Clinical Neurophysiology 136 (2022) 13–38

Contents lists available at ScienceDirect

Clinical Neurophysiology

journal homepage: www.elsevier.com/locate/clinph

# Review of techniques useful for the assessment of sensory small fiber neuropathies: Report from an IFCN expert group

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ARTICLE INFO

*Article history:*
Accepted 6 January 2022
Available online 19 January 2022

*Keywords:*
Sensory small fibers
Aδ fibers
C fibers
Neurophysiological assessment

## HIGHLIGHTS

- Nerve conduction studies do not evaluate function of small afferent fibers.
- Different techniques have been developed for evaluation of these fibers, but none of them alone is sufficient for a complete evaluation.
- Each clinical laboratory should develop its own set of tests for evaluation of small afferent fibers.

## ABSTRACT

Nerve conduction studies (NCS) are an essential aspect of the assessment of patients with peripheral neuropathies. However, conventional NCS do not reflect activation of small afferent fibers, including Aδ and C fibers. A definitive gold standard for laboratory evaluation of these fibers is still needed and therefore, clinical evaluation remains fundamental in patients with small fiber neuropathies (SFN). Several clinical and research techniques have been developed for the assessment of small fiber function, such as (i) microneurography, (ii) laser evoked potentials, (iii) contact heat evoked potentials, (iv) pain-related electrically evoked potentials, (v) quantitative thermal sensory testing, (vi) skin biopsy-intraepidermal nerve fiber density and (vii) corneal confocal microscopy. The first five are physiological techniques, while the last two are morphological. They all have advantages and limitations, but the combined use of an appropriate selection of each of them would lead to gathering invaluable information for the diagnosis of SFN. In this review, we present an update on techniques available for the study of small afferent fibers and their clinical applicability. A summary of the anatomy and important physiological aspects of these pathways, and the clinical manifestations of their dysfunction is also included, in order to have a minimal common background.



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https://doi.org/10.1016/j.clinph.2022.01.002
1388-2457/

**Contents**



## 1. Introduction

Conventional sensory nerve conduction studies (NCS) are the most commonly used neurophysiological tests for the evaluation of peripheral sensory fibers. Techniques used for NCS are well established and standardized, and normal and abnormal values have been clearly defined (Stålberg et al., 2019). Nevertheless, it is well known that conventional NCS studies leave the function of small afferent fibers (SAFs) untested. This group of sensory fibers includes thinly myelinated Aδ fibers mediating nociception and cold sensation, and unmyelinated C fibers, also mediating nociception, as well as warm sensation. The study of these fibers is important because their impairment generates a variety of sensory symptoms, including neuropathic pain and also trophic changes in the affected regions. Additionally, they may be affected without significant abnormalities in the function of large afferent fibers (LAFs). Therefore, neurophysiologic testing of SAFs is clinically relevant.

A definitive gold standard for the diagnosis of small fiber neuropathy (SFN) is still needed. In the past decades, a number of techniques have been used for the physiological or morphological study of thinly myelinated Aδ and unmyelinated C fibers, including: (i) microneurography (MNG), (ii) laser evoked potentials (LEPs), (iii) contact heat evoked potentials (CHEPs), (iv) pain-related electrically evoked potentials (PREPs), (v) quantitative thermal sensory testing (QTT), (vi) skin biopsy-intraepidermal nerve

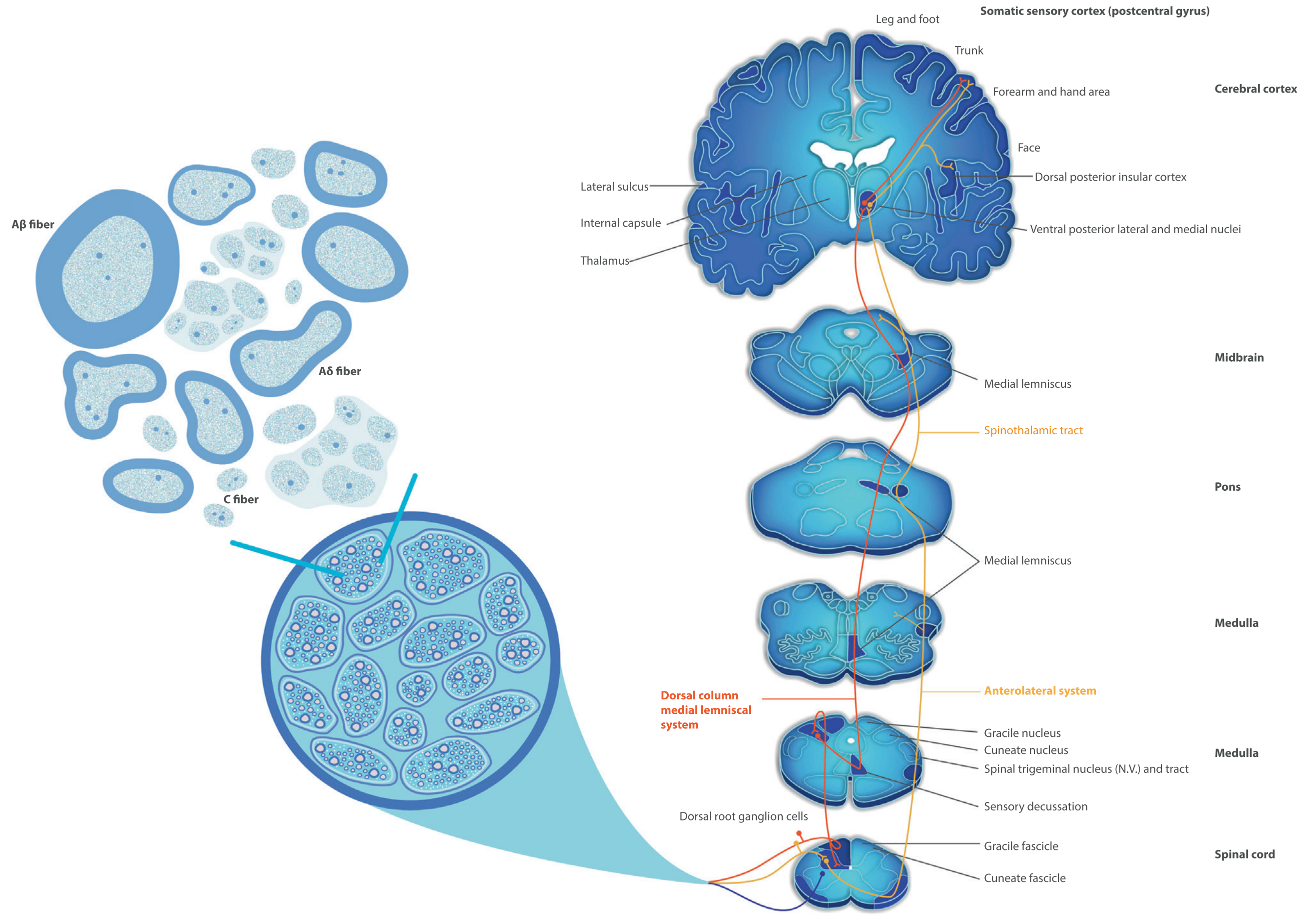


**Fig. 1.** Schematic representation of the peripheral and central sensory pathways. In the Central Nervous System the information conveyed by small afferent fibers is carried mostly by the spinothalamic system. On the left side there is a representation a peripheral sensory nerve. The inlet shows large myelinated afferents (Aβ), thinly myelinated afferents (Aδ) and unmyelinated C fibers. C fibers can be isolated or grouped by one Schwan cell.

fiber density (IENFD) and (vii) corneal confocal microscopy (CCM), among others. These techniques have different advantages and disadvantages, and the combined use of an appropriate selection of each of them would lead to gathering invaluable information for the diagnosis of SAFs impairment, as well as for understanding the pathophysiology underlying the disorder. This article aims at providing an updated review on the currently available neurophysiological and morphological diagnostic techniques for SFN, including the state of the art on their usefulness, and limitations.

## 2. Brief summary of anatomy and physiology of small afferent fibers and central pathways

Pain and temperature senses are mediated at primary afferent level by small diameter fibers, thinly myelinated Aδ and unmyelinated C fibers (Fig. 1). At the periphery, these fibers are associated with specialized receptors which differ in morphology, receptive fields and stimulus selectivity (Dubin and Patapoutian, 2010). Nociceptors have been described in skin, muscle, joints and viscera, and thermorreceptors involved in cutaneous temperature sensation are localized in different layers of the skin. (McGlone and Reilly, 2010; Schepers and Ringkamp, 2009). These first order sensory neurons perform two main functions: (i) the transduction and encoding of stimuli into electrical signals and (ii) the transmission of those signals into the central nervous system (Julius and Basbaum, 2001; Ringkamp et al., 2008).

### *2.1. Characteristics of small afferent fibers*

Nociceptive C fibers are classified according to the kind of stimulus activating their receptors: mechanical (M), thermal, cold (C) and heat (H), and polymodal. There are also silent C nociceptors, normally insensitive to mechanical and heat stimuli (MiHi units), which become sensitive to them only after being sensitized by inflammatory or chemical mediators. Unmyelinated C fibers have nerve conduction velocity of 0,4–1,4m/s, and they transmit a second pain of longer duration, which is not as well localized (Basbaum et al. 2009; Ringkamp and Meyer, 2008).

Aδ nociceptive fibers are subdivided into two principal classes: Type I Aδ, respond to both mechanical and chemical stimulation, but have relatively high heat thresholds (>50–53°C). Type II Aδ nociceptive fibers have a much lower heat threshold (47°C), but a very high mechanical threshold. Aδ fibers have nerve conduction velocity of 5–30 m/s and they are responsible for the initial perception of acute pain. (Basbaum et al. 2009; Iannetti et al.2006).

Afferent fibers of small diameter also transmit non-nociceptive thermal information: Aδ for cold and C for warm sensation. Aδ fibers have a maximum frequency of discharge between 20°C and 30°C; at temperatures > 40°C or < 17°C, these fibers maintain low frequency of discharge or become silent (Darian-Smith,1984; Schepers and Ringkamp, 2009). Human C fibers that respond to cold, have been identified through microneurography, they discharge continuously at skin temperature of 30°C and reach maximum discharge at 15°C. It has been hypothesized that they have a thermoregulatory role. (Campero et al.,1996; Campero et al., 2001).

Warm fibers correspond to C fibers and have a small receptive field, with conduction velocities of 1 m/s and do not respond to mechanical stimuli. They present continuous activity at 30°C, with maximum discharge frequency between 40°C and 43°C and minimal activity at 50°C, and with low temperatures (Duclaux and Kenshalo, 1980; Schepers and Ringkamp, 2009).

### 2.2. Primary afferent input to dorsal spinal cord

The soma of first order sensory neurons is clustered in the dorsal root and trigeminal ganglia. Small primary afferents from limbs and the trunk project to the tract of Lissauer, and divide into ascending and descending branches before contacting second order neurons in the dorsal lamina of the spinal cord. Craniofacial small afferents fibers terminate in the trigeminal nucleus of the spinal tract (Almeida et al., 2004; Willis and Westlund, 1997).

Nociceptive specific neurons are located mainly in Rexed's laminae I, II and V. Lamina I is the only region that receives monosynaptic input from A delta and C primary afferent fibers. Lamina II contains inhibitory and excitatory interneurons which respond to Aδ and C nociceptive afferents and to other non-noxious stimuli. Lamina V contains the wide dynamic range neurons (WDR): they respond more vigorously to inputs from nociceptive afferents, but also discharge in response to non-noxious stimuli and inputs from visceral nociceptors. (Basbaum and Jessell, 2013)

### 2.3. Spinal cord ascending projections

The spinothalamic tract (STT), which is the principal ascending nociceptive and temperature pathway, transmits this information to the thalamus and cerebral cortex. It originates mainly from lamina I and laminae III to VI. Approximately 85–90% of fibers cross the midline, just ventral to the central canal, and reaches the white matter of the contralateral spinal cord one or two segments rostral to the cells of origin. There are two ascending tracts: the lateral spinothalamic and the anterior spinothalamic. At the spinomedullary junction, the lateral and anterior STT join in the ventrolateral aspect of the medulla. Also, trigeminothalamic axons join to the medial aspect of STT at this level. (Almeida et al., 2004; Willis and Westlund, 1997; Dostrovsky and Craig 2013).

The spinothalamic pathway projects to the sympathetic columns of the toracolumbar spinal cord, and sends collateral branches to the reticular formation of the medulla, pons and midbrain including nucleus reticularis magnocellularis and periaqueductal gray matter, which are areas involved in the descending modulation system of pain. (Craig 2002).

STT terminates in six distinct regions of the thalamus, and spinothalamic neurons from lamina I project mainly to the posterior part of the ventromedial nucleus (VMpo), (Craig 2002; Dostrovsky and Craig 2013).

Third order sensory neurons project from the thalamus to the dorsal insular cortex, which is activated by temperature, pain and numerous interoceptive modalities that relate to the physiological condition of the entire body (Craig, 2002).

The parietal somatosensory cortex receives input from the ventral posterior nuclei and ventroposterior superior nucleus. The parietal cortex has a somatotopic contralateral body representation of cutaneous mechanorreceptors in primary sensory areas 3b and 1; proprioception from joints and muscles are represented in areas 3a and 2. (McGlone and Reilly, 2010; Dostrovsky and Craig, 2013).

Finally, several areas of the neuroaxis participate in the modulation of pain. These include the prefrontal cortex, cingulate area, insular cortex, hypothalamus, periaqueductal gray matter (PAG), dorso lateral pons, the nucleus of raphe magnus and the nucleus reticularis magnocellularis (RVMM). Descending monoaminergic pathways use serotonin, norepinephrine or dopamine as neurotransmitters, and exert the principal modulatory effect over nociception at the dorsal horn level, mainly in lamina I (Verdugo et al.2007; Benarroch, 2008; Basbaum and Jessell, 2013; Dostrovsky and Craig, 2013).

## 3. Clinical manifestations of small fiber neuropathy

SFN is a heterogeneous group of peripheral nerve disorders that selectively involve thinly myelinated Aδ and unmyelinated C fibers, leaving the LAFs unaffected (Terkelsen et al. 2017). Even though there are multiple disorders associated with small nerve fiber degeneration, including metabolic disorders, immune-mediated disorders, infectious diseases, exposure to drugs and toxins, and genetic causes, a definitive etiology remains unknown in up to 50% of patients (Farhad et al. 2016). It is important to recognize that small fiber impairment may also be present in neuropathies that affect LAFs and motor fibers. In fact, SFN is an inherent part of almost all large fiber neuropathies, and conventional NCS should still be performed with the aim of at least characterizing the degree of involvement of LAFs in SFN. Readers interested in NCS techniques to characterize polyneuropathies in general are directed to recent reviews on the topic (Tankisi et al., 2019; 2020; Haque et al., 2020; McCorquodale and Smith, 2019; Kincaid et al., 2017).

SFN is nowadays recognized as two distinct entities: 1) length-dependent typical/classical small fiber polyneuropathy, and 2) non-length dependent SFN (Chan and Wilder-Smith, 2016). The clinical presentation is characterized by sensory and autonomic dysfunction, leading to a significant reduction in quality of life (Devigili et al. 2008). No single patient fits with the classical clinical patterns, giving a high clinical heterogeneity. Classically, sensory dysfunction involves two types of symptoms: "negative" and "positive" sensory phenomena. A negative sensory phenomenon is an expression of deficit in sensory function, such as loss of warm or cold sensation. These symptoms and signs are sometimes difficult to verify in clinical sensory testing, where pain or allodynia may mask the sensory deficits. On the other hand, a positive sensory phenomenon is an expression of abnormally increased function of the sensory system, such as paresthesias or neuropathic pain. Neuropathic pain, which is usually chronic and recurrent, is commonly described as painful paraesthesia (burning sensation, and pins and needles) and spontaneous shooting or stabbing pain. Some patients describe spontaneous burning pain, usually projected to the skin, which is the expression of ectopic discharges of peripheral C nociceptors (Jensen and Finnerup 2014). Hyperalgesia and allodynia are also common, and they are usually presented in patients with loss of warm or cold sensation. Positive phenomena interfere with sleep, as they usually are worse at night, probably because the absence of external inputs allows for more salience of those generated ectopically in the damaged nerves. Dysautonomic complaints may include sweating, flushing, palpitations, dry eyes or mouth, erectile dysfunction, orthostatic dizziness, and bowel and urinary disturbances, among others (Thaisetthawatkul et al. 2014). Pruritus has also been reported as a symptom of SFN, which adds to the complexity and heterogeneity of the clinical profile (Misery et al. 2014). Some patients also have nightly deep cramp-like pains, restless legs, or involuntary foot movements.

