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TRPA1 rare variants in chronic neuropathic and nociplastic pain patients

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Abstract

Missing aspects of the heritability of chronic neuropathic pain, as a complex adult-onset trait, may be hidden within rare variants with low effect on disease risk, unlikely to be resolved by a single-variant approach. To identify new risk genes, we performed a next-generation sequencing of 107 pain genes and collapsed the rare variants through gene-wise aggregation analysis. The optimal unified sequence kernel association test was applied to 169 patients with painful neuropathy, 223 patients with nociplastic pain (82 diagnosed with chronic widespread pain and 141 with fibromyalgia), and 216 healthy controls. Frequency and features of variants in *TRPA1*, which was the most significant gene, were further validated in 2 independent cohorts of 140 patients with chronic pain (90 with painful neuropathy and 50 with chronic widespread pain) and 34 with painless neuropathy. The effect of aminoacidic changes were modeled in silico according to physicochemical characteristics. *TRPA1* was significantly enriched of rare variants which significantly discriminated chronic pain patients from healthy controls after Bonferroni correction ($P = 6.7 \times 10^{-4}$, $\rho = 1$), giving a risk of 4.8-fold higher based on the simple burden test ($P = 0.0015$, OR = 4.8). Among the 32 patients harboring *TRPA1* variants, 24 (75%) were diagnosed with nociplastic pain, either fibromyalgia (12; 37.5%) or chronic widespread pain (12; 37.5%), whereas 8 (25%) with painful neuropathy. Irrespective of the clinical diagnosis, 12 patients (38%) complained of itch and 10 (31.3%) of cold-induced or cold-accentuated pain, mostly episodic. Our study widens the spectrum of channelopathy-related chronic pain disorders and contributes to bridging the gap between phenotype and targeted therapies based on patients' molecular profile.

Keywords: TRPA1, Neuropathic pain, Painful neuropathy, Fibromyalgia, Chronic widespread pain, Nociplastic pain

1. Introduction

Chronic pain, one of the most common noncommunicable disorders, is a distinct clinical entity caused by a large variety of underlying etiologies and influenced by multiple factors, which the biopsychosocial model recapitulates.[11] It was formerly classified as either *neuropathic* or *nociceptive*, which excluded patients without obvious activation of nociceptors or a proven lesion or disease of the somatosensory nervous system. The International Association for the Study of Pain has recently introduced a third new descriptor called *nociplastic* pain to classify patients complaining of pain for at least 3 months, with regional rather than discrete distribution, that is not adequately explained by nociceptive or neuropathic mechanisms, and showing clinical signs of hypersensitivity (ie, evoked pain hypersensitivity phenomena such as static or dynamic mechanical allodynia, heat or cold allodynia, or painful after sensations after any of the

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mentioned evoked pain hypersensitivity assessments) that are at least present in the region of pain.[40] Nociplastic pain shares different syndrome categories, which include chronic widespread pain and fibromyalgia, 2 overlapping conditions estimated to affect up to 11% of the general population.[27] Fibromyalgia, which is diagnosed based on established criteria,[3] typically associates with other somatic, mood, cognitive symptoms, and rheumatic symptoms and has been reported to include small fiber pathology in some patients, although this association is unclear.[43]

These different pain syndromes can have overlapping features, many of which are subjective, resulting in a further fragmentation of small and nonhomogeneous phenotypic classes. In recent years, the discovery of variants in voltage-gated sodium channel (VGSC) genes, whose pathogenicity was demonstrated by cell electrophysiological studies,[25,26,33] allowed expansion of the spectrum of pain-related channelopathies from rare Mendelian, early-onset conditions, such as congenital insensitivity to pain (CIP), inherited erythromelalgia (IE), and paroxysmal extreme pain disorder (PEPD), to more common disorders such small fiber neuropathy (SFN).[66] However, VGSC gene variants do not necessarily in themselves cause adult-onset painful neuropathies but rather appear to require additional insults to produce axonal injury[24,46,56,59] and, within the context of this multihit model, have been characterized as "risk factors."[6,19]

Moreover, the missing heritability for complex adult-onset traits, such as chronic pain, is unlikely to be resolved by single-variant analysis approach and may be hidden within rare variants that have low-to-moderate effect on disease risk.[51] To address this issue, the best alternative is collapsing sets of qualifying rare variants within genes through gene-wise aggregation analyses[45] and comparing the allele frequency between patients and healthy individuals to enable reliable interpretation of the findings.

Our gene-wise aggregation analysis identified *TRPA1* as the gene with the most robust differential enrichment of rare and potentially disruptive variants both in patients with widespread chronic pain or fibromyalgia and with painful neuropathy, with cold pain and itch as predominant symptoms. These findings, overcoming the limitations of a single variant approach, suggest a basis for targeted therapeutic strategies in a selected subgroup of patients with neuropathic and nociplastic pain.

2. Materials and methods

2.1. Study population

Three-hundred ninety-two patients were recruited at the Fondazione IRCCS Istituto Neurologico "Carlo Besta" of Milan (FINCB) and the Applied Neurophysiology and Pain Unit of the University Hospital of Bari, Italy, between 2016 and 2022. Phenotypic classification was performed according to the diagnostic criteria for neuropathic pain,[61] widespread chronic pain,[53] and fibromyalgia.[3,32] In particular, neuropathic pain was diagnosed when there was evidence of a lesion or disease of the somatosensory nervous system,[61] widespread chronic pain in the presence of diffuse musculoskeletal pain in at least 4 of 5 regions of the body persisting for at least 3 months and not directly attributable to a nociceptive process,[53] and fibromyalgia in the presence of multisite pain defined as 6 or more pain sites from a total of 9 possible sites associated with sleep problems or fatigue for at least 3 months.[3,32]

Two-hundred sixteen healthy controls with no neurological and pain symptoms were collected among blood donors in the HYPERGENES project (94.9%)[60] and the in-house repository (5.1%). Furthermore, 90 chronic painful neuropathy, 50 patients with chronic widespread pain, and 34 painless neuropathy patients, recruited between 2006 and 2019, were used to validate the incidence of single variants. All subjects were unrelated and of Anglo-American ancestry. The study was approved by the local ethic committee, and all subjects gave their written informed consent.

