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Small fibre pathology in patients with fibromyalgia syndrome

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Fibromyalgia syndrome is a clinically well-characterized chronic pain condition of high socio-economic impact. Although the pathophysiology is still unclear, there is increasing evidence for nervous system dysfunction in patients with fibromyalgia syndrome. In this case-control study we investigated function and morphology of small nerve fibres in 25 patients with fibromyalgia syndrome. Patients underwent comprehensive neurological and neurophysiological assessment. We examined small fibre function by quantitative sensory testing and pain-related evoked potentials, and quantified intraepidermal nerve fibre density and regenerating intraepidermal nerve fibres in skin punch biopsies of the lower leg and upper thigh. The results were compared with data from 10 patients with monopolar depression without pain and with healthy control subjects matched for age and gender. Neurological and standard neurophysiological examination was normal in all patients, excluding large fibre polyneuropathy. Patients with fibromyalgia syndrome had increased scores in neuropathic pain questionnaires compared with patients with depression and with control subjects ($P < 0.001$ each). Compared with control subjects, patients with fibromyalgia syndrome but not patients with depression had impaired small fibre function with increased cold and warm detection thresholds in quantitative sensory testing ($P < 0.001$). Investigation of pain-related evoked potentials revealed increased N1 latencies upon stimulation at the feet ($P < 0.001$) and reduced amplitudes of pain-related evoked potentials upon stimulation of face, hands and feet ($P < 0.001$) in patients with fibromyalgia syndrome compared to patients with depression and to control subjects, indicating abnormalities of small fibres or their central afferents. In skin biopsies total ($P < 0.001$) and regenerating intraepidermal nerve fibres ($P < 0.01$) at the lower leg and upper thigh were reduced in patients with fibromyalgia syndrome compared with control subjects. Accordingly, a reduction in dermal unmyelinated nerve fibre bundles was found in skin samples of patients with fibromyalgia syndrome compared with patients with depression and with healthy control subjects, whereas myelinated nerve fibres were spared. All three methods used support the concept of impaired small fibre function in patients with fibromyalgia syndrome, pointing towards a neuropathic nature of pain in fibromyalgia syndrome.

Keywords: fibromyalgia syndrome; small fibre neuropathy; skin biopsy; quantitative sensory testing; pain related evoked potentials

Abbreviations: ADS = 'Allgemeine Depressionsskala', i.d. German version of the Centre for Epidemiologic Studies Depression Scale; IENFD = intraepidermal nerve fibre density; NPSI-G = German version of the Neuropathic Pain Symptom Inventory; PREP = pain-related evoked potential; QST = quantitative sensory testing

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Introduction

Fibromyalgia syndrome is a clinically well-characterized condition that involves chronic widespread pain and additional symptoms like fatigue and sleep disturbance (Wolfe *et al.*, 1990). Fibromyalgia syndrome imposes a substantial burden on the individual quality of life and on the general socio-economy. No specific pathogenic agents or morphological alterations have been identified to date.

Whereas the CNS has been investigated extensively in patients with fibromyalgia syndrome (Staud, 2011), research on peripheral nervous involvement is less advanced. One study described a subgroup of patients with fibromyalgia syndrome with a chronic polyneuritis resembling chronic inflammatory demyelinating neuropathy partially responding to immunomodulatory therapy (Caro *et al.*, 2008). The small thinly-myelinated and unmyelinated nerve fibres (A-delta- and C-fibres) mainly conducting pain, temperature, light touch and itch, and their central connections have been investigated to determine structural changes (Sprott *et al.*, 1997; Kim *et al.*, 2008), thermal and pain thresholds (Hurtig *et al.*, 2001; Klauenberg *et al.*, 2008; Pfau *et al.*, 2009; Blumenstiel *et al.*, 2011), and A-delta pain pathways (Gibson *et al.*, 1994; Granot *et al.*, 2001; de Tommaso *et al.*, 2011). These studies gave conflicting results, and an overall concept on the impact of the PNS on pain in fibromyalgia syndrome is missing. Hurtig *et al*. (2001) described lower cold and heat pain thresholds at the dorsal hand of patients with fibromyalgia syndrome using quantitative sensory testing (QST), whereas thermal perception thresholds were not different compared with control subjects. In the study by Blumenstiel *et al*. (2011) patients with fibromyalgia syndrome displayed higher mechanical pain sensitivity and higher cold pain sensitivity compared with healthy control subjects. Pfau *et al*. (2009) found no difference in thermal perception and pain thresholds of patients with fibromyalgia syndrome and control subjects when applying QST at the hand. In the study by Klauenberg *et al*. (2008) QST at the hand of patients with fibromyalgia syndrome showed reduced perception of temperature differences; thermal pain thresholds were not different from control subjects. Gibson *et al*. (1994) showed reduced heat pain thresholds at the dorsal surface of the hand in patients with fibromyalgia syndrome using CO_2-laser stimulation. Assessment of laser-evoked potentials with bilateral stimulation of tender and control points in upper limbs of patients with fibromyalgia syndrome gave hints for C-fibre sensitization and ultra-late laser-evoked potentials could be recorded in the majority of patients (Granot *et al.*, 2001).

We hypothesized that pain in fibromyalgia syndrome is related to small fibre dysfunction. To test this hypothesis we applied three different methods and provide first evidence for functional and morphological small fibre impairment in fibromyalgia syndrome.

Patients and methods

Subjects

The study was approved by the Würzburg Medical School Ethics Committee. The study was conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all study participants. In our case-control study we prospectively enrolled 25 patients with fibromyalgia syndrome (23 females, two males; median age: 59 years, range: 50–70 years) diagnosed according to the 1990 American College of Rheumatology criteria (Wolfe *et al.*, 1990). Patients were recruited between 2007 and 2011 from all over Germany by contacting fibromyalgia syndrome self-help organizations. A telephone interview was performed first in 47 patients who were interested in participating in our study. During this interview the patients were asked about their medical history and current symptoms; inclusion and exclusion criteria were checked and the patients were informed about the study. Inclusion criteria were: male and female patients >18 years of age; medically confirmed diagnosis of fibromyalgia syndrome according to the American College of Rheumatology criteria; other possible differential diagnoses excluded (e.g. rheumatologic, orthopaedic); and willingness to participate in all tests during the study. Exclusion criteria included: other differential diagnoses explaining the pain (e.g. rheumatologic, orthopaedic); other and additional pain sources (e.g. pain due to arthritis); or abnormalities in routine blood tests. After the telephone interview, 20/47 patients either were not willing to take part in the study or were excluded because the inclusion criteria were not fulfilled or exclusion criteria were present. The main reason why patients refused to participate was that they lived too far away and were unwilling to travel long distances to our department. Twenty-seven patients were then invited to the Department of Neurology, University of Würzburg, Germany. Patients were investigated as detailed below. Additionally, all medical records from previous investigations of the patients leading to the diagnosis of fibromyalgia syndrome were checked, as well as the American College of Rheumatology criteria. After this examination, two further patients were excluded; in one the American College of Rheumatology criteria were not fulfilled and the second patient was suffering from acute major depression. Since depressive symptoms are frequently observed in fibromyalgia syndrome and in order to control for possible confounding effects, we additionally investigated a group of 10 patients (nine females, one male; median age: 50 years, range 39–75) with unipolar major depression without pain at present or pain history. These patients were enrolled between 2010 and 2011 at the Department of Psychiatry at the University of Würzburg. We recruited a healthy control group for each investigation, as detailed below.

