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The analysis of epidermal nerve fibre spatial distribution improves the diagnostic yield of skin biopsy

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The analysis of epidermal nerve fibre spatial distribution improves the diagnostic yield of skin biopsy

Aim: Small fibre neuropathy (SFN) diagnosis represents a challenge for neurologists. The diagnostic gold standard is intraepidermal nerve fibre (IENF) density, but in about 10–20% of patients with symptoms/signs and abnormalities on functional tests, it remains within normal range. We propose an adjunctive parameter to improve the efficiency of skin biopsy diagnosis. **Methods**: We recruited 31 patients with SFN symptoms/signs, normal nerve conduction study, abnormal quantitative sensory testing and normal IENF density. We also included 31 healthy controls and 31 SFN patients with reduced IENF density as control groups. **Results**: We measured the distance between consecutive IENFs in the three groups. Mean inter-fibre distances did not differ between patients with normal counts and healthy controls (66.7 ± 14.5 μm *vs.* 76.7 ± 13.4 μm; $P = 0.052$), while the relative standard deviation was significantly ($P < 0.001$) higher in patients (79.3 ± 29.9) compared to controls (51.6 ± 12.2). Using ROC analysis, we identified an inter-fibre distance of 350 μm as the measure that better differentiated patients from controls (AUC = 0.85, sensitivity: 74%, specificity: 94%). At least one such segment was also observed in all patients with reduced IENF count. **Conclusion**: Irregular spatial distribution is an SFN intrinsic feature preceding actual nerve loss. The presence of a stretch of denervated epidermis longer than 350 μm is a parameter able to increase the diagnostic efficiency of skin biopsy.

Keywords: immunohistochemistry, intraepidermal nerve fibres, neuropathic pain, neuropathology, skin biopsy, small fibre neuropathy

Introduction

Small fibre neuropathy (SFN) selectively or most prevalently affects thinly myelinated Aδ-fibres and unmyelinated C-fibres that are mainly involved in nociception, thermal and autonomic function. This condition typically manifests as a symmetrical length-dependent sensory-autonomic disorder, with pain as the most disabling symptom. It complicates the course of metabolic, toxic, immune-mediated and genetic conditions, although in about half of the cases, the cause remains unknown [1–2]. Several tests, such as quantitative sensory testing (QST), laser-evoked potentials, contact-heat-evoked potentials, microneurography and autonomic testing, are available to explore small fibre (SF) function. Morphological analysis of SF in the sural nerve is applied nowadays to a few selected conditions. An alternative approach to quantify small fibres *in vivo*

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is corneal confocal microscopy [3]. This technique is not invasive but its reliability in the diagnosis of SFN still needs to be proven on a large scale. Skin biopsy has been used in the last few decades to assess SF through the count of intraepidermal nerve fibres (IENF). Other aspects such as epidermal fibre length or subepidermal plexus quantification have been considered [4–5]. Morphological changes such as varicosities have been shown to predict nerve loss [6]. Although the diagnostic criteria for SFN are still an object of debate, clinical, functional and morphological evidence of SF impairment are crucial for the diagnosis, and the quantification of IENFs remains the most reliable tool for confirmation [7–10]. Nevertheless, in a percentage (10–20%) of patients with clinical and functional evidence of SFN, the IENF count remains within the normal range [9]. Often a loss of the regular and homogeneous distribution of IENFs along the epidermis is observed in SFN [11-12]. This abnormal spatial distribution may occur early in SFN [11,13].

The aim of our study was to assess IENF spatial distribution in patients with clinical and functional evidence of SFN but normal IENF counts, to identify further morphological markers of SF pathology, able to distinguish patients from controls.

Patients and methods

From January 2013 to July 2018, we recruited 238 patients with symptoms and signs of SFN and normal nerve conduction study (Table 1). A flowchart of our study design and results is outlined in Figure 1. All patients underwent QST performed according to previously published procedures [14]. Briefly, thermal thresholds to innocuously cold and warm stimuli and to painfully hot and cold stimuli were recorded on the dorsum of the hand, the distal thigh, distal leg and the dorsum of the foot using a computerized system (TSA2001 thermal sensory analyser; Medoc Advanced Medical Systems, Ramat Yishai, Israel) and the method of limits. Tactile detection thresholds and mechanical pain perception were assessed at the same sites. Tactile thresholds were assessed using a series of calibrated monofilaments (Semmes–Weinstein) and defined as the weight (in grams) exerted by the thinnest filament, perceived at least five times in the 10 attempts. Mechanical pain perception was tested with a sharp non-penetrating probe (50-μm diameter tip) attached to a

