

Intraepidermal nerve fibre density, quantitative sensory testing and nerve conduction studies in a patient material with symptoms and signs of sensory polyneuropathy

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Small diameter nerve fibre (SDNF) neuropathy is an axonal sensory neuropathy affecting unmyelinated (C) and thin myelinated (A-delta) fibres. We have evaluated 75 patients with symptoms and signs suggesting SDNF dysfunction with or without symptoms and signs of co-existing large diameter nerve fibre involvement. The patients were examined clinically and underwent skin biopsy, quantitative sensory testing (QST) and nerve conduction studies (NCS). The purpose of this study was to compare the relationship between the different methods and in particular measurements of thermal thresholds and intraepidermal nerve fibre (IENF) density in the same site of the distal leg. The main subdivision of the patient material was made according to the overall NCS pattern. Patients with normal NCS (38) had 6.4 ± 3.8 and patients with abnormal NCS (37) had 4.4 ± 3.4 IENF per mm ($P = 0.02$). Limen (difference between warm and cold perception thresholds) was significantly higher (more abnormal) in those with abnormal than in those with normal NCS (22.1 ± 9.1 vs. 13.4 ± 5.6 , $P < 0.0001$). Cold perception threshold was more abnormal ($P < 0.0001$) than warm perception threshold ($P = 0.002$). Correlation between IENF and QST was statistically significant only when NCS was abnormal, and thus dependent of a more severe neuropathic process in SDNFs.

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Introduction

Small diameter nerve fibre (SDNF) neuropathy may occur isolated or, more commonly, in mixed polyneuropathies affecting all categories of sensory and often motor nerve fibres. Since most SDNFs are unmyelinated, SDNF neuropathy is an axonopathy. Patients with isolated SDNF neuropathy typically complain of burning pain, tingling and numbness. Symptoms are most often symmetrical and distal. Frequently, clinical examination reveals no other neurologic deficit in these patients than impairment of pain and temperature sensation. SDNF neuropathy may have different causes; the most common is considered to be diabetes mellitus. Among other causes are alcohol abuse, Sjögren's syndrome, amyloidosis, hereditary sensory neuropathy, AIDS and Fabry's disease. In the majority of patients, especially over the age of 60, the cause is rarely found [1].

Routine investigations of the peripheral nervous system most often include techniques that evaluate large diameter myelinated fast conducting fibres. Nerve conduction studies (NCS) give an accurate measure of large diameter nerve fibre function. In isolated small fibre dysfunction, NCS will be normal. Thin myelinated (A-delta type) and unmyelinated fibres (C type) require special diagnostic investigations. Quantitative sensory testing (QST) is used for measuring the perception as well as pain thresholds for heat and cold. QST is a psychophysiological test, requiring patient cooperation. In several studies, the sensitivity ranges from 60 to 85% [2–5].

Quantification of intraepidermal nerve fibres (IENF) in immunostained skin punch biopsies is a technique that can be used to confirm the presence of SDNF involvement. Multiple sites can easily be examined and studied serially, rendering this technique favourable compared to the more invasive, complicated and time-consuming sural nerve biopsy. IENF density may even be more sensitive than sural nerve biopsy in identifying SDNF neuropathy [6]. The technique and reference values of IENF from the distal leg obtained in our laboratory have recently been published [7]. In this study there was a significant decrease of IENFs with

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increasing age, and males had lower IENF density than females. The variation of IENF values in healthy persons has been reported to be large [8], and was in our laboratory in the range of 4.5–26.5/mm [7].

The purpose of this study was to compare results of IENF density and QST (warm and cold perception thresholds) in the same site of the distal leg of patients with mixed polyneuropathy and symptoms and signs suggesting SDFN dysfunction. We also wished to evaluate possible differences in these parameters for patients classified into normal and abnormal using conventional NCS. In a subpopulation of the material, we also wanted to compare IENF density and QST with recordings (sensory amplitudes) of the nerve innervating the same skin area (the superficial peroneal nerve).