Clinical examination and laboratory tests

All patients were seen by the same investigator for neurological examination, for confirming the diagnosis of fibromyalgia syndrome, and for excluding clinical signs of an ongoing systemic infection. Laboratory tests included whole blood and differential cell counts, electrolytes, renal and liver function tests, blood glucose levels and C-reactive protein.

Pain and depression questionnaires

We applied the German version of the Neuropathic Pain Symptom Inventory (NPSI-G) (Bouhassira *et al.*, 2004; Sommer *et al.*, 2011). The NPSI-G investigates pain intensity and quality resulting in a sum score between 0 (no pain) and 1 (maximum pain) and additional subscores for different pain characteristics. We additionally calculated the NPSI-G discriminative score to distinguish neuropathic from non-neuropathic pain (Sommer *et al.*, 2011). This weighted score ranges from 42.4 to 80 and has a cut-off value of 53.5 for neuropathic

pain with a sensitivity/specificity ratio of 80/82%. To address depressive symptoms we used the 'Allgemeine Depressionsskala' (ADS, German version of the Centre for Epidemiologic Studies Depression Scale) (Radloff, 1977). The ADS ranges from 0 to 60 with a score $\geqslant$16 being regarded as clinically significant. The control group for questionnaire assessment consisted of 25 healthy volunteers (three males, 22 females; median age: 56 years, range 49–70 years).

Electrophysiological measurements

To exclude large fibre polyneuropathy the right sural and tibial nerves were investigated using surface electrodes and following standard procedures (Kimura, 2001). The results were compared with laboratory normal values: antidromic sural nerve sensory nerve action potential amplitude $\geqslant$10 μV for age $<$65 years, $\geqslant$5 μV for age $>$65 years; sural nerve conduction velocity $>$40 m/s for all ages; tibial nerve compound motor action potential $\geqslant$10 mV, nerve conduction velocity $\geqslant$40 m/s for all ages.

Quantitative sensory testing

QST was performed following a standardized protocol (Somedic) (Rolke *et al.*, 2006). All subjects were investigated at the left dorsal foot. Based on the log transformed raw values for each QST item a *z*-score sensory profile was calculated: *z*-score = (value of the subject − mean value of control subjects)/standard deviation of control subjects. Negative *z*-scores indicate loss of perception, positive *z*-scores indicate gain of perception. For individual analysis values were compared with published reference data (Magerl *et al.*, 2010). For group analysis patients' data were compared with values of age- and gender-matched healthy control subjects from our laboratory. The control group for patients with fibromyalgia syndrome consisted of 25 healthy subjects (23 females, two males; median age: 59 years, range 49–72 years) and for patients with depression, of 10 healthy subjects (nine females, one male; median age: 50 years, range 39–73 years). We determined cold and heat detection thresholds and pain thresholds (cold pain threshold, heat pain threshold), and the ability to detect temperature changes (thermal sensory limen). Paradoxical heat sensation was recorded if the subject experienced cold as heat. We determined the mechanical detection threshold and pain threshold, mechanical pain sensitivity, pressure pain threshold, and vibration detection threshold. All QST-control subjects also underwent sural nerve conduction studies.

Pain-related evoked potentials

Superficial electrical stimulation with a concentric planar electrode can be used to record potentials evoked by activity of A-delta fibres (Lefaucheur *et al.*, 2012). Stimulation with these electrodes induces a pin-prick sensation, which is a typical A-delta phenomenon. In pilot experiments we investigated the nerve conduction velocity of fibres recruited after stimulation with the concentric electrodes. When stimulating at two sites at the lower leg and calculating the nerve conduction velocity (distance between these two sites divided by difference in N1-latencies) we found a median nerve conduction velocity of 4 m/s (range 3–9). This conduction velocity is characteristic for A-delta fibres (Kakigi *et al.*, 1991) and similar values have been reported by others (Lefaucheur *et al.*, 2012). The procedure followed a published protocol (Kaube *et al.*, 2000) with modifications. Pain-related evoked potentials (PREPs) were elicited bilaterally by stimulation at the face (above eyebrow), hands (medial phalanx, second and third digit), and feet (dorsum) using concentric electrodes (Inomed Medizintechnik) and a stimulator (Digitimer DS7A). Potentials were recorded from above Cz by a subcutaneously placed needle electrode referred to linked earlobes (A1–A2) of the international 10-20 system using Signal Software (Version 2-16; Cambridge Electronic Design). Twenty triple pulses were applied. Intensity: double individual pain threshold, duration: 0.5 ms, random interstimulus interval: 15–17 s. Potential recording: gain: $\times$5000, bandwidth: 1–1000 Hz, sweep length: 400 ms, sampling rate: 2.5 kHz. The individual pain threshold was determined by stimulating the area of interest twice with increasing and decreasing current intensities until the subject reported a pin-prick sensation. The average value was taken as the individual pain threshold. Two sets of averaged PREP curves (from 10 single swipes each) were compared for reproducible N1 (first positive peak), P1 (subsequent negative peak) latencies and peak-to-peak amplitudes using MATLAB software (Version 7.7.0.471, The MathWorks). All records were individually evaluated to exclude technical or blink artefacts by the same investigator who was unaware of the diagnosis. The control group consisted of 32 females and 23 males (median age: 44 years, range 21–72 years). All healthy control subjects investigated with PREP were also asked to fill in the questionnaires.

