Corneal Nerve Tortuosity in Diabetic Patients with Neuropathy

Panagiotis Kallinikos,¹ Michael Berhanu,² Clare O’Donnell,¹ Andrew J. M. Boulton,³ Nathan Efron,¹ and Rayaz A. Malik⁴

PURPOSE. Corneal confocal microscopy is a reiterative, rapid, noninvasive in vivo clinical examination technique capable of imaging corneal nerve fibers. Nerve fiber tortuosity may indicate a degenerative and attempted regenerative response of nerve fibers to diabetes. The purpose of this study was to define alterations in the tortuosity of corneal nerve fibers in relation to age, duration of diabetes, glycemic control, and neuropathic severity.

METHODS. The cornea and collected images of the subbasal nerve plexus of 18 diabetic patients (stratified into mild, moderate, and severe neuropathic groups using conventional clinical measures of neuropathy) and 18 age-matched nondiabetic control subjects were scanned, and a novel mathematical paradigm was applied to quantify the extent of nerve tortuosity, which was termed the tortuosity coefficient (TC).

RESULTS. TC was significantly different between the four clinical groups (F₂,¹⁷ = 12.2, P < 0.001). It was significantly greater in the severe neuropathic group than in control subjects (P < 0.003) and in the mild (P < 0.004) and moderate (P < 0.01) neuropathic groups. TC did not correlate significantly with the age (r = −0.003, P > 0.05), duration of diabetes (r = −0.219, P > 0.05), or hemoglobin Alc (HbA1c; r = 0.155, P > 0.05) of diabetic patients.

CONCLUSIONS. Corneal confocal microscopy allows rapid, noninvasive in vivo evaluation of corneal nerve tortuosity. This morphologic abnormality relates to the severity of somatic neuropathy and may reflect an alteration in the degree of degeneration and regeneration in diabetes. (Invest Ophthalmol Vis Sci. 2004;45:418–422) DOI:10.1167/iovs.03-0637

The accurate quantification of diabetic polyneuropathy is important to define at risk patients, anticipate deterioration, and assess new therapies.¹ Electrophysiology and quantitative sensory tests both separately²–⁵ and as summated scores⁴ quantify neuropathic severity. However, these tests cannot discriminate damage and particularly repair to specific fiber types after intervention.²–⁴

Quantitative sensory tests of thermal and pain perception are proposed to assess small-fiber damage.²–⁴ However, we have recently shown no relationship between quantitative sensory tests and small myelinated or unmyelinated fiber damage and repair.⁵ Alternative, more accurate measures of nerve fiber damage and repair include nerve biopsy with electron microscopy⁶ and ex vivo confocal microscopy of skin biopsy specimens,⁷ but both are invasive procedures.

The cornea represents one of the most densely innervated tissues of the body.⁸–⁹ Corneal innervation provides protective and trophic functions¹⁰–¹³ for corneal repair in relation to disease, trauma, or surgery.¹⁴ Defining alterations in the corneal nerves has been limited. We have recently used corneal confocal microscopy to quantify corneal nerve morphology in normal subjects¹⁵ and have developed this application to show that alterations in fiber density and branching relate to the severity of somatic neuropathy in diabetic patients.¹⁶

Corneal nerves course through the stroma which is composed of collagen and substances such as fibronectin and proteoglycans.¹⁷–¹⁸ These substances are known to be upregulated in diabetes¹⁹ and influence axonal outgrowth and regeneration.²⁰ Much of our knowledge on nerve regeneration is based on experiments after peripheral sciatic nerve crush which have demonstrated increased tortuosity of regenerating nerves particularly in older animals.²¹ Any direct comparison between a peripheral and cranial nerve must be interpreted with caution, as the regenerative response may differ in the two sites. In the present study, we used corneal in vivo confocal microscopy to quantify corneal nerve tortuosity and relate it to the severity of somatic diabetic neuropathy.

METHODS

Eighteen diabetic patients aged 58 ± 12 (mean ± SD) years underwent neuropathic severity evaluation and corneal in vivo confocal microscopic examination. Patients with any other known cause of neuropathy or previous corneal abnormality were excluded. In vivo confocal microscopy was performed on a further 18 age- and sex-matched control subjects. The research adhered to the tenets of the Declaration of Helsinki. Informed consent was obtained from all subjects after explanation of the nature and possible consequences of the study. The protocol used was approved by the Local Research Ethics Committee (Central) of Manchester Health Authority.

