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 induce 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.

Keywords: corneal confocal microscopy, corneal nerve, diabetic neuropathy, diabetic peripheral neuropathy, diabetes mellitus

Established diabetic neuropathy leads to pain and foot ulceration. Early detection of neuropathy may allow treatment interventions to slow or reverse these conditions.[1] 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.[2–4] 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,[5] is now used to evaluate diabetic polyneuropathy because the cornea is the most richly innervated bodily tissue.[6] 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[7] and those with impaired glucose tolerance,[8] and the corneal nerve fibers also decrease in association with progression of diabetic retinopathy (DR),[9,10] nephropathy,[11] and neuropathy.[12–18] Therefore, the abundance of the corneal nerve fibers is considered to reflect the status of diabetic peripheral neuropathy.

In previous studies, images of the subbasal nerve plexus around the central cornea were used to assess the corneal nerve fibers. However, it was not known definitively which corneal area was being captured by IVCM because the technology captures a small area and no marker of the corneal center is available during the measurement. Accordingly, the areas used for assessment may differ between patients and may change over time in the same patient. Despite the good repeatability of the IVCM parameters,[19–21] reproducibility and reliability remain an issue.[22]

The inferocentral corneal subbasal nerve plexus contains a distinctive whorl-like pattern[23–25] (Fig. 1A), which may be a more suitable parameter for evaluating the corneal subbasal

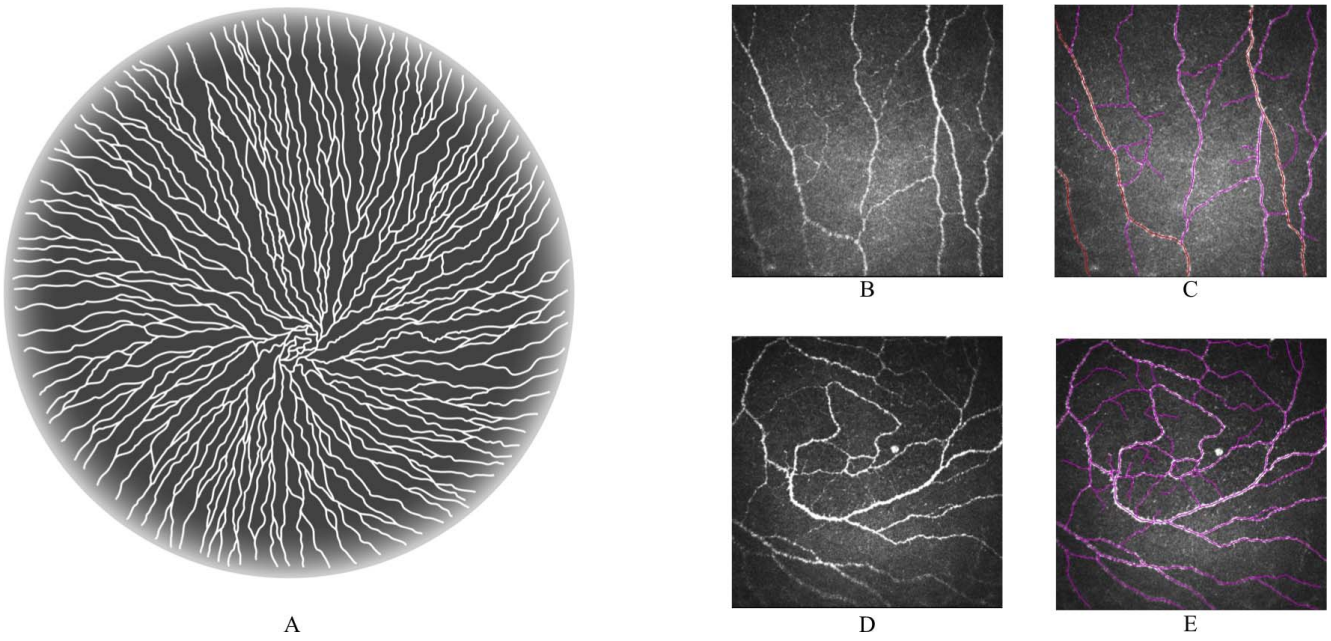

Figure 1. Corneal subbasal nerve plexus. (**A**) A schema of the entire corneal subbasal nerve plexus. The inferocentral cornea contains a distinctive whorl-like pattern of the subbasal nerve plexus. The corneal subbasal nerve plexus appears as a whorl-like pattern in the inferocentral cornea. One image 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.