2.2. Neurological evaluation

All patients underwent clinical examination, diagnostic screening for common and rare causes of acquired neuropathy,[22] nerve conduction study, and 3 mm punch skin biopsy at the distal site of the leg for intraepidermal nerve fiber density quantification based on the published protocol and normative reference values.[42,44] Detailed description of "positive" (allodynia and hyperalgesia) and "negative" (reduced sensation) symptoms and signs, chronic pain duration, and factors triggering or exacerbating their pain (eg, cold, warm, physical activity, etc.) were recorded following previously published protocol.[17]

The Douleur Neuropathique 4 questions[8] and the 9-item adjusted PainDetect[28] were used to screen those individuals with higher probability of having neuropathic pain. The 11-point Likert Pain Intensity Numeric Rating Scale (PI-NRS) was used to score their mean intensity of pain experienced over the 2 previous weeks at 3 time points (morning, afternoon, and evening) and the mean pain intensity of the whole day. Patients reporting PI-NRS $>$ 0 for more than 3 months were defined as having chronic pain.

Response to first-line (ie, tricyclic antidepressants, pregabalin, gabapentin, duloxetine, and venlafaxine) and second-line drugs (ie, tramadol, oxycodone, carbamazepine or oxcarbazepine, and lacosamide) was defined as $>$50% pain reduction on the PI-NRS at 3 months.[12]

2.3. Next-generation sequencing

All subjects underwent blood withdrawal after providing specific informed consent for genetic analyses. Genomic DNA was extracted from whole blood. Samples used for the aggregated analysis (392 cases and 216 healthy controls) were all tested by Illumina Nextera Flex for Enrichment (NFFE-NGS, now named Illumina DNA prep with enrichment). We constructed a targeted enrichment kit to capture the coding and 20 bp flanking intron sequences of 107 pain-related genes. The genetic screening included 5 VGSC genes expressed in dorsal root ganglion neurons and other 102 candidate genes known to be involved in neuropathic pain; their first order interacting partners, ion channel, and receptor genes expressed in dorsal root ganglion; and genes selected by comparative and integrative genomics during the PROPANE project (European Union seventh Framework Program FP7/2007-2013: PROPANE study, grant agreement number: 602273). All 107 genes were grouped by functional categories (Supplementary Table 1, available at http://links.lww.com/PAIN/B804).

2.4. Data preprocessing, mapping, and variant calling

A first quality control step was performed with FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) to identify base quality drops across cycles and adapter contamination and to evaluate overall data quality. We performed both adapter trimming and quality trimming using Trimmomatic (version 0.36).[7] High-quality reads were mapped to the hg19 reference genome using bwa v. 0.7.17-r1188 (mem algorithm).[47] Hence, we

performed duplicated read marking, local realignment, and base quality score recalibration as suggested by GATK best practices.[16] We performed single-nucleotide variant and insertion or deletion calling using the GATK module Haplotype Caller (version 4.1.9) over the target region. For the variant annotation, we used the SnpEff software[10] that annotates and predicts the effects of genetic variants on genes and proteins. Variants that did not pass the variant filtering (total reads count ≤20, alternative allele depth ≤10, and allele balance of 25%) were removed. Visual inspection of bam files was performed using IGV software.[65]

2.5. Gene-wise aggregation analysis

Gene-wise aggregation analysis evaluates the cumulative effects of multiple rare variants in a gene to test whether there was a higher excess of variants in patients with chronic pain compared with healthy controls. All nonsynonymous variants, reaching at least 80% of call rate, with minor allele frequency < 0.01 in cases and controls combined for each phenotype, were collapsed into a single gene. We selected only rare variants, with impact on the protein product, ie, small insertion or deletion, missense, nonsense, and splicing variants. Only genes with at least 2 variants that passed the filters were included, whereas genes mapped by only one rare nonsynonymous variant were not considered. The combined burden and variance-component test, the optimal unified sequence kernel association test (SKAT-O), was applied. The SKAT-O test combines a standard collapsing burden test with the sequence kernel-based variance component test, encompassing all the possible underlying biological models. The burden test maximizes power, assuming that all rare variants collapsed in a specific gene are causal and affect the phenotype in the same direction, whereas SKAT is more robust when a large proportion of the variants are noncausal or if causal variants have effects on different directions. The unified test (ie, SKAT-O) enables maintaining the power in both the scenarios.

Rho statistic indicates the direction of the effects, with $\rho = 1$ referring to high percentage of causality in the same direction and $\rho = 0$ to the simultaneous presence of causal and noncausal variants with opposing directions. Analysis was performed per the entire cohort and per phenotype groups, which included painful neuropathy, chronic widespread pain, and fibromyalgia. Phenotypes were not additionally partitioned in smaller groups to not lose power. However, the clinical features of each patient carrying rare variants were recorded. All the analyses were corrected for sex and age as covariates and performed using Efficient and Parallelizable Association Container Toolbox software. The Bonferroni test was applied for multiple-comparison correction.

2.6. Best candidate gene variants analysis and validation

Single genetic variants identified in the burdened gene were annotated according to the Human Genome Variation Society (http://www.hgvs.org) and classified according to current American College of Medical Genetics and Genomics (ACMG) or Association for Molecular Pathology guidelines.[58] Variant analysis was supported by Alamut Visual Plus (SophiaGenetics, Saint Sulpice, Switzerland and Boston, MA, USA) for annotations on splicing prediction, amino acid physical–chemical differences, and conservation among species. For a descriptive analysis of the potential variant pathogenicity in VGSC genes, we referred to Waxman recommendation to assign the classes "possibly pathogenic," "probably pathogenic," or "pathogenic."[66]

The incidence of rare variants in the candidate genes was further tested on a cohort of 90 patients with chronic neuropathic pain and in 34 patients diagnosed with painless neuropathy previously sequenced. Type and localization of the variants were compared among the cohorts investigating any phenotype–genotype correlation.

2.7. Data availability

The genetic variation data of all the individual participating in the study are publicly available at: https://zenodo.org/deposit/6913835.

3. Results

3.1. Clinical features

A total of 392 patients with chronic pain (ie, PI-NRS > 0) were included. Their age ranged between 14 and 87 years (mean 52.4 $\pm$ 14.2), and 260 (66%) were women. Patients were classified into the following groups: (1) length-dependent painful neuropathy (n = 169) diagnosed on the presence of plausibly distributed symptoms, clinical signs, Douleur Neuropathique 4 questions score >4, PainDETECT score >15.2, reduced age and sex-adjusted intraepidermal nerve fiber density, or reduced sensory nerve action potential (SNAP) of sural nerve (normal value >7 μV) and (2) nociplastic pain, clustered as chronic widespread pain (n = 82) and fibromyalgia (n = 141). All participants were unrelated, collected in Italy, and had Anglo-American ancestry.

