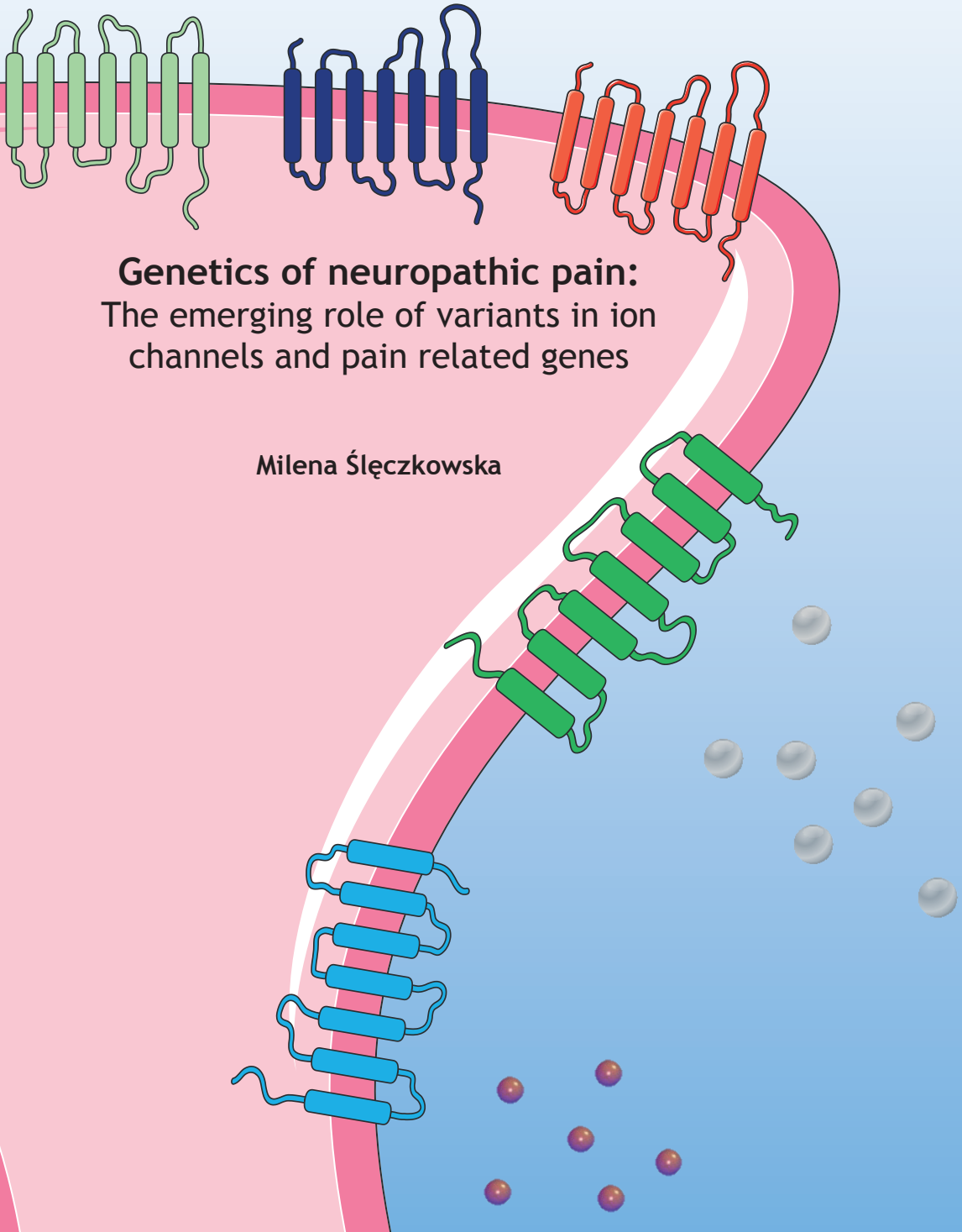
Genetics of neuropathic pain



Genetics of neuropathic pain:
The emerging role of variants in ion channels and pain related genes

Milena Ślęczkowska

Genetics of neuropathic pain:

the emerging role of variants in ion channels and pain-related genes

Milena Ślęczkowska

ISBN: 978-94-6469-381-2
Layout: Milena Ślęczkowska and Proefschriftmaken
Cover design Milena Ślęczkowska, Misra Kaalindi and Proefschriftmaken
Printed by Proefschriftmaken

Genetics of neuropathic pain:

the emerging role of variants in ion channels and pain-related genes

DISSERTATION

to obtain the degree of Doctor at Maastricht University,
on the authority of the Rector Magnificus Prof. dr. Pamela Habibović
in accordance with the decision of the Board of Deans,
to be defended in public
on Tuesday, 27th of June, 2023 at 13:00 hours

by
Milena Agata Ślęczkowska
born on 1st of May 1990, in Końskie, Poland

Promotors:

Prof. dr. H.J.M. Smeets

Prof. dr. C.G. Faber

Co-promotor:

Dr. M.M. Gerrits

Assessment committee:

Prof. dr. J.H.M. van Zundert (chair)

Prof. dr. D.L. Bennett, University of Oxford, UK

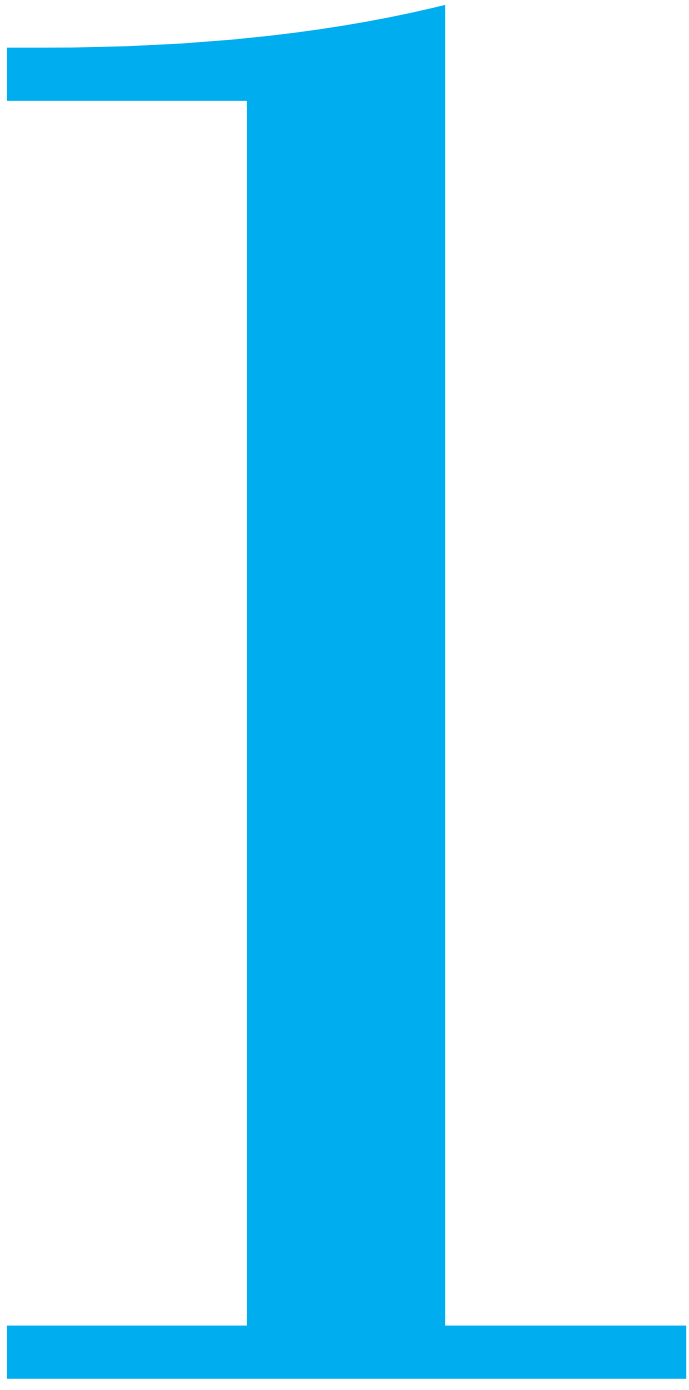
Prof. dr. E-J. Speel

Prof. dr. K. Talavera Pérez, KU Leuven, Belgium

Prof. dr. M.P. Weijenberg

The research described in this thesis was funded by the Marie-Skłodowska Curie Actions foundation, from the Molecule-to-Man Pain Network, a European Commission Multi-Center Collaborative Projects through the European Union's Horizon 2020 research and innovation program under grant agreement No. 721841. The research was conducted at School for Mental Health and Neuroscience (MHENS), Maastricht University.

Table of Contents



Chapter 1

General introduction and outline of the thesis

1.1 Neuropathic pain

The first widely accepted definition of pain was established in 1979 after two years of debate by the International Association for the Study of Pain (IASP) and remained unchanged until 2020 [1]. The current definition of pain revised by IASP to better convey the pain complexity is "an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage" [1]. Although pain is necessary for survival, acute, prolonged pain is a huge problem negatively influencing quality of life and mental health of affected individuals [2]. Neuropathic pain (NeP) is usually chronic and may arise as an effect of damage or disease of the somatosensory nervous system [3]. It is estimated that prevalence of NeP reached up globally to 10% of people presenting in primary care [4]. Multiple diseases are associated with neuropathic pain including peripheral neuropathies and polyneuropathies; metabolic and nutritional (e.g. diabetes), drug and toxin related, hereditary diseases (e.g. Amyloid neuropathy, Fabry's disease, Charcot-Marie-Tooth), malignant and other polyneuropathies such as Small Fibre Neuropathy (SFN) or erythromelalgia [5]. NeP is very difficult to treat, since many first line analgesics are ineffective or their use is limited by dosage or side effects [5]. The underlying mechanism of NeP is complex and despite of big progress in pain research in the last two decades, still remains poorly understood [3].

1.2 Peripheral diabetic neuropathy

Diabetes mellitus (DM) is the most common metabolic disorder, affecting 422 million people worldwide [6]. DM is characterized by chronic hyperglycaemia that over time can lead to serious complications such as nerve damage and peripheral diabetic neuropathy (DN) [7]. According to the estimations up to 50% of patients with DN develop neuropathic pain, usually manifested as spontaneous, length-dependent burning, predominately present in the feet [8]. Despite intensive research to identify the cause of the painful form of diabetic neuropathy, it is still not clear why some patients with DN develop NeP, whereas others do not [9]. Multiple epidemiological studies have addressed this question and identified potential risk factors such as obesity, smoking, female sex, long diabetes duration and increasing age [9-11]. Interestingly, the NeP was found to be heritable in 37% of 1357 individuals in a twin study, highlighting the importance of genetic factor in NeP pathophysiology [12].

1.3 Small Fibre Neuropathy

Small fibre neuropathy is a type of peripheral neuropathy that affects the small nerve fibres: thinly myelinated Aδ-fibers and unmyelinated C-fibers in the skin [13]. These afferent fibres respond to thermal, mechanical, and chemical stimuli and are involved in pain sensation [14]. SFN manifests by abnormal painful sensations, typically tingling, burning, prickling, shooting pain, or aching expressed mainly in the length-dependent manner [15, 16]. Next to that, patients often report autonomic complaints including dry mouth and/or eyes, orthostatic dizziness, bowel disruption, micturition disturbances, hyper- or hypohidrosis, impotence, diminished ejaculation or lubrication, hot flushes and cardiac palpitations [13]. Although several underlying causes; diabetes mellitus, HIV, hyperlipidemia, amyloidosis, Fabry disease, coeliac disease, sarcoidosis and other systemic diseases, and voltage-gated sodium channel gene (VGSC or Nav) mutations are known, approximately half of SFN cases remain idiopathic [13, 17]. This impedes the treatment of the underlying condition, which in some cases is possible [14]. Moreover, lack of knowledge about molecular mechanism of SFN largely limits the therapy to symptomatic treatment of NeP [18].

1.4 Ion channels

Ion channels are polypeptides responsible for conduction of electrical signals through the body [19]. Ion channels expressed in afferent nerve fibres are critically involved in transmission and processing of pain signals (Figure 1), therefore they are considered as promising analgesic targets [20]. Despite the fact that the dysfunction of multiple ion channels have been linked to spectrum of pain disorders, we still do not fully understand pathological mechanism of pain [3, 19, 21-23]. The role of calcium channels, potassium channels, hyperpolarization-activated cyclic nucleotide-gated channels, anoctamins, transient receptor potential cation channels, and acid-sensing ion channels in painful neuropathies will be discussed in detail in chapter 2 of this thesis.

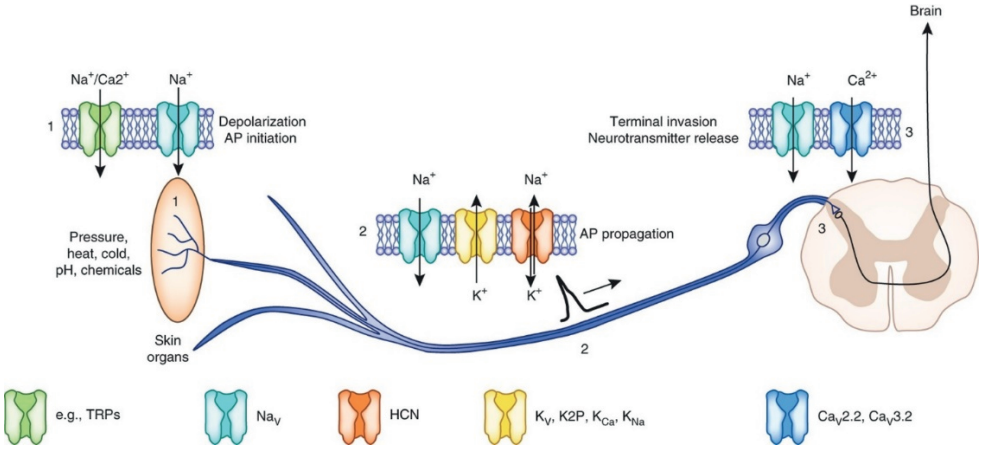


Figure 1. Ion channels and pain processing pathway. 1. Ion channels (e.g. TRPs) in nociceptive nerves are activated by noxious stimuli leading to membrane depolarization and initiation of action potentials. 2. Voltage-gated sodium channels and voltage-gated potassium channels propagate action potential along the axons to synaptic nerve terminals in the spinal dorsal horn. 3. Depolarization of the presynaptic terminal induces opening of voltage-gated calcium channels and release of neurotransmitters that activate central nervous system neurons that project to the brain. TRP, transient receptor potential–activated channel; Na_V, voltage-gated sodium channel; HCN, hyperpolarization-activated cyclic nucleotide–gated channel; K_V, voltage-gated potassium channel; K2P, two-pore potassium channel; K_{Ca}, calcium-activated potassium channel; K_{Na}, sodium-activated potassium channel; Ca_V, voltage-gated calcium channel. Figure and description adapted and modified from references [20, 24].

1.5 Voltage-gated sodium ion channels

Voltage-gated sodium ion channels are integral membrane proteins expressed in central and peripheral nervous system [25]. Their activity is crucial in initiation and propagation of action potentials in sensory neurons, therefore they play important role in pain pathways [26]. In mammals nine α subunits have been identified, encoded by *SCN1A, SCN2A, SCN3A, SCN4A, SCN5A, SCN8A, SCN9A, SCN10A*, and *SCN11A* [27]. In VGSCs, large α subunits with ion-selective pore are associated with one or two β subunits, that are involved in VGSCs modulation and protein targeting in plasma membrane (Figure 2) [28].

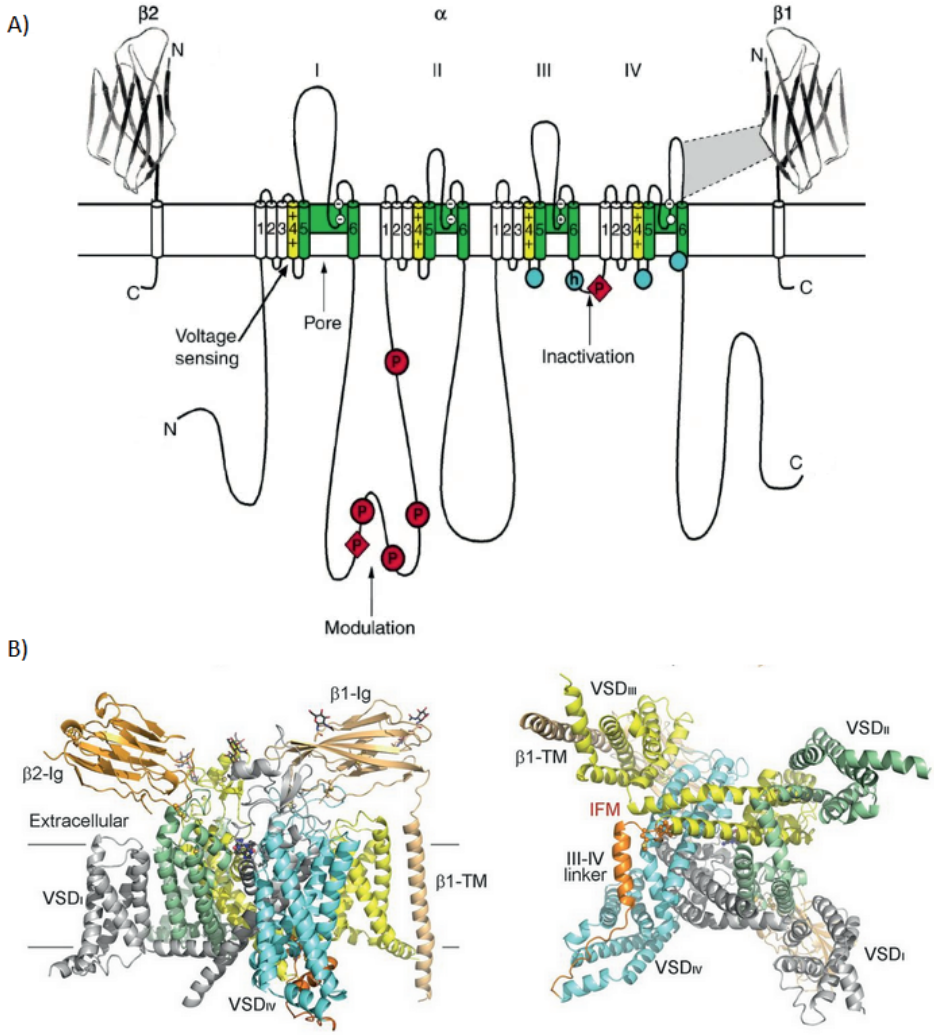
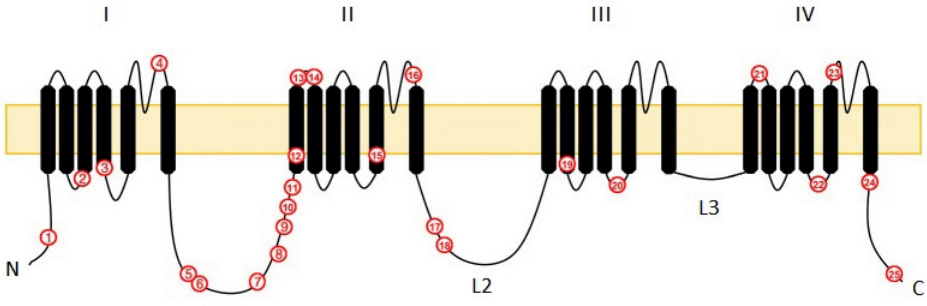


Figure 2. Structure of voltage-gated sodium channels. A) Schematic representation of the sodium channel α subunit together with the β1 and β2 subunits; I, II, III, IV, the domains of the α subunit; blue circles indicate the fast-inactivation motif Ile-Phe-Met (IFM) and its receptor (h, inactivation gate); P, phosphorylation sites. B) Overall 3D structure of Nav1.7 in complex with β1 and β2. The III-IV linker with IFM marked in orange. Figure adapted and modified from references [29, 30].

The most studied of VGSCs are Nav1.7, Nav1.8 and Nav1.9, which are expressed in nociceptive neurons [24]. Bi-allelic loss-of-function (LOF) mutation in *SCN9A* (Nav1.7) has been identified in extremely rare condition called congenital insensitivity to pain (CIP), in which person is unable to feel any physical pain [31].

In contrast, *SCN9A* gain-of-function mutations have been found to increase spontaneous activity and excitability in sensory neurons in pain disorders [32-34]. Similarly, GOF mutations of *SCN11A (*Nav1.9) and *SCN10A* (Nav1.8) have been associated with pain symptoms in painful peripheral neuropathies and familial episodic pain [24]. Later studies, have shown the involvement of sodium channel β subunit mutations in idiopathic small fibre neuropathy and painful diabetic neuropathy [35, 36].



	DI
1	K40E
2	R185H
3	I228M
4	N336T

	L1
5	V518F
6	E519K
7	T531N
8	D623N
9	A678E
10	F710V
11	I720K

	DII
12	I739V
13	P756T
14	M757W
15	G856D
16	M932L

	L2
17	Y990C
18	V991L

	DIII
19	M1230T
20	R1279Q

	DIV
21	M1532I
22	R1620L
23	Y1657S
24	V1754F
25	D1971V

Figure 3. *SCN9A* mutations in heritable SFN. Schematic linear representation of Nav1.7, showing the 24 tans-membrane regions, contained within four homologous domains (I-IV), joined by three intracellular loops (L1-L3). Most of the SFN associated mutation are clustered around L1. Figure adopted and modified from [37].

1.6 Diagnosis of Neuropathy and Neuropathic Pain

1.6.1 Clinical and physical examination

Diagnosis of neuropathic pain is based on a detailed clinical history and physical and neurological examination, however often extra tests are needed [38]. Clinical examination should aim to identify or rule out treatable conditions, confirm the diagnosis of NeP and recognize individual clinical features, that may accompany NeP and/or contribute to NeP, such as insomnia, depression, anxiety, or autonomic

complains, that can help to personalize the treatment strategy [39]. There are several symptoms that are often associated with NeP including abnormal sensation or hypersensitivity; hyperalgesia, allodynia, hypoalgesia, paresthesia, dysesthesia, and hypoesthesia (Table 1) [40]. Therefore, it is important to investigate pain onset, location and distribution, as well to ask patient to describe the pain, since shooting, burning, aching, tingling, stabbing are common indicators of NeP [38]. There are screening tools for instance the Neuropathic Pain Questionnaire (NPQ), Neuropathic Pain Symptom Inventory (NPSI), the Neuropathic Pain Diagnostic Questionnaire (DN4) and the Neuropathic Pain Scale (NPS), that may help to classify pain based on verbal report provided by the patient [5].

Negative symptoms	Definition	Bedside assessment	Expected pathological response
Hypoaesthesia	Reduced sensation to non-painful stimuli	Touch skin with painter's brush, cotton swab, or gauze	Reduced perception, numbness
Pall-hypoaesthesia	Reduced sensation to vibration	Apply tuning fork on bone or joint	Reduced perception threshold
Hypoalgesia	Reduced sensation to painful stimuli	Prick skin with single pin stimulus	Reduced perception, numbness
Thermal hypoaesthesia	Reduced sensation to cold or warm stimuli	Contact skin with objects of 10°C (metal roller, glass with water, coolants such as acetone); contact skin with objects of 45°C (metal roller, glass with water)	Reduced perception
Spontaneous sensations or pain			
Paraesthesia	Non-painful ongoing sensation (skin crawling sensation)	Grade intensity (0–10); area in cm^2	..
Paroxysmal pain	Shooting electrical attacks for seconds	Number per time; grade intensity (0–10); threshold for evocation	..
Superficial pain	Painful ongoing sensation, often a burning sensation	Grade intensity (0–10); area in cm^2	..
Evoked pain			
Mechanical dynamic allodynia	Pain from normally non-painful light moving stimuli on skin	Stroke skin with painter's brush, cotton swab, or gauze	Sharp burning superficial pain; present in the primary affected zone but spreads beyond into unaffected skin areas (secondary zone)
Mechanical static hyperalgesia	Pain from normally non-painful gentle static pressure stimuli on skin	Apply manual gentle mechanical pressure to skin	Dull pain; present in the area of affected (damaged or sensitised) primary afferent nerve endings (primary zone)
Mechanical punctate, pin-prick hyperalgesia	Pain from normally stinging but non-painful stimuli	Prick skin with a safety pin, sharp stick, or stiff von Frey hair	Sharp superficial pain; present in the primary affected zone but spreads beyond into unaffected skin areas (secondary zone)

Negative symptoms	Definition	Bedside assessment	Expected pathological response
Temporal summation	Increasing pain sensation (wind-up-like pain) from repetitive application of identical single noxious stimuli	Prick skin with safety pin at intervals of <3 s for 30 s	Sharp superficial pain of increasing intensity
Cold hyperalgesia	Pain from normally non-painful cold stimuli	Contact skin with objects of 20°C (metal roller, glass with water, coolants such as acetone); control: contact skin with objects of skin temperature	Painful, often burning, temperature sensation; present in the area of affected (damaged or sensitised) primary afferent nerve endings (primary zone)
Heat hyperalgesia	Pain from normally non-painful heat stimuli	Contact skin with objects of 40°C (metal roller, glass with water); control: contact skin with objects of skin temperature	Painful burning temperature sensation; present in the area of affected (damaged or sensitised) primary afferent nerve endings (primary zone)
Mechanical deep somatic hyperalgesia	Pain from normally non-painful pressure on deep somatic tissues	Apply manual light pressure at joints or muscles	Deep pain at joints or muscles

Table 1. Definition and assessment of negative and positive sensory symptoms in patients with neuropathic pain. Adopted from [5].

1.6.2 Skin biopsy

Punch skin biopsy is minimally invasive tool, used in diagnosis of SFN and generally well accepted by patients [5]. Skin biopsies are helpful for assessment of C-fibers, sympathetic fibers innervating the cutaneous sweat glands, and Aβ and Aδ fibers present in the dermis [5, 41]. The immunostaining with the cytoplasmic PGP9.5 antibodies is specific for neurons and visualises the intra-epidermal nerve fibers [41]. The length-dependent decrease in fiber density is common in neuropathies, including diabetes mellitus, HIV-associated sensory neuropathy, Fabry's disease, restless legs syndrome and small fiber neuropathy [5, 14].

1.6.3 Quantitative sensory testing (QST)

Quantitative sensory testing (QST) is a method to assess the sensory function using mechanical and thermal stimuli in quantitative way [42]. This tool is used in patients with symptoms of nerve damage, neuropathic pain or neurologic dysfunction in addition to a clinical neurological examination [43]. Standard testing includes series of innocuous or noxious stimuli such as touch, pressure, vibration and temperature (warm and cold) [44]. Although the sensory stimuli are objective, the patient response is subjective, therefore this method demands cooperation and alert from the

patient [44]. Therefore, QST differs from nerve conduction and evoked potential testing were the measurement is independent of subject cooperation [44]. It is important to realize that QST is a tool to detect abnormalities along the somatosensory system, but cannot be used to localize the injury.

1.6.4 Genetics

Undoubtedly, the development of Sanger sequencing technique in 1977 was a breakthrough in determining DNA sequences [45]. Although the method had limited throughput and high cost, it enabled sequencing the human genome. New sequencing technologies, known as next-generation sequencing (NGS), were developed, some of them based on Sanger sequencing [46] during last two decades. The rapid technological advancements and increasing throughput combined with decreasing price contributed to broad use of NGS in research and diagnostics [47]. Multiple studies have been using NGS in pain research to resolve the genetic architecture of NeP [48]. These efforts included both targeted sequencing of pre-defined gene panels (i.a. Molecular Inversion Probe- Next Generation Sequencing) [49] and (whole) exome sequencing [50, 51]. Several genes have been identified as potential pain candidates and specific pathogenic mutations have been discovered using this approach [33, 48, 52]. The clinical application of NGS is limited to some extent, because many of identified variants are variants of uncertain significance (VUS), for which it is unclear if they are the cause of the pain [53]. Nevertheless, VUS remain relevant findings, since the literature shows that a significant number of VUS have been proved to be pathogenic over the time [53]. This highlight, the importance of functional confirmation of the impact of the variants identified [53].

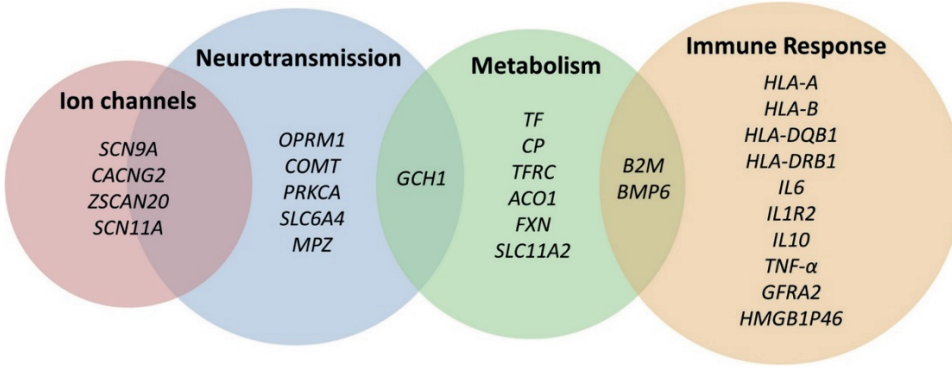


Figure 4. Genes associated with neuropathic pain, grouped according to their function and biological pathways. Figure adapted from reference [22].

1.6.5 Functional characterisation of genetic variants in pain research

Functional validation of a VUS is challenging. The pathogenicity of the variant can be established by targeted functional assays in model systems, such as patient-derived cells, organoids, cell culture and animal models [54]. In the case of ion channel variants the common assay is electrophysiology, which is highly laborious and therefore not possible to validate every VUS [33, 55]. It is performed using transgenic expression in model system or in Induced Pluripotent Stem Cells (IPSCs) derived from variant carrier [56, 57]. The first option includes genetic engineering techniques (e.g. Site Directed Mutagenesis) [58] to obtain construct containing gene of interest with specific variant which is later transfected to the cells or, alternatively, other techniques allowing genome editing in the cell (e.g. CRISPR-Cas9) [58-61]. Human cell cultures have been utilized for testing genetic variants, for example dorsal root ganglion (DRG) [31, 55] and GOF or LOF variants have been characterized this way [62, 63]. However, cell cultures might not fully reflect the *in vivo* conditions, complexity of pain pathways and genetic background of the patient (as IPSCs) [22]. Additional functional knowledge about pain genes is coming from animal studies involving mice or rats [22, 64]. These studies mostly provide information about gene function rather the single variant [22], however a models expressing specific variant such as mice with *SCN10A* p.G1662S point mutation have been characterized [59]. This type of testing cannot be performed on a large scale, therefore other model organism such as *Drosophila melanogaster* or Zebrafish are being explored [22, 65, 66].

1.7 Therapy for NeP

The heterogeneity and amount of possible underlying mechanisms in neuropathic pain make it very difficult to specifically treat a patient [3]. First line treatment strategies recommended by the Neuropathic Pain Special Interest group of IASP include tricyclic antidepressants (e.g. (nor)triptyline), serotonin-noradrenaline reuptake inhibitors (e.g. duloxetine) and α2-δ subunit blockers of voltage-gated calcium channel (pregabalin and gabapentin) [19, 67]. Second-line medication include topical agents such as lidocaine, capsaicin, and tramadol (a weak opioid), while third-line constitute strong opioids (e.g. morphine and oxycodone) [68]. Although, several treatment options are available (Figure 5), in general their effectivity is poor with a majority of patient that do not achieve pain relief [5]. The efficacy of pregabalin, duloxetine, nortriptyline and mexiletine was investigated in a 12- weeks randomized clinical trial [69]. The efficacious result was defined as at least 50% reduction in NeP and the best outcome was achieved for nortriptyline with 25% of patients reported such improvement [69]. In case of pregabalin it was only

15% of patients [69]. Another important point and disadvantage of NeP analgesics are common adverse effects, which limit the dosage and the duration of usage in the patients [67, 69]. Also, symptomatic treatment rarely brings complete pain alleviation and therapy may reduce pain by 30-50% at best [70]. As VGSC GOF mutations are known to contribute to neuropathic pain, the VGSC blockers seem to be good candidates as potential treatment strategy for this patient group. Non-selective VGSC inhibitors, such as local anesthetics (e.g. mexiletine) and antiepileptic drugs have been used. However, they are not specific and inhibit also VGSC expressed in the other organs, for example heart, therefore side effects might occur [36]. It has been shown that also other factors can influence drug effectiveness, for instance responsiveness to lacosamide is altered by Nav1.7 mutations [71]. Accordingly, specific targeting voltage-gated channels and other ion channel candidates is of great interest, since it might open possibility to develop new, personalized options for NeP alleviation.

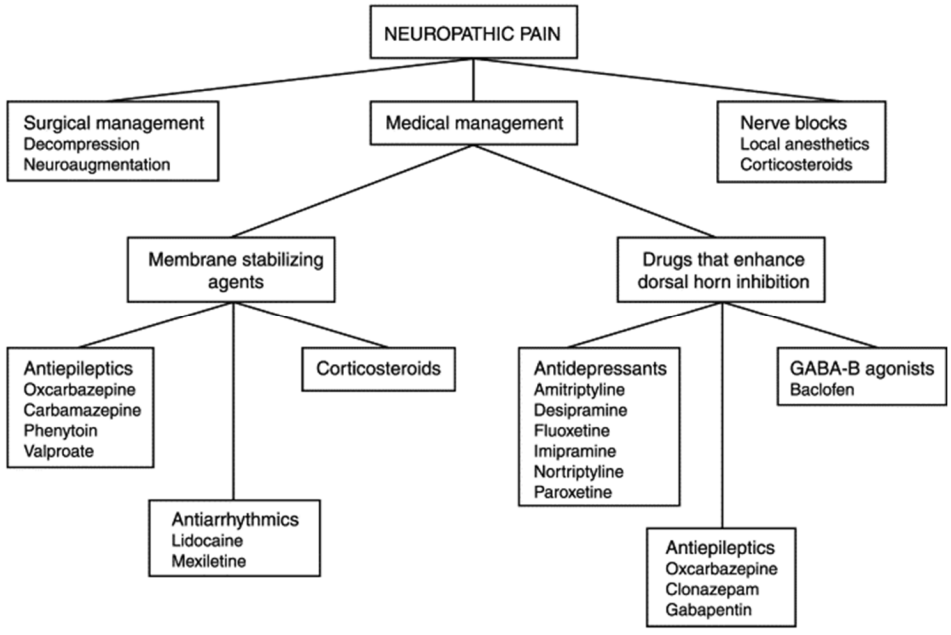


Figure 5. Neuropathic pain treatment strategies. Adapted from [38]

1.8 Aims and outline of the thesis

Neuropathic pain is a huge problem with serious economic, social and psychological impact and great burden for affected individuals and their families. High prevalence of NeP in neuropathy patients combined with poor therapy outcome demands better understanding of the underlying mechanisms to develop novel treatment strategies. Resolving the genetic causes of neuropathic pain may elucidate new molecular pain-targets from which affected individuals could benefit and which can open possibilities for future personalized medicine, treating the patient instead of the disease.

