In Vivo Confocal Microscopy of Corneal Nerves: An Ocular Biomarker for Peripheral and Cardiac Autonomic Neuropathy in Type 1 Diabetes Mellitus

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Purpose. We investigated the relationship between corneal subbasal nerve (SBN) plexus density, corneal sensitivity, and peripheral and cardiac autonomic neuropathy in patients with type 1 diabetes mellitus.

Methods. We recruited 53 patients with type 1 diabetes mellitus and 40 normal control participants. Corneal in vivo confocal microscopy (IVCM) and sensitivity testing were performed on one eye of each subject. Autonomic function testing was done and an overall neuropathy score obtained from a combination of a symptomatic neuropathy score, clinical assessment, biothesiometry, and nerve conduction tests.

Results. The corneal SBN density ($P < 0.001$) and corneal sensitivity ($P < 0.001$) were significantly lower in subjects with diabetes compared to controls. A modest negative correlation between total neuropathy score and SBN density was observed ($r = -0.33$, $P = 0.01$). A negative correlation between corneal sensitivity and expiration/inspiration component of the autonomic nerve analysis (ANS-EI) also was noted ($r = -0.36$, $P = 0.008$). Corneal SBN density was abnormal in 50% of diabetic subjects classified as "Normal" by the clinical and electrophysiological based tests of total neuropathy score.

Conclusions. The correlation of corneal SBN density with total neuropathy score suggests that reduced corneal nerve density reflects peripheral neuropathy in diabetes. Corneal SBN changes precede other clinical and electrophysiology tests of neuropathy supporting a possible role for corneal IVCM and corneal sensitivity testing as surrogate markers in the assessment of diabetic peripheral and cardiac autonomic neuropathy.

Keywords: in vivo confocal microscopy, diabetes, corneal subbasal nerves, peripheral neuropathy, cardiac autonomic neuropathy

Peripheral and autonomic neuropathies are common accompaniments of diabetes and lead to significant morbidity and mortality. Early diagnosis could, thus, initiate interventions that delay or potentially reverse the process before significant tissue damage has occurred. Unfortunately, current tests of peripheral neuropathy are insensitive and become abnormal only when neuropathy is well established. Typically, electrophysiological measurement of nerve conduction velocity and amplitude is used to detect nerve dysfunction objectively, and can be combined with subjective neuropathic symptoms and signs that detect the presence and severity of distal length-dependent neuropathy, to form a total neuropathy score.[1,2]

Nerve conduction studies (NCS) enable only large fiber dysfunction to be detected at later stages of diabetic neuropathy.[3] Identification of abnormalities of distal small nerve fibers is otherwise difficult by standard objective neuropathic tests.[4] The gold standard assessment of severity of small fiber neuropathy is via a skin punch biopsy, but this enables examination of morphology only, not function,[5] and its invasive nature limits repeat testing and adoption as a standard screening tool.[5] Conversely, in vivo corneal confocal microscopy (IVCM) allows direct noninvasive visualization of small fiber corneal nerve microstructure.[6–8] The technique of IVCM enables examination of the living human cornea at a cellular level.[9,10] More recently, IVCM has been shown to correlate well with other evidence of peripheral neuropathy in patients with diabetes and has demonstrated reproducibility as a technique.[11–13] A general threat to the ocular surface integrity in diabetes mellitus has been identified, especially with reduced tear production and tear stability, and its association with peripheral neuropathy.[14]

The primary aim of this study was to examine, in detail, the relationships between corneal nerve microstructure and corneal sensitivity, and indices of peripheral and autonomic neuropathy in patients with type 1 diabetes.

Methods

The study was conducted in the University of Auckland Cornea and Anterior Segment Unit based in the Department of

Ophthalmology, Greenlane Clinical Centre, Auckland District Health Board, New Zealand. This study adhered to the tenets of the Declaration of Helsinki and ethical approval was granted by the Regional Ethics Committee (NTX/09/12/122).