Painful neuropathies were associated with diabetes (n = 25), hypothyroidism (n = 7), B12 vitamin deficiency (n = 10), HIV (n = 1), hepatitis B or C (n = 11), and systemic connective tissue disorders (n = 42). Nociplastic pain patients had diabetes (n = 13), hypothyroidism (n = 16), B12 vitamin deficiency (n = 10), HIV (n = 1), hepatitis B or C (n = 6), and systemic connective tissue disorders (n = 28).

In the validation cohort, length-dependent painful neuropathies were associated with diabetes (n = 12), hypothyroidism (n = 7), B12 vitamin deficiency (n = 3), hepatitis B (n = 1), and systemic connective tissue disorders (n = 12). Painless neuropathies were associated with diabetes (n = 2), hypothyroidism (n = 4), and systemic connective tissue disorders (n = 1); one patient was diagnosed with sensory variant of chronic demyelinating polyradiculoneuropathy, and the others were idiopathic.

3.2. Gene-wise aggregation analysis

We applied the SKAT-O test to investigate whether there was an excess of rare variants in patients respect to healthy controls. Variants were chosen based on allele frequency, high genotyping quality, and consequence to the transcript as detailed in the methods. We stratified the study population into 2 cohorts: length-dependent painful neuropathy (n = 169) and nociplastic pain (widespread chronic pain and fibromyalgia, n = 223). Then, we applied the association analysis both to the entire population and separately to the 2 cohorts and compared with the 216 healthy controls.

No gene achieved the Bonferroni-adjusted significance ($P < 7.9^{e-04}$ for the number of considered genes) both for the entire study population and the painful neuropathy cohort compared with the healthy control population. Conversely, the transient receptor potential cation channel subfamily A member 1 (TRPA1) gene showed a Bonferroni-adjusted significant difference ($P = 6.7^{e-04}$) of rare variant distribution in nociplastic pain patients

when compared with healthy controls, with a risk of 4.8-fold higher based on the simple burden test ($P = 0.0015$, OR = 4.8). The $\rho = 1$ corresponded to the high percentage of causality in the same direction. None of the VGSC genes (SCN9A, SCN10A, and SCN11A) were found significantly associated to any cohort (Supplementary Table 2, available at http://links.lww.com/PAIN/B804).

3.3. TRPA1 single-variant description

Considering both gene-wise and validation cohorts, we identified 26 variants, of which 17 were exclusively present in the nociplastic pain group, 4 exclusively in the painful neuropathy cohort, 1 both in nociplastic and in painful neuropathy patients, 3 both in cases and healthy controls, and 1 (c.993+7A>C) in a healthy control. The single variant analysis on the validation cohort of 140 subjects (90 with painful neuropathy and 50 with chronic widespread pain) replicated 4 variants identified in the gene-wise cohort, including one found in 1 healthy control and highlighted one new variant (p.Thr598Asnfs*10) (**Table 1**).

According to the consequence on the transcription, 15 of the 22 variants exclusively found in patients were missense (p.L118V, p.T311N, p.N373I, p.D412N, p.R458H, p.I469V, p.D495G, p.M626I, p.C651F, p.A789V, p.M844V, p.N954T, p.R996H, p.K1001I, and p.K1046E), 2 were frameshift variants: one was a dinucleotide deletion (c.174-175delAT) resulting in a nonsense codon and one p.T598Nfs*10. According to the topological distribution on the protein, 13 of the 15 missense mutations were clustered in the N-terminal cytoplasmic domain, also known as ankyrin-rich domain, which is essential for channel function and regulation, 6 of which mapping within one of the 16 ankyrin domains (**Fig. 1**). The small dinucleotide deletion c.174_175delAT is predicted to change the protein sequence starting at position 58 and inserting a stop codon (p.Cys59*), thus encoding a premature truncated protein or a transcript doomed to the nonsense-mediated decay.

The variants identified exclusively in patients had lower frequencies in the general population if compared with those found in healthy controls, with a maximum of 0.35% in the European–NonFinnish GnomAD database, and according to the ACMG standards for variants, interpretation[58] had at least one line of moderate evidence supportive for pathogenicity (PM1 or PM2). Conversely, the variants carried also or exclusively by the healthy controls had a higher minimum allele frequency in the general population (GnomAD-NFE) and a more benign ACMG classification (BP6), thus appearing more likely to be rare polymorphism (**Table 1**).

3.4. Phenotype of TRPA1 variant carriers

Among the 32 patients harboring *TRPA1* variants, 24 (75%) were diagnosed with nociplastic pain, either fibromyalgia (12; 37.5%) or chronic widespread pain (12; 37.5%), whereas 8 (25%) with length-dependent painful neuropathy (**Table 2**). Irrespective of the clinical diagnosis, 12 (37.5%) patients complained of itch and 10 (31.3%) of cold-induced or cold-accentuated pain, mostly episodic.

As an example, the 20-year-old girl (p753) harboring the missense variant p.T311N inherited from the mother who reported a similar, albeit milder phenotype, complained of episodic severe painful cold, electric shock-like, burning sensation, and deep pain lasting many hours up to a few days with a generalized distribution. Her clinical image was further aggravated by comorbidity with TNF receptor-associated periodic syndrome (OMIM 191190) due to the heterozygous p.Arg92Gln in *TNFRSF-1A* gene.

The 2 patients (p518 and p123), one male and one female, harboring the missense variant p.I469V, reported extreme pain exacerbated by cold, mainly over the trunk and lower limbs, episodic unbearable itch, and Raynaud phenomenon. Other 2 patients (p052 and p833) carrying the missense variant p.D495G both complained for about 15 years of diffuse episodic burning pain, associated with cold pain, pungent, and electric shock sensations over the trunk and shoulders (p052) and swelling and redness in legs and feet (p833). Genetic tests for hereditary transthyretin amyloidosis, Fabry disease, and porphyria as well as clinical and radiological screening for malignancies were negative.

