doi:10.1093/brain/awz333 BRAIN 2019: 0; 1–9 | 1

BRAIN
A JOURNAL OF NEUROLOGY

Diagnostic criteria for small fibre neuropathy in clinical practice and research

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The diagnostic criteria for small fibre neuropathy are not established, influencing the approach to patients in clinical practice, their access to disease-modifying and symptomatic treatments, the use of healthcare resources, and the design of clinical trials. To address these issues, we performed a reappraisal study of 150 patients with sensory neuropathy and a prospective and follow-up validation study of 352 new subjects with suspected sensory neuropathy. Small fibre neuropathy diagnostic criteria were based on deep clinical phenotyping, quantitative sensory testing (QST) and intraepidermal nerve fibre density (IENFD). Small fibre neuropathy was ruled out in 5 of 150 patients (3.3%) of the reappraisal study. Small fibre neuropathy was diagnosed at baseline of the validation study in 149 of 352 patients (42.4%) based on the combination between two clinical signs and abnormal QST and IENFD (69.1%), abnormal QST alone (5.4%), or abnormal IENFD alone (20.1%). Eight patients (5.4%) had abnormal QST and IENFD but no clinical signs. Further, 38 patients complained of sensory symptoms but showed no clinical signs. Of those, 34 (89.4%) had normal QST and IENFD, 4 (10.5%) had abnormal QST and normal IENFD, and none had abnormal IENFD alone. At 18-month follow-up, 19 of them (56%) reported the complete recovery of symptoms and showed normal clinical, QST and IENFD findings. None of those with one single abnormal test (QST or IENFD) developed clinical signs or showed abnormal findings on the other test. Conversely, all eight patients with abnormal QST and IENFD at baseline developed clinical signs at follow-up. The combination of clinical signs and abnormal QST and/or IENFD findings can more reliably lead to the diagnosis of small fibre neuropathy than the combination of abnormal QST and IENFD findings in the absence of clinical signs. Sensory symptoms alone should not be considered a reliable screening feature. Our findings demonstrate that the combined clinical, functional and structural approach to the diagnosis of small fibre neuropathy is reliable and relevant both for clinical practice and clinical trial design.

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Keywords: small fibre neuropathy; skin biopsy; diagnostic criteria; neuropathic pain; quantitative sensory testing

Abbreviations: C/WDT = cold/warm detection threshold; IEFND = intraepidermal nerve fibre density; NCS = nerve conduction study; QST = quantitative sensory testing; SFN = small fibre neuropathy

Received May 30, 2019. Revised July 6, 2019. Accepted September 4, 2019

Introduction

Small fibre neuropathy (SFN) is a sensory, typically painful, disease of thin myelinated and unmyelinated nerve fibres. It occurs early in the course of several systemic illnesses such as diabetes, amyloidosis and connective tissue disorders, can be genetically determined or idiopathic (Cazzato and Lauria, 2017), and is a model to investigate the efficacy of new targeted analgesics (Eijkenboom *et al.*, 2019*b*). The somatic compartment of this class of fibres conveys thermosensation, nociception and itch from cutaneous fields through sensory peripheral nerves to the dorsal horns in a hierarchical fashion determined by molecular-driven coding of sensory neurons (Lallemend and Ernfors, 2012; LaMotte *et al.*, 2014; Lou *et al.*, 2015). Distinct molecular profiles have been identified as key for the neurogenesis of nociceptors (Bartesaghi *et al.*, 2019) and axon ending targeting to the ectodermic or mesodermic/endodermic tissues (Yang *et al.*, 2013). Thus, phylogenetically conserved peripheral signalling such as thermosensation and nociception are conveyed towards the integrative brain areas through a complex class of nerve fibres whose function is driven by precise molecular ontogenesis.

Somatic and autonomic functional assessment of small nerve fibres is achieved by assaying the psychophysical sensory thresholds (e.g. cold, heat) by quantitative sensory testing (QST), pain-related and laser evoked potential recording, single axon recording using microneurography and tests encompassing sympathetic and parasympathetic autonomic functions (Terkelsen *et al.*, 2017). Their structural assessment relies on skin biopsy and corneal confocal microscopy. The first combines quantification of intraepidermal nerve fibre density (IENFD), dermal nerve bundles, and autonomic organ innervation with analysis of pain-related receptors and myelin protein expression, and has become a routine method (Lauria *et al.*, 2004, 2006, 2011; Lauria and Lombardi, 2007; Provitera *et al.*, 2007; Zhao *et al.*, 2008; Gibbons *et al.*, 2009; Nolano *et al.*, 2010). The second provides various morphometric parameters to quantify corneal nociceptors and their changes over time, and is currently mostly applied in research (Kalteniece *et al.*, 2018; Petropoulos *et al.*, 2019). Overall, these techniques have replaced sensory nerve biopsy that, although it allows identification of small nerve fibres enwrapped into Remak bundles in semi-thin sections, and their ultrastructural quantification, it cannot differentiate afferent and efferent autonomic from somatic axons and is much more invasive (Sommer, 2018).

