Clinical pain research

Structural and functional characterization of nerve fibres in polyneuropathy and healthy subjects

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**Highlights**

- We present a rigorous comparison between functional and morphological parameters.
- There was a less relationship between nerve fibre structure and function in patients.
- Combining small fibre parameters may improve the diagnostic accuracy of DSP.

**Article Info**

**Keywords:** IENFD, Nerve fibre length density, Skin biopsy, Global spatial sampling, Polyneuropathy

**Abstract**

**Objectives:** Quantification of intraepidermal nerve fibre density (IENFD) is an important small fibre measure in distal symmetric polyneuropathies (DSP), but quantitative evaluation of additional structural and functional factors may help in elucidating the underlying mechanisms, and in improving the diagnostic accuracy in DSP. The literature reports a weak or moderate relationship between IENFD and spontaneous and evoked pain in neuropathies, but the relationship between functional and structural small fibre parameters in patients with DSP is unclear. The objectives of the current study, therefore, were to determine morphological and functional parameters related to small nerve fibres in subjects with distal symmetric polyneuropathy (DSP) and healthy controls, and to characterize the interplay among these parameters in these two groups.

**Materials and Methods:** 17 patients with painful DSP (≥4 on 0–10 numerical rating scale) and with symptoms and signs of small fibre abnormality (with or without large fibre involvement) and 19 healthy control subjects underwent comprehensive functional and structural small fibre assessments that included quantitative sensory testing, response to 30 min topical application of 10% capsaicin and analysis of skin biopsy samples taken from the distal leg (IENFD, epidermal and dermal nerve fibre length densities (eNFLD, dNFLD) using global spatial sampling and axonal swelling ratios (swellings/IENFD and swellings/NFLD)).

**Results:** DSP patients had reduced sensitivity to cold (median −11.07 °C vs. −2.60, \( P \leq 0.001 \)) and heat (median 46.7 vs. 37.70, \( P \leq 0.001 \)), diminished neurovascular (median 184 vs. 278 mean flux on laser Doppler, \( P = 0.0003 \)) and pain response to topical capsaicin (median 10 vs. 35 on 0–100 VAS, \( P = 0.0002 \)), and lower IENFD, eNFLD and dNFLD values combined with increased swelling ratios (all \( P < 0.001 \)) compared to healthy controls. The correlation between structural and functional parameters was poor in DSP patients, compared with healthy controls. In healthy controls eNFLD and dNFLD, IENFD and eNFLD, IENFD and dNFLD all correlated well with each other (\( r = 0.81; P < 0.001, r = 0.58; P = 0.009, r = 0.60; P = 0.007, \) respectively). In DSP, on the other hand, only eNFLD and dNFLD showed significant correlation (\( r = 0.53, P = 0.03 \)). A diagnostic approach of combined IENFD and eNFLD utilization increased DSP diagnostic sensitivity from 82.0% to 100% and specificity from 84.0% to 89.5%.

DOI of refers to article: http://dx.doi.org/10.1016/j.sjpain.2015.09.005.

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http://dx.doi.org/10.1016/j.sjpain.2015.08.007

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Conclusions: This study presents a rigorous comparison between functional and morphological parameters, including parameters such as eNFDL and dNFDL that have not been previously evaluated in this context. The correlation pattern between functional and structural small fibre parameters is different in patients with DSP when compared to healthy controls. The findings suggest a more direct relationship between structure and function of nerve fibres in healthy controls compared to DSP. Furthermore, the findings suggest that combining IENFD with measurement of NFLD improves the diagnostic sensitivity and specificity of DSP.

Implications: Combining small fibre parameters may improve the diagnostic accuracy of DSP.

1. Introduction

Painful distal sensory polyneuropathies (DSP), with exclusive or preferential involvement of small nerve fibres of the A-delta and C types are very common in clinical practice [1].

