# Impaired Skin Microvascular Reactivity in Painful Diabetic Neuropathy

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**OBJECTIVE** — The pathogenesis of painful diabetic neuropathy (PDN) is not clear. Following our in vivo observations of increased sural nerve epineurial blood flow in patients with PDN, we investigated the cutaneous microcirculation of the foot by laser Doppler flowmetry to determine if the epineurial findings were just confined to the nerve or more widespread in other vascular beds.

**RESEARCH DESIGN AND METHODS** — We measured foot skin vasodilator responses to acetylcholine (Ach) and sodium nitroprusside (SNP) and vasoconstrictor responses to sympathetic (deepest possible gasp) stimulation in 5 healthy control subjects, 10 non-neuropathic diabetic (NND) patients, 10 diabetic patients with painless neuropathy (PLDN), and 8 diabetic patients with PDN.

**RESULTS** — In PDN, there were significantly reduced responses to Ach (ANOVA $P = 0.003$) and vasoconstrictor inspiratory gasp (ANOVA $P < 0.001$) but not to SNP (NS). Post hoc analysis showed significant differences in Ach-induced vasodilation between PDN and nondiabetic control subjects ($P < 0.05$) as well as between PDN and NND ($P < 0.05$) but not PDN and PLDN (NS). There were no significant differences for SNP-induced vasodilation. However, there were significant differences in the vasoconstrictor response between PDN and control, NND, and PLDN ($P < 0.01$).

**CONCLUSIONS** — We found an impairment of cutaneous endothelium-related vasodilation and C-fiber–mediated vasoconstriction in PDN. Inappropriate local blood flow regulation may have a role in the pathogenesis of pain in diabetic neuropathy. Prospective studies are required to determine the temporal relationship of these changes in relation to the emergence of neuropathic pain.

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Diabetic peripheral neuropathy is one of the most common long-term complications of diabetes (1) and is associated with cardiovascular risk factors and mortality (2,3). Pain is one of the manifestations of diabetic neuropathy that often prompts the patient to seek medical attention and occurs in ~30% of patients with diabetic neuropathy (4).

Painful diabetic neuropathy (PDN) is difficult to treat and results in depression and a marked reduction in quality of life (5). Despite considerable research, a complete understanding of the mechanisms of neuropathic pain remains elusive. Consequently, many patients respond poorly to drug therapies. Elucidation of the pathophysiological mechanisms underlying PDN may lead to more effective treatments (3).

Many years ago, Archer et al. (6) showed that diabetic patients with PDN have altered blood flow patterns in the lower limbs. More recently, we found an increase in sural nerve epineurial blood flow in patients with PDN (7). We have also shown that patients with severe pain due to insulin neuritis have abnormal epineurial nutrient vessel anatomy and increased epineurial shunt flow, which may lead to a reduction in endoneurial nutritive blood flow (8). These observations linking PDN with alterations in blood flow are supported by two recent trials that have demonstrated significant benefits of pain relief from the use of vasodilators, isosorbide dinitrate spray, and glyceryl trinitrate patches (9,10).

Previous studies have investigated microvascular abnormalities invasively, by nerve biopsy (11–13), epineurial videomicroscopy (7), and hydrogen polarography (14). In this study, we have used a noninvasive laser Doppler technique to *1*) explore further the underlying mechanisms that may be amenable to therapeutic intervention and *2*) determine if the abnormality in vasoregulation is only present in the nerve or also in the skin vessel beds (15,16).

## RESEARCH DESIGN AND METHODS

— The study was approved by the South Sheffield Research Ethics Committee, and all subjects gave informed consent. A total of 28 type 1 and type 2 diabetic patients aged between 18 and 70 years were recruited. Diabetes was diagnosed on the basis of a casual plasma blood glucose >200 mg/dl, a fasting plasma glucose >126 mg/dl, or a 2-h postload glucose >200 mg/dl.

