Corneal Confocal Microscopy Detects Early Nerve Regeneration in Diabetic Neuropathy After Simultaneous Pancreas and Kidney Transplantation

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Diabetic neuropathy is associated with increased morbidity and mortality. To date, limited data in subjects with impaired glucose tolerance and diabetes demonstrate nerve fiber repair after intervention. This may reflect a lack of efficacy of the interventions but may also reflect difficulty of the tests currently deployed to adequately assess nerve fiber repair, particularly in short-term studies. Corneal confocal microscopy (CCM) represents a novel noninvasive means to quantify nerve fiber damage and repair. Fifteen type 1 diabetic patients undergoing simultaneous pancreas–kidney transplantation (SPK) underwent detailed assessment of neurologic deficits, quantitative sensory testing (QST), electrophysiology, skin biopsy, corneal sensitivity, and CCM at baseline and at 6 and 12 months after successful SPK. At baseline, diabetic patients had a significant neuropathy compared with control subjects. After successful SPK there was no significant change in neurologic impairment, neurophysiology, QST, corneal sensitivity, and intraepidermal nerve fiber density (IENFD). However, CCM demonstrated significant improvements in corneal nerve fiber density, branch density, and length at 12 months. Normalization of glycemia after SPK shows no significant improvement in neuropathy assessed by the neurologic deficits, QST, electrophysiology, and IENFD. However, CCM shows a significant improvement in nerve morphology, providing a novel noninvasive means to establish early nerve repair that is missed by currently advocated assessment techniques. ***Diabetes*** **62:254–260, 2013**

Diabetic polyneuropathy is one of the most common long-term complications of diabetes and underlies the development of painful neuropathy in 21% of both type 1 and type 2 diabetic patients (1). It is the main initiating factor for foot ulceration and lower extremity amputation (2). At present we have no treatment to repair nerve fibers and improve diabetic neuropathy. Even in the Diabetes Control and Complications Trial (DCCT) and follow-up Epidemiology of Diabetes Interventions and Complications (EDIC) study, improved glycemic control only delayed the progression of clinical diabetic neuropathy and indeed nerve conduction studies at closeout showed no significant risk reduction (3). Furthermore, the Steno-2 study demonstrated that although multifactorial intervention showed an improvement in retinopathy, nephropathy, and cardiac autonomic neuropathy, there was no benefit for somatic neuropathy (4). Even in the most dramatic example of "curing" type 1 diabetes with pancreas transplantation, in 115 patients followed over 10 years, neurologic function, nerve conduction studies, and autonomic function were only prevented from worsening and failed to show an improvement (5). This is in keeping with the lack of improvement in heart rate variability, 43 months after simultaneous pancreas–kidney transplantation (SPK) (6) and intraepidermal nerve fiber density (IENFD) 2.5 years after SPK (7). Neuropathy is of course extremely severe at this stage, as evidenced by severe intraepidermal nerve fiber depletion in pancreas transplant recipients, suggesting either a point of no return or the need for long-term follow-up to identify posttransplant nerve fiber regeneration (8). However, IENFD and corneal nerve morphology have been shown to improve in subjects with impaired glucose tolerance neuropathy (9) and in patients with type 2 diabetes (10), respectively, after improvement in metabolic risk factors.

To establish efficacy of a new treatment, ideally an improvement in diabetic neuropathy has to be shown. Although current end points have a good ability to diagnose diabetic neuropathy (11), their ability to define a therapeutic response may have significant limitations (12). This may indeed be a major reason why clinical trials in human diabetic neuropathy have failed to reach prespecified primary end points such as neuropathic deficits and electrophysiology (13). The assessments of neurologic symptoms and deficits have recently been shown to have poor diagnostic reproducibility (14). Although electrophysiology correlates with large fiber damage, it does not assess small fibers, which are the earliest to be damaged (15) and demonstrate repair even in advanced neuropathy (12). Nerve fiber morphology in sural nerve biopsies (16) and IENFD in skin-punch biopsies (17) can accurately quantify nerve fiber damage and repair, but both are invasive procedures.

We and others (18,19) have used corneal confocal microscopy (CCM) to detect subclinical diabetic neuropathy and relate it to the severity of somatic neuropathy (20) and IENFD (21) with good sensitivity and specificity (20). This led us to propose that CCM, a noninvasive and reiterative test, might be an ideal surrogate end point for evaluating

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therapeutic efficacy in clinical trials of human diabetic neuropathy (22). In a preliminary study, we have previously shown a significant improvement in corneal nerve fiber density (CNFD) and length 6 months after SPK (23), but at that time we did not compare CCM with established end points of diabetic neuropathy. In the current study we have compared CCM with neurologic deficits, quantitative sensory testing (QST), electrophysiology, and IENFD at baseline and 6 and 12 months after SPK to help define the measures that may best detect an improvement in diabetic neuropathy after intervention.