The diagnosis process of symptoms suggesting small fiber impairment has two main goals: (i) to establish the presence or absence of a neurological lesion and (ii) to determine the localization of the disease in the nervous system (central or peripheral). In the neurological examination, the clinician must consider the dis-

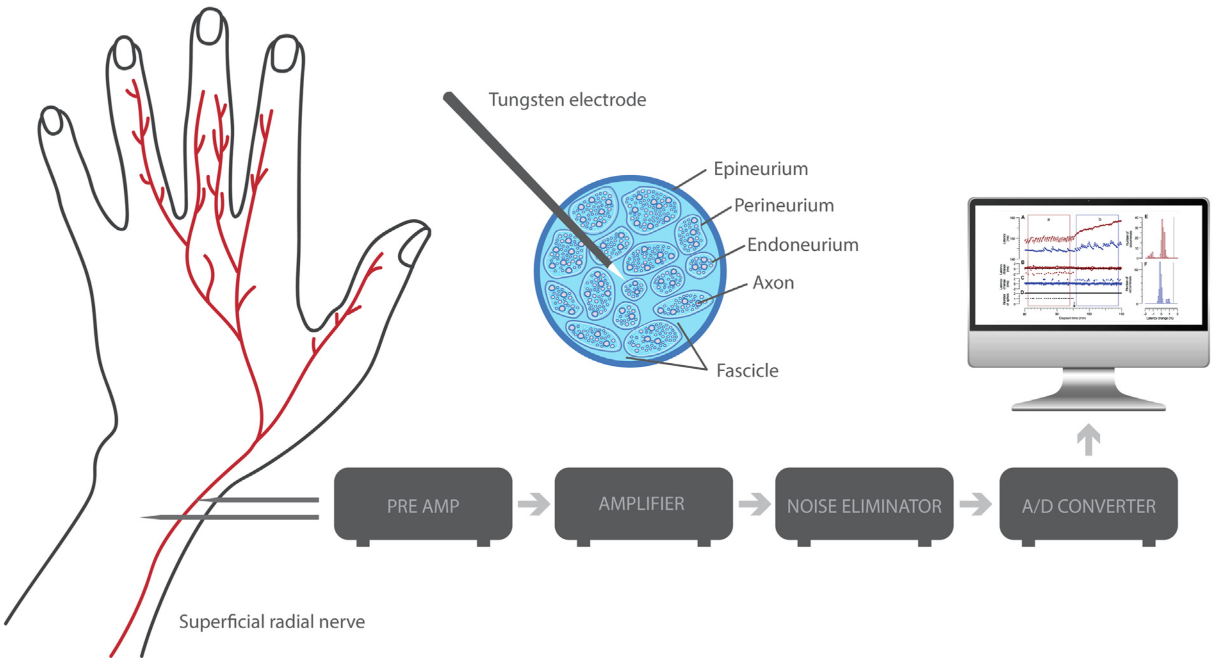


**Fig. 2.** A simple schematic for microneurography setting. A nerve to be impaled is chosen. Those superficial skin nerves are particularly easy to recognise, such as the superficial peroneal nerve at the ankle or the superficial radial nerve at the distal forearm. The nerve is impaled by a high impedance "active" tungsten electrode (~10–100 KΩ) and referred to a low impedance "reference" tungsten electrode placed subcutaneously some centimeters apart. The signal feeds a preamplifier near the recording site and is further filtered and amplified by a commercially available device. Depending on the electrical environment, the signal could go through a "noise eliminator" before being digitized by a standard A/D converter. Finally, the digitised signal feeds a PC where it is processed through a custom made or commercially available software.

tribution of negative and positive sensory manifestations. Typically, in length-dependent SFN, warm and cold hypoesthesia and hypoalgesia are referred in hands and feet, i.e.,stocking and glove distribution, and may associate to allodynia and hyperalgesia (Tesfaye et al. 2010; Lolignier et al. 2015). The non-length-dependent pattern of SFN accounts for 25 to 35% of all SFN cases. This type of SFN is typically present in young women with immune-mediated diseases, and it is characterized by a patchy distribution of symptoms and signs involving the face, upper limbs and trunk. This presentation tends to be under-recognized, and is commonly misdiagnosed as a psychogenic disorder (Khan and Zhou, 2012). The clinical phenomenon of an increase in pain perception with repeated stimulation, known as the "wind-up" phenomenon (Price, 1972), can also be a useful clinical sign. Pure SFN does not present with dysfunction of LAFs (such as touch-pressure, sense of vibration, joint position), muscle strength and tendon reflexes. In this sense, it's not surprising that conventional NCS, which primarily test large myelinated fiber function, are mostly normal in these patients. This phenomenon is explained by the fact that Aδ and C fibers have a high depolarization threshold and slow conduction velocities that are beyond the resolution of conventional NCS techniques. (Kimura, 2013). According to England et al. (2005), diagnostic accuracy of polyneuropathy is rather poor when it is based on symptoms alone. While it improves when signs are taken into account, the common signs of loss of of function suggesting polyneuropathy may be overlooked in SFN. It is, therefore, necessary to include objective diagnostic tests in the study of patients with suspected SFN. Finally, it is essential to point out that the techniques available to assess SFN mainly detect loss of function (negative signs). Only QTT and microneurography can evaluate positive sensory signs quantitatively.

The high clinical heterogeneity of SFN patients explains why not all patients fit with the classical clinical pattern, resulting in difficulties in the diagnostic process if we do not have the proper diagnostic tools (Chan and Wilder-Smith, 2016; Colloca et al. 2017). Until now the diagnostic criteria of SFN remained a matter of debate in the scientific community, although there has been an evolution from the first structured proposal to a new grading system. Briefly, SFN can be diagnosed in patients with symptoms and signs of small fiber dysfunction, who present with normal NCS but show reduced IENFD in skin biopsy and/or abnormal thermal thresholds in the QST. However, there is no gold standard for the diagnosis of SFN. A graded diagnostic criteria for use in all forms of SFN, regardless of etiology, include the following categories: (i) possible: presence of length-dependent symptoms and/or clinical signs of small-fiber damage; (ii) probable: presence of length-dependent symptoms, clinical signs of small-fiber damage, and normal sural NCS; and (iii) definite: Presence of length-dependent symptoms, clinical signs of small-fiber damage, normal sural NCS, and reduced IENFD at the ankle and/or abnormal QTT at the foot (Tesfaye et al. 2010; Sopacua et al. 2019). However, this definition is restrictive because: (i) it excludes the non-length-dependent SFN patterns, and (ii) it only considers SFN in patients with pure impairment of Aδ and C fibers. Therefore, the criteria exclude a frequent group of mixed sensory neuropathies with co-existing damage of SAFs and LAFs. Thus, a correct classification of SFN is critical, helping in the diagnostic workup for an underlying disease and the design of therapeutic clinical trials.

## 4. Tests for the study of small afferent fibers

### *4.1. Microneurography*

Recording action potentials from single afferent fibers in awake human volunteers and patients was achieved in the mid-1960 s in Sweden (Ackerley and Watkins, 2018; Vallbo et al., 2004; Vallbo, 2018). The term MNG, coined by Yngve Zotterman some decades earlier, has been reserved for this technique, that requires the percutaneous insertion of a microelectrodes into a nerve in a human

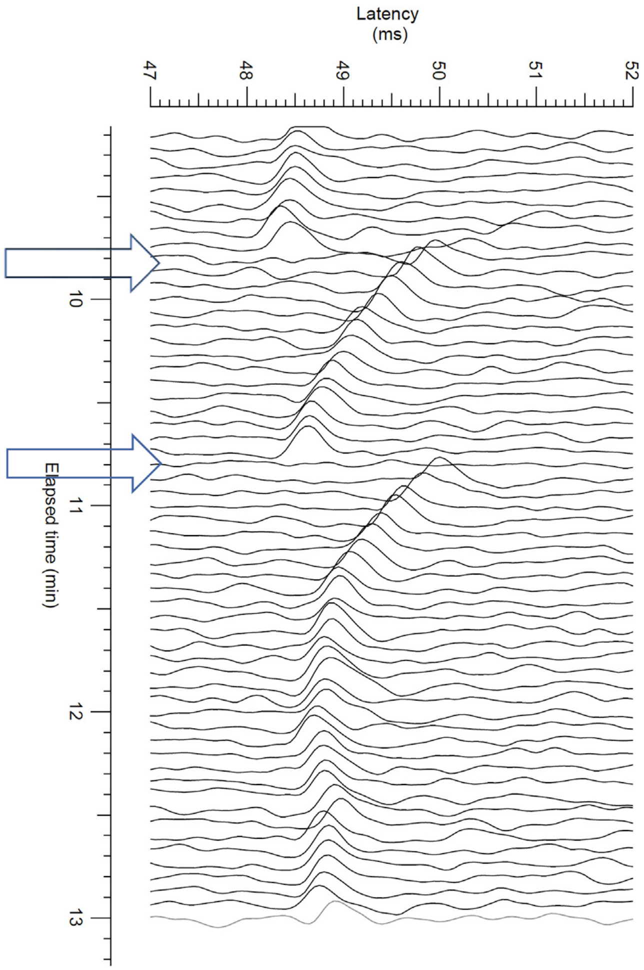


**Fig. 3.** Single fiber recording from the superficial peroneal nerve on a healthy subject. Regular low frequency stimulation (0.25 Hz) of the cutaneous receptive field 35 mm distal to the recording site evokes a time-locked action potential at a peak latency of 48.5 ms (conduction velocity 0.72 m/s). The punctiform receptive field or this cold unit was touched with a cold metal rod ~ 1 mm2 in diameter (blue arrows). A sudden contact with the receptive field induces a train of action potentials, causing an initial collision of anti and orthodromic volley, then a transient hyperpolarisation leading to conduction slowing, and then recovering conduction time in about 1 minute.

subject. In general, with this method it has been possible to describe the properties of receptors in skin and muscles of normal individuals, the conduction properties of myelinated and unmyelinated axons (Schmelz and Schmidt, 2010) and, importantly, it has been possible to recognize abnormal behavior of afferent fibers in experimental and disease states, thus providing some of the bases for understanding sensory symptoms (Serra et al., 2012). Since this issue is connected to the assessment of SFN, functions related to sensory aspects mediated by LAFs will not be discussed here.

*4.1.1. Principles of the technique*

MNG is the electrophysiological recording of action potentials from a myelinated or unmyelinated nerve fiber, obtained by manually introducing a tungsten microelectrode of 200 μm diameter with a pointed tip (5 μm) into an accessible nerve in upper or lower limbs (Vallbo, 2018), and occasionally from superficial branches of the trigeminal nerve (Nordin, 1990). The recording electrode is coated with insulating lacquer, except for the tip, providing a nominal impedance of ~ 1 MΩ. A reference, low impedance electrode, is inserted subcutaneously some centimeters away. The neural signal is amplified by a differential amplifier and filtered by an adjustable analogue filter, typically in a band between 100 and 5000 Hz. The line interference could be additionally removed by an online noise eliminator. The amplified and filtered signals are then digitized by an analogue-to-digital board and stored for offline analysis. Laboratories use different type of tungsten recording and reference electrodes, amplifiers, analogue-to-digital converter boards and software to analyze nerve signals (Serra et al., 1999). A typical scheme of the recording device is provided in Fig. 2.

In general, the signal-to-noise ratio must be above 2:1 to allow unequivocal recognition of action potential shape (Macefield and Wallin, 2018; Schmelz and Schmidt, 2010). Myelinated fibers are typically recorded extracellularly at the node of Ranvier, or through an impalement of the myelin sheath, with a triphasic waveform and a large positive deflection, whereas unmyelinated fibers have a large negative deflection recorded from the proximity of a Remak bundle. In different laboratories the signal is inverted for analysis and display. Two main physiological aspects can be assessed with the study of single nerve fibers through MNG. First, the receptor-response features can be characterized for mechano, thermo and chemoreceptors. This has allowed classification of receptors responding to different energy modalities at a given intensity, revealing their adaptation properties (slowly or rapidly adapting), their cutaneous receptive fields, their relative spatial distribution, and their activation thresholds. Second, single fiber conduction velocity can be estimated using electrical stimulation of the cutaneous innervating territory, which helps in recognizing the type of fibers responding to the stimuli (Aβ, Aδ, or C). Furthermore, electrical stimulation allows for the study of activity-dependent behavior of single axons. Activity-dependent slowing (or speeding) is a phenomenon occurring when the axon's membrane potential level has been modified after the propagation of a preceding action potential, generated either at the receptor or at the axon. This has led to the development of the 'marking technique' (Fig. 3; Schmelz et al., 1995; Torebjörk and Hallin, 1974), where the stable response of a single action potential to a regular low frequency stimulation is altered when modifying the stimulation frequency (frequency-dependent change). The pattern revealed by such activation changes is specific for different types of fibers.

*4.1.2. Recording responses and reference values*

In MNG there are no proper reference values, because it is still an experimental technique. It has been very useful in the characterization of single afferent fibers in humans, but it has no routine clinical application at present. A brief summary of the main findings follows.

