Research Paper

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# Increased peptidergic fibers as a potential cutaneous marker of pain in diabetic small fiber neuropathy

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## Abstract

Diabetic polyneuropathy (DPN) is a common complication of diabetes and is often associated with neuropathic pain. The mechanisms underlying development and maintenance of painful DPN are largely unknown, and quantification of intraepidermal nerve fiber density from skin biopsy, one of the neuropathological gold standard when diagnosing DPN, does not differentiate between patients with and without pain. Identification of possible pain pathophysiological biomarkers in patients with painful DPN may increase our knowledge of mechanisms behind neuropathic pain. Animal models of painful DPN have been shown to have an increased density of peptidergic nerve fibers (substance P and calcitonin gene-related peptide). In this study, we performed a detailed skin biopsy analysis in a well-characterized group of DPN patients with primarily small fiber involvement, with and without pain, and in healthy controls and test for correlation between skin biopsy findings and pain intensity and quantitative sensory testing. We found that although there was no difference in intraepidermal nerve fiber density using protein gene product 9.5 between patients with and without pain, patients with pain had increased density of dermal peptidergic fibers containing substance P and calcitonin gene-related peptide compared with patients with painless DPN and healthy controls. Peptidergic nerve fiber density correlated with pain ratings in patients with pain ($R = 0.33$; $P = 0.019$), but not with quantitative sensory testing results. In this article, we show, for the first time in humans, an increased density of dermal peptidergic fibers in painful DPN. These findings provide new insight in the pathophysiological mechanisms of pain in diabetes and open the research towards new therapeutic targets.

**Keywords:** Neuropathic pain, Skin biopsy, Peptidergic, SP, CGRP, IENFD

## 1. Introduction

Diabetic polyneuropathy (DPN) is the most common microvascular complication of diabetes with a lifetime prevalence of up to 50%.[18] In addition, DPN is in 30% to 40% of the cases accompanied by neuropathic pain, a condition referred to as painful DPN.[1,8,11,15] The sensory symptoms in neuropathy can be ascribed to an affection of either small fibers causing a small fiber neuropathy (SFN), large fibers, termed large fiber neuropathy, or a mixture of both small and large fibers, termed mixed nerve fiber neuropathy; for review, see Terkelsen et al.[47] The mechanisms underlying DPN and especially development and maintenance of painful DPN are still largely unknown. Symptoms of DPN may be either positive, such as tingling, pins and needles, and burning or cold pain sensation, or negative, such as numbness and sensory loss to one or several sensory modalities or both.[20,24]

It is possible to distinguish between affection of the different nerve fiber types using neuropathological and neurophysiological methods. One neuropathological evidence of DPN with small nerve fiber involvement is a decrease in intraepidermal nerve fiber density (IENFD) (also known as epidermal neurite density),[38] as detected by punch skin biopsy and immunostaining using the pan neuronal marker protein gene product (PGP) 9.5.[28,32] Using nerve conduction studies (NCSs), it is possible to detect damage to the large fibers (large fiber neuropathy).[44,46] The function of both small and large nerve fibers can be assessed by quantitative sensory testing (QST), a psychophysical measure.[42] There is, however, poor relationship between functional measures, such as QST, the presence or the intensity of neuropathic pain, and the IENFD.[26,27]

Identification of possible pain pathophysiological biomarkers in patients with painful and nonpainful SFN is important because it can increase our limited knowledge of the mechanisms driving the pain in neuropathies. Estimating small nerve fiber by only counting PGP 9.5-positive fibers in skin biopsies does not allow for visualizing specific fiber subtypes and may be one of the reasons for the lack of correlation between morphological findings and functions, such as pain.[2,27,39]

Nociceptive C fibers can be divided into peptidergic and nonpeptidergic fibers.[43] The peptidergic population of C nociceptors expresses the tropomyosin receptor kinase A (TrkA) receptor and

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release substance P (SP) and calcitonin gene-related peptide (CGRP). We hypothesized that by specifically mapping the peptidergic nerve fibers for the nociceptive substances SP and CGRP using designated antibodies, we could differentiate patients with neuropathic pain from patients without pain. Although there are studies that have performed analysis of peptidergic nerve fibers,[6,10] no study has compared peptidergic nerve fiber density between SFN patients with and without neuropathic pain.

In this study, we addressed the above issues by performing a detailed skin biopsy analysis in the following 2 subgroups of DPN patients: one with painless SFN and one with painful SFN compared with healthy controls. The objectives were to compare IENFD as well as dermal SP-positive, CGRP-positive, and myelin basic protein (MBP, for identification of myelinated nerve fibers)-positive nerve fibers between diabetic SFN patients with and without pain and to correlate the morphological findings from skin biopsies with QST findings.

## 2. Materials and methods

### 2.1. Study design and participant populations

Fifty-three patients were prospectively recruited among those referred to Istituti Clinici Scientifici Maugeri Spa SB, Department of Neurology, Telese Terme (BN), Italy, with suspected DPN between March 2015 and December 2018. Patients with a definite diagnosis of SFN according to the Toronto Consensus criteria, ie, a combination of symptoms and signs, abnormal IENFD or QST, and normal NCSs, were enrolled.[48] The presence of chronic neuropathic pain due to DPN was determined at the time of the clinical assessment, following the IASP definition of neuropathic pain, further specified by the IASP special interest group on neuropathic pain (NeuPSIG).[50] The pain had to be symmetrical and located at the distal legs, to be present for a minimum of 3 months, and to have an intensity of at least 4 on a numerical ranking scale (0-10, where 0 = no pain at all and 10 = worst possible pain).