RESEARCH DESIGN AND METHODS

Selection of patients. Fifteen type 1 diabetic patients were evaluated at baseline and 6 and 12 months after SPK and compared with 10 age/sex-matched nondiabetic healthy control subjects. The healthy volunteers were recruited from the general population. Both patients and control subjects underwent full neurologic and medical assessments. Those patients with any history of systemic (apart from diabetes for patient group) or neurologic conditions or history of ocular trauma and those wearing contact lens or those who have had ocular surgery were excluded. The study was approved by the Central Manchester Ethics Committee, and written informed consent was obtained according to the Declaration of Helsinki.

Assessment of neuropathy. All patients and control subjects underwent a detailed evaluation of neurologic symptoms according to the neuropathy symptom profile (NSP), and the McGill pain analog score was used to assess the severity of painful neuropathy. Neurologic deficits were assessed using the modified neuropathy disability score (NDS), which includes evaluation of vibration, pin prick, and temperature perception as well as the presence or absence of ankle reflexes to establish the severity of neuropathy: NDS 0–2, no neuropathy; NDS 3–5, mild neuropathy; NDS 6–8, moderate neuropathy; and NDS 9–10, severe neuropathy. Quantitative sensory testing included an assessment of vibration perception threshold (VPT), measured on the first toe using a Neurothesiometer (Horwell, Scientific Laboratory Supplies, Wilford, Nottingham, U.K.), cold sensation (CS) (A_δ fibers) and warm sensation (WS) (C fibers) thresholds using the method of limits with the MEDOC *TSA II* (Medoc, Ramat Yishai, Israel) on the dorsum of the left foot (24).

Computer-Aided Sensory Evaluator (CASE IV) was used to measure the heart rate response to deep breathing. In this test, the patient was asked to inhale and exhale deeply eight times in a row in the supine position while following the rhythm of a "breathing cue," and the changes in heart rate were displayed on an ECG monitor. Two eight-cycle breathing series' were completed interspersed by a 5-min period of normal breathing. The acquired data were analyzed by calculating the mean difference between the highest and lowest heart rate for five consecutive, artifact-free cycles in each eight-cycle series.

Electro-diagnostic studies were undertaken using a Dantec "Keypoint" system (Dantec Dynamics, Bristol, U.K.) equipped with a Dansk Industri Syndikat temperature regulator to keep limb temperature constantly between 32°C and 35°C. Peroneal motor and sural sensory nerves were assessed in the right lower limb by a consultant neurophysiologist. The motor study was performed using silver-silver chloride surface electrodes at standardized sites defined by anatomical landmarks, and recordings for the sural nerve were taken using antidromic stimulation over a distance of 100 mm.

Corneal sensitivity. Corneal sensitivity was quantified using a noncontact corneal aesthesiometer (NCCA) (Glasgow Caledonian University, Glasgow, Scotland, U.K.), which uses a puff of air through a bore 0.5 mm in diameter lasting 0.9 s and exerting a force expressed in millibars (mbars) (25). The stimulus jet is mounted on a slit lamp and is positioned 1 cm from the eye, and the air jet is aligned to the center of the cornea. Each subject was presented with a supramaximal stimulus, and the staircase method was used by reducing the stimulus strength until the patient did not feel the jet on three occasions, to establish the threshold. The coefficient of variation for NCCA was 5.6%.

CCM. Patients underwent examination with the Heidelberg retina tomograph III in vivo corneal confocal microscope. The subject's eyes were anesthetized using a drop of 0.4% benoxinate hydrochloride, and Viscotears were applied on the front of the eye for lubrication. A drop of viscoelastic gel was placed on the tip of the objective lens, and a sterile disposable Perspex cap was placed over the lens allowing optical coupling of the objective lens to the cornea. The patient was instructed to fixate on a target with the eye not being examined. Several scans of the entire depth of the cornea were recorded by turning the fine focus of the objective lens backward and forward for ~2 min using the section mode, which enables manual acquisition and storage of single images of all corneal layers. This provides en face two-dimensional images with a lateral resolution of ~2 μm/pixel and final image size of 400 × 400 pixels of the subbasal nerve plexus of the cornea from each patient and control subject. This layer is of particular relevance for defining neuropathic changes since it is the location of the main nerve plexus that supplies the overlying corneal epithelium. Each nerve fiber bundle contains unmyelinated fibers, which run parallel to Bowman's layer before dividing and terminating as individual axons underneath the surface epithelium (26). Five images per patient from the center of the cornea were selected and examined in a masked and randomized fashion (27). Three corneal nerve parameters were quantified: *1*) CNFD, the total number of major nerves per square millimeter of corneal tissue; *2*) corneal nerve branch density (CNBD), the number of branches emanating from all major nerve trunks per square millimeter of corneal tissue; and *3*) corneal nerve fiber length (CNFL), the total length of all nerve fibers and branches (mm/mm^2) within the area of corneal tissue. CNFD and CNFL are considered to reflect overall nerve fiber degeneration, whereas CNBD reflects nerve fiber regeneration, which is partially also captured by CNFL.