*4.1.2.1. Low threshold C mechanoreceptors.* The afferents activated by a gentle brush have been characterized as responding to a less than 5 mN brush, compared to C mechanoresponsive nociceptors that respond to punctiform mechanical stimuli above 10 mN. These afferents have been implied to mediate affective touch (McGlone et al., 2012; Vallbo et al., 1999; Vallbo et al., 1995; Wessberg et al., 2003)

*4.1.2.2. Fibers responding to innocuous low temperature.* Fibers responding to innocuosus cold (e.g. < 32C) have been described in humans (Campero et al., 2009; Campero and Bostock, 2010; Campero et al., 2001; Hensel and Boman, 1960). Their receptive field comprises one, or a few spots, no larger than 1 mm in diameter. The mean cold threshold for fibers responding to innocuous low temperature was 31.7°C. Two-thirds of this sample also responded to innocuous heating with a mean threshold of 36.4°C. All fibers tested with menthol at the receptive field became sensitized to cold. Conduction velocity of this sample of cold-sensitive

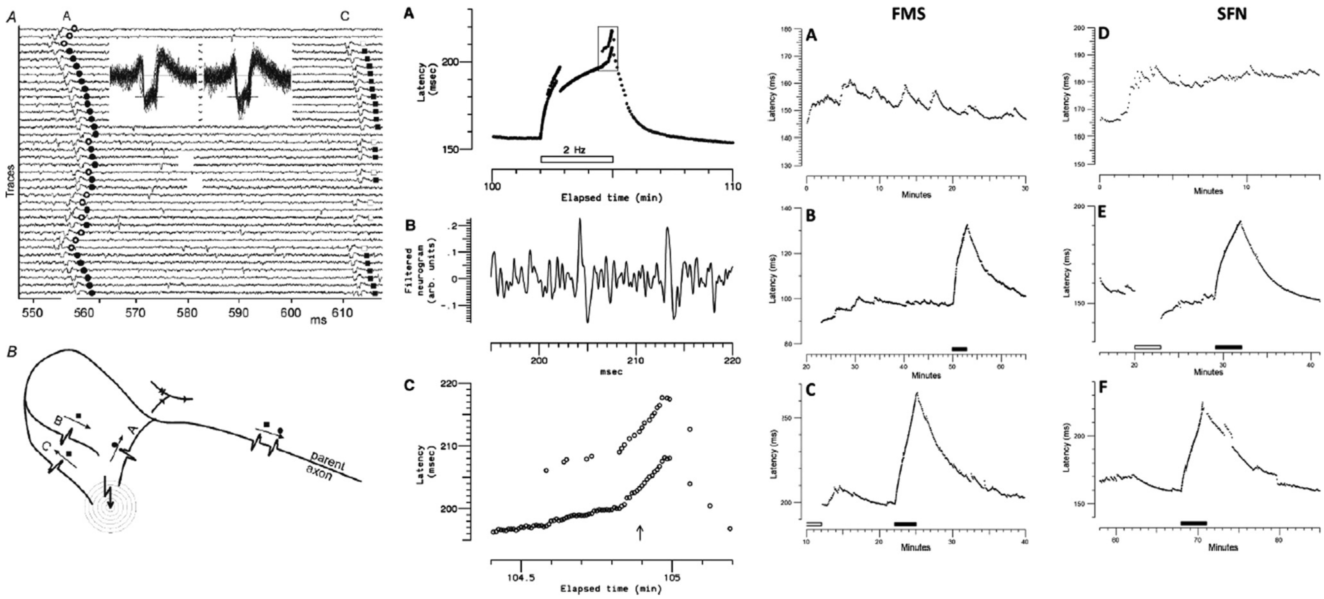


**Fig. 4.** Activity dependent slowing of C nociceptors. Examples of activity dependent slowing due to double firing of C nociceptors in asymptomatic subjects (left panel) and patients with painful neuropathy (middle panel) as the consequence of unidirectional conduction block at the site of arborisation of the nerve terminal (illustrated in left, inferior panel). Right panel shows spontaneous burst in nociceptors inducing recurrent episodes of activity dependent slowing in examples of nociceptors from patients with small fiber neuropathy, a proportion of them fitting the diagnosis of fibromyalgia syndrome. Figure modified from Weidner et al., 2003; Bostock et al., 2005; Serra et al., 2014.

afferents was 1.55 m/s (±1.2) with only two units having conduction velocities in the low Aδ range (4.4 and 3.7 m/s.; Campero et al., 2009). These fibers have been implicated in cold hyperalgesia in neuropathic pain (Serra et al 2009).

*4.1.2.3. Fibers carrying inputs from warm receptors have been seldom described in humans.* Hallin et al (1982) described a sample of 5 of them, all with punctiform receptive fields. Their threshold was 33°C and fired at a peak frequency of ~ 18 Hz at 42°C. The authors did not mention cold responsiveness of these units. Their role in positive sensory symptoms in SFN is not known.

*4.1.2.4. Nociceptors.* In the human skin at least two main types of nociceptors have been described: mechano-heat sensitive nociceptors (also known as polymodal nociceptors; Perl, 1996) and mechano-insensitive nociceptors (Schmidt et al., 2002; Weidner, et al., 1999). The former have a receptive field up to 2 cm in diameter (Schmidt et al., 1997; Schmidt et al., 2002) with a very low mechanical threshold of ~ 59 mN (von Frey Hairs) and a mean heat threshold of 40oC, very close to the heat pain threshold (Campero et al., 1996; Yarnitsky et al., 1992). Nearly one-third of polymodal nociceptors respond to cold in the noxious range with few action potentials (Campero et al., 2009; Campero et al., 1996). Mechanoinsensitive nociceptors, also described as ‘sleeping’ nociceptors (Weidner et al., 1999), are largely distributed in the skin, have a large electrical receptive field (~6 cm), and acquire heat and mechanical sensitivity as the result of local application of pungent chemicals, such as capsaicin (Ochoa et al., 2005; Serra et al., 2004), or by repeated skin stimulation. These silent nociceptors are probably engaged in pain sensitivity during neurogenic inflammation. It has been shown that during experimental inflammation only some of the branches of the cutaneous territory may become sensitised (Schmelz et al., 1996). Abnormal spontaneous activity has been well documented in patients with painful polyneuropathy and in a subgroup of painful conditions such as fibromyalgia.

*4.1.2.5. Itch fibers.* Two types of primary nerve afferents have been related to histamine and cowhage-induced itch (Namer et al., 2008). The discharge of these fibers, activated by agonists that evoke itch, match the frequency and time duration of the itching period. These fibers have similar conduction properties described for silent nociceptors and have been identified behind neuropathic itch.

*4.1.2.6. Sympathetic efferents.* Vasomotor and sudomotor sympathetic efferent fibers have been largely studied through MNG (Carter, 2019). Although this technique provides evidence to understand numerous physiological processes no relation to potential activation of C nociceptors nor interaction with large diameter tactile afferents have been identified (Campero et al., 2010; Elam, 1998; Elam, 2001).

#### *4.1.3. Abnormal behavior of single nerve afferents in small fiber neuropathy*

Namer et al (2009) analyzed the distribution of types of C-fibers in young adults, compared to aged healthy subjects. Young subjects have a slightly larger proportion of mechano-sensitive nociceptors in comparison to aged subjects, while the opposite occurred with silent nociceptors. A small proportion of C nociceptive afferents from aged subjects had evidence of spontaneous activity (see below), which was not evident in the younger population. Several MNG studies of nociceptors in healthy volunteers and patients has led to identifying the possible surrogates of spontaneous pain and hyperalgesia in the presence of spontaneous activity or increased response to natural stimulation (Bostock et al., 2005; McDermott et al., 2019; Schmidt et al., 2012; Serra et al., 2012; Serra et al., 2014; Serra et al., 2009). The differences in peak latency of consecutive action potentials triggered by electrical stimulation of the cutaneous receptive field that could be attributed to variations of background electrophysiological noise, is much less than the slowing induced by a single extra stimuli, thus allowing unequivocal separation between spontaneous activity, or fluctuations of random electrical noise (Serra et al., 2012). Spontaneous ectopic activity should not be mistaken for a rather common phenomenon, which are latency jumps from electrical excitation of different branches contributing to one parent axon.

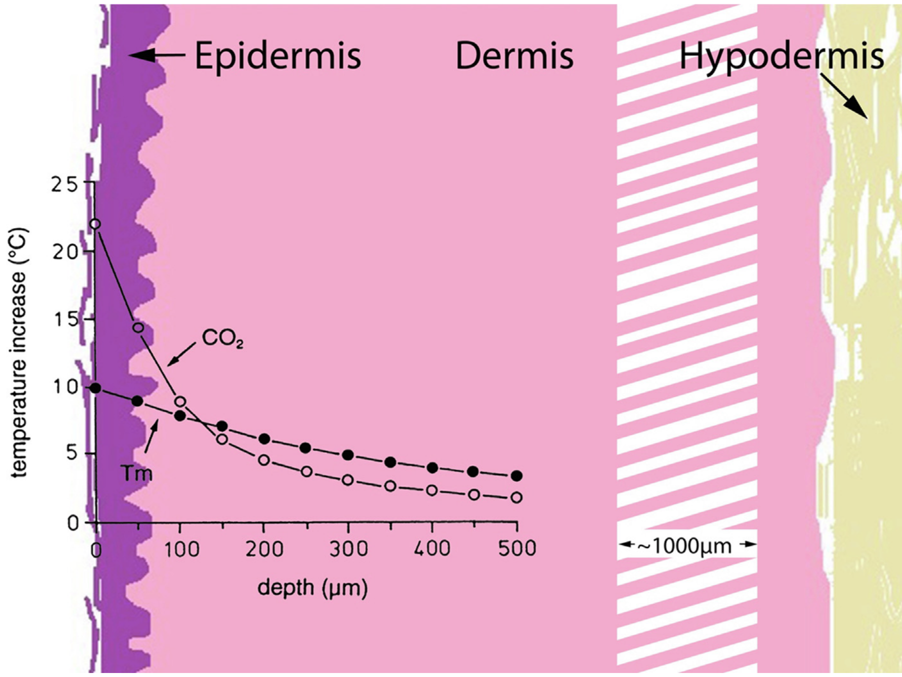


**Fig. 5.** Temperature profiles induced in hairy skin by laser pulses at pain threshold level. Temperature vs. depth profiles are superimposed onto a sketch of the skin layers (epidermis, dermis, hypodermis), to scale. A larger part of the dermis (approx. 1000 μm) is spared, indicated by the intersection to the right. $CO_2$ laser wavelength is absorbed more superficially than Thulium laser wavelength. The two curves intersect at 130 μm below skin surface (i.e. within the dermis), where both lasers increase local temperature by about 8 °C (adapted from Spiegel et al. 2000).

This phenomenon is seen as two parallel flat peak latencies on a raster plot. A different phenomenon, reported originally by Weidner et al (2003), refers to the propagation of more than one action potential triggered by a single stimuli at the cutaneous receptive field of one nociceptor because, at the site of branching of the nociceptor terminal, the expected collision of two action potentials reaching a parental axon does not occur, due to unidirectional conduction failure. Thus, a sudden increase in conduction time is reflected in the raster plot as depicted in Fig. 4. While this phenomenon was described in ~ 3% of a population of C nociceptors in normal volunteers (Weidner et al., 2003), this feature seems far more frequent in patients with SFN (Bostock et al., 2005), and may be one mechanism of hyperalgesia.

*4.1.4. Advantages*

MNG is the only technique that makes it possible to directly correlate stimulus attributes (modality, intensity) with the activity induced in single primary afferents and the evoked sensation. It also provides an unrivalled opportunity to recognize the presence of physiological and pathological propagation of action potentials, and their correlation with spontaneous pain and hyperalgesia in experimental and disease states. In human experimental models, MNG has also brought up an explanation for allodynia and hyperalgesia from peripheral mechanisms (LaMotte et al., 1992).

*4.1.5. Limitations*

The technique of MNG, although simple, requires a highly trained team with sophisticated and, usually, specially designed recording equipment and software for analysis of neural signals. Recording from healthy volunteers may take up to several hours, and even in this group of subjects, obtaining a reliable recording is not always possible. During an MNG session, subjects are required to stay still, since minute movements can displace the recording electrode, losing the recording site. This is not always possible in patients, particularly in those with ongoing pain. Considering these limitations MNG has not become a routine diagnostic test, remaining an experimental technique limited to a few well-established neurophysiology laboratories around the world. Nevertheless, because the method is very powerful for the assessment of abnormal behavior in primary afferents and nociceptors in particular, the role of MNG in understanding the physiology of sensory and autonomic fibers is of much value, and it has potential for contribution to diagnosis.

*4.2. Laser evoked potentials*

The following recommendations were updated and adapted for application to SFNs from a previous IFCN publication dedicated to somatosensory evoked potentials (SEPs) and their general clinical applications (Cruccu et al. 2008).

*4.2.1. Principles of the technique*

Time-locked evoked potentials require rapid activation of the sensory system under study. In case of the nociceptive system, the primary nociceptive afferents are responsive to thermal, mechanical and chemical stimuli; they can also be activated non-specifically with electrical stimuli. Nociceptive afferents are the largest functional subgroup of small fibers in peripheral nerves (Aδ and C fibers); thermoreceptive afferents cover a similar range of fiber sizes (afferent), and postganglionic fibers of the autonomic nervous system are also among the small fibers (efferent).

Since its introduction to pain research (Carmon et al. 1976), rapid skin heating by infrared laser radiation that elicits LEPs has become a reference standard of clinical neurophysiological assessment of nociceptive pathways in humans (Treede et al 2003; Cruccu et al. 2004; Kakigi et al 2005; Garcia-Larrea 2006). Alternative methods include CHEPs (Granovsky et al., 2016), pinprick evoked potentials (Iannetti et al. 2013), chemosensory evoked potentials (Stuck et al. 2006), and intraepidermal electrical stimulation (IES; Inui and Kakigi 2012, Mouraux et al. 2010). The term

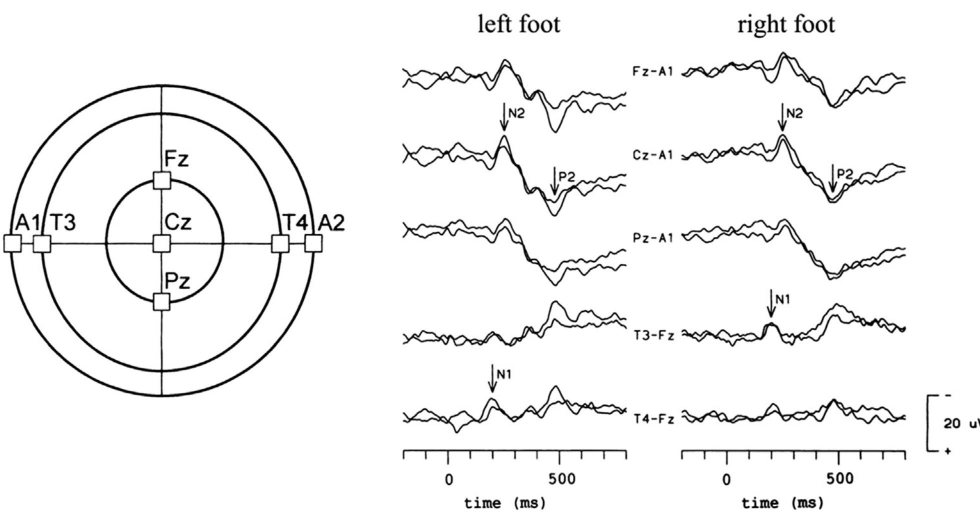


**Fig. 6.** Recording montage for clinical use of laser-evoked potentials. Five recording sites (midline vs. earlobes, parasylvian vs. Fz), 2 × 20 stimuli, 0.2–70 Hz bandpass, 200 Hz sampling rate (modified from Spiegel et al. 1996).

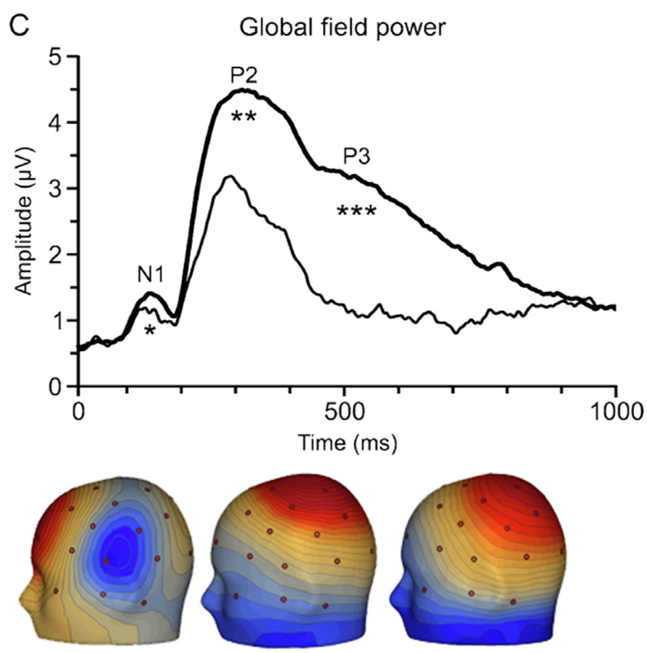


**Fig. 7.** Global field power assessment of laser-evoked potentials. A global estimate of electrical brain activity can be obtained by calculating the spatial standard deviation across many scalp electrodes (global field power). This way, an early N1 component can be distinguished from the vertex potential P2 and the endogenous P3. Thick line: spatial discrimination task for laser location, thin line: distraction by mental arithmetic (from Schlereth et al. 2003). Surface maps illustrate the spatial distribution of the electrical field at the time of the different Global Field Power peaks (N1, P2, P3), and can lead to subsequent dipole source analyses.

"pain related evoked potentials" (PREPs) comprises all sorts of potentials in response to nociceptive stimulation of different modalities in principle, however its use has primarily been associated with electrical stimulation (see section 6).