Skin biopsies

Five-millimetre diameter skin punch biopsies (Stiefel) were obtained for histological analysis as previously described (Üçeyler *et al.*, 2010). Two biopsies were taken from each participant (lateral calf and thigh). One patient with fibromyalgia syndrome refused a biopsy at the thigh and another patient with fibromyalgia syndrome refused skin punch biopsy. For immunohistochemistry skin samples were fixed in fresh 4% buffered paraformaldehyde (pH 7.4) for 2–4 h. The samples were then washed in phosphate buffer and stored in 10% sucrose with 0.1 M phosphate buffer. Tissue was embedded in Tissue-Tek® O.C.T., frozen in 2-methylbutane cooled in liquid nitrogen, and stored at −80°C before further processing. To visualize nerve fibres, 50 μm cryostat sections were immunoreacted with the antibody to pan-axonal marker protein-gene product (PGP) 9.5 (Ultraclone, UK, 1:800) with goat anti-rabbit IgG labelled with cyanine 3.18 fluorescent probe (Amersham, 1:100; Cy3). For quantification of intraepidermal nerve fibre density (IENFD), three immunoreacted sections per site were assessed (Axiophot 2 microscope, Zeiss) using Spot Advanced Software (Windows Version 4.5) by an observer unaware of the identity of the specimen. IENFD was determined following published counting rules (Lauria *et al.*, 2005). As reference values for PGP9.5 at the lower leg we took data of normal samples collected in our laboratory (n = 98; median age: 49 years, range 23–86 years, median IENFD 9.5, range 3.2–17.4). As reference values for the upper thigh we took data obtained from 23 control subjects who agreed to a proximal biopsy (median age: 45 years, range 23–69 years, IENFD 11.7 fibres/mm, range 6.7–21.5). Myelinated nerve fibres were visualized with antibodies against myelin basic protein (MBP; GeneTex, 1:200) as described previously (Doppler *et al.*, 2012). The entire dermis was screened for nerve bundles containing at least five PGP9.5 immunoreactive axons excluding nerves of the subepidermal plexus. The number of dermal PGP9.5 immunoreactive nerve bundles containing MBP immunoreactive myelinated nerve fibres was quantified on PGP9.5-MBP double-stained skin sections. Results are expressed as nerve bundles per mm^2.

To visualize regenerating nerve fibres we applied antibodies against growth-associated protein 43 (GAP43, 1:500, Abcam). The assessment of GAP43 stains was performed as with PGP9.5. To visualize macrophages (CD68; 1:3000, Abcam) and T lymphocytes (CD3, 1:200, Abcam), 10-μm frozen sections were stained using standard

immunohistochemistry (ABC kit, Vector). The numbers of T cells and macrophages per dermal vessel were counted in two complete sections. All histological assessments were performed by an examiner who was masked as to the allocation of the specimens.

Statistical analysis

IBM PASW Statistics 19.0 software (IBM) was used for statistical analysis. For data comparison of non-normally distributed data the non-parametric Kruskal-Wallis test was applied; for data comparison of normally distributed data the parametric student's *t*-test was used. Data distribution was tested with the Kolmogorov–Smirnov test and by observing data histograms. Results of non-normally distributed data are given as median and range; results of normally distributed data are given as mean ± standard deviation. For correlation analyses we used the bivariate Spearman correlation. $P < 0.05$ was considered significant.

Results

Clinical and laboratory findings

Table 1 gives demographic and baseline data of the patient groups; Supplementary Table 1 shows individual data and current medication of patients with fibromyalgia syndrome. Patients with depression were on the following treatment: amitriptyline ($n = 4$), venlafaxine ($n = 3$), mirtazapine ($n = 3$), lithium ($n = 2$), quetiapine ($n = 2$), tranylcypromine, maprotiline, nortriptyline, clomipramine, escitalopram ($n = 1$ each). The majority of the depressive patients were on one or two drugs; one patient each was on three and four drugs. Age difference between patients and control subjects was not significant. Tender point count reached ⩾11 in all patients with fibromyalgia syndrome. Neurological and standard neurophysiological examination was normal in all patients. No patient had indicators of ongoing infection.

Patients with fibromyalgia syndrome had a higher NPSI-G median sum score (0.3; range 0.1–0.8) than patients with depression (0; range 0–0.3; $P < 0.001$) and healthy control subjects (0; 0–0.4; $P < 0.001$). In the NPSI-G discriminative score 20 patients with fibromyalgia syndrome (80%) were at or above the cut-off value for neuropathic pain, only one patient with depression (10%) and three healthy control subjects (12%) reached or exceeded this limit (Fig. 1A, $P < 0.001$ each). More depressive symptoms were present in the fibromyalgia syndrome ($P < 0.01$) and depression group ($P < 0.001$) compared with control subjects (Fig. 1B).

Patients with fibromyalgia syndrome have impaired small fibre function in quantitative sensory testing

Patients with fibromyalgia syndrome had elevated cold and warm detection thresholds and increased detection thresholds for temperature changes compared with control subjects ($P < 0.001$ each; Fig. 2A). These alterations indicate functional impairment of A-delta- and C-fibres. Patients with fibromyalgia syndrome also had elevated mechanical detection thresholds but increased mechanical pain sensitivity. As expected, they had reduced pressure pain thresholds ($P < 0.001$ for all items, Fig. 2A). Patients with depression showed a tendency toward elevated thermal perception thresholds, however, differences to control subjects were not significant (Fig. 2B). The fibromyalgia syndrome subgroups with an ADS score ⩾16 or <16 did not differ ($P > 0.05$).

Table 1 Demographics and clinical data of patients with fibromyalgia syndrome and depression

	Fibromyalgia syndrome	Depression
Male, female	2, 23	1, 9
Median age (range) [years]	59 (50–70)	50 (39–75)
Median disease duration (range) [years]	21 (3–50)	23 (3–35)
Questionnaires		
Median NPSI-G sum score (range)	0.3 (0.1–0.8)	0 (0–0.3)
Median NPSI-G discriminative score (range)	57.9 (43.8–79.4)	42.4 (42–56)
Median ADS score (range)	20 (2–44)	29 (4–50)
Electrophysiology		
Sural nerve:		
Sensory nerve action potential [μV]	14 (9–27)	23 (9–37)
Nerve conduction velocity [m/s]	47 (42–52)	51 (46–55)
Tibial nerve:		
Compound motor action potential [mV]	19 (12–30)	18 (8–20)
Distal motor latency [ms]	4 (3–5)	3 (2–4)
Nerve conduction velocity [m/s]	46 (40–52)	47 (41–54)

ADS = 'Allgemeine Depressionsskala', i.d. German version of the Centre for Epidemiologic Studies Depression Scale; NPSI-G = German version of the Neuropathic Pain Symptom Inventory.