Patients with neuropathy from other causes, on vasoactive drugs, and with hypertension, active proliferative retinopathy, established renal disease, or macroalbuminuria and peripheral vascular disease (defined as the absence of both posterior tibial and dorsalis pedis pulses on palpation) were excluded from the study (17). The clinical assessment included sex, age, height and weight, BMI (kg/m$^2$), duration of diabetes, metabolic control (A1C; normal range 4.5–5.5%), lipid profile (total cholesterol, HDL cholesterol), and the presence of other chronic complications (retinopathy status and the presence of microalbuminuria).

All patients underwent neurophysiologic assessment, which included *1*) full history and neurological examination to assess the neuropathy disability score

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**Abbreviations:** Ach, acetylcholine; NDS, neuropathy disability score; NND, non-neuropathic diabetic; PDN, painful diabetic neuropathy; PLND, painless diabetic neuropathy; SNP, sodium nitroprusside; VAS, visual analogue scale.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

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**Table 1—*Characteristics of study subjects***

| | Control | NND | PLDN | PDN |
|---|---|---|---|---|
| *n* | 5 | 10 | 10 | 8 |
| Male:female | 2:3 | 4:6 | 6:4 | 4:4 |
| Age (years) | 50 ± 14 | 53 ± 13 | 56 ± 8 | 54 ± 15 |
| Duration diabetes (years) | | 10 ± 7 | 18 ± 15 | 16 ± 6 |
| Type 1:type 2 diabetes | | 2:8 | 5:5 | 4:4 |
| Height (cm) | 174 ± 11 | 163 ± 11 | 172 ± 9 | 169 ± 12 |
| Weight (kg) | 72 ± 14 | 81 ± 4 | 80 ± 18 | 79 ± 17 |
| BMI (kg/m$^2$) | 23 ± 3 | 30 ± 4 | 27 ± 5 | 28 ± 5 |
| A1C (%) | | 8.3 ± 1.2 | 9.0 ± 1.1 | 9.4 ± 2.4 |
| Foot temperature (°C) | 28.5 ± 3.1 | 29.4 ± 1.8 | 28.9 ± 2.3 | 28.6 ± 3.2 |
| Retinopathy (N/B/P) | | 6/3/1 | 6/3/1 | 5/2/1 |
| Albuminuria (N/m/M) | | 9/1/0 | 8/1/0 | 6/2/0 |
| NDS | | 1.8 ± 2.0 | 5.5 ± 1.8 | 5.5 ± 3.5 |
| Total cholesterol:HDL ratio | | 5.0 ± 1.2 | 4.0 ± 1.9 | 4.0 ± 1.2 |
| Peroneal nerve conduction velocity (m/s) | 45.1 ± 3.6 | 45.2 ± 6.9 | 36.8 ± 6.0 | 38.9 ± 14.7 |
| Sural nerve conduction velocity (m/s) | 47.2 ± 3.1 | 51.6 ± 9.1 | 31.1 ± 5.5 | 37.7 ± 10.9 |
| VAS (0–10) | 0 | 0 | 0 | 6.4 ± 2.4 |
| Baseline dorsum (arbitrary units of volts) | 0.28 ± 0.16 | 0.23 ± 0.12 | 0.45 ± 0.42 | 0.30 ± 0.23 |
| Baseline plantar (arbitrary units of volts) | 1.72 ± 2.07 | 1.25 ± 1.17 | 1.86 ± 1.80 | 1.23 ± 1.25 |

Data are means ± SD of anthropometric, metabolic, and neuropathy variables of subjects participating in the study. Retinopathy: N = normal, B = background, P = proliferative/laser treated. Albuminuria: N = normal albumin/creatinine ratio, m = microalbuminuric, M = macroalbuminuric. VAS: 0–10, average intensity of pain as measured by the patient. There were no significant differences between the groups in age, weight, duration of diabetes, A1C, foot temperature, and proportion of patients with retinopathy and albuminuria. There were significantly more type 2 diabetic patients in the NND group. Differences in BMI are only marginally significant between groups ($P = 0.06$). Longer duration of diabetes in patients with neuropathy is not statistically significant. The neuropathic groups (PLDN and PDN) were matched for severity of neuropathy.