The introduction of skin biopsies to examine small nerve fibre morphology together with functional measures such as quantitative sensory testing (QST) has led to an improvement in diagnosing patients with small fibre neuropathy [2–10]. A correlation between loss of epidermal nerve fibres and reduction in sensitivity to thermal stimuli has been documented for a variety of conditions such as diabetic neuropathy [5], HIV neuropathy [11], Fabry’s disease [12] and other inherited and genetic conditions [13]. Several studies have indicated a weak or moderate relationship between intraepidermal nerve fibre density (IENFD) and spontaneous, as well as evoked pain in neuropathies [14–18], but the correlations between sensory thresholds and loss of skin innervation are not straightforward and are currently debated. It is especially unclear how this relationship between functional and structural small fibre parameters in patients with DSP compares to healthy subjects.

Previous studies have primarily relied on one morphological measure, the IENFD, to determine nerve fibre structural pathology. More recently, additional morphological parameters have been introduced and reported to be abnormal in polyneuropathies. These additional measures include small fibre axonal swellings and the epidermal and dermal nerve length density (eNFDL and dNFDL, respectively) [19–24]. It is currently unclear if the combination of two or more morphological or functional measures may yield higher sensitivity and/or specificity rates in diagnosing painful DSP with diseased small fibres.

Here we report the results of a study designed at determining whether patients with DSP and healthy controls have differential patterns of correlations between structural and functional nerve measurements. Additionally, we investigated if combinations of structural and functional measurements can improve the diagnostic accuracy of DSP with small fibre involvement.

2. Materials and methods

This study was approved by the regional ethics committee (No. 20090234), and all participants gave written consent to participate. We enrolled patients aged 18–80 years with a confirmed diagnosis of painful DSP with symptoms and signs of small fibre abnormality (with or without large fibre involvement) based on clinical characteristics and assessment by a trained neurologist [4], and “probable” or “definite” neuropathic pain [25,26] with intensity ≥4 on 0–10 numerical rating scale (NRS). The patients underwent a thorough clinical neurological examination and a routine lab screening including endocrine, renal, and hepatic functions, IgG, IgM, IgA and M-component, vitamin B12, and methylmalonate determination. Patients were excluded from the study if they had any previous application of capsaicin or near the skin biopsy area (distal leg). Healthy subjects aged 18–80 with no prior history of chronic pain, neurological diseases, diabetes (or other metabolic disorders), alcohol- or substance misuse served as controls. Healthy subjects were recruited using flyers posted at Aarhus University and Aarhus University Hospital, and advertising on the social media.

2.1. Quantitative sensory testing (QST)

Quantitative sensory testing was performed on the dorsal foot to evaluate sensory nerve fibre function. Mechanical detection threshold (MDT), cold and warm detection thresholds (CDT and WDT, respectively) and cold and heat pain thresholds (CPT and HPT, respectively) were determined. Von Frey filaments graded from 0.25 mN to 512 mN applied force were used to assess tactile detection threshold to mechanical stimuli using method of limits [27]. Thermal threshold parameters, i.e. CDT, WDT, CPT, and HPT were determined using a Thermal Sensory Analyser device (Medoc, Israel) to evaluate thermal function of C and A-delta nerve fibres as previously described [28,29]. The thresholds were determined by the method of limits, by continuous ramping of temperature by 1 °C’s from a baseline temperature of 32 °C up until first detection of change to warm (WDT) or first sensation of painful heat (HPT); or ramping down until first detection of change to cold (CTD) or first sensation of painful cold (CPT), as described in detail elsewhere [30]. Cut-off temperatures were 0 °C for cold and 50 °C for heat to avoid thermal skin damage. An average threshold was calculated from three measurements in each area. Cold detection threshold was determined as change from baseline (CTD temperature [°C] = Baseline temperature [32 °C]). For each participant, we calculated the warm sensitivity index (WSI), which represents the range in which non-noxious heat is perceived [31], calculated with the following formula: WSI = 1 – (WDT-BL)/(HPT-BL), where BL is the baseline temperature (32 °C in the current study).