Inclusion criteria for patients were as follows: age >18 years, nerve conduction study results within normal limits, and the presence of type II diabetes, as defined by the American Diabetes Association. Exclusion criteria for patients were neuropathy or neuropathic pain due to other causes than diabetes (including alcohol abuse, nonlength dependent syndrome, other neurologic diseases, exposure to neurotoxins, B12 vitamin deficiency, and a family history of neuropathy). Patients with concomitant pathologies of the central nervous system were also excluded.

The patients were divided into 2 groups as follows: (1) patients with painless diabetic SFN (PL-SFN, 22 patients) (symptoms and/or signs of small fiber impairment but no pain, abnormal IENFD or QST, and normal NCSs) and (2) patients with painful diabetic SFN (PF-SFN, 31 patients) (symptoms and/or signs of small fiber impairment, including pain, abnormal IENFD or QST, and normal NCSs). All PF-SFN patients were either on gabapentin/pregabalin or duloxetine; some occasionally used nonsteroidal anti-inflammatory drugs or acetaminophen. None of the patients used opioids. In addition, 45 healthy controls were recruited concurrently with the patients at Istituti Clinici Scientifici Maugeri Spa SB. The controls consisted of relatives to the study patients and colleagues and physicians who were not directly involved in this study and volunteered to participate. The inclusion criterion for healthy controls was age >18 years. Exclusion criteria were diabetes or other metabolic disorders, symptoms or signs of neuropathy, any psychiatric or neurological illness, and chronic pain. In this study, we compared the test results (neurological examination, NCS, QST, and skin biopsy) of the patients with the test results of the healthy controls.

All study participants underwent a structured neurological examination, NCS, QST, and a skin biopsy at the distal leg (see further below). In addition, laboratory and clinical investigation data were obtained, including current treatment, pain descriptors, pain duration and pain type, HbA1c, weight, and height. Pain intensity was rated on the numerical rating scale (NRS) ranging from 0 to 10.

The study was conducted according to the Helsinki criteria and approved by the institutional review board (protocol 418 "Istituto di Ricovero e Cura a Carattere Scientifico Pascale" Ethical Committee), and all patients and controls signed informed consent before the study started.

### 2.2. Nerve conduction studies

Each participant underwent a conventional nerve conduction study with surface electrodes. We examined the median, ulnar, peroneal, and sural nerves. Nerve conduction studies were performed using a Keypoint.net EMG machine, and we determined distal motor latency, conduction velocity, compound muscle action potential (CMAP) amplitude, and sensory nerve action potential (SNAP) amplitude.[44,45]

### 2.3. Quantitative sensory testing

All tests were performed at the right side in all study participants using a truncated version of the standardized protocol developed by the German Research Network for Neuropathic Pain.[42] Mechanical pain sensation, tactile thresholds, and thermal thresholds were assessed at the following 4 body sites: the foot and hand dorsum and distal thigh and distal leg on the right side.

#### 2.3.1. Mechanical pain sensation

Stimuli were applied 10 times with a sharp nonpenetrating probe (50-μm diameter tip) attached to a calibrated nylon filament with a bending force of 95 mN. Stimulus duration was 1 to 2 seconds. The percent of applications perceived as painful was recorded, and the magnitude of evoked pain was assessed using a visual analog scale ranging from 0 (no pain) to 10 (most severe imaginable pain).

#### 2.3.2. Tactile threshold

Stimuli were given with calibrated Semmes-Weinstein monofilaments. Beginning with a suprathreshold stimulus, each monofilament was applied 10 times. Threshold was defined as the smallest monofilament perceivable at least 5 of 10 applications.

#### 2.3.3. Thermal thresholds

Cold, warm, heat pain, and cold pain thresholds were assessed using a thermal analyzer (TSA-2001, Medoc Ltd., Israel) with the method of limits and using a thermal probe of 3 × 3 cm following the standardized protocols.[4]

### 2.4. Skin biopsies

Skin biopsies were taken in accordance with the published guidelines.[32] Biopsies were taken under sterile conditions after intradermal injection of 1% lidocaine 10 cm proximal to the lateral malleolus with a disposable 3-mm punch (Miltex, York, PA).

Samples were fixed with Zamboni fixative overnight and then cryoprotected in 20% sucrose 0.1 M phosphate buffer.

Specimens were cut into 50-μm thick sections using a freezing sliding microtome and processed with an indirect immunofluorescence technique using a large panel of antibodies as primary markers (see Supplementary Table 1, available at http://links.lww.com/PAIN/B161) and appropriate secondary antibodies on free-floating sections. After some pilot trials using rabbit CGRP, rabbit SP, and double CGRP-SP (rabbit CGRP and mouse SP) staining, we found that the optimal staining results were achieved using SP and CGRP antibodies raised in rabbit and visualized with anti-rabbit Cy3 fluorophore in consecutive sections. This allowed more reliable quantification of the nerve fibers.