Subjects and methods

Patient selection

The patient material consists of individuals with symptoms and signs of sensory polyneuropathy seen in our department during the time period from September 2001 to September 2004. Due to a clinical presentation suggesting involvement of SDFN, QST and skin biopsy in addition to clinical neurological examination and NCS were performed in 80 patients. All examinations of an individual patient were performed within 1 year (mean 1.5 ± 2.6 months, range 0–11). Two female patients (79 and 81 years old) were excluded because of insufficient co-operation and inconsistent responses in QST. One of them had parietal infarctions on MRI that could influence recognition of sensory thresholds in QST. One patient with mononeuritis multiplex was excluded because of asymmetric symptoms and findings, and two patients were excluded because of central symptoms and findings (one had multiple sclerosis and one had probable hereditary spastic paraplegia). Thus 75 patients (34 females and 41 males), were included. Ages ranged from 7 to 82 years (mean 53.2 ± 12.3).

All QST measurements were performed by the same neurologists (SIM and SL), and processing of skin biopsies and fibre counting were done by the same technician. For each patient, information about duration of symptoms, presence of neuropathic pain, autonomic symptoms, presence of distal sensory loss, distal weakness and reduced or absent Achilles tendon reflex were recorded. Relevant blood tests including immunologic tests were also performed.

The polyneuropathies were classified into five categories: idiopathic, diabetic (including cases with impaired glucose tolerance), inflammatory/immunologic, hereditary and other causes (Table 1).

Table 1 Classification and number of patients in each group of neuropathies

Classification	Number of patients
Idiopathic	31 (41%)
Diabetes/impaired glucose tolerance	10 (13%)
Inflammatory/immunologic ^a	12 (16%)
Hereditary ^b	13 (17%)
Other causes ^c	9 (12%)

^aChronic inflammatory demyelinating polyneuropathy (CIDP) ($n = 2$), polyneuropathy with anti-GM1 ($n = 2$) and anti-MAG ($n = 2$) antibodies, Sjögren's syndrome ($n = 4$), mixed connective tissue disease ($n = 1$), Wegeners granulomatosis ($n = 1$).

^bHereditary motor and sensory polyneuropathy; axonal ($n = 4$) or demyelinating ($n = 5$), familial burning feet ($n = 4$).

^cVitamin B₁₂ deficiency ($n = 2$), hypothyroidism ($n = 4$), alcohol abuse ($n = 1$), occupational organic solvent exposure ($n = 1$), sequelae from critical illness polyneuropathy ($n = 1$).

Skin biopsy

The skin biopsies were taken with a 3-mm disposable circular needle (Biopsy Punch; Stiefel Laboratories Ltd., Sligo, Ireland) implementing a sterile technique and local anaesthetic (2% lidocaine with adrenaline). Two biopsies were obtained from each patient on the distal part of the leg approximately 5 cm above the lateral malleolus. This area is innervated by the superficial peroneal nerve. In four patients with suspected dorsal ganglionitis two biopsies were obtained also from the lateral aspect of the thigh in the same limb (data not reported). The specimens were fixed immediately in 2% paraformaldehyde, lysine and periodate fixative at 4°C. The remaining procedure is described elsewhere [7,9]. After immunostaining the sections were counterstained with eosin and haematoxylin (the latter to better visualize the dermal-epidermal junction). Fifty-micron freezing microtome sections were immunostained with the panaxonal marker PGP 9.5. The number of separate IENFs in at least three sections from each biopsy was counted, and the total length of epidermis was measured using the NIH Image 1.61 morphometry program. The number of nerve fibres per millimetre was then reported as the mean of counts in six sections. Only single fibres and not branches from the same IENF were counted. A skilled technician assessed all the biopsies in a blinded fashion. The method performed in our laboratory has been evaluated earlier for inter- and intraobserver agreement, and normative reference data for distal leg IENF density has been established [7].

Quantitative sensory testing

The recordings were performed under standard conditions in a silent room at 23°C with a ThermoTest type I

(Somedic AB, Sweden). A thermode of 50 × 25 mm was continuously applied to the skin at the distal part of the leg at the same place as the skin biopsy was taken afterwards. The QST was always done prior to the skin biopsy. In 72 patients, QST was also performed in the middle of the thigh's outer surface in the same limb, but these data are not reported as only four patients had a skin biopsy from the same location. Skin reference temperature on the ThermoTest was set at 30°C. The temperature was then continuously decreased with 1°C/s until the patient perceived a feeling of cold and pushed a button changing the current in the thermode. Then the temperature was increased (with the same speed as used for cooling), and in the same way the patient pushed the button when there was a feeling of warmth. Ten measurements were obtained for both cooling and warmth with random time interval of 1-2s. The limits of temperature were 5°C (maximum cold) and 50°C (maximum hot). Mean thresholds for perception of warmth (WPT) and cold (CPT) and the difference between both of them (limen) were recorded. Values obtained when the patient indicated that the button was pushed too early or too late were omitted from calculation.