3.5. TRPA1 in painless neuropathy patients

Consistently, none of the variants found in the 2 pain cohorts were identified in painless neuropathy patients, in 3 of whom we identified 3 missense variants, the novel p.R919L and the rare p.A561T and p.Y419C. The substitution of the arginine residue with leucine in position 919 affects a conserved residue within the pore-delimiting region, with a strong potential effect on channel permeability; the same patient carried a second rare missense variant in *TRPV1* (p.R116G), which is coexpressed with *TRPA1* to form functional heterotetrameric channels. The 2 variants might exert a combined loss-of-function effect on the TRP channel. A previous study[9] investigating the neighbor variants (D915A, D915E, E920A, and E920Q) showed an increased rectification, reduced conductance, and reduced block by ruthenium, demonstrating the importance of this region for channel permeability. The patient carrying the p.R919L variant reported low frequency episodes (up to 3-4 per year) of painless tingling in the hands and the lower limbs with cold sensation, sometimes occurring also in the face, accompanied by headache lasting 2 to 4 days with spontaneous resolution. The variant p.A561T has a frequency of 0.002% in the NFE-GNOMEX population, localizes in the ANK15 repeat in the N-terminus, and affects a moderately conserved amino acid inducing small physiochemical difference between residues. Both variants have predicted evidence of pathogenicity (PM1, PM2, and BP4). The carrier reported symmetrical numbness and painless tingling in the soles since he was 55 years, which extended to thighs and hands. The variant p.Y419C is very rare in the European population (0.0009% in NFE-GNOMEX) and affects the N-terminal ANK11 repeat. The patient complained of paresthesia and numbness in the soles.

3.6. TRPA1 in silico modelling

Residue substitutions were evaluated on TRPA1 tetramer ligand-free structure, bound with calcium (PDB ID: 6v9w),[69] using PyMOL "Mutagenesis" tool (https://pymol.org/2/) to obtain protein variants localized in the resolved structure (447-1079). Mutants in the first residues (1-446) were evaluated on AlphaFold prediction monomer (AF-O75762-F1),[37] and amino acid and related substitutions were classified based on Livingstone et al.[4,50] H-bonds were estimated by the Visual Molecular Dynamics 1.9.2.[34] We assigned a class as neutral (N) or damaging (D) based on the Grantham score[31,48] and physiochemical differences (Supplementary Table 3, available at http://links.lww.com/PAIN/B804). Variants were defined neutral if evolutionarily less conserved, with Grantham score lower or equal to 50, and with small physiochemical differences. Damaging variants were those with Grantham score higher than

Table 1

TRPA1 rare variants frequencies and distribution in the study cohorts.

rsID	gnomAD-NFE	Effect	c.pos	p.pos	Localization	Predicted changes	Pathogenicity classes	Gene-wise cohorts			Validation cohort	
								HC (N = 216)	NocP patients (N = 223)	NeuP patients (N = 169)	NeuP patients (N = 90)	NocP (n = 50)
rs766398528	0.004%	Frameshift	c.174_175delAT	p.C59*	C		PM2		1 (0.45%)			
rs61753713	0.440%	Missense	c.327C>A	p.N109K	C (ANK2)		PM1, BP6	2 (0.9%)	2 (1%)			
rs201061221	0.026%	Missense	c.352C>G	p.L118V	C (ANK2)		PM1, PM2		1 (0.45%)			
rs564619453	0.014%	Splicing	c.444+5G>T		C (ANK3)	−15.0% (DS)	PM2		1 (0.45%)			
rs561796522	0.025%	Missense	c.932C>A	p.T311N	C (ANK8)		PM1, PM2		1 (0.45%)			
rs751673891	0.001%	Splicing	c.944+4A>T		C (ANK8)	−10.1% (DS)	PM2			1 (0.6%)	1 (1.1%)	
rs757559276	0.000%	Splicing	c.993+7A>C		C			1 (0.5%)				
rs61736313	0.005%	Missense	c.1118A>T	p.N373I	C		PM1, BP4		1 (0.45%)			
rs749200395	0.000%	Splicing	c.1195-3T>C		C (ANK10)	+6.6% (AS)	PM2			1 (0.6%)		
rs530978468	0.004%	Missense	c.1234G>A	p.D412N	C (ANK11)		PM1, PM2, BP4		1 (0.45%)			
rs144498143	0.009%	Missense	c.1373G>A	p.R458H	C (ANK12)		PM1, PM2		1 (0.45%)			
rs61758118	0.148%	Missense	c.1405A>G	p.I469V	C (ANK12)		PM1, BP4		1 (0.45%)			1 (2%)
rs756703385	0.010%	Missense	c.1484A>G	p.D495G	C (ANK13)		PM1, PM2, BP4			1 (0.6%)		1 (2%)
rs147715599	0.000%	Frameshift	c.1792dupA	p.T598Nfs*10	C (ANK16)		PM2				1 (1.1%)	
rs61753709	0.348%	Missense	c.1878G>A	p.M626I	C		PM2, BP4, BP6		1 (0.45%)			
rs767859469	0.003%	Missense	c.1952G>T	p.C651F	C		PM2		1 (0.45%)			
rs143973551	0.689%	Splicing	c.1965T>C	p.Y655Y	C	+13.8% (DS)	BP6	1 (0.5%)	2 (1%)	1 (0.6%)	1 (1.1%)	
rs886397498		Splicing	c.1965+3A>G		C	−16.9% (DS)	PM2		1 (0.45%)			
rs1193285296	0.001%	Missense	c.2366C>T	p.A789V	C		PM1, PM2, BP4		1 (0.45%)			
8:72948548		Missense	c.2530A>G	p.M844V	TM4		PM1, PM2, BP4		1 (0.45%)			
rs760812691	0.003%	Missense	c.2861A>C	p.N954T	TM6		PM1, PM2, PP3		1 (0.45%)			
rs186828882	0.490%	Splicing	c.2937+6T>C		C	−5.0% (DS)	BP6	2 (0.9%)	3 (1.3%)			
rs142468969	0.011%	Missense	c.2987G>A	p.R996H	C		PM2, BP4		1 (0.45%)			
8:72938244		Missense	c.3002A>T	p.K1001I	C		PM2, BP4			1 (0.6%)		
rs180680340	0.037%	Splicing	c.3052-6C>T		C	+13.4% (AS)			2 (1%)			
rs757559276	0.002%	Missense	c.3136A>G	p.K1046E	C, IP		PM2					1 (2%)

Variants are sorted according to chromosome position. Localization indicates the variant's position in the protein domains. Predicted changes and pathogenicity classes are assigned using Alamut Visual Plus (SophiaGenetics), according to ACMG guidelines. cpos refers to the transcript NM_007332. The symbol * in ppos refers to the introduction of a STOP signal in the protein product. The mutation nomenclature has been validated through https://variantvalidator.org according to the HGVS recommendations. Alt, alternative; ANK, ankyrin domain; AS, acceptor splice site; BP, supporting evidence of benign impact; C, cytoplasmic; CHRPOS, chromosome position; c.pos, coding position; DS, donor splice site; IP, inositol-phosphate binding (1046-1052), coiled-coil; HC, healthy controls, NocP, nociplastic pain (ie, chronic widespread pain and fibromyalgia); NeuP, painful neuropathy; NFE, European Non-Finland; PM, moderate evidence of pathogenicity; p.pos, protein position; rsID, univocal locus code in dbSNP; TM, transmembrane; TRPA1, Transient receptor potential cation channel subfamily A member 1.

