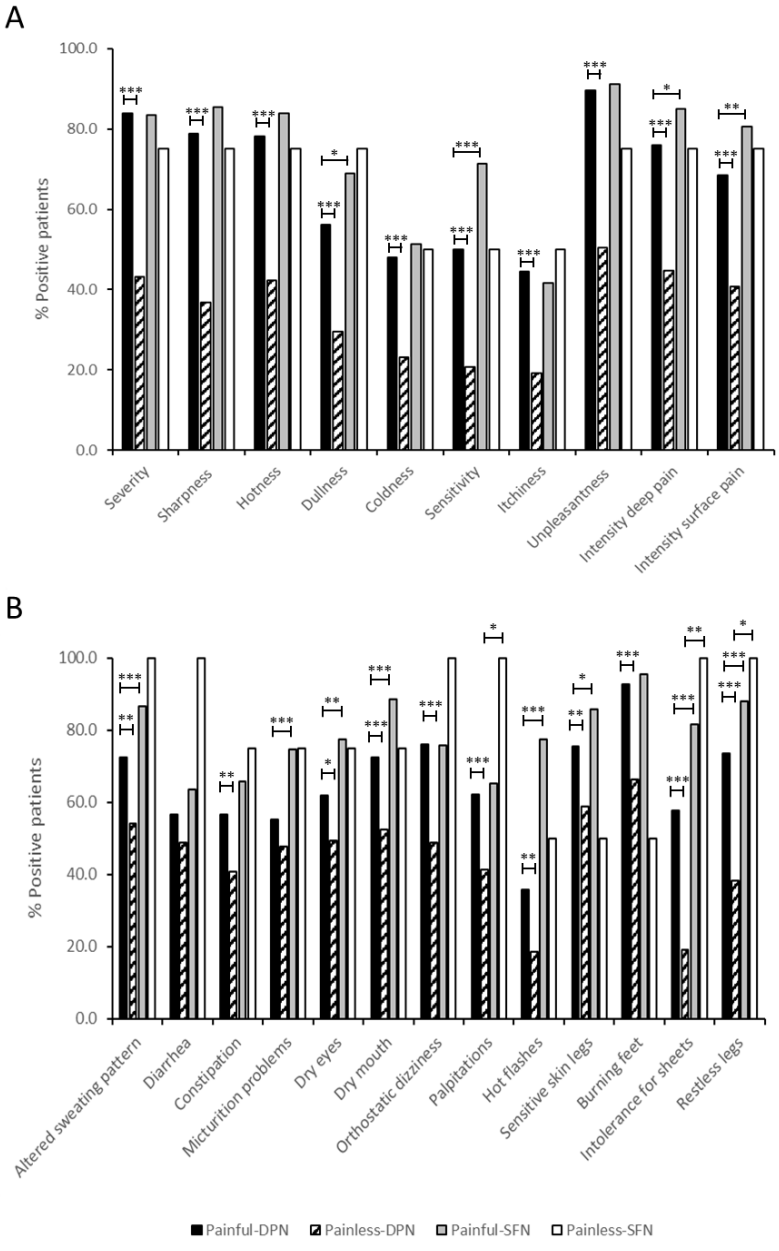


Figure 7.2 Clinical features in patients with painful and painless diabetic peripheral neuropathy (DPN) and idiopathic small fiber neuropathy (SFN). (A) Neuropathy Pain Scale (NPS) of painful-DPN (n=149), painless-DPN (n=125), painful-SFN (n=247) and painless-SFN (n=4) patients. Each item is scored on an 11-point scale (0=not applicable to the experienced pain and 10=in the most severe form applicable to the experienced pain).

An NPS score >3 is considered as a relevant pain quality. (B) Symptoms inventory questionnaire (SFN-SIQ) of painful-DPN (n=152), painless-DPN (n=172), painful-SFN (n=266) and painless-SFN (n=4) patients. The answer options of the SFN-SIQ include 'never=1','sometimes=2', 'often=3' and 'always=4'. A symptom is considered to be present when the score is >1. Statistically significant differences are shown as * for $p<0.05$, **for $p<0.01$ and *** $p<0.001$ ***. Painful-DPN, painful diabetic peripheral neuropathy; Painless-DPN, painless diabetic peripheral neuropathy; Painful-SFN, painful-idiopathic small fiber neuropathy; Painless-SFN, painless-idiopathic small fiber neuropathy.

For the SFN-SIQ, 12 of the 13 symptoms were more often reported by patients with painful neuropathy compared to painless neuropathy, with only diarrhea being reported equally for both patient groups. Nine peripheral neuropathy related symptoms were reported significantly more often by patients with painful-SFN compared to painful-DPN. Bowel problems, orthostatic dizziness and burning feet were reported equally in both painful neuropathy groups (Figure 7.2B).

Observations for the painless-SFN patients are shown in Figure 7.2, but were not formally compared as they may have been biased due to the low number of patients with painless-SFN.

Performance of SCG- smMIPs-NGS

To profile our study population for ten SCG variations, a smMIPs-NGS targeted enrichment kit was constructed smMIPs which contains 320 probes to capture all exons and intron-exon junctions (±20 bp) of ten sodium channel genes, *SCN3A, SCN7A-SCN11A*, and SCN1B-SCN4B. Targeted regions enrichment, capture, and library sequencing were performed for all patients with painful and painless diabetic and idiopathic SFN. To calculate the performance and capture efficiency of the sodium channel genes- smMIPs-NGS only data of patients with complete clinical and diagnostic evaluation and sufficient smMIPs-NGS data were included (n=1125 patients, Figure 7.1).

The performance and capture efficiency for the ten genes smMIPs-NGS was 99.1% (n=317/320 smMIPs) and on average, 97.6% of the targeted regions were covered >30x. The average coverage of *SCN3A*=99.6%, *SCN7A*=100%, *SCN8A*=97.6%, *SCN9A*=98.7%, *SCN10A*=99.9%, *SCN11A*=99.9%, *SCN1B*=93.6%, *SCN2B*=100%, *SCN3B*=100%, and *SCN4B*=86.5%. No sequence reads were obtained for three smMIPs (0.9%, n=3/320). We were not able to capture and sequence the first exon of *SCN1B*, *SCN4B* and *SCN11A* genes by smMIPs-NGS. Furthermore, exon 2 of *SCN3B*, exons 13, 16, 21 and 26 of *SCN8A*, exon 13 of *SCN10A* and exon 14 of *SCN11A* showed high variation in sequencing reads. To

provide full coverage for *SCN3A*, *SCN8A-SCN11A*, and *SCN1B,* and *SCN4B*, 2321 bp were subsequently tested by Sanger sequencing.

SCG variants in painful-DPN patients

In our cohort of 237 patients with painful-DPN, smMIP-NGS of *SCN3A*, *SCN7A-11A*, and *SCN1B-SCN4B* genes revealed 36 different potentially pathogenic heterozygous variants in 41 patients (17.3%, n=41/237, Figure 7.1). Twelve variants were previously associated with neuropathic pain[9-12,24,25], of which six have been reported in patients with painful-DPN.[12,13] Twenty-four variants were novel for neuropathic pain, and classified as VUS (Supplementary Table S7.1).

Five variants were found in >1 patient. Two patients were positive for three heterozygous *SCN9A* variants; c.2794A>C, c.2971G>T and c.4612T>C. Cell electrophysiology has shown a gain-of-function of *SCN9A* variants, c.2215A>G, c.2794A>C/c.2971G>T[10] and c.4612T>C[24], *SCN11A* variant c.3473T>C[25], and *SCN2B* variant c.325G>A[12], and DRG neuron hyperexcitability for *SCN10A* variant c.4568G>A[9]. For patients with an SCG variant co-segregation data was not available.

Twelve (potentially) pathogenic variants detected in our cohort of patients with painful-DPN have been reported in patients with epilepsy with or without neurodevelopment delay, autism spectrum disorder, atrial fibrillation, and sudden unexplained/infant death syndrome.[26-30] None of our patients carrying ≥1 of these SCG variants reported a cardiac disorder. Other neurological complaints were not registered in this study.

SCG variants in painless-DPN patients

Among our cohort of 309 painless-DPN patients, 42 different potentially pathogenic heterozygous SCG variants were detected in 46 patients (14.9%, n=46/309, Figure 7.1) (Supplementary Table S7.2).

Seven patients harboured more than one SCG variant. One patient was heterozygous for the pathogenic *SCN9A* variants, c.2794A>C and c.2971G>T, and two patients were heterozygous for the likely pathogenic *SCN10A* variants, c.472T>G and c.2441G>A. Four other patients were heterozygous for the VUSs *SCN7A* c.3461G>A and *SCN10A* c.1489C>T, *SCN8A* c.4748T>C and *SCN10A* c.41G>T, *SCN10A* c.2367C>A and c.4736G>A, and *SCN10A* c.5657C>T and *SCN2B* VUS c.625_626delinsCC, respectively. Co-segregation data was not available for any of the variants.

Eight variants have previously been reported in painful-DPN or painful-SFN; *SCN9A* c.2215A>G variants, c.2794A>C/c.2971G>T and c.3799C>G, *SCN10A* variants, c.41G>T, c.3803G>A and c.4568G>A, and *SCN11A* variant c.4282G>A.[9-11,13] Except for *SCN11A* c.4282G>A, these variants have also been seen in patients with Dravet syndrome, Autism, Brugada syndrome, atrial fibrillation and long QT syndrome.[26,28,31-33]

Five (potentially) pathogenic, *SCN10A* variants c.472T>G, c.2441G>A and c.5657C>T, *SCN1B* variant c.457G>A, and *SCN3B* variant c.583G>A, have not been seen in patients with painful-DPN and SFN, but three of them have functionally been tested and reported as disease causing for atrial fibrillation[29] and three VUSs have been reported in patients with Brugada syndrome, atrial fibrillation and long QT syndrome.[34,35] Only one patient heterozygous for *SCN10A* variant c.5657C>T and *SCN2B* variant c.625_626delinsCC reported cardiac related complaints.

SCG variants in painful-SFN patients

For the 547 painful-SFN patients, 71 different potentially pathogenic heterozygous variants were found in 101 patients (18.5%, n=101/547, Figure 7.1). Fourty-six variants were previously described in patients with a neuropathic pain phenotype, including SFN.[9-11,13,24,25,32,36,37] Seven variants have already been published as pathogenic[9-11], nine variants as likely pathogenic[9,11,25,32], and 28 variants as VUS for a pain phenotype.[11,25,38] Twenty-seven variants were novel for painful-SFN, and classified as VUS (Supplementary Table S7.3).

Twenty variants were seen in >1 patient. Eight patients harboured more than one SCG variant, of which one was heterozygous for *SCN3A* c.5583G>T, *SCN9A* c.2794A>C, c.2971G>T, c.4612T>C and *SCN11A* c.1560G>T. Two patients were heterozygous for *SCN8A* c.1426A>C and *SCN9A* c.2794A>C, c.2971G>T, c.4612T>C, one patient was heterozygous for *SCN9A* c.2794A>C, c.2971G>T and c.4612T>C, three patients were heterozygous for *SCN9A* c.2794A>C and c.2971G>T, one patient was heterozygous for *SCN9A* c.1555G>A and c.2271G>A and *SCN11A* c.3473T>C, and one patient was heterozygous for *SCN9A* c.3799C>G and *SCN11A* c.1142T>C.

Sixteen variants were tested by voltage- and/or current-clamp electrophysiology and 13 showed a gain-of-function of $Na_v1.7$, $Na_v1.8$ or $Na_v1.9$. Segregation with the disease was seen for 7 variants.

Sixteen (potentially) pathogenic variants detected in our patients with painful-SFN have been reported in patients with other neurological and cardiac disorders like epilepsy with and without neurodevelopmental delay, autism spectrum disorder, Brugada syndrome, atrial fibrillation and sudden infant death syndrome.[26-28,31,32,39] Two patients carrying *SCN10A* variant c.4568G>A are known with a cardiac disorder.

SCG variants in painless-SFN patients

Ten different potentially pathogenic heterozygous variants were detected in six patients with painless-SFN (18.8%, n=6/32, Figure 7.1). Eight variants were reported previously in patients with a painful neuropathy.[9-12,24,25]

SCN3A c.2077A>G and *SCN4B* c.298C>T were novel for a painful and painless neuropathy, and classified as VUS. Five variants were found in one patient (Supplementary Table S7.4).

Seven (potentially) pathogenic SCG variants detected in our cohort of patients with painless SFN have been reported in patients with epilepsy with or without neurodevelopmental delay, autism spectrum disorder, and several cardiac disorders.[27,28,31,32,40,41] None of the patients carrying ≥1 of these SCG variants reported a cardiac disorder.

Shared SCG variants in painful and painless DPN and SFN

In our cohort of 1125 patients with painful and painless-DPN and SFN, 74 potentially pathogenic variants in 92 patients were specific for a painful neuropathy, and 28 potentially pathogenic variants in 26 patients were specific for a painless neuropathy. Twenty-three potentially pathogenic variant were shared, either between painful-DPN and SFN or painful-DPN/SFN and painless-DPN. However, *SCN9A* c.2215A>G, c.3799C>G and c.4612T>C, *SCN10A* c.2972C>T and *SCN11A* c.3473T>C were more often seen in patients with painful neuropathy, while *SCN10A* c.41G>T and c.4568G>A were more often seen in patients with painless neuropathy.

Mutation frequencies and distribition of SCG variants in painful and painless DPN and SFN patients

In total 125 different (potential) pathogenic SCG variants were detected in 194 patients (17.2%, n=194/1125). The overall SCG mutation frequency for painful-DPN patients was 17.3% (n=41/237), for patients with painless-DPN

14.9% (n=46/309), painful-SFN 18.5% (n=101/547), and painless-SFN 18.8% (n=6/32).

More than 70% of identified (potential) pathogenic variants were found in *SCN7A, SCN9A, SCN10A* and *SCN11A*. The frequency of *SCN9A* and *SCN11A* variants in patients with painful-SFN was higher than in painful-DPN and painless-DPN, while the frequency of *SCN10A* variant was lower in patients with painful-DPN and painful-SFN compared to painless-DPN. No significant differences in mutation frequencies were seen for the other tested genes (*SCN3A, SCN8A, SCN1B-SCN4B)* between patients with painful DPN, painless-DPN and painful-SFN (Figure 7.3A). Data from the patients with painless-SFN was excluded from analysis due to the low number of patients included in the study.

The majority of *SCN9A* variants identified in our cohort of patients with painful SFN were clustered in transmembrane domains I (DI) and II (DII), and in the intracellular linker between DI and DII (Supplementary Figure S7.1A). The distribution of *SCN9A* variants in patients with painful-DPN was all across the gene (Supplementary Figure S7.1B), while in patients with painless-DPN they were more localized to DII and domain III (DIII) (Supplementary Figure S7.1C).

For *SCN10A* variants, the majority of variants were localized to DIII and domain IV (DIV), the intracellular linker between DIII-DIV and C-terminus of the protein in the patients with painful-SFN (Supplementary Figure S7.1D). In the patients with painless-DPN, the identified variants were localized to DI and DII (Supplementary Figure S7.1E). For patients with painful-DPN, no hotspot region for *SCN10A* variants could be identified (Supplementary Figure S7.1D).

When the above data was corrected for variants that were not specific for a painful or painless phenotype the overall mutation frequency was 11.8% (n=28/237) for patients with painful-DPN, 7.4% (n=23/309) for painless-DPN, 11.7% (n=64/547) for painful SFN, and 9.4% (n=3/32) for painless SFN. For patients with painful-DPN and painful-SFN, around 80% of the (potentially) pathogenic variants were identified in *SCN7A, SCN9A, SCN10A,* and *SCN11A,* while for patients with painless-DPN most variants were identified in *SCN10A* (Figure 7.3B).

The localization of *SCN9A* variants detected in the patients with painful-SFN and painless-DPN did not change after exclusion of shared SCG variants. For painful-DPN, only 3 variants were unique for a painful phenotype. One was localized in the N-terminus and the other 2 were localized in the linker between DI and DII. For *SCN10A*, the majority of the variants identified in the patients with painful-

SFN were localized to domain IV of the protein. No localization of *SCN10A* variants to specific domains was seen in patients with painless-DPN and painful DPN. For *SCN1*1A, most variants were unique for patients with painful-SFN. The distribution of these variants was all across the gene. For the others tested SCG, the mutation frequency were too low to identify possible hotspot regions.

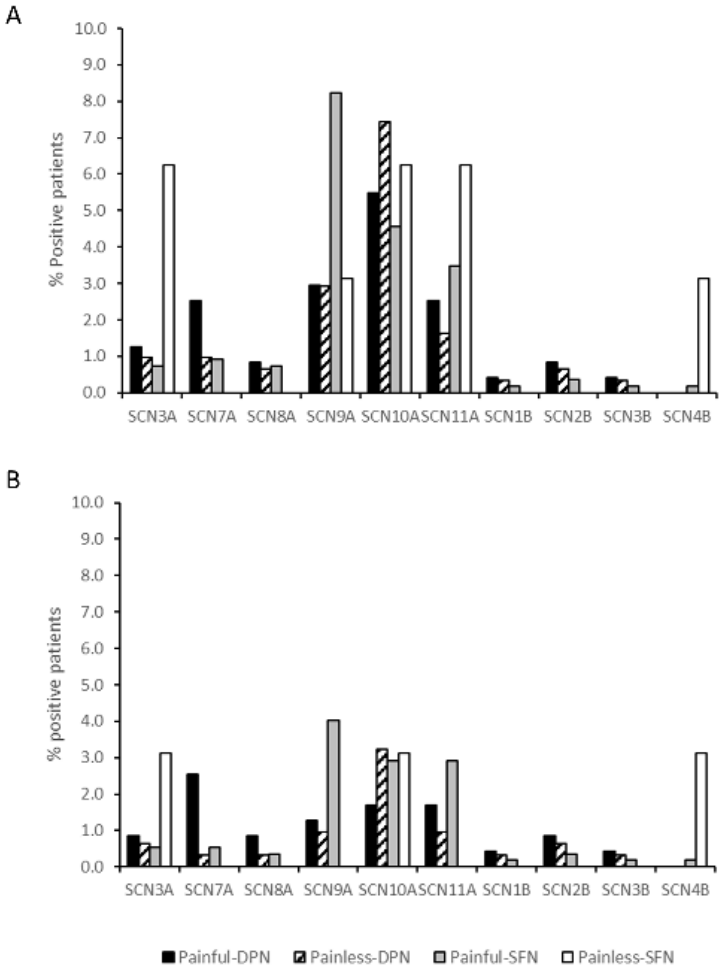


Figure 7.3 SCG variant positive patients with painful and painless diabetic and idiopathic small fiber neuropathy. (A) Percentage of *SCN3A*, *SCN7A-SCN11A* and *SCN1B-SFN4B* positive painful-DPN (n=237), painless-DPN (n=309), painful-SFN (n=547) and painless-SFN (n=32) patients with ≥1 variant(s). (B) Percentages of *SCN3A*, *SCN7A-SCN11A* and *SCN1B-4B* positive patients with ≥1 variant(s) corrected for variants that were not specific for a painful or painless diabetic/idiopathic neuropathy. Painful-DPN, painful diabetic peripheral neuropathy; Painless-DPN, painless diabetic peripheral neuropathy; Painful-SFN, painful-idiopathic small fiber neuropathy; Painless-SFN, painless-idiopathic small fiber neuropathy; SCG , voltage gated sodium channel.

Clinical features of painful-DPN, painless-DPN and SFN patients with and without SCG variants

To provide a rationale for genetic profiling in patients with painful or painless DPN or SFN, clinical features of the 4 different subgroups with and without SCG variants were compared. Except family history in patients with painful-DPN with and without SCG variants (36.0% vs. 15.3%, p=0.023), no signifance differences were seen for any of the patients characteristics for any of the subgroups.

When the comparison was corrected for variants that were not specific for a painful or painless phenotype, and the main patients characteristics were analyzed for all patient with or without SCG variants regardless of their phenotype, the patients with SCG variants more often reported a family history of neuropathy (33.3% vs. 18.8%, p=0.008).

Patients with and without SCG variants were grouped into those with painful or painless neuropathy. In this analysis, the occurrence of coldness and hot flashes was significantly higher in patients with painful neuropathy and SCG variants versus patients with painful neuropathy without SCG variants (coldness, 65.1% vs. 48.1%, p=0.036; hot flashes (77.1% vs. 60.3%, p=0.024). The data was further analysed according to the presence or absence of SCG variants. The duration of neuropathy was shorter for patients with painful-DPN with SCG variants than for patients with painful-DPN without SCG variants (3.19 [SD +/- 2.34] vs. 7.07 [SD +/- 6.00), p<0.001), while dry eyes was reported more often in patients with painful-SFN and painless-DPN with SCG variants than patients with painful-SFN and painless-DPN without SCG variants (painful-SFN, 91.2% vs. 75.0%, p=0.036; painless DPN 87.5% vs. 47.6%, p=0.033). All features analysed after correction for non-painful or painless specific SCG variants are given in Supplementary Table S7.5.

Discussion

There is a strong body of evidence implicating sodium channel genes in human painful disorders.[42] Gain-of-function mutations in *SCN9A, SCN10A,* and *SCN11A* genes are associated with painful neuropathy as a consequence of dorsal root ganglion (DRG) neuron hyperexcitability. The pathogenic role of mutations in *SCN9A, SCN10A,* and *SCN11A* genes has triggered substantial interest in other sodium channels genes such as *SCN3A, SCN7A*, *SCN8A* and *SCN1B-SCN4B.* To date, this is the first study that has investigated in a large cohort of painful and painless diabetic and idiopathic small fibre neuropathy for

the presence of genetic variants in ten SCG (*SCN3A, SCN7A-SCN11A, SCN1B-SCN4B*).

In our cohort of 1125 patients we identified 125 different (potential) pathogenic *SCN3A, SCN7A-SCN11A* and *SCN1B-SCN4B* variants in 194 patients (17.2%, n=194/1125). Of the patients with painful neuropathy, 18.1% (n=142/784) were heterozygous for ≥1 SCG variant versus 15.2% (n=52/341) of those with painless neuropathy. Ten patients harbored (potentially) pathogenic sodium channel variants in more than one sodium channel gene (painless-DPN n=4, painful-SFN n=5, painless-SFN n=1). Considering the presence of SCG variants, the duration of neuropathy was significantly shorter and dry eyes and hot flashes were significantly more often reported by patients with SCG variants.

For painful-SFN, (potentially) pathogenic *SCN9A* variants were found more frequently than sodium channel variants in other genes, followed by *SCN10A* and *SCN11A* variants. These mutation frequencies for SFN patients were similar to those reported previously.[2,4,11,25]

For painful-DPN and painless-DPN, (potential) pathogenic variants were found more frequently in *SCN10A* than in other genes (painful-DPN, 5.5%, n=13/237; painless-DPN, 7.4%, n=23/309), followed by *SCN9A* (painful-DPN, 3.0%, n=7/237; painless-DPN, 2.9%, n=9/309), *SCN7A* (painful-DPN, 2.5%, n=6/237) and *SCN11A* (painful-DPN, 2.5%, n=6/237; painless-DPN, 1.6.%, n=5/309). Less frequent variants (<6 variants) were identified in *SCN3A, SCN8A,* and *SCN1B-SCN4B.* The mutation frequencies for *SCN3A, SCN8A,* and *SCN1B-SCN4B* did not differ significantly between painful and painless diabetic and idiopathic SFN.

