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Influence of polymorphisms of three TRP genes on pain sensitivity in neuropathic pain patients

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Abbreviations:

NMDA	N-methyl-Daspartate receptor
CNS	central nervous system
BK	bradykinin
PGs	prostaglandis
5-HT	serotonin
PKC	protein kinase C
NO	nitric oxide
CWT	cold withdrawal time
PCR	Polymerase Chain Reaction
APS	adenosine 5’ phosphosulfate
PNS	peripheral nervous system
ALL	dynamic mechanical allodynia
WDT	warm detection threshold
CDT	cold detection threshold
CPT	cold pain threshold
WDT	warm detection threshold
PHS	paradoxical heat sensation
MDT	mechanical detection threshold
MPT	mechanical pain threshold
TSL	thermal sensory limen
CPT	cold pain threshold
PHS	paradoxical heat sensation
PPT	pressure pain threshold
VDT	vibration detection threshold
CRPS	complex regional pain syndrome
PHN	post-herpetic neuralgia
NE	norcpinephrinc
COMT	catechol-O-methyltransferase
CNS	central nerve system
SNP	single nucleatide polymorphism
PI(3)K	phosphatidylinositol-3-kinase
TRP	transient receptor potential channel

DRG	dosal root ganglion
TG	trigeminal ganglion
NG	nodose ganglian
mGluRs	metabotropic glutamate receptors
MAPK	mitogen-activated protein kinase pathway
NGF	nerve growth factor
PSQ	Pyrosequencing
GABA	gamma-aminobuytric acid
OPRM	μ-opioid receptor
TrkA	receptor tyrosine kinases

1. INTRODUCTION

1.1 Neuropathic pain overview

1.1.1 Definition of neuropathic pain

According to the International Association for the Study of Pain (IASP), neuropathic pain is defined as pain caused by damage of the neural structures that disrupts the ability of the sensory nerves to transmit correct information to the brain. Therefore, neurogenic pain syndromes arise as consequence of central and peripheral nerve damage, which generally characterized by three different properties [1]

(a) it is often experienced in parts of the body that otherwise appear normal,

(b) it is generally chronic, severe and resistant to over-the-counter analgesics,

(c) it is further aggravated by allodynia (touch-evoked pain).

1.1.2 Etiology of neuropathic pain

Neuropathic pain may result from various causes that affect the brain, spinal cord and peripheral nerve system, including cervical or lumbar radiculopathy, diabetic neuropathy, cancer-related neuropathic pain, HIV-related neuropathy, spinal cord injury, trigeminal neuralgia, complex regional pain syndrome type (CRPS) II and post-herpetic neuralgia (PHN) [2,3]. Based on the location of the nervous system lesion, neuropathic pain syndromes may be divided into two groups, central and peripheral. Alterations of central nervous system physiology may play a significant role in many of the neuropathic pain conditions associated with peripheral pathology, making the distinction between peripheral and central pain less distinct than once believed. PHN is a typical example of neuropathic pain with mixed central and peripheral components [3]. Neuropathic pain, in contrast to nociceptive pain (i.e. post–operative pain), is described as "burning", "electric", "tingling", and "shooting" in nature. It can be continuous or paroxysmal in presentation. Neuropathic pain brings tremendous direct and indirect costs to patients and their families in terms of pain and suffering, health care expenditures as well as the quality of life.

The hallmarks of neuropathic pain are chronic allodynia and hyperalgesia. Allodynia is defined as pain resulting from a stimulus that ordinarily does not elicit a painful

response (i.e. light touch). Hyperalgesia is defined as an increased sensitivity to a normally painful stimulus [4]. Primary hyperalgesia, caused by sensitization of C–fibers, occurs immediately within the area of the injury. Secondary hyperalgesia, caused by sensitization of dorsal horn neurons, occurs in the undamaged area surrounding the injury. However, clinically, both negative and positive signs may be present on examination. Negative signs are evidence of nerve damage such as sensory deficits, weakness, and reflex changes. Positive signs, also indicative of nerve damage, include hyperalgesia and allodynia. However, the epidemiology of neuropathic pain has not been adequately studied, partly because of the diversity of the associated conditions. Demonstrating a lesion of the nervous system compatible with particular symptoms and signs could provide strong support for considering the pain to be neuropathic.

1.1.3 Mechanisms of neuropathic pain

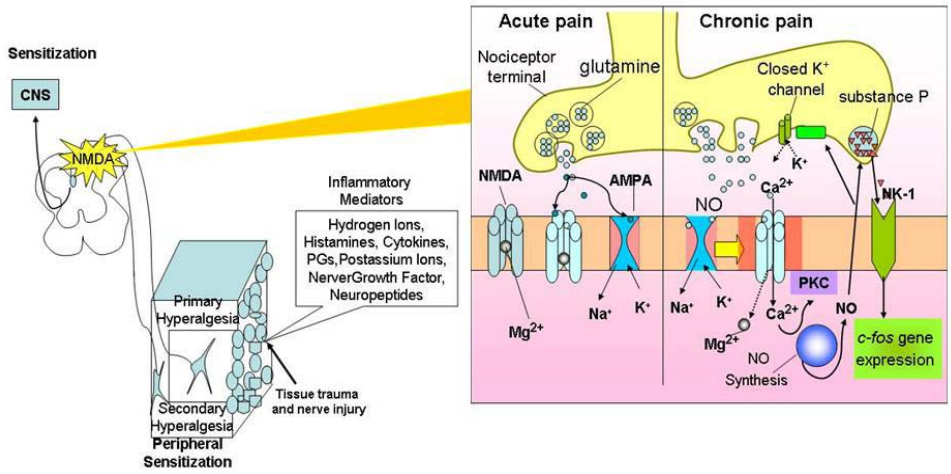
With the development of various experimental models of nerve injury, it has become increasingly clear that both peripheral and central mechanisms could be relevant to the pathogenesis of neuropathic pain (Figure 1a). Initially, injury or disease affecting peripheral nerves induces axonopathy and demyelization in injured afferents and perhaps also in uninjured neighbours, leading to a phenomena termed membrane remodelling which consequently increases neuronal excitability, and greatly contribute to the formation of peripheral sensitization. This is due in a large part to subtype-selective abnormalities in the expression and trafficking of Na^+ channels and perhaps also to altered kinetic properties of unitary channels. The resulting excess discharge constitutes a primary neuropathic pain signal [5, 6].

Recently, there has been increasing evidence that hypersensitivity and pain that occurring under various pathological conditions is often due to up-regulated expression and /or increased sensitivity of transient receptor potential (TRP) channels. Several lines of evidence point to the involvement of members of TRPV (vanilloid receptor) family in the aetiology of neuropathic pain [7].

Sustained painful stimuli result in central sensitization, which is defined as heightened sensitivity of spinal neurons, reduced activation thresholds and enhanced responsiveness to synaptic inputs. Under neuropathy status, sustained ectopic discharge can maintain central sensitization indefinitely and with it “Aβ pain “and tactile allodynia. At present, it is widely accepted that central sensitization is largely mediated by the glutamatergic *N*-methyl-D-aspartate (NMDA) receptor (Figure 1b). In addition, some evidence has been demonstrated that the sympathetic nervous system also plays an important role in neuropathic pain by analgesia following sympathectomy in animals and humans [8, 9]. Furthermore, several lines of evidence indicated that immune cells, such as endoneurotrophilis and macrophages, might be involved in the initiation of neuropathic pain and its maintenance [10, 11].

1.1.4 Therapy of neuropathic pain

The pharmacotherapy is currently the mainstay of treatment in patients with neuropathic pain. Since hyperexcitability is associated with abnormal sodium channel regulation in neuropathic pain, alternative treatment strategies have targeted activity against specific cellular components involved in the pain-processing loop, either by blocking Na^+, Ca^{2+} and K^+ channels as to suppress neuronal excitability at the level of the spinal cord or by potentiating the effects of the various antinociceptive substances released via the central descending systems [12].



a b

Figure 1: (a) Tissue trauma or nerve injury leads to the release of inflammatory mediators as well as increased neuronal excitability at the site of injury, resulting in a reduction in the pain threshold at the site (peripheral hyperalgesia). Central sensitization is an increase in the excitability of spinal neurons as a result of persistent exposure to afferent input from peripheral neurons, CNS = central nervous system, BK = bradykinin, PGs = prostaglandins and 5-HT = serotonin

(b) Schematic representation of the activation of NMDA receptor in relation to pain. The activation of NMDA in response to the released glutamine results in the removal of Mg^{2+} which leads to the ca^{2+} influx; ca^{2+} ions flowing into cell activates the protein kinase C (PKC), an enzyme required for the synthetics of NO, followed by the activation of substance P that binds to the NK-1 receptor triggering *c-fos* gene expression and hypersensitivity.

A wide variety of drugs, such as 5% lidocaine patches, nonsteroidal anti-inflammatory drugs (NSAIDs), anticonvulsants (e.g. carbamazepine, gabapentin, lamotrigine), and tricyclic antidepressants (TCAs), e.g. amitriptyline hydrochloride, desipramine hydrochloride), could be used as first-line therapy in the treatment of neuropathic pain. The second-line drug, which mainly encompasses narcotics analgesics or NMDA receptor antagonist, could be applied to patients who do not respond to treatment with first line agents. The choice of medication should be directed toward the type of painful symptom described. Patients who do not respond to monotherapy

with any of the first- or second-line agents may respond to combination therapy or may be candidates for invasive therapy in pain clinic [13, 14].

Nowadays, pain research is directing on new molecular methods, such as gene therapy, stem cell therapy and viral vectors for delivery of biologic antinociceptive molecules. These methods could provide a new therapeutic approach to neuropathic pain relief. Pharmacological treatment for the symptoms of painful neuropathy, however, is still considered to be difficult, as the treatment effect is frequently variable between individuals. Traditionally, this variation has been explained by variable bioavailability, differences in intensities of pain stimuli and individual differences in pain perception. Recently, there is increasing evidence supporting that polymorphisms in some candidate genes may affect the expression and function in target product, leading to variability in pain perception or dose requirement of drugs used for pain treatment [15,16].

1.2 TRP channels and pain

Transient receptor potential (TRP) channels have been found in many cell types. Three TRP channels including TRPV1, TRPM8 and TRPA1, which have been shown to be expressed in primary afferent nociceptors, are gradually emerging as sensory transducers that may participate in the generation of pain sensations evoked by chemical, thermal and mechanical stimuli (as shown in Figure 2).

1.2.1 TRPV1

Since the molecular identification of the capsaicin receptor, now known as TRPV1, transient receptor potential (TRP) channels have occupied an important place in the understanding of sensory nerve function in the context of pain. Functional TRPV1 is expressed both in different level of neuron tissues like brain, dorsal root ganglion (DRG), trigeminal ganglion (TG), nodose ganglian (NG) neurons and in non-neuronal tissues which include gastric epithelial cells, airway epithelial cells, liver, vascular endothelium, mast cells and bladder. The increase in TRPV1 expression was suggested to contribute to the pathogenesis of various disease states, such as

multiple types of pain, vulvar allodynia as well as inflammatory bowel disease, pancreatitis and migraine [17, 18]. TRPV1 function has also been investigated in models of neuropathic pain. Several lines

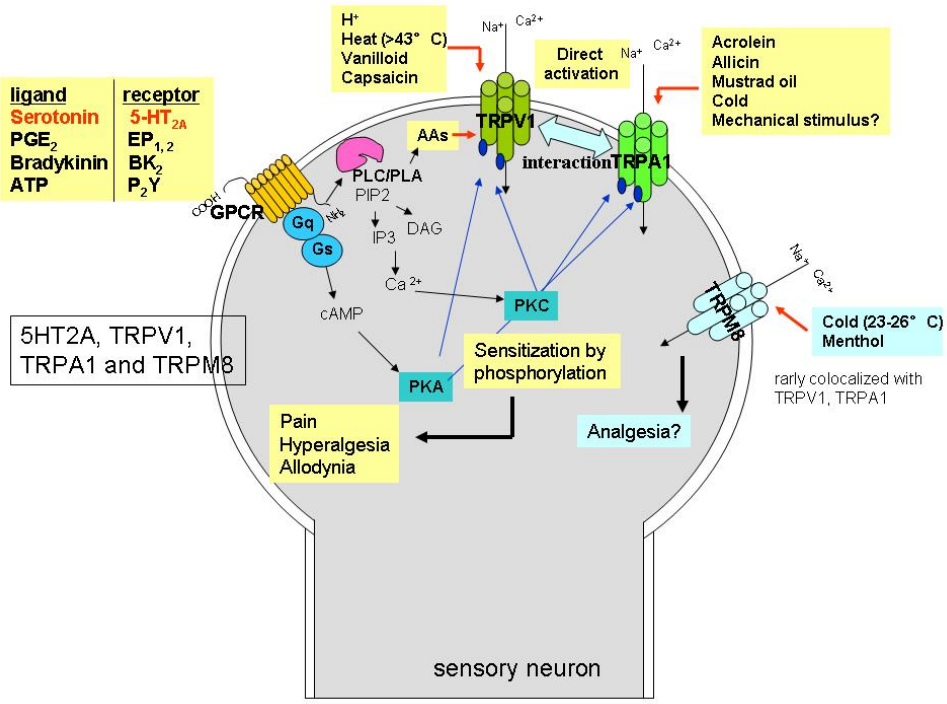


Figure2. Selected stimuli and intracellular pathways that contribute to the sensitization of TRP function in terminal primary sensory neurons. Besides activation by endogenous activating lipids via PLC and PLA pathways, the TRPV1 receptor can be activated by heat (>43 °C), acid and pungent vanilloid compounds. In contrast to the TRPV1 receptor, The TRPM8 receptor can be activated by many cooling compounds (23 °C-26 °C) and odorants like menthol. In addition, the TRPA1 receptor can be activated by endogenous inflammatory mediators and by diverse exogenous irritants, including mustard oil, garlic and allicin. Probably, TRPA1 is functionally coupled to TRPV1 and regulated by similar PLC-dependent sensitization pathways. However, whether the TRPA1 receptor respond to mechanical stimuli is unidentified. BK = bradykinin, PGs = prostaglandins and 5-HT = serotonin, PLA/PLC pathway = phospholipase A / phospholipase C, PGE2 = Prostaglandin E2, AA = arachidonic acid, GPCR = G-protein-coupled receptor, 5HT-2A = serotonin receptor.