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-Bonnet esthesiometer.[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- $\times$ 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.

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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 previously described.[32] The following equation from the Modification of the Diet in Retinal Disease Study Group compiled for Japanese individuals was recommended by the Japanese Society of Nephrology:

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

The absence of chronic kidney disease was defined as eGFR > 90 mL/min/1.73 m^2.[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 nerve plexus (whorl-like CNFL) were compared using Pearson's correlation coefficient. Coefficient of variation of 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 A_{1c} 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 1. The IVCM Parameters Around the Center Cornea in All Subjects

		n	Conventional CNFL, mm/mm^2	*P* Value vs. Control	*P* Value ANOVA	Linear Trend	*P* Value	Conventional CNFD, n/mm^2	*P* Value vs. Control	*P* Value ANOVA	Linear Trend	*P* Value
Control		21	25.90 ± 1.08					217.5 ± 16.3				
DM												
Total		47	20.86 ± 0.81	0.0007				153.6 ± 8.0	0.0002			
Neuropathy	(−)	29	21.31 ± 1.19	0.0081	0.0025	−2.884	0.002	158.1 ± 11.1	0.0025	0.0008	−35.63	0.0006
	(+)	18	20.13 ± 0.92	0.0029				146.3 ± 10.6	0.0012			
DR	NDR	19	21.46 ± 1.47	0.0307	0.0042	−1.047	0.001	155.1 ± 13.9	0.006	0.0017	−11.77	0.0017
	Mild–moderate NPDR	17	21.44 ± 1.29	0.0367				163.0 ± 13.3	0.0232			
	Severe NPDR–PDR	11	18.92 ± 1.28	0.0026				136.4 ± 12.4	0.0021			
eGFR	90	16	21.80 ± 1.23	0.0676	0.0063	−0.7924	0.027	160.8 ± 12.3	0.0206	0.0026	−10.12	0.0137
	60 < 90	23	20.09 ± 1.16	0.0021				148.3 ± 12.4	0.0013			
	<60	8	21.19 ± 2.49	0.1025				154.2 ± 19.4	0.0442			
Corneal sensation	Normal	38	21.57 ± 0.88	0.0068	0.0016	−3.617	0.002	162.5 ± 8.9	0.0024	0.0003	−48.05	0.0003
	Abnormal	8	18.67 ± 1.81	0.0029				121.4 ± 13.4	0.0005			

The data are expressed as the means ± standard errors. NDR, no diabetic retinopathy; NPDR, nonproliferative diabetic retinopathy; PDR, proliferative diabetic retinopathy.

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TABLE 2. The IVCM Parameters in Subjects in Whom We Could Capture the Whorl-like Nerve Plexus

