E stablished diabetic neuropathy leads to pain and foot ulceration. Early detection of neuropathy may allow treatment interventions to slow or reverse these conditions. The current diagnostic approach for diabetic peripheral neuropathy involves medical interviews and examinations, nerve conduction velocity tests, and vibration perception tests. However, previous studies have revealed that small nerve fibers are damaged early in diabetic polyneuropathy and can be detected only using invasive examinations such as skin biopsies. Therefore, a noninvasive and repeatable diagnostic approach targeting small nerve fibers is necessary to detect diabetic polyneuropathy early.

In vivo corneal confocal microscopy (IVCM), a novel noninvasive technique to obtain repeated images of the small nerve fibers that compose the corneal subbasal nerve plexus, is now used to evaluate diabetic polyneuropathy because the cornea is the most richly innervated bodily tissue. The advantage of IVCM is that it is a noninvasive objective test that targets the small nerve fibers. Fewer corneal nerve fibers are present in patients with diabetes and those with impaired glucose tolerance, and the corneal nerve fibers also decrease in association with progression of diabetic retinopathy (DR) and nephropathy, and decreased corneal sensation. There was a significant positive correlation between the CNFL values obtained with the conventional method and those obtained with the study method. The coefficient of variation of the CNFL values in the study method was significantly smaller than in the conventional method.

**CONCLUSIONS.** Our findings indicated that IVCM measurements of the whorl-like patterns may accurately define the extent of corneal nerve damage in order to monitor diabetic peripheral neuropathy. Keywords: corneal confocal microscopy, corneal nerve, diabetic neuropathy, diabetic peripheral neuropathy, diabetes mellitus

Imaging of the Corneal Subbasal Whorl-like Nerve Plexus: More Accurate Depiction of the Extent of Corneal Nerve Damage in Patients With Diabetes

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**PURPOSE.** To show that noninvasive in vivo corneal confocal microscopy (IVCM) can make more accurate imaging of the corneal subbasal nerve plexus possible. This diagnostic technique monitors the status of diabetic peripheral neuropathy. However, it is difficult to accurately confirm the corneal area captured by IVCM, which can cause measurement errors. Because the whorl-like characteristic pattern of the corneal subbasal nerve plexus is in the inferocentral cornea, we evaluated whether IVCM images of the whorl-like patterns can accurately evaluate the corneal nerve fibers in diabetic neuropathy.

**METHODS.** Forty-seven patients with diabetes (DM group) and 21 healthy control subjects underwent IVCM examination to compare the characteristics of the corneal subbasal nerve plexus around the central cornea (conventional method) and the whorl-like pattern in the inferocentral cornea (study method). We measured the total corneal nerve fiber and branch length (CNFL).

**RESULTS.** The total CNFL were significantly shorter in the DM group than in the control group and tended to decrease with progression of diabetic retinopathy, nephropathy, neuropathy, and decreased corneal sensation. There was a significant positive correlation between the CNFL values obtained with the conventional method and those obtained with the study method. The coefficient of variation of the CNFL values in the study method was significantly smaller than in the conventional method.

**CONCLUSIONS.** Our findings indicated that IVCM measurements of the whorl-like patterns may accurately define the extent of corneal nerve damage in order to monitor diabetic peripheral neuropathy.
nerve plexus. Therefore, the aim of the current study was to determine whether the whorl-like pattern of the subbasal nerve plexus in the inferocentral cornea is a more reliable landmark to evaluate the corneal subbasal nerve plexus compared with the conventional method.

**METHODS**

**Study Subjects**

This observational study included 47 patients with diabetes and 21 healthy control subjects seen in the Department of Ophthalmology of Asahikawa Medical University Hospital. Diabetes was diagnosed based on the criteria of the Japan Diabetes Society.26 Subjects were considered to have diabetes if they were undergoing treatment with insulin or oral hypoglycemic agents. The exclusion criteria were previous ocular trauma, ocular surgery, any corneal disorder, active ocular disease, or any other systemic disease that might affect the cornea. Patients with any other known cause of neuropathy also were excluded. The current study adhered to the tenets of the Declaration of Helsinki. The local ethics committee approved the study protocol. All subjects provided informed consent after they received a detailed explanation of the study.