After correcting our data for variants that were not specific for a painful or painless phenotype we show that the frequencies of (potentially) pathogenic variants in *SCN9A, SCN10A* and *SCN11A* genes were 3.1% (painful-DPN, 1.3%, n=3/237; painful-SFN, 4.0%, n=22/547), 2.6% (painful-DPN, 1.7%, n=4/237; painful-SFN, 2.9%, n=16/547) and 2.6% (painful-DPN, 1.7%, n=4/237; painful-SFN, 2.9%, n=16/547) respectively, in the patients with painful neuropathy, while they were 0.9% (painful-SFN, 1.0%, n=3/309; painless-SFN, 0%, n=0/32), 3.2% painful-SFN, 3.2%, n=10/309; painless-SFN, 3.1%, n=1/32) and 0.9% (painful-SFN, 1.0%, n=3/309; painless-SFN, 0%, n=0/32) respectively, in the patients with painless neuropathy. Our data contrast with the study by Wadhawan et al. 2017, who in a cohort of 457 patients showed no significant difference in the missense variant allele frequencies for *SCN9A*, *SCN10A*, and *SCN11A* between patients with painful and non-painful neuropathy.[43] Several rare (potential)

pathogenic *SCN9A* variants were identified in patients with both painful-DPN and painless-DPN. Whereas, previously Blesneac et al., 2018 found that 10 out of 111 patients with painful-DPN harboured rare Na_v 1.7 variants (potentially pathogenic) compared to none of their patients with painless-DPN (n=78).[13] These differences may be explained by the use of different criteria to select patients, different populations and applying different variant filtering strategies.

Shared SCG variants were identified between painful-DPN and painful-SFN, painless-DPN and painless-SFN, as well as painful-DPN/SFN and painless-DPN/SFN. These findings suggest that a single genetic variant may be associated with different phenotypes depending on the presence or absence of other genetic variants in the SCG or other genomic regions and environmental factors. As several of these variants have been reported as gain-of-function mutations[10,24,25], we suggest that these rare genetic variants in the SCG genes may act as risk factors and/or play a role in predisposing patients to develop a specific phenotype.

A number of studies have shown that rare variants in genes belonging to sodium, calcium, potassium and transient receptor potential channel gene families are implicated in determining pain risk threshold and sensitivity[44]; while others have shown that specific polymorphisms in genes, like *KCNS1, SCN9A, ADRB2, H2TRA, CACNG2,* and *IL16* play a role in increasing the risk of chronic pain or an increase in pain sensitivity.[2,45-47] There are also reports of genetic variants in e.g. *KCNQ2, KCNQ3, KNQ3*, *COMT, OPRM1, TRPV1, MC1R, GCH1,* and *CACNA2D3* genes that have been associated with pain protection or pain resilience.[47-49] Moreover, it appears likely that development of peripheral neuropathy associated with voltage-gated sodium channel mutations involves "multi-hit" pathophysiology in which factors such as energetic run-down and changes in transmembrane ionic gradients interact with presence of the mutation.[50,51] The localization of identified *SCN9A* and *SCN10A* variants in our painful-SFN patient cohort to specific protein domains was similar to previously published data.[11]

Clustering of *SCN9A* and *SCN10A* variants was also seen in patients with painless-DPN, however, at different regions of the *SCN9A* and *SCN10A* variants identified in our painful-SFN cohort, respectively, in DII and DIII of Na_v1.7, and DI and DII of Na_v1.8. In our cohort with painful-DPN, no hotspot regions were identified for *SCN9A* and *SCN10A* variants. After exclusion of the non-painful and painless specific SCG variants, the localization of *SCN9A* variants detected in the patients with painful-SFN and painless-DPN did not change. For *SCN10A*, the majority of variants identified in the patients with painful-SFN were localized to

DIV, instead of DIII, DIV, the linker between DIII-DIV and C-terminus of the protein, whilst in patients with painless-DPN, the variants were located across the whole gene. Functional studies have shown that SCG variants are associated with both gain-of-function and loss-of-function leading to impaired inactivation and hyperexcitability or hypoexcitability of neurons.[11,46,52,53] Unfortunately, functional data of variant localization in hotspot regions identified in this study are too limited to correlate channel characteristics with the clinical phenotype.

In our cohort, males were represented more in DPN than SFN and the patients with DPN were older compared to patients with SFN. There was no difference in the duration of neuropathy or VAS between patients with DPN or SFN, although the duration of neuropathy was shorter in patients with painless compared to painful neuropathy. Of note, coldness and hot flashes were experienced more often by patients with painful neuropathy with SCG variants compared to those with painful neuropathy without SCG variants. Also VAS and unpleasantness of symptoms was higher in patients with painless neuropathy and SCG variants compared to patients with painless neuropathy without SCG variants. Furthermore, a complaint of dry eyes was reported more often in painful-SFN and painless-DPN patients with SCG variants rather than those without SCG variants. The duration of neuropathy was shorter in patients with painful-DPN and SCG variants than in patients with painful-DPN without SCG variants. This finding is in line with the results reported by Blesneac et al., who found that participants carrying rare $Na_v1.7$ variants had been diagnosed for a significantly shorter duration.[13] However, the difference we have seen with the SFN-SIQ for those with painful-SFN contrasts with Eijkenboom et al. 2018, who reported comparable NPS and SFN-SIQ in 1139 pure-SFN patients with and without (potentially) pathogenic *SCN9A, SCN10A* and *SCN11A* variants.[11] This difference could be explained by the smaller cohort size in our study, testing of more VGCS genes (10 versus 3), and correction for variants not specific for a painful or painless neuropathy phenotype.

Conclusion

In this large cohort of 1125 patients with painful-DPN and painless-DPN and painful-SFN and painless-SFN, 18.1% of patients with painful neuropathy and 15.2% of patients with painless neuropathy carry a potential pathogenic SCG genetic variant. The presence of SCG variants in patients with painful neuropathy was associated with symptoms of coldness and hot flashes. Furthermore, VAS and the occurrence of unpleasantness was higher in patients with painless neuropathy with SCG variants.

The phenotypic expression and management of neuropathic pain is undoubtedly complex. Whilst SCG variants appear to play a key role in the development of neuropathic pain, identification of additional pain loci may be necessary to identify optimal therapeutic modulation and pain relief.

References

1. Jensen TS, Baron R, Haanpää M, Kalso E, Loeser JD, Rice ASC, et al. A new definition of neuropathic pain. Pain. 2011;152:2204–5.
2. Brouwer BA, Merkies ISJ, Gerrits MM, Waxman SG, Hoeijmakers JGJ, Faber CG. Painful neuropathies: The emerging role of sodium channelopathies. J Peripher Nerv Syst. 2014;19: 53–65.
3. Hoeijmakers JG, Faber CG, Lauria G, Merkies IS, Waxman SG. Small-fibre neuropathies-advances in diagnosis, pathophysiology and management. Nat Rev Neurol. 2012;8: 369–79.
4. de Greef BTA, Hoeijmakers JGJ, Gorissen-Brouwers CML, Geerts M, Faber CG, Merkies ISJ. Associated conditions in small fiber neuropathy – a large cohort study and review of the literature. Eur J Neurol. 2018;25:348–55.
5. Bril V, Breiner A, Perkins BA, Zochodne D. Neuropathy. Can J Diabetes. 2018;42:S21S221.
6. Tesfaye S, Boulton AJM, Dyck PJ, Freeman R, Horowitz M, Kempler P, et al. Diabetic neuropathies: Update on definitions, diagnostic criteria, estimation of severity, and treatments. Diabetes Care. 2010;33:2285–93.
7. Davies M, Brophy S, Williams R, Taylor A. The prevalence, severity, and impact of painful diabetic peripheral neuropathy in type 2 diabetes. Diabetes Care. 2006;29:1518–22.
8. Gore M, Brandenburg NA, Dukes E, Hoffman DL, Tai KS, Stacey B. Pain severity in diabetic peripheral neuropathy is associated with patient functioning, symptom levels of anxiety and depression, and sleep. J Pain Symptom Manage. 2005;30:374–85.
9. Faber CG, Lauria G, Merkies ISJ, Cheng X, Han C, Ahn HS, et al. Gain-of-function Nav1.8 mutations in painful neuropathy. Proc Natl Acad Sci U S A. 2012;109: 19444–9.
10. Faber CG, Hoeijmakers JGJ, Ahn HS, Cheng X, Han C, Choi JS, et al. Gain of function Na V1.7 mutations in idiopathic small fiber neuropathy. Ann Neurol. 2012;71:26–39.
11. Eijkenboom I, Sopacua M, Hoeijmakers JGJ, De Greef BTA, Lindsey P, Almomani R, et al. Yield of peripheral sodium channels gene screening in pure small fibre neuropathy. Journal of Neurology, Neurosurgery and Psychiatry. 2019;90: 342–352. doi:10.1136/jnnp-2018-319042
12. Alsaloum M, Estacion M, Almomani R, Gerrits MMM, Bönhof GJGJ, Ziegler D, et al. A gain-of-function sodium channel β2-subunit mutation in painful diabetic neuropathy. Mol Pain. 2019;15:1744806919849802
13. 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;159:469–80.
14. Wang J, Ou SW, Wang YJ. Distribution and function of voltage-gated sodium channels in the nervous system. Channels. 2017;11:534–54.
15. Hiatt JB, Pritchard CC, Salipante SJ, O'Roak BJ, Shendure J. Single molecule molecular inversion probes for targeted, high-accuracy detection of low-frequency variation. Genome Res. 2013;23: 843–54.
16. 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;70:1630–5.
17. Farrar JT, Young JP, LaMoreaux L, Werth JL, Poole RM. Clinical importance of changes in chronic pain intensity measured on an 11-point numerical pain rating scale. Pain. 2001;94: 149–58.
18. Maxwell C. Sensitivity and accuracy of the visual analogue scale: a psycho-physical classroom experiment. Br J Clin Pharmacol.1978;6:15–24.
19. Galer BS, Jensen MP. Development and preliminary validation of a pain measure specific to neuropathic pain: The Neuropathic Pain Scale. Neurology. 1997;48:332–8.
20. Bakkers M, Merkies ISJ, Lauria G, Devigili G, Penza P, Lombardi R, et al. Intraepidermal nerve fiber density and its application in sarcoidosis. Neurology. 2009;73:1142–8.
21. O'Roak BJ, Vives L, Fu W, Egertson JD, Stanaway IB, Phelps IG, et al. Multiplex targeted sequencing identifies recurrently mutated genes in autism spectrum disorders. Science. 2012;338:1619–22.

22. Almomani R, Marchi M, Sopacua M, Lindsey P, Salvi E, de Koning B, et al. Evaluation of molecular inversion probe versus TruSeq® custom methods for targeted next-generation sequencing. PLoS One. 2020;15:1–15.
23. Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, et al. Standards and guidelines for the interpretation of sequence variants: A joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genet Med. 2015;17: 405–424.
24. Cregg R, Laguda B, Werdehausen R, Cox JJ, Linley JE, Ramirez JD, et al. Novel mutations mapping to the fourth sodium channel domain of nav1.7 result in variable clinical manifestations of primary erythromelalgia. Neuromolecular Med. 2013;15: 265–78.
25. Huang J, Han C, Estacion M, Vasylyev D, Hoeijmakers JGJ, Gerrits MM, et al. Gain-of-function mutations in sodium channel NaV1.9 in painful neuropathy. Brain. 2014;137:1627–42.
26. Singh NA, Pappas C, Dahle EJ, Claes LRF, Pruess TH, De Jonghe P, et al. A role of SCN9A in human epilepsies, as a cause of febrile seizures and as a potential modifier of Dravet syndrome. PLoS Genet. 2009;5:1–12.
27. Fernández-Marmiesse A, Roca I, Díaz-Flores F, Cantarín V, Pérez-Poyato MS, Fontalba A, et al. Rare Variants in 48 Genes Account for 42% of Cases of Epilepsy With or Without Neurodevelopmental Delay in 246 Pediatric Patients. Front Neurosci. 2019;13:1–17.
28. Rubinstein M, Patowary A, Stanaway IB, McCord E, Nesbitt RR, Archer M, et al. Association of rare missense variants in the second intracellular loop of Na v 1.7 sodium channels with familial autism. Mol Psychiatry. 2018;23:231–9.
29. Savio-Galimberti E, Weeke P, Muhammad R, Blair M, Ansari S, Short L, et al. SCN10A/Nav1.8 modulation of peak and late sodium currents in patients with early onset atrial fibrillation. Cardiovasc Res. 2014;104:355–63.
30. Scheffer IE, Harkin LA, Grinton BE, Dibbens LM, Turner SJ, Zielinski MA, et al. Temporal lobe epilepsy and GEFS+ phenotypes associated with SCN1B mutations. Brain. 2007;130:100–109.
31. Hu D, Barajas-Martínez H, Pfeiffer R, Dezi F, Pfeiffer J, Buch T, et al. Mutations in SCN10A are responsible for a large fraction of cases of brugada syndrome. J Am Coll Cardiol 2014;64: 66-79.
32. van Lint FHM, Mook ORF, Alders M, Bikker H, Lekanne dit Deprez RH, Christiaans I, et al. The G1662S NaV1.8 mutation in small fibre neuropathy: Impaired inactivation underlying DRG neuron hyperexcitability. Brain. 2014;2:771–82.
33. Abou Ziki MD, Seidelmann SB, Smith E, Atteya G, Jiang Y, Fernandes RG, et al. Deleterious protein-altering mutations in the SCN10A voltage-gated sodium channel gene are associated with prolonged QT. Clinical Genetics. 2018;93:741–51.
34. Watanabe H, Darbar D, Kaiser DW, Jiramongkolchai K, Chopra S, Donahue BS, et al. Mutations in Sodium Channel β1- and β2-Subunits Associated With Atrial Fibrillation. Circ Arrhythm Electrophysiol. 2009;2:268–75.
35. van Lint FHM, Mook ORF, Alders M, Bikker H, Lekanne dit Deprez RH, Christiaans I. Large next-generation sequencing gene panels in genetic heart disease: yield of pathogenic variants and variants of unknown significance. Neth Heart J. 2019;27:304–9.
36. Meglič A, Perkovič-Benedik M, Trebušak Podkrajšek K, Bertok S. Painful micturition in a small child: an unusual clinical picture of paroxysmal extreme pain disorder. Pediatr Nephrol. 2014;29:1643–6.
37. Di Stefano G, Yuan JH, Cruccu G, Waxman SG, Dib-Hajj SD, Truini A. Familial trigeminal neuralgia – a systematic clinical study with a genomic screen of the neuronal electrogenisome. Cephalalgia. 2020;40: 767–77.
38. Labau JIR, Estacion M, Tanaka BS, de Greef BTA, Hoeijmakers JGJ, Geerts M, et al. Differential effect of lacosamide on Nav1.7 variants from responsive and non-responsive patients with small fibre neuropathy. Brain. 2020;143:771–82.
39. Kambouris M, Thevenon J, Soldatos A, Cox A, Stephen J, Ben-Omran T, et al. Biallelic SCN10A mutations in neuromuscular disease and epileptic encephalopathy. Ann Clin Transl Neurol. 2017;4:26–35.

40. Mulley JC, Hodgson B, McMahon JM, Iona X, Bellows S, Mullen SA, et al. Role of the sodium channel SCN9A in genetic epilepsy with febrile seizures plus and Dravet syndrome. Epilepsia. 2013;54:122–6.
41. Neubauer J, Lecca MR, Russo G, Bartsch C, Medeiros-Domingo A, Berger W, et al. Post-mortem whole-exome analysis in a large sudden infant death syndrome cohort with a focus on cardiovascular and metabolic genetic diseases. Eur J Hum Genet. 2017;25:404–9.
42. Dib-Hajj SD, Geha P, Waxman SG. Sodium channels in pain disorders: Pathophysiology and prospects for treatment. Pain. 2017;158:S97–107.
43. Wadhawan S, Pant S, Golhar R, Kirov S, Thompson J, Jacobsen L, et al. Na V channel variants in patients with painful and nonpainful peripheral neuropathy . Neurol Genet. 2017;3:e207.
44. Lauria G, Ziegler D, Malik R, Merkies ISJ, Waxman SG, Faber CG. The Role of Sodium Channels in Painful Diabetic and Idiopathic Neuropathy. Curr Diab Rep. 2014;14:538.
45. Estacion M, Harty TP, Choi JS, Tyrrell L, Dib-Hajj SD, Waxman SG. A sodium channel gene SCN9A polymorphism that increases nociceptor excitability. Ann Neurol. 2009;66:862–6.
46. Bennett DLH, Woods CG. Painful and painless channelopathies. Lancet Neurol. 2014;13: 587-99.
47. Young EE, Lariviere WR, Belfer I. Genetic basis of pain variability: Recent advances. J Med Genet. 2012;49:1–9.
48. Yuan JH, Estacion M, Mis MA, Tanaka BS, Schulman BR, Chen L, et al. KCNQ variants and pain modulation: a missense variant in Kv7.3 contributes to pain resilience. Brain Commun. 2021;3:1–15.
49. Mis MA, Yang Y, Tanaka BS, Gomis-Perez C, Liu S, Dib-Hajj F, et al. Resilience to pain: A peripheral component identified using induced pluripotent stem cells and dynamic clamp. J Neurosci. 2019;39:382–92.
50. Estacion M, Vohra BPS, Liu S, Hoeijmakers J, Faber CG, Merkies ISJ, et al. Ca2+ toxicity due to reverse Na+/Ca2+ exchange contributes to degeneration of neurites of DRG neurons induced by a neuropathy-associated Nav1.7 mutation. J Neurophysiol. 2015;114:1554–64.
51. Lee S Il, Hoeijmakers JGJ, Faber CG, Merkies ISJ, Lauria G, Waxman SG. The small fiber neuropathy NaV1.7 I228M mutation: Impaired neurite integrity via bioenergetic and mitotoxic mechanisms, and protection by dexpramipexole. J Neurophysiol. 2020;123:645–57.
52. Waxman SG, Merkies ISJ, Gerrits MM, Dib-Hajj SD, Lauria G, Cox JJ, et al. Sodium channel genes in pain-related disorders: Phenotype-genotype associations and recommendations for clinical use. Lancet Neurol. 2014;13:1152–60.
53. Huang J, Vanoye CG, Cutts A, Goldberg YP, Dib-Hajj SD, Cohen CJ, et al. Sodium channel NaV1.9 mutations associated with insensitivity to pain dampen neuronal excitability. J Clin Invest. 2017;127:2805–14.
54. Han C, Yang Y, de Greef BTA, Hoeijmakers JGJ, Gerrits MM, Verhamme C, et al. The Domain II S4-S5 Linker in Nav1.9: A Missense Mutation Enhances Activation, Impairs Fast Inactivation, and Produces Human Painful Neuropathy. Neuromolecular Med. 2015;17:158–69.

Supplementary data

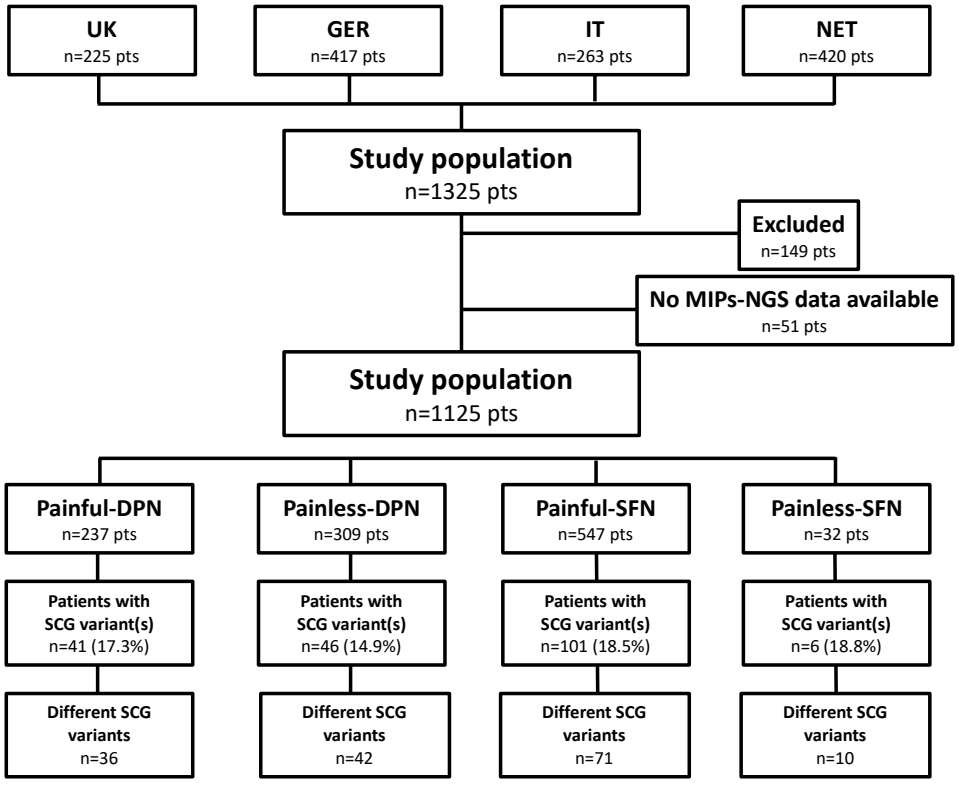


Figure S7.1 PROPANE study patients analysed for potentially pathogenic SCG variants by single molecule Molecular Inversion Probe-Next generation sequencing (smMIP-NGS). Between June 2014 and September 2016, patients with painful and painless diabetic peripheral neuropathy (DPN) and idiopathic small fiber neuropathy (SFN), recruited in four different centres, were screened for *SCN3A, SCN7A-SCN11A*, and *SCN1B-SCN4B* variants by smMIP-NGS. Variants' pathogenicity was classified according to to current classification guidelines.[23] UK, University of Manchester, United Kingdom; GER, Deutsche Diabetes Forschungsgesellschaft EV, Germany; IT, Fondazione IRCCS Instituto Neurologico "Carlo Besta", Italy; NET, Maastricht University Medical Centre+, the Netherlands; n, number; pts, patients; Painful-DPN, painful diabetic peripheral neuropathy; Painless-DPN, painless diabetic peripheral neuropathy; Painful-SFN, painful-idiopathic small fiber neuropathy; Painless-SFN, painless-idiopathic small fiber neuropathy; SCG, sodium channel genes.

