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SYMPOSIUM

Corneal Confocal Microscopy to Assess Diabetic Neuropathy: An Eye on the Foot

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Abstract

Accurate detection and quantification of human diabetic peripheral neuropathy are important to define at-risk patients, anticipate deterioration, and assess new therapies. Easily performed clinical techniques such as neurological examination, assessment of vibration perception or insensitivity to the 10 g monofilament only assess advanced neuropathy, i.e., the at-risk foot. Techniques that assess early neuropathy include neurophysiology (which assesses only large fibers) and quantitative sensory testing (which assesses small fibers), but they can be highly subjective while more objective techniques, such as skin biopsy for intra-epidermal nerve fiber density quantification, are invasive and not widely available. The emerging ophthalmic technique of corneal confocal microscopy allows quantification of corneal nerve morphology and enables clinicians to diagnose peripheral neuropathy in diabetes patients, quantify its severity, and potentially assess therapeutic benefit. The present review provides a detailed critique of the rationale, a practical approach to capture images, and a basis for analyzing and interpreting the images. We also critically evaluate the diagnostic ability of this new noninvasive ophthalmic test to diagnose diabetic and other peripheral neuropathies.

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Diabetic neuropathy (DN) is a global problem affecting ~50% of the 26 million Americans and more than 366 million people worldwide with diabetes. It is the most common and costly complication of diabetes, leading to painful neuropathy (~21%)[1] and a 23.3-fold increased relative risk of foot ulceration and amputation.[2] It has been previously shown that foot ulceration is much more common in patients with diabetic peripheral neuropathy (DPN) with the annual incidence rising from <1% in those without neuropathy to >7% in those with established neuropathic deficits.[3] Furthermore, it has been shown to be an independent predictor for all-cause (hazard ratio = 4.4) and diabetes-related (hazard ratio = 11.82) mortality.[4]

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Abbreviations: (DN) diabetic neuropathy, (DPN) diabetic peripheral neuropathy, (IENFD) intra-epidermal nerve fiber density, (IVCCM) *in vivo* corneal confocal microscopy, (QST) quantitative sensory testing

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Management is difficult, as even tight glycemic control, a cornerstone for the management of diabetes, has been shown, at best, to limit progression of neuropathy in patients with type 1 diabetes,[5] but not type 2 diabetes.[6–9] There is no Food and Drug Administration-approved therapy to prevent or reverse human DN. Moreover, the development of disease-modifying drugs for DPN has stalled completely. Of course, there are many potential reasons/excuses for the multiple failed trials. However, it is increasingly apparent that there are significant issues with the end points deployed in clinical trials of human DN. Indeed a two-step hierarchical cluster analysis has revealed that neurophysiological tests do not aggregate by typical "small," "large," or "autonomic" nerve fiber subtypes.[10] Yet the latest recommendations continue to advocate a combination of symptoms and signs, quantitative sensory testing (QST), and electrophysiology for the diagnosis of DN.[11] By default, rather than design, these same measures have been adopted as surrogate end points to establish the benefits of therapeutic intervention and yet have clearly failed in several major clinical trials.[12,13]

While symptoms and neurological deficits have direct relevance to patients, the assessment is excessively variable with poor reproducibility.[14] Similarly, QST is subjective, is highly variable, and has limited reproducibility.[15] Neurophysiology is objective and reproducible but does not assess small fibers, which are the earliest to be damaged and show repair.[16] Small fibers can be assessed objectively by quantifying intra-epidermal nerve fiber density (IENFD) in skin biopsies; however, this is an invasive procedure that requires expert laboratory assessment and has considerable variability even among control.[17,18] Therefore, effective treatments may have failed not because of a lack of efficacy, but because of an inability of the currently advocated end points to detect improvement in clinical trials of DN.[19] A summary of the advantages and limitations of the present techniques to quantify nerve fiber damage in DN is presented in **Table 1**.