Table 1. Nerve conduction study in patients and controls

	Ulnar					*Peroneal*			*Sural*	
	Sensory		*Motor*			*Motor*			*Sensory*	
	VCS (m/s)	*Snap (μV)*	*Lat dist (ms)*	*VCM (m/s)*	*CMAP (mV)*	*Lat Dist (ms)*	*VCM (m/s)*	*CMAP (mV)*	*VCS (m/s)*	*SNAP (μV)*
CTRL	51.4 ± 3.7	36.3 ± 13.8	2.8 ± 0.3	61.8 ± 3.7	16.6 ± 3.3	4.3 ± 0.6	50.8 ± 3.4	6.6 ± 4.1	52.8 ± 2.7	15.0 ± 5.0
SFN-NC	52.9 ± 2.6	32.7 ± 16.9	2.5 ± 0.3	64.4 ± 4.7	12.1 ± 3.8	4.2 ± 0.4	49.2 ± 3.0	6.9 ± 3.6	51.8 ± 4.3	14.3 ± 4.1
SFN-LC	53.8 ± 3.3	32.1 ± 11.6	2.6 ± 0.3	61.5 ± 5.6	13.8 ± 3.7	4.1 ± 0.5	49.3 ± 3.4	6.7 ± 3.3	53.4 ± 4.0	13.0 ± 5.2

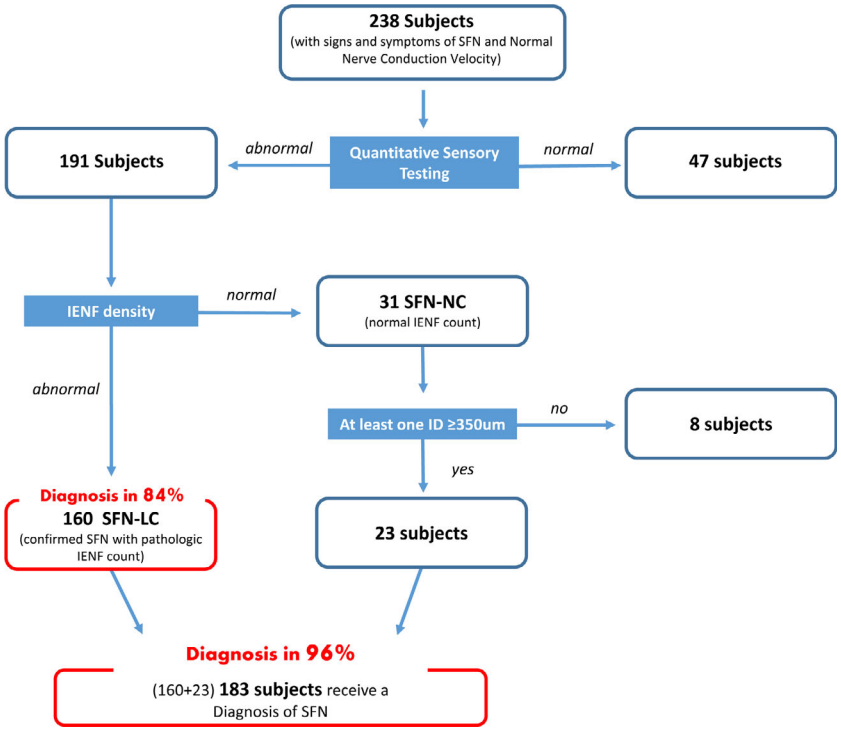

Figure 1. Study design. Flow diagram summarizing the study design and results.

calibrated nylon filament with a bending force of 95 mN. The percentage of stimuli perceived as painful was recorded. We selected 191 patients who presented definite QST abnormalities (at least two abnormal sensory modalities) and therefore, according to previously published diagnostic criteria [7,8,15] reached the diagnosis of SFN. All of them underwent a 3-mm punch skin biopsy from the distal leg to confirm histologically the diagnosis of SFN. Skin samples were processed as previously reported [16]. IENF density, measured according to current counting rules [9], was below the sex and age adjusted [17] normal cut-off in 160 out of 191 patients (84%). In the remaining 31 subjects (M/F: 8/23, age: 45.7 ± 15.2, range: 18–72 years), IENF density was within normal range, thus preventing a morphological confirmation of SFN. We defined this group as SFN with normal count (SFN-NC). To match this group, we selected on the basis of sex and age criteria, 31 healthy subjects (M/F: 10/21; age: 46.1 ± 16.3, range: 34–79 years) as a control group (HC), and 31 patients (M/F: 17/14; age 48.5 ± 15.8, range: 21–80 years) from the group of 160 SFN patients with low count (SFN-LC) as a disease control group. The HC group did not have any potential cause of neuropathy, did not report any SFN symptoms, showed a normal clinical examination and had normal electrophysiological and QST investigation.