Despite the advances allowed by new techniques, the diagnostic criteria for SFN are yet to be fully established (Terkelsen *et al.*, 2017). This limitation has several implications both for clinical practice in terms of correct access of patients to treatments and research for the definition of entry criteria in trials. However, it does not arise from the lack of knowledge on the diagnostic performance of tests for small nerve fibre functioning or morphometric assay, but rather from how their combination meets the diagnostic requirements for individual patients at the clinical level. Indeed, abnormal findings in some small nerve fibre-related tests, such as skin biopsy, QST or laser-evoked potentials can occur in painful clinical conditions irrespective of the localization, nature and aetiology of pain (Devigili *et al.*, 2008; Backonja *et al.*, 2013; Terkelsen *et al.*, 2017; Uceyler *et al.*, 2018) and even in painless neuropathies or systemic diseases (Nolano *et al.*, 2001, 2008; Bennett and Woods, 2014; Dalla Bella *et al.*, 2016; Marchi *et al.*, 2018). In such a frame, patients' symptoms and signs of small nerve fibre dysfunction are crucial to the reliable interpretation of the findings obtained by the diagnostic tests.

In the past decade, two sets of diagnostic criteria have been proposed. The first (Besta criteria) (Devigili *et al.*, 2008) is based on the combination of at least two abnormal findings of the following: (i) clinical signs of small fibre impairment (pinprick and thermal sensory loss and/or allodynia and/or hyperalgesia); (ii) abnormal warm or cold thresholds, or both, at the foot as assessed by QST; and (iii) reduced IENFD at the distal leg. Exclusion criteria were any clinical sign of large fibre impairment (e.g. light touch and vibratory sensation, deep tendon reflexes, limb or gait ataxia) and any abnormality at nerve conduction studies (NCSs). The second, within the revised guideline of the Diabetic Neuropathy Study Group of the European Association for the Study of Diabetes (NEURODIAB) (Tesfaye *et al.*, 2010) based on a grading as: (i) possible, if symptoms or clinical signs of small fibre damage, or both; (ii) probable, if clinical signs of small fibre damage, and normal sural NCS; and (iii) definite, if clinical signs of small fibre damage, normal sural NCS, and abnormal QST thresholds at the foot or reduced IENFD at the ankle, or both. The NEURODIAB criteria do not require specification of the clinical signs of small fibre damage.

Our study aimed to address three key questions: (i) the agreement between the two proposed criteria; (ii) the weight of each of the three main components of the proposed criteria, i.e. symptoms and signs (clinical), QST (functional), and IENFD (structural); and (iii) the most reliable approach to individual patients suspected to have SFN. To this end, based on the comparison between the NEURODIAB and Besta criteria, we performed a reappraisal study of a cohort of sensory neuropathy patients (Devigili *et al.*, 2008), analysed how patients were reclassified, and conducted a prospective and follow-up validation study on a new large cohort of patients to confirm the reliability of the diagnostic criteria.

Materials and methods

Part 1 Reappraisal study

We retrospectively re-evaluated the clinical files of 150 sensory neuropathy patients included in the study performed to develop the Besta criteria (Devigili *et al.*, 2008). For all, we

reassigned the value of IENFD at the distal leg using the normative values (Lauria *et al.*, 2010*a*), which were not available at the time the study was carried out. The diagnosis of definite SFN based on the NEURODIAB criteria (Tesfaye *et al.*, 2010) was used as the gold standard to reclassify the SFN patients formerly diagnosed using the Besta criteria. Thereafter, we compared the diagnostic power between skin biopsy and warm detection QST thresholds at foot and hand between the two groups of definite SFN based both on Besta and NEURODIAB criteria. Data from 99 age and sex-matched healthy subjects were used as controls for the QST findings.

