Original Article

Surrogate Markers of Small Fiber Damage in Human Diabetic Neuropathy

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Surrogate markers of diabetic neuropathy are being actively sought to facilitate the diagnosis, measure the progression, and assess the benefits of therapeutic intervention in patients with diabetic neuropathy. We have quantified small nerve fiber pathological changes using the technique of intraepidermal nerve fiber (IENF) assessment and the novel in vivo technique of corneal confocal microscopy (CCM). Fifty-four diabetic patients stratified for neuropathy, using neurological evaluation, neurophysiology, and quantitative sensory testing, and 15 control subjects were studied. They underwent a punch skin biopsy to quantify IENFs and CCM to quantify corneal nerve fibers. IENF density (IENFD), branch density, and branch length showed a progressive reduction with increasing severity of neuropathy, which was significant in patients with mild, moderate, and severe neuropathy. CCM also showed a progressive reduction in corneal nerve fiber density (CNFD) and branch density, but the latter was significantly reduced even in diabetic patients without neuropathy. Both IENFD and CNFD correlated significantly with cold detection and heat as pain thresholds. Intraepidermal and corneal nerve fiber lengths were reduced in patients with painful compared with painless diabetic neuropathy. Both IENF and CCM assessment accurately quantify small nerve fiber damage in diabetic patients. However, CCM quantifies small fiber damage rapidly and noninvasively and detects earlier stages of nerve damage compared with IENF pathology. This may make it an ideal technique to accurately diagnose and assess progression of human diabetic neuropathy. *Diabetes* 56:2148–2154, 2007

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CCM, corneal confocal microscopy; CDT, cooling detection threshold; CNBD, corneal nerve branch density; CNF, corneal nerve fiber; CNFD, corneal nerve fiber density; CNFL, corneal nerve fiber length; DB-HRV, deep breathing heart rate variability; HP-VAS 0.5, 5.0, and 0.5–5.0, heat-as-pain visual analog score minimal threshold, intermediate threshold, and differential threshold expressing pain tolerance, respectively; IENF, intraepidermal nerve fiber; IENFBD, intraepidermal nerve fiber branch density; IENFD, intraepidermal nerve fiber density; IENFL, intraepidermal nerve fiber length; NDS, neuropathy disability score; PNAP, peroneal nerve amplitude potential; PNCV, peroneal nerve conduction velocity; PNFL, peroneal nerve F-wave latency; PNOL, peroneal nerve onset latency; QST, quantitative sensory testing; SNAP, sural nerve amplitude potential; SNCV, sural nerve conduction velocity; SNOL, sural nerve onset latency; TNAP, tibial nerve amplitude potential; TNCV, tibial nerve conduction velocity; TNFL, tibial nerve F-wave latency; TNOL, tibial nerve onset latency.

Somatic polyneuropathy is one of the most common long-term complications of diabetes and is the main initiating factor for foot ulceration and lower extremity amputation (1,2). As 80% of amputations are preceded by foot ulceration, an effective means of detecting and treating peripheral neuropathy would have a major medical, social, and economic impact. The attributable costs, 2 years after a new foot ulcer in a middle-aged male diabetic patient, are ~$28,000 (3), and strategies that reduce amputation by earlier detection of neuropathy may potentially save $2–3 million over 3 years (4). With the exception of optimal glycemic control, there are currently no U.S. Food and Drug Administration–licensed treatments that prevent, slow, or arrest the development of neuropathy (1).

Although electrophysiology, quantitative sensory testing (QST), and assessment of neurological disability are advocated to define neuropathic severity (1), they have limitations when they are used to define therapeutic efficacy in clinical intervention trials (5). Only biopsy of the sural nerve (6,7) and skin biopsy (8,9) permit a direct examination of nerve fiber damage and repair. However, both are invasive procedures, and assessment of therapeutic efficacy in clinical trials requires repeat biopsy. The earliest nerve fibers to undergo damage (8,10,11) and subsequent repair (12) are the small unmyelinated fibers, as evidenced by studies in patients with impaired glucose tolerance and diabetic patients with minimal neuropathy (7).

Regulatory authorities favor clinically relevant surrogate end points. The development of a surrogate end point for diabetic neuropathy is vital to accurately define at-risk patients, anticipate deterioration, and, in particular, assess the efficacy of new therapies. Thus, an alternative surrogate end point for neuropathy has been proposed using punch skin biopsies, which directly quantify pathological changes in C nerve fibers. Reliable and reproducible processing and measurement techniques have been established for assessing intraepidermal nerve fiber (IENF) pathology, and normative ranges have been published (13). This technique has also been shown to be useful in identifying patients with small fiber neuropathy from a variety of causes (14,15), including those with painful neuropathy due to impaired glucose tolerance (8). However, the disadvantage of the skin biopsy technique is that it is an invasive procedure, which requires a dedicated

laboratory for processing and quantifying IENF pathological changes.

As an alternative, in vivo imaging of small fibers may be achieved in a totally noninvasive way using corneal confocal microscopy (CCM). Several authors have already investigated the cornea in vivo using CCM (16–18). One may argue that corneal innervation has little relevance to somatic innervation of the lower limbs and therefore has limited application as a surrogate marker for peripheral neuropathy in diabetic patients. However, of clinical relevance is the loss of corneal innervation that has been shown to lead to neurotrophic keratopathy and corneal ulceration in diabetic patients (16). Furthermore, CCM shows a reduction in the number of corneal nerve fiber (CNF) bundles that correlates significantly with a reduction in corneal sensitivity and most importantly with the severity of somatic neuropathy, evaluated using the Michigan Neuropathy Screening Instrument (19). We have refined and significantly improved the quantification of corneal C fibers in normal subjects (17) and also demonstrated that CCM is a promising means to quantify the severity of human diabetic neuropathy (20–22).