of evidence point to the involvement of members of the TRPV family, especially TRPV1 in the aetiology of neuropathic pain [19]. It can be speculated that up-regulated expression of TRPV1 in myelinated fibers (A-neurons) is part of the

neuropathic pain phenotypic switch and contributes to hyperalgesia. TRPV1 also plays an important role in chemical and thermal hyperalgesia in a model of diabetic neuropathy. The application of *Trpv1*-specific siRNA VsiR1 for rats with neuropathic pain showed that the *Trpv1*-specific siRNA VsiR1 diminishes the frequency of cold-induced paw lifting by approximately 50%, indicating a reduction of cold allodynia [18]. More interestingly, animal experiment showed this receptor may have a protective role in the development of mechanical hyperalgesia [20].

The TRPV1 channel is encoded by the *TRPV1* gene, located on chromosome 17p13.3. Several exonic *TRPV1* polymorphisms in coding region have identified recently, including the two common single nucleotide polymorphism (SNP) rs222747 and rs8065080, also known as *TRPV1* Met315Ile and Ile585Val, which substitutes methionine to isoleucine at codon 315 and isoleucine to valine at codon 585, respectively. To date, there have been few studies in the literature reporting the association between genomic variations of the *TRPV1* and the pain perception or the development of the pathologic pain in humans. An *in vitro* study by Kayes et al [21] failed to confirm that the Ile585Val substitution alters the receptor function. Another study by Kim et al, however, suggested that this substitution at codon 585 significantly influences the cold withdrawal time (CWT) in Caucasian females [20]. Haplotypes analysis including these two polymorphisms were recently identified by Kim et al in a European Americans, but there was no significant association between short duration cold pain sensitivity and variations in *TRPV1* in a gender dependent manner [22]. Until now, the detailed mechanism explaining how these SNPs influence the mRNA expression and their relevant function *in vivo* is unclear. In addition, it remains open whether these polymorphisms in human could influence pain perception on the condition of neuropathic pain. Hence, further investigation to explore these associations between polymorphism and pain perception is necessary.

1.2.2 TRPM8

The TRPM8 channel is encoded by *TRPM8*, which is located on the chromosome 2q37.1. TRPM8 is expressed in prostate and in small-diameter trigeminal and dorsal

root ganglion (DRG) neurons, suggesting its specific expression in C-and possibly A-fibers, where TRPM8 functions as cold sensing pain pathway and sensor [20, 23]. TRPM8, also known as the menthol receptor, can be activated by many cooling compounds and odorants, which are dependent on different factors such as intra and extracellular Ca^{2+} concentration and PH [19, 20]. It has been previously proposed that TRPM8 is a candidate as sensory transducer contributing to pain hypersensitivity due to its property of responding to both innocuous and noxious temperature. Recently, it has been shown that ethanol is capable of inhibiting this channel as one of known TRPM8 modulators, but the exact mechanism how the ethanol affect or modulate the activity of TRPM8 channel is still poorly understood.

More interestingly, it has been demonstrated that activation of TRPM8 in sensory afferents of neuropathic pain rat models by either cutaneous or intrathecal application of pharmacological agent or modest cooling causes inhibition of sensitized pain response, thereby inhibiting the characteristic sensitization of dorsal-horn neurons and behavioural-reflex facilitation. The mechanism of analgesic effect in TRPM8 activation could be due to the central mediation and possibly relies on group II/III metabotropic glutamate receptors (mGluRs), which very likely respond to glutamate released form TRPM8-containing neurons to suppress nociceptive inputs [24].

There is increasing evidence that mutation in coding regions could alter the function of the TRPM8 channel. Colburn et al showed that sensory neurons derived from *trpm8* null mice lack detectable levels of TRPM8 mRNA and protein and that the number of these neurons responding to cold (18°C) and menthol (100 mM) is greatly decreased. Furthermore, compared with WT mice, null mice display deficiencies in certain behaviours; these results suggest that TRPM8 may play an important role in certain types of cold-induced pain [25]. However, in a study in humans, Kim et al could not find any association between *TRPM8* and cold or heat pain sensitivity and speculated that TRPM8 receptor was possibly not activated by cold temperature (2-4°C) used [22].

1.2.3 TRPA1

The TRPA1 protein encoded by the *TRPA1* gene, is located on the chromosome 8q13. As a member of the TRP family of ion channels, TRPA1 is mainly expressed in the inner ear, trigeminal and DRG neurons, where there have been proposed roles in sensing sound, painful cold, and irritating chemicals [19].

Traditionally, the TRPA1 was proposed as a candidate for the hair-cell transduction channel, but Kwan and colleagues found that TRPA1 seems to be unnecessary for hair-cell transduction [26]. The observation of *trpa1*−/−(lacking *trpa1*) mice revealed that neither hearing behaviour nor transduction currents changed in response to bundle deflections, further confirming the possibility that another TRP channel can compensate the loss of TRPA1.

The majority of studies point to an important role for TRPA1 in the pain response to endogenous inflammatory mediators and to diverse exogenous irritants, including mustard oil, garlic, wintergreen oil, clove oil, ginger and cinnamon oil, all of which elicit acute painful burning or pricking sensation [20]. Some behavioural studies in mice lacking *trpa1* confirmed these effects in nociception. More interestingly, being consistent with a role in nociception, TRPA1 is highly co-expressed with TRPV1 in small-diameter peptidergic nociceptors while it is rarely co-expressed with TRPM8.

TRPA1 has also been linked to cold hyperalgesia in the condition of neuropathic pain. Both inflammation and nerve injury increase the expression of TRPA1 in DRG neurons. Such over-expression, which appeared to be driven by nerve growth factor (NGF)-engaging the p38mitogen-activated protein kinase pathway (MAPK), contributes to injury-induced cold hyperalgesia because *trpa1* knock down by siRNA strategies suppressed cold hyperalgesia in a rat nerve injury model. Obata et al further confirmed that NGF-induced TRPA1 increase in sensory neurons via p38 activation is necessary for cold hyperalgesia [27]. Therefore, blocking TRPA1 in sensory neurons might provide a fruitful strategy for treating cold hyperalgesia caused by inflammation and nerve damage.

From some animal experiments, it has been obviously shown that mice *trpa1* gene knock out or *trpa1* antagonist exhibited behavioural change in response to the pain stimuli, which illustrated the important role of TRPA1 in pain perception. Genomic variations may alter protein expression or possibly have significant consequences on the function of the protein. Up to date, there have been few data suggesting that polymorphism in the *trpa1* gene might contribute to the interindividual variation in pain perception. For example, in the study by Kim et al [22], it could be demonstrated that homozygote carriers of the intronic *TRPA1* G38218A variant (rs1198795) have less pain tolerance to the cold stimuli compared to homozygote G carriers. But detailed knowledge of regulation is less available. Hence, more research work intended to clarify clinical impact of these genetic variations is required.

1.3 Aim of the study

Candidate gene studies on the basis of biological hypotheses have been a practical approach to identify relevant genetic variation in complex traits. There is growing evidence showing that single nucleotide polymorphisms (SNPs) in candidate genes, including TRPV1, TRPA1 and TRPM8, may influence pain sensitivity in animal models of neuropathic pain. However, the exact role of these SNPs in pain perception, interpretation and behavioural expression in humans are currently unknown. As we know, the research for predictive SNPs within genes may require the examination not only of the exons, but also of the promoters and/or the area of intron-exons boundaries and mRNA processing signals. Therefore, in this study, two SNPs were selected from TRPV1, 3 SNPs from TRPA1 and 6 SNPs from TRPM8. The SNPs were located in exonic and promoter regions and were examined for their frequency in a large sample of patients with different neuropathic pain entities and healthy controls. They were further investigated for their effects on pain sensitivity.

2 MATERIALS AND METHODS

2.1 Patients

The study was carried out in cooperation with the German Research Network on Neuropathic Pain headed by Prof. Dr. Ralf Baron, Division of Neurologic Pain Research, Department of Neurology at the University Hospital Schleswig-Holstein Campus Kiel and Prof. Dr. Dr. Thomas Toelle, Department of Neurology at University Medical Centre Rechts der Isar of the Technical University Munich. The study was performed in accordance to the principles of the Helsinki declaration. All patients gave their written informed consent and the ethics committee of the medical faculty of the University of Kiel and the local ethics committees of the participating centres approved the study. We analyzed the neuropathic pain patient population in a primary study. All patients were Caucasians and unrelated to each other. So, a total of 296 patients were recruited to the current study for genotyping. Due to the failed collection of clinical data in some cases, there were 236 patients with a mean age of 57.4 years (140 women, 96 men) investigated by further statistic analysis (Tab. 1).

Table 1: Demographic data of 236 neuropathic pain patients

Subgroup	number (male/female)	Age (years)
Central pain	9/8	51.1 ± 11.7
Ccomplex regional pain syndrome	9/47	51.1 ± 13.3
Peripheral nerve injury	13/14	52.6 ± 15.3
Post-herpetic neuralgia	5/22	52.2 ± 16.7
Polyneuropathy	42/29	67.4 ± 12.0
Trigeminal pain	13/17	60.9 ± 12.7
Other neuropathy	5/2	56.5 ± 15.7

Data expressed as the number of patients, mean ± SD

2.2 Volunteers

For comparison of the genotype distribution, 252 healthy volunteers were enrolled at the Institute of Pharmacology of the Kiel University under a clinical protocol approved by ethics committee of the medical faculty of the University of Kiel. All volunteers gave their written informed consent and were German Caucasians.

2.3 Experimental pain test measurements

In each participating centre, all 13 QST procedures were performed by trained observers using the same equipment and standardized instructions to the patients. All test techniques are based on the quantitative sensory testing of the German Research Network on Neuropathic Pain (DFNS) [28, 29].

2.3.1 Thermal detection, thermal pain thresholds and paradoxical heat sensation

The thermal test includes CDT (cold detection threshold), WDT (warm detection threshold) , PHS (paradoxical heat sensation), TSL (thermal sensitivity limen) and CPT (cold pain threshold). Cold and warm detection thresholds were firstly measured. In addition, patients were asked about paradoxical heat sensations (PHS) during the thermal sensory limen (TSL) procedure of alternating three times of warm and cold stimuli. Then cold pain and heat pain thresholds were determined (CPT, HPT).

2.3.2 Mechanical detection threshold for modified von Frey filaments

The mechanical detection threshold (MDT) was measured with a standardized set of modified von Frey hairs that exert forces upon bending between 0.25 and 512 mN. Five threshold determinations were made by using the “methods of limits”, the final threshold was the geometric mean of these five series. Similarly, the mechanical pain threshold (MPT) was measured using custom-made weighted pinprick stimuli as a set of seven pinprick mechanical stimulators, which usually were applied at a rate of 2 s on, 2 s off in an ascending order until the fist percept of sharpness was reached.

2.3.3 Mechanical pain threshold for pinprick stimuli and dynamic mechanical allodynia

Mechanical pain sensitivity (MPS) was assessed using the same set of pinprick stimuli to obtain a stimulus-response function for pinprick-evoked pain. Patients were asked to give a pain rating for each stimulus on a “0-100” numerical rating scale. Furthermore, dynamic mechanical allodynia (ALL) was assessed as part of the test

above, using a set of three light tactile stimulators (cotton wisp, cotton wool and brush) as moving innocuous stimuli.

2.3.4. Windup ratio-temporal pain summation for repetitive pinprick stimuli

In this test, after a single pinprick stimulus and a series of 10 repetitive pinprick stimuli of the same physical intensity separately applied to the patients, patients were asked to give a pain rating representing the single stimuli, and the estimated mean over the whole series of 10 stimuli using a "0-100" numerical rating scale. Wind-up ration (WUR) was calculated as the ratio: mean rating of the five series divided by the mean rating of the five single stimuli.