Table 1.
Summary of Advantages and Disadvantages of Tests to Assess Diabetic Neuropathy

Method	Advantage	Disadvantage
Clinical/neurological examination	Simple, easy to perform, does not require special equipment	Not sensitive, not reproducible
Nerve conduction studies	Sensitive, objective, currently the gold standard for diagnosis	Assesses only large fibers, requires special equipment
QST	Evaluates both large and small nerve fibers, quantitative, relatively easy to perform	Subjective, moderate reproducibility, requires special equipment
Sympathetic skin response	Simple, fast, objective	Semiquantitative, low sensitivity
Quantitative sudomotor axon reflex test	Sensitive, objective, reproducible	Requires special equipment, time consuming
Autonomic testing	Objective, quantitative	Moderate sensitivity, requires special equipment
Neuropad™ (sudomotor function assessment)	Noninvasive, easy to perform, does not require special equipment	Subjective, moderate sensitivity, uncertain interpretation
Sural nerve/skin biopsy	Quantitative, sensitive, currently the gold standard to quantify small fibers	Invasive, costly, risk of infection at the site of biopsy, requires specialist histological technique to quantify IENFD
Noncontact Corneal Aesthesiometry	Noninvasive, quantitative	Subjective, moderate sensitivity
IVCCM	Reproducible, rapid, sensitive, noninvasive, reiterative, quantitative	Requires special equipment and expertise

Hence, there is an urgent need for a noninvasive, sensitive surrogate marker in clinical trials of DN. There is strong evidence that the ophthalmic technique of *in vivo* corneal confocal microscopy (IVCCM) might be such an ideal surrogate end point for DPN.

Morphology of Human Corneal Innervation

The cornea is the most densely innervated tissue in the body.[20] Corneal nerves are derived from the ophthalmic division of the trigeminal nerve and enter the cornea in the middle third of the stroma and run forward anteriorly in

a radial fashion toward the center, where they lose their myelin sheath. The human cornea contains myelinated Aδ fibers, which are large-diameter (6 μm), straight nerves that respond primarily to mechanical stimuli, and unmyelinated C fibers, which are small-diameter (2–4 μm), beaded nerves that respond to thermal and chemical stimuli[21] (**Figure 1**). Detailed knowledge of corneal nerve architecture and morphology has been provided by studies employing light[22–24] and electron[21,25] microscopy and, later, IVCCM.[26–31]

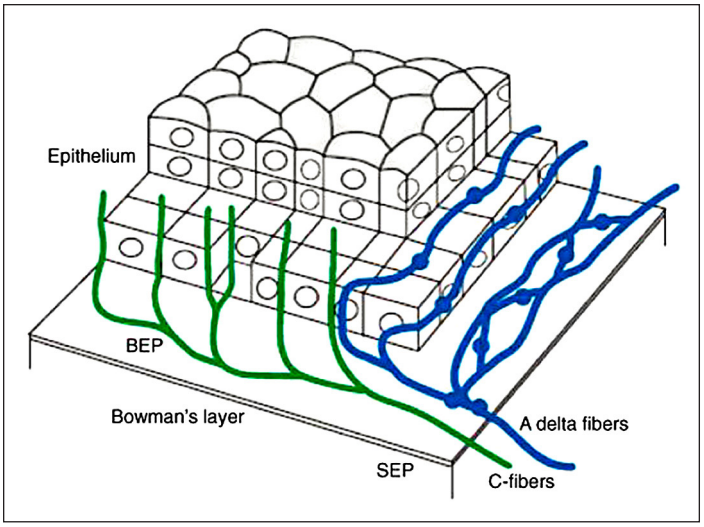

Figure 1. Three-dimensional representation of the innervation of the human cornea. BEP, basal epithelial plexus; SEP, subepithelial plexus.[32]

Corneal innervation plays an important role in regulating epithelial cell growth, proliferation, and differentiation in normal physiological states or in response to corneal disease, trauma, or surgery through the release of several growth factors, cytokines, and neurochemicals.[33] Although there are numerous studies on the anatomy and physiology of corneal innervation, its complete and complex physiological role remains unclear. *In vitro* coculture studies suggest that neurons and epithelial cells provide each other trophic support through the release of soluble substances. Neurons release substance P that stimulates epithelial cell growth, proliferation, differentiation, and type VII collagen production.[20] Thus patients with impaired corneal innervation may be at increased risk of ulceration due to impaired trophic support provided by the corneal nerves.[34,35]

In Vivo Corneal Confocal Microscopy

In vivo corneal confocal microscopy is an established technique, which has evolved rapidly from a predominantly research application to a diagnostic tool with a variety of clinical applications in ocular and neurological diseases. The noninvasive nature and rapid image acquisition time of the technique has made it an ideal method to extensively study all microstructures of the cornea, including the epithelial cell layer, Bowman's membrane, sub-basal nerve plexus, stroma, and endothelium.