Skin biopsy and immunohistochemistry. A 3-mm punch skin biopsy was taken from the dorsum of the foot ~2 cm above the second metatarsal head after local anesthesia (1% lidocaine). The biopsy site was closed using Steristrips, and the specimen was immediately fixed in PBS-buffered 4% paraformaldehyde. After 18–24 h, it was rinsed in Tris-buffered saline and soaked in 33% sucrose (2–4 h) for cryoprotection. It was then embedded in optimal cutting temperature–embedding compound, rapidly frozen in liquid nitrogen, and cut into 50-μm sections using a cryostat (model OTF; Bright Instruments, Huntington, U.K.). Four floating sections per subject were subjected to melanin bleaching (0.25% $KMnO_4$ for 15 min followed by 5% oxalic acid for 3 min), a 4-h protein block with a Tris-buffered saline solution of 5% normal swine serum, 0.5% powdered milk, and 1% Triton X-100, and overnight incubation with 1:1,200 Biogenesis polyclonal rabbit anti-human PGP9.5 antibody (Serotec, Oxford, U.K.). Biotinylated swine anti-rabbit secondary antibody (1:300; DakoCytomation, Ely, U.K.) was then applied for 1 h; sections were quenched with 1% H_2O_2 in 30% MeOH-PBS (30 min) before a 1-h incubation with 1:500 horseradish peroxidase–streptavidin (Vector Laboratories, Peterborough, U.K.). Nerve fibers were demonstrated using 3, 3̀-diaminobenzidine chromogen (Sigma-Aldrich, Manchester, U.K.). Sections were mildly counterstained with eosin to better localize the basement membrane to identify nerve fibers passing through it. Negative control subjects consisted of replacing the anti-PGP9.5 antibody with rabbit immunoglobulin (DakoCytomation) at a concentration matching that of the primary antibody, which showed no immunostaining. IENFD, i.e., the number of fibers per millimeter of basement membrane, was quantified in accord with established criteria and techniques and expressed as number per millimeter (28).

Statistics. SPSS 16.05.0 for Windows was used to compute the results. Analysis included descriptive and frequency statistics. All data are expressed as means ± SEM. A paired sample *t* test was used to test whether a sample mean (of a normally distributed interval variable) differed between control subjects and diabetic patients at baseline and at follow-up 6 and 12 months after SPK.

RESULTS

The clinical characteristics and detailed assessment of neuropathy in diabetic patients and age-matched control subjects are summarized in Table 1. BMI was nonsignificantly lower in diabetic patients and showed an increase after SPK. HbA_{1c} was higher in diabetic patients compared with control subjects and improved into the normal range at 6 and 12 months after SPK, but this was not statistically significant. The total cholesterol was significantly lower (P = 0.01) in diabetic patients and remained the same at 6 and 12 months after SPK. Both HDL and triglycerides were comparable between diabetic patients and control subjects, and remained unchanged after SPK. The estimated glomerular filtration rate was lower in diabetic patients at baseline (P = 0.02) and did not change significantly at 6 and 12 months after SPK.

