Corneal Confocal Microscopy: A Novel Non-invasive Test to Diagnose and Stratify the Severity of Human Diabetic Neuropathy

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Submitted 12 February 2010 and accepted 24 April 2010.

This is an uncopyedited electronic version of an article accepted for publication in Diabetes Care. The American Diabetes Association, publisher of Diabetes Care, is not responsible for any errors or omissions in this version of the manuscript or any version derived from it by third parties. The definitive publisher-authenticated version will be available in a future issue of Diabetes Care in print and online at http://care.diabetesjournals.org.
Objective: The accurate quantification of human diabetic neuropathy is important to define at risk patients, anticipate deterioration, and assess new therapies.

Research Design and methods: 101 diabetic patients and 17 age-matched control subjects underwent neurological evaluation, neurophysiology, quantitative sensory testing and evaluation of corneal sensation and corneal nerve morphology using corneal confocal microscopy (CCM).

Results: Corneal sensation decreased significantly ($P=0.0001$) with increasing neuropathic severity and correlated with the neuropathy disability score (NDS) ($r=0.441$, $P<0.0001$). Corneal nerve fibre density (NFD) ($P<0.0001$), length (NFL), ($P<0.0001$) and branch density (NBD), ($P<0.0001$) decreased significantly with increasing neuropathic severity and correlated with NDS (NFD ($r=-0.475$, $P<0.0001$), NBD ($r=-0.511$, $P<0.0001$); NFL ($r=-0.581$, $P<0.0001$). NBD and NFL demonstrated a significant and progressive reduction with worsening heat pain thresholds ($P=0.01$). ROC curve analysis for the diagnosis of neuropathy (NDS>3) defined an NFD of <27.8/mm$^2$ with a sensitivity of 0.82 (95% CI: 0.68-0.92) and specificity of 0.52 (95% CI: 0.40-0.64) and for detecting patients at risk of foot ulceration (NDS>6) a NFD cut off of <20.8/mm$^2$ with a sensitivity of 0.71 (95% CI: 0.42-0.92) and specificity of 0.64 (95% CI: 0.54-0.74).

Conclusions: Corneal Confocal microscopy is a non-invasive clinical technique which may be used to detect early nerve damage and stratify diabetic patients with increasing neuropathic severity.
Established diabetic neuropathy leads to pain and foot ulceration. Detecting neuropathy early may allow intervention with treatments to slow or reverse this condition (1). Recent studies suggest that small unmyelinated c-fibres are damaged early in diabetic neuropathy (2-4), but can only be detected using invasive procedures such as sural nerve biopsy (4; 5), or skin-punch biopsy (6-8). Our studies have shown that corneal confocal microscopy (CCM) can identify early small nerve fibre damage and accurately quantify the severity of diabetic neuropathy (9-11). We have also shown that CCM relates to intra-epidermal nerve fibre loss (12), a reduction in corneal sensitivity (13) and detects early nerve fibre regeneration following pancreas transplantation (14). Recently we have also shown that CCM detects nerve fibre damage in patients with Fabry disease (15) and idiopathic small fibre neuropathy (16) when electrophysiology and QST are normal.

This study assessed corneal sensitivity and corneal nerve morphology using CCM in diabetic patients stratified for the severity of diabetic neuropathy using neurological evaluation, electrophysiology and QST are normal. This study assessed corneal sensitivity and corneal nerve morphology using CCM in diabetic patients stratified for the severity of diabetic neuropathy using neurological evaluation, electrophysiology and quantitative sensory testing. This enabled us to compare CCM and corneal aesthesiometry with established tests of diabetic neuropathy and define their sensitivity and specificity to detect diabetic patients with early neuropathy and those at risk of foot ulceration.

RESEARCH DESIGN AND METHODS

Subjects. 101 diabetic patients and 17 non-diabetic healthy volunteers participated in the study. Patients were excluded if they had another cause of neuropathy, absent pedal pulses, wore contact lenses or had a history of corneal trauma or surgery. The protocol was approved by the local research ethics committee of the Greater Manchester Health Authority and all subjects gave written informed consent.