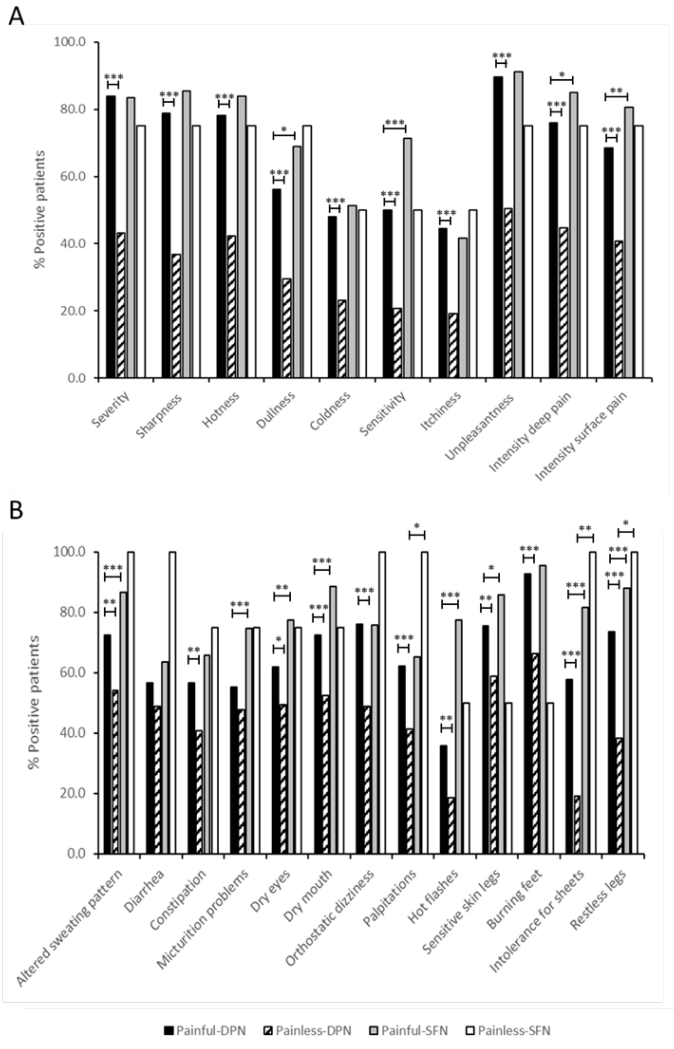


Figure S7.2 Clinical features of painful and painless diabetic peripheral neuropathy (DPN) and idiopathic small fiber neuropathy (SFN) patients. (A) Neuropathy Pain Scale (NPS) of painful-DPN (n=149), painless-DPN (n=125), painful-SFN (n=247) and painless-SFN (n=4) patients. Each item is scored on an 11-point scale (0=not applicable to the experienced pain and 10=in the most severe form applicable to the experienced pain). An NPS score >3 is considered as a relevant pain quality. (B) Symptoms inventory questionnaire (SFN-SIQ) of painful-DPN (n=152), painless-DPN (n=172), painful-SFN (n=266) and painless-SFN (n=4) patients. The answer options of the SFN-SIQ include 'never=1','sometimes=2', 'often=3' and 'always=4'. A symptom is considered to be present when the score is >1. Statistical significant differences of are shown as * for $p<0.05$, **for $p<0.01$ and *** $p<0.001$ ***. Painful-DPN, painful diabetic peripheral neuropathy; Painless-DPN, painless diabetic peripheral neuropathy; Painful-SFN, painful-idiopathic small fiber neuropathy; Painless-SFN, painless-idiopathic small fiber neuropathy.

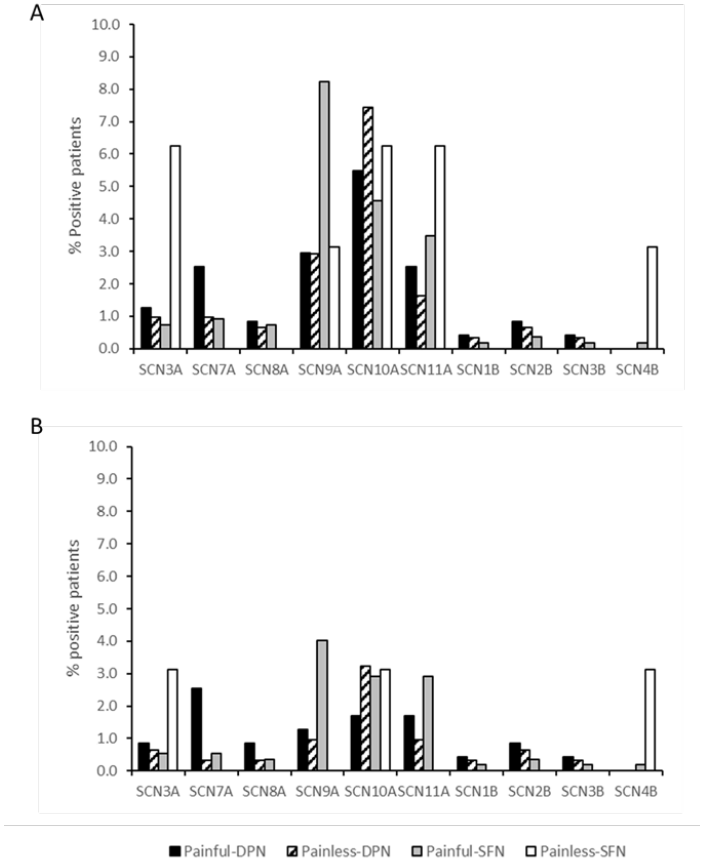


Figure S7.3 SCG variant positive painful and painless diabetic and idiopathic small fiber neuropathy patients. (A) Percentages of *SCN3A*, *SCN7A-SCN11A* and *SCN1B-SFN4B* positive painful-DPN (n=237), painless-DPN (n=309), painful SFN (n=547) and painless-SFN (n=32) patients with ≥1 variant(s). (B) Percentages of *SCN3A*, *SCN7A-SCN11A* and *SCN1B-4B* positive patients with ≥1 variant(s) corrected for variants that were not specific for a painful or painless diabetic/idiopathic neuropathy. Painful-DPN, painful diabetic peripheral neuropathy; Painless-DPN, painless diabetic peripheral neuropathy; Painful-SFN, painful-idiopathic small fiber neuropathy; Painless-SFN, painless-idiopathic small fiber neuropathy; SCG , voltage gated sodium channel

Table S7.1 Potentially pathogenic SCG variants (*SCN3A*, *SCN7A-11A*, and *SCN1B-SCN4B*) identified in patients with painful diabetic peripheral neuropathy (painful-DPN, n=237 patients).

Gene	c.position &	p.position	Number of patients	Location	MAF gnomAD (%)	Additional variant characteristics	Variant classification	Ref.
SCN3A	c.132T>G	p.(Asp44Glu)	1	N-terminus	0.0021	-	VUS	-
	c.634G>A	p.(Val212Ile)	1	Loop DI/S3-DI/S4	-	-	VUS	-
	c.1619C>T	p.(Ser540Phe)	1	Linker DI/S6-DII/S1	0.15	-	VUS	-
SCN7A	c.538T>A	p.(Trp180Arg)	1	DI/S3	0.0051	-	VUS	-
	c.2612C>T	p.(Ser871Leu)	1	Linker DII/S6-DIII/S1	0.0027	-	VUS	-
	c.2795A>C	p.(Glu932Ala)	1	Linker DII/S6-DIII/S1	0.027	-	VUS	-
	c.3782T>C	p.(Ile1261Thr)	1	DIV/S1	0.0022	-	VUS	-
	c.3791A>G	p.(Gln1264Arg)	1	DIV/S1	-	-	VUS	-
	c.3855G>T	p.(Trp1285Cys)	1	DIV/S2	0.065	-	VUS	-
SCN8A	c.160A>G	p.(lys54Glu)	1	N-terminus	-	-	VUS	-
	c.3529G>A	p.(Glu1177Lys)	1	Linker DII/S6-DIII/S1	0.0012	-	VUS	-
SCN9A	c.185T>C	p.(Ile62Thr)	1	N-terminus	-	-	VUS	-
	c.1208T>C	p.(Met403Thr)	1	Linker DI/S6-DII/S1	0.0046	-	VUS	-
	c.1555G>A	p.(Glu519Lys)	1	Linker DI/S6-DII/S1	0.047	-	VUS	-
	c.2215A>G	p.(Ile739Val)	1	DII/S1	0.25	Gain-of-function Na_v 1.7 channel	VUS (risk factor)	[13]
	c.2794A>C /	p.(Met932Leu) /	2	DII/S6	3.39	Gain-of-function Na_v1.7 channel	Pathogenic	[13]
	c.2971G>T*	p.(Val991Leu)		Linker DII/S6-DIII/S1	3.01			
	c.3799C>G	p.(Leu1267Val)	1	DIII/S3	0.13	?	VUS	[13]
	c.4612T>C*	p.(Trp1538Arg)	2	Loop DIV/S1-DIV/S2	0.20	Gain-of-function Na_v1.7 channel	Pathogenic	[13]
SCN10A	c.1138G>A	p.(Val380Ile)	2	DI/S6	0.058	-	VUS	-
	c.1249_1251del	p.(Lys417del)	2	Linker DI/S6-DII/S1	0.081	-	VUS	-
	c.1492C>T	p.(Arg498Trp)	1	Linker DI/S6-DII/S1	0.0052	-	VUS	'
	c.2972C>T	p.(Pro991Leu)	3	Linker DII/S6-DIII/S1	0.0.094	-	VUS	
	c.3061C>T	p.(Gln1021*)	1	Linker DII/S6-DIII/S1	0.0012	-	VUS	
	c.3803G>A	p.(Arg1268Gln)	1	Loop DIII/S4-DIII/S5	0.19		VUS	-
	c.3859G>A	p.(Val1287Ile)	1	DIII/S5	0.059	-	VUS	-
	c.4379G>A	p.(Arg1460Gln)	1	Linker DIIIS6-DIV/S1	0.100	-	VUS	-
	c.4568G>A	p.(Cys1523Tyr)	1	DIV/S2	0.11	DRG neuron hyperexcitability	Likely pathogenic	-

Table S7.1 (continued)

Gene	c.position &	p.position	Number of patients	Location	MAF gnomAD (%)	Additional variant characteristics	Variant classification	Ref.
SCN11A	c.3473T>C	p.(Leu1158Pro)	3	DIII/S4	0.047	Gain-of-function $Na_v1.9$ channel	Likely pathogenic	-
	c.4282G>A	p.(Gly1428Ser)	1	DIV/S3	0.020		VUS	-
	c.4499T>C	p.(Ile1500Thr)	1	DIV/S5	-	-	VUS	-
	c.4607C>T	p.(Thr1536Ile)	1	Loop DIV/S5-DIV/S6	0.0028	-	VUS	-
SCN1B	c.254G>T	p.(Arg85Leu)	1	β-subunit	-	-	VUS	-
SCN2B	c.319A>C	p.(Lys107Gln)	1	β-subunit	-	-	VUS	-
	c.325G>A	p.(Asp109Asn)	1	β-subunit	0.00071	Gain-of-function $Na_v1.7$ channel	Likely pathogenic	[12]
SCN3B	c.161T>G	p.(Val54Gly)	1	β-subunit	0.0028	-	VUS	-

c.position, location cDNA; p.position, location in protein; MAF gnomAD, Minor Allele Frequency Genome Aggregation Database; VUS, Variants with uncertain clinical significance. & Variants detected were annotated according to the guidelines of the Human Genome Variation Society using reference sequence GRCh37 and transcript numbers, NM_006922.3 (*SCN3A)*; NM_002976.3 (*SCN7A);* NM_014191.3 (*SCN8A);* NM_002977.3 (*SCN9A)*; NM_006514.2 (*SCN10A)*; NM_014139.2 (*SCN11A)*; NM_001037.4 (*SCN1B)*; NM_004588.4 (*SCN2B)*; NM_018400.3 (*SCN3B).* * Two patient were heterozygous for *SCN9A* c.2794A>C, c.2971G>T and c.4612T>C.

Table S7.2 Potential pathogenic SCG variants (*SCN3A*, *SCN7A-11A*, and *SCN1B-SCN4B*) identified in patients with painless diabetic peripheral neuropathy (painless-DPN, n=309 patients).

Gene	c.position [&]	p.position	Number of patients	Location	MAF gnomAD (%)	Additional variant characteristics	Variant classification	Ref.
SCN3A	c.1619C>T	p.(Ser540Phe)	1	Linker DI/S6-DII/S1	0.15	-	VUS	-
	c.4679G>A	p.(Arg1560Gln).	1	DIV/S2	0.0011	-	VUS	-
	c.5589G>C	p.(Glu1863Asp)	1	C-terminus	0.0014	-	VUS	-
SCN7A	c.3461C>A*	p.(Ser1154Tyr)	1	Loop DIII/S5-DIII/S6	0.010	-	VUS	-
	c.4072C>T	p.(Arg1358Cys)	1	DIV/S4	0.026	-	VUS	-
	c.4865G>A	p.(Arg1622Gln)	1	C-terminus	0.17	-	VUS	-
SCN8A	c.1475G>A	p.(Arg492His)	1	Linker DI/S6-DII/S1	0.0021	-	VUS	-
	c.4748T>C*	p.(Ile1583Thr)	1	Loop DIV/S2-DIV/S3	0.020	-	VUS	-
SCN9A	c.2215A>G	p.(Ile739Val)	2	DII/S1	0.25	Gain-of-function	VUS (risk factor)	-
	c.2794A>C /	p.(Met932Leu) /	1	DII/S6	3.39	Gain-of-function $Na_v1.7$ channel	Pathogenic	-
	c.2971G>T	p.(Val991Leu)		Linker DII/S6-DIII/S1	3.01			
	c.2819T>C	p.(Val940Ala)	1	Loop DII/S5-DII/S6	0.024	-	VUS	-
	c.2969A>G	p.(Tyr990Cys)	1	Linker DII/S6-DIII/S1	0.060	-	VUS	-
	c.3799C>G	p.(Leu1267Val)	2	DIII/S3	0.13	-	VUS	-
	c.4288A>G	p.(Ile1430Val)	1	Loop DIII/S5-DIII/S6	0.00076	-	VUS	
	c.5458G>T	p.(Val1820Phe).	1	C-terminus	0.0014	-	VUS	-
SCN10A	c.41G>T*	p.(Arg14Leu)	3	N-terminus	0.19	-	VUS	-
	c.368C>T	p.(Ala123Val)	1	N-terminus	0.017	-	VUS	-
	c.472T>G /	p.(Tyr158Asp) /	2	DI/S2	0.026	Gain-of-function $Na_v1.8$ channel	Likely pathogenic	-
	c.2441G>A	p.(Arg814His)		Loop DII/S5-DII/S6	0.031			

Table S7.2 (continued)

Gene	c.position [&]	p.position	Number of patients	Location	MAF gnomAD (%)	Additional variant characteristics	Variant classification	Ref.
SCN10A	c.1079G>A	p.(Arg360His)	1	Loop DI/S5-DI/S6	0.025	-	VUS	-
	c.1138G>A	p.(Val380Ile)	1	DI/S6	0.058	-	VUS	-
	c.1141A>G	p.(Ile381Val)	2	DI/S6	0.062	-	Likely pathogenic	-
	c.1249_1251del	p.(Lys417del)	1	Linker DI/S6-DII/S1	0.081	-	VUS	-
	c.1489C>T*	p.(Arg497Cys)	1	Linker DI/S6-DII/S1	0.0048	-	VUS	-
	c.2367C>A*	p.(Asn789Lys)	1	Loop DII/S4-DII/S5	0.0021	-	VUS	-
	c.2530C>T	p.(Arg844Cys)	1	Loop DII/S5-DII/S6	0.0032	-	VUS	-
	c.3803G>A	p.(Arg1268Gln)	2	Loop DIII/S4-DIII/S5	0.19	-	VUS	-
	c.3757C>T	p.(Arg1253Cys)	1	DIII/S4	0.0032	-	VUS	-
	c.4568G>A	p.(Cys1523Tyr)	4	DIV/S2	0.11	DRG neuron hyperexcitability	Likely pathogenic	-
	c.4736G>A*	p.(Arg1579Gln)	1	DIV/S4	0.0085	-	VUS	-
	c.5216A>T	p.(Asp1739Val)	1	C-terminus	0.00040	-	VUS	-
	c.5657C>T*	p.(Ala1886Val)	1	C-terminus	0.12	Gain-of-function $Na_v1.8$ channel	VUS	-
SCN11A	c.712C>T	p.(Arg238Cys)	1	DI/S4	0.019	-	VUS	-
	c.1730C>T	p.(Pro577Leu)	1	DII/S1	0.018	-	VUS	-
	c.2379G>T	p.(Leu793Phe)	1	DII/S6	-	-	VUS	-
	c.4282G>A	p.(Gly1428Ser)	1	DIV/S3	0.020	-	VUS	-
	c.4607C>T	p.(Thr1536Ile)	1	Loop DIV/S5-DIV/S6	0.0028	-	VUS	-
SCN1B	c.457G>A	p.(Asp153Asn)	1	β-subunit	0.0067	-	VUS	-
SCN2B	c.526G>T	p.(Val176Leu)	1	β-subunit	-	-	VUS	-
	c.625_626 delinsCC*	p.(Asn209Pro)	1	β-subunit	-	-	VUS	-
SCN3B	c.583G>A	p.(Ala195Thr)	1	β-subunit	0.0099	-	VUS	-

c.position, location cDNA; p.position, location in protein; MAF gnomAD, Minor Allele Frequency Genome Aggregation Database; VUS, Variants with uncertain clinical significance. [&] Variants detected were annotated according to the guidelines of the Human Genome Variation Society using reference sequence GRCh37 and transcript numbers, NM_006922.3 (*SCN3A)*; NM_002976.3 (*SCN7A);* NM_014191.3 (*SCN8A);* NM_002977.3 (*SCN9A)*; NM_006514.2 (*SCN10A)*; NM_014139.2 (*SCN11A)*; NM_001037.4 (*SCN1B)*; NM_004588.4 (*SCN2B)*; NM_018400.3 (*SCN3B).* * Four patients were respectively heterozygous for *SCN7A* c.3461G>A and *SCN10A* c.1489C>T, *SCN8A* c.4748T>C and *SCN10A* c.41G>T, *SCN10A* c.2367C>A and *SCN10A* c.4736G>A, and *SCN10A* c.5657C>T and *SCN2B* c.625_626delinsCC.

Table S7.3 Potential pathogenic SCG variants (*SCN3A*, *SCN7A-11A*, and *SCN1B-SCN4B*) identified in patients with painful idiopathic small fiber neuropathy (painful-SFN, n=547 patients).

Gene	c.position [&]	p.position	Number of patients	Location	MAF gnomAD (%)	Additional variant characteristics	Variant classification	Ref.
SCN3A	c.2610G>C	p.(Met870Ile)	1	Loop DII/S4-DII/S5	-	-	VUS	-
	c.3072G>C	p.(Glu1024Asp)	1	Linker DII/S6-DIII/S1	-	-	VUS	-
	c.4467_4475 delinsCAAAAATT	p.(Gln1489Hisfs*8	1	Linker DIII/S6-DIV/S1	-	-	VUS	
	c.5583G>T*	p.(Leu1861Phe)	1	C-terminus	0.0012		VUS	
SCN7A	c.1501A>C	p.(Ile501Leu)	1	DII/S1	0.01	-	VUS	
	c.2795A>C	p.(Glu932Ala)	1	DIII/S1	0.027	-	VUS	-
	c.3461C>A,	p.(Ser1154Tyr)	1	Loop DIII/S5-DIII/S6	0.010		VUS	-
	c.4072C>T	p.(Arg1358Cys)	1	DIV/S4	0.026		VUS	-
	c.4865G>A	p.(Arg1622Gln)	1	C-terminus	0.17		VUS	-
SCN8A	c.1426A>C*	p.(Ser476Arg)	2	Linker DI/S6-DII/S1	-	-	VUS	-
	c.4719G>A	p.(Met1573Ile)	1	DIV/S2	0.0056	-	VUS	-
	c.4748T>C	p.(Ile1583Thr)	1	DIV/S3	0.020		VUS	-
SCN9A	c.554G>A	p.(Arg185His)	1	Loop DI/S2-DI/S4	0.31	Co-segregation inconclusive, gain-of-function Na_v 1.7 channel	VUS (risk factor)	[9,11]
	c.684C>G	p.(Ile228Met)	4	DI/S4	0.086	Co-segregation with disease, gain-of-function Na_v 1.7 channel	Pathogenic	[9,11]
	c.910T>C	p.(Tyr304His)	1	Loop DI/S5-DI/S6	-	-	VUS	-
	c.1007A>C	p.(Asn336Thr)	1	Loop DI-S5-DI/S6	-		VUS	[11]
	c.1555G>A*	p.(Glu519Lys)	1	Linker DI/S6-DII/S1	0.047		VUS	[11]
	c.1592C>A	p.(Thr531Asn)	1	Linker DI/S6-DII/S1	-	-	VUS	[11]
	c.1859G>A	p.(Ser620Asn)	1	Linker DI/S6-DII/S1	0.00080	-	VUS	-
	c.2033C>A	p.(Ala678Glu)	1	Linker DI/S6-DII/S1	-	-	VUS	[11]
	c.2157G>C	p.(Trp719Cys)	1	Linker DI/S6-DII/S1	0.098	-	VUS	[11]
	c.2159T>A	p.(Ile720Lys)	1	Linker DI/S6-DII/S1	0.016	Co-segregation with disease, gain-of-function Na_v 1.7 channel	Pathogenic	[9,11]
	c.2215A>G	p.(Ile739Val)	7	DII/S1	0.25	Co-segregation inconclusive, gain-of-function Na_v 1.7 channel	VUS (risk factor)	[9,11]
	c.2266C>A	p.(Pro756Thr)	1	Loop DII/S1-DII/S2	0.0029	-	VUS	[11]
	c.2271G>A*	p.(Met757Ile)	1	Loop DII/S1-DII/S2	0.0041	-	VUS	[11]

Table S7.3 (continued)