Part 2 Validation study

We conducted a prospective and follow-up study on new patients referred to our centre for suspected sensory neuropathy between January 2009 and September 2017. The study was designed according to the standards for reporting of diagnostic accuracy (STARD) and approved by the local Ethic Committee. All patients underwent a standardized protocol comprehensive of questionnaires and pain maps, clinical and neurophysiological tests, multimodal QST battery and skin biopsy, as detailed below. Inclusion criteria were age >18 years and symptoms suggesting sensory peripheral neuropathy (positive and/or negative symptoms with length-dependent distribution at lower limbs and autonomic symptoms) not otherwise explained.

Clinical evaluation and questionnaires

From all patients, we recorded a detailed clinical history including time and features of symptoms onset and any condition potentially causing neuropathy (e.g. known systemic diseases, neurotoxic drugs, etc.). Baseline screening included haematological assays for diabetes and impaired glucose tolerance, thyroid diseases, vitamin B_{12} and folate, hepatitis C and HIV makers, autoantibodies, serum and urine protein immunofixation, antibodies against gangliosides and sulphatide, onconeuronal antibodies and neoplastic markers.

Patients were asked to report any type of annoying or painful sensation either spontaneous (e.g. paraesthesia, cold or warm feeling, tightening feeling, cramps) or evoked (e.g. touching the sheets, warm or cold water, wearing shoes, walking). As part of the baseline evaluation, all patients were asked to fill the SFN Symptom Inventory Questionnaire (SFN-SIQ) (Bakkers *et al.*, 2014) for somatic and autonomic symptoms recording and the Neuropathic Pain Symptoms Inventory (NPSI) questionnaire (Padua *et al.*, 2009), and to draw their pain distribution on a picture of a human.

All patients underwent a thorough bedside clinical evaluation. Presence and distribution of negative and positive sensory and signs were recorded using a comparative assessment of affected and non-affected skin areas to differentiate the quality of the altered sensation and define dermatomeric, mono/multineuropathic and polyneuropathic distribution. Vibratory sensation was quantified using the 128 Hz graduated tuning fork (Martina *et al.*, 1998). Cutaneous sensory signs were assessed asking the patient to keep the eyes closed and to report the sensation induced by tactile stimuli and gently brushing with cotton bud and flat tip brush (dynamic allodynia), punctate skin stimulation with a stick or pin (punctate allodynia), prickling with disposable needle (hyperalgesia), cooling/warming with cold/warm water tube (thermal allodynia), superficial and deep mechanical sensation by finger pressure applied to skin and underlying tissue (static allodynia and hyperalgesia) (Jensen and Finnerup, 2014). The feeling of distorted sensation (e.g. spreading, increased and/or persistent, electric shock) in the affected areas (e.g. soles, dorsal feet, legs, fingertips, palms, forearms, peri-nipple) compared with the neat (normal) sensation in non-affected areas were recorded as allodynia, hyperalgesia or aftersensation based on the type of stimulus used (Backonja *et al.*, 2013). Each clinical test was performed at least twice. Sensory signs were graded as $+2$, $+1$ (gain of function), 0, -1, -2 (loss of function) to allow the comparison with QST findings. Signs of dysautonomia were also recorded (e.g. pupil abnormalities, abnormal sweating, skin flushing or discolouration, orthostatic hypotension, heart frequency).

Nerve conduction studies

Sensory and motor NCSs were performed using surface recording electrodes with standard placement. Compound muscle action potential, motor nerve conduction velocity, distal motor latency and F-wave latencies were recorded for ulnar, peroneal, and tibial nerves bilaterally. Sensory nerve conduction velocity and sensory nerve action potential were assessed for radial, median, ulnar, superficial peroneus, sural nerves and sural dorsal nerves.

Skin biopsy

All patients underwent skin biopsies at distal leg and proximal thigh according to standardized procedures for bright-field immunohistochemistry (Lauria *et al.*, 2010*c*). IENFD was quantified according to standardized guidelines and individual reports based on age and sex-adjusted normative values (Lauria *et al.*, 2010*b*).

Quantitative Sensory Testing

QST examination was performed with a comprehensive method of threshold determination and a multimodal approach including thermal and mechanical stimuli, in order to improve the sensitivity. Stimuli were tested bilaterally to correct borderline findings.