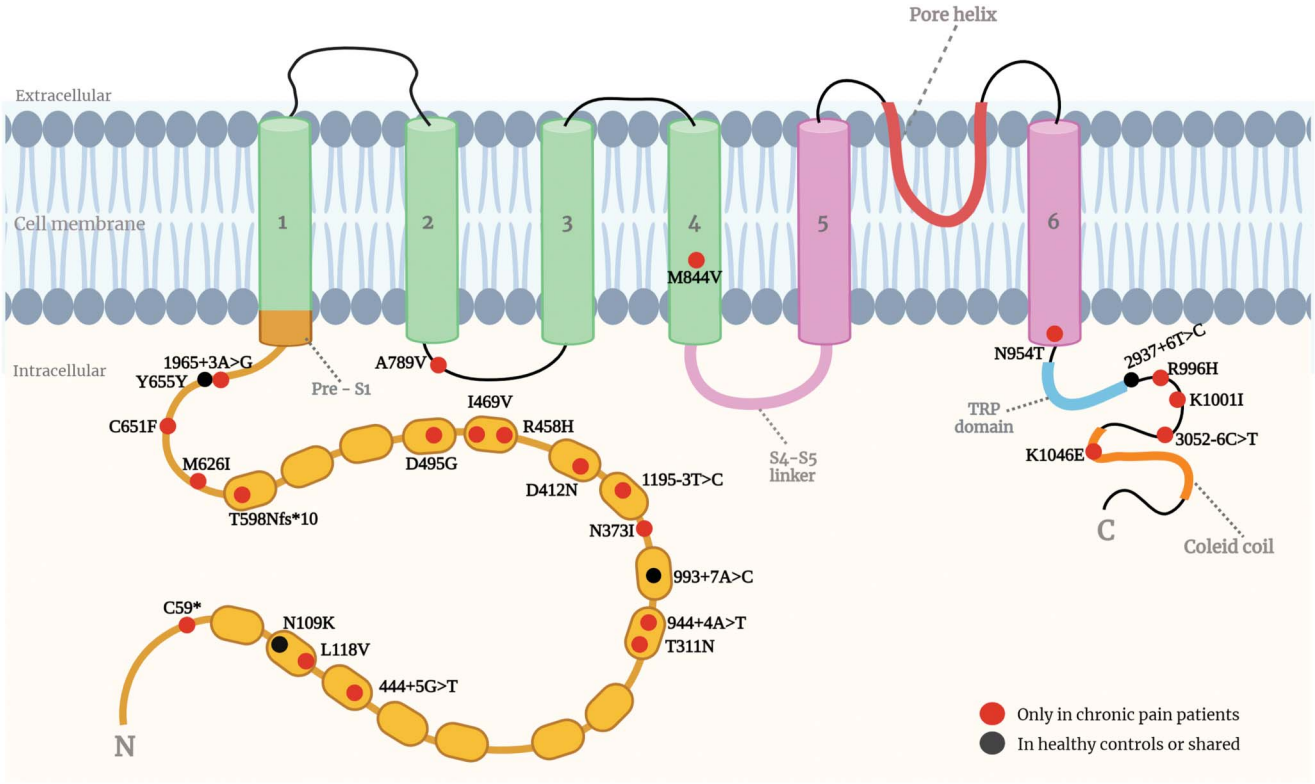

Figure 1. Schematic representation of human TRPA1 structure and genetic variants. Red dots indicate the variants exclusively carried by chronic pain patients, and black dots refer to variants identified also in healthy controls. Variants are mapped according to O75762 UniProtKB human template (https://www.uniprot.org/uniprotkb/O75762/entry). Created with BioRender.com (agreement number: *XZ257JVKR*).

100 or with large physiochemical changes. The physicochemical changes induced by the substitution were assessed performing integrative analysis by Alamut Visual Plus (SophiaGenetics, Bidart, France).

The impact on protein structure was evaluated on the human tetrameric resolved ligand-free structure (**Fig. 2A**). In particular, the 2 substitutions p.N373I and p.D412N, carried by the same patient, are both localized in highly conserved loci; the substitution of the asparagine 373 remove an H-bond probably involved in the maintenance of the ANK domain (**Fig. 2B**). The p.D495G, found in 2 patients with similar phenotype, is localized in the conserved ANK13 domain, where the H-bond with K496 is likely involved in the maintenance of the typical ankyrin helix-turn-helix secondary structure (**Fig. 2C**). The p.M626I is localized in the cytosol helixes close to residue C633 (**Fig. 2D**) where it can perturb the disulphide bridge and to P622 and M634 which are key residues for the activation by the scorpion wasabi receptor toxin.[49] The p.R919L is localized internally, in the pore lumen, and induces the change of a positively charged into a hydrophobic amino acid (**Fig. 2E**), with a strong potential impact on channel selectivity and permeability to Ca^{2+}.

3.7. SCN9A rare variants and neanderthal haplotype frequency

Genetic research on Mendelian heritable pain disorders revealed the involvement of genes encoding VGSC expressed in the peripheral nervous system, with *SCN9A* being the most frequent represented in the spectrum of pain disorders, from CIP to IE, PEPD, and SFN.[18] Although *SCN9A* variants in families with the early-onset EM and PEPD reported to date have full penetrance,[20] those identified in SFN patients showed incomplete penetrance and their impact on diseases pathogenesis is challenging because many nucleotides substitutions have also been identified in healthy carriers,[21] suggesting a modulatory role in the development of neuropathic pain.

In our study, 14% (n = 55/392) of patients and 11% (n = 23/216) of healthy individuals carried rare variants in *SCN9A*, resulting not significantly enriched (P = 0.9) even when subgrouping the cohort in painful neuropathy and nociplastic pain (P = 0.9 and 0.86, respectively). Among them, we identified 19 rare nonsynonymous variants exclusively carried by 21 patients, 6 variants present in 31 patients and 12 healthy controls, and 10 rare variants carried exclusively by 11 healthy controls. The N1245S variant was carried by 6 painful patients and 1 painless patient (Supplementary Table 4, available at http://links.lww.com/PAIN/B804).