2.2. Topical capsaicin response

Capsaicin, which causes a painful response and local vasodilation due to activation of TRPV1 (Transient Receptor Potential Vanilloid 1) channels on nociceptors [32], was used as an additional measure of small fibre function. Pain and local vasodilation induced by topical application of capsaicin were assessed by the following procedure at the same site where QST was performed. The skin in the painful area on the dorsal foot was heated to 34 °C using a feedback lamp [33,34], and baseline spontaneous pain intensity was assessed on 0–100 numerical rating scale (NRS) (0 = no pain, 100 = worst pain imaginable). On both dorsal feet, measurement of cutaneous blood flow was performed on with laser Doppler, followed by application of 100 μL of 1% capsaicin cream on a circular area of 2 cm in diameter for 30 min. Participants’ ratings of spontaneous pain intensity on the 0–100 NRS were recorded at baseline (0 min), with 5-min intervals up to 30 min, and again 10 min after capsaicin had been removed (40 min). The skin temperature was kept constant at 34 °C during the 30-min period,
since skin temperature can affect both the capsaicin penetration rate through the skin and the local capsaicin-induced vasodilation response [35,36]. Doppler blood flow measurement was performed again immediately after removal of the cream.

The Doppler system (LDI-2, Laser Doppler Imager, Moor Instruments Ltd, Devon, UK; software version 3.11) was configured as follows: Scan area was 7.5 cm x 7.5 cm, scan speed was 50 ms/pixel, and a visible red laser at a wavelength of 690 nm was used. The scanning was performed in a raster pattern, and a Doppler shift caused by the moving blood in the microvasculature was processed to create a colour-coded image of the skin perfusion. For analysis, the full scan area was defined as the region of interest, and the mean and standard deviation (SD) for the flux in the baseline scan image was calculated using the LDI software. The net increase in the area and intensity of flux after capsaicin application was calculated by subtracting the baseline flux (mean +2 SD value) from the second measurement [33]. Data presented as mean flux and a composite score of mean flux multiplied by the area of capsaicin-induced vasodilation (pixels).

2.3. Skin biopsy

2.3.1. Skin biopsy and staining

One skin biopsy from the distal leg (10 cm above the lateral malleolus) was taken from each participant under sterile conditions, using a 3-mm disposable biopsy punch (Miltex, York, PA, USA). Immediately after obtaining, the biopsy was fixed in 2% paraformaldehyde-lysine-periodate for 18–24 h, followed by an overnight cryoprotection in 20% glycerol and 0.08 M Sorensen’s PO₄ buffer.

Each biopsy was cut into 50 μm cryostat sections perpendicular to the skin surface (Microm Cryostat HM 500 OM, Zeiss, Germany), and three sections were sampled systematically and immunostained, using the free floating protocol as described elsewhere using rabbit anti-human PGP 9.5 (1:1000; AbD Serotec, Düsseldorf, Germany) as a primary antibody and horseradish peroxidase-marked goat anti-rabbit as secondary antibody (1:200; DakoCytomation, Glostrup, Denmark) [23,37].

2.3.2. Analysis

All measurements from skin biopsies were performed using a light microscope (Olympus BX51 microscope) connected to an Olympus DP70 camera (Olympus, Tokyo, Japan), a Heidenhain ND 281 encoder (Heidenhain, Schaumburg, IL, USA), and a Prior Proscans II motorized stage (Prior Scientific Inc., Rockland, MA, USA). Length measurement of each section, length measurement of swellings, NFLD estimations as well as control of the hardware was performed using the newCAST software (Visiopharm, Hoersholm, Denmark).

The epidermal length of three sections was measured, and small fibres crossing the basal membrane to the epidermis were counted under a normal light microscope (60 x immersion lens, Olympus UPlanSapo, NA = 1.35) to obtain IENFD, see Fig. 1, right. All sections were counted twice with 4 weeks apart by the same investigator (PRK), who was blinded to the origin of the biopsies during both counts.

2.3.3. Global spatial sampling

The method is described in detail elsewhere [23]. Briefly, to get an unbiased estimation of NFLD, there must be an equal probability for every single fibre in the sample to be hit by a test plane. In global spatial sampling, software generated, randomly orientated and parallel isotropic virtual planes within a virtual 3D box are superimposed over the region of interest on the computer screen, having an equal probability of intersecting the nerve fibres. Every nerve fibre in focus that intersects with the virtual planes is counted, and the length is estimated by the equation below, see Fig. 1, left. In the next field of view, the orientation of the box is changed at random.