Gene	c.position &	p.position	Number of patients	Location	MAF gnomAD (%)	Additional variant characteristics	Variant classification	Ref.
SCN9A	c.2567G>A	p.(Gly856Asp)	1	Loop DII/S4-DII/S5	-	Co-segregation with disease, gain-of-function Na_v 1.7 channel	Pathogenic	9,11
	c.2759C>A	p.(Thr920Asn)	1	Loop DII/S5-DII/S6	-	-	VUS	-
	c.2794A>C /	p.(Met932Leu) /	7	DII/S6	3.39	Co-segregation with disease, gain-of-function Na_v1.7 channel	Pathogenic	9,11
	c.2971G>T*	p.(Val991Leu)		Linker DII/S6-DIII/S1	3.01			
	c.2969A>G	p.(Tyr990Cys)	2	Linker DII/S6-DIII/S1	0.060		VUS	11
	c.3689T>C	p.(Met1230Thr)	1	DIII/S2	-	-	VUS	11
	c.3799C>G*	p.(Leu1267Val)	9	DIII/S4	0.13	?	VUS	9,38
	c.3836G>A	p.(Arg1279Gln)	1	DIII/S4	0.0036	-	VUS	11
	c.4612T>C*	p.(Trp1538Arg)	5	DIV/S2	0.20	Co-segregation with disease, gain-of-function Na_v 1.7 channel	Pathogenic	11,24
	c.4970A>C	p.(Tyr1657Ser)	1	DIV/S5	-	-	Likely pathogenic	11
SCN10A	c.41G>T	p.(Arg14Leu)	2	N-terminus	0.19	Co-segregation inconclusive	VUS	11
	c.1138G>A	p.(Val380Ile)	2	DI/S6	0.058	-	VUS	-
	c.1141A>G	p.(Ile381Val)	1	DI/S6	0.062		Likely pathogenic	11
	c.2221C>G	p.(Leu741Val)	1	DII/S3	0.0088	-	VUS	11
	c.2737G>A	p.(Ala913Thr)	1	Linker DII/S6-DIII/S1	0.033	-	VUS	11
	c.2972C>T	p.(Pro991Leu)	4	Linker DII/S6-DIII/S1	0.094	Co-segregation inconclusive (controleren)	VUS	11
	c.3482T>C	p.(Met1161Thr)	1	DIII/S1	0.019	-	Likely pathogenic	11
	c.3803G>A	p.(Arg1268Gln)	1	Loop DIII/S4-DIII/S5	0.19	-	VUS	11
	c.3910G>A	p.(Ala1304Thr)	2	DIII/S5	0.0046	Gain-of-function Na_v1.8 channel	Likely pathogenic	9,11
SCN10A	c.4562G>A	p.(Gly1521Asp)	1	DIV/S2	-	-	VUS	11
SCN11A	c.4568G>A	p.(Cys1523Tyr)	3	DIV/S2	0.11	DRG neuron hyperexcitability	Likely pathogenic	9,11
	c.4745G>A	p.Arg1582His	1	DIV/S4	-	-	VUS	-
	c.4878G>A	p.(Met1626Ser)	1	DIV/S5	0.0046	-	VUS	11
	c.4984G>A	p.(Gly1662Ser)	1	Loop DIV/S5-DIV/S6	0.14	Co-segregation with disease, gain-of-function Na_v1.8 channel	Pathogenic	[9,11]
	c.5116A>G	P.(Ile1706Val)	1	DIV/S6	-	Gain-of-function Na_v1.8 channel	Likely pathogenic	11,25
	c.5263G>A	p.Ala1755Thr	1	C-terminus	0.00080	-	VUS	-
	c.5588G>A	p.(Arg1863Gln)	1	C-terminus	0.0024	-	VUS	-

Table S7.3 (continued)

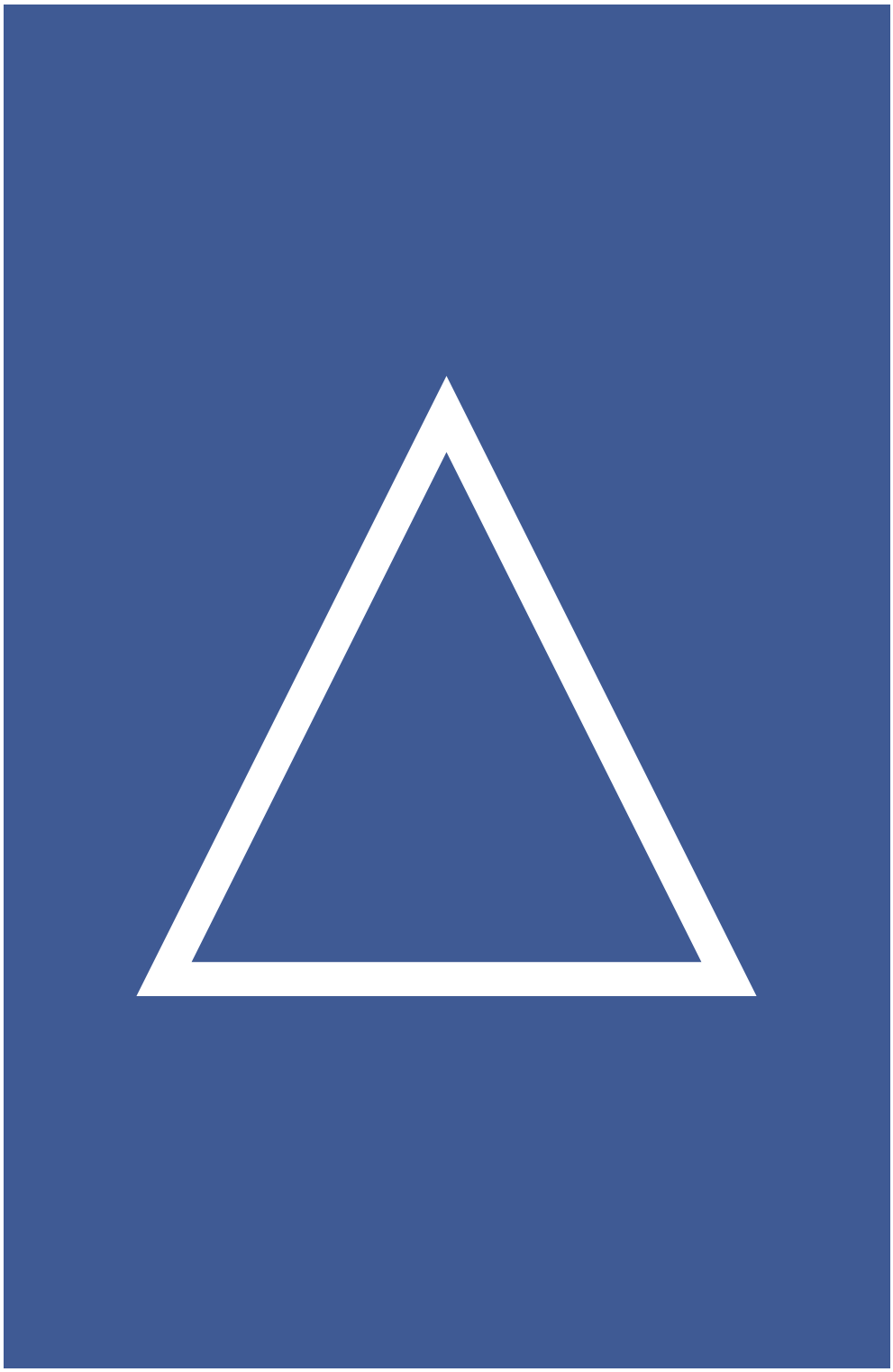
Gene	c.position [&]	p.position	Number of patients	Location	MAF gnomAD (%)	Additional variant characteristics	Variant classification	Ref.
SCN10A	c.95C>T	p.(Ala32Val)	1	N-terminus	0.063	-	VUS	[11]
SCN11A	c.1142T>C*	p.(Ile381Thr)	1	DI/S6	0.00080	Gain-of-function $Na_v1.9$ channel	Likely pathogenic	[11,25]
	c.1257G>T	p.(Lys419Asn)	3	Linker DI/S6-DII/S1	0.056	-	VUS	[11,25]
	c.1560G>T*	p.(Gln520His)	1	Linker DI/S6-DII/S1	0.0028	-	VUS	[11]
	c.1744G>A	p.(Ala582Thr)	2	DII/S1	0.015		VUS	[11,25]
	c.2042C>A	p.(Ala681Asp)	1	DII/S4	-	Loss-of-function $Na_v1.9$ channel	VUS	[11,25]
	c.2045A>T	p.(Lys682Ile)	1	DII/S4	-		VUS	-
	c.2095G>A	p.(Gly699Arg)	1	Loop DII/S4-DII/S5	0.016	Gain-of-function $Na_v1.9$ channel	Likely pathogenic	[11,54]
	c.2524G>C	p.(Ala842Pro)	1	Linker DII/S6-DIII/S1	0.0012	-	VUS	[11,25]
SCN11A	c.3473T>C*	p.(Leu1158Pro)	2	DIII/S4	0.047	Gain-of-function $Na_v1.9$ channel	Likely pathogenic	[11,25]
SCN1B	c.3506A>G	p.Asn1169Ser	1	Loop DIII/S4-DIII/S5	0.033	-	VUS	-
SCN2B	c.4049G>A	p.(Arg1350Gln)	1	Linker DIII/S6-DIV/S1	0.0039	-	VUS	[11]
SCN3B	c.4057-1G>A	p.?	2	Linker DIII/S6-DIV/S1	-	-	VUS	[11,25]
SCN4B	c.5327G>A	p.(Gly1776Glu	1	C-terminus	-	-	VUS	-
	c.632G>A	p.(Cys211Tyr)	1	β-subunit	0.039	-	VUS	-
	c.205T>C	p.(Tyr69His)	1	β-subunit	0.0049	DRG neuron hyperexcitability	Likely pathogenic	-
	c.502G>T	p.(Gly168Cys)	1	β-subunit	0.00080	-	VUS	-
	c.147G>A	p.(Met49Ile)	1	β-subunit	-	-	VUS	-
	c.592_593+1 delAAG	p.(Lys198 Glufs*11)	1	β-subunit	0.0020	-	VUS	-

c.position, location cDNA; p.position, location in protein; MAF gnomAD, Minor Allele Frequency Genome Aggregation Database; VUS, Variants with uncertain clinical significance. [&] Variants detected were annotated according to the guidelines of the Human Genome Variation Society using reference sequence GRCh37 and transcript numbers, NM_006922.3 (*SCN3A)*; NM_002976.3 (*SCN7A)*, NM_014191.3 (*SCN8A),* NM_002977.3 (*SCN9A)*; NM_006514.2 (*SCN10A)*; NM_014139.2 (*SCN11A)*; NM_001037.4 (*SCN1B)*; NM_004588.4 (*SCN2B)*;, NM_018400.3 (*SCN3B)*; NM_174934.4(*SCN4B)*. * One patient was heterozygous for *SCN3A* c.5583G>T, *SCN9A* c.2794A>C, c.2971G>T, c.4612T>C and *SCN11A* c.1560G>T; two patients were heterozygous for *SCN8A* c.1426A>C and *SCN9A* c.2794A>C, c.2971G>T,c.4612T>C, one patient was heterozygous for *SCN9A* c.2794A>C, c.2971G>T and c.4612T>C, three patients were heterozygous for *SCN9A* c.2794A>C and c.2971G>T, one patient was heterozygous for *SCN9A* c.1555G>A and c.2271G>A and *SCN11A* c.3473T>C, and one patient was heterozygous for *SCN9A* c.3799C>G and *SCN11A* c.1142T>C.

Table S7.4 Potential pathogenic SCG variants (*SCN3A*, *SCN7A-11A*, and *SCN1B-4B*) identified in patients with painless small fiber neuropathy (painless-SFN, n=32 patients).

Gene	c.position [&]	p.position	Number of patients	Location	MAF gnomAD (%)	Additional variant characteristics	Variant classification	Ref.
SCN3A	c.2077A>G	p.(Met693Val)	1	Linker DI/S6-DII/S1	0.032	-	VUS	-
	c.5583G>T*	p.(Leu1861Phe)	1	C-terminus	0.0012		VUS	-
SCN9A	c.2794A>C /	p.(Met932Leu) /	1	DII/S6	3.39	Gain-of-function $Na_v1.7$ channel	Pathogenic	[9,11]
	c.2971G>T*	p.(Val991Leu)		Linker DII/S6-DIII/S1	3.01			
	c.4612T>C*	p.(Trp1538Arg)	1	DIV/S2	0.20	Gain-of-function Na_v 1.7 channel	Pathogenic	[11,24]
SCN10A	c.3674T>C	p.(Ile1225Thr)	1	DIII/S3	0.055	-	VUS	[11]
	c.4568G>A	p.(Cys1523Tyr)	1	DIV/S2	0.11	DRG neuron hyperexcitability	Likely pathogenic	[9,11]
SCN11A	c.1560G>T*	p.(Gln520His)	1	Linker DI/S6-DII/S1	0.0028	-	VUS	[11]
	c.1744G>A	p.(Ala582Thr)	1	DII/S1	0.015		VUS	[11,25]
SCN4B	c.298C>T	p.(Arg100Cys)	1	β-subunit	0.0032	-	VUS	-

c.position, location cDNA; p.position, location in protein; MAF gnomAD, Minor Allele Frequency Genome Aggregation Database; VUS, Variants with uncertain clinical significance. [&] Variants detected were annotated according to the guidelines of the Human Genome Variation Society using reference sequence GRCh37 and transcript numbers, NM_006922.3 (*SCN3A)*; NM_002977.3 (*SCN9A)*; NM_006514.2 (*SCN10A)*; NM_014139.2 (*SCN11A);* NM_174934.4(*SCN4B).* * One patient was heterozygous for *SCN3A* c.5583G>T, *SCN9A* c.2794A>C, c.2971G>T, *SCN9A* c.4612T>C and *SCN11A* c.1560G>T.



CHAPTER 8

PAIN TRIANGLE PHENOMENON IN POSSIBLE ASSOCIATION WITH SCN9A: A CASE REPORT

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Abstract

Background

Voltage-gated sodium channels are essential for the generation and conduction of electrical impulses in excitable cells. Sodium channel $Na_v1.7$, encoded by the *SCN9A*-gene, has been of special interest in the last decades because missense gain-of-function mutations have been linked to a spectrum of neuropathic pain conditions, including inherited erythermalgia (IEM), paroxysmal extreme pain disorder (PEPD), and small fiber neuropathy (SFN).

Methods

In this case report, we present a 61-year old woman who was referred to our tertiary referral center in a standard day care setting with a suspicion of SFN. We performed additional investigations: skin biopsy to determine the intra-epidermal nerve fiber density (IENFD), quantitative sensory testing (QST) and blood examination (including DNA-analysis) for possible underlying conditions.

Results

The patient showed a clinical picture that fulfilled criteria of IEM, PEPD, and SFN. DNA-analysis revealed the heterozygous variant c.554G>A in the *SCN9A*-gene (OMIM 603415). This variant has already been described in all three human pain conditions separately, but never in one patient having symptoms of all three conditions. Because its pathogenicity has never been functionally confirmed, the variant is classified as a variance of unknown significance (VUS)/risk factor. This suggests that another genetic and/or environmental substrate play a role in the development in neuropathic conditions like described.

Conclusion

We have described this as the *SCN9A*-pain triangle phenomenon. Treatment should focus on pain management, genetic counseling, and improving/maintaining quality of life by treating symptoms and, if indicated, starting a rehabilitation program.

Introduction

Previous research showed that changes in the expression of voltage-gated sodium channels (VGSC) play a key role in the pathogenesis of neuropathic pain and that drugs that block these channels are potentially therapeutic.[1] These channels are found in the peripheral dorsal root ganglion (DRG), trigeminal ganglion, and sympathetic ganglion neurons.

Gain-of-function mutations in VGSC $Na_v1.7$, that promote neuronal hyperexcitability, can cause human pain conditions. These pain syndromes are inherited erythermalgia (IEM), paroxysmal extreme pain disorder (PEPD), and small fiber neuropathy (SFN). IEM is a rare disorder characterized by recurrent attacks of red, warm and painful hands, and/or feet, with onset of symptoms during the first decade.[2-7] PEPD is known for its severe pain episodes and skin flushing starting in infancy and induced by perianal probing or bowel movement. The pain progresses to ocular and mandibular areas with age.[8-12] SFN patients typically manifest neuropathic pain in distal extremities, as well as autonomic dysfunction.[13,14] All these conditions are linked to *SCN9A* missense variants. Pathogenic mutations in *SCN9A*, which encodes the VGSC $Na_v1.7$, have been found. IEM is usually caused by enhanced $Na_v1.7$ channel activation, whereas mutations that alter steady-state fast inactivation often lead to PEPD.

In SFN, functional variants of $Na_v1.7$ impairs channel slow inactivation and produces DRG neuron hyperexcitability that contributes to pain. Characteristics of *SCN9A* variants differ between and within the mentioned pain syndromes. Moreover, the same clinical phenotype may be associated with multiple different variants, and a single $Na_v1.7$ variant can be associated with a range of clinical phenotypes.[14] In this case report, we present a patient with a clinical picture of IEM, PEPD and SFN, which never have been reported in this combination. Symptoms could be explained by an *SCN9A*-variant, c.554G>A (OMIM 603415).

Case report

A 61-year old woman was referred to the neurological outpatient clinic because she experienced neuropathic pain in her feet, which started four years ago. The pain in her feet became worse during walking. She described the pain as a burning and tingling sensation. A couple of months after initiation, the complaints extended to her legs. Furthermore, she developed complaints of itch all over her

body, especially in her lower back and lower legs. Dysautonomic symptoms were present, such as hyperhidrosis during the night, hot flashes and palpitations. Sometimes she had complaints of obstipation.

Furthermore, she described attacks of dull rectal pain, particularly during the nights and after defecation. Sometimes, she has attacks of allodynia in her legs and feet, only present on the left side of her body.

She preferred cold circumstances and walking barefoot at home. She sleeps with air-conditioning at the footboard of her bed; even more, she daily brings cold packs to her working place to put these occasionally on her legs in order to reduce the burning sensation. Pressure on her feet during lying or walking with shoes are painful for her. She is not able to use a conventional bike because of the pressure on the pedals and rides more comfortably with an e-bike.

Three years ago, the patient experienced painful feet with attacks of redness. Attacks last for 90 minutes and disappear with rest. Situational factors such as warm weather and/or exercise worsened these pain attacks. She was diagnosed with primary erythromelalgia. Clonidin was started to reduce the pain; however, it had no effect. Subsequently, amitriptylin was started, up to 25 mg ante noctem. She experienced side-effects such as nausea and vertigo. The dosage was lowered to 15 mg which resulted in some pain relief during the night. Yet, she was not able to sleep more than 3-4 hours, because pain intensity remained relatively high.

The patient has a daughter and three sisters, without similar complaints. Her grandmother had diabetes mellitus type II without neuropathic pain symptoms.

Level of daily functioning

The patient works for more than 40 years in a factory where she has been exposed to toxic fluids and gases. She still works despite of her pain. She is not able to walk longer than 12 minutes due to painful feet. Driving a car is possible but may lead to dangerous situations if she gets into a pain attack. She and her husband adjusted their lives to the pain by avoiding activities that aggravate the pain, such as long walks, dining out, and city trips. However, they do everything to maintain their Quality of Life.

Clinical assessment and additional investigations

During neurological examination, there were no indication for a central nervous system problem or involvement of large nerve fiber pathology. During examination, red feet and a dry skin of the lower legs were noticed.

Besides a high Vitamin B12 (>1000 pmol/L), other laboratory abnormalities that could be associated with SFN-like symptoms were not found.[15]

Nerve conduction studies to test large nerve fibers were normal. Quantitative sensory testing (QST), according to the Levels method, showed an abnormal warmth sensation in both feet. Furthermore, the IENFD was 5.6/mm, which was normal according to the reported normative values (5th percentile: 3.2/mm; median: 8.7/mm).[16]

DNA analysis of the *SCN9A*, *SCN10A* and *SCN11A*, by means of the Molecular Inversion Probes-Next generation sequencing (MIPs-NGS)[17], showed the following variant: c.554G>A (version number AB839087.1) Iin the *SCN9A* gene, which is a gain-of-function variant which enhanced resurgent currents within the DRG neurons and renders DRG neurons hyperexcitable.[18,19] The variant is classified as a variant of unknown significance(VUS)/risk factor[13], according to the Practice Guidelines by the Association for Clinical Genetic Science (ACGS) and recommendations by Waxman *et al.*.[20]

Diagnosis

The patient was diagnosed with the combination of IEM, PEPD, and SFN, based on clinical symptoms and an abnormal QST, and could be associated with the variant c.554G>A in the *SCN9A*-gene.

Discussion

In literature, the *SCN9A*-variant c.554G>A is known to cause several pain syndromes, namely IEM[21], PEPD[10] and SFN.[22] In this case, signs and symptoms of all three pain syndromes that are associated with the missense mutation (c.554G>A) were present in the patient. Situational factors such as warm weather and/or exercise worsened the total package of her complaints. Therefore, this concept could be described as an '*SCN9A* pain triangle phenomenon' (see Figure 8.1).

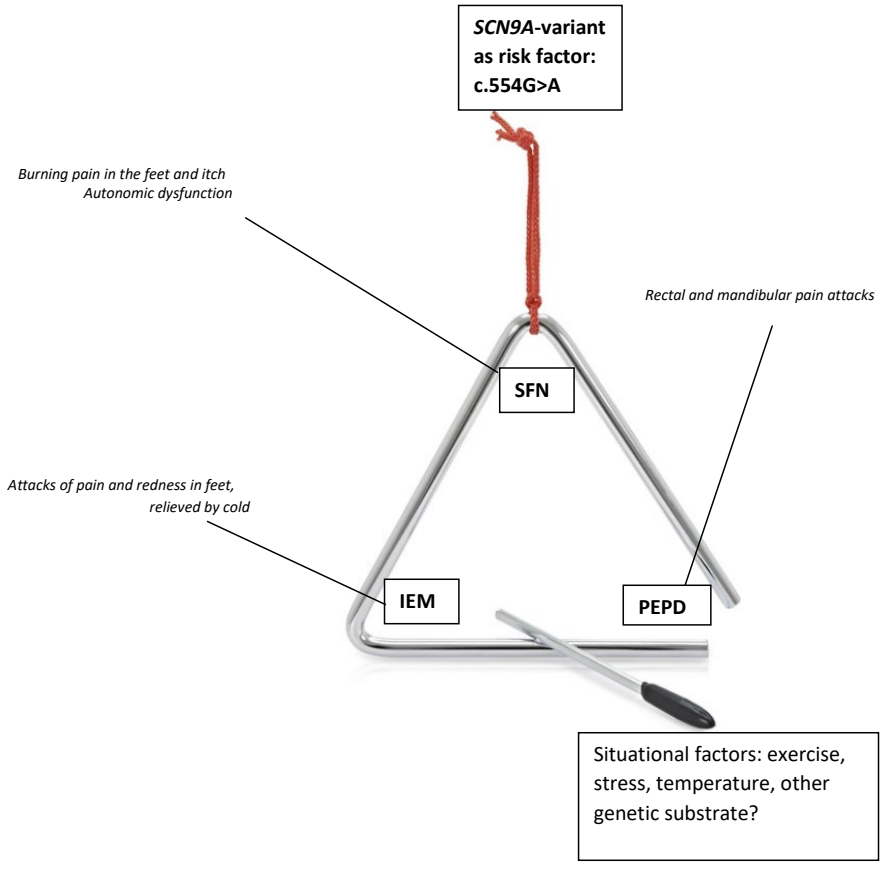


Figure 8.1 The SCN9A-pain triangle phenomena.

However, some doubt on its pathogenicity could bel raised due to its relatively high frequency in the general population (around 0.3% in total population and around 0.24% in European-non Finnish population. Data from GnomADv2.1.1) and in healthy heterozygous carriers. Moreover, it is still an open question how the same genetic variant could cause early-onset diseases, like EM and PEPD, as well as a late-onset condition, like SFN.