Recently, a subset of 3 *SCN9A* amino acid substitutions (M932L, V991L, and D1908G), previously associated with increased pain sensitivity and SFN,[23,57] have been shown to have introgressed the modern humans' genome from Neanderthal ancestors to enhance pain sensitivity in the general population.[68] These variants are reported in the global population (GnomAD 2.1 total populations), respectively, as 3% for M932L/V991L and 5.8% for D1908G, with a maximum frequency of 33% in the population of Latin origin (Supplementary Table 5, available at http://links.lww.com/PAIN/B804).

We identified this haplotype in 6 patients (1.3%) and 2 healthy individuals (0.9%). Furthermore, another 5 patients carried the D1908G without the haplotype M932L/V991L, for a total of 11 patients (2.4%). Zeberg et al.[68] did not mention the missense W1538R, which we found in strong linkage with the Neanderthal

Table 2

Clinical features of patients carrying TRPA1 variants.

Sample	Diagnosis	Pain features	Itch	Positive/ negative signs	Sural SNAP amplitude	IENFD at distal leg	TRPA1 variants
p526	Fibromyalgia	Cold pain; stinging; Electric shock-like; burning	Yes	Yes	Normal	Normal	p.C59*
p669	Fibromyalgia	Stinging; electric shock-like; burning	Yes	No	Normal	Normal	p.N109K,c.3052-6C>T
p357	Fibromyalgia	Cold pain; electric shock-like; burning	Yes	Yes	Normal	Normal	p.N109K
p388	Fibromyalgia	Cold pain; stinging; electric shock-like; burning	No	Yes	Normal	Normal	p.N373I, p.D412N
p798	Fibromyalgia	Burning	Yes	Yes	ND	Normal	p.R458H
p290	Fibromyalgia	Stinging; electric shock-like; burning	No	No	ND	Normal	p.M626I
p197	Fibromyalgia	Stinging; electric shock-like	No	No	Normal	Normal	p.Y655Y (SS)
p619	Fibromyalgia	Electric shock-like	Yes	No	Reduced	Normal	p.M844V, c.1965+3A>G
p387	Fibromyalgia	Stinging; electric shock-like	Yes	No	Normal	Normal	p.A789V
p274	Fibromyalgia	Cold pain; stinging; electric shock-like; burning	Yes	No	Reduced	Normal	p.N954T
p270	Fibromyalgia	Stinging; electric shock-like; burning	No	Yes	Normal	Normal	c.2937+6T>C
p417	Fibromyalgia	Stinging; electric shock-like; burning	Yes	No	Normal	Normal	p.R996H
p657	WCP	Cold pain; stinging; burning	No	Yes	Normal	Normal	p.L118V, p.V299M
p481	WCP	Burning	No	No	Normal	Below cut-off	c.444+5G>T
p753	WCP	Cold pain; stinging; electric shock-like; burning	Yes	No	ND	Below cut-off	p.T311N
p518	WCP	Cold pain	Yes	Yes	ND	Normal	p.I469V
p123	WCP	Cold pain	Yes	Yes	ND	ND	p.I469V
p052	WCP	Cold pain; stinging; electric shock-like; burning	No	No	ND	ND	p.D495G
p660	WCP	Constricting; Stinging	No	Yes	ND	Normal	p.C651F
p905	WCP	Stinging; Electric shock-like; Burning	No	Yes	ND	Normal	p.Y655Y (SS)
p644	WCP	Constricting; stinging; electric shock-like; burning	No	No	Normal	Below cut-off	c.2937+6T>C
p946	WCP	Stinging; electric shock-like	No	No	Normal	Normal	c.2937+6T>C
p504	WCP	Cold pain; stinging; electric shock-like; burning	Yes	Yes	Normal	Below cut-off	c.3052-6C>T
127215	WCP	Burning	No	No	Normal	Below cut-off	p.K1046E
p783	Painful neuropathy	Burning	No	Yes	Normal	Normal	c.944+4A>T
p205	Painful neuropathy	Stinging; burning	No	Yes	Normal	Below cut-off	c.944+4A>T
p699	Painful neuropathy	Stinging; electric shock-like	No	Yes	Normal	Below cut-off	c.1195-3T>C
p833	Painful neuropathy	Burning, swelling and redness in feet and legs	No	Yes	ND	ND	p.D495G
119600	Painful neuropathy	Constricting; burning	No	Yes	Reduced	Below cut-off	p.T598Nfs*10
p085	Painful neuropathy	Constricting; stinging; electric shock-like	No	Yes	Normal	Below cut-off	p.Y655Y (SS)
p591	Painful neuropathy	Stinging; burning	No	Yes	ND	Normal	p.Y655Y (SS)
p686	Painful neuropathy	Stinging; electric shock-like; burning	No	Yes	Normal	Normal	p.K1001I

Three patients had multiple variants of TRPA1 gene. The phasing of multiple variants was not detectable by the used short reads NGS approach. The symbol * refers to the introduction of a STOP signal in the protein product. The mutation nomenclature has been validated through https://variantvalidator.org according to the HGVS recommendations.

IENFD, intraepidermal nerve fiber density; ND, not done; SNAP, sural nerve conduction; SS, splice site; WCP, widespread chronic pain; TRPA1, Transient receptor potential cation channel subfamily A member 1.