Thus, given the need to establish novel surrogate markers of human diabetic neuropathy, we have compared the ability of CCM and skin biopsy to quantify small nerve fiber pathological changes to diagnose and assess progression of human diabetic neuropathy.

RESEARCH DESIGN AND METHODS

Patients attending the Manchester Diabetes Centre and nondiabetic volunteers who underwent neurological examination and electrophysiology were invited to participate unless they had a neuropathy of another cause or absent pedal pulses or wore contact lenses.

Assessment of neuropathy. Symptoms were assessed by using the diabetic neuropathy symptom score (23) and painful symptoms were quantified with a visual analog score ranging between 0 and 10. Neurological deficits were assessed using the neuropathy disability score (NDS) (24), quantitative sensory tests (cooling detection threshold [CDT] and heat-as-pain visual analog score minimal threshold, intermediate threshold, and differential threshold expressing pain tolerance (HP-VAS 0.5, 5.0, and 0.5–5.0, respectively) (1), autonomic function (deep breathing heart rate variability [DB-HRV]) with a CASE IV system (WR Medical Electronics, Stillwater, MN), and electrodiagnostic studies with a Dantec "Keypoint" system (Dantec Dynamics, Bristol, U.K.) equipped with a DISA temperature regulator to keep limb temperature constantly between 32 and 35°C. The NDS included tuning fork vibration perception, pin prick perception, and temperature perception as well as the presence or absence of ankle reflexes and ranged between 0 and 10; neuropathy was diagnosed if the NDS was ≥3 of 10. Patients were deemed to have mild neuropathy when the NDS was between 3 and 5, moderate neuropathy when the NDS was between 6 and 8, and severe neuropathy when the NDS was 9 or 10. Electrophysiological measures included peroneal (PNOL), sural (SNOL), and tibial (TNOL) nerve onset latencies, amplitude potential (PNAP, SNAP, and TNAP, respectively), conduction velocity (PNCV, SNCV, and TNCV, respectively), and peroneal (PNFL) and tibial (TNFL) F-waves. For small fiber evaluation we adopted the CASE IV normality range. For thermal detection thresholds the 95th percentile and for autonomic function (DB-HRV) the 5th percentile was considered abnormal. We used DB-HRV, CDT, HP-VAS 0.5, and HP-VAS 5.0 to stratify the severity of small fiber dysfunction, and we chose the following criteria: normal (all tests normal), mild (one abnormal test), moderate (two abnormal tests), and severe (≥3 abnormal tests).

CCM. Patients were examined with a Confoscan corneal confocal microscope model P4 (Tomey, Erlangen, Germany). Each eye being examined was anesthetized with 1 drop of 0.4% benoxinate hydrochloride (oxybuprocaine hydrochloride; Minims). The objective lens of the confocal microscope was disinfected (isopropyl alcohol 70% vol/vol swabs). A large drop of Viscotears liquid gel (carbomer 940; Ciba Vision Ophthalmics) was applied onto the tip of the lens and advanced slowly forward until the gel touched the cornea, allowing optical but not physical contact between the objective lens and corneal epithelium during the examination. One eye of each subject was selected at random for examination. Several scans of the entire depth of the cornea were recorded by turning the fine focus of the objective lens backward and forward for ~2 min to acquire satisfactory images of all corneal layers, providing en face two-dimensional images with a lateral resolution of ~1–2 μm and final image size of 768 × 576 pixels. On average, three to five high-quality images of Bowman's layer were used for investigation in all patients and control subjects. This layer is of particular relevance for defining neuropathic changes because it is the location of the main nerve plexus that supplies the overlying corneal epithelium. The investigator (M.T.) who examined the cornea with the confocal microscope and who obtained the morphometric measurements of the images was masked with respect to the severity of neuropathy of the diabetic patients. By using the images collected, the following three parameters were quantified to define CNF damage and repair: *1*) corneal nerve fiber density (CNFD), the total number of major nerves per square millimeter of corneal tissue; *2*) corneal nerve fiber length (CNFL), the total length of all nerve fibers and branches (millimeters per square millimeter) of corneal tissue; and *3*) corneal nerve branch density (CNBD), the number of branches emanating from major nerve trunks per square millimeter of corneal tissue. Measures *1* and *3* were determined using morphometric software incorporated within the Tomey instrument. Measure *2* was determined using third-party image analysis software (Scion Image for Windows; Scion, Frederick, MD).

To estimate the error in measuring CNFD, CNFL, and CNBD, we acquired images and determined each of these parameters in 15 subjects on two occasions separated by at least 48 h. The coefficients of variation for these parameters were 12% for CNFD, 9% for CNFL, and 24% for CNBD.

