Abnormal LDI flare but normal quantitative sensory testing and dermal nerve fiber density in patients with painful diabetic neuropathy

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Objectives: 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 intra-epidermal nerve fibre density (IENFD). We recently described a reproducible physiological technique, the LDIflare, which assesses small fiber function and as such may reflect early dysfunction prior to structural damage. The value of this technique in painful neuropathy was assessed by comparing it with QST and dermal NFD.

Methods: Fifteen healthy controls (HC), 10 subjects with type 2 diabetes and painful (PFN) and 12 with painless (PLN) neuropathy were studied. LDIflare, QST and dermal NFD were determined on the dorsum of the foot.

Results: Both large and small fiber quantitative sensory tests were abnormal in patients with painless but not painful neuropathy compared to controls. Dermal NFD was also significantly reduced in the painless neuropathy group compared with controls (205.8 +/- 165.3 v 424.9 +/- 176.3; Mean +/- SD; p=0.003) but not in the painful group (307.6 +/- 164.5). In contrast, the LDIflare (cm2) was reduced in both painful (1.59 +/- 0.41) and painless (1.51 +/- 0.56) groups compared to controls (4.38 +/- 1.4), p<0.001 for both. NFD correlated significantly with the LDIflare (r=0.57, p<0.0001).

Conclusion: The LDIflare demonstrated impaired small fiber function in patients with painful neuropathy when other assessments revealed no abnormality. We believe that this method has potential diagnostic value, particularly as it is non-invasive, has excellent reproducibility and correlates with NFD. Furthermore, it may have an important role in assessing preventative therapies in early neuropathy.
Peripheral neuropathy affects between 40 and 60% of people 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 pathology 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 quantified small fiber function. Quantitative sensory testing used to define thermal and pain thresholds using the Computer Aided Sensory Evaluator – IV [Case IV] [WR Medical Electronics, Minnesota] or the TSA-II NeuroSensory Analyzer [Medoc Advanced Medical Systems Ltd, Israel] have been employed primarily in clinical research (5,6). However, they are dependent on subjective responses and therefore have a high inter-observer variability and poor reproducibility (7,8). We recently described a novel and reproducible (CV<15%) technique to assess small fiber dysfunction, the “LDIflare”, which measures axon-reflex mediated vasodilatation in response to skin heating (15). 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 non-invasive methods (9). However, the structural basis for an abnormal LDIflare response has not been established.

Although, intraepidermal nerve fiber density, with good intra-observer reproducibility, has been increasingly used to diagnose small fiber neuropathies it is an invasive procedure (10,11). In the present study we assessed small fiber function, using QST and the LDIflare and compared these with the results of dermal NFD in foot skin biopsied from the same area. Dermal 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. Additionally, as there is no current consensus as to whether an abnormality in small fiber dysfunction and damage underlies diabetic painful neuropathy, we compared diabetic patients with painful and painless neuropathy.

STUDY DESIGN

Subjects: Type 2 diabetic patients with painful neuropathy (PFN) (n=10), painless neuropathy (PLN), (n=12) and 15 healthy control subjects (HC) were studied. Patients with diabetes were recruited from the outpatient clinics of the Ipswich Hospital Diabetes Centre. 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 ethical committee and all the subjects gave informed written consent.

METHODS

Assessment of LDIFlare: Subjects were allowed to acclimatize for 30 minutes in a temperature-controlled room where the temperature was maintained at 25+/–1°C. The foot temperature was measured proximal to the 1st and 2nd metatarsal heads using an infrared thermometer [Linear Laboratories, California]. The axon-reflex mediated LDIflare was examined using a laser Doppler imager [LDI] [Moor instruments, Devon U.K] and our established methodology (9). Skin proximal to the 1st and 2nd metatarsal heads on the dorsum was heated with a circular skin heater (diameter – 1.0 cm) [Moor instruments, Devon U.K] to 44°C for 20 minutes. An area of 3.5cm x 3.5cm surrounding the heated skin was scanned with the laser Doppler imager.
immediately after careful removal of the heater probe. We have shown previously the removal of the heater along with the holder does not impact on the size of the flare (9). 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 the MoorLDI version 3.11 software and expressed in cm$^2$.

