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ORIGINAL COMMUNICATION

Asymptomatic small fiber neuropathy in diabetes mellitus: investigations with intraepidermal nerve fiber density, quantitative sensory testing and laser-evoked potentials

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Abstract This study aimed at evaluating the performance of a battery of morphological and functional tests for the assessment of small nerve fiber loss in asymptomatic diabetic neuropathy (DNP). Patients diagnosed for ≥10 years with type 1 ($n = 10$) or type 2 ($n = 13$) diabetes mellitus (DM) without conventional symptoms or signs of DNP were recruited and compared with healthy controls ($n = 18$) and patients with overt DNP ($n = 5$). Intraepidermal nerve fiber density (IENFd) was measured with PGP9.5 immunostaining on punch skin biopsies performed at the distal leg. Functional tests consisted of quantitative sensory testing (QST) for light-touch, cool, warm and heat pain detection thresholds and brain-evoked potentials with electrical (SEPs) and CO_2 laser stimulation [laser-evoked potentials (LEPs)] of hand dorsum and distal leg using small (0.8 mm^2) and large (20 mm^2) beam sizes. Results confirmed a state of asymptomatic DNP in DM, but only at the distal leg. Defining a critical small fiber loss as a reduction of IENFd ≤−2 z scores of healthy controls, this state prevailed in type 2 (30%) over type 1 DM (10%) patients despite similar disease duration and current glycemic control. LEPs with the small laser beam performed best in terms of sensitivity (91%), specificity (83%) and area-under-the ROC curve (0.924). Although this performance was not statically different from that of warm and cold detection threshold, LEPs offer an advantage over QST given that they bypass the subjective report and are therefore unbiased by perceptual factors.

Keywords Diabetic neuropathy · Skin punch biopsy · Intraepidermal nerve fiber density · Laser-evoked potentials · Quantitative sensory testing

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Introduction

Early recognition of incipient DNP is justified as it offers the opportunity to alter the course of suboptimal glycemic control and to take proactive preventative measures prior to the development of clinical DNP [1–3] Moreover, the availability in the future of newer agents providing neuroprotection and/or stimulating nerve regeneration makes a rationale for developing sensitive screening methods to assess their efficacy in patients enrolled into prospective randomized clinical trials [4].

At present, no early indicator of presymptomatic small nerve fiber impairment or damage has definitely been identified for DNP. Light-touch testing with monofilaments and standard neurophysiologic explorations lack sensitivity, as they focus predominantly on the large-fiber nerve system (Aβ-fibers), whereas patients at early stages of DNP mostly present with defects in the small-fiber nerve system

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(Aδ- and C-fibers). Despite this, the current level of evidence in the medical literature for simple screening methods for DNP still focuses primarily on the use of the Semmes–Weinstein 10 g monofilament (SWME) [4, 5]. Thermal detection and pain thresholds using a probe that operates on the Peltier principle permit more detailed assessment of small-fiber dysfunction, albeit limited by the psychophysical nature and weak specificity of the method.

Recent studies suggest that intraepidermal nerve fiber density (IENFd) assessment on punch skin biopsies using PGP9.5 immunostaining can detect neuropathy at an earlier stage than other tests currently recommended, and might specifically reflect painful neuropathy [6–8]. Despite its minimal invasiveness, IENFd assessment by skin punch biopsy may be considered as a predictive biomarker in future clinical trials related to DNP [9].

According to the latest EFNS guidelines [10], nociceptive laser-evoked brain potentials (LEPs) remain the easiest and most reliable of the neurophysiological methods for assessing function of nociceptive pathways (mainly Aδ-fibers). LEP studies have already been performed in asymptomatic type 1 DM patients (T1DM) [11], in both asymptomatic T1DM and T2DM patients [12] and in both types of DM patients with different degrees of peripheral nerve damage [13, 14]. However, in these studies, the diagnostic performance of LEPs was not compared with that of other methods for investigating small-fiber neuropathies (SFNs).

The present study, exploratory in nature, investigated a group of patients diagnosed for at least 10 years with DM (type 1 or type 2), but without any conventional symptoms or signs of peripheral neuropathy. The first aim was to explore the state of asymptomatic DNP using a battery of tests, including morphological (IENF density in punch skin biopsies) and functional tests [quantitative sensory testing (QST) and event-related potentials (ERPs)]. The second aim was to compare the diagnostic sensitivity and specificity of these methods for the detection of asymptomatic SFN in DM.

Methods

Subjects

Seventy-two patients were selected from a clinical database of diabetic outpatients followed in the Department of Internal Medicine of the *Cliniques universitaires Saint-Luc* in Brussels, Belgium. The selection was based on the following main criteria: (1) T1DM or T2DM with a minimum of 10 years elapsed since DM diagnosis, (2) age range 30–40 years (T1DM) or 50–60 years (T2DM), (3) at least an annual follow-up in the outpatient clinic including blood sample analyses, and if indicated, cardiovascular screening and ophthalmologic exam, and finally (4) a score <4 at the Toronto Clinical Scoring System for Diabetic Neuropathy. Given the first criterion enrolment of patients would inevitably have resulted in a bimodal frequency distribution of ages, since T1DM affects patients much earlier in their lives than T2DM. This was also the case in the study of Pozzessere et al. [12]. The second criterion, introducing the specified age groups, was used to reduce the difference in age between the two groups. This cohort of 72 patients (40 T1DM and 32 T2DM) was invited by letter to participate in a study intended to compare different diagnostic and screening procedures for diabetic neuropathies. Ten patients called back asking for more information or to participate, the remainder were contacted by phone and informed on the purposes of the study. Most patients refused to participate for simple reasons like anxiety about the biopsy procedure, traveling distance and personal convenience (working population).

From this cohort, a total of 10 T1DM and 18 T2DM patients were recruited over a period extending from June 2009 to December 2009. Eighteen healthy volunteers, with a mean age of 43 years (age range 30–55; 12 men) were also included in the study as controls. The first 12 healthy volunteers had previously participated in a neurophysiological study [15]. None of the 18 control subjects had a history of alcohol or drug abuse, significant illnesses, or clinical findings suggestive of peripheral or central nervous system disorders. Signed informed consent was obtained from all subjects and patients. The local ethics committee approved the study protocol.