Laser radiant heat stimulation selectively activates Aδ and C nociceptors via thermal gating of the capsaicin receptor TRPV1 (Greffrath et al. 2002). There is no concomitant activation of mechanoreceptors that are innervated by large Aβ fibers. Heat-sensitive nociceptors activated by the laser beam include those that are mechanically insensitive or "silent" (Dusch et al. 2016). Desensitization of the skin by high-concentration topical capsaicin abolishes laser-evoked pain, but not pinprick evoked pain (Henrich et al. 2015); thus, there is a subpopulation of TRPV1-negative cutaneous nociceptive afferents that are not assessed by LEPs (van den Broeke et al. 2016).

The laser wavelength has a strong effect on its penetration depth, and hence the depth range of activated nociceptive afferents. Studies in monkeys indicated that heat sensitive nociceptors are found in both epidermis and dermis in a depth range of 20–570 μm (Tillman et al. 1995) with an average depth around 130 μm (Spiegel et al. 2000). Both, intraepidermal and subepidermal nerve fibers express TRPV1 in human skin (Narayanaswamy et al. 2012). $CO_2$ laser pulses, the most frequently used laser type with a wavelength of 10.6 μm, are absorbed within the epidermis, while those of solid-state lasers (mainly Thulium or Neodymium-based with wavelengths of 1–2 μm) penetrate into the dermis (Fig. 5). Solid-state laser radiation is easily conducted via optic fibers, and due to their deeper penetration, they reduce superficial burns, which may be an advantage for clinical use. Near infrared radiation of sufficient intensity has also been generated by modern diode lasers (Tzabazis et al. 2011).

Unlike the traditional median nerve N20 or tibial nerve P40 somatosensory evoked potentials, the currently clinically useful LEPs do not reflect the primary evoked potential of the primary somatosensory cortex (SI). LEPs should rather be considered event related potentials that reflect activation of a network of nociceptive brain areas including primary and secondary somatosensory cortex, insula and mid-cingulate cortex (Tarkka and Treede, 1993; Garcia-Larrea et al. 2003). The most prominent LEP component is the vertex potential N2-P2. As part of the orienting response, vertex potentials may also be elicited by non-painful somatosensory stimuli or by auditory stimuli; topographic differences in scalp distribution between these different vertex potentials are subtle (Treede et al. 1988, Mouraux and Iannetti 2009). Therefore, the clinical specificity of LEPs relies on specificity of the stimulus, not the response.

The LEP vertex potential is preceded by a small negativity N1, with scalp maxima at bilateral temporal electroencephalography (EEG) leads, that inverts its polarity over the midline (Fig. 6, right; Fig. 7). EEG – magnetoencephalography (MEG) dipole analysis and intracortical recordings indicate that this signal is mainly generated in the upper bank of the sylvian fissure, encompassing the secondary somatosensory area (SII) and the posterior insula, also called operculoinsular cortex (Garcia-Larrea et al. 2003; Schlereth

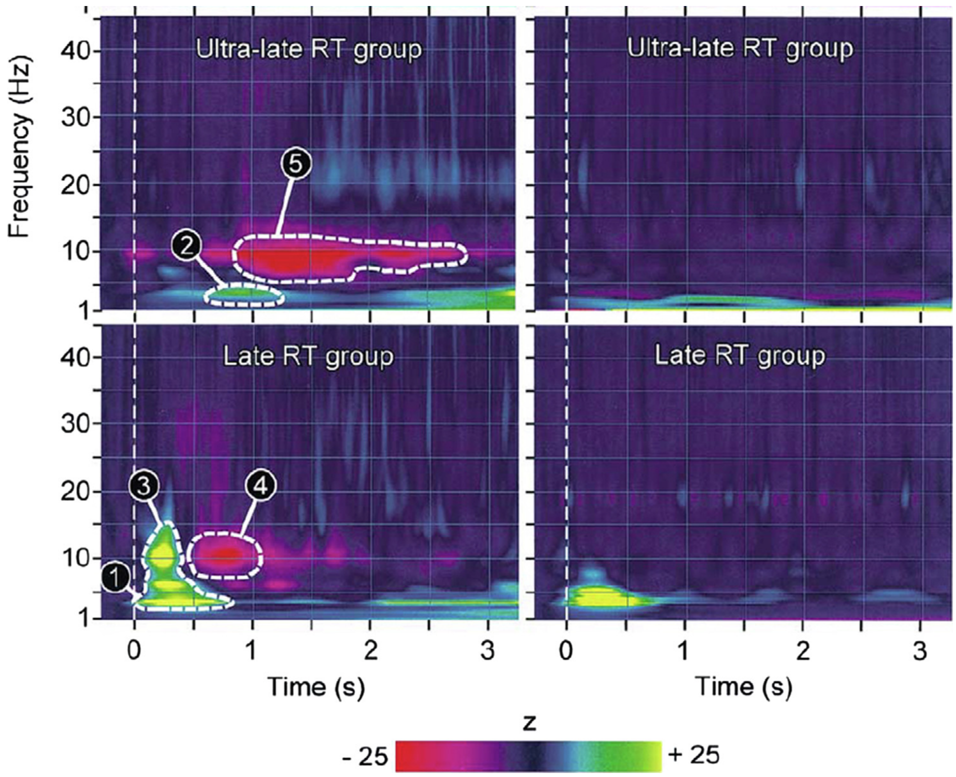


**Fig. 8.** Time-frequency analysis of laser-evoked potentials. Time-frequency estimation of oscillation amplitude following $CO_2$ laser stimulation. Left panels display the TF transform of time averaged trials enhancing only activities both time and phase locked. Right panels display the average of Time-Frequency transforms of single trials enhancing in addition the non-phase-locked stimulus related EEG changes, which are more pronounced. Results are displayed as increases and decreases of oscillation amplitude (z-scores), relative to a reference period (2350 – 2150 ms). Foci of stimulus-related EEG changes are circumscribed by dashed lines. Late RT (Reaction Time): trials of responses with short reaction times related to A-delta fiber activity; ultralate RT: trials of responses with long reaction times related to C-fiber activity (from Mouraux et al. 2003).

**Table 1**
Data from publications on normative values for Laser Evoked Potentials.

| Laser types | Nd YAP | Tm YAG | $CO_2$ (1) | $CO_2$ (2) |
|---|---|---|---|---|
| **Face** | | | | |
| N1 latency (ms) | 112.3 +/- 6.7 | | | 131.1 +/- 21.4 |
| N1 amplitude (uV) | 6.5 +/- 1.8 | | | 7.9 +/- 5.2 |
| N2 latency | 163.0 +/- 13.3 | | 164.0 +/- 13.8 | 201.1 +/- 29.3 |
| P2 latency | | | 241.3 +/- 20.2 | 320.2 +/- 32.5 |
| N2-P2 amplitude (uV) | 26.2 +/- 6.0 | | 21.9 +/- 8.5 | 26.8 +/- 22.1 |
| **Hand** | | | | |
| N1 latency (ms) | 165.7 +/- 11.6 | | | 179.1 +/- 23.3 |
| N1 amplitude (uV) | 5.3 +/- 1.6 | | | 6.3 +/- 5.0 |
| N2 latency | 221.5 +/- 13.4 | 208.0 +/- 18.0 | 236.0 +/- 18.0 | 225.2 +/- 25.4 |
| P2 latency | | 329.0 +/- 34.0 | 314.5 +/- 23.1 | 335.1 +/- 31.9 |
| N2-P2 amplitude (uV) | 22.1 +/- 6.1 | 30.3 +/- 10.9 | 18.3 +/- 8.5 | 24.6 +/- 19.5 |
| **Foot** | | | | |
| N1 latency (ms) | 213.3 +/- 11.2 | | | |
| N1 amplitude (uV) | 4.1 +/- 2.3 | | | |
| N2 latency | 265.6 +/- 14.8 | 248.0 +/- 27.0 | 275.4 +/- 16.7 | |
| P2 latency | | 381.0 +/- 41.0 | 361.0 +/- 26.3 | |
| N2-P2 amplitude (uV) | 18.4 +/- 5.0 | 22.5 +/- 6.7 | 16.0 +/- 5.5 | |

Data for Nd YAP from Di Stefano et al. 2017 (n = 73), data for Tm YAG from Spiegel et al. 2000 (n = 23), data for $CO_2$ (1) from Truini et al. 2005 (n = 100), data for $CO_2$ (2) from de Tommaso et al. 2017 (n = 170). Data are expressed as mean +/- SD. Nd YAP = Neodymium-doped Yttrium Aluminum Perovskite, Tm YAG = Thulium-doped Yttrium Aluminium Grenat.

et al. 2003; Kakigi et al 2005; Frot et al., 2007); and it also would contain a component from SI (Valentini et al 2012). Due to its lower susceptibility to attention and vigilance effects (Garcia-Larrea et al., 1997), the N1 has theoretical advantages over the vertex potential, but its small amplitude hinders its clinical use.

The main vertex potential (N2/P2) contains bilateral activity from the operculoinsular cortex (earlier part of the N2); the greatest part, however, is generated in the mid cingulate cortex. The source localizations were found to be more or less the same for LEP and CHEPs (at different latencies; Garcia-Larrea et al 2003, Valeriani et al 2002). Due to the faster transduction and steeper temperature rise of laser heat compared to contact heat (1000°/s vs. nominally 70°/s), LEP display shorter latencies and clearer waveforms than CHEPs. Although the positivity of the LEP vertex potential has a peak latency near 300 ms, it is clearly distinct from the classical P300 or P3 endogenous evoked potential. Using oddball paradigms with laser stimuli of different task-relevance, the laser-evoked P3 was shown to occur after the vertex potential (Siedenberg and Treede, 1996), and in turn the laser-evoked vertex potential can also be recorded under distraction by mental arithmetics (Schlereth et al. 2003).

Although laser radiant heat stimuli excite both Aδ and C nociceptors (Bromm and Treede, 1983), the cortical responses mentioned so far only reflect Aδ-fiber activation. Specific modifications of the experimental paradigm are needed to obtain evoked potentials specific of C-fiber stimulation: a) the Aδ component of the afferent volley is suppressed by nerve compression, b) large skin areas are stimulated with low power density, c) laser stimuli are focused to tiny skin spots (Magerl et al. 1999, Plaghki and Mouraux 2003, Cruccu et al. 2003, Kakigi et al 2005). All these modifications yield so-called "ultra-late" LEPs, occurring 800–1000 ms after stimulating the dorsal hand.

*4.2.2. Recording parameters and normal values*

As indicated in the previous section, the main component (N2/P2) is generated in the midcingulate cortex with radial orientation pointing towards the vertex. Therefore, the minimum EEG montage for LEP recording consists of a vertex lead vs. an earlobe reference plus a vertical electrooculography (EOG) montage to control for blink artefacts. In order to capture the N1 component, which is elicited bilaterally after unilateral stimulation, two additional electrodes (T3 and T4) referenced to Fz are recommended. By adding Fz vs. earlobes and Pz vs. earlobes, the LEP vertex potential N2-P2 can be distinguished from laser-evoked P3 potentials (Fig. 8). The recording bandpass should be 0.1–200 Hz; it may be narrowed when signal-to-noise ratio is poor but should not be narrower than 0.2–30 Hz. Sample rate for digitization should be at least 250 Hz. Two runs of 20–30 stimuli are usually sufficient to measure the vertex potential; more stimuli are needed to record N1. The attentional state of the subjects should be standardized (e.g. by a rating task) and eyes should either be consistently closed (reducing blink artefacts) or open with steady gaze toward a fixed point (reducing alpha EEG contamination).

Adequate laser intensity depends on wavelength, beam diameter and pulse duration; these parameters have not yet been standardized. Unlike the case of SSEPs in response to electrical stimulation of peripheral nerve trunks, where stimulus intensity must be related to individual detection thresholds, the intensity of the laser test pulses may be fixed per laboratory. For $CO_2$ laser stimulation, an energy density of 20 mJ/mm$^2$ was found to be sufficient (Treede et al. 1988). The stimulated spot should be slightly moved between consecutive pulses to avoid skin lesions and fatigue of peripheral nociceptors. Stimuli should not be delivered at intervals less than 6 sec to minimize habituation.

LEP peak latencies depend on distance from the stimulating point to the recording site and to a lesser extent on pulse duration (peak skin temperature is reached at the end of the pulse). Due to their shorter pulse duration, solid-state lasers give rise to earlier responses than $CO_2$ lasers (Perchet et al. 2008). For this and other reasons, each laboratory should obtain its own set of reference data for LEP latencies and amplitudes. An overview of normative values from a number of laboratories for different laser types is given in Table 1. LEP vertex amplitudes decrease, and latencies tend to increase with age, but do not show significant differences with respect to gender in larger studies (Truini et al. 2005, de Tommaso et al. 2017, Di Stefano et al. 2017; but see Staikou et al. 2017). Studies in multiple sclerosis have shown that LEP latencies are sensitive to detecting demyelination, but the most frequent abnormality is loss or reduction of amplitude of the LEP vertex potential (Spiegel et al. 2003). The vast majority of studies used laser stimulation of hairy skin. It has been concluded from monkey studies, that glabrous skin may not contain the same nociceptive fibers as hairy skin, since heat stimulation using $CO_2$ lasers did not yield the same responses from glabrous as from hairy skin. However, when a laser wavelength with higher penetration depth was used, bypassing the heat transduction time, the same LEP were obtained from both skin types in humans (Iannetti et al., 2006). This has not been clinically tested but may prove useful in future studies.

Based on differences in the distribution of C and Aδ nociceptors (Aδ < C), Plaghki's group reported the successful stimulation of C nociceptors by laser beams for the first time (Bragard et al., 1996). They stimulated a tiny area of the skin with a laser beam that was expected to hit C nociceptors exclusively: the results supported this. The difference in the threshold of the response to thermal stimuli between C (40°C) and Aδ nociceptors (46°C) is also useful for the selective activation of C nociceptors by laser beams.

More recent analysis techniques include the calculation of global field power (spatial variance), and time–frequency analysis of single trials. Global field power improves the signal-to-noise ratio by including a larger set of scalp electrodes; the resulting waveform may be used to distinguish N1, N2-P2 and P3 components in the time domain, and in a second step the scalp distribution or dipole source structure can be analyzed according to global field power peaks (Fig. 7). Time-frequency analysis improves signal detection by being invariant to latency jitter of the vertex potential; the vertex potential is visible as theta power (Aδ-fiber LEP) or delta power (C-fiber LEP) in time–frequency analysis of averaged evoked potentials, but time–frequency analysis of single trials also makes stimulus-induced changes which are not phase-locked, clearly visible (Fig. 8; Mouraux et al., 2003). These techniques have mostly been used in experimental studies, but with modern digital technology they should be easily implemented in clinical neurophysiology laboratories.

*4.2.3. Advantages*

LEP abnormalities are closely correlated to hypoalgesia to heat; they provide an objective correlation of functional alterations in nociceptive pathways. Co-activation of thermosensitive pathways is of little diagnostic relevance, because peripheral afferents (small fibers) and central processing (STT pathways) of thermosensitive pathways are highly similar to those in nociceptive pathways. LEPs can detect signaling abnormalities at any point along the nociceptive pathways, from periphery to cortex, including very small lesions, provided that (a) these lesions impair STT conduction and (b) the laser test stimuli are applied to the anatomically appropriate skin region. Even small lesions in the thalamus, brainstem or nerve roots are easily detected with LEPs if they alter conduction in pain pathways (Treede et al 2003, Kakigi et al 2005, Montes et al 2005, Garcia-Larrea 2006, Quante et al. 2007, Hüllemann et al. 2017). LEP are less sensitive in peripheral lesions where latency prolongations or a complete loss of the potentials can be found only when almost all thinly myelinated fibers are affected. The technique is most sensitive when a lesion affects densely bundled fibers, as found in CNS tracts. In contrast, LEPs are unchanged in psychogenic hypalgesia. Dissociation between normal SSEPs and abnormal LEPs reflects the dissociated sensory loss in STT lesions such as syringomyelia, brainstem syndromes or SFNs (Treede et al 1991, Kakigi et al 1992, Garcia-Larrea 2006). Thus, LEPs are a valuable tool to assess functional spinothalamic small fiber abnormalities in clinically doubtful cases.