		n	Conventional CNFL, mm/mm²	*P* Value vs. Control	*P* Value ANOVA	Linear Trend	*P* Value	Conventional CNFD, n/mm²	*P* Value vs. Control	*P* Value ANOVA	Linear Trend	*P* Value	Whorl-like CNFL, mm/mm²	*P* Value vs. Control	*P* Value ANOVA	Linear Trend	*P* Value
Control		7	28.70 ± 1.94					243.6 ± 30.4					35.98 ± 2.93				
DM																	
Total		20	21.86 ± 1.04	0.0032				159.3 ± 11.6	0.0037				26.87 ± 1.87	0.0041			
Neuropathy	(−)	12	23.02 ± 1.38	0.0333	0.0059	−4.29	0.0017	171.4 ± 15.4	0.0329	0.0089	−51.2	0.0029	28.62 ± 1.87	0.0435	0.0061	−5.87	0.0017
	(+)	8	20.11 ± 1.47	0.0033				141.3 ± 16.7	0.0056				24.24 ± 1.71	0.0032			
DR	NDR	8	21.61 ± 1.76	0.0152	0.0042	−1.67	0.0020	152.2 ± 17.2	0.0128	0.0051	−19.7	0.0033	28.03 ± 2.56	0.0822	0.0363	−1.80	0.0197
	Mild–moderate NPDR	9	23.75 ± 1.30	0.0971				185.0 ± 15.5	0.1252				26.64 ± 1.85	0.0304			
	Severe NPDR–PDR	3	16.87 ± 1.28	0.0025				101.3 ± 11.8	0.0037				24.44 ± 3.58	0.0542			
eGFR	90	8	23.70 ± 1.65	0.0895	0.0053	−4.03	0.0014	178.6 ± 19.7	0.0816	0.0082	−48.6	0.0022	28.03 ± 2.82	0.0543	0.0145	−4.95	0.0046
	<90	12	20.64 ± 1.27	0.0026				146.5 ± 13.6	0.0042				26.09 ± 1.39	0.0086			
Corneal sensation	Normal	18	22.45 ± 1.05	0.0104	0.0035	−6.09	0.0030	166.0 ± 11.7	0.0122	0.0052	−72.4	0.0049	27.45 ± 1.43	0.0188	0.0089	−7.17	0.0113
	Abnormal	2	16.52 ± 2.13	0.0057				98.8 ± 20.0	0.0093				21.65 ± 3.89	0.0293			

The data are expressed as the means ± standard errors. NDR, no diabetic retinopathy; NPDR, nonproliferative diabetic retinopathy; PDR, proliferative diabetic retinopathy.

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 significantly shorter in the DM group than in the control group.

shorter in the DM group than in the control group, regardless of neuropathy ($P = 0.0025$), severity of DR ($P = 0.0042$), or corneal sensation ($P = 0.0016$) (Table 1). In contrast, only patients with diabetes with an eGFR of 60 to 90 mL/min/1.73 m² had significantly ($P = 0.0021$) shorter conventional CNFLs than the healthy subjects. The CNFLs showed a decreasing trend associated with disease progression of diabetic neuropathy, DR, nephropathy, and decreased corneal sensation ($P = 0.0015$, $P = 0.0014$, $P = 0.0268$, and $P = 0.0015$, respectively).

The CNFD was significantly ($P = 0.0002$) 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 ($P = 0.0006$, $P = 0.0017$, $P = 0.0137$, and $P = 0.0003$, respectively).

Comparison of IVCM Parameters Around the Central Cornea and Whorl-like Nerve Plexus

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 ($P = 0.0032$) lower conventional CNFL values than the control group (Fig. 2). Conventional measurement of the CNFL showed a decreasing trend associated with disease progression of diabetic neuropathy, DR, nephropathy, and decreased corneal sensation ($P = 0.0017$, $P = 0.0020$, $P = 0.0014$, and $P = 0.0030$, respectively). The CNFD 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 ($P = 0.0029$, $P = 0.0033$, $P = 0.0022$, and $P = 0.0049$, respectively).

The CNFL of the whorl-like nerve plexus was significantly ($P = 0.0041$) shorter in the DM group than in the control group (Fig. 2). The whorl-like CNFL showed a decreasing trend associated with progression of diabetic neuropathy, DR, nephropathy, and decreased corneal sensation ($P = 0.0017$, $P = 0.0197$, $P = 0.0046$, and $P = 0.0113$, respectively) (Fig. 3). The whorl-like CNFD is not shown in Table 2 because corneal nerves cannot be counted in the whorl-like nerve plexus.