Patients with fibromyalgia syndrome have prolonged distal pain-related evoked potential latencies and a generalized reduction of pain-related evoked potential amplitudes

For all subject groups, investigated areas and parameters, there was no difference between the right and left side, therefore data from both sides were pooled. The median stimulation current (i.e. the double individual pain threshold) was not different between groups (Table 2). N1 and P1 latencies did not differ at face and hands between groups (Fig. 3A and C). Patients with fibromyalgia syndrome had prolonged N1 latencies at the feet compared to healthy control subjects ($P < 0.01$) and to patients with depression ($P < 0.05$; Fig. 3E). The most striking finding was that patients with fibromyalgia syndrome had reduced peak-to-peak-amplitudes in all investigated areas compared to healthy control subjects and to patients with depression ($P < 0.001$ each; Fig. 3B, D and F). These alterations indicate an impairment of pathways carrying A-delta fibre pain signals. The fibromyalgia syndrome subgroups with an ADS score ⩾16 or <16 did not

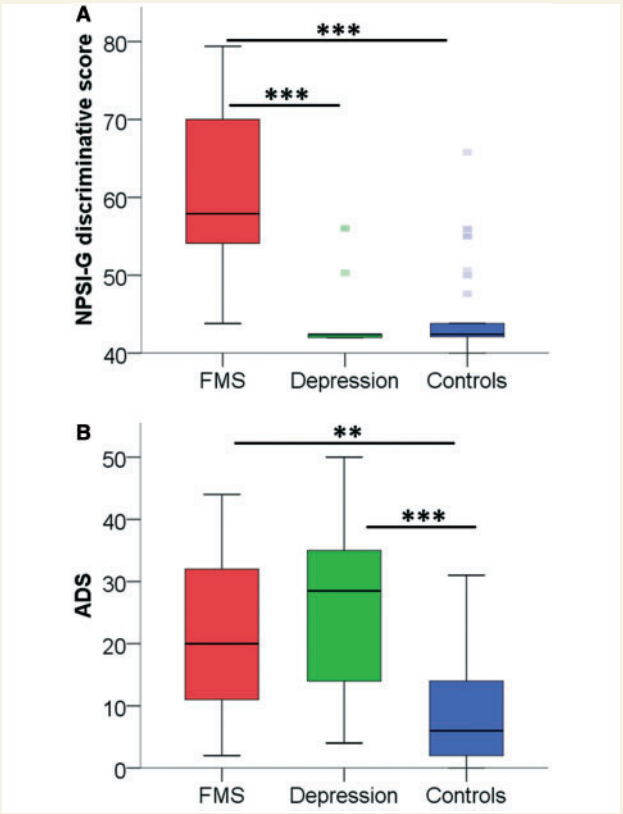

Figure 1 The boxplots give (**A**) the NPSI-G discriminative score for the assessment of neuropathic pain and (**B**) the ADS assessing depressive symptoms. (**A**) Patients with fibromyalgia syndrome have higher NPSI-G discriminative scores compared to patients with depression and to healthy control subjects. The median and range values are as follows: fibromyalgia syndrome = 58 (44–79), patients with depression = 42 (42–56), healthy control subjects = 42 (40–66). The higher score in patients with fibromyalgia syndrome points towards neuropathic pain. Dots above the bar graphs indicate outlier values. (**B**) Patients with fibromyalgia syndrome and patients with depression have elevated scores in the ADS compared with healthy control subjects. The median and range values are as follows: fibromyalgia syndrome = 20 (2–44), patients with depression = 29 (4–50), healthy control subjects = 6 (0–31). $^{**}P < 0.01$, $^{***}P < 0.001$. ADS = 'Allgemeine Depressionsskala', i.d. German version of the Centre for Epidemiologic Studies Depression Scale; FMS = fibromyalgia syndrome; NPSI-G = German version of the Neuropathic Pain Symptom Inventory.

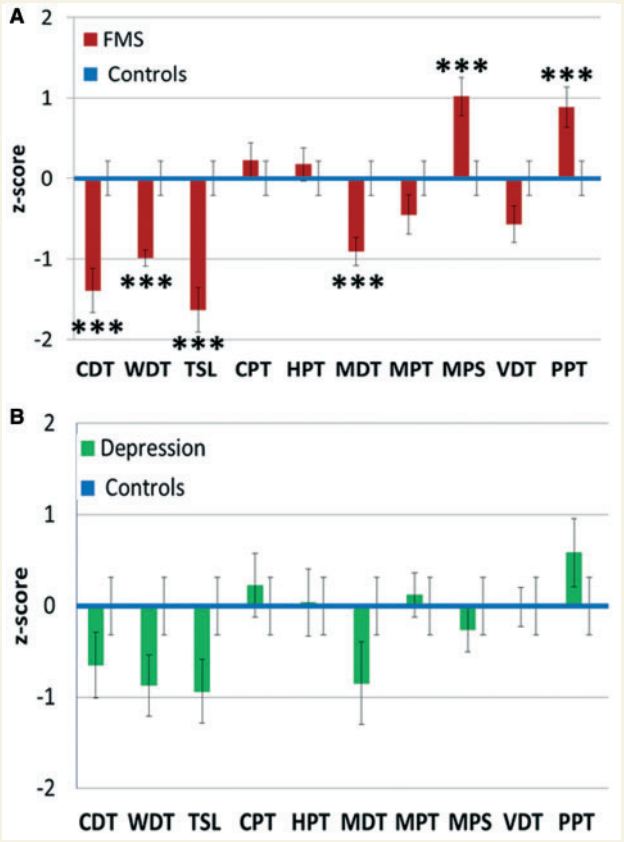

Figure 2 Sensory profiles measured with QST at the left foot of patients with fibromyalgia syndrome (**A**) and depression (**B**) compared with age- and gender-matched control subjects (blue zero-line in **A** and **B**). (**A**) Patients with fibromyalgia syndrome have elevated warm and cold detection thresholds (WDT, CDT) and impaired ability to distinguish temperature changes (i.e. increased thermal sensory limen, TSL). Also the mechanical detection threshold (MDT) is elevated, while mechanical pain sensitivity (MPS) and pressure pain thresholds (PPT) are decreased. (**B**) Patients with depression do not differ from healthy control subjects. $^{***}P < 0.001$. CPT = cold pain threshold; HPT = heat pain threshold; FMS = fibromyalgia syndrome; MPT = mechanical pain threshold; VDT = vibration detection threshold.

differ for any parameter ($P > 0.05$). Table 2 summarizes the median values of the measured PREP parameters.