#### 4.2.4. Limitations

In contrast to standard SSEPs, subcortical LEPs have not been identified yet. Therefore, LEPs can only assess the overall functional state of the nociceptive system, but cannot suggest peripheral, spinal or brain lesion sites. The level of the lesion cannot be identified using a single stimulation level. Only in spinal lesions, stimulation of different segments may indicate the level of the lesion; identification of the lesion site beyond spinal level using LEPs alone is not possible. At higher age, LEP amplitude reductions or abolished signals become unspecific, since amplitudes decay with age. However, this has been shown to become really relevant at an age of 80 years and beyond (Gibson et al., 1991; Creac'H et al., 2015).

The earliest LEP component N1 reflects activation of parasylvian brain regions. However, due to its small amplitude, it cannot be identified in all healthy subjects. Clinical use of N1 is hence limited to group comparisons for pathophysiological studies, but not for judgement of individual patients. In contrast, a missing vertex potential N2-P2 is considered sufficiently robust to be used as a clinical biomarker in individual patients, by many neurophysiologists. Like all vertex potentials, this waveform of the LEP is modulated by attentional factors. Those factors need to be maintained constant in comparative studies using LEPs.

### 4.3. Contact heat evoked potentials

Activation of specialized skin nociceptors is the only way in which neurophysiological techniques can provide reliable responses related to nociception. This is relatively easy, as free terminals reach the epidermis all over the body. Out of the various methods used to stimulate skin nociceptors, contact heat provides a non-invasive type of stimulation. Once the nociceptors have been activated, a sensory volley that travels towards the central nervous system is generated. This volley generates neither recordable nerve action potentials in peripheral nerves, nor reliable short-latency cerebral evoked potentials. However, it gives rise to long-latency event-related brain evoked potentials, which are referred to in the previous chapter as N1, N2 and P2, to laser stimuli. In fact, these are endogenously generated potentials resulting from the integration of nociceptive inputs on cerebral circuits that respond to the stimulus salience. Despite these apparent drawbacks, CHEPs are easy to record and measure; the N2 and P2 appear at a latency compatible with conduction in SAFs; they are obtained using non-invasive methods, and have normative values of reference for their application in clinical practice (Cruccu et al., 2010; Haanpää et al., 2011, Madsen et al., 2014; Lagerburg et al., 2015; Granovsky et al., 2016).

#### 4.3.1. Principles of the technique

For contact heat stimuli to produce pain-related evoked potentials, they must activate nociceptors in such a way that the volley of afferent inputs is synchronous enough. This can be accomplished these days with a device manufactured by Medoc Ltd. (PATHWAY, Ramat-Yishai, Israel), which has been approved by the US Food and Drug Administration (FDA) for the study of pain. The system contains a thermofoil that can increase temperature at a rate sufficiently fast to give a sensation of pinprick rather than heat. The thermofoil is covered with a 25 μm layer of thermoconductive plastic (thermal conductivity of 0.1–0.35 w/m/k at 23̊C), which also contains two thermocouples embedded in a layer of 10 μm, to estimate skin temperature at the thermode surface. This stimulator is usually supplied with a round thermode of 27-mm diameter, covering an area for skin contact of 572.5 mm$^2$. Typically, the stimulus is delivered by increasing the temperature to around 52°C at a rate of 70°C/sec. Even with such fast increase of temperature, it takes a relatively long time for the stimulus to reach the target temperature (it takes 286 ms from 32°C to 52°C). For a better synchronization of afferent inputs to thermoalgesic stimuli, some authors have proposed shortening the time to peak temperature by increasing the baseline temperature of the thermode (Kramer et al., 2012). These authors studied healthy subjects and patients with spinal cord injuries, and demonstrated that the CHEPs obtained with a baseline temperature of 42 °C improved detection of the stimuli in patients and enhanced the amplitude (and shortened the latency) in healthy subjects and patients, in comparison to lower baseline temperature. Various studies by the same group have demonstrated the better outcomes with this approach for detection of CHEPs to upper (Jutzeler et al., 2016) and lower limbs (Rosner et al. 2018). One important achievement with this technique is the improvement in recording the N1 waveform (Kramer et al., 2013).

The slope of increasing the temperature of the thermode is not perfectly smooth. A small hump appears where there is a small alteration in the speed of temperature change, which is necessary for the control of peak temperature and is unnoticeable in practice (Fig. 9). However, even though the CHEPs are, in the majority of cases, smooth waveforms of large amplitude, it is not infrequent to obtain responses with two peaks. In theory, afferent volleys of inputs sufficiently synchronized as to activate the brain structures responsible for the generation of event-related evoked potentials, can be produced at any point beyond 42°C, which is considered the lower limit of temperature at which pain receptors are activated (Treede et al. 1998; Harkins et al. 2000; Arendt-Nielsen and Chen 2003). This leads to some degree of jitter between responses and to the possibility of generating many ascending volleys out of a single stimulus. Although this may complicate recordings, scalp surface mapping and dipole source analysis suggest generators of the CHEPs similar to those described with laser stimuli, at the operculo-insular and mid-cingulate cortices (Valeriani et al., 2002; Garcia-Larrea et al., 2003). It may be helpful to record the sudomotor skin response of the palm of the hand, simultaneously with the CHEPs, as shown in Fig. 9. Its presence would bode in favor of the stimulus being above threshold for perception, and

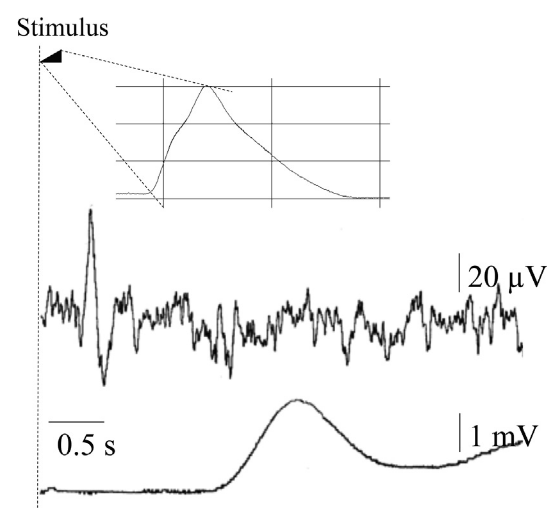


**Fig. 9.** Contact Heat Evoked Potentials (CHEPs): Stimulation and recording considerations. Schematic representation of the stimulus in real time in the small triangle marked as 'stimulus' and amplified in the upper graph. The stimulus characteristics were: rise time of 70°C/s from 32°C to 52°C. Observe the small hump at the rising phase of the stimulus. The evoked potential, recorded from Cz with reference to the ears, resulted from a single stimulus, at a latency of 451 ms and a peak-to-peak amplitude of 93 μV. After averaging 10 responses, this healthy subject had a CHEPs of an amplitude of 62 μV (due to jitter and variability of response amplitude). We recorded simultaneously the sudomotor skin response of the palm of the hand (bottom trace), which appears in healthy subjects at about 2 s after stimulus onset.

**Table 2**
Data from publications on normative values for Contact Heat Evoked Potentials (CHEPs).

| | | Chen et al.,2006 | Lagerburg et al., 2015 | Granovsky et al., 2016 | Jutzeler et al., 2016 | Jutzeler et al., 2016[d] |
|---|---|---|---|---|---|---|
| Number of subjects | | 35 | 97 | 226 | 101 | 101 |
| Body sites | | 5 | 2 | 5 | 3 | 3 |
| Temperature range | | 32 °C to 51 °C | 35 °C to 51 °C | 32 °C to 51 °C | 35 °C to 52 °C | 45 °C to 52 °C |
| CHEPs parameters[a] | Latency (ms) | 370.1+/- 20.3 | 399 (45) | 463.7 (48.9) | 361.5 (31.6) | 267.3 (22.9) |
| | Amplitude (μV) | 10.2 +/-4.9 | 29.5 +/- 11.0 | 23.9+/-14.6 | 19.0 +/-6.4 | 35.0 +/-11.8 |
| Side-to-side differences | | No | Not reported | No | Not reported | Not reported |
| Gender differences[b] | | No | Yes | Yes | Yes | Yes |
| Age differences[c] | | No | Yes | Yes | Yes[e] | Yes[e] |

a. To stimulation applied at volar forearm, a site common to all studies. They refer to N2 peak latency and the N2/P2 amplitude.
b. Corrected by height. In all studies reporting gender differences, CHEPs have larger amplitude and shorter latency in females than in males.
c. In all studies reporting age differences, there is a tendency for elder people to show smaller amplitudes and longer latencies of CHEPs than younger people.
d. Data on increased baseline protocol.
e. Differences only present for N2/P2 amplitude when using 35°C as baseline and for both, latency and amplitude when using increased baseline method (45°C).

rule out technical problems related to stimulation (Cervera et al., 2002).

Thermoalgesic stimuli heat up the skin under the thermode so fast that the subject experiences pinprick pain rather than heat. This activates mechano-heat skin receptors, mainly TRPV1 (Baumgärtner et al., 2012), and generates ascending volleys in thinly myelinated and unmyelinated axons. In fact, the role of skin receptors is important for the generation of CHEPs, as they are very sensitive to changes in stimulation conditions. Depending on activation history, receptors will be more or less predisposed to deal with a new stimulus. This is one of the reasons why it is recommended that the thermode position is modified from one stimulus to another. In doing that, the examiner must also consider the pressure exerted by the thermode over the skin and the inter-stimulus interval, after having placed the thermode in contact with the skin. Changes in pressure would make pressure receptors fire, and immediately after thermode-to-skin contact, tactile receptors will be firing. In both instances, there will be ascending volleys generated by corpuscles responding to touch or pressure, which could theoretically cause some gating on the afferent thermoalgesic volley. Therefore, it is appropriate to wait for a few seconds while maintaining the thermode quiet at the same slight pressure, before applying the thermoalgesic stimulus.

Obviously, the thermofoil needs cooling after the temperature increase, which is done with a Peltier element, at a rate of 40°C/s. This has been considered too slow to elicit reliably evoked potentials in relation to cold stimuli. However, some technical improvement and a rigorous control of artifacts, including those derived from EEG recordings, have recently allowed Rosner et al. (2019), to obtain a good enough signal-to-noise ratio to distinguish the cold evoked potentials (CEPs) from background activity. These are smaller than CHEPs, but the technique holds good promise for future clinical application.

*4.3.2. Recording parameters and normal values*

For CHEPs to be reliably measured, they have to be free of artifacts and movements. To avoid subjects' reactions, it is advisable to have them familiarized with the type of sensation by receiving a few stimuli prior to testing. CHEPs can be recorded from many scalp sites, but it is recommended to record at least from midline, over both hemispheres (i.e., Cz, FPz, C3 and C4). Surface electrodes in these sites are usually referenced to the earlobes or the nasion. The waveforms obtained from these electrodes are similar to those obtained with laser stimuli and receive the same name: N2 and P2. The largest amplitude and most consistent waveform is usually obtained in Cz; it is the waveformmost commonly used for measuring CHEPs. The N1 component of the nociceptive evoked potentials is not obtained reliably with CHEPs, but Kramer et al. (2013) reported better recordings using the increased baseline temperature stimulation technique, and recording from the temporal lobe (Tc) with reference to Fz. The earth electrode can be placed around the head or at the neck. An EOG electrode should also be used to monitor blinking. Such movement may contaminate the cerebral evoked potentials and, therefore, traces should be rejected if there is blinking on them. This is one of the reasons why the best way to proceed is to apply single pulses, and average the artifact-free recordings off-line. Gain for representation of the signal in the EEG/EMG screen should be about 20 μV/div, as the waveform expected is of some 50 μV. The frequency bandpass must be selected to record a slowly developing waveform, i.e., from 0.1 Hz to 50 Hz. Impedance should be tested and kept below 5 kOhm. Room temperature must be kept comfortable, between 20 and 24°C.

There are many reports on normative values (Chen et al., 2006; Lagerburg et al., 2015; Granovsky et al., 2016; Jutzeler et al., 2016), with slightly different methodological approaches (Table 2). Observations common to the majority of reports are the dependence of CHEPs latency and amplitude on age and gender. CHEPs are usually smaller and of longer latency in men than in women, even after correction for the differences in height between genders. Also, most studies report on a tendency for peak latency and amplitude to become, respectively, longer and smaller with age.

The mean N2 peak latency is longer in Granovsky et al. (2016) than in the other reports. However, this study was based on a very large sample where subjects of all ages were equally represented, which may be a bias towards the influence of older persons in the mean value. Granovsky et al's study (2016) contains normograms for all age decades, from 20 to 79 years, for males and females. Interestingly, differences with age were significant in Jutzeler et al., 2016 only when the authors used the increased baseline protocol, i.e., when the baseline temperature was 45 °C.

There is currently little evidence on the number of stimuli needed to achieve reproducible signals. Most commonly, authors collect between 10 and 15 artifact-free responses per stimulation point before off-line averaging. Obviously, the number of averaged traces influences the amplitude of the averaged response, as CHEPs habituate because of fatigue of peripheral receptors. Reliability of CHEPs was examined by Ruscheweyh et al.(2013), who found that, in healthy volunteers, the most reliable parameter of CHEPs recorded in two successive sessions, separated by 6 months, was the area of the response, while peak amplitude showed a tendency to increase, and peak latency to decrease, in the second session. Nevertheless, peak amplitude and N2 latency are considered the standard measures of CHEPs, as they have been reported to reveal the degree of skin denervation in patients with painful neuropathies (Casanova-Molla, 2011; Wu et al., 2017).

#### 4.3.3. Advantages and limitations

Both advantages and limitations of the technique are based on whether they reflect specific features of contact heat stimuli. On one hand, these can be compared to other techniques available for the study of nociceptive evoked potentials. On the other hand, the usefulness of CHEPs can be compared, with a broader perspective, to the utility of other techniques available for the study of thermoalgesic sensation in health and disease, e.g. QTT.

The form of nociceptive evoked potentials that was first described with applicability to clinical studies is radiant heat through a laser beam (Bromm and Treede, 1983; Cruccu et al., 2010). A large amount of data has been accumulated on LEPs for the assessment of the nociceptive pathway, and are valued by some researchers as more representative of the activation of pain pathways than CHEPs. The main difference is that stimuli with radiant heat may not involve fibers other than those conveying the pinprick sensation, as it is not necessary to touch the skin to activate pain receptors with the laser beam. This is, indeed, an important difference that requires certain precautions with the application of the thermode for CHEPs, as outlined above. The counterpart is that laser stimuli can cause burns, either superficially (with $CO_2$) or in deep tissues (with other lasers such as argon or neodymium), and regulations apply for their use in certain countries. Repetitive stimulation over the same spot is unacceptable with laser because of the increased risk of severe burns, while it is feasible with CHEPs. Because of this and other features, contact heat may be more convenient than radiant heat for studies requiring repetitive stimulation.

For the assessment of painful neuropathies, contact heat has the same limitations as LEPs heat, i.e., they do not provide information on the level or the cause of an eventual abnormality, they are relatively less suited for the assessment of peripheral nerve than CNS lesions, they cannot activate pain receptors well enough in glabrous skin, and they may give rise to false negatives in otherwise healthy individuals (see Treede et al., 2003).