Adult patients with a history of type 1 diabetes, as described in the expert committee report,[15] ($n = 207$) were identified by a specialist endocrinologist (GB). Of these, 53 fulfilled the inclusion criteria (type 1 diabetes with duration of diabetes > 5 years, age 18–70, no evidence of neuropathy from a cause other than diabetes, and no history of retinal laser therapy for diabetic retinopathy, contact lens wear, ocular trauma, or ocular surgery in both eyes) and provided written, informed consent to participate in the study. We recruited, as controls, 40 healthy subjects with no history of diabetes (and hemoglobin A_{1c} [HbA_{1c}] <5.9%, 41 mmol/mol), by poster advertisements at the University of Auckland and the Department of Ophthalmology, Greenlane Clinical Centre, Auckland District Health Board, New Zealand. None of the patients or controls had a history of contact lens wear, ocular surgery, or trauma in the eye to be examined, or ipsilateral leg amputation. Ocular assessments were performed on the right eye only ($n = 81$), with the exception of individuals with a history of uni-ocular surgery or trauma to the right eye, in which case the left eye ($n = 12$) was examined. A detailed medical history was obtained, including time since diabetes diagnosis, medications used, pack years of smoking, and alcohol use estimated in units/wk.

Laser scanning IVCM was performed on the central cornea with the Heidelberg Retina Tomograph II, Rostock corneal module (Heidelberg Engineering GmBH, Heidelberg, Germany).[7] This clinical technique uses a confocal arrangement to eliminate out-of-focus light, thus, enabling noninvasive optical sectioning of the living human cornea. Each IVCM image represents 400 × 400 μm with a lateral resolution of 2 μm and optical section thickness of 4 μm. Three high-quality focussed individual images of subbasal nerves (SBNs) were selected, then randomized for subsequent analysis. The SBN density was evaluated by tracing visible nerves with an electronic pen (Wacom Technology Group, Vancouver, BC, Canada) and measuring the total length of nerves per frame with a digital calliper tool (analySIS 3.1; Soft Imaging System, Münster, Germany).[7,8]

The central corneal sensitivity threshold was evaluated by noncontact corneal aesthesiometry or NCCA (Glasgow Caledonian University, Glasgow, UK) using the double staircase method.[16] Only participants with diabetes underwent a peripheral neuropathy assessment, and a total neuropathy score (TNS) was generated from a combination of the symptomatic neuropathy score, clinical neuropathic assessment by an experienced neurologist (DK), biothesiometry (quantitative sensory testing) and NCS.[1] The brief and validated, questionnaire, of three questions, asked participants about numbness, pain, and sensitivity in their extremities. Administration of the questionnaire was followed by a clinical examination of peripheral nerves. The vibration threshold of the nerves of the feet was established using a Biothesiometer (Biomedical Instrument Company, Newbury, OH, USA) before the final test of NCS.[17] An overall score then was summated with a possible maximum TNS score of 40. A score of more than 1 was considered abnormal. Cardiovascular autonomic nervous system (CAN) assessment evaluated heart rate variability and reflex responses during resting, breathing, paced breathing (including expiration/inspiration ratio, or ANS-EI), Valsalva maneuver, and the orthostatic response. The data obtained from these responses were compared to reference data obtained from normal subjects (unpublished local data) and a correlation score was created. Digital images of the central and peripheral retina were captured using a nonmydriatic retinal camera (Non-Mydriatic Retinal Camera DR-DGi; Canon, Inc., Melville, NY, USA) for diabetic retinopathy grading by an independent, fellowship-trained, medical retina specialist (MP). The grading was based on the Early Treatment Diabetic Retinopathy Study research group (ETDRS) scale.[18]

