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Skin denervation in type 2 diabetes: correlations with diabetic duration and functional impairments

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Summary

Sensory neuropathy is a prominent component of diabetic neuropathy. It is not entirely clear how diabetes influences skin innervation, and whether these changes are correlated with clinical signs and laboratory findings. To investigate these issues, we performed skin biopsies on the distal leg of 38 consecutive type 2 diabetic patients with sensory symptoms in lower limbs (25 males and 13 females, aged 56.2 ± 9.4 years) and analysed the correlations of intraepidermal nerve fibre (IENF) densities in skin with glycaemic status (duration of diabetes, HbA_{1C}, and fasting and post-prandial glucose levels), and functional parameters of small fibres (warm and cold thresholds) and large fibres (vibratory threshold and parameters of nerve conduction studies). Clinically, 23 patients (60.5%) had signs of small-fibre impairment, and 19 patients (50.0%) had signs of large-fibre impairment. IENF densities were much lower in diabetic patients than in age- and gender-matched controls (1.794 ± 2.120 versus 9.359 ± 3.466 fibres/mm, $P < 0.0001$), and 81.6% (31/38) of diabetic patients had reduced IENF densities. IENF densities were negatively associated with the duration of diabetes (standardized coefficient: –0.422, $P = 0.015$) by analysis with a multivariate linear regression model. Abnormal results of functional examinations were present in 81.6% (warm threshold), 57.9% (cold threshold), 63.2% (vibratory threshold) and 49% (amplitude of sural sensory action potential) of diabetic patients. Among the three sensory thresholds, the warm threshold temperature had the highest correlation with IENF densities (standardized coefficient: –0.773, $P < 0.0001$). On nerve conduction studies in lower-limb nerves, there were abnormal responses in 54.1% of sural nerves, and 50.0% of peroneal nerves. Of neurophysiological parameters, the amplitude of the sural sensory action potential had the highest correlation with IENF density (standardized coefficient: 0.739, $P < 0.0001$). On clinical examination, 15 patients showed no sign of small-fibre impairment, but seven of these patients had reduced IENF densities. In conclusion, small-fibre sensory neuropathy presenting with reduced IENF densities and correlated elevation of warm thresholds is a major manifestation of type 2 diabetes. In addition, the extent of skin denervation increases with diabetic duration.

Keywords: diabetic neuropathy; epidermal nerves; skin biopsy; ubiquitin; quantitative sensory testing; nerve conduction studies

Abbreviations: 95% CI = 95% confidence interval; CMAP = compound muscle action potential; IENF density = intraepidermal nerve fibre density; NCV = nerve conduction velocity; PGP 9.5 = protein gene product 9.5; QST = quantitative sensory testing; SAP = sensory action potential

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Introduction

Diabetes mellitus is a common cause of peripheral nerve disorders in adults (Singleton *et al.*, 2001). Among different subtypes of diabetic neuropathy, sensory neuropathy is a frequent cause of painless injury, and is related to various complications including the necessity for the amputation of limbs (McNeely *et al.*, 1995; Adler *et al.*, 1999). Thus, early detection of sensory nerve impairment is an important issue and a great challenge in evaluating diabetic neuropathy (Dyck

Table 1 *Sensory manifestations of diabetic patients*

Sensory symptom	*n* (%)	Neurological sign	*n* (%)
Character		Small-fibre signs	23 (60.5)
Paraesthesia	38 (100)	Impairment of sensation to pinprick	19 (50.0)
Painful neuropathy	9 (23.7)	Impairment of sensation to heat	15 (39.5)
Distribution		Impairment of sensation to cold	14 (36.8)
Sensory pattern A	13 (34.2)	Large-fibre signs	19 (50.0)
Sensory pattern B	18 (47.4)	Impairment of vibratory sense	9 (23.7)
Sensory pattern C	6 (15.8)	Impairment of joint senses	11 (28.9)
Sensory pattern D	1 (2.6)		

Patterns of sensory symptoms: A, toes only; B, toes + feet; C, B + distal leg; D, C + entire leg or hands. Clinical signs: small-fibre type (impairment of at least one sensation to pinprick, warm stimulus and cold stimulus); large-fibre type (impairment of at least one kinaesthetic sensation).

et al., 1999; Polydefkis *et al.*, 2003). The perception of nociceptive stimuli begins with sensory receptors in the skin with unmyelinated sensory nerves terminating in the epidermis (Kennedy and Wendelschafer-Crabb, 1993; McCarthy *et al.*, 1995; Hsieh *et al.*, 2000). Evaluation of these sensory structures in the skin should be useful for diagnosis of diabetic sensory neuropathy. Epidermal nerves in the skin are readily demonstrated by immunohistochemistry with various neuronal markers, particularly protein gene product 9.5 (PGP 9.5), a ubiquitin C-terminal hydrolase (Wilson *et al.*, 1988; Karanth *et al.*, 1991; Kennedy and Wendelschafer-Crabb, 1993; Periquet *et al.*, 1999; Griffin *et al.*, 2001). Sensory nerve terminals in the epidermis of the skin degenerate after cutaneous denervation by mechanical or chemical insults, and epidermal nerve fibres are depleted earlier than their nerve trunks in the dermis (Hsieh *et al.*, 2000; Rajan *et al.*, 2003). Skin biopsy together with quantification of epidermal nerve fibres has therefore become a novel pathological approach to diagnose small-fibre sensory neuropathy (Kennedy and Said, 1999; Griffin *et al.*, 2001).

Intraepidermal nerve fibre (IENF) densities are reduced in patients with impaired glucose tolerance and clinically overt diabetes (Levy *et al.*, 1989; Kennedy and Wendelschafer-Crabb, 1996; Smith *et al.*, 2001; Sumner *et al.*, 2003). These results clearly suggest that epidermal nerves are affected in diabetes, and skin biopsy is useful in evaluating small-fibre sensory neuropathy of diabetes (Kennedy and Wendelschafer-Crabb, 1999; Polydefkis *et al.*, 2001). Apparently, epidermal denervation is related to diabetic status. However, there is a lack of direct evidence regarding the correlation between epidermal denervation and diabetic parameters, such as diabetic duration and hyperglycaemic control.

Small-diameter sensory nerves are presumably responsible for detection of thermal stimuli to the skin, and thermal thresholds are altered in diabetic subjects (Dyck *et al.*, 2000). Quantitative sensory testing examines the function of the thermal stimuli-detecting system, but provides limited information regarding the pathology of small-diameter nerves. It remains unanswered whether changes in IENF densities parallel changes in thermal thresholds in diabetic patients (Kennedy *et al.*, 1996; Hirai *et al.*, 2000; Yasuda *et al.*, 2000; Sumner *et al.*, 2003).

