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ORIGINAL ARTICLE

Morphological changes of the peripheral nerves evaluated by high-resolution ultrasonography are associated with the severity of diabetic neuropathy, but not corneal nerve fiber pathology in patients with type 2 diabetes

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ABSTRACT

Aims/Introduction: To evaluate the morphological changes of the median and posterior tibial nerve using high-resolution ultrasonography, and the corneal C fiber pathology by corneal confocal microscopy in type 2 diabetic patients.

Materials and Methods: The cross-sectional area, hypoechoic area and maximum thickness of the nerve fascicle of both nerves were measured by high-resolution ultrasonography in 200 type 2 diabetic patients, stratified by the severity of diabetic neuropathy, and in 40 age- and sex-matched controls. These parameters were associated with corneal C fiber pathology visualized by corneal confocal microscopy, neurophysiological tests and severity of diabetic neuropathy.

Results: The cross-sectional area, hypoechoic area and maximum thickness of the nerve fascicle of both nerves in patients without diabetic neuropathy were larger than those in control subjects ($P < 0.05$ to $P < 0.001$), and further increased relative to the severity of neuropathy ($P < 0.0001$). All morphological changes of both nerves were negatively associated with motor and sensory nerve conduction velocity ($P = 0.01$ to $P < 0.0001$), and directly associated with 2,000-Hz current perception threshold ($P = 0.009$ to $P < 0.001$). The significant corneal C fiber pathology occurred before developing the neuropathy, and deteriorated only in patients with the most severe neuropathy. The association between the morphological changes of both nerves and corneal C fiber pathology was poor.

Conclusions: The morphological changes in peripheral nerves of type 2 diabetic patients were found before the onset of neuropathy, and were closely correlated with the severity of diabetic neuropathy, but not with corneal C fiber pathology.

INTRODUCTION

Diabetic neuropathy (DN) is diagnosed primarily through the presence of characteristic signs and symptoms, and confirmed by neurophysiological tests[1,2]. Corneal confocal microscopy (CCM) has been used to diagnose small C fiber neuropathy[3], which shows a good correlation with the DN stage[4]. Because an invasive nerve biopsy is required for the histological diagnosis of DN of peripheral nerves[5], it is not routinely carried out. The development of high-resolution ultrasonography (HRU) and high-frequency linear probe (HFLP) has enabled evaluation of the fine structures of peripheral nerves by imaging[6]. As only

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one study has previously used HRU and HFLP, the results of ultrasonography in DN vary regarding the use of the nerve cross-sectional area (CSA) as a possible marker of DN[7,8]. The differences in the CSA of peripheral nerves between controls and patients without DN have never been fully examined. An increase in the hypoechoic area (HA)[9] in transverse sections of the median nerve (MN) and the maximum thickness of the nerve fascicle (MTNF)[8] in sural nerve was found in patients with DN. Because these studies comprised small numbers of participants, the results were inconsistent. As none of the previous reports examining the fine structures of peripheral nerves in DN simultaneously assessed corneal C fiber morphology by CCM, the correlation of the morphological changes (CSA, HA and MTNF) in peripheral nerves and corneal C fibers is unknown.

The aim of the present study was to determine the CSA, HA, and MTNF of the MN and posterior tibial nerve (PTN) by HRU equipped with HFLP, and to associate them with corneal C fiber pathology visualized by CCM, neurophysiological tests and the DN severity in large numbers of age- and sex-matched controls and patients with type 2 diabetes stratified by the DN severity.

MATERIALS AND METHODS

Participants

Japanese patients with type 2 diabetes attending the Ishibashi Clinic, and age- and sex-matched non-diabetic controls (glycated hemoglobin [HbA1c] <5.7% and fasting plasma glucose <5.5 mmol/L or casual postprandial plasma glucose <7.7 mmol/L) were enrolled in the present study. Subjects were excluded if they consumed >20 g alcohol/day, had been diagnosed with neuropathy due to another cause, were symptomatic due to carpal tunnel syndrome (CTS) or tarsal tunnel syndrome, or wore contact lenses. Written informed consent was obtained from all participants. The ethics committee of the Ishibashi Clinic approved the protocol of this research.