**Corneal Sensation and IVCM**

Corneal sensitivity was assessed using the Cochet-Bonnetesthesiometer.27 This test mechanically stimulates the corneal nerves by pressing a retractable 60-mm-long monofilament nylon thread 0.12 mm in diameter against the anterior corneal surface. The subjects put their chin on the chin rest of the slit-lamp and indicated when they felt the stimulus. Starting from 60 mm, the filament length was progressively reduced in 5.0-mm increments to increase its rigidity until the first response was obtained. The longest filament length resulting in a positive response was recorded as the indicator of corneal sensitivity. If the filament was shorter than 50 mm, the corneal sensitivity was considered to be abnormal. Laser scanning IVCM was performed using the Rostock Corneal Module/Heidelberg Retina Tomograph III (Heidelberg Engineering GmbH, Dossenheim, Germany). The corneal subbasal nerve plexus layers were scanned using the sequence mode, with a frame rate of 10 frames per second. A 400-×400-μm frame was used for the images of the nerve plexus. The corneal subbasal nerve plexus around the central cornea was scanned (conventional method). To identify the whorl-like pattern, a fixation lamp was raised slightly and the scanned sites were slowly shifted close to the whorl-like pattern along the nerve. If the distinctive whorl-like pattern was identified, it was scanned (study method). The five clearest images of the subbasal nerve plexus around the central cornea and the clearest image of the whorl-like pattern of the inferocentral cornea were selected. In addition, the three clearest images of the whorl-like pattern were selected to compare the coefficients of variation (CV) between the conventional and the study methods. When both eyes of a patient were eligible for the study, we selected the eye with the best image of the whorl-like nerve plexus. Nerve analysis was performed using the semiautomated tracing program NeuronJ (a plug-in for ImageJ; Erik Meijering, Rotterdam, The Netherlands), which is image analysis software in the public domain distributed by the National Institute of Health (Bethesda, MD, USA).28 The IVCM parameters measured were the total corneal nerve fiber and branch length (CNFL) and corneal nerve fiber and branch density (CNFD). The CNFL was calculated by adding the lengths of all corneal nerve fibers and branches captured in one image and dividing by the square measure of one image. The CNFD was calculated by adding the number of all corneal nerve fibers and branches captured in one image and dividing by the square measure of one image.

**FIGURE 1.** Corneal subbasal nerve plexus. (A) A schema of the entire corneal subbasal nerve plexus. The inferocentral cornea contains a distinctive whorl-like pattern of the subbasal nerve plexus. The corneal subbasal nerve plexus appears as a whorl-like pattern in the inferocentral cornea. One image captured by IVCM covers only a small area. (B–E) In vivo corneal confocal microscopy images of the corneal subbasal nerve plexus in the same patient. (B) An image of corneal subbasal nerve plexus around the central cornea. (C) Tracing image of (B) using the NeuronJ software. (D) An image of the whorl-like nerve complex in the inferocentral cornea. (E) Tracing image of (D) using the NeuronJ software.
Diagnosis of Diabetic Neuropathy, DR, and Nephropathy

Two internal medicine specialists (AA, MH) diagnosed diabetic neuropathy using the abbreviated diagnostic criteria for diabetic polyneuropathy in Japan.29,30 Diabetic neuropathy was diagnosed when two or more of the following were present: symptoms, no Achilles tendon reflexes, and abnormal scores of the vibration perception threshold using a C128 tuning fork, where bilateral spontaneous pain, hypoesthesia, or paresthesia of the legs was considered a neuropathic symptom. The patients were divided into one of three groups according to the retinal findings based on the Early Treatment Diabetic Retinopathy Study.31 Renal function was evaluated based on the estimated glomerular filtration rate (eGFR) calculated as

\[ eGFR (\text{mL/min}/1.73\text{m}^2) = 194 \times \text{Scr}^{-1.094} \times \text{Age}^{-0.287} \times 0.739 \] (for women).

The absence of chronic kidney disease was defined as eGFR > 90 mL/min/1.73 m².33

Statistical Analysis

The data are reported as the means ± standard errors. Statistical analysis was performed using GraphPad Prism version 6.0 for Mac (GraphPad Software, San Diego, CA, USA). An unpaired t-test was used to compare the variables between the patients with diabetes (DM group) and the healthy subjects (control group). Variables within subgroups were compared using one-way analysis of variance followed by Dunnett’s multiple comparisons test and posttest for linear trend. The CNFL around the central cornea (conventional CNFL) and the CNFL at the whorl-like corneal nerve plexus (whorl-like CNFL) were compared using Pearson’s correlation coefficient. Coefficient of variation of conventional nerve plexus (whorl-like CNFL) were compared using an unpaired t-test and posttest for linear trend. The CNFL around the central cornea (conventional CNFL) and that of whorl-like CNFL were compared using an unpaired t-test. P < 0.05 was considered statistically significant.