2.3.5 Vibration detection threshold and pressure pain threshold

The vibration detection threshold (VDT) was recorded at a time point when patients could not feel the vibration of tuning fork placed over a bony prominence. VDT was determined as a disappearance threshold with three stimulus repetitions. In addition, the Pressure pain threshold (PPT) was measured by using a pressure gauge device. The PPT was determined with three series of ascending stimulus intensities, each applied as a slowly increasing ramp of 50 kPa/s (~5 kg/cm^2 s).

2.3.6 Test side and control side

This test stimuli were applied in runs alternating between the affected test side and a contralateral non-afftected control site.

2.3.7 Data evaluation

In order to describe which measure is more sensitive to detect sensory plus and minus signs, the absolute reference data were used to normalize test results of individual patients by calculating the z-transform: $Z=(value_{patient}–mean_{controls})/SD_{controls}$. Subjects were blinded with regard to the temperature of the stimulus.

2.4 Genotyping

To study possible association between neuropathic pain and single nucleotide polymorphism (SNP) in candidate genes (Table1), a total of 11 single nucleotide polymorphism in 296 patients and in 253 volunteers was genotyped by PSQ, respectively. Besides volunteers, prepared DNA was obtained from Department of Neurology of the Munich University Medical Centre and all DNA sample stored at -4 °C until PCR reactions were performed. Primer and pyrosequencing primer were designed by PSQ assay design software (Biotage, Uppsala, Sweden)

2.4.1 Polymerase Chain Reaction (PCR)

DNA fragment containing polymorphic sites were amplified from genomic DNA using specific primers (as showed in Tab. 2), which were designed by pyrosequencing PSQ assay design software (Biotage, Uppsala, Sweden). DNA amplifications for these SNPs were typically performed under similar conditions for each reaction with 1µl of genomic DNA, 0.2 µM of forward and reverse primers (TIB Molbiol, Berlin, Germany), 2.5 µM magnesium chloride, 1.0 µM dNTPs (Biotage) and 0.15 µl of *Taq* DNA polymerase (Invitrogen, Karlsruhe, Germany) , in a total volume of 25 µl (Tab. 3). PCR was performed under the following conditions: initial denaturation for 4 min at 94 °C, followed by 50 cycles at 94 °C for 30 s, annealing at 63 °C (61 °C for SNP rs13268757) for 30s and at 72 °C for 30 s, and 7 min at 72 °C (Tab. 4). All PCR amplifications were carried out using a GeneAmp 9700 thermocycler (Applied Biosystems, Darmstadt, Germany). For controlling the PCR reaction, the DNA fragments were separated on a 2.0% agarose gel (AppliChem, Darmstadt, Germany) and visualized after ethidium bromide staining by a digital image station (Kodak, Stuttgart, Germany).

Table 2: Primers used for genotyping of polymorphisms in three TRP genes

Gene	SNP ID	mRNA location	Primer name	Primer sequence
TRPV1	rs222747	G1103C	TRPV1-1	F:5'ACG AAG TTT GTG ACG AGC ATG TA
				R:5'Bio-TCC CTT CTT GTT GGT GAG CT
				S:5'-ATG TAC AAT GAG ATT CTG AT
	rs8065080	A1911G	TRPV1-2	F:5'-Bio-GCG CTG ACC AAG CTC ATT ACC T
				R:5'-TGG CCT CGG CTC TGA TGA
				S:5'-CCG TTT CAT GTT TGT CTA
TRPM8	rs17862932	C2235T	TRPM8-4	F:5'-GGG TCC AGG AAG AAA CCT GTC
				R:5'-Bio-GCG ATC TAG AAG ACC ACA TTC C
				S:5'-GTA CTA TGTTGT GGT GTT CTT
	rs7593557	G1296A	TRPM8-3	F:5'-Bio-GAG TCT TTT CCT GCC CTC
				R:5'-CGG TCA TTG GTG AAA ATC TCA T
				S:5'-CAG TTA TCC TTG TCT TGC
	rs13004520	G780C	TRPM8-1	F:5'-TTT AGC CCA GTA CCT TAT GGA TG
	rs28901637	A787T		R:5'-Bio-CTA GCT GAT TCC GGA GCT TTG
	rs11562975	G790C		
	rs17868387	A792G		S:5'-TTA TGG ATG ACT TCA CAA
TRPA1	rs13268757	C182T	TRPA1-1	F:5'-Bio-CTC CAG GGC GCC ACA TCT
				R:5'-TTT CGC TGC CTG TGA GCT G
				S:5'-GGT GGG GTC AAT GAA
	rs920829	G710A	TRPA1-2	F:5'-TGA TCA TTG CGT GCA CCA C
				R:5'-Bio-TCC TCT TAA GCG GGG AGT ACA TAC
				S:5'-CGT GCA CCA CAA ATA AT
	rs959976	A3228G	TRPA1-3	F:5'-Bio-TTG TCT TAT TTC CCC AGT GCA A
				R:5'-TGG CAC ATG CTT AAG AAA AAG TTC
				S:5'-ATT TTT TAT CCG ACA GC

Abbreviations: F, forward primer; R, reverse primer; S, sequencing primer; dNTP, deoxynucleotide triphosphates; Dispensing order: sequence order given by Pyrosequencing software according to each analyzed template sequence.

Formatierte Tabelle

Table 3: PCR reaction reagents used for amplification of SNPs in three *TRP* genes**.**

Reagent	Volume [µl]	Concentration
Taq-buffer (10x)	2.5	1 x
$MgCl_2$ (50 mM)	1.25	2.5 mM
dNTPs (10 mM)	2.5	1 mM
Primer forward (10 mM)	0.5	200 nM
Primer reverse (10 mM)	0.5	200 nM
Taq-DNA-polymerase (5U/µl)	0.15	0.03 U/µl
DNA (30-100 ng/µl)	1.0	
H_2O	16.9	
Total volume	25.0	

Table 4: Thermocycler conditions of PCR-reaction used for amplification of SNPs in three *TRP* genes**.**

Denaturation	Denaturation	Annealing	Elongation	Terminal elongation
4 min	30 s	30 s	30 s	7 min
94 °C	94 °C	63 °C*	72 °C	72 °C
		45 cycles		

* 61 °C for SNP rs13268757

2.4.2 Pyrosequencing (PSQ)

10 µl of PCR-reaction product were aliquoted in each well with 30 µl of pure water (Biochrom AG; Berlin, Germany) and 37 µl of binding buffer (Biotage) as well as 3µl streptavidine-coated sepharose beads (GE Healthcare Bio-Sciences, Uppsala, Sweden), the mixture were incubated in a shaker for 20 min. Afterwards using the Vacuum Prep workstation, biotinylated single-stranded PCR fragments were treated with 70% ethanol (Merck, Darmstadt, Germany), 0.1%mol/l NaOH and washing buffer (Biotage),and transferred to 96-well plates, containing annealing buffer and 10 pmol of sequencing primer in a total volume of 12 µl each. Hybridization was applied by incubation at 80 °C for 2 min, the plate was cooled to room temperature.

According to the volume information, the enzymes, which include DNA polymerase, ATP sulfurylase, luciferase and apyrase, the substrates including adenosine 5' phosphosulfate (APS) and luciferin, as well as the nucleotides were filled in the tips stored in a dispensing tip holder, from where they were added automatically into each well of the 96 wells plate containing the sequencing primer /PCR product mix.

The pyrosequencing technique is based on the detection of pyrophosphate released during polymerization of DNA as illustrated in Figure 3.

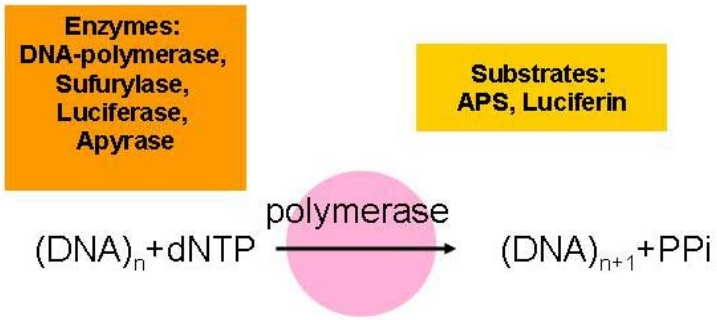


Figure 3a: Step 1. A polymerase catalyzes incorporation of nucleotide acid chain. As result of the incorporation, a pyrophosphate molecule (ppi) is released and subsequently converted to ATP by ATP sulfurylase.

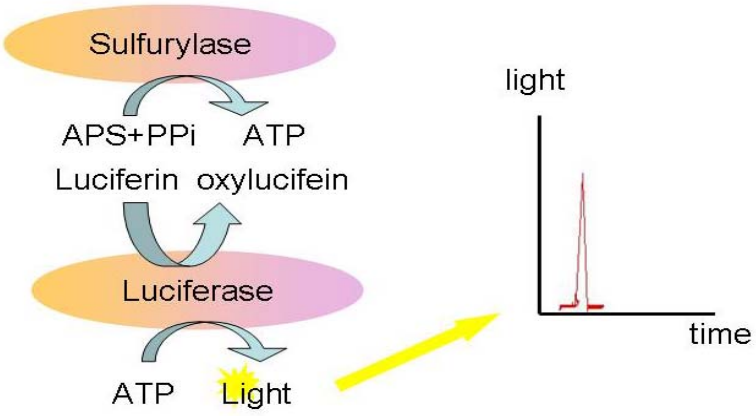


Figure 3b: Step 2. Meanwhile, light is produced in the luciferin reaction during which a luciferin molecule is oxidized. The light is proportional to the amount of incorporated nucleotide and seen as a peak in the program

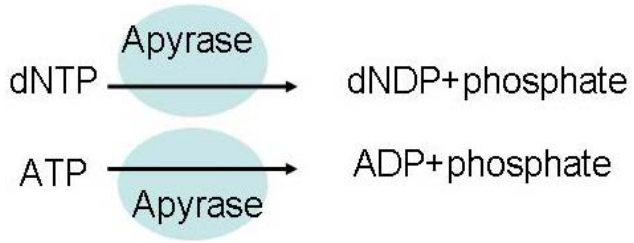


Figure 3: Step 3. The excess of the added nucleotide wil be degraded by apyrase, the produced ATP also will rapidly be degraded by apyrase.

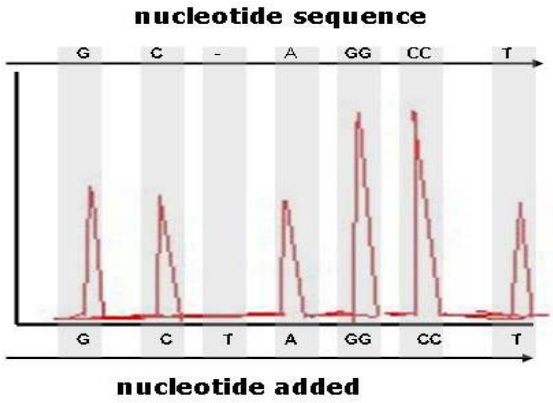


Figure 3c: Step 4. Pyrosequencing raw data obtained as pyrogramme, in which proportional peak signals represent one or two base incorporations. Nucleotide addition, according to the dispending order, is indicated below the program and the obtained sequence in indicated above the program.

2.5 Statistical analysis

Descriptive data are presented as mean±SD. The Kruskal-Wallis test and the Jonckheere-Terpstra test were performed to test the association between SNPs and 13 pain test parameters, using computer software SPSS (version 11.5). A Kruskal-Wallis test was used to determine whether continuously measured QST parameters differed according to variants location, and to determine the association between the candidate SNPs and the QST parameters. The Jonckheere-Terpstra test was used to assess the statistical significance of the trend in continuously measured

QST parameters across genotypes. In addition, Mann-Whitney U-test (multiple comparison) was used to compare the difference dependent on genotype either on test side or on control side. To exam the equality of the allele and genotype frequencies between the patients and controls, Statcalc (version 6) was used for calculation of odds ratios. Haplotype analysis was performed using the SHEsis software, which provided free service from online. A P-value below 0.05 was considered to be statistical significant and calculated as two-sided significance.

3. RESULTS

3.1. The characteristics of seven subgroups of neuropathic pain

Mean values of all quantitative sensory testing (QST) parameters of test and control sides are shown in Figure 4. These QST parameters were objectively designed to detect pain patterns after partial nerve lesion, which is indirectly consistent with the characteristics of different afferent nerve fibers (nociceptive C-fibers and non-nociceptive myelinated A-fibers). Theoretically, cutaneous sensitivity is mediated by various populations of Aβ, Aδ, and C-fiber afferents. Brush and touch by a blunt von Frey type probe predominately activate Aβ-fiber low–threshold mechanoreceptors. Gently cooling the skin activates Aδ-fiber thermoreceptors. Contact with sharp objects such as pinpricks predominantly activates Aδ-fiber nociceptors. Gently warming the skin activates C-fiber thermoreceptors. Painful heat stimuli activate both Aδ and C-fiber nociceptors, but due to their lower thresholds only C-fibers are involved in heat pain threshold. Therefore, based on the quantitative sensory testing (QST), the Aβ-fiber can be represented by VDT and MDT, whereas the C-fiber function is represented by the WDT and HPT. The presence of abnormal cold pain threshold (CPT) indicates a disturbance of Aδ-cold sensation.