The aims of the thesis are:

1. Identifying candidate genetic variants for pain in specific patient cohorts, using next-generation sequencing
2. Elucidating novel pathophysiological mechanisms by functional characterization of novel variants/genes

Chapter 2 provides an overview of the emerging role of ion channel genes in painful neuropathies. Voltage-gated sodium channel mutations have been demonstrated as genetic contributors to neuropathic pain, however, they are only present in part of the patients, 18% in the case of SFN. As also other ion channels might be involved, we summarize and discuss the current knowledge about calcium channels, potassium channels, hyperpolarization-activated cyclic nucleotide-gated channels, anoctamins, transient receptor potential cation channels, and acid-sensing ion channels and their involvement in pain pathophysiology.

Chapter 3 describes the screening of 15 ion channel genes in 222 painful- and 304 painless-DN patients using MIP-NGS. In this study, we sequenced 5 transient receptor potential cation channels, 8 potassium channels and 2 calcium-activated chloride channel genes, identifying potentially pathogenic variants in ICG in both phenotypes. Moreover, we linked detected variants with clinical features, if possible and compared them between patients' group, with specific focus on painful-DN patients with ICG variants and painful-DN without ICG variant.

In **Chapter 4** we sequenced 414 patients with painful SFN using MIP-NGS with the same gene panel of 15 ICG (chapter 3). The cohort consisted of well-characterized patients, in which genetic variants in VGSC (*SCN3A, SCN7A-SCN11A* and *SCN1B-SCN4B)* were excluded. In 20 patients a potentially pathogenic ICG variant was identified. Clinical features such as pain intensity, thermal sensitivity and autonomic

complaints were compared between patients with ICG variant and patients without ICG variant.

In **Chapter 5,** a panel of 592 pain genes was sequenced in 50 patients with painful SFN. To select pain related gene candidates for this panel, literature and genetics pain databases were screened for genes, associated with pain. In addition, Online Mendelian Inheritance in Man (OMIM) was checked for genes, associated with peripheral neuropathy, neuropathic pain and associated symptoms. In this study, we identified (potentially) pathogenic variants that might contribute to neuropathic pain in genes other than VGSC and ICG.

In **Chapter 6** we focused on functional testing of potentially pathogenic variants in the Anoctamin 3 (ANO3) gene, which were present in painful-DN and SFN. We generated plasmids containing the ANO3 wild type gene, in which the variants of interest were introduced. ANO3 is novel gene, not linked with painful neuropathy before, with largely unknown function. Therefore, we investigated the ANO3 protein expression in several cell lines to choose a suitable model for further experiments. We tested three cell lines, performing transfection of HEK293, HT-29 and mesoangioblasts (MABs) using created plasmids. Moreover, a potential read out based on calcium imaging has been explored.

In **Chapter 7** the findings of the thesis are discussed and put in the context of future prospectives**. Chapter 8** elaborates about significance of this work in the light of future implementation, potential benefits and improvements.

References

1. Raja SN, Carr DB, Cohen M, Finnerup NB, Flor H, Gibson S, et al. The revised International Association for the Study of Pain definition of pain: concepts, challenges, and compromises. Pain. 2020;161(9):1976-82. doi: 10.1097/j.pain.0000000000001939. PubMed PMID: WOS:000571186100006.
2. Hunt SP, Mantyh PW. The molecular dynamics of pain control. Nat Rev Neurosci. 2001;2(2):83-91. doi: 10.1038/35053509. PubMed PMID: 11252998.
3. Colloca L, Ludman T, Bouhassira D, Baron R, Dickenson AH, Yarnitsky D, et al. Neuropathic pain. Nat Rev Dis Primers. 2017;3:17002. Epub 2017/02/17. doi: 10.1038/nrdp.2017.2. PubMed PMID: 28205574; PubMed Central PMCID: PMCPMC5371025.
4. Smith BH, Hébert HL, Veluchamy A. Neuropathic pain in the community: prevalence, impact, and risk factors. Pain. 2020;161 Suppl 1:S127-s37. Epub 2020/10/23. doi: 10.1097/j.pain.0000000000001824. PubMed PMID: 33090746.
5. Baron R, Binder A, Wasner G. Neuropathic pain: diagnosis, pathophysiological mechanisms, and treatment. Lancet Neurol. 2010;9(8):807-19. doi: Doi 10.1016/S1474-4422(10)70143-5. PubMed PMID: WOS:000280657800012.
6. Lovic D, Piperidou A, Zografou I, Grassos H, Pittaras A, Manolis A. The Growing Epidemic of Diabetes Mellitus. Curr Vasc Pharmacol. 2020;18(2):104-9. doi: 10.2174/1570161117666190405165911. PubMed PMID: WOS:000509755000002.
7. Grisold A, Callaghan BC, Feldman EL. Mediators of diabetic neuropathy: is hyperglycemia the only culprit? Curr Opin Endocrinol Diabetes Obes. 2017;24(2):103-11. doi: 10.1097/MED.0000000000000320. PubMed PMID: 28098594; PubMed Central PMCID: PMCPMC5831542.
8. Feldman EL, Callaghan BC, Pop-Busui R, Zochodne DW, Wright DE, Bennett DL, et al. Diabetic neuropathy. Nature Reviews Disease Primers. 2019;5. doi: 10.1038/s41572-019-0092-1. PubMed PMID: WOS:000472462700001.
9. Spallone V, Greco C. Painful and painless diabetic neuropathy: one disease or two? Current diabetes reports. 2013;13(4):533-49. Epub 2013/05/17. doi: 10.1007/s11892-013-0387-7. PubMed PMID: 23677582.
10. Ponirakis G, Elhadd T, Chinnaiyan S, Dabbous Z, Siddiqui M, Al-Muhannadi H, et al. Prevalence and risk factors for painful diabetic neuropathy in secondary healthcare in Qatar. J Diabetes Investig. 2019;10(6):1558-64. doi: 10.1111/jdi.13037. PubMed PMID: 30860314; PubMed Central PMCID: PMCPMC6825939.
11. Abbott CA, Malik RA, van Ross ER, Kulkarni J, Boulton AJ. Prevalence and characteristics of painful diabetic neuropathy in a large community-based diabetic population in the U.K. Diabetes care. 2011;34(10):2220-4. Epub 2011/08/20. doi: 10.2337/dc11-1108. PubMed PMID: 21852677; PubMed Central PMCID: PMCPMC3177727.
12. Momi SK, Fabiane SM, Lachance G, Livshits G, Williams FMK. Neuropathic pain as part of chronic widespread pain: environmental and genetic influences. Pain. 2015;156(10):2100-6. doi: 10.1097/j.pain.0000000000000277. PubMed PMID: WOS:000363362200029.
13. Hoeijmakers JG, Faber CG, Lauria G, Merkies IS, Waxman SG. Small-fibre neuropathies--advances in diagnosis, pathophysiology and management. Nature reviews Neurology. 2012;8(7):369-79. Epub 2012/05/30. doi: 10.1038/nrneurol.2012.97. PubMed PMID: 22641108.
14. Lauria G, Merkies IS, Faber CG. Small fibre neuropathy. Current opinion in neurology. 2012;25(5):542-9. Epub 2012/09/04. doi: 10.1097/WCO.0b013e32835804c5. PubMed PMID: 22941266.

15. Strand N, Wie C, Peck J, Maita M, Singh N, Dumbroff J, et al. Small Fiber Neuropathy. Curr Pain Headache R. 2022;26(6):429-38. doi: 10.1007/s11916-022-01044-8. PubMed PMID: WOS:000778915300001.
16. Lauria G. Small fibre neuropathies. Current opinion in neurology. 2005;18(5):591-7. doi: DOI 10.1097/01.wco.0000177330.35147.70. PubMed PMID: WOS:000232823800018.
17. 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. European journal of neurology. 2018;25(2):348-55. Epub 2017/11/08. doi: 10.1111/ene.13508. PubMed PMID: 29112785; PubMed Central PMCID: PMCPMC5814938.
18. Chan ACY, Wilder-Smith EP. Small fiber neuropathy: Getting bigger! Muscle Nerve. 2016;53(5):671-82. doi: 10.1002/mus.25082. PubMed PMID: WOS:000375116300001.
19. Finnerup NB, Kuner R, Jensen TS. Neuropathic Pain: From Mechanisms to Treatment. Physiological reviews. 2021;101(1):259-301. Epub 2020/06/26. doi: 10.1152/physrev.00045.2019. PubMed PMID: 32584191.
20. Waxman SG, Zamponi GW. Regulating excitability of peripheral afferents: emerging ion channel targets. Nature neuroscience. 2014;17(2):153-63. doi: 10.1038/nn.3602. PubMed PMID: WOS:000330910000007.
21. Zhang J, Yao J, Rong M. Editorial: Role of Ion Channels in Pain. Frontiers in pharmacology. 2022;13:884665. doi: 10.3389/fphar.2022.884665. PubMed PMID: 35734416; PubMed Central PMCID: PMCPMC9207521.
22. Calvo M, Davies AJ, Hébert HL, Weir GA, Chesler EJ, Finnerup NB, et al. The Genetics of Neuropathic Pain from Model Organisms to Clinical Application. Neuron. 2019;104(4):637-53. Epub 2019/11/22. doi: 10.1016/j.neuron.2019.09.018. PubMed PMID: 31751545; PubMed Central PMCID: PMCPMC6868508.
23. Levine JD, Alessandri-Haber N. TRP channels: targets for the relief of pain. Biochimica et biophysica acta. 2007;1772(8):989-1003. Epub 2007/02/27. doi: 10.1016/j.bbadis.2007.01.008. PubMed PMID: 17321113.
24. Goodwin G, McMahon SB. The physiological function of different voltage-gated sodium channels in pain. Nature Reviews Neuroscience. 2021;22(5):263-74. doi: 10.1038/s41583-021-00444-w. PubMed PMID: WOS:000634638700002.
25. Bennett DL, Clark AJ, Huang J, Waxman SG, Dib-Hajj SD. The Role of Voltage-Gated Sodium Channels in Pain Signaling. Physiological reviews. 2019;99(2):1079-151. Epub 2019/01/24. doi: 10.1152/physrev.00052.2017. PubMed PMID: 30672368.
26. Rogers M, Tang L, Madge DJ, Stevens EB. The role of sodium channels in neuropathic pain. Seminars in cell & developmental biology. 2006;17(5):571-81. Epub 2006/11/25. doi: 10.1016/j.semcdb.2006.10.009. PubMed PMID: 17123847.
27. de Lera Ruiz M, Kraus RL. Voltage-Gated Sodium Channels: Structure, Function, Pharmacology, and Clinical Indications. J Med Chem. 2015;58(18):7093-118. doi: 10.1021/jm501981g. PubMed PMID: 25927480.
28. Catterall WA, Goldin AL, Waxman SG. International Union of Pharmacology. XLVII. Nomenclature and structure-function relationships of voltage-gated sodium channels. Pharmacological reviews. 2005;57(4):397-409. Epub 2005/12/31. doi: 10.1124/pr.57.4.4. PubMed PMID: 16382098.
29. Yu FH, Catterall WA. Overview of the voltage-gated sodium channel family. Genome Biol. 2003;4(3). doi: 10.1186/gb-2003-4-3-207. PubMed PMID: WOS:000182694200003.
30. Shen H, Liu D, Wu K, Lei J, Yan N. Structures of human Nav1.7 channel in complex with auxiliary subunits and animal toxins. Science. 2019;363(6433):1303-8. doi: 10.1126/science.aaw2493. PubMed PMID: 30765606.

31. 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. Epub 2006/12/15. doi: 10.1038/nature05413. PubMed PMID: 17167479; PubMed Central PMCID: PMCPMC7212082.
32. 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 : a journal of neurology. 2005;128(Pt 8):1847-54. Epub 2005/06/17. doi: 10.1093/brain/awh514. PubMed PMID: 15958509.
33. Faber CG, Hoeijmakers JG, Ahn HS, Cheng X, Han C, Choi JS, et al. Gain of function Nav1.7 mutations in idiopathic small fiber neuropathy. Annals of neurology. 2012;71(1):26-39. Epub 2011/06/24. doi: 10.1002/ana.22485. PubMed PMID: 21698661.
34. Estacion M, Han CY, Choi JS, Hoeijmakers JGJ, Lauria G, Drenth JPH, et al. Intra- and interfamily phenotypic diversity in pain syndromes associated with a gain-of-function variant of Na(V)1.7. Molecular pain. 2011;7. doi: Artn 92 10.1186/1744-8069-7-92. PubMed PMID: WOS:000298672100001.
35. Alsaloum M, Estacion M, Almomani R, Gerrits MM, Bönhof GJ, Ziegler D, et al. A gain-of-function sodium channel β2-subunit mutation in painful diabetic neuropathy. Molecular pain. 2019;15:1744806919849802. Epub 2019/05/02. doi: 10.1177/1744806919849802. PubMed PMID: 31041876; PubMed Central PMCID: PMCPMC6510061.
36. Alsaloum M, Labau JIR, Sosniak D, Zhao P, Almomani R, Gerrits M, et al. A novel gain-of-function sodium channel beta2 subunit mutation in idiopathic small fiber neuropathy. J Neurophysiol. 2021;126(3):827-39. doi: 10.1152/jn.00184.2021. PubMed PMID: 34320850; PubMed Central PMCID: PMCPMC8461825.
37. Baker MD, Nassar MA. Painful and painless mutations of SCN9A and SCN11A voltage-gated sodium channels. Pflugers Archiv : European journal of physiology. 2020;472(7):865-80. doi: 10.1007/s00424-020-02419-9. PubMed PMID: 32601768; PubMed Central PMCID: PMCPMC7351857.
38. Chong MS, Bajwa ZH. Diagnosis and treatment of neuropathic pain. J Pain Symptom Manage. 2003;25(5 Suppl):S4-S11. doi: 10.1016/s0885-3924(03)00064-2. PubMed PMID: 12694987.
39. Gilron I, Baron R, Jensen T. Neuropathic Pain: Principles of Diagnosis and Treatment. Mayo Clin Proc. 2015;90(4):532-45. doi: 10.1016/j.mayocp.2015.01.018. PubMed PMID: WOS:000352197900018.
40. McCarberg B, D'Arcy Y, Parsons B, Sadosky A, Thorpe A, Behar R. Neuropathic pain: a narrative review of etiology, assessment, diagnosis, and treatment for primary care providers. Curr Med Res Opin. 2017;33(8):1361-9. doi: 10.1080/03007995.2017.1321532. PubMed PMID: WOS:000405213700002.
41. 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 Fe-deration of Neurological Societies and the Peripheral Nerve Society. European journal of neurology. 2010;17(7):903-E49. doi: 10.1111/j.1468-1331.2010.03023.x. PubMed PMID: WOS:000279021700005.
42. Pfau DB, Geber C, Birklein F, Treede RD. Quantitative Sensory Testing of Neuropathic Pain Patients: Potential Mechanistic and Therapeutic Implications. Curr Pain Headache R. 2012;16(3):199-206. doi: 10.1007/s11916-012-0261-3. PubMed PMID: WOS:000303880500002.
43. Backonja M, Attal N, Baron R, Bouhassira D, Drangsholt M, Dyck PJ, et al. Value of quantitative sensory testing in neurological and pain disorders: NEUPSIG consensus (vol 154, pg 1807,

2013). Pain. 2014;155(1):205-. doi: 10.1016/j.pain.2013.09.026. PubMed PMID: WOS:000329210000033.
44. Shy ME, Frohman EM. Quantitative sensory testing: Report of the Therapeutics and Technology Assessment Subcommittee of the American Academy of Neurology - Reply. Neurology. 2003;61(11):1630-. PubMed PMID: WOS:000187070400044.
45. Schuster SC. Next-generation sequencing transforms today's biology. Nat Methods. 2008;5(1):16-8. doi: 10.1038/Nmeth1156. PubMed PMID: WOS:000252120600009.
46. Hu TS, Chitnis N, Monos D, Dinh A. Next-generation sequencing technologies: An overview. Hum Immunol. 2021;82(11):801-11. doi: 10.1016/j.humimm.2021.02.012. PubMed PMID: WOS:000711185600002.
47. Vinksel M, Writzl K, Maver A, Peterlin B. Improving diagnostics of rare genetic diseases with NGS approaches. J Commun Genet. 2021;12(2):247-56. doi: 10.1007/s12687-020-00500-5. PubMed PMID: WOS:000607980200001.
48. Zorina-Lichtenwalter K, Parisien M, Diatchenko L. Genetic studies of human neuropathic pain conditions: a review. Pain. 2018;159(3):583-94. Epub 2017/12/15. doi: 10.1097/j.pain.0000000000001099. PubMed PMID: 29240606; PubMed Central PMCID: PMCPMC5828382 the end of this article.
49. Almomani R, Marchi M, Sopacua M, Lindsey P, Salvi E, Koning B, et al. Evaluation of molecular inversion probe versus TruSeq® custom methods for targeted next-generation sequencing. PloS one. 2020;15(9):e0238467. Epub 2020/09/03. doi: 10.1371/journal.pone.0238467. PubMed PMID: 32877464; PubMed Central PMCID: PMCPMC7467307.
50. Williams FMK, Scollen S, Cao DD, Memari Y, Hyde CL, Zhang BH, et al. Genes Contributing to Pain Sensitivity in the Normal Population: An Exome Sequencing Study. Plos Genet. 2012;8(12). doi: 10.1371/journal.pgen.1003095. PubMed PMID: WOS:000312905600011.
51. Kurth I, Baumgartner M, Schabhuttl M, Tomni C, Windhager R, Strom TM, et al. Whole exome sequencing in congenital pain insensitivity identifies a novel causative intronic NTRK1-mutation due to uniparental disomy. Am J Med Genet B. 2016;171(6):875-8. doi: 10.1002/ajmg.b.32458. PubMed PMID: WOS:000382871600013.
52. 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(6). doi: ARTN e207.doi:10.1212/NXG.0000000000000207. PubMed PMID: WOS:000425033200011.
53. Shashi V, McConkie-Rosell A, Schoch K, Kasturi V, Rehder C, Jiang YH, et al. Practical considerations in the clinical application of whole-exome sequencing. Clinical genetics. 2016;89(2):173-81. doi: 10.1111/cge.12569. PubMed PMID: 25678066.
54. Rodenburg RJ. The functional genomics laboratory: functional validation of genetic variants. J Inherit Metab Dis. 2018;41(3):297-307. doi: 10.1007/s10545-018-0146-7. PubMed PMID: WOS:000432732200003.
55. Asmild M, Oswald N, Krzywkowski KM, Friis S, Jacobsen RB, Reuter D, et al. Upscaling and automation of electrophysiology: Toward high throughput screening in ion channel drug discovery. Receptor Channel. 2003;9(1):49-58. doi: 10.1080/10606820308258. PubMed PMID: WOS:000186344000007.
56. 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. Epub 2018/11/22. doi: 10.1523/jneurosci.2433-18.2018. PubMed PMID: 30459225; PubMed Central PMCID: PMCPMC6335750.
57. Dib-Hajj SD. Human pain in a dish: Native DRG neurons and differentiated pluripotent stem cells. Pain. 2014;155(9):1681-2. doi: 10.1016/j.pain.2014.07.010. PubMed PMID: 25047782.

58. Yang W, Jiang LH. Site-directed mutagenesis to study the structure-function relationships of ion channels. Methods Mol Biol. 2013;998:257-66. doi: 10.1007/978-1-62703-351-0_20. PubMed PMID: 23529436.
59. Chidiac C, Xue Y, Muniz Moreno MDM, Bakr Rasheed AA, Lorentz R, Birling MC, et al. The Human SCN10A(G1662S) Point Mutation Established in Mice Impacts on Mechanical, Heat, and Cool Sensitivity. Frontiers in pharmacology. 2021;12:780132. doi: 10.3389/fphar.2021.780132. PubMed PMID: 34925037; PubMed Central PMCID: PMCPMC8671994.
60. Newell JG, Davies M, Bateson AN. The use of site-directed mutagenesis, transient transfection, and radioligand binding. A method for the characterization of receptor-ligand interactions. Mol Biotechnol. 2000;14(1):25-45. doi: 10.1385/MB:14:1:25. PubMed PMID: 10911613.
61. Bayerer B, Stamer U, Hoeft A, Stuber F. Genomic variations and transcriptional regulation of the human mu-opioid receptor gene. European journal of pain (London, England). 2007;11(4):421-7. doi: 10.1016/j.ejpain.2006.06.004. PubMed PMID: 16843022.
62. Faber CG, Lauria G, Merkies IS, Cheng X, Han C, Ahn HS, et al. Gain-of-function Nav1.8 mutations in painful neuropathy. Proceedings of the National Academy of Sciences of the United States of America. 2012;109(47):19444-9. Epub 2012/11/02. doi: 10.1073/pnas.1216080109. PubMed PMID: 23115331; PubMed Central PMCID: PMCPMC3511073.
63. 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 : a journal of neurology. 2014;137(Pt 6):1627-42. Epub 2014/04/30. doi: 10.1093/brain/awu079. PubMed PMID: 24776970.
64. Mogil JS. Animal models of pain: progress and challenges. Nature Reviews Neuroscience. 2009;10(4):283-94. doi: 10.1038/nrn2606. PubMed PMID: WOS:000264402500011.
65. Milinkeviciute G, Gentile C, Neely GG. Drosophila as a tool for studying the conserved genetics of pain. Clinical genetics. 2012;82(4):359-66. doi: 10.1111/j.1399-0004.2012.01941.x. PubMed PMID: WOS:000308646100007.
66. Eijkenboom I, Sopacua M, Otten ABC, Gerrits MM, Hoeijmakers JGJ, Waxman SG, et al. Expression of pathogenic SCN9A mutations in the zebrafish: A model to study small-fiber neuropathy. Exp Neurol. 2019;311:257-64. doi: 10.1016/j.expneurol.2018.10.008. PubMed PMID: WOS:000452937300023.
67. 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. doi: 10.1111/jns.12298. PubMed PMID: 30569495.
68. Shinu P, Morsy MA, Nair AB, Al Mouslem AK, Venugopala KN, Goyal M, et al. Novel Therapies for the Treatment of Neuropathic Pain: Potential and Pitfalls. J Clin Med. 2022;11(11). doi: ARTN 300210.3390/jcm11113002. PubMed PMID: WOS:000808866400001.
69. Barohn RJ, Gajewski B, Pasnoor M, Brown A, Herbelin LL, Kimminau KS, et al. Patient Assisted Intervention for Neuropathy: Comparison of Treatment in Real Life Situations (PAIN-CONTRoLS) Bayesian Adaptive Comparative Effectiveness Randomized Trial. Jama Neurol. 2021;78(1):68-76. doi: 10.1001/jamaneurol.2020.2590. PubMed PMID: WOS:000610675700009.
70. Bassel W. Abou-Khalil PA, Bela Ajtai,Jeffrey C. Allen,Brandon Ally,Andrea A. Almeida,Anthony A. Amato,Michael J. Aminoff,Nicolaas C. Anderson,Tetsuo Ashizawa,Hatim Attar,Alon Y. Avidan,Joachim M. Baehring,Asim K. Bag,Laura J. Balcer et al. Bradley and Daroff's Neurology in Clinical Practice. 8th Edition ed: Elsevier; 2022. p. 1853-929.
71. 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 : a journal of neurology. 2020;143(3):771-82. doi: 10.1093/brain/awaa016. PubMed PMID: 32011655; PubMed Central PMCID: PMCPMC7089662.

Chapter 2

The emerging role of ion channel genes in painful neuropathies

Milena Ślęczkowska[1,2#], Kaalindi Misra[3#], Silvia Santoro[3], Federica Esposito[3,4], Massimo Filippi[4,5,6,7], Janneke G J Hoeijmakers[2], Monique M Gerrits[8], Giuseppe Lauria[9,10], Hubert J M Smeets[1,2], Catharina G Faber[2*]

# Contributed equally

Chapter 3

Peripheral ion channel gene screening in painful- and painless- diabetic neuropathy

Milena Ślęczkowska[1,2], Rowida Almomani[1,3,4], Margherita Marchi[5], Bianca T A de Greef[3], Maurice Sopacua[3], Janneke G J Hoeijmakers[3], Patrick Lindsey[1], Erika Salvi[5], Gidon J Bönhof[6,7], Dan Ziegler[6], Rayaz A Malik[8,9], Stephen G Waxman[10,11], Giuseppe Lauria[5], Catharina G Faber[3], Hubert J M Smeets[1,2], Monique M Gerrits[12]

Published: Int J Mol Sci. 2022 Jul; 23(13): 7190; doi.org/10.3390/ijms23137190

ABSTRACT

Neuropathic pain is common in diabetic peripheral neuropathy (DN), probably caused by pathogenic ion channel gene variants. Therefore, we performed Molecular Inversion Probes-Next Generation Sequencing of 5 transient receptor potential cation channel, 8 potassium channel and 2 calcium-activated chloride channel genes in 222 painful- and 304 painless-DN patients. Twelve painful-DN (5.4%) patients showed potentially pathogenic variants (5 nonsense/frameshift, 7 missense, 1 out-of-frame deletion) in ANO3 (n=3), HCN1 (n=1), KCNK18 (n=2), TRPA1 (n=3), TRPM8 (n=3) and TRPV4 (n=1) and 14 painless-DN patients (4.6% - 3 nonsense/frameshift, 9 missense, 1 out-of-frame deletion) in ANO1 (n=1), KCNK18 (n=3), KCNQ3 (n=1), TRPA1 (n=2), TRPM8 (n=1), TRPV1 (n=3) and TRPV4 (n=3). Missense variants were present in both conditions, presumably with loss- or gain-of-function. KCNK18 nonsense/frameshift variants were found in painless/painful-DN, making a causal role in pain less likely. Surprisingly, premature stop-codons with likely nonsense-mediated RNA-decay were more frequent in painful-DN. Although limited in number, painful-DN patients with ion channel gene variants reported higher maximal pain during night and day. Moreover, painful-DN patients with TRP variants had abnormal thermal thresholds and more severe pain during night and day. Our results suggest a role of ion channel gene variants in neuropathic pain, but functional validation is required.

INTRODUCTION

Neuropathic pain (NeP) is caused by damage or disease of the somatosensory nervous system [1]. Around 50% of patients with diabetes develop diabetic peripheral neuropathy (DN) [2]. NeP impacts quality of life negatively in affected individuals and is associated with anxiety and depression [1]. Unfortunately, available treatment for neuropathic pain is largely unsatisfying with less than 50% of patients achieving 50% pain relief [3].

Several small-scale studies have investigated why some patients with diabetic neuropathy develop painful DN, while others do not [4]. Female sex, longer diabetes duration, older age, higher body mass index and smoking have been reported as risk factors [5-7]. However, these results were not confirmed in large cross-sectional studies performed in patients with type 2 diabetes, except for the association with smoking [8]. The complexity of the condition, limited number of participants, different assessment methods and definition of DN make it difficult to conclude if painful-DN and painless-DN represent different disorders or are different manifestations of the same disease [3, 4].

In recent years, genetic causes of NeP have partially been resolved, and Next-Generation Sequencing (NGS) has been increasingly performed to identify genes and genetic variants associated with NeP [6]. Pathogenic variants have been identified in voltage-gated sodium ion channels (VGSCs), especially the α-subunits. Voltage-gated potassium channels are a large group of channels, opening in response to membrane depolarization [9]. VGSCs are transmembrane proteins expressed in the central and peripheral nervous system that play important role in cells' electrical signaling [9]. Loss-of-function mutation in *SCN9A* gene encoding $Na_v1.7$, in contrast, led to congenital insensitivity to pain, while gain-of-function mutations have been linked to a spectrum of pain disorders, including painful-DN [9]. Patients with painful-DN and rare $Na_v1.7$ variants report more severe burning pain and increased pressure stimuli sensitivity compared to painful- DN patients without the *SCN9A* variant [10, 11]. However, the majority of genetic factors contributing to painful-DN still remains to be identified.

Apart from VGSCs, other ion channels are also essential for proper functioning of the nervous system [11]. It has been reported that transient receptor potential (TRP) cation channels, voltage-gated potassium (Kv) channels and hyperpolarization-activated and cyclic nucleotide-gated channels (HCN) may play a role in pain modulation and processing and/or painful neuropathies [11-14]. TRP channels are responsible for thermal, chemical and mechanical sensation [15]. HCN channels contribute to electrical excitability and pace-making activity in neuronal cells and have been linked to neuropathic pain [11, 13]. In addition, anoctamins have been

reported in pain processing and modulation of neuropathic pain [16, 17]. ANO1 is the most studied Ca^{2+}-activated Cl- channel from the ANO family, that interact with TRPV1 leading to pain enhancement in sensory neurons, while ANO3 regulate pain processing via increasing the Slack channels activity in DRG neurons [16-18].
Our hypothesis is that in addition to VGSCs, variants in other ion channels are involved in painful DN. Therefore, we analyzed seven Kv, five TRP, two ANO and one HCN ion channel genes expressed in peripheral nerves, using single molecule Molecular Inversion Probes-Next Generation Sequencing (smMIPs-NGS) in patients with painful-DN and painless-DN. The painless-DN group was included to identify variants linked to absence of pain, which may play role in pain resilience and to exclude common variants present frequently in both patient groups. We also assessed if these variants were linked to specific clinical manifestations.

METHODS

Study population and clinical assessment

Patients with painful-DN and painless-DN were enrolled in the Deutsche Diabetes Forschungsgesellschaft (DDFG), Heinrich Heine University, Düsseldorf, Germany or at the University of Manchester, Manchester, United Kingdom. They were examined between June 2014 and September 2016. Only patients aged above 18 years old and diagnosed with diabetes mellitus type 1 or type 2 (World Health Organization criteria) were eligible for this study. Demographic data, medical history, age of onset of complaints, duration of symptoms, altered pain sensation and family history were recorded. Pain intensity was evaluated using the 11-point Numerical Rating Scale (PI-NRS) from 0-10, where 0 means “no pain” and 10 refers to “the worst pain imaginable.” For all patients, informed consent was obtained and a blood sample for genetic analysis was drawn.
For patients with painful-DN quantitative sensory testing of the lower limb was performed on the dorsum of the foot using the Medoc® device. NeP was diagnosed as reported before [19]. The diagnosis of DN was established according to international criteria, based on typical clinical symptoms, nerve conduction studies (NCS) and IENFD determined by skin biopsy and/or abnormal TTT, after excluding all other known causes of neuropathy [20]. The DN patients were divided according to pain sensitivity into two groups: painful-DN subpopulation and painless-DN subpopulation. The patients having pain for at least 1 year, who reported score PI-NRS ≥ 4 despite analgesic or before starting treatment were defined as painful-DN, those with pain score ≤ 3 were defined as painless-DN.

DNA isolation and storage

Genomic DNA was extracted from whole blood using the NucleoSpin8 Blood Isolation kit (Macherey-Nagel, Düren, Germany) or QIAamp DNA Blood Maxi Kit, Puregene® Blood Core Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions at Maastricht UMC+, and IRCCS Neurological Institute Carlo Besta. DNA samples were coded and stored in the central DNA bank at Maastricht UMC+ and IRCCS Neurological Institute Carlo Besta in -20°C.

Gene panel and smMIPs-NGS protocol

Gene candidates for smMIPs-NGS were selected based on their role with pain in OMIM and literature, high expression in peripheral nerve DRG and/or trigeminus cells, and by comparative and integrative genomics with SCN9A co-expression described by Szklarczyk et al. [21]. Fifteen ion channel gene panel included: Anoctamin 1 and 2 (ANO1, ANO3), Hyperpolarization activated cyclic nucleotide-gated potassium channel 1 (HCN1), Potassium voltage-gated channel, shaker-related subfamily, member 2 and member 4 (KCNA2, KCNA4), Potassium channel, subfamily K, member 18 (KCNK18), Potassium intermediate/small conductance calcium-activated channel, subfamily N, member 1 (KCNN1), Potassium voltage-gated channel, KQT-like subfamily, member 3 and member 5 (KCNQ3, KCNQ5), Potassium voltage-gated channel, delayed-rectifier, subfamily S, member 1 (KCNS1), Transient receptor potential cation channel, subfamily A, member 1 (TRPA1), Transient receptor potential cation channel, subfamily M, member 8 (TRPM8), Transient receptor potential cation channel, subfamily V, member 1, member 3 and member 4 (TRPV1, TRPV3, TRPV4) (Table 1S).

In total 295 single-molecule molecular inversion probes (smMIPs) were designed to capture exon and exon-flanking intron sequences (+/- 20 bps) of 15 ion channel gene candidates (Table 1S), using a modified version of MIPgen tool (http://shendurelab.github.io/MIPGEN/). The number smMIPs per gene and size of targeted coding region per gene have been given in Table 3S. All probes, synthesized by Integrated DNA Technologies (IDT, Iowa, USA), were 77-80-mers long, containing extension and ligation arm, joined by common linker with two universal PCR primer sites as described before [22]. The smMIPs with high arm copy count (>5x) and/or common nucleotide polymorphisms (SNPs) (>1%) in the extension and ligation arms were excluded. The gap-fill length was 220-230 nt. Each probe contains a 5 nt unique molecular identifier (UMI), which enables removal of duplicates introduced during PCR amplification and sequencing.

SmMIP-NGS was performed as described before [22]. In brief, 50-100 ng genomic DNA was used for hybridization. After gap filling and ligation, circularized DNA

molecules served as PCR template. In the PCR reaction universal primers complementary to the linker were used. Amplified samples were pooled, purified by Ampure XP beads (Beckman Coulter, Inc, Brea, California) and paired-end sequenced (2 x 250 bp) using NextSeq 500 (Illumina, Inc., San Diego, CA, USA).