(NDS) (18); *2*) sural sensory and peroneal motor and sensory nerve conduction, measured at a mean ± SD skin temperature of 32 ± 2°C at a room temperature of 22 ± 1°C, using a Dantec 2000 MC electrophysiological system with signal averaging (Dantec, Bristol, U.K.); *3*) vibration perception threshold at the great toe using the computer-assisted sensory evaluation system (CASE IV; WR Medical Electronics, Stillwater, MN); and *4*) five standard cardiac autonomic function tests, carried out with a computer-assisted technique according to the O'Brien protocol (19). Neuropathy was defined by NDS ≥4 and at least one other abnormal parameter from the following: *1*) sural nerve conduction velocity <40 m/s; *2*) peroneal nerve conduction velocity <40 m/s; *3*) vibration perception threshold >95th percentile (20); and *4*) autonomic function tests according to the O'Brien protocol (19). For inclusion in the PDN group, typical painful neuropathic symptoms with a visual analogue scale (VAS) score >4 for more than 6 months were required (21).

Four groups were studied, including one group of five nondiabetic volunteers and three carefully characterized groups of diabetic patients: 10 non-neuropathic diabetic (NND) patients, 10 with painless diabetic neuropathy (PLDN), and 8 with PDN affecting their lower limbs in a symmetrical fashion. Clinical and biochemical characteristics of the patients are provided in Table 1.

## Skin blood flow assessment

All subjects were evaluated after a 20-min acclimatization period in a room with the temperature controlled at 22 ± 1°C. Patients were made to lie down with one leg semi-flexed and supported so that the foot rested comfortably on a couch, at the level of the heart. A flat area on the dorsum of the foot was then localized and the skin temperature measured using an infrared thermometer. The skin was gently cleaned with NuPrep abrasive paste (Weaver, Aurora, CO) to lower skin resistance. Two MIC-ION 3 iontophoresis chambers were then firmly attached by means of double-sided adhesive rings, and two optic probes (DP7c; Moor Instruments, Devon, U.K.) 2 cm apart were fitted. Solutions of MIOCHOL (1% acetylcholine [Ach] wt/vol water solution) in the first chamber and sodium nitroprusside (SNP) dihydrate (20 mg, diluted in 2 ml water:1% solution) in the second chamber were used. For Ach, anodal currents were used to transfer the cation ($Ach^+$); for sodium nitroprusside ($SNP^-$), cathodal currents were used to pass the anion. After a baseline laser Doppler (MoorLAB; Moor Instruments) blood flow recording for 60 s, a continuous direct electric current stimulation was applied for 60 s at an intensity of 100 μA. After iontophoresis was stopped, the flux recording was continued for 10 min.

Reflex vasoconstriction was tested by measuring blood flow responses to maximal inspiratory gasp on the pulp of the great toe (22). The laser Doppler probe was positioned on the pulp of the great toe (right foot) and fixed using a suitable holder and a double-sided sticker. Patients were asked to rest supine on the couch and relax with closed eyes. Then the blood flux recording was started. When a stable baseline was detectable, patients were asked to take the deepest possible inspiration and then breathe normally. The flux rapidly decreased and maximal changes from baseline were recorded. The recording was continued until baseline flux was reached again.

## Data analysis

All data are means ± SD. Statistical analysis was performed using SPSS version 13 for Windows. Logarithmic transformation was used to normalize data. One-way ANOVA and Bonferroni post hoc test

**Table 2—*Response to iontophoresis of Ach***

| Group | Response (%) |
|---|---|
| Control | 757 ± 571 |
| NND | 605 ± 400 |
| PLDN | 476 ± 395 |
| PDN | 113 ± 68 |
| ANOVA *P* | 0.003 |

Data are means ± SD. Response % = maximum flux increase from baseline, expressed as a percentage. Bonferroni analysis showed the PDN group to be significantly different from control ($P = 0.014$) and NND ($P = 0.005$) but not PLDN groups (NS).

were used to compare between each group. Spearman's analysis was used to study correlation between the NDS and VAS scores and each peak response.