Warm and cold detection thresholds (WDT, CDT) were assayed combining limits and levels methods (LIM + LEV) at the dorsal foot bilaterally and at the dorsal aspect of the non-dominant hand. Then, the limits method (LIM) was used alone for WDT and CDT at proximal thigh, and for cold and heat pain threshold determination at all the sites. Abnormal sensations including errata sensation, thermal allodynia or hyperalgesia, and aftersensation were recorded for all the tests. The sites were evaluated in the following order: non-dominant dorsal hand, right dorsal foot, proximal thigh and left dorsal foot. Thermal stimuli were assessed by the Medoc^{TM} device (Medoc^{TM} Thermal Sensory Analyser, TSA-2001) using a 30 $\times$ 30 mm probe, with ramp stimuli of 1°C/s from 32°C. Results were compared with published reference normative values (Magerl *et al.*, 2010) and for direct comparison with a cohort of 99 age- and gender-matched healthy subjects who underwent the same QST protocol. We used as cut-off *Z*-values above $+1.96$ or below -1.96.

Mechanical detection threshold was measured with a standardized set of modified von Frey hairs (SenseLab, Somedic von Frey Aesthesiometer; range 0.26–490 mN) using the method of limits in five determinations. The test was conducted at all the sites where thermal stimuli were tested.

Statistical analysis

We used the unpaired *t*-test and the Mann-Whitney test to compare the normally and the non-normally distributed variables, respectively. Patient categorization based on clinical variables was correlated with thermal thresholds (WDT and CDT) at both feet, and IENFD at the distal leg.

Sensory modalities assessed by the clinical exam and graded as mentioned above and QST findings were compared using the paired *t*-test and Pearson R^2 coefficient test. We used the Spearman's rank correlation coefficient for the correlation analysis between clinical and laboratory variables. Where applicable $P < 0.05$ was considered statistically significant. For logistic regression studies we used the group of definite SFN from the reclassification study as the validation gold standard to be compared with healthy subjects group. Receiver operating characteristic (ROC) curves were built for distal leg IENFD and several combinations of thermal QST at foot i.e. WDT by method of limits unilaterally (WDT LIM foot) or bilaterally (WDT LIM R + L), WDT by method of levels unilaterally (WDT LEV foot) or bilaterally (WDT LEV R + L), WDT combined with CDT by method of levels (WDT + CDT LEV) and limits (WDT + CDT LIM). Sensitivity, specificity and diagnostic accuracy were calculated by ROC findings for the different techniques, including combination of modality for thermal thresholds detection. There were no missing data in either the reappraisal or validation studies. All analyses were performed using the SPSS for Mac release, 21.0.0.0.

Data availability

The data that support the findings of this study are available from the corresponding author.

Results

Part 1 Reappraisal study

The assignment of IENFD values based on the normative study (Lauria *et al.*, 2010*a*) changed the diagnosis in 5 of 150 patients: SFN was ruled out in one patient, whereas four mixed (large and small sensory fibre) neuropathies were reclassified as large fibre neuropathy. Based on the Besta criteria, 25 of 66 patients eventually diagnosed with definite SFN had abnormal IENFD and QST findings but lacked clinical signs showing only one of the two negative sensory signs required (i.e. pinprick and thermal hypoesthesia). Conversely, the NEURODIAB criteria for definite SFN require the evidence of 'clinical signs of small fibre damage', without further specification in number and quality. Therefore, when we compared the reliability of the Besta criteria for definite SFN against the NEURODIAB criteria used as the gold standard, all except one patient met the criteria. The ROC analysis for diagnostic efficacy of the Besta criteria based on the comparison with the NEURODIAB showed an area under the curve (AUC) of 0.98, with 100% sensitivity and 98.5% specificity.

Part 2 Validation study

A total of 352 patients (184 females and 168 males) met the entry criteria. Ten patients were diagnosed with vascular stenosis, somatoform disorder, lumbar stenosis, plantar fasciitis and ruled out. Sensory neuropathy was hypothesized in 342 patients (176 females and 166 males; age range 19–79 years, mean 58 ± 13.3) complaining of symptoms. All patients underwent clinical, neurophysiological, QST, skin biopsy, and laboratory tests. The diagnostic classification was axonal large sensory fibre neuropathy (43; 12.6%), mixed large and small sensory fibre neuropathy (81; 23.7%), sensory neuronopathy (16; 4.7%), demyelinating neuropathy (5; 1.5%), mononeuropathy (3; 0.8%), and multiplex mononeuropathy (7; 2%).