Image Acquisition

The type of IVCCM used can significantly affect the quality of images. As a result, studies using a laser-scanning confocal microscope (e.g., Heidelberg Retina Tomograph III Rostock Cornea Module, Heidelberg GmbH, Heidelberg, Germany) have reported higher sub-basal nerve densities compared with studies using a tandem-scanning confocal microscope or a slit-scanning confocal microscope (e.g., Nidek Confoscan 4, Nidek Technologies, Padova, Italy, and Tomey Confoscan P4, Tomey, Erlangen, Germany) due to differences in the light source, contrast, and resolution.[32] Furthermore, studies have employed a range of scanning, image sampling, and quantification methodologies. There is no consensus regarding the minimum number of images required for representative quantitative analysis. The majority of published studies have used up to five images per layer per eye for analysis, and one study has suggested that 5–8 images are optimal, depending on the parameter being assessed.[36]

Image Quantification

The quality of the selected images is vital, and once image selection is complete, all images should be deidentified and randomized by an independent investigator prior to analysis to avoid observer bias. The majority of studies have defined sub-basal nerve density as the total number of nerves in each image, which allows quantification of the nerve density in an area (number/mm^2).[37–43] Others have presented the data as the number of nerves per image[44] or the total length of the nerves within a frame[45,46] but have nevertheless referred to the measure as a nerve density, which can be confusing to the nonexpert reader.

Adaptation of a global protocol to quantify corneal nerve morphology is of paramount importance, as it will enable a direct comparison of the results from different studies and allow multicenter studies. To date, most studies have employed semiautomated image analysis to assess sub-basal nerve alterations, which is a labor-intensive, subjective, and time consuming task. Studies from several different centers have assessed the impact of interobserver and intra-observer variability on the quantification of corneal nerve morphology using IVCCM and have reported excellent reproducibility among patients with diabetes and controls.[47–49] Very good reproducibility has been shown using a clinically relevant "study-level" protocol of subject re-examination (intraobserver intraclass correlation coefficient, 0.72; interobserver intraclass correlation coefficient, 0.73).[49] Inherent interobserver differences and experience were identified as the main causes of variation, especially for the parameter of nerve branch density, suggesting the need for a fully automated image analysis system to eliminate inconsistencies and expedite image analysis time. Such software has been developed.[50–52]

Stromal nerves have been studied less extensively with IVCCM. A few studies have quantified stromal nerves and quantified the density and the diameter of the nerves.[26,53,54] However, a wide range of results have been reported that may be due to the inconsistency in capturing stromal nerves because of their orientation and sparse distribution.[53] Stromal rather than sub-basal nerves appear more robust in surviving postmortem change,[54] therefore *in vitro* studies should focus on stromal nerves, whereas *in vivo* studies using IVCCM should focus on sub-basal nerves.

Corneal Nerve Changes in Diabetic Neuropathy

There has been increasing research interest in modeling the relationship between corneal nerve fiber loss and neuropathy. An association between neurotrophic corneal ulcers and diabetes was reported as early as 1977.[55] Subsequently, a reduction in corneal nerve density was demonstrated in experimental diabetes *ex vivo*.[35] The cornea, due to the unique property of transparency, allows direct, noninvasive, *in vivo* imaging of the small unmyelinated nerve fiber bundles. The first study using noncontact corneal esthesiometry in diabetes was by Rosenberg and colleagues[56] in 2000, showing sub-basal nerve alterations and a reduction in corneal sensitivity in patients with DN. However, since then, a burgeoning literature shows that IVCCM can quantify DN (**Table 2**).[39–42,44,56,57]

We have demonstrated that IVCCM quantifies early small nerve fiber damage[39,42,65] with good sensitivity and specificity[39] (**Figure 2**). Others have confirmed that IVCCM detects mild neuropathy,[38] and corneal nerve fiber length in particular has a high sensitivity (91%) and specificity (93%) for identifying diabetic sensorimotor polyneuropathy.[43]