Global nerve fibre length density, \( L_{v}(nerve\ fiber/area\ of\ interest) \) is estimated by:

\[
L_{v}(nerve\ fiber/area) = \frac{\sum Q(nerve\ fiber)}{\sum a(\ plane)}
\]

\[
= \frac{2p(box)}{avg(\ plane)} \frac{\sum Q(nerve\ fiber)}{\sum P(area)}
\]

where \( \sum Q(nerve\ fiber) \) is the sum of marked nerve fibres in the area of interest (epidermis or dermis), \( p(box) \) is the number of corners in one sampling box, \( avg(\ plane) \) is the average of the sum of areas of isotropic oriented planes in one sampling box, and \( \sum P(area) \) is the number of sampling box corners hitting the region of interest. The sampling box volume divided by the plane distance is \( a(\ plane) \). The sampling box height was set to 15 μm, the counting box area was set to 7200 μm², the \( x- \) and \( y- \) sampling steps were 127 μm x 113 μm, and the plane separation distance was 25 μm.

2.4. Statistics

T-test was used to compare continuous variables between DSP patients and controls. Non-normally distributed data were compared using Mann–Whitney test, and presented as medians with interquartile range (IQR) Associations between structural and functional parameters were determined using linear regression (Sigmplot version 12.0, Systat Software, Inc., Chicago, IL, USA). We calculated the coefficient of variation (CV), which is defined as the ratio of the standard deviation to the mean. Based on previous experience, we determined that groups of at least 15 subjects would be required for examining the correlation between structural and functional parameters.

3. Results

Seventeen patients, female/male: 4/13 (aged 32–79 yrs, mean age 58.2 yrs), with painful DSP and diseased small nerve fibres and meeting the criteria of definite or probable neuropathic pain, and 19 healthy controls, female/male: 12/7 (aged 33–64, mean age 48.3 yrs) were enrolled in the study. Demographics and clinical characteristics are presented in Table 1. All DSP patients had symmetric symptoms and sensory disturbances, and their QST and topical capsaicin response measurements are shown in Figs. 2 and 3. Cold detection thresholds (median –11.07°C, [(IQR) = 8.9] vs. −2.60 [IQR = −1.9], \( \text{P} < 0.001 \)) and warm sensibility index (median 0.00 [IQR = 0.24] vs. 0.48 [IQR = 0.26], \( \text{P} < 0.001 \)) were lower and warm detection thresholds were higher (median 46.7 [IQR = 5.72] vs. 37.70 [IQR = −1.73], \( \text{P} < 0.001 \)) in DSP patients compared to healthy controls. Following capsaicin application, DSP patients also reported lower pain intensity (median 10 [IQR = 15] vs. 35 [IQR = 40] on 0–100 VAS, \( \text{P} = 0.0002 \)) and displayed less local vasodilation response (median 184 [IQR = 103] vs. 278 [IQR = 69] mean flux on laser Doppler, \( \text{P} = 0.0003 \)).

Quantitative measurements results from DSP and healthy skin biopsies are presented in Table 2, and in Figs. 4 and 5. All parameters (IENFD, eNFD, dNFD and swelling ratios) were substantially different between DSP and controls, with patients having lower nerve fibre densities but higher swelling ratios.

In healthy controls eNFD and dNFD, IENFD and eNFD, IENFD and dNFD all correlated well with each other (\( r = 0.81; \ P = 0.001, r = 0.58; \ P = 0.009, r = 0.60; \ P = 0.007 \), respectively). In DSP, on the other hand, only eNFD and dNFD showed significant correlation (\( r = 0.53, P = 0.03 \)). This suggests that while in healthy controls small fibre density and lengths are closely correlated, in DSP they may represent different, not necessarily correlated, measures of fibre density.
pathology. In addition, we found that while epidermal fibre length (eNFLD) was inversely correlated with heat pain threshold (HPT) in healthy controls \((r=0.47, P=0.04)\), fibre density (IENFD) was the parameter more closely linked with HPT in DSP \((r=0.58, P=0.01)\) further suggesting that length and density parameters represent different, perhaps supplementary, measures of fibre dysfunction. Interestingly, both in DSP and healthy controls, mechanical detection threshold (MDT) was inversely related to capsaicin local capsaicin-induced vasodilation composite score \((r=0.88, P<0.0001\) and \(r=0.45, P=0.05\), respectively), but not to capsaicin-induced pain response. This finding may suggest that local functional parameters of nociceptor stimulation by capsaicin (e.g. vascular response) may present a different measure than centrally-mediated responses such as pain.