The remaining 187 patients with no clinical and NCS evidence of large sensory and motor nerve fibre impairment were considered affected by possible SFN. Of those, 38 (20.3%; 29 females, nine males; mean age 45.6 ± 11.9) complained of sensory symptoms but did not show any clinical signs. Thirty-four (89.4%) had normal QST and IENFD, four (10.5%) had abnormal QST and normal IENFD, and none had abnormal IENFD only. Eventually, using the Besta criteria, 149 patients were diagnosed with definite SFN based on the combination of two clinical signs plus abnormal QST and IENFD (103; 69.1%), or abnormal QST alone (8; 5.4%), or abnormal IENFD alone (30; 20.1%), whereas eight patients (5.4%) did not present clinical signs but had abnormal QST and IENFD. This latter subgroup of patients would have been ruled out based on the NEURODIAB criteria that require the evidence of clinical signs.

We compared the diagnostic efficiencies of the NEURODIAB criteria and the Besta criteria. To this end, diagnostic efficiency was calculated using the Besta criteria as the gold standard, which identified a group of true positive patients (n = 149). The diagnosis of definite SFN had a sensitivity of 94.6%, specificity of 99% [95% confidence interval (CI) = 0.649–0.775]; positive predictive value (PPV) 0.993 (95% CI 0.97–0.99), negative predictive value (NPV) 0.925 (95% CI 0.882–0.953). For the diagnosis of probable SFN, values did not differ from those of definite SFN because in this cohort no patient had negative signs alone without abnormal QST and/or IENFD findings. The diagnosis of possible SFN had sensitivity 100% (all 187 patients had symptoms of SFN and normal NCS), specificity 71.5% (95% CI 0.965–0.998), PPV 0.793 (95% CI 0.74–0.837) and NPV 1.0 (95% CI 0.98–1.0).

Sensory symptoms were reported to have unilateral onset in 69 patients (46.3%), whereas 38 (25.5%) described their complaints as asymmetric at the neurological examination. We recorded autonomic symptoms in 52 patients (34.9%)

Table 1 Intensity and frequency of pain features in 149 small fibre neuropathy patients using the NPSI questionnaire

Painful symptoms	Patients, *n* (%)	Mean NRS 11 point
Spontaneous pain	56 (39.7)	6.6
Evoked pain	21 (14.9)	6.2
Spontaneous and evoked pain	64 (45.3)	8.6
Main quality of pain		
Burning pain	71.9 (51)	6.5
Sharp pain	31.0 (22)	7.8
Deep aching pain	15.5 (11)	7.3
Pinprick	14.1 (10)	5.8
Cold pain	4.2 (3)	6.9
Itching	4.2 (3)	8.5
Frequency of pain features at NPSI		
Q1 Burning	102 (72.3)	5.0 ± 1.7
Q2 Squeezing	75 (51.0)	4.1 ± 2.3
Q3 Pressure	69 (48.9)	3.6 ± 1.0
Q4 Electric shocks	11 (7.8)	1.6 ± 2.1
Q5 Stabbing	42 (29.8)	1.7 ± 2.0
Q6 Evoked by brushing	52 (36.9)	3.2 ± 2.4
Q7 Evoked by pressure	47 (33.3)	2.8 ± 2.4
Q8 Evoked by cold stimuli	39 (27.7)	1.6 ± 2.0
Q9 Pins and needles	71 (50.3)	4.7 ± 1.6
Q10 Tingling	18 (12.8)	2.4 ± 2.1

NPSI = Neuropathic Pain Symptoms Inventory.

Table 2 Negative and positive sensory signs in 141 patients with SFN

	Patients, *n* (%)	Stimulus
Negative signs		
Total	141	
Pinprick and thermal hypoaesthesia	141 (100)	Disposable needle; cold/warm water tube
Mechanical hypoaesthesia	31 (22)	Cotton ball
Positive signs		
Total	111 (78.7)	
Allodynia		
Mechanical – punctate (static)	69 (62)	Stick or pin
Mechanical (dynamic)	46 (41.4)	Flat tip painter's brush
Thermal	55 (49.5)	Cold/warm water tube
Pressure	66 (59.4)	Gentle finger pressure
Hyperalgesia		
Pinprick hyperalgesia	91 (82)	Disposable needle
Pressure-evoked hyperalgesia	79 (71)	Finger pressure
Aftersensation	88 (79)	

In 111 patients we found both negative and positive signs, whereas 30 patients had only negative signs.

using the SFN-SIQ (Bakkers *et al.*, 2014). The NPSI questionnaire (Padua *et al.*, 2009) findings are summarized in Table 1.