Sensory neuropathies can be classified as large-fibre type and small-fibre type according to clinical signs. In addition to clinical assessment, nerve conduction studies are performed to evaluate large-fibre neuropathy, and skin innervation can be used as a parameter of small-fibre sensory neuropathy (Sumner *et al.*, 2003). Diabetic neuropathy is conceptually considered a mixed neuropathy of both large- and small-fibre types (Thomas and Tomlinson, 1993). It would be intriguing to investigate whether large- and small-diameter nerves are affected in diabetes to similar degrees, and how clinical assessments and laboratory diagnoses are related.

To address the issues of small-diameter sensory nerve degeneration and the extent of large- and small-fibre neuropathies in human diabetes, we studied diabetic patients by evaluating skin innervation, measuring sensory thresholds with quantitative sensory testing, and comparing results from nerve conduction studies.

Material and methods

Patients and control subjects

The study population exclusively consisted of diabetic patients with sensory symptoms referred to an out-patient neuropathy clinic of National Taiwan University Hospital, Taipei, Taiwan between January 2000 and August 2001. Symmetric sensory symptoms in the foot with a graded stocking pattern of distribution were the prominent feature of all these patients. All patients were able to ambulate without clinical weakness or signs of injury. Neurological examinations followed routine procedures, including detailed examinations of sensations to hot, cold, vibratory and kinaesthetic stimuli. Analysis of these signs was modified from several clinical forms, including the Neuropathy Symptom Score, Neuropathy Disability Score and Total Neuropathy Score (Dyck *et al.*, 1980; Cornblath *et al.*, 1999), and results are listed in Table 1. Painful neuropathy was defined as burning or shooting pains disturbing sleep and work activities (Eaton *et al.*, 2003). No medication was given for painful neuropathy before neurophysiological, psychophysical and pathological evaluations. Hypoglycaemic or insulin-induced neuropathy was excluded by (i) a history of hypoglycaemic events and cerebral manifestations; and (ii) the absence of muscle weakness

(Thomas and Tomlinson, 1993). All patients completed an initial evaluation that revealed no history of familial neuropathy or toxin exposure, and normal results on blood tests including electrolytes, liver function, complete blood count, thyroid function tests, vitamin B_{12} and folic acid levels, and serum protein electrophoresis with immunofixation analysis.

A diagnosis of type 2 diabetes was based on the revised American Diabetes Association recommendations (American Diabetes Association, 1997). These patients were regularly followed-up and received oral hypoglycaemic agents and/or insulin. Patients usually visited the diabetic clinic once every 1–3 months for adjustment of diabetic control by regular measurements of fasting and 2 h post-prandial glucose levels and glycated haemoglobin (HbA_{1C}). Data of hyperglyacemic control 3 years before the skin biopsy were analysed for patients with diabetic duration longer than 3 years. For those patients with diabetic duration shorter than 3 years, all glycaemic data before the skin biopsy were collected for analysis.

There were 38 diabetic patients (25 males and 13 females), aged 56.2 ± 9.4 years (range, 36–72) with a diabetic duration of 10.55 ± 7.20 years (range, 1–23). The wide range reflected the various sources of referral and the diversity of patients. Subjects in the control group were recruited from a previously described cohort (Pan *et al.*, 2001*a*) matched by gender and age (stratified by decade), including 25 males and 13 females, aged 55.5 ± 11.0 years (range, 31–73).

Skin biopsy

A skin specimen of 3 mm in diameter was taken with a biopsy punch from the lateral side of the distal leg under 2% lidocaine local anaesthesia (Chien *et al.*, 2001). All subjects tolerated the procedure with no obvious signs of discomfort. No suturing was required, and the wounds were covered with a piece of gauze. Wound healing took 7–10 days, similar to a typical abrasion wound. The Ethics Committee of National Taiwan University Hospital had approved this study. Informed consent was obtained from each patient before the skin biopsy.

Immunohistochemistry

For immunohistochemistry on microtome sections (Hsieh and Lin, 1999), skin tissues were fixed with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS) pH 7.4 for 48 h. Sections at 50 µm were quenched with 1% H_2O_2, blocked with 5% normal goat serum, and incubated with rabbit antiserum to PGP 9.5 (UltraClone, UK, diluted 1 : 1000 in 1% normal serum/Tris) at 4°C for 16–24 h. After rinsing in Tris, sections were incubated with biotinylated goat anti-rabbit immunoglobulin G at room temperature for 1 h, followed by incubation with the avidin–biotin complex (Vector, Burlingame, CA) for another hour. The reaction product was demonstrated with chromogen SG (Vector), and counterstained with eosin (Sigma, St Louis, MO).

Quantification of epidermal innervation

Epidermal innervation was quantified following established protocols, and slides were coded to ensure that measurements were blinded (Chiang *et al.*, 1998). PGP 9.5-immunoreactive nerve fibres in the epidermis of each section were counted at a magnification of 40× with an Olympus BX40 microscope (Tokyo, Japan) through the depth of the entire section. Each individual nerve fibre with branching points inside the epidermis was counted as one. For epidermal nerve fibres with branching points in the dermis, each individual nerve fibre was counted separately. The length of the epidermis along the upper margin of the stratum corneum in each section was measured using Image-Pro PLUS software (Media Cybernetics, Silver Spring, MD). IENF density was derived and expressed as fibres/mm. For each tissue, there were 48–50 sections after sectioning, and all sections were labelled sequentially. Every fourth section of each tissue sample was immunostained. The mean of epidermal nerve fibre densities of the stained sections was defined as the IENF density of that tissue specimen. In the distal leg, normative values of IENF density from our laboratory were 11.16 ± 3.70, 5.88 and 4.2 fibres/mm (mean ± SD, the 5th percentile value, and the 1st percentile value, respectively) for those aged <60 years, and 7.64 ± 3.08, 2.50 and 2.2 fibres/mm for those aged 60 years. These values are similar to those of McCarthy *et al.* with the same staining methods and quantitation criteria (McCarthy *et al.*, 1995, 1998; Chien *et al.*, 2001). The cut-off point of IENF density was 5.88 and 2.50 fibres/mm according to the respective age group, and skin denervation was defined as an IENF density lower than the cut-off value for that patient.

Quantitative sensory testing

We performed quantitative sensory testing (QST) with a Thermal Sensory Analyzer and Vibratory Sensory Analyzer (Medoc Advanced Medical System, Minneapolis, MN) to measure sensory thresholds of warm, cold and vibratory sensations. The facilities and procedures were described previously (Yarnitsky and Ochoa, 1991; Pan *et al.*, 2001*a*, 2003). Maintenance procedures followed the manufacturer's suggestions and were done every 4 weeks, including filling water and adjustment of pump voltage. The analyser was calibrated with two methods every 2 months: (i) using the default test programs of the sensory analyser, including the patient response unit test programme and the thermode test program; and (ii) measuring the surface temperature of the thermode stimulator by attaching an MC 8700 type digital thermometer (Exacon A/S, Roskilde, Denmark) to the thermode.

The stimulator was applied to the skin of the foot dorsum for thermal and vibratory stimuli. The examiner explained the procedures to the subjects, and the subjects underwent several trials to become familiar with the test.