The normal range from plus 2 to minus 2 in patients profile was presented by grey zone. The minus z-score represents loss of sensory function, those minus z-score outside of normal range shows significant loss of sensory function such as hypoesthesia. In contrast, the plus mean z-score above normal range represents the

gain of sensory function like hyperalgesia or allodynia.

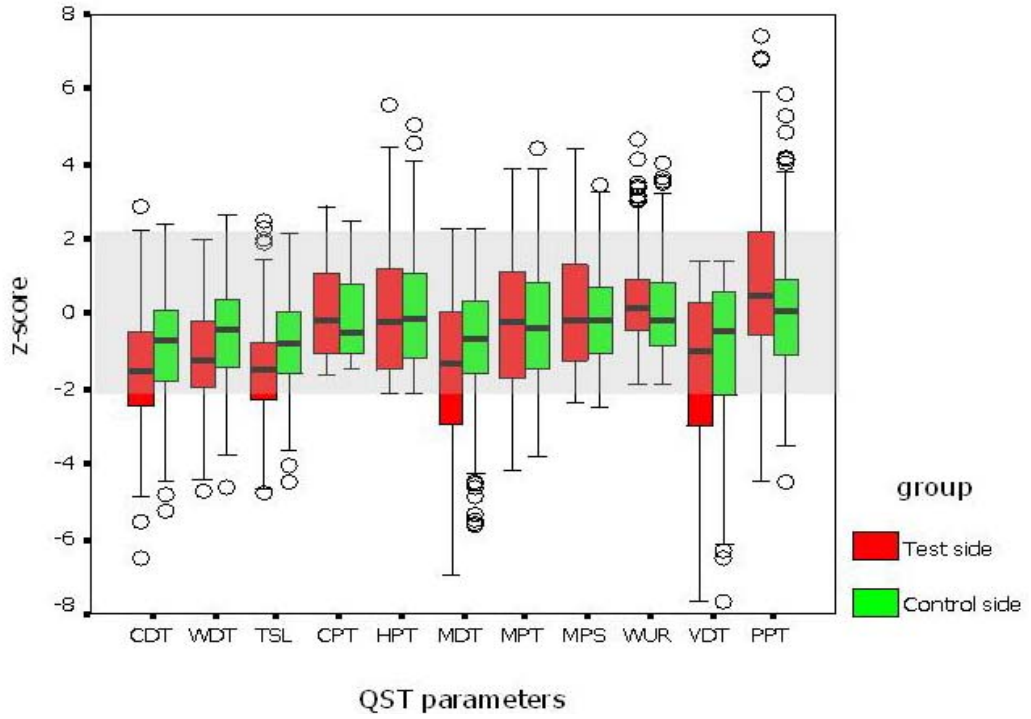


Figure 4: Z- score sensory profiles of 235 patients on both test and control sides (test side represented the affected side of neuropathic pain, whereas the control side signified the non-affected side of neuropathic pain). These patients, who are being scattered among seven types of neuropathic pain, exhibited different response to 11 items of QST parameters applied. Since the majority of mean value of z-score was distributed within the normal range, the patients were subsequently divided into subgroups for further investigation.

To further illustrate the characteristics of subgroups of neuropathic pain, patients were divided into seven subgroups, including central pain, complex regional pain syndrome(CRPS), peripheral nerve injury, polyneuropathy, trigeminal pain, post herpetic neuralgia and other neuropathy. The comparison data on both test and control side among subgroups are shown in Figures 5 a-g. Compared to the control side, central pain patients apparently exhibited hypoesthesia in CDT, WDT, TSL MDT and VDT on test side (Figure 5a). Similarly, hypoesthesia with regard to MDT and VDT was also observed in post herpetic neuralgia (PHN) (Figure 5e), polyneuropathy (Figure 5f) and peripheral nerve injury patients (Figure 5d), respectively. On the

contrary, the CRPS patients showed static hyperalgesia to blunt pressure (PPT) on the test side (Figure 5b). In the trigeminal pain (Figure 5g) and other neuropathy groups (Figure 5c), there is no obvious loss or gain of sensory function by comparing test and control side.

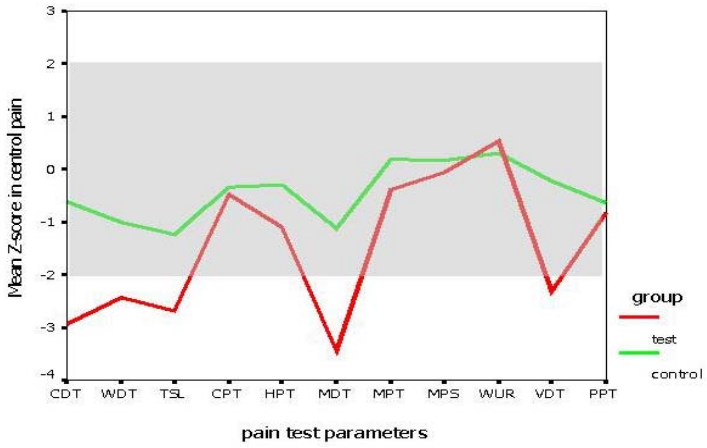


Figure.5a. Pain patterns of test and control side in central pain patients. Compared to the control side, central pain patients apparently exhibit hypoesthesia to thermal stimuli (CDT, WDT, TSL) and VDT at test side.

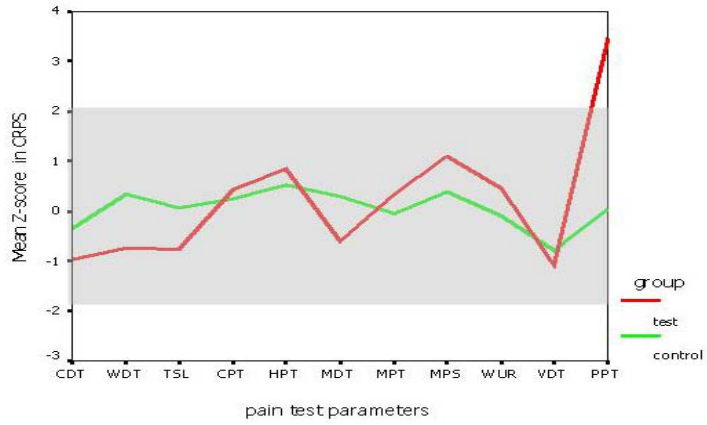


Figure 5b: Pain patterns of test and control side in complex regional pain syndrome (CPRS) patients. QST-profile of test side shows static hyperalgesia to blunt pressure (PPT). There was neither allodynia nor hyperalgesia on the control side.

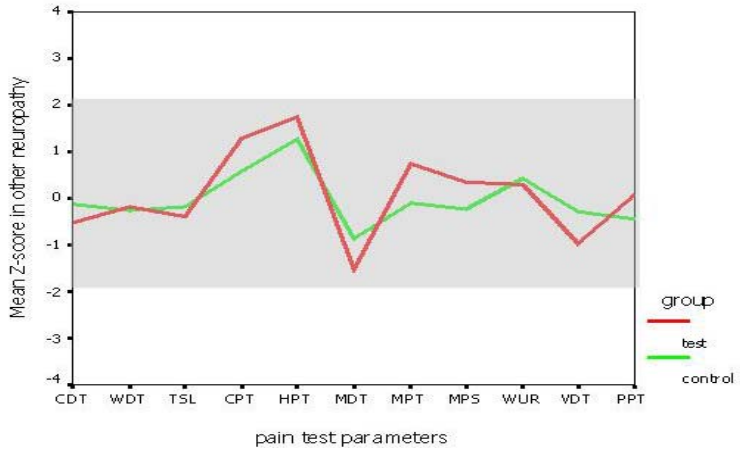


Figure 5c: Pain patterns of test and control side in other neuropathy subgroup. Neither hypralgesia nor alodynia was observed on test side and control site.

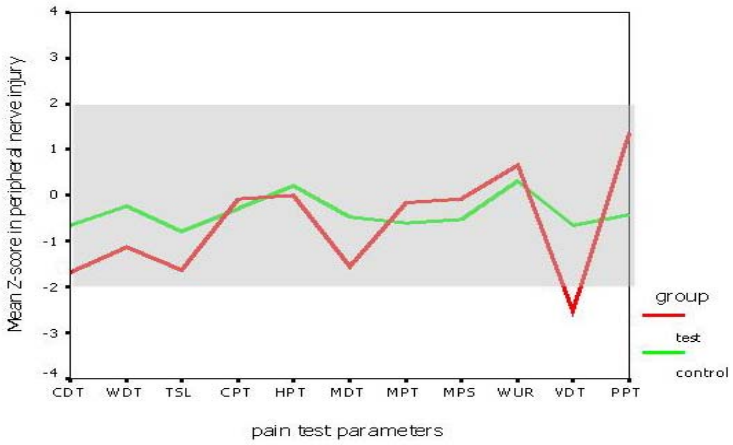


Figure 5d: Pain patterns of test and control side in peripheral nerve injury patient. QST-profile of the test side referred to the affected side confirms hypoesthesia in vibration detection threshold (VDT).

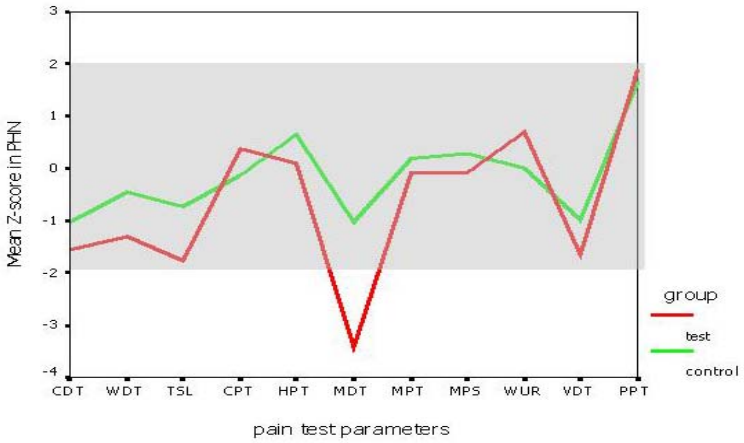


Figure 5e. Pain patterns of test and control side in post-herpetic neuralgia (PHN) patient, Comparing the control side, the mechanical detection threshold (MDT) of test side decreased dramatically, exhibiting tactile hypoesthesia.

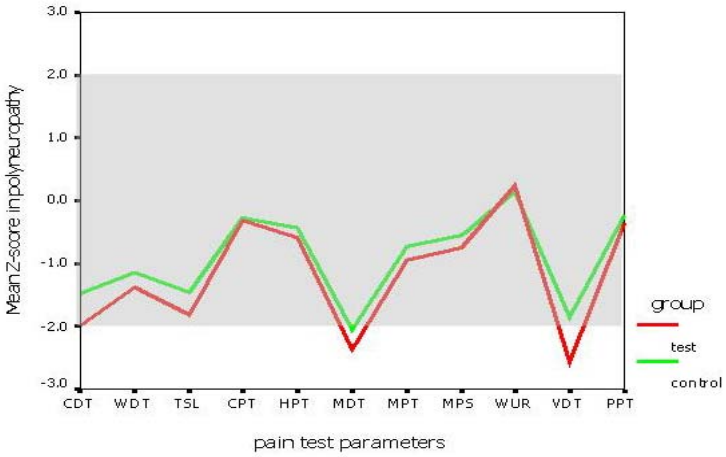


Figure 5f: Pain patterns of test and control side in polyneuropathy patients. QST-profile of test side compared to the control side suggests tactile hypoesthesia (MDT) and hypoesthesia in vibration detection threshold (VDT), even though the pattern of control side is more close and similar to the test side.

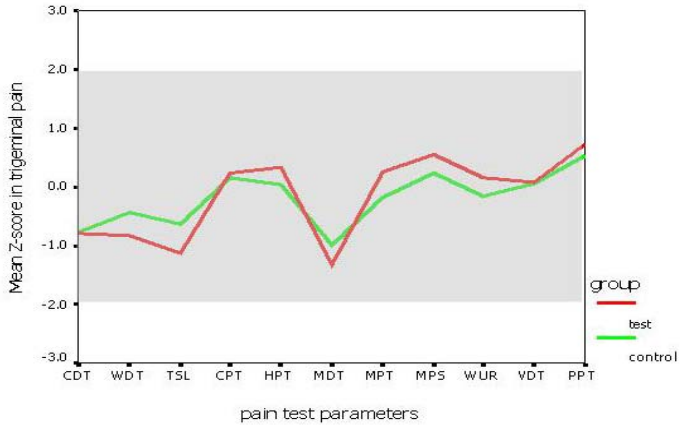


Figure 5g: Pain patterns of test and control side in trigeminal pain patients. QST-profile of the control side versus test side shows no significant difference.