Immunohistochemistry. A 3-mm punch skin biopsy was taken from the dorsum of the foot, ~2 cm above the second metatarsal head, with the use of 1% lidocaine local anesthesia. The biopsy site was closed using Steri-strips, and the specimen was immediately fixed in PBS-buffered 4% paraformaldehyde. After 18–24 h, it was rinsed in Tris-buffered saline and soaked in 33% sucrose (2–4 h) for cryoprotection. It was then embedded in OCT (optimum cutting temperature embedding compound), rapidly frozen in liquid nitrogen, and cut into 50-μm sections using a cryostat (model OTF; Bright Instruments, Huntington, U.K.). Four floating sections per subject were subjected to melanin bleaching (0.25% $KMnO_4$ for 15 min followed by 5% oxalic acid for 3 min), a 4-h protein block with a Tris-buffered saline solution of 5% normal swine serum, 0.5% powdered milk, and 1% Triton X-100, and overnight incubation with 1:1,200 Biogenesis polyclonal rabbit anti-human PGP9.5 antibody (Serotec, Oxford, U.K.). Biotinylated swine anti-rabbit secondary antibody (1:300; DakoCytomation, Ely, U.K.) was then applied for 1 h; sections were quenched with 1% H_2O_2 in 30% MeOH-PBS (30 min) before a 1-h incubation with 1:500 horseradish peroxidase–Streptavidin (Vector Laboratories, Peterborough, U.K.). Nerve fibers were demonstrated using 3,3′-diaminobenzidine chromogen (Sigma-Aldrich, Manchester, U.K.). Sections were mildly counterstained with eosin to better localize the basement membrane and hence the nerve fibers passing through the basement membrane. Negative controls consisted of replacing the anti-PGP9.5 antibody with rabbit immunoglobulin (DakoCytomation) at a concentration matching that of the primary antibody and showed no immunostaining. Basement membrane length (micrometers), average nerve fiber length (micrometers), and number of branches were measured using computer image analysis (Nikon digital camera and Leica QWin Standard V2.4 program). Intraepidermal nerve fiber linear density (IENFD), i.e., the number of fibers per millimeter of basement membrane, was expressed as number per millimeter, intraepidermal nerve fiber branch density (IENFBD), the number of branches in a selected area of epidermis, was expressed as number per square millimeter, and intraepidermal nerve fiber length (IENFL), the average length of epidermal nerve fibers in a given specimen, was expressed as micrometers.

Statistical analysis. Statistical analysis was performed using SPSS 14.0 for Windows (SPSS, Chicago, IL). The data are presented as means ± SE. Skewed data underwent logarithmic transformation before ANOVA and data without homogenous variance underwent nonparametric ANOVA (Kruskal-Wallis). Post hoc analysis for multiple comparisons between groups was performed using the Tukey test (parametric) or Dunnett T3 test (nonparametric). Correlations were performed using the Spearman rank test and expressed as a coefficient (r_s) with level of significance. For comparison between patients with and without neuropathic pain, the unpaired t test or the Mann-Whitney test was used. $P < 0.05$ was considered statistically significant.

RESULTS

Neuropathic severity. Table 1 shows the clinical and demographic data with measures of clinical neuropathy in control subjects and diabetic patients matched for age, duration of diabetes, and glycemic control. Fifty-four diabetic patients (no [$n = 10$], mild [$n = 18$], moderate [$n = 15$], and severe [$n = 11$] neuropathy) were compared

TABLE 1
Demographics and clinical neuropathy evaluation in control subjects and diabetic patients

	Control subjects	No neuropathy	Mild	Moderate	Severe
n	15	10	18	15	11
Age (years)	55.0 ± 4.78	53.5 ± 3.23	58.6 ± 3.07	57.5 ± 2.79	61.9 ± 1.89
Duration (years)	0	16.7 ± 4.44	18.4 ± 2.64	25.2 ± 3.28	20.4 ± 3.41
A1C (%)	—	7.16 ± 0.40	7.85 ± 0.38	8.30 ± 0.41	7.88 ± 0.27
Type 1/type 2	—	3/7	6/12	5/10	2/9
Sex (male/female)*	6/9	6/4	15/3	11/4	10/1§
SNOL (ms)†	2.43 ± 0.12	2.63 ± 0.14	3.03 ± 0.13§	3.66 ± 0.48	3.03 ± 0.36
SNAP (μV)¶	20.25 ± 3.76	14.44 ± 2.36	5.74 ± 0.79§	5.65 ± 1.19§	2.65 ± 0.76§
SNCV (m/s)†	46.52 ± 1.87	42.99 ± 1.42	41.20 ± 1.28	38.19 ± 2.03§	35.18 ± 2.14§
PNOL(ms)†	4.09 ± 0.28	4.72 ± 0.31	4.62 ± 0.18	5.42 ± 0.27§	6.41 ± 0.88
PNAP (mV)‡	4.27 ± 0.64	4.26 ± 0.59	2.91 ± 0.43	1.54 ± 0.22§	1.10 ± 0.75§
PNCV (m/s)‡	45.71 ± 0.99	44.15 ± 0.79	40.82 ± 0.84§	36.88 ± 1.41§	32.37 ± 2.48§
PNFL (ms)‡	47.36 ± 1.58	53.26 ± 1.82	57.43 ± 1.80§	57.07 ± 2.17§	63.37 ± 5.73§
TNOL (ms)‡	4.76 ± 0.23	5.11 ± 0.42	5.67 ± 0.22	6.89 ± 0.43§	8.29 ± 0.62§
TNAP (mV)‡	8.70 ± 1.02	6.00 ± 0.72	4.82 ± 0.57§	3.46 ± 0.99§	0.83 ± 0.24§
TNCV (m/s)‡	45.94 ± 0.98	40.24 ± 1.31§	38.67 ± 0.87§	36.06 ± 0.78§	31.18 ± 3.82§
TNFL (ms)‡	48.59 ± 1.21	55.86 ± 1.95	57.73 ± 1.85§	58.94 ± 2.16§	72.23 ± 4.72§
CDT (percentile)‡	—	66.90 ± 8.99	74.22 ± 7.12	96.67 ± 0.59‖	98.00 ± 0.91‖
HP-VAS 0.5 (percentile)*	—	41.38 ± 8.87	34.06 ± 8.49	52.20 ± 9.68	81.20 ± 11.20¶
HP-VAS 5.0 (percentile)*	—	46.13 ± 13.19	34.67 ± 7.87	48.33 ± 9.23	75.80 ± 9.40¶
HP-VAS 0.5–5.0 (percentile)	—	55.00 ± 11.62	40.00 ± 7.81	52.85 ± 9.19	44.17 ± 18.10
DB-HRV (percentile)‡	—	82.88 ± 4.97	34.00 ± 7.91‖	14.00 ± 5.26‖	14.38 ± 8.67‖