We used two testing strategies: the method of limits and the method of level. Results of these two algorithms were correlated; correlation coefficients were 0.89–0.92 among different sensory modalities (Lin *et al.*, 1998). The method of level is independent of reaction time. Briefly, the sensory analyser delivered a stimulus of constant intensity set by the algorithm. The intensity of the next stimulus was either increased or decreased by a fixed ratio according to the response of the subject, i.e. whether or not the subject had perceived the stimulus. Such procedures were repeated until a predetermined difference in intensity was reached. The mean intensity of the final two stimuli was the threshold for the level method. Thermal thresholds were expressed as a warm threshold temperature and cold threshold temperature. Vibratory thresholds were measured with similar algorithms, and are expressed in micrometres.

We have established normative data for Taiwanese using descriptive functions, including percentiles, of the statistical software SPSS (Chicago, IL). This database was set up initially in 1996, and has been updated every 6 months. Currently, there are data

on >400 normal Taiwanese subjects in the database. A staff neurologist had examined each subject of the control database to ensure the absence of neurological symptoms and signs. Systemic diseases were excluded by laboratory examinations including plasma glucose levels and kidney and liver functions. We previously compared the normative values of this database with those of other databases, and no significant ethnic differences were detected (Yarnitsky and Ochoa, 1991; Yarnitsky and Sprecher, 1994; Yarnitsky, 1997; Lin *et al.*, 1998). The normative values of this database are listed in the Appendix. Cut-off values were defined as the 95th percentile value (for warm threshold and vibratory threshold) and the 5th percentile value (for cold threshold). Thresholds beyond the cut-off value were considered abnormal.

Nerve conduction studies

Nerve conduction studies were performed with a Nicolet Viking IV Electromyographer (Madison, WI) in all patients following standardized methods recommended by the Consensus Development Conference on Standardized Measures in Diabetic Neuropathy (Consensus Development Conference, 1992; Pan *et al.*, 2003). Amplitudes of the sensory action potential (SAP) and compound muscle action potential (CMAP) as well as the nerve conduction velocity (NCV) were recorded for analysis. Studied nerves included sural, peroneal and median (motor and sensory) nerves. Criteria for abnormality of each individual nerve were based on the lower limits of amplitudes and conduction velocities of SAP and CMAP (Pan *et al.*, 2001*b*).

Statistical analysis

Numerical variables are expressed as the mean ± SD, and compared with *t* tests if the data followed a Gaussian distribution. In the first step, graphic evaluation of the correlations between variables was analysed with the slope of the regression line, including the 95% confidence interval (95% CI), using GraphPad Prism (GraphPad Software, San Diego, CA). The correlation was explored further with multiple linear regression analysis using SPSS software. To evaluate the influence of diabetes on skin innervation, IENF density was set as the dependent variable. Age, gender and diabetic parameters (including diabetic duration, fasting and post-prandial glucose levels, and HbA_{1C}) were independent variables. To understand whether sensory thresholds were correlated with IENF densities in diabetic patients, IENF density, age, gender and significant diabetic parameters from the previous analysis were independent variables. Each sensory threshold was a dependent variable. In addition to entering all independent variables for analysis, forward and backward stepwise linear regressions were applied. The significance of each model was determined by R^2 (goodness-of-fit for the model), and the significance of each variable in the tested model was judged by the coefficient for that variable (standardized coefficients β, *t,* and *P*). Results were considered significant at $P \leqslant 0.05$.

Results

Clinical features of diabetic patients

All 38 patients had constant, symmetric paraesthesia in the lower limbs but with various distributions: limited to the toes in 13 patients (34.2%), involving the entire foot in 18 patients (47.4%), affecting the foot and the distal leg in six patients (15.8%), and affecting the leg and foot in one patient (2.6%) (Table 1). None had motor weakness as assessed by neurological examinations. Ankle jerk was absent in 11 patients (28.9%); and ankle jerk and knee jerk were absent in nine of those patients (23.7%). Generalized areflexia was noted in seven patients (18.4%). Nine patients (23.7%) had painful neuropathy, such as burning and shooting pain. Patients without painful neuropathy tended to have distribution of sensory symptoms limited to the toes compared with patients with painful neuropathy ($P < 0.0072$). Painful neuropathy was not related to diabetic duration ($P = 0.54$).

Clinically, sensory neuropathy according to signs on neurological examinations was divided into small-fibre type (impairment of at least one sensation to pinprick, warm stimulus and cold stimulus), and large-fibre type (impairment of at least one kinaesthetic sensation). Twenty-three patients (60.5%) had signs of small-fibre impairment, and 19 patients (50.0%) had signs of large-fibre impairment. Ten patients (26.3%) had no clinical sign of either type: eight of these had symptoms limited to the toes, and the other two patients had symptoms in the foot.

Skin innervation in diabetic patients

In normal skin, typical epidermal nerve fibres immunoreactive for PGP 9.5 ascended through the epidermal–dermal border, occasionally with a varicose appearance (Fig. 1A). PGP 9.5 (+) nerves formed dense subepidermal nerve plexuses in control subjects. In the epidermis of diabetic patients, the abundance of epidermal nerves was markedly reduced. In many patients, the skin was completely denervated (Fig. 1B).

In the dermis of normal skin, individual dermal nerves with dense immunoreactivities in linear patterns were grouped together as nerve bundles (Fig. 1C). The immunoreactive pattern of dermal nerves became fragmented in the dermis of diabetic patients, suggesting nerve degeneration (Fig. 1D).

Quantitative pathology in diabetic skin

To quantify the pathology of epidermal innervation, we compared IENF densities of diabetic patients with those of age- and gender-matched normal subjects serving as controls. IENF densities of diabetic patients were significantly lower than those of age- and gender-matched control subjects (1.794 ± 2.120 versus 9.359 ± 3.466 fibres/mm, $P < 0.0001$, Fig. 2), with 81.6% (31 out of 38) of diabetic patients having IENF densities lower than the 5th percentile value of the norm (Fig. 3).

