Abnormal LDIflare but Normal Quantitative Sensory Testing and Dermal Nerve Fiber Density in Patients with Painful Diabetic Neuropathy

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OBJECTIVE — Abnormal small nerve fiber function may be an early feature of diabetic neuropathy and may also underlie painful symptoms. Methods for assessing small-fiber damage include quantitative sensory testing (QST) and determining intraepidermal nerve fiber density. We recently described a reproducible physiological technique, the LDIflare, which assesses small-fiber function and thus may reflect early dysfunction before structural damage. The value of this technique in painful neuropathy was assessed by comparing it with QST and dermal nerve fiber density (NFD).

RESEARCH DESIGN AND METHODS — Fifteen healthy control subjects, 10 subjects with type 2 diabetes and painful neuropathy (PFN), and 12 subjects with type 2 diabetes and painless neuropathy (PLN) were studied. LDIflare and QST were performed on the dorsum of the foot, and dermal NFD was determined.

RESULTS — Results of both large- and small-fiber quantitative sensory tests were abnormal in patients with PLN but not those with PFN compared with control subjects. Dermal NFD was also significantly reduced in the PLN group compared with control subjects (205.8 ± 165.3 vs. 424.9 ± 176.3 [mean ± SD], P = 0.003) but not in the PFN group (307.6 ± 164.5). In contrast, the LDIflare (square centimeters) was reduced in both PFN (1.59 ± 0.41) and PLN (1.51 ± 0.56) groups compared with control subjects (4.38 ± 1.4) (P < 0.001 for both). NFD correlated significantly with the LDIflare (r = 0.57, P < 0.0001).

CONCLUSIONS — The LDIflare demonstrated impaired small-fiber function in patients with PFN when other assessments revealed no abnormality. We believe that this method has potential diagnostic value, particularly because it is noninvasive, has excellent reproducibility, and correlates with NFD. Furthermore, it may have an important role in assessing preventative therapies in early neuropathy.

P eripheral neuropathy affects between 40 and 60% of individuals with diabetes and is commonly diagnosed by assessing large-fiber sensory modalities. However, detection of small-fiber neuropathy may be of equal or more importance for several reasons. Structural and functional changes in small fibers precede large-fiber pathological changes and have been implicated in foot ulceration and delayed wound healing (1–3). Furthermore, C-fiber dysfunction may be involved in the genesis of neuropathic pain (4).

Until recently, few objective methods have been available to quantify small-fiber function. Quantitative sensory tests to define thermal and pain thresholds using the Computer Aided Sensory Evaluator–IV (CASE IV; WR Medical Electronics, Stillwater, MN) or the TSA-II NeuroSensory Analyzer (Medoc Advanced Medical Systems, Ramat Yishai, Israel) have been used primarily in clinical research (5,6). However, they are dependent on subjective responses and therefore have a high interobserver variability and poor reproducibility (7,8). We recently described a novel and reproducible (coefficient of variation <15%) technique to assess small-fiber dysfunction, the “LDIflare,” which measures axon reflex–mediated vasodilation in response to skin heating (9). We have also demonstrated that LDIflare detects early C-fiber dysfunction in type 2 diabetes before small-fiber neuropathy can be detected by other currently available noninvasive methods (10). However, the structural basis for an abnormal LDIflare response has not been established.

Although intraepidermal nerve fiber density (IENFD), with good intraobserver reproducibility, has been increasingly used to diagnose small-fiber neuropathies, it is an invasive procedure (11,12). In the present study we assessed small-fiber function using quantitative sensory testing (QST) and the LDIflare and compared these results with the results of dermal NFD in foot skin biopsy specimens from the same area. Dermal NFD as opposed to IENFD was quantified to define the underlying structural basis of the LDIflare, as this depends on an abnormality in dermal blood flow. In addition, as there is no current consensus as to whether an abnormality in small-fiber dysfunction and damage underlie painful diabetic neuropathy, we compared diabetic patients with painful neuropathy (PFN) and painless neuropathy (PLN).

RESEARCH DESIGN AND METHODS — Type 2 diabetic patients with PFN (n = 10) and PLN (n = 12) and 15 healthy control subjects were studied. Patients with diabetes were recruited from the outpatient clinics of the Ipswich Hospital Diabetes Centre (Ipswich, U.K.). Subjects with absent pedal pulses or evidence of peripheral
vascular disease were excluded, and all subjects had an ankle brachial pressure index (ABPI) of >0.8. The study was approved by the local ethics committee, and all the subjects gave informed written consent.