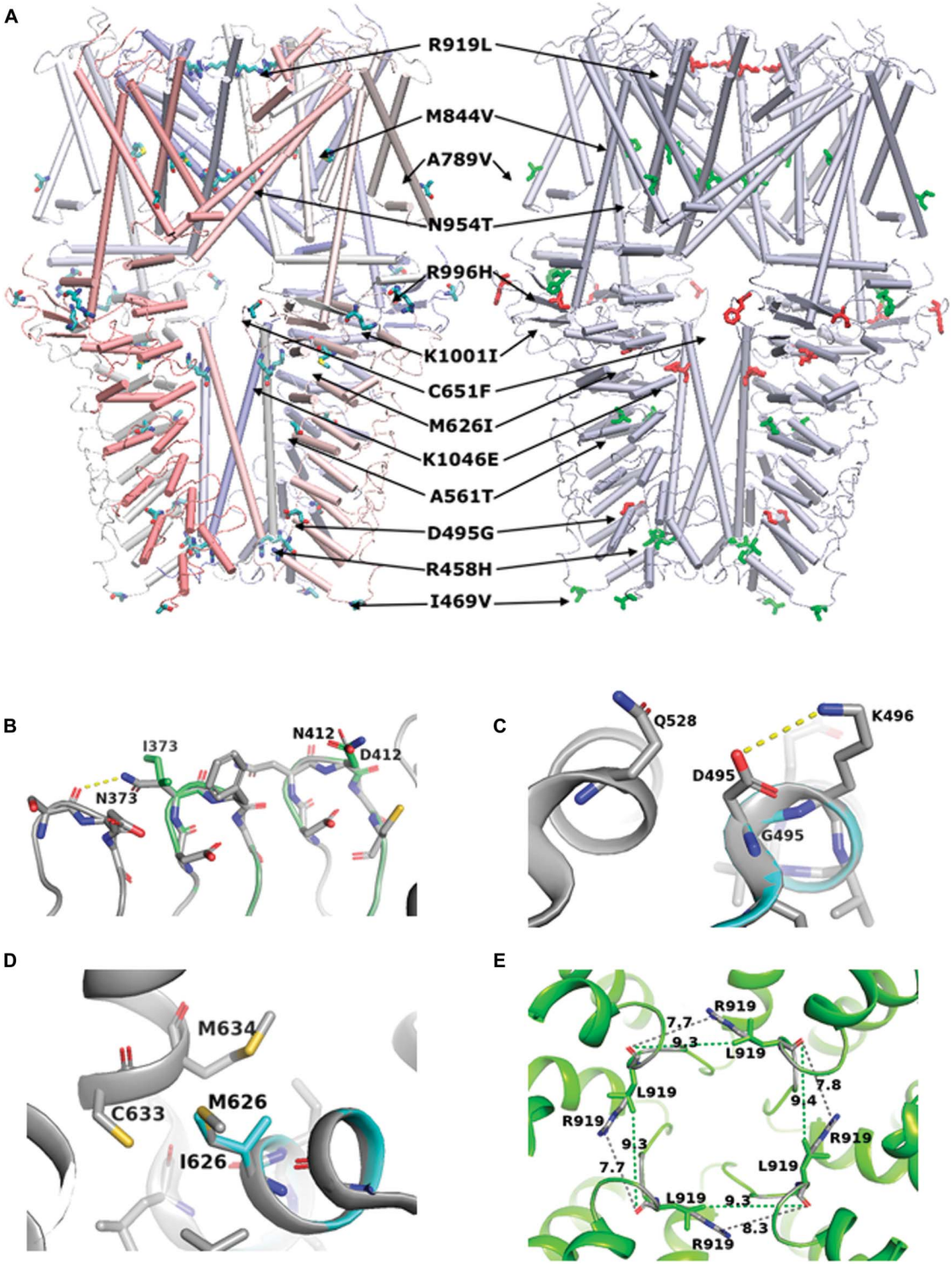

Figure 2. Residue substitutions on TRPA1 tetramer. (A) The residues are modelled on the solved structure (PDB ID: 6v9w, residues: 447-1079). In sticks the identified variants. The amino acidic changes are indicated with different colours according to their impact on the channel, taking into consideration the physical–chemical differences and the context of neighbour residues: red is used for a more deleterious predicted effect and green for substitutions with a milder effect, as indicated in Table S3, available at http://links.lww.com/PAIN/B804. (B) The double substitution carried by the same patient p.N373I and p.D412N, localized in a highly conserved loci; substitutions are visualized on the AF-O75762-F1 model; in grey sticks, the wild type side chains and in green, the mutants; in yellow is evidenced the H-bond lost in the mutant. (C) p.D495G localizes in the conserved ANK13 domain, wild type amino acid is in grey and mutant in light blue; in yellow is evidenced the H-bond lost in the mutant. (D) p.M626I is in the cytosol helixes, close to the residue C633, involved in disulphide bridge, whose position can be perturbed, and near to P622 and M634, involved in the activation by the scorpion wasabi receptor toxin; in grey sticks the wild type side chains and in green the mutants. (E) p.R919L localizes internally, in the pore lumen, and induces a change from a positively charged amino acid into a hydrophobic one, with a strong potential impact on the channel selectivity and permeability to Ca^{2+}; in green the mutated side chains and in grey the wild type; distances between wild type side chains and mutated one are in evidence. PDB, Protein Data Bank.

subset. Indeed, 5 of 7 patients and 2 of 3 healthy controls carrying the 3 substitutions (M932L, V991L, and D1908G), also carried W1538R, with a global frequency in chronic pain patients nearly twice as high as in healthy individuals (0.9%; OR = 1.9, $P = 0.43$, not significant), and about 8 times more frequent than in the general population (0.2%; OR = 8.6, $P = 2.08E-9$) and non-Finnish population (OR = 7.1, $P = 5.4E-8$; Supplementary Tables 5 and 6, available at http://links.lww.com/PAIN/B804).

4. Discussion

The clinical classification of patients with chronic neuropathic pain is highly complex and frequently results in a mixed image with substantial mechanistic overlap precluding enrolment in clinical trials, reducing their quality of life, and increasing healthcare costs. This underscores the need to cluster patients based on variables other than their phenotype. To overcome this issue, we implemented the phenotype-driven approach with a gene-wise aggregated analysis, with the aim of improving the characterization of patients by adding the genetic risk factors to the clinical assessment. A previous study used a gene-based association test to investigate a small group of 20 patients with corneal neuralgia after refractive surgery against GnomAD general population.[67] This is the first to apply a burden test approach in the largest cohort of patients with chronic pain compared with healthy individuals. Notably, we identified a significant enrichment of rare genetic variants in *TRPA1* in patients presenting with diffuse pain (ie, chronic widespread pain and fibromyalgia), even if some rare variants were present also in patient with classical length-dependent painful neuropathy. Irrespective of the clinical diagnosis, 38% of mutated patients complained of itch and 31.3% of cold-induced or cold-accentuated pain, mostly episodic. The variants exclusively carried by patients had lower frequencies in the general population compared with healthy controls and according to the ACMG standards had higher pathogenicity scores. Conversely, the variants carried also or exclusively by the healthy controls had a higher minimum allele frequency in the general population and a more benign classification, thus being more likely rare polymorphisms.