To explore further the influence of diabetes on skin innervation, we plotted the relationship between IENF densities and diabetic parameters (Fig. 4). IENF densities were negatively correlated with diabetic duration (slope = –0.1220 ± 0.052, 95% CI = –0.2272 to –0.0169, $P = 0.024$, Fig. 4A). Other parameters (HbA_{1C}, fasting and post-prandial

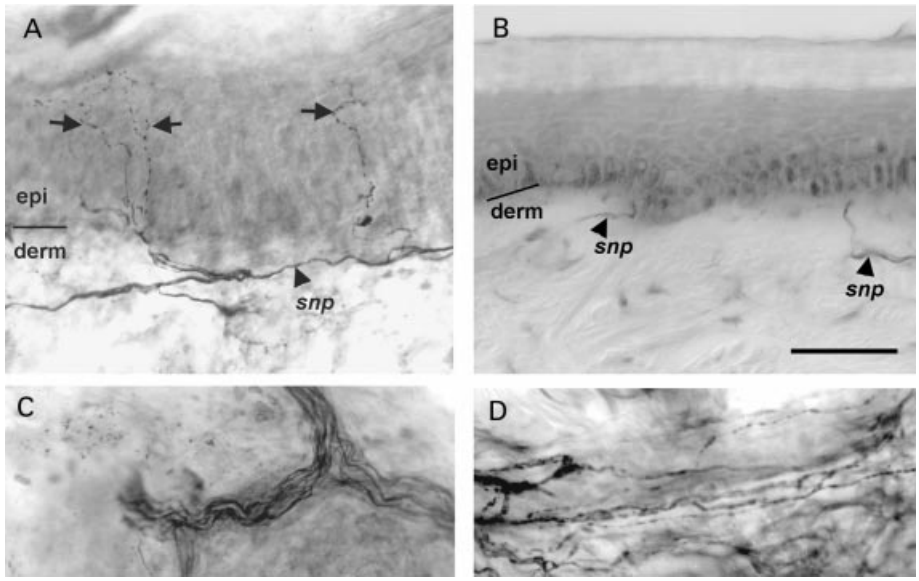

Fig. 1 Skin innervation in diabetic patients. Skin tissues from control (**A** and **C**) and diabetic (**B** and **D**) subjects were immunostained with protein gene product 9.5 (PGP 9.5). The boundary between the epidermis (epi) and the dermis (derm) is marked by a line. (**A**) In the skin of a normal subject, PGP 9.5 (+) nerves appear in the epidermis and dermis. Typical epidermal nerves (arrows) arise from the subepidermal nerve plexuses (snp). (**B**) In the skin of a diabetic patient, the epidermis is completely denervated. The staining of the subepidermal nerve plexus (snp) has become faint, and the number of dermal nerve fascicles is markedly reduced. (**C**) In the deep dermis of normal skin, dermal nerve fascicles exhibit a pattern of linear and dense staining. (**D**) In the deep dermis of diabetic skin, individual nerves are usually separated and have become fragmented (bar = 80 μm).

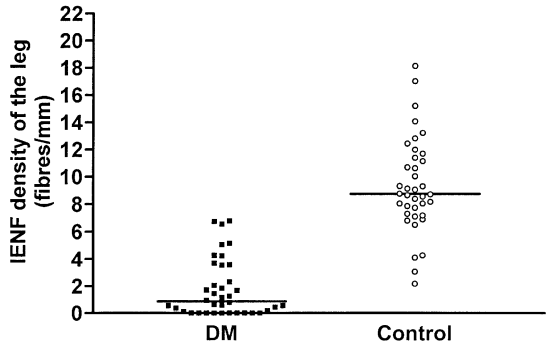

Fig. 2 Epidermal innervation in diabetes. The intraepidermal nerve fibre density (IENF density) of the leg in diabetic patients (filled squares) is markedly reduced compared with that in age- and gender-matched control subjects (open circles) (1.794 ± 2.120 versus 9.359 ± 3.466 fibres/mm, $P < 0.0001$) (bar = mean value).

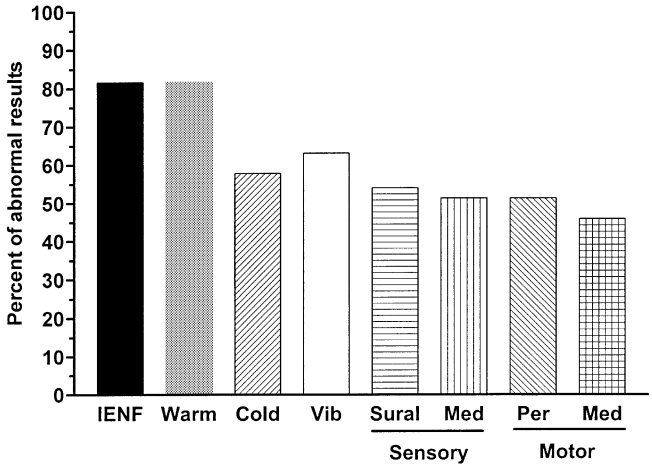

Fig. 3 Comparison of abnormal rates on various examinations in diabetic patients. The graph shows the proportion of diabetic patients with abnormal results on the intraepidermal nerve density of the leg (IENF); warm threshold at the foot dorsum (Warm); cold threshold at the foot dorsum (Cold); vibratory threshold (Vib); and nerve conduction studies on motor nerves (Motor) and sensory nerves (Sensory). The latter include sural (Sural), peroneal (Per) and median (Med) nerves.

glucose levels) were not linearly correlated with IENF densities (Fig. 4B–D).

Because age and gender influence IENF densities in normal subjects (McArthur *et al.*, 1998), we performed a multiple linear regression analysis (Table 2). Diabetic duration was significantly associated with IENF density after age and gender were controlled for, and had a standardized coefficient of –0.422 ($P = 0.015$). The standardized coefficient of post-prandial glucose level with IENF density was 0.367, but this value did not reach statistical significance ($P = 0.056$).

Thermal thresholds in diabetes

To understand the functional correlations of cutaneous nerve degeneration, sensory thresholds of various modalities in diabetic patients were compared with those of age- and gender-matched control subjects. With regard to the functions

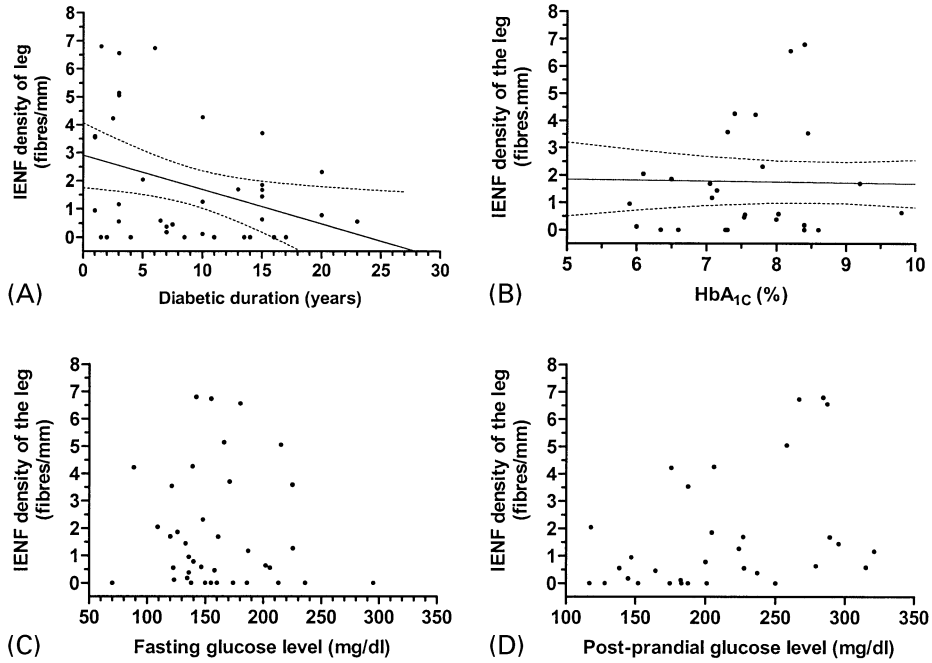

Fig. 4 Relationships between intraepidermal nerve fibre densities (IENF densities) of the leg and diabetic parameters. IENF densities are plotted with diabetic duration (**A**), HbA_{1C} (**B**), fasting glucose level (**C**) and post-prandial glucose level (**D**). The linear regression line is only significant for IENF density and diabetic duration (slope = –0.1220 ± 0.052, $P = 0.024$) (solid line = regression line; dotted lines = 95% confidence interval).