**Assessment of LDIflare**
Subjects were allowed to acclimatize for 30 min in a temperature-controlled room in which the temperature was maintained at 25 ± 1°C. The foot temperature was measured proximal to the first and second metatarsal heads using an infrared thermometer (Linear Laboratories, Fremont, CA). The axon reﬂex-mediated LDI flare was examined using a laser Doppler imager (LDI) (Moor Instruments, Devon U.K.) and our established methodology (10). Skin proximal to the first and second metatarsal heads on the dorsum was heated with a circular skin heater (diameter 1.0 cm; Moor Instruments) to 44°C for 20 min. An area of 3.5 × 3.5 cm surrounding the heated skin was scanned with the LDI immediately after careful removal of the heater probe. We have shown previously that removal of the heater along with the holder does not have an impact on the size of the flare (10). On the ﬂux image, the region of interest demarcated by the edge of the ﬂare was drawn, and the area of the LDI ﬂare was calculated using Moor LDI software (version 3.11) and expressed in square centimeters.

**Clinical neuropathy assessment**
Vibration perception threshold (VPT) was assessed using the Neurothesiometer (Horwell Scientiﬁc Laboratory Supplies, Nottingham, U.K.) at the pulp of the great toe using the ascending method of limits. The results were expressed in volts, and a value of 51 was assigned if the subjects could not feel the maximum vibration. The right foot was assessed using the Neuropen (Owen Mumford, Oxford, U.K.), which contains a 10-g monofilament to assess pressure perception and a Neurotip (Owen Mumford) for pinprick sensation (13,14). Ten-gram monofilaments were applied for 2 s on the plantar aspect of the ﬁrst, third, and ﬁfth metatarsal heads, and the Neurotip was applied at the eponychium of the ﬁrst toe. Subjects with an abnormal response using the Neuropen assessment and/or impaired VPT (≥15 V, i.e., >95th percentile for this age-group) were classiﬁed as having neuropathy. Subjects with typical painful neuropathic symptoms with a visual analog scale score >4 for >6 months were classiﬁed as having PFN (15). Quantitative sensory tests using the CASE IV, including vibration detection threshold (VDT), cold detection threshold (CDT), warmth detection threshold (WDT), and heat pain onset (HPO) were performed with software CASE IV (version 4.27.1; WR Medical Electronics). VDT, CDT, and WDT were measured using the 4, 2, 1 stepping algorithm with null stimuli (5). The VDT was obtained on the dorsal aspect of the hallux, and CDT and WDT were examined on the dorsum of the midfoot. For each test, the computer calculated the “just noticeable difference” (JND) from the subject’s responses, with a higher JND reﬂecting a larger amplitude of the stimulus (vibration) or larger change in temperature (thermal). A value of 26 was given if the JND was greater than the maximum of 25.

**Skin biopsy**
On a different day, skin biopsies were performed using a sterile 3-mm biopsy punch (Stiefel Laboratories, Bucks, U.K.) in the same area where the LDI flare had been assessed previously. All subjects tolerated the biopsy, and there was no infection or other adverse event.

**Fixation immunostaining protocol**
The biopsy specimen was immersed in 4 ml of 4% buffered paraformaldehyde for 18–24 h, washed with Tris-buffered saline (TBS) buffer for 15 min, and autolyzed at 30 min in a temperature-controlled room at 5°C. After addition of secondary antibody (swine anti-rabbit for PGP), a streptavidin-horseradish–conjugated peroxidase and 3′,3′-diaminobenzidine chromogen substrate were used to detect binding of the primary antibodies. Negative controls comprised sections that underwent the same runs except that the primary antibody was omitted. Developing time was exactly the same for all sections in each separate run, and in each run the sections were processed synchronously.

**Image analysis**
Patterns of immunostaining were examined by light microscopy (Leitz DM RB microscope). Digital images were captured at ×400 magniﬁcation with a Nikon digital camera and analyzed with Leica QWin Standard V2.4 (Leica Microsystems Imaging, Cambridge, U.K.) to detect color intensities in a ﬁxed and constant range. Every image was evaluated using a standardized Leica program to quantify the amount of stained and total areas (Leica QWin Standard V2.4). The PGP 9.5 positively stained proﬁles and blood vessel cross-sections were counted manually and divided by the dermal area to obtain a density (number per square millimeter). Because PGP 9.5 ubiquitously stains all nerve ﬁbers, both sensory and autonomic C ﬁbers in the dermis were included. The blood vessels counted were predominantly capillaries, although some precapillary arterioles or postcapillary venules may have been included as cross-sections were studied. Large arterioles and venules were not counted. All observations were performed on coded slides to prevent observer bias.