Results

Baseline Characteristics of the Study Subjects

No significant differences in age or sex were seen between the DM and control groups. In the DM group, which included six patients with type 1 diabetes and 41 patients with type 2 diabetes, the mean duration of diabetes mellitus was 9.3 ± 6.8 years and the mean level of hemoglobin A₁c was 7.6 ± 1.5%.

Application of the IVCM Technique to the Whorl-like Nerve Plexus

The IVCM technique clearly distinguished the neural patterns of the subbasal corneal nerve plexus around the central cornea (Figs. 1B, 1C) and the whorl-like nerve plexus in the inferocentral cornea (Figs. 1D, 1E) of the same patient with DM. The CNFL and CNFD values were determined based on tracings performed with the NeuronJ software (Figs. 1C, 1E).

Quantitative Analysis of IVCM Images of the Area Around the Central Cornea

Parametric analysis of the IVCM images indicated that the CNFL around the central cornea (conventional CNFL) was significantly...
### Table 2. The IVCM Parameters in Subjects in Whom We Could Capture the Whorl-like Nerve Plexus

<table>
<thead>
<tr>
<th></th>
<th>Conventional CNFL, n/mm²</th>
<th></th>
<th>P Value</th>
<th>Linear ANOVA</th>
<th>Trend</th>
<th>Conventional CNFL, n/mm²</th>
<th></th>
<th>P Value</th>
<th>Linear ANOVA</th>
<th>Trend</th>
<th>Whorl-like CNFL, n/mm²</th>
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<th>P Value</th>
<th>Linear ANOVA</th>
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<tbody>
<tr>
<td>Control</td>
<td>28.70 ± 1.94</td>
<td>243.6 ± 30.4</td>
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<td></td>
<td></td>
<td>35.98 ± 2.93</td>
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<td>DM</td>
<td>21.86 ± 1.04</td>
<td>0.0032</td>
<td>159.3 ± 11.6</td>
<td>0.0037</td>
<td></td>
<td>26.87 ± 1.87</td>
<td>0.0041</td>
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<tr>
<td>Neuropathy (-)</td>
<td>23.02 ± 1.38</td>
<td>0.0033</td>
<td>171.4 ± 15.4</td>
<td>0.0029</td>
<td></td>
<td>28.62 ± 1.87</td>
<td>0.0045</td>
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<td>(+)</td>
<td>20.11 ± 1.47</td>
<td>0.0033</td>
<td>141.3 ± 16.7</td>
<td>0.006</td>
<td></td>
<td>24.24 ± 1.71</td>
<td>0.0032</td>
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<td>DR</td>
<td>21.61 ± 1.76</td>
<td>0.0052</td>
<td>152.2 ± 17.2</td>
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<td>28.03 ± 2.56</td>
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<td>Mild-moderate</td>
<td>23.75 ± 1.30</td>
<td>0.0071</td>
<td>185.0 ± 15.5</td>
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<td>26.64 ± 1.85</td>
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<td>Severe Neuropathy</td>
<td>16.87 ± 1.28</td>
<td>0.0025</td>
<td>101.3 ± 11.8</td>
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<td>Severe NPDR</td>
<td>23.70 ± 1.65</td>
<td>0.0095</td>
<td>178.6 ± 19.7</td>
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<td>28.03 ± 2.82</td>
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<tr>
<td>eGFR &lt;90</td>
<td>20.64 ± 1.27</td>
<td>0.0026</td>
<td>146.5 ± 13.6</td>
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<td>26.09 ± 1.39</td>
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<td>Abnormal</td>
<td>22.45 ± 1.05</td>
<td>0.0014</td>
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<tr>
<td>Normal</td>
<td>16.52 ± 2.13</td>
<td>0.0057</td>
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<td>0.0093</td>
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<td>21.65 ± 3.89</td>
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The data are expressed as the means ± standard errors. NDR, no diabetic retinopathy; NPDR, nonproliferative diabetic retinopathy; PDR, proliferative diabetic retinopathy.

The conventional and whorl-like CNFL measurements were compared using paired analysis on each subject. A significant decreasing trend was noted in the whorl-like CNFL in all study groups compared with the control group. The CNFL of the whorl-like nerve plexus was significantly lower in the DM group than in the control group and also showed a decreasing trend associated with progression of diabetic neuropathy, DR, nephropathy, and decreased corneal sensation.