Table 2 *Intraepidermal nerve fibre density and diabetic parameters*

Model: R^2, P	Standardized coefficients β, t, P		
	Diabetic parameter	Age	Gender
A: 0.236, 0.042*	Diabetic duration; –0.422, –2.580, 0.015*	0.261, 1.624, 0.115	–0.199, –1.211, 0.235
B: 0.072, 0.460	HbA_{1C}; 0.004, 0.020, 0.984	0.237, 1.258, 0.219	–0.154, –0.826, 0.416
C: 0.058, 0.562	AC glucose; –0.037, 0.220, 0.827	–0.152, –0.911, 0.369	0.190, 1.123, 0.269
D: 0.201, 0.127	PC glucose; 0.367, 2.002, 0.056	–0.283, –1.572, 0.129	0.061, 0.332, 0.743

Model: IENF density as the dependent variable with a diabetic parameter (diabetic duration, HbA_{1C}, AC glucose or PC glucose), age and gender as independent variables; i.e. in model A with diabetic duration, age and gender; in model B with HbA_{1C} age and gender; in model C with AC glucose, age and gender; in model D with PC glucose, age and gender. Standardized coefficient β with t and P for each independent variable; AC glucose = fasting glucose; PC glucose = 2 h post-prandial glucose. *Statistically significant.

of small-diameter sensory nerves, diabetic patients had abnormal thresholds to warm and cold stimuli. Warm threshold temperatures of the foot dorsum (the method of level) were significantly higher in diabetic patients than in control subjects (44.43 ± 4.26 versus 37.58 ± 1.60°C, $P < 0.0001$, Fig. 5A). In summary, 81.6% of diabetic patients had elevated warm thresholds (Fig. 3). A similar trend was observed by using the method of limits, with higher warm threshold temperatures in diabetes than in control subjects (45.94 ± 3.87 versus 39.25 ± 2.32°C, $P < 0.0001$).

Diabetic patients also had reduced cold threshold temperatures (the method of level) in the foot dorsum (22.66 ± 11.02 versus 30.31 ± 0.79°C, $P = 0.001$, Fig. 5B). The abnormal rate of altered cold thresholds in the diabetic group was 57.9% (Fig. 3). Cold threshold temperatures measured by the method of limits were also markedly reduced in diabetic patients compared with normal subjects (20.07 ± 11.02 versus 28.50 ± 1.88°C, $P < 0.0001$).

Large-fibre neuropathy in diabetes

To evaluate the function and physiology of large-diameter nerves, we compared the results of vibratory thresholds and nerve conduction studies between diabetic and control subjects. Vibratory thresholds (the method of level) were higher in diabetic patients than in control subjects (25.34 ± 33.93 versus 4.10 ± 1.64 μm, $P <$ 0.0001, Fig. 5C), with 63.2% of diabetic patients having elevated vibratory thresholds (Fig. 3). Similarly, vibratory thresholds using the method of limits were elevated in diabetic patients

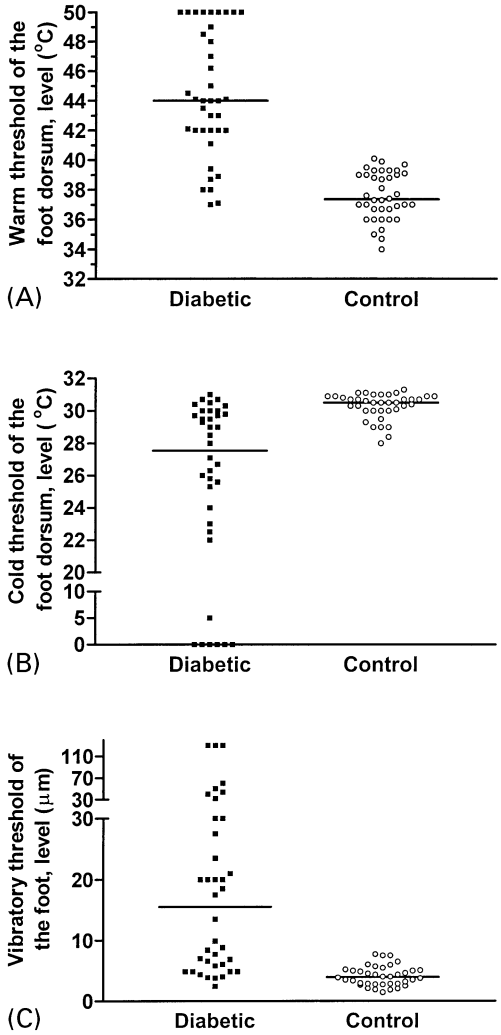

Fig. 5 Sensory thresholds in diabetic patients. Thresholds were measured using the algorithm of level. (**A**) Warm threshold temperatures of diabetic patients were higher than those of age- and gender-matched control subjects (44.43 ± 4.26 versus 37.58 ± 1.60°C, $P < 0.0001$). (**B**) Cold threshold temperatures of diabetic patients were much lower than those of age- and gender-matched control subjects (22.66 ± 11.02 versus 30.31 ± 0.79°C, $P = 0.001$). (**C**) Vibratory thresholds were higher in diabetic patients than in control subjects (25.34 ± 33.93 versus 4.10 ± 1.64 μm, $P < 0.0001$) (bar = mean value).