### 2.5. Omission of the primary antibodies was used as negative control experiment

Protein gene product 9.5-immunoreactive nerve fibers penetrating the basal membrane and crossing the epidermis were counted under a ×20 objective using a Zeiss Imager M.2 microscope and the Zen 2.3 Pro software (Carl Zeiss, Oberkochen, Germany). The epidermal length of the section was also measured. Intraepidermal nerve fiber density was assessed using established counting rules and expressed as fibers per linear millimeter (fibers/mm). Intraepidermal nerve fiber density was considered abnormal if it was below the fifth percentile for age-matched and sex-matched healthy controls.[40]

The SP innervation was assessed by superimposing a grid consisting of 30 vertical lines over the tissue using the Grid function in the Zen 2.3 software. The distance between the lines was 64 μm, and the counting was performed under a ×20 objective. The number of SP+ fibers crossing the lines was divided by the dermal area taken into account (the entire length of the section and 563 μm down from the basal membrane). The density of the SP+ fibers is then expressed as the number of intercepts per square millimeter of dermal tissue (intercepts/mm$^2$) (**Fig. 1**). The same setting was applied for MBP assessment, using a ×10 magnification to ensure quantification of myelinated fibers in the dermis, usually located at the base of hair follicles (up to 1127 μm depth). Because of the different pattern of distribution of CGRP, a grid consisting of 20 horizontal lines and a distance of 60.8 μm was used under a ×20 objective to assess CGRP density. We used horizontal lines for CGRP and vertical lines for SP because of the natural orientation of the nerve fibers and to create intercepts between the fibers and the lines. Substance P-positive fibers were primarily horizontal in orientation, whereas CGRP-positive fibers were primarily vertical. The number of intercepts between the fibers and the grid provides an estimation of the surface of the nerve fibers, and the results are presented as intercepts/mm$^2$.[30]

To verify the repeatability of the method, a second operator blinded to disease or control state performed the same quantification on 20 subjects (5 PL-SFN, 6 PF-SFN, and 9 controls). All counting was performed at Istituti Clinici Scientifici Maugeri Spa SB in a blinded fashion.

### 2.6. Statistics

Data are presented as mean ± SD. Median and ranges are used when reporting data with non-normal distribution. We used *t* test for unpaired data, and Mann–Whitney test (when analyzing nonparametric data) to compare morphological and functional findings from patients and controls. Comparisons among 3 groups were performed using the analysis of variances, followed by Bonferroni post hoc tests to identify the pairs of groups with significant differences. Correlations between variables were assessed using the Pearson correlation coefficient.

Intraclass correlation coefficient between measures performed on the quantification of SP, CGRP, and MBP nerve fibers by different operators was used to assess interrater repeatability.

A 2-sided *P*-value of $<0.05$ was considered statistically significant. Statistical analysis was performed using STATA.

## 3. Results

Demographic and clinical characteristics of patients are reported in **Table 1**. Neurophysiological assessment showed that the NCS values of all study participants were within the normal range (**Table 2**). However, comparison of mean values between the groups revealed lower SNAP and CMAP amplitude of median and ulnar nerves and lower conduction velocity of peroneal nerve in patients compared with controls ($P < 0.05$). Therefore, although the individual NCS assessment was congruent with the diagnosis of SFN, the overall analysis revealed a subclinical involvement of large fibers, indicating that some patients in our cohort may be defined as having DPN with prevalent SFN involvement. Lower SNAP amplitude of ulnar and sural nerves and lower CMAP amplitude of median nerve were observed in PL-SFN compared with PF-SFN ($P < 0.05$), possibly attributable to age differences (**Tables 1 and 2**).

There was no significant difference in body mass index or Hb1Ac levels between the patient groups (see **Table 1**). Patients with pain were younger (mean age 59.1 ± 14.1 vs 66.9 ± 10.7) than patients without pain. Painful SFN patients complained of a moderate-to-high intensity pain (NRS = 7.1 ± 1.7) distributed distally at feet and, in some cases, hands as well. Among them, 18 of 31 (58%) complained of mixed, spontaneous, and evoked pain; 9 of 31 (29%) complained of spontaneous pain; and 4 of 31 (13%) complained of evoked pain. A detailed description of pain quality among patients with PF-SFN is reported in **Table 3**. Main complaints of PL-SFN patients were numbness in 15 of 22 (68%) and nonpainful paresthesias in 9 of 22 (41%).

Quantitative sensory testing showed a length-dependent loss of function with increased numbers of abnormal sensory modalities from proximal to distal sites, as shown in **Figure 2**. All the sensory modalities were abnormal on the foot, except tactile sensation. None of the sensory modalities could distinguish between PF-SFN and PL-SFN (**Fig. 2**).

All patients showed a reduction in IENFD with values below the fifth percentile cutoff,[38] whereas all controls had normal IENFD (see **Table 4**). The mean values of IENFDs were significantly lower than those of controls ($P < 0.01$), whereas they did not differ between PF-SFNi and PL-SFN patients ($P = 0.67$; **Table 4**). The mean MBP nerve fiber density (intercepts/mm$^2$) was lower in both PF-SFN and PL-SFN groups than controls, but the difference did not reach statistical significance ($P = 0.307$ and $P = 0.268$, respectively). The densities (intercepts/mm$^2$) of both SP-ir and CGRP-ir nerve fibers were significantly increased in PF-SFN compared to PL-SFN patients ($P < 0.01$). The density of total peptidergic (CGRP-ir + SP-ir) fibers was also significantly higher ($P < 0.001$) in PF-SFN than PL-SFN patients. The density of total peptidergic fibers and SP-ir fibers in PF-SFN was also significantly higher than controls. All morphological data are reported in **Table 4**. Examples of PGP 9.5-ir, CGRP-ir, and SP-ir fibers are shown in **Figures 3 and 4**.