Assessment of DN

DN was diagnosed in patients with type 2 diabetes based on the presence of two of the following three factors, as recommended in the simplified diagnostic criteria proposed by the DN Study Group in Japan[10]: (i) subjective symptoms in bilateral lower limbs or feet; (ii) loss of or decreased ankle jerk; and (iii) decreased vibration perception, assessed using a C128 tuning fork and bilaterally measured at the medial malleoli. Diabetic patients were classified into one of five stages of DN, according to the DN Study Group in Japan criteria[10]: stage I, patients who do not meet the diagnostic criteria for DN; stage II, patients meet the diagnostic criteria, but are asymptomatic; stage III, patients are symptomatic, but either the ankle jerk reflex or vibration sensation is normal; stage IV, patients show the clinical manifestations of autonomic neuropathy; and stage V, motor neuropathy appears. The last two stages were represented by small numbers of patients; we therefore grouped stages IV and V together (IV+V).

Determination of the CSA, HA and MTNF of Peripheral Nerves

The CSA of MN and PTN were measured by HRU using HFLP (18.0 MHz, HIVISION Ascendus; Hitachi, Tokyo, Japan). Ultrasonographic transverse scans were obtained 5 cm proximal to the distal wrist crease for MN[9] and 3 cm proximal to the cephalad border of the medial malleolus for PTN[11]. The CSA was evaluated by direct tracing. The pixel numbers were counted using Photoshop Elements 8.0 (Adobe Systems Inc., San Jose, CA, USA). The percentage (%) of HA with an echo intensity <70% of the mean echo level of the CSA was determined by histogram analysis in Photoshop Elements 8.0. The MTNF[8] was measured along the short axis of the nerve fascicle using the measure tool of Photoshop 5.0 (Adobe Systems Inc.; shown in Figure 1).

The reproducibility (intrarater variability) in assessing the morphological parameters of peripheral nerves by HRU was evaluated by repeating the examinations on three occasions with the same examiner for 16 healthy volunteers, and calculating the coefficient of variation (CV) for the individual parameters.

Corneal Confocal Microscopy

Participants were examined using a Heidelberg Retina Tomograph-3 equipped with a Rostock Cornea Module (Heidelberg Engineering, Heidelberg, Germany)[12,13]. The examined eye was anesthetized by instilling 0.4% benoxinate hydrochloride (Santen Pharmaceutical Co., Osaka, Japan). Comfort Gel (Dr. Mann Pharma, Berlin, Germany) was applied to the lens, and a disposable sterilized Tomocap was mounted on the holder to cover the objective lens. After applying Comfort Gel to the Tomocap, the lens was slowly advanced until the gel touched the cornea. More than 50 images of the subbasal nerve plexus were captured using the section mode, and we analyzed at least six high-clarity images per participant to quantify the following parameters to define changes in the corneal C fiber: (i) corneal nerve fiber density/mm^2 (CNFD); (ii) corneal nerve fiber length mm/mm^2 (CNFL); (iii) corneal nerve branch density/mm^2 (CNBD); (iv) corneal nerve branch length mm/mm^2 (CNBL) emanating from the major nerves; (v) tortuosity grade (TG); and (vi) frequency/0.1 mm of beading (BF). The TG was measured using the grading system of Oliveira-Soto and Efron[14]; all other measurements were carried out using ImageJ (Texelcraft, Tokyo, Japan).

Assessment of Nerve Function

Motor (MCV) and sensory (SCV) nerve conduction velocities and action potential amplitudes in the forearm segment were determined by conventional procedures with an electromyography instrument (Neuropak S1; NIHON KOHDEN, Tokyo, Japan). A motor and sensory nerve study was carried out on the MN and ulnar nerve, respectively. The current perception threshold (CPT) was measured using a neurometer (Neurotron, Baltimore, MD, USA). The lowest stimulus perceivable by the participant was defined as the CPT for that current frequency.