### Table 1
Demographics and clinical characteristics of DSP patients.

<table>
<thead>
<tr>
<th>#</th>
<th>Age (yrs)</th>
<th>Gender</th>
<th>Aetiology</th>
<th>Nerve fibre involvement</th>
<th>Disease duration (yrs)</th>
<th>Neuropat. pain probability</th>
<th>Pain duration (yrs)</th>
<th>Pain frequency</th>
<th>Average daily pain intensity (NRS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>66</td>
<td>F</td>
<td>CIPN</td>
<td>SFN + LFN</td>
<td>5</td>
<td>Probable</td>
<td>5</td>
<td>Constant</td>
<td>7</td>
</tr>
<tr>
<td>2</td>
<td>57</td>
<td>M</td>
<td>Type 2 DM</td>
<td>SFN + LFN</td>
<td>6</td>
<td>Probable</td>
<td>6</td>
<td>Daily, not constant</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>46</td>
<td>M</td>
<td>Type 2 DM</td>
<td>SFN + LFN</td>
<td>4</td>
<td>Definite</td>
<td>4</td>
<td>Constant</td>
<td>8</td>
</tr>
<tr>
<td>4</td>
<td>43</td>
<td>F</td>
<td>CIPN and CTD</td>
<td>SFN + LFN</td>
<td>&gt;10</td>
<td>Probable</td>
<td>&gt;10</td>
<td>Daily, not constant</td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td>72</td>
<td>M</td>
<td>Idiopathic PN</td>
<td>SFN*</td>
<td>9</td>
<td>Probable</td>
<td>9</td>
<td>Constant</td>
<td>6</td>
</tr>
<tr>
<td>6</td>
<td>62</td>
<td>M</td>
<td>Type 2 DM</td>
<td>SFN</td>
<td>5</td>
<td>Definite</td>
<td>2</td>
<td>Constant</td>
<td>7</td>
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<tr>
<td>7</td>
<td>41</td>
<td>M</td>
<td>Idiopathic PN</td>
<td>SFN</td>
<td>1.5</td>
<td>Probable</td>
<td>1.5</td>
<td>Constant</td>
<td>7</td>
</tr>
<tr>
<td>8</td>
<td>67</td>
<td>M</td>
<td>Idiopathic PN</td>
<td>SFN*</td>
<td>2</td>
<td>Probable</td>
<td>2</td>
<td>Constant</td>
<td>8</td>
</tr>
<tr>
<td>9</td>
<td>56</td>
<td>M</td>
<td>Idiopathic PN</td>
<td>SFN + LFN</td>
<td>3</td>
<td>Definite</td>
<td>3</td>
<td>Daily, not constant</td>
<td>7</td>
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<tr>
<td>10</td>
<td>57</td>
<td>M</td>
<td>Type 2 DM</td>
<td>SFN*</td>
<td>3</td>
<td>Definite</td>
<td>3</td>
<td>Constant</td>
<td>4</td>
</tr>
<tr>
<td>11</td>
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<td>SFN</td>
<td>2</td>
<td>Probable</td>
<td>2</td>
<td>Daily, not constant</td>
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<tr>
<td>12</td>
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<td>Type 2 DM</td>
<td>SFN</td>
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<td>5</td>
<td>Daily, not constant</td>
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<tr>
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<td>M</td>
<td>Idiopathic PN</td>
<td>SFN*</td>
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<td>Daily, not constant</td>
<td>6</td>
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<td>Idiopathic PN</td>
<td>SFN</td>
<td>6</td>
<td>Probable</td>
<td>6</td>
<td>Daily, not constant</td>
<td>7.5</td>
</tr>
<tr>
<td>15</td>
<td>53</td>
<td>F</td>
<td>Type 2 DM</td>
<td>SFN + LFN</td>
<td>0.5</td>
<td>Probable</td>
<td>0.5</td>
<td>Daily, not constant</td>
<td>8</td>
</tr>
<tr>
<td>16</td>
<td>77</td>
<td>M</td>
<td>Idiopathic PN</td>
<td>SFN + LFN</td>
<td>3</td>
<td>Probable</td>
<td>3</td>
<td>Daily, not constant</td>
<td>7</td>
</tr>
<tr>
<td>17</td>
<td>64</td>
<td>M</td>
<td>Idiopathic PN</td>
<td>SFN</td>
<td>2</td>
<td>Probable</td>
<td>2</td>
<td>Daily, not constant</td>
<td>7.5</td>
</tr>
</tbody>
</table>