We acquired digital confocal images using AxioImager M2-microscope with a magnification of 20X from three non-consecutive 50-μm thick sections, double-marked with anti-PGP 9.5 (nerve fibres) and anti-ColIV (basement membrane) antibodies according with standard procedures. [9] We used a dedicated image analysis software (ZenPro Zeiss) to measure along the entire sections, all the distance between consecutive fibres, immediately after they cross the basement membrane (inter-fibre distance, ID) in the 31 SFN-NC, in the 31 SFN-LC patients and in 11 HC (M/F: 5/6; age 49.3 ± 13.5, range: 34–79 years). We built a distribution curve of the IDs for the three groups. Among the longest ID measurements, we chose the length that better differentiated SFN-NC patients from controls. Then, we evaluated directly through the microscope oculars, the presence of such ID length in three skin sections of the remaining 20 HC. To verify the inter-rater reproducibility a second operator blind to the diagnosis repeated the assessment.

Nine patients from the 31 SFN-NC group were revaluated at follow-up (SFN-NC-F), with a second skin

biopsy after an average of 38.1 months (range: 6–62). IENF density as well as all the ID along the entire length of three skin sections were measured and compared with the first assessment.

Statistical analysis

The data are reported as mean ± standard deviation. Student's *t*-test for unpaired data and paired data and the Mann–Whitney test for nonparametric data were used when appropriate. The receiving operating characteristics (ROC) curves, after logistic regression, were used to discriminate groups using the ID measure. The inter-rater reproducibility of the test was assessed using Cohen's kappa coefficient (κ). All analyses were performed using STATA 14.1 software (Stata Corp LP, CA, USA) with 0.05 type I error threshold.

Results

Clinical findings of SFN-NC

In these patients, screening for causes revealed dysmetabolism in 5 (diabetes or glucose intolerance), a toxic insult in 3 (chemotherapy, alcohol), an immune-mediated condition in 4 (systemic lupus erythematous, Sjögren syndrome), a genetic disease in 6 (early stage Fabry disease, familial amyloid neuropathy), while in 13 patients (41%) we could not identify any cause. Nearly 80% complained of spontaneous pain, mostly burning (48%) or cold pain (30%). Evoked pain was present in 28% and paraesthesia in 68% of patients. Negative signs such as thermal and mechanical hypoaesthesia were present in 19 out of 31 patients (61%). The duration of symptoms was 3.4 ± 3.2 years. QST findings, reported in Table 2, showed a length-dependent impairment with the dorsum of the foot as the most involved site. Warm (abnormal in 73%) and cold (abnormal in 67%) thresholds and pinprick perception (abnormal in 69%) were the most involved sensory modalities. QST abnormalities in SFN-NC were similar to SFN-LC patients, although to a lesser extent (Table 2).

Skin biopsy findings

Overall, we analysed cutaneous innervation in 102 skin samples from 31 HC, 31 SFN-LC, 31 SFN-NC and 9 SFN-NC-F patients. We acquired a total of 885 digital images. IENF linear density was 13.5 ± 2.6 fibre/mm in HC, 15.7 ± 3.6 fibre/mm in SFN-NC ($P = 0.03$), 11.9 ± 3.6 in SFN-NC-F and 6.7 ± 1.9 in SFN-LC patients.

The distribution of IENFs was quite irregular in SFN-NC patients with the frequent occurrence of long segment of denervated epidermis alternated with areas of clustered nerve fibres (Figure 2). Frequent pre-degenerative features such as nerve varicosities and axonal