Data analysis

Sequenced data was analyzed using a smMIPs-NGS pipeline developed by Maastricht UMC+ in collaboration with Radboud University [22]. The smMIP arms were trimmed by our pipeline and then the sequences were aligned to human reference sequence GRCh37. Duplicates were removed based on UMIs probe sequences. Variant calling was performed using GATK (haplotype caller and unified genotyper) following best practices [23]. Variants were annotated based on information and frequencies from ExAC, dbSNP, cadd and Gencode [24]. Furthermore, the pipeline calculates coverage per sample, number of bases, mean and median coverage per MIP target [22].

Variant interpretation

Exonic variants and exonic flanking regions (+/- 20 bps) with >20x coverage and an alternative variant call of >30% were analyzed in further detail. Common SNPs were excluded from the analysis; only variants with dbSNP <5%, ExAC <5% and in house database of 12244 exomes <1% were included. Alamut Visual (Interactive Biosoftware, Rouen, France) software was used for variant interpretation. Variants were classified according the practice guidelines of the Association for Clinical Genetic Science (ACGS) [25]. Potentially pathogenic variants were checked manually, using BAM visualization in the Alamut Visual software and confirmed by Sanger sequencing. Due to the structure of the cohort co-segregation of potentially pathogenic variants with the disease was not possible.

Statistical analysis

The independent Student's t-test was used to analyze continuous variables and chi-square test was applied for categorical variables. The significance level was defined as 0.05.

RESULTS

Patient characteristics

In total 612 patients with DN were included, 393 from the Heinrich Heine University (Düsseldorf, Germany), and 219 from the University of Manchester (Manchester, UK). Of these, 86 patients were, due to low DNA quality, MIP failure, incomplete

clinical data or withdrawal of formal consent. Among the enrolled patients, 222 had painful-DN and 304 had painless-DN. The mean age at recruitment was 64.5 years old (SD +/-11.2 years). Sex distribution in the study group was 372 (70.7 %) males versus 154 (29.3 %) females. The higher male prevalence was present in both phenotypes. Clinical characteristics of the study population are presented in Table 2S.

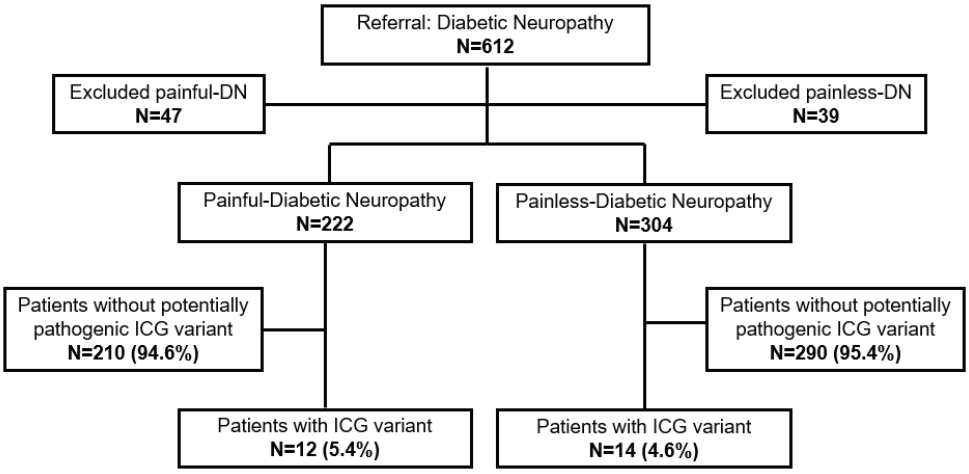


Figure 1. Patients with painful- and painless- Diabetic Neuropathy screened for potentially pathogenic variants in 15 ion channel gene panel. ICG, ion channel gene; DN, diabetic peripheral neuropathy; N, number

Performance of smMIPs-NGS

To assess the performance, capture efficiency and coverage of exons and exon-flanking sequences (+/- 20 bps) of 15 ICG, data of 50 samples from 5 different NGS runs were used. An overall coverage of more than 30x/bp was obtained for 93% of the on-target regions. Exons and exon-flanking sequences of ten gene areas have an average coverage >90%. Four exons had a poor coverage (<20x/bp) or were completely missing (exon 1, exon 12 and exon 20 of *ANO1*, and exon 3 of *TRPV1*), and MIPs for 14 exons covered the targeted region >20x/bp only partially (Table 3S). For the remaining exons and exon-flanking sequences the average coverage <90%, but at least an average coverage of 84% was reached.

Genetic screening of 15 ion channels in painful diabetic neuropathy

MIP-NGS data analysis of 222 patients with painful-DN resulted in identification of 13 different potentially pathogenic variants in 6 ICG genes (*ANO3, HCN1, KCNK18, TRPA1, TRPM8, TRPV4,* Table 1*).* Eleven patients were positive for one heterozygous variant, and one patient harbored two heterozygous variants (n=12/222, 5.4%, Fig. 1). All 13 variants were classified as variant of uncertain clinical significance (VUS, Table 1). Nine out of 13 identified ICG variants were novel (not reported in the literature, the Human Gene Mutation Database (HGMD) and ClinVar). Two frameshift mutations localized in the *KCNK18* gene c.414_415del and c.361dup have been functionally linked to migraine [26-28]. The *ANO3* missense variant c.638C>T was reported in ClinVar as VUS without phenotype and *TRPV4* c.2336+1G>A as VUS for Charcot-Marie-Tooth disease. Most of the variants were located in genes from the TRP family, responsible for mechanical, chemical and thermal sensation. The painful-DN patients with an ICG variant have been previously tested for variants in *SCN3A*, *SCN7A-SCN11A* and *SCN1B-SCN4B*. None of them was harboring a VUS, likely pathogenic or pathogenic variant in one of these genes (unpublished data). The clinical characteristics of painful- DN patients with ICG VUS are presented in Table 2 and the autonomic complaints are summarized in supplementary materials (Table 4Sb, supplementary data). Most of the patients (7 out of 9, for 3 patients TTT is missing) with potentially causative ICG variant had an abnormal TTT, especially patients carrying variants in one of TRP genes (4 out of 4, for 2 patients TTT is missing). The majority of patients with painful-DN with an ICG variant reported a negative family history for neuropathy and 6 out of 12 patients had identified a possible non-genetic underlying cause (Table 2). Patients with painful-DN with identified ICG variants (n=9) reported higher mean maximal pain scores during 24 hours (PI-NRS 7.2 +/- 2.4) compare to patients with painful-DN without an ICG variant (PI-NRS 6.0 +/- 3.0, n=150). However due to the small sample size, statistical analysis was not possible (Table 4Sa). Interestingly, a patient with painful-DN and a potentially causative variant in the TRP gene had severe or very severe pain during night (PI-NRS 8.5 +/-1.29) and day (PI-NRS 8.0 +/-0.82) (Table 2) and impaired thermal sensation.

Table 1: Potentially pathogenic variants of ion channel genes identified in patients with painful-Diabetic Neuropathy (n=222).

Gene	c.position [&]	p.position	Number of patients	Classification based Richards et al [25]	Location	MAF gnomAD (%)	Ref.
ANO3	c.638C>T	p.(Ser213Phe)	1	VUS	N-terminus	0	-
	c.1357A>G	p.(Ile453Val)	1	VUS	N-terminus	0	-
	c.2950C>T	p.(Leu984Phe)	1	VUS	Transmembrane domain VIII	0.0046	-
HCN1	c.1214G>A	p.(Arg405Gln)	1	VUS	C-terminus	0.0004	-
KCNK18	c.414_415del	p.(Phe139Trpfs*25)	1	VUS	Exon 3, the new reading frame ends in a STOP codon at position 25	0.043	[26-29]
	c.361dup	p.(Tyr121Leufs*44)	1	VUS	Exon 3, the new reading frame ends in a STOP codon at position 44	0.024	[26]
TRPA1	c.2481del	p.(Ala828Leufs*17)	1	VUS	Exon 21, the new reading frame ends in a STOP codon at position 17	0	-
	c.352C>G	p.(Leu118Val)	1	VUS	Ankyrin repeat II-containing domain	0.047	-
	c.1954C>T	p.(Arg652*)	1	VUS	Cytoplasmic domain between ANK repeats and 1st transmembrane domain	0.015	-
TRPM8	c.2114del	p.(Val705Glyfs*79)	1	VUS	Exon 16, The new reading frame ends in a STOP codon at position 79	0.0004	-
	c.1437G>T	p.(Glu479Asp)	1	VUS	N-terminus	0.012	-
	c.2195C>T	p.(Thr732Ile)	1	VUS	Linker between transmembrane domain I and II	0.069	-
TRPV4	c.2336+1G>A	p.? [#]	1	VUS	Donor splice site of intron 14	0.0012	-

c. position, location cDNA; p. position, location in protein; MAF gnomAD, Minor Allele Frequency The Genome Aggregation Database; n/a, not applicable;
[&] Variants detected were annotated according to the guidelines of the Human Genome Variation Society using reference sequence GRCh37 and transcript numbers, NM_001313726.1 (*ANO3)*; NM_021072.4 (*HCN1)*, NM_181840.1 (*KCNK18),* NM_007332.2 (*TRPA1)*; NM_024080.4 (*TRPM8),* NM_021625.4 (*TRPV4)*;
* One patient was heterozygous for *TRPA1* c.2481del and *TRPM8* c.1437G>T; [#]predicted skip of exon 14 would lead to a premature stop codon

Table 2: Clinical characteristic of patients with painful-Diabetic Neuropathy and ion channel gene variant.

Sex	Age	Diabetes type	Age of onset (diabetes)	Age of onset (neuropathy)	Max pain during night	Mean pain during night	Max pain during day	Mean pain during day	Potential underlying cause of neuropathy	TTT	Positive family history (neuropathy)	variant
F	61	DM2	34	54	6	4	5	3	adenoma of the thyroid gland, hypothyroidism	abnormal (feet)	yes	ANO3 p.(Ser213Phe)
F	68	DM2	43	60	6	4	10	4	unknown	normal	no	ANO3 p.(Ile453Val)
M	43	DM2	34	37	0	0	8	2	slipped disk (three times)	normal	no	ANO3 p.(Leu984Phe)
M	65	DM2	57	65	9	9	4	3	back surgery after car crash, slipped disk	abnormal (feet+hands)	no	HCN1 p.(Arg405Gln)
F	75	DM2	49	72	8	3	7	3	unknown	abnormal (feet+hands)	no	KCNK18 p.(Phe139Trpfs*25)
M	77	DM2	60	-	-	-	-	-	-	-	-	KCNK18 p.(Tyr121Leufs*44)
M	48	DM2	33	-	-	-	-	-	-	-	no	TRPA1 p.(Ala828Leufs*17) TRPM8 p.(Glu479Asp)
M	65	DM2	65	65	10	10	8	8	spine injury	abnormal (feet)	no	TRPA1 p.(Leu118Val)
M	73	DM2	58	69	8	6	8	6	unknown	abnormal (feet)	no	TRPA1 p.(Arg652*)
M	57	DM2	18	46	7	0	7	0	hypothyroidism	abnormal (feet+hands)	yes	TRPA8 p.(Val705Glyfs*79)
F	77	DM2	75	75	9	5	9	6	hypothyroidism	abnormal (feet+hands)	no	TRPM8 p.(Thr732Ile)
M	73	DM1	8	-	-	-	-	-	-	-	-	TRPV4 p.?

DM1, Diabetes mellitus type 1; DM2, Diabetes mellitus type 2; F, female; M, male; -, data incomplete; TTT, thermal threshold testing. Age given at the day of recruitment. Neuropathic pain assessed using pain intensity numerical rating scale

Genetic screening of 15 ion channels in painless diabetic neuropathy

In 304 patients with painless-DN, 13 different potentially pathogenic heterozygous variants were identified. Fourteen (4.6%) patients had a potentially pathogenic variant in one of the 15 ICG, but none had more than one ICG variant (Table 3). In two individuals with an ICG variant, previously a potentially pathogenic SCN variant was reported; *SCN10A* c.368C>T, VUS and *ANO1* c.1892G>A, VUS, and *SCN10A* c.1141A>G, possibly pathogenic and *TRPV4* c.958C>T, VUS. Potentially pathogenic ICG variants were located in 7 genes: *ANO1, KCNK18, KCNQ3, TRPA1, TRPM8, TRPV1* and *TRPV4* (Table 3). Seven out of 13 detected variants were novel. Six variants have been reported in ClinVar as pathogenic, VUS or variant with conflicting interpretations of pathogenicity. Both *KCNK18* variants have been linked to migraine and reported respectively as pathogenic, but with conflicting interpretations of pathogenicity. *KCNQ3* c.1226C>G was reported as VUS in benign familial neonatal seizures. *TRPV4* c.711A>G and c.1039G>T were reported as VUS for Charcot-Marie-Tooth disease type 2C and *TRPV4* c.958C>T as a variant with conflicting interpretations of pathogenicity for skeletal dysplasias, spinal muscular atrophies and Charcot-Marie-Tooth. Five painless-DN patients with an ICG variant filled out pain questionnaire and all of them reported no pain during 24 hours; max and mean pain during night and day (PI-NRS 0). It seems that patients with painless-DN with an ICG VUS variant reported less often autonomic complains, especially diarrhea, dry mouth, orthostatic dizziness, sheet intolerance and restless leg, however the group size (n = 4) is too small to draw a definite conclusion. The clinical characteristic and autonomic complaints in patients with painless- DN and ICG variant are presented in Table 4 and Table 5S in supplementary materials.

Table 3: Potentially pathogenic variants of ion channel genes identified in patients with painless-Diabetic Neuropathy (n=304).

Gene	c.position [&]	p.position	Number of patients	Classification based Richards et al [25]	Location	MAF gnomAD (%)	Ref.
ANO1	c.1892G>A	p.(Arg631Gln)	1	VUS	Linker between transmembrane domain V and VI	0.012	-
KCNK18	c.414_415del	p.(Phe139Trpfs*25)	1	VUS	Exon 3, the new reading frame ends in a STOP codon at position 25	0.043	[26-29]
	c.361dup	p.(Tyr121Leufs*44)	2	VUS	Exon 3, the new reading frame ends in a STOP codon at position 44	0.024	[26]
KCNQ3	c.1226C>G	p.(Pro409Arg)	1	VUS	C-terminus	0.067	-
TRPA1	c.352C>G	p.(Leu118Val)	1	VUS	Ankyrin repeat II-containing domain	0.047	-
	c.1980C>A	p.(Phe660Leu)	1	VUS	Cytoplasmic domain between ANK repeats and transmembrane domain I	0.01	-
TRPM8	c.2956G>A	p.(Val986Ile)	1	VUS	C-terminus	0.002	-
TRPV1	c.1450G>C	p.(Gly484Arg)	1	VUS	Transmembrane domain II	0	-
	c.1781C>T	p.(Ala594Val)	1	VUS	Transmembrane domain V	0.042	-
	c.1790C>T	p.(Thr597Met)	1	VUS	Transmembrane domain V	0.0021	-
TRPV4	c.711A>G	p.? [#]	1	VUS	Ankyrin repeat I-containing domain	0.0008	-
	c.958C>T	p.(Arg320*)	1	VUS	N-terminus	0.0039	-
	c.1039G>T	p.(Asp347Tyr)	1	VUS	N-terminus	0.018	-

c. position, location cDNA; p. position, location in protein; MAF gnomAD, Minor Allele Frequency The Genome Aggregation Database; n/a, not applicable; 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_018043.5 (*ANO1)*; NM_181840.1 (*KCNK18),* NM_004519.3 (*KCNQ3),* NM_007332.2 (*TRPA1)*; NM_024080.4 (*TRPM8)*; NM_080706.3 (*TRPV1);* NM_021625.4 (*TRPV4)*; [#] predicted alternative splice sites would lead to a premature stop codon

Table 4: Clinical characteristic of patients with painless-Diabetic Neuropathy and ion channel gene variant.

Sex	Age	Diabetes type	Age of onset (diabetes)	Age of onset (neuropathy)	Potential underlying cause of neuropathy	TTT	Positive family history (neuropathy)	variant
M	78	DM2	55	-	-	-	no	ANO1 p.(Arg631Gln)
M	68	DM2	64	-	-	-	-	KCNK18 p.(Phe139Trpfs*25)
M	50	DM2	46	-	-	-	no	KCNK18 p.(Tyr121Leufs*44)
M	73	DM2	57	-	-	-	-	KCNK18 p.(Tyr121Leufs*44)
M	69	DM1	13	-	-	-	-	KCNQ3 p.(Pro409Arg)
F	61	DM1	32	61	thyroidectomy	abnormal (feet)	yes	TRPA1 p.(Leu118Val)
F	39	DM2	26	-	-	-	-	TRPA1 p.(Phe660Leu)
M	81	DM2	66	78	hypothyroidism	abnormal (hands and feet)	no	TRPM8 p.(Val986Ile)
M	54	DM1	7	-	-	-	-	TRPV1 p.(Gly484Arg)
F	62	DM2	60	-	-	-	-	TRPV1 p.(Ala594Val)
M	43	DM1	17	35	unknown	abnormal (hands and feet)	yes	TRPV1 p.(Thr597Met)
M	76	DM1	23	66	slipped disc LS 5/S1	Normal	no	TRPV4 p.?
M	52	DM2	50	-	-	-	-	TRPV4 p.(Arg320*)
M	67	DM2	67	68	-	normal	no	TRPV4 p.(Asp347Tyr)

DM1, Diabetes mellitus type 1; DM2, Diabetes mellitus type 2; F, female; M, male; -, data incomplete. Age given at the day of recruitment visit.

DISSCUSSION

Summary of detected variants

In our cohort of patients with DN, twelve patients with painful- DN (5.4%) and fourteen patients with painless- DN (4.6%) harbored at least one potentially pathogenic variant in ICG gene, respectively linked to pain or no pain. Only one patient with painful-DN had two heterozygous variants TRPA1 c.2481del and TRPM8 c.1437G>T. In total, we identified 13 different potentially pathogenic variants in painful- DN located in *ANO3, HCN1, KCNK18, TRPA1, TRPM8, TRPV4*. Three patients (1.4%) had a potentially pathogenic variant in *ANO3*, one (0.5%) in *HCN1*, two (0.9%) in *KCNK18*, three (1.4%) in *TRPA1*, three (1.4%) in *TRPM8*, one (0.5%) in *TRPV4*. In patients with SFN and NeP, the frequency of (potentially) pathogenic variants in the sodium ion channels was slightly higher; 5.1% for *SCN9A*, 3.7% for *SCN10A* and 2.9% for *SCN11A* [30]. A total of 13 different potentially pathogenic variants was detected in painless-DN in *ANO1, KCNK18, KCNQ3, TRPA1, TRPM8, TRPV1* and *TRPV4.* Potentially causative variants were identified in one patient (0.3%) in *ANO1*, three (1%) in *KCNK18*, one (0.3%) in *KCNQ3*, two (0.7%) in *TRPA1*, one (0.3%) in *TRPM8*, three (1%) in *TRPV1* and three (1%) in *TRPV4*. Four of these genes: *ANO1, ANO3, HCN1* and *KCNQ3* have not been linked to a painful phenotype or to pain insensitivity before in HGMD. We did not find any potentially pathogenic variant in 6 genes from our gene panel: *KCNA2, KCNA4, KCNNA, KCNQ5, KCNS1, TRPV3*.

The same missense variants were not present in any of the ICG genes in painful- and painless-DN with the exception of migraine related *KCNK18* variants c.414_415del and c.361dup and *TRPA1* c.352C>G. Our patients did not report migraine during clinical investigation. However, questions about headache or migraine were not included in the standard questionnaires and we were not able to contact the patients to confirm the lack of migraine symptoms. The presence of these variants in both phenotypes might be explained by other mutations in genes not included in our gene panel or non-genetic risk factors contributing to a painful phenotype [6, 31]. In two individuals with an ICG variant, previously a potentially pathogenic SCN variant was reported; *SCN10A* c.368C>T, VUS and *ANO1* c.1892G>A, VUS, and *SCN10A* c.1141A>G, possibly pathogenic and *TRPV4* c.958C>T, VUS.

Effect of VUS on protein function

All variants meeting our criteria were variants of uncertain clinical significance, making it difficult to draw a definite conclusion on a causative role. However, this is not surprising, since most of the variants we identified were novel and we could only rely on in-silico predictions without segregation analysis in families or

functional studies. The development of NGS and more affordable cost of sequencing have resulted in recent years in identification of a significant number of VUS and part of them have been proved to be pathogenic over the time [32].
The VUS detected in our study either change the protein via substitutions of conserved amino acids (missense VUS) or are generally predicted to lead to the absence of the protein, because of nonsense mediated decay, triggered by a premature stop codon (nonsense/frameshift VUS). For the latter group, the KCNK18 gene contained premature stop codons in both painful and painless DN, making a causal role of haploinsufficiency of these genes on the pain phenotype unlikely. For the other nonsense/frameshift variants this is not the case. Although NMD is most likely, also truncated non-functional protein or alternative processes, such as frameshift mutation-induced alternative translation initiation (fsATI) have been reported [26], requiring functional validation of frameshift/nonsense mutation to be sure of the mechanism.
Overlap exists between genes with missense mutations in painful and painless-DN, but this does not disqualify the role they might have, as it has been reported that missense mutations can lead to gain or loss-of-function with possible opposite effects on pain sensitivity, highlighting the importance of functional effect rather the variation itself. Additional support for functional relevance is for the 3 VUS in TRPV1 and 1 VUS in ANO3, located in the transmembrane domain, which can directly affect channel properties e.g., channel gating [33]. Two missense VUS were located in Ankyrin repeat II-containing domain, responsible for protein-protein interactions and protein folding [34]. Dysfunction in ANK repeat proteins have been reported in many human diseases [34]. Seven missense VUS were located in the N- and the C- terminal part of the protein, which play an important role in protein stability and turnover [35]. Two missense VUS were found in intracellular linkers between transmembrane domains in TRPM8 and ANO1. Interestingly most of the potentially pathogenic SCN9A variants were localized in the intracellular linkers [30]. Therefore, the VUS identified are expected to have a negative quantitative effect on protein function (nonsense/frameshift) or a GOF or LOF (missense), although the direction or magnitude can not be predicted by bioinformatic tools.

Possible link between VUS and clinical manifestations

- ***Ca^{2+} channels***

Three missense heterozygous variants in *ANO3* gene were detected in painful-DN and one missense heterozygous variant in *ANO1* gene in painless-DN. These genes

belong to Ca^{2+}-activated Cl- channels and they are highly expressed in dorsal root ganglia (DRG) [17, 18]. ANO3 is known to regulate pain processing via interaction with Slack channels and it has been shown that ANO3 knockdown lead to reduced mechanical allodynia and pain attenuation [18]. Therefore, GOF ANO3 variants potentially increase protein activity causing enhanced pain sensitivity in painful-DN. ANO1 has been described as a heat sensor and a knockout of the ANO1 gene reduces thermal nociceptive responses [17, 36]. A LOF of the ANO1 VUS would confirm a role in the painless-DN phenotype.

- ***K^+ channels***

Voltage-dependent potassium channels are crucial for controlling the excitability of nociceptors and pain processing [37]. A heterozygous missense variant HCN1 c.1214G>A was identified in a patient with painful-DN. It has been reported that both inflammatory and neuropathic pain are rapidly inhibited by blocking HCN-dependent repetitive firing in peripheral nociceptive neurons [38-40]. Furthermore, the specific blockade of HCN1 attenuates hyperalgesia and allodynia in neuropathic pain animal model [41]. If the HCN1 VUS increases HCN-dependent firing, this would explain the opposite effect. Recent studies have demonstrated that gain of function mutations of KCNQ2/3 channels contribute to pain resilience [42, 43], which might explain the KCNQ3 c.1226C>G VUS variant present in a patient with painless-DN.

- ***TRP channels***

We identified several variants located in TRP channels both in painful- and painless-DN. TRPV1, TRPM8, and TRPA1 are thermal and chemical detectors that activate sensory neurons to produce pain, while TRPV4 mediates nociceptive behaviors by hyper- and hypotonic stimuli [44]. Three TRPA1 VUS variants were detected in painful-DN: one missense c.352C>G and two variants (c.2481del and c.1954C>T) leading to a premature stop codon and most likely nonsense mediated decay, predicted specifically in case of c.1954C>T variant. This shows that TRPA1 loss of function is linked to increased pain sensitivity. Moreover, the c.1954C>T was also detected in a patient with SFN and burning pain in the hands and lower legs (unpublished data). Interestingly, the TRPA1 c.2755C>T variant leading to a premature stop codon at position 919 has been found to co-segregate with pain in cram-fasciculation syndrome [45]. As mechanism, a toxic gain-of-function was proposed, which was consistent with a marked clinical improvement with carbamazepine, a cation (sodium) channel blocker. However, this would require

expression of the truncated protein, for which no evidence was provided. Increased TRPA1 expression in DRG has been observed after nerve injury and has been linked to cold hyperalgesia, that was reversed after TRPA1 blockade [46]. Consistent with that, GOF mutation Asn855Ser caused cold hyperalgesia in familial episodic pain syndrome [47]. Two out of three painful-DN patients with a TRPA1 VUS (one missense, one nonsense) had an abnormal TTT, for one patient data was incomplete. In painless-DN, one missense TRPA1 VUS out of 2 has an abnormal TTT. This suggests a role of TRPA1 variants in thermal sensation, however based on the limited number of samples it is not possible to draw a definite conclusion, especially as abnormal thermal sensation is common in patients with diabetic neuropathy.

Similarly, to TRPA1, TRPM8 modulates cold sensation and its activation produces analgesia in chronic neuropathic pain states [48, 49]. In painful-DN, we detected one frameshift mutation leading to pre-mature stop codon and two missense TRMP8 VUS and 2 patients had abnormal TTT values. One TRPM8 VUS c. 2956G>A was present a in patient with painless-DN and abnormal TTT, again stressing that for the missense mutations GOF or LOF has to be defined first before drawing conclusions.

TRPV1 and TRPV4 belong to the transient receptor potential vanilloid family and most of the VUS in these genes were present in painless-DN. In total three missense VUS in TRPV1 were present in painless-DN, all located in the functional domain of the channel; c.1450G>C in transmembrane domain II, c.1781C>T and c.1790C>T both in transmembrane domain IV. Activation of TRPV1 results in generation of action potentials and in many cases pain [50]. Moreover the GOF TRPV1 mutation at position p.Q85R have been identified in 3 cases with persistent neuropathic corneal pain after refractive surgery [51], therefore variants reducing TRPV1 activity are expected in the painless-DN group. TRPV4 is involved in mechanical hyperalgesia and lead to pain behaviors in animal model in response to hypo-osmotic stimulation and inflammation [52]. As indicated above, a TRPV4 VUS variants leading to premature stop codons and most likely NMD was found in painless-DN. The splice-site variant c.2336+1G>A, r.spl? detected in painful-DN, is predicted to skip exon 14, leading to a frameshift and premature stop codon (Table 1), whereas splice-site variant c.711A>G, r.spl? detected in pain-less DN, most likely resulted in incorporation of intronic sequences, in all cases leading to a frame-shift and premature stop codon as well. Therefore, both variants can be considered LOF function mutations as well, which would preclude a role in the pain phenotype. Obviously, confirmation at mRNA or protein level is required before drawing a definite conclusion.

Painful-DN with ICG variant have more pain than painful-DN without ICG variant

Patients with painful-DN with an ICG VUS (n=9) had higher mean pain scores compared to patients with painful-DN without potentially causative ICG variant (n=150). Determining clinically important difference of pain is challenging [53], but applying a pain classification proposed by Serlin *et al.,* where PI-NRS 0-4 is considered as mild pain, 5-6 moderate and 7-10 severe [54] we found that patients harboring an ICG variant had severe maximal pain during the day and night, while patients without an ICG variant reported moderate pain. Moreover, four patient with painful-DN with a TRP variant had severe max pain during night (PI-NRS 8.5 +/- 1.29) and day (PI-NRS 8.0 +/-0.82) together with abnormal TTT. These observations have been made in our DN patient population, including more male than female patients, and therefore, they might be less relevant for female patients. Confirmation in cohort of female patients should definitely be considered.

Conclusions and future perspectives

Painful neuropathy is a heterogeneous, multifactorial disease with many potential genetic contributors and risk factors [31]. According to Eijkenboom *et al.* screening of SCN9A, SCN10A and SCN11A may reveal an underlying genetic cause in 11,6% of pure SFN patients, comparable with previous studies [30]. The same set of genes was used for screening painful- and painless- idiopathic peripheral neuropathy and diabetic polyneuropathy, identifying of low-frequency nonsynonymous missense variants in at least one of the 3 genes in 47.7% of patients [55]. Interestingly, there were no significant differences in missense variant allele frequencies of SCN9A, SCN10A and SCN11A between patients with painful- or painless- peripheral neuropathy [55]. This overlap corroborates our findings in 15 other ICG in this group of patients. In a well characterized cohort of patients with diabetic neuropathy, we have identified potentially pathogenic variants in 5.4% of patients with painful- DN and 4.6% of patients with painless- DN. Co-segregation analysis or functional analysis was not possible, limiting pathogenicity scores to predominantly VUS. However, our results suggest that other ion channels, including TRPs can be genetic contributors to neuropathic pain, making them interesting candidates for further investigation and potential targets for pain therapy.

Ethics approval and patients consent

Committee for this study. Informed consent was obtained from all subjects involved in the study. Patient information was anonymized and de-identified prior to analysis.

Supplementary materials

Table 1S. Peripheral ion channels panel

Gene	OMIM number	Full gene name
ANO1	610108	Anoctamin 1, calcium activated chloride channel
ANO3	610110	Anoctamin 3
HCN1	602780	Hyperpolarization activated cyclic nucleotide-gated potassium channel 1
KCNA2	176262	Potassium voltage-gated channel, shaker-related subfamily, member 2
KCNA4	176266	Potassium voltage-gated channel, shaker-related subfamily, member 4
KCNK18	613655	Potassium channel, subfamily K, member 18
KCNN1	602982	Potassium intermediate/small conductance calcium-activated channel, subfamily N, member 1
KCNQ3	602232	Potassium voltage-gated channel, KQT-like subfamily, member 3
KCNQ5	607357	Potassium voltage-gated channel, KQT-like subfamily, member 5
KCNS1	602905	Potassium voltage-gated channel, delayed-rectifier, subfamily S, member 1
TRPA1	604775	Transient receptor potential cation channel, subfamily A, member 1
TRPM8	606678	Transient receptor potential cation channel, subfamily M, member 8
TRPV1	602076	Transient receptor potential cation channel, subfamily V, member 1
TRPV3	607066	Transient receptor potential cation channel, subfamily V, member 3
TRPV4	605427	Transient receptor potential cation channel, subfamily V, member 4

87

Table 2S. Characteristics of patients with painful- and painless- Diabetic Neuropathy

	Painful-DN	Painless-DN	Total	p
Mean age at recruitment [years +/- SD]	64.2 (+/- 10.3)	64.8 (+/- 11.9)	64.5 (+/- 11.2)	0.56*
Females (n, %)	82 (36.9)	72 (23.7)	154 (29.3)	< 0.0001&
Males (n, %)	140 (63.1)	232 (76.3)	372 (70.7)	
DM1 (n, %)	34 (30.4)	78 (69.4)	112 (21.3)	0.0032&
DM2 (n, %)	190 (45.9)	224 (54.1)	414 (78.7)	
Mean age of onset DM1 [years +/- SD]	25.9 (+/- 15.2)	27.8 (+/- 16.1)	27.2 (+/- 11.2)	0.56*
Mean age of onset DM2 [years +/- SD]	52.1 (+/- 12.1)	55.1 (+/- 10.9)	53.7 (+/- 11.6)	0.007*
Mean age of onset neuropathy [years +/- SD]	59.1 (+/-11.1)	62.3 (+/-11.7)	60.8 (+/-11.5)	0.012*
Duration of neuropathy [years +/- SD]	6.9 (+/- 5.9)	5.5 (+/-6.5)	6.2 (+/- 6.2)	0.25*
Positive family history for neuropathy (n, %)	30 (18.6)	159 (56.8)	189 (42.9)	< 0.0001&
Negative family history for neuropathy (n, %)	131 (81.4)	121 (43.2)	252 (57.1)	
Max pain during night [PI-NRS]	6.2 (+/- 3.0)	0.5 (+/- 1.5)	3.2 (+/- 3.7)	< 0.0001*
Mean pain during night [PI-NRS]	3.7 (+/- 2.8)	0.2 (+/- 0.8)	1.8 (+/- 2.7)	< 0.0001*
Max pain during day [PI-NRS]	5.8 (+/- 3.0)	0.6 (+/-1.6)	3.1 (+/- 3.5)	< 0.0001*
Mean pain during day [PI-NRS]	3.4 (+/-2.6)	0.2 (+/- 0.8)	1.7 (+/- 2.5)	< 0.0001*
Normal TTT (n, %)	32 (20.1)	44 (24.3)	76 (22.4)	0.36&
Abnormal TTT (n, %)	127 (79.9)	137 (75.7)	264 (77.6)	

*Independent Student's t-test, &chi-square test, significance level <0.05

Table 3S. Average coverage of MIP-NGS of 15 ICG

Gene name	Number of MIP [n]	Targeted coding region [bp]	Total number of nucleotides not covered by MIP <20x/bp [bp]	Location of missing area	Average coverage >30x/bp [%]
ANO1	32	2961	441	ex1*, ex2, ex12*, ex20*, ex26	85.1
ANO3	30	2946	0	-	100
HCN1	18	2673	425	ex1*	84.1
KCNA2	10	1500	0	-	100
KCNA4	10	1962	0	-	100
KCNK18	7	1155	0	-	100
KCNN1	14	1632	249	ex6, ex10, ex11	84.7
KCNQ3	21	2619	256	ex1	90.2
KCNQ5	23	2856	192	ex1	93.3
KCNS1	9	1581	181	ex4	88.6
TRPA1	31	3360	111	ex1	96.7
TRPM8	32	3315	0	-	100
TRPV1	20	2520	153	ex3*	93.9
TRPV3	19	2376	348	ex2, ex3, ex7	85.4
TRPV4	19	2616	214	ex3, ex9	91.8

bp, base pair, ex, exon, n, number; * large part of exon <20x coverage/bp or sequence data is missing completely

Table 4Sa. Mean pain score in patients with painful- Diabetic Neuropathic with and without an ion channel variant

	Max pain during night	Mean pain during night	Max pain during day	Mean pain during day
patients with ICG variant (n=9) [+/- SD]	7.00 [+/- 2.96]	4.56 [+/- 3.47]	7.33 [+/-1.87]	3.89 [+/-2.42]
patients without ICG variant (n=150) [+/- SD]	6.2 [+/-3.0]	3.7 [+/-2.8]	5.8 [+/-3.0]	3.4 [+/-2.6]

Pain intensity was evaluated using numerical rating scale PI-NRS.