Mean 58.23
Std dev 12.85

DM, diabetes mellitus; CIPN, chemotherapy induced peripheral neuropathy; CTD, connective tissue disease; LFN, large fibre neuropathy \((* = \text{yes}, \_ = \text{no})\). Nerve fibre involvement is based on clinical examination and nerve conduction studies. SFN* = the patient was not referred to nerve conduction studies.

### Table 2
Quantitative skin biopsy measurements from DSP and healthy controls. NA = not applicable.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>DSP, mean (\pm SD)</th>
<th>Control, mean (\pm SD)</th>
<th>(P) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IENFD ((\text{mm}^{-1})) CV</td>
<td>3.32 (\pm 2.23)</td>
<td>8.04 (\pm 2.86)</td>
<td>(&lt;0.0001) NA</td>
</tr>
<tr>
<td>eNFLD ((\text{mm}^{-2})) CV</td>
<td>151 (\pm 118)</td>
<td>523 (\pm 251)</td>
<td>(&lt;0.0001) NA</td>
</tr>
<tr>
<td>dNFLD ((\text{mm}^{-2})) CV</td>
<td>163 (\pm 177)</td>
<td>392 (\pm 201)</td>
<td>(&lt;0.0001) NA</td>
</tr>
<tr>
<td># Swellings/IENFD CV</td>
<td>0.57 (\pm 0.55)</td>
<td>0.06 (0.10)</td>
<td>(&lt;0.0004) NA</td>
</tr>
<tr>
<td># Swellings/eNFLD CV</td>
<td>0.02 (\pm 0.02)</td>
<td>0.001 (0.002)</td>
<td>(&lt;0.0002) NA</td>
</tr>
</tbody>
</table>
Fig. 2. Warm sensibility index (WSI), warm detection threshold (WDT), heat pain threshold (HPT), cold detection threshold (CDT, baseline temperature – CDT) and cold pain threshold (CPT) for healthy controls and pain DSP patients. Median values, interquartile range and 95% confidence intervals are shown. Baseline temperature was set to 32°C.

Fig. 3. Flux intensity after 30 min topical capsaicin application and highest capsaicin pain response measured with numerical rating scale (NRC) for healthy controls and pain DSP patients. Median values, interquartile range and 95% confidence intervals are shown.

Sensitivity and specificity of IENFD in diagnosing polyneuropathy in this study was 82.4% (95% CI: 56.6–96.0%) and 84.2% (95% CI: 60.4–96.4%), respectively, with clinician-based diagnosis of small fibre involvement in DSP serving as the standard. It is important to achieve as high sensitivity and specificity as possible to correctly diagnose painful DSP. By combining IENFD and eNFLD values, a sensitivity of 100% (95% CI: 80.3–100%) where at least one of the two parameters was abnormal and a specificity of 89.5% (95% CI: 66.8–98.4%) were obtained. Combining other test results or adding a third measurement to IENFD and eNFLD did not result in a higher specificity. By combining IENFD and WSI the diagnostic sensitivity was increased from 82.4% to 94.1% while the diagnostic specificity remained unchanged at 84.2%.

4. Discussion

This study assessed the relationship between structural and functional small fibre measures in patients with distal polyneuropathy and healthy controls, and investigated strategies to improve the diagnostic accuracy of small fibre neuropathy in DSP.
We observed that correlations between structural measurements were much stronger among control subjects than in DSP patients, with heat pain threshold correlating with IENFD in both groups. We observed a differential pattern of correlation between skin biopsy parameters in DSP patients as compared with healthy controls, with controls presenting more direct relationship between various measures of small fibre morphology. By combining epidermal nerve fibre length with IENFD, the diagnostic sensitivity and specificity of DSP is improved and may contribute to improved diagnosis of neuropathy with small fibre involvement.