Table 2. Quantitative sensory testing in patients and controls

		CS	*WS*	*CP*	*HP*	*PP*	*TT*
HAND	CTRL	1.4 ± 0.5	1.8 ± 0.6	8.5 ± 2.7	7.9 ± 1.9	90.9 ± 18.1	0.3 ± 0.3
	SFN-NC	1.3 ± 0.9	2.6 ± 2.9	10.2 ± 7.2	8.0 ± 4.0	48.8 ± 43.9*	0.8 ± 1.0
	SFN-LC	3.1 ± 3.2§	4.7 ± 3.9*	13.7 ± 8.2	9.9 ± 3.8	37.2 ± 33.7*	0.5 ± 0.2
THIGH	CTRL	2.4 ± 1.4	2.6 ± 0.6	6.6 ± 1.6	8.7 ± 2.7	87.3 ± 15.6	0.5 ± 0.3
	SFN-NC	3.0 ± 1.7	3.0 ± 1.5	10.9 ± 8.3	8.3 ± 3.3	37.5 ± 33.3*	1.5 ± 2.8
	SFN-LC	3.3 ± 2.2	3.5 ± 2.0	10.3 ± 6.5	10.7 ± 4.2	35.6 ± 36.3*	0.7 ± 1.2
LEG	CTRL	2.2 ± 0.8	4.3 ± 2.1	7.7 ± 3.2	10.4 ± 2.2	79.1 ± 17.6	0.9 ± 0.7
	SFN-NC	4.1 ± 2.1*	7.8 ± 5.6	10.6 ± 6.5	13.2 ± 3.2	32.5 ± 36.5*	3.1 ± 4.8
	SFN-LC	4.0 ± 2.5	7.6 ± 4.1	12.8 ± 7.4	13.7 ± 3.5*	36.7 ± 32.0*	1.1 ± 0.5
FOOT	CTRL	2.5 ± 1.1	2.8 ± 0.9	8.9 ± 2.6	8.5 ± 3.3	81.8 ± 15.4	0.6 ± 0.6
	SFN-NC	4.1 ± 4.0	6.1 ± 3.5*	12.3 ± 8.7	12.0 ± 2.8*	42.5 ± 29.6*	1.6 ± 2.8
	SFN-LC	6.4 ± 4.4*	8.0 ± 4.0*	15.7 ± 7.0	13.7 ± 3.2*	50.0 ± 39.6*	0.6 ± 0.4

Thermal threshold values are expressed in degree Celsius; tactile threshold values are expressed in grams; and pinprick values are expressed in percentage as number of stimuli perceived as painful out of 10. CP, cold pain; CS, cold threshold; HP, heat pain; TT, tactile threshold; PP, mechanical pain perception; WS, warm threshold.

*$P < 0.05$ *vs.* CTRL.

§$P < 0.05$ *vs.* SFN-NC.

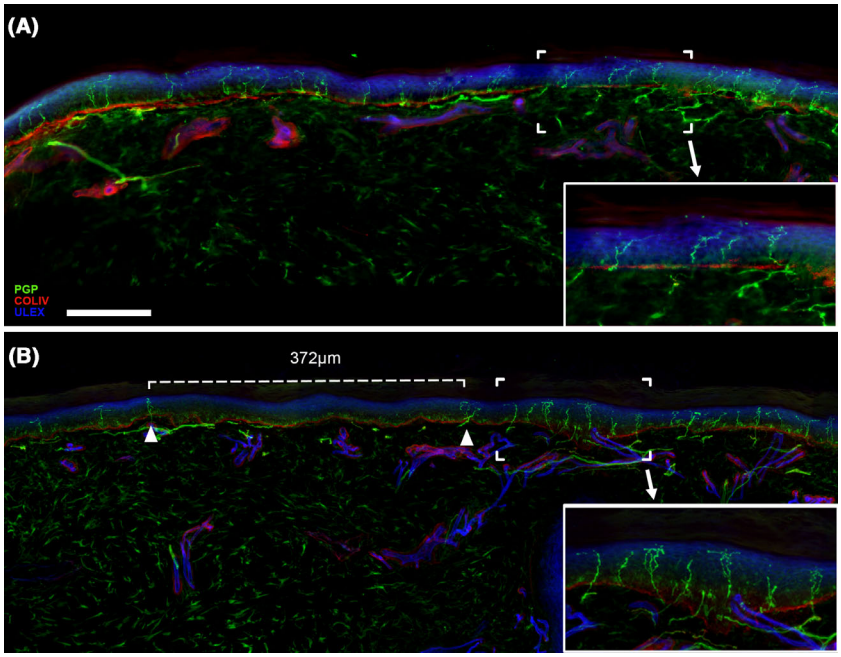

Figure 2. Distribution of epidermal innervation in a healthy subject and in an SFN patient with IENF density within normal range. (**A**) Regular nerve fibre distribution in the skin of a healthy control. (**B**) Long segment of denervated epidermis (dashed line) alternated to clustered fibres (see magnification). Space bar = 100 μm.

swellings were also observed. The mean ID was not significantly different between SFN-NC patients and HC (66.7 ± 14.5 μm *vs.* 76.7 ± 13.4 μm; $P = 0.052$), while the mean standard deviation of ID was significantly ($P < 0.001$) higher in patients (79.3 ± 29.9 μm) than in controls (51.6 ± 12.2 μm). The SFN-LC patients had a mean ID of 171.1 ± 59.9 μm, significantly longer than SFN-NC and HC ($P < 0.001$).