Nerve conduction studies

For NCS recording the Keypoint EMG equipment (Medtronic, Skovlunde, Denmark) was used. Surface electrodes were utilized for recording and stimulation in the motor and sensory nerves. NCS were performed in at least two limbs (including at least one symptomatic leg). In the upper limb two motor and three sensory NCS were made unilaterally, and in the lower limb NCS included two motor and minimum one sensory nerve (the sural nerve in all patients and in addition the superficial peroneal nerve in the last 36 patients of the series). For motor nerves the following parameters were analysed; conduction velocity (CV), negative amplitude, proximal/distal amplitude decay, distal latency and *F*-wave latency. For sensory nerves the following parameters were analysed: CV, peak latency and peak-peak amplitude. Neurography of the sural and the superficial peroneal nerve was performed antidromically. In 70 patients the NCS was done on the same limb as the skin biopsy and the QST. In five patients the neurography had been performed on the opposite leg from that used for biopsy and QST, but all had bilateral symmetrical symptoms.

The NCS recordings for each nerve was compared with age matched reference material. For motor nerves, control values for latencies and CV were taken from a large material from other laboratories [10] and have been used routinely in our laboratory. For sensory nerve studies in the leg, the first 33 patients were

examined with a reusable recording electrode (Oxford Instruments no. 16934) for sural nerve and the values were compared with a separate smaller reference material ($n = 20$) in our laboratory. In the last 42 patients another surface electrode (Nihon Kohden NM-420S, Nihon Kohden Corporation, Tokyo, Japan) was used for the sural nerve ($n = 42$) and for the superficial peroneal nerve ($n = 36$). The same surface electrode has been used in the large available reference material [11], and these reference values were used. For all parameters, the degree of abnormality is expressed as relative deviation from normal, or in terms of *Z*-score (difference between obtained and expected value expressed as number of SDs dependent of the patient's age and height). In this way, the results are normalized and not dependent on change in method during the study.

The over all results of NCS were grouped into normal (including borderline pathology in one nerve) and abnormal. Patients with NCS only suggesting unilateral or bilateral carpal tunnel syndrome were grouped as borderline and thus normal.

The patients were also grouped into normal and abnormal depending on the result of sural nerve amplitude and CV and tibial and peroneal *F*-responses.

Ethical committee

The study was approved by the regional ethical committee at the University of Tromsø.

Statistics

Results are presented as mean ± SD. Unpaired *t*-test (two tailed) was used for testing difference between two groups of quantitative data. Simple regression analysis with IENF as the dependent variable was applied to test associations with QST and NCS recordings. Equivalent non-parametric statistics (Mann-Whitney and Spearman rank correlation) produced confirmatory results. For comparison of categorical data, chi-square calculations were performed.

Results

Patient material

Patient demographics, symptoms and clinical findings are shown in Table 2. Thirty-eight patients (23 females and 15 males) had normal NCS and 37 patients (11 females and 26 males) were classified as abnormal. NCS abnormalities were more prevalent in men than in women. As expected, hyporeflexia and distal weakness were most common within the group with abnormal NCS. Otherwise the two groups did not differ.

Table 2 Characteristics of patients with normal NCS and abnormal NCS

Characteristics	Total	Normal NCS	Abnormal NCS	<i>P</i> -value
Number of patients	75	38	37	
Age (years), mean \pm SD	53.2 \pm 12.3	51.9 \pm 13.6	54.6 \pm 11.0	NS ^a
Female:male	34:41	23:15	11:26	0.007 ^b
Duration of symptoms (years), mean \pm SD	10.8 \pm 12.4	8.2 \pm 9.4	13.4 \pm 14.6	NS ^a
Painful neuropathy, <i>n</i> (%)	53 (71)	29 (76)	24 (65)	NS ^b
Autonomic symptoms, <i>n</i> (%)	25 (33)	15 (39)	10 (27)	NS ^b
Hyporeflexia of ankle reflex, <i>n</i> (%)	43 (58)	14 (38)	29 (78)	0.0004 ^b
Distal sensory loss, <i>n</i> (%)	60 (80)	28 (74)	32 (86)	NS ^b
Distal weakness, <i>n</i> (%)	19 (25)	3 (8)	16 (43)	0.0004 ^b

SD, standard deviation; NS, not significant.