Table 1. Demographics of Patients With Type 1 Diabetes and Controls

	Patients	Controls	ANOVA *P* Values
Demographics			
Subjects, n	53	40	–
M:F ratio	26:27	17:23	–
Age, y	48.6 ± 11.8	44.3 ± 14.7	0.12
BMI, kg/m^2	26.9 ± 4.6	25.0 ± 3.8	0.03
Smoking history, pack y	4.2 ± 10.3	1.7 ± 1.0	<0.001
Alcohol units/wk	3.8 ± 2.3	2.1 ± 0.2	<0.001
HbA_{1c}, %	7.8 ± 3.2	5.4 ± 0.2	<0.01
HbA_{1c}, mmol/mol	61 ± 12	35 ± 2	–
Mean DM Duration, y	25.8 ± 11.3	–	–
<10, $n = 5$	–	–	–
10–20, $n = 10$	–	–	–
21–30, $n = 22$	–	–	–
>30, $n = 16$	–	–	–
Corneal subbasal nerve density, mm/mm^2	11.04 ± 3.8	21.17 ± 4.2	<0.001
Corneal sensitivity threshold, mBAR	1.3 ± 1.3	0.2 ± 1.3	<0.001
Diabetic retinopathy Grade, No. (%)			
Nil	32 (60.3)	–	–
Mild	11 (20.7)	–	–
Moderate	10 (18.8)	–	–
Severe	0 (0)	–	–
Neuropathy score			
TNS	5.3 ± 5.1	–	–
Symptoms	0.5 ± 0.9	–	–
Clinical	1.1 ± 1.7	–	–
Biothesiometry	0.8 ± 1.4	–	–
NCS	2.7± 2.8	–	–

The mean values of corneal SBN density (mm/mm^2) and CST (mBAR) in patients with type 1 diabetes and control subjects also are stated. Data are mean ± SD. Patients, type 1 diabetes; BMI, body mass index; TNS, total neuropathy score; NCS, nerve conduction study.

The Shapiro-Wilk test was performed to confirm normality of the data distributions for those with diabetes. In collaboration with a biostatistician, and on the basis of previously reported data,[7] a power calculation for this study determined a sample size of 50 patients and 40 controls was required to detect a difference between the groups, with 80% power and 95% confidence. One-factor ANOVA was performed to determine the statistical difference between the groups. A Pearson correlation (2-tailed) analysis was applied to determine the relationship between variables. A P value of <0.05 were considered significant. Data are presented as means ± SD.

Results

Patient demographics describing sex, age, body mass index (BMI), duration of diabetes, smoking history (pack years), alcohol history as units/wk, HbA_{1c}, and severity of diabetic retinopathy are documented in Table 1. We studied 53 patients with type 1 diabetes (26 male and 27 female), with a mean age

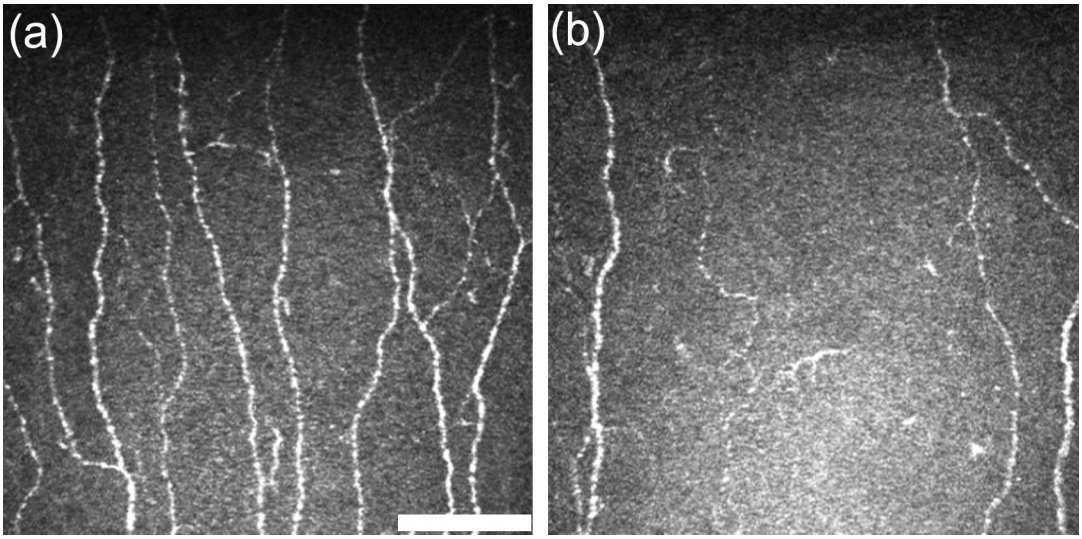

FIGURE 1. Representative in vivo confocal images showing the corneal SBN plexus of (**a**) a 47-year-old female control subject and (**b**) a 45-year-old female with 30-year history of DM. *Scale bar*: 100 μm.

of 48 ± 11 years (age range, 19–70 years). The mean duration since diabetes diagnosis was 26 ± 11 years. Mean data from neuropathic assessments also are presented in Table 1. An age-matched cohort of 40 controls (17 male and 23 female) with an age range of 22 to 73 years, with a mean age of 44 ± 14 years, and no previous ophthalmic history or contact lens wear, were assessed for comparison. The control and patient group were age-matched ($P = 0.12$).