Symptoms and neurologic deficits. Neuropathic symptoms as assessed with the NSP were significantly greater in diabetic patients than in control subjects at baseline (P = 0.005), but there was no significant improvement at 6 (P = 0.1) or 12 (P = 0.9) months after transplantation. The McGill pain index was significantly (P = 0.01) greater at baseline compared with control subjects and did not show a significant change at 6 (P = 0.9) or 12 (P = 0.9) months after transplantation. The modified NDS was significantly

TABLE 1
Clinical demographic results in control subjects and type 1 diabetic patients undergoing SPK at baseline and follow-up visits at 6 and 12 months

Parameter	Control subjects	Baseline	Follow-up	
			6 months	12 months
n (female/male)	10 (3/7)	15 (5/10)	15	15
Age (years)	47 ± 3	47 ± 3	—	—
Diabetes duration (years)	0	27 ± 3.5	—	—
BMI (kg/m^2)	27 ± 1	22 ± 2	25.5 ± 1	25.5 ± 1
HbA_{1c} (%)	5.7 ± 0.1	7.4 ± 0.8	5.9 ± 0.3	5.9 ± 0.4
Cholesterol (mmol/L)	5.1 ± 0.2	4.0 ± 0.3*	4.3 ± 0.3	4.5 ± 0.3
HDL (mmol/L)	1.5 ± 0.1	1.3 ± 0.2	1.5 ± 0.2	1.6 ± 0.2
Triglycerides (mmol/L)	1.3 ± 0.2	1.4 ± 0.1	1.2 ± 0.1	1.03 ± 0.1
Estimated glomerular filtration rate (mL/min/L)	86.22 ± 2.13	60.53 ± 8.64†	64.0 ± 7.5	66.0 ± 6.19

Data are presented as mean ± SEM in diabetic patients and control subjects unless otherwise indicated. All symbols represent statistically significant differences using paired sample *t* test. *$P < 0.01$. †$P < 0.02$ (baseline vs. control).

($P = 0.003$) greater at baseline compared with control subjects, indicating a mild to moderate neuropathy, and did not change significantly at 6 ($P = 0.7$) or 12 ($P = 0.8$) months after transplantation (Table 2).

Quantitative sensory tests. VPT was significantly greater in diabetic patients compared with control subjects at baseline ($P = 0.01$) and did not change significantly at 6 ($P = 0.1$) or 12 ($P = 0.6$) months after transplantation. CS was significantly greater in diabetic patients compared with control subjects at baseline ($P = 0.004$) and did not change significantly at 6 ($P = 0.5$) or 12 ($P = 0.5$) months after transplantation. WS was significantly greater in diabetic patients compared with control subjects at baseline ($P = 0.005$) and did not change significantly at 6 ($P = 0.9$) or 12 ($P = 0.4$) months after transplantation.

Autonomic function. Average heart rate variability was significantly lower in diabetic patients compared with control subjects at baseline ($P = 0.01$) and did not change significantly at 6 ($P = 0.9$) or 12 ($P = 0.8$) months after SPK.

Electrophysiology. Peroneal nerve conduction velocity and amplitude were significantly lower in diabetic patients compared with control subjects at baseline ($P = 0.0001$, $P = 0.0001$, respectively) and did not change significantly at 6 ($P = 0.6$, $P = 0.5$) or 12 ($P = 0.3$, $P = 0.2$) months after transplantation. Sural nerve conduction velocity and amplitude were significantly lower in diabetic patients compared with control subjects at baseline ($P = 0.003$, $P = 0.001$, respectively) and did not change significantly at 6 ($P = 0.7$, $P = 0.9$) or 12 ($P = 0.6$, $P = 0.3$) months after transplantation (Table 2).

IENFD. IENFD was significantly lower in diabetic patients compared with control subjects at baseline ($P < 0.0001$) and did not show a significant improvement 12 months after transplantation ($P = 0.9$) (Fig. 1 and Table 3).

Corneal sensation. The corneal sensation threshold was significantly greater in diabetic patients compared with control subjects at baseline ($P = 0.03$) and did not change at 6 ($P = 0.9$) or 12 ($P = 0.9$) months after transplantation (Table 3).

CCM. Representative images from a diabetic patient at baseline show a marked reduction in subbasal corneal nerves with a progressive repair at 6 and 12 months after SPK. CNFD was significantly lower in diabetic patients compared with control subjects at baseline ($P < 0.0001$), did not improve at 6 months ($P = 0.7$), but reached significance at 12 months ($P = 0.02$). Similarly, CNFL was significantly lower in diabetic patients compared with control subjects at baseline ($P < 0.0001$) and did not improve at 6 months ($P = 0.2$) but reached statistical significance at

TABLE 2
Clinical neuropathy evaluation in control subjects and type 1 diabetic patients undergoing SPK at baseline and follow-up visits at 6 and 12 months