TRPA1 encodes for a member of the transient receptor potential (TRP) superfamily of ion channels, which is gated by temperature and is believed to participate in the initiating phase of nociceptive signaling. The channel can be activated by various noxious stimuli, chemical agents, and intense cold.[64] Most of the functionally relevant parts of the TRPA1 channel were discovered using in vitro mutagenesis, chimeras of different species isoforms, or deletion constructs of the channel. Despite many of such artificial variants and constructs being reported, the only genetic disease associated to *TRPA1* is the familial episodic pain syndrome (FEPS, OMIM #615040) due to the p.N855S substitution, which we did not find in any of the subjects of this study. Furthermore, high-throughput studies on the frequency of *TRPA1* single nucleotide polymorphisms found no association with neuropathic pain susceptibility.[5] Conversely, our study focused on rare coding *TRPA1* variants, suggesting their strong association particularly with nociplastic pain, which includes chronic widespread pain and fibromyalgia, with cold-induced pain and itch as the predominant symptoms.

Recent models of human TRPA1 channel structure obtained from cryo-electron microscopy imaging revealed a tetrameric arrangement[55] with monomer consisting of 6 transmembrane domains (TM), a pore-forming loop between TM5 and TM6, and a large intracellular NH2 and COOH terminal.[13] The most distinctive characteristic of *TRPA1* is the presence of 16 ankyrin repeat domains in the large NH2-terminal portion of the protein.[29, 63] In this study, we evaluated the residue substitutions on the TRPA1 tetramer ligand-free structure, bound with calcium (PDB ID: 6v9w) while mutants in the first residues (1-446) were modelled on an AlphaFold prediction monomer (AF-O75762-F1). The in silico predictions of damage to the protein were performed according to chemical–physical characteristics of amino acidic changes and those with higher differences were modelled. In particular, the substitution p.N373I removes an H-bond which is probably involved in the maintenance of the ANK domain. The mutation p.D495G, found in 2 patients with a similar phenotype, localizes in the conserved ANK13 domain, where the H-bond with K496 is likely involved in the maintenance of the typical ankyrin helix-turn-helix secondary structure. The missense p.M626I in the cytosol helixes, close to the residue C633, could perturb the disulphide bridge and is near to P622 and M634 key residues for activation by the scorpion wasabi receptor toxin.[49] The substitution p.R919L localizes in the pore lumen where it could impact on channel selectivity and permeability to Ca^{2+}. Interestingly, this novel p.R919L was identified in a patient with painless neuropathy together with a second rare missense variant (p.R116G) in *TRPV1*, which is coexpressed with *TRPA1* to form functional heterotetrameric channels. The 2 variants might exert a combined loss-of-function effect on TRP channels which is consistent with the observed phenotype.

Recently, Meents et al.[52] reviewed the literature on *TRPA1* functional studies, reporting more than 90 mutagenized loci in human *TRPA1* and over 100 amino acid variants of the channel. However, most of the substitutions have never been found in humans, and only 7 were reported in the dbSNP database. In this study, we describe new human genetic variants identified in *TRPA1*, which are promising candidates for functional studies and potential druggable targets. Moreover, we identified both missense and potentially protein truncating variants. Remarkably, a stop variant (p.R919*) in *TRPA1* has been previously linked to cramp-fasciculation syndrome (CFS),[54] a rare muscular hyperexcitability disorder. The functional consequences of this variant have not yet been addressed; however, the 2 related patients, father and son, also suffered from an array of other hyperexcitability–hypersensitivity syndromes such as asthma and cold hyperalgesia, which have previously been related to TRPA1 function.[36,62] We identified one stop and one frameshift variant, but carrier patients did not report cramp-fasciculation syndrome. We did not find any protein truncating variant in the cohort of 216 healthy individuals. It cannot be excluded that loss-of-function variants, even if per se tolerated, could affect the functioning of the tetrameric channel, because other variants in the second allele, even if not rare and with small effect, would be expressed hemizygously, amplifying their effects. Furthermore, it is unknown to what extent a *TRPA1* null allele would be replaced by *TRPV1* subunits in the heterotetrameric TRP channel. For this reason, patients carrying *TRPA1* variants were also investigated for TRPV1 variants, to have a more complete image of the possible combination of variants in the TRP channel (Supplementary Table 7, available at http://links.lww.com/PAIN/B804).

Surprisingly, *SCN9A* aggregated variants did not show an increased incidence in patients when compared with healthy individuals. The Neanderthal haplotype associated with heightened pain sensitivity in the UK Biobank (M932L, V991L, D1908G)[68] was underrepresented in our cohort of chronic pain patients compared with healthy controls and the general population. Conversely, single variant analysis revealed that the W1538R was significantly enriched in patients and frequently linked to the haplotype (M932L + V991L) or to the variant D1908G

(Supplementary Table 5, available at http://links.lww.com/PAIN/B804). This finding suggests that the weight of the Neanderthal haplotype could be more likely related to the presence of the W1538R, which is frequently inherited together with the haplotype. It should be recalled in this context that although strong mutations of VGSC genes are causative for rare early-onset (infancy or early childhood) Mendelian disorders such as CIP, IE, and PEPD, VGSC variants in more common adult-onset SFN[66] likely require additional insults possibly involving metabolic or energetic stress[24,46,56,59] to produce clinical manifestations and have thus been explicitly characterized as "risk factors."[6,19] Moreover, differences in the inclusion criteria for subjects assessed in different studies[21,25] may affect the yield of VSCG variants.

The development of personalized therapies for neuropathic pain treatment has recently been advanced by Nav1.7-selective blockers[1] and their efficacy in patients harboring specific gain-of-function variants in *SCN9A*. Importantly, recent studies have demonstrated that variants in *SCN9A*, such as S241T, can enhance responsiveness to carbamazepine,[30] whereas W1538R can affect the capability of lacosamide, a nonspecific sodium channel blocker, to modulate the gating of the channel,[2,41] explaining the lack of response of SFN patients enrolled in a clinical trial.[14] These findings support the hypothesis that a genotype-first analysis of patients could improve the outcome in clinical trials and translate into better management in clinical practice. Our study demonstrated the strength of the gene risk burden approach in widening the spectrum of genes relevant for guiding personalized pain treatment.

The TRP channel family has attracted substantial attention in pain pharmacogenomics,[39] and preclinical studies are providing proof-of-concept for its use.[15,38] One proof-of-concept RCT failed to demonstrate the efficacy of an orally available inhibitor of TPRA1 in 138 patients with painful diabetic neuropathy at 4 weeks of treatment, although highlighted a statistically significant and clinically meaningful improvement in pain in a subgroup of patients with preserved small nerve fiber function defined by quantitative sensory testing.[35]

In addition to widening the spectrum of channelopathy-related chronic pain disorders, our study suggests that new clinical trials targeting TRPA1 should be designed including patients based on their molecular profile.

Conflict of interest statement

The authors disclose no conflict of interest.

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Supplemental digital content

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