Skin innervation and regenerating fibres are reduced in patients with fibromyalgia syndrome

Median IENFD as quantified through PGP9.5 immunofluorescence was reduced at the lower leg in patients with fibromyalgia syndrome compared with healthy control subjects (fibromyalgia syndrome: 5 fibres/mm, range 0.3–11.3, control subjects: 9.5 fibres/mm, 3.2–17.4; $P < 0.001$; Fig. 4A). IENFD of patients with depression (7.5 fibres/mm, range 3.5–14.5) was not different from control subjects (Fig. 4A). At the upper thigh IENFD of patients with fibromyalgia syndrome was also lower than in control subjects (fibromyalgia syndrome: 8.0 fibres/mm, range 0–18.0, control subjects: 11.6 fibres/mm, 6.7–21.5; $P < 0.001$, Fig. 4B). Median thigh IENFD of patients with depression (9.7 fibres/mm, range 4.9–15.1) was not different from control subjects (Fig. 4B). The fibromyalgia syndrome subgroups with an ADS score $\geqslant 16$ or < 16 did not differ in proximal and distal IENFD ($P > 0.05$).

Intraepidermal GAP43 immunoreactivity, indicating regenerating nerve fibres, was lower in distal skin samples of patients with fibromyalgia syndrome compared with healthy control subjects (fibromyalgia syndrome: 5.2 fibres/mm, range 0–11.3; control subjects: 8.0 fibres/mm, range 0–17.9; $P < 0.01$; Fig. 4C) and also in the

Table 2 PREP measurements in patients and controls

	FMS ($n = 25$) FMS + depression ($n = 16$) FMS no depression ($n = 9$)	Monopolar depression ($n = 10$)	Controls ($n = 55$)	*P*-value (FMS versus controls)
Face	PREP obtained: 22/25	PREP obtained: 7/10	PREP obtained: 43/55	
N1 latency (ms)	135.0 (49.3–179.3) 134.1 (49.3–179.3) 135.9 (58.1–144.2)	132.3 (109.2–155.3)	135.5 (44.7–173.3)	ns
P1 latency (ms)	183.4 (95.4–267.7) 182.9 (95.4–267.7) 183.9 (111.5–249.3)	203.7 (175.1–241)	193.1 (90.8–269.6)	ns
Amplitude (μV)	8.7 (2.6–35.6) 11.7 (2.6–35.6) 8 (4.1–13.8)	40.3 (18.7–74.7)	32.0 (5.3–74.2)	<0.001
Current intensity (mA)	1.1 (0.6–2.1) 1.1 (0.6–2.1) 1 (0.6–1.5)	1.0 (0.6–1.6)	0.9 (0.4–1.4)	ns
Hand	PREP obtained: 20/25	PREP obtained: 7/10	PREP obtained: 43/55	
N1 latency (ms)	146.8 (71.4–186.6) 150.9 (71.4–173.7) 134.6 (97.7–186.6)	150.2 (104.6–176)	148.8 (103.7–204.1)	ns
P1 latency (ms)	197.2 (131.3–290.3) 205.5 (131.3–229) 183.4 (133.6–290.3)	197.7 (165.4–274.7)	209.7 (143.8–302.8)	ns
Amplitude (μV)	6.7 (2.4–31.4) 8 (2.7–31.4) 3.5 (2.4–19.9)	32.3 (18.5–54.5)	23.3 (3.4–105.3)	<0.001
Current intensity (mA)	1.3 (0.5–2.4) 1.2 (0.7–2.4) 1.4 (0.5–1.8)	1.3 (0.7–2.4)	1.2 (0.5–2.1)	ns
Foot	PREP obtained: 20/25	PREP obtained: 5/10	PREP obtained: 45/55	
N1 latency (ms)	211.5 (130.4–245.6) 195.4 (130.4–245.6) 228.8 (206–245.6)	168.9 (124.9–194.5)	174.7 (124.9–269.6)	<0.01
P1 latency (ms)	261.8 (161.4–361.8) 243.5 (161.4–361.8) 289.4 (245.6–312.4)	216.6 (171–287.1)	248.4 (164.5–316.6)	ns
Amplitude (μV)	5.9 (1.7–35.2) 7.7 (1.7–35.2) 4.9 (1.9–18.5)	27.6 (16.6–42.9)	22.1 (2.7–70.2)	<0.001
Current intensity (mA)	1.7 (0.9–2.4) 1.7 (1–2.4) 1.8 (0.9–2.4)	1.7 (0.9–2.4)	1.7 (0.6–2.4)	ns

All values are given as medians; ranges are given in brackets. All values are averaged from the values of both body sides.
FMS = fibromyalgia syndrome; ns = not significant.

proximal skin sample (fibromyalgia syndrome: 3.7 fibres/mm, range 0–19.1; control subjects: 8.0 fibres/mm, range 3.8–17.0; $P < 0.01$; Fig. 4D). Patients with depression did not differ from healthy control subjects (distal: 6.0 fibres/mm, range 0.8–17.4; proximal: 6.7 fibres/mm, range 1.6–11.6). The fibromyalgia syndrome subgroups with an ADS score $\geqslant 16$ or <16 did not differ in the proximal and distal GAP43 immunoreactivity ($P > 0.05$).

The mean number of PGP9.5 immunoreactive dermal nerve fibre bundles was reduced in patients with fibromyalgia syndrome compared with healthy control subjects (fibromyalgia syndrome: 2.9 ± 1.3 bundles/mm^2; healthy control subjects 4.4 ± 1.5 bundles/mm^2; $P = 0.018$), while patients with depression (3.4 ± 1.6 bundles/mm^2) did not differ from control subjects (Fig. 5A–C). The mean number of MBP immunoreactive dermal nerve fibre bundles (i.e. myelinated nerve fibres) was not different between groups (fibromyalgia syndrome: 1.1 ± 0.6 bundles/mm^2; depression 1.2 ± 0.9 bundles/mm^2; healthy control subjects 1.6 ± 1.1 bundles/mm^2; Fig. 5D–F). Also the mean percentage of PGP9.5 immunoreactive nerve bundles containing MBP immunoreactive myelinated nerve fibres did not differ between groups (fibromyalgia syndrome 36%, depression 32%, healthy control subjects 37%). These results underline that patients with fibromyalgia syndrome have a reduction in PGP9.5 immunoreactive unmyelinated nerve fibres, while the MBP immunoreactive myelinated nerve fibre population is spared.

There was no inflammation in skin samples of patients with fibromyalgia syndrome and depression. Cell counts for dermal T cells and macrophages were not different between groups (data not shown).