The assessment of peptidergic and myelinated fibers' density performed on a subset of 20 subjects by 2 operators blinded to each other's results and the origin of the biopsies showed good

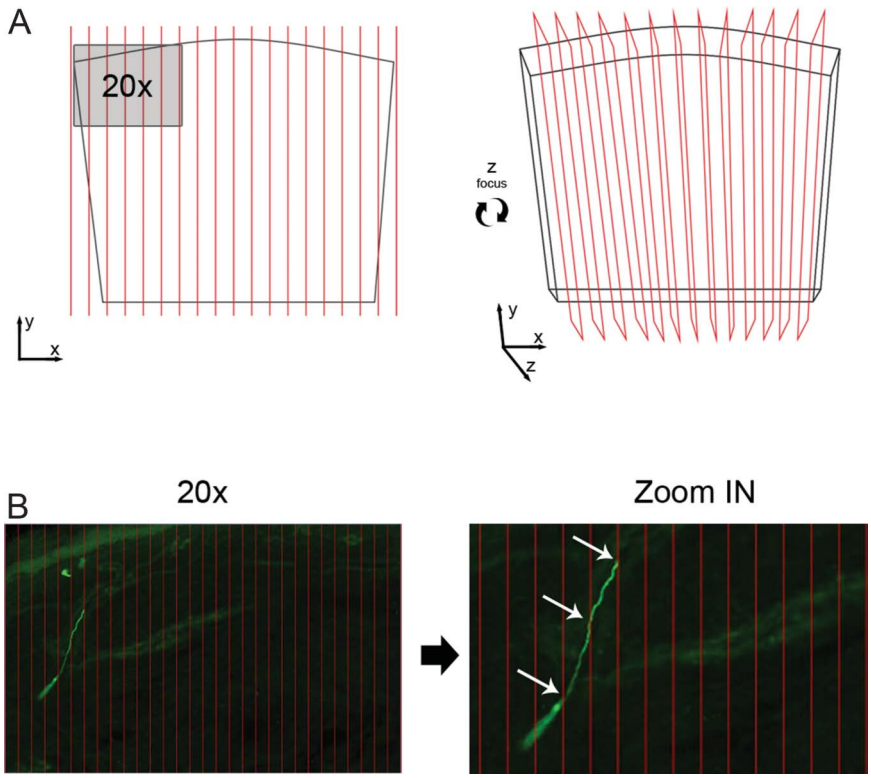


**Figure 1.** Method for the quantification of SP-positive, CGRP-positive, and MBP-positive nerve fibers. (A) The grid is superimposed over the specimen. A magnification of ×20 for CGRP and SP and ×10 for MBP was used. The count of the intercepts was performed by focusing through the entire specimen in live mode. We moved along the "x"-axis to obtain the entire width of the specimen (typically 3 video frames at ×20 lens in our configuration). For the "y"-axis (depth), we used the maximum measure of the video frame (563 μm at ×20 and 1127 μm at ×10). (B) A frame from a live image with grid lines shown in red. The arrows indicate the intercepts between the grid lines and the fiber. CGRP, calcitonin gene-related peptide; MBP, myelin basic protein; SP, substance P.

repeatability of the procedure (intraclass correlation coefficient = 0.9, $P = 0.001$).

Interestingly, a significant ($R = 0.33$; $P = 0.019$; coef. = 1.44) correlation was found between pain NRS and the density of total peptidergic fibers. In turn, no correlations were found between QST and morphological findings (IENF and density of peptidergic fibers).

## 4. Discussion

The main finding of this study was a significant increase of peptidergic fibers containing SP and CGRP in DPN patients with pain compared with patients without pain. Conversely, the 2 patient populations were similar for body mass index, diabetes duration, HbA1c, QST impairment, MBP nerve fiber, and IENFD.

Although we recruited only patients with normal NCSs, thus meeting the criteria for small fiber impairment, we observed that mean values of SNAP and CMAP were lower in patients than controls, implying a subclinical involvement of large fibers in our patient population. Therefore, some of the patients may be defined as having DPN with primary involvement of small fibers. The sensory profile also revealed that mainly small fibers were affected. The QST documented the presence of a length-dependent impairment of all sensory modalities at the foot, except for touch. No significant differences in sensory detection thresholds were observed between the 2 patient groups. Patients with neuropathic pain complained of moderate-to-high intensity pain symptoms located distally at feet and hands. Most of the patients described the pain as being burning, often associated with different types of sensory symptoms such as prickling, itching, or paresthesia. A minority of the patients reported cold pain as the main symptom. Although some patients complained of itching, none of them had clinical signs of prurigo, which has been associated with increased CGRP in the skin.[33]

Overall, the patients seemed to have an involvement of small fibers based on the loss of IENF and on the sensory impairment to

**Table 1**

**Demographic and clinical characteristics.**

| | Age | Sex | HbA1c | BMI | Disease (diabetes) duration (y) | NRS | Pain duration (y) |
|---|---|---|---|---|---|---|---|
| PL-SFN | 66.9 ± 10.7*† | 18M/4F | 6.3 ± 1.0 | 28.2 ± 3.3 | 9.4 ± 7.1 | 0 (±0) | n.a. |
| PF-SFN | 59.1 ± 14.1 | 19M/12F | 7.5 ± 1.3 | 26.8 ± 3.6 | 13.2 ± 10.4 | 7.1 ± 1.7 | 2.8 ± 3.0 |
| Control | 61.2 ± 8.7 | 17M/28F | n.a. | 27.1 ± 4.6 | n.a. | n.a. | n.a. |

* $P < 0.05$ vs PF-SFN.