Study design

Two outpatient visits to the clinical unit were planned on a time-span of maximum 2 weeks, with a minimum interval of 3 days. At the first visit, patients underwent a screening evaluation including medical history, concomitant medication recording, physical and neurological examination. The Toronto Clinical Scoring System was used to screen for diabetic (large-fiber) neuropathy. All volunteers underwent on visit 1 Quantitative Sensory Testing (tactile sensitivity pressure thresholds using calibrated Semmes–Weinstein filaments and thermal thresholds testing using a TSAII-thermotest—see below). Five patients with mild-to-moderate DNP according to the Toronto Clinical Scoring System were also enrolled as positive controls for studying diagnostic performance of different assessment procedures (see section "Diagnostic performance of functional tests"). One patient declined to participate further in this study and did not present at visit 2. At visit 2, the 27 remaining subjects were assessed with electrical (somatosensory-evoked potentials, SEPs) and CO_2 laser stimulation testing

(laser-evoked potentials, LEPs). Skin punch biopsies were obtained from 25 subjects (two patients declined), each of whom were familiarized with the procedures prior to biopsy.

Clinical and neurologic examinations

After physical examination including height, weight and blood pressure measurements, and peripheral (pedious) pulses, all patients and healthy volunteers had a neurologic examination focused on the identification of clinical signs of neuropathy. Presence and distribution of positive (paresthesias, allodynia) or negative (hypoesthesia) sensory symptoms at touch and pin-prick, muscle weakness, reflex loss and abnormal features at joint position or vibration testing at upper and lower limbs were actively sought. Symptoms and signs of dysautonomy were recorded.

All volunteers were assessed with the Toronto Clinical Scoring System for Diabetic Polyneuropathy, a validated instrument to reflect the presence and severity of DNP [16]. Mild DNP was defined from a score of 5 onwards, and severe DNP as a score >10.

Quantitative sensory testing (QST)

Semmes–Weinstein monofilament examination (SWME)

Light-touch sensitivity thresholds were assessed using calibrated Semmes–Weinstein monofilaments (Semmes–Weinstein Aesthesiometer; Senselab, Somedic, Sweden) at the first dorsal web space of the non-dominant hand and at the lateral aspect of the non-dominant calf, approximately 10 cm above the lateral malleolus. A validated adaptive staircase algorithm was used as described elsewhere [17]. In short, after having been informed on the purposes and modality of the measurement, comfortably seated with their hands on a table or lying on a bed, subjects were blinded and asked to report by yes or no if they had felt the filament touching their skin. When necessary, the cutaneous site to be tested was gently shaved to avoid stimulating skin hairs with the filament. A double interlaced stepping algorithm adapted from the "4, 2, 1 staircase algorithm" [18] was applied using a total number of 35 stimuli per testing site. Touch-pressure detection thresholds were adjusted for relative room humidity.

Thermal thresholds assessments (thermotest)

Thermal perception and thermal pain thresholds were assessed using a contact thermode measuring 30 × 30 mm (TSA 2001-II, Medoc, Israel). Stimuli were successively delivered at the dorsum of the non-dominant hand and laterally at the distal non-dominant calf. A baseline thermode temperature of 32°C and a heating rate of 1°C/s were used. Thresholds were measured according to the method of limits previously described by Fruhstorfer et al. [19]. Briefly, increasing or decreasing heating ramps were applied to the skin. For each stimulus the subject was instructed to press a button that reversed the thermal stimulation as soon as he/she detected a change in skin temperature, either as cool detection threshold (CDT) or as warm detection threshold (WDT), or as soon as the stimulation became painful (heat pain threshold, HPT). CDT and WDT were estimated as the mean of four successive stimulus presentations. HPT was estimated as the mean of three successive stimulus presentations. An inter-stimulus interval of 6–8 s was used when testing perception thresholds, whereas intervals of 15–20 s were used for HPT. To prevent tissue damage, the maximum and minimum temperatures were set at 50 and 5°C, respectively.

Event-related brain potentials (ERPs)

Electrical stimulation

Somatosensory stimuli were constant-current square-wave electrical pulses (0.5 ms duration; DS7A, Digitimer Ltd, UK) delivered through a pair of skin electrodes (2.5 cm inter-electrode distance) placed on the same sites as described in the QST section (stimulating consecutively the *nervus radialis superficialis* and the *nervus suralis*). Stimulus intensity (2 ± 0.7 mA for the hand, 3 ± 1.5 mA for the leg) was twice the absolute detection threshold and set to avoid any painful or uncomfortable sensation.

CO_2-laser heat stimulation

CO_2 laser stimuli were applied to the same sites used for QST. The CO_2 laser was designed and built in the Department of Physics of the *Université catholique de Louvain*. The system generates a highly collimated infrared beam (wavelength 10.6 μm). Two stimulus surface areas were used (0.8 and 20 mm^2) with respective stimulus durations of 10 (small area) and 50 ms (large area) but with a similar energy density (10 mJ/mm^2), supraliminal for Aδ-nociceptor activation (threshold 7.5 mJ/mm^2). In healthy subjects, stimuli were perceived as a clear pricking sensation (or first pain related to Aδ-nociceptor activation) usually followed by a dull burning sensation (or second pain related to C-nociceptor activation) when the larger stimulus was used. The laser beam was slightly shifted between each trial to avoid skin overheating and minimize nociceptor sensitization or habituation.

Data acquisition and identification of ERP components

After familiarization with both kinds of stimuli, absolute detection threshold, defined as the lowest stimulus intensity detected with a probability of 0.5, and pinprick detection threshold (Aδ-nociceptor-related activation) were determined using the method of limits. Next, a series of 30 stimuli were applied on each site with an inter-stimulus interval of 6–12 s.

Reaction times (RTs) were measured by instructing the subject to press a micro-switch mounted on a hand-controller as soon as any type of sensation at the stimulation site was perceived. The trade-off between RTs ascribed to Aδ-nociceptor activations and those in response to C-nociceptor activations after CO_2-laser heat stimuli was set at 750 ms according to previous studies [15, 20]. Detection rates were deduced from RTs; when a RT exceeded 2,500 ms, the stimulus was considered as undetected.

Brain-evoked potentials were recorded from 19 Ag–AgCl scalp electrodes, based on the International 10–20 system, with linked earlobes (A1A2) as reference. Impedance was kept below 5 kΩ. In addition, electro-oculogram (EOG) of the right eye was recorded with disposable Ag–AgCl surface electrodes (Nessler Medizintechnik, Innsbruck, Austria). Evoked potentials, EOG, RTs and laser trigger signals were recorded on a PL-EEG (Walter Graphtek, Germany). Signals were filtered (low-pass 75 Hz) and digitized (sampling rate 167 cps with a 10-bit resolution). Data were stored on disk and analyzed off-line with the BrainVision Analyzer (Brain Products GmbH, Germany). The time window for analysis was −500 to 2,500 ms according to laser stimulus onset. All trials containing EOG artifacts were rejected from subsequent analysis after visual inspection. Artifact-free trials were time-averaged and corrected for baseline offset using the pre-stimulus interval of 500 ms for each channel.