In the distribution curve of the IDs, the IDs longer than 150 μm were more frequently observed in SFN-NC patients than in HC. In SFN-LC patients, the distribution curve was further shifted towards the longer measures (**Figure 3A**).

Using ROC analysis, we established that the ID length that best separated SFN-NC from HC was 350 μm (AUC = 0.85, **Figure 3B**). Sensitivity/specificity analysis showed that the identification of one single 350-μm long stretch of denervated epidermis achieved a sensitivity of 74% and a specificity of 94%. In particular, in our cohort at least one 350-μm long stretch of denervated epidermis was present in 23 out of 31 patients (74%) and 2 out of 31 controls (6%). The agreement in the assessment of all the measures between two independent operators was excellent ($\kappa = 0.98$).

In all nine SFN-NC-F patients, IENF density fell compared to the first assessment (mean loss 3.6 ± 2.5 fibres/mm). In three cases, the count was below the relative sex-and age-adjusted cut-off allowing the morphological confirmation of SFN. The ID distribution at second assessment compared to the first one showed a significant ($P < 0.05$) increase in the number of segments of denervated epidermis longer than 350 μm (5.1 ± 2.4 *vs.* 3.0 ± 4.1) (**Figure 3C**). In particular, all patients at the second time point had at least one segment of 350 μm. This was true only in seven out nine patients at first assessment. At least one segment of denervated epidermis longer than 350 μm was observed also in the 31 SFN-LC patients.

Discussion

The diagnosis of SFN is indubitable if typical symptoms and signs, abnormalities of QST and loss of IENF are present. However, diagnostic uncertainty arises when patients show clinical and functional evidence of SFN, but the IENF count remains within the normal range [15]. We tested the hypothesis that the analysis of IENF spatial distribution may provide further morphological features that increase the ability of skin biopsy to detect an SFN.