Parameter	Control subjects	Baseline	Follow-up	
			6 months	12 months
NSP (0–38)	0	6.7 ± 1.8†	7.6 ± 2.2	7.3 ± 2.0
NDS (0–10)	0.3 ± 0.2	4.6 ± 0.9†	5.0 ± 1.1	5.4 ± 0.7
McGill pain index	0	1.7 ± 0.6*	1.9 ± 0.8	1.3 ± 0.5
VPT (volts)	6.7 ± 1.8	19.4 ± 3.7*	17.4 ± 3.3	16.9 ± 3.4
CS (°C)	29.3 ± 0.4	17.5 ± 3.1†	19.8 ± 2.9	20.0 ± 2.7
WS (°C)	38.1 ± 0.8	43.7 ± 1.4†	43.8 ± 1.2	42.3 ± 1.1
Heart rate variability (average bpm)	15.3 ± 2.1	7.1 ± 1.7†	5.7 ± 1.7	4.9 ± 2.1
Sural nerve conduction velocity (m/s)	47.9 ± 0.5	40.6 ± 2.2†	41.5 ± 1.6	41.8 ± 1.9
Sural amplitude (μA)	20.7 ± 3.4	5.1 ± 0.9†	5.1 ± 0.9	4.0 ± 0.6
Peroneal nerve conduction velocity (m/s)	47.7 ± 0.9	35.9 ± 1.8‡	37.7 ± 1.2	38.5 ± 1.8
Peroneal amplitude (mV)	12.2 ± 0.9	2.4 ± 0.4‡	1.9 ± 0.4	1.7 ± 0.3

Data are presented as mean ± SEM in diabetic patients and control subjects. All symbols represent statistically significant differences using paired sample *t* test. *$P < 0.05$. †$P < 0.01$. ‡$P < 0.001$ (baseline vs. control; 6 months vs. baseline; 12 months vs. baseline).