Paired Comparison of Conventional and Whorl-like CNFL

The conventional and whorl-like CNFL measurements were compared using paired analysis on each subject. A significant

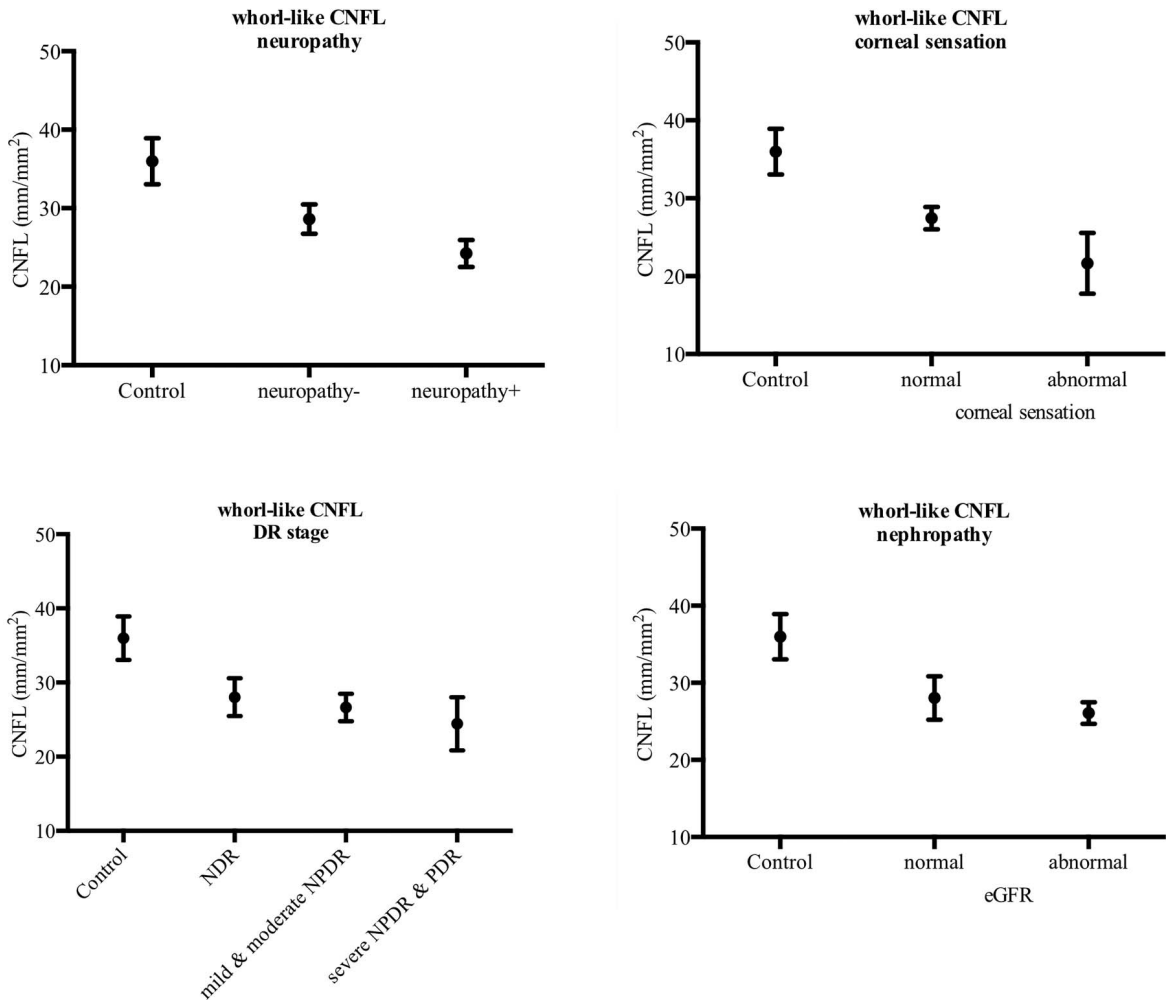

Figure 3. Relationships of the whorl-like CNFL with diabetic complications. The data are expressed as the means ± standard errors. The whorl-like CNFL shows a decreasing trend associated with progression of diabetic neuropathy, DR, nephropathy, and decreased corneal sensation ($P = 0.0017$, $P = 0.0197$, $P = 0.0046$, and $P = 0.0113$, respectively).