**RESULTS** — Subject groups were similar for age and sex composition (Table 1). NND patients had a higher proportion of type 2 diabetic patients than the other diabetic groups. There were no significant differences between the groups in age, weight, duration of diabetes, A1C, lipid profile, foot temperature, and proportion of patients with retinopathy and microalbuminuria. PLDN and PDN patients were matched for severity of neuropathy. With regard to autonomic neuropathy, 2 of 8 PDN patients and 2 of 10 PLDN patients had at least two abnormal tests according to the O'Brien protocol (21). Peroneal and sural nerve conduction velocity were reduced in both neuropathy groups. The VAS score was abnormal in PDN (6.8 ± 2.3) and was normal (0) in both PLDN and NND patients (Table 1).

### Iontophoresis responses

Tables 2 and 3 show the peak vasodilatory responses to Ach and SNP, respectively. No significant differences were observed between groups for baseline blood flow, and this was most likely due to the high coefficient of variation of 65.5%. Peak responses were significantly reduced after Ach ($P = 0.003$) but not SNP (NS) iontophoresis because of the high variability of the SNP tests. The patients with PDN had a marked reduction in the response to Ach. There was no correlation between baseline blood flow and any of the responses to iontophoresis for either Ach or SNP. Bonferroni post hoc test showed significant differences between PDN and both control ($P = 0.014$) and NND groups ($P < 0.01$), but there was no difference between PDN and PLDN.

**Table 3—*Response to iontophoresis of SNP***

| Group | Response (%) |
|---|---|
| Control | 777 ± 797 |
| NND | 685 ± 1,020 |
| PLDN | 816 ± 1,015 |
| PDN | 178 ± 174 |
| ANOVA *P* | 0.095 |

Data are means ± SD. Response % = maximum flux increase from baseline, expressed as a percentage. Bonferroni post hoc analysis was not performed, since the ANOVA *P* value was not significant.

**Table 4—*Vasoconstriction response to inspiratory gasp***

| Group | Maximum flux decrease from baseline (%) |
|---|---|
| Control | 46 ± 23 |
| NND | 45 ± 14 |
| PLDN | 46 ± 17 |
| PDN | 14 ± 9 |
| ANOVA *P* | 0.0004 |

Data are means ± SD. Response % = maximum flux decrease from baseline, expressed as a percentage. Bonferroni $P < 0.05$ for PDN vs. control; $P < 0.01$ for PDN vs. NND and PLDN. Bonferroni post hoc analysis showed the PDN group to be significantly different from control ($P = 0.007$), NND ($P = 0.002$), and PLDN ($P = 0.001$).

In all diabetic groups, there was a statistically significant inverse correlation between the NDS score and Ach peak response ($r = -0.532$, $P < 0.01$) but not with the SNP response. It was noted that, in the PDN group, both the Ach and SNP responses correlated inversely to the VAS score ($r = -0.581$, $P < 0.01$, and $r = -0.399$, $P = 0.02$, respectively).

### Vasoconstrictor response

Table 4 shows the peak vasoconstrictor response to deep inspiration in the different groups. The peak vasoconstrictor response was significantly reduced in patients with PDN compared with the other groups (ANOVA $P < 0.001$). The Bonferroni post hoc test showed that PDN was different from all other groups (control, NND, and PLDN) ($P < 0.01$). There was no correlation between NDS, postural hypotension, and the lying-to-standing blood pressure response with the vasoconstrictor response. In the PDN group, there was a significant correlation between the VAS score and the vasoconstrictor response ($r = 0.694$, $P < 0.01$).