Furthermore, a reduction in corneal nerve fiber length has been related to elevated hemoglobin A1c even in normal subjects, suggesting that IVCCM may detect early subclinical prediabetic nerve injury.[66] In a study of patients with idiopathic small fiber neuropathy, we have demonstrated significant corneal nerve damage, which was related to higher triglycerides.[67] We have also shown that IVCCM can be performed in children with diabetes.[58] Importantly, we have shown that corneal nerve damage assessed using IVCCM relates to the severity of intra-epidermal nerve fiber loss (gold standard for small fiber damage) in foot skin biopsies.[16] Corneal nerve fiber length has been shown to correlate significantly with three independent measures of small fiber function: cold detection thresholds, laser Doppler imager flare, and heart rate variability.[68] The further significant potential of IVCCM as a viable surrogate end point has been evidenced by demonstrating that IVCCM detects nerve fiber regeneration within 6 months of simultaneous pancreas kidney transplantation, while neurological deficits, QST, nerve conduction studies, and IENFD remain unchanged in diabetes patients (**Figures 3** and **4**).[37,69]

Of immediate clinical relevance, we have also demonstrated an improvement in corneal nerve fiber density after improvement in glycemia, blood pressure, and lipids in diabetes patients.[61] A potential limitation of IVCCM is the speed of analysis; however, automated image analysis has been developed for the rapid quantification of corneal nerve images.[70,71] Indeed we have developed an automated image analysis system that shows high correlation with manually assessed corneal nerve fiber density and length.[52,72] Our automated algorithm uses a dual model feature descriptor with a neural network classifier for dynamic detection and quantification whereas others approaches are

Table 2.
Summary of the Results of Quantitative Corneal Nerves Assessment with IVCCM in Diabetic Neuropathy[a]

Studies, first author	*n*	Age, years	Type of IVCCM	Acquisition method/images assessed per subject	Corneal nerve fiber density	Corneal nerve branch density	Corneal nerve fiber length	Study limitations
Sellers[58]	12	14.8 ± 2.1	LSCM	Section/5 images	24.1 ± 3.1 no/mm^2	43.7 ± 13.7 no/mm^2	18.2 ± 2.4 mm/mm^2	Small sample size
Zhivov[59]	18	68.8 ± 8.8	LSCM	Section/not specified	0.006 ± 0.002 mm/mm^2	25.3 ± 28.6 no/frame	6222 ± 2419 µm	Small sample size
Ahmed[43]	33	50 ± 14.3	LSCM	Volume/2 images	28.0 ± 9.0 no/mm^2	17.0 ± 12.0 no/mm^2	11.1 ± 3.6 mm/mm^2	Image selection criteria
Edwards[38]	88	58 ± 9	LSCM	Section/8 images	—	Graphical	Graphical	Corneal nerve fiber density not presented
Nitoda[60]	139	63 ± 2	LSCM	Sequence/3–5 images	23.3 ± 0.8 no/mm^2	31.8 ± 2.6 no/mm^2	12.5 ± 2.6 mm/mm^2	—
Tavakoli[61]	25	52 ± 2	SSCM	Section/3–5 images	18.8 ± 2.1 no/mm^2	6.9 ± 1.5 no/mm^2	8.3 ± 0.9 mm/mm^2	Small sample size
Hertz[49]	26	43 ± 16.9	LSCM	Volume/2 images	32.5 ± 9.7 no/mm^2	26.0 ± 17.1 no/mm^2	13.6 ± 3.5 no/mm^2	Image selection criteria
Ishibashi[62]	38	46.7 ± 1.6	LSCM	Not specified/4–5 images	25.3 ± 1.0 no/mm^2	—	9.8 ± 0.3 mm/mm^2	—
Tavakoli[39]	101	58.3 ± 2.2	SSCM	Section/3–5 images	24.1 ± 2.6 no/mm^2	10.3 ± 1.7 no/mm^2	4.9 ± 0.5 mm/mm^2	—
Messmer[40]	67	54	LSCM	Volume and sequence/ 5 images	16.5 no/mm^2	17.5 no/mm^2	10.2 mm/mm^2	Sample demographics
De Cillà[63]	50	62.6 ± 6	LSCM	Not specified/1 image	2.4 ± 1.0 no/frame	—	—	Image selection and analysis criteria
Midena[44]	42	—	SSCM	—	2.2 ± 0.3 no/frame	0.8 ± 0.1 (degree)	—	—
Chang[41]	42	63.8 ± 7.2	SSCM	Not specified	16.1 ± 5.7 no/mm^2	24.9 ± 7.7 no/mm^2	—	Image selection and analysis criteria
Quattrini[16]	54	58 ± 10.9	SSCM	Section/3–5 images	23.7 ± 3.2 no/mm^2	7.31 ± 1.98 no/mm^2	3.94 ± 0.63 mm/mm^2	—
Mocan[64]	35	58.4 ± 10	SSCM	Not specified/1 image	28.3 ± 10.4	39.7 ± 13.2 no/mm^2	—	Image analysis criteria
Malik[42]	18	57 ± 12.8	SSCM	Section/3–5 images	27.8 ± 6.5 no/mm^2	27.2 ± 13.2 no/mm^2	7.5 ± 1.1 mm/mm^2	Sample size
Rosenberg[56]	23	46 ± 8.3	TSCM	Section/2 images	3.1 ± 1.2 no/frame	—	—	Type of IVCCM