For every subject, latency (in ms) and amplitude (in μV) of evoked potentials was measured on the averaged waveform for each site. Latencies were measured from stimulus onset to peak. Amplitudes were measured from peak to the averaged amplitude of the 500-ms pre-stimulus interval.

Three distinct SEP components (P100, N120, P240) were individualized. First, component P240 was identified at the vertex electrode C_Z as a positive component peaking between 150 and 300 ms after stimulus onset. The preceding N120 and P100 peaks were then identified as follows. The N120 was defined as a negative peak preceding the P240 at electrode C_Z and occurring between 80 and 160 ms after stimulus onset. The P100 component was defined as a positive peak preceding the N120 component and occurring between 70 and 130 ms after stimulus onset at electrodes C_Z and P4.

Three well-known late LEP components were individualized: N170, N240 and P350. Component P350 was identified at electrode C_Z as the positive component with maximal amplitude between 300 and 500 ms after stimulus onset. N240 was then defined at electrode C_Z as the negative component preceding P350 and occurring between 150 and 300 ms after stimulus onset. N170 was then defined at the temporal electrode contralateral to the stimulation site as well as by trace superposition, as the negative deflection preceding N240 between 120 and 200 ms after stimulus onset.

Skin punch biopsies

The method for taking skin biopsies and the procedures for immunofluorescence staining and linear density of intra-epidermal nerve fibers (IENF) estimation of the present study are described in detail in a previous study [15]. In short, skin punch biopsies (diameter 3 mm) were performed under local anesthesia and aseptic techniques. Two samples were taken from the non-dominant calf, 10 cm above the lateral malleolus, within the *nervus suralis* territory. Sixteen micrometer-thick freezing microtome sections were immunoassayed with anti-human PGP9.5 (RA 95101, Ultraclone, Cambridge, UK; 1:5,000) and visualized by a Cy_3-labeled secondary antibody (Jackson Immunoresearch Laboratories, Inc., PA, USA). Counterstain was performed using Hoechst (Invitrogen Corporate, Molecular Probes, California, USA).

The quantification procedure of linear IENF density (IENFd) was modified from the EFNS guidelines [21] as thinner tissue sections were used. IENF included all single stained nerve fibers crossing the dermal-epidermal junction. Clearly-branched epidermal nerve fragments that did not cross the basement membrane were also counted. As in a previous study [15], tissue sections of skin biopsies were processed at 15–16 μm thickness to allow staining for multiple markers in the same biopsy, with a number of sections for each marker [22–24]. Only the results for PGP9.5 immunofluorescence staining are presented here.

Statistical analysis

Analysis of variance (ANOVA) was used for estimating differences in morphological, psychophysical and electrophysiological data between groups. A Bonferroni test for multiple comparisons was used for comparing treatment group means with controls when the ANOVA null hypothesis for equal means was rejected. When normality tests failed, a Kruskal–Wallis one-way analysis of variance

on ranks was used, with Dunn's test for multiple comparisons with controls. Bivariate correlations were assessed using Pearson's correlation coefficient. When testing for equality of means of IENFd, CDT/WDT or LEPs between different skin areas, paired t tests or non-parametric Wilcoxon signed-rank tests were used (α-level = 0.05). Statistical analyses were performed with SigmaStat software version 3.5 (Systat Software, San Jose, CA) and ROC analyses with NCSS 2007 Statistical Software version 07.1.19 (Kaysville, UT).

Results

Demographic data

Patients and controls are divided into four groups (Table 1): T1DM patients, T2DM patients, DM patients with overt DNP (as positive controls) and healthy controls. Mean age was significantly different between groups. This resulted as a direct consequence of the inclusion criteria. The groups did not differ in height. Mean BMI was significantly higher in T2DM ($p < 0.05$). Known diabetes duration, current glycated hemoglobin level (HbA_{1C}) and serum creatinine were similar between both subtypes of diabetic patients, including patients with overt DNP. Regarding chronic complications of DM, i.e., retinopathy, nephropathy (creatininaemia and albuminuria), and macroangiopathy (coronary heart disease and lower limb arteriopathy), one T1DM and three T2DM patient(s) had microalbuminuria (albuminuria between 30 and 300 mg/24 h), whereas one T1DM patient had macroalbuminuria (419 mg/24 h). Two patients had proliferative retinopathy (one in each DM subtype), whereas non-proliferative retinopathy was reported in one T1DM and in four T2DM patients. Both coronary heart disease and proliferative retinopathy were reported in two (different) patients with DNP.

As per protocol, none of both subtypes of DM patients had neuropathic symptoms. They all had preserved ankle reflexes, albeit weak in seven of them (two within T1DM). All these patients scored <4 on the Toronto Clinical Scoring System for Diabetic Polyneuropathy, indicating that there was no clinical evidence for DNP. Conversely, all patients with DNP had per protocol a Toronto Clinical Scoring System >5 and <10 (mild to moderate DNP).

Quantitative sensory testing (QST)

As a rule, all patients performed QST, including SWME and thermal thresholds assessments. All controls performed the latter, but only six performed the SWME, the 12 remaining were recruited from a previous study where only thermotests were performed. Results from QST in the four groups are shown in Table 2.