4.1. Morphological changes

There is currently no gold standard for diagnosing small fibre involvement in DSP. The diagnosis is typically based on patient’s history, pain distribution and characteristics, and neurological examination including sensory assessment of thermal and mechanical thresholds, as determined in our study by a neurologist (TSJ). While skin biopsies are useful, the literature reports that up to 12% of patients with small fibre neuropathy diagnosed by history and neurologist examination, have normal IENFD [5]. It is therefore important to improve the ability to diagnose these patients based on objective parameters. Among the 17 subjects diagnosed by the above clinical criteria, 13 had abnormalities in all skin biopsy parameters (adjusted for age for IENFD), and additional three subjects were in the lower end of normal values for IENFD, eNFD, dNFD, and had higher axonal swelling ratios, indicating that both epidermal and dermal nerve fibres undergo degeneration in DSP with small fibre symptomatology [24,38]. One patient had normal skin biopsy results. These results are consistent with previous findings, where we demonstrated a reduced eNFD and dNFD in small fibre neuropathy patients compared with healthy controls [23,24].

Nerve conduction evaluation was available from 13/17 patients, which was a part of their clinical evaluation. Seven of the 13 patients had normal nerve conduction studies (NCS), while the remaining six patients showed signs of large fibre involvement (mild-moderate sensorimotor axonal polyneuropathy). The patients with normal NCS had higher IENFD values compared to the patients with large fibre involvement (4.23 vs. 2.74, respectively), a finding that confirms the results from a previous study [41]. These findings suggest that detectable damage to the larger nerve fibres occurs first when there is pronounced damage in the nociceptors.

Comparing morphological changes such as IENFD between studies can be difficult. Immunohistological stainings are difficult to standardize and staining protocols and counting rules may not be identical between studies, all variations that are likely to cause variations between laboratories.

4.2. Correlations between structure and function

Several studies have demonstrated a correlation between IENFD, dNFD and/or eNFD; one showed that a combination of IENFD and dermal NFD increases diagnostic specificity and sensitivity in diagnosing small fibre neuropathy [1,23,38]. In the present study, a higher diagnostic sensitivity and specificity were obtained by combining the determined values of both IENFD and epidermal NFD, thus allowing a better differentiation between DSP and healthy controls. Combining a functional measure (the WSI) and a structural measure (IENFD) improved the sensitivity from 82.4% to 94.2% while the specificity remained unchanged at 84.2%. As shown by Nebuchennyk and colleagues, the choice of reference values is important when considering the diagnostic value of IENFD [42]. The diagnostic sensitivity and specificity of IENFD in a cohort of patients with both pure small fibre damage and also large nerve fibre involvement and different etiologies changed substantially based on different reference values, with ROC analysis showing the best sensitivity (0.77) but Z-score and 5th percentile analysis yielded the best specificity (0.98 and 0.95, respectively).

In healthy controls, the structural skin biopsy parameters correlated with each other better than in DSP patients; it was only in healthy controls that IENFD showed a significant correlation with both epidermal and dermal fibre lengths. These are important findings, as we currently do not know whether pathology that involves nerve fibre density loss is reflected in a similar change in nerve fibre length in the dermis and the epidermis. These may represent different, perhaps independent, measures of small fibre pathology mechanisms.

In DSP patients, HPT correlated with IENFD, consistent with the notion that degeneration of C-fibres (resulting in low IENFD) impairs the ability to detect noxious heat and thus results in increased HPT. In controls, HPT correlated better with eNFD, further suggesting that epidermal fibre length and density may represent somewhat different small fibre pathology parameters. Surprisingly, other QST parameters such as cold detection and cold pain thresholds did not correlate well with morphological small fibre parameters. This may indicate that the correlation between morphology and function is more direct in heat-sensing C-fibres than in cold-sensing Aδ-fibres. In addition, this phenomenon might be explained by the fact that degeneration and regeneration occur in concert among neuropathy and healthy control subjects. The skin is by nature constantly being replaced and epidermal nerves have a strong capacity to regenerate [39]. One possibility is that hypersensitive regenerating sprouts may counteract sensory loss that would be expected to occur in nerve fibre loss. Alternatively, there may be other structures within the skin that contribute to functional measurement but are not reflected by IENFD, NFD or axonal swellings. An additional possibility is that while morphological small fibres measures are entirely objective, the assessments of threshold or supra-threshold patient-reported parameters inherently involve central processing and modulation components. Further investigation is needed to elucidate whether psychological, cognitive and other patient-related factors may interfere with peripheral stimulation processing in QST.