3.2. The distribution of genetic polymorphisms

The genotype distribution of all investigated polymorphisms showed no deviations from Hardy-Weinberg equilibrium neither in patients nor in controls. To evaluate whether the genotypes investigated may act itself as susceptibility factors for neuropathic pain, the genotype and allele frequencies were compared between the case group and healthy volunteers. For most genotypes and alleles, there was lack of evidence of an association, since there was no statistical difference (P>0.05). However, the G variant of the 1911A>G polymorphism in *TRPV1* was more frequent in patients than in controls (42.8%) than in the controls group (36.0%, OR=1.28, 95% CI 1.00-1.65, *P*=0.04) (Table 5).

In a second approach, the linkage disequilibrium (LD) between each pair in *TRPV1* as well as *TRPA1* was checked by using SHEsis software. Two polymorphisms (2235 C>T and A787T) in *TRPM* 8 were not included into the haplotype calculation due to the low frequency. All the *P*-value corresponding to haplotypes are shown in Table 6. There was significant difference between patients and controls groups in the haplotype C-A consisting of *TRPV1* 1103 T>C and 1911A>G. The frequency of haplotype C-A was lower in patients (46.1%) than in controls (53.8%, ,OR=0.73,95%CI 0.57-0.94) (p=0.02). The global haplotype frequency,

however, showed no significant differences between patients and controls in *TRPA1* (χ^2=6.44, P=0.09*), TRPV1* (χ2=1.62, P=0.65) and *TRPM8 (*χ^2=0.67, P=0.72*)*.

Tab. 5: Allele frequencies and frequencies of genotypes in 296 patients and in 253 volunteers

Gene	Location SNP		Allele	Allele Frequency (%)		p-value	Odds ratio	Geno-type	Frequency of genotype(%)		p
	mRNA	Protein		Pat	Controls	d.f.=1	(95%)CI		Pat	Controls	d.f.=2
TRPV1	G1103C	Met315Ile	C	75.0	72.8	0.4	1.122	CC	56.6	52.4	0.63
			G	25.0	27.2		(0.85-1.48)	CG	36.8	40.7	
								GG	6.6	6.9	
	A1911G	Ile585Val	A	63.2	57.2	0.04	1.285	AA	41.0	33.2	0.14
			G	36.8	42.8		(1.00-1.65)	AG	44.4	48	
								GG	14.6	18.8	
TRPM8	C2235T	Thr762Ile	C	100	99.8	0.93	-	CC	100	99.6	0.93
			T	0.0	0.2			CT	0.0	0.4	
								TT	0.0	0.0	
	G1296A	Ser419Asn	G	93.6	94.9	0.48	0.83	GG	87.5	91.1	0.43
			A	6.4	5.1		(0.49-1.38)	GA	12.2	7.6	
								AA	0.3	1.3	
	G780C	Arg247Thr	G	94.2	95.8	0.22	0.42	GG	88.3	92.4	0.05
			C	5.8	4.2		(0.81-2.47)	GC	11.7	6.8	
								CC	0.0	0.8	
	A787T	Pro249Pro	A	99.5	99.4	0.82	1.17	AA	99.0	98.8	0.82
			T	0.5	0.6		(0.19-7.26)	AT	1.0	1.2	
								TT	0.0	0.0	
	G790C	Leu250Leu	G	88.9	90.4	0.41	1.17	GG	77.7	81.2	0.31
			C	11.1	9.6		(0.79-1.75)	GC	22.3	18.4	
								CC	0.0	0.4	
	A792G	Tyr251Cys	A	94.3	95.6	0.35	0.77	AA	88.7	92	0.08
			G	5.7	4.4		(0.44-1.38)	GA	11.3	7.2	
								GG	0.0	0.8	
TRPA1	C182T	Arg3Cys	C	86.0	85.0	0.66	1.08	CC	70.1	72.3	0.54
			T	14.0	15.0		(0.76-1.52)	CT	29.9	27.3	
								TT	0.0	0.0	
	G710A	Glu179Lys	G	89.0	89.4	0.83	1.04	GG	79.7	80.4	0.98
			A	11.0	10.6		(0.70-1.53)	GA	18.6	18.0	
								AA	1.7	1.6	
	A3228G	His1018Arg	A	83.3	83.7	0.84	0.97	AA	70.0	69.1	0.36
			G	16.7	16.3		(0.70-1.33)	AG	26.6	29.3	
								GG	3.4	1.6	

P-value of genotype was calculated by using wild-type homozygotes vs. heterozygotes and variant homozygotes

Tab. 6: Estimated haplotype frequencies in 296 patients and 253 controls

TRPV1 G1103C	A1911G		*Frequency* (%)		*P*-value	OR (95% CI)	Global result
haplotype			Pat	Control			
1	C	A	53.6	46.1	0.02	1.35(1.06-1.73)	χ^2=6.44
2	C	G	21.4	26.7	0.05	0.75(0.56-1.00)	df=3
3	G	A	9.6	11	0.44	0.85(0.57-1.28)	*P*=0.09
4	G	G	15.4	16.2	0.74	0.94(0.67-1.32)	

TRPA1 C182T	G710A	A3228G	*Frequency* (%)		*P*-value	OR (95% CI)	
haplotype			Pat	Control			
1	C	A	A	0.4	1.3	-	-	χ^2=0.98
2	C	A	G	10	9.2	0.67	1.10(0.72-1.66)	df=3
3	C	G	A	75	73.6	0.92	1.01(0.76-1.35)	*P*=0.81
4	C	G	G	0.4	1.4	-	-	
5	T	A	A	0	0	-	-	
6	T	A	G	0	0	-	-	
7	T	G	A	9.1	7.7	0.38	0.82(0.53-1.27)	
8	T	G	G	5.9	5.4	0.74	1.09(0.64-1.85)	

TRPM8 G1296A	G780C	G790C	A792G	*Frequency (*%)		*P*-value	OR (95% CI)	
haplotype								
1	G	G	G	A	82.9	84.7	0.44	0.88(0.62-1.23)	χ^2=0.67
2	G	G	C	A	10.4	9.6	0.66	1.10(0.73-1.64)	df=2
3	A	C	G	G	5	4.2	0.51	1.21(0.68-2.15)	*P*=0.72
4	G	C	G	G	0.2	-	-	-	
5	G	C	G	A	0.2	-	-	-	
6	G	G	G	G	-	0.2	-	-	
7	A	C	C	G	0.6	-	-	-	
8	A	G	C	A	0.1	-	-	-	
9	A	G	G	A	0.6	1.2	-	-	

The estimated haplotype frequency of patients and controls was compared by using SHEsis software platform.

3.3. Impact of polymorphisms on pain perception

To explore the association between selected SNPs and 13 QST parameters, Kruskal-Wallis test and Jonckheere-Terpstra test were performed to test for subgroups. The results are presented from Table 7 to Table 12. There was a clear correlation between *TRPA1* polymorphisms 710G>A and MPT and MPS,

as well as between 3228A>G and MPT and MPS of test side in central pain subgroup

(710G>A, *P*=0.021 and *P*=0.021, respectively, Kruskal wallis test; 3228A>G, *P*=0.007 and *P*=0.007, respectively, Kruskal wallis test, as shown in
Table 6).The homozygote A-allele carrier in 710G>A showed less sensitivity to pain perception than GA allele at locus 710 in mechanical pain sensitivity (MPS) measurement (P=0.028 Mann-Whitney U test) (Figure 6a). Moreover, in machincal pain thresholds (MPT) measurement, the heterozygote 710GA seems less sensitive to pain perception than homozygote G allele carrier (Figure 6b). The MPT z-score of test side in *TRPA1* polymorphism A3228G indicated that the homozygote 3228G carriers were more likely to be loss of sensitivity than homozygote 3228A and heterozygote GA carriers (P=0.032 and P=0.048, respectively (Figure 6C). Similarly, the comparison of MPS z-score of test side apparently showed that the *TRPA1* homozygote 3228G carriers were also more insensitive to the pain perception than homozygote 3228A but not heterozygote carriers(P=0.03 Mann-Whitney U test).

In CRPS patients, statistically significant associations were found between the *TRPV1* polymorphism 1911A>G and MPS on test side (P=0.003). The homozygote 1911G carrier showed less sensitive to pain perception than homozygote 1911A (P=0.011) (Figure 6e). Compared the cold detection threshold (CDT) and thermal sensitivity limen (TSL) with each SNP, there was also markedly significant difference in *TRPA1* polymorphisms 1911A>G (P=0.03 and P=0.046, respectively, Kruskal wallis test) on control side, the further comparison depending on the genotypes indicating patients carrying homozygote 3228G was more sensitive to cold stimui than heterozygote 3228AG and homozygote 3228A carriers (P=0.026 and P= 0.024, respectively, Mann-Whitney U test) (Figure 6g).

Tab. 7: Association between pain perception and SNPs from 3 TRP genes in 17 central pain patients.

		CDT		WDT		TSL		CPT		HPT		MDT	
		T	C	T	C	T	C	T	C	T	C	T	C
Genes	SNPs												
TRPV1	G1103C	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
	A1911G	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
TRPM8	C2235T	a	a	a	a	a	a	a	a	a	a	a	a
	G1296A	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
	G780C	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
	A787T	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
	G790C	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
	A792G	a	a	a	a	a	a	a	a	a	a	a	a
TRPA1	C182T	a	a	a	a	a	a	a	a	a	a	a	a
	G710A	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
	A3228G	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s

		MPT		MPS		WUR		VDT		PPT	
		T	C	T	C	T	C	T	C	T	C
Genes	SNPs										
TRPV1	G1103C	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
	A1911G	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
TRPM8	C2235T	a	a	a	a	a	a	a	a	a	a
	G1296A	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
	G780C	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
	A787T	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
	G790C	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
	A792G	a	a	a	a	a	a	a	a	a	a
TRPA1	C182T	a	a	a	a	a	a	a	a	a	a
	G710A	0.021 (K-W) 0.004 (J-T)	0.043 (K-W)	0.021 (K-W) 0.004 (J-T)	n.s	n.s	n.s	n.s	n.s	n.s	n.s
	A3228G	0.007 (K-W). 0.001 (J-T)	n.s	0.007 (K-W) 0.002 (J-T)	n.s	n.s	n.s	n.s	n.s	n.s	n.s

K: Kruskal-Wallis test; J: Jonckheere-Terpstra test; T: test side; C: control side; n.s : no significance

a .The Kruskal Wallis Test was conducted to compare 13 items of QST parameters with selected SNPs from TRP gene. b: The Kruskal wallis test cannot be performed in “a” row due to lack of valid cases.

In this subgroup, there is a significant correlation between TRPA1 polymorphisms 710G>A and MPT and MPS, the significant correlation between *TRPA1* A3228G and MPT and MPS. Furthermore, the Jonckheere-Terpstra test on test side suggests there is a z-score trend dependent on genotypes.

Tab. 8: Association between pain perception and SNPs from 3 TRP genes in 56 CRPS patients

		CDT		WDT		TSL		CPT		HPT		MDT	
		T	C	T	C	T	C	T	C	T	C	T	C
Genes	SNPs												
TRPV1	G1103C	n.s	0.03(K) 0.045(J)	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
	A1911G	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
TRPM8	C2235T	a	a	a	a	a	a	a	a	a	a	a	a
	G1296A	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
	G780C	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
	A787T	a	a	a	a	a	a	a	a	a	a	a	a
	G790C	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
	A792G	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
TRPA1	C182T	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
	G710A	n.s	0.035(K) 0.014(J)	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
	A3228G	n.s	0.03(k) 0.022(J)	n.s	n.s	n.s	0.03(K) 0.022(J)	n.s	n.s	n.s	0.016(K)	n.s	n.s

		MPT		MPS		WUR		VDT		PPT	
		T	C	T	C	T	C	T	C	T	C
Genes	SNPs										
TRPV1	G1103C	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
	A1911G	n.s	n.s	0.003(K) 0.001(J)	n.s	n.s	n.s	n.s	n.s	n.s	n.s
TRPM8	C2235T	a	a	a	a	a	a	a	a	a	a
	G1296A	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
	G780C	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
	A787T	a	a	a	a	a	a	a	a	a	a
	G790C	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
	A792G	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
TRPA1	C182T	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
	G710A	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
	A3228G	n.s	n.s	n.s	n.s	0.019(K)	n.s	n.s	n.s	n.s	n.s

K: Kruskal-Wallis test; J: Jonckheere-Terpstra test; T: test side; C: control side; n.s : no significance

In CRPS patients, there is a significant correlation was found between CPT of control side and *TRPV1* G1103C, *TRPA1* G710A and A3228G, there are also statistically significant correlation between TSL of control side and *TRPA1* A3228G, MPS of test side and *TRPV1*A1911G. Furthermore, Jonckheere-Terpstra test shows significant trends in data mentioned above. However, the significant trends were not found between HPT of control side and *TRPA1* A3228G, WUR of test side and *TRPA1* A3228G.