CHEPs, like LEPs, are a surrogate measure of the integration of inputs from nociceptive afferents in brain circuits. Therefore, they are not really dependent on the elicitation of pain, but on the salience that the inputs have in the environment (external and internal) in which they have been generated. The evoked potential will be outstanding from the background depending on the relationship between the number of fibers activated by the contact heat stimulus and the density of other simultaneous inputs. In fact, when subjects were asked to evaluate the timing at which they perceived the thermoalgesic stimulus, using Libet's clock, Valls-Solé et al. (2012) observed a negative correlation between the time of subjective awareness of the stimulus and the amplitude of CHEPs, but not with their latency or the intensity of the stimulus, indicating that inputs that were more salient were perceived earlier.

A specific limitation for contact heat is that there is a maximum temperature, for safety reasons . This maximum allows for activation of a sufficient number of receptors in healthy subjects and patients with most neurological disorders. However, in patients with severe polyneuropathies, stimuli applied to leg sites may be insufficient for elicitation of a suitable afferent volley, and no evoked potentials would be recorded. It is in these occasions when psychophysical testing may be more helpful, as a study on the evaluation of perception can be done faster in various sites. In fact, nociceptive evoked potentials are dominated by the inputs induced in thinly myelinated Aδ fibers, whereas the unmyelinated fibers, although they are certainly activated by the nociceptive stimulus, are not usually represented in the response, unless certain methodological precautions are taken (Plaghki and Mouraux, 2003). In contrast, the results of psychophysical studies involve representation of all types of fibers.

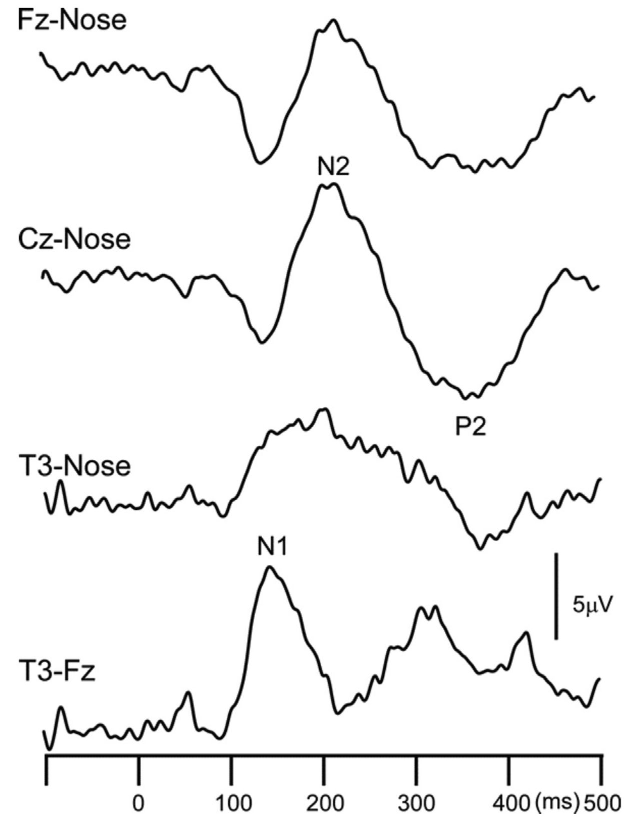


**Fig. 10.** Intraepidermal electrical stimulation evoked potentials. Early N1/P1, N2, and P2 components following IES to the dorsum of the right hand from a single subject. Average 25 sweeps, 0.1–70 Hz bandpass filter.

In conclusion, recording of CHEPs provides for an objective, noninvasive, physiological evaluation of SAFs. In combination with psychophysical testing, CHEPs offer the possibility to document small-fiber sensory deficit.

### 4.4. Pain-related intraepidermal electrically evoked potentials

#### 4.4.1. Principles of the technique

There are various ways to activate the nociceptive system including chemical, thermal, electrical, and mechanical stimulation. Each method has its own advantages and disadvantages, but ideally, the stimulation should be selective, safe, reproducible, and quantifiable. Electrical stimulation is the easiest method to activate cutaneous fibers, but it has a serious problem in selectivity. Electrical stimuli always co-activate mechanoreceptors of the tactile system at a noxious intensity, because their electrical threshold is lower than nociceptors. This unselective stimulation occurs particularly with superficial electrical stimulation of the skin. This is the reason why this summary is limited to intraepidermal electrical stimulation (IES):

IES is a method that can selectively stimulate cutaneous nociceptors (Inui et al., 2002a). This method is based on the fact that nociceptive fiber terminals are located in the epidermis and superficial layer of the dermis, while other fibers run more deeply in the dermis. Therefore, when the superficial layer of the skin is electrically stimulated, the localized current is expected to selectively activate nociceptors. A concentric bipolar configuration is used for IES. The cathode is an outer ring 1.3 mm in diameter, and the anode is an inner needle that protrudes 0.02 mm from the outer ring. By using the concentric bipolar electrode, the current is expected to localize in the epidermis, because it can be regarded as a radial assembly of infinite tripolar arrays that can reduce undesired loop currents (Ohsawa and Inui, 2009). Like laser beams and contact heat (Treede et al., 2003; Garcia-Larrea, 2012; Jutzeler et al., 2016), IES can be used to elicit PREPs.

The electric stimulus can be a conventional square wave pulse of 0.2–1.0 ms but a slowly rising pulse, such as a triangular wave, is better (Otsuru et al., 2009). Double pulses with a 10–25 ms interval are usually used to obtain clear responses, but a single pulse is also used when a precise response latency is necessary. The current intensity is 1.5–2 times the threshold at which stimulation causes a definite pain sensation, 3–6 on the visual analogue scale. A sharp pricking sensation, an indication of Aδ nociceptor activation, is elicited without any other sensation. The pricking sensation is abolished by the local application of lidocaine (Otsuru et al., 2010). To increase the response, two or three electrodes 10 mm apart are used. In recent studies, a triple-electrode type (NM-983 W, Nihon Kohden, Tokyo, Japan) has been used.

By using specific stimulation parameters, IES activates C-fibers selectively. The stimulus is a train of 10 triangular pulses of 0.2–1 ms at an interstimulus interval of 20 ms (Otsuru et al., 2009; Kodaira et al., 2014; Motogi et al., 2014).

*4.4.2. Recording parameters and normal values*

Cortical responses to IES are large vertex evoked potentials consisting of a negative (N2) followed by a positive (P2) potential. Sometimes they are preceded by an earlier component (N1/P1), similar to laser stimulation (Fig. 10). A Cz electrode referred to the ear lobe is necessary to record the vertex potential. If possible, one temporal electrode referred to Fz is used to record the N1/P1 component arising from the opercular region. An average of 10–15 sweeps is usually enough to observe clear components. Similar to other stimulation methods, stimuli have to be delivered with an interval longer than 5 s to avoid habituation.

Electrical stimulation using a conventional bipolar or concentric surface electrode in the hand area elicits an N2/P2 peaking at 140/250 ms, as a result of activation of Aβ fibers, while the IES-induced N2/P2 peaks at about 200/300 ms, because of the slower conduction velocity of Aδ fibers (Otsuru et al., 2010). As for the foot, the IES-induced N2/P2 latency is about 230/370 ms (Kodaira et al., 2014). Since the latency of EP components is longer following IES of a distal rather than proximal site due to the distance traveled, the peripheral conduction velocity (CV) can be calculated by dividing the difference in latency between the EP components, by the distance between the two sites. With this method, the mean CV was 15.1 m/s using EPs (Inui et al., 2002a) in the hand and upper arm, and 15.6 m/s using evoked magnetic fields with the MEG recording technique (N1/P1) in the hand and elbow (Inui et al., 2002b).

IES with anodal inner needle elicits EPs similar to those obtained by laser stimulation. Because of the slow conduction of C fibers, N2/P2 components are very late, compared to Aδ stimulation. For example, in a study by Otsuru et al. (2009), the P2 latency was 783 and 1007 ms for forearm and hand stimulation respectively, yielding a conduction velocity of 1.5 m/s. In a study using MEG, the mean conduction velocity for the lower limb was 1.1 m/s with an N1/P1 latency for the knee and foot of 903 and 1302 ms, respectively (Motogi et al., 2014).

*4.4.3. Advantages*

IES has several advantages over other noxious stimuli. Unlike laser stimulation, electrical stimulation does not induce undesired skin effects such as heat burn or erythema, inducing minimal discomfort. The method allows for selective activation of Aδ nociceptors, without the need for expensive equipment. The stimulus is easy to control, requiring no specialized skills, and can be applied to any part of the body (Omori et al., 2013). Since IEs is an electrical method, it provides a good time locked stimulus, which is important when analyzing responses in the order of milliseconds (Inui et al., 2003). For clinical testing, IES has been used for SFN such as diabetic neuropathy, and hereditary transthyretin amyloidosis (Omori et al., 2017; Kodaira et al., 2019).

*4.4.4. Limitations*

IES activates tactile mechanoreceptors in the dermis when the current is too strong. In fact, results of a study by Mouraux et al. (2010) using a nerve conduction blockade showed that IES at 2.5 mA activates Aβ mechanoreceptors in addition to Aδ nociceptors. Therefore, one cannot use a strong current even when intense sensations of pain are necessary. Usually, the threshold for stimulation of Aδ nociceptors by IES with double pulses is below 0.1 mA, and 2–3 times the threshold is enough to obtain clear cortical responses. At around this intensity, IES selectively activates Aδ nociceptors. For a stronger sensation, spatial summation by use of multiple electrodes or temporal summation by a long duration pulse or pulse train should be considered instead of an increase in intensity. To validate the usefulness of IES and to establish normative data, studies using a large group of normal subjects are necessary.

*4.5. Quantitative thermal sensory testing*

Although consensus guidelines on the diagnosis of small fiber neuropathy are not available, psychophysical quantitative testing of non-nociceptive and nociceptive thermal perception thresholds has been suggested to be part of the diagnostic work-up (Themistocleous et al., 2014, Terkelsen et al., 2017). The method has been used clinically to demonstrate small fiber pathology in a wide range of conditions, eg. trigeminal neuropathy (Jääskeläinen et al., 2005), diabetic neuropathy (Løseth et al., 2016), and non-freezing cold injury (Jørum and Opstad, 2019). Other quantitative sensory tests can also be used, e.g quantitative assessment of painful pin prick and pressure pain, which are a part of the German research network protocol for quantitative sensory testing profiling (Rolke et al., 2006). However, these techniques are less validated for the purpose of diagnosing small fiber neuropathy, and are usually not suggested to be part of the diagnostic work-up.

*4.5.1. Principles of the technique.*

The approach consists of measuring psychophysical perception threshold responses to non-painful and painful cold and warm stimuli controlled by an automated device, where the patient can terminate a stimulus when experiencing a specified percept (Fig. 11). A commercially available Peltier element based thermode, included in a computerized system, is used with a unidirectional stimulation technique (Hansson et al., 1988; Verdugo and Ochoa, 1992). Quantitative thermal testing (QTT) is based on precise definition of the thermal stimulus properties (modality, intensity, spatial and temporal characteristics), analysis of the quality of evoked sensation, as well as quantification of its intensity. Loss and gain of function can both be assessed. Non-noxious warm and cold stimuli activate a subgroup of C- and A-delta fibers respectively, while such stimuli in the noxious range trigger a mixture of nociceptive A- and C-fibers (Backonja et al., 2013). This information is conveyed further in the spino (trigemino)-thalamo-cortical tract, after synaptic transmission in the dorsal horn of the spinal cord or the spinal trigeminal nucleus in the pons. In an elaborate protocol developed by the German Network on Neuropathic Pain (Rolke et al., 2006), QTT performed by trained examiners had good inter-observer and test–retest reliability for use in patients with sensory disturbances of different etiologies (Backonja et al.,

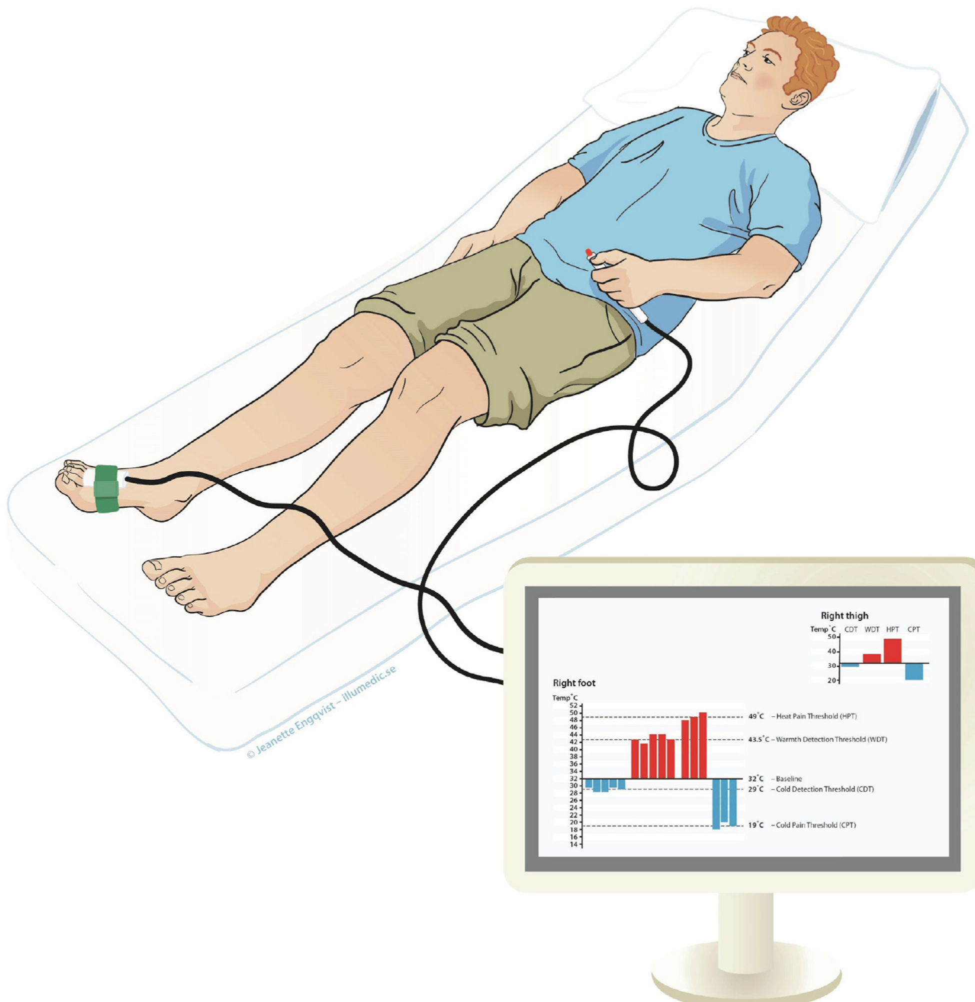


**Fig. 11.** General setting for Quantitative Thermal Sensory Testing. The thermode is applied on the foot of the patient who holds the switch to signal the temperature felt. The screen displays the thresholds signaled for cold, warmth, heat pain and cold pain.

2013). Reliability of QTT has been studied extensively with fair to good reliability (Moloney et al., 2012; Heldestad Lilliesköld and Nordh, 2018).

Temperature bedside testing should always precede QTT, e.g., using Lindblom rollers for testing cold and warmth perception (Marchettini et al., 2003), and a pin (pin prick) to map the distribution of possible dysfunction and to guide the application site of quantitative measures, i.e., the area of most pronounced hypoesthesia. The main advantage of QTT over bedside examination would be greater precision in assessing the functionality of sensory channels although, importantly, differences in outcome between QTT and bedside testing of small fibers have been reported (Leffler and Hansson, 2008). This inconsistency might be explained by the categorization criteria of what are normal and pathological outcomes, and differences in physical properties of the employed stimuli, or the way the stimuli are applied. Nevertheless, for QTT one should also test different areas and compare, e.g., one or more distal test areas with a proximal test area when investigating a patient with a possible length-dependent small fiber neuropathy.

Instructions to the patients should be standardized, preferably using written formal instructions. Examiners need extensive training to optimally introduce and follow-up stimulus-induced percepts reported by the patient when terminating a stimulus (Backonja et al., 2013). This schooling can be provided by renowned laboratories across the world.