Evaluation of neuropathic severity. The neuropathy deficit score (NDS) was established by neurological examination and the severity of neuropathy was determined: NDS = 0-2: ‘no neuropathy’; NDS = 3-5: ‘mild neuropathy’; NDS = 6-8: ‘moderate neuropathy’; and NDS = 9-10: ‘severe neuropathy’(17; 18). Quantitative sensory testing included assessment of: vibration perception threshold (VPT), using a Neuroesthesiometer (Horwell, Scientific Laboratory Supplies, Wilford, Nottingham, UK); heat as pain thresholds (HP-VAS, C fibres) and cooling detection thresholds (CDT, Aδ fibres) using the CASE IV (WR Medical Electronics, Minn., USA) with the thresholds for abnormality set at the 95th percentile. The DANTEC Keypoint™ EMG system (software version 1.4) was used to quantify sural sensory and peroneal motor nerve conduction velocity and amplitude in all subjects.

Corneal sensitivity. Corneal sensitivity was assessed using a non-contact corneal aesthesiometer (NCCA) which uses a puff of air on the centre of the cornea, lasting 0.9 seconds and exerting a force expressed in millibars (mbars) (13). The coefficient of variation for NCCA is 5.6%.

Corneal Confocal Microscopy. One eye of each subject was selected at random and examined with a Tomey Confoscan model P4 using previously described methodology (10; 12). Three to five high quality images of the sub-basal nerve plexus from the centre of the cornea were assessed from each diabetic patient and control subject in a randomised masked fashion.
Three parameters were quantified (9; 10; 12): Corneal nerve fiber density (NFD) - total number of major nerves/mm²; nerve fiber length (NFL) - total length of all nerve fibers and branches (mm/mm²); and nerve branch density (NBD) - number of branches emanating from major nerve trunks/mm².

**Statistical methods.** SPSS 11.0.5.0 was used to compute the results. Data are presented as Mean ± SEM. The data were not normally distributed, hence one-way analysis of variance (ANOVA) with Scheffe Post-hoc tests were used to establish differences between the five (control, none, mild, moderate and severe diabetic neuropathy) groups. The Pearson coefficient test was used to analyze correlations between variables. Receiver operating characteristic (ROC) analysis established AUC to determine the optimal threshold, sensitivity and specificity values to estimate precision for NFD, NBD, NFL and NCCA in defining the presence of neuropathy (NDS >3) and risk of foot ulceration (NDS > 6). The ROC curves were used to compare the four tests, as well as define the optimum cut-off points, whereby sensitivity and specificity were equally weighted.

**RESULTS**

101 diabetic patients aged 58 ± 2.0 yrs and 17 age matched (55 ± 4.8 yrs) control subjects were studied (Table 1). Diabetic patients were stratified according to NDS: None (1.4 ± 0.1, n=34), mild (3.8 ± 0.1, n=37), moderate (6.5 ± 0.1, n=16) and severe (9.7 ± 0.1, n=14). Age, duration of diabetes and HbA1c did not differ between groups and there was no correlation between NDS and HbA1c (r= 0.098, P=0.36 ).

Peroneal and sural nerve conduction velocities did not differ in patients without neuropathy but were significantly reduced in mild (P<0.001, P<0.05), moderate (P<0.001, P<0.01) and severe (P<0.001, P<0.001) neuropathy. Similarly sural nerve amplitude did not differ in diabetic patients without neuropathy but was significantly reduced in mild, moderate and severe (P<0.001) neuropathy. Peroneal nerve amplitude was significantly reduced in diabetic patients without (P<0.05) and with mild, moderate and severe (P<0.001) neuropathy. VPT increased significantly with severity of neuropathy (P<0.0001) but did not differ in patients without neuropathy (P=1.0) and was only significant in those with mild (P= 0.01), moderate (P<0.0001) and severe (P<0.0001) neuropathy. Cold detection threshold was significantly increased in mild (P= 0.01), moderate (P<0.0001) and severe (P<0.0001) neuropathy. Heat pain perception thresholds increased significantly with increasing neuropathic severity (P<0.05). Corneal sensitivity decreased in diabetic patients compared to control subjects (P<0.0001) but this was significant only in patients with severe neuropathy (P<0.001) (Table 1).

Qualitatively there was a reduction in nerve fibre and branch density in diabetic patients compared to control subjects (Fig. 1). Intra-individual variability was established by repeating CCM in 15 subjects on two occasions and the coefficient of variation was: 12% for NFD, 9% for NFL and 24% for NBD. Corneal nerve fibre density (P<0.0001); branch density (P<0.0001) and length (P<0.0001) were significantly and progressively reduced in diabetic patients (Table 1, Fig. 2). NFD was significantly reduced in diabetic patients with no (P<0.02), mild (P<0.001), moderate (P<0.0001) and severe (P<0.0001) neuropathy when compared to control subjects (Fig. 2a). Similarly, NBD was
significantly reduced in diabetic patients with no (P=0.03), mild (P<0.001), moderate (P<0.0001) and severe (P<0.0001) neuropathy compared to control subjects (Fig. 2b). NFL was reduced in diabetic patients with no neuropathy (P= 0.07), mild (P<0.0001), moderate (P<0.0001) and severe (P<0.0001) neuropathy compared to control subjects (Figure 2c). NDS correlated significantly with corneal sensitivity (r=0.44, P=0.0001); NFD (r=-0.44, P<0.0001); NBD (r=-0.44, P<0.0001) and NFL (r=-0.57, P<0.0001).