**Statistical analysis**
Descriptive statistics were used to describe subject characteristics. Nonparametric analysis (Kruskal-Wallis test and Mann-Whitney U test) was used to determine differences between the groups. Pearson’s correlation coefﬁcient was used to correlate the variables. The results are expressed as means ± SD. P < 0.05 was considered to be signiﬁcant. SPSS (version 11.0, SPSS, Chicago, IL) was used for the statistical analysis.

**RESULTS** — Clinical characteristics of the subjects with diabetes and control subjects are shown in Table 1. All subjects were Caucasian and were matched for
The duration of diabetes in the diabetic groups was similar. As expected, the 
BMI was lower in the control group but 
was similar in the two diabetic groups. A1C was not significantly different in 
the two diabetic groups. ABPIs were similar 
in all three groups.

The neurological assessments are shown in Table 2. VPT, VDT, WDT, 
CDT, and HPO were significantly higher in the PLN group but not in the PFN 
group compared with healthy control subjects (Table 2). However, the LDIflare 
was significantly reduced in both diabetic groups compared with the healthy 
control subjects (healthy control subjects 4.38 ± 1.4 cm²; PLN group 1.59 ± 0.41 
cm², and PFN group 1.51 ± 0.56 cm²; P < 0.0001). In contrast, the NFD was 
significantly reduced in the PLN group compared with that in healthy control 
subjects (205.8 ± 165.3 vs. 424 ± 176.3 mm²; P = 0.003) but not in the PFN 
group (307.6 ± 164.5 vs. 424 ± 176.3 mm²; P = 0.13). There was no significant 
difference between the PFN and PLN groups for either LDIflare or NFD (LDI-
flare: PLN 1.59 ± 0.41 cm² vs. PFN 1.51 ± 0.56 cm²; P = 0.49; NFD: PLN 
205.8 ± 165.3 mm² vs. PFN 307.6 ± 164.5 mm²; P = 0.12). There was also no 
significant difference in dermal vascular density among any groups (Table 2). The 
LDIflare correlated significantly with dermal NFD (Fig. 1) (r = 0.57; P < 0.0001) in 
all subjects combined and also within control subjects (r = 0.53; P < 0.05) and 
in the PFN group (r = 0.71; P < 0.05) but not in the PLN group (r = 0.38; P = 0.22).

CONCLUSIONS — A significant number 
of patients with diabetic neuropathy present with pain as their first neuro-
pathic symptom. Many of these patients have no objective clinical signs. It has 
been suggested that this is because conventional bed-side tests such as reflexes,

**Table 1—Clinical characteristics of subjects**

| Healthy control subjects | Type 2 diabetic subjects |
|---------------------------|---------------------------|
| Sex (male/female)         | PFN                       | PLN                       |
| Age (years)               | 5/10                      | 5/5                       | 6/6                       |
| Duration (years)          | 54.4 ± 9.7                | 61.0 ± 11.2               | 62.9 ± 10.2               |
| BMI (kg/m²)               | 25.4 ± 2.4                | 30.7 ± 3.1                | 32.3 ± 2.8                |
| A1C (%)                   | 1.1 ± 0.1                 | 8.2 ± 3.8                 | 8.6 ± 3.5                 |
| ABPI                      | 7.08 ± 2.8                | 3.8 ± 1.0                 | 1.2 ± 0.1                 |
| VAS (0–10)                | 0                         | 5.7 ± 1.1                 | 37.1 ± 12.9               |

Data are means ± SD. There were no significant differences in age between the healthy control, PFN, and PLN groups. BMI was lower in the control subjects than in the PFN and PLN groups (P = 0.001 and P = 0.0001, respectively). Duration of diabetes and A1C were not significantly different between the PFN and PLN groups. ABPI was not different among the three groups. VPT was not significantly different between the healthy control and PFN groups but high in the PLN group (P < 0.0001). Visual analog scale (VAS) was high in the PFN group.