Data are means ± SE for age, duration of diabetes, A1C, and measures of electrophysiology and QSTs in diabetic patients and control subjects. Statistically significant difference using ANOVA: *$P < 0.05$; †$P < 0.01$; ‡$P < 0.001$. §Post hoc results significantly different from control subjects. ‖Post hoc results significantly different from the group with no neuropathy. ¶Post hoc results significantly different from the group with mild neuropathy.

with 15 control subjects using ANOVA. SNOL increased ($P < 0.01$) and SNAP ($P < 0.001$) and SNCV ($P < 0.01$) decreased significantly with increasing neuropathic severity. Similarly, PNOL and TNOL (respectively, $P < 0.01$ and $P < 0.001$) and PNFL and TNFL (respectively, $P < 0.001$ and $P < 0.001$) increased, and PNAP and TNAP (respectively, $P < 0.001$ and $P < 0.001$) and PNCV and TNCV (respectively, $P < 0.001$ and $P < 0.001$) decreased with increasing neuropathic severity. The CDT ($P < 0.001$) and minimal ($P < 0.05$) and intermediate ($P < 0.05$) pain thresholds increased with increasing neuropathic severity. Autonomic function (DB-HRV) demonstrated a progressive decrease with increasing neuropathic severity ($P < 0.001$).

IENF pathology. IENF loss was demonstrated in diabetic patients compared with control subjects (Table 2 and Fig. 1*a* and *b*). IENFD (ANOVA $P < 0.0001$) showed a progressive reduction with increasing neuropathic severity, which was significant in diabetic patients with mild ($P < 0.05$), moderate ($P < 0.05$), and severe ($P < 0.01$) neuropathy compared with control subjects using post hoc analysis (Fig. 2*a*). IENFBD (ANOVA $P < 0.001$) also showed a progressive reduction with increasing neuropathic severity, which was significant for mild (post hoc $P < 0.05$), moderate ($P < 0.01$), and severe ($P < 0.01$) neuropathy compared with that in control subjects (Fig. 2*b*). IENFL (ANOVA $P < 0.05$) only showed a significant reduction in patients with severe neuropathy (post hoc $P < 0.05$) compared with control subjects (Fig. 2*c*).

CCM. CNF loss was also demonstrated in diabetic patients compared with control subjects (Table 2 and Fig. 3*a* and *b*). CNFD (ANOVA $P < 0.001$) showed a progressive reduction with increasing neuropathic severity, which was significant in patients with mild ($P < 0.01$), moderate ($P < 0.05$), and severe ($P < 0.01$) neuropathy compared with control subjects using post hoc analysis (Fig. 4*a*). CNFBD was significantly reduced (ANOVA $P < 0.001$) with increasing neuropathic severity, but the reduction was significant even in patients without neuropathy ($P < 0.001$) and in those with mild, moderate, and severe ($P < 0.001$) neuropathy compared with control subjects using post hoc

TABLE 2
CCM compared with IENF morphology in control subjects and diabetic patients stratified into different clinical severities of neuropathy according to NDS

	Control subjects	No neuropathy	Mild	Moderate	Severe
CNFD (no/mm^2)*	43.20 ± 5.05	29.05 ± 3.07	22.95 ± 2.39§	22.59 ± 4.05§	20.13 ± 3.14§
IENFD (no/mm)*	11.21 ± 0.84	7.72 ± 1.28	5.56 ± 0.86§	5.84 ± 0.94§	2.54 ± 0.76§‖
CNBD (no/mm^2)*	27.39 ± 3.31	8.10 ± 1.80†	7.91 ± 1.70§	7.71 ± 2.24§	5.55 ± 2.19§
IENFBD (no/mm^2)†	139.66 ± 23.42	57.55 ± 14.41	39.92 ± 10.53§	35.48 ± 10.81§	13.47 ± 5.79§
CNFL (mm/mm^2)	6.14 ± 1.22	4.59 ± 0.92	3.87 ± 0.62	3.64 ± 0.56	3.69 ± 0.44
IENFL (μm)‡	42.10 ± 4.31	38.00 ± 3.32	31.47 ± 2.46	29.59 ± 4.53	22.61 ± 7.12§

Data are means ± SE in diabetic patients and control subjects. Statistically significant difference using ANOVA: *$P < 0.001$; †$P < 0.01$; ‡$P < 0.05$. §Post hoc results significantly different from control subjects. ‖Post hoc results significantly different from the group with no neuropathy.