According to the heat pain threshold (HPT) patients were classified into four groups: normal (percentile 0-25); mild (percentile 26-50); moderate (percentile 51-75) and severe (percentile 76-100) neuropathy (Table 2). NCCA increased and NFD decreased with increasing heat pain thresholds but were not significant. However, NBD and NFL demonstrated a progressive reduction with worsening HPT (P= 0.01).

**CCM sensitivity and specificity.**
According to the ROC curves (Fig. 3) for the three CCM parameters for NDS >3 AUC was: NFD- 0.76, NBD- 0.79, NFL- 0.84 and for NDS >6 AUC was: NFD-0.76, NBD- 0.79, NFL- 0.81. As the ROC curve (Fig. 3a) for NFL was above the other two variables it is considered to be the better test for diagnosing patients with diabetic neuropathy, though for high values of specificity NFD and NBD may be considered a better test. For those at risk of foot ulceration the ROC curves for the three CCM parameters (Fig. 3b) are more comparable, though NFL has a higher specificity than the other tests. For the diagnosis of neuropathy the sensitivity and specificity were: NFD-82%, 52%; NBD-91%, 45%; NFL- 64%, 79%, and NCCA-60%, 61%, respectively. For detecting those at risk of foot ulceration the sensitivity and specificity were: NFD-71%, 64%; NBD-71%, 71%; NFL- 64%, 71%, and NCCA-23%, 89%, respectively.

**CONCLUSIONS**
It is important to detect nerve damage at the earliest stage of diabetic neuropathy as intervention at this stage with improved glycaemic control (DCCT) (19) or improvement of other risk factors (20; 21) may prevent nerve degeneration or promote regeneration. Although diabetic patients with established neuropathy have increased vibration and thermal perception and decreased nerve conduction velocity, early detection of neuropathy is difficult (22). Recent studies show significant IENF (Intra Epidermal Nerve Fibre) loss in skin biopsies, despite normal electrophysiology and QST (2; 3) suggesting that IENF assessment may be important in the early diagnosis of neuropathy (23). However, as skin biopsy is an invasive procedure this study has assessed the utility of 2 novel non-invasive measures of neuropathy, namely corneal aesthesiometry and corneal confocal microscopy.

An early study of Type 1 diabetic patients demonstrated a reduction in corneal sensitivity, and the number of corneal nerve fibre bundles, which correlated significantly with the severity of neuropathy (24). We have previously demonstrated a significant reduction in corneal sensitivity using two independent measures and both correlated with neuropathic severity (13). Our studies (9; 10) using CCM demonstrated corneal nerve fibre abnormalities which were related to the severity of somatic neuropathy. More recently we have shown that CCM reflects IENF loss in skin biopsies from the dorsum of the foot in diabetic patients (12) and may also
show nerve repair following pancreas transplantation (14). Furthermore, we have recently demonstrated that CCM detects small fibre damage in patients with Fabry disease (25) and idiopathic small fibre neuropathy (16) indicating that CCM is a direct surrogate of peripheral neuropathy.

We now demonstrate a progressive reduction in corneal sensitivity and increasing corneal nerve degeneration with increasing severity of diabetic neuropathy. Importantly, corneal nerve fibre damage was present in patients deemed to have no evidence of neuropathy based on neurological evaluation, QST and neurophysiology, consistent with the recent studies showing IENF loss in diabetic patients without neuropathy (2; 3). We also establish for the first time that corneal aesthesiometry and CCM have reasonable sensitivity and specificity to detect diabetic patients with minimal neuropathy and those at risk of foot ulceration. A limitation of this study is that the data are derived from a cross-sectional study. Ideally a longitudinal study would provide more robust data regarding the ability of CCM to diagnose patients at risk of developing neuropathy.