The CNFD was significantly lower in the DM group than in the control group, regardless of whether images of the whorl-like nerve plexus at the inferocentral cornea were available. Around the central cornea, the DM group had significantly shorter CNFDs than the control group. The CNFD showed a decreasing trend associated with progression of diabetic neuropathy, DR, nephropathy, and decreased corneal sensation.

**Figure 2.** The whorl-like CNFL and conventional CNFL in the control group and DM group. The data are expressed as the means ± standard errors. Both the whorl-like CNFL and conventional CNFL are shorter in the DM group than in the control group. The CNFL of the whorl-like nerve plexus shown in Table 2 shows the IVCM parameters of the subjects for whom images of the whorl-like nerve plexus at the inferocentral cornea were available. Around the central cornea, the DM group had significantly shorter CNFDs than the control group. The CNFD showed a decreasing trend associated with progression of diabetic neuropathy, DR, nephropathy, and decreased corneal sensation.
A significant ($r = 0.79, P < 0.0001$) correlation was seen between the findings for conventional CNFL and whorl-like CNFL measurements. Whorl-like CNFL values were consistently significantly ($P < 0.0001$) higher than conventional CNFL values in the control and DM groups (Fig. 4).

**Paired Comparison of the CVs**

The CV of whorl-like CNFL value was significantly ($P = 0.0002$) smaller than that of the conventional method (Fig. 5).

**DISCUSSION**

The current study showed that whorl-like CNFL values were consistently higher than the conventional CNFL values in healthy subjects and patients with diabetes. These data suggested that the CNFL values depend on the corneal area examined by IVCM. Previous reports have shown that the epithelial nerve density in the central area was higher than in the periphery. Because the CNFL values are affected by the area of the subbasal nerve plexus captured by IVCM, it is important to capture the same area for an accurate comparison between patients and over time in the same patient. The whorl-like CNFL value may be an ideal parameter for accurately
In vivo corneal confocal microscopy analysis of the whorl-like nerve plexus may accurately define the extent of corneal nerve damage. Nonetheless, more advanced imaging technologies are necessary to facilitate imaging of the whorl-like nerve plexus.

**Acknowledgments**

Disclosure: T. Utsunomiya, None; T. Nagaoka, None; K. Hanada, None; T. Omae, None; H. Yokota, None; A. Abiko, None; M. Haneda, None; A. Yoshida, None

**References**

12. Petropoulos IN, Alam U, Fadavi H, et al. Corneal nerve loss detected with confocal microscopy is symmetrical and more sensitive than those in previous studies. Although previous reviews showed that the CNFL and CNFD values varied by study even when using the same microscope,22,39 this would be because we traced even minute nerve filaments in the clearest images. We found a positive correlation between the whorl-like CNFL and conventional CNFL values and the same trend as conventional CNFL. In addition, whorl-like CNFL values varied less than conventional CNFL, suggesting that IVCM measurements of the whorl-like patterns of the corneal subbasal nerve plexus may accurately define the extent of corneal nerve damage.

The strength of the current study was that whorl analysis was as valid as the conventional measurement and may be more useful because the whorl-like pattern can be reidentified as a distinct corneal landmark for accurate measurement of the corneal subbasal nerve plexus.

The limitations of the current study were the small number of subjects and the fact that we captured the whorl-like pattern in only half of the subjects. However, we would be able to capture the whorl-like pattern in more subjects if we captured this area first because visual fixation became unstable with fatigue. In addition, patients with type 1 and type 2 diabetes were combined for analysis in the current study. Additional research is needed to confirm our findings in the whorl-like nerve plexus. In vivo corneal confocal microscopy analysis of the whorl-like nerve complex remains challenging. Considerable work and time are required to search for the whorl-like nerve complex, because IVCM captures only a small area. However, less variability occurs when capturing the whorl-like nerve complex. In the near future, capturing the whorl-like nerve complex may be facilitated by new imaging technologies, such as the image–montage technique,40,41 wide imaging technology,34,42 autofocus function, and automatic analysis systems.43,44 In addition, a simpler IVCM analyzing technology would allow this platform to become a common diagnostic and monitoring tool for patients with diabetic peripheral neuropathy.

In conclusion, the current study showed that the whorl-like nerve plexus can be analyzed by IVCM. The whorl-like CNFL value is as sensitive as the conventional CNFL value for the diagnosis and monitoring of peripheral neuropathy in patients with diabetes. Furthermore, our findings indicated that IVCM measurements of the whorl-like patterns may accurately define the extent of corneal nerve damage. Nonetheless, more advanced imaging technologies are necessary to facilitate imaging of the whorl-like nerve plexus.