Clinically we determined an index for warm sensibility termed WSI, a sensory parameter derived from thermal threshold measurement. It represents the range in which non-noxious heat is perceived and can be used for early diagnosis of small fibre neuropathy [30]. Nine of our 17 DSP patients showed WSI = 0, i.e.,
complete inability to perceive non-noxious heat, compared to none of the healthy controls. Given that the combination of IENFD and WSI increased the diagnostic sensitivity up to 94.1%, WSI, similarly to HPT, may be another small fibre (predominantly C-fibre) specific measure that better correlates with small fibre loss in DSP.

Topically applied capsaicin activates the TRPV1 receptors located on the nerve endings of C-fibres and causes pain and a local vasodilation response. The response to topical capsaicin was different between DSP patients and controls, with 8 DSP subjects not experiencing any pain from capsaicin application, as opposed to only one such control. Two DSP patients (vs. 6 controls) experienced moderate to severe pain from capsaicin application. It is important to note that the local vasodilation response (flux area) correlated positively with the intensity of capsaicin pain in controls, suggesting an association between the local and centrally mediated effects of small fibre TRPV1 activation by capsaicin. Such correlation, however, was not observed in DSP patients, nor did the morphological small fibre parameters correlate with capsaicin pain response in DSP. The reason for this is not known. While TRPV1 expression in C-fibres may be relatively homogenous in a healthy population, its expression in neuropathy may be substantially altered [40], so that TRPV1-mediated responses may not correlate with small fibre morphology in DSP. One possibility is that hypersensitivity in remaining nerve fibres, exhibit an exaggerated response to TRPV1 stimulation [33]. However, as long as the contribution of degenerating and regenerating fibres to a functional outcome is unknown it is hypothetical what the result of a reduction in nerve fibre count may cause in terms of functional change. A double immunohistological staining of PGP 9.5 and an antibody against the TRPV1 channel would further clarify this, but a reliable antibody against TRPV1 in human skin is not commercially available.

In addition, since the pain response, as opposed to the local vasodilation response, is centrally mediated, the ongoing pain in DSP patients may affect the intensity of pain experience following capsaicin application [34]. The fact that MDT correlated with local vasodilation response but not pain response to capsaicin, supports this notion.

This study presents a rigorous comparison between functional and morphological nerve parameters from skin biopsies in patients with DSP and healthy controls, including parameters such as ENFDL and dNFDL that have not been previously evaluated in this context. Cut-off values or normative reference values are needed for the length densities. While all DSP patients had signs of small fibre involvement, one limitation of the study is that the group is heterogeneous in terms of DSP aetiology and large nerve fibre involvement; therefore these findings need to be confirmed in further, larger studies. However, such heterogeneity is common in neuropathy clinics, as only a fraction of patients present with pure small fibre neuropathy. Another disadvantage of the study is the difference in gender ratios between patients (24% female) and controls (63% female) and that the mean age of the patients is 10 years higher compared to controls, but normative IENFD reference values are higher in females compared to males and nerve fibre density declines with age [6]. Supporting the generalizability of our results is the fact that the thermal sensory thresholds observed in this study are in concordance with previously reported results from larger studies [1,7].

This study has several important findings. We observed a differential pattern of correlation between structural and functional parameters in control subjects compared to DSP patients, suggesting a more direct relationship in healthy controls. Furthermore, our findings suggest that combining IENFD with measurement of epidermal nerve fibre length improves the diagnostic sensitivity and specificity of distal symmetric polyneuropathies with small fibre involvement.

Funding

This study was supported by The Danish Diabetes Academy, supported by the Novo Nordisk Foundation (PK), and Villum Foundation grant to the Centre for Stochastic Geometry and Advanced Bioimaging, Aarhus University and an unrestricted grant from Astellas.

Conflict of interest

The authors declare that they have no conflict of interest.

References