Ideally, a test for detecting neuropathy in the early stages should be noninvasive, quantitative, and detect changes with time or in response to therapeutic interventions (2). CCM appears to fulfill these attributes, especially as it is noninvasive, directly quantifies small fibre pathology and stratifies neuropathic severity. CCM may therefore be an ideal surrogate marker for early diagnosis, stratification of severity and the assessment of therapeutic efficacy of new treatments in human diabetic neuropathy.

Author Contributions: Contributed to discussion, Wrote manuscript: MT, RAM. Reviewed/edited manuscript: MT, RAM, AJMB. Researched data.: MT, CQ, CA, PK, AM, JF, PM. Contributed to discussion: NE.

ACKNOWLEDGMENTS
This work was supported by Juvenile Diabetes Research Foundation International Grant 5-2002-185 and National Eye Institute Grant 1 R01 NS46259-01.

The authors would like to thank Annie Herbert from Pennine Acute Hospitals NHS Trust for statistical advice.

Disclosure: The authors have no conflict of interest to disclose.

REFERENCES
Table 1. Clinical demographics, clinical neuropathy evaluation, QST and electrophysiology and corneal sensitivity with NCCA and corneal confocal microscopy of the nerve fibers in Bowman’s layer of the cornea in control subjects and diabetic patients with increasing neuropathic severity.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>No neuropathy</th>
<th>Mild</th>
<th>Moderate</th>
<th>Severe</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>17</td>
<td>34</td>
<td>37</td>
<td>16</td>
<td>14</td>
</tr>
<tr>
<td>Age (years)</td>
<td>55 ± 4.8</td>
<td>55 ± 1.9</td>
<td>58 ± 2.1</td>
<td>59 ± 2.5</td>
<td>61 ± 2.05</td>
</tr>
<tr>
<td>Diabetes duration (years)</td>
<td>0</td>
<td>10.7 ± 1.82</td>
<td>15.5 ± 2.08</td>
<td>18.6 ± 3.06</td>
<td>19.3 ± 2.85</td>
</tr>
<tr>
<td>Diabetes Type (I/II)</td>
<td>-</td>
<td>2 / 32</td>
<td>9 / 28</td>
<td>4 / 12</td>
<td>2 / 12</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>8/9</td>
<td>19/15</td>
<td>32/5</td>
<td>12/4</td>
<td>10/4</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>&lt;6.5</td>
<td>8.1 ± 0.27</td>
<td>7.9 ± 0.23</td>
<td>8.4 ± 0.37</td>
<td>8.3 ± 0.38</td>
</tr>
<tr>
<td>Sural CV (m/s) (≥40)*</td>
<td>47.85 ± 2.62</td>
<td>42.88 ± 0.92</td>
<td>41.10 ± 0.83†</td>
<td>36.78 ± 1.88‡</td>
<td>37.26 ± 2.60†</td>
</tr>
<tr>
<td>Sural Amplitude (µA) (≥5)</td>
<td>18.62 ± 2.55</td>
<td>13.74 ± 1.46</td>
<td>6.13 ± 0.73¶</td>
<td>4.05 ± 0.67†</td>
<td>6.16 ± 3.25†</td>
</tr>
<tr>
<td>Peroneal Amplitude (&gt;2)</td>
<td>5.58 ± 1.02</td>
<td>3.58 ± 0.28†</td>
<td>2.10 ± 0.25¶</td>
<td>1.62 ± 0.28¶</td>
<td>1.16 ± 0.50¶</td>
</tr>
<tr>
<td>VPT (volts) *</td>
<td>9.58 ± 0.93</td>
<td>9.56 ± 0.84</td>
<td>18.18 ± 1.65†</td>
<td>25.35 ± 2.85†</td>
<td>42.29 ± 3.83¶</td>
</tr>
<tr>
<td>CDT (percentile)</td>
<td>-</td>
<td>54.64 ± 3.89</td>
<td>76.08 ± 4.1‡</td>
<td>89.60 ± 4.9¶</td>
<td>98.40 ± 8.9¶</td>
</tr>
<tr>
<td>NCCA (milli bar)*</td>
<td>0.72 ± 0.36</td>
<td>1.16 ± 0.07</td>
<td>1.34 ± 0.10</td>
<td>1.49 ± 0.20</td>
<td>2.23 ± 0.51‡</td>
</tr>
<tr>
<td>NFD (<em>number/mm²</em>)</td>
<td>45.60 ± 4.47</td>
<td>31.63 ± 2.33†</td>
<td>28.36 ± 1.95‡</td>
<td>18.57 ± 3.63¶</td>
<td>17.84 ± 2.49¶</td>
</tr>
<tr>
<td>NBD (<em>number/mm²</em>)</td>
<td>25.38 ± 2.99</td>
<td>17.42 ± 2.02†</td>
<td>13.28 ± 1.79¶</td>
<td>5.63 ± 1.33¶</td>
<td>4.95 ± 1.79¶</td>
</tr>
<tr>
<td>NFL (mm²/mm²)*</td>
<td>11.21 ± 0.88</td>
<td>8.05 ± 0.71</td>
<td>5.48 ± 0.45‡</td>
<td>3.01 ± 0.39¶</td>
<td>2.99 ± 0.34¶</td>
</tr>
</tbody>
</table>