**Clinical neuropathy assessment:**
Vibration perception threshold [VPT] was assessed using the Neurothesiometer [Horwell Scientific Laboratory Supplies, Nottingham, UK] 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 Ltd, Oxford U.K] which contains a 10g monofilament to assess pressure perception and a Neurotip™ [Owen Mumford Ltd, Oxford U.K] for pinprick sensation (12,13). 10g monofilaments were applied for 2 seconds on the plantar aspect of the 1$^{st}$, 3$^{rd}$ and 5$^{th}$ metatarsal heads, and Neurotip™ was applied at the epinychium of the 1$^{st}$ toe. Subjects with an abnormal response using the Neuropen™ [Owen Mumford Ltd, Oxford U.K] assessment and/or impaired vibration perception threshold [VPT ≥ 15 Volts i.e. >95$^{th}$ percentile for this age group] were classified as neuropathic. Subjects with typical painful neuropathic symptoms with a visual analogue scale (VAS) score > 4 for more than 6 months were classified as painful diabetic neuropathy (PFN) (14). Quantitative sensory tests using CASE IV including VDT, CDT, WDT and HPO were carried out using the Computer Aided Sensory Evaluator IV (CASE IV) with software version 4.27.1 (WR Medical Electronics, Minnesota). Vibration (VDT), cold (CDT) and warmth (WDT) detection thresholds were measured using the 4, 2, 1 stepping algorithm with null stimuli (5). The VDT was performed on the dorsal aspect of the hallux and CDT and WDT were examined on the dorsum of the mid foot. For each test the computer calculated the “just noticeable difference” or 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 above the maximum of 25.

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

**Fixation immunostaining protocol:**
The biopsy specimen was immersed in 5 mls of 4% buffered paraformaldehyde for 18-24 hours, washed with TBS buffer for 15 minutes and transported to the lab to be embedded in paraffin wax. Wax blocks were cut on the microtome into thin sections (5µm) which were mounted on positively charged slides (three per slide), dewaxed in xylene, and gradually rehydrated through decreasing ethanol dilutions. In all cases, epidermal melanin was bleached with 0.25% KMnO4 and 5% oxalate before serum protein block. Before applying primary antibody, enzymatic antigen retrieval pre-treatment with trypsin was used for anti-CD31 and anti-CD34 for blood vessels. Tissue was washed with tween20 detergent/TBS buffer before starting the run. 3% Hydrogen peroxide was used to block endogenous peroxidase and a TBS buffered-solution of 1:10 normal serum for protein block. Dilution of anti-PGP 9.5 rabbit anti-human polyclonal antibody was 1:100 and it was applied for 60-72 hours at 5 ºC. CD34 (dilution 1:300) and CD31 (dilution 1:50) polyclonal mouse anti-human antibodies were applied overnight at 5ºC. After adding secondary antibody (swine anti-rabbit for PGP), a streptavidin-HRP conjugated peroxidase and DAB chromogen
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substrate was 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 x400 magnification with a Nikon digital camera and analyzed with Leica QWin Standard V2.4 (Leica Microsystem Imaging, Cambridge, U.K.) set 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). As 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 post capillary 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. Non parametric analysis (Kruskal-Wallis Test and Mann-Whitney U Test) was used to determine the differences between the groups. Pearson’s correlation coefficient was used to correlate the variables. The results were expressed as Mean +/- Standard Deviation (Mean+/−SD). A “p” value of < 0.05 was considered to be significant. SPSS version 11.0 software package 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 age. The duration of diabetes in the diabetic groups was similar. As expected the body mass index [BMI] was lower in the control group but was similar in the two diabetic groups. HbA1c was not significantly different in the two diabetic groups. Ankle brachial pressure indices [ABPI] 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 painless but not the painful neuropathy group compared to healthy controls (Table 2). However, the LDIflare was significantly reduced in both diabetic groups compared to the healthy controls (HC - 4.38+/−1.4; PLN - 1.59+/−0.41; PFN - 1.51+/−0.56; p <0.0001). In contrast, compared with healthy controls the NFD was significantly reduced in the painless neuropathy group (PLN - 205.8+/−165.3 v HC -- 424+/−176.3; p = 0.003) but not in the painful neuropathy group (PFN - 307.6+/−164.5 v HC -- 424+/−176.3; p = 0.13). There was no significant difference between the painful and painless neuropathy groups for either LDIflare or NFD [LDIflare - (PLN - - 1.59+/−0.41 v PFN --1.51+/−0.56; p = 0.49); NFD (PLN-- 205.8+/−165.3 v PFN -- 307.6+/−164.5; p = 0.12)]. There was also no significant difference in dermal vascular density (DVD) between any group (Table 2). The LDIflare correlated significantly with dermal nerve fiber density (Fig 1. (r = 0.57; p < 0.0001) in all studied subjects combined and also within controls (r = 0.53; p < 0.05) and PFN group (r = 0.71; p < 0.05) but not the PLN group (r = 0.38, p = 0.22); Fig 1.