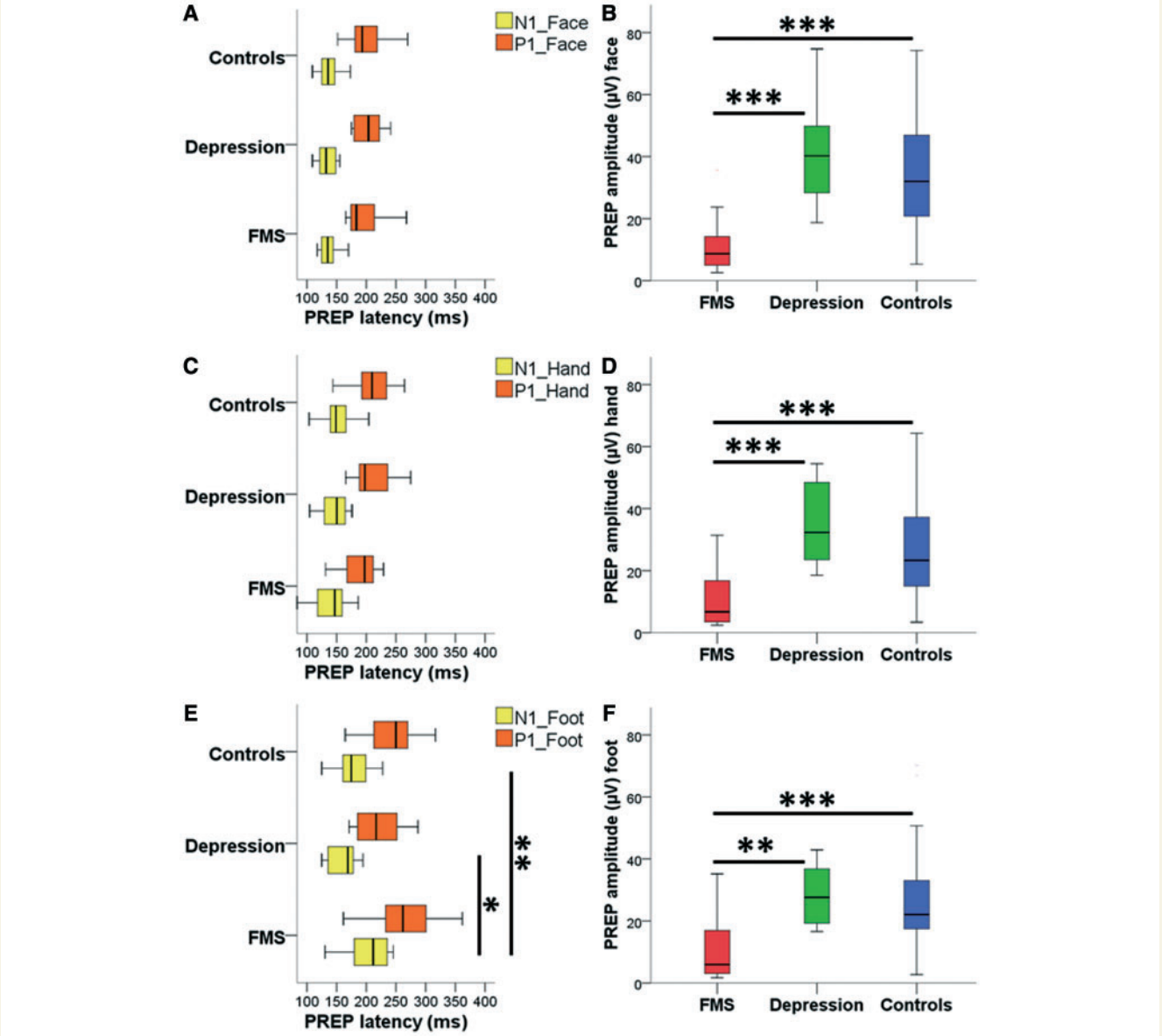

Figure 3 PREP in patients with fibromyalgia syndrome, depression and in healthy control subjects. (**A**, **C** and **E**) N1 and P1 latencies of patients with fibromyalgia syndrome are not different after eliciting PREP at the face and the hand; N1 latencies are prolonged in patients with fibromyalgia syndrome compared with patients with depression and to healthy control subjects when elicited at the foot. (**B**, **D** and **F**) Peak-to-peak amplitudes of PREP are reduced in patients with fibromyalgia syndrome when PREP is elicited at the face, the hand, or the feet. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$. FMS = fibromyalgia syndrome.

The majority of patients with fibromyalgia syndrome have pathological findings in more than two small fibre tests

A subgroup of 8/20 patients with fibromyalgia syndrome (referring to patients with recordable PREP) showed pathological findings in all three and a subgroup of 10/20 patients with fibromyalgia syndrome in two small fibre tests (QST, IENFD and PREP). Two of 20 patients had only one test pathological. None of the patients had normal values in all three tests. These findings show that besides group differences, small fibre affection is present in the majority of the investigated patients with fibromyalgia syndrome also on an individual basis. No correlation was found when comparing IENFD and PREP parameters or pain phenotype in our study cohort.

Discussion

Using three different standardized methods, we consistently showed abnormalities in small nerve fibre properties in patients with fibromyalgia syndrome. Neurophysiological and

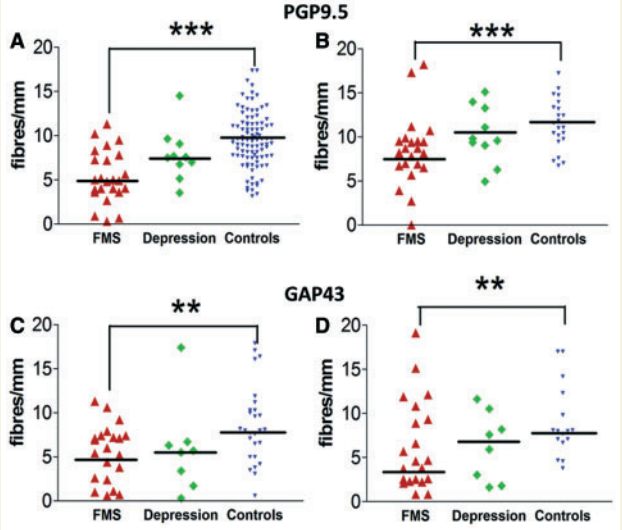

Figure 4 IENFD at the lower leg (**A**) and the proximal thigh (**B**) of patients with fibromyalgia syndrome, depression, and of healthy control subjects investigated with the pan-axonal marker PGP9.5. Patients with fibromyalgia syndrome have lower PGP9.5-positive IENFD compared with healthy control subjects at both biopsy sites. Density of regenerative intraepidermal nerve fibres immunoreacted with GAP43 at the lower leg (**C**) and the proximal thigh (**D**) of patients with fibromyalgia syndrome, depression, and of healthy control subjects. Patients with fibromyalgia syndrome have lower immunoreactivity for GAP43 compared with healthy control subjects at both biopsy sites. $^{**}P < 0.01$; $^{***}P < 0.001$. FMS = fibromyalgia syndrome. GAP43 = growth-associated protein 43. PGP9.5 = protein-gene product 9.5.

psychophysical hypofunction were morphologically paralleled by a significant decrease in distal total and regenerating small fibre density.