† $P < 0.05$ vs controls.

BMI, body mass index; HbA1c, glycosylated hemoglobin type A1c; NRS, numerical rating scale; n.a., not applicable; PL-SFN, painless small fiber neuropathy; PF-SFN, painful small fiber neuropathy.

**Table 2**

**Neurophysiological data on study participants.**

**Antidromic sensory nerve conduction studies**

| | Median | | Ulnar | | Sural | |
|---|---|---|---|---|---|---|
| | NCV | SNAP (μV) | NCV | SNAP (μV) | NCV | SNAP (μV) |
| PL-SFN | 51.0 (50.0-61.0) | 13.9 (10.2-23.0)* | 51.0 (50.1-52.0) | 10.3 (10.0-19.9)*§ | 48.4 (45.0-55.9) | 6.1 (7.2-18.0)*§ |
| PF-SFN | 52.3 (50.5-61.0) | 17.6 (10.0-36.4)* | 50.6 (50.3-55.3) | 12.9 (10.0-27.0)* | 48.0 (40.0-57.1) | 10.6 (5.0-27.0) |
| Control | 54.0 (50.3-64.0) | 25.0 (16.0-53.0)† | 52.5 (50.6-55.0) | 27.5 (12.5-45.0)† | 48 (42.0-60.0) | 12.5 (7.0-23.0)† |

**Motor nerve conduction studies**

| | Median | | | Ulnar | | | Peroneal | | |
|---|---|---|---|---|---|---|---|---|---|
| | DML | NCV | CMAP (mV) | DML | NCV | CMAP (mV) | DML | NCV | CMAP (mV) |
| PL-SFN | 3.7 (3.3-3.8) | 54.2 (50.0-65.0) | 8.5 (6.1-16.0)*§ | 2.7 (2.5-2.9) | 62.1 (54.7-74.0) | 11.3 (7.2-18.0)* | 4.1 (3.1-4.8) | 46.9 (45.0-55.9) | 7.2 (3.2-13.0) |
| PF-SFN | 3.6 (2.9-4.6) | 54.2 (50.0-67.4) | 14.0 (6.7-19.9) | 2.6 (2.0-3.1) | 61.6 (54.0-66.7) | 11.3 (7.2-20.0)* | 3.6 (2.2-4.9) | 46.0 (44.0-52.4)* | 7.3 (3.5-14.8) |
| Control | 3.5 (2.9-3.9) | 57.0 (50.0-67.0) | 16.0 (7.0-28.0) | 2.7 (2.5-3.5) | 62.0 (56.0-67.0) | 17.0 (12.0-23.0)† | 4.2 (3.4-5.1) | 51.5 (44.0-54.0)† | 9.0 (5.0-26.0) |

Data are expressed as median and range (in parentheses).

* $P < 0.05$ vs controls.

† $P < 0.05$ vs PF-SFN.

‡ $P < 0.05$ patients (PL-SFN + PF-SFN) vs controls.

CMAP, compound muscle action potential; DML, distal motor latency; NCV, nerve conduction velocity; PL-SFN, painless small fiber neuropathy; PF-SFN, painful small fiber neuropathy; SNAP, sensory nerve action potential.

thermal and mechanical painful stimuli. A subgroup of the patients with pain reported both burning pain (C-fiber gain of function) and cold pain (Aδ-fiber gain of function). There was no correlation between IENFD and sensory thresholds, type of pain, or intensity of pain. This lack of correlation between IENFD and presence and intensity of neuropathic pain reported in this article and in the literature[21,49] highlights the need to identify better biomarkers of pain pathophysiology.

In fact, in the past 3 decades, skin biopsy has been largely applied to diagnose SFN and other neuropathies (for recent reviews on SFN and other distal symmetric polyneuropathies, see Refs. 27, 38, 47). According to the Toronto Diabetic Neuropathy Expert Group statements, a loss in PGP 9.5-ir IENF at the distal leg, and/or QST abnormality, is the gold standard to reach a diagnosis of definite diabetic SFN.[48] However, skin biopsy through the count of IENFD generally does not seem to be able to discriminate between painful-diabetic and painless-diabetic neuropathies,[12,27] albeit a few studies have found significant differences, eg, in patients previously affected by shingles with or without postherpetic neuralgia[37] and in patients with and without painful DPN.[41] Several groups have tried to acquire new insight in the pathophysiology of pain through the study of cutaneous innervation.[12,23,47] So far, the search for pain-related pathology in the skin has been inconclusive. Contradictory findings have been reported on the severity of epidermal denervation, on IENF morphological changes assessed by measuring IENF length or axonal swellings (varicosities) as markers of nerve degeneration, and on the quantification of markers of nerve regeneration, such as growth-associated protein-43 (GAP-43).[12,13] A recent study applied topical capsaicin to a small group of patients with painless and painful polyneuropathies to test whether hyperactive nociceptors contribute to neuropathic pain.[21] Quantitative sensory testing revealed that although the capsaicin application induced localized heat hyperalgesia in all study subjects, there was no difference in the degree of heat hyperalgesia between patients with and without pain, but the axon reflex flare and blood perfusion were greater in patients with pain. These findings are in agreement with the increased density of peptidergic fibers, involved in vasodilatation,[9] that we observed in the group of patients with pain.