**CONCLUSION**

A significant number of patients with diabetic neuropathy present with pain as their
first neuropathic symptom. Many of these patients have no objective clinical signs. It has been suggested that this is because conventional bedside tests such as reflexes, pressure sensation and vibration relate to large fiber function, whereas pain sensation conveyed by small unmyelinated C-fibers and A-delta fibers, is not examined by these tests (14). This study compared the ability to define an underlying abnormality in small fiber dysfunction, using the novel technique of LDIflare with established quantitative sensory testing using CASE IV and dermal skin nerve fiber density in patients with diabetic neuropathy.

In diabetic patients with painless neuropathy, as expected, all neurological tests including VPT, QSTs as well as LDIflare and NFD were abnormal, consistent with the extensive nerve damage in this group (9). However, in patients with painful neuropathy there was no bedside evidence of neuropathy; i.e. they had intact reflexes and 10 gm monofilament sensation. Furthermore, large (VPT, VDT) and more surprisingly small fiber function (WDT, CDT and HPO) assessed by QST did not differ significantly from the controls.

In contrast in the painful neuropathy group, the LDIflare was the only test which was abnormal and indeed this was as severely impaired as that in the painless neuropathy group. The NFD in the painful neuropathy group though lower was not significantly different to that of the control subjects and lay between that of patients with painless neuropathy and the control group.

A reduced LDIflare response may occur due to, impaired C-fiber function; loss of C fibers, reduced microvascular vasodilatation (including in response to vasoactive peptides) or reduced blood vessel density. The latter is unlikely as there was no significant difference in the dermal blood vessel density between the three groups and indeed in previous studies we have found no reduction in blood vessel density in people with diabetes (15). Although it is widely recognized that hyperemic responses are reduced in people with diabetes this only relates to maximal hyperemia; we have previously shown that the flare response in terms of the area over which the flare spreads following skin heating is clearly demonstrable even in subjects with severely impaired maximal hyperemia (9). Thus, the smaller flares are not a result of either reduced blood vessel density or reduced maximal vasodilatation response. We specifically assessed dermal as opposed to intraepidermal nerve fiber density as this provides a direct measure of the structural integrity of the innervation of the dermal blood vessels and hence the LDIflare. We found no significant difference in dermal nerve fiber density between those with painful neuropathy and controls; this suggests that the reduced flare reflects functional as opposed to structural impairment in the axon reflex pathway. This observation further supports the paradigm that impaired small fiber function occurs before the development of large fiber neuropathy, but in addition we now demonstrate that this precedes structural defects to the small fibers.