Significantly lower corneal sensitivity (higher threshold to sensation) compared to controls ($P < 0.0001$) was noted in those with diabetes (Table 1). Typical IVCM images of the SBN plexus from a normal control subject and from a patient with type 1 diabetes are shown in Figure 1. Participants with diabetes had a significantly lower SBN density compared to healthy controls ($P < 0.0001$; Table 1, Fig. 2). The corneal SBN density showed an inverse correlation with total neuropathy score ($r = -0.33$, $P = 0.01$; Fig. 3), neuropathy symptoms ($r = -0.34$, $P = 0.01$), and nerve conduction velocity ($r = -0.34$, $P = 0.01$). Of patients with diabetes, 60% had no active retinopathy, 21% had mild, and 19% moderate retinopathy.

Pearson correlation analysis of BMI did not correlate with the neuropathy scores or ocular assessments. Longer disease duration showed modest correlation with total neuropathy score ($r = 0.33$, $P = 0.01$), clinical neuropathy assessment score ($r = 0.27$, $P = 0.04$), and nerve conduction velocity ($r = 0.34$, $P = 0.01$). Elevated HbA_{1c} was associated with higher smoking score ($r = 0.29$, $P = 0.03$), total neuropathy scores ($r = 0.30$, $P = 0.03$), and neuropathy clinical assessment ($r = 0.38$, $P = 0.004$). Pack years of smoking was associated with greater TNS ($r = 0.47$, $P < 0.001$; Table 2). Hemogobin A_{1c} was associated with diabetic retinopathy grading ($r = 0.46$, $P = 0.001$), as were NCS-clinical ($r = -0.32$, $P = 0.02$) and ANS-EI ($r = -0.40$, $P = 0.003$) scores.

There was an inverse correlation between corneal sensitivity threshold (CST) and corneal SBN density, which was statistically significant ($r = -0.36$, $P = 0.008$). The clinical component of NCS also showed a positive correlation with corneal sensitivity ($r = 0.31$, $P = 0.02$).

Autonomic function assessment was performed successfully on 52 participants with diabetes; data from one participant could not be analyzed due to excessive ectopy. A total of 19

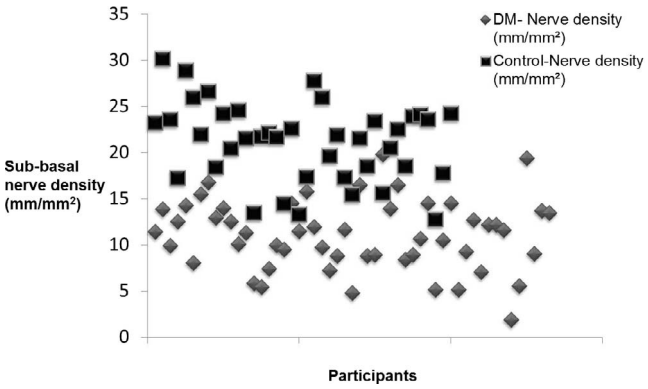

FIGURE 2. A data point graph displaying separation in corneal SBN density between control participants (control, 21.17 ± 4.2 mm/mm²) and patients with type 1 diabetes (DM, 11.0 ± 3.8 mm/mm²; $P < 0.0001$).