^aUnpaired *t*-test; ^bChi-squared test.

IENF density and QST in patients with normal and abnormal NCS

Images of IENFs in a healthy person and in a patient are shown in Fig. 1. The data are presented in Table 3, and show that there was more depletion of IENFs when NCS was abnormal. Comparing the results of IENF density in this study with our laboratory's normal material, fibre density also in those with normal NCS was significantly lower than in the controls [mean 6.4 \pm 3.8/mm (range 1.5–18.9) vs. 12.4 \pm 4.6 (4.5–26.5), *P* < 0.0001].

Among patients with definite fibre loss [IENF < 3.2/mm (defined as mean value in healthy persons – 2 SD)], there were nine with normal [9 of 38 (24%)] and 15 with

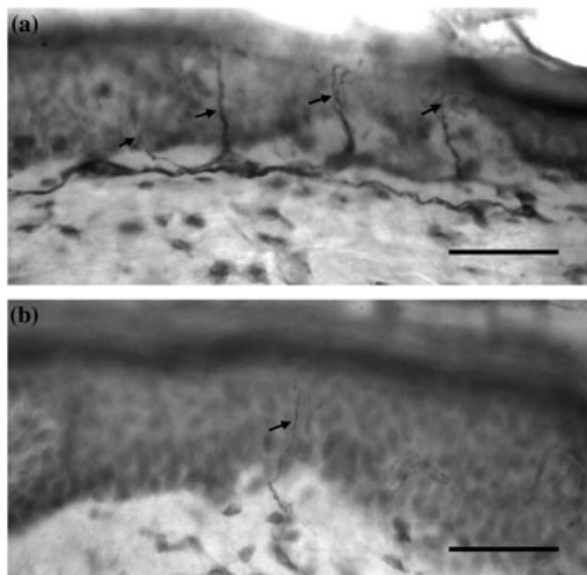


Figure 1 Skin sections (50 μ m) immunostained with the panaxonal marker PGP 9.5. Image A is from a normal subject and image B from a patient. The numbers of separate IENFs (arrows) per millimetre are counted. Scale bars = 50 μ m.

Table 3 Comparison of groups of normal and abnormal NCS with IENF density and QST parameters

	Normal NCS	Abnormal NCS	<i>P</i> -value (unpaired <i>t</i> -test)
IENF (mean \pm SD)	6.4 \pm 3.8	4.4 \pm 3.4	0.02
WPT (mean \pm SD)	10.1 \pm 4.3	13.3 \pm 4.0	0.002
Limen (mean \pm SD)	13.4 \pm 5.6	22.1 \pm 9.1	< 0.0001
CPT (mean \pm SD)	3.3 \pm 1.8	8.8 \pm 6.9	< 0.0001

IENF, intraepidermal nerve fibres; WPT, warmth perception threshold; CPT, cold perception threshold; limen, difference between WPT and CPT; SD, standard deviation.

abnormal NCS [15 of 37 (41%)], but the difference was not statistically significant.

The QST parameters (limen, CPT and WPT) were significantly different in the two groups with normal and abnormal NCS respectively. This was especially the case for CPT (Table 3).

The NCS results were also subclassified into groups of normal and abnormal sural nerve amplitude and velocity, and normal and abnormal *F*-responses in the peroneal and tibial nerve. IENF density, limen and CPT were significantly more abnormal in those with decrease of sural nerve amplitudes (Table 4), but not with sural nerve velocity (data not shown). We did not find it meaningful to make a linear regression between sural nerve sensory action potential versus IENF and QST in the whole material due to change of the method with use of different electrodes (see above). However, the last 42 patients were studied with a new electrode for recordings in the sural (*n* = 42) and the superficial peroneal nerve (*n* = 36).