[a] LSCM, laser-scanning confocal microscope; SSCM, slit-scanning confocal microscope; TSCM, tandem-scanning confocal microscope

based primarily on the reflectivity of structures.[60] This particular method has also been chosen from a number of possible combinations, following evaluation of its clinical effectiveness in a cohort of patients with DPN.[51]

Arguments against IVCCM have revolved around the relatively short nerves being studied and the fact that the cornea is avascular, which is in contrast with the long somatic nerves and the compelling evidence for a vascular basis of DN.[73,74] However, reassuringly, corneal nerve pathology has been found to correlate with IENFD loss in biopsies from the dorsum of the foot[16] and a range of small fiber measures of DN.[68] Furthermore, studies in animal models of DN using IVCCM have shown a significant reduction in blood flow in the posterior ciliary artery and corneal nerve

fiber loss with an improvement in both blood flow and corneal innervation after intervention with a vasopeptidase inhibitor.[75,76]

In vivo corneal confocal microscopy has shown a decrease in the number and density of sub-basal nerves in a variety of abnormal ocular and systemic conditions, including dry eyes not related to Sjögren's syndrome and dry eyes related to primary Sjögren's syndrome.[77–79] There is also a burgeoning literature on the use of IVCCM to quantify not only DN,[39–42,44,56] but also idiopathic small fiber neuropathy,[69] Fabry disease,[80] hereditary sensory and autonomic neuropathy,[81] autoimmune neuropathy,[82] Crohn's disease,[78] and neuropathy associated with chemotherapy.[78,83]