Neuropathic Severity Evaluation

All patients underwent a clinical history and neurologic examination to rule out any other cause of neuropathy or previous corneal abnormality. The neuropathy disability score (NDS) was based on a clinical scoring system obtained from a neurologic examination that defined abnormalities of vibration perception threshold (VPT) using a tuning fork, pin-prick perception, and temperature perception threshold (TPT), as well as the presence or absence of ankle reflexes, producing a score ranging from 0 to 10.²²,²³ Quantitative vibration and thermal assessment were performed with a sensory evaluator (Computer Aided Sensory Evaluator IV [CASE IV]; WR Medical Electronics Co., Stillwater, Minnesota, USA).
TC Computation

Using the MatLab built-in function “im2double,” we converted the image to an array (matrix) of numbers. The elements of the matrix were either zeros (background) or ones (nerve fiber). The coordinates of the nerve were the indices of the “nonzero” entries in the matrix, which were returned by the MatLab built-in function “find.” A straight line that connected the end points of the nerve fiber was plotted, and the image was translated and rotated to the origin, to align the straight line with the x-axis.

The computation of TC of corneal nerves was based on the approach presented in a previous study, where the researchers proposed a quantitative index for evaluating arterial tortuosity, based on the second differences of the coordinates of the vessel midline.

In the present study we calculated TC for corneal nerves based on a series of simple mathematical calculations. Each corneal nerve was represented as the graph of a function. The derivative of a function \( f \) at a point \( x \) is a measure of the rate at which that function is changing as (one of) its independent variables change. This corresponds to the slope of the tangent to the graph of the function at that point. If we increase \( x \) by a small amount, \( dx \), we can calculate \( f(x + dx) \).

We first considered equally spaced points \( x_j \) on the straight line that connected the ends of the nerve. The approximation of the first derivative is given by the difference of two consecutive points on the nerve, divided by the step size (\( dx \)). The second derivative is calculated as the difference of two consecutive values of the first derivative, divided by the step size. The step size \( dx \) is the distance between the projections on the \( x \)-axis of two consecutive pixels of the nerve fiber. The value of \( dx \) is constant and equal to 1 pixel, because the number of columns in the matrix that have a nonzero entry is always the same as the number of \( x \) coordinates of the nerve fiber. The following equations give an approximation of the first and second derivatives in the interval \((x_j, x_{j+1})\), respectively:

\[
\frac{df(x_j)}{dx} = \frac{f(x_{j+1}) - f(x_j)}{dx}
\]

\[
\frac{d^2f(x_j)}{dx^2} = \frac{f(x_{j+1}) - 2f(x_j) + f(x_{j-1})}{(dx)^2}
\]

The first and second derivatives are squared and added. The sum is multiplied by the length of the interval \((x_j, x_{j+1})\), to estimate the change in the direction of the nerve, within that interval. The sum of all the values is obtained and the square root taken. Once all the quantities have been computed, TC is calculated by the following formula:

\[
TC = \sqrt{\sum_{j=1}^{n-1} (x_{j+1} - x_j)\left[\left(\frac{df(x_j)}{dx}\right)^2 + \left(\frac{d^2f(x_j)}{dx^2}\right)^2\right]}
\]

### Table 1. Clinical Details and Indices of Neuropathic Severity in Diabetic Patients and Nondiabetic Control Subjects

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Nondiabetic Control Subjects (n = 18)</th>
<th>Diabetic Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mild Neuropathy (n = 4)</td>
<td>Moderate Neuropathy (n = 7)</td>
</tr>
<tr>
<td>Age (y)</td>
<td>57.8 ± 11.5</td>
<td>53.0 ± 18.5</td>
</tr>
<tr>
<td>Diabetes duration (y)</td>
<td>21.3 ± 3.6</td>
<td>20.8 ± 5.1</td>
</tr>
<tr>
<td>Diabetes (type 1/type 2)</td>
<td></td>
<td>2/2</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>6.5</td>
<td>7.8 ± 0.8</td>
</tr>
<tr>
<td>NDS</td>
<td>0</td>
<td>1.2 ± 0.6</td>
</tr>
<tr>
<td>PMNCV (ms⁻¹)</td>
<td>&gt;50.0</td>
<td>37.6 ± 3.4</td>
</tr>
<tr>
<td>VPT (V)</td>
<td>&lt;14.0</td>
<td>11.2 ± 4.3</td>
</tr>
<tr>
<td>TPT (JND)</td>
<td>&lt;15.0</td>
<td>17.6 ± 2.2</td>
</tr>
</tbody>
</table>