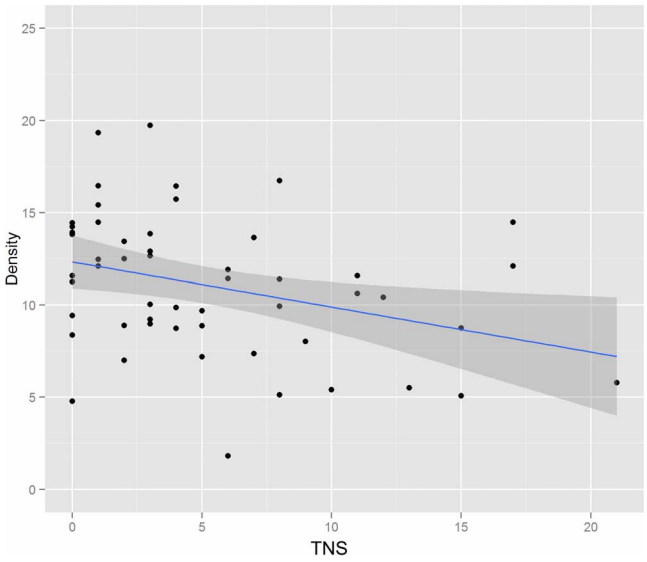

FIGURE 3. A dot plot elucidating association between corneal SBN density (mm/mm²) and TNS ($r = -0.33$) in patients with type 1 diabetes.

Table 2. Pearson Correlation Analysis Between Smoking (Pack Years), HbA_{1c}, Corneal Sensitivity (mBAR), SBN Densities (mm/mm^2), and TNS

	Correlation, *r* Values	Significance, *P* Values
Smoking score vs. HbA_{1c}	0.29*	0.03
Smoking score vs. TNS	0.47†	<0.001
HbA_{1c} vs. TNS	0.30*	0.03
Corneal sensitivity vs. SBN	−0.36†	0.01
TNS vs. SBN	−0.33*	0.01

* Correlation significant at the 0.05 level (2-tailed).
† Correlation significant at the 0.01 level (2-tailed).

patients had a composite score outside the 95% confidence interval derived from 60 normal subjects (unpublished data). There was poor correlation between this measure and corneal SBN density, but corneal sensitivity was noted to be negatively correlated with the ANS-EI ($r = –0.36$, $P = 0.008$).

Discussion

Currently, nerve electrophysiology, sural nerve, and skin punch biopsy are the gold standards for diagnosing and quantifying diabetic peripheral neuropathy.[5] However, the ability of these tests to detect small nerve fiber damage is limited, and they are invasive, suitable neither for routine diagnostic, nor repeat, testing. In randomized controlled trials, subjective assessments including health surveys canvassing symptoms, mood states, or pain scores are used.[19] Clinical evaluation of diabetic peripheral neuropathy with invasive tests, such as skin punch biopsy, is neither practical for the purpose of routine screening nor as an objective measure in randomized controlled trials to test the efficacy of various treatment strategies.

Unmyelinated C-class and Aδ small nerve fibers are adversely affected in diabetic peripheral neuropathy leading to hyperesthesia, paraesthesia, and loss of pain and temperature sensation.[20] Small corneal nerve fibers also can be affected at this early stage.[21] These early changes are identified using IVCM, which allows noninvasive detection of small nerve fiber damage in vivo. Under typical physiological conditions, the corneal SBN plexus contributes to a healthy epithelial surface by maintaining corneal sensitivity, normal epithelial metabolism, and by liberating neuropeptides and growth factors.[22] The corneal nerves are known to be affected by diabetes.[11,23] Changes include a reduction in SBN density, decreased nerve branching, and increased nerve tortuosity, the latter possibly reflecting nerve regeneration in diabetic keratopathy.[24–26]

The SBN density was markedly reduced in the patients with type 1 diabetes in the current study compared to age-matched controls ($P < 0.001$). Subbasal nerve branching and tortuosity were not considered nor analyzed in the present study due to the previously established lack of reproducibility of these measurements in patients with diabetes.[27] The SBN density is the most sensitive and repeatable measurement of SBN plexus in those with diabetes, and thus was used in the current study.[27] However, tandem and slit-scanning confocal microscopes report lower SBN density compared to laser scanning IVCM, which was used in this research.[28]

Corneal sensitivity exhibited an inverse relationship with corneal SBN density in this study ($r = 0.36$ and $P = 0.008$). Such a decrease in corneal sensitivity in patients with diabetes is unsurprising ($P < 0.001$), since corneal sensitivity in these patients is reported to be 70% sensitive and 75% specific for accuracy.[29,30] The decrease in corneal sensitivity and abnormal neural regulation in the diabetic cornea has been recognized to result in delayed epithelial wound healing and recurrent corneal erosions[31] and, potentially, to be valuable as an ophthalmic marker of diabetic peripheral neuropathy.[30] A prospective longitudinal study would significantly aid in cementing IVCM measurement of the SBN plexus as an ophthalmic marker for peripheral neuropathy in diabetes.