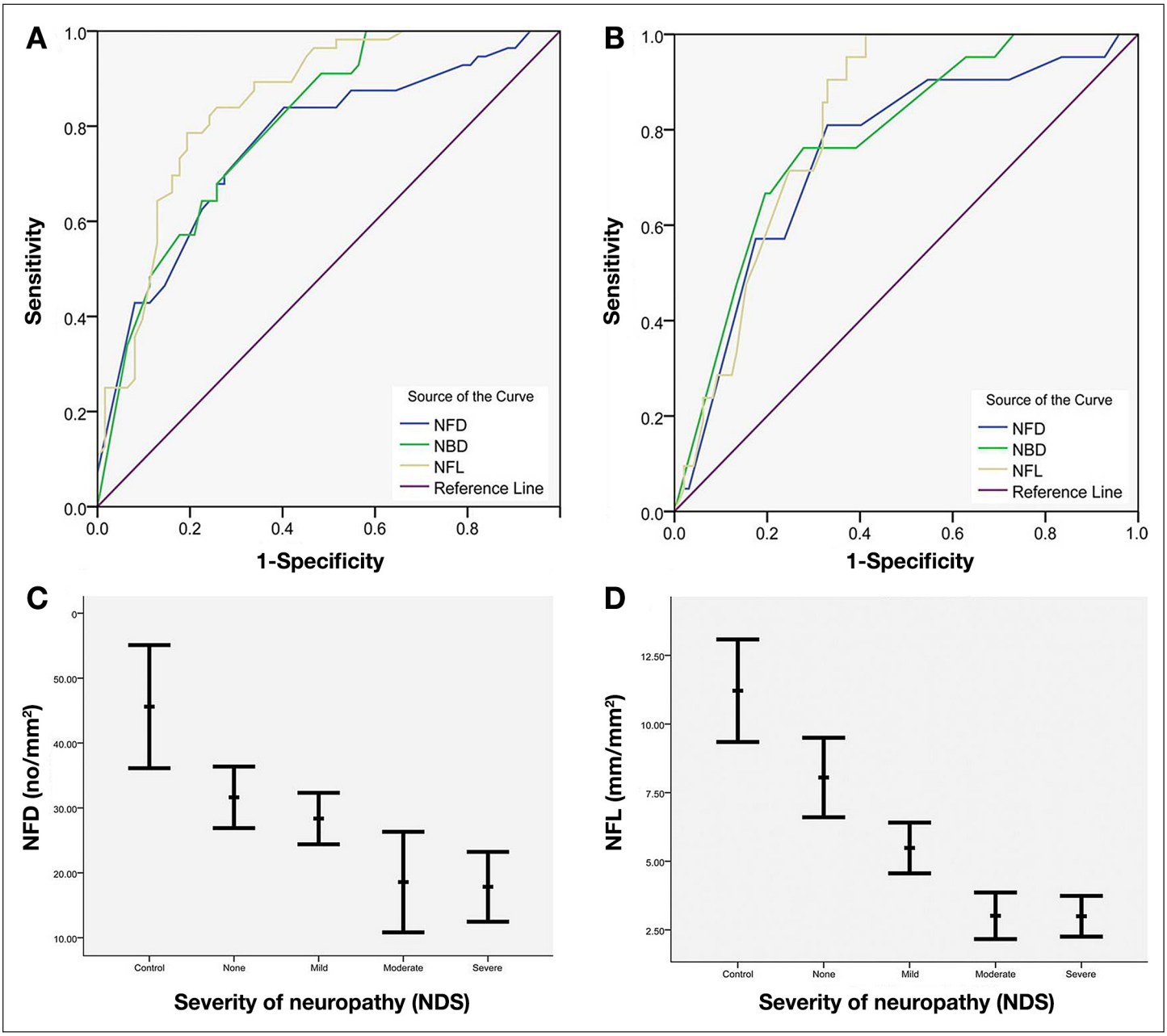

Figure 2. Receiver operating characteristic curves for the diagnostic validity of nerve fiber density, nerve branch density, and nerve fiber length for **(A)** NDS > 3 and **(B)** NDS > 6. Corneal nerve morphology in control subjects and diabetes patients with increasing neuropathic severity: **(C)** nerve fiber density ($p < .0001$) and **(D)** nerve fiber length ($p < .0001$). NFD, nerve fiber density; NBD, nerve branch density; NDS, neuropathy disability score; NFL, nerve fiber length.[39]