Several studies have shown that in diabetes the degree of intra epidermal nerve fiber loss correlates with the severity of the neuropathy (16-18). However, few studies have focused specifically on dermal nerve fiber density in painful diabetic neuropathy. Lauria et al found reduced intra epidermal nerve fiber density (IENF) in a study of six patients with painful diabetic neuropathy compared to normal controls (19). In another study of patients with neuropathic pain, Sorenson et al paradoxically found more severe IENF loss compared to those with painless neuropathy. From this they concluded that loss of IENF cannot explain genesis of pain in all cases (20). Loseth et al also reported significantly lower IENF density and higher cold perception thresholds in
patients with diabetes and normal nerve conduction studies whether they had painful symptoms or not (21).

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 examined intra-epidermal as opposed to dermal nerve fiber density. From previous reports it would appear that IENF may be a more useful as a diagnostic test for detecting early structural nerve damage, as they are more distal than dermal nerves.

However, the assessment of dermal nerve fiber density 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 cutaneous 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 pathogenesis of pain in diabetic neuropathy (22). A recent study confirms the validity of assessing alterations in dermal nerve fiber density in thin sections and has specifically demonstrated a reduction in arteriolar innervation in patients with small fiber neuropathy (23). Furthermore, the assessment of dermal nerve fiber density in addition to IENF density has been shown to improve the diagnostic sensitivity for detecting painful sensory neuropathy (24).

Finally, functional defects in unmyelinated C–fibers may precede structural defects (25); which would be detected by an abnormal LDI flare but with no effect on nerve fiber density, 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 painful neuropathy groups. This would be expected as the size of the flare response should relate not only 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 painless neuropathy group as 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 conclusion, using the LDI flare technique we have demonstrated abnormal C-fiber function in subjects with symptomatic painful neuropathy in whom conventional quantitative sensory tests were normal and in whom there was no significant reduction in nerve fiber density. Due to the small sample size in the current study further studies with larger numbers of patients are required to confirm these findings and to determine the sensitivity and specificity of the LDI flare as a diagnostic modality in painful diabetic neuropathy. As the LDI flare detects small fiber dysfunction before the occurrence of potentially irreversible structural loss of nerve fibers, in addition to its potential diagnostic value, it may have an important role in assessing preventative therapies in early neuropathy.
REFERENCES

1. Levitt NS, Stansberry KB, Wynchang S, Vinik AI: The natural progression of autonomic neuropathy and autonomic function tests in a cohort of people with IDDM. *Diabetes Care* 19:751-4, 1996


Table 1

<table>
<thead>
<tr>
<th></th>
<th>HC</th>
<th>PFN</th>
<th>PLN</th>
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<tbody>
<tr>
<td>Male/female</td>
<td>5/10</td>
<td>5/5</td>
<td>6/6</td>
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<tr>
<td>Age (years)</td>
<td>54.4 +/- 9.7</td>
<td>61.0 +/- 11.2</td>
<td>62.9 +/- 10.2</td>
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<td>Duration (years)</td>
<td>--</td>
<td>12.1 +/- 4.2</td>
<td>13.3 +/- 4.29</td>
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<tr>
<td>BMI (Kg/m²)</td>
<td>25.4 +/- 2.4</td>
<td>30.7 +/- 3.1</td>
<td>32.3 +/- 2.8</td>
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<td>HbA1c (%)</td>
<td>--</td>
<td>8.2 +/- 3.8</td>
<td>8.6 +/- 3.5</td>
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<tr>
<td>ABPI</td>
<td>1.1 +/- 0.1</td>
<td>1.0 +/- 0.2</td>
<td>1.2 +/- 0.1</td>
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<tr>
<td>VPT</td>
<td>7.08 +/- 2.8</td>
<td>8.6 +/- 2.2</td>
<td>37.1 +/- 12.9</td>
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<tr>
<td>VAS (0-10)</td>
<td>5.7 +/- 1.1</td>
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</table>

Mean +/- SD

HC – Healthy control subjects; PFN - Type 2 diabetic patients with painful neuropathy; PLN – Type 2 diabetic patients with painless neuropathy.