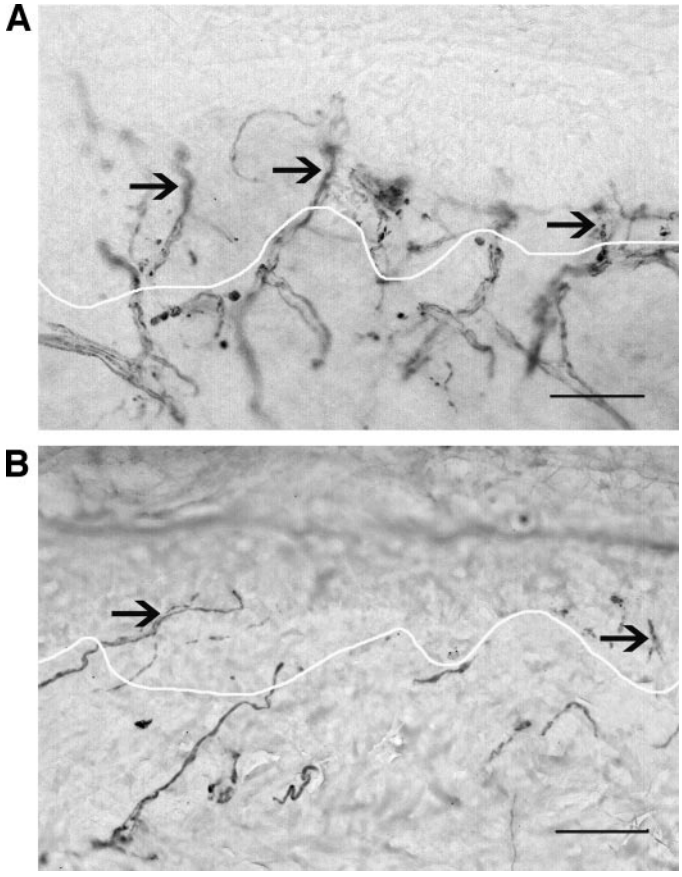

FIG. 1. Skin sections (50-μm-thick) immunostained for PGP9.5 from a control subject (*a*) with numerous dermal and intraepidermal nerve fibers (stained dark gray to black) compared with a diabetic patient with severe neuropathy (*b*) with depletion of dermal and particularly IENFs. Sections were not counterstained for image clarity, all visible staining indicates nerve fibers (bar, 100 μm). The line illustrates the position of dermal-epidermal junction. The large black arrows indicate the IENFs.

analysis (Fig. 4*b*). CNFL did not differ between diabetic patients and control subjects (ANOVA NS) (Fig. 4*c*).

Relationship between IENF and corneal nerve pathology with neuropathic severity. IENFD correlated with CNFD ($r_s = 0.385$, $P = 0.001$) and IENFBD correlated with CNFBD ($r_s = 0.416$, $P = 0.001$). IENFL did not correlate with CNFL.

Both IENFD and IENFBD correlated with NDS, electrophysiology of the peroneal, tibial, and sural nerves, and thermal perception thresholds for cold and heat (Table 4). IENFL correlated with NDS, electrophysiology of peroneal

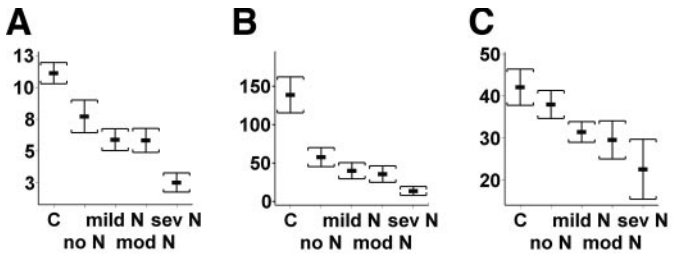

FIG. 2. IENF (number per millimeter), IENFBD (number/square millimeter) and intraepidermal nerve fiber length (μm) in control subjects (C) and diabetic patients with no neuropathy (no N), mild neuropathy (mild N), moderate neuropathy (mod N), and severe neuropathy (sev N). For each group, horizontal bars illustrate the mean and vertical bars illustrate the SE.

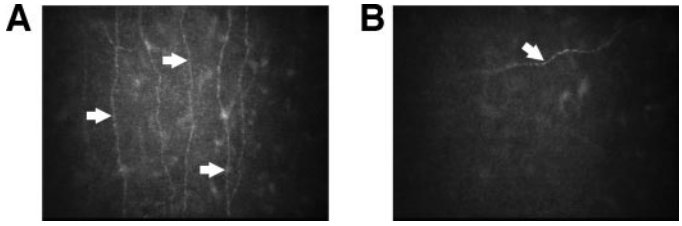

FIG. 3. Frame from CCM scan, original magnification ×700. *a*: Five fibers and two major branches are present in a control subject; *b*: this is reduced to one fiber with no branches in a diabetic patient with severe neuropathy. The large white arrows indicate nerve fibers.

and sural nerves, and cold perception threshold only (Table 4). CCM demonstrated fewer correlations but CNFD correlated with NDS, thermal perception, and DB-HRV and CNFBD correlated with SNOL. There was no correlation between CNFL and any measure of neuropathic severity (Table 4).