The late onset of IEM and PEPD is rare. Both conditions have been linked to symptoms that start in childhood.[23,24] In this case, symptoms started with the clinical picture of SFN (burning feet after walking) and probably triggered the IEM and PEPD symptoms. Interestingly, no family members were known with identical clinical symptoms. For this reason, co-segregation research has not been

conducted. However, it would be interesting to investigate whether other genetic factors (by whole exome sequencing) could be involved in the possible missing hereditability in healthy carriers of this *SCN9A* variant and provide novelty in the explanation of genotype-phenotype correlation.

Nevertheless, it is important as a consultant to recognize (parts of) this triangle in order to put in the appropriate additional investigations and discuss treatment options. Genetic counseling is important to find out the underlying cause and/or the get informed about the future perspective and inheritance pattern for patients and their families with a pathogenic *SCN9A* mutation. Furthermore, clinical studies with $Na_v1.7$-selective blockers have started to test pain relief[1], but its clinical use is still scarce. Nowadays, the treatment of neuropathic pain should follow an algorithm not focusing on the physical aspects but also on psychological and socials aspects.[25]

Patients' perspective

Our patient was complimented and encouraged to keep her life-style as active as possible. She accepted the illness as it is. Other pain medication such as pregabaline and carbamazepine was offered, but she was reluctant in taking pain medication due to its side effects. She tries to focus on activities which give her relaxation and energy such as playing the piano, reading books and working in the garden. She was informed to contact a rehabilitation consultant when she experienced significant limitations in her daily activities within her household and/or on society level.

Main conclusion and relevance

This is the first case that describes a combination of signs and symptoms of IEM, PEPD, and SFN. With genetic diagnostic screening, a missense variant in the *SCN9A* gene was found, which is classified as VUS/risk factor. This means that other genetic factors might play a role in this genotype-phenotype correlation. Previously, these three pain conditions have been linked separately to the same variant. In complicated pain conditions, it is evident to focus on the sound and loudness that the triangle produces. Perhaps more importantly, attention for factors that determine its tone and volume and its position in the societal orchestra are inevitable.

The patient gave informed consent to share her clinical story for educational and/or scientific purposes.

References

1. de Greef BT, et al. Efficacy, safety, and tolerability of lacosamide in patients with gain-of-function Nav1.7 mutation-related small fiber neuropathy: study protocol of a randomized controlled trial-the LENSS study. Trials. 2016;17(1):306.
2. Drenth JP, et al. SCN9A mutations define primary erythermalgia as a neuropathic disorder of voltage gated sodium channels. J Invest Dermatol. 2005;124(6):1333-8.
3. Arthur L, et al. Pediatric Erythromelalgia and SCN9A Mutations: Systematic Review and Single-Center Case Series. J Pediatr. 2019;206:217-224 e9.
4. Cheng X, et al. Mutation I136V alters electrophysiological properties of the Na(v)1.7 channel in a family with onset of erythromelalgia in the second decade. Mol Pain. 2008;4:1.
5. Dib-Hajj SD, et al. Gain-of-function mutation in Nav1.7 in familial erythromelalgia induces bursting of sensory neurons. Brain. 2005;128(Pt 8):1847-54.
6. Estacion M, et al. A new Nav1.7 mutation in an erythromelalgia patient. Biochem Biophys Res Commun. 2013;432(1):99-104.
7. Harty TP, et al. Na(V)1.7 mutant A863P in erythromelalgia: effects of altered activation and steady-state inactivation on excitability of nociceptive dorsal root ganglion neurons. J Neurosci. 2006;26(48):12566-75.
8. Dib-Hajj SD, et al. Paroxysmal extreme pain disorder M1627K mutation in human Nav1.7 renders DRG neurons hyperexcitable. Mol Pain. 2008;4:37.
9. Jarecki BW, et al. Alternative splicing of Na(V)1.7 exon 5 increases the impact of the painful PEPD mutant channel I1461T. Channels (Austin). 2009;3(4):259-67.
10. Meglic A, et al. Painful micturition in a small child: an unusual clinical picture of paroxysmal extreme pain disorder. Pediatr Nephrol. 2014;29(9):1643-6.
11. Suter MR, et al. p.L1612P, a novel voltage-gated sodium channel Nav1.7 mutation inducing a cold sensitive paroxysmal extreme pain disorder. Anesthesiology. 2015;122(2):414-23.
12. Yiangou Y, et al. Voltage-gated ion channel Nav1.7 innervation in patients with idiopathic rectal hypersensitivity and paroxysmal extreme pain disorder (familial rectal pain). Neurosci Lett. 2007; 427(2):77-82.
13. Eijkenboom I, et al. Yield of peripheral sodium channels gene screening in pure small fibre neuropathy. J Neurol Neurosurg Psychiatry. 2019;90(3):342-52.
14. Sopacua M, et al. Small-fiber neuropathy: Expanding the clinical pain universe. J Peripher Nerv Syst. 2019;24(1):19-33.
15. de Greef BTA, et al. Associated conditions in small fiber neuropathy - a large cohort study and review of the literature. Eur J Neurol. 2018;25(2):348-55.
16. Lauria 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.
17. Almomani R, et al. Evaluation of molecular inversion probe versus TruSeq(R) custom methods for targeted next-generation sequencing. PLoS One. 2020;15(9):e0238467.
18. Faber CG, et al. Gain of function Nanu1.7 mutations in idiopathic small fiber neuropathy. Ann Neurol. 2012;71(1):26-39.
19. Han C, et al. Functional profiles of SCN9A variants in dorsal root ganglion neurons and superior cervical ganglion neurons correlate with autonomic symptoms in small fibre neuropathy. Brain. 2012;135(Pt 9):2613-28.
20. Waxman SG, et al. Sodium channel genes in pain-related disorders: phenotype-genotype associations and recommendations for clinical use. Lancet Neurol. 2014;13(11):1152-60.
21. Goldberg YP, et al. Treatment of Na(v)1.7-mediated pain in inherited erythromelalgia using a novel sodium channel blocker. Pain. 2012;153(1):80-5.
22. Han C, et al. Nav1.7-related small fiber neuropathy: impaired slow-inactivation and DRG neuron hyperexcitability. Neurology. 2012;78(21):1635-43.
23. Arthur L, et al. Pediatric Erythromelalgia and SCN9A Mutations: Systematic Review and Single-Center Case Series. J Pediatr. 2019;206:217-24.e9.

24. Fertleman CR, et al. Paroxysmal extreme pain disorder (previously familial rectal pain syndrome). Neurology. 2007;69(6):586-95.
25. Brouwer BA, et al. Neuropathic Pain due to Small Fiber Neuropathy in Aging: Current Management and Future Prospects. Drugs Aging. 2015;32(8):611-21.

IV

PART 4

GENERAL DISCUSSION, IMPACT AND SUMMARY

CHAPTER 9

GENERAL DISCUSSION, CONCLUSIONS AND FUTURE PERSPECTIVES

According to the international criteria, the diagnosis small fiber neuropathy (SFN) is based on clinical symptoms and signs in combination with a reduced intra-epidermal nerve fiber density (IENFD) in skin biopsy and/or abnormal temperature threshold testing (TTT) levels.[1]

There is no golden standard for diagnosing SFN. To test whether assessments can improve the diagnostic algorithm, we intended to test non-invasive tools and determine whether they could contribute to the diagnosis of SFN (part I). In part II, we explored the significance of genetic screening in SFN.

Summary

After the general introduction and the outline of this thesis, as presented in **chapter 1**, we gave a review of the clinical presentations, actual diagnostic tools and genetic mutations, presenting SFN as part of a continuum in the genetic pain landscape, with Paroxysmal Extreme Pain Disorder (PEPD) and Inherited Erythermyalgia (IEM) as other diseases (**chapter 2**).[2]

In **part I & part II**, we outlined techniques that could be part of the diagnostic tools, to set the diagnosis of SFN.

In **chapter 3,** we tested whether cornea confocal microscopy (CCM), a non-invasive ophthalmic diagnostic technique, can identify peripheral neurodegeneration in patients with the suspicion of SFN. We found a moderate to high reproducibility for the manual analysis method; a high correlation was found with the full automated analysis for corneal nerve fiber length (CNFL) and corneal nerve fiber density (CNBD). However, we concluded that the CCM has no additional value in the diagnostic process to detect pure SFN, according to its moderate specificity (60.5%) and low sensitivity (39.3%) in comparison to the actual reference tests.

In **chapter 4**, we concluded that the 5-point scale method to assess the degree of skin wrinkling of the fingertips after application with EMLA cream is not reliable for clinical use. Therefore, we introduced the Digit Wrinkle Scan to test whether this could increase the reliability of the examination of the wrinkles that are present. After evaluation in 82 participants, we concluded that this digitalized technique does not work optimally (yet) for the application in clinical practice in order to detect autonomic dysfunction.

In **part III** of this thesis, genetic variants' frequencies were reported in both a national, and international setting. We presented the prevalence of genetic variants in pure SFN patients in the Netherlands and the prevalence of genetic variants in diabetic painless, diabetic painful, and non-diabetic SFN patients on European level. Lastly, we presented a case study with an unusual clinical picture in the presence of an *SCN9A*-gene variant.

In **chapter 5**, we aimed to develop a reliable method to re-sequence multiple genes in a large cohort of painful neuropathy patients at low cost.[3] We compared sensitivity, specificity, targeting efficiency, performance and cost effectiveness of Molecular Inversion Probes-Next generation sequencing (MIPs-NGS) and TruSeq® Custom Amplicon-Next generation sequencing (TSCA-NGS). Capture probes were designed to target nine sodium channel genes (*SCN3A, SCN8A-SCN11A*, and *SCN1B-SCN4B*). The average targeted regions coverage for MIPs-NGS was 97.3% versus 93.9% for TSCA-NGS. MIPs-NGS has a more versatile assay design and is more flexible than TSCA-NGS. The cost of MIPs-NGS is >5 times cheaper than TSCA-NGS when 500 or more samples are tested. In conclusion, MIPs-NGS is a reliable, flexible, and relatively inexpensive method to detect genetic variations in a large cohort of patients.

In **chapter 6,** we described the frequency of *SCN9A*, *SCN10A* and *SCN11A* variants in 1139 patients with pure SFN.[4] We analyzed their clinical features and provided a rationale for genetic screening. Pathogenicity of variants was classified according to established guidelines of the Association for Clinical Genetic Science (ACGS) and frequencies were determined.[5] Patients with SFN were grouped according to the VGSC variants detected, and clinical features were compared. Among 1139 patients with SFN, 132 (11.6%) patients harbored 73 different (potentially) pathogenic voltage gated sodium channel (VGSC) variants, of which 50 were novel and 22 were found in ≥1 patient. The frequency of (potentially) pathogenic variants was 5.1% (n=58/1139) for *SCN9A,* 3.7% (n=42/1139) for *SCN10A* and 2.9% (n=33/1139) for *SCN11A*. (Potentially) pathogenic VGSC variants are present in 11.6% of patients with pure SFN. Only erythromelalgia-like symptoms and warmth-induced pain were significantly more common in patients harbouring VGSC variants. Therefore, genetic screening of *SCN9A, SCN10A* and *SCN11A* should be considered in patients with pure SFN, independently of clinical features or underlying conditions.

In **chapter 7**, we explored the genetic differences between painful diabetic neuropathy, painless diabetic neuropathy and painful idiopathic SFN. Diabetes is the most important cause of neuropathic pain. Resolving the genetic architecture of painful/painless diabetic and idiopathic SFN patients will lead to better disease

management strategies. We reported genetic variants of ten sodium channel genes (*SCN3A, SCN7A-SCN11A*, and *SCN1B-4B*), expressed in the nociceptive pathway by means of molecular Inversion Probes targeted Next Generation Sequencing approach (MIP-targeted NGS). In total, 1094 patients with painful (n=237), painless diabetic neuropathy (n=309) and painful idiopathic SFN (n=548) were included from four centers, namely the University of Manchester (United Kingdom), Deutsche Diabetes Forschungsgesellschaft EV (Germany), Fondazione IRCCS Instituto Neurologico "Carlo Besta" (Italy) and the Maastricht University Medical Center+ (the Netherlands). Variants' pathogenicity was classified according to the practice guidelines of the ACGS and co-segregation analysis. Variants were found in the three groups, with a prevalence varying between 14.9% and 18.4%. Functional assessment is essential for definitive variant pathogenicity classification.

In **chapter 8**, we presented a patient with an SCN9A-gene variant who has clinical features of erythermalgia, PEPD and SFN and introduced the concept of the *SCN9A*-triangle phenomenon.[6]

General discussion

Improving understanding of the clinical picture of SFN

The variability of the clinical presentation of SFN makes it difficult to set a diagnosis. Although the overall prevalence of SFN in the Netherlands in 2012 was estimated on 53:100.000[7], it is thought that this might be an underestimation of the real number, which makes it more important to recognize the clinical picture and its diversity for a physician or general practitioner.

Fibromyalgia (FM) is a heterogeneous syndrome that has many symptoms that overlap with those found in SFN. Last years, publications have been increased where SFN is linked with fibromyalgia.[8-10] A growing body of research has shown that approximately 40-60% of patients carrying a diagnosis of FM have evidence of small nerve fiber involvement on skin punch biopsy. However, underlying mechanisms are still unknown, and it remains unclear why symptoms may overlap in both conditions. Diagnostic tests with high specificity should be developed which help clinicians to differentiate between FM and SFN.

Besides neuropathic pain symptoms, itch is a symptom that is present in a relatively high percentage of SFN patients. Itch is defined as 'an unpleasant sensation that leads to scratch or desire to scratch', a physiological behaviour

aimed to expel mechanical and chemical irritants.[11] However, it can be pathological due to do an abnormal function of neurons.[12] Several research groups reported paroxysmal and acute itch in patients with an *SCN9A* and *SCN11A* mutation.[13,14] Pruralgia (itch along with painful sensations) on normal appearing skin, occurrence in attacks and symptomatic alleviation with cold/ice application should alert physicians for a possible neuropathic SFN-related origin of itch. A reduced IENFD can confirm the diagnosis of SFN.

Another issue, relevant for pathophysiological and clinical purposes, regards the presence and entity of autonomic abnormalities. Some patients can have subclinical autonomic impairment, whereas others show a severe invalidating involvement of gastrointestinal or cardiovascular systems. Several tests have been proposed to improve the characterization of the autonomic involvement and increase the diagnostic efficacy.[2]

A frequently asked question of patients concerns the prognosis of SFN. The natural course is still largely unknown. In a recently published study, which included 101 patients with biopsy proven SFN, with an average follow-up of 6.2 years (2 to 12 years), it was concluded that SFN is a relatively static disease with rare progression to involve large nerve fibers (11.9%) or lead to significant long-term disability (5.3%) and gait dysfunction (6.3%). Furthermore, the average number of neuropathic pain medication used per patient was more than 4 and long-term pain control remained the main challenge.[15] However, the prognosis depends on many factors, such as the presence/absence of triggers and underlying disorders. Longitudinal studies with diagnostic testing over time can provide more information about the natural course and may support development of lead to disease-modifying strategies.

Improving assessments in SFN

Since the clinical picture and the aetiology of SFN greatly vary, we should reconsider the use of the best diagnostic tools to assess SFN. Ultimately, we may strive to an algorithm of diagnostic tools, where the clinical picture forms the base of the tests that will be applied to confirm the diagnosis SFN. Furthermore, for research purposes, we should look for another classification where the diagnosis SFN is accompanied with the main clinical outcome (autonomic/sensory) and a class of aetiology (genetic, idiopathic, obtained). In this case, we could not rule out the CCM and DWS in the assessment of SFN, because of the diversity of our study population. In this case, well-defined groups with respect to clinical picture and aetiology are needed. For example, CCM as diagnostic tool should be

considered in patients with neuropathy complaints and a medical history of diabetes mellitus or an impaired glucose intolerance.[16-18]

Because a golden standard for diagnosing and classifying SFN is lacking, a series of different definitions and classifications have been suggested[1,19,20], which may explain the large variation in frequencies of SFN in different samples reported in the literature.[19,21-23]

The IENFD reflects the severity of distal axonal degeneration and is an objective and reliable tool. However, it has a moderate sensitivity to confirm the diagnosis SFN, resulting in false negative findings. It is known that IENFD decreases with age, but only one study has been performed to monitor IENFD during disease course.[24] It is conceivable that the decrease of IENFD occurs faster in SFN than in healthy people. Besides, at the moment, only the distal IENFD is used to establish the diagnosis SFN. This location is chosen because of the length-dependent general acceptance of its pattern, although non-length dependent forms have been published.[25] To show length-dependency, the proximal/distal IENFD ratio could be used. A study regarding this ratio is ongoing in our center. It is hypothesized that this ratio is higher in SFN than in healthy subjects. Both follow-up of IENFD and determination of the proximal/distal IENFD ratio may have implications for the diagnostic strategy in patients with possible SFN, because IENFD may be normal at presentation and decrease to abnormal over time, or will remain within the normal range, but might show an increased proximal/distal ratio compared with healthy people.[26] These additional approaches may increase the sensitivity of skin biopsy for the diagnosis SFN.

Publications on CCM as a diagnostic tool are rapidly increasing. However, CCM has been mainly used in patients with diabetes mellitus type 1 and type 2.[16,27-32] In the study, described in Chapter 3, we excluded patients with diabetic neuropathy, since there is a relatively high incidence of both large and small nerve fiber impairments. Our findings suggested that the CCM in an SFN group with a variety of underlying causes does not add value to existing diagnostic tools.

CCM is an elegant method to visualize nerve fibers in the cornea. However, the morphology is complicated which can cause difficulties in the validity, in terms of the differentiation of the ultrastructure of nerve bundles. It might be a tool to evaluate therapeutic outcome, which have been shown in patients with diabetes and in patients with SFN.[33,34]

Testing autonomic (dys)function in SFN is challenging. Several studies have been conducted to test whether available tests that investigate autonomic function can

be used in the diagnosis of SFN. In Chapter 2, an overview was presented of several tests that are used to examine autonomic function.[2] Until now, there is no test available that correlates with sensory information, such as IENFD. We have explored the potential value of the skin wrinkling test and examined whether it could contribute to the diagnostic pallet of SFN. Current clinical practice recognizes a 5-grade visual scale of wrinkling. However, in our study (Chapter 4), we reported that the 5-grade visual scale is not reliable enough to use it as diagnostic tool.[35] We tried to quantify wrinkle changes by using a new software program approach, the Digit Wrinkle Scan. This method showed slightly better reliability scores, but reliability was still moderate to the best and thus not useful for diagnostic purposes. Recently, ultrasound imaging might be a valuable adjunct to the visual assessment of skin wrinkling of fingertips.[36] The change of volume loss of skin wrinkling can be detected. The ultrasound measurements provide an alternative means of quantifying skin wrinkling at the level of fingertip. The count should correspond to the number of hyper-echogenic regions each surrounded by one hypo-echogenic area. When no visible wrinkling is seen after water immersion, the reduction in area of the hypoechogenic structures visualized on ultrasound provides an indirect estimate of fluid loss in the distal phalanx.

Another test that has been suggested is the Sudoscan, which is a simple, non-invasive, easy-to-perform sudomotor test.[37] It was developed to allow the measurement of sweat gland function. The test is based on the electrochemical reaction between the chloride ions in sweat and stainless steel-based plate electrodes, on which the subject's hands or feet are placed. A low-voltage current (<4 V) is applied through the electrodes, attracting chloride ions from the sweat glands (which are densely concentrated on the palms and soles). A measurement of conductance for the hands and feet is generated from the derivative current associated with the applied voltage. Sudoscan testing is entirely painless, can be conducted in 3 min, and requires no special patient or equipment preparation. Several studies have described promising results in detecting small and/or large fiber dysfunction in diabetes mellitus.[37-39] No studies have been done yet in pure SFN to determine the value of the sudoscan as a diagnostic tool. This study has recently been performed in our center.
Despite the various tests examined, and efforts made to enrich the diagnostic arsenal of tools in SFN, to date the IENFD +/- QST approach remain the adopted "gold standard". The proximal to distal ratio examination ongoing in our center, could increase as a variation the value of IENFD even further, but the results are still pending.

Understanding the pathophysiology

Understanding the pathophysiology of pain and identifying plausible specific pain genotype-phenotype relations, may lead to improved and more personalized treatment strategies. Therefore, the focus should extend beyond the scope of peripheral mechanisms of the nervous system and should include searching for pain complex central, thus brain-based, mechanisms and patterns (a pain network of somatosensory, limbic and associate structures). Previous findings suggest that SFN is not a pure peripheral nervous system disorder. Pain-related brain networks tend to break into functionally independent components, with severity linked to the degree of skin nerve degeneration.[13]

Furthermore, functional imaging studies have displayed specific brain activity patterns in patients with different types of pain syndromes.[40-42] Also, one study with a small number of SFN patients reported changes in functional connectivity.[43] However, whether a specific activation pattern can be seen depends on many factors, such as type of brain imaging modality. It is conceivable that a particular type of pain (stimulus) may enhance a specific pain brain pattern, but also specific person factors (for example gender and genetic factors) may influence the pain activation network. Psychological modulation as well as chronicity of pain may influence the activation network, and should therefore be taken into account. Furthermore, the specific location of pain can display different kinds of patterns because of partially somatotopic organization, such as described in the S1 cortex. In our research group, functional MRI is used to study differences between SFN patients with abnormal IENFD, with and without *SCN9A* mutations, and healthy controls. It would be very interesting to determine the magnitude of a peripheral nerve disorder like SFN being centrally driven.

Improving genetic assessments, functional testing and level of pathogenicity

The science of pain has witnessed tremendous advances in the last decades. In particular, studies of pain genetics have identified markers for pain sensitivity and susceptibility to chronic pain. We found that 11.6% of our SFN population has a genetic variant which could be the underlying cause or risk factor for SFN.[4]

The complexity of the relationship between hyperexcitability, neuropathy and pain in sodium channel gene mutations has recently been underscored. The $Na_v1.7$ I228M mutation is interesting because patients carrying this mutation experience its pain onset within the first three decades[44,45] and it causes a significant reduction in neurite length.[46] Also, reverse operation of the Na^+/Ca^{2+} exchange and damaged mitochondrial energy metabolism may contribute to the impaired

neurite integrity present in I228M-transfected DRG neurons.[46,47] However, all these potential pathophysiological and pathogenetic observations could be intertwined. Recently, by means of mouse lines, no evidence was found that a functional change alone induces genetically driven pain and neuropathy phenotype seen in patients with SFN carrying this mutation.[48] A multi-hit model has been proposed to underlie the length and age-dependence of onset of peripheral neuropathies. Other underlying factors, such as systemic diseases, modifier genes or environmental factors, may be required, together with a mutation, for the onset of clinical signs and symptoms of SFN.

Understanding the structure and function of the Na_v-channels is of major biophysical, as well as clinical, importance given their key role in cellular pathophysiology. Modelling the single-channel structure and its atomic porous environment.[49]

The PAIN-Net consortium aims to analyze in vitro the pathogenic potential of mutations in sodium channel genes identified in patients with neuropathic pain through clinical and genetic assessment. Also, the group has as purpose to use functional proteomics for identification and characterization of peripheral sodium channel signaling complexes.