Comparison with regression analysis between sural and superficial peroneal amplitudes showed a strong correlation (*r* = 0.8, *P* < 0.0001). Sural and superficial peroneal nerve amplitudes versus IENF density and QST parameters are presented in Table 5. The *r*-values

Table 4 Comparison of groups of normal and abnormal amplitude of sural nerve sensory action potential with IENF and QST parameters

	Normal sural amp	Abnormal sural amp	P-value (unpaired <i>t</i> -test)
IENF (mean \pm SD)	5.9 \pm 3.8	3.9 \pm 3.2	0.04
WPT (mean \pm SD)	11.1 \pm 4.3	13.1 \pm 4.6	NS
Limen (mean \pm SD)	15.9 \pm 7.6	22.2 \pm 9.8	0.004
CPT (mean \pm SD)	4.8 \pm 4.5	9.1 \pm 7.2	0.003

IENF, intraepidermal nerve fibres; WPT, warmth perception threshold; CPT, cold perception threshold; limen, difference between WPT and CPT; SD, standard deviation; NS, not significant.

Table 5 Comparison of amplitude of sural ($n = 42$) and superficial peroneal nerve ($n = 36$) sensory action potential with IENF and QST parameters

Sural nerve versus IENF	$r = 0.37, P = 0.01$
Sural nerve versus WPT	$r = -0.38, P = 0.01$
Sural nerve versus limen	$r = -0.43, P = 0.004$
Sural nerve versus CPT	$r = -0.39, P = 0.01$
Superficial peroneal nerve versus IENF	$r = 0.37, P = 0.03$
Superficial peroneal nerve versus WPT	$r = -0.44, P = 0.007$
Superficial peroneal nerve versus limen	$r = -0.50, P = 0.002$
Superficial peroneal nerve versus CPT	$r = -0.44, P = 0.007$

IENF, intraepidermal nerve fibres; WPT, warmth perception threshold; CPT, cold perception threshold; limen, difference between WPT and CPT.

tend to be somewhat higher for superficial peroneal amplitudes and QST than for sural amplitudes.

The patients with delayed *F*-responses of the peroneal and tibial nerve did not have a statistically lower IENF density than those with normal *F*-responses. However, QST perception thresholds were generally more abnormal in those with delayed *F*-responses (data not showed).

Correlation between IENF density and QST

Linear regression analysis did not show a significant correlation between IENF density and WPT, but a statistically significant association between IENF and limen ($r = -0.32, P = 0.006$) and with CPT ($r = -0.33, P = 0.004$). This correlation was only present within the group of patients with abnormal NCS [IENF versus limen ($r = -0.39, P = 0.02$) and IENF versus CPT ($r = -0.35, P = 0.03$)] and in those with reduced Achilles tendon reflex [IENF versus limen ($r = -0.39, P = 0.001$) and IENF versus CPT ($r = -0.38, P = 0.001$)].

Discussion

Different methods are available for the study of neuropathy, including isolated SDNF neuropathy. Deter-

mination of IENF density, QST and NCS and their relationship are in focus of this study. It is well known that the NCS assess both slowing of conduction, corresponding to demyelination, and decrease in amplitude, corresponding to axonal loss. NCS generally reflect conduction in the fastest axons (large diameter axons), which are normal in isolated SDNF neuropathies. Special techniques have been developed for small fibre neurography (collision techniques, near nerve recording, signal shape analysis), but these have not been used in this study. Neither has autonomic testing been performed systematically in our material.

In this series of patients with mixed polyneuropathies, we found that the two subpopulations defined as neurographically normal, respectively abnormal, differed both in IENF density and in QST. When there was a combination of small and large diameter fibre neuropathy, the process involved small diameter fibres more extensively. This was also recently demonstrated by Herrmann *et al.* [12] comparing plantar nerve action potentials and skin biopsy. Only in patients with abnormal NCS, there was a statistically significant association between IENF and QST. As IENF density was lower in those with abnormal NCS, this indicates that a more profound involvement of SDNFs is necessary to demonstrate a correlation. In our material, the cold perception thresholds correlated better than warmth with NCV abnormalities as cold modality includes small myelinated fibres. This is in accordance with the interpretation that small diameter myelinated A-delta fibre involvement is associated with myelinopathy also of larger nerve fibres.