One hundred and eleven patients showed both negative and positive signs, whereas 30 patients had only negative signs (Table 2). In 79 patients there were signs of peripheral venous-arteriolar dysfunction, including erythromelalgia-like in 22 patients. The aetiology of SFN was identified in 87 patients (58.3%), whereas it remained unknown in 62 patients. Aetiologies included diabetes (22; 25.4%), impaired glucose tolerance (26; 29.9%), mixed connective tissue disease (17; 19.5%), hypothyroidism (6; 6.9%), sarcoidosis (2; 2.3%), Fabry disease (6; 6.9%), rheumatoid arthritis (2; 2.3%), progressive systemic sclerosis (2; 2.3%), Sjögren syndrome (2; 2.3%), HCV infection (1; 1.1%) and *Borrelia burgdorferi* infection (1; 1.1%).

Diagnostic accuracy and comparison studies

Table 3 details sensitivity, specificity and diagnostic efficiency in the 149 SFN patients. ROC analysis showed a higher performance of IENFD compared to QST. Moreover, it showed that QST achieved the highest performance when both warm and cold thresholds were performed at the feet using with both limit and level test. The comparison between clinically determined positive and negative signs and QST *Z*-scores showed good agreement for all the sensory modalities tested: warm (r^2 = 0.91), cold (r^2 = 0.68), mechanical (r^2 = 0.73) and pressure sensation (r^2 = 0.81).

Follow-up study

Over a period of 2.6 ±1.4 years, we followed up 104 of 149 patients with definite SFN, 54 of them with unknown aetiology, and all 38 patients with symptoms but no clinical signs of SFN (Fig. 1). All patients underwent haematological screening, clinical evaluation with SIQ-SFN and NPSI, QST, NCS, and/or other instrumental evaluation when clinically appropriate.

In 28 of 104 SFN patients, abnormal findings at NCS changed the diagnosis of SFN in mixed large and small fibre neuropathy. SFN remained idiopathic in 40 of 54 patients (74%), whereas 12 patients (22%) were diagnosed with impaired glucose tolerance (4), hypothyroidism (4), coeliac disease (3) and diabetes (1). The pair sample *t*-test did not show significant changes in the pain scales and NPSI findings. Follow-up skin biopsy was performed in 62 of 104 patients and showed a mean loss of 1.23 fibres/mm yearly.

At 18-month follow-up (range 1–6 years), 19 of 34 (56%) patients with symptoms alone reported complete recovery and had normal clinical examination, QST and IENFD. In 14 of them an alternative diagnosis was achieved (e.g. foot osteoarthritis, chronic venous insufficiency). Of the three of the four patients with symptoms and abnormal QST alone at baseline, none showed clinical signs or abnormal IENFD. All eight patients diagnosed

Table 3 Diagnostic accuracy using of skin biopsy and various combination of thermal thresholds test comparing SFN and healthy subjects

	AUC ROC	Sensitivity	Specificity	Efficiency
IENF density distal leg	0.93	94.3	91.9	93.3
Thermal QST combination				
Method of limits				
WDT foot LIM	0.606	73.7	50.5	64.2
WDT feet LIM R + L	0.76	75.1	74.7	75
Method of levels				
WDT foot LEV	0.716	67.3	78.7	72
WDT feet LEV R + L	0.809	78.7	78.8	78.7
WDT + CDT feet (LEV)	0.783	85.8	76.7	82.8
Method of limits and levels combined				
WDT + CDT feet (LIM + LEV)	0.836	85.1	80.8	82.9%

L = left; LEV = levels; LIM = limits; R = right.