**CONCLUSIONS** — PDN continues to pose a major clinical problem because there are few effective therapies. An understanding of the pathophysiological mechanisms underlying neuropathic pain in diabetes is therefore important. However, many previous studies have been beset by problems related to selection criteria and confounding factors such as concomitant medication. We have carefully selected patients with classic chronic distal symmetrical painful neuropathy and excluded patients with hypertension and those on vasoactive drugs. Ach and SNP iontophoresis were used to stimulate the release of endothelial-derived factors and nitric oxide, respectively, to help characterize vascular dysfunction in PDN. Endothelial dysfunction and variable responses to SNP have been previously demonstrated in diabetic and prediabetic patients (23–26). In the present study, we have extended these observations to carefully selected patients with and without PDN. We have shown a marked impairment in the cutaneous response to iontophoresed Ach in patients with PDN compared with control and NND patients but not against individuals with PLDN. However, endothelial dysfunction was associated with the severity of neuropathy, and both endothelium-dependent and -independent responses were related to the severity of pain in patients with PDN. Our findings of altered foot skin vascular reactivity as evidenced by a significantly reduced Ach-induced vasodilation and sympathetically mediated vasoconstriction may therefore be of pathophysiological relevance, not only to the etiology of diabetic neuropathy, but also PDN. With regard to the relevance of vascular dysfunction in the pathogenesis and treatment of diabetic neuropathy, Reja et al. (27) and Malik et al. (28) previously demonstrated an improvement in nerve function after treatment with ACE inhibitors in patients with diabetic neuropathy. Furthermore, even though mean SNP responses were reduced in individuals with PDN, high SDs limited the ability of this test to discriminate between the groups, and we therefore cannot exclude a type 2 error. Indeed, several previous studies with larger numbers of patients have shown a significant difference in the SNP response between patients with and without neuropathy (15,24). Additionally, other factors such as deficits in prostanoids as opposed to nitric oxide may be of importance (29).

In patients with complex regional pain syndrome, a condition in some aspects similar to PDN, Gorodkin et al. (30)

found normal Ach and SNP responses, whereas Ludwig et al. (31) recently described a reduction in Ach-induced vasodilation with normal responses to SNP. In this syndrome, the sympathetic system may play a specific pathogenetic role and has been linked to the concept of sympathetically maintained pain (32).

We studied the sympathetic system functionally and observed a normal sympathetically mediated vasoconstrictor response in NND patients and in patients with PLDN. Our novel finding is that of a markedly abnormal vasoconstrictor response in PDN. The involvement of the sympathetic system in PDN has been investigated previously by Ahlgren and Levine (33), who suggested that the sympathetic nervous system was not involved in pain generation in animal models. However, Sabroe et al. (34) demonstrated high toe blood flow in diabetic patients with both painful and painless neuropathy, suggestive of increased shunting and hence peripheral sympathetic denervation. The high blood flow could only be reduced in patients with PDN by using arousal stimuli, suggesting partially preserved sympathetic innervation. Their selection criteria were different from ours, as they found differences in foot temperature and baseline blood flow not found in our study. Tsigos et al. (35) assessed the levels of circulating norepinephrine and showed that PDN patients matched the levels seen in control patients, but the levels in PLDN were decreased, and this was attributed to increased sympathetic activity. This would be analogous to the concept of sympathetically maintained pain but has not been confirmed (32). More recently, Tack et al. (36), using tritiated norepinephrine spillover and positron emission therapy scanning, provided indirect evidence for the loss of sympathetic fibers in the feet of patients with PDN.

In the present study, we investigated sympathetically mediated peripheral vasoconstriction (37) using the inspiratory gasp, which has been recently validated (22). We found evidence of impaired peripheral sympathetic response in PDN. Such a finding would be in keeping with the study of Tack et al. (36), indirectly supporting a peripheral sympathetic deficit in PDN. We propose local sympathetic dysfunction may lead to increased cutaneous shunting and reduced dermal nutritional blood flow. Hypoxia could trigger the symptoms in patients with PDN. Although a number of other mediators undoubtedly play a role, the relief of painful symptoms obtained by local vasodilators, isosorbide dinitrate patches (9), and glyceryl trinitrate spray (10) supports the role of altered blood flow in the genesis of PDN.

Our study is limited by the small number of subjects recruited because of the stringent selection criteria, deemed essential to ensure that the observed changes in blood flow would not be altered by confounding factors, particularly vasodilator drugs. Additionally, this was not an interventional study, so we are not showing evidence that the disappearance of the symptoms coincide with an improvement in microvascular reactivity in PDN.

Laser Doppler flowmetry provides a noninvasive means to define endothelial dysfunction and sympathetic denervation, which may be important in the pathogenesis of PDN. Larger prospective studies are required to determine the temporal relationship of these changes in relation to emergence of neuropathic pain.

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