Until this time, sodium channels were of specific interest. However, potassium channels could also be of interest. Electrical signaling is terminated by activation of voltage-gated potassium channels which activate and inactivate over a longer time domain.[50-53] Furthermore, a recent study found I228M neurons show a significant reduction in mitochondrial density and size, indicating dysfunctional mitochondria and as a consequence degeneration and loss of nerve fibers in SFN.[47]

Consequences for pharmacological treatment

Small nerve fibers have the highest regenerative capacity and prompt diagnosis and initiation of disease-modifying treatment of SFN may result in substantial regeneration and recovery, particularly in young and otherwise healthy patients.[54] Current treatments for painful neuropathies are largely inadequate. Less than 50% of patients achieve 50% of pain relief with available drugs. This disappointing effect in painful neuropathies is due to the lack both of drugs acting on target sites for which there is strong evidence of pathogenicity and the inability to identify responder patients. Most of the available analgesics act at different levels (e.g. sodium channels, noradrenergic system, opioidergic system) and are prescribed to patients suffering from pain without any selection in terms of

pathogenesis and etiology. The choice of the medication is usually empirical following a "trial and error" process, and guided by safety profile, comorbidities, and concomitant medication. New strategies are urgently needed to achieve a better control of neuropathic pain.

Immunologic mechanisms have been linked to the pathophysiology of SFN, because several immune-mediated disorders such as sarcoidosis, Sjögren disease, and systemic lupus erythematosus can be associated with SFN.[55-59] Because of the possible role of auto-immune mechanisms, treatment with intravenous immunoglobulins (IVIg) was propagated as a potential treatment.[60] However, a randomized placebo-controlled trial by our group showed no significant effect of IVIg compared to placebo in patients with painful idiopathic SFN. No significant differences were found in 1-point and 2-point responders on pain, patients' global impression of change (PGIC), overall disability, autonomic symptoms, and pain relief. Statistically significant differences were found in maximum night pain, daily sleep interference, intense and hot pain, and some domains of the quality of life; however they were inconsistent and were not considered to be clinically meaningful in the treatment goal of this highly expensive drug.[61] In a small group of patients with diabetic neuropathy, pain reduction (> 50%) four weeks after IVIG-treatment was shown in 63.6% of the experimental group.[62] However, this study had a small sample size and a short duration. The significant reduction of pain did not last at 8 weeks, and it is conceivable that the early significant difference came from other aspects.

Many chronic pain syndromes are peripheral in origin and reflect hyperactivity of peripheral pain-signalling neurons. Current treatments are ineffective or only partially effective.

Signatures of individual susceptibility to pain and analgesic responsiveness are urgently needed to improve patients´ management. For example, lacosamide was effective in pain treatment of SFN in patients with an SCN*9A*-mutation.[64] The PAIN-Net programme will contribute to tailor-made pain treatment based on next generation sequencing, whole exome sequencing, epigenetics and pharmacogenomics studies, nociceptor and sodium channel functioning based on biophysics and proteomics studies, targeted analgesics based on high-throughput screening, targeted analgesic delivery based on encapsulated cell bioreactor implants, and to the development and extensive characterization of the first knock-in mouse models of sodium channel-related neuropathic pain based on the CRISP-Cas technology.

From the clinical perspective, the goal in future research should focus on the identification and development of biomarkers for pain to enable better patient stratification, mechanism-based treatment selection and targeted prevention strategies for high-risk individuals.

Consequences for non-pharmacological treatment

In a review, it was suggested that transcranial magnetic stimulation (TMS) can produce exitatory/inhibitory stimulation of the cerebral cortex or local areas (i.e., the primary motor cortex[66,67]), promote the remodelling of the nervous system, and that is has good application prospects for the localization of the injury, neuroprotection, and the promotion of nerve regeneration. TMS can also alleviate pain symptoms by changing the cortical threshold and inhibiting the conduction of sensory information in the thalamo-spinal pathway which can have a therapeutic potential effect.[68]This will be the topic of future research in Maastricht UMC+.

Rehabilitation programme

Chronic pain is not static. It may progress or remit. Over time, the initial events lead to alternations in sensation, central sensitization and changes in affective, cognitive and emotional states.[69] Transformation from a healthy state to one that limits a patient's ability to work and enjoy life may result in a negative cascade of failure of systems that worsen the condition, launching a negative feed-forward cycle. Therefore, it is important to define chronification of pain and develop treatments that halt or reverse it at the earliest possible time. To initiate the most appropriate treatment, it is important for the clinician to clarify the request for help. In most cases, patients would like to get rid of the pain. (Partial) pain reduction can be achieved by treating the underlying cause or starting symptomatic pain treatment with medication. Additionally, a start with a rehabilitation program, specialized on chronic pain can help patients from a holistic perspective, when pain is (still) significantly disturbing their daily living on several domains, such as mobility, house-hold activities, duties at work and/or social activities. SFN patients demonstrated a severe overall reduction in QoL.[70] Pain intensity, male gender and fear of falling were positively associated with disability in patients with painful diabetic neuropathy.[71] Rehabilitation programs can focus on pain education and have continued oversight on the potential evolution of the pain condition with the help of a physical therapist, occupational therapist and/or behaviour therapist.

Patients with a chronic pain syndrome, such as SFN, are helped with a multidisciplinary approach in a rehabilitation programme, using the ICF-model

(see Figure 9.1). This model conceptualises a person's level of functioning as a dynamic interaction between her or his health conditions, environmental factors, and personal factors. It is a biopsychosocial model of disability, based on an integration of the social and medical models of disability. Follow up studies have shown that permanent changes over time only appear when there is a treatable cause of a health condition. In all other cases, patients have to live with the pain syndrome. There is no evidence for the efficacy of a rehabilitation programme for SFN; however, if SFN is considered a chronic pain syndrome and patients are focusing on functional goals which are important in their daily lives, programs such as graded activity or graded exposure could be helpful. A treatment protocol for patients with painful diabetic neuropathy has recently been suggested.[72] Integrative therapies such as mindfulness meditation or cognitive behavioural therapy (CBT) can also be part of a comprehensive pain management plan. Benefits include reduction of pain-related medication consumption, better treatment outcomes, improvement in comorbid conditions, such as anxiety and depression, as well as the lack of risk of addiction or abuse.[73-75]

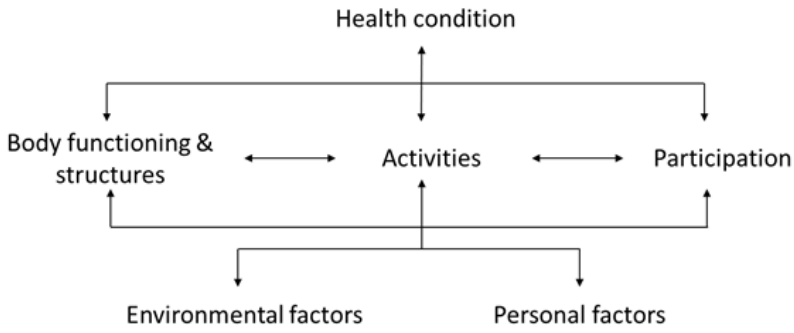


Figure 9.1 The International Classification of Functioning (ICF)-model.

Future perspectives

Improving assessments in a multifactorial way

SFN is a chronic neuropathic pain condition which is more recognized nowadays. Neuropathic pain is to be distinguished from nociceptive or inflammatory pain, although in many cases both nociceptive and neuropathic components may contribute to pain. Because of this, patients with pure SFN may be misdiagnosed with another pain syndrome. The variety of clinical symptoms (i.e. the presence of autonomic symptoms) urge for a better and more intensive collaboration between

medical specialists. Patients with the suspicion of SFN are usually referred to neurologist. Indeed, the main symptoms (neuropathic pain) are most appropriate to the field of the neurologist, but other specialists should be aware that the clinical picture of SFN can start with autonomic symptoms. For example, if patients have symptoms of idiopathic obstipation and/or diarrhea, SFN should be in the differential diagnosis. In many cases, these patients are diagnosed with irritable bowel syndrome. Furthermore, pain syndromes like FM may overlap with the clinical picture of SFN. Cooperation with the rheumatologist is important to improve knowledge and understanding of the different chronic pain syndromes. Lastly, itch can be a manifestation of neuropathy (neuropathic itch), for which patients can initially visit the dermatologist.

Understanding the natural history of SFN could be improved. For example, digital healthcare can help patients to monitor the course of the pain/disease which help physicians and/or researchers to understand the relationship between physical and additional examinations. Moreover, digital healthcare can contribute to self-management and participatory health care.

Overall, the ideal pain clinician would have expertise in pain neurobiology, behavioral medicine (including psychiatry/psychology), pharmacology and interventional procedures. Integrative care should target treatments for both symptom management and disease modification and be specific to individual patients. Moreover, greater cross-fertilization between clinicians and basic researchers would advance health care.

Genetic structure and linkage to the clinical picture

In this thesis, we presented the epidemiology of genetic mutations in our cohorts, nationally and internationally. A better description of pain phenotype-genotype can form the basis for personalized pain management, as reasons for some patients responding to treatment while other remain resistant will be better understood. Although pain can be linked to multiple genetic factors with its susceptibility, complex environmental factors also influence the individual variation in pain, such as race, ethnicity, gender and social context and interpretation of the pain experience.

Although we are a long way from understanding what triggers neuropathic pain as a disease or a symptom or from knowing how to cure it, we have an exciting opportunity to use this new knowledge about chronic pain to guide new approaches to both understanding and treating chronic pain more efficiently.

Improving pain management

Future research should focus on the identification and development of biomarkers for pain to enable better patient stratification, mechanism-based treatment selection and targeted prevention strategies for high-risk individuals. Furthermore, the use of uniform outcome measures in peripheral neuropathies, such as SFN, is important to improve the quality of randomized controlled trials, enabling comparison between studies.

While attempts at personalized medicine for chronic pain may be tailored to the molecular and genomic profile of an individual, other issues including systems biology, psychological manifestations, therapeutic interactions (placebo response) and social issues all contribute to the chronic pain state and need to be considered. Future integrative approaches need to be performed in the context of defined, quantitative or objective measures. Understanding the factors that set individual risk levels for chronic neuropathic pain, including phenotypes such as anxiety, fear avoidance and catastrophizing, is central to improve pain management.

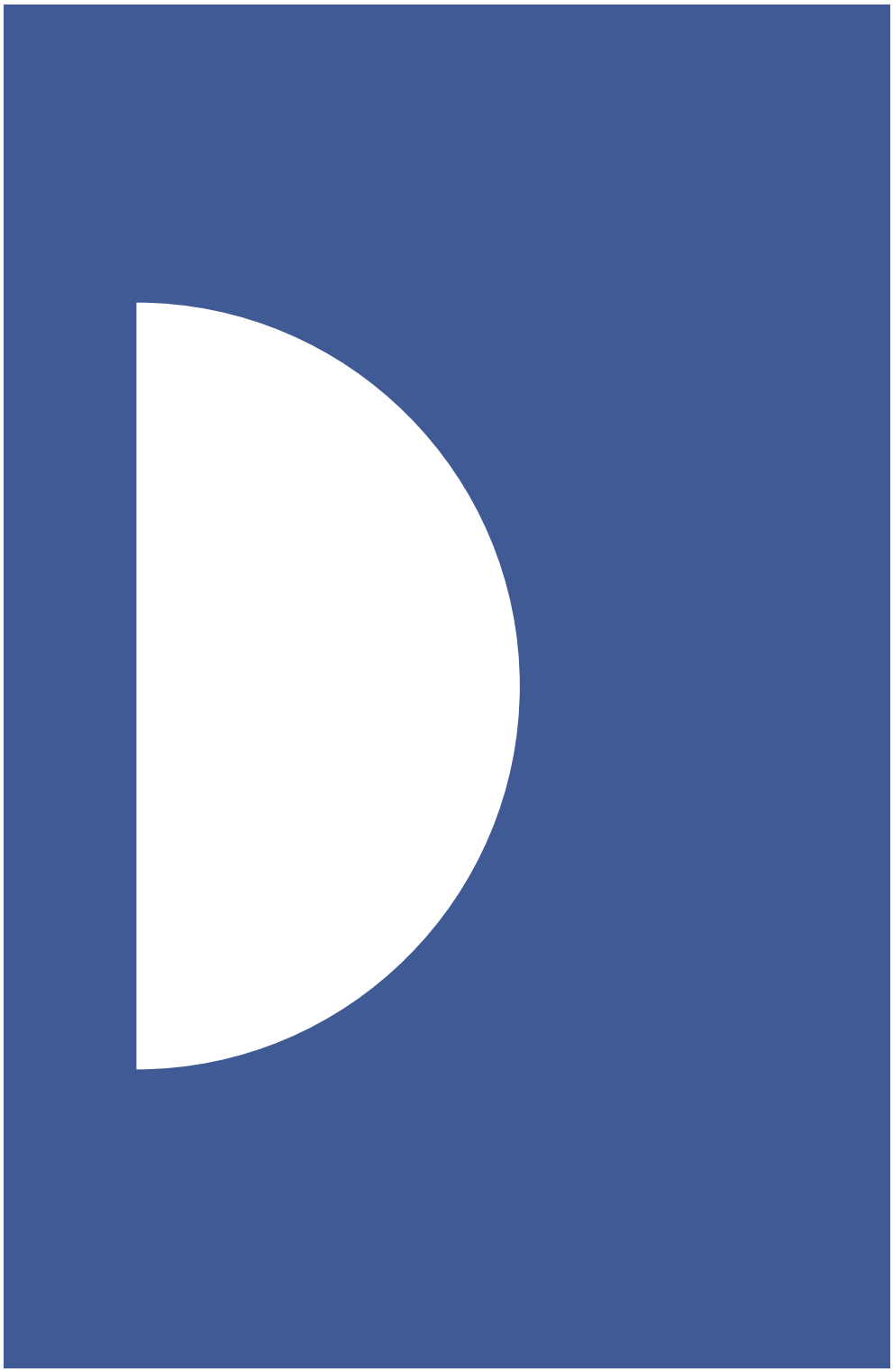
References

1. Devigili G, et al. Diagnostic criteria for small fibre neuropathy in clinical practice and research. Brain. 2019;142(12):3728-36.
2. Sopacua, M., et al., Small-fiber neuropathy: Expanding the clinical pain universe. J Peripher Nerv Syst. 2019;24(1):19-33.
3. Almomani R, et al. Evaluation of molecular inversion probe versus TruSeq(R) custom methods for targeted next-generation sequencing. PLoS One. 2020;15(9):e0238467.
4. Eijkenboom I, et al. Yield of peripheral sodium channels gene screening in pure small fibre neuropathy. J Neurol Neurosurg Psychiatry. 2019;90(3):342-52.
5. Waxman SG, et al. Sodium channel genes in pain-related disorders: phenotype-genotype associations and recommendations for clinical use. Lancet Neurol. 2014;13(11):1152-60.
6. Sopacua M, et al. Pain triangle phenomenon in possible association with SCN9A: A case report. Mol Genet Genomic Med. 2022;10(10):e2026.
7. Peters MJ, et al. Incidence and prevalence of small-fiber neuropathy: a survey in the Netherlands. Neurology. 2013;81(15):1356-60.
8. Martinez-Lavin M. Fibromyalgia and small fiber neuropathy: the plot thickens! Clin Rheumatol. 2018;37(12):3167-71.
9. Di Carlo M, Cesaroni P, Salaffi F. Neuropathic pain features suggestive of small fiber neuropathy in fibromyalgia syndrome: a clinical and ultrasonographic study on female patients. Clin Exp Rheumatol. 2021;39 Suppl 130:102-7.
10. Viceconti A, et al. Neuropathic pain and symptoms of potential small-fiber neuropathy in fibromyalgic patients: A national on-line survey. Joint Bone Spine. 2021;88(4):105153.
11. Ikoma A, et al. The neurobiology of itch. Nat Rev Neurosci. 2006;7(7):535-47.
12. Bernhard JD. Itch and pruritus: what are they, and how should itches be classified? Dermatol Ther. 2005;18(4):288-91.
13. Devigili G, et al. Paroxysmal itch caused by gain-of-function Nav1.7 mutation. Pain. 2014; 155(9):1702-7.
14. Salvatierra J, et al. A disease mutation reveals a role for NaV1.9 in acute itch. J Clin Invest. 2018;128(12):5434-47.
15. MacDonald S, et al. Longitudinal follow-up of biopsy-proven small fiber neuropathy. Muscle Nerve. 2019;60(4):376-81.
16. Azmi S, et al. Corneal Confocal Microscopy Identifies Small-Fiber Neuropathy in Subjects With Impaired Glucose Tolerance Who Develop Type 2 Diabetes. Diabetes Care. 2015;38(8):1502-8.
17. Alam U, et al. Diagnostic utility of corneal confocal microscopy and intra-epidermal nerve fibre density in diabetic neuropathy. PLoS One. 2017;12(7):e0180175.
18. Tavakoli M, et al. Corneal sensitivity is reduced and relates to the severity of neuropathy in patients with diabetes. Diabetes Care. 2007;30(7):1895-7.
19. Devigili G, et al. The diagnostic criteria for small fibre neuropathy: from symptoms to neuropathology. Brain. 2008;131(Pt 7):1912-25.
20. Freeman R, et al. Idiopathic distal sensory polyneuropathy: ACTTION diagnostic criteria. Neurology. 2020;95(22):1005-14.
21. de Greef BTA, et al. Associated conditions in small fiber neuropathy - a large cohort study and review of the literature. Eur J Neurol. 2018;25(2):348-55.
22. Truini A, et al. A cross-sectional study investigating frequency and features of definitely diagnosed diabetic painful polyneuropathy. Pain. 2018;159(12):2658-66.
23. Sumner CJ, et al. The spectrum of neuropathy in diabetes and impaired glucose tolerance. Neurology/ 2003;60(1):108-11.
24. Lauria G, et al. Side and time variability of intraepidermal nerve fiber density. Neurology. 2015; 84(23):2368-71.
25. Gemignani F, et al. Non-length-dependent small fiber neuropathy: Not a matter of stockings and gloves. Muscle Nerve. 2022;65(1):10-28.

26. Khoshnoodi MA, et al. Longitudinal Assessment of Small Fiber Neuropathy: Evidence of a Non-Length-Dependent Distal Axonopathy. JAMA Neurol. 2016;73(6):684-90.
27. Ahmed A, et al. Detection of diabetic sensorimotor polyneuropathy by corneal confocal microscopy in type 1 diabetes: a concurrent validity study. Diabetes Care. 2012;35(4):821-8.
28. Chen X, et al. Small nerve fiber quantification in the diagnosis of diabetic sensorimotor polyneuropathy: comparing corneal confocal microscopy with intraepidermal nerve fiber density. Diabetes Care. 2015;38(6):1138-44.
29. Ferdousi M, et al. Diagnosis of Neuropathy and Risk Factors for Corneal Nerve Loss in Type 1 and Type 2 Diabetes: A Corneal Confocal Microscopy Study. Diabetes Care. 2021;44(1):150-6.
30. Roszkowska AM, et al. Corneal nerves in diabetes-The role of the in vivo corneal confocal microscopy of the subbasal nerve plexus in the assessment of peripheral small fiber neuropathy. Surv Ophthalmol. 2021;66(3):493-513.
31. Dhage S, et al. Corneal confocal microscopy identifies small fibre damage and progression of diabetic neuropathy. Sci Rep 2021;11(1):1859.
32. Kalteniece A, et al. Corneal confocal microscopy detects small nerve fibre damage in patients with painful diabetic neuropathy. Sci Rep. 2020;10(1):3371.
33. Azmi S, et al. Corneal confocal microscopy shows an improvement in small-fiber neuropathy in subjects with type 1 diabetes on continuous subcutaneous insulin infusion compared with multiple daily injection. Diabetes Care. 2015;38(1):e3-4.
34. Mehra S, et al. Corneal confocal microscopy detects early nerve regeneration after pancreas transplantation in patients with type 1 diabetes. Diabetes Care. 2007;30(10):2608-12.
35. Sopacua M, et al. The applicability of the digit wrinkle scan to quantify sympathetic nerve function. Clin Neurophysiol Pract. 2022;7:115-9.
36. Gechev A. Fluid Containing Structures in the tips of the fingers and toes delineated by Ultrasound Imaging before and after Induced Skin Wrinkling. Sci Rep. 2019;9(1):1640.
37. Casellini CM, et al. Sudoscan, a noninvasive tool for detecting diabetic small fiber neuropathy and autonomic dysfunction. Diabetes Technol Ther. 2013;15(11):948-53.
38. Mao F, et al. Sudoscan is an effective screening method for asymptomatic diabetic neuropathy in Chinese type 2 diabetes mellitus patients. J Diabetes Invest. 2017;8(3):363-8.
39. Ma JJ, et al. [The clinical value of SUDOSCAN in diagnosis of diabetic distal symmetrical peripheral neuropathy]. Zhonghua Yi Xue Za Zhi. 2017;97(15):1170-4.
40. Moisset X, Bouhassira D. Brain imaging of neuropathic pain. Neuroimage. 2007;37 Suppl 1: S80-8.
41. Cauda F, et al. Altered resting state attentional networks in diabetic neuropathic pain. J Neurol Neurosurg Psychiatry. 2010;81(7):806-11.
42. Tseng MT, et al. fMRI evidence of degeneration-induced neuropathic pain in diabetes: enhanced limbic and striatal activations. Hum Brain Mapp. 2013;34(10):2733-46.
43. Hsieh PC, et al. Imaging signatures of altered brain responses in small-fiber neuropathy: reduced functional connectivity of the limbic system after peripheral nerve degeneration. Pain. 2015;156(5):904-16.
44. Faber CG, et al. Gain of function Nanu1.7 mutations in idiopathic small fiber neuropathy. Ann Neurol. 2012;71(1):26-39.
45. Estacion M, et al. Intra- and interfamily phenotypic diversity in pain syndromes associated with a gain-of-function variant of NaV1.7. Mol Pain. 2011;7:92.
46. Persson AK, et al. Neuropathy-associated Nav1.7 variant I228M impairs integrity of dorsal root ganglion neuron axons. Ann Neurol. 2013;73(1):140-5.
47. Lee SI, et al. The small fiber neuropathy NaV1.7 I228M mutation: impaired neurite integrity via bioenergetic and mitotoxic mechanisms, and protection by dexpramipexole. J Neurophysiol. 2020;123(2):645-57.
48. Chen L, et al. Two independent mouse lines carrying the Nav1.7 I228M gain-of-function variant display dorsal root ganglion neuron hyperexcitability but a minimal pain phenotype. Pain. 2021; 162(6):1758-70.