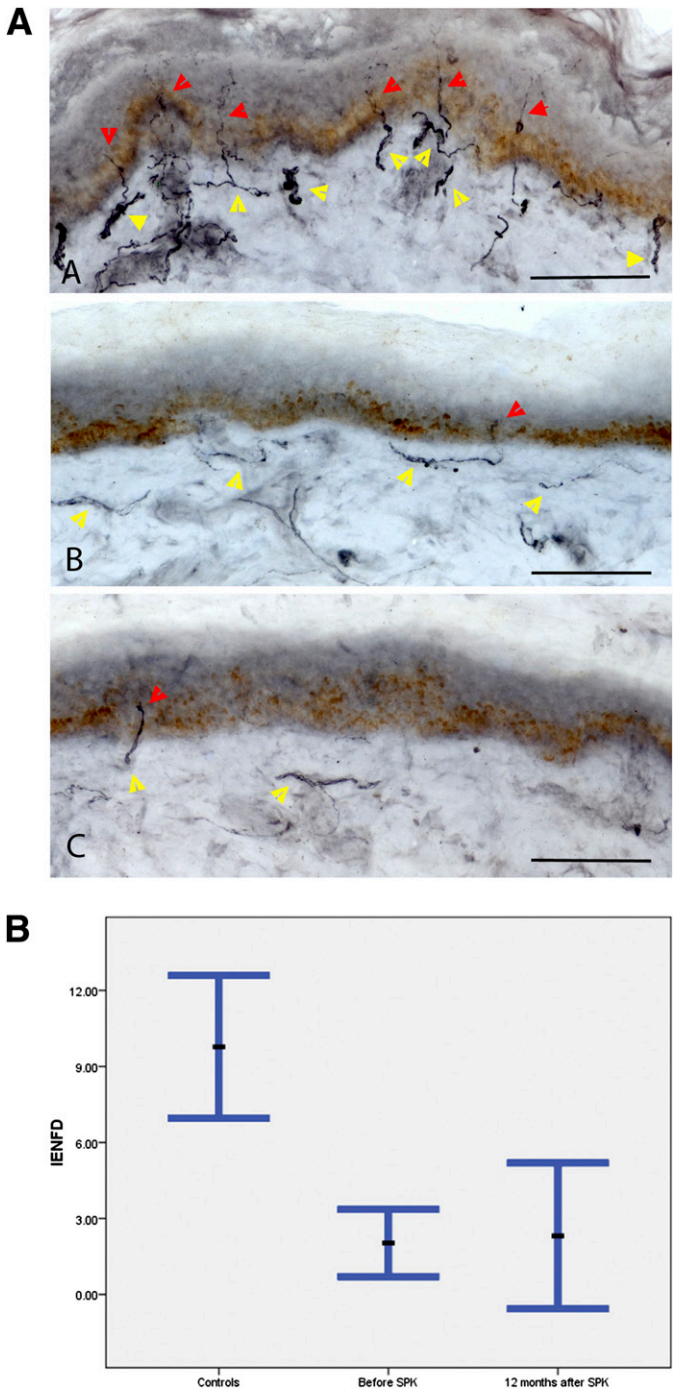

FIG. 1. *A*: **Skin biopsies immunostained for PGP9.5. Healthy control (A) shows numerous intraepidermal nerve fibers (red arrowheads) reaching upper levels of epidermis with a well-developed subepidermal nerve plexus (yellow arrowheads) in a healthy subject (A) compared with scant subepidermal and minimal intraepidermal nerve fibers in the diabetic patient both at baseline (B) and at follow-up (C). Scale bar = 100 μm.** *B*: **IENFD in control subjects and in diabetic patients at baseline and 12 months after SPK. Data are mean ± SEM. (A high-quality digital representation of this figure is available in the online issue.)**

12 months (P = 0.03). CNBD was significantly lower in diabetic patients compared with control subjects at baseline ($P < 0.0001$) but showed a significant improvement (P = 0.03) at 6 months and continued to improve significantly (P = 0.008) at 12 months (Figs. 2 and 3).

Although IENFD did not show an improvement at 12 months, it showed a significant correlation with corneal nerve parameters including CNFD (P = 0.656, $r < 0.0001$), CNBD (P = 0.709, $r < 0.0001$), and CNFL (P = 0.695, $r < 0.0001$).

DISCUSSION

The natural history of nerve damage in patients with type 1 diabetes is not entirely clear. Longitudinal data from the Rochester cohort support the contention that the duration and severity of exposure to hyperglycemia are related to the progression and hence severity of neuropathy rather than its onset (29). In type 1 diabetes the development of diabetic neuropathy has been related not only to glycemic control but also to conventional cardiovascular risk factors such as hypertension and lipids (30). The Toronto consensus identified clinical and neurophysiologic evaluation combined with quantitative sensory and autonomic function testing as well as small fiber evaluation to diagnose neuropathy (11). However, there is no clear consensus as to the critical end points, which should be used to define the benefits of therapeutic intervention.

The cure for type 1 diabetes is via pancreas transplantation, which normalizes blood glucose. Over the past 20 years, the survival and mortality of SPK transplants has improved significantly (31); therefore, it provides the ideal intervention to assess whether the long-term complications of diabetes are reversible. Some studies show that retinopathy can deteriorate in 10–35% of patients with unstable eye disease immediately after pancreas transplantation, but benefits do become apparent after several years (32,33). Other studies demonstrate an improvement and/or stabilization of diabetic retinopathy after a median follow-up of only 17 months (34,35). For nephropathy, normoglycemia can stop the progression of diabetic glomerulopathy, but does not reverse it (36,37). Similarly, pancreas transplantation alone can limit further reduction in glomerular filtration rate (33), and SPK protects the graft kidney from developing diabetic nephropathy (38).

With regard to neuropathy, pancreas transplantation has previously been shown to improve nerve conduction and motor and sensory action potentials in the upper but not the lower limb as well as sudomotor function (5),within 1 year, but with no impact on autonomic function (5–7). SPK has been shown to improve gastric emptying and symptoms related to gastroparesis compared with kidney transplantation alone (39), although gastrointestinal symptoms and autonomic deficits do not correlate with each other. In a recent study in 18 type 1 diabetic patients there was no improvement in IENFD 21–40 months post-SPK (7). However, most patients receiving transplantation had severe nerve fiber damage as evidenced by marked depletion of intraepidermal nerve fibers (8).

Although nerve conduction studies and quantitative sensory testing are useful and well-validated measures to help diagnose and assess the progression of diabetic neuropathy, their utility in evaluating a therapeutic response may be limited (40). More detailed and reproducible measures, which accurately quantify small fiber neuropathy via skin or nerve biopsy, may be more sensitive but are invasive (15–17). There is now an increasing literature on the potential for CCM to quantify C-fiber pathology in peripheral neuropathies (18,41,42). Detailed morphometric and immunohistological studies have demonstrated that the subbasal nerve fiber bundles studied by CCM are

TABLE 3
Corneal sensitivity, corneal nerve morphology, and IENFD in control subjects and type 1 diabetic patients at baseline and after SPK at 6 and 12 months

Parameter	Control subjects	Baseline	Follow-up	
			6 months	12 months
NCCA (mbars)	0.56 ± 0.1	1.78 ± 0.42*	1.83 ± 0.73	1.84 ± 0.89
CNFD (no./mm^2)	35.77 ± 1.53	14.44 ± 1.20‡	15.22 ± 1.63	19.27 ± 1.57*
CNBD (no./mm^2)	100.92 ± 13.1	21.46 ± 3.78‡	36.85 ± 6.04*	43.02 ± 6.48†
CNFL (mm/mm^2)	27.93 ± 1.26	11.35 ± 1.04‡	13.35 ± 1.50	15.63 ± 1.56*
IENFD (no./mm)	9.77 ± 1.24	2.03 ± 0.61‡	—	2.31 ± 1.17