Tactile stimulation of the dorsum of the non-dominant hand and distal leg with calibrated monofilaments (mechanical detection threshold, MDT) did not reveal significant differences between the groups of both subtypes of DM patients and healthy controls. As a rule, the dorsum

Table 1 Demographic and clinical data of patients and controls (mean ± SD)

	Patients			Healthy controls
	T1DM ($n = 10$)	T2DM ($n = 13$)	DNP ($n = 5$)	$n = 18$
Gender, M/F	6/4	9/4	5/0	12/6
Age (years)	33.2 ± 3.1	55.6 ± 3.3	55.4 ± 3.3	42.5 ± 9.1
[range]	[30:39]	[50:60]	[51:59]	[30:55]
Height (cm)	174 ± 9	170 ± 11	172 ± 7	176 ± 9
[range]	[160:186]	[150:185]	[166:183]	[156:191]
BMI (kg/m^2)	25.1 ± 4.5	29.8 ± 2.8	28.2 ± 2.6	25.3 ± 4
DM duration (years)	19.0 ± 7.9	14.8 ± 4.0	16.2 ± 4.6	
[range]	[10:32]	[10:23]	[10:22]	
HbA_{1C} (%)	8.1 ± 1.1	8.2 ± 1.4	9.2 ± 0.9	
Creatinine (μmol/L)	76.2 ± 8.2	82.5 ± 13.1	68.8 ± 15.1	
Albuminuria ≥30 mg/24 h, n (%)	2 (20)[a]	3 (23)	3 (60)	
Retinopathy, n (%)	2 (20)[b]	5 (38)[b]	2 (40)[c]	
Coronary heart disease, n (%)	0 (0)	4 (31)	2 (40)	
Lower limb arteriopathy, n (%)	0 (0)	0 (0)	0 (0)	

[a] One patient ≥300 mg/24 h

[b] One patient with proliferative retinopathy

[c] Both patients with proliferative retinopathy

Table 2 QST and IENFd in patients and healthy controls (mean ± SD)

	Patients			Healthy controls
	T1DM	T2DM	DNP	
SWME (hand dorsum)	$n = 10$	$n = 13$	$n = 5$	$n = 6$
MDT (g/mm^2)	6.2 ± 1.0	6.8 ± 2.8	6.7 ± 1.0	7.4 ± 2.0
SWME (distal leg)	$n = 10$	$n = 13$	$n = 5$	$n = 6$
MDT (g/mm^2)	17.2 ± 5.9	17.4 ± 8.0	15.0 ± 2.4	16.0 ± 4.1
Thermotest (hand dorsum)	$n = 10$	$n = 13$	$n = 5$	$n = 18$
CDT (°C)	−1.1 ± 0.2	−1.1 ± 0.5	−2.3 ± 1.4	−1.2 ± 0.4
WDT (°C)	1.5 ± 0.9	2.1 ± 1.3	4.0 ± 3.0	1.5 ± 0.5
HPT (°C)	11.5 ± 3.9	13.3 ± 3.4	14.8 ± 3.1	13.7 ± 2.2
Thermotest (distal leg)	$n = 10$	$n = 13$	$n = 5$	$n = 18$
CDT (°C)	−2.8 ± 1.1	−4.1 ± 2.0	−7.4 ± 2.7	−2.2 ± 1.2
WDT (°C)	5.7 ± 2.3	6.4 ± 3.5	11.7 ± 3.7	4.7 ± 3.0
HPT (°C)	12.6 ± 3.0	14.1 ± 3.0	16.7 ± 1.2	14.8 ± 2.0
Skin biopsy (distal leg)	$n = 10$	$n = 11$	$n = 4$	$n = 18$
IENFd (F/mm)	8.7 ± 5.4	6.4 ± 3.3	1.9 ± 3.4	12.1 ± 6.5
Median (IQR)	8.3 [5.5]	6.1 [7.5]	0.4 [5.4]	11.1 [11.7]

SWME Semmes–Weinstein monofilament examination, *MDT* mechanical detection threshold, *CDT* cool detection threshold, *WDT* warm detection threshold, *HPT* heat pain threshold, *IENFd* intraepidermal nerve fiber density, *IQR* interquartile range

of the hand was more sensitive to light-touch than the distal leg ($t = -10.816$; $p < 0.001$).

Concerning CDT, WDT and HPT, statistical analysis did not reveal any significant difference between the groups, except for CDT at the distal leg between T2DM patients and control group ($p < 0.05$). As for MDT, the hand dorsum was always more sensitive for CDT and WDT than the distal leg ($t = 7.704$, $p < 0.001$ for CDT; $t = -8.837$, $p < 0.001$ for WDT). This was also true for HPT in the paired comparison between hand dorsum and distal leg ($t = -2.814$, $p = 0.004$).

After z-transformation of the QST data and setting a trade-off at $z \geq 2$ for cases considered as pathological [25], 10% (1/10) of T1DM patients and 31% (4/13) of T2DM patients were considered as having an abnormal MDT. None of the T1DM patients had abnormal thermal detection thresholds. CDT or WDT was pathological in 31% (4/13) of T2DM patients. Only 1 T2DM patient had an abnormal score for both thermal detection thresholds. HPT was abnormal in 20% (2/10) of T1DM and in 8% (1/13) of T2DM patients.

Stimulus detection rates and reaction times

Detection rates and reaction times were obtained from all patients (10 T1DM and 13 T2DM) and from 18 healthy controls (except for the data concerning electrical stimulation, where only six controls were stimulated).

The % detection rates of electrical stimuli were complete or near complete in all groups; healthy controls = 100% for the hand and 99% for the distal leg, T1DM = 100% for the hand and 97% for the leg and T2DM = 100% for hand and leg.

RT frequency distributions were slightly right-skewed, but still clearly monomodal. Mean RTs upon hand stimulation ranged from 232 ± 69 ms in controls, 242 ± 48 ms in T1DM and 271 ± 59 ms in T2DM, but was not significantly different between groups. For the distal leg, mean RTs ranged from 269 ± 35 ms in controls, 308 ± 66 ms in T1DM and 322 ± 69 ms in T2DM, respectively. As for stimulation of the hand dorsum, no statistically significant differences between mean RTs of the three groups were found ($p > 0.05$). However, for all groups, there was a significant ($t = -8.223$, $p < 0.001$) increase of the average RTs obtained at the distal leg as compared to those obtained at the hand dorsum.

The % detection rates for laser stimulations are reported in Table 3. In healthy controls and as compared to the hand, stimulation of the distal leg resulted in a slightly lower % detection rate, irrespective of laser beam size. However, in contrast to RT distributions with electrical stimulation, RT distributions with laser stimulation were right-skewed though clearly bimodal, the first peak being related to detections by fast conducting Aδ-fibers and the second peak to detections related to the much slower-conducting C-fibers. By taking a cut-off of 750 ms [15, 20] between the two distributions, we obtained an estimate of Aδ-fiber-related detection rates reaching 85% in the healthy controls for stimulation of the hand dorsum, 79% for the distal leg with the large laser beam and 82% with the

Table 3 Detection rates and reaction times (RTs mean ± SD) to laser stimulation

	Patients			Healthy controls
	T1DM	T2DM	DNP	
Hand dorsum	$n = 10$	$n = 13$	$n = 4$	$n = 18$
Total detection rate (%)	99	96	89	98
RTs ≤ 750 ms (%)	88	78	73	85
RT (ms)	442 ± 215	556 ± 342	586 ± 331	472 ± 281
Distal leg (large laser beam)	$n = 10$	$n = 13$	$n = 4$	$n = 18$
Total detection rate (%)	95	89	74	94
RTs ≤ 750 ms (%)	81	69	48	79
RT (ms)	536 ± 337	624 ± 337	784 ± 492	512 ± 319
Distal leg (small laser beam)	$n = 10$	$n = 13$	$n = 4$	$n = 18$
Total detection rate (%)	83	84	54	94
RTs ≤ 750 ms (%)	68	60	33	82
RT (ms)	562 ± 329	684 ± 461	797 ± 492	502 ± 346

small laser beam. The remaining detections, with longer latency, were thus related to C-fiber afferent input and, to a lesser extend, to misses of Aδ-fiber afferent input.