**Table 3**

**Pain descriptors in patients with painful symptoms.**

| | Patients |
|---|---|
| Burning | 23 (74%) |
| Prickling/itching | 14 (45%) |
| Allodynia/hyperalgesia | 9 (29%) |
| Tightening | 7 (22%) |
| Stabbing | 6 (19%) |
| Cramps | 6 (19%) |
| Cold pain | 5 (16%) |
| Electrical shocks | 3 (9%) |

Protein gene product-ir fibers in the epidermis are the terminal endings of axons originating in dorsal root ganglion neurons. These can be subdivided into peptidergic and nonpeptidergic fibers according to the expression of peptides.[52] Physiologically, peptidergic and nonpeptidergic fibers terminate in different epidermal layers and have different projection patterns to the spinal cord.[5] Peptidergic fibers contain one or both of the neuropeptides SP and CGRP and are mostly present in the dermis of hairy skin and are more abundantly present in glabrous skin.[36] A higher density of peptidergic fibers running along vasoactive intestinal peptide-ir fibers in the subepidermal neural plexus[35] is observed in a peculiar type of hairy skin: the facial skin. This anatomical pattern may be related to the peculiar functions of craniofacial structures, such as emotional blushing, and may contribute to the susceptibility to pain of this district.[35] Peptidergic fibers also express the high affinity nerve growth factor (NGF) receptor TrkA, which regulates sensory neuropeptide expression.[5] Nonpeptidergic fibers encode a receptor tyrosine kinase that binds glial cell line–derived neurotrophic factors and often coexpress the purinergic P2X3 receptor.[5] Studies in animal models of painful DPN have demonstrated elevated activity along the NGF–TrkA pathway with elevated levels of NGF and increased proportion of IENFs expressing TrkA and CGRP in diabetic rat hind paw skin.[16] This pattern of elevated

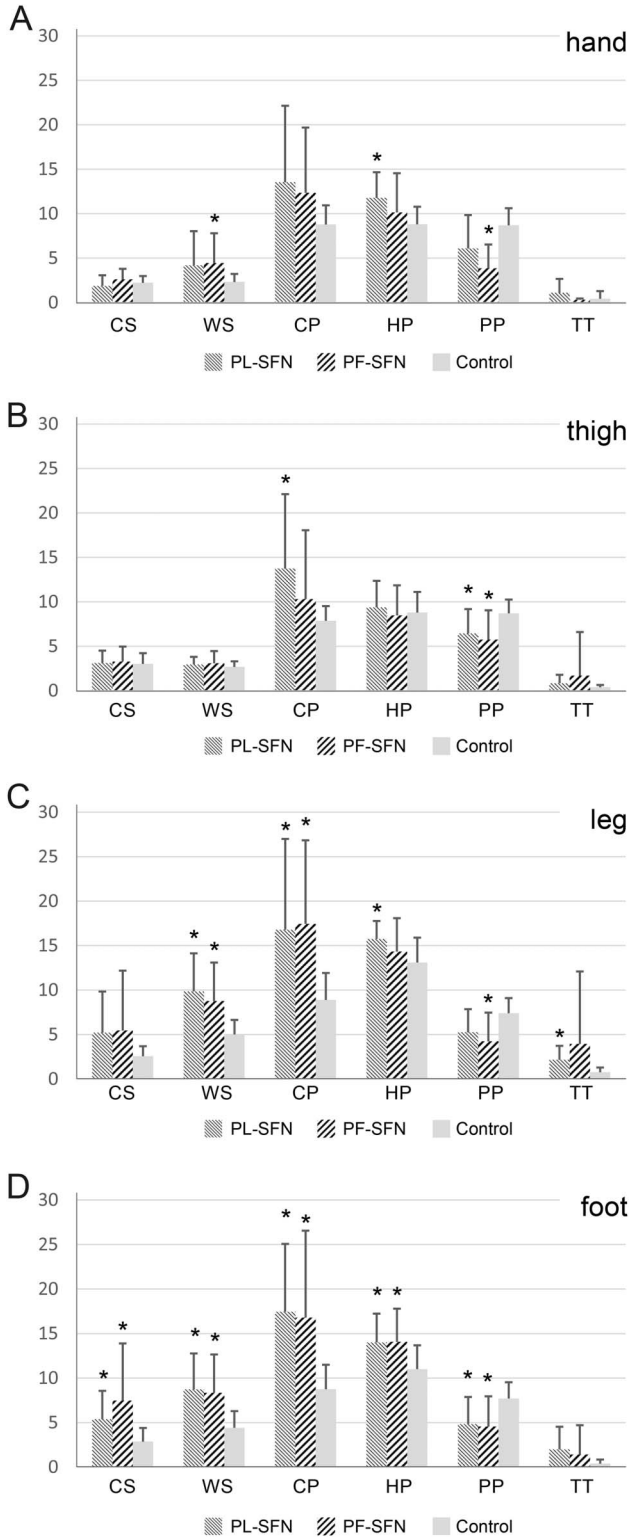


**Figure 2.** Quantitative sensory testing results. Tests were performed at the right side in all participants. Thermal threshold values are expressed as the delta from 32°C; tactile threshold values are expressed in grams; and pinprick values are expressed as number of stimuli out of as painful. None of the sensory modalities differentiates PF-SFN from PL-SFN. CS, cold threshold; CP, cold pain; HP, heat pain; PF-SFN, painful small fiber neuropathy; PL-SFN, painless small fiber neuropathy; PP, mechanical pain perception; TT, tactile threshold; WS, warm threshold. *$P < 0.05$ vs control.