Small fiber deficits. As both CNF and IENF pathological changes assess small fiber damage, we specifically assessed changes in CNF and IENF pathology with the degree of small fiber dysfunction. Subjects were stratified for the severity of small fiber deficits in accordance with the number of abnormal test results from CDT, HP-VAS 0.5, HP-VAS 5.0, and DB-HRV. Of the patients, 30% had normal small fiber function, 28% had mild dysfunction, 25% had moderate dysfunction, and 17% had severe dysfunction.

IENF pathology. Post hoc analysis of IENFD demonstrated a reduction in diabetic patients even without small fiber dysfunction and also in patients with mild, moderate, and severe small fiber dysfunction ($P < 0.001$ for all) compared with control subjects (Table 3). IENFBD was also significantly reduced in diabetic patients without ($P < 0.05$) and with mild and severe ($P < 0.01$) dysfunction compared with control subjects. IENFL did not differ between groups (NS).

CCM. CNFD was significantly reduced in diabetic patients without ($P < 0.05$), and with mild ($P < 0.05$) and severe ($P < 0.01$) small fiber dysfunction using post hoc analysis (Table 3). With post hoc analysis, CNFBD was reduced in diabetic patients without ($P < 0.01$) and with mild ($P < 0.01$), moderate ($P < 0.01$), and severe ($P < 0.001$) small fiber dysfunction compared with control subjects. CNFL did not differ significantly between groups (NS).

Relationship between small fiber function and structure. As might be expected with regard to the relationship between small fiber function and structure (Table 4), the

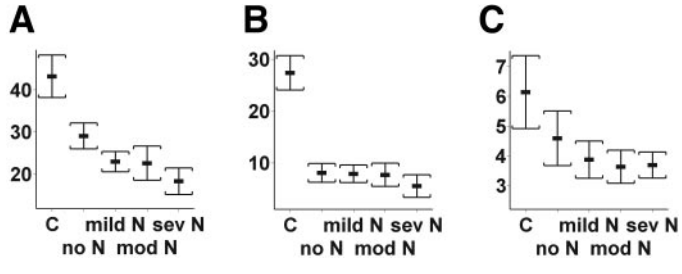

FIG. 4. CCM nerve fiber density (number per square millimeter), nerve fiber branch density (number per square millimeter), and nerve fiber length (millimeter per square millimeter) in control subjects (C) and diabetic patients with no neuropathy (no N), mild neuropathy (mild N), moderate neuropathy (mod N), and severe neuropathy (sev N). For each group, horizontal bars illustrate the mean and vertical bars illustrate the SE.

TABLE 3
CCM compared with IENF pathology in diabetic patients stratified according to severity of small fiber dysfunction

	Control subjects	None	Mild	Moderate	Severe
CNFD (no/mm^2)*	43.20 ± 5.05	24.88 ± 2.41†	22.13 ± 3.05†	26.71 ± 4.76	16.20 ± 1.64†
IENFD (no/mm)*	11.21 ± 0.84	7.22 ± 1.04	5.75 ± 0.85†	5.12 ± 1.16†	3.20 ± 0.77†,‡
CNBD (no/mm^2)*	27.39 ± 3.31	6.87 ± 1.60†	8.33 ± 2.07†	9.26 ± 2.63†	4.63 ± 1.49†
IENFBD (no/mm^2)*	139.66 ± 23.42	44.99 ± 8.93†	34.47 ± 9.52†	40.38 ± 15.98†	27.19 ± 6.56†
CNFL (mm/mm^2)	6.14 ± 1.22	3.97 ± 0.80	3.48 ± 0.47	4.61 ± 0.64	3.36 ± 0.44
IENFL (μm)	42.10 ± 4.31	32.64 ± 2.78	30.21 ± 3.98	24.96 ± 4.47	34.84 ± 8.39

Data are means ± SE for patients were stratified according to the severity of small fiber dysfunction which included CDT, HP-VAS 0.5, HP-VAS 5.0, DB-HRV. Statistically significant difference using ANOVA: *$P < 0.001$. †Post hoc results significantly different from control subjects. ‡Post hoc results significantly different from patients with no neuropathy.

CDT correlated inversely with IENFD ($r_s = -0.466$, $P < 0.001$) and IENFBD ($r_s = -0.408$, $P < 0.01$). The HP-VAS 0.5 also correlated with IENFD ($r_s = -0.311$, $P < 0.05$). The CDT showed an inverse correlation with CNFD ($r_s = -0.399$, $P < 0.01$) but not with CNFBD (NS). The HP-VAS 0.5 correlated inversely with CNFD ($r_s = -0.291$, $P < 0.05$). Heart rate variability correlated with CNFD ($r_s = 0.348$, $P < 0.05$) but not IENFD (NS).