RTs in response to hand dorsum stimulation were significantly (Dunn's test $Q = 5.654$; $p < 0.05$) increased in T2DM patients compared to the RTs of T1DM and healthy controls. The two latter groups had similar mean RTs. In T2DM, the RTs in response to stimulation of the leg with a large laser beam were significantly ($Q = 7.679$; $p < 0.05$) delayed compared to the RTs of T1DM patients and healthy controls. However, using the small stimulus surface area, the RTs of both T1DM and T2DM patients were on average similar and significantly delayed ($Q = 4.290$ for T1DM and $Q = 6.975$ for T2DM; $p < 0.05$) as compared to healthy controls.

Event-related potentials (ERPs)

ERP components were obtained from all patients (10 T1DM and 13 T2DM) and from 18 healthy controls (except for the SEPs, since only six healthy controls were electrically-stimulated).

SEP components (N120 and P240 latency and N120-P240 peak-to-peak amplitude) upon stimulation of the non-dominant hand and of the distal leg are summarized in Table 4a and b respectively. Statistical analysis did not reveal significant differences between DM patients and healthy controls ($p > 0.05$). In one patient out of each DM subgroup, there was no clear SEP present in the time-averaged records at C_Z indicating dysfunctional large fiber afferents (e.g., demyelination).

The grand time-averaged LEPs are shown in Fig. 1. Group averages of latencies and amplitudes of late LEP components obtained after stimulation of the hand dorsum and distal leg (with large and small laser beam) are reported in Table 4a and b, respectively. Well-recognizable vertex late LEPs, related to Aδ-nociceptor activations, were identified among all healthy controls and T1DM patients. However, in four out of 13 (30%) T2DM patients, no LEP components could be identified in the time-averaged records for both large and small stimulus surface areas. This was also the case for two out of four patients of the DNP group. Of note, ultra-late LEPs, i.e., related to selective isolated activation of C-nociceptors [26], were never observed.

Latencies of LEP components (N240 and P350) did not differ among different groups, whatever the stimulation site or the size of the stimulus surface area ($p > 0.05$). Regarding amplitudes of the N240–P350 complex, there was a significant difference between groups ($F_2 = 4.639$; $p = 0.015$) after allowing for effects of differences in site and laser beam sizes (as repeated-measure factor). There was also a clear difference in mean values among the different levels of site and laser beam size ($F_2 = 11.572$; $p < 0.001$), but without significant interaction with the groups ($F_4 = 0.288$; $p = 0.885$). Amplitudes after stimulation of the hand did not statistically differ among groups, but they were distinctly larger than those obtained by stimulation of distant leg with the large ($t = 3.958$; $p < 0.001$) or with the small beam ($t = 4.168$; $p = <0.001$). When stimulating the distal leg with the large laser beam, amplitudes in T2DM patients were reduced but not significantly ($t = 2.463$; $p = 0.097$) as compared to T1DM patients, who in turn did not statistically differ from healthy controls. However, with the small laser beam, amplitudes in T2DM patients were significantly reduced in comparison with T1DM patients ($t = 2.686$; $p = 0.022$) and healthy controls ($t = 2.936$; $p = 0.027$).

Table 4 ERP analysis in patients and healthy controls

	Patients			Healthy controls
	T1DM	T2DM	DNP	
A. Stimulation of the hand dorsum				
SEP	$n = 10$	$n = 13$	$n = 4$	$n = 6$
N120 latency (ms)	143 ± 25	153 ± 45	146 ± 30	136 ± 30
P240 latency (ms)	236 ± 50	249 ± 66	241 ± 87	217 ± 49
N120-P240 amplitude (μV)	35 ± 11[b]	20 ± 10	26 ± 17	23 ± 11
LEP large laser beam	$n = 10$	$n = 13$	$n = 4$	$n = 18$
N240 latency (ms)	246 ± 25	267 ± 40	293 ± 60[a]	244 ± 39
P350 latency (ms)	347 ± 42	373 ± 41	390 ± 71	374 ± 53
N240–P350 amplitude (μV)	33 ± 9	25 ± 22	22 ± 11	33 ± 16
B. Stimulation of the distal leg				
SEP	$n = 10$	$n = 13$	$n = 4$[d]	$n = 6$
N120 latency (ms)	169 ± 42	185 ± 65	144	166 ± 45
P240 latency (ms)	272 ± 77	267 ± 72	192	246 ± 65
N120-P240 amplitude (μV)	25 ± 12	14 ± 7	7	20 ± 10
LEP large laser beam	$n = 10$	$n = 13$	$n = 4$	$n = 18$
N240 latency (ms)	291 ± 34	318 ± 59	326 ± 88	281 ± 42
P350 latency (ms)	400 ± 62	401 ± 74	401 ± 85	428 ± 64
N240–P350 amplitude (μV)	25 ± 13[c]	14 ± 9[a]	3 ± 13[a]	26 ± 13
LEP small laser beam	$n = 10$	$n = 13$	$n = 4$	$n = 6$
N240 latency (ms)	272 ± 48	303 ± 36	287 ± 42	261 ± 28
P350 latency (ms)	384 ± 63	406 ± 44	350 ± 13	403 ± 24
N240–P350 amplitude (μV)	23 ± 10[b]	11 ± 9[a]	4 ± 7[a]	27 ± 14

Values expressed as mean ± 1 SD

SEP somatosensory-evoked brain potentials, *LEP* laser-evoked brain potentials Level of statistical significance (p level < 0.05): [a]compared to controls, [b]compared to T2DM patients and patients with DNP, [c]compared to patients with DNP, [d]only one subject had identifiable SEPs

Skin biopsies

Linear IENF densities for each group (DM patients and healthy controls) are summarized in Table 2 (lower part). Representative images of PGP9.5-immunofluorescent fibers in epidermis from the different groups are shown in Fig. 2. The IENF densities of T2DM patients were on average significantly reduced compared to those of T1DM patients and healthy controls ($F_2 = 3.77$; $p = 0.033$). Of note, IENF density reached on average 1.9 ± 3.4 fibers/mm in the four biopsied patients with overt DNP. The IENF densities of the controls covered a wide range of values, extending from 4.7 to 26 fibers/mm, the consequence of which further analyses were performed on Log-transformed values of IENF densities.