49. Xenakis MN, et al. Non-extensitivity and criticality of atomic hydropathicity around a voltage-gated sodium channel's pore: a modeling study. J Biol Phys. 2021;47(1):61-77.
50. Busserolles J, et al. Potassium channels in neuropathic pain: advances, challenges, and emerging ideas. Pain. 2016;157 Suppl 1:S7-S14.
51. Tsantoulas C, McMahon SB. Opening paths to novel analgesics: the role of potassium channels in chronic pain. Trends Neurosci. 2014;37(3):146-58.
52. Tsantoulas C. Emerging potassium channel targets for the treatment of pain. Curr Opin Support Palliat Care. 2015;9(2):147-54.
53. Tsantoulas C, et al. Sensory neuron downregulation of the Kv9.1 potassium channel subunit mediates neuropathic pain following nerve injury. J Neurosci. 2012;32(48):17502-13.
54. Bautista J, et al. Regenerative plasticity of intact human skin axons. J Neurol Sci. 2020;417: 117058.
55. Hoitsma E, et al. Improvement of small fiber neuropathy in a sarcoidosis patient after treatment with infliximab. Sarcoidosis Vasc Diffuse Lung Dis. 2006;23(1):73-7.
56. Descamps E, et al. Small fiber neuropathy in Sjogren syndrome: Comparison with other small fiber neuropathies. Muscle Nerve. 2020;61(4):515-20.
57. Gaillet A, et al. Intravenous immunoglobulin efficacy for primary Sjogren's Syndrome associated small fiber neuropathy. Autoimmun Rev. 2019;18(11):102387.
58. Tekatas A, et al. Small fiber neuropathy and related factors in patients with systemic lupus erythematosus; the results of cutaneous silent period and skin biopsy. Adv Rheumatol 2020; 60(1):31.
59. Goransson LG, et al. Small-diameter nerve fiber neuropathy in systemic lupus erythematosus. Arch Neurol. 2006;63(3):401-4.
60. Oaklander AL. Immunotherapy Prospects for Painful Small-fiber Sensory Neuropathies and Ganglionopathies. Neurotherapeutics. 2016;13(1):108-17.
61. Geerts M, et al. Intravenous Immunoglobulin Therapy in Patients With Painful Idiopathic Small Fiber Neuropathy. Neurology. 2021;96(20):e2534-45.
62. Jann S, et al. High-Dose Intravenous Immunoglobulin Is Effective in Painful Diabetic Polyneuropathy Resistant to Conventional Treatments. Results of a Double-Blind, Randomized, Placebo-Controlled, Multicenter Trial. Pain Med. 2020;21(3):576-85.
63. Brown WA. Expectation, the placebo effect and the response to treatment. R I Med J. 2015; 98(5):19-21.
64. de Greef BT, et al. Efficacy, safety, and tolerability of lacosamide in patients with gain-of-function Nav1.7 mutation-related small fiber neuropathy: study protocol of a randomized controlled trial-the LENSS study. Trials. 2016;17(1):306.
65. Rosenberg ML, et al. Evidence for Dietary Agmatine Sulfate Effectiveness in Neuropathies Associated with Painful Small Fiber Neuropathy. A Pilot Open-Label Consecutive Case Series Study. Nutrients. 2020;12(2):576.
66. Attal N, et al. Repetitive transcranial magnetic stimulation for neuropathic pain: a randomized multicentre sham-controlled trial. Brain. 2021;144(11):3328-39.
67. Lefaucheur JP, Nguyen JP. A practical algorithm for using rTMS to treat patients with chronic pain. Neurophysiol Clin. 2019;49(4):301-7.
68. Xu X, Xu DS. Prospects for the application of transcranial magnetic stimulation in diabetic neuropathy. Neural Regen Res. 2021;16(5):955-62.
69. Maihofner C, Nickel FT, Seifert F. [Neuropathic pain and neuroplasticity in functional imaging studies]. Schmerz. 2010;24(2):137-45.
70. Bakkers M, et al. Small fibers, large impact: quality of life in small-fiber neuropathy. Muscle Nerve. 2014;49(3):329-36.
71. Geelen CC, et al. Anxiety affects disability and quality of life in patients with painful diabetic neuropathy. Eur J Pain. 2017;21(10):1632-41.
72. Van Laake-Geelen CCM, et al. Biopsychosocial Rehabilitation Treatment "Exposure In Vivo" for Patients with Painful Diabetic Neuropathy: Development of a Treatment Protocol. J Rehabil Med Clin Commun. 2019;2:1000015.

73. Hussain N, Said ASA. Mindfulness-Based Meditation Versus Progressive Relaxation Meditation: Impact on Chronic Pain in Older Female Patients With Diabetic Neuropathy. J Evid Based Integr Med. 2019;24:2515690X19876599.
74. Bai Y, et al. Effect of Cognitive-Behavioral Therapy or Mindfulness Therapy on Pain and Quality of Life in Patients with Diabetic Neuropathy: A Systematic Review and Meta-Analysis. Pain Manag Nurs. 2022;23(6):861-70.
75. Rozworska KA, et al. Mediators and moderators of change in mindfulness-based stress reduction for painful diabetic peripheral neuropathy. J Behav Med. 2020;43(2):297-307.



CHAPTER 10

IMPACT PARAGRAPH

In this chapter, a reflection is presented on the scientific impact of the results of the research described in this thesis.

The field of chronic neuropathic pain medicine is currently facing enormous challenges. The incidence of chronic neuropathic pain is increasing worldwide, particularly in the developed world. Epidemiological studies have shown that the prevalence in the general population may be as high as 7 to 8%[1,2], accounting for 20 to 25% of individuals with chronic pain. As a result, chronic neuropathic pain is imposing a growing burden on Western societies in terms of cost of medical care and loss of productivity. From a health care perspective, the total average annual costs of clinically referred patients undergoing skin biopsy and/or quantitative sensory testing (QST) in proven small fiber neuropathy (SFN) in 2020 amounted to €29.8 million and from a societal perspective €147.7 million (submitted, own data). The development of objective measurements of neuropathic pain is critical to improve pain management in the long-term to determining modulation of the nervous system that may take place over time. Due to the current inadequate treatments for pain currently available, these patients represent a major concern for the public health system.

Diabetes is one of the main causes of painful neuropathy, up to 25% of diabetic patients with moderate to severe pain in most. Patients are limited in their general functioning and their ability to sleep and often experience anxiety and depression.[3] As a consequence, high health care costs due to hospitalizations and outpatient visits are seen. The total annual cost of diabetic peripheral neuropathy (DPN) and its complications in the United States was estimated to be between 4.6 and 13.7 billion US dollars. Up to 27% of the direct medical cost of diabetes may be attributed to DPN.[4] Moreover, (severe) painful DPN had significantly greater healthcare resource utilization and costs than patients with diabetes only.[5] In addition, the painful symptoms cause impaired work productivity and annual lost productivity cost increased with pain severity.[6] Estimated annual productivity losses based on several European countries were €5.646, €10.552, and €16.597 for mild, moderate, and severe painful DPN, respectively.[6] Previous retrospective studies show that 1-year excess health costs for DPN, can be high as $8500.[7]

Listening to patients

Medicine and medical research starts with questions and concerns that are originated from experiences of people's life. Without a patients' story or request for help, we have no work to do. Therefore, the patients' clinical picture is the

starting point for medical research and ask questions like 'why', 'how', 'when' etc, the so-called gold circle but now applied in health care.

Improving assessments

It is important to recognize as general practitioner and consultant the clinical picture of SFN and/or pain related syndromes. Doing research and publishing both negative and positive results will help to develop more knowledge and to get more publicity. If a clinical picture is not recognized, patients undergo additional investigations that might be irrelevant and misdiagnosis arises driving costs of health care. From a patients' perspective, the overuse of diagnostic tests is considered as annoying and time-consuming. Patients frequently report that, even the pain is still present after diagnostics, clarity of the clinical picture and its diagnosis is more important for them.

In this thesis, we intended to explore the value of additional diagnostic tests in order to improve the diagnostic algorithm of SFN. Nowadays, patients are referred to our center, Maastricht University Medical Center+ (Maastricht UMC+), since we are the most experienced center with a unique setting in the Netherlands for diagnosing SFN. Even more, patients from abroad are seen in our center. Because of this, the waiting list for patients before they are invited for diagnostic testing can run up to 12 months. Ideally, if we find more simple, cheaper, sensitive and easily applicable tests to diagnose SFN, diagnostics does not have to take place in our expertise center but could be applied in every center in our country, or internationally. In the literature, CCM and SSW seemed promising existing diagnostic tools to quantify small nerve fibers in the eyes and test the autonomic function of these fibers, respectively. We concluded in this thesis that both tests do not have an additional value in the diagnostic process of SFN. Further investigation should be done in order to find their diagnostic position. Specifically, research has to be focused on the improvement of the skin wrinkling analysis and/or the applicability of CCM in SFN subgroups (i.e. those with glucose intolerance) or as a biomarker after pharmacological treatments.

However, until now, we still need to focus on the skin biopsy and temperature threshold testing since this is the 'golden standard' for the diagnosis of SFN and no other test to date have changed this pattern.

Treatment options

The current treatment approaches in neuropathic pain are essentially 'educated trial and error' where specific treatments are offered, usually within the focus of a clinical domain (e.g. interventional, alternate, behavioral etc). Even within treatment domains (e.g. pharmacological), medications are tried, nearly a random trial for each individual, beginning with the least invasive treatment and progressing along an increasing gradient of treatment side effects to find a strategy that provides some measure of relief. In addition to be inefficient, this 'trial and error' approach may create conditions under which subsequent treatments are less or more effective than if administered to naïve patients, so effective solutions may be missed or not fully understood.

Understanding pathogenicity

The knowledge in genetics is growing. Previous studies showed that in more than 50% of SFN is idiopathic.[8] Patients should be explained that idiopathic SFN does not mean that their complaints does not have a cause. However, they should be told that our knowledge is limited, meaning that not all causes ("cryptogenic status") are detected yet. In this thesis, we showed that a variety of genetic components can be an underlying condition and/or risk factor for SFN. This means that SFN might have a familial component, which can help diagnosing SFN in family members with the same complaints. Zebra-fish models can help us to understand the relation between specific DNA change and pain severity. Furthermore, these models help us to figure out whether existing and new pharmacological treatments could relief pain (behavior). We also reported that new, valid and rapid techniques are applicable for the detection of genetic mutations and should therefore be implemented in the overall diagnostics for SFN.

Overall, the PROPANE-study had a strong translational value that can be identified both in the diagnostic and treatment approaches to neuropathic pain. The approach to neuropathic pain as a whole condition influenced by a genetic background, irrespective of the clinical feature, led to include subgroups of patients with peculiar phenotypes, like itch or diffuse pain, in whom the diagnosis has been defined and a new gene has been identified thus far.

Respecting social impact

Although it is important to focus on the pathogenicity of neuropathic pain, we should bear in mind that patients with neuropathy cannot wait until we have found personalized solutions in pain relief. So, attention for their daily functioning in life is inevitable.

Chronic neuropathic pain, which means pain more than six weeks, is a society problem with important consequences for the functioning of the human being. For many patients, pain interferes with social activities such as household, work or family activities. A rehabilitation process could help patients to adjust with chronic neuropathic pain, such as therapies with Graded Exposure and/or Graded Activity. However, there is no literature to support this and more research should be done on integrated biopsychosocial interventions in patients with SFN.

Research and innovation might focus on creating effective ways of monitoring and evaluating neuropathic pain symptoms. For example, fill out diaries on a mobile device with the possibility to monitor *on distance* the pain and level of functioning (activities and participation) might help to intervene when it is needed and most efficient. This method is not expensive (beside data storage), costs a patient little time and helps interventions being more effective than before.

Nowadays, value-based healthcare is important, where we try to provide new diagnostic tests or other ways to improve health care, and in particular with low costs. Still, the emphasize of good clinical practice should be in the contact with a patient and not in lowering health care costs.

References

1. Bouhassira D, et al. Prevalence of chronic pain with neuropathic characteristics in the general population. Pain. 2008;136(3):380-7.
2. Torrance N, et al. The epidemiology of chronic pain of predominantly neuropathic origin. Results from a general population survey. J Pain. 2006;7(4):281-9.
3. Kalra S, Jena BN, Yeravdekar R. Emotional and Psychological Needs of People with Diabetes. Indian J Endocrinol Metab. 2018;22(5):696-704.
4. Gordois A, et al. The health care costs of diabetic peripheral neuropathy in the US. Diabetes Care. 2003;26(6):1790-5.
5. Sadosky A, et al. Healthcare utilization and costs in diabetes relative to the clinical spectrum of painful diabetic peripheral neuropathy. J Diabetes Complications. 2015;29(2):212-7.
6. Alleman CJ, et al. Humanistic and economic burden of painful diabetic peripheral neuropathy in Europe: A review of the literature. Diabetes Res Clin Pract. 2015;109(2):215-25.
7. Dworkin RH, et al. Impact of postherpetic neuralgia and painful diabetic peripheral neuropathy on health care costs. J Pain. 2010;11(4):360-8.
8. de Greef BTA, et al. Associated conditions in small fiber neuropathy - a large cohort study and review of the literature. Eur J Neurol. 2018;25(2):348-55.

CHAPTER 11

NEDERLANDSE SAMENVATTING

Dunnevezelneuropathie (DVN) wordt gekenmerkt door neuropathische pijn en autonome stoornissen. De functies die aangedaan zijn verlopen automatisch en kunnen we niet actief en/of bewust beïnvloeden, zoals het hartritme, het zweetpatroon en problemen met mictie en defecatie. Op dit moment wordt de diagnose DVN gesteld op basis van het klinische beeld in combinatie met een afwijkend temperatuurdrempelonderzoek en/of afwijkend huidbiopt, zonder afwijkingen op elektromyografie (EMG). Soms zien we patiënten die wel de klachten hebben van neuropathische pijn en autonome klachten passende bij DVN maar kunnen we het helaas niet met de genoemde onderzoeken vaststellen. We kunnen derhalve op dit moment dus nog niet spreken over een gouden standard. Om die reden onderzoeken we of bestaande technieken een (aanvullende) bijdrage kunnen leveren bij het stellen van deze diagnose. Daarnaast hebben we in dit proefschrift gekeken naar de genetische bijdrage aan DVN.

In **Deel I en deel II** werd een overzichtsartikel gepresenteerd waarin wetenschappelijke literatuur omtrent diagnostiek bij DVN; daarnaast werd in dit deel twee bestaande technieken onderzocht om na te gaan wat hun toepasbaarheid is bij het vaststellen van DVN en/of autonome dysfunctie.

In **Hoofdstuk 1** wordt een overzicht gegeven van het ziektebeeld DVN en een uiteenzetting van het proefschrift: de noodzaak van verbetering van de huidige diagnostiek en een toevoeging van genetische diagnostiek als onderdeel van de analyse naar DVN.

Hoofdstuk 2 geeft een overzicht van het ziektebeeld DVN met daarbij verschillende klinische testen die worden verricht om zowel de kwantificering als de functie van de dunne zenuwvezels te testen. In dit hoofdstuk werd een beknopt overzicht gegeven over varianten die zijn gevonden en die gerelateerd lijken te zijn aan DVN. Daarnaast werd uiteengezet dat DVN deel uitmaakt van een continuüm in het pijnlandschap met een genetisch onderliggende oorzaak. De pijnsyndromen Paroxysmal Extreme Pain Disorder (PEPD) en Inherited Erythermyalgie (IEM) zijn onderdeel van dit continuüm.

In **Hoofdstuk 3** werd een prospectieve studie beschreven waarbij 183 proefpersonen werden onderzocht naar de aanwezigheid van dunne zenuwvezels in het hoornvlies (cornea) van beide ogen door middel van een speciale microscoop, genaamd de corneale confocale microscoop (CCM). Voorafgaand aan de inclusie van de patiëntengroep met de verdenking DVN, is gekeken of gezonde proefpersonen voldeden aan de normaalwaarden die eerder door een onderzoeksgroep uit Manchester zijn gepubliceerd. Dit bleek het geval te zijn. De

patiëntengroep werd vóór het onderzoek met de CCM onderzocht door een oogarts (in opleiding) om oogafwijkingen uit te sluiten. Indien hier geen noemenswaardige afwijkingen naar voren kwamen, werden de ogen voorbereid met een verdovingsdruppel en ooggel in beide ogen. De ooggel vormt een barrière tussen het oog en de microscoop waardoor er geen direct contact plaatsvindt tussen de lens van de microscoop en het oogoppervlak. Door de verdovingsdruppels is het tevens een niet-pijnlijk onderzoek. Het duurt per oog maximaal twee minuten om de dunne zenuwvezels in kaart te brengen. Deze werden nadien beoordeeld op onder andere de lengte, dichtheid en krommingen. Er werd een middelmatig tot hoge reproduceerbaarheid van de handmatige analyse gevonden. Daarnaast werd een hoge correlatie gevonden met de volledig automatische analyse voor enkele parameters, te weten de zenuwvezel lengte en -dichtheid. Er werd geen verschil in corneale parameters gevonden in de groep waarbij, op basis van de gouden standaard, DVN werd aangetoond ten opzichte van patiënten waarbij het (nog) niet werd aangetoond. Er werd gevonden dat patiënten met een onderliggende glucose intolerantie mogelijk vaker afwijkingen op dit onderzoek vertoonden. De relatie tussen afwijkingen in zenuwen en glucose-intolerantie of diabetes mellitus type 2 werd door eerder onderzoek al aangetoond. Uit dit onderzoek werd geconcludeerd dat de CCM geen meerwaarde heeft binnen de huidige diagnostiek naar DVN.

In **Hoofdstuk 4** werd de studie beschreven van de digitale huidrimpeltest. Met deze studie werd geprobeerd een betere methode te vinden dan de huidige methode waarop de vingers op dit moment wordt beoordeeld, middels een categorische 5-puntsschaal. Tijdens dit onderzoek werd er normaalwaarden verzameld en de interbeoordelaarsbetrouwbaarheid bepaald. In totaal werden er 82 gezonde proefpersonen geïncludeerd. Er werd een verdovingscrème op acht vingertoppen gesmeerd en na een half uur gekeken wat de mate van rimpeling was in de vingers. Vóór het aanbrengen en na het verwijderen van de crème werden er foto's van de vingertoppen worden gemaakt. Deze foto's werden met een nieuw computerprogramma (Digit Wrinkle Scan©) geanalyseerd om zo de mate van rimpeling op een kwantitatieve wijze te beoordelen. Er werd geconcludeerd dat de 5-puntsschaal geen betrouwbare methode is voor de klinische praktijk om de mate van rimpeling na te gaan. Middels de nieuwe software, vonden we voor twee vingers redelijke betrouwbaarheid. Echter is dit niet voldoende om deze methode in de klinische praktijk toe te kunnen passen. Daarnaast is de vraag hoe specifiek deze test zal zijn, om bijvoorbeeld DVN aan te tonen en te onderscheiden van andere ziektebeelden met een autonome dysfunctie.

In **Deel III** van dit proefschrift werd gekeken naar de methode om genetische varianten te meten en de frequenties van genetische varianten, op zowel nationaal als internationaal niveau. De prevalentie van genetische varianten in DVN in Nederland werd onderzocht evenals de genetische varianten in diabetische en non-diabetische patiënten, met én zonder pijnklachten in vier Europese centra. Tenslotte werd een casus-omschrijving gepresenteerd met een ongebruikelijk klinisch beeld bij een *SCN9A*-variant.

In **Hoofdstuk 5** werd een diagnostische, relatief goedkope, methode beschreven die meerdere genen kan achterhalen in een grote groep patiënten met pijnlijke neuropathie. Twee technieken (*Molecular Inversion Probes-Next generation sequencing* (MIPs-NGS) en *TruSeq® Custom Amplicon-Next generation sequencing* (TSCA-NGS)) werden met elkaar vergeleken op gebied van sensitiviteit, specificiteit, efficiëntie, uitvoering en kosteneffectiviteit. Beide methoden zijn ontwikkeld om negen natrium-kanaalgenen te achterhalen (*SCN3A, SCN8A-SCN11A* en *SCN1B-SCN4B*). Ten opzichte van de TSCA-NGS, zagen we dat de MIPs-NGS een grotere dekkingsgraad bood (97.3% versus 93.9%). Daarnaast zagen we dat MIPs-NGS een veelzijdiger ontwerp heeft; is flexibeler dan TSCA-NGS. De kosten van MIPs-NGS zijn meer dan 5 maal goedkoper dan TSCA-NGS wanneer 500 of meer DNA-monsters worden getest. Kortom, MIPs-NGS is een betrouwbare, flexibele en relatief goedkope methode om genetische variaties in een groot cohort patiënten te detecteren.

In **Hoofdstuk 6** beschreven we de frequentie van *SCN9A*, *SCN10A* en *SCN11A* varianten bij 1139 patiënten met DVN. We analyseerden hun klinische kenmerken en voerden een genetische screening uit. Pathogeniciteit van varianten werd geclassificeerd volgens vastgestelde richtlijnen van de Association for Clinical Genetic Science (ACGS) en frequenties werden bepaald. Patiënten met DVN werden gegroepeerd volgens de gevonden genetische varianten en klinische kenmerken werden vergeleken. Van de 1139 patiënten met DVN hadden 132 (11.6%) patiënten 73 verschillende (potentieel) pathogene varianten in de spanningsafhankelijke natriumkanalen, waarvan 50 nieuwe varianten waren en 22 varianten werden gevonden bij meer dan één patiënt. De frequentie van (potentieel) pathogene varianten was 5.1% (n=58/1139) voor *SCN9A*, 3.7% (n=42/1139) voor *SCN10A* en 2.9% (n=33/1139) voor *SCN11A*. Klinisch vonden we dat bij patiënten met een genetische variant er vaker sprake was van erythermyalgie-achtige klachten en pijnklachten die opgewekt wordt door warmte. We concludeerden dat screenend onderzoek naar deze drie genen belangrijk is om na te gaan of er sprake is van een genetisch onderliggende oorzaak/risicofactor voor het ontwikkelen van klachten passende bij DVN.

In **Hoofdstuk 7** onderzochten we de genetische verschillen tussen pijnlijke diabetische neuropathie, pijnloze diabetische neuropathie en pijnlijke idiopathische DVN. Diabetes is de belangrijkste oorzaak van neuropathische pijn. Het oplossen van de genetische architectuur van pijnlijke/pijnloze diabetische en idiopathische DVN-patiënten zal leiden tot betere behandelingsstrategieën.
We rapporteerden genetische varianten van tien natriumkanaalgenen (*SCN3A*, *SCN7A-SCN11A* en *SCN1B-4B*), die allen onderdeel zijn van de nociceptieve route. Het onderzoek vond plaats door middel van het in door hoofdstuk 5 beschreven methode, de MIPs-NGS. In totaal werden 1094 patiënten met pijnlijke (n = 237), pijnloze diabetische neuropathie (n = 309) en pijnlijke idiopathische DVN (n = 548) geïncludeerd uit vier centra in Europa, te weten University of Manchester (Verenigd Koninkrijk), Deutsche Diabetes Forschungsgesellschaft EV (Duitsland), Fondazione IRCCS Instituto Neurologico "Carlo Besta" (Italië) en Maastricht University Medical Center+ (Nederland). Er werd een prevalentie binnen de drie groepen, liggende tussen de 14.9% en 18.4%. Functioneel testen is essentieel voor een definitieve classificatie van een genetische variant.

In **Hoofdstuk 8** presenteerden we een patiënt met een variant in het *SCN9A*-gen. De casus presenteerde zich met de klinische kenmerken van erythermalgie, PEPD (Paroxysmal Extreme Pain Disorder) en DVN.