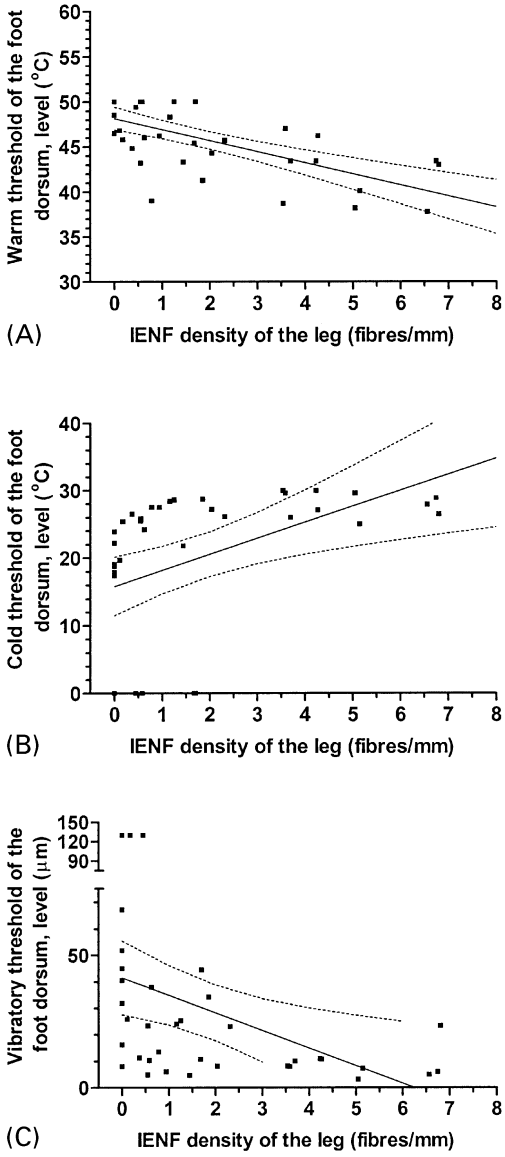

Fig. 6 Correlations between sensory thresholds and intraepidermal nerve fibre densities (IENF densities). Each sensory threshold is plotted against IENF densities: (**A**) warm threshold, (**B**) cold threshold and (**C**) vibratory threshold. All sensory thresholds are linearly correlated with IENF densities (solid line = regression line; dotted lines ± 95% confidence interval).

Table 3 *Findings of nerve conduction studies in diabetic patients*

Nerve	Amplitude		Velocity (m/s)		Abnormal rate (%)
	Control	Diabetes	Control	Diabetes	
Sural nerve (sensory)	17.93 ± 6.16	7.54 ± 6.03*	54.9 ± 5.23	37.2 ± 19.0*	54.1
Peroneal nerve (motor)	5.67 ± 2.47	3.34 ± 2.41*	50.1 ± 3.5	38.8 ± 11.7*	51.4
Median nerve (sensory)	39.32 ± 13.09	23.59 ± 14.66*	58.7 ± 8.1	49.0 ± 7.8*	51.4
Median nerve (motor)	8.75 ± 1.88	7.19 ± 1.98*	56.2 ± 3.6	49.1 ± 5.1*	45.9

Amplitude = amplitudes of sensory action potential for sensory nerves (μV), or compound muscle action potential for motor nerves (mV).
*Statistically significant reduction compared with the control group.

compared with control subjects (29.24 ± 34.00 versus 6.55 ± 2.81 μm, $P < 0.0001$).

In nerve conduction studies, both amplitude and conduction velocity of motor and sensory nerves in diabetic patients were significantly reduced compared with age- and gender-matched control subjects (Table 3). Consistent with previous findings, a trend for higher abnormal rates was noted in lower-limb nerves versus upper-limb nerves, and in sensory nerves versus motor nerves (Albers *et al.*, 1996). Although this difference was small due to the small sample size, the findings indicated that the current study group was not a biased population.

Parallel changes in IENF density with sensory thresholds and neurophysiological parameters

To investigate whether sensory thresholds paralleled IENF densities in diabetes, each sensory threshold was plotted against the IENF density (Fig. 6). All sensory thresholds were linearly correlated with IENF densities with a slope of –1.226 ± 0.225 (95% CI = –1.683 to –0.768, $P < 0.0001$) for the warm threshold (Fig. 6A), 2.375 ± 0.771 (95% CI = 0.8113–3.939, $P = 0.0039$) for the cold threshold (Fig. 6B), and –6.633 ± 2.458 (95% CI = –11.630 to –1.640, $P = 0.0106$) for the vibratory threshold (Fig. 6C).

Because diabetic duration was significantly associated with IENF densities, the correlation between sensory thresholds and IENF density was refined using multiple linear regression analysis (Table 4). After controlling for diabetic duration, age and gender, IENF densities were linearly correlated with all these sensory thresholds, particularly the warm threshold of the foot dorsum (standardized coefficient: –0.773 for the method of level, and –0.779 for the method of limits, $P < 0.0001$). A similar analysis was performed for parameters of nerve conduction studies (Table 5). Among these, sural SAP amplitude had the highest standardized coefficient (0.739, $P < 0.0001$).

Comparison of clinical and laboratory assessments

To understand the clinical value of skin biopsy, we compared the relationship between clinical and laboratory assessments (Table 6). We employed objective parameters as criteria of laboratory neuropathy: reduced IENF density for small-fibre neuropathy, and reduced sural SAP amplitude and/or NCV for large-fibre neuropathy. A substantial proportion of patients (seven out of 15) had subclinical small-fibre neuropathy on laboratory assessment: two from the subgroup of 10 patients without clinical signs, and five of all patients with signs of large-fibre impairment only.

We then examined the data to see whether laboratory parameters were related to symptoms (Table 6). The biopsy sites were at symptomatic areas of seven patients (Table 1). In the 10 patients without clinical signs, eight had symptoms limited to toes, and symptoms of the other two patients were limited to the foot. For patients with symptoms extending to the leg, IENF densities were much lower than those with symptoms limited to the toes and feet ($P = 0.0116$), as was the sural SAP amplitude ($P = 0.0034$) (Table 7). The presence of neuropathic pain, however, was not correlated with IENF density or sural SAP amplitude ($P = 0.1137$ and 0.1962, respectively) (Table 7).

Discussion

The present study demonstrates significant skin denervation in type 2 diabetes, occurring in 81% of the current study group. Epidermal denervation was associated with pathological evidence of dermal nerve degeneration. The degree of epidermal denervation was negatively associated with diabetic duration. In this group of diabetic patients, IENF densities in the leg were highly correlated with warm thresholds of the foot; the high proportion of skin denervation together with functional impairments indicates that small-fibre sensory neuropathy is a major manifestation of diabetic neuropathy.

Epidermal denervation in diabetic skin

Skin biopsy has become a new approach to investigate small-fibre sensory neuropathy (Kennedy and Said, 1999; Polydefkis *et al.*, 2003). The current study focused on skin innervation and parallel changes in psychophysical parameters. Characteristic findings in diabetic skin include marked denervation of the epidermis, a decrease in subepidermal nerve abundance, and the presence of fragmented dermal nerves, indicating ongoing nerve degeneration (Hsieh *et al.*, 2000; Pan *et al.*, 2003). These pathological hallmarks clearly suggest that nerve degeneration is responsible for epidermal denervation and that small-diameter sensory neuropathy is a major manifestation of diabetic neuropathy. This finding extends previous observations of small-fibre sensory neuropathy in diabetes; for example, epidermal nerve densities and lengths are reduced in type 1 diabetes (Kennedy and Wendelschafer-Crabb, 1996). The reduction in epidermal nerve density is an early event in diabetes, as demonstrated in recent studies on subjects with impaired glucose tolerance and clinical diabetes (Smith *et al.*, 2001; Sumner *et al.*, 2003). This is in contrast to another report indicating that epidermal innervation is increased in the early stages of type 1 diabetes (Properzi *et al.*, 1993). Despite quantitative changes in epidermal innervation, several issues remain to be explored: for example, direct evidence of dermal nerve degeneration as a cause of epidermal denervation, relationships to hyperglycaemic control, and functional correlations of epidermal denervation. The current study suggests that morphometric changes in epidermal innervation may be related to the progression of neuropathy, as recently demonstrated in