Data are presented as mean ± SEM in diabetic patients and control subjects. Note that skin biopsy was not performed at 6 months. All symbols represent statistically significant differences using paired sample t test. *$P < 0.05$. †$P < 0.01$. ‡$P < 0.001$ (baseline vs. control; 6 months vs. baseline; 12 months vs. baseline).

predominantly nociceptive C fibers (43,44). Indeed, CCM has been applied to evaluate diabetic neuropathy (19,20), idiopathic small fiber neuropathy (45), and Fabry disease (46).We have shown that corneal nerve damage assessed using CCM relates to the severity of intraepidermal nerve fiber loss (21) and is related to a loss of corneal sensitivity (25) in diabetic neuropathy. CCM detects very early small-fiber damage even in subjects with an elevated HbA_{1c}, still within the normal range (18), and HbA_{1c} levels 7–10 years before CCM correlate with the severity of nerve damage (47). Furthermore, an improvement in HbA_{1c} by optimizing medical therapy (10) and pancreas transplantation (23) led to corneal nerve regeneration, shown using CCM. However, in these studies the evaluation of neuropathy was limited to CCM.

The present study allowed us to evaluate the relative ability of CCM to detect nerve fiber repair compared with all other established measures for assessing neuropathy, including neurologic deficits, QST, neurophysiology, and IENFD. The results demonstrate a severe neuropathy in diabetic patients before SPK as evidenced by significant abnormalities in electrophysiology, QST, IENFD, and corneal nerve fibers, confirming previous studies (5–8). However, despite this considerable baseline damage, we now show a significant improvement in corneal nerve branch density within 6 months of transplantation. This improvement confirms our previous work (23) indicating an early nerve-fiber repair process with the restoration of euglycemia, followed by a significant improvement in nerve-fiber density and nerve-fiber length 12 months after SPK. This is in contrast to all other standard measures of neuropathy, including detailed QST, autonomic function, electrophysiology, and IENFD, all of which failed to show an improvement 12 months after SPK. These findings support previous studies in diabetic neuropathy where at best a prevention of progression in nerve damage was shown only after several years of euglycemia (5–8,48–51). However, these studies focused heavily on electrophysiology and quantitative sensory assessment, which predominantly assessed large fiber function. It is relevant that where small fiber function was assessed in the form of sudomotor function, a significant improvement was demonstrated within 1 year of SPK (5,7). The main limitations of this study are the small number of subjects studied, the

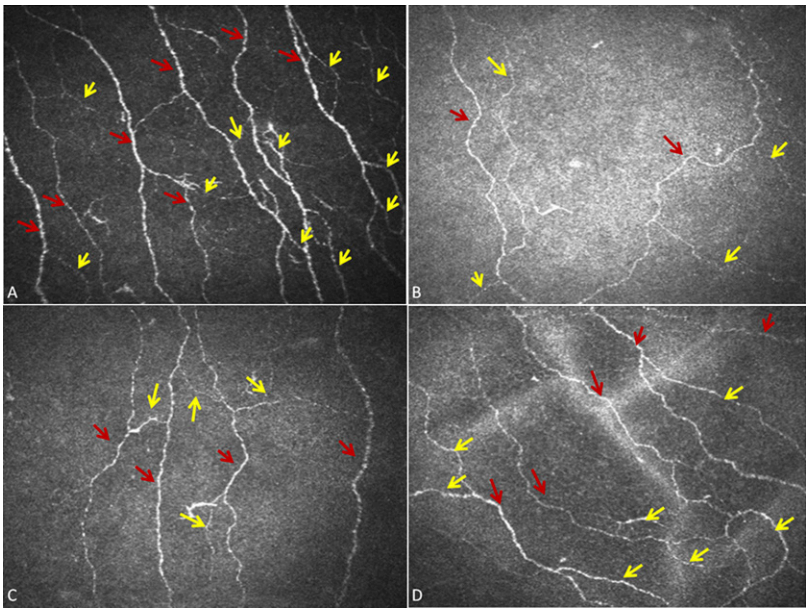

FIG. 2. CCM images from Bowman's layer of cornea: a control subject (A) and patient with type 1 diabetes at baseline (B) and at 6 (C) and 12 (D) months after SPK. The red arrows indicate main nerve fibers, and yellow arrows indicate branches. (A high-quality color representation of this figure is available in the online issue.)