The relationship between corneal SBN density and TNS (Fig. 3) illustrates the clinically important feature that corneal SBN density already was abnormal in 50% of patients with diabetes classified as "Normal" by the clinical and electrophysiological based tests of total neuropathy score. Analysis of the corneal SBN density, therefore, is likely to be a more sensitive marker of early diabetic neuropathy.

Importantly, the inverse correlation of corneal SBN density with total neuropathy score emphasizes the parallel involvement of the trigeminal (cranial) nerve in the context of diabetic peripheral neuropathy ($r = –0.33$, $P = 0.01$). Interestingly, an independent study of 25 patients with a history of idiopathic small fiber neuropathy also showed significant corneal SBN density damage.[32] It has been suggested that corneal and intraepidermal nerve measurements are significantly lower in patients with painful neuropathy compared to those with painless neuropathy.[33] Not only does IVCM detect the involvement of corneal nerves in patients with diabetic peripheral neuropathy, but in prospective observational studies it has revealed improvements in corneal nerve morphology occurring with better glycemic control.[12,23] Semiautomated analysis of IVCM images previously has shown repeatable results in those with diabetes,[6] which would make clinical use of the technique more readily accessible.

Although a previous study has shown that reduced corneal SBN density is correlated with worsening diabetic retinopathy, the current study did not identify any significant correlation between retinopathy grading and SBN density, despite the presence of peripheral neuropathy.[11] However, it must be noted that patients with retinopathy beyond a moderate level were excluded from this study due to the potential for adverse effects of laser photocoagulation on corneal nerve structure.[34] The observations of the present study are supported by the recent publication by Zhivov et al.,[35] which reported reduced SBN density in patients with peripheral neuropathy, irrespective of the presence of retinopathy. The purely microvascular nature of diabetic retinopathy as opposed to the combined neuropathic and microvascular contribution to peripheral neuropathy could be a possible explanation.[36,37]

The current study confirmed previously established relationships between elevated HbA_{1c}, diabetic retinopathy, higher total neuropathy score, and aberrant cardiovascular autonomic control, corroborating the importance of glycemic control.[38–41] A greater smoking history correlated with peripheral small fiber neuropathy, CAN, and retinopathy,[40,42] consistent with previous reports showing that patients with a history of over 30 pack-years are at 3-fold increased risk of developing neuropathy.[43]

Abnormalities of heart rate variability are regarded as an early sign of CAN. A few studies have reported dysfunctional heart rate variability in patients with diabetes and CAN using the instantaneous cardiac rate-meter.[44,45] Patients with uncontrolled glucose levels are reported to suffer from some degree of neural dysfunction with small fiber neuropathy and CAN.[46] We believe this is the first study to explore relationships between CAN, total neuropathy score, retinopathy and corneal SBN damage. Typically, CAN with reduced heart rate variability and parasympathetic loss is observed within 2 years of the diagnosis of type 1 diabetes.[47] Therefore, correlation between total neuropathy score and autonomic nerve abnormality was not surprising. However, the inverse correlation of corneal sensitivity with autonomic nerve analysis is a novel observation. The assessment of this ocular variable could be helpful in

predicting cardiac autonomic neuropathy, a feature that may be associated with an increased risk of mortality.[48]

In conclusion, diabetes-related corneal abnormalities, including reduced SBN density and altered corneal sensitivity, occur in parallel with peripheral and cardiac autonomic neuropathy. Both of these ocular parameters, therefore, have a potential role as noninvasive biomarkers for diabetic neuropathy allowing earlier prediction of these microvascular complications. "At risk" patients with diabetes who are enlisted for regular diabetic retinal screening programs and are more prone to complications, thus, could benefit with complementary corneal IVCM and corneal sensitivity assessment. Importantly, these noninvasive ocular assessments, as distinct from invasive skin punch biopsies, could also have a valuable role in monitoring the efficacy of novel treatments for ocular as well as peripheral complications of diabetes in randomized controlled trials.

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