Table 9: Association between pain perception and SNPs from 3 TRP genes in 27 peripheral nerve injury patients

		CDT		WDT		TSL		CPT		HPT		MDT	
		T	C	T	C	T	C	T	C	T	C	T	C
Genes	SNPs												
TRPV1	G1103C	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
	A1911G	n.s	n.s	n.s	0.008(k)	n.s	n.s	0.024(k)	n.s	n.s	n.s	n.s	n.s
TRPM8	C2235T	a	a	a	a	a	a	a	a	a	a	a	a
	G1296A	n.s	n.s	n.s	n.s	n.s	n.s	0.035(k) 0.033(J)	n.s	n.s	n.s	n.s	n.s
	G780C	n.s	n.s	n.s	n.s	n.s	n.s	0.033(k) 0.033(J)	n.s	n.s	n.s	n.s	n.s
	A787T	a	a	a	a	a	a	a	a	a	a	a	a
	G790C	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
	A792G	a	a	a	a	a	a	a	a	a	a	a	a
TRPA1	C182T	0.04(k) 0.004(J)	0.024(k) 0.024(J)	n.s	n.s	n.s	n.s	0.005(k) 0.005(J)	n.s	n.s	n.s	n.s	n.s
	G710A				n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
	A3228G	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s

		MPT		MPS		WUR		VDT		PPT	
		T	C	T	C	T	C	T	C	T	C
Genes	SNPs										
TRPV1	G1103C	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
	A1911G	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
TRPM8	C2235T	a	a	a	a	a	a	a	a	a	a
	G1296A	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
	G780C	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
	A787T	a	a	a	a	a	a	a	a	a	a
	G790C	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
	A792G	a	a	a	a	a	a	a	a	a	a
TRPA1	C182T	0.027(k) 0.027(J)	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
	G710A	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
	A3228G	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s

K: Kruskal-Wallis test; J: Jonckheere-Terpstra test; T: test side; C: control side; n.s : no significance

In peripheral nerve injury patients, z-score of CPT of test side is significantly associated with *TRPM8* G1296A, G780C and *TRPA1* C182T. There is significant correlation between CDT of test side and *TRPA1* C182T. Similarly, the z-score of MPT of test side is significantly associated with *TRPA1* C182T. Morover, Jonckheere-Terpstra test shows significant trends in data mentioned above , but there is no significant trends comparing TRPV1A1911G with WDT and CPT.

Table 10: Association between pain perception and SNPs from 3 TRP genes in 27 PHN patients

		CDT		WDT		TSL		CPT		HPT		MDT	
		T	C	T	C	T	C	T	C	T	C	T	C
Genes	SNPs												
TRPV1	G1103C	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
	A1911G	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
TRPM8	C2235T	a	a	a	a	a	a	a	a	a	a	a	a
	G1296A	n.s	n.s	n.s	n.s	n.s	n.s	0.024(k)	n.s	n.s	n.s	n.s	n.s
	G780C	n.s	n.s	n.s	n.s	n.s	n.s	0.021(k)	n.s	n.s	n.s	n.s	n.s
								0.021(J)					
	A787T	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
	G790C	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
	A792G	n.s	n.s	n.s	n.s	n.s	n.s	0.021(k)	n.s	n.s	n.s	n.s	n.s
								0.021(J)					
TRPA1	C182T	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
	G710A	n.s	n.s	n.s	n.s	n.s	n.s	0.044(k)	n.s	n.s	n.s	0.049(k)	n.s
								0.044(J)				0.049(J)	
	A3228G	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s

		MPT		MPS		WUR		VDT		PPT	
		T	C	T	C	T	C	T	C	T	C
Genes	SNPs										
TRPV1	G1103C	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
	A1911G	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
TRPM8	C2235T	a	a	a	a	a	a	a	a	a	a
	G1296A	n.s	n.s	n.s	n.s	n.s	n.s	0.037(k)	n.s	n.s	n.s
	G780C	n.s	n.s	n.s	n.s	n.s	n.s	0.029(k)	n.s	n.s	n.s
								0.029 (J)			
	A787T	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
	G790C	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
	A792G	n.s	n.s	n.s	n.s	n.s	n.s	0.029(k)	n.s	n.s	n.s
								0.029(J)			
TRPA1	C182T	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
	G710A	0.031(k)	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
		0.031(J)									
	A3228G	0.022(k)	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
		0.022(J)									

K: Kruskal-Wallis test; J: Jonckheere-Terpstra test; T: test side; C: control side; n.s : no significance

In post-herpetic neuralgia patients, z-score of CPT of test side is significantly associated with *TRPM8* G1296A, G780C and *TRPA1* G710A. A significant correlation was found between VDT of test side and *TRPM8* G780C as well as A792G. Meanwhile, the z-score of MPT of test side is significantly associated with *TRPA1* G710A as well as A3228G. Additinally, there is a significant correlation between MDT of test side and *TRPA1* G710A. Morover, Jonckheere-Terpstra test shows significant trends in data mentioned above. The significant trend was not observed between CPT of test side and *TRPM8* G1296A.

Table 11: Association between pain perception and SNPs from 3 TRP genes in 71 polyneuropathy patients

		CDT		WDT		TSL		CPT		HPT		MDT	
		T	C	T	C	T	C	T	C	T	C	T	C
Genes	SNPs												
TRPV1	G1103C	n.s	n.s	n.s	n.s	n.s	0.037(k)	n.s	n.s	n.s	n.s	0.029(k)	n.s
	A1911G	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
TRPM8	C2235T	a	a	a	a	a	a	a	a	a	a	a	a
	G1296A	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
	G780C	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
	A787T	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
	G790C	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
	A792G	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
TRPA1	C182T	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
	G710A	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
	A3228G	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s

		MPT		MPS		WUR		VDT		PPT	
		T	C	T	C	T	C	T	C	T	C
Genes	SNPs										
TRPV1	G1103C	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
	A1911G	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
TRPM8	C2235T	a	a	a	a	a	a	a	a	a	a
	G1296A	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
	G780C	n.s	n.s	n.s	0.041(k) 0.041(J)	n.s	n.s	n.s	n.s	n.s	n.s
	A787T	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
	G790C	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
	A792G	n.s	n.s	n.s	0.045(k) 0.045(J)	n.s	n.s	n.s	n.s	n.s	n.s
TRPA1	C182T	n.s	n.s	n.s	n.s	0.045(k) 0.045(J)	0.045(k) 0.045(J)	n.s	n.s	n.s	n.s
	G710A	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
	A3228G	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s

K: Kruskal-Wallis test; J: Jonckheere-Terpstra test; T: test side; C: control side; n.s : no significance

In polyneuropathy patients, the MPS of test side is significantly associated with *TRPM8* G780C as well as A792G. A significant correlation was found between WUR of both sides and *TRPA1* G780C.Furthermore, further Jonckheere-Terpstra test suggests significant trends in data mentioned above. There is significant association without trend between *TRPV1* G1103C and TSL of control side as well as MDT of test side

Tab. 12: Association between pain perception and SNPs from 3 TRP genes in 30 trigeminal patients

		CDT		WDT		TSL		CPT		HPT		MDT	
		T	C	T	C	T	C	T	C	T	C	T	C
Genes	SNPs												
TRPV1	G1103C	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
	A1911G	0.035(k)	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
TRPM8	C2235T	a	a	a	a	a	a	a	a	a	a	a	a
	G1296A	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
	G780C	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
	A787T	a	a	a	a	a	a	a	a	a	a	a	a
	G790C	n.s	n.s	n.s	n.s	0.024(k) 0.024(J)	n.s	n.s	n.s	n.s	n.s	n.s	n.s
	A792G	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
TRPA1	C182T	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
	G710A	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
	A3228G	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s

		MPT		MPS		WUR		VDT		PPT	
		T	C	T	C	T	C	T	C	T	C
Genes	SNPs										
TRPV1	G1103C	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
	A1911G	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
TRPM8	C2235T	a	a	a	a	a	a	a	a	a	a
	G1296A	n.s	n.s	n.s	n.s	n.s	n.s	0.041(k) 0.041(J)	n.s	n.s	n.s
	G780C	n.s	n.s	n.s	n.s	n.s	n.s	0.04(k) 0.04(J)	n.s	n.s	n.s
	A787T	a	a	a	a	a	a	a	a	a	a
	G790C	n.s	n.s	0.008(k) 0.008(J)	0.012(k) 0.012(J)	n.s	n.s	n.s	n.s	n.s	n.s
	A792G	n.s	n.s	n.s	n.s	n.s	n.s	0.04(k) 0.04(J)	n.s	n.s	n.s
TRPA1	C182T	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
	G710A	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s
	A3228G	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s	n.s

K-W:Kruskal-Wallis test;J-T:Jonckheere-Terpstra test; T: test side; C: control side; n.s : no significance

In trigeminal patients, the TSL of test side is significantly associated with *TRPM8* G790C. A significant correlation was found between MPS of both sides and *TRPM8* G790C. Similarly, there is significant association between the VDT of test side and *TRPM8* G1296A, G780C as well as A792G. Furthermore, further Jonckheere-Terpstra test suggests significant trends in data mentioned above. There is significant association but trend between *TRPV1* A1911G and CDT of test side.

Considering the TSL, the homozygote G-allele carriers in *TRPA1* SNP 3288A>G seems also less tolerance to thermal stimuli than homozygote carriers 3288 A (P=0.045) (Figure 6h). However, there was no significant influence on pain perception dependent on genotypes in the other SNPs compared with QST parameters.

(a)

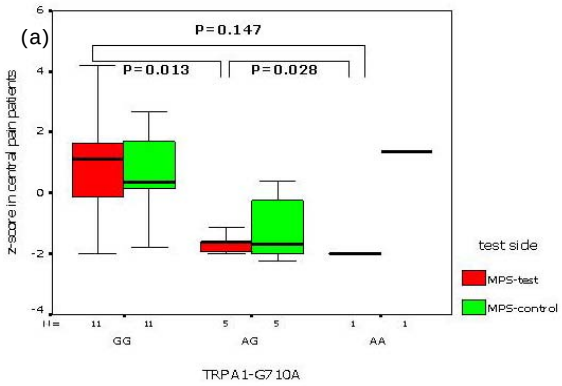


Figure 6a: Comparison of MPS of test side depending on *TRPA1* G710A genotypes in central pain patients

(b)

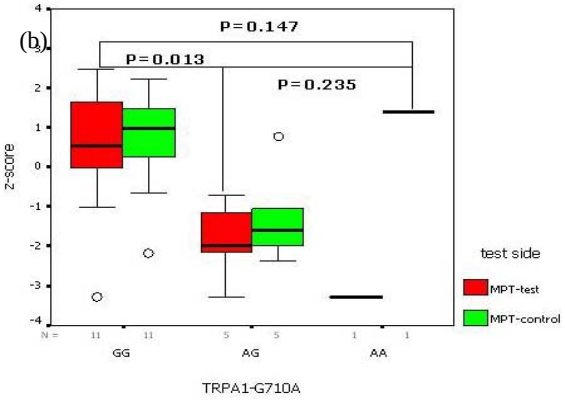


Figure 6b: Comparison of MPT of test side depending on *TRPA1* G710A genotypes in central pain patients

(c)

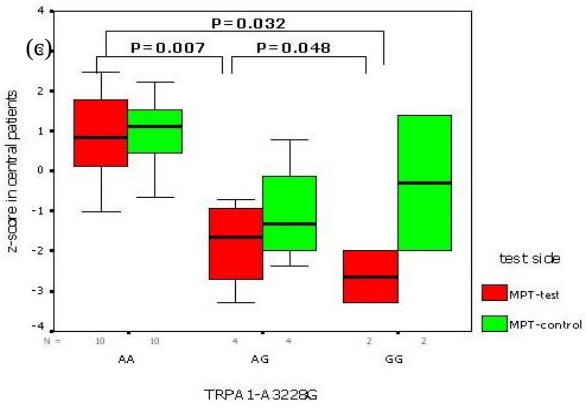


Figure 6c: Comparison of MPT of test side depending on *TRPA1* A3228G genotypes in central pain patients

(d)

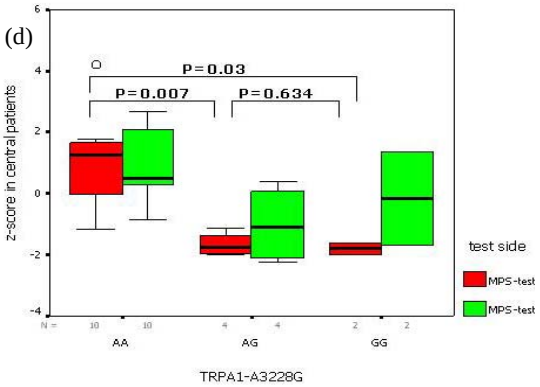


Figure 6d: Comparison of MPS of test side depending on *TRPA1* A3228G genotypes in central pain patients

(e)