($r = 0.79$, $P < 0.0001$) 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.[34] 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

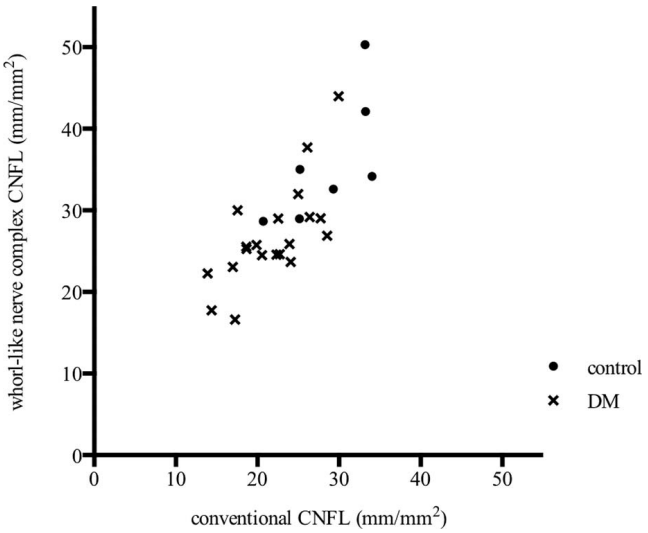

Figure 4. Correlation between conventional CNFL and whorl-like CNFL. A significant ($r = 0.79$, $P < 0.0001$) correlation exists between the conventional CNFL and whorl-like CNFL. The whorl-like CNFL is significantly ($P < 0.0001$) greater than the conventional CNFL in the control group and DM group.

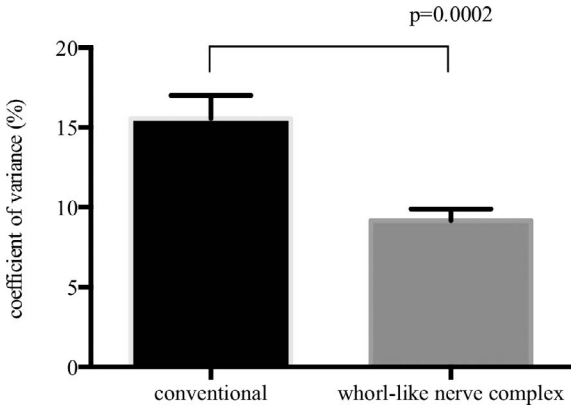

FIGURE 5. The CV of the whorl-like CNFL and conventional CNFL. The data are expressed as the means ± standard errors. The CV of the whorl-like CNFL is significantly smaller than that of the conventional method.

diagnosing peripheral diabetic neuropathy, because the target site is identified easily in the whorl-like nerve complex.

In addition, the most common pattern of diabetic peripheral neuropathy is distal neuropathy following fiber length–dependent progression.[35–37] The whorl-like complex is the most distal corneal nerve in that the subbasal nerve plexus runs toward the whorl-like complex in the inferocentral cornea.[23–25] In fact, the inferior whorl and surrounding area were reported to show the greatest loss of nerve fibers in patients with diabetic neuropathy,[38] although that report included only two cases. Our method may be favorable for sensitivity, although additional research is needed.

The current study showed significant reductions in the conventional CNFL and CNFD values in patients with diabetes compared with healthy subjects. Furthermore, a decreasing trend was detected with progression of DR, nephropathy, neuropathy, and decreased corneal sensation. This was consistent with previous studies reporting that corneal nerve fibers decreased in patients with diabetes[7] and in association with progression of DR,[9,10] nephropathy,[11] and neuropathy.[12–18] The current CNFL and CNFD values were higher 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,[34,41,42] 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.

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