Relationship to painful symptoms. Of the patients, 38 (72%) had a positive diabetic neuropathy symptom score (≥1 of 4). Corneal and intraepidermal nerve fiber pathological changes were compared between diabetic patients stratified into those with (visual analog score ≥3 of 10) ($n = 28$) and without ($n = 26$) painful neuropathy. There was no significant difference in IENFD (painful 4.8 ± 0.7 vs. painless 6.3 ± 0.7, NS) and IENFBD (painful 27.5 ± 5.7 vs. painless 46.6 ± 9.4, NS). IENFL was significantly reduced in those with painful (25.3 ± 2.8) compared with painless (35.9 ± 3.3) neuropathy ($P < 0.05$). There was no significant difference for CNFD (painful 23.0 ± 2.3 vs. painless 23.1 ± 2.4, NS) and CNBD (painful 6.5 ± 1.1 vs. painless 8.52 ± 1.7, NS), but

TABLE 4
Relationship between IENF and CNF morphology and measures of neuropathic severity

	IENFD (no/mm)	IENFBD (no/mm^2)	IENFL (μm)	CNFD (no/mm^2)	CNFBD (no/mm^2)	CNFL (mm/mm^2)
NDS (0–10)	**−0.425**	**−0.376**	**−0.343**	**−0.299**	−0.107	−0.088
	0.001	**0.006**	**0.012**	**0.028**	NS	NS
SNOL (ms)	0.011	0.092	0.086	−0.003	**0.459**	0.056
	NS	NS	NS	NS	**0.002**	NS
SNAP (μV)	**0.351**	**0.394**	**0.295**	0.176	−0.114	−0.018
	0.015	**0.007**	**0.047**	NS	NS	NS
SNCV (m/s)	0.246	**0.286**	**0.333**	0.176	−0.075	0.083
	NS	**0.054**	**0.024**	NS	NS	NS
PNOL (ms)	−0.147	**−0.340**	−0.259	−0.035	0.072	−0.070
	NS	**0.018**	NS	NS	NS	NS
PNAP (mV)	0.242	0.219	**0.315**	0.084	−0.072	−0.070
	NS	NS	**0.027**	NS	NS	NS
PNCV (m/s)	**0.406**	**0.391**	**0.511**	0.250	−0.200	0.181
	0.003	**0.005**	**<0.001**	NS	NS	NS
PNFL (ms)	**−0.364**	−0.160	−0.029	0.016	0.272	−0.130
	0.032	NS	NS	NS	NS	NS
TNOL (ms)	**−0.406**	**−0.324**	−0.242	−0.144	0.130	−0.001
	0.004	**0.025**	NS	NS	NS	NS
TNAP (mV)	0.202	0.269	0.196	0.259	0.027	0.219
	NS	NS	NS	NS	NS	NS
TNCV (m/s)	**0.370**	0.225	0.221	0.127	−0.236	0.193
	0.014	NS	NS	NS	NS	NS
TNFL (ms)	**−0.589**	**−0.473**	−0.128	−0.035	0.313	−0.017
	< 0.001	**0.005**	NS	NS	NS	NS
CDT (percentile)	**−0.466**	**−0.408**	**−0.285**	**−0.399**	−0.025	−0.081
	< 0.001	**0.003**	**0.041**	**0.003**	NS	NS
HP-VAS 0.5 (percentile)	**−0.311**	**−0.297**	−0.223	**−0.291**	−0.269	−0.134
	0.028	**0.039**	NS	**0.040**	NS	NS
HP-VAS 5.0 (percentile)	**−0.357**	−0.220	−0.146	−0.264	−0.267	−0.221
	0.010	NS	NS	NS	NS	NS
HP-VAS 0.5–5.0 (percentile)	0.009	0.255	0.148	0.032	0.029	−0.069
	NS	NS	NS	NS	NS	NS
DB-HRV (percentile)	0.268	0.204	0.148	**0.348**	0.073	0.202
	NS	NS	NS	**0.024**	NS	NS

Data are Spearman correlations (r_s) and significance (P) between intraepidermal and corneal nerve morphology and NDS, electrophysiology, and quantitative sensory tests. Significant correlations are in bold type.

there was a significant reduction in CNFL in painful (3.3 ± 0.4) compared with painless (4.6 ± 0.5) neuropathy ($P < 0.05$).

DISCUSSION

The National Institutes of Health has deemed the development of a surrogate end point for diabetic neuropathy as a priority area for the complications of diabetes. The main aim of establishing a surrogate marker is to define those at risk of developing a complication, anticipate deterioration, and assess the efficacy of new therapies. Although electrophysiology, QST, and assessment of neurological disability are advocated to define neuropathic severity (1), there is debate as to which test, if any, is appropriate in the early stages of neuropathy (25) and also whether the same tests should be used to define therapeutic efficacy in clinical intervention trials (5).

Regulatory authorities favor clinically relevant surrogate end points. Electrophysiology and assessment of vibration perception threshold quantify large fiber deficits, yet the earliest nerve fibers to undergo damage (8,10,11) and subsequent repair (12) are the small unmyelinated fibers, as evidenced by studies in patients with impaired glucose tolerance (8) and diabetic patients with minimal neuropathy (7). Although functional measures of small fiber damage include assessment of thermal perception thresholds, these tests show considerable variability and are not considered to be sufficiently robust to be included as major end points for clinical trials (1). Sural nerve (6,7) and skin biopsies (8,9) directly quantify small nerve fiber damage and repair and have been proposed as surrogate markers for human diabetic neuropathy, but both are invasive procedures. As an alternative noninvasive test, we (20–22) and others (19) have shown that CCM can also quantify small fiber pathological changes and stratify the severity of somatic neuropathy in diabetic patients. The present study compared in detail CCM and skin biopsy to quantify small fiber pathological changes in diabetic patients stratified in accordance with the severity of neuropathy using established clinical, electrophysiological, and QST techniques.