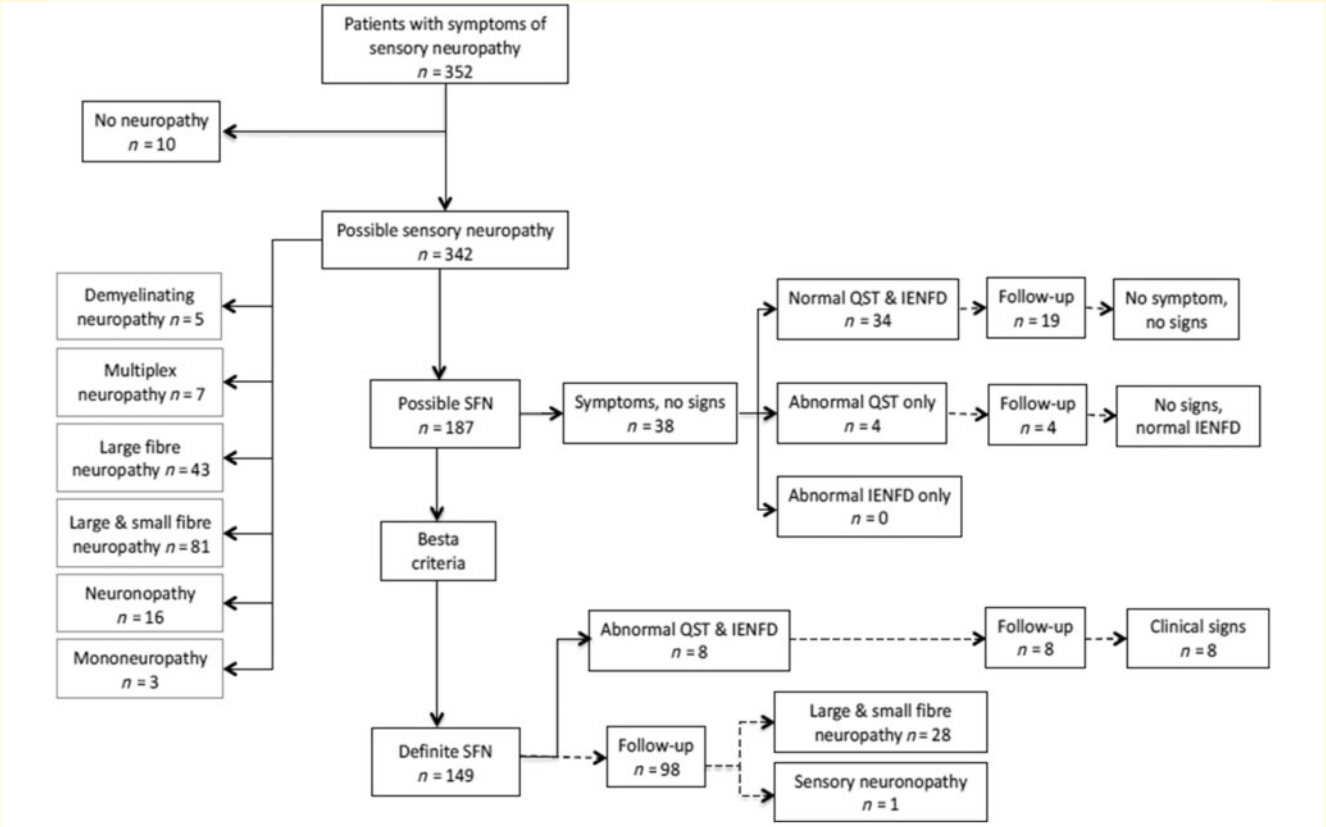

Figure 1 Flow-chart of the diagnostic assessment of patients included in the validation study.

with definite SFN based on abnormal QST and IENFD alone showed clinical signs.

Discussion

SFN is an emergent, intriguing disorder with relevant impact on patients' well-being and research in neuropathic pain (Terkelsen *et al.*, 2017). Small nerve fibres are the largest class of peripheral nerves in mammals and encompass highly conserved functions in the domain of thermosensation, nociception and autonomic responses. Their endings in epidermis and epithelia widely express the TRPV1 receptor and are the most distal peripheral nociceptors in humans (Lauria *et al.*, 2005, 2006). The reason why they can selectively degenerate and sometimes cause

neuropathic pain is largely unknown. Indeed, they also degenerate in painless conditions such as amyotrophic lateral sclerosis (Weis *et al.*, 2011; Dalla Bella *et al.*, 2016) in which a potential mechanism has been discovered in the SOD^{G93A} mouse model to be the accumulation of a toxic splicing variant of peripherin that is not expressed in large size sensory neurons (Sassone *et al.*, 2016).

The assessment of small fibre nerves should be in the frame of a well-defined clinical context (Cazzato and Lauria, 2017). The early diagnosis of SFN is important to identify patients at risk of developing more generalized neuropathy such as that associated with amyloidosis (Chao *et al.*, 2019) and diabetes (Lauria *et al.*, 2003; Devigili *et al.*, 2008; Khoshnoodi *et al.*, 2016; Loseth *et al.*, 2016). The recent identification of sodium channel gene mutations in idiopathic and diabetic SFN patients (Huang *et al.*, 2014; Waxman *et al.*, 2014; Alsaloum *et al.*, 2019; Eijkenboom *et al.*, 2019*a*) has enlarged the spectrum of neuropathic pain disorders including new phenotypes (Faber *et al.*, 2012*a*, *b*; Hoeijmakers *et al.*, 2012; Bennett and Woods, 2014; Devigili *et al.*, 2014; Serra *et al.*, 2014; Doppler *et al.*, 2015; Martinelli-Boneschi *et al.*, 2017), allowing the development of new innovative *in vitro* (Persson *et al.*, 2013; Rolyan *et al.*, 2016) and *in vivo* (Eijkenboom *et al.*, 2019*b*) models, and has prompted randomized clinical trials with new targeted compounds. In this scenario, the need for clearly defined and reliable diagnostic criteria for SFN appears crucial (Terkelsen *et al.*, 2017).