Table 4 *Correlation of intraepidermal nerve fibre density with sensory thresholds*

Functional parameter		Standardized coefficients β, *t*, *P*			
Model	R^2, *P*	IENF density	Diabetic duration	Age	Gender
Warm threshold, level	0.500, <0.0001*	–0.773, –5.485, <0.0001*	0.304, 2.151, 0.039*	–0.031, –0.240, 0.812	–0.181, –1.374, 0.179
Cold threshold, level	0.386, 0.002*	0.414, 2.648, 0.012*	0.056, 0.361, 0.720	0.400, 2.813, 0.008*	0.121, 0.828, 0.414
Vibratory threshold, level	0.253, 0.061	–0.566, –3.028, 0.005*	–0.154, –0.824, 0.417	0.159, 0.952, 0.349	–0.050, –0.287, 0.776
Warm threshold, limits	0.511, <0.0001*	–0.779, –5.590, <0.0001*	–0.259, –1.854, 0.073	–0.026, –0.206, 0.838	–0.132, –1.009, 0.320
Cold threshold, limits	0.381, 0.003*	0.393, 2.505, 0.017*	–0.013, –0.081, 0.936	0.400, 2.804, 0.008*	0.101, 0.687, 0.497
Vibratory threshold, limits	0.222, 0.082	–0.536, –2.899, 0.007*	–0.261, –1.428, 0.163	0.045, 0.270, 0.789	–0.082, –0.475, 0.638

Model: each functional parameter as dependent variable, with IENF density, diabetic duration, age and gender as independent variables. For example, in the first model: with warm threshold temperature of the foot dorsum as the dependent variable, and with IENF density, diabetic duration, age and gender as independent variables. Level: method of level; limits: method of limits. Standardized coefficient β with *t*, and *P* for each independent variable. *Statistically significant.

Table 5 *Correlation of electrophysiological parameters with intraepidermal nerve fibre density*

Functional parameter		Standardized coefficients β, *t*, *P*			
Model	R^2, *P*	IENF density	Diabetic duration	Age	Gender
Sural SAP	0.661, <0.0001*	0.739, 6.253, <0.0001*	–0.043, 0.358, 0.723	–0.251, 2.342, 0.026*	0.201, 1.816, 0.079
Sural SNCV	0.507, <0.0001*	0.497, 3.494, 0.01*	–0.060, –0.481, 0.679	0.366, 2.831, 0.008	0.220, 1.647, 0.109
Peroneal CMAP	0.674, 0.001*	0.648, 4.324, <0.0001*	0.065, 0.434, 0.667	0.156, 1.145, 0.261	0.179, 1.275, 0.211
Peroneal MNCV	0.541, 0.022*	0.447, 2.619, 0.013*	0.031, 0.180, 0.859	0.206, 1.329, 0.193	–0.113, –0.709, 0.483
Median SAP	0.771, <0.0001*	0.638, 4.971, <0.0001*	–0.101, –0.803, 0.428	–0.273, 2.323, 0.027*	0.020, 0.169, 0.867
Median SNCV	0.565, 0.013*	0.464, 2.792, 0.009*	–0.046, –0.280, 0.781	0.203, 1.334, 0.192	0.174, 1.132, 0.266
Median CMAP	0.437, 0.137	0.395, 2.178, 0.037*	–0.019, –0.106, 0.916	–0.071, –0.430, 0.670	0.265, 1.579, 0.124
Median MNCV	0.607, 0.004*	0.447, 2.794, 0.009*	–0.132, –0.836, 0.409	0.263, 1.797, 0.082	–0.021, –0.144, 0.887

Model: each functional parameter as dependent variable, with IENF density, diabetic duration, age and gender as independent variables. For example, in the first model: with amplitude of sural sensory action potential (SAP) as the dependent variable, and with IENF density, diabetic duration, age and gender as independent variables. Standardized coefficient β with *t*, and *P* for each independent variable. SAP = amplitude of sensory action potential; SNCV = sensory nerve conduction velocity; CMAP = amplitude of compound muscle action potential; MNCV = motor nerve conduction velocity. *Statistically significant.

Table 6 *Comparison between clinical and laboratory assessments*

Type	No.	Small-fibre	Large-fibre	Combined
Clinical signs	10 (26.3)*	9 (23.7)	5 (13.2)	14 (36.8)
Laboratory neuropathy	8 (21.1)	11 (28.9)	0 (0)	19 (50.0)

**n* (%). Clinical signs: small-fibre type (impairment of at least one sensation to pinprick, warm stimulus and cold stimulus); large-fibre type (impairment of at least one kinaesthetic sensation) as in Table 1. Laboratory neuropathy: small-fibre type (reduced IENF densities); large-fibre type (reduced sural nerve amplitude and/or velocity).

Table 7 *Relationship between intraepidermal nerve fibre density and amplitude of sural sensory action potential with clinical parameters*

		IENF density (fibres/mm)		Sural SAP amplitude (μV)	
		Median (range)	*P*	Median (range)	*P*
Painful neuropathy	Yes (9)+	0.17 (0–4.23)	0.1137	4.2 (0–13.1)	0.1962
	No (29)	1.44 (0–6.80)		8.95 (0–18.65)	
Symptomatic site	Yes (7)	0 (0–1.68)	0.0116*	0 (0–4.96)	0.0034*
	No (31)	1.25 (0–6.8)		8.95 (0–18.65)	

+*n*. *Statistically significant.

acrylamide neurotoxicity and painful neuropathy (Ko *et al.*, 2002; Lauria *et al.*, 2003).

Diabetes exerts a profound influence on sensory neurons; potential mechanisms include vascular, metabolic and immunological defects (Said *et al.*, 2003). Neurological manifestations in most patients with diabetic neuropathy exhibit a stocking or glove-stocking pattern, suggesting that nerve terminals may be early targets of diabetic neuropathy, and skin biopsy can demonstrate lesions in the distalmost parts of nerve terminals. Similar approaches have been reported for diseases in which sensory nerves are predominantly affected, e.g. leprosy (Facer *et al.*, 1998).