Skin biopsy is a reliable and reproducible way of quantifying small nerve fiber pathological changes and has been advocated for diagnosing small fiber neuropathy from a variety of causes (14,15), including painful neuropathy due to impaired glucose tolerance (8). Furthermore, in a recent study evaluating the therapeutic benefits of improving multiple risk factors on nerve damage in patients with impaired glucose tolerance, IENF pathology, as opposed to electrophysiology and QST, was the most sensitive measure of neuropathy change over 1 year (12), although, it is interesting that a recent study was unable to show a significant difference in IENFD between diabetic patients with and without neuropathy (26). An additional morphological feature of IENF that has been identified recently is terminal axonal swelling, which occurs with greater frequency in those with paresthesias and also predicts accelerated nerve fiber loss in patients with idiopathic painful neuropathy (27). To further define the ability of IENF assessment to diagnose and assess progression of human diabetic neuropathy and to compare it with corneal nerve pathology (20), we have additionally quantified IENFL and IENFBD.

We evaluated IENF pathology in relation to increasing neuropathic severity. As expected IENFD on the dorsum of the foot, a distal site, was slightly lower than that for other studies in the leg (13,28) and thigh (26). However, neither IENFD, IENFBD, nor IENFL was significantly reduced in diabetic patients without neuropathy compared with control subjects. This result is in keeping with recent observations in diabetic patients without neuropathy assessed using conventional measures of neuropathic severity (26) and suggests that IENF pathological changes may not be an early marker of nerve damage in diabetic patients. This finding is, of course, in contrast with several studies that have demonstrated a reduction in IENF density in patients with impaired glucose tolerance, but it must be remembered that these patients also had painful neuropathy (12). Indeed, it may be that pain is related to loss of IENFs as shown in a recent study in which diabetic patients with painful neuropathy had a significant reduction in IENFD compared with diabetic patients with painless neuropathy (29). Several studies have attempted to address whether there is a relationship between the severity of neuropathy and the degree of IENF loss. In one of the earliest studies to define IENF abnormalities in diabetic patients, Kennedy et al. (30) showed a marked reduction in IENFD and related it to the severity of neuropathy. However, it is important to note that all patients had severe neuropathy as they were undergoing pancreas transplantation. Similarly, a recent study has also demonstrated an association between IENFD and vibration perception threshold; however, the majority of patients in that study also had moderate to severe neuropathy (29). Another study has shown a correlation between loss of IENF and the severity of neuropathy assessed using electrophysiology and QST (28). In the present study, we demonstrate a significant reduction in IENFD and IENFBD in patients with mild neuropathy, which progressed with increasing neuropathic severity. IENFL was only reduced in those with severe neuropathy.

With regard to CNF pathology, whereas CNFD was reduced in diabetic patients with mild neuropathy and progressively worsened with increasing neuropathic severity, CNFBD was significantly reduced in patients even without evidence of neuropathy and showed a progressive reduction with increasing neuropathic severity. Although there was a trend for a reduction in CNFL, this was not significant. One may interpret this reduction as a possible compensatory elongation of remaining fibers; however, we believe that the increased variability in this measure also contributed to the lack of significant difference. The finding that corneal nerve pathology is related to neuropathic severity is in keeping with our previously published study (21) and other data from type 1 diabetic patients in whom a reduction in the number of CNF bundles correlated significantly with the severity of somatic neuropathy (19). A recent study has also shown that CNF loss is associated with the severity of retinopathy in diabetic patients (31).

Although CNF pathology showed fewer correlations to electrophysiology than IENF pathology, it did correlate with the clinically relevant measure of neuropathic severity, namely the NDS. Moreover, it also showed a correlation with autonomic dysfunction. In the present study when we stratified the diabetic patients in accordance with the severity of small fiber dysfunction, both CNF and IENF showed abnormal pathology even in patients without apparent small fiber dysfunction and in those with mild, moderate, and severe dysfunction. Furthermore, we also showed an association between CNFD and IENFD and both cold and heat as pain detection thresholds. A previous study has demonstrated an association between IENFD and cold but not warm detection thresholds (29). Shun et al. (28) have also shown an association between IENFD and both cold and warm detection thresholds.

Painful diabetic neuropathy is debilitating, yet both its

etiology and treatment are not clear. As pain is mediated via the C and Aδ fibers, it has been assumed that more specific testing of small fiber dysfunction or damage may help to differentiate patients with and without painful neuropathy. As IENFs and CNFs are C fibers, both techniques provide an ideal opportunity to study in detail any changes, which may occur in painful diabetic neuropathy. Several studies assessing small fiber dysfunction (32,33) and sural nerve unmyelinated nerve fiber pathology (6,34) have failed to show any difference between diabetic patients with and without painful diabetic neuropathy. However, in the present study we show that both corneal and intraepidermal nerve fiber length were significantly reduced in patients with painful compared with painless neuropathy. Sorensen et al. (29) have previously demonstrated a reduction in intraepidermal nerve fiber density in diabetic patients with painful neuropathy. Furthermore, in a lifestyle intervention study in patients with impaired glucose tolerance, an improvement in painful neuropathic symptoms was associated with an improvement in IENF density (12). These data support the contention that small nerve fiber damage may be associated with pain, and this novel finding merits further study.

In conclusion, an ideal surrogate marker for diabetic neuropathy should be easy to use, reliable, sensitive, and noninvasive to enable repeated assessment as often or as long as necessary to define progression or response to therapeutic intervention. We have demonstrated that whereas both techniques of CNF and IENF assessment accurately reflect the severity of somatic neuropathy, CCM provides a significant further advantage as it detects damage before detectable nerve dysfunction, but most importantly it is entirely noninvasive.

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