Table 4Sb: Autonomic complaints reported by patients with painful- Diabetic Neuropathy (n=9) carrying variant in ICG.

Variant	sweating change	diarrhea	consti-pation	micturition problems	dry eyes	dry mouth	orthostatic dizziness	palpita-tions	hot flashes	hypersensitivity of leg's skin	burning feet	sheet intolerance	restless leg
ANO3 p.(Ser213Phe)	1	2	0	0	1	0	2	3	1	3	3	0	1
ANO3 p.(Ile453Val)	1	0	0	0	3	0	1	1	1	3	2	3	1
ANO3 p.(Leu984Phe)	3	1	1	0	2	2	1	0	0	2	1	0	0
HCN1 p.(Arg405Gln)	2	1	1	0	2	2	1	2	0	2	2	2	1
KCNK18 p.(Phe139Trpfs*25)	1	1	1	1	0	2	2	0	0	2	1	1	0
TRPA1 p.(Leu118Val)	1	1	1	0	0	1	1	0	0	1	2	3	3
TRPA1 p.(Arg652*)	0	1	1	2	2	2	1	1	0	1	2	1	0
TRPA1 p.(Val705Glyfs*79)	0	1	0	0	0	1	1	0	0	1	1	0	0
TRPM8 p.(Thr732Ile)	2	0	2	0	2	2	1	1	1	1	1	0	1

Numeric scale (1-4) expresses frequency of complaints; 0, never; 1, sometimes; 2, often; 3, always; -, not determined. Three painful-DN patients not shown in the table, data incomplete

Table 5S: Autonomic complaints reported by patients with painless- Diabetic Neuropathy (n=4) carrying a variant in ICG.

Variant	sweating change	diarrhea	consti-pation	micturition problems	dry eyes	dry mouth	orthostatic dizziness	palpita-tions	hot flashes	hypersensit ivity of leg's skin	burning feet	sheet intolerance	restless leg
TRPA1 p.(Leu118Val)	1	0	1	1	3	1	0	1	0	2	0	0	0
TRPM8 p.(Val986Ile)	1	0	1	2	1	0	0	1	1	1	1	0	0
TRPV4 p.(Thr597Met)	0	1	0	0	0	0	1	1	0	1	0	0	0
TRPV4 p.?	2	0	1	2	3	1	1	1	0	0	1	0	0

Numeric scale (1-4) expresses frequency of complaints; 0, never; 1, sometimes; 2, often; 3, always; -, not determined. Data presented only for patients with ICG that fully completed the autonomic complaints questionnaire

References

1. Colloca L, Ludman T, Bouhassira D, Baron R, Dickenson AH, Yarnitsky D, et al. Neuropathic pain. Nat Rev Dis Primers. 2017;3:17002. Epub 2017/02/17. doi: 10.1038/nrdp.2017.2. PubMed PMID: 28205574; PubMed Central PMCID: PMCPMC5371025.
2. Singh R, Kishore L, Kaur N. Diabetic peripheral neuropathy: current perspective and future directions. Pharmacological research. 2014;80:21-35. Epub 2014/01/01. doi: 10.1016/j.phrs.2013.12.005. PubMed PMID: 24373831.
3. Spallone V, Greco C. Painful and painless diabetic neuropathy: one disease or two? Current diabetes reports. 2013;13(4):533-49. Epub 2013/05/17. doi: 10.1007/s11892-013-0387-7. PubMed PMID: 23677582.
4. Shillo P, Sloan G, Greig M, Hunt L, Selvarajah D, Elliott J, et al. Painful and Painless Diabetic Neuropathies: What Is the Difference? Current diabetes reports. 2019;19(6):32. Epub 2019/05/09. doi: 10.1007/s11892-019-1150-5. PubMed PMID: 31065863; PubMed Central PMCID: PMCPMC6505492.
5. Abbott CA, Malik RA, van Ross ER, Kulkarni J, Boulton AJ. Prevalence and characteristics of painful diabetic neuropathy in a large community-based diabetic population in the U.K. Diabetes care. 2011;34(10):2220-4. Epub 2011/08/20. doi: 10.2337/dc11-1108. PubMed PMID: 21852677; PubMed Central PMCID: PMCPMC3177727.
6. Smith BH, Hébert HL, Veluchamy A. Neuropathic pain in the community: prevalence, impact, and risk factors. Pain. 2020;161 Suppl 1:S127-s37. Epub 2020/10/23. doi: 10.1097/j.pain.0000000000001824. PubMed PMID: 33090746.
7. Van Acker K, Bouhassira D, De Bacquer D, Weiss S, Matthys K, Raemen H, et al. Prevalence and impact on quality of life of peripheral neuropathy with or without neuropathic pain in type 1 and type 2 diabetic patients attending hospital outpatients clinics. Diabetes & metabolism. 2009;35(3):206-13. Epub 2009/03/20. doi: 10.1016/j.diabet.2008.11.004. PubMed PMID: 19297223.
8. Gylfadottir SS, Christensen DH, Nicolaisen SK, Andersen H, Callaghan BC, Itani M, et al. Diabetic polyneuropathy and pain, prevalence, and patient characteristics: a cross-sectional questionnaire study of 5,514 patients with recently diagnosed type 2 diabetes. Pain. 2020;161(3):574-83. Epub 2019/11/07. doi: 10.1097/j.pain.0000000000001744. PubMed PMID: 31693539; PubMed Central PMCID: PMCPMC7017941 interests that may be relevant to content are disclosed at the end of this article.
9. Bennett DL, Clark AJ, Huang J, Waxman SG, Dib-Hajj SD. The Role of Voltage-Gated Sodium Channels in Pain Signaling. Physiological reviews. 2019;99(2):1079-151. Epub 2019/01/24. doi: 10.1152/physrev.00052.2017. PubMed PMID: 30672368.
10. 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. Epub 2017/11/28. doi: 10.1097/j.pain.0000000000001116. PubMed PMID: 29176367; PubMed Central PMCID: PMCPMC5828379 the end of this article.
11. Finnerup NB, Kuner R, Jensen TS. Neuropathic Pain: From Mechanisms to Treatment. Physiological reviews. 2021;101(1):259-301. Epub 2020/06/26. doi: 10.1152/physrev.00045.2019. PubMed PMID: 32584191.
12. Marwaha L, Bansal Y, Singh R, Saroj P, Bhandari R, Kuhad A. TRP channels: potential drug target for neuropathic pain. Inflammopharmacology. 2016;24(6):305-17. Epub 2016/10/21. doi: 10.1007/s10787-016-0288-x. PubMed PMID: 27757589.

13. Rivolta I, Binda A, Masi A, DiFrancesco JC. Cardiac and neuronal HCN channelopathies. Pflugers Archiv : European journal of physiology. 2020;472(7):931-51. doi: 10.1007/s00424-020-02384-3. PubMed PMID: 32424620.
14. Busserolles J, Tsantoulas C, Eschalier A, López García JA. Potassium channels in neuropathic pain: advances, challenges, and emerging ideas. Pain. 2016;157 Suppl 1:S7-s14. Epub 2016/01/20. doi: 10.1097/j.pain.0000000000000368. PubMed PMID: 26785158.
15. Basso L, Altier C. Transient Receptor Potential Channels in neuropathic pain. Current opinion in pharmacology. 2017;32:9-15. Epub 2016/11/12. doi: 10.1016/j.coph.2016.10.002. PubMed PMID: 27835802.
16. Takayama Y, Uta D, Furue H, Tominaga M. Pain-enhancing mechanism through interaction between TRPV1 and anoctamin 1 in sensory neurons. Proceedings of the National Academy of Sciences of the United States of America. 2015;112(16):5213-8. Epub 2015/04/08. doi: 10.1073/pnas.1421507112. PubMed PMID: 25848051; PubMed Central PMCID: PMCPMC4413337.
17. Cho H, Yang YD, Lee J, Lee B, Kim T, Jang Y, et al. The calcium-activated chloride channel anoctamin 1 acts as a heat sensor in nociceptive neurons. Nature neuroscience. 2012;15(7):1015-21. Epub 2012/05/29. doi: 10.1038/nn.3111. PubMed PMID: 22634729.
18. Huang F, Wang X, Ostertag EM, Nuwal T, Huang B, Jan YN, et al. TMEM16C facilitates Na(+)-activated K+ currents in rat sensory neurons and regulates pain processing. Nature neuroscience. 2013;16(9):1284-90. Epub 2013/07/23. doi: 10.1038/nn.3468. PubMed PMID: 23872594; PubMed Central PMCID: PMCPMC4034143.
19. 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. Epub 2007/11/16. doi: 10.1212/01.wnl.0000282763.29778.59. PubMed PMID: 18003941.
20. Hoeijmakers JG, Faber CG, Lauria G, Merkies IS, Waxman SG. Small-fibre neuropathies--advances in diagnosis, pathophysiology and management. Nature reviews Neurology. 2012;8(7):369-79. Epub 2012/05/30. doi: 10.1038/nrneurol.2012.97. PubMed PMID: 22641108.
21. Szklarczyk R, Megchelenbrink W, Cizek P, Ledent M, Velemans G, Szklarczyk D, et al. WeGET: predicting new genes for molecular systems by weighted co-expression. Nucleic acids research. 2016;44(D1):D567-73. Epub 2015/11/20. doi: 10.1093/nar/gkv1228. PubMed PMID: 26582928; PubMed Central PMCID: PMCPMC4702868.
22. Almomani R, Marchi M, Sopacua M, Lindsey P, Salvi E, Koning B, et al. Evaluation of molecular inversion probe versus TruSeq® custom methods for targeted next-generation sequencing. PloS one. 2020;15(9):e0238467. Epub 2020/09/03. doi: 10.1371/journal.pone.0238467. PubMed PMID: 32877464; PubMed Central PMCID: PMCPMC7467307.
23. McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernytsky A, et al. The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. Genome research. 2010;20(9):1297-303. Epub 2010/07/21. doi: 10.1101/gr.107524.110. PubMed PMID: 20644199; PubMed Central PMCID: PMCPMC2928508.
24. Nicolas G, Acuña-Hidalgo R, Keogh MJ, Quenez O, Steehouwer M, Lelieveld S, et al. Somatic variants in autosomal dominant genes are a rare cause of sporadic Alzheimer's disease. Alzheimer's & dementia : the journal of the Alzheimer's Association. 2018;14(12):1632-9. Epub 2018/08/17. doi: 10.1016/j.jalz.2018.06.3056. PubMed PMID: 30114415; PubMed Central PMCID: PMCPMC6544509.
25. 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. Genetics in medicine:

official journal of the American College of Medical Genetics. 2015;17(5):405-24. Epub 2015/03/06. doi: 10.1038/gim.2015.30. PubMed PMID: 25741868; PubMed Central PMCID: PMCPMC4544753.
26. Royal P, Andres-Bilbe A, Ávalos Prado P, Verkest C, Wdziekonski B, Schaub S, et al. Migraine-Associated TRESK Mutations Increase Neuronal Excitability through Alternative Translation Initiation and Inhibition of TREK. Neuron. 2019;101(2):232-45.e6. Epub 2018/12/24. doi: 10.1016/j.neuron.2018.11.039. PubMed PMID: 30573346.
27. Lafrenière RG, Cader MZ, Poulin JF, Andres-Enguix I, Simoneau M, Gupta N, et al. A dominant-negative mutation in the TRESK potassium channel is linked to familial migraine with aura. Nature medicine. 2010;16(10):1157-60. Epub 2010/09/28. doi: 10.1038/nm.2216. PubMed PMID: 20871611.
28. Pettingill P, Weir GA, Wei T, Wu Y, Flower G, Lalic T, et al. A causal role for TRESK loss of function in migraine mechanisms. Brain : a journal of neurology. 2019;142(12):3852-67. Epub 2019/11/20. doi: 10.1093/brain/awz342. PubMed PMID: 31742594; PubMed Central PMCID: PMCPMC6906598.
29. Liu P, Xiao Z, Ren F, Guo Z, Chen Z, Zhao H, et al. Functional analysis of a migraine-associated TRESK K+ channel mutation. J Neurosci. 2013;33(31):12810-24. Epub 2013/08/02. doi: 10.1523/jneurosci.1237-13.2013. PubMed PMID: 23904616; PubMed Central PMCID: PMCPMC3728689.
30. 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(3):342-52. Epub 2018/12/17. doi: 10.1136/jnnp-2018-319042. PubMed PMID: 30554136.
31. Zorina-Lichtenwalter K, Parisien M, Diatchenko L. Genetic studies of human neuropathic pain conditions: a review. Pain. 2018;159(3):583-94. Epub 2017/12/15. doi: 10.1097/j.pain.0000000000001099. PubMed PMID: 29240606; PubMed Central PMCID: PMCPMC5828382 the end of this article.
32. Shashi V, McConkie-Rosell A, Schoch K, Kasturi V, Rehder C, Jiang YH, et al. Practical considerations in the clinical application of whole-exome sequencing. Clinical genetics. 2016;89(2):173-81. doi: 10.1111/cge.12569. PubMed PMID: 25678066.
33. Grandl J, Kim SE, Uzzell V, Bursulaya B, Petrus M, Bandell M, et al. Temperature-induced opening of TRPV1 ion channel is stabilized by the pore domain. Nature neuroscience. 2010;13(6):708-14. Epub 2010/04/24. doi: 10.1038/nn.2552. PubMed PMID: 20414199; PubMed Central PMCID: PMCPMC2876202.
34. Mosavi LK, Cammett TJ, Desrosiers DC, Peng ZY. The ankyrin repeat as molecular architecture for protein recognition. Protein science : a publication of the Protein Society. 2004;13(6):1435-48. Epub 2004/05/21. doi: 10.1110/ps.03554604. PubMed PMID: 15152081; PubMed Central PMCID: PMCPMC2279977.
35. Krishna MM, Englander SW. The N-terminal to C-terminal motif in protein folding and function. Proceedings of the National Academy of Sciences of the United States of America. 2005;102(4):1053-8. Epub 2005/01/20. doi: 10.1073/pnas.0409114102. PubMed PMID: 15657118; PubMed Central PMCID: PMCPMC545867.
36. Lee B, Cho H, Jung J, Yang YD, Yang DJ, Oh U. Anoctamin 1 contributes to inflammatory and nerve-injury induced hypersensitivity. Molecular pain. 2014;10:5. Epub 2014/01/24. doi: 10.1186/1744-8069-10-5. PubMed PMID: 24450308; PubMed Central PMCID: PMCPMC3929161.
37. Wang JJ, Li Y. KCNQ potassium channels in sensory system and neural circuits. Acta pharmacologica Sinica. 2016;37(1):25-33. Epub 2015/12/22. doi: 10.1038/aps.2015.131. PubMed PMID: 26687932; PubMed Central PMCID: PMCPMC4722976.

38. Papp I, Holló K, Antal M. Plasticity of hyperpolarization-activated and cyclic nucleotid-gated cation channel subunit 2 expression in the spinal dorsal horn in inflammatory pain. The European journal of neuroscience. 2010;32(7):1193-201. Epub 2010/08/24. doi: 10.1111/j.1460-9568.2010.07370.x. PubMed PMID: 20726890.
39. Descoeur J, Pereira V, Pizzoccaro A, Francois A, Ling B, Maffre V, et al. Oxaliplatin-induced cold hypersensitivity is due to remodelling of ion channel expression in nociceptors. EMBO molecular medicine. 2011;3(5):266-78. Epub 2011/03/26. doi: 10.1002/emmm.201100134. PubMed PMID: 21438154; PubMed Central PMCID: PMCPMC3377073.
40. Young GT, Emery EC, Mooney ER, Tsantoulas C, McNaughton PA. Inflammatory and neuropathic pain are rapidly suppressed by peripheral block of hyperpolarisation-activated cyclic nucleotide-gated ion channels. Pain. 2014;155(9):1708-19. Epub 2014/05/28. doi: 10.1016/j.pain.2014.05.021. PubMed PMID: 24861581.
41. Resta F, Micheli L, Laurino A, Spinelli V, Mello T, Sartiani L, et al. Selective HCN1 block as a strategy to control oxaliplatin-induced neuropathy. Neuropharmacology. 2018;131:403-13. Epub 2018/01/18. doi: 10.1016/j.neuropharm.2018.01.014. PubMed PMID: 29339292.
42. 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. Epub 2018/11/22. doi: 10.1523/jneurosci.2433-18.2018. PubMed PMID: 30459225; PubMed Central PMCID: PMCPMC6335750.
43. 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 communications. 2021;3(3):fcab212. Epub 2021/09/25. doi: 10.1093/braincomms/fcab212. PubMed PMID: 34557669; PubMed Central PMCID: PMCPMC8454204.
44. Alessandri-Haber N, Joseph E, Dina OA, Liedtke W, Levine JD. TRPV4 mediates pain-related behavior induced by mild hypertonic stimuli in the presence of inflammatory mediator. Pain. 2005;118(1-2):70-9. Epub 2005/10/11. doi: 10.1016/j.pain.2005.07.016. PubMed PMID: 16213085.
45. Nirenberg MJ, Chaouni R, Biller TM, Gilbert RM, Paisán-Ruiz C. A novel TRPA1 variant is associated with carbamazepine-responsive cramp-fasciculation syndrome. Clinical genetics. 2018;93(1):164-8. Epub 2017/04/25. doi: 10.1111/cge.13040. PubMed PMID: 28436534; PubMed Central PMCID: PMCPMC5654709.
46. Obata K, Katsura H, Mizushima T, Yamanaka H, Kobayashi K, Dai Y, et al. TRPA1 induced in sensory neurons contributes to cold hyperalgesia after inflammation and nerve injury. The Journal of clinical investigation. 2005;115(9):2393-401. Epub 2005/08/20. doi: 10.1172/jci25437. PubMed PMID: 16110328; PubMed Central PMCID: PMCPMC1187934.
47. Kremeyer B, Lopera F, Cox JJ, Momin A, Rugiero F, Marsh S, et al. A gain-of-function mutation in TRPA1 causes familial episodic pain syndrome. Neuron. 2010;66(5):671-80. Epub 2010/06/16. doi: 10.1016/j.neuron.2010.04.030. PubMed PMID: 20547126; PubMed Central PMCID: PMCPMC4769261.
48. Proudfoot CJ, Garry EM, Cottrell DF, Rosie R, Anderson H, Robertson DC, et al. Analgesia mediated by the TRPM8 cold receptor in chronic neuropathic pain. Current biology : CB. 2006;16(16):1591-605. Epub 2006/08/22. doi: 10.1016/j.cub.2006.07.061. PubMed PMID: 16920620.
49. Su L, Wang C, Yu YH, Ren YY, Xie KL, Wang GL. Role of TRPM8 in dorsal root ganglion in nerve injury-induced chronic pain. BMC neuroscience. 2011;12:120. Epub 2011/11/25. doi: 10.1186/1471-2202-12-120. PubMed PMID: 22111979; PubMed Central PMCID: PMCPMC3235975.
50. Levine JD, Alessandri-Haber N. TRP channels: targets for the relief of pain. Biochimica et biophysica acta. 2007;1772(8):989-1003. Epub 2007/02/27. doi: 10.1016/j.bbadis.2007.01.008. PubMed PMID: 17321113.

51. Yuan JH, Schulman BR, Effraim PR, Sulayman DH, Jacobs DS, Waxman SG. Genomic analysis of 21 patients with corneal neuralgia after refractive surgery. Pain Rep. 2020;5(4):e826. doi: 10.1097/PR9.0000000000000826. PubMed PMID: 32766464; PubMed Central PMCID: PMCPMC7390595.
52. Darby WG, Grace MS, Baratchi S, McIntyre P. Modulation of TRPV4 by diverse mechanisms. The international journal of biochemistry & cell biology. 2016;78:217-28. Epub 2016/07/19. doi: 10.1016/j.biocel.2016.07.012. PubMed PMID: 27425399.
53. Farrar JT. What is clinically meaningful: outcome measures in pain clinical trials. The Clinical journal of pain. 2000;16(2 Suppl):S106-12. Epub 2000/06/28. doi: 10.1097/00002508-200006001-00018. PubMed PMID: 10870749.
54. Serlin RC, Mendoza TR, Nakamura Y, Edwards KR, Cleeland CS. When is cancer pain mild, moderate or severe? Grading pain severity by its interference with function. Pain. 1995;61(2):277-84. Epub 1995/05/01. doi: 10.1016/0304-3959(94)00178-h. PubMed PMID: 7659438.
55. 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(6). doi: 10.1212/NXG.0000000000000207. PubMed PMID: WOS:000425033200011.

Chapter 4

Peripheral ion channel genes screening in painful Small Fiber Neuropathy

Milena Ślęczkowska[1,2#], Rowida Almomani[2,3#], Margherita Marchi[4], Erika Salvi[4], Bianca T A de Greef[2], Maurice Sopacua[2], Janneke G J Hoeijmakers[2], Patrick Lindsey[1], Stephen G Waxman[5,6], Giuseppe Lauria[4], Catharina G Faber[2*], Hubert J M Smeets[1,2], Monique M Gerrits[7]

[#] These authors contributed equally to this work

Published: Int. J. Mol. Sci. 2022, 23(22), 14095; doi.org/10.3390/ijms232214095

ABSTRACT

Neuropathic pain is a characteristic feature of Small Fiber Neuropathy (SFN), which in 18% of the cases is caused by genetic variants in voltage-gated sodium ion channels. In this study, we assessed the role of fifteen other ion channels in neuropathic pain. Patients with SFN (n=414) were analysed for *ANO1, ANO3, HCN1, KCNA2, KCNA4, KCNK18, KCNN1, KCNQ3, KCNQ5, KCNS1, TRPA1, TRPM8, TRPV1, TRPV3, TRPV4* variants by single molecule Molecular inversion probes-Next Generation Sequencing. These patients did not have genetic variants in *SCN3A, SCN7A-SCN11A* and *SCN1B-SCN4B*. In twenty patients (20/414, 4.8%) a potentially pathogenic heterozygous variant was identified in an ion-channel gene. Variants were present in seven genes, for two patients (0.5%) in *ANO3*, one (0.2%) in *KCNK18*, two (0.5%) in *KCNQ3*, seven (1.7%) in *TRPA1*, three (0.7%) in *TRPM8*, three (0.7%) in *TRPV1* and two (0.5%) in *TRPV3*. Variants in the TRP gene were most frequent (n=15, 3.6%), partly in patients with high mean maximal pain scores VAS=9.65 +/- 0.7 (n=4). Patients with ion-channel gene variants reported more severe pain compared to patients without such variants (VAS=9.36+/-0.72 vs. VAS=7.47+/-2.37). This work supported previous findings that ICG may contribute to neuropathic pain and provided novel SFN phenotype specific list of variants for further investigation.

INTRODUCTION

Neuropathic pain (NeP) is defined as a pain condition usually caused by progressive nerve disease [1]. NeP symptoms are often described as a shooting or burning pain, accompanied by allodynia, hyperalgesia, sensory dysfunction and autonomic complaints [2]. Chronic pain is common in peripheral neuropathy, including diabetic neuropathy and small fiber neuropathy (SFN), where Aδ-fibres and C-fibres are affected [3, 4]. Patients suffering from neuropathic pain report major negative impact on quality of life [5]. Unfortunately, the currently available treatment has moderate effect and often does not bring expected pain relief [2, 6]. Several conditions such as diabetes mellitus, autoimmune disorders, viral infections, inflammatory disorders and chemotherapy have been linked to NeP, but the pathophysiology is largely unresolved [2]. An increasing number of reports highlight a role for genetic factors involved in pain development [1, 7, 8], still, in more than 80% of cases a possible genetic factor is unknown [9].

The last two decades, alterations of the voltage-gated sodium ion channels (VGSCs) have been reported caused by genetic mutations in the underlying genes [10-13]. VGSCs are transmembrane polypeptides responsible for the generation and conduction of action potentials in excitable cells [14]. Gain-of-function (GOF) variants of *SCN9A, SCN10A* and *SCN11A* have been reported in several pain related diseases including SFN [10-12, 14, 15], adding up to 12% in patients with pure SFN [3]. Screening of all VGSCs genes including *SCN3A, SCN7A-SCN11A, SCN1B-SCN4B* increased the number of patients with NeP with identified (potential) underlying cause to 18.1% [9].

In literature, also other ion channels genes (ICG) have been reported in pain modulation, mainly transient receptor potential (TRP) cation channels [16], potassium voltage-gated (Kv) channels [17], hyperpolarization-activated and cyclic nucleotide-gated channels (HCN) [18] and Ca^{2+}-activated Cl- channels, also known as Anoctamins (ANO) [19]. TRP channels function as thermo-, chemical- and mechanical sensors [16]. Kv are group of potassium channels, involved in modulation of sensory neuron excitability and pain processing [17]. HCN channels exhibit wide expression in peripheral nerves and their impaired functioning have been linked with neuropathic pain [18]. The most studied member of ANO family, ANO1 has been found to interact with TRPV1 leading to increased pain in sensory neurons [20]. Moreover, ANO3 modulates nociception in dorsal root ganglion (DRG) via enhancement of the sodium-activated potassium channel Slack activity [21].

Our previous work investigated the genetic variation in ICG in painful- and painless-DN revealing different GOF or LOF variants present in both groups, providing more insight into genetic differences of both phenotypes but also suggesting that certain variants of ICG may contribute to NeP [22]. Therefore, in this study we aimed to compare these finding and if possible to extend relevance of previous work to another, well characterized phenotype. However, here we focused specifically on patients with SFN without defined underling genetic cause as *SCN3A, SCN7A-SCN11A, SCN1B-SCN4B* mutations. To elucidate, possible genetic variants in the remaining SFN patients we decided to sequence 15 ion channel genes, which were expressed in peripheral nerves [Kv (7), TRP (5), ANO (2) and HCN (1)]. We performed single molecule Molecular Inversion Probes- Next Generation Sequencing (smMIPs-NGS) to analyze exons and exon-intron junctions and classified the variants identified.

MATERIAL AND METHODS

Study population

From June 2014 and September 2016, 553 patients diagnosed with SFN were recruited at Maastricht University Medical Center+ (MUMC+), Maastricht, The Netherlands and at Fondazione I.R.C.C.S. Istituto Neurologico Carlo Besta (FINCB), Milan, Italy for this study. MUMC+ is a tertiary referral center for SFN in the Netherlands and FINCB is a national referral centre for several neurological conditions including SFN. Medical history and clinical data were collected and recorded as described previously [12]. The diagnosis of SFN was confirmed based on clinical symptoms of SFN combined with a reduced intraepidermal neve fiber density (IEND) in skin biopsy without signs of large fiber involvement and/or abnormal temperature threshold testing (TTT) and/or neve conduction study (NCS) [23]. The assessment of neuropathic pain was performed using the visual analog scale (VAS), where the scores are recorded by marking a value on 10-cm line, in which 0 represents “no pain” and 10 is “the worst pain that you can imagine” and the 11-point Numerical Rating Scale (PI-NRS) ranging from 0-10, where 0 means “no pain” and 10 referring to “the worst pain imaginable” [24, 25]. Only patients with diagnosed SFN and neuropathic pain with VAS>3 were included. In this study, we focused on patients negative for *SCN3A, SCN7A-SCN11A* and *SCN1B-SCN4B* variant screening. Most of the included patients (n=348, 69%) were Dutch, while 156 (31%) patients were from Italy. The mean age of recruited SFN patients was 54.1 years (SD 14.2 years) with age of onset of complaints 42.2 years (SD 13.6 years). More females than males were present in study population. Females consisted

59.5% (n=300) and males 40.5% (n=204). In both populations females were overrepresented, respectively 59.8% (n=208) females were in Dutch cohort and 59% (n=92) females in Italian cohort. Twenty-three percent of patients (n=65) reported positive cases of neuropathy in the family, while negative family history was registered for 77% (n=217). The data for familial cases of neuropathy was incomplete for two-hundred-twenty-two patients. More than one-third of recruited patients had abnormal skin biopsy (n=120, 36.5%).

DNA extraction and storage

Genomic DNA was isolated for genetic analysis from blood samples using Nucleo-Spin8 Blood Isolation kit (Macherey-Nagel, Düren, Germany) or QIAamp DNA Blood Maxi Kit, Puregene® Blood Core Kit (Qiagen, Hilden, Germany). DNA extraction was performed according to manufacturer's instructions and stored at Maastricht UMC+, and IRCCS Neurological Institute Carlo Besta and placed in the central DNA storage at Maas-tricht UMC+ and IRCCS Neurological Institute Carlo Besta in -20°C.

smMIPs-NGS of peripheral ion channels and data analysis

Table 1. Gene panel

Ion channel family	Gene	OMIM number	Full gene name
Anoctamins	*ANO1*	610108	Anoctamin 1, calcium activated chloride channel
	ANO3	610110	Anoctamin 3
Non-selective cation channels	*HCN1*	602780	Hyperpolarization activated cyclic nucleotide-gated potassium channel 1
Potassium channels	*KCNA2*	176262	Potassium voltage-gated channel, shaker-related subfamily, member 2
	KCNA4	176266	Potassium voltage-gated channel, shaker-related subfamily, member 4
	KCNK18	613655	Potassium channel, subfamily K, member 18
	KCNN1	602982	Potassium intermediate/small conductance calcium-activated channel, subfamily N, member 1
	KCNQ3	602232	Potassium voltage-gated channel, KQT-like subfamily, member 3
	KCNQ5	607357	Potassium voltage-gated channel, KQT-like subfamily, member 5
	KCNS1	602905	Potassium voltage-gated channel, delayed-rectifier, subfamily S, member 1
Transient receptors	*TRPA1*	604775	Transient receptor potential cation channel, subfamily A, member 1
	TRPM8	606678	Transient receptor potential cation channel, subfamily M, member 8

	TRPV1	602076	Transient receptor potential cation channel, subfamily V, member 1
	TRPV3	607066	Transient receptor potential cation channel, subfamily V, member 3
	TRPV4	605427	Transient receptor potential cation channel, subfamily V, member 4

The smMIPs-NGS of exonic regions with +/- 20 bps exon-flanking sequences of the 15 ion channel genes (Table 1) was performed according previously established protocol [26]. The detailed description of the technique, smMIPs characteristic, number of probes per targeted coding region per gene and smMIPs-NGS pipeline used for data analysis have been described before [22].

Variant classification

Exonic and +/-20 bp exon-flanking intron variants with >20x coverage and an alternative variant call of >30% were included for the analysis. The SNPs with high frequency defined as dbSNP >5%, ExAC >5% and >1% in our Dutch in-house database of 12244 exomes were excluded from the analysis. All variants were examined individually, including BAM file visualization and variant interpretation performed in Alamut Visual software (Interactive Biosoftware, Rouen, France). Variants were classified according the practice guidelines of the Association for Clinical Genetic Science (ACGS) [27].

Statistical analysis

The analysis of continuous variables was performed using the independent Student's t-test. Categorical variables were analyzed using chi-square test or Fisher exact test in the case of small counts <5. The statistical significance of <0.05 was applied.

RESULTS

smMIPs-NGS of patients with SFN

The performance of smMIPs-NGS, capture efficiency and coverage of targeted exons and exon-flanking sequences (+/- 20 bps) for 15 ICG panel was assessed as described previously [22]. The average coverage of these regions (>30x/bp) was 93%. The targeted sequences of *ANO3* (2946 bp), *KCNA2* (1500 bp), *KCNA4* (1962 bp), *KCNK18* (1155 bp), *KCNQ3* (2619 bp), *KCNQ5* (2856 bp), *TRPA1*

(3360 bp), *TRPM8* (3315 bp), *TRPV1* (2520 bp) and *TRPV4* (2616 bp) have an average coverage >90%, for five targeted gene areas [*ANO1* (2961 bp), *HCN1* (2673 bp), *KCNN1* (1632 bp), *KCNS1* (1581 bp), *TRPV3* (2376 bp)] the average coverage was at least 84%. Four exons (exon 1, exon 12 and exon 20 of *ANO1*, and exon 3 of *TRPV1*) have poor coverage (<20x/bp) or were completely missing. The calculated capture efficiency of each probe and coverage of on-target regions was reproducible per region, per run and between samples. A total of 49 out of 553 samples were excluded due to low quality of DNA, not sufficient number of reads or incomplete clinical information (Figure 1).

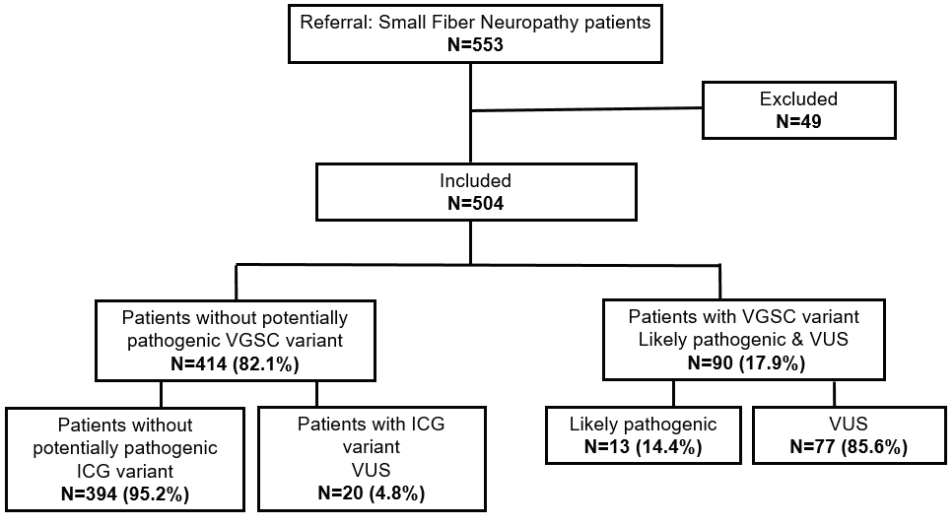


Figure 1. SFN patients analyzed using smMIP-NGS for (potentially) pathogenic ICG and VGSC variants. ICG, ion channel gene; VGSC, voltage-gated sodium ion channel; VUS, variant with uncertain clinical significance

Genetic variants identified in ion channel genes

Among 414 patients negative for *SCN3A, SCN7A-SCN11A* and *SCN1B-SCN4B* screening, 19 different potentially pathogenic variants have been identified in ICGs. All detected variants have been classified as VUS and they have been localized in seven ICG genes: *ANO3, KCNK18, KCNQ3, TRPA1, TRPM8, TRPV1, TRPV3*. In total, two missense variants have been detected in *ANO3,* one frameshift variant leading to shorten protein in *KCNK18,* two missense in *KCNQ3,* four missense and two nonsense variants in *TRPA1*, three missense in *TRPM8,* three missense in

TRPV1, one splice variant in donor site of intron 9 and one missense in *TRPV3* (Table 2). All of them were heterozygous. The detected nonsense/ frameshift variants are predicted either to causing shortening of the protein length due to premature stop codon, or, most likely, cause nonsense-mediated decay (NMD) of the mRNA, while splice site variant in donor site of intron 9 (*TRPV3* c.1242+1G>A) is predicated to alter protein sequence via alternative splicing.