CGRP has not, until now, been replicated in human subjects with chronic neuropathic pain. Conversely, a reduction of CGRP-ir fibers in skin biopsies of diabetic subjects has been reported in an old and small study, in which a correlation with pain was not evaluated.[34] Increased levels of the neurotrophin NGF within the skin of patients with DPN and sensory symptoms, including pain, compared with painless DPN have been reported.[3] Moreover, NGF has recently been shown to sensitize nociceptors in human skin, and it has been hypothesized that the remaining IENF in painful DPN may be exposed to excessive levels of NGF (the so-called "over-trophing"), resulting in hypersensitivity and neuropathic pain.[17] Our data of increased peptidergic nerve fiber population in diabetic patients with painful SFN, as well as the correlation between pain intensity and total peptidergic fibers density, support this hypothesis. We did not quantify the whole subepidermal plexus (that includes peptidergic and nonpeptidergic fibers). Therefore, we cannot directly report the proportion of peptidergic fibers. However, it has been demonstrated that the density of nerves in the subepidermal plexus correlates with IENFD.[31] There was no difference in IENFD between patients with and without pain, suggesting no significant differences between the 2 groups in the subepidermal neural plexus. The density of peptidergic fibers in patients with pain was instead more than two-fold higher, implying a higher proportion of peptidergic fibers and, consequently, a lower proportion of the nonpeptidergic nociceptors in the dermal plexus compared with patients without pain and with controls. A reduction of the nonpeptidergic nociceptors has been proposed to have a relevant role in the development of neuropathic pain in a rat model of nerve injury–induced neuropathic pain[7] and in patients with bortezomib-induced peripheral neuropathy.[6] We hypothesize that a loss of the physiological balance between peptidergic and nonpeptidergic nociceptors may be the trigger to induce pain. In fact, nerve degeneration in diabetes induces a series of events that might compensate for the nerve fiber loss. Therefore, in the skin, signs of nerve remodeling with the coexistence of degenerative and regenerative processes can be observed. To counteract nerve loss, an upregulation of NGF-dependent response leading to an increased expression of neuropeptides in surviving cutaneous unmyelinated C fibers or in newly generated fibers may occur, and this may have a relevant role in the development of pain as demonstrated in animal models of neuropathic pain[14,25,29] through the higher expression of TRPV1[19] and sodium channels.[22]

Why only some, but not all, diabetic patients develop neuropathic pain is unknown, but other contributing factors in addition to glucose dysmetabolism may play an important role in the development of pain in diabetic patients.

The inclusion of the MBP staining in our work aimed to look at the relationship between pain, the large myelinated fibers, and the small fibers. We have previously observed under a condition of neuropathic facial pain a severe loss of myelinated fibers and a normal density of IENF.[51] This was not the case in this work. In fact, we did not find differences in both IENF and MBP density between patients with and without pain. However, the distal leg skin contains considerably less dermal myelinated fibers compared to trigeminal skin, and therefore, it may not be the optimal site to look for possible changes in myelinated fiber density.

This study shows, for the first time in humans, an increased density of peptidergic fibers in patients with painful DPN compared with patients without pain and supports similar findings in animal models of diabetic neuropathy,[16] adding new insight in the pathophysiology of neuropathic pain. Moreover, our

**Table 4**

**Morphological data from skin biopsies.**

| | IENFD leg (fibers/mm) | MBP (intercepts/mm$^2$) | CGRP (intercepts/mm$^2$) | SP (intercepts/mm$^2$) | Peptidergic total (intercepts/mm$^2$) |
|---|---|---|---|---|---|
| PL-SFN | 6.5 ± 4.6* | 1.7 ± 1.5 | 15.2 ± 7.7† | 9.5 ± 7.3† | 24.8 ± 11.0† |
| PF-SFN | 7.0 ± 3.9* | 1.7 ± 1.8 | 22.3 ± 14.3 | 15.7 ± 12.8* | 37.9 ± 16.8* |
| Controls | 12.4 ± 2.7 | 2.2 ± 2.0 | 16.6 ± 10.6 | 10.2 ± 7.5 | 26.8 ± 13.7 |

Peptidergic total represents the total number of CGRP and SP intercepts/mm$^2$.
* $P < 0.05$ vs controls.
† $P < 0.05$ vs PF-SFN.
CGRP, calcitonin gene-related peptide; IENFD, intraepidermal nerve fiber density; MBP, myelin basic protein; PL-SFN, painless small fiber neuropathy; PF-SFN, painful small fiber neuropathy; SP, substance P.