JND, just noticeable difference. Data are the mean ± SD.
where $dx = x_{i+1} - x_{i}$ and $f'(x_{i})$ and $f''(x_{i})$ are the first and second derivatives at the point $x_{i}$, respectively.

To test the validity of this approach, TC was calculated for four simple functions: (1) $f(x) = \sin(x)$; (2) $f(x) = \sin(2x)$; (3) $f(x) = \sin(4x)$; and (4) $f(x) = x$.

The TCs obtained for each function were (1) 2.5066, (2) 7.9265, (3) 29.2292 and (4) 0, respectively. This analysis verified that higher TCs are obtained for curves of greater tortuosity (frequency), whereas the TC for a straight line ($f(x) = x$) equals zero. The same TCs are obtained when the graphs of these functions are rotated at various angles, indicating that TC is independent of the angle of the nerve axis.

**Statistical Analysis**

A univariate analysis of variance (U-ANOVA) was conducted to compare the tortuosity of corneal nerves for the four clinical groups. Where differences within the clinical groups were established at $P = 0.05$ level, post hoc analysis was conducted using the least significant difference (LSD) test. Spearman’s correlation coefficient was computed to test for significant associations between the TC and age, duration of diabetes, and HbA1c of the diabetic patients. Correlation was set to be significant at $P = 0.05$.

**RESULTS**

The groups of patients were matched for age, type, and duration of diabetes and degree of glycemic control. The clinical details of study subjects and the measures of neuropathic severity assessed are shown in Table 1. Diabetic patients demonstrated a progressive increase in vibration and thermal perception and a decrease in nerve conduction velocity with increasing neuropathic severity.

Qualitative assessment of the subbasal nerve plexus layer of a control subject demonstrates three fibers with a typical beaded appearance and normal tortuosity (Fig. 1). In comparison, the subbasal nerve plexus layer of a diabetic patient with severe neuropathy demonstrates only one nerve fiber with a single branch and increased tortuosity (Fig. 2).

The TC was significantly different between the four clinical groups ($F_{3, 122} = 12.2, P < 0.001$). Post hoc analysis demonstrated that the TC was significantly increased in the severely neuropathic group compared with control subjects ($P < 0.003$) and the mild ($P < 0.004$) and moderate ($P < 0.01$) neuropathic groups. In addition, TC for patients with moderate neuropathy was greater than that of the control subjects and the mild neuropathy group, but these differences were not statistically significant (Fig. 3). The descriptive statistics for the TCs for the four clinical groups are presented in Table 2.

The TC was not significantly correlated with age ($r = -0.003, P > 0.05$), duration of diabetes ($r = -0.219, P > 0.05$), or HbA1c ($r = 0.155, P > 0.05$) among the diabetic patients.

**DISCUSSION**

The application of confocal microscopy to imaging the cornea provides a new approach to the study of corneal nerve morphology.26–27 It allows rapid, in vivo, noninvasive evaluation enabling prospective and reiterative examination of the human cornea in healthy subjects, contact lens wearers, patients who have had refractive surgery,27 and those with ocular and systemic disease.16–28

Corneal nerves have protective and trophic functions in the cornea.10–13 Anatomically, they extend from the ophthalmic division of the trigeminal nerve through the anterior ciliary nerves entering the middle third of the stroma to form the

![Figure 1](https://example.com/image1)

**Figure 1.** Confocal microscope image of Bowman’s layer in a control subject. Corneal nerve fibers demonstrate normal tortuosity.

![Figure 2](https://example.com/image2)

**Figure 2.** Confocal microscope image of Bowman’s layer in a diabetic patient with severe neuropathy. Corneal nerve displays greater tortuosity.