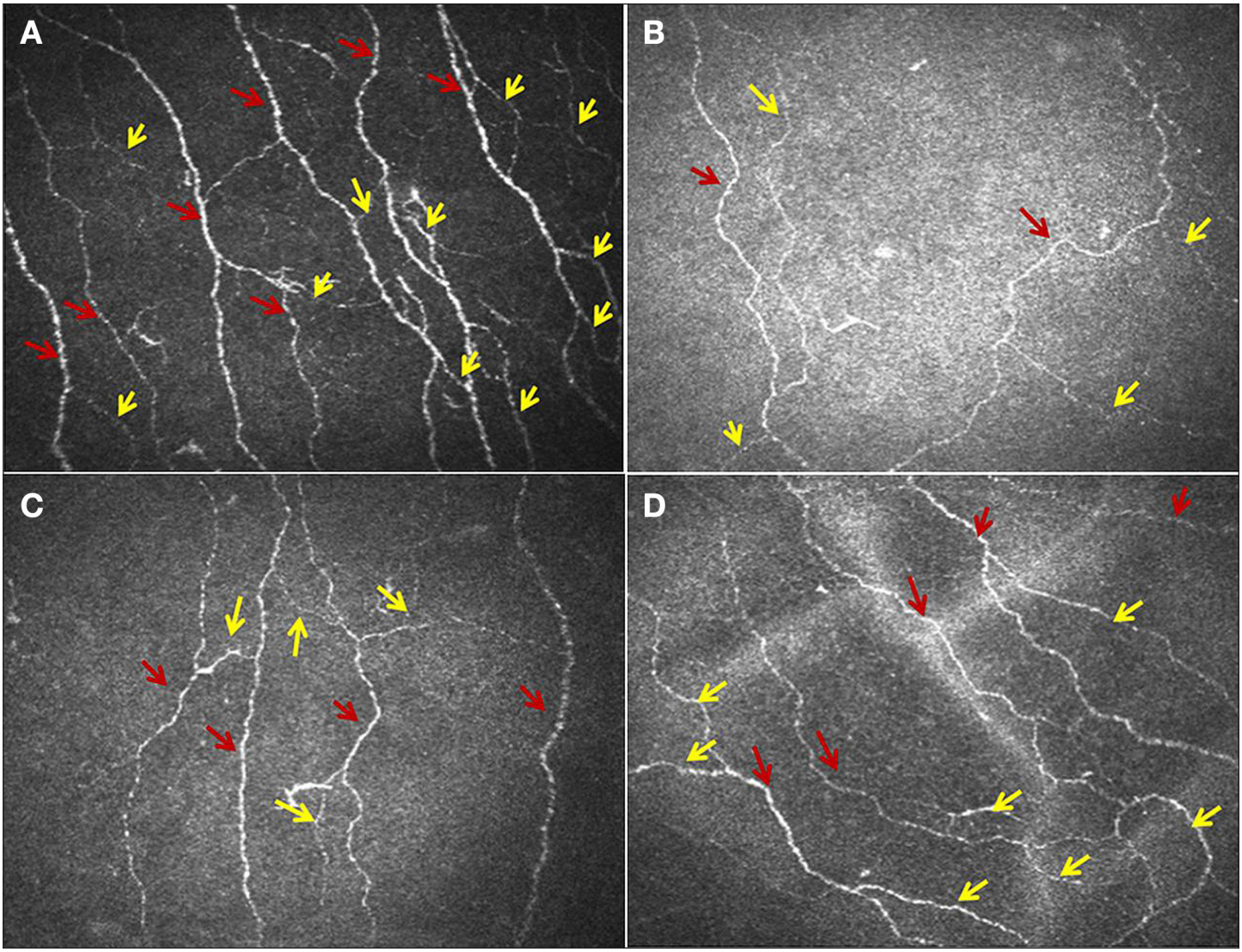

Figure 3. Sub-basal nerve images from the cornea of **(A)** a control subject and **(B)** a patient with type 1 diabetes at baseline and at **(C)** 6 and **(D)** 12 months after simultaneous pancreas kidney transplantation. The red arrows indicate main nerve fibers, and yellow arrows indicate branches.[37]

Summary

In conclusion, IVCCM appears to be an ideal noninvasive clinical technique that can assess alterations in corneal cellular pathology and, in particular, has been used to quantify small nerve fiber pathology in relation to DN. With the development of automated image analysis, we predict a rapid increase in the clinical utility of IVCCM in the assessment of DN and a range of peripheral neuropathies. In this review, we have summarized the potential of this powerful technique to undertake detailed morphological analysis of corneal nerves to act as a surrogate measure of peripheral neuropathy. It appears that the widest application of IVCCM may well be in the field of metabolic or neurological disease, particularly as it may provide a noninvasive means to identify patients with minimal neuropathy, quantify the severity of neuropathy, and follow progression of or assess therapeutic response, in not only DN, but also a range of other neuropathies. There is clearly a need to standardize the method of capturing, sampling, and analyzing the images in order to use IVCCM in longitudinal prospective or interventional multicenter studies. Finally, "prevention is better than cure." Hence, preventing foot ulceration may well require a paradigm shift from identifying advanced neuropathy (monofilament)—which may be too late for intervention)—to minimal neuropathy, which may be amenable to intervention; hence keeping an "eye on the foot."