Although the best predictable functional effect will be lack of the protein due to NMD or truncated, non-functional polypeptides (*KCNK18* c.1107del, *TRPA1* c.1177C>T and c.1954C>T), the substitution in conserved functional areas of the protein may affect proper functioning. Three variants (*TRPV1* c.1348A>G and c.1735C>T and *TRPV3* c.2006T>C) have been found in transmembrane domains. One variant (*ANO3* c.2656A>T) was detected in linker between transmembrane domains, three variants were located in ankyrin repeats (ANK) (*TRPA1* c.932C>A and c.980A>G and *TRPV1* c.914T>G), three in N- terminus (*TRPM8* c.665A>G and c.1102C>T), four in C- terminus (*ANO3* c.3100G>C, *KCNQ3* c.1885G>A and c.1706A>G, *TRPA1* c.3136 A>G and *TRPM8* c.2945C>T) and one in inositol-phosphate binding site of TRPA1 (c.3136A>G).

Each ICG variant was detected in one patient, with exception of *TRPA1* c.3136A>G, which was present in two siblings affected with SFN (Table 2). Most of the reported ICG variants were novel (n=16/19, 84%). *TRPA1* c.1954C>T variant has been seen before in German painful- diabetic peripheral neuropathy patient [22]. The majority of identified (n=14/19, 73.7%) variants were located in TRP genes. No potential causing variant has been identified in eight genes from our gene panel: *ANO1, HCN1, KCNA2, KCNA4, KCNQ5, KCNN1, KCNS1, TRPV4*. None of patient with identified VGSC variant had an additional variant in one of the other ICG (Figure 1).

Table 2: Potentially pathogenic variants of ion channel genes identified in patients with SFN (n=504).

Gene	c.position [&]	p.position	Number of patients	Classification according to Richards et.al 2015	Location	MAF gnomAD (%)
ANO3	c.2656A>T	p.(Ile886Phe)	1	VUS	Linker between transmembrane domain VII and VIII	0
	c.3100G>C	p.(Gly1034Arg)	1	VUS	C-terminus	0.011
KCNK18	c.1107del	p.(Met370Cysfs*?)	1	VUS	Frame shift starting at codon Met370	0.0016
KCNQ3	c.1885G>A	p.(Val629Ile)	1	VUS	C-terminus	0.052
	c.1706A>G	p.(Asp569Gly)	1	VUS	C-terminus	0.0018
TRPA1	c.932C>A	p.(Thr311Asn)	1	VUS	Ankyrin repeat VIII-containing domain	0.044
	c.980A>G	p.(Tyr327Cys)	1	VUS	Ankyrin repeat VIII-containing domain	0
	c.1177C>T	p.(Arg393*)	1	VUS	Stop codon in Ankyrin repeat X –containing domain	0.018
	c.1954C>T	p.(Arg652*)	1	VUS	Cytoplasmic domain between ANK repeats and transmembrane domain I	0.015
	c.2065A>G	p.(Met689Val)	1	VUS	Cytoplasmic domain between ANK repeats and transmembrane domain I	0.0068
	c.3136A>G	p.(Lys1046Glu)	2[*]	VUS	inositol-phosphate binding site in C-terminus	0.0008
TRPM8	c.665A>G	p.(Asn222Ser)	1	VUS	N-terminus	0.0004
	c.1102C>T	p.(Arg368Trp)	1	VUS	N-terminus	0.0016
	c.2945C>T	p.(Thr982Met)	1	VUS	C-terminus	0.005
TRPV1	c.914T>G	p.(Phe305Cys)	1	VUS	Ankyrin repeat V-containing domain	0.00054
	c.1348A>G	p.(Thr450Ala)	1	VUS	Transmembrane domain I	0
	c.1735C>T	p.(Arg579Cys)	1	VUS	Transmembrane domain V	0.0017
TRPV3	c.1242+1G>A	p.? [^]	1	VUS	Donor splice site of intron 9	0.0029
	c.2006T>C	p.(Leu669Pro)	1	VUS	Transmembrane domain VI	0.0019

c. position, location cDNA; p. position, location in protein; MAF GnomAD, Minor Allele Frequency; VUS, variant 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_001313726.1 (*ANO3)*; NM_181840.1 (*KCNK18),* NM_004519.3 (*KCNQ3),* NM_007332.2 (*TRPA1)*; NM_024080.4 (*TRPM8),* NM_080706.3 (*TRPV1);* NM_001258205.1 *(TRPV3).* All detected variants are heterozygous.[*]variant detected two times in siblings, [^]changed protein length due to splicing event (loss of donor splice site of intron 9).

Patients with SFN with an ICG variant compared to patients without ICG/VGSC variant

We did not observed statistically significant differences between patients with a potentially pathogenic ICG variant compared to patients without such a variant (no ICG/VGSC variant) in relation to mean age of onset of neuropathy (52.2 +/- 13.3 vs. 54.3 +/-13.9 years old, p=0.786), neuropathy duration (n= 12, 4.4 +/- 5.0 years vs. n=277, 8.1 +/-9.3, p=0.183), positive family history (n=5, 38.5% vs. n=50, 22.2%, p= 0.088), abnormal TTT (n=9, 100% vs. n=280, 91.8%, p= 1) and abnormal skin biopsy (n=14, 77.8% vs. n=129, 42%, p=0.0056) (Table 3). Detailed clinical characteristics of patients with SFN and an ICG variant is available in supplementary materials.

During the clinical assessment multiple questionnaires, including the SFN Symptom Inventory Questionnaire (SFN-SIQ) [28] and the Visual Analogue Scale have been given to the patients (Table 4). All analyzed patients that completed the questionnaire reported severe pain, however individuals carrying a potentially pathogenic ICG variant (n=7) have higher maximal pain scores VAS=9.36 +/-0.72 comparing to the group without such ICG/VGSC variant [n=204, VAS=7.47 +/- 2.37 (Table 4)]. Each individual with ICG reported maximal pain above the mean maximal pain calculated for patients without ICG/VGSC variant. The patient with an *ANO3* variant reported maximal pain (VAS=9.7), the patient with the *KCNQ3* c.1885G>A variant reported VAS=8.7 and the patient with the *KCNQ3* c.1706A>G VAS=8.5. Interestingly three out of four *TRPA1* positive patients evaluated maximal pain as VAS=10, which is the highest possible value in the scale. Only one patient carrying *TRPA1* variant, specifically c.2065A>G reported VAS= 8.6. So, the maximal pain scores in group of patients having variants in the same gene were higher than in subgroup without ICG/VGSC variant (Table 4).

Several features including SFN specific symptoms, like changed sweating pattern, diarrhea, constipation, micturition problems dry eyes, dry mouth, orthostatic dizziness, palpitations, hot flashes, sensitive legs' skin, burning feet, sheet intolerance legs, restless legs were evaluated for the SFN patients (Table 5 and Table 7). All patients with an ICG variant for which this data was available (n=6) reported often or always burning feet and restless legs symptom. Next to that, the most commonly mentioned complained among patients with ICG variants was sheet intolerance on legs, which presents often or always (n=5/6, 83.3%) and sometimes (n=1/6, 16,7%) (Table 6). However, burning feet, sheet intolerance and restless legs were also the most commonly mentioned features in patients without an ICG/VGSC variant. Burning feet present always and often (n=213, 79.1%), restless legs present

always and often (n=156, 57,4%) and sheet intolerance present always and often [n=126, 46.3% (Table 8)]. Statistical significance was not reached for any of features investigated.

Table 3. Comparison of clinical features of patients with Small Fiber Neuropathy and ICG variant vs. no ICG/VGSC variant

	Patients with SFN and ICG variant N=20	**Patients with SFN without ICG/ VGSC variant N=394**
Mean age at recruitment [years +/- SD]	52.2 (+/-13.3)	54.2 (+/- 13.9)
Females (n, %)	11 (55.0)	240 (+/- 60.9)
Males (n, %)	9 (45.0)	154 (+/- 39.1)
Mean age of onset neuropathy [years +/- SD]	46.2 (+/-12.2)	47.1 (+/-13.4)
Duration of neuropathy [years +/- SD]	4.4 (+/- 5.0)	8.1 (+/-9.3)
Positive family history for neuropathy (n, %)	5 (38.5)	50 (22.2)
Negative family history for neuropathy (n, %)	8 (61.5)	175 (77.8)
Normal skin biopsy (n, %)	4 (22.2)	178 (58)
Abnormal skin biopsy (n, %)	14 (77.8)	129 (42)
Normal TTT (n, %)	0 (0)	25 (8.2)
Abnormal TTT (n, %)	9 (100)	280 (91.8)

ICG, ion channel gene; VGSC, voltage-gated sodium ion channel; SD, standard variation. Patients with incomplete data not included in the table. Differences between patients with SFN and ICG variant vs. patients with SFN without ICG and VGSC variant were not statistically significant.

Table 4. Mean pain scores in patients with Small Fiber Neuropathy

	Patient with ANO3 variant (n=1)	Patients with KCNQ3 variant (n=2)	Patient with TRPA1 variant (n=4)	Patients with ICG variant (n=7)	Patients without ICG variant and without VGSC variant (n=204)
Maximal pain [VAS] [+/- SD]	9.7	8.6 (+/-0.14)	9.65 (+/- 0.7)	9.36 (+/-0.72)	7.47 (+/- 2.37)

ICG, ion channel gene; VGSC, voltage-gated sodium ion channel; SD, standard variation. Pain evaluated using the visual analog scale (VAS), where 0 represents “no pain” and 10 is “the worst pain that you can imagine”. Patients with incomplete data not included in the table.

Table 5: Clinical features of SFN patients carrying ICG variant.

Gene	Variant	Gender	Onset complaints	NCS	TTT	IENFD	Itch	Muscle cramps	Warmth influence	Cold influence	Exercise influence	Rest influence	Temperature sensation	Pain sensation	Allodynia	Sleep pattern
ANO3	(Gly1034Arg)	M	18	n	a/n	n	N	N	-	-	N	N	n	↑	Y	n
KCNQ3	(Val629Ile)	M	49	a/n	a/n	a/n	N	N	-	Y ↑	Y ↑	Y ↑	n	-	Y	n
KCNQ3	(Asp569Gly)	M	46	n	a/n	n	N	Y	-	-	-	Y ↑	-	-	Y	-
TRPA1	(Arg393*)	M	50	a/n	a/n	a/n	N	Y	N	N	Y ↑	-	n	-	Y	a/n
TRPA1	(Arg652*)	F	17	n	a/n	n	N	N	Y ↑	Y ↑	Y ↑	N	↓	↓	Y	a/n
TRPA1	(Met689Val)	F	34	n	a/n	a/n	Y	N	Y ↑	-	Y ↑	Y ↑	-	↑	Y	a/n
TRPA1	(Tyr327Cys)	M	45	n	-	a/n	N	Y	-	-	-	-	-	-	-	-
TRPM8	(Arg368Trp)	M	45	n	a/n	a/n	N	N	-	-	-	-	↓	n	-	-
TRPV1	(Arg579Cys)	M	43	n	a/n	n	Y	Y	N	N	-	Y ↑	↓	n	Y	a/n

p. position, location in protein; M, male; F, female; NCS, Nerve Conduction Study; n, normal; a/n, abnormal; -, data incomplete; TTT, Thermal Threshold Testing; IENFD, Intraepidermal Nerve Fiber Densities; Y, yes; N, no; ↓, decreased; ↑, increased. Patients with incomplete data not included in the table.

Table 6: Complaints reported in Small Fiber Neuropathy Symptom Inventory Questionnaire (SFN-SIQ) by SFN patients carrying variant in ICG.

Gene	variant	sweating change	diarrhea	constipation	micturition problem	dry eyes	dry mouth	dizziness on standing	palpitations	hot flashes	hypersensitivity of leg's skin	burning feet	sheet intolerance	restless leg
ANO3	(Gly1034Arg)	4	1	1	3	3	4	2	-	1	4	4	4	4
KCNQ3	(Val629Ile)	2	2	2	2	1	2	1	1	2	1	3	2	3
KCNQ3	(Asp569Gly)	3	2	2	3	2	2	2	2	2	3	4	3	4
TRPA1	(Arg393*)	-	2	3	3	1	3	2	1	2	4	4	4	4
TRPA1	(Arg652*)	3	2	3	2	1	3	3	2	2	3	3	3	3
TRPA1	(Met689Val)	3	3	3	2	4	4	3	3	3	4	3	4	4

Number value 1-4 expresses frequency of complaints; 1, never; 2, sometimes; 3, often; 4, always; -, not determined

Table 7: Clinical features of SFN patients without ICG/VGSC variant.

Feature	NCS N=308	TTT N=305	IENFD N=307	Temperature sensation N=204	Pain sensation N=145	Sleep pattern N=130	
Normal	297, 96.4%	25, 8.2%	178, 58%	120, 58.8%	73, 50.3%	35, 26.9%	
Abnormal	11, 3.6 %	280, 91.8%	129, 42%	84,41,2% (1, 0.5% ↑, 83, 40.7% ↓)	72, 49.7% (33, 22.8% ↑, 39, 26.9% ↓)	95, 73.1%	
Feature	**Itch N=308**	**Muscle cramps N=308**	**Warmth influence N=172**	**Cold influence N=169**	**Exercise influence N=195**	**Rest influence N=123**	**Allodynia N=211**
yes	25, 8.1%	56, 18.2%	84, 48.8% (63, 36.6% complains ↑, 21, 12.2 % complains ↓)	82, 48.5% (54,32% complains ↑, 28,16.6 % complains ↓)	161,82.6% (144,. 73.8% complains ↑, 17, 8.7% complains ↓)	54, 68.3% (45, 36.6% complains ↑, 39,31.7% complains ↓)	193, 91.5%
no	283, 91.9%	252, 81.8%	88, 51.2%	87, 51.5%	34, 17.4%	39, 31.7%	18, 8.5%

NCS, Nerve Conduction Study; n, normal; a/n, abnormal; -, not determined; TTT, Thermal Threshold Testing; IENFD, Intraepidermal Nerve Fiber Densities; Y, yes; N, no; ↓, decreased; ↑, increased

Table 8: Patients with SFN without (potentially) pathogenic ICG/VGSC variant reporting Small Fiber Neuropathy Symptom Inventory Questionnaire (SFN-SIQ) complaints.

Frequency of complaint	Sweating change N=268	Diarrhea N=272	Constipation N=275	Micturition problem n=273	Dry eyes n=273	Dry mouth N=273	Orthostatic dizziness N=271	Palpitations N=268	Hot flashes N=270	Hypersensitivity of leg's skin N=273	Burning feet n=269	Sheet intolerance N=272	Restless leg N=272
never	56, 20.9%	114, 41.9%	115, 41.8%	91, 33.3%	95, 34.8%	54, 19.8%	73, 26.9%	105, 39.2%	86, 31.9%	52, 19.0%	21, 7.8%	70, 25.7%	36, 13.2%
sometimes	94, 35.1%	110, 40.4%	86, 31.3%	75, 27.5%	87, 31.9%	97, 35.5%	130, 48.0%	108, 40.3%	94, 34.8%	75, 27.5%	35, 13.0%	76, 27.9%	80, 29.4%
often	85, 31.7%	44, 16.2%	55, 20.0%	80, 29.3%	65, 23.8%	91, 33.3%	59, 21.8%	54, 20.1%	86, 31.9%	70, 25.6%	101, 37.5%	68, 25.0%	91, 33.5%
always	33, 12.3%	4, 1.5%	19, 6.9%	27, 9.9%	26, 9.5%	31, 11.4%	9, 3.3%	1, 0.4%	4, 1.5%	76, 27.8%	112, 41.6%	58, 21.3%	65, 23.9%

N, number of patients that completed SFN-SIQ related question.

DISCUSSION

Summary of ICG screening in patients with Small Fiber Neuropathy

In 414 patients with no (potentially) pathogenic variants in the *SCN3A, SCN7A-SCN11A* and *SCN1B-SCN4B* genes, twenty patients (4.8%) had one potentially pathogenic heterozygous variant in ICG. Detected variants were located in 7 genes and were present in two patients (0.5%) in *ANO3*, one (0.2%) in *KCNK18*, two (0.5%) in *KCNQ3*, 7 (1.7%) in *TRPA1*, 3 (0.7%) in *TRPM8*, 3 (0.7%) in *TRPV1* and two (0.5%) in *TRPV3*. Most of the detected variants were novel, however 3 variants have been reported before. The *ANO3* c.3100G>C variant is present in the Human Gene Mutation Database (HGMD) and described as likely disease-causing with questionable pathogenicity in primary torsion dystonia [29]. Two *KCNQ3* variants have been reported in ClinVar; the c.1885G>A was reported two times, once as VUS in benign familial neonatal seizures and as once as likely benign without linking to specific phenotype, while the c.1706A>G variant was reported as VUS, the phenotype was not provided. Our patients do not have complaints typical for dystonia and familial neonatal seizures.

In our study population each ICG variant was detected only once, with exception of the *TRPA1* c.3136A>G variant present in brother and sister with SFN, which was considered as one independent finding. None of the identified ICG variants have been reported before in neuropathic pain, except for the *TRPA1* c.1954C>T variant, present in a 73 years old male diagnosed with painful-DN [22]. Although, painful-DN and SFN patients suffered from NeP, only one VUS was present in both cohorts. Obtained data, may suggest that different variants underlay SFN and painful-DN. However, we cannot definitely conclude it based on these limited in numbers cohorts. Nevertheless, this study provides list of novel painful-SFN phenotype specific variants for further investigation together with detailed clinical description of the patients. This might be helpful for phenotype-genotype follow up study and for clinicians providing additional SFN/ICG case related information.

There was no overlap between variants detected in SFN and previously screened painless-DN, which may preclude their role in pain pathophysiology [22]. No potentially pathogenic variants were detected in eight genes; *ANO1, HCN1, KCNA2, KCNA4, KCNQ5, KCNN1, KCNS1, TRPV4*. Five of them; *KCNA2, KCNA4, KCNQ5, KCNN1, KCNS1* were also negative for screening of diabetic neuropathy population [22]. Interestingly in SFN cohort, variants have been detected in 3 genes; *KCNQ3, TRPV1, TPRV3* which did not appear in painful-DN [22], extending previous list of first choice gene candidates for NGS in NeP.

The frequency of potentially pathogenic ICG variants in our population (4.8%) was slightly lower than the frequency in patients with painful-DN (5.4%) [22]. The most frequent variants in the SFN cohort were located in TRP genes, in total in fifteen patients (3.6%), which is consistent with data obtained for painful-DN, where seven individuals (3.3%) had identified VUS TRP variants, respectively three (1.4%) in *TRPA1*, three (1.4%) in *TRPM8*, one (0.5%) in *TRPV4*. Different heterozygous missense VUS were also present in three (1.4%) painful-DN patients in *ANO3* and two different *KCNK18* variants leading to premature stop codon in two (0.9%) individuals with painful-DN [22].

VUS meaning in context of protein function

All detected variants were classified as VUS, which reflects the limitations of bioinformatics analysis of novel variants in absence of functional data or segregation in the family [30, 31]. In this study, we only relied on in-silico prediction of pathogenicity, therefore it is difficult to draw a definite conclusion about variant causality, which demands further validation. However, it is likely that a number of these VUS will change to (possibly) pathogenic, as they clearly have a strong impact on the protein. The nonsense/ frameshift/splice site variants cause an altered protein length or protein absence due to nonsense-mediated decay (NMD). Moreover, substitutions are present in conserved, functionally relevant regions of the protein, most likely affecting protein function, like channel opening and heat sensitivity (variants in transmembrane domain) [32], channel gating and desensitization (variants in linker between transmembrane domains) [33], protein-protein interactions (variation in ANK) [34, 35], protein stability and folding (variants in N- and C- terminus) [36], binding sites, such as inositholphospathe binding site in the case of *TRPA1* c.3136 A>G, leading to channel inactivation [37].

ICG variants in relation to patients' clinical manifestations

- **Anoctamin 3**

Anoctamin 3 is member of the Ca^{2+}-activated chloride channel family involved in pain processing and thermoregulation [38]. *ANO3* indirectly inhibits pain signaling via enhancing sodium-activated potassium (SLACK) channels in DRG [21]. Since *ANO3* knockout resulted in decreased pain threshold in tested rats, GOF variants will be expected in the pain related phenotypes [21]. Consistent with that, two heterozygous missense variants with predicted GOF were detected in individuals

affected with painful-DN [22]. In our study cohort of SFN patients, two heterozygous missense variants (*ANO3* c.2656A>T and c.3100G>C) have been identified in two unrelated subjects. Both of them reported burning pain, allodynia and sheet intolerance. TTT was abnormal in both cases, moreover female patient identified cold temperature as pain provoking factor. Altogether, it may link patients' clinical manifestation with disrupted thermoregulation.

- **Potassium channels**

Potassium channels are important in regulation of nociceptor excitability in sensory neurons [39]. A heterozygous deletion of one nt in *KCNK18* c.1107del leading to frameshift and a shortened C-terminus has been identified in an SFN patient. Although, missense and frameshift mutations with premature stop codon in *KCNK18* are linked mainly to migraine, some studies indicate that downregulation of *KCNK18* contributes to neuropathic pain [40, 41]. Two patients from our cohort harbored two different heterozygous missense variants in *KCNQ3* c.1885G>A and c.1706A>G. Interestingly, both of them had similar phenotype with numb sensation and burning pain in the feet. In both cases, pain complaints were getting worse at rest. Additionally to that, both patients had disturbed warm and cold sensation at feet. However, one patient reported cold increasing pain and warmth reducing symptoms, while the other patient did not indicated temperature influence on pain. GOF mutations in *KCNQ3* are recognized as contributors to pain resilience [42], therefore a loss-of-function (LOF) effect of detected variants would be expected in our patients with SFN and neuropathic pain.

- **TRP channels**

Variants located in the TRP genes constitute the largest group of variants identified in our study cohort. TRP channels constitute a large, divergent gene family with a well-documented role in mediating nociceptive behaviors in response to thermal, chemical and osmotic stimuli [43]. In total 6 variants have been detected in *TRPA1* (two nonsense and 4 missense), three missense variants in *TRPM8*, three missense in *TRPV1* and two variants in *TRPV3* (one missense and one splice variant). TRPA1 channel activity has been functionally linked to pain hypersensitivity, cold hyperalgesia and mechanical nociception in inflammatory and neuropathic pain models [44-46]. The GOF mutation N855S in *TRPA1* has been associated with familial episodic pain syndrome. The TRPA1 R919* variant was segregating in a

family with the cram-fasciculation syndrome [47, 48]. Interestingly, for R919* GOF effect was suggested based on clinical improvement with carbamazepine, however it was not demonstrated whether the truncated TRPA1 protein was expressed at all [48]. In our study, one patient heterozygous for the missense VUS c.980A>G has complaints of numb sensation and painful cramps in fingers, lower legs and feet, worsening after exercise. As several VUS leading to premature stop-codons or nonsense-mediated RNA-decay variants have been reported in painful-DN [22] and the presence of two nonsense variants in our patient cohort (c.1177C>T and c.1954C>T in painful-DN), a LOF mechanism would in our opinion be more likely. The clinical manifestation in the patients with *TRPA1* VUS is diverse in our cohort, however the most common complaints are numb sensation, tingling and burning pain. Four out of seven patients reported pain increase after exercise and all the patients with performed TTT, had abnormal TTT values.

Three different heterozygous VUS have been detected in patients with disturbed thermal sensation (abnormal TTT or QST) in *TRPM8*. Additionally, the patient with the c.665A>G VUS reported increased pain due to warm temperature. As *TRPM8* is modulator of cold sensation and potential target for neuropathic pain treatment [43], those variants remain interesting candidates for functional validation and determine a GOF and LOF effect.

TRPV1 and *TRPV3* are heat-activated proteins, involved in thermosensation and pain perception [43]. In addition to that, the *TRPV3* activation play a role in enhanced itch sensation [49]. In our study two patents had *TRPV3* variant (c.20006T>C combined with itch episodes and splice-site variant c.1242+1G>A *r.spl?*) and three patients were heterozygous for a missense variant in *TRPV1* (c.914T>G or c.1348A>G or c.1735C>T). Two of them reported increased pain complaints during rest, which might interfere with night rest. Interestingly no pain change was reported in response of warm/cold temperature. The literature indicate that upregulation of *TRPV1* increases amplification of pain signals [43]. Consistent with that GOF Q85R mutation of *TRPV1* have been link with neuropathic pain [50]. On the other hand, *TRPV1* missense variants localized in transmembrane domains have been reported in diabetic neuropathy patients without pain [22], which highlight the need of functional variant validation before drawing a definite conclusion.

Patients with ICG variant have more severe clinical manifestations and higher pain scores

Due to small number of patients with ICG variant, it is difficult to draw a definite conclusion whether these patients have more severe clinical manifestations in relation to basic clinical features or SFN-SIQ comparing to patients without ICG/VGSC variant. It seems that patients with ICG variant tend to have more often abnormal skin biopsy and shorter duration of neuropathy, however the statistical significance was not reached for any of these features. There is evidence implicating enhanced Na^+ influx produced by gain-of-function Nav1.7 mutations as a trigger for calcium-importing reverse Na^+/Ca^{2+} exchange that can contribute to axonal degeneration [51]; further work will be needed to determine whether ionic imbalances due to any of the variants found in this study are injurious to axons. Nevertheless, patients with ICG variant reported higher maximal pain VAS=9.36+/-0.72 than patients without ICG/VGSC variant VAS=7.47+/-2.37. All of the patients with ICG variant had severe mean maximal pain VAS≥8.5, always higher than the mean calculated for patients without ICG/VGSC variant. Interestingly, patients with *TRPA1* report very severe pain (mean max pain VAS=9.65+/-0.7) and three of them gave VAS=10, which is the highest possible score. This data is consistent with previous study showing that mean max pain was higher for painful-DN patients with ICG variant vs. painful-DN without ICG variant [22].

Conclusions

In a well-characterized cohort of patients with SFN we identified twenty patients (4.8%) carrying potentially pathogenic variant in ICG. Patients with ICG variant might have more severe pain than patients without (potentially) pathogenic ICG/VGSC variant, which is in agreement with data previously obtained for diabetic peripheral neuropathy, but the numbers are small. These findings expands the relevance of ICG screening for SFN patients, providing novel phenotype specific list of variants for further investigation. Moroever, it extends the list of potential pain related genes, reporting variants in KCNQ3, TRPV1 and TPRV3 that were not present in painful-DN. Although, all of the variants were classified as VUS and the consequences of the variation cannot be certainly predicted in-silico, they remain valid finding indicating future direction for research, with potential benefit for patients with SFN who cannot be diagnosed based on VGSC screening.

Ethics approval and patients consent

All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Local Medical Ethical Committee for all of the centres involved in this work. Patient information was anonymized prior data analysis.

Conflict of Interest

Professor Stephen Waxman received SGW reports grants from European Union's Horizon 2020 research and innovation programme Marie Sklodowska-Curie grant for PAIN-Net, Molecule-to-Man Pain Network (grant no. 721841) and European Union 7th Framework Programme (grant no. 602273) for the PROPANE study, was supported in part by the Rehabilitation Research and Development Service and Biomedical Laboratory Research Service, Department of Veterans Affairs, has served as a consultant to ChromoCell Corp. and Sangamo Therapeutics, and has served on the Scientific Advisory Board of OliPass Corporation, MedTronics and Navega Therapeutics, outside the submitted work.

Funding

This work was supported by funding from the Molecule-to-Man Pain Network, a European Commission Multi-Center Collaborative Projects through the European Union's Horizon 2020 research and innovation program under grant agreement No. 721841 and the European Union seventh framework programme for the PROPANE study under grant agreement No. 602273). The funding source had no involvement in study design, data collection, analysis and interpretation of data.

Supplementary materials

Clinical characteristics of patients with Ion Channel variant

Patient with ANO3 c.2656A>T variant

The patient is in their 50s presented with burning and aching pain with severe tactile allodynia at lower limbs, lateral and medial thigh at distal leg, since 3 years. The complaints are increasing during rest. Wearing clothes, tactile stimuli and cold temperature were reported as pain triggers. The mean pain at recruitment was VAS>3. The mean pain intensity was PI-NRS=5.

Patient suffered from dysautonomia: skin discoloration, distal vasoparalisis and orthostatic dizziness.

No family members reported similar complaints.

The patient has normal NCS and reduced IEND both at distal leg and proximal thigh (IEND value=2.2, IEND 5e percentile value =3.4). TTT was abnormal. Quantitative Sensory Testing (QST) at foot and at proximal thigh indicated increased warm detection thresholds, and reduced heat pain thresholds (allodynia).

Patient took pain medications: Duloxetine 60 mg (45% pain relief) and Gabapentin 2400 mg.

Patient with ANO3 c.3100G>C variant

The patient is in their 60s with onset of complaints at age 18. Patient has allodynia, hypersensitivity of leg's skin, burning feet, sheet intolerance and restless leg syndrome. Patient does not report any trigger influencing pain, however rest has been reported to reduce complaints. Temperature sensation was normal, pain sensation was increased. Maximal pain was VAS=9.7.

Patient suffered from dysautonomia: dry mouth, dry eyes, sweating change, micturition problems and sometimes orthostatic dizziness.

The positive family history has been reported. Mother had painful arms and legs without underlying reason. Cousin, son of mothers' sister, also have painful arms and legs. No underlying reason was found.

The patient has normal NCS and normal IEND, IEND value=7.6, IEND 5e percentile value =2.8. Thermal Threshold Testing (TTT) was abnormal.

The patient took pain medication: amitriptylin and pregabalin, which were effective against the pain. However, with the medication, the patient is less alert (probably due to amitriptylin).

Patient with KCNK18 c.1107del variant

The patient is in their 60s presented with progressive complaints since three years. It started with a tight feeling in both feet (at the right side more than on the left side), subsequently, developed a burning sensation and tingling in the feet, which extended to both legs, hands and arms. The complaints increased at night, during exercise and stressful events. During the day, warmth alleviated the complaints. At night, it was the opposite. Therefore, sometimes patient used icepacks at body. Some days feet discolored red.

The patient suffered from dysautonomia: hot flashes, dry eyes and mouth, constipation and increased transpiration.

No family members reported similar complaints.

Neurological examination showed absent Achilles tendon reflexes. NCS showed no large nerve fiber involvement. The diagnosis of SFN was confirmed in different center. TTT and IENFD have not been performed at our centre.

Patient took pain medication: amitriptylin, gabapentin, pregabalin, tegretol and oxycontin without any effect. Amitriptylin caused weight increase. Cannabis on medical receipt improved sleep quality.

Patient with KCNQ3 c.1706A>G variant

The patient is in their 40s presented with a stabbing pain in the right foot since two years, which has extended to both feet, legs, hands, lower back, groins, buttocks, shoulders and cheeks. Both feet felt numb. Touching the skin was sometimes annoying. Patient experienced itch at different parts of the body. Patient reported burning feet and restless legs. The complaints were worse at rest, leading to the sleeping problems. Temperature did not influence the pain. Pain intensity was evaluated in VAS score, the maximal pain was 8.5.

Besides increased transpiration and micturition problems, patient did not report other autonomic complaints.

No family members reported similar complaints.

Neurological examination was without any abnormalities. NCS showed no signs of large nerve fiber damage. TTT showed abnormal cold and warmth sensation in both feet. The IENFD in skin biopsy was normal (5.0/mm, 5th percentile 4.4/mm).

Patient took amitriptylin 75 mg twice a day, which had a positive effect on sleep, but did not relief the pain.

Patient with KCNQ3 c.1885G>A variant

The patient is in their 60s presented with an annoying feeling in both feet since 15 years. Gradually this feeling had changed to a numb sensation. The right foot felt continuously tight, with alternating burning, cold and painful tingling sensations. Sometimes the complaints extended to the knees. In addition, the patient experienced tingling in both hands, but those were well tolerated. Exercise and cold temperatures increased the complaints, while warmth decreases the pain. Also during the day, the complaints worsened at rest. Pain intensity was evaluated in VAS score, the maximal pain was 8.7.

The patient suffered from dysautonomia: sometimes orthostatic dizziness and impotence.

NCS showed abnormalities compatible with a severe axonal sensorimotor peripheral neuropathy. The temperature levels for warmth and cold were disturbed at both feet. Skin biopsy showed a decreased IENFD of 0.7/mm (5th percentile 2.8/mm). Neurological examination showed an abnormal sensation (vibration sense, pain sensation and propriocepsis) at the toes and an areflexia of all tendon reflexes.

Additional blood tests revealed a positive antinuclear antibodies (ANA), elevated gamma-GT and triglycerides, and a monoclonal gammopathy IgG type Lambda.

Patient took pregabalin, which was effective for pain treatment.

Patient with TRPA1 c.932C>A variant

The patient is in their 60s. Patient was diagnosed with SFN, and suffered from Ehlers-Danlos syndrome and neuropathic pain. The IENFD 10 cm above the ankle was decreased.

Patient with TRPA1 c.980A>G variant

The patient is in their 50s presented with a numb sensation in both legs since four years. Three years after this presentation patient developed painful cramps in fingers, lower legs and feet. Exercise worsened the cramps. Pain intensity was evaluated in VAS score, the maximal pain was 10.

The patient suffered from dysautonomia: dry mouth and dizziness (probably side effect of medication).

Family history showed no persons with similar complaints.

Neurological examination showed a hypoesthesia at both feet and ankles. Repetitive NCS showed no signs of large nerve fiber involvement. The IENFD 10 cm above the ankle was decreased: 2.1/mm (5^{th} percentile 4.0/mm). TTT was not performed.

Patient took pain medication: gabapentin gave too much side effects (300 mg in the morning and 100 mg in the evening), duloxetine 30 mg twice a day did not have effect,

amitripylin 100 mg a day did not relief the complaints, pregabalin 150 mg twice day caused dizziness, tramadol and methadone gave too much side effects.