With the concept that NCS reflect large fibres, and IENF and QST small fibres, their information should be seen as complementary, and not redundantly reflecting the same process. Patients with abnormal thermal thresholds and normal IENF should in particular be evaluated to exclude involvement of central sensory systems. Those with CNS abnormalities on MRI, were excluded from this study. However MRI was not used as a standard routine procedure in the present series, and there might have been CNS lesions in some causing seemingly neuropathic symptoms.

In the whole patient material, there was a statistically significant association between IENF/QST and abnormal sural amplitude. Sural neurography is a standard technique to study neuropathies [3,6,13,14]. However, only few studies have been published to compare the sensitivity of sural neurography with other neurophysiologic measures. One such study was performed in diabetic patients, showing the tibial nerve *F*-responses to be more abnormal than sural parameters [15]. One of the reasons is the fact that a neuropathy with conduction slowing is methodologically easier to detect over

long nerve segments [*F*-responses (1.5 m) compared with short segments (sural nerve, 14 cm)]. The test-retest variability is smaller in *F*-latency measurements than in sural nerve measurements [16]. In our study there were significantly more abnormal QST parameters in the patients with delay of peroneal and tibial *F*-responses but this was not the case for IENF density. This is not unexpected in these patients with predominantly sensory symptoms and signs as *F* latencies reflect motor fibre function.

The QST and IENF density measurements were performed in the skin area innervated by the superficial peroneal nerve. In a subpopulation of the material, we therefore included recordings of the superficial peroneal nerve to also compare these data with QST recordings and IENF density. The superficial peroneal and sural nerve amplitudes correlated strongly when compared directly. *R*- and *P*-values indicated a somewhat better correlation between superficial peroneal nerve sensory amplitudes than for the sural nerve versus QST parameters. However, overall the two nerves seemed to be involved approximately to the same extent. Especially, we could not demonstrate a stronger association between IENF density versus sensory amplitudes of the superficial peroneal nerve than for the neighbouring sural nerve. A larger patient material may clarify whether correlation between superficial peroneal nerve amplitudes versus IENF density/QST recordings from the skin innervated by this nerve is more relevant than using the sural nerve for comparisons. Another nerve that recently has been showed to reflect a more sensitive measure of distal sensory neuropathy than sural nerve action potentials is the medial plantar nerve [12].

In contrast to most previous studies (except Pittenger *et al.* [17]), we took skin biopsy at the same location at the distal part of the leg as had been used for QST measurements. We therefore expected a correlation between IENF density and QST parameters in a group of patients with neuropathy. This was also the case in our patients, but rather weak in the whole material and only significant in the group with NCS abnormalities. In those presumed to have pure small fibre involvement, there was no statistically significant association between IENF density and QST. One reason for the overall weak correlation is the interindividual variability in measurements, particularly of IENF density. The methods are quantitative, but both have inherent errors. Thermotesting includes a psychophysiological component. In addition, sensory thresholds depend on both the peripheral and the central nervous system, and abnormal thresholds can be caused by disturbances of any level of the somatosensory nervous system. Cooling may be a more accurate measure than warming due to a low density of warm receptors in some normals [18].

Another reason for the modest correlation between IENF density and QST may depend upon the use of 50 × 25 mm thermode reflects fibres in a larger area and in deeper structures of the skin than the IENFs in 3 mm biopsies. Finally, our material includes patients with mixed polyneuropathies; both axonal and demyelinating or a combination, and with different aetiologies. This may explain a generally weaker correlation between IENF density and QST than in the diabetic neuropathy patients of Shun *et al.* [19].

In conclusion, our results support and confirm that in patients with polyneuropathy, the small diameter fibre loss, detected by measurement of IENF density and QST, is more pronounced in patients with large diameter fibre involvement as demonstrated by NCS. There was also only a statistically significant association between IENF density and QST in patients with large diameter fibre involvement. The methods (NCS, IENF quantification and QST) are complementary as they reflect different fibre populations. We therefore consider it valuable in a work up of patients with sensory loss to include both QST and determinations of IENF density when neurography is normal. On the other hand, in polyneuropathy patients with abnormal NCS and dominating symptoms and clinical findings suggesting small fibre involvement, QST and IENF quantification may provide additional information. Although not systematically explored in this study, we also suggest that when there is a discrepancy with normal IENF density and abnormal QST, a CNS lesion in sensory pathways should be searched for.

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