The morphological appearance of intra-epidermal nerve fibers did not differ much in diabetic subjects compared to healthy controls. Although axonal swellings were observed in some cases their presence was not abundant. As for IENF, the dermal nerve fiber plexus seemed affected in type 2 DM patients as well as the autonomic nerve fibers innervating sweat glands, hair follicles, etc. These fibers were clearly reduced and remainders showed a clumped appearance (data not shown).

Among DM patients, a significant negative correlation was found between the Log-IENF densities versus albuminuria ($p < 0.001$, $R = -0.780$) and to a lesser extent versus the presence and severity of retinopathy, i.e., presence or not of a proliferative retinopathy ($p = 0.020$, $R = -0.499$). No significant correlations were found with other potential clinical or biological covariates including age, height and weight, DM duration, glycated hemoglobin, serum creatinine and vascular complications.

Correlations of IENF density with QST and LEP variables

Results of correlation analyses of IENF densities versus QST and LEP variables are reported in Table 5. No significant correlation ($p > 0.05$) was found between SWME findings and Log-IENF density. There was a trend toward a positive correlation ($p = 0.07$) with SEP-related N120–P240 amplitudes. In contrast, significant correlations were found between Log-IENFd versus WDT (negative) and IENFd versus CDT (positive). These relationships are illustrated as a scatter plot in Fig. 3, where the gray area represents the domain of healthy controls.

The Aδ-related detection rate (RTs < 750 ms) of laser stimuli correlated significantly ($p < 0.01$) and positively with Log-IENFd, the correlation was slightly larger with the small ($R = 0.54$) than with the large laser beam ($R = 0.48$).

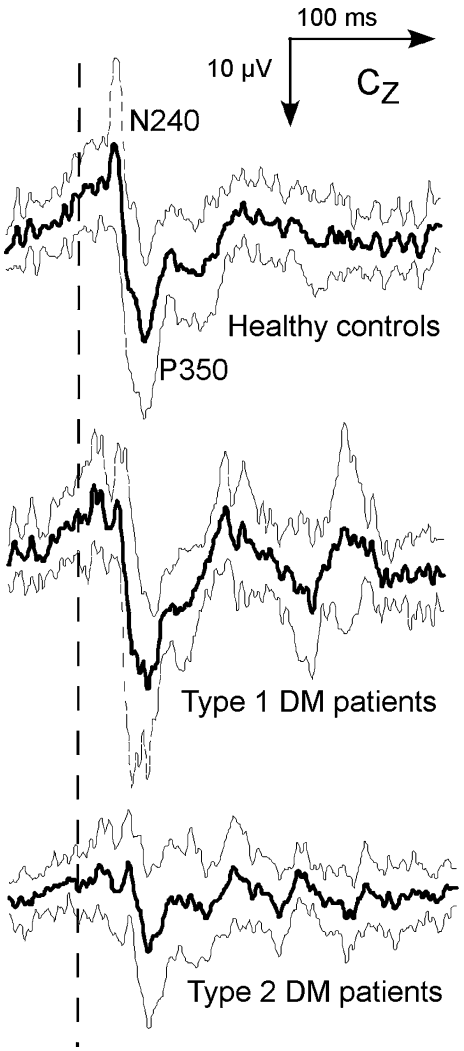

Fig. 1 Grand time-averaged LEPs (±1 SD in *greyish* traces) for the different groups (T1DM, T2DM patients, and controls). T2DM patients had a significantly decreased N240–P350 complex at C_Z compared to healthy controls. The dotted vertical line corresponds to laser stimulus onset

Concerning LEPs, the N240–P350 amplitudes correlated significantly with IENF density, particularly with the large laser beam. In contrast, latencies of LEPs correlated significantly and negatively with IENF density, but only for LEPs with the small laser beam.

Diagnostic performance of functional tests

To compare the different screening or diagnostic methods used in the present work, one must in the first place define a "critical small-fiber loss". Such categorization of IENF densities in either normal or pathological states was needed to perform ROC analyses (binormal method) based on a morphological criterion as reference. To fulfill this purpose, a patient was considered as pertaining to the category named "critical small-fiber loss" when his/her Log-IENF density was ≤ -2 z scores of the normalized Log-IENF density distribution. This criterion was exceeded in 1/10 (10%) T1DM patients, in 3/11 (27%) T2DM patients and in 3/4 (75%) DNP patients. Although not customary in operating characteristics analysis for diagnostic tools, the healthy volunteers were included in the dataset for power reasons.

As shown in Fig. 4, ROC analyses of thermal threshold testing and LEP amplitudes revealed a high overall diagnostic performance in terms of sensitivity, specificity and area under the ROC curve (AUC). The best-performing test, i.e., WDT and LEP N240–P350 amplitude using a small laser beam, was defined with parameters that maximized the AUC. For CDT, the best performances were obtained after selecting a cut-off value of −4.6°C, with sensitivity 76%, specificity 78% and AUC 0.840 ± 0.081 (mean ± SE). With a cut-off value of +7.6 and +13.9°C, respectively, WDT yielded an AUC of 0.915 ± 0.048 (sensitivity 84% and specificity 84%) and HPT yielded an AUC of 0.7616 ± 0.086 (sensitivity 59% and specificity 58%). When stimulating with the large laser beam and using a cut-off value of +15 µV for the N240–P350 amplitude of LEPs, AUC was 0.735 ± 0.168 (sensitivity 74% and specificity 64%). With the small laser beam and a cut-off of +10 µV for the N240–P350 amplitudes, the diagnostic performances were significantly increased ($z = -2.27$, $p = 0.023$), yielding an AUC of 0.924 ± 0.032 (sensitivity 91%, specificity 83%). When AUCs were compared, LEPs diagnostic performance with the small laser beam did not significantly differ from those based on CDT ($z = 0.74$, $p = 0.458$) or WDT ($z = 0.85$, $p = 0{,}394$). Comparison of the AUC of the best-performing test with the others needs confirmation in a future larger dataset.