Data are expressed as Mean ± SEM for diabetic patients and control subjects. Statistically significant difference between diabetic patients and controls using ANOVA: * P<0.001. Post hoc results with significant difference between control subjects and diabetic patients with differing severity of neuropathy: †P<0.05, ‡ P<0.01, ¶P<0.001 (CDT: Cold Detection Threshold; HPT: Heat Perception Threshold; PMNCV: Peroneal Motor Nerve Conduction Velocity; VPT: Vibration Perception Threshold)
Table 2. Results of corneal nerve parameters in diabetic patients stratified for severity of neuropathy according to the heat pain threshold (HPT).

<table>
<thead>
<tr>
<th></th>
<th>No neuropathy (n=34)</th>
<th>Mild neuropathy (n=37)</th>
<th>Moderate neuropathy (n=16)</th>
<th>Severe Neuropathy (n=14)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>NFD (number/mm²)</td>
<td>28.63 ± 2.51</td>
<td>25.73 ± 2.27</td>
<td>22.00 ± 4.99</td>
<td>23.26 ± 3.26</td>
<td>0.44</td>
</tr>
<tr>
<td>NBD (number/mm²)</td>
<td>15.13 ± 2.09</td>
<td>13.79 ± 2.13</td>
<td>8.60 ± 2.20</td>
<td>5.65 ± 1.37</td>
<td>0.01</td>
</tr>
<tr>
<td>NFL (mm²/mm²)</td>
<td>6.54 ± 0.65</td>
<td>5.75 ± 0.74</td>
<td>4.90 ± 1.18</td>
<td>3.27 ± 0.24</td>
<td>0.01</td>
</tr>
<tr>
<td>NCCA (mbar)</td>
<td>1.19 ± 0.07</td>
<td>1.38 ± 0.11</td>
<td>1.83 ± 0.43</td>
<td>1.75 ± 0.38</td>
<td>0.11</td>
</tr>
</tbody>
</table>

Table 3 (a): Diagnostic efficiency of corneal nerve parameters presented as Area under the curve (AUC), P values with CCM and NCCA cut-offs with sensitivity and specificity for diagnosis of patients with neuropathy (NDS > 3), and for diagnosis of patients at risk of foot ulceration (NDS > 6)

<table>
<thead>
<tr>
<th>Variable</th>
<th>NDS&gt;3</th>
<th>NDS&gt;6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AUC</td>
<td>Optimum cut-off</td>
</tr>
<tr>
<td>NFD</td>
<td>0.764... P&lt;0.0001</td>
<td>&lt; 27.81</td>
</tr>
<tr>
<td>NBD</td>
<td>0.801 P&lt;0.0001</td>
<td>&lt; 13.89</td>
</tr>
<tr>
<td>NFL</td>
<td>0.849 P&lt;0.0001</td>
<td>&lt; 3.39</td>
</tr>
<tr>
<td>NCCA</td>
<td>0.678 P=0.001</td>
<td>&gt; 1.12</td>
</tr>
</tbody>
</table>

Figure Legends:

Figure 1. Images of corneal nerves in Bowman’s layer, showing abundant nerve fibres and adequate branching in a control subject (a) with a typical image from a diabetic patient with mild (b) moderate (c) and severe (d) neuropathy showing a progressive loss of nerve fibres.

Figure 2. Corneal nerve morphology in control subjects and diabetic patients with increasing neuropathic severity (a) Nerve fiber density (NFD) (P<0.0001); (b) Nerve branch density (NBD) (P< 0.0001); (c) Nerve fiber length (NFL) (P< 0.0001).  

Figure 3. ROC curves for NFD, NBD and NFL for (a) NDS >3 (b) NDS >6.
Figure 2a

Figure 2b
Figure 2c
Figure 3a

ROC Curve

Diagonal segments are produced by ties.

Figure 3b

ROC Curve

Diagonal segments are produced by ties.