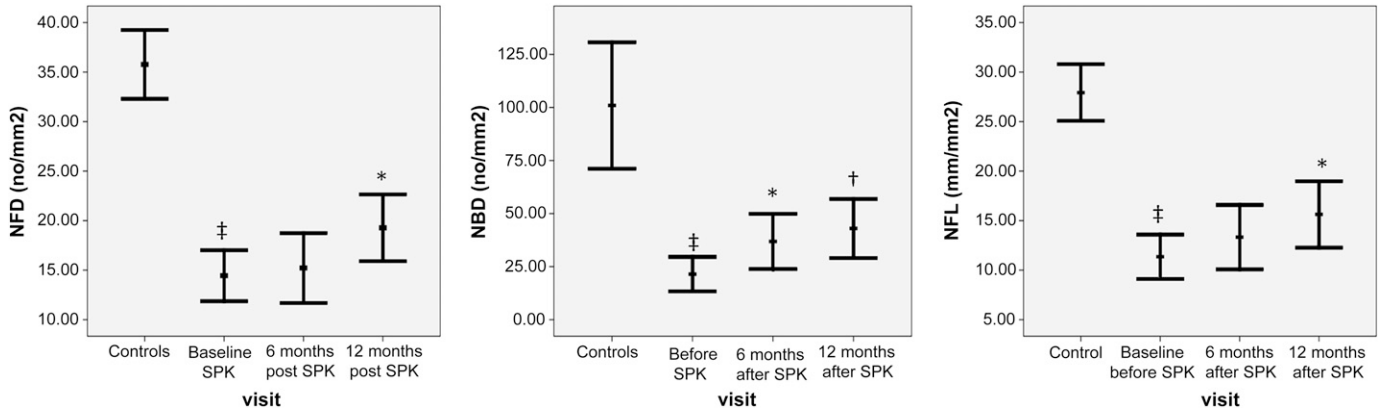

FIG. 3. CNFD (*left*), CNBD (*middle*), and CNFL (*right*) in diabetic patients at baseline and at 6 and 12 months after SPK. *$P < 0.05$; †$P < 0.01$; ‡$P < 0.001$ (baseline vs. control; 6 months vs. baseline; 12 months vs. baseline).

possibility of false-positive results based on the number of comparisons, the lack of sudomotor testing given its previous improvement in these patients, and the lack of blinding given that all patients were known to have had a SPK during the follow-up period. Furthermore, with regard to the lack of improvement in IENFD, this may reflect the location of the skin biopsy as we assessed this on the dorsum of the foot, whereas a previous study (9) has shown that proximal IENFD assessment in the thigh is more responsive to intervention. Similarly, for neurophysiological assessment it has been suggested that upper limb neurophysiology may show a better response to intervention as a result of lesser severity of damage (52).

We now confirm and extend the results of our previous study using the latest generation Heidelberg retina tomograph III, which provides enhanced small fiber imaging and detects earlier nerve fiber repair, particularly reflected in the increase in nerve branch density, followed by significant improvements in nerve fiber density and length. We believe these data provide further support for the need to study small fibers as surrogate markers and end points in intervention trials of diabetic neuropathy. An important issue with regard to the utility of CCM or indeed any surrogate end point has to be that these alterations in corneal nerve morphology predict deterioration of neuropathy and ultimately clinically meaningful outcomes such as foot ulceration. An alternative interpretation of this data could of course be that CCM is measuring something unique that is not an accurate biomarker of how other peripheral nerves are faring or indeed that corneal nerves respond well to restoration of insulin and normoglycemia, whereas other peripheral nerves do not. Nevertheless, CCM appears to represent a promising noninvasive and hence reiterative test with high sensitivity, which may represent an ideal surrogate end point for assessing the benefits of pancreas transplantation and indeed other therapies in clinical trials of human diabetic neuropathy.

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M.T. researched and analyzed the data and wrote the manuscript. M.M.-P. and T.A. were the transplant surgeons. I.N.P. researched data and analyzed CCM images. H.F., O.A., and U.A. undertook clinical and neurological assessment, skin biopsy, and QST. G.P. was the study coordinator. M.J. undertook IENFD assessments. A.M. undertook neurophysiology. N.E. reviewed and revised the manuscript. A.J.B. reviewed and revised the manuscript. R.A.M. supervised the project, undertook IENFD assessment, and reviewed and revised the manuscript. R.A.M. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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