Discussion

The present study brought additional and new evidence for an asymptomatic stage of SFN that is readily identified using objective CO_2 laser-evoked measurements in a group of patients diagnosed with diabetes (type 1 and type 2) for at least 10 years but without any conventional symptoms and signs of peripheral neuropathy. In this respect, light-touch assessment and SEPs were clearly not contributive. As expected for a length-dependent process for neuropathy [27], functional assessment based on WDT, CDT and LEPs showed that the distal leg seemed to be much more affected than the hand. As a group, T2DM patients were more affected than T1DM patients. Taking the IENF density as reference with a criterion for "critical small-fiber loss" defined as the Log-IENF density at ≤ -2 z scores and

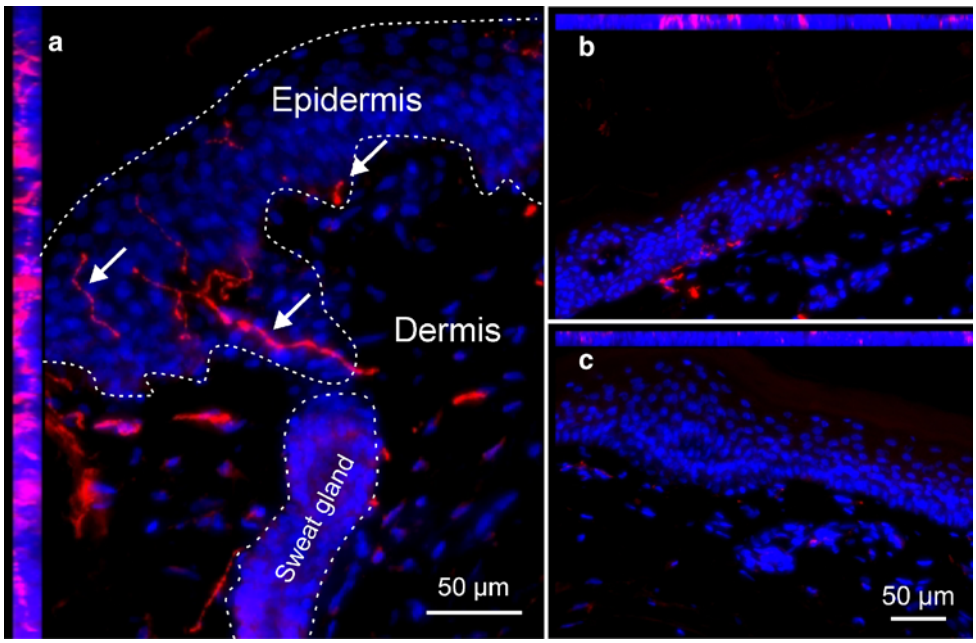

Fig. 2 Representative Z-stack images of PGP9.5. Immunofluorescence in skin epidermis of healthy control (**a**); T2DM patient without SFN (**b**) and patient with overt DNP (**c**). PGP9.5 immunoreactive nerve fibers (*red*) and Hoechst nuclear counterstain (*blue*). The left and upper image edge represents a cut view through the thickness (z-stacks) of the image. *Arrows* indicate IENFs

Table 5 Correlations of Log-transformed IENFd with QST and LEP variables

	Log-IENFd
SWME	
MDT	ns
Thermotest	
CDT	$P < 0.01$, $R = 0.42$
WDT	$P < 0.001$, $R = -0.54$
HPT	ns
LEP (large laser beam)	
N240 latency	ns
P350 latency	ns
N240–P350 amplitude	$P < 0.05$, $R = 0.34$
LEP (small laser beam)	
N240 latency	$P < 0.001$, $R = -0.61$
P350 latency	$P < 0.05$, $R = -0.50$
N240–P350 amplitude	$P < 0.05$, $R = 0.16$

SWME Semmes–Weinstein monofilament examination, *MDT* mechanical detection threshold, *CDT* cool detection threshold, *WDT* warm detection threshold, *HPT* heat pain threshold, *LEP* laser-evoked brain potentials (large or small indicates the stimulus surface area: 20 or 0.8 mm^2 respectively), *IENFd* intraepidermal nerve fiber density

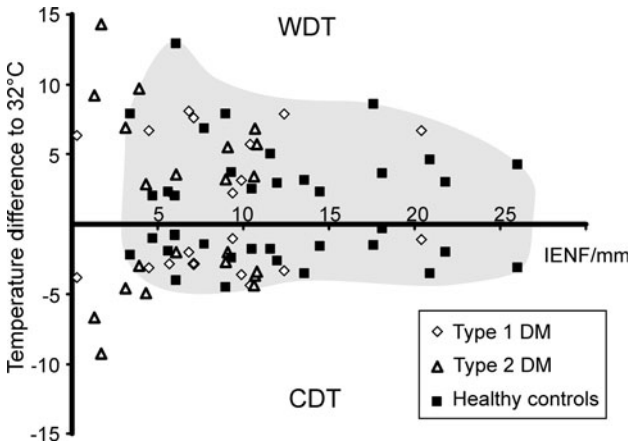

Fig. 3 Bivariate scatterplot with IENF density versus CDT and WDT in the groups of T1DM (*tiles*) and T2DM (*dots*) patients and healthy controls (*squares*). The gray area represents the domain of healthy controls

including the healthy controls in the analysis for power reasons, it appeared that the best diagnostic performance, in terms of sensitivity and specificity, was achieved with the recordings of cortical potentials evoked by intense CO_2 laser stimuli (LEPs) of short duration (ms) applied upon a small surface area (<1 mm^2). However, the results concerning sensitivity/specificity should be put in context of the researchers aims. To derive a very sensitive diagnostic tool for asymptomatic DPN then they could compromise specificity for sensitivity. On the other hand, if the test advocated is used to definitely confirm patients with early DPN (e.g., for recruitment into drug trials) then specificity is paramount. It would be imperative that patients without disease are not included by too sensitive criterion. However, the small sample size and large biological variation of some of the tests used in the study makes this task difficult.