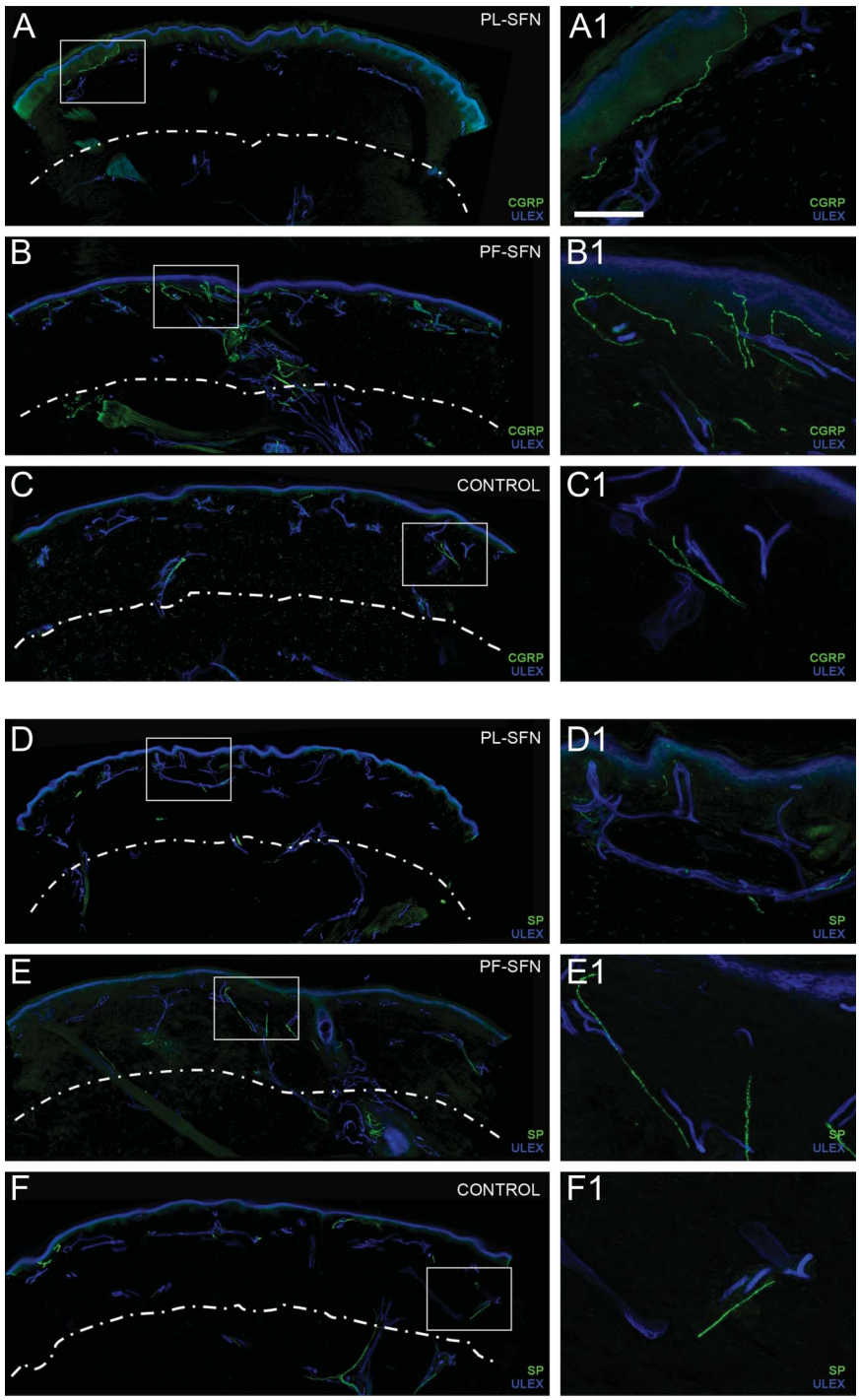


**Figure 3.** Peptidergic fibers in diabetic painful and painless SFN and controls. Confocal images of the entire skin section marked with CGRP (green in A, B, and C) and SP (green in D, E, and F) from PL-SFN (A and D), PF-SFN (B and E), and control (C and F). The white dashed lines indicate the depth used for the quantification. In A1, B1, C1, D1, E1, and F1 details of peptidergic nerve fibers at higher magnification are shown. Scale bar = 100 μm. CGRP, calcitonin gene-related peptide; PF-SFN, painful small fiber neuropathy; PL-SFN, painless small fiber neuropathy.

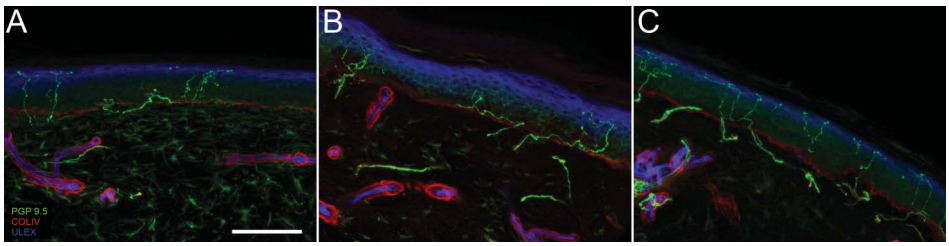


**Figure 4.** Confocal digital images showing a similar loss of IENF in a diabetic patient with SFN without pain (A) and in a diabetic patient with SFN with pain (B) compared with a healthy control (C). Nerve fibers are in green (protein gene product 9.5), basal membrane and vessels in red (Collagen type IV, Col IV), and epidermis and endothelium in blue (ULEX). Scale bar = 100 μm. IENFD, intraepidermal nerve fiber density; SFN, small fiber neuropathy.

finding may have important implications pointing towards new pharmacological targets.

Our study has the following limitations: First, the study includes a relatively small number of patients. Second, patients without pain were almost 7 years older than patients with pain and healthy controls, and they have had diabetes for a shorter duration. Further studies on larger patient cohort and with more complex immunohistochemical protocols are needed to support and strengthen our findings.

## 5. Conclusions

Our work demonstrated, for the first time in humans, an increased density of dermal peptidergic fibers in diabetic neuropathy as a morphological signature of pain in the skin. Furthermore, there was a significant correlation between peptidergic fiber density and pain scores. These findings provide new insight in the pathophysiological mechanisms of pain in diabetes and open the research toward new therapeutic targets.

## Conflict of interest statement

The authors have no conflicts of interest to declare.

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## Appendix A. Supplemental digital content

Supplemental digital content associated with this article can be found online at http://links.lww.com/PAIN/B161.

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