Patient with TRPA1 c.1177C>T variant

The patient is in their 50s presented with a disturbed sensation, tingling and pain in the right foot that has extended to the knee and left leg. The complaints has started eight years ago after a nerve root block. The pain was continuously present, but differed in intensity. Exercise increased the pain. During the weekend the pain decreased. The sleeping pattern was disturbed by the pain. The patient could stand the blankets on feet, but touching the mattress was painful. The maximal pain score was VAS=10.

Patient suffered from dysautonomia: dry eyes, hyperhidrosis, alternating diarrhea and constipation, micturition problems, orthostatic dizziness.

No family members with similar complaints.

Neurological examination was normal. NCS showed signs of a mild axonal sensorimotor peripheral neuropathy (decreased CMAP amplitude peroneal and tibial nerve, decreased conduction velocity sural nerve, prolonged F-response peroneal nerve). TTT showed abnormal levels for warmth and cold sensation in both feet. The IENFD 10 cm above the ankle was decreased: 3.2/mm (5^{th} percentile 3.5/mm).

Patient took pain medication: pregabalin (caused side effects), amitripylin (no effect on the pain), duloxetin (stopped because of hypertension, no effect on the pain, also side effects of impotence and obstipation).

Patient with TRPA1 c.1954C>T variant

The patient is in their 20s presented with tingling, numbness and sometimes a burning sensation in both hands (right hand most affected) and lower legs (left leg most affected) since three years. Exercise and temperature changes increased the complaints. Rest alleviated the sensations. Blankets and shoes were painful at the skin. The night rest was disturbed by an annoying feeling in the legs. Pain intensity was evaluated in VAS score, the maximal pain was 10.

Patient reported several autonomic complaints: dry mouth, hyperhidrosis, facial flushing, constipation, orthostatic dizziness, gastroparesis and diminished lubrication.

Daughter of father has same complaints and was diagnosed with Multiple Sclerosis.

Neurological examination was normal. NCS showed no signs of large nerve fiber involvement. TTT showed only a disturbed level for warmth sensation at the right foot. Skin biopsy showed a normal IENFD of 12.0/mm (5^{th} percentile 8.4/mm). Additional blood tests showed an elevated Sol-Il 2 receptor value.

Patient took diclofenac that did not have effect.

Patient with TRPA1 c.2065A>G variant

The patient is in their 30s presented with a burning sensation, tingling and a numbness feeling in both hands and feet since two years. Besides, the patient experienced itch in arms, lower legs and neck. The symptoms were continuously present, but most intense during rest. Exercise and a warm shower increased the pain. A cold shower alleviated the complaints. During coldness, hands become white. Shoes and blankets were painful to the skin. The night rest was disturbed by the pain. The maximal pain was VAS=8.6.

Patient suffered from dysautonomia: dry eyes and mouth, hyperhidrosis, hot flashes, cardiac palpitations, orthostatic dizziness, swallowing difficulties, micturation problems and alternation diarrhea and constipation.

The mother reported palpitations and burning sensations in hands and feet.

Neurological examination and NCS were normal. TTT showed abnormal levels for cold sensation at both feet. The IENFD 10cm above the ankle was decreased: 6.5/mm (5^{th} percentile 7.1/mm).

Additional blood tests showed ANA and decreased vitamin B12.

Patient took pregabalin and tramadol, but the effect is unclear.

Patient with TRPA1 c.3136A>G variant

The patient is in their 40s. In 2003 patient started to complain of pain in the left knee that could not be connected with an orthopedic diagnosis. The pain was described as burning and eventually spread to left calf and later to the foot. The pain increased with movement. The mean pain at recruitment was VAS>3.

Autonomic complaints not reported.

The patient is one of two siblings with *TRPA1* c.932C>A variant and burning pain worsening with movement and responding to neuropathic pain medications.

The patient has normal NCS and reduced IEND (IEND value=3.6, IEND 5e percentile value =4.4).

The patient took pain medication: Tolep 300, Lioresal 25 mg, Neurontin 100 mg, Lyrica 150, all poorly tolerated. Good response with Laroxyll, but urinary retention appears, later developed intolerance to Lyrica (tachycardia and general malaise), Xeristar suspended for ineffectiveness.

Second patient with TRPA1 c.3136A>G variant

The patient is in their 40s presented with burning in the thighs with progressive worsening (acute onset in May 2012). The mean pain at recruitment was VAS>3. Severe pain causing awakening was reported during night and increased activity was recognized as pain provoking factor.

No autonomic complaints were reported.

One of the sibling of the patient has similar complaints (included in this study, also carrying *TRPA1* c.3136A>G).

The patient carried out in Motor Evoked Potential (MEP), Somatosensory Evoked Potentials (SEPs), Electroneuronography (ENoG), Electromyography (EMG) which resulted in the norm (2012), venous doppler in the norm. The IENFD was decreased.

The patients is ongoing hypothyroidism therapy. Lumbar discopathies (from L2 to L5 bulging discs without impingement, in L5-S1 small not severe hernia).

The patient was undergoing steroid therapy with Muscoril with good response, but quick reoccurrence of symptoms appeared after suspension. Patient took pain medication: Lyrica 25 mg, lexotan 10 drops x 3, zoloft 25 drops were recommended under psychiatric counseling, with a moderate benefit. The patient suspended zoloft on their own.

Patient with TRPM8 c.665A>G variant

The patient is in their 50s presented with complaints of sharp and stabbing pain, numbness at feet and distal legs, since 8 years. Pain was increased at night and due to movement and hot temperature.

Patient suffered from dysautonomia: distal anhidrosis, orthostatic hypotension, signs of peripheral vasoparalysis, constipation.

No family members with similar complaints.

The patient has normal NCS and reduced IEND at distal leg (IEND value=1.1/mm), not at proximal thigh. Quantitative Sensory Testing (QST) displayed high warm and cold detection thresholds, and reduced heat pain thresholds (hyperalgesia).

The patient took pain medication: gabapentin 2400 mg + amytriptiline 25 mg with mild pain improvement (30%).

Patient with TRPM8 c.1102C>T variant

The patient is in their 50s presented with a burning sensation at the dorsal side of the left upper leg since one year. Besides, the patient experienced burning pain alternating in both legs, knees, calves and feet, every evening both feet were burning. The patient could

not stand the blankets at the feet. The patient suffered from diabetes type II and had a history of alcohol abuse.

The patient reported various autonomic complaints including dry mouth, hyperhidrosis and constipation.

Neurological examination showed allodynia at the dorsal side of the left leg. NCS were normal. TTT showed disturbed warmth sensation at both hands and an abnormal cold sensation at the left hand. Skin biopsy showed a decreased IENFD of 2.6/mm (5^{th} percentile 3.5/mm).

Patient took medication: pregabalin 75 mg twice a day, but after one week stopped because of dizziness. It is unclear whether it influenced the pain intensity.

Patient with TRPM8 c.2945C>T variant

The patient is in their 30s presented with pelvic pain (stabbing and electric shock-like pain and itch) and feet dorsum. The symptoms started at 19 years old and worsened in the last 4 years. Patient was diagnosed with SFN, without known underlying case.

Patient with TRPV1 c.914T>G variant

The patient is in their 40s and was diagnosed with SFN, without known underlying case. Patient complained for burning mouth, since 2 years. The mean pain at recruitment was VAS>3. The mean pain intensity was PI-NRS=6.

The patient suffered from dysautonomia: dry mouth and restless leg syndrome.

The IENFD 10 cm above the ankle was decreased

The patient took Gabapentin, which was suspended due to intolerance (gastrointestinal disturbances and vertigo).

Patient with TRPV1 c.1348A>G variant

The patient is in their 40s presented with pain in the hands from the beginning of 2014 in the right hand and after three months, also in the left hand. Pain was prevalent at rest and affected predominantly the intrinsic musculature of the hand (palm), not the joints. No difficulty in opening the fingers after clench fists. Since 2015 the pain has spread to all fingers, it gets worse in position firm, slight improvement if moving fingers. Pain is worsening at rest. No pain changes in warm or cold temperature was observed. The mean pain at recruitment was VAS>3. Usually, the pain oscillates between VAS=3-4, when worsened it reached VAS=7-8.

Performed ENoG indicated damage at C6C7 bilateral. SEP laser, Nuclear Magnetic Resonance (NMR), Laser-evoked potentials (LEPs) were normal. Patient has reduced IENFD, IEND value=3.0, IEND 5e percentile value =4.4.

Patient took pain medications: Lyrica 25 mg, withdrawn due to excess sleep.

Patient with TRPV1 c.1735C>T variant
The patient is in their 50s presented with restless legs and tingling in both lower legs since six years. After a tibia fracture operation in 2009 the patient experienced an increase of symptoms. The lower legs felt like they were tangled with stretch bandage. During rest, tingling started in both legs. The patient experienced continuously a pins and needles sensation in the hands, arms and neck. The complaints increased at night interfering with night rest. Temperature did not influence the tingling or pain. After a warm shower the patient experienced severe itch at lower legs. The temperature sensation was decreased. Blankets, shoes and socks were painful to the skin. Pain intensity was evaluated in VAS score, the maximal pain was 6.
Patient reported the following autonomic symptoms: hyperhidrosis and urine incontinence.

Family history revealed no persons with similar complaints.

Neurological examination and NCS were normal. TTT showed abnormal levels for warmth and cold sensation in both feet. The IENFD in skin biopsy was normal: 7.6/mm (5th percentile 4.4/mm).

Patient took oxycontin 5 mg, which reliefs the pain at night.

Patient with TRPV3 c.1242+1G>A variant
The patient is in their 80s presented with complaints of swollen legs sensation followed by intense burning, heat and sensation of constriction. At the same time, the patient had hypertensive episodes with facial flushing, associated with a sensation of intense heat with fecal sphincter urgency. The patient has tactile allodynia.

The patient has normal NCS and reduced IENFD, IENFD value=1.2, IENFD 5e percentile value=1.7.

The patent took pain medications: Lyrica, which was suspended and substituted with duloxetine (no data on drug response).

Patient with TRPV3 c.2006T>C variant
The patient is in their 50s presented with intra-auricular itch and pain and burning in the feet soles, diffusing till the legs. The complaints stared in 2010 and subsequently pain symptoms were spreading in the arms and trunk. Patient reported episodes of itching in the cervical region, in the auricles, in the head and in the genital area.

No family members with similar phenotype.

The patient has normal NCS and reduced IENFD, IENFD value=2.4.

The patient has hypothyroidism since 2009, vitamin D deficiency and osteopenia in therapy with cholecalciferol.

The patent took pain medication: duloxetine with partial benefit in the beginning, later it was discontinued due to ineffectiveness. Lyrica was ineffective. The patients is taking Palexia 150 mg x 2 with partial benefit, Diazepam 5-6 drops in the evening, with benefit on sleep and Atarax in the evening.

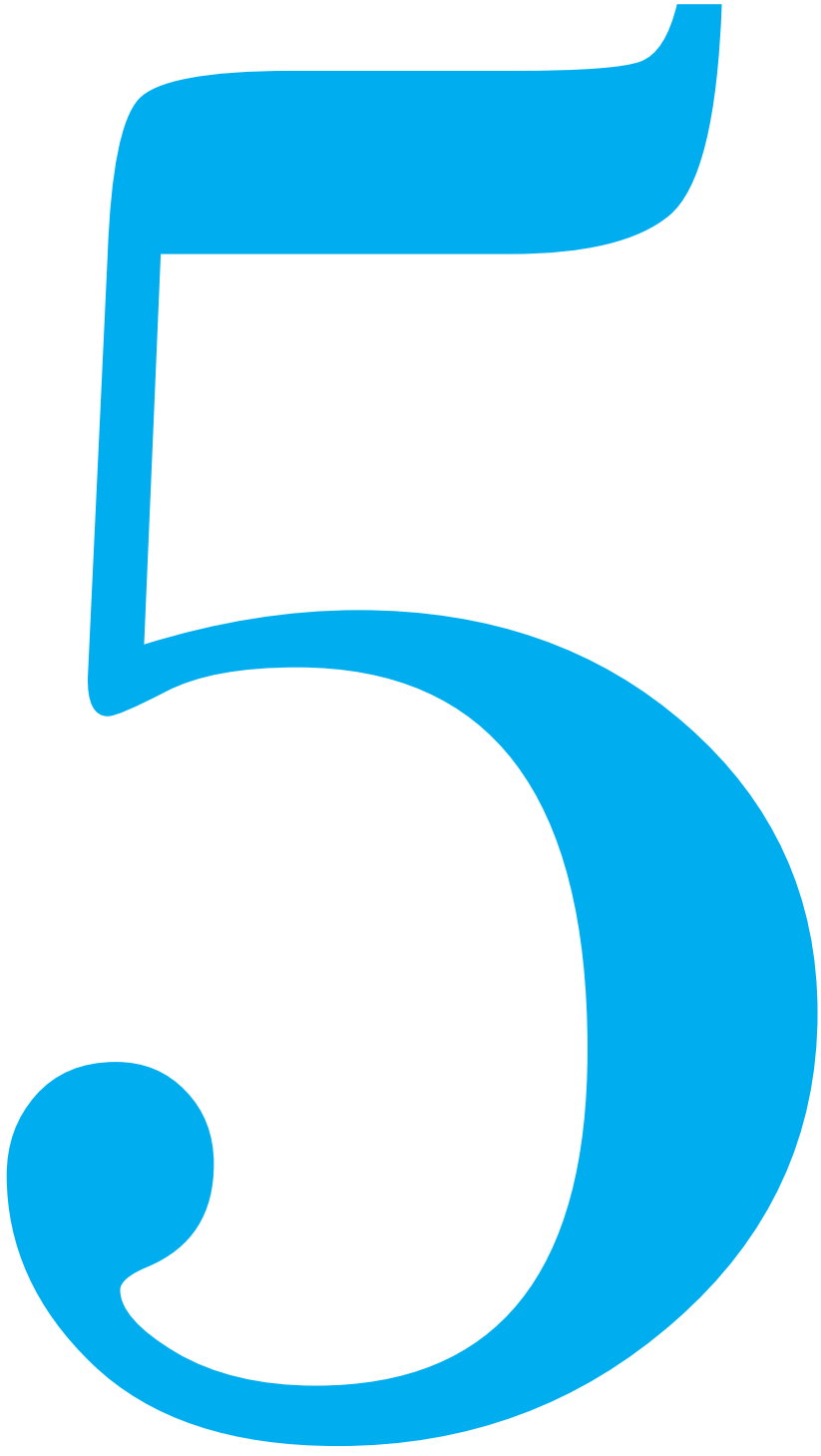
References

1. Calvo M, Davies AJ, Hébert HL, Weir GA, Chesler EJ, Finnerup NB, et al. The Genetics of Neuropathic Pain from Model Organisms to Clinical Application. Neuron. 2019;104(4):637-53. Epub 2019/11/22. doi: 10.1016/j.neuron.2019.09.018. PubMed PMID: 31751545; PubMed Central PMCID: PMCPMC6868508.
2. Cavalli E, Mammana S, Nicoletti F, Bramanti P, Mazzon E. The neuropathic pain: An overview of the current treatment and future therapeutic approaches. International journal of immunopathology and pharmacology. 2019;33:2058738419838383. Epub 2019/03/23. doi: 10.1177/2058738419838383. PubMed PMID: 30900486; PubMed Central PMCID: PMCPMC6431761.
3. 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(3):342-52. Epub 2018/12/17. doi: 10.1136/jnnp-2018-319042. PubMed PMID: 30554136.
4. Colloca L, Ludman T, Bouhassira D, Baron R, Dickenson AH, Yarnitsky D, et al. Neuropathic pain. Nat Rev Dis Primers. 2017;3:17002. Epub 2017/02/17. doi: 10.1038/nrdp.2017.2. PubMed PMID: 28205574; PubMed Central PMCID: PMCPMC5371025.
5. McCarberg BH, Billington R. Consequences of neuropathic pain: quality-of-life issues and associated costs. The American journal of managed care. 2006;12(9 Suppl):S263-8. Epub 2006/06/16. PubMed PMID: 16774458.
6. Spallone V, Greco C. Painful and painless diabetic neuropathy: one disease or two? Current diabetes reports. 2013;13(4):533-49. Epub 2013/05/17. doi: 10.1007/s11892-013-0387-7. PubMed PMID: 23677582.
7. Nguyen PT, Yarov-Yarovoy V. Towards Structure-Guided Development of Pain Therapeutics Targeting Voltage-Gated Sodium Channels. Frontiers in pharmacology. 2022;13:842032. doi: 10.3389/fphar.2022.842032. PubMed PMID: 35153801; PubMed Central PMCID: PMCPMC8830516.
8. Mogil JS. Sources of Individual Differences in Pain. Annu Rev Neurosci. 2021;44:1-25. doi: 10.1146/annurev-neuro-092820-105941. PubMed PMID: 34236890.
9. Almomani R, Sopacua M, Marchi M, Ślęczkowska M, Lindsey P, Greef BTd, et al. Genetic profiling of sodium channels in painful and painless diabetic and idiopathic small fiber neuropathy (in preparation).
10. 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 : a journal of neurology. 2014;137(Pt 6):1627-42. Epub 2014/04/30. doi: 10.1093/brain/awu079. PubMed PMID: 24776970.
11. Faber CG, Lauria G, Merkies IS, Cheng X, Han C, Ahn HS, et al. Gain-of-function Nav1.8 mutations in painful neuropathy. Proceedings of the National Academy of Sciences of the United States of America. 2012;109(47):19444-9. Epub 2012/11/02. doi: 10.1073/pnas.1216080109. PubMed PMID: 23115331; PubMed Central PMCID: PMCPMC3511073.
12. Faber CG, Hoeijmakers JG, Ahn HS, Cheng X, Han C, Choi JS, et al. Gain of function Nav1.7 mutations in idiopathic small fiber neuropathy. Annals of neurology. 2012;71(1):26-39. Epub 2011/06/24. doi: 10.1002/ana.22485. PubMed PMID: 21698661.
13. Alsaloum M, Estacion M, Almomani R, Gerrits MM, Bönhof GJ, Ziegler D, et al. A gain-of-function sodium channel β2-subunit mutation in painful diabetic neuropathy. Molecular pain. 2019;15:1744806919849802. Epub 2019/05/02. doi: 10.1177/1744806919849802. PubMed PMID: 31041876; PubMed Central PMCID: PMCPMC6510061.

14. Hoeijmakers JG, Faber CG, Merkies IS, Waxman SG. Painful peripheral neuropathy and sodium channel mutations. Neuroscience letters. 2015;596:51-9. Epub 2015/01/06. doi: 10.1016/j.neulet.2014.12.056. PubMed PMID: 25556685.
15. 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 : a journal of neurology. 2005;128(Pt 8):1847-54. Epub 2005/06/17. doi: 10.1093/brain/awh514. PubMed PMID: 15958509.
16. Basso L, Altier C. Transient Receptor Potential Channels in neuropathic pain. Current opinion in pharmacology. 2017;32:9-15. Epub 2016/11/12. doi: 10.1016/j.coph.2016.10.002. PubMed PMID: 27835802.
17. Busserolles J, Tsantoulas C, Eschalier A, López García JA. Potassium channels in neuropathic pain: advances, challenges, and emerging ideas. Pain. 2016;157 Suppl 1:S7-s14. Epub 2016/01/20. doi: 10.1097/j.pain.0000000000000368. PubMed PMID: 26785158.
18. Rivolta I, Binda A, Masi A, DiFrancesco JC. Cardiac and neuronal HCN channelopathies. Pflugers Archiv : European journal of physiology. 2020;472(7):931-51. doi: 10.1007/s00424-020-02384-3. PubMed PMID: 32424620.
19. Cho H, Yang YD, Lee J, Lee B, Kim T, Jang Y, et al. The calcium-activated chloride channel anoctamin 1 acts as a heat sensor in nociceptive neurons. Nature neuroscience. 2012;15(7):1015-21. Epub 2012/05/29. doi: 10.1038/nn.3111. PubMed PMID: 22634729.
20. Takayama Y, Uta D, Furue H, Tominaga M. Pain-enhancing mechanism through interaction between TRPV1 and anoctamin 1 in sensory neurons. Proceedings of the National Academy of Sciences of the United States of America. 2015;112(16):5213-8. Epub 2015/04/08. doi: 10.1073/pnas.1421507112. PubMed PMID: 25848051; PubMed Central PMCID: PMCPMC4413337.
21. Huang F, Wang X, Ostertag EM, Nuwal T, Huang B, Jan YN, et al. TMEM16C facilitates Na(+)-activated K+ currents in rat sensory neurons and regulates pain processing. Nature neuroscience. 2013;16(9):1284-90. Epub 2013/07/23. doi: 10.1038/nn.3468. PubMed PMID: 23872594; PubMed Central PMCID: PMCPMC4034143.
22. Ślęczkowska M, Almomani R, Marchi M, de Greef BTA, Sopacua M, Hoeijmakers JGJ, et al. Peripheral Ion Channel Gene Screening in Painful- and Painless-Diabetic Neuropathy. International Journal of Molecular Sciences. 2022;23(13):7190. PubMed PMID: doi:10.3390/ijms23137190.
23. 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. European journal of neurology. 2018;25(2):348-55. Epub 2017/11/08. doi: 10.1111/ene.13508. PubMed PMID: 29112785; PubMed Central PMCID: PMCPMC5814938.
24. Delgado DA, Lambert BS, Boutris N, McCulloch PC, Robbins AB, Moreno MR, et al. Validation of Digital Visual Analog Scale Pain Scoring With a Traditional Paper-based Visual Analog Scale in Adults. Journal of the American Academy of Orthopaedic Surgeons Global research & reviews. 2018;2(3):e088. Epub 2018/09/14. doi: 10.5435/JAAOSGlobal-D-17-00088. PubMed PMID: 30211382; PubMed Central PMCID: PMCPMC6132313
25. Williamson A, Hoggart B. Pain: a review of three commonly used pain rating scales. J Clin Nurs. 2005;14(7):798-804. doi: DOI 10.1111/j.1365-2702.2005.01121.x. PubMed PMID: WOS:000230291200003.
26. Almomani R, Marchi M, Sopacua M, Lindsey P, Salvi E, Koning B, et al. Evaluation of molecular inversion probe versus TruSeq® custom methods for targeted next-generation sequencing. PloS one. 2020;15(9):e0238467. Epub 2020/09/03. doi: 10.1371/journal.pone.0238467. PubMed PMID: 32877464; PubMed Central PMCID: PMCPMC7467307.

27. 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. Genetics in medicine : official journal of the American College of Medical Genetics. 2015;17(5):405-24. Epub 2015/03/06. doi: 10.1038/gim.2015.30. PubMed PMID: 25741868; PubMed Central PMCID: PMCPMC4544753.
28. 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. doi: 10.1111/jns.12128. PubMed PMID: 26114213.
29. Zech M, Gross N, Jochim A, Castrop F, Kaffe M, Dresel C, et al. Rare sequence variants in ANO3 and GNAL in a primary torsion dystonia series and controls. Movement disorders : official journal of the Movement Disorder Society. 2014;29(1):143-7. Epub 2013/10/24. doi: 10.1002/mds.25715. PubMed PMID: 24151159.
30. Perrone F, Cacace R, van der Zee J, Van Broeckhoven C. Emerging genetic complexity and rare genetic variants in neurodegenerative brain diseases. Genome Med. 2021;13(1):59. doi: 10.1186/s13073-021-00878-y. PubMed PMID: 33853652; PubMed Central PMCID: PMCPMC8048219.
31. Federici G, Soddu S. Variants of uncertain significance in the era of high-throughput genome sequencing: a lesson from breast and ovary cancers. J Exp Clin Cancer Res. 2020;39(1):46. doi: 10.1186/s13046-020-01554-6. PubMed PMID: 32127026; PubMed Central PMCID: PMCPMC7055088.
32. Grandl J, Kim SE, Uzzell V, Bursulaya B, Petrus M, Bandell M, et al. Temperature-induced opening of TRPV1 ion channel is stabilized by the pore domain. Nature neuroscience. 2010;13(6):708-14. Epub 2010/04/24. doi: 10.1038/nn.2552. PubMed PMID: 20414199; PubMed Central PMCID: PMCPMC2876202.
33. Papke D, Grosman C. The role of intracellular linkers in gating and desensitization of human pentameric ligand-gated ion channels. J Neurosci. 2014;34(21):7238-52. doi: 10.1523/JNEUROSCI.5105-13.2014. PubMed PMID: 24849357; PubMed Central PMCID: PMCPMC4028499.
34. Gaudet R. A primer on ankyrin repeat function in TRP channels and beyond. Mol Biosyst. 2008;4(5):372-9. doi: 10.1039/b801481g. PubMed PMID: WOS:000254998800002.
35. Mosavi LK, Cammett TJ, Desrosiers DC, Peng ZY. The ankyrin repeat as molecular architecture for protein recognition. Protein science : a publication of the Protein Society. 2004;13(6):1435-48. Epub 2004/05/21. doi: 10.1110/ps.03554604. PubMed PMID: 15152081; PubMed Central PMCID: PMCPMC2279977.
36. Krishna MM, Englander SW. The N-terminal to C-terminal motif in protein folding and function. Proceedings of the National Academy of Sciences of the United States of America. 2005;102(4):1053-8. Epub 2005/01/20. doi: 10.1073/pnas.0409114102. PubMed PMID: 15657118; PubMed Central PMCID: PMCPMC545867.
37. Brewster MSJ, Gaudet R. How the TRPA1 receptor transmits painful stimuli: Inner workings revealed by electron cryomicroscopy. Bioessays. 2015;37(11):1184-92. doi: 10.1002/bies.201500085. PubMed PMID: WOS:000363481200009.
38. Wang TFA, Chen C, Huang F, Feng SJ, Tien JS, Braz JM, et al. TMEM16C is involved in thermoregulation and protects rodent pups from febrile seizures. Proceedings of the National Academy of Sciences of the United States of America. 2021;118(20). doi: 10.1073/pnas.2023342118. PubMed PMID: WOS:000656222000007.

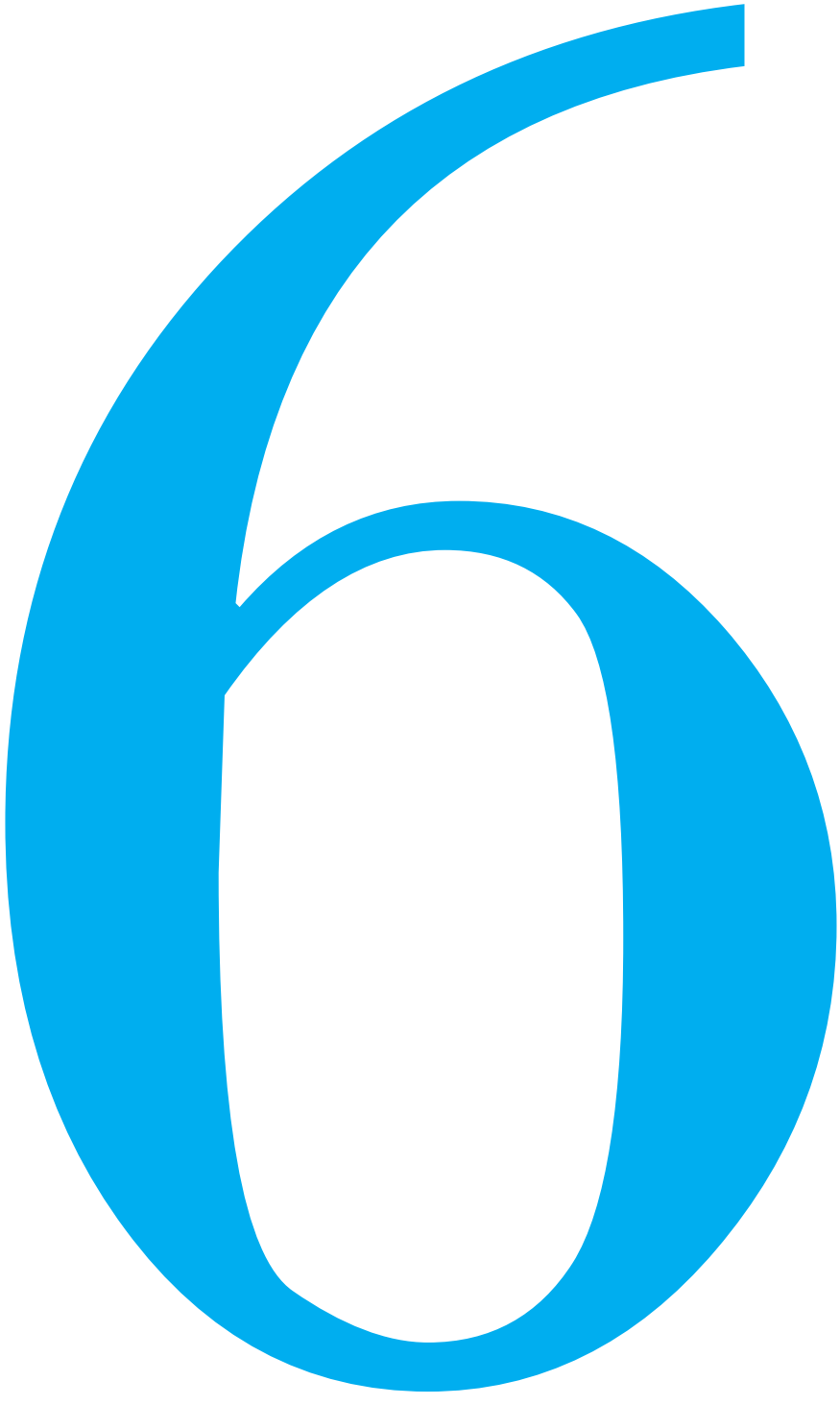
39. Tsantoulas C. Emerging potassium channel targets for the treatment of pain. Curr Opin Support Pa. 2015;9(2):147-54. doi: 10.1097/Spc.0000000000000131. PubMed PMID: WOS:000353841000010.
40. Zhou J, Yang CX, Zhong JY, Wang HB. Intrathecal TRESK gene recombinant adenovirus attenuates spared nerve injury-induced neuropathic pain in rats. Neuroreport. 2013;24(3):131-6. doi: 10.1097/WNR.0b013e32835d8431. PubMed PMID: 23370493.
41. Zhou J, Chen HT, Yang CX, Zhong JY, He WY, Xiong QM. Reversal of TRESK Downregulation Alleviates Neuropathic Pain by Inhibiting Activation of Gliocytes in the Spinal Cord. Neurochem Res. 2017;42(5):1288-98. doi: 10.1007/s11064-016-2170-z. PubMed PMID: WOS:000399906000002.
42. 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 communications. 2021;3(3):fcab212. Epub 2021/09/25. doi: 10.1093/braincomms/fcab212. PubMed PMID: 34557669; PubMed Central PMCID: PMCPMC8454204.
43. Gonzalez-Ramirez R, Chen Y, Liedtke WB, Morales-Lazaro SL. TRP Channels and Pain. In: Emir TLR, editor. Neurobiology of TRP Channels. Frontiers in Neuroscience. Boca Raton (FL)2017. p. 125-47.
44. Obata K, Katsura H, Mizushima T, Yamanaka H, Kobayashi K, Dai Y, et al. TRPA1 induced in sensory neurons contributes to cold hyperalgesia after inflammation and nerve injury. The Journal of clinical investigation. 2005;115(9):2393-401. Epub 2005/08/20. doi: 10.1172/jci25437. PubMed PMID: 16110328; PubMed Central PMCID: PMCPMC1187934.
45. Dai Y, Wang S, Tominaga M, Yamamoto S, Fukuoka T, Higashi T, et al. Sensitization of TRPA1 by PAR2 contributes to the sensation of inflammatory pain. Journal of Clinical Investigation. 2007;117(7):1979-87. doi: 10.1172/Jci30951. PubMed PMID: WOS:000247837700030.
46. McNamara CR, Mandel-Brehm J, Bautista DM, Siemens J, Deranian KL, Zhao M, et al. TRPA1 mediates formalin-induced pain. Proceedings of the National Academy of Sciences of the United States of America. 2007;104(33):13525-30. doi: 10.1073/pnas.0705924104. PubMed PMID: WOS:000248899600056.
47. Kremeyer B, Lopera F, Cox JJ, Momin A, Rugiero F, Marsh S, et al. A gain-of-function mutation in TRPA1 causes familial episodic pain syndrome. Neuron. 2010;66(5):671-80. Epub 2010/06/16. doi: 10.1016/j.neuron.2010.04.030. PubMed PMID: 20547126; PubMed Central PMCID: PMCPMC4769261.
48. Nirenberg MJ, Chaouni R, Biller TM, Gilbert RM, Paisán-Ruiz C. A novel TRPA1 variant is associated with carbamazepine-responsive cramp-fasciculation syndrome. Clinical genetics. 2018;93(1):164-8. Epub 2017/04/25. doi: 10.1111/cge.13040. PubMed PMID: 28436534; PubMed Central PMCID: PMCPMC5654709.
49. Cui TT, Wang GX, Wei NN, Wang K. A pivotal role for the activation of TRPV3 channel in itch sensations induced by the natural skin sensitizer carvacrol. Acta pharmacologica Sinica. 2018;39(3):331-5. doi: 10.1038/aps.2017.152. PubMed PMID: 29094727; PubMed Central PMCID: PMCPMC5843833.
50. Yuan JH, Schulman BR, Effraim PR, Sulayman DH, Jacobs DS, Waxman SG. Genomic analysis of 21 patients with corneal neuralgia after refractive surgery. Pain Rep. 2020;5(4):e826. doi: 10.1097/PR9.0000000000000826. PubMed PMID: 32766464; PubMed Central PMCID
51. 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. Journal of Neurophysiology. 2015;114(3):1554-64. doi: 10.1152/jn.00195.2015. PubMed PMID: WOS:000362045100021.