It is also essential to train the subject to be examined before commencing definitive clinical testing. Patients with neuropathy may have severely distorted percepts in the affected area, and may need to have a normal site tested for the tuning of normal perceptual criteria. In an area of neuropathy qualitative (e.g., allodynia, dysesthesia), temporal (abnormal latency, aftersensation), and spatial (spread of sensation, faulty localization) aberrations of the induced percept should also be sought during QTT and bedside testing, to have a full-blown picture of the functional status of the small fiber system (Hansson, 1994). In patients with painful symptoms from the neuropathy, allodynia to warmth and cold may be found. In other instances, loss of function may be found in all four percepts.

An alternative to the method of limits is the method of levels (Dyck et al., 2005). In this case, subjects are presented with temperature stimuli that resolve on their own and, after a signal, they are requested to respond if they have noticed the requested percept or not. If the answer is no, the relative temperature deviation from baseline is increased; if the answer is yes, it is decreased. The procedure is repeated several times until an accurate level of the sensation, corresponding to threshold, is defined. The method of levels is not dependent on reaction time and offers the advantage of automatization among laboratories (Dyck et al., 1993). The main drawback is the time required to do the whole process, which is significantly longer than with the method of limits.

*4.5.2. Recording parameters and reference values*

Numerous algorithms have been employed for the assessment of sensory thresholds. The method of limits (Fruhstorfer et al., 1976) has proven to be quick, reliable, and easy to use (Heldestad et al., 2010). The temperature applied to the skin is increased or decreased from a baseline temperature of 32°C, until the subject perceives the first sensation of the requested percept (i.e., warmth perception). The subject then stops the stimulus by a feedback control, e.g., a button. The outcome is thus dependent on the reaction time, which again is dependent on both the motor ability and attention of the subject under investigation, as well as the rate of the changing temperature (Hilz et al., 1999). For safety reasons, temperatures above 50°C are avoided. For warm and cold perception thresholds, it is common to perform three or five repetitions with randomly varying inter-stimulus intervals (e.g., 3–6 seconds), and, e.g., three repetitions if assessing pain perception thresholds. There is significant habituation to repeated stimulation, in particular for heat pain threshold. Therefore, averaging the values obtained from three repetitions may be sufficient (Agostinho et al., 2009).

The outcome may be reported as deviation from baseline (i.e., relative change) or as absolute temperature. Assuming a baseline temperature of 32°C, a heat perception threshold of 42°C may thus be reported as a relative change of 10°C, or as the absolute temperature (42°C). Reporting the deviation from baseline has the advantage that a high value always indicates a large deviation from baseline, and may be easier to use when reporting or discussing deviations from thresholds, uniformly for warm and cold perception; reporting the absolute temperature may be more intuitive for readers with less experience with QTT. When comparing reliability studies of QTT, it is important to be aware of whether relative or absolute temperature measures are used.

Cultural and subtle language differences underline the need for laboratory-specific reference values. Other factors, like probe size, velocity of temperature change, initial skin temperature, environmental factors, gender, age, the site of stimulation, as well as differences in written formal instructions, all may influence the reference values [Chong and Cros, 2004; Moloney et al., 2012). Nevertheless, several reference values have been published. However, due to the above-mentioned factors reference values may diverge between laboratories [e.g. Blankenburg et al., 2010; Magerl et al., 2010; Hafner et al., 2015; van den Bosch et al., 2017; Heldestad Lillieskold and Nordh, 2018) and should be used with caution. Local normative values stratified on age and gender should be considered mandatory for clinical practice. Interestingly, there are few reports on reference values for intra-individual side differences (Zwart and Sand, 2002). Reports of wide normal limits for side-differences (Dunker et al. 2021), as well as the potential for contralateral changes, possibly due to plasticity in central processing (Enax-Krumova et al. 2017) or contralateral affection in presumably unilateral conditions (Krumova et al. 2021, Konopka et al. 2012; Oaklander et al. 1998), limits the potential use of the method in patients with unilateral conditions.

For the purpose of diagnosing SFN, findings of increased warm and cold perception thresholds (relative change) may indicate involvement of unmyelinated and small myelinated sensory nerves, respectively. Lowered heat pain and cold pain thresholds (relative change) may be used to document allodynia. However, aiming at documenting increased heat and cold pain thresholds, i.e., hypoalgesia, is seldom valuable, as the upper reference values most often will be very high, and close to the upper margin. Accordingly, pathological warm and cold perception thresholds will be more readily detected than heat and cold pain thresholds. Moreover, increased heat and cold pain thresholds (relative change) with normal warm and cold perception thresholds is a rare testing outcome, difficult to interpret, and will most often not be considered a clinically meaningful finding when aiming specifically at diagnosing small fiber neuropathy.

*4.5.3. Advantages*

The equipment is commercially available and easy to use. The results are available immediately, and may be conveyed to the patient during the same consultation by the treating physician. In addition, and as mentioned above, all four thermal percepts may be investigated with the method, and both loss and gain of function may be assessed in one session. Equipment assessing QTT is a low risk medical device, but damaged thermodes may pose a risk for burn injury, and therefore require maintenance and calibration at regular intervals

*4.5.4. Limitations*

As a psychophysical measure, QTT is dependent on close interaction between the examiner and a fully cooperative subject. Monotonous information on how the test is run, and continuous communication to assure that reported sensations are according to protocol are mandatory. Furthermore, the approach cannot detail the level of the lesion along the neuraxis. Alterations in temperature and pain sensitivity may be due to pathology in peripheral nerves or the spino (trigemino) –thalamo-cortical pathway. Medical history and focused neurological bedside examination, in conjunction with other investigations, like NCS, may jointly indicate the level of disease or lesion of the neuraxis (Shy et al., 2003). Although NCS only tests for large fiber pathology, this is still the first line neurophysiological investigation for peripheral neuropathy. As small fiber neuropathy is an inherent part of almost all large fiber neuropathies, the additive value of QTT is often minor if NCS has demonstrated pathology.

QTT done arbitrarily in a small part of the area suspected to be pathological may not be "representative". This is why the faster bedside examination that allows testing of a larger part of the area of interest offers guidance as to where to perform QTT. The placement of the QTT must also be guided by the anatomical areas where the reference material is collected. These considerations also apply to other methods, e.g., skin biopsy and evoked potentials.

Altered attention, motivation and cognition may influence results, and interspersed null stimuli may be used to assess this, as well as the potential contamination of outcome by malingering.

From a differential diagnostic perspective with special emphasis on painful small fiber neuropathy, sensory aberrations are not pathognomonic for neurological lesions. In non-neuropathic pain conditions, somatosensory thermal abnormalities have been reported in the focal or referred pain area of subgroups of patients (Nathan, 1960; Leffler et al., 2008), which may indicate the possibility of activity in the nociceptive system interacting with function in other sensory systems (Treede and Magerl, 2000). Hence, QTT is never a standalone strategy in diagnosing neuropathy, but is a complement to bedside examination of somatosensory function based on a careful medical history and a focused neurological examination, as well as additional objective investigations.

Although the equipment is easy to use and readily implemented in the clinic, problems with proper standardization and interpretation of the results may be difficult to deal with for a clinician without long experience with these issues. The tedious work of collecting body-site specific reference values from a proper normal population should not be underestimated.

### *4.6. Skin biopsy - intraepidermal nerve fiber density*

*4.6.1. Principles of the technique*

The epidermis is composed of four layers of keratinocytes that, from the basal layer, progress towards the stratum corneum,

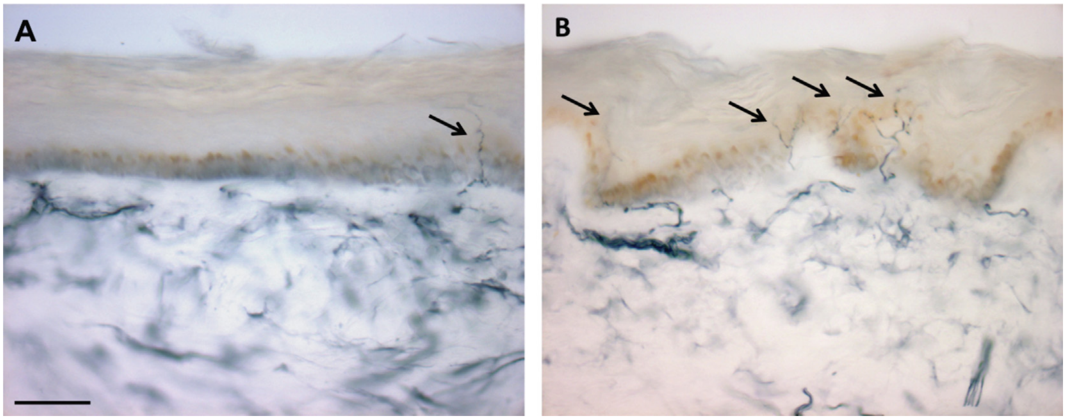


**Fig. 12.** Skin biopsy - intraepidermal nerve fiber density. Intraepidermal nerve fibers (arrows) immunostained for neuronal marker PGP 9.5 in skin sections of a representative patient with Small Fiber Neuropathy (A) compared with an age-matched healthy subject (B). Original magnification × 40, scale bar = 50 μm.

undergoing a gradual differentiation in the course of 3 weeks. Other resident cells in the epidermis are Langerhans cells, melanocytes, and Merkel cells. The autonomic structures include pilomotor muscles, blood vessels, and sweat glands. In the glabrous skin, the apexes of the papillae contain the Meissner corpuscles. These structures have a density of about 30 per millimeter in the fingertip (Nolano et al., 2003). Pacini and Ruffini corpuscles reside in the deeper layers of the dermis. From nerve bundles in the subpapillary dermis arise individual unmyelinated nerves that cross the dermal–epidermal junction, lose the Schwann cell ensheathment, and enter the epidermis (Lauria et al., 2004).

Intraepidermal nerve fibers (IENF) have exclusive somatic functions, as demonstrated by their normal appearance and density after experimental sympathectomy (Li et al., 1997). They widely express the capsaicin receptor, which makes them the most distal nociceptors (Lauria et al., 2006). The same occurs in the epithelium of the mucosae (Lauria et al., 2005, Borsani et al., 2014). Also, keratinocytes and other non-neuronal resident cells might be relevant in the homeostasis of temperature sensations, and in the pathogenesis of mechanical hyperalgesia and inflammatory pain through pain-related receptors, which could influence IENF excitability (Peier et al., 2002, Denda and Tsutsumi, 2011, Fernandes et al., 2012). In this perspective, the whole skin, in particular the epidermis, can be considered as a huge polymodal receptor, whose functions are based on the relationship between resident cells and nerves (Lumpkin and Caterina, 2007).

IENF and autonomic nerves regenerate within 3–6 months after chemical damage induced by capsaicin, and this correlates with loss and recovery of sensation and cutaneous autonomic organ functioning (Simone et al., 1998, Gibbons et al., 2010b). The same can occur in clinical condition after disease-modifying treatments or spontaneous recovery, such as truncal diabetic neuropathy, hypothyroidism, steroid-responsive neuropathy, and impaired glucose tolerance-related SFN (Lauria et al., 1998, Nodera et al., 2003, Smith et al., 2006, Penza et al., 2009). IENF regeneration is affected in clinical conditions like diabetes and HIV infection, even when patients do not have any sign of peripheral neuropathy (Polydefkis et al., 2004, Hahn et al., 2007), emphasizing the utility of skin biopsy for studying axon degeneration and regeneration in humans (Khoshnoodi et al., 2017).

Over the last 20 years, skin biopsy has become a popular tool to quantify somatic IENF (Gasparotti et al., 2017), dermal nerve bundles (Lauria et al., 2011), autonomic nerve fibers of sweat glands (Gibbons et al., 2010a), and pilomotor muscles (Nolano et al., 2010). This approach has expanded the diagnostic work-up of patients with peripheral neuropathies, and procedures have been published in European and USA guidelines (England et al., 2009, Lauria et al., 2010b).

The most common application of skin biopsy is in the diagnosis of SFN (Cazzato et al., 2017), a condition that -albeit typically diffuse- can involve terminal branches of sensory nerves like in notalgia (Lauria and Lombardi, 2007). In essence, this tool provides evidence of terminal somatic and autonomic nerve degeneration, when routine sensory nerve conduction studies may give normal results. Furthermore, it allows investigating the involvement of myelinated nerve fibers in inherited and acquired demyelinating neuropathies (Li et al., 2005, Lombardi et al., 2005, Saporta et al., 2009, Stalder et al., 2009, Ruts et al., 2012, Manganelli et al., 2015).

Skin biopsy, when performed using a 3-mm disposable punch, as usually done in clinical setting, is a minimally invasive and safe procedure, with healing occurring within 7–10 days. The estimated frequency of undesirable effects is less than 2 per 1,000, most commonly mild infections due to improper management of the wound, that recovered with topical antibiotic therapy, or excessive bleeding that does not require suture (Lauria et al., 2010b). Biopsy is performed in a sterile field after topical anesthesia with 2% lidocaine, and no suture is required, using a 3-mm punch. When a larger punch is used (5-mm), steri-strip tape is required.

The specimen includes the epidermis and the dermis, and allows the analysis of sweat glands, piloerector muscles, and arterial-venous anastomosis. To increase the probability of sampling these structures, it is recommended to perform the biopsy including a hair in the punch biopsy. A less invasive sampling method is removing the epidermis by applying an aspiration capsule to the skin. This "blister technique" does not cause bleeding, nor does it require local anesthesia (Kennedy et al., 1999).

After the punch biopsy is performed, the specimen should be immediately fixed for approximately 24 h, at 4°C, in 2% paraformaldehyde-lysine-sodium periodate (PLP) or Zamboni's solution (2% paraformaldehyde, picric acid). Formalin fixation should be avoided, because it could cause a fragmentation of the nerve fibers. The specimen is then kept in a cryoprotectant solution overnight, and cut with a cryostat in vertical sections of 50 μm thickness. A 3-mm punch biopsy can provide about 45–50 sections. At least three sections should be immunostained, using the polyclonal antibody against the cytoplasmatic neuronal marker protein gene product 9.5 (PGP 9.5), a ubiquitin carboxyl-terminal hydrolase. Other antibodies targeting the cytoskeleton (e.g tubules and neurofilaments) could be used and be of help in assessing nerve regeneration (Lauria et al., 2004).

#### 4.6.2. Measurement parameters of skin biopsy and reference values

All IENF crossing the dermal-epidermal junction are counted, the length of the epidermis is measured using a software for biological measure, and IENF density per millimeter is calculated (Fig. 12). IENF are counted under the light microscope at 40 × magnification, or using a software for image analysis. Either bright-field immunohistochemistry or immunofluorescence, with or without confocal microscopy, has been used, but the technique does not affect the reliability of skin biopsy in assessing IENF loss (Lauria et al. 2010a). Using the confocal microscope immunofluorescence technique, density is usually calculated based on evaluation of image, sum of consecutive 2 μm optical sections, for a standard linear length of epidermis. The guidelines of the European Federation of the Neurological Societies and Peripheral Nerve Society reports technical procedures and methods to assess IENF density (Lauria et al., 2010b). Even though the blister method is not commonly used in clinical practice, the quantification of IENF was reported to be similar to that obtained from punch skin biopsy (Panoutsopoulou et al., 2009).

The availability of normative references for IENFD assessment, adjusted to age decade and sex (Lauria et al., 2010a, Provitera et al., 2016), has contributed to the diffusion of skin biopsy as a diagnostic tool for SFN in clinical practice.

Bright-field and indirect immunofluorescence techniques for IENFD assessment (Lauria et al., 2010a, Provitera et al., 2016) have been compared, showing high agreement (Nolano et al., 2015a). Moreover, in both healthy subjects and SFN patients, IENFD is consistent when compared between the left and right distal leg, and at 3-week follow up, which is the period of keratinocyte turnover from the most basal epidermal layer to the stratum corneum (Lauria et al., 2015). Normal values have also been established for the face (Nolano et al., 2013).