**Table 2—Neurological assessments**

| Healthy control subjects | Type 2 diabetic subjects |
|---------------------------|---------------------------|
| LDIflare (cm²)            | 4.38 ± 1.4                | 1.59 ± 0.4               | 1.51 ± 0.56               |
| Dermal nerve density (mm²) | 424 ± 176.3              | 307.6 ± 164.5           | 205.8 ± 165.3            |
| Dermal vascular density (mm²) | 115.8 ± 23.7          | 129.9 ± 23.8            | 103.4 ± 27.1             |
| VPT (V)                   | 7.0 ± 2.8                 | 8.7 ± 2.2                | 37.0 ± 12.9              |
| VDT (JND)                 | 18.4 ± 3.2                | 19.5 ± 3.2               | 23.0 ± 3.7               |
| CDT (JND)                 | 10.4 ± 5.0                | 13.8 ± 5.1               | 19.5 ± 4.6              |
| WDT (JND)                 | 17.5 ± 2.0                | 18.3 ± 6.1               | 25.2 ± 1.8              |
| HPO (JND)                 | 21.6 ± 1.8                | 21.3 ± 3.0              | 25.3 ± 1.6              |

Data are means ± SD. Except for LDIflare, none of the neurovascular parameters were significantly different in the PFN group compared with the healthy control subjects. P values compared with healthy control subjects: *P < 0.0001; †P = 0.003; ‡P = 0.005.
Abnormal LDIflare in PDN

They concluded that loss of IENFD cannot

more severe IENFD loss compared with

rensen et al. (20) paradoxically found

normal control subjects. In another study

Lauria et al. (19) found reduced IENFD in

mal NFD in painful diabetic neuropathy. 

However, few studies have focused specifically on der-

mal NFD in painful diabetic neuropathy. Lauria et al. (19)

found reduced IENFD in a study of six patients with painful dia-

abetic neuropathy compared with that in normal control sub-

jects. In another study of patients with neuropathic pain,

Sorensen et al. (20) paradoxically found more severe IENFD loss compared with

that in those with PLN. From this finding, they concluded that loss of IENFD cannot

explain genesis of pain in all patients. Los-

etz et al. (21) also reported significantly lower IENFD and higher cold perception

thresholds in patients with diabetes and normal nerve conduction studies whether

they had painful symptoms or not. There are several reasons why we did

not find a significant difference in nerve fiber density in comparison with the

above studies. Patient selection may be important, as our patients had no clinical

signs of neuropathy and may thus represent an earlier phase in the pathological

process. The majority of studies have ex-

amined IENFD as opposed to dermal

NFD. From previous reports it would ap-

pear that IENFD may be a more useful
diagnostic test for detecting early struc-
tural nerve damage, as intraepidermal

fibers are more distal than dermal nerves. However, the assessment of dermal NFD

may provide more mechanistic insights into the pathogenesis of painful diabetic neuropathy, as it provides a measure of

dermal blood vessel innervation and, hence, any potential impact on dermal blood flow. Indeed, we have previously
demonstrated an impairment of cutane-

ous endothelium-related vasodilatation and C-fiber–mediated vasoconstriction in

painful diabetic neuropathy and suggested that inappropriate local blood flow

regulation may have a role in the patho-

genesis of pain in diabetic neuropathy

(22). A recent study confirms the valid-

ity of assessing alterations in dermal NFD in thin sections and has specifi-
cally demonstrated a reduction in arte-

riolar innervation in patients with small-

fiber neuropathy (23). Furthermore, the

assessment of dermal NFD in addition to IENFD has been shown to improve the
diagnostic sensitivity for detecting painful sensory neuropathy (24). Fi-

nally, functional defects in unmyeli-
nated C-fibers may precede structural
defects (25), which would be detected by an abnormal LDIflare but with no
effect on NFD, as demonstrated in this study. It is of importance that the LDI-
flare results correlated with NFD in the groups combined as well as separately in the control and PFN groups. This re-
sult would be expected because the size of the flare response should relate not
to neural function but also to the actual number of functioning nerves. It was not unexpected that there would be no correlation between the flare response and NFD in the PLN group because whether or not dermal nerve fibers were identified, all modalities of nerve function were severely impaired or absent with little or no graduation in this group.

In summary, using the LDIflare tech-
nique, we have demonstrated abnormal C-fiber function in subjects with symp-
tomatic PFN in whom results of conven-
tional quantitative sensory tests were normal and in whom there was no signif-
icant reduction in NFD. Because of the small sample size in the current study,
further studies with larger numbers of pa-
tients are required to confirm these find-
ings and to determine the sensitivity and

specificity of the LDIflare as a diagnostic

modality in painful diabetic neuropathy. Because the LDIflare detects small-fiber
dysfunction before the occurrence of po-
tentially irreversible structural loss of

nerve fibers, in addition to its potential
diagnostic value, it may have an impor-
tant role in assessing preventative thera-
pies in early neuropathy.

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