Skin biopsy and diagnosis of small-fibre sensory neuropathy

This technique of skin biopsy with PGP 9.5 immunohistochemistry has been demonstrated by ultrastructural studies to label the terminal portions of both small myelinated and unmyelinated nerves in the epidermis (Kennedy and Wendelschafer-Crabb, 1993; Hilliges *et al.*, 1995; Hsieh *et al.*, 1996, 2000). Two issues merit discussion regarding the pathology and biology of sensory nerves in the skin. First, IENF densities recorded by the current quantitation method include normal, pre-degenerating and regenerating epidermal nerves. For normal and pre-degenerating epidermal nerves, there is a significant correlation between changes at the light microscopic level and those under electronic microscopy (Hsieh *et al.*, 2000). Our group previously has demonstrated pathological evidence of pre-degenerating epidermal nerves: swelling and an increased number of branches (Ko *et al.*, 2002). Future ultrastructural studies are required to delineate the relative proportion of normal versus pre-degenerating epidermal nerves, and to investigate the significance of these nerve pathologies (Lauria *et al.*, 2003). In contrast, there are no correlated light and electron microscopic findings in regenerating nerves. Determining this will require further immunohistochemical analyses with different neuronal markers of regenerating nerves, such as growth-associated protein 43 (Woolf *et al.*, 1990).

Secondly, encapsulated sensory receptors, such as Meissner corpuscles and Merkel cells in the human skin, are not labelled with PGP 9.5 immunohistochemistry (Hsieh *et al.*, 2000). It is an open question whether receptors for conducting thermal stimuli are located exclusively in epidermal nerve terminals or in keratinocytes surrounding nerve terminals. This issue will require further studies with new markers against temperature-sensitive molecules, such as transient receptor potential channels (Clapham, 2003); this type of study may elucidate the relationship between clinical symptoms and skin innervation.

Skin innervation and hyperglycaemic control

The present study demonstrates that skin innervation was reduced with increased diabetic duration. This finding supports the concept that long-term impaired glucose metabolism is related to changes in structural organization and function of small-diameter sensory neurons (Perkins *et al.*, 2001). Although previous studies indicated that epidermal nerve fibres and dermal nerve fibre lengths are reduced in diabetes (Kennedy *et al.*, 1996), it has not been demonstrated whether skin denervation was related to diabetic parameters. Beyond diabetic duration, the current study showed no correlation between epidermal innervation and HbA_{1C} levels.

This indicates that mechanisms for skin denervation in diabetes may be complicated issues. For example, parameters in the blood are not necessarily correlated with changes at the tissue level, which directly contribute to nerve damage, such as oxidative stress (Feldman, 2003). Alternatively, HbA_{1C} data of 3 years before skin biopsy were available in the current study, and this interval is short compared with a diabetic duration of 10.55 ± 7.20 years of the study population (Ziegler *et al.*, 1991). The limited sample size is also a factor given the dispersed values of HbA_{1C} in the current study group. These latter two issues are important for addressing the effects of glycaemic control (Dyck *et al.*, 1999; Perkins *et al.*, 2001). A larger scale prospective study is necessary, and skin biopsy offers the opportunity to evaluate the influence of diabetes on skin innervation longitudinally, as in a DCCT (Diabetes Control and Complications Trial) study on nerve conduction (Albers *et al.*, 1995).

In addition to metabolic and vascular effects of diabetes, sensory neurons of the small type with their processes depend on various specific neurotrophins for survival and maintenance, particularly nerve growth factor (Snider, 1994; Lentz *et al.*, 1999). In diabetic skin, the transcript level of nerve growth factor is increased (Diemel *et al.*, 1999), while the protein content of nerve growth factor is reduced (Anand *et al.*, 1996). Diabetes reduces the retrograde transport of nerve growth factor, and this effect can be ameliorated by exogenous replacement of the sonic hedgehog protein (Calcutt *et al.*, 2003; Feldman, 2003). These findings suggest that the altered balance of nerve growth factor by modifications at the levels of transcription, translation and post-translation may underlie skin denervation in diabetes, and skin biopsy provides a new approach to test this hypothesis.

Pathology of skin innervation and functional changes

The present report documents parallel psychophysical changes with IENF densities. Previous studies on sural nerves of diabetics indicated that both myelinated and unmyelinated nerve densities were reduced in diabetes (Llewelyn *et al.*, 1991; Malik *et al.*, 2001). However, unmyelinated nerve densities of sural nerves were not correlated with parameters of nerve conduction studies or quantitative sensory testing (Malik *et al.*, 2001). Several possibilities may underlie this discrepancy. These include the presence of autonomic nerves and difficulties in differentiating regenerating nerve sprouts from unmyelinated axons in sural nerve biopsies, which may have influenced the precise counting of unmyelinated nerves (Bickel *et al.*, 2000; Malik *et al.*, 2001).

In the current report, IENF densities, which reflect the abundance of skin innervation, clearly demonstrated linear correlations with thermal thresholds when diabetic duration and age were controlled for. Changes in thermal sensitivities are common in diabetic neuropathy (Dyck *et al.*, 2000). Quantitative sensory testing evaluates the entire pathway of the sensory system, while skin biopsy provides complementary information about sensory nerve terminal changes in diabetic neuropathy.

Most studies on diabetic neuropathy have focused mainly on a certain subtype of neuropathy, and rarely compared the degree of involvement among various subtypes of neuropathy (Albers *et al.*, 1995; Sands *et al.*, 1997). The current report provides laboratory evidence that diabetic neuropathy encompasses both large-fibre and small-fibre neuropathies, and the abnormal rates of small-fibre tests were higher than those of large-fibre tests. Several possibilities may underlie this observation. Patients were at a relatively advanced stage of diabetes in the current series, because all of them had clinically overt diabetes and sensory symptoms. Alternatively, epidermal denervation is a rather early event for a subset of diabetic patients (Sumner *et al.*, 2003). Nevertheless, parallel changes in epidermal innervation and thermal thresholds suggest that skin biopsy together with quantitative sensory testing could potentially play a role in screening and managing diabetic neuropathy. In the management of diabetic neuropathy, different strategies are necessary to target different components of clinical impairment, i.e. large versus small fibres. Given the high proportion of small-fibre sensory neuropathy in the current report, instructions on preventing painless injury due to the reduced sensitivity to thermal stimuli in diabetes need to be stressed.

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Appendix

Table A1 *Normative values of quantitative sensory testing on the foot dorsum*

Group by age	<60 years, n = 444	⩾60 years, n = 114
Method of level		
Warm threshold temperature (°C)	39.30 (36.52 ± 1.79)*	40.00 (37.85 ± 1.52)
Cold threshold temperature (°C)	29.50 (30.70 ± 0.62)	28.50 (30.29 ± 0.83)
Vibratory threshold (μm)	5.30 (2.64 ± 1.43)	7.85 (4.84 ± 1.88)
Method of limits		
Warm threshold temperature (°C)	42.10 (38.11 ± 2.88)	43.20 (40.00 ± 2.12)
Cold threshold temperature (°C)	26.40 (29.17 ± 1.37)	25.60 (28.76 ± 1.52)
Vibratory threshold, limits (μm)	8.40 (4.43 ± 2.02)	11.80 (7.43 ± 2.89)

*Cut-off value (mean ± SD); cut-off value was the 95th percentile value (for warm threshold and vibratory threshold) and the 5th percentile value (for cold threshold).