The small myelinated Aδ fibers lose their myelin before entering the dermis, and become indistinguishable from C fiber axons (Provitera et al., 2007). Instead, dermal myelinated endings are thinner terminal branches of large Aβ fibers. The quantification of dermal nerve fibers has been proposed using different approaches that, in general, agreed on the correlation with the measurement of IENFD in differentiating healthy subjects from SFN patients (Vlckova-Moravcova et al., 2008, Lauria et al., 2011). Nevertheless, the contribution that dermal nerve fiber assessment can provide to the diagnosis of sensory neuropathy in patients with normal IENF count is not yet established, although the morphometric analysis of dermal myelinated nerve fibers may be useful in patients with inherited and acquired demyelinating neuropathies (Nolano et al., 2003; Nolano et al., 2015b; Saporta et al., 2009, Manganelli et al., 2015; Lombardi et al., 2005; Cortese et al., 2019).

#### 4.6.3. Advantages

Quantitation of IENF density gives direct information on the damage that the neuropathic disorder is causing in Aδ terminals (and in the other structures that can be analyzed after skin biopsy). The diagnostic yield of IENFD assessment in SFN has been evaluated by a reappraisal and validation study that involved 150 and 352 patients, respectively (Devigili et al., 2019). Skin biopsy showed higher diagnostic efficiency (93.3%), as compared to the best performance of QST (82.9%) obtained measuring warm and cold thresholds at both feet, and combining the methods of limits and levels.

#### 4.6.4. Limitations

The quantification of IENFD remains a morphometric parameter that, while useful to define the presence of SFN, does not provide information on its nature, course, response to treatment, and correlation with the clinical picture. Indeed, a loss of IENF can be found in painful and painless SFN, and in congenital insensitivity to pain syndromes, whereas density can be normal in patients with inherited erythromelalgia or other genetic paroxysmal painful disorders. Thus, the diagnosis of SFN should rely on the combination of findings from clinical neurological examination and the additional tests deemed convenient.

One further limitation is the lack of extensive normative reference values for IENFD at sites other than the distal leg. Moreover, despite several approaches used, there is no information on the usefulness of the morphometric assessment of dermal nerve fibers in combination with IENFD quantification, for the diagnosis of SFN. Finally, cutaneous nerves can regenerate, but no reliable marker of regeneration has been validated.

### 4.7. Corneal confocal microscopy

CCM is another morphological technique to evaluate SAFs. This is a non-invasive technique that allows in vivo imaging of all layers of the cornea, enabling quantitation of corneal nerve fibers (CNFs). This novel technique is increasingly considered in SFN evaluation.

#### 4.7.1. Principles of the technique

Corneal nerve axons arise from their corresponding cell bodies in the trigeminal ganglion, and pass via the ophthalmic division of the trigeminal nerve and long ciliary nerves, to enter the cornea as stromal nerves. Then, they run anteriorly as the sub-basal plexus, comprising 1–10 unmyelinated axons, and converge at the inferior whorl to terminate as intraepithelial nerves (~7000/mm$^2$) (Müller et al., 2003). These nerves respond to touch, temperature and pain, and are modulated by a range of neurotrophic factors including substance P, nerve growth factor, calcitonin gene-related peptide, and neurotrophin-3 (Al-Aqaba et al., 2019). CCM has been employed extensively to image the corneal sub-basal plexus, using the HRT III Rostock corneal module (Heidelberg Engineering GmBh, Germany), which uses a class 1 laser system from a 670 nm diode (Tavakoli and Malik, 2011). Most investigators use the section mode to capture 2D digital images (384x384 pixels) with an optical resolution of 10 μm/pixel. Central (6–8 images) and inferior whorl (4 images) images are selected, taking into account the depth, orientation, contrast and quality of the image (Kalteniece et al., 2017). Manual (CCM image Analysis Tools v 0.6′; CCMetrics) and automated (ACCMetrics) software (Dabbah et al., 2011) can be used for measurement.

#### 4.7.2. Measurement parameters and reference values

The following corneal nerve parameters are measured:

1. Corneal nerve fiber density (CNFD), as the number of main nerve fibers per frame (no/mm$^2$).
2. Corneal nerve branch density (CNBD), as the number of primary nerve branches per frame (no/mm$^2$).
3. Corneal nerve fiber length (CNFL), as the sum of nerve length per frame (mm/mm$^2$). This parameter may be standardized for corneal nerve fiber tortuosity (CNFT), which measures the number and amplitude of the changes in direction of fibers.
4. Inferior whorl length (IWL), as the sum of the inferior whorl nerve length (mm/mm$^2$).

Automated artificial intelligence-based deep learning algorithms have been used for unbiased corneal nerve quantification, and show enhanced diagnostic ability for diabetic peripheral neuropathy (DPN) (Williams et al., 2020).

Age-adjusted normative values have been published for CCM (see Petropoullos et al., 2013 and Tavakoli et al., 2015a). There is a significant linear age-dependent decrease in CNFD and CNFL, and increase in CNFT, for men and women, but there is no age-dependent change for CNBD. Height, weight, and BMI did not significantly influence any corneal nerve parameter.

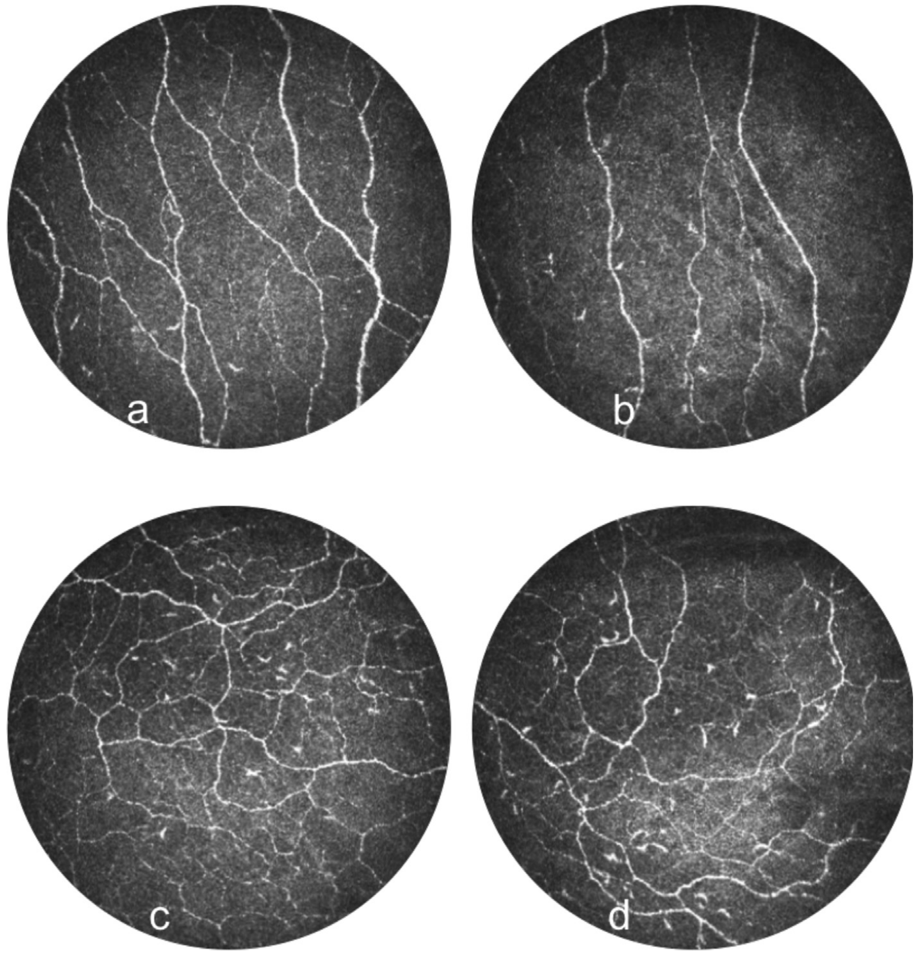


**Fig. 13.** Corneal confocal microscopy in healthy control and patient with diabetic neuropathy. Corneal nerve images from the central (top) and inferior whorl (bottom) regions in a healthy control subject (a,c) and patient with diabetic neuropathy (b,d) showing a loss of corneal nerve fibers.

CCM has been studied repeatedly in disorders related to glucose metabolism: Decreased CNFD occurs in subjects with impaired glucose tolerance (Asghar et al., 2014), recently diagnosed type 2 diabetes mellitus (see Fig. 13; Ziegler et al., 2014), and children and adolescents with type 1 diabetes mellitus (Fedousi et al., 2019). The decrease in CNFD is associated with increasing severity of polyneuropathy in diabetic patients (Malik et al., 2003; Quattrini et al., 2007; Li et al., 2019; Yan et al., 2020; Hafner et al., 2020). Several authors have reported on good sensitivity and specificity for CNFD and CNFL, for diagnosing DPN (Ahmed et al., 2012; Petropoulos et al., 2013a; Brines et al., 2018; Perkins et al., 2018; Tavakoli et al., 2015b). Greater corneal nerve loss (Kalteniece et al., 2018), and augmented branching (Puttgen et al., 2019) have been reported in patients with painful diabetic neuropathy. In a cohort of patients with idiopathic SFN, there was a significant reduction in CNFD and CNFL, which was related to the severity of sensory symptoms (Tavakoli et al., 2010).

CCM has demonstrated corneal nerve loss in various conditions associated with SFN, such as in diabetes (Pritchard et al., 2015; Lovblom et al., 2015; Edwards et al., 2017), chemotherapy-induced polyneuropathy (Ferdousi et al., 2015), HIV (Kemp et al., 2017), Fabry's disease (Bitirgen et al., 2018a), nerve growth factor-β mutation (Perini et al., 2016) or amyloidosis (Rousseau et al., 2016). Decreased CNFD has also been reported in patients with fibromyalgia (Evdokimov et al., 2019), Behcet's disease (Bitirgen et al., 2018b), Charcot Marie Tooth's disease Type 1A (Tavakoli et al., 2012), neurofibromatosis (Barnett et al., 2019), Friedreich's ataxia (Pagovich et al., 2018), CIDP, multifocal motor neuropathy and monoclonal gammopathy of unknown significance (Stettner et al., 2015), and in a group of healthy subjects with elevated HbA1c, triglycerides, and body mass index (Sharma et al., 2018a).

*4.7.3. Advantages*

CCM is a rapid non-invasive ophthalmic imaging technique with high reproducibility, and excellent intraclass correlation coefficient (ICC) for interobserver (e.g. CNFD: 0.92, 95% CI-0.85–0.96) and intra-observer (e.g. CNFD: 0.95, 95% CI-0.91–0.97) reliability (Hertz et al., 2011; Petropoulos et al., 2013b). CNFD has a diagnostic performance for DPN comparable to that of IENFD (Chen et al., 2015; Alam et al., 2017; Moulton and Borsook, 2019), and similar data have been reported in diabetic patients with autonomic neuropathy from Italy (Maddaloni et al., 2015), China (Wang et al., 2015), Japan (Ishibashi et al., 2017) and New Zealand (Misra et al., 2015).

CNFD may be a good indicator of the effect of therapy on terminal nerve fiber damage, as it has shown improvement after simultaneous pancreas and kidney transplantation in type 1 diabetes (Mehra et al., 2007; Tavakoli et al., 2013), after treatment with Cibinetide -a novel first-in-class erythropoietic peptide- in patients with sarcoidosis-related neuropathy (Culver et al., 2017; Dahan et al., 2013), after omega-3 polyunsaturated fatty acid supplementation in patients with T1DM (Lewis et al., 2017), and after

12 months of treatment with thyroxine in patients with hypothyroidism (Sharma et al., 2018b).

The use of CCM in peripheral neuropathies appears as compelling (Petropoulos et al., 2020) as it is a rapid, non-invasive, objective and reproducible technique for quantification of small nerve fiber degeneration and regeneration.

#### 4.7.4. Limitations

CCM is an ophthalmic technique, which requires training to undertake the procedure. Training courses are available in several centres, and the company providing the equipment offers onsite training when installing the device. The availability of the CCM device may be a limitation, although there are currently ~ 500 HRTIII devices across the world and another ~ 3000 retinal modules, which can be modified to undertake corneal nerve imaging. Corneal dystrophy, keratoconus and severe dry eye disease should be accounted for when interpreting corneal nerve pathology.

It has been argued that CCM lacks specificity due to abnormalities found in a wide range of neurodegenerative diseases. However, the same criticism may be applicable to all techniques evaluating SAFs pathology.

## 5. Concluding remarks

There is no single technique or procedure available nowadays that fulfills the requirements for diagnosis, approach to the pathophysiological mechanisms, and follow-up, in disorders presenting with small afferent fiber impairment. In addition to good reliability, such technique would have to be minimally invasive, affordable in many centers, easy to apply, and reproducible. Although they have limitations, NCS and needle EMG are the standard techniques for the evaluation of large caliber sensory and motor fibers impairment. For small fibers, a combination of techniques is necessary and, as in other neurological disorders but particularly in this case, their results must be interpreted strictly in light of the clinical picture.

MNG is a procedure able to directly record the activity of small afferents showing the substrate for positive sensory phenomena. It is still an investigational technique, but can be applied exceptionally to selected patients. The recording of evoked potentials to nociceptive stimulation, whether laser, contact heat or electrical stimuli, allows for evaluation of the peripheral and central pain pathway, is reproducible, and correlates with the presence of hypoesthesia/hypoalgesia rather than hyperalgesia. Nevertheless, there are limitations, some of them common to all types of nociceptive stimulation, such as the lack of localization of the level of the lesion, and some others particular to each type of stimulation, such as safety precautions in the case of laser stimulation, equipment cost in the case of laser and contact heat stimulation, and potential coactivation of large fibers in the case of electrical stimulation. QTT has the advantages of being noninvasive and easy to apply. It is able to assess both negative and positive signs in patients. Nevertheless, as a psychophysical test, it depends on the collaboration of the patient and does not have a localizing diagnostic value. Skin biopsy allows for the evaluation of IENFD, a quantitative method to assess the number and morphology of cutaneous small afferents, but the technique cannot provide information regarding the functional status of the remaining afferents, and requires an adequate laboratory. CCM has the advantage of being noninvasive but, as a morphological method, it does not provide information on the functional status of the fibers, requires special equipment, and is rather unspecific. Further studies showing specificity and sensitivity of the method are expected. In the near future new techniques, such as the use of the micropatterned interdigitated electrode for selective stimulation of nociceptive fibers (Di Stefano et al., 2020), will certainly become very useful.

In present days it is recommended to use more than one procedure for the study of SAFs. The specific techniques to be used depend on the experience of the center but it would be advantageous to combine neurophysiological and morphological techniques. We hope that this review helps the reader to evaluate the advantages and disadvantages of each of the methods presently available.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Glossary

*CNS:* Central nervous system
*CEPs:* Cold evoked potentials
*CHEPs:* Contact heat evoked potentials
*CCM:* Corneal confocal microscopy
*CNFs:* Corneal nerve fibers
*DPN:* Diabetic peripheral neuropathy
*WDR:* Dynamic range neurons
*EOG:* Electrooculography
*IES:* Intraepidermal electrical stimulation
*IENFD:* Intraepidermal nerve fiber density
*IENF:* Intraepidermal nerve fibers
*LAFs:* Large afferent fibers
*LEPs:* Laser evoked potentials
*MNG:* Microneurography
*NCS:* Nerve conduction studies
*PREPs:* Pain-related electrically evoked potentials
*SI:* Primary somatosensory cortex
*QTT:* Quantitative thermal sensory testing
*SII:* Secondary somatosensory cortex
*SAFs:* Small afferent fibers
*SFN:* Small fiber neuropathy
*STT:* Spinothalamic tract
*TRP:* Transient receptor potential
*FDA:* US Food and Drug Administration