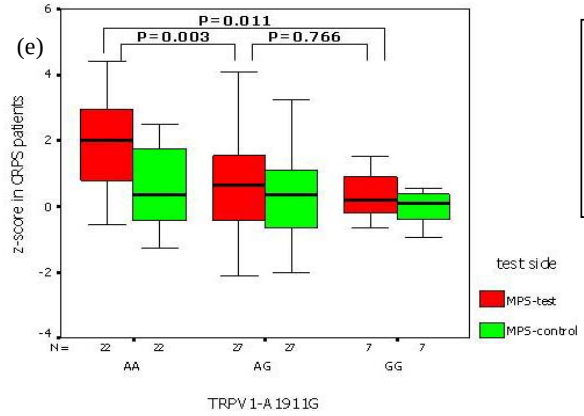


Figure 6e: Comparison of MPS of test side depending on *TRPV1* A1911G genotypes in complex regional pain symdrom (CRPS) patients

(f)

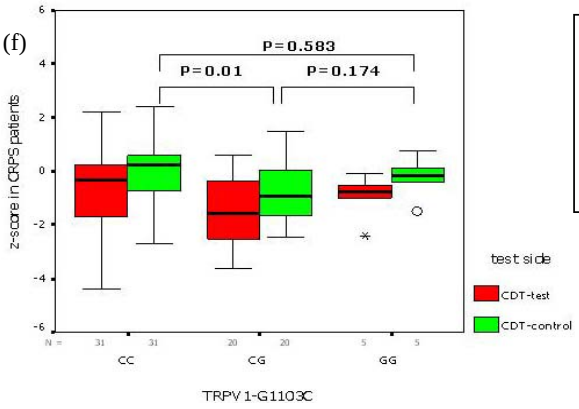


Figure 6f: Comparison of CDT of control side depending on *TRPV1* G1033C genotypes in centrol pain patients

(g)

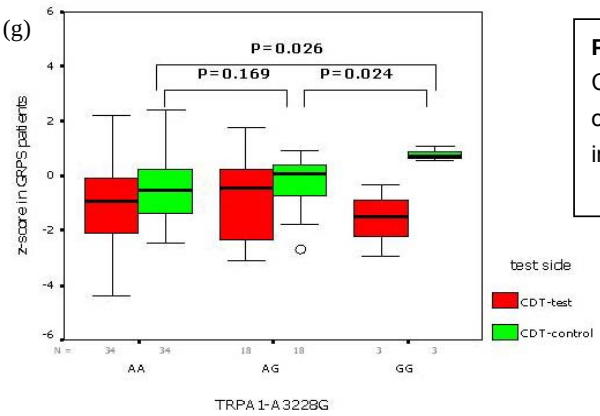


Figure 6g: Comparison of CDT of control side depending on *TRPA1* A3228G genotypes in GRPS patients

(h)

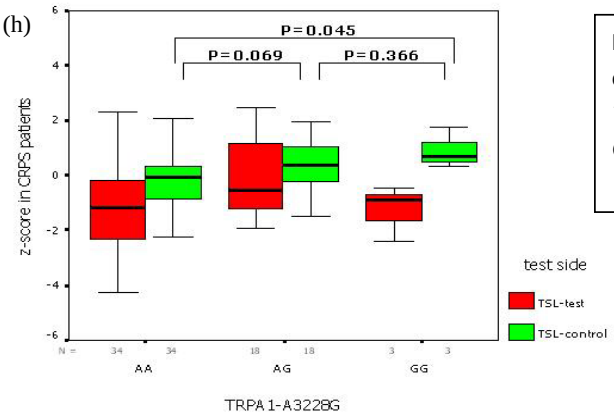


Figure 6h: Comparison of TSL of control side depending on *TRPA1* A3228G genotypes in GRPS patients

Figure 6: Comparison of z-score of QST parameters in some associated SNPs dependent on genotypes. (P-values calculated by Mann-Whitney U-test.)

4. DISCUSSION

4.1. The characteristics of neuropathic pain in the seven subgroups

Neuropathic pain is a chronic pain syndrome that has been associated with drug-, disease-, or injury-induced damage or destruction of the sensory afferent fibers of the peripheral nervous system (PNS), and has a substantial impact on quality of life and mood [30]. Our clinical data, which include seven types of neuropathic pain, showed that sensory alternations diversified depending on these different diseases. For example, in central pain patient, post-herpetic neuralgia PHN as well as polyneuropathy patient, the pain patterns characterized by loss of sensory function are apparently distinct from the complex regional pain syndrome type (CRPS) patients, who often exhibited the pain pattern of gain of sensory of function like hyperalgesia. Our findings are consistent with the phenomenon in previously published literature, in which the type of pain could manifest not only with positive sensory phenomena, such as pain, dysesthesia, and different types of hyperalgesia, but also with negative sensory phenomena and negative and positive motor symptoms and signs [31, 32]. Among thirteen QST parameters applied, the z-score of cold detection threshold (CDT), mechanical detection threshold (MDT), thermal sensitivity limen (TSL), mechanical detection threshold (MDT) and vibration detection threshold (VDT) in central pain showed negative alterations in sensory function, suggesting that small and large sensory fiber like afferent C- or Aδ-fiber could be implicated as a consequence of long-duration pathological lesion. More interestingly, the VDT z-score of CRPS patients showed hyperalgesia to blunt pressure, a phenomenon of feeling vibration response to in-noxious stimuli. In contrast, the VDT z-score in peripheral nerve injury and polyneuropathy subgroups exhibited the loss of sensory function. Similarly, the MDT z-score of PHN and polyneuropathy patient indicated loss of function in mechanical stimuli sensitivity. It is reported that Aβ-fiber is represented by the VDT and MDT, May be lesions involving this type of afferent fiber probably result in the gain or loss of function of mechanical sensitivity. Since the ability of peripheral nervous system (PNS) to sense and perceive pain is an essential protective

mechanism that warns against threatened or ongoing tissue damage. Disruption of this protective mechanism may result in the development of chronic pain. On the other hand, the lower efficiency of endogenous pain modulation induced by alternation in CNS pain processing, which in part attenuate the pain inhabitation, probably play a crucial role in the origin of the neuropathic pain. In addition, several molecular mechanisms, such as abnormal release of neurotransmitter (e.g. norepinephrine, NE), increased neuronal hyperexcitability as well as unbalanced between excitatory (e.g. glutamate) and inhibitory (e.g gamma-aminobuytric acid, GABA) transmitter system, commonly lead to the dysfunction on pain signalling pathway, and creating a state of allodynia and hyperalgesia or hypoesthesia [33, 34].

4.2 Neuropathic pain and single nucleotide polymorphism

Due to the multifactorial aetiology of neuropathic pain, a variety of treatment algorithms were adopted by clinicians to alleviate the symptoms. But the overall successful rate for treatment seems low because of the universal individual variability and needs to be improved urgently. Meanwhile, this intriguing phenomenon draws lots of attention to explore the underlying mechanism for relevant genes or SNPs, which is recently suspected to play a crucial role in the variability of pain perception. Up to now, there have been few genetic studies on human suggesting that single nucleotide polymorphisms of specific genes could contribute significantly to basal pain sensitivity. For example, Single-nucleotide polymorphisms of the μ-opioid receptor gene like *OPRM1 a*nd the catechol-O-methyltransferase (COMT) gene were shown to be associated with pain ratings in response to standardized noxious stimuli and with pain-induced μ-opioid receptor binding in the CNS [22, 35, 36]. The polymorphism *OPRM1*A118G, however, leading to a lower affinity of ligands to the extracellular binding domain of the μ-opioid-receptor, seems more likely to be associated with the acute pain [37]. It is commonly reported that postoperative patients carrying homozygously 118G, exhibit a higher pain sensitivity and require higher opioids dosages than those being homozygous for 118A. Another common functional polymorphism 1947G>A in *COMT* gene cause substitution from valine to methionine

at amino acid position 158, which is suggested to increase pain sensitivity and to decrease opioid system activation in the brain [38]. Haplotypes including *COMT* Val^{158}Met were recently identified and an association was suggested with experimental pain sensitivity and a chronic pain condition [22]. However, there are still controversial findings concerning this SNP in acute pain patients or in different ethnicities [15, 33].

Recently, there is increasing evidence supporting that hypersensitivity and pain that occurs under various pathological conditions is often due to up-regulated expression and/or increased sensitivity of TRP channels. Several lines of evidence point to the involvement of members of the TRPV family in the aetiology of neuropathic pain [39,40], So far, considering the potentially functional role of polymorphism in TRP gene, only few information on humans has been explored regarding the association between pain perception and TRP genetic polymorphisms. Recently, a study led by Xu et al [41] suggested that *TRPV1* Met^{315}Ile variant might lead to functional difference at the level of expression at the cell surface, on a HEK293 cells model. Furthermore, a body of previous literature focuses on racial differences in the pain perception response to painful stimuli [42]. For example, two clinical studies found that Asian patients may require less post-operative analgesia than European patients [43]. This phenomenon, however, has not been replicated in all reported studies [44].

4.3 Genetic variability of TRPV1 and their influence on pain perception

In this study, the genotype distribution of 11 selected TRP gene polymorphisms were examined in neuropathic pain patients and volunteers, trying to explore whether there has been a biologically relationship between these SNP and alterations of pain sensitivity. Among these markers, two common single nucleotide polymorphisms *TRPV1* 1103 C>G and 1911 A>G, also known as TRPV1 Met315Lle and Lle585Val, result in substitution from methionine to isoleucine at codon 315 and isoleucine to valine at codon 585,repectively[22, 45]. They are 12.7 kb apart from each other in the genomic sequence. We found the allelic frequency for the 1911 A>G polymorphism was statistically significant higher in patients compared with controls (P=0.04). The

overall G allelic frequency in patients was 36.8%, which was similar to those reported in the HapMap database (34.2%) and in the study led Kim et al (36.0%). More interestingly, the overall allelic frequency of the 1911A>G polymorphism in control group approaches 42.8%. Although the genotype frequency of 1911GG carriers in control group was 18.8%, slightly higher than the patient group (14.6%), there is no significant difference (P=0.14, $\chi 2=3.86$) between patient and control group. Our data still indicated that neuropathic pain patients were less likely to carry the *TRPV1* 1911G allele at this position.

Until now, conflicting findings over *TRPV1* polymorphisms still exist. For the *TRPV1* 1911A>G polymorphism, no different functional response to capsaicin, pH and temperature between the different alleles has been detected in whole-cell patch-clamp and calcium imaging experiments, suggesting that the substitution does not critically alter receptor structure or function, at least in vitro[46]. Conversely, Kim et al [47] observed that this polymorphism alters cold withdrawal times (CWT i.e, increased sensitivity to cold-induced pain). From several animal experiments, there was some evidence derived supporting that TRPV1 after long-term activation could exert a protective role in the development of mechanical hyperalgesia [48], even though the detailed mechanism was not finally clarified. Considering our control group with the higher allele frequency in *TRPV1* 1911A>G, the question remains open as to whether the carrier with G allele in neuropathic pain subject have a potentially protective role from aggravating into further stage. So, further study should be investigated to confirm this hypothesis.

TRPV1 likely plays a major role in integrating nociceptive signals, particularly in the contexts of inflammatory and neuropathic pain [49, 50]. When comparing genotype data in patients with 13 of the PSQ parameters, a statistically significant correlation was found between mechanical pain sensitivity (MPS), cold detection threshold (CDT) and polymorphism *TRPV1* 1911A>G in CRPS patients (P=0.003 and P=0.03,respectively). Our results are not consistent with the previously published finding by Kim et al [22], in which they failed to find any significant association between these two polymorphisms in *TRPV1* and cold/heat pain sensitivity in

American female subjects of European descent. In their study, the different experimental environment used, which includes taking healthy subjects, different methods of pain measurement, pain parameters selected as well as the racial difference may explain this inconsistency. In our study, MPS was designed to detect an typical phenomenon, termed pinprick hypoesthesia, which was often complicated with the neuropathic pain and was believed to be consistent with a large fiber sensory deafferentation like afferent Aδ-fiber, and the presence of abnormal cold pain threshold (CDT) in GRPS subgroup also indicates a disturbance of Aδ-cold sensory function, which might indirectly reflect the damage extent on the afferent pain signalling pathway, and evaluate the influence on the pain sensitivity. However, the relative contribution of C- and Aδ-fiber nociceptors to cold and pressure pain threshold is less clear [28, 51]. Based on our results, we could conclude that *TRPV1* 1103G>C and 1911A>G was closely associated with the pain perception in certain type of neuopathic pain, especially for thermal and mechanical sensitivity. Many evidences suggested TRPV1 plays an important role in chemical and thermal hyperalgesia in a model of diabetic neuropathy [52]. Its role may be associated with decreased cell-specifically TRPV1 protein expression in C-fibers paralleled by an increase in A-fibers, leading to an increase in its function. Furthermore, a possible “cooperation” between TRPA1 and TRPV1 channels has also been reported in recent vitro study, in which the canabinoid agonist WIN 55,212-2 dephosphorylates, therefore desensitizing TRPV1 in trigeminal sensory neurons via the activation of TRPA1.