Previous studies using QST in patients with fibromyalgia syndrome gave conflicting results on thermal perception and thermal pain thresholds (Hurtig *et al.*, 2001; Desmeules *et al.*, 2003; Geisser *et al.*, 2003; Klauenberg *et al.*, 2008; Smith *et al.*, 2008; Pfau *et al.*, 2009; Blumenstiel *et al.*, 2011). These studies are diverse in methodology, reference values and investigated body regions. The latter is of major importance, because large and small fibre impairment typically displays a distal-to-proximal spread and impairment in small fibre function would be first and most intensively expected at distal parts of the body i.e. the feet. We here applied a standardized and validated QST protocol (Rolke *et al.*, 2006) comparing the individually obtained data with published control values and with values of individually recruited age- and gender-matched healthy control subjects. To assess small fibre function we applied QST at the dorsal feet. The only other study that tested this body area (Klauenberg *et al.*, 2008) found a similar trend of increased perception thresholds for heat and cold, indicating A-delta and C-fibre malfunction.

Small fibre involvement has recently been described in the more localized complex regional pain syndrome (Üçeyler *et al.*, 2007; Oaklander and Fields, 2009); these data were partly confirmed in a recent study investigating a large number of CRPS patients using QST (Gierthmühlen *et al.*, 2012).

PREPs have not been investigated in fibromyalgia syndrome to date. The activation of A-delta afferents is achieved by the high current density in superficial skin layers when applying low current intensities, sparing deeper skin areas containing A-beta fibres. This is obtained by the small anode–cathode distance of the concentric electrodes. Upon stimulation the subject experiences a pin-prick sensation that is typical for the activation of A-delta afferents. Additionally, the nerve conduction velocity of fibres depolarized by these concentric electrodes is typical for A-delta fibres (unpublished data; Kakigi *et al.*, 1991; Müller *et al.*, 2010; Lefaucheur *et al.*, 2012). A-delta and C-fibre pathway function in patients with fibromyalgia syndrome has been investigated with laser-evoked potentials (Gibson *et al.*, 1994; Lorenz *et al.*, 1996; Granot *et al.*, 2001; de Tommaso *et al.*, 2011). These studies, investigating small patient groups of 10 to 14 patients with fibromyalgia syndrome without disease control subjects, mainly report on increased potential amplitudes (Gibson *et al.*, 1994; Lorenz *et al.*, 1996; de Tommaso *et al.*, 2011), in one study with a positive correlation to pain (Granot *et al.*, 2001). However, data cannot be directly compared between studies: in addition to the different stimulation methods (laser-evoked potentials versus PREP), different numbers of stimuli and interstimulus intervals were used. Our finding of reduced PREP amplitudes is in accordance with impaired small fibre function and reduced IENFD, as has been reported in patients with diabetes (Müller *et al.*, 2010) and in HIV-associated small fibre neuropathy (Obermann *et al.*, 2008).

Our study is the first to quantitatively show a reduction in intraepidermal innervation and regeneration sparing myelinated nerve fibres in patients with fibromyalgia syndrome. Electron microscopy has previously revealed unusual patterns of unmyelinated nerve fibres and their associated Schwann cells (Kim *et al.*, 2008). Together with the present study, this strongly points to a pathological process involving C-fibres in fibromyalgia syndrome.

Neuropathic pain is defined as pain caused by a disease or a lesion of the somatosensory nervous system (Treede *et al.*, 2008). In addition to small fibre pathology, our patients with fibromyalgia syndrome had neuropathic pain characteristics in the NPSI-G. However, the clinical presentation of fibromyalgia syndrome (deep and generalized muscle pain) is quite distinct from pain in small fibre neuropathy (acral burning pain). A potential mechanism of how diseased C-fibre afferents may produce neuropathic pain symptoms was recently shown in diabetic rats. While healthy small fibres have a filtering function that allows them to conduct only a small fraction of all incoming volleys elicited by painful stimuli, diseased small fibres have lost this barrier function and unselectively conduct the majority of elicited action potentials (Sun *et al.*, 2012). In view of the generalized mainly deep tissue pain in fibromyalgia syndrome, it may be that this deficit in filtering not only occurs in the skin but also in muscle and joint afferents. An alternative potential mechanism relates to a specialised subgroup of C-fibre tactile afferents that are responsible for the conduction of pleasant touch (Morrison *et al.*, 2011). A reduction of this type of C-fibres in patients with fibromyalgia syndrome might lead to a relative increase in pain perception and in the worst case, to fibromyalgia syndrome pain. A reduction in these