BMI – Body mass index, ABPI – Ankle brachial pressure index, VPT – Vibration perception threshold, VAS – Visual analogue scale score.

There were no significant differences in age between the HC, PFN and PLN groups. BMI was lower in the control subjects (HC) than in the PFN and PLN groups (p = 0.001 and p = 0.0001, respectively). Duration of diabetes and HbA1c were not significantly different between the PFN and PLN groups. Ankle brachial pressure index was not different between the three groups. VPT was not significantly different between the HC and PFN group but high in the PLN group (p < 0.0001). VAS was high in the PFN group.

Table 2

<table>
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<tr>
<th></th>
<th>HC</th>
<th>PFN</th>
<th>PLN</th>
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<tbody>
<tr>
<td>LDI flare (cm²)</td>
<td>4.38 +/- 1.4</td>
<td>1.59 +/- 0.4¹</td>
<td>1.51 +/- 0.56¹</td>
</tr>
<tr>
<td>Dermal Nerve Density (mm²)</td>
<td>424 +/- 176.3</td>
<td>307.6 +/- 164.5</td>
<td>205.8 +/- 165.3*</td>
</tr>
<tr>
<td>Dermal Vascular Density (mm²)</td>
<td>115.8 +/- 23.7</td>
<td>129.9 +/- 23.8</td>
<td>103.4 +/- 27.1</td>
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<tr>
<td>Vibration Perception Threshold (Volts)</td>
<td>7.0 +/- 2.8</td>
<td>8.7 +/- 2.2</td>
<td>37.0 +/- 12.9¹</td>
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<tr>
<td>Vibration Detection Threshold (JND)</td>
<td>18.4 +/- 3.2</td>
<td>19.5 +/- 3.2</td>
<td>23.0 +/- 3.7¹</td>
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<tr>
<td>Cold Detection Threshold (JND)</td>
<td>10.4 +/- 5.0</td>
<td>13.8 +/- 5.1</td>
<td>19.5 +/- 4.6¹</td>
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<td>Warmth Detection Threshold (JND)</td>
<td>17.5 +/- 2.0</td>
<td>18.3 +/- 6.1</td>
<td>25.2 +/- 1.8¹</td>
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<tr>
<td>Heat Pain Onset (JND)</td>
<td>21.6 +/- 1.8</td>
<td>21.3 +/- 3.0</td>
<td>25.3 +/- 1.6¹</td>
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Mean +/- SD

¹ - p < 0.0001; * - p = 0.003; ¹ - p = 0.005

Symbols indicate p value when compared to HC.

HC – Healthy control subjects; PFN - Type 2 diabetic patients with painful neuropathy; PLN – Type 2 diabetic patients with painless neuropathy.

JND – Just noticeable difference.

Except LDI flare none of the neurovascular parameters were significantly different in the PFN group when compared with the Healthy controls (HC).

Figure Legend: Figure 1. Correlation of LDI flare and NFD

NFD – Dermal nerve fiber density

- Control group
- - Painless neuropathy group
- ▲ - Painful neuropathy group

The LDI flare correlated significantly with dermal nerve fiber density. (r = 0.57; p < 0.0001) in all studied subjects combined and within controls (r = 0.53; p < 0.05) and PFN group (r = 0.71; p < 0.05) but not the PLN group (r = 0.38, p = 0.22).
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Figure 1  Correlation of LDIflare and NFD

NFD – Dermal nerve fiber density

- Control group
- Painless neuropathy group
- Painful neuropathy group

The LDIflare correlated significantly with dermal nerve fiber density. \((r = 0.57; p < 0.0001)\) in all studied subjects combined and within controls \((r = 0.53; p < 0.05)\) and PFN group \((r = 0.71; p < 0.05)\) but not the PLN group \((r = 0.38, p = 0.22)\).