With regard to the comparison of the MPS z-value with *TRPV1* polymorphisms, the results of test side clearly showed that homozygote 1911G carriers are more likely to exhibit the loss of sensory function than homozygote A1911 carriers. Similarly, the comparison of mechanical pain threshod (MPT) z-score on control side indicated that subjects carrying *TRPV1* 1911GA are less sensitive to the cold stimuli than those who carrying homozygote 1911A, representing loss of sensory function. Impaired pain sensation might result from distorted TRPV1 regulation in the peripheral nervous system, there is quite a little vivo data recently supported that TRPV1 protein

expression could be enhanced by IGF-I via PI(3)K signal pathway [53]. Moreover, as a target of the cAMP/PKA signal pathway, the effect of TRPV1 under tissue inflammation or nerve injury might also be influenced by alteration of PKA-mediated phosphorylation [54].So, it can also be speculated that the substitution of amino acid caused by SNP *TRPV1* 1103G>C and 1911A>G probably alters biological function on afferent fiber or primary sensory neuron by altering secondary structure of mRNA or TRPV1 protein expression, leading to individual differences of pain sensitivity.

4.4 Association of TRPM8 genotypes with neuropathic pain

We found no difference in the genotype or allelic frequencies for these five SNPs in TRPM8 gene between patients or controls, indicating that there was no evidence for an association of risk of developing neuropathic pain. The genotype or allelic frequencies in our patients and controls were also similar to those reported in HapMap database. Interestingly, some SNPs in the TRPM8 gene, namely 2235C>T ($Thr^{762}Tle$), 780G>C ($Arg^{247}Thr$), 787A>T (synonymous), 790G>C (synonymous) and 792A>G ($Tyr^{251}Cys$), could not be found in our patients. Among these SNPs, two out of them do not seem to be polymorphic in our volunteers. From the HapMap, we found that the minor allele frequency of 780 A>C and 790G>C in Asia population but not in European population was always polymorphic, indicating the existence of an ethnic difference in these two SNPs. TRPM8 is a non-selective, outwardly rectifying channel that can be activated by cold (8-28 °C) [55], Up to date, there is increasing evidence that mutation in coding regions could contribute to the function of the TRPM8 channel. Dragoni et al [56] have identified two potential *N*-glycosylation sites in TRPM8 (Asn-821 and Asn-934) in rats, and observed the decreased efficiency of synthesis of mature, glycosylated TRPM8 as a result of mutation. It is widely accepted now that TRPM8 may play a major role in certain types of cold-induced pain [57].

In this study, comparing five *TRPM8* SNPs with 13 QST parameters, no significant association was found dependent on the genotypes mostly because of no valid case in each subgroup. Also Kim et al failed to find any association between *TRPM8* variant and cold as well as heat pain sensitivity in their healthy European Americans.

However, considering the relatively higher range of threshold temperature to TRPM8 (8-28 °C), it is likely that the TRPM8 receptor was not activated by the painful cold temperature they used (2-4 °C) [22]. Nevertheless, it still raises the intriguing possibility that genetic polymorphisms, such as those investigated here, may account for or contribute to a racial difference in pain threshold or tolerance. In addition, animal experiments showed that mutation in *TRPM8* gene could contribute to the function of this TRPM8 channel [57]. It is possible, therefore, that one SNP may be in linkage with another functional SNP in the *TRPM8* gene, inducing an impact on behavioural phenotype such as pain perception difference through an epigenetic mechanism related to this nucleotide substitution.

Duan et al [58] showed that synonymous SNPs within haplotypes may have functional consequences drastically different from those of each isolated variant. This effect was attributed to alterations in the secondary structure of mRNA, which result in weakened mRNA stability or protein translation. Furthermore, it has been well documented that TRPM8 is mostly present in small diameter sensory neurons, and all TRPM8-expressing neurons also expressed receptor tyrosine kinases (TrkA), which is a NGF receptor [59]. For those *TRPM8* SNPs involving amino acid substitution, such as 1296G>A and 780G>C, the question remains open whether these polymorphisms might alter the expression of TRPM-8 receptor through the nerve growth factor (NGF) pathway.

4.5 Genetic variability of TRPA1 and association to pain perception

The genotype and allele distribution of three *TRPA1* SNPs investigated was identical in our patients and controls, and there was no evidence for an association to risk of neuropathic pain. All the genotype and allelic frequencies were similar to those reported in Hapmap database in European population supporting the generalizability of the findings. Regardless of *TRPA1* 182C>T, the minor allelic and genotype frequency of *TRPA1* 710G>A and 3228A>G reported in HapMap database showed significant difference among ethnic groups, which should also be considered in association studies with SNPs and phenotypes.

Considering the results of pain perception in central pain subgroup, we found that *TRPA1* SNPs 710G>A and 3228A>G were significantly associated with MPT and MPS on affected side of the patient. Compared mechanical pain threshold (MPT) z-score depending on the genotype in SNPs A3228G, central pain patients carrying homozygously the 3288G allele, tended to be insensitive to pain perception than homozygote 3288A carriers (*P*=0.032 in MPT and *P*=0.03 in MPS, respectively, Mann-Whitney U-test) and heterozygote GA carriers (*P*=0.048 in MPT). A similar result was found for *TRPA1* 710G>A, but possibly due to the low allelic frequency - the difference was only significant in heterozygote GA carriers (*P*=0.013 in MPT and *P*=0.013 in MPS, respectively, Mann-Whitney U-test). The decreased mechanical pain thresholds or pain sensitivity in central pain patients could be linked to the disturbance of afferent Aδ-fiber. Furthermore, the MPT or MPS parameter could be used to detect the mechanical pain stimuli and to determine whether patients with pain processing abnormalities (i.e. neuropathic pain) show exaggerated temporal summation of second pain intensity [49]. The mechanism involving *N*-methyl-D-aspartate (NMDA) receptor within the dorsal horn is thought to be responsible for temporal summation of pain [60]. This finding was partially in agreement with the receptor function in previously published literature [61], in which the TRPA1 was believed to be a sensor for mechanical stimuli. Under neuropathic pain status, alterations in neuronal function, chemistry, and structure, which often occurred secondary to nerve injury, might result in abnormal expression of TRPA1 receptor, leading to loss of sensory function. It is still unclear which mechanism gets involved in the decreased afferent fibers function in the homozygote 710A. The amino acid substitution caused by this polymorphism from histidine to arginine at position 1018 could be one possible reason to contribute to this different sensitivity. In addition, one interesting property of TRPA1 channels, which inactivate in hyperpolarized cells but remain open in depolarized cells, also provides a mechanism for the lack for desensitization, coincidence detection, and allodynia [63]. However, whether the amino acid change induced by polymorphism alters these depolarized cells leading to influence on iron influx still need to be considered in future studies.

The comparison data of QST parameters like thermal sensitivity limen (TSL) and cold detection threshold (CDT) on the non-affected control side of the patient for *TRPA1* A3228G also indicated that homozygote 3288G carriers of CRPS patients were more sensitive to pain perception than homozygote A3288 (in CDT) and heterozygote AG carriers (both CDT and TSL). A great amount of evidence supporting that TRPA1 has also been linked to cold hyperalgesia behind neuropathic pain [38, 64]. Inflammation and nerve injury increase the expression of TRPA1 in DRG neurons. Furthermore, the co-expression of TRPA1 and TRPV1 and the flexibility of the TRP family channels raise the possibility that TRPA1 channel might interact to influence the properties of one another [19, 64]. Animal experiments showed that an NGF-induced TRPA1 increase in sensory neurons via p38 activation is necessary for cold hyperalgesia [38, 65]. Other studies further demonstrated that mice following *trpa1* gene knock out or *trpa1* antagonist exhibited behavioural changes in response to the pain stimuli, which illustrated the important role of TRPA1 in pain perception.

4.6 Limitation of our study

There are still some limitations in the study. Firstly, the volunteers were not investigated by the QST test panel, which consequently failed to powerfully evaluate the variability in pain perception among healthy subjects. Secondly, in the study, patients in subgroups apparently showed different pain pattern dependent on the aetiology of the neuropathic pain. Therefore we necessarily further compared z-score with associated SNPs for each subgroup based on the genotype, which probably increases the ability to study phenotype-genotype association. But owing to no valid cases in some of subgroups, larger sample are necessary in order to get stronger statistic power. Finally, functional test based on the *vivo* or *vitro* study should be performed to directly measure the effect of these SNPs as to confirm the association and to provide a stronger rationale for the relationship with pain sensitivity. Our association analyses have identified several loci associated with thermal and mechanical pain sensitivity, but their functional importance has yet to be confirmed.

4.7 Conclusion

In conclusion, our results have a considerable clinical significance and represent the first study of this kind which demonstrated an association between several genetic polymorphisms like *TRPV1* 1103C>G, 1911A>G, 710G>A as well as 3228A>G and pain sensitivity. These SNPs produce different influences on pain perception dependent on the genotype in patients on the condition of neuropathic pain. As far as complex regional pain syndrome (CRPS) patients are concerned, the subjects carrying the minor homozygote 3228G exhibited more sensitivity in response to thermal pain stimuli, than those with wild-type homozygote carriers. In contrast, homozygote 1103C, 1911G carriers were more insensitive to mechanical and thermal stimuli than homozygote G1103, A1911carriers, which were consistent with the influence of homozygote 3288G carriers on WPT and WPS in central pain subgroup. These polymorphisms exert major effects maybe by influencing the level of expression of the gene product, dependent of its function. Finally, these polymorphisms alone or interaction between SNPs may alter transcription, mRNA stability, or the protein half-life, leading to the association with a certain clinical status. For those polymorphisms without involving amino acid substitution, the observed relationship between associated SNPs and pain perception could be caused by other functional polymorphisms in the neighbouring genes that posses high LDs with tested SNPs. Although there was no evidence of an association between genotypes and the risk of being neuropathic pain, for the TRPV1 1911A>G, the relative lower proportion in homozygote 1911G in patient compared to healthy volunteers possibly indicated that subjects likely carrying the common G allele at this position have a potentially protective role, preventing the aggravation in the further stages.

5. SUMMARY

Neuropathic pain is a chronic pain syndrome that has been associated with drug-, disease-, or injury-induced damage or destruction of the sensory afferent fibers of the peripheral nervous system (PNS). The type of pain could be manifested not only with positive sensory phenomena, such as pain, dysesthesia, and different types of hyperalgesia, but also with negative sensory phenomena and negative and positive motor symptoms and signs. The pharmacological treatment of the symptoms of painful neuropathy, however, is still considered to be difficult. Nowadays, much attention is focused on the genetic polymorphisms, some of the single nucleotide polymorphisms can be speculated to be associated with the pain sensitivity as a result of amino acid substitution in crucial position. But the detailed knowledge of individual variability on pain perception under neuropathic pain is still poorly understood. Candidate gene studies on the basis of biological hypothesis have been a practical approach to identify relevant genetic variation in complex traits. There is growing evidence showed that single nucleotide polymorphisms in related genes, including *TRPV1, TRPA1 and TRPM8*, may influence pain sensitivity in animal model of neuropathic pain.

In our study, we selected 2 of the SNPs from*TRPV1*, 3 of the SNPs from *TRPA1* and 6 SNPs from *TRPM8* to examine the effect of these variations on clinical neuropathic pain responses, to investigate the contribution of genetic factors on pain sensitivity in humans. There were a total of 296 Germany patients and 253 healthy volunteers recruited in our study for investigation. Owing to the failed collection of clinical data in some cases, finally only 237 patients were enrolled to carry out the association analysis. The results show that patients exhibited markedly gain of sensory function along with the application of cold detection (CDT), thermal sensitivity limen (TSL) on control side. The hypoesthesia, however, was occurred on test side when patients were giving mechanical pain threshold (MPT) and mechanical pain sensitivity (MPS) measure. The comparison of seven subgroups between test and control side showed that there are different pain patterns based on the subgroups. Besides the trigeminal pain and other neuropathy patients, the five other subgroups exhibiting either the loss

of sensory function in CDT, WDT, TSL and VDT on central pain, or the gain of sensory function in pressure pain threshold (PPT) in CPRS patient. Referring to the genotype and allele frequencies on patients and controls, there are no significant differences between the two groups, except the *TRPV1* polymorphism A1911G.

With regard to the association between SNPs and 13 QST parameters, there were some significant correlations related to *TRPV1* polymorphisms 1103 C>G and 1911A>G, as well as to *TRPA1* polymorphism 710G>A and 3228A>G with several QST parameters in two neuropathic pain subgroups. The CRPS patients carrying homozygote G in *TRPA1* 3228A>G were more sensitive to pain perception than those patients carrying the heterozygote genotype in the TSL and CDT test, conversely, in some subgroups homozygote TRPV1 1911G carriers were more likely to be insensitive to sensory pain than homozygote 1911A carriers; Similarly, further comparison of the data indicated that patients carrying homozygote TRPA1 710A or 3228G were less sensitive to pain perception than heterozygote and homozygote G710 or 3228A carriers.

The results suggest that at least some of these genetic polymorphisms may exert major effects on pain perception possibly by influencing the level of expression of the gene product, depending on its function. Finally, polymorphisms alone or interaction between SNPs may alter transcription, mRNA stability, or the protein half-life, leading to a specific clinical status. For those polymorphisms which do not involve amino acid substitution, the observed association with pain perception could be caused by other functional polymorphisms in the neighbouring genes that posses high LDs with tested SNPs.

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