![Figure 3](https://example.com/image3)

**Figure 3.** TC in control subjects and diabetic patients with mild, moderate and severe neuropathy. Shaded box: interquartile range (50% of the values); whiskers: lines that extend from the box to the highest and lowest values; midline: median. The TC was significantly different between the four clinical groups ($F_{3, 122} = 12.2, *P < 0.001$).
subbasal epithelial plexus anterior to Bowman’s layer and finally innervate the basal and superficial epithelial cell layer. Anatomic and immunohistological studies confirm the presence of catecholaminergic, adrenergic, and primarily nociceptive C fibers.13–15 These nerves respond primarily to noxious mechanical, thermal, and chemical stimuli; for example, application of topical capsaicin results in a 70% reduction in corneal nerve fiber density.29 Furthermore, recent studies in mutant mice in which TrkA-the high-affinity receptor for nerve growth factor (NGF) has been inactivated, demonstrate a marked reduction in response to mechanical, thermal, and chemical noxious stimuli and the number of nerve terminals in the cornea.20

After LASIK, the number of subbasal and stromal nerve fiber bundles decreases by 90% and, during the first year, reinnervation occurs but the number remains less than half of that before LASIK.31 These findings are of particular relevance to diabetic patients; Rosenberg et al.28 demonstrated a reduction in corneal nerve bundles and related it to loss of corneal sensation and severity of neuropathy in patients with type 1 diabetes. We have recently refined and extended these observations by demonstrating a significant reduction in corneal nerve fiber density suggestive of enhanced degeneration, together with a reduction in branching, suggestive of limited regeneration, which relates to measures of somatic neuropathy in diabetic patients.16 Corneal epithelial metabolism, cell adhesion, and wound healing depend on adequate corneal innervation.52 This may explain the significantly higher risk of development of postoperative epithelial complications and poorer refractive results in diabetic patients who undergo LASIK.14

The mechanisms governing corneal nerve integrity and hence their structure are potentially complex. In the corneal stroma, physical structures such as collagen, fibronectin, and proteoglycans19,53 as well as a number of growth factors including TGF-β,54 fibroblast growth factor,55 and NGF56 have been shown to regulate nerve fiber damage and repair. This may be relevant, as many of these factors are upregulated in diabetes.19 The morphologic features of corneal nerve fiber degeneration and regeneration remain to be clearly delineated. However, recent studies have demonstrated a reduction in total nerve fiber and branch density, which has been related to loss of both somatic16 and corneal26 sensation. With regard to regeneration, sciatic nerve crush experiments have demonstrated increased tortuosity of regenerating nerves, particularly in older animals.21 Thus, increased tortuosity may represent a morphologic marker of nerve regeneration. The present work demonstrates increased tortuosity of corneal nerve fibers, which is independent of age, duration of diabetes, or glycemic control in diabetic patients with increasing severity of somatic neuropathy. Caution is advised on the functional and clinical relevance of this finding in relation to corneal sensation, especially with the small number of patients studied. Nevertheless, these observations provide further support for a significant impact of diabetes on corneal nerve integrity.

### References


### Table 2. Descriptive Statistics for TC, for Diabetic Patients with Mild, Moderate and Severe Neuropathy and Control Subjects

<table>
<thead>
<tr>
<th>Tortuosity Coefficient (Descriptive Statistics)</th>
<th>Nondiabetic Control Subjects (n = 18)</th>
<th>Mild Neuropathy (n = 4)</th>
<th>Moderate Neuropathy (n = 7)</th>
<th>Severe Neuropathy (n = 7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>25.86</td>
<td>25.61</td>
<td>32.28</td>
<td>48.59</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>9.89</td>
<td>1.26</td>
<td>1.76</td>
<td>4.47</td>
</tr>
<tr>
<td>Median</td>
<td>25.23</td>
<td>24.56</td>
<td>32.94</td>
<td>49.46</td>
</tr>
<tr>
<td>Minimum</td>
<td>9.56</td>
<td>23.90</td>
<td>23.40</td>
<td>54.61</td>
</tr>
<tr>
<td>Maximum</td>
<td>43.47</td>
<td>29.43</td>
<td>40.66</td>
<td>52.62</td>
</tr>
</tbody>
</table>