Chapter 5

Novel and rare variants identified by screening 592 pain-related candidate genes in Small Fiber Neuropathy patients

Milena Ślęczkowska[1,2], Kaalindi Misra[3], Silvia Santoro[3], Federica Esposito[3,4], Rowida Almomani[1,5],, Margherita Marchi[6], Janneke G J Hoeijmakers[2], Giuseppe Lauria[6], Catharina G Faber[2], Hubert J M Smeets[1,2], Monique M Gerrits[7]



Chapter 6

Exploring the Role of Anoctamin 3 in painful Diabetic Neuropathy and Small Fiber Neuropathy

Milena Ślęczkowska[1,2], Monique M Gerrits[3], Rossella Avagliano Trezza[1], Farhad Jahanfar[4], Laura Carraresi[4], Massimo D'Amico[4], Catharina G Faber[2], Hubert J M Smeets[1,2]

Chapter 7

General discussion

General discussion

7.1 Personalized pain management is a challenge for modern medicine

Personalized pain management is one of the major challenges for health care providers, still being largely ineffective despite of joined effort of clinicians and scientists [1]. Together with psychological comorbidities it remains a significant public health problem and a major concern for affected individuals, their families and society as a whole [2]. It is difficult to treat a condition we do not fully understand, therefore more knowledge about the underlying pathophysiological mechanisms is required. Increasing numbers of reports, highlight the importance of genetic factors in neuropathic pain and targeting the specific defect caused by a gene mutation might provide a highly accurate and effective treatment [3-7]. Progress in the genetics of neuropathic pain enabled the development of ICG-targeted pain therapy [8-10]. For instance, it has been demonstrated that certain mutations in Nav1.7 affect the analgesic effect of lacosamide [11], revealing a group of patients that can benefit from personalized treatment. But the picture still remains complex and variants in the same gene might have different effects, requiring more studies in well-characterized cohorts of fully characterized patients to resolve the genetic landscape of pain. Technological developments, such as NGS, have made these studies technically and financially possible. Screening of patients with painful Diabetic-Neuropathy and Small Fiber Neuropathy for variants in ion channel genes (Chapter 3-4) and NGS of 592 pain-relates genes (Chapter 5) revealed several (likely) pathogenic variants and VUS being potential pain treatment targets, however the second demands further validation.

7.2 Functional testing of genetic variants

Rapid developments in clinical application of NGS allow for extensive sequencing both in a diagnostic and research setting providing valuable data on genetic variants in patients and patient cohorts [12]. However, the numbers of variants with a possible pathogenic role, including massive number of VUSes, exceed the functional testing capacity of diagnostic and research centers [13]. A VUS is not reported to patients, because it is an ambiguous result, not suitable for health-care [13]. But a functional role cannot be excluded and part of them will have a functional role, so validation is highly desirable. A VUS that can be classified as pathogenic based on additional evidence, will increase the number of potential treatment targets. Designing assays for functional read outs is often challenging, especially in the field of neurological disease and particularly when the function of the gene in question is not established yet, for instance Anoctamin 3 (Chapter 6). Another difficulty is that these are often

low-throughput models, which are suitable for studying pathophysiological pathways, but cannot keep up with the speed of variant identification. Although high-throughput approaches are possible [14, 15], they also have limitations. Both model types will be discussed below.

7.3 Models for functional testing of pain related genetic variants

7.3.1 *In vitro* models

7.3.1.1 Cell culture

Two-dimensional (2D) cell culture is an *in vitro* model that can be used for multiple assays in pain research, focusing among others on basic cell biology, biochemistry, protein expression, toxicity testing, genetic defects and disease modeling [16]. This model allows for growing cells under controlled conditions and for performing genetic manipulation, therefore, it may be used to understand the pathophysiology and linking the genetic defect to the patient's phenotype, if at least the cellular phenotype is a biomarker for the clinical phenotype [16]. The limitations of 2D cell cultures might be cell flattening affecting nuclear shape, which in consequence results in differences in gene/protein expression and limited ability to study complex cell-to-cell interactions [17]. Still, cellular models have been used to characterize VUS in ion channels, based on a defined cellular phenotype. There are three main methods for evaluation of ion channel function: ligand binding, membrane potential measurements and flux measurements [18]. Binding assays detect changes influencing specific ligand-binding site [18, 19], therefore they will be suitable for validation of VUS located in binding site or affecting this protein area. Membrane potential assays illustrate changes in ion channel conductance caused by mutation or drugs [18, 20]. Finally, ion flux assays, including ion-selective fluorescent probes and voltage-clamp measurement are designed to trace the cellular influx or efflux of specific ions through ion channels [18, 19]. Multiple 2D cell lines are available commercially, for instance human embryonic kidney 293 (HEK293) cells that are easy to transfect and widely applied in assays testing VUS functional effect, including mutations in ion channel genes [21-23]. Additionally, Chinese hamster ovary (CHO) cells and Xenopus oocytes have been used for studying ion channels as well [18]. These models have the possibility of high-throughput drug screening or VUS testing, for instance by using an automated patch clamp [18-20]. Although using these cell lines is easy and cost effective, testing specific variant in a cell type that is close to the native tissue (e.g. DRG for mutations in peripheral sodium ion channels) is highly desirable and recommended [24, 25]. DRG cultures reflect the characteristics of nociceptors very well, moreover their size allows for electrophysiology recordings and therefore they are suitable to study ICG and ICG

VUS involved in pain pathways [26]. Obviously, there are extra costs and efforts to be made, since DRG are more difficult to transfect. Moreover, in the case of ICG, transfected and endogenous channels need to be distinguished in DRG [24, 25, 27]. This makes it more difficult to perform VUS testing in DRG on large scale. Patient-derived DRG cells would enable to study VUS in the full genetic background of the patient, however obtaining material for DRG isolation is problematic, since it requires a surgical procedure [28, 29]. The other option to obtain human DRG is differentiation of other cell types such as fibroblasts or blood cells into DRGs (see below).

7.3.1.2 Induced pluripotent stem cells

Human induced pluripotent stem cells (iPSCs) have the ability to differentiate into all somatic cell types of the body [30]. This feature opens opportunities for many research disciplines including neurosciences research, since isolation of primary human tissue is very invasive [30]. Moreover, converting adult patient-derived somatic cells into iPSCs provided option for individual-specific variant testing and personalized therapy [31]. Patient derived stem cells maintain their full genetic landscape during the differentiation [32], which not only provides a native environment for testing but also overcomes the difficulties and challenges of genome editing. Electrophysiology recordings demonstrate that iPSCs-derived sensory neurons (iPSCs-SN) exhibit the pathological features associated with neuropathic pain in patients with inherited erythromelalgia (IEM) and SFN [33, 34]. iPSCs-SN have been successfully generated from patients with pain episodes, affected with IEM, and used for NaV1.7 I848T variant characterization by patch-clamp technology, showing abnormal ectopic activity explaining the pain phenotype [33]. A similar strategy was applied to study LOF variants in NaV1.7 found to cause congenital insensitivity to pain [35]. Moreover the iPSCs model has been shown to be suitable for studying other ion channels and more complex genetic interactions, for example revealing that *KCNQ2* and *KCNQ3* GOF variants modulate neurons excitability and therefore contribute to pain resilience in patients with pathogenic Nav1.7 variant [36, 37]. Cao *et al.* utilized iPSC-derived sensory neurons from patients with IEM to demonstrate that the PF-05153462 Nav1.7 sodium channel blocker is effective in decreasing heat-induced pain [38], while other studies indicate lactosamide to decrease spontaneous nociceptor activity in iPSC- derived neurons from SFN patients [34]. These examples demonstrate that iPSCs have huge potential for personalized medicine and drug screening, especially in the context of complex genotype-phenotype interactions.

Although, iPSCs-SN are a powerful model for both VUS characterization and individual-specific drug screening, there are some limitations. The main challenge is the cost of iPSCs generation, maintenance and differentiation into the appropriate neuronal cell type [39]. This work includes intensive cell culturing and time-consuming steps, making the whole procedure lasting from weeks to months to obtain material for further investigation [31]. iPSCs have weak proliferation capacity and decline in function with aging [31, 40]. Moreover, genetic and environmental factors might negatively affect sample-to-sample reproducibility [31, 39]. Therefore, iPSCs-SN are not currently used on large scale either for VUS characterization or drug screening, mainly due to time and cost constraints.

7.3.1.3 Organoids

Organoids are self-organized three-dimensional (3D) tissue structures generated from stem cells [41]. In contrast to adherent monolayer cultures, they exhibit another level of complexity, that better reflects *in vivo* conditions [41, 42]. Dorsal root ganglia organoid (DRG organoids) derived from iPSCs from patients' fibroblasts contain the sensory neural cells and glial cells present in somatic DRGs [43]. Obtained sensory neurons were characterized by functional excitability and ability to organize into complex structures closely resembling mature peripheral sensory neurons [42, 43]. Moreover, resulting cutaneous nociceptive and thermoceptive neurons expressed *TRPA1, TRPM8, TRPV1/2/3, SCN11A, KCNQ2, CACNA1A* and *CANCA2D1*, and neurotransmitter receptor genes *GRIA1* and *NK1R* [42]. Organoids are highly suitable for disease modeling, small-scale drug screening and toxicity assays and they may limit the use of animal models [41, 44]. Due to the 3D structure, organoids allow for more precise studies of drug penetration [45], therefore they are superior over 2D cell culture for toxicity and efficacy evaluation of drugs. However, organoids are also powerful tools for personalized medicine in context of VUS effect assessment and possible treatment, since patients' derived organoids contain the original genetic environment of the donor. On the other hand, this model is laborious, adding extra cost for organoid generation and readouts, also restricting the throughput capacity [45]. Moreover, limited control over organoids formation may lead to reduced reproducibility, as organoids derived from the same patient may display phenotypic differences [44, 45].

7.3.2 *In vivo* models

7.3.2.1 *Drosophila melanogaster*

Drosophila melanogaster also known as a fruit fly is a model organism widely used in genetic and developmental studies [46]. It is an easy and inexpensive model in maintenance, with short life span and big number of offspring [47]. Almost 75% of the human disease-related genes are present in the *Drosophila* genome [46]. The fruit fly shows complex behaviors and represents whole-body physiological process [47, 48], therefore it is commonly used to study central and peripheral nervous system disorders, as well nociception and pain processing [48]. The nociceptors of *Drosophila* are preserved throughout metamorphosis and are present in adult individuals, exhibiting morphological and functional similarities to nociceptors of vertebrates [46]. Additional advantages compared to mammal models are relatively easy genome editing, the possibility to perform experiments on large scale and no major consideration according to animal protection law [46, 49]. However, certainly there are some limitations such as different physiology and being no vertebrates, that might limit application of this model in specific research areas and translation of the results into human [50]

Nevertheless, the *Drosophila* model significantly contributed to our understanding of molecular mechanism of electrical activity in nervous system [51]. Interestingly, the nociceptor family TRP channels were first identified in *D. melanogaster* for phototransduction and this discovery allowed the first *Drosophila's* TRP channel to be cloned in the 1980s [52, 53]. The initial experimental assays of thermal and mechanical nociception used heat and von Frey fibers stimulations to induce rolling responses in the fly larvae [54]. Other fruit fly models were developed for investigating sensitization induced by inflammation produced by UV exposure [55, 56]. Finally, the *Drosophila* nerve injury model for neuropathic sensitization [57] and fly larvae model resembling painful diabetic neuropathy were successfully applied in pain research [58].

Multiple variants affecting ion channels have been characterized in *Drosophila* [51]. Those include causative variants in voltage-gated sodium channels and several types of potassium channels that have been identified based on reversible temperature-sensitive paralysis or leg shaking phenotype [51]. A big part of ion channel variants studied in fruit fly were generated by chemical mutagenesis, however nowadays other genome editing techniques including advanced and specific CRISPR/Cas9 technology can be applied [51, 59]. Another advantage of the model is the possibility to use electrophysiology recordings for ion channels characterization, also using automated patch clamp technology [60]. Easy genetic manipulation combined with

multiple assays for pain testing makes this model suitable for VUS characterization, also including variants in ion channels, since electrophysiology recording have been successfully utilized for *Drosophila* before.

7.3.2.2 Zebrafish

The zebrafish (*Danio rerio*) is a powerful tool in multiple fields of science, including genetics, neuroscience and pharmacology [61]. Zebrafish are vertebrates and zebrafish have a large genetic and physiological homology with human genes and functional domains, estimated as ~70% of similarity at protein level [50]. However, the Zebrafish genome is for a large part duplicated, meaning that for many genes two copies are present [62]. This is the case for multiple ion channels such as calcium channel CaV2.1 encoded by *cacna1aa* and *cacna1ab* and VGSC [62-64]. Therefore, the human ortholog needs to be identified, which for multiple genes is difficult, for example it is not clear which of the Zebrafish VGSC have the function of the human VGSC [65]. Zebrafish are powerful due to rapid embryonic development and large numbers of offspring [50]. They are easy to manipulate due to external fertilization, transparent and cost- and space-effective [66]. Although, there are some anatomical differences comparing with humans' central nervous system, the model displays electrical activity and responds to various nociceptive stimuli similarly to mice and humans [61].

Several nociceptive assays and zebrafish models have been developed including: complete Freund's adjuvant (CFA), formalin injection and acetic acid administration, where pain behaviors can be inhibited by analgesics [66-68]. Therefore, zebrafish provide a promising strategy to perform high-throughput screening of novel pain medications in zebrafish [61]. Moreover, a glucose-treated zebrafish model was used to study glucose-induced axon degeneration observed in diabetic neuropathy [69] and overexpression of gain-of function mutations in human *SCN9A* resulted in decreased small fiber density and hypersensitivity to temperature changes in tested fish [65]. Although, improvements in methodical approaches are still required, for instance there is no assay for allodynia assessment [61], these results highlight the utility of zebrafish for studying SFN pathophysiology and functional testing of VUS, although for high-throughput screening the model needs to be further automated [70].

7.3.2.3 Mice

The mouse *(Mus musculus)* genome and physiology are closely related to human counterparts and therefore they are widely used in basic research and pre-clinical pain studies [71]. This is not surprising taking into consideration the availability of multiple models of inflammatory and neuropathic pain [72]. Neuropathic pain can be initiated in rodents centrally via contusive spinal cord injury or peripherally via sciatic nerve total transection, chronic constriction injury, (partial) sciatic nerve injury or spinal nerve ligation [72]. There are also metabolic mice models for DM1 and DM2, among which the common diabetic neuropathy mouse with neurotoxicity induced by streptozotocin [72]. Noxious stimuli trigger pain behavior in tested animals that can be objectively scored [73]. Several assays help to determine both mechanical (von Frey and paw pressure test) and thermal hypersensitivity (cold: acetone evaporation test and cold plate; warm: hot plate, Hargreaves or plantar test and tail flick test) [72]. As pain is not only a physical response of the body, a further advantage is the possibility to assess the psychological and emotional comorbidities based on mice anxiety- and depression-like behaviors [72].

A wide range of validated pain-related assays make the mouse model attractive to study the functional effect of genetic variants, including sex differences and highly specific subphenotypes. For example, specific variants may lead to heat and mechanical hypersensitivity, but no hypersensitivity to noxious cold temperatures [74]. Recently, mice with VUS of Nav1.7 and Nav1.8 have been successfully created and characterized, demonstrating that these VUS indeed caused an SFN phenotype [74, 75]. Mice with the G1662S point mutation in the *SCN10A* gene, generated by homologous recombination in embryonic stem cells, exhibited increased sensitivity to touch [74]. Interestingly, female homozygous mutants tended to be more sensitive to cooling stimuli, while male homozygous mutants were more sensitive to radiant heat in the Hargreaves, showing the possible differences between sexes [74]. Altered nociception in the mice corresponded to pain symptoms present in SFN individuals carrying G1662S variant [74]. The mouse model created by CRISPR/Cas9 technology was successful to understand the impact of the *SCN9A* R185H variant, since the transgenic animals displayed pain behaviors and spontaneous pain, resembling the phenotype of patients with SFN [75].

These studies confirm that transgenic mouse models are of great value for pain research, however generation of VUS, knockin/knockout transgenic lines using traditional methods require multiple costly and time consuming steps [74, 76]. Application of CRISPR/Cas9 technology enabled the creation of genetic mouse models faster and less expensive, however off-target effects are one major concern

of this technology [77]. Although, the mouse model enables to study complex pain behaviors, it is time-consuming and demands high financial outlays, therefore large scale VUS testing in this model is not yet feasible.

Table 1. Summary of *in vitro* and *in vivo* models for functional variant testing with their main advantages and limitations.

***In vitro* models**		
model	**advantages**	**limitations**
2D cell culture	• Controlled conditions of cell culture • Cost effective and easy in maintenance • Multiple cell lines from different tissues can be used • Easy genetic manipulation • Multiple functional assays can be applied	• Limited cell-to-cell interactions • Do not reflect fully biological conditions of the body • Cell flattening affects nuclear shape and gene/protein expression
Induced pluripotent stem cells	• Donor cell can be easily and non-invasively obtained • Do not rise ethical issues unlike embryotic stem cells • Can be differentiated in any type of somatic cell • Patient-specific cells with original genetic background • Ability to personalize the treatment	• Higher cost, longer and more difficult generation procedure comparing to 2D cell culture • Donor-based variability • Declining function with aging • Weak proliferation capacity
Organoids	• Composed of different cell types better reflecting endogenous conditions • Patient specific model • Limitation of animal model usage	• More expensive and advanced culture system • Limited high-throughput capacity • Diversity due to limited control of organoid conditions

In vivo models		
model	**advantages**	**limitations**
Drosophila melanogaster	• Short developmental time and big number of offspring • Inexpensive in maintenance • Compact genome and conserved genes • Relatively easy genome editing • Developed pain assays	• Different physiology than human • Difficult to measure complex behaviours
Zebrafish	• Easy and cheap maintenance • High number of offspring • External fertilization • Fast development and transparent embryos • Several nociceptive assays present	• Different physiology comparing to human and anatomical differences in central nervous system • Many genes present in two copies • Methodology in pain assessment needs improvement (no assays for allodynia)
Mice	• Mammalian model genetically and physiologically close to humans • Multiple models and pain assays developed • Possibility to study complex behaviours • Availability of transgenic mouse models • CRISPR/Cas gene editing technology can be applied	• High cost • Small scale model • Slow development and long mutant line generation • Ethical considerations

7.4 Conclusions

Next Generation Sequencing is a highly powerful technology to identify novel genes and mutations involved in the development or maintaining of neuropathic pain. Genetic screening of affected individuals may be applied for diagnostics, identification of disease predisposing genetic factors and to indicate patient-targeted specific therapy, such as ion channel blockers. Performing NGS of patients with painful neuropathies, we identified several variant being potential targets for pain treatment, including number of VUS, that need to be further investigated in laboratory setting to provide evidence for their causality, which in many cases is challenging and time consuming.

It is difficult to select the best model for testing the effect of VUS in different genes. Due to high demand of variant validation, high-throughput models would be favorable. For instance, in case of ion channels; 2D cell culture combined with automated patch-clamp, might be the strategy of first choice, as it is relatively simple and inexpensive. On the other hand, this model may lack sensitivity and may not or only partly reflect pain behaviors present in *in vivo* models, so *Drosophila* or zebrafish would be better in this aspect. It is important to perform the functional assays in these models, being aware of their limitations and purpose of the study. It is clear that a good model should properly reflect the specific research question in humans. Mice seem to most appropriate from that point of view, but mice models are laborious, expensive and can be performed only on small scale. Therefore high-throughput approaches for variant testing and drug screening might require further optimization of more simple models that can keep up with the speed of genetic discovery. This work contributed to better understanding of the genetic landscape of neuropathic pain and provides direction for further research aiming to develop personalized pain therapy and disease prognosis.

References

1. Colloca L, Ludman T, Bouhassira D, Baron R, Dickenson AH, Yarnitsky D, et al. Neuropathic pain. Nat Rev Dis Primers. 2017;3:17002. Epub 2017/02/17. doi: 10.1038/nrdp.2017.2. PubMed PMID: 28205574; PubMed Central PMCID: PMCPMC5371025.
2. Thomas MA. Pain management - the challenge. Ochsner J. 2003;5(2):15-21. PubMed PMID: 22826679; PubMed Central PMCID: PMCPMC3399329.
3. Abbott CA, Malik RA, van Ross ER, Kulkarni J, Boulton AJ. Prevalence and characteristics of painful diabetic neuropathy in a large community-based diabetic population in the U.K. Diabetes care. 2011;34(10):2220-4. Epub 2011/08/20. doi: 10.2337/dc11-1108. PubMed PMID: 21852677; PubMed Central PMCID: PMCPMC3177727.
4. Gonzalez-Ramirez R, Chen Y, Liedtke WB, Morales-Lazaro SL. TRP Channels and Pain. In: Emir TLR, editor. Neurobiology of TRP Channels. Frontiers in Neuroscience. Boca Raton (FL)2017. p. 125-47.
5. Marchettini P, Lacerenza M, Mauri E, Marangoni C. Painful peripheral neuropathies. Current neuropharmacology. 2006;4(3):175-81. Epub 2008/07/11. doi: 10.2174/157015906778019536. PubMed PMID: 18615140; PubMed Central PMCID: PMCPMC2430688.
6. Rogers M, Tang L, Madge DJ, Stevens EB. The role of sodium channels in neuropathic pain. Seminars in cell & developmental biology. 2006;17(5):571-81. Epub 2006/11/25. doi: 10.1016/j.semcdb.2006.10.009. PubMed PMID: 17123847.
7. Zorina-Lichtenwalter K, Parisien M, Diatchenko L. Genetic studies of human neuropathic pain conditions: a review. Pain. 2018;159(3):583-94. Epub 2017/12/15. doi: 10.1097/j.pain.0000000000001099. PubMed PMID: 29240606; PubMed Central PMCID: PMCPMC5828382 the end of this article.
8. 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. doi: 10.1371/journal.pone.0152405. PubMed PMID: 27050761; PubMed Central PMCID: PMCPMC4822888.
9. McDonnell A, Collins S, Ali Z, Iavarone L, Surujbally R, Kirby S, et al. Efficacy of the Nav1.7 blocker PF-05089771 in a randomised, placebo-controlled, double-blind clinical study in subjects with painful diabetic peripheral neuropathy. Pain. 2018;159(8):1465-76. doi: 10.1097/j.pain.0000000000001227. PubMed PMID: 29578944.
10. Stevens EB, Stephens GJ. Recent advances in targeting ion channels to treat chronic pain. Br J Pharmacol. 2018;175(12):2133-7. doi: 10.1111/bph.14215. PubMed PMID: 29878335; PubMed Central PMCID: PMCPMC5980455.
11. 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 : a journal of neurology. 2020;143(3):771-82. doi: 10.1093/brain/awaa016. PubMed PMID: 32011655; PubMed Central PMCID: PMCPMC7089662.
12. Hu TS, Chitnis N, Monos D, Dinh A. Next-generation sequencing technologies: An overview. Hum Immunol. 2021;82(11):801-11. doi: 10.1016/j.humimm.2021.02.012. PubMed PMID: WOS:000711185600002.
13. Hoffman-Andrews L. The known unknown: the challenges of genetic variants of uncertain significance in clinical practice. J Law Biosci. 2017;4(3):648-57. doi: 10.1093/jlb/lsx038. PubMed PMID: WOS:000423557400015.
14. Neely GG, Keene AC, Duchek P, Chang EC, Wang QP, Aksoy YA, et al. TrpA1 Regulates Thermal Nociception in Drosophila. PloS one. 2011;6(8). doi: 10.1371/journal.pone.0024343. PubMed PMID: WOS:000294680800085.
15. Toh MF, Brooks JM, Strassmaier T, Haedo RJ, Puryear CB, Roth BL, et al. Application of High-Throughput Automated Patch-Clamp Electrophysiology to Study Voltage-Gated Ion Channel Function in Primary Cortical Cultures. Slas Discov. 2020;25(5):447-57. doi: 10.1177/2472555220902388. PubMed PMID: WOS:000511556400001.

16. Anju Verma MV, Anchal Singh. Animal tissue culture principles and applications. Animal Biotechnology. 2020:269–93. Epub 2020 Jun 26. doi: 10.1016/B978-0-12-811710-1.00012-4. PubMed Central PMCID: PMCPMC7325846.
17. Knight E, Przyborski S. Advances in 3D cell culture technologies enabling tissue-like structures to be created in vitro. J Anat. 2015;227(6):746-56. doi: 10.1111/joa.12257. PubMed PMID: 25411113; PubMed Central PMCID: PMCPMC4694114.
18. Bennett PB, Guthrie HRE. Trends in ion channel drug discovery: advances in screening technologies. Trends Biotechnol. 2003;21(12):563-9. doi: 10.1016/j.tibtech.2003.09.014. PubMed PMID: WOS:000187014000011.
19. Yu HB, Li M, Wang WP, Wang XL. High throughput screening technologies for ion channels. Acta pharmacologica Sinica. 2016;37(1):34-43. doi: 10.1038/aps.2015.108. PubMed PMID: WOS:000367514700006.
20. Dunlop J, Bowlby M, Peri R, Tawa G, LaRocque J, Soloveva V, et al. Ion channel screening. Comb Chem High T Scr. 2008;11(7):514-22. doi: Doi 10.2174/138620708785204117. PubMed PMID: WOS:000259446200004.
21. Gando I, Williams N, Fishman GI, Sampson BA, Tan YY, Coetzee WA. Functional characterization of SCN10A variants in several cases of sudden unexplained death. Forensic Sci Int. 2019;301:289-98. doi: 10.1016/j.forsciint.2019.05.042. PubMed PMID: WOS:000473261300043.
22. He W, Young GT, Zhang BH, Cox PJ, Cho LTY, John S, et al. Functional confirmation that the R1488*variant in SCN9A results in complete loss-of-function of Na(v)1.7. Bmc Med Genet. 2018;19. doi: 10.1186/s12881-018-0643-4. PubMed PMID: WOS:000439504300001.
23. Parks TA, Bahia PK, Taylor-Clark TE. Functional evidence of distinct electrophile-induced activation states of the ion channel TRPA1. Biochem Biophys Rep. 2021;27:101044. doi: 10.1016/j.bbrep.2021.101044. PubMed PMID: 34189279; PubMed Central PMCID: PMCPMC8219651.
24. 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. doi: 10.1523/JNEUROSCI.2695-04.2004. PubMed PMID: 15385606; PubMed Central PMCID: PMCPMC6729696.
25. 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 : a journal of neurology. 2012;135(Pt 9):2613-28. doi: 10.1093/brain/aws187. PubMed PMID: 22826602.
26. Passmore GM. Dorsal root ganglion neurones in culture: a model system for identifying novel analgesic targets? J Pharmacol Toxicol Methods. 2005;51(3):201-8. doi: 10.1016/j.vascn.2004.08.007. PubMed PMID: 15862465.
27. Li Y, North RY, Rhines LD, Tatsui CE, Rao G, Edwards DD, et al. DRG Voltage-Gated Sodium Channel 1.7 Is Upregulated in Paclitaxel-Induced Neuropathy in Rats and in Humans with Neuropathic Pain. J Neurosci. 2018;38(5):1124-36. doi: 10.1523/JNEUROSCI.0899-17.2017. PubMed PMID: 29255002; PubMed Central PMCID: PMCPMC5792474.
28. Anand U, Yiangou Y, Akbar A, Quick T, MacQuillan A, Fox M, et al. Glucagon-like peptide 1 receptor (GLP-1R) expression by nerve fibres in inflammatory bowel disease and functional effects in cultured neurons. PloS one. 2018;13(5). doi: 10.1371/journal.pone.0198024. PubMed PMID: WOS:000433521800040.
29. Li Y, North RY, Rhines LD, Tatsui CE, Rao G, Edwards DD, et al. DRG Voltage-Gated Sodium Channel 1.7 Is Upregulated in Paclitaxel-Induced Neuropathy in Rats and in Humans with Neuropathic Pain. Journal of Neuroscience. 2018;38(5):1124-36. doi: 10.1523/Jneurosci.0899-17.2017. PubMed PMID: WOS:000423817800008.
30. Bellin M, Marchetto MC, Gage FH, Mummery CL. Induced pluripotent stem cells: the new patient? Nat Rev Mol Cell Biol. 2012;13(11):713-26. doi: 10.1038/nrm3448. PubMed PMID: 23034453.
31. Labau JIR, Andelic M, Faber CG, Waxman SG, Lauria G, Dib-Hajj SD. Recent advances for using human induced-pluripotent stem cells as pain-in-a-dish models of neuropathic pain. Exp Neurol. 2022;358:114223. doi: 10.1016/j.expneurol.2022.114223. PubMed PMID: 36100046.

32. Inoue H, Nagata N, Kurokawa H, Yamanaka S. iPS cells: a game changer for future medicine. EMBO J. 2014;33(5):409-17. doi: 10.1002/embj.201387098. PubMed PMID: 24500035; PubMed Central PMCID: PMCPMC3989624.
33. Meents JE, Bressan E, Sontag S, Foerster A, Hautvast P, Rosseler C, et al. The role of Nav1.7 in human nociceptors: insights from human induced pluripotent stem cell-derived sensory neurons of erythromelalgia patients. Pain. 2019;160(6):1327-41. doi: 10.1097/j.pain.0000000000001511. PubMed PMID: 30720580; PubMed Central PMCID: PMCPMC6554007.
34. Namer B, Schmidt D, Eberhardt E, Maroni M, Dorfmeister E, Kleggetveit IP, et al. Pain relief in a neuropathy patient by lacosamide: Proof of principle of clinical translation from patient-specific iPS cell-derived nociceptors. Ebiomedicine. 2019;39:401-8. doi: 10.1016/j.ebiom.2018.11.042. PubMed PMID: WOS:000456677400048.
35. McDermott LA, Weir GA, Themistocleous AC, Segerdahl AR, Blesneac I, Baskozos G, et al. Defining the Functional Role of NaV1.7 in Human Nociception. Neuron. 2019;101(5):905-19 e8. doi: 10.1016/j.neuron.2019.01.047. PubMed PMID: 30795902; PubMed Central PMCID: PMCPMC6424805.
36. 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. Epub 2018/11/22. doi: 10.1523/jneurosci.2433-18.2018. PubMed PMID: 30459225; PubMed Central PMCID: PMCPMC6335750.
37. 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 communications. 2021;3(3):fcab212. Epub 2021/09/25. doi: 10.1093/braincomms/fcab212. PubMed PMID: 34557669; PubMed Central PMCID: PMCPMC8454204.
38. Cao LS, McDonnell A, Nitzsche A, Alexandrou A, Saintot PP, Loucif AJC, et al. Pharmacological reversal of a pain phenotype in iPSC-derived sensory neurons and patients with inherited erythromelalgia. Science Translational Medicine. 2016;8(335). doi: 10.1126/scitranslmed.aad7653. PubMed PMID: WOS:000374412300005.
39. Lampert A, Bennett DL, McDermott LA, Neureiter A, Eberhardt E, Winner B, et al. Human sensory neurons derived from pluripotent stem cells for disease modelling and personalized medicine. Neurobiol Pain. 2020;8:100055. doi: 10.1016/j.ynpai.2020.100055. PubMed PMID: 33364527; PubMed Central PMCID: PMCPMC7750732.
40. Mertens J, Reid D, Lau S, Kim Y, Gage FH. Aging in a Dish: iPSC-Derived and Directly Induced Neurons for Studying Brain Aging and Age-Related Neurodegenerative Diseases. Annu Rev Genet. 2018;52:271-93. doi: 10.1146/annurev-genet-120417-031534. PubMed PMID: WOS:000453889500014.
41. Corsini NS, Knoblich JA. Human organoids: New strategies and methods for analyzing human development and disease. Cell. 2022;185(15):2756-69. doi: 10.1016/j.cell.2022.06.051. PubMed PMID: 35868278.
42. Xiao DC, Deng QQ, Guo YN, Huang XT, Zou M, Zhong JW, et al. Generation of self-organized sensory ganglion organoids and retinal ganglion cells from fibroblasts. Sci Adv. 2020;6(22). doi: 10.1126/sciadv.aaz5858. PubMed PMID: WOS:000537238200018.
43. Mazzara PG, Muggeo S, Luoni M, Massimino L, Zaghi M, Valverde PTT, et al. Frataxin gene editing rescues Friedreich's ataxia pathology in dorsal root ganglia organoid-derived sensory neurons. Nat Commun. 2020;11(1). doi: 10.1038/s41467-020-17954-3. PubMed PMID: WOS:000569766000004.
44. Heydari Z, Moeinvaziri F, Agarwal T, Pooyan P, Shpichka A, Maiti TK, et al. Organoids: a novel modality in disease modeling. Biodes Manuf. 2021;4(4):689-716. doi: 10.1007/s42242-021-00150-7. PubMed PMID: 34395032; PubMed Central PMCID: PMCPMC8349706.
45. Perkhofer L, Frappart PO, Muller M, Kleger A. Importance of organoids for personalized medicine. Pers Med. 2018;15(6):461-5. doi: 10.2217/pme-2018-0071. PubMed PMID: WOS:000451754700004.
46. Milinkeviciute G, Gentile C, Neely GG. Drosophila as a tool for studying the conserved genetics of pain. Clinical genetics. 2012;82(4):359-66. doi: 10.1111/j.1399-0004.2012.01941.x. PubMed PMID: WOS:000308646100007.