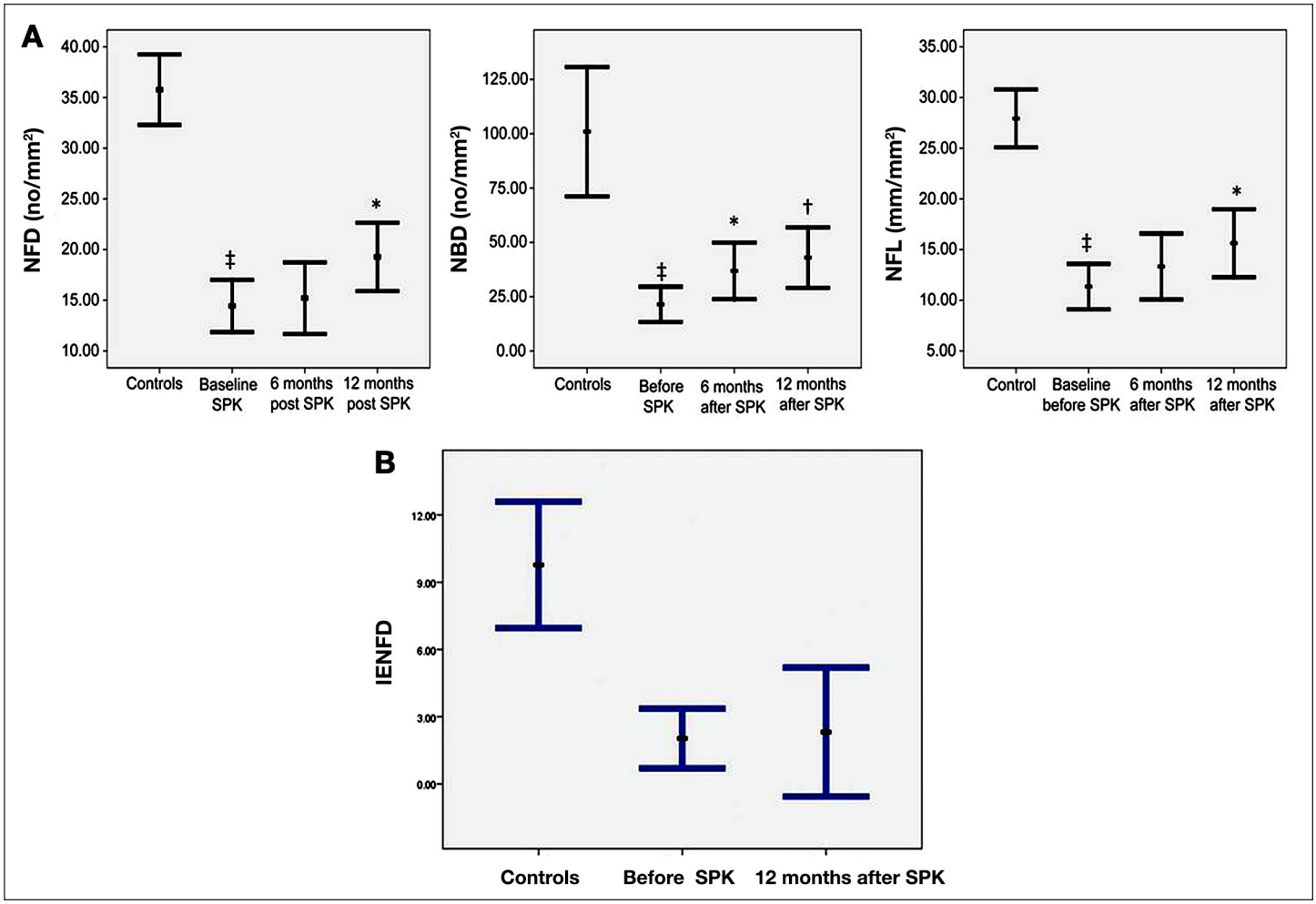

Figure 4. (A) Corneal nerve fiber density **(left)**, corneal nerve branch density **(middle)**, and corneal nerve fiber length **(right)** in diabetes patients at baseline and at 6 and 12 months after simultaneous pancreas kidney transplantation, where significant regeneration is recorded. **(B)** Intra-epidermal nerve fiber density in control subjects and in diabetes patients at baseline and 12 months after simultaneous pancreas kidney transplantation (SPK) showed no significant improvement. NFD, nerve fiber density; NBD, nerve branch density; NFL, nerve fiber length.[37]

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