Algemene conclusie en discussie

DVN is een chronisch pijnsyndroom die tegenwoordig meer wordt (h)erkend. Neuropathische pijn moet worden onderscheiden van nociceptieve of inflammatoire pijn, hoewel in veel gevallen zowel nociceptieve als neuropathische componenten kunnen bijdragen aan pijn. Hierdoor kunnen patiënten met DVN onterecht gediagnosticeerd worden met een ander pijnsyndroom. De verscheidenheid aan klinische symptomen dwingt ons tot een betere en intensievere samenwerking tussen medisch specialisten, wanneer er bijvoorbeeld ook sprake is van autonome symptomen. Patiënten met het vermoeden van DVN worden meestal doorverwezen naar de neuroloog. De belangrijkste symptomen (neuropathische pijn) zijn inderdaad het meest geschikt voor het vakgebied van de neuroloog, maar andere specialisten moeten zich ervan bewust zijn dat het klinische beeld van DVN kan beginnen met autonome symptomen.
Deze variabiliteit van de klinische presentatie van DVN maakt het dus moeilijk om een diagnose te stellen. Hoewel de totale prevalentie van DVN in Nederland in 2012 werd geschat op 53:100.000, wordt aangenomen dat dit een onderschatting is van het werkelijke aantal, waardoor het belangrijker is om het

klinische beeld en de diversiteit ervan te herkennen voor de huisarts en medisch specialist.
Een veel gestelde vraag van patiënten betreft de prognose van DVN. Het natuurlijke beloop is nog grotendeels onbekend. In een onlangs gepubliceerde studie, waaraan 101 DVN patiënten met een afwijkend huidbiopt aan deelnamen werd geconcludeerd dat DVN een relatief statische ziekte is met zeer langzame progressie waarbij grote zenuwvezels betrokken zijn (11.9%) of leiden tot aanzienlijke langdurige invaliditeit (5.3%) en loopstoornissen (6.3%). In deze studie was er sprake van een gemiddelde follow-up duur van 6.2 jaar. Bovendien was het gemiddelde aantal neuropathische pijnmedicatie dat per patiënt werd gebruikt meer dan vier en bleef pijnbestrijding op de lange termijn de grootste uitdaging. De prognose hangt echter van veel factoren af, zoals de aanwezigheid/afwezigheid van triggers en onderliggende aandoeningen. Longitudinale studies kunnen meer informatie verschaffen over het natuurlijke beloop en kunnen de ontwikkeling van ziekte-modificerende strategieën helpen. Daarnaast kan digitale zorg patiënten de mogelijkheid bieden om het beloop van de pijn/ziekte te monitoren, waardoor artsen en/of onderzoekers inzicht krijgen in de relatie tussen lichamelijk en aanvullend onderzoek. Bovendien kan digitale zorg bijdragen aan zelfregie en participatieve zorg.

Toepassen van diagnostiek

Aangezien het klinische beeld en de etiologie van DVN sterk variëren, moeten we het gebruik van de beste diagnostische technieken om DVN te beoordelen, heroverwegen. Uiteindelijk wordt gestreefd naar een algoritme van diagnostische mogelijkheden, waarbij het klinisch beeld de basis vormt van de tests die zullen worden toegepast om de diagnose DVN te bevestigen. Bovendien moeten we voor onderzoeksdoeleinden zoeken naar een andere classificatie waarbij de diagnose DVN gepaard gaat met de belangrijkste klinische uitkomst (autonoom/sensorisch) en een klasse van etiologie (genetisch, idiopathisch, verworven). In dit proefschrift kwamen we tot de conclusie dat de CCM en huidrimpeltest in de huidige vorm geen toegevoegde waarde heeft binnen de diagnostiek naar DVN. Hiervoor zijn beter gedefinieerde groepen in het klinisch beeld en de etiologie nodig. Zo moet CCM als diagnostisch hulpmiddel worden overwogen bij patiënten met neuropathische pijn en een medische voorgeschiedenis van diabetes mellitus of een gestoorde glucose-intolerantie.
Echter, in de literatuur is er ook sprake van een diversiteit aan DVN classificatie. De reeks aan verschillende definities en classificaties maakt dat er een grote variatie ontstaat waardoor studies moeizaam met elkaar te vergelijken zijn.
Publicaties over CCM als diagnostisch hulpmiddel nemen snel toe. CCM wordt echter voornamelijk gebruikt bij patiënten met diabetes mellitus type 1 en type 2.

In de studie, beschreven in hoofdstuk 3, hebben we patiënten met diabetische neuropathie uitgesloten, omdat er een relatief hoge incidentie is van zowel grote als kleine zenuwvezelstoornissen. CCM is een elegante methode om zenuwvezels in het hoornvlies zichtbaar te maken. De morfologie is echter gecompliceerd, wat problemen kan veroorzaken bij de validiteit, in termen van de differentiatie van zenuwbundels op micro-niveau. Het kan mogelijk wel een goede test zijn om therapeutische uitkomsten te evalueren.
Tot nu toe is er geen test beschikbaar die correleert met sensorische informatie. We hebben de potentiële waarde van de huidrimpeltest onderzocht en bekeken of deze zou kunnen bijdragen aan het diagnostisch palet van DVN. De huidige klinische praktijk past een visuele schaal voor rimpels toe, in vijf categorieën. In ons onderzoek (Hoofdstuk 4) hebben we echter beschreven dat deze categorische schaal niet betrouwbaar genoeg is om deze als diagnostische test te gebruiken. We hebben geprobeerd rimpelveranderingen te kwantificeren door een nieuwe software te gebruiken, de Digit Wrinkle Scan© (DWS). Deze methode liet iets betere betrouwbaarheidsscores zien, maar dit varieerde tussen matig tot zeer goed. Dit lijkt dus ook geen verbetering te zijn op het huidige diagnostische palet.
Het is belangrijk om als medici het ziektebeeld van DVN en/of pijngerelateerde syndromen te herkennen. Het doen van onderzoek en het publiceren van zowel negatieve als positieve resultaten zal helpen om meer kennis te ontwikkelen en meer publiciteit te krijgen. Als een klinisch beeld niet wordt herkend, ondergaan patiënten aanvullende onderzoeken die mogelijk niet relevant zijn en ontstaat er een verkeerde diagnose die de kosten van de gezondheidszorg verhoogt. Vanuit het perspectief van de patiënt wordt het overmatig gebruik van diagnostische tests als vervelend en tijdrovend ervaren. Patiënten geven vaak aan dat, zelfs als de pijn na diagnostiek nog steeds aanwezig is, duidelijkheid van het ziektebeeld en de diagnose voor hen belangrijker is.

Genetische pathofysiologie van neuropathische pijn

Het begrijpen van de pathofysiologie van pijn en het identificeren van specifieke pijn genotype-fenotype relaties kan leiden tot verbetering van en meer gepersonaliseerde behandelstrategieën. De wetenschap van pijn heeft de afgelopen decennia enorme vooruitgang geboekt. We ontdekten dat 11.6% van onze DVN-populatie een genetische variant heeft die de onderliggende oorzaak of risicofactor voor DVN zou kunnen zijn. Er is een zogenoemd *multi-hit-model* voorgesteld die ten grondslag ligt aan de lengte- en leeftijdsafhankelijkheid van perifere neuropathieën. Andere onderliggende factoren, zoals systemische ziekten, modificerende genen of omgevingsfactoren, kunnen nodig zijn, in combinatie met een mutatie, voor het ontstaan van klinische tekenen en symptomen van DVN. Het begrijpen van de structuur en functie van de

spanningsafhankelijke natrium-kanalen is van groot biofysisch en klinisch belang gezien hun sleutelrol in cellulaire pathofysiologie. Tot op heden is voornamelijk onderzoek verricht naar in de invloed van natriumkanalen. Kaliumkanalen kunnen echter ook van belang zijn. Elektrische signalering wordt beëindigd door activering van spanningsafhankelijke kaliumkanalen die gedurende een langere tijdsdomein worden geactiveerd en gedeactiveerd. Ook werd in eerder onderzoek aangetoond dat een significante vermindering van de mitochondriale dichtheid en grootte bij een *SCN9A*-mutatie kan leiden tot degeneratie en verlies van zenuwvezels in DVN.
Eerdere studies toonden aan dat in meer dan 50% van SFN idiopathisch is. Aan patiënten moet worden uitgelegd dat idiopathische SFN niet betekent dat hun klachten geen oorzaak hebben. Ze moeten echter worden verteld dat onze kennis beperkt is, wat betekent dat nog niet alle oorzaken ("cryptogene status") zijn gedetecteerd. In dit proefschrift hebben we aangetoond dat een verscheidenheid aan genetische componenten een onderliggende aandoening en/of risicofactor voor DVN kan zijn. Dit betekent dat DVN mogelijk een familiale component heeft, wat kan helpen bij het diagnosticeren van DVN bij familieleden met dezelfde klachten. Zebravis-modellen kunnen ons helpen de relatie tussen specifieke DNA-verandering en de ernst van de pijn te begrijpen. Bovendien helpen deze modellen ons te achterhalen of bestaande en nieuwe farmacologische behandelingen pijn(gedrag) kunnen verlichten. In dit proefschrift is ook een nieuwe, valide en snelle techniek beschreven die toepasbaar is voor de detectie van genetische varianten. Deze zou dus geïmplementeerd moeten worden in de algehele diagnostiek naar DVN.

Medicamenteuze behandelmogelijkheden

Dunne zenuwvezels hebben het hoogste regeneratieve vermogen en een snelle diagnose en start van een bijbehorende behandeling van DVN kan resulteren in substantiële regeneratie en herstel, vooral bij jonge, relatief gezonde patiënten. De huidige behandelingen voor pijnlijke neuropathieën, gericht op symptomatische pijnbestrijding, zijn grotendeels ontoereikend. Minder dan 50% van de patiënten bereikt 50% pijnverlichting met beschikbare medicijnen. De meeste beschikbare analgetica werken op verschillende niveaus (bijv. natriumkanalen, noradrenerge systeem, opioïderge systeem).
In dit proefschrift hebben we de epidemiologie van genetische mutaties in onze cohorten beschreven, zowel nationaal en internationaal. Een betere beschrijving van pijnfenotype-genotype kan de basis vormen voor gepersonaliseerde pijnbehandeling, omdat de redenen waarom sommige patiënten op de behandeling reageren terwijl andere resistent blijven, beter zullen worden begrepen. Hoewel pijn kan worden gekoppeld aan meerdere genetische

factoren, beïnvloeden complexe omgevingsfactoren ook de individuele variatie in pijn, zoals ras, etniciteit, geslacht en sociale context en interpretatie van de pijnervaring.
De huidige behandeling van neuropathische pijn is in wezen '*trial-and-error*' waarbij behandelingen worden aangeboden, bijna op basis van een willekeurige proef voor een individu, beginnend met de minst ingrijpende behandeling om vervolgens een strategie te vinden die enige mate van verlichting biedt.

Niet-medicamenteuze behandelingen

Na verloop van tijd kan chronische pijn leiden tot wisselingen in sensatie, centrale sensitisatie en veranderingen in affectieve, cognitieve en emotionele toestand. De transformatie van een gezonde toestand naar een toestand die het vermogen van een patiënt beperken op het fysieke/mentale/sociale domein, kan resulteren in een negatieve cascade van falen van systemen die de toestand verergeren, waardoor een negatieve feed-forward-cyclus op gang komt. Daarom is het belangrijk om de ontwikkeling van deze pijn te definiëren en behandelingen te ontwikkelen die deze pijn zo snel mogelijk kunnen stoppen of omkeren. Om de meest geschikte behandeling te starten, is het belangrijk dat de behandelaar de hulpvraag verduidelijkt. In de meeste gevallen willen patiënten van de pijn af. (Gedeeltelijke) pijnvermindering kan worden bereikt door de onderliggende oorzaak te behandelen of door symptomatische pijnbehandeling te starten met medicatie. Daarnaast kan een start met een revalidatieprogramma, gespecialiseerd in chronische pijn, patiënten vanuit een holistisch perspectief helpen wanneer pijn hun dagelijks leven (nog) aanzienlijk verstoort op verschillende domeinen, zoals mobiliteit, huishoudelijke activiteiten, taken op het werk en/of in sociale activiteiten. Een eerdere studie beschreef dat DVN-patiënten een ernstige algehele vermindering van kwaliteit van leven ervaren. Revalidatieprogramma's kunnen gericht zijn op pijneducatie en houden voortdurend toezicht op het opheffen van beperkingen in het dagelijks functioneren, ondanks de aanwezigheid van pijnklachten, onder begeleiding van een revalidatiearts, fysiotherapeut, ergotherapeut en/of gedragstherapeut.

Toekomstperspectief

Over het algemeen zou de ideale pijnbehandelaar expertise hebben op het gebied van pijnneurobiologie, gedragsgeneeskunde (inclusief psychiatrie/ psychologie), farmacologie en behandelprocedures. Integratieve zorg moet gericht zijn op behandelingen voor zowel symptoombeheersing als ziekte-modificatie en moet specifiek zijn voor individuele patiënten. Bovendien zou een grotere kruisbestuiving tussen clinici en onderzoekers de gezondheidszorg vooruit kunnen helpen.

Hoewel we nog lang niet begrijpen wat neuropathische pijn als ziekte of symptoom veroorzaakt, of weten hoe we het kunnen genezen, hebben we een geweldige kans om deze nieuwe kennis over chronische pijn te gebruiken om nieuwe benaderingen uit te werken voor zowel het begrijpen als behandelen van chronische pijn.

Impact op de samenleving

Het gebied van chronische neuropathische pijngeneeskunde staat voor enorme uitdagingen. De incidentie van chronische neuropathische pijn neemt wereldwijd toe, vooral in de beter ontwikkelde landen. Epidemiologische studies hebben aangetoond dat de prevalentie in de algemene bevolking kan oplopen tot 7 tot 8%, wat neerkomt op 20 tot 25% van de personen met chronische pijn. Als gevolg hiervan legt chronische neuropathische pijn een steeds grotere last op de Westerse samenlevingen in termen van kosten van medische zorg en productiviteitsverlies. Vanuit zorgperspectief bedroegen de totale gemiddelde jaarlijkse kosten van klinisch doorverwezen patiënten die een huidbiopsie en/of kwantitatief sensorisch onderzoek ondergaan bij bewezen DVN in 2020 € 29.8 miljoen en vanuit maatschappelijk perspectief € 147.7 miljoen (gegevens Maastricht UMC+). De ontwikkeling van objectieve metingen van neuropathische pijn is van cruciaal belang om pijnbeheersing op de lange termijn te verbeteren voor het bepalen van modulatie van het zenuwstelsel die in de loop van de tijd kan plaatsvinden. Vanwege de huidige ontoereikende behandelingen voor pijn die momenteel beschikbaar zijn, vormen deze patiënten een groot probleem voor de volksgezondheid.

V

PART 5

ADDENDUM

DANKWOORD
ABOUT THE AUTHOR
LIST OF PUBLICATIONS

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Curriculum Vitae

Maurice Sopacua was born on October 16th, 1984 in Maastricht, the Netherlands. In 2003, he graduated from highschool (Euro College, Maastricht). He went to Amsterdam to study Human Movement Sciences at the VU University (Amsterdam). He graduated for his Master's degree in the field of Rehabilitation & Physiotherapy (January, 2008). After one year off, he decided to study Medicine (shortened trajectory) at the Rijksuniversiteit Groningen. He received his Medical Degree in June 2013. He returned to his hometown to work as non-resident for one year in the Rehabilitation department in Maastricht University Medical Center+ (MUMC+). Because of his ambitions to obtain a PhD, he started his PhD-research in the Neurology department of MUMC+, under supervision of Prof. C.G. Faber, with the title 'Improving the assessments of the diagnosis of Small Fiber Neuropathy'. From June 2018, he started as resident in Rehabilitation Medicine in Adelante Zorggroep. Since March 2022, he is a PM&R specialist in Libra Revalidatie & Audiologie. His daily practice is in the hospitals Sint Jans Gasthuis (Weert, The Netherlands) and Elkerliek Ziekenhuis (Helmond, the Netherlands).

Maurice Sopacua werd geboren op 16 oktober 1984 te Maastricht. In 2003 behaalde hij zijn VWO-diploma aan het Euro-College te Maastricht. Hij begon zijn studentenleven in Amsterdam en studeerde Bewegingswetenschappen aan de Vrije Universiteit (Amsterdam) waar hij zijn Master haalde met afstudeerrichting Revalidatie & Fysiotherapie (januari, 2008). Na een tussenjaar besloot hij Geneeskunde (Zij-Instroom) te studeren aan de Rijksuniversiteit Groningen. Hij ontving zijn artsenbul in juni 2013. Daarna keerde hij terug naar zijn geboortestad om een jaar als arts-assistent-niet-in-opleiding-tot-specialist (ANIOS) te werken op de afdeling Revalidatiegeneeskunde van het Maastricht Universitair Medisch Centrum+ (MUMC+). Omdat hij de ambitie had om te promoveren, begon hij met een promotieonderzoek op de afdeling Neurologie van het MUMC+, onder leiding van Prof. C.G. Faber, getiteld 'Improving the assessments of the diagnosis of Small Fiber Neuropathy'. Vanaf juni 2018 volgde hij als arts-assistent (AIOS) de opleiding tot revalidatiearts bij Adelante Zorggroep. Vanaf maart 2022 is hij werkzaam als revalidatiearts voor Libra Revalidatie & Audiologie, in de ziekenhuizen Sint Jans Gasthuis te Weert en het Elkerliek Ziekenhuis te Helmond.

List of publications

Almomani R, **Sopacua M**, Marchi M, Ślęczkowska M, Patrick Lindsey P, De Greef BTA, Hoeijmakers JG, Salvi E, Merkies ISJ, Fadavi H, Malik RA, Ziegler D, Derks KWJ, Boenhof G, Martinelli-Boneschi F, Cazzato D, Lombardi R, Dib-Hajj S, Waxman SG, Smeets HJM, Gerrits MM, Faber CG, Lauria G. **Propane Study Group**. Genetic profiling of sodium channels in painful and painless diabetic and idiopathic small fiber neuropathy. *Submitted.*

Sopacua M, Hoeijmakers JGJ, De Greef BTA, Colonna A, Scarpa F, Nuijts RMMA, Merkies ISJ, Faber CG, Dickman MM, Berendschot TTJM. No additional value for corneal confocal microscopy in the diagnosis of small fiber neuropathy. *Submitted.*

Ślęczkowska M, Almomani R, Marchi M, Salvi E, De Greef BTA, **Sopacua M**, Hoeijmakers JGJ, Lindsey P, Waxman SG, Lauria G, Faber CG, Smeets HJM, Gerrits MM. Peripheral Ion Channel Genes Screening in Painful Small Fiber Neuropathy. *Int J Mol Sci.* 2022;23(22):14095. doi: 10.3390/ijms232214095.

Ślęczkowska M, Almomani R, Marchi M, De Greef BTA, **Sopacua M**, Hoeijmakers JGJ, Lindsey P, Salvi E, Bönhof GJ, Ziegler D, Malik RA, Waxman SG, Lauria G, Faber CG, Smeets HJM, Gerrits MM. Peripheral Ion Channel Gene Screening in Painful- and Painless-Diabetic Neuropathy. *Int J Mol Sci.* 2022;23(13):7190. doi: 10.3390/ijms23137190.

Sopacua M, Gorissen-Brouwers CML, De Greef BTA, Joosten IBT, Faber CG, Merkies ISJ, Hoeijmakers JGJ. The applicability of the Digit Wrinkle Scan to quantify sympathetic nerve function. *Clin Neurophysiol Pract.* 2022;7:115-119.

Sopacua M, Hoeijmakers JGJ, van der Kooi AJ, Merkies ISJ, Faber CG. Pain triangle phenomenon in possible association with SCN9A: A case report. *Mol Genet Genomic Med.* 2022;10(10):e2026.

Geerts M, De Greef BTA, **Sopacua M**, Van Kuijk SMJ, Hoeijmakers JGJ, Faber CG, Merkies ISJ. Intravenous immunoglobulin therapy in patients with painful idiopathic small fiber neuropathy. *Neurology.* 2021;96(20):e2534-e254.

Almomani R, Marchi M, **Sopacua M**, Lindsey P, Salvi E, Koning B, Santoro S, Magri S, Smeets HJM, Martinelli Boneschi F, Malik RR, Ziegler D, Hoeijmakers JGJ, Bönhof G, Dib-Hajj S, Waxman SG, Merkies ISJ, Lauria G, Faber CG, Gerrits MM, on behalf on the **Propane Study Group**. Evaluation of molecular

inversion probe versus TruSeq® custom methods for targeted next-generation sequencing. *PloS One.* 2020;15 (9):e0238467.

Alsaloum M, Estacion M, Almomani R, Gerrits MM, Bönhof GJ, Ziegler D, Malik R, Ferdousi M, Lauria G, Merkies ISJ, Faber CG, Dib-Hajj S, Waxman SG, **Propane Study Group.** A gain-of-function sodium channel B2-subunit mutation in painful diabetic neuropathy. *Mol Pain.* 2019;15:1744806919849802..

Sopacua M, Hoeijmakers JGJ, Merkies ISJ, Lauria G, Waxman SG, Faber CG. Small-fiber neuropathy: expanding the clinical pain universe. *J Peripher Nerv Syst.* 2019;24(1):19–33.

Eijkenboom I, **Sopacua M**, Hoeijmakers JGJ, De Greef BTA, Lindsey P, Almomani R, Marchi M, Vanoevelen J, Smeets HJM, Waxman SG, Lauria G, Merkies ISJ, Faber CG, Gerrits MM. Yield of peripheral sodium channels gene screening in pure small fibre neuropathy. *J Neurol Neurosurg Psychiatry.* 2019;90(3):342–52.

Eijkenboom I, **Sopacua M**, Otten ABC, Gerrits MM, Hoeijmakers JGJ, Waxman SG, Lombardi R, Lauria G, Merkies ISJ, Smeets HJM, Faber CG, Vanoevelen JM, **Propane Study Group**. Expression of pathogenic SCN9A mutations in the zebrafish: a model to study small-fiber neuropathy. *Exp Neurol.* 2019;311:257–64.

Kapetis D, Sassone J, Yang Y, Galbardi B, Xenakis MN, Westra RL, Szklarczyk R, Lindsey P, Faber CG, Gerrits MM, Merkies ISJ, Dib-Hajj SD, Mantegazza M, Waxman SG, Lauria G, **Propane Study Group**. Network topology of Nav1.7 mutations in sodium channel-related painful disorders. *BMC Syst Biol.* 2017; 11(1):28.

Hoeijmakers JGJ, **Sopacua M**, Merkies ISJ, Faber CG. Dunnevezelneuropathie, van diagnose tot behandeling. *Nervus.* 2017;3:27–34.

Sanz-Barbero B, **Sopacua M**, Otero-García L, Borda-Olivas A, Zunzunegui MV. Inequalities in the use of mammography in Spain: effect of caring for disabled family. *Int J. Public Health.* 2012;57(6):953–7.

M. Sopacua