A reduction in IENF density of skin biopsy samples in T1DM and T2DM patients with asymptomatic neuropathy was already observed by Umapathi et al. [7] and Loseth et al. [8]. The prevalence of small fiber loss in T2DM over T1DM patients with asymptomatic neuropathy was not yet reported. Although no variables correlated with age in the present study, one can speculate that this difference may

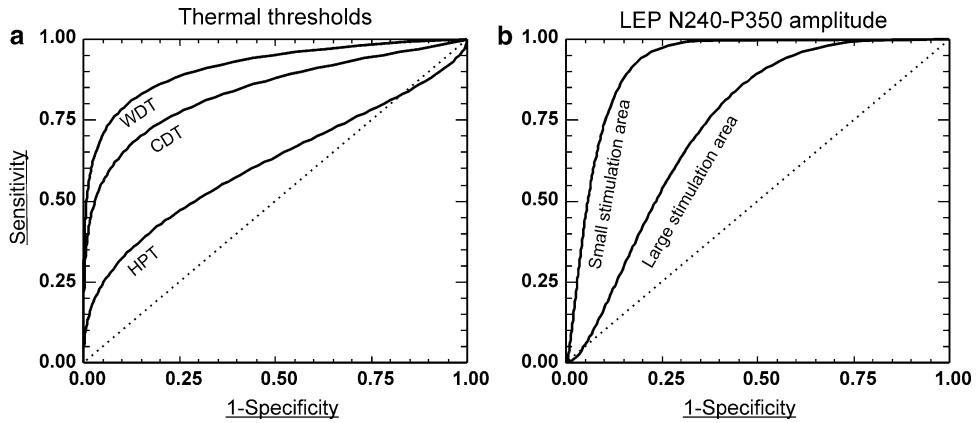

Fig. 4 ROC analysis of thermal threshold assessments (thermotest) (**a**) and laser-evoked potentials (LEPs) with small and large stimulus surface areas (**b**) at distal leg in 18 volunteers and in 25 patients, of whom seven fulfilled the criterion of "critical small-fiber loss" defined as a Log-IENF density ≤ -2 z scores. Area under the ROC curve (AUC) for CDT = 0.840; for WDT = 0.915; for HPT = 0.761. AUC for N240–P350 amplitudes using a large laser beam = 0.735 and using a small laser beam = 0.924

nevertheless result from a difference in age between the two groups, remembering that it was a direct consequence of our selection criteria. Indeed, IENF densities [28–30], temperature perception thresholds measured with the method of limits [31] and LEP amplitudes [32, 33] are all age-dependent. Yet, correction for age was not introduced, as these variables were, according to the above mentioned studies, relatively homogeneous within the range of 30–59 years of age. For instance, Periquet et al. [34], using also the immunofluorescence technique, reported that IENF density was significantly reduced only for healthy subjects above 60 years of age. Another confounding factor is the fact that in T2DM patients it is difficult to determine the duration of disease, as it may be present well before a diagnosis of DM is made and even at the stage of impaired glucose tolerance [35–37]. These may have confounded the relationship between age or DM duration and IENF density, sensory thresholds or LEP amplitudes.

There was no significant correlation between distal IENFd of diabetic patients and age, height, weight, duration of diabetes and level of glycemic control, although these factors are well-established risk factors for overt DNP [38, 39]. Our observations are in concordance with recent studies of Shun et al. [40] and Umapathi et al. [7]. In other studies [41], no correlation was observed between duration of diabetes or baseline HbA_{1c} and the reduction in regenerative capacity of epidermal nerve fiber after topical capsaicin in diabetic patients.

The absence of significant correlation between DM duration and distal IENFd in the present work may be related to our inclusion criteria and small sample size, but also to the variable delay in the definite diagnosis of diabetes as mentioned here above. Differences in the physiopathology and history of nerve lesions at early and later stages of DNP had already been suggested by Umapathi and coworkers [7] and may also explain why correlations are observed between certain risk factors and distal IENF loss in early or well-established DNP.

Although significant relationships are observed between IENF densities and performances on functional tests (including QST), the strength, as expressed by the coefficient of determination, is in general rather modest. However, these weak relationships should not come as a surprise, since IENF density encompasses all types of nerve terminals and only a fraction of these are involved during functional testing. The more, the morphological presence of nerve endings does not entail their functional integrity (e.g., defective transduction processes and/or impaired nerve conduction velocity). Indeed, Beiswenger et al. [42] showed that, in diabetic rodents, "functional changes relating to sensory processing precede structural changes in SFN or current microscopic techniques are not sufficiently sensitive to detect early structural damage". Ragé et al. [15] reported a similar mismatch between morphological and functional changes.

Another critical factor for the diagnostic performance of LEPs in SFN seemed to be the surface area exposed to the CO_2 laser stimulus. It is reasonable to assume that the quantity of nociceptive information send to the central nervous system is dependent on the product of Aδ-nociceptors IENFd times the surface area of skin exposed to the intense laser beam, allowing for spatial recruitment. To permit detection of the stimulus, the quantity of sensory information conveyed to the CNS must exceed some sensorial threshold. As a consequence, the lower the IENFd and the smaller the surface area, the more difficult it is to reach such threshold. This combination is likely to contribute to the superiority of the small laser beam in detecting asymptomatic DNP, as it was also shown to be the case with small versus large thermodes [43]. The

optimal laser stimulus surface area for diagnostic purposes remains to be determined by further research.

Conclusions

Present investigations, although exploratory in nature given the small number of diabetic patients in each group, confirm that intraepidermal nerve fibers are affected in patients with long-standing DM even in the complete absence of conventional symptoms, signs and routine laboratory tests for small-fiber neuropathy. This asymptomatic stage of SFN seems to prevail in T2DM over T1DM patients despite similar estimates of disease duration and glycemic control. This has to be confirmed in a larger cohort of patients.

A most suitable method for assessing early neuropathy should be noninvasive quantitative, sensitive and specific. LEPs (assessing Aδ pathways) and QST seem to achieve some of these goals as they were equally performing in terms of sensitivity and specificity regarding the diagnosis of SFN in DM. However, the main advantage of LEPs over WDT is that it relies less on the active cooperation of the patients and by consequence is less influenced by perceptual or cognitive factors (e.g., decision criterion or psychological bias). The main disadvantage of LEPs is that few laboratories are equipped with laser stimulators as they are not at present readily available on the market and are rather expensive. Besides being non-invasive and quantitative, a most suitable method should also be sensitive enough to detect meaningful changes over time in response to therapeutic interventions. CO_2 laser stimulation seems to fulfill most of these requirements, but not all. Indeed its potentiality for detecting changes over time was recently confirmed [15], although its capacity to prospectively measure responses following therapeutic intervention remains to be determined by further research.

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Conflict of interest The authors declare that they have no competing interests, but MT, JS and TM are employees of Janssen Pharmaceutica N.V.

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