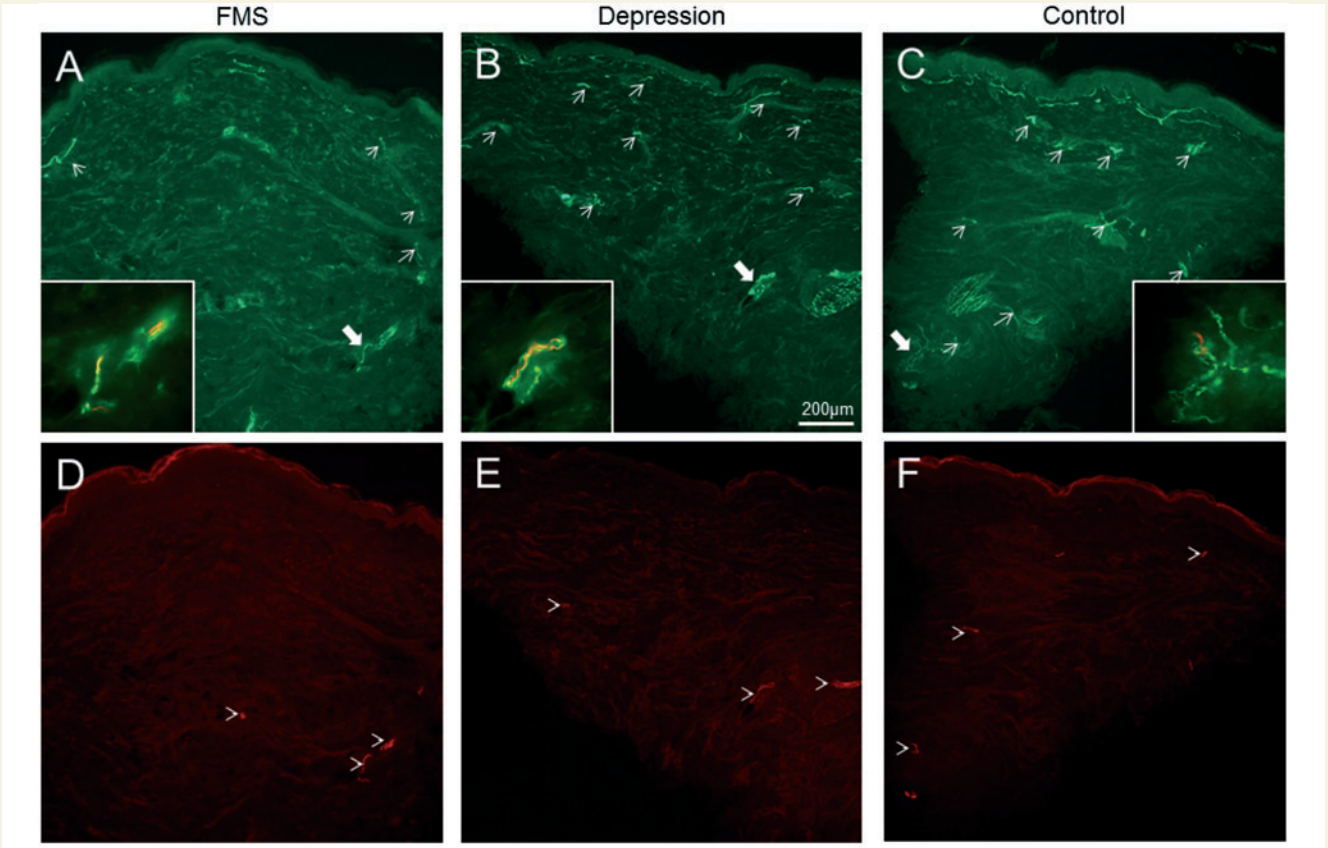

Figure 5 Representative photomicrographs of PGP9.5 stained skin punch biopsies of a patient with fibromyalgia syndrome (**A** and **D**), a patient with depression (**B** and **E**), and of a healthy control subject (**C** and **F**). (**A–C**) PGP9.5 stain for dermal nerve fibre bundles; (**D–F**) MBP stain for myelinated nerve fibres. The thin arrows indicate PGP9.5 immunoreactive dermal nerve fibre bundles (**A–C**); arrowheads indicate MBP immunoreactive myelinated nerve fibres (**D–F**); the thick arrows (**A–C**) indicate the respective nerve fibre bundle that is shown in the inset. The inset illustrates a double-stain of PGP9.5 (red) and MBP (green) immunoreactive fibre bundles. FMS = fibromyalgia syndrome; GAP43 = growth-associated protein 43; PGP9.5 = protein-gene product 9.5. Scale bar = 200 µm.

mechanosensitive C-fibres might also be a reason for the observed increase in mechanical detection thresholds when applying QST.

The lack of correlation between the histologically and electrophysiologically obtained data and the clinical manifestation of pain in our study cohort is in line with the literature (Lauria *et al.*, 2005; Chao *et al.*, 2010; EFNS/PNS, 2010; Petersen *et al.*, 2010; Üçeyler *et al.*, 2010; Haanpää *et al.*, 2011; Buonocore *et al.*, 2012; Saperstein *et al.*, 2013). There are several possibilities why the IENFD does not correlate with pain. First, the degenerated fibres may be not the ones 'causing' pain and the fibres still present may induce pain by spontaneous activity or by another type of pathological discharge (Serra *et al.*, 2011, 2012). Second, nerve fibres that appear normal morphologically may be sensitized by inflammatory mediators in the skin as has previously been suggested for small fibre neuropathy (Üçeyler *et al.*, 2010). Third, the missing nerve fibres may in part not be nociceptors, but C-touch fibres as discussed above. The lack of a correlation between the IENFD and PREP parameters is also not surprising. The major issue here is that intraepidermal nerve fibres are mostly C-fibres, and PREPs measure A-delta fibres. Only one study investigated PREP parameters and IENFD and found a positive correlation. The study was conducted in a very homogeneous population of young males with HIV-associated small fibre neuropathy (Obermann *et al.*, 2008).

In this study, patients with fibromyalgia syndrome not only differed from healthy control subjects but also from patients with monopolar depression without pain. This strengthens the notion that fibromyalgia syndrome is not a variant of depression but rather represents an independent entity that may be associated with depressive symptoms. The finding is compatible with fibromyalgia syndrome and depression being independent disorders although concomitant in a proportion of patients. Also with regard to the persistent somatoform pain disorder that is sometimes assumed to be underlying in patients with fibromyalgia syndrome, our study shows a clear distinction to fibromyalgia syndrome: persistent somatoform pain disorder (ICD-10 F45.40) may be present in patients with fibromyalgia syndrome, however, in the majority of cases the definition of pain starting in connection with an emotional conflict situation or psycho-social stress strong enough to be taken as a crucial aetiological influence and pain in the course of a primary depressive disorder or schizophrenia in addition to chronic widespread pain lasting longer than 6 months is not fulfilled.

In our cohort only four patients reported an emotional conflict situation at the beginning of fibromyalgia syndrome symptoms; none of these patients were diagnosed with primary depression or schizophrenia.

The major limitation of our study is the small sample of patients with fibromyalgia syndrome, which did not allow for analysing subgroups and also precluded stratification for ongoing medical treatments. Another aspect is the potential bias introduced by recruiting patients who are obviously motivated to participate in an extensive experimental study with no other benefit than the attention they get. We were struck, however, by the homogeneity of findings in the fibromyalgia syndrome group and clear-cut differences from the control groups and therefore regard our observations as valid.

This study has major implications regarding the nosological status of fibromyalgia syndrome: (i) our results challenge the current concept of fibromyalgia syndrome as a variant of depression or merely psychological disorder. Rather, objective small fibre impairment is shown on the psychophysical, neurophysiological, and morphological level in fibromyalgia syndrome; (ii) small fibre pathology may be a PNS contributor to the complex pathophysiology of pain in fibromyalgia syndrome. The combination of 'lesion of the somatosensory nervous system' (i.e. fibre loss) and pain defines 'neuropathic pain' (Treede *et al.*, 2008). Thus, pain in fibromyalgia syndrome appears to be closely related to neuropathic pain; and (iii) by definition, the combination of an identifiable group of signs and symptoms and a consistent anatomical alteration (i.e. small fibre impairment) would qualify fibromyalgia syndrome as a disease that would deserve to be recognized accordingly.

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Supplementary material

Supplementary material is available at *Brain* online.

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