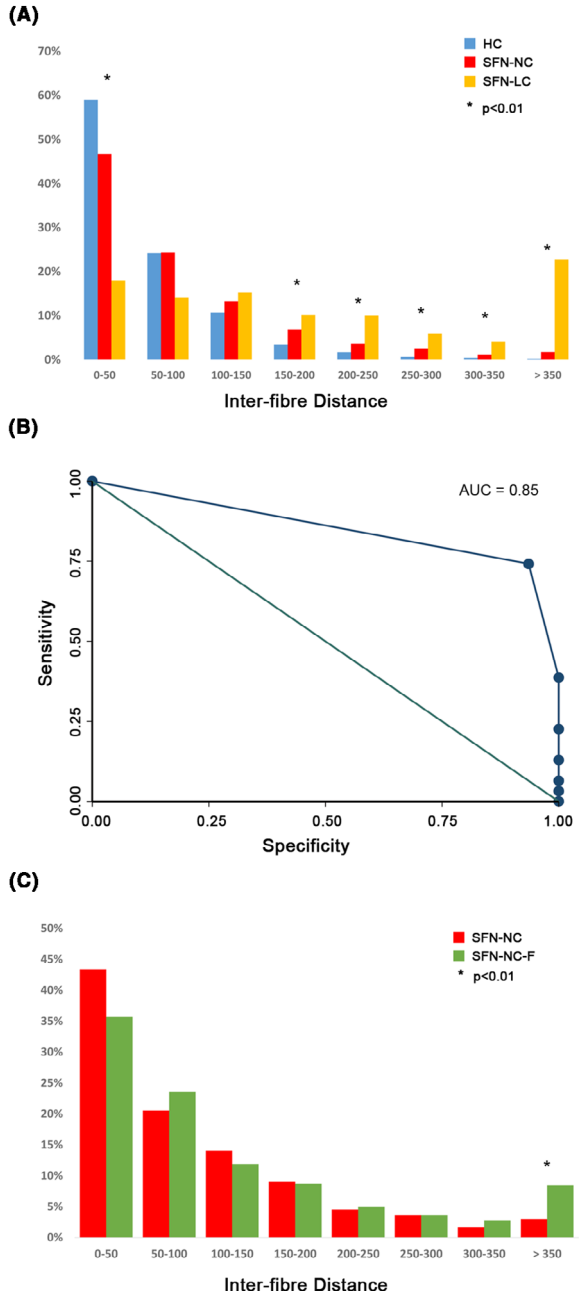

Figure 3. Inter-fibre distances distribution and ROC curve. (**A**) Distribution of inter-fibre distance length in healthy controls (HC), small fibre neuropathy with normal (SFN-NC) and lower IENF count (SFN-LC) patients. Inter-fibre distances longer than 150 μm were more frequent in both patient groups than in healthy subjects. Moreover, in SFN-LC patients, the distribution curve was further shifted towards the longer measures. (**B**) ROC curves analysis showing the AUC considering a single segment of 350 μm of denervated epidermis. (**C**) Distribution of inter-fibre distances length in 9 SFN-NC patients at baseline and follow-up (SFN-NC-F). The graphic of the distribution curve shows a shift towards longer IDs at follow-up.

We found in SFN-NC patients (definite diagnosis of SFN according to recognized criteria) [8] in contrast to HC, an irregular distribution of IENF, with clustered fibres alternating with long segments of denervated epidermis. We identified a value of ID longer than 350 μm is a feature that is able to distinguish efficiently patients with clinical and functional evidence of SFN from controls (AUC = 0.85). In our laboratory, this adjunctive parameter allowed an increase in the diagnostic rate from 84% (160 out of 191 patients diagnosed through IENF count alone) to 96% (183 out of 191 combining the two methods) (Figure 1). We found a single 350 μm ID only in two HC out of 31, thus confirming the high specificity (94%) of this parameter. The method proved to be reliable with an excellent agreement between the measures obtained by two blinded operators ($\kappa = 0.98$). This parameter can be easily and quickly assessed directly at the microscope oculars adding few minutes to the time needed for the routine count of IENF [9]. The same method can be applied with the brightfield technique, provided the assessment of the relative ID, due to the differences in the IENF count between brightfield and fluorescence [18].

The abnormal spatial distribution found in SFN-NC patients appears to be an intrinsic feature of the chronic degenerative process that underlies small nerve pathology. In fact, the loss of IENF is counterbalanced by regenerative attempts with early evidence of clusters [11] that prevents modifications of IENF count. The higher standard deviation of ID in SFN-NC patients compared to HC is the expression of such abnormal spatial distribution.

The presence of a denervated segment of epidermis longer than 350 μm indicates the loss of 4–5 consecutive IENF considering that the mean ID in controls was 76.7 ± 13.4 μm. Therefore, the loss of several consecutive fibres reveals the presence of a pathological process. In fact, at least one segment of denervated epidermis longer than 350 μm was observed also in all the 31 SFN-LC patients although subjects with various degrees of IENF loss were included. As expected, these segments of denervated epidermis were more frequent and longer in patients with more severe denervation, with an overall shift of the distribution curve towards the longer IDs in patients with SFN-LC compared to SFN-NC patients (**Figure 3A**). A further confirmation of the degenerative meaning of such segments of denervated epidermis was the observation of a loss of IENFs in the patients who repeated skin biopsy over time. This IENF loss occurred

at a higher rate compared to the physiological age-related fibre loss (1 fibre in 20 years) [17]. In addition, all the SFN-NC-F had at least one segment of 350 μm denervated epidermis and the distribution curve was shifted towards longer IDs (**Figure 3C**). Such findings suggest that at one point along the disease course there is a loss balance between degenerative and regenerative processes, with the former overcoming the latter.

This work is based on a relatively small group of patients and only few of them were followed up, and those were heterogeneous in the duration of their follow-up. However, it points to an important morphological characteristic that is helpful in obtaining a definite diagnosis of SFN in patients with normal IENF count.

In conclusion, our work suggests that the abnormal spatial distribution is due to the nerve remodelling caused by SF pathology. Therefore, it should be considered an intrinsic feature of SFN and its analysis may increase the diagnostic efficiency of skin biopsy when IENF count remains normal. This may be particularly relevant in the clinical practice to correctly offer patients disease modifying and symptomatic treatment and to better stratify patients in clinical trials.

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Disclosures

The authors report no disclosures.

Ethical approval

The study was approved by the local ethical committee (Fondazione IRCCS 'G. Pascale') and all subjects signed an informed consent.

Data availability

Anonymized data will be shared by request from any qualified investigator.

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