47. Yamaguchi M, Yoshida H. Drosophila as a Model Organism. Adv Exp Med Biol. 2018;1076:1-10. doi: 10.1007/978-981-13-0529-0_1. PubMed PMID: WOS:000453864300002.
48. Calvo M, Davies AJ, Hébert HL, Weir GA, Chesler EJ, Finnerup NB, et al. The Genetics of Neuropathic Pain from Model Organisms to Clinical Application. Neuron. 2019;104(4):637-53. Epub 2019/11/22. doi: 10.1016/j.neuron.2019.09.018. PubMed PMID: 31751545; PubMed Central PMCID: PMCPMC6868508.
49. Baenas N, Wagner AE. Drosophila melanogaster as an alternative model organism in nutrigenomics. Genes Nutr. 2019;14. doi: 10.1186/s12263-019-0641-y. PubMed PMID: WOS:000467082000001.
50. Pandey UB, Nichols CD. Human Disease Models in Drosophila melanogaster and the Role of the Fly in Therapeutic Drug Discovery. Pharmacological reviews. 2011;63(2):411-36. doi: 10.1124/pr.110.003293. PubMed PMID: WOS:000289885400007.
51. Ganetzky B. Genetic analysis of ion channel dysfunction in Drosophila. Kidney Int. 2000;57(3):766-71. doi: 10.1046/j.1523-1755.2000.00913.x. PubMed PMID: 10720927.
52. Cosens DJ, Manning A. Abnormal electroretinogram from a Drosophila mutant. Nature. 1969;224(5216):285-7. doi: 10.1038/224285a0. PubMed PMID: 5344615.
53. Montell C, Rubin GM. Molecular Characterization of the Drosophila Trp Locus - a Putative Integral Membrane-Protein Required for Phototransduction. Neuron. 1989;2(4):1313-23. doi: Doi 10.1016/0896-6273(89)90069-X. PubMed PMID: WOS:A1989U274400004.
54. Tracey WD, Wilson RI, Laurent G, Benzer S. painless, a Drosophila gene essential for nociception. Cell. 2003;113(2):261-73. doi: Doi 10.1016/S0092-8674(03)00272-1. PubMed PMID: WOS:000182508500014.
55. Babcock DT, Landry C, Galko MJ. Cytokine Signaling Mediates UV-Induced Nociceptive Sensitization in Drosophila Larvae. Current Biology. 2009;19(10):799-806. doi: 10.1016/j.cub.2009.03.062. PubMed PMID: WOS:000266469800019.
56. Babcock DT, Shi SP, Jo J, Shaw M, Gutstein HB, Galko MJ. Hedgehog Signaling Regulates Nociceptive Sensitization. Current Biology. 2011;21(18):1525-33. doi: 10.1016/j.cub.2011.08.020. PubMed PMID: WOS:000295423400026.
57. Khuong TM, Wang QP, Manion J, Oyston LJ, Lau MT, Towler H, et al. Nerve injury drives a heightened state of vigilance and neuropathic sensitization in Drosophila. Sci Adv. 2019;5(7). doi: 10.1126/sciadv.aaw4099. PubMed PMID: WOS:000478770400069.
58. Im SH, Patel AA, Cox DN, Galko MJ. Drosophila Insulin receptor regulates the persistence of injury-induced nociceptive sensitization. Dis Model Mech. 2018;11(5). doi: 10.1242/dmm.034231. PubMed PMID: WOS:000433908200008.
59. Nyberg KG, Carthew RW. CRISPR-/Cas9-Mediated Precise and Efficient Genome Editing in Drosophila. Methods Mol Biol. 2022;2540:135-56. doi: 10.1007/978-1-0716-2541-5_6. PubMed PMID: 35980576.
60. Brockmann A, Ulrich Ebbinghaus-Kintscher. Characterization of Drosophila melanogaster slo1 channel using mutagenesis and automated patch clamp measurements on syncropatch384 (nanion technologies). Biophysical Journal 2022;121(3):389a.
61. Costa FV, Rosa LV, Quadros VA, de Abreu MS, Santos ARS, Sneddon LU, et al. The Use of Zebrafish as a Non-traditional Model Organism in Translational Pain Research: The Knowns and the Unknowns. Current neuropharmacology. 2022;20(3):476-93. doi: 10.2174/1570159x19666210311104408. PubMed PMID: WOS:000811614400003.
62. Kolesnikova TO, Demin KA, Costa FV, Zabegalov KN, de Abreu MS, Gerasimova EV, et al. Towards Zebrafish Models of CNS Channelopathies. International Journal of Molecular Sciences. 2022;23(22). doi: 10.3390/ijms232213979. PubMed PMID: WOS:000887378400001.
63. Weuring WJ, Hoekman JW, Braun KPJ, Koeleman BPC. Genetic and Functional Differences between Duplicated Zebrafish Genes for Human SCN1A. Cells-Basel. 2022;11(3). doi: 10.3390/cells11030454. PubMed PMID: WOS:000760020900001.
64. Novak AE, Taylor AD, Pineda RH, Lasda EL, Wright MA, Ribera AB. Embryonic and larval expression of zebrafish voltage-gated sodium channel alpha-subunit genes. Dev Dyn. 2006;235(7):1962-73. doi: 10.1002/dvdy.20811. PubMed PMID: 16615064.

65. Eijkenboom I, Sopacua M, Otten ABC, Gerrits MM, Hoeijmakers JGJ, Waxman SG, et al. Expression of pathogenic SCN9A mutations in the zebrafish: A model to study small-fiber neuropathy. Exp Neurol. 2019;311:257-64. doi: 10.1016/j.expneurol.2018.10.008. PubMed PMID: WOS:000452937300023.
66. Taylor JC, Dewberry LS, Totsch SK, Yessick LR, DeBerry JJ, Watts SA, et al. A novel zebrafish-based model of nociception. Physiol Behav. 2017;174:83-8. doi: 10.1016/j.physbeh.2017.03.009. PubMed PMID: WOS:000401387800011.
67. Costa FV, Rosa LV, Quadros VA, Santos ARS, Kalueff AV, Rosemberg DB. Understanding nociception-related phenotypes in adult zebrafish: Behavioral and pharmacological characterization using a new acetic acid model. Behav Brain Res. 2019;359:570-8. doi: 10.1016/j.bbr.2018.10.009. PubMed PMID: WOS:000456222600065.
68. Magalhaes FEA, de Sousa CAPB, Santos SAAR, Menezes RB, Batista FLA, Abreu AO, et al. Adult Zebrafish (Danio rerio): An Alternative Behavioral Model of Formalin-Induced Nociception. Zebrafish. 2017;14(5):422-9. doi: 10.1089/zeb.2017.1436. PubMed PMID: WOS:000412338600004.
69. Waldron AL, Schroder PA, Bourgon KL, Bolduc JK, Miller JL, Pellegrini AD, et al. Oxidative stress-dependent MMP-13 activity underlies glucose neurotoxicity. J Diabetes Complicat. 2018;32(3):249-57. doi: 10.1016/j.jdiacomp.2017.11.012. PubMed PMID: WOS:000427810500002.
70. Wlodkowic D, Campana O. Toward High-Throughput Fish Embryo Toxicity Tests in Aquatic Toxicology. Environ Sci Technol. 2021;55(6):3505-13. doi: 10.1021/acs.est.0c07688. PubMed PMID: WOS:000636727200007.
71. Mogil JS. Animal models of pain: progress and challenges. Nature Reviews Neuroscience. 2009;10(4):283-94. doi: 10.1038/nrn2606. PubMed PMID: WOS:000264402500011.
72. Abboud C, Duveau A, Bouali-Benazzouz R, Masse K, Mattar J, Brochoire L, et al. Animal models of pain: Diversity and benefits. J Neurosci Meth. 2021;348. doi: 10.1016/j.jneumeth.2020.108997. PubMed PMID: WOS:000611826600003.
73. Jaggi AS, Jain V, Singh N. Animal models of neuropathic pain. Fund Clin Pharmacol. 2011;25(1):1-28. doi: 10.1111/j.1472-8206.2009.00801.x. PubMed PMID: WOS:000285970500001.
74. Chidiac C, Xue Y, Muniz Moreno MDM, Bakr Rasheed AA, Lorentz R, Birling MC, et al. The Human SCN10A(G1662S) Point Mutation Established in Mice Impacts on Mechanical, Heat, and Cool Sensitivity. Frontiers in pharmacology. 2021;12:780132. doi: 10.3389/fphar.2021.780132. PubMed PMID: 34925037; PubMed Central PMCID: PMCPMC8671994.
75. Xue Y, Kremer M, Muniz Moreno MDM, Chidiac C, Lorentz R, Birling MC, et al. The Human SCN9A (R185H) Point Mutation Induces Pain Hypersensitivity and Spontaneous Pain in Mice. Front Mol Neurosci. 2022;15:913990. doi: 10.3389/fnmol.2022.913990. PubMed PMID: 35769334; PubMed Central PMCID: PMCPMC9234669.
76. Scuteri D, Hamamura K, Watanabe C, Tonin P, Bagetta G, Corasaniti MT. Transgenic Mice for the Translational Study of Neuropathic Pain and Dystonia. Int J Mol Sci. 2022;23(15). doi: 10.3390/ijms23158580. PubMed PMID: 35955710; PubMed Central PMCID: PMCPMC9368787.
77. Sun L, Lutz BM, Tao YX. The CRISPR/Cas9 system for gene editing and its potential application in pain research. Transl Perioper Pain Med. 2016;1(3):22-33. PubMed PMID: 27500183; PubMed Central PMCID: PMCPMC4971521.

Impact paragraph

Pain is a complex sensory and emotional experience, in some cases lasting over a life-time with no improvement despite medication used [1]. Chronic pain affects approximately one fourth of adults and it is often associated with numerous complications, including anxiety and depression dramatically influencing patients' quality of life [1]. Moreover, it has huge economic and societal impact [2]. In 2020, the the health care costs of patients with painful neuropathic disorders in US were estimated at $17,355, which is 3-fold higher comparing to age-matched controls without this condition [2]. Neuropathic pain management includes non-pharmacological, pharmacological (mainly anticonvulsants and antidepressants), and interventional therapies focusing largely on symptomatic treatment [3]. This might be the reason of poor therapy outcome, resulting in a significant number of patients struggling with pain during their lifespan [4]. This creates an urgent need for novel treatments, targeting mechanistically the specific causes of pain in an individual patient. For example, different specific blockers have been used to treat dysfunctional VGSC channels as a pain source, but they can be applied only in patients carrying VGSC mutation, and they also have variable efficacy for instance between Nav1.7- mutations-carrying SFN individuals [5]. Therefore, it is of medical and societal importance to fully characterize the molecular and genetic mechanisms underlying NeP and understand the individual-to-individual differences.

Therefore, in **Chapter 3,** we investigated 15 ion channel genes for variants that could influence channel function and explain pain features. Screening of two patients group, painful- and painless-Diabetic Neuropathy (DN), resolved a number of candidate variants in novel genes. Moreover, our findings revealed that painful-DN patients with ICG variants had more pain than painful-DN without ICG variant, indicating these patients might benefit from ion channel targeted treatment. These results were confirmed in **Chapter 4**, where we have shown that patients with SFN and ion-channel gene variants reported more severe pain compared to patients with SFN and without ICG variants, expanding the relevance of this work for SFN patients. Most of the potentially causative variants have been localized in the TRP genes, remaining promising group of therapeutic targets especially in the light of developing TRP channel pharmacology [6].

Although VSCG are known to be associated with NeP and screening of ICG extended the list of potential gene candidates, there is still a large number of individuals with unresolved pain pathogenicity. Therefore, we screened 592 pain-related genes in SFN patients, not only including ion channels, but involved in a

broad variety of pain-associated processes. In **Chapter 5** we identified pathogenic and likely pathogenic variants and VUS in multiple genes; n=17, 42.5% variants in genes involved in neurotransmission, n=16, 40% in ion channel genes, n=6, 15% in metabolism related genes and n=1, 2.5% variant in gene involved in immune response. This work provided new insight into pain-related genetic markers and pathways associated with NeP and revealed novel gene candidates for further investigation.

Undoubtedly, NGS is a valuable tool in pain diagnostics and research, providing large numbers of potentially relevant variant, however the NGS outcome cannot be validated by bioinformatics only, and should be confirmed in system mimicking physiological conditions, especially in the case of VUS and novel genes. Unfortunately, models for functional read out are limited, costly and highly laborious, requiring novel systems with a higher throughput at lower cost. In **Chapter 6** we explored a novel cell model to functionally test ANO3 variants. ANO3 protein expression was analysed in several cell lines, as creating a model overexpressing wild-type and mutant *ANO3* gene would bring new information about gene function and its role in pain pathophysiology.

In conclusion, this work provides more knowledge about genetic landscape of neuropathic pain, highlighting important role of ion channels and novel pain related genes identified in this study. Findings presented in this thesis may contribute in a future perspective to improve diagnostics of NeP, disease prognosis and development of new therapeutic interventions and more effective pain-targeting personalized treatment.

References

1. Yong RJ, Mullins PM, Bhattacharyya N. Prevalence of chronic pain among adults in the United States. Pain. 2022;163(2):E328-E32. doi: 10.1097/j.pain.0000000000002291. PubMed PMID: WOS:000742403300026.
2. Berger A, Dukes EM, Oster G. Clinical characteristics and economic costs of patients with painful neuropathic disorders. J Pain. 2004;5(3):143-9. doi: 10.1016/j.jpain.2003.12.004. PubMed PMID: WOS:000221363600001.
3. Gilron I, Baron R, Jensen T. Neuropathic Pain: Principles of Diagnosis and Treatment. Mayo Clin Proc. 2015;90(4):532-45. doi: 10.1016/j.mayocp.2015.01.018. PubMed PMID: WOS:000352197900018.
4. Blackburn-Munro G, Blackburn-Munro RE. Chronic pain, chronic stress and depression: Coincidence or consequence? J Neuroendocrinol. 2001;13(12):1009-23. doi: DOI 10.1046/j.0007-1331.2001.00727.x. PubMed PMID: WOS:000172422700001.
5. de Greef BTA, Hoeijmakers JGJ, Geerts M, Oakes M, Church TJE, Waxman SG, et al. Lacosamide in patients with Nav1.7 mutations-related small fibre neuropathy: a randomized controlled trial. Brain : a journal of neurology. 2019;142(2):263-75. doi: 10.1093/brain/awy329. PubMed PMID: 30649227.
6. Koivisto AP, Belvisi MG, Gaudet R, Szallasi A. Advances in TRP channel drug discovery: from target validation to clinical studies. Nat Rev Drug Discov. 2022;21(1):41-59. doi: 10.1038/s41573-021-00268-4. PubMed PMID: WOS:000696168700001.

Summary

Neuropathic pain (NeP) is a complex condition usually resulting from damage or disease of the central and/or peripheral nervous system. It is a common manifestation in several disorders altering the somatosensory nervous system, including Small Fiber Neuropathy (SFN) and peripheral diabetic neuropathy (DN), affecting up to 10% of general population. NeP is associated with multiple comorbidities and especially its psychiatric and emotional component negatively influences patients' quality of life and well-being, often leading to anxiety and depression. Current pain management is based on symptomatic treatment and many of the patients cannot achieve pain relief with available analgesics. This creates a huge need for novel, targeted therapies for better clinical outcome, however lack of knowledge about underlying mechanism and pathophysiology impedes effective drug development. It is also clear that genetic predisposition is playing a role and multiple genes have been shown to be associated or causally involved with pain. Voltage gated sodium ion channels (VGSC) are the best studied genes with a clearly documented role in pain pathophysiology, however only part of the patients have dysfunctional VGSC due to a pathogenic genetic variant. For the majority of NeP patients the underlying genetic cause is not known. Therefore, the work presented in this thesis aimed to identify novel genetic variants and potential NeP related genes via NGS of patient cohorts and to establish the functional role of possible pathogenic variants in novel genes.

Chapter 1 provides a general introduction of neuropathic pain. This chapter reviews different aspects of NeP, including clinical manifestations, pathophysiology, causes, diagnostics and treatment strategies. Moreover, two phenotypes associated with neuropathic pain; SFN and DN are being discussed in more detail. The second part of the chapter focusses on the genetic background of NeP and elaborates on the involvement of VGSCs in pain etiology. It also covers the detection of novel disease genes using NGS technology and the challenges of functionally testing Variants of Unknown Significance (VUS) role. The chapter ends with the aims and an outline of the thesis.

In the past years, a role for ion channel genes in painful neuropathy has become evident, making them important novel therapeutic targets. **Chapter 2** elaborates about current state of knowledge on the involvement of ICG. Ion channels expressed in dorsal root ganglion neurons are potentially analgesic pharmacological targets in sensory signalling (e.g., *TRPV1*), regulation of neuronal excitation (potassium

channels), action potential transmission (sodium channels), generation of involuntary action potentials (HCN channels), mediating thermal pain (Anoctamin), pH modulation (ASIC channels), and neurotransmitter release (calcium channels). Therefore, understanding the role of variants in ion channel genes in neuropathic pain is important to personalize this broad range of potential molecular pain-control targets. The most investigated channels in relation to pain are voltage-gated sodium ion channels, however, they are involved in less than 20% of the patients with neuropathic pain. In this chapter, we focus on other ion channel genes and their involvement in painful neuropathies. The calcium auxiliary subunits and TRP channel genes, particularly *TRPV1*, have been thoroughly investigated as cause of and therapeutic target for pain. However, pain alleviation has not been achieved, probably due the heterogeneous genetic background in these patients. Studies on the genetic factors involving these ion channels may improve our knowledge of the common molecular mediators of pain and neuropathy, leading to personalized therapeutic approaches for painful neuropathies and paving the way toward new targets to be investigated.

The possible implication of 15 ion channel genes (ICG) and related genetic variants potentially affecting channel function have been investigated in **Chapter 3.** We performed molecular inversion probes-next generation (MIP-NGS) sequencing of 5 transient receptor potential cation channels, 8 potassium channels and 2 calcium-activated chloride channel genes in 222 painful- and 304 painless-DN patients, showing 12 painful-DN (5.4%) patients showed potentially pathogenic variants (five nonsense/frameshift, seven missense, one out-of-frame deletion) in *ANO3* (n = 3), *HCN1* (n = 1), *KCNK18* (n = 2), *TRPA1* (n = 3), *TRPM8* (n = 3) and *TRPV4* (n = 1) and 14 painless-DN patients (4.6%-three nonsense/frameshift, nine missense, one out-of-frame deletion) in *ANO1* (n = 1), *KCNK18* (n = 3), *KCNQ3* (n = 1), *TRPA1* (n = 2), *TRPM8* (n = 1), *TRPV1* (n = 3) and *TRPV4* (n = 3). We observed that painful-DN patients with ion channel gene variants reported higher maximal pain and painful-DN patients with TRP variants had severe pain combined with abnormal thermal thresholds. In **Chapter 4**, we used the same gene panel to perform MIP-NGS of 414 SFN patients that did not have VSCG variant. The sequencing resulted in identification of potentially pathogenic heterozygous variants in twenty patients (4.8%) in *ANO3* (n = 2), *KCNK18* (n=2), *KCNQ3* (n=2), *TRPA1* (n=7), *TRPM8* (n=3), *TRPV1* (n=3) and *TRPV3* (n=2). Again, patients with ion-channel gene variants, including individuals with TRP variants, reported more severe pain compared to patients without such variants. Our results described in **Chapter 3** and

Chapter 4 strongly suggest extensive ICG variants implication in neuropathic pain, however these findings require functional validation.

To identify novel pain genes and specific genetic variants (**Chapter 5**), we created panel of 592 gene candidates, selected from the literature because of their association with pain, genes from genetic pain databases and Human Phenotype Ontology database and Online Mendelian Inheritance in Man (OMIM) based on association with (painful) peripheral neuropathy symptoms. We identified 4 different heterozygous pathogenic variants (c.684C>G, c.2159T>A, c.2567G>A and a combination of c.2794A>C, c.2971G>T and c.5756A>G) in *SCN9A*, and 3 different heterozygous likely pathogenic variants in 3 genes; *AR* (c.1792A>G), *MFN2* (c.1384T>C) and *SCN11A* (c.1744G>A). In total, 40 different variants of uncertain clinical significance (VUS) in 37 genes; n=17, 42.5% variants in genes involved in neurotransmission, n=16, 40% in ion channel genes, n=6, 15% in metabolism related genes and n=1, 2.5% variant in gene involved in immune response. As variant classification is largely based on pathogenicity prediction tools and literature data, the experimental studies of these variants is of great interest.

Using next-generation sequencing, we identified possible pathogenic variants [VUS; c.638C>T, p.(S213F) and c.1357A>G, p.(I453V)] in the Anoctamin 3 (ANO3) gene in NeP patients. To get further insight in the possible role of these VUS in pain pathophysiology, we worked on an experimental model and functional read outs for these ANO3 VUS (**Chapter 6**). First, using Site Directed Mutagenesis we generated mutated ANO3 in an expression vector. In order to functionally test these variants, we selected HEK293 and HT-29 cell lines, in which according to literature and databases ANO3 was not expressed. Surprisingly, Western blot did not show significant protein overexpression, but unexpectedly also the untransfected cells displayed ANO3 protein. Therefore, we tested a number of cell lines for ANO3 expression and muscle stem cells, called mesoangioblasts (MABs) were the only cell line not expressing ANO3 protein at all. The MABs were transfected, but we did not obtain sufficient material for WB to confirm protein expression, probably due to a poor transfection efficiency. As ANO3 belongs to Ca^{2+}-activated Cl- channels, we have been developing a functional assay based on calcium measurement. In conclusion, awaiting the results of the functional studies, we cannot draw a definite conclusion on the role of these ANO3 variants in NeP, but everything is now in place to be able to perform the required experiments.

Chapter 7 discusses the key findings of this thesis and elaborates on the challenges of functional validation of VUS detected during NGS. Several *in vitro* and *in vivo* models are being described including their advantages and limitations in terms of VUS testing. **Chapter 8** provides overview of the research chapters together with scientific and social impact of this work.

Acknowledgements

My PhD was an exciting journey that would not be that nice and successful without a support of great people that I had around. Therefore, I would like to acknowledge my colleagues, friends and family.

At the first place, I would like to thank my supervision team; **Prof. Bert Smeets, Prof. Karin Faber** and **Dr. Monique Gerrits**. **Bert,** thank you for your guidance and support provided during my PhD. You shared your scientific knowledge and guided me during my research in the way that triggers creativity without giving 'ready solutions'. Your support helped me to better find myself in scientific world and to finalize this thesis. Thank you for patience, especially at the writing stage. **Karin**, thank you for trust and for possibility to be a part of **Pain-Net project**. That was very fruitful period that stimulated my scientific and personal development. I am impressed, that you could manage so many things at once, still having time to help me, when needed. **Monique,** I am grateful for your invaluable input in this thesis. You introduced me to the world of NGS and variant interpretation. Thank you for your generous help.

I would like to acknowledge all the members of my assessment committee: **Prof. dr. J.H.M. van Zundert, Prof. dr. D.L. Bennett, Prof. dr. E-J. Speel, Prof. dr. K. Talavera Pérez** and **Prof. dr. M.P. Weijenberg.** Thank you for assessing my thesis.

Herein, I thank all the members of the **Pain-Net project** for their contributions and successful collaborations. Special thanks go to **Prof. Giuseppe Lauria, Prof. Stephen Waxman** and **Dr. Sulayman Dib-Hajj** for their invaluable input in development of this project and my research. I also thank **Prof. Augusto Caraceni** and **Prof. Paolo Garagnani** for hosting me and mentoring during my secondments. It was a pleasure to work with colleagues from IRCCS Ospedale San Raffaele, therefore I thank **Prof. Federica Esposito** and **Dr. Silvia Santoro.** I am grateful for **Dr. Massimo D'Amico** and **Farhad** for great support with stable cell line generation and patch clamp experiments. I thank **Prof. J.W.M. Heemskerk** and **Delia** for their kind help with setting up the FlexStation 3 Multi-Mode Microplate Reader experiments.

I would like to thank **Rossella** for guidance in practical work, related to chapter 6 of my thesis and **Rowida** for help with preparation of NGS chapters. I am very grateful for **Florence** and **Mike** for all precious suggestions that helped me with lab work. I also thank **Janneke, Bianca** and **Maurice** for help and assistance provided along my PhD course.

A special word goes to my paranymphs, my dear friends **Somaieh** and **Toni** that shared with me a bright and dark moments of my PhD journey. Both of you, are such a warm and caring personalities. **Somaieh,** you are very strong woman and most likely the kindest person that I have ever meet. **Toni,** I love your optimism and positive life attitude. Thank you, guys, for endless chats about life, for sharing disappointments and hopes, and hectoliters of coffee drunk together, that made me a coffee addict.

Ivo, Leo and **Ruby**, it was nice to be your officemate. Thank you for a great atmosphere and all the fun that we had together. **Ivo**, I still have dreams (nightmares?) about you singing in the office. **Leo**, your calmness is as a gold, I should learn from you. **Ruby,** thank you for friendly and inspiring talks. I guess that you might be responsible for my coffee addiction too.

I thank my colleagues from Pain-Net for being part not only of the research, but also for sharing all the great moments. **Julie**, our trip to Rome was amazing. We were JMMM quartet (Julie-**Mirna**-Milena-Morena) in Paris, having so much fun. **Morena,** thank you for making my stay in Milan nice and memorable. Thank you for the trips around Milano and all the Russian drinks drunk together at Navigli. **Kaalindi,** it was a pleasure to work with you, extending the friendly atmosphere outside the office hours.

Lynne, thank you for being kind, quiet and respectful roommate at my last stage of my PhD. This definitely helped me to finalize the writing process. I would like to thank all the colleagues from UM for friendly talks and nice work environment. **Nikolaj, Jelmar, Marta, Nikki, Julia, Daniela**, **Nhan, Marcha, Brian, Saad, Juan, Wen, Yueqin, Kaidi, Na, Amir, Li-Yen** thank you all!

It is a pleasure to thank **Erika, Iris, Rick, Marcel** and **Evelien** for supporting my work by their help and advice. I am extending my gratitude to **Sean, Danyel** and **Patrick**, who rescued me many times, when my computer decided to "go on strike". I thank **Christa**, **Margot** and **Diana** for assistance in arranging all formalities during my PhD.

I cannot forget about the friendships started outside the university. I thank **Gijs**, **Ghislaine, Monika, Jules, Enzo, Sofiya** for all the fun activities. With you I have never been bored.

Coming to the end, I would like to thank all the colleagues not mentioned by name here, for creating a nice work environment during the past years. I will switch to polish to acknowledge my friends and family.

Podziękowania kieruję do moich kolegów z UMEDu, a w szczególności do **Łukasza**, którego wsparcie pozwoliło mi rozwinąć skrzydła i rozpocząć doktorat w Maastricht. Dziękuję za bezinteresowną pomoc, bez której nie byłoby mnie tutaj. Dziękuję, również **Wioli** za niezliczoną ilość godzin, które spędziłyśmy razem w pracy i poza nią. Dzięki Tobie mój pobyt w Łodzi nabrał jasnych barw. Dzięki za wszystko! Dziękuję **Emi** i **Sielwanowi,** za niekończącą się gościnność i cierpliwość do mnie i mojego męża ;)

Niniejszą pracę doktorską dedykuję moim rodzicom. **Mamo, Tato**, jesteście wspaniałymi ludźmi. Dziękuję za trud włożony w moje wychowanie i niekończącą się pomoc. Zawsze powtarzaliście, że nauka jest najważniejsza i staraliście się stworzyć jak najlepsze warunki dla mojego rozwoju, dlatego ta praca jest również Waszą zasługą.

Dziękuję mojej siostrze **Ewelinie**. To zaskakujące, że można tak dobrze się rozumieć. Dla nas nie ma rzeczy niemożliwych! Tylko Ty byłaś w stanie spakować moje rzeczy podczas videorozmowy i wysłać je ciężarówką do Holandii, gdy okazało się, że jedna walizka, którą zabrałam to zdecydowanie za mało. Dziękuję za zrozumienie, chwile pełne radości i ratowanie z opresji w wielu kryzysowych sytuacjach.

Oczywiście nie może zabraknąć specjalnego podziękowania dla mojego męża, mojej bratniej duszy. **Marcinie**, dziękuję, że zawsze we mnie wierzyłeś i motywowałeś mnie do działania, nawet wtedy, gdy brakowało mi sił i wiary w siebie. Razem możemy wszystko, a bez nas nie byłoby nic. Dziękuję za rzeczy małe i wielkie. Za wspaniałe podróże, pełne przygód. To zaskakujące z jakim urokiem wpędzasz nas w kłopoty, ale tak przecież tworzą się najlepsze wspomnienia. To wielkie szczęście mieć takiego człowieka przy boku.

Curriculum Vitae

Milena Ślęczkowska (Molasy) was born on the 1st of May of 1990 in Końskie, Poland. In 2009, she started her Bachelor in Biotechnology at the University of Silesia in Poland. As a part of the programme, she did an internship at University of Southampton in UK, where she investigated formation and stability of DNA triple helices.

She finished her BSc degree in 2012 and followed her Master programme in Biotechnology at the University of Silesia. In 2013 she was awarded with the British Society of Plant Pathology grant for talented students, that covered research project performed at University of Oxford. As a visiting student, she investigated *SHM6* gene role in plant defense against *Pseudomonas syringae*. She graduated with MSc in 2014. During her master project, she studied cadmium-induced changes on activity of photosystem II.

After graduation, she worked as research assistant at the Department of Clinical Chemistry and Biochemistry, Medical University of Lodz. Her work was focused on microRNA influence on matrix metalloproteinases in pathogenesis of open angle glaucoma. In 2015, she was awarded the grant for outstanding young scientists.

In October 2017, she started her PhD at University of Maastricht within Horizon 2020 - Pain-Net Project. Part of the research she performed at the National Cancer Institute of Milan and University of Bologna. Under supervision of Prof. Bert Smeets, Prof. Karin Faber and Dr. Monique Gerrits she evaluated genes and genetic variants implicated in neuropathic pain using next-generation sequencing and functional studies. The most important results are presented in this dissertation.

List of publications

From this thesis:

Ślęczkowska M, Almomani R, Marchi M, Salvi E, et. al. Peripheral Ion Channel Genes Screening in Painful Small Fiber Neuropathy. Int J Mol Sci. 2022 Nov 15;23(22):14095. doi: 10.3390/ijms232214095.

Ślęczkowska M, Almomani R, Marchi M, de Greef BTA, et. al. Peripheral Ion Channel Gene Screening in Painful- and Painless-Diabetic Neuropathy. Int J Mol Sci. 2022 Jun 28;23(13):7190. doi: 10.3390/ijms23137190.

Marchi M, Salvi E, Andelic M, Mehmeti E, D'Amato I, Cazzato D, Chiappori F, Lombardi R, Cartelli D, Devigili G, Bella ED, Gerrits M, Almomani R, Malik RA, **Ślęczkowska M**, et. al. TRPA1 gene rare variants in chronic neuropathic and nociplastic pain patients. PAIN. 2023 Apr 19. doi: 10.1097/j.pain.0000000000002905.

Almomani R, Sopacua M, Marchi M, **Ślęczkowska M**, Lindsey P, et. al. Genetic profiling of sodium channels in painful and painless diabetic and idiopathic small fiber neuropathy. Int. J. Mol. Sci. 2023, 24(9), 8278. doi: 10.3390/ijms24098278

In preparation:

Ślęczkowska M, Misra K, Santoro S, Gerrits MM, Hoeijmakers JGJ. The emerging role of ion channel genes in painful neuropathies.

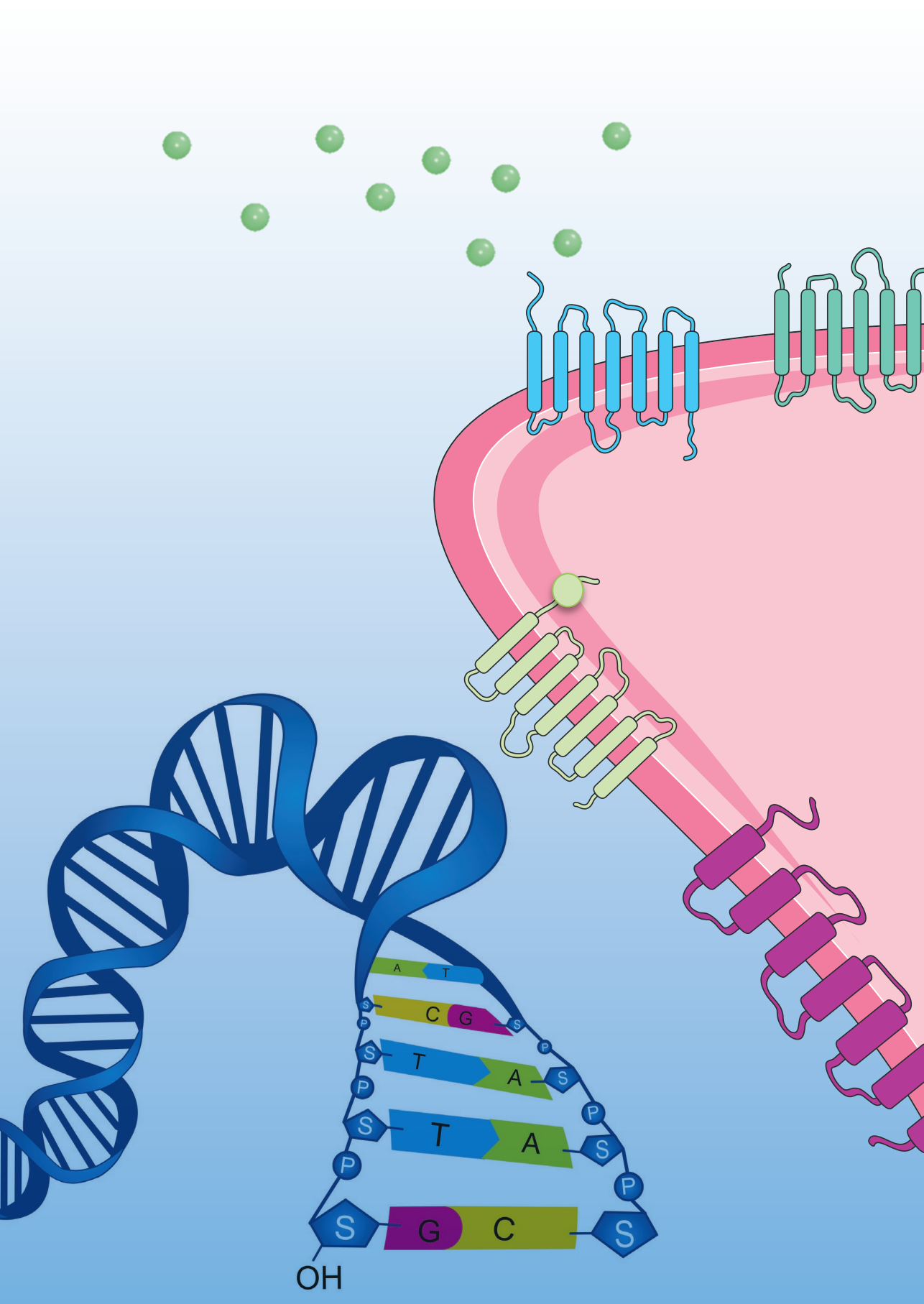
Ślęczkowska M, Misra K, Santoro S, Esposito F, et al. Novel and rare variants identified by screening 592 pain-related candidate genes in Small Fiber Neuropathy patients.

Ślęczkowska M, Gerrits MM, Avagliano Trezza R, Jahanfar F, et. al. Exploring the Role of Anoctamin 3 in painful Diabetic Neuropathy and Small Fiber Neuropathy.

Previous publications:

Molasy M, Walczak A, Przybylowska-Sygut K, Zaleska-Za A, et. al. Association of polymorphic variants of RAN and GEMIN3 genes with clinical parameters and the Primary Open-Angle Glaucoma risk. Ophthalmic Genetics 2018 Apr;39(2):180-188. doi: 10.1080/13816810.2017.1381978.

Molasy M, Walczak A, Szaflik J, Szaflik JP, Majsterek I. MicroRNAs in glaucoma and neurodegenerative diseases. Journal of Human Genetics 2017;62,105-112; doi: 10.1038/jhg.2016.91.


A
T
C
G
T
A
T
A
G
C
OH