The combination of various somatic and autonomic nerve testing has been proposed to increase the diagnostic ability (Terkelsen *et al.*, 2017) but none of them has been validated or concretely applied in clinical practice. As a matter of fact, the process toward the definition of the diagnosis of SFN in individual patients, which begins from complaints of sensory symptoms, is based on clues from skin biopsy and/or QST results, whose reliability has been investigated in a huge number of studies (Gasparotti *et al.*, 2017). Conversely, the weight of the clinical signs, albeit emphasized (Tesfaye *et al.*, 2010; Malik *et al.*, 2011; Edwards *et al.*, 2016), remains unaddressed, as they are felt to be difficult to analyse objectively at the bedside. Specific questionnaires such as the Utah Early Neuropathy Scale can be used to detect subtle sensory disturbances (Singleton *et al.*, 2008) and could differentiate patients with neuropathy from controls (Zilliox *et al.*, 2015), but they have not been included in guidelines and recommendations, and only one study has tested specificity and sensitivity of the clinical examination (Devigili *et al.*, 2008).

The aim of this work was to provide conclusive evidence on the diagnostic criteria for SFN to be used in clinical practice and trial design. One challenging issue regards the potential circularity of the analysis of efficacy of three approaches used to achieve the diagnosis, namely clinical examination, IENFD and QST findings. Acknowledging the lack of a 'gold standard' with which to compare specificity and sensitivity of the diagnostic criteria, we first proposed the 'two of three' combinatory approach (Devigili *et al.*, 2008). The 'Besta criteria' are based on the evidence of at least two abnormal findings among the three used to assess small fibre damage, which include the presence of two negative clinical signs (pinprick and thermal sensory loss) possibly associated with positive clinical signs (allodynia and/or hyperalgesia), abnormal warm or cold thresholds, or both, at the foot assessed by QST, and reduced IENFD at the distal leg. To confirm the validity of our criteria, we performed a reappraisal and a validation study keeping as 'gold standard' the NEURODIAB criteria that essentially define the diagnosis of SFN in the presence of the combination of clinical signs not further specified and abnormal QST or reduced IENFD at the distal leg. Our results showed not only a strict agreement between the two diagnostic approaches, but demonstrated the validity of the clinical assessment both for negative and positive signs when compared with the QST findings, indicating the reliability of focused bedside assessment of small fibre functioning in individual patients. These findings could contribute in better defining the clinical profile of patient's phenotype (von Hehn *et al.*, 2012) and meeting the needs of clinical trial design (Farrar, 2010).

Sensory symptoms, most commonly spontaneous and positive thus in the spectrum of neuropathic pain, are the reason why patients seek help and neurologists schedule investigations for possible neuropathy. Nevertheless, sensory symptoms belong to a completely subjective domain and their reliability cannot be directly tested. To overcome this issue, we separately analysed the course of 38 of 187 patients presenting with symptoms but showing no clinical signs at the neurological examination (Fig. 1). In 89.5% of them, QST and IENFD both at baseline and 18-month follow-up were normal, whereas in the remaining 10.5% abnormal QST thresholds remained the unique finding.

QST remains a valid test to assess the diagnosis of SFN, though its diagnostic accuracy is lower than that of IENFD (Table 3). Our study demonstrated that its specificity and sensitivity increases if warm and cold thresholds are measured combining the methods of limits and levels at both the feet. However, such comprehensive testing is extremely time consuming and the determination of warm threshold alone using the method of levels could be a reasonable compromise.

In conclusion, to increase the reliability of the diagnosis and reduce the number of screening failure in clinical trials, patients should be suspected to have SFN when at least two clinical signs are present. Sensory symptoms alone are not reliable. Quantification of IENFD remains the most reliable tool to confirm the diagnosis.

Funding

The study received intramural funds from the "Ricerca Corrente" program of the Italian Ministry of Health.

Competing interests

The authors report no competing interests.

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