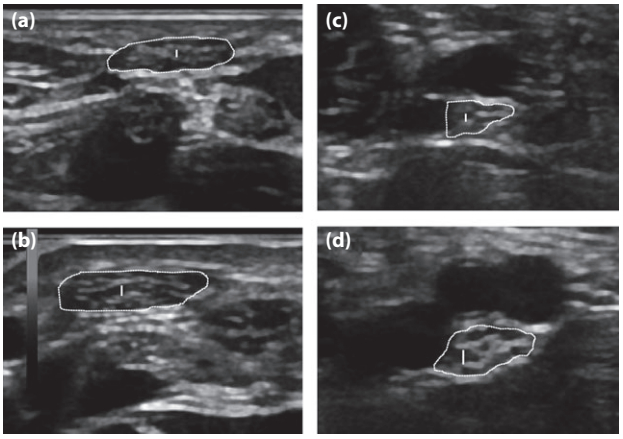

Figure 1 | Transverse sectional image of the median nerve of (a) a control subject or (b) type 2 diabetic patient, and the posterior tibial nerve of (c) a control subject or (d) type 2 diabetic patient, visualized by high-resolution ultrasonography using an 18.0-MHz linear array probe (HIVISION Ascendus; Hitachi, Tokyo, Japan). The control subject was a healthy 41-year-old man with a normal neurophysiological examination and normal parameters of the corneal nerve fibers, as determined by corneal confocal microscopy. The type 2 diabetic patient was a 43-year-old man with stage III diabetic neuropathy, as defined by the Diabetic Neuropathy Study Group in Japan[10], and reduced morphological parameters of the corneal nerve fibers. The cross-sectional areas of the peripheral nerves were determined by tracing the hyperechoic inner rim and counting the pixel numbers using Photoshop Elements 8.0 (Adobe Systems Inc.). The vertical white solid lines represent the maximum thickness of the nerve fascicles, and their lengths were determined using the measure tool of Photoshop 5.0 (Adobe Systems Inc.).

To assess the cardiovagal function of the autonomic nervous system, the $CV_{R\text{-}R}$ was calculated from R-R intervals of 200 samples on an electrocardiogram.

Laboratory Data

HbA1c levels (converted to National Glycohemoglobin Standardization Program [NGSP] units by adding 0.4% to the measured values[15]), serum creatinine, lipid levels and urinary albumin creatinine ratio (ACR) were determined.

Statistical Analyses

All statistical analyses were carried out using the SPSS medical package (SPSS, Chicago, IL, USA). The data are presented as the mean ± standard error of the mean. Analysis of variance (ANOVA) was used to compare the controls and type 2 diabetic patients with stratified DN. Multivariate regression analysis was used to determine the independent relationship between the CSA, HA or MTNF of peripheral nerves, and clinical factors, neurophysiological examinations, DN stages or corneal C fiber pathology. The multicollinearity in the correlation analyses was examined by the variance inflation factor (VIF). A P-value <0.05 was considered significant.

RESULTS

Table 1 shows the clinical and demographic data of the controls and type 2 diabetic patients with stratified DN. The sex ratio and age were similar between the controls and diabetic subgroups. The body mass index in patients at stage II or III was higher than in controls. The blood pressures of patients at stage IV+V were higher than those in other groups. Angiotensin receptor blocker or angiotensin-converting enzyme inhibitor was prescribed more frequently for diabetic patients than for controls. HbA1c levels in patients at stage III were higher than in those without neuropathy, and higher in patients at stage IV+V than in those at stage I or II. Low-density lipoprotein cholesterol in patients at stage III was higher than in controls. High-density lipoprotein cholesterol in controls was higher than in patients at stage I or II. The ACR in patients at stage IV+V increased compared with other four subgroups.

Figure 1 shows the representative HRU images of the MN and PTN in a control and a diabetic patient at stage III. The CSA of the MN (Figure 1b) and PTN (Figure 1d) in the diabetic patient was larger than that of the MN (Figure 1a) and PTN (Figure 1c) in the control. The vertical white solid lines indicate the MTNF, which are thicker in the diabetic patient than in the control.

The intrarater variability (averaged CV) in the measurement of morphological parameters by HRU for the MN and PTN, respectively, was as follows: 5.8, 9.0% for the CSA; 13.6, 15.9% for the HA; and 9.3, 10.3% for the MTNF.

The CSA of the MN (Figure 2a) and PTN (Figure 2b) in patients without neuropathy was greater than that in controls with further enlargements related to the DN severity. There was no difference in the CSA of the MN between the right and left hands in 20 controls (6.4 ± 0.4 vs 6.2 ± 0.3 mm^2, $P = 0.17$) and in 20 diabetic patients (9.1 ± 0.4 vs 8.9 ± 0.4 mm^2, $P = 0.36$).

The % HA of the MN (Figure 2c) and PTN (Figure 2d) was compared between controls and diabetic patients stratified by DN. When the HA was defined as less than the mean echo level, the % HAs in the MN (58.6 ± 0.64 to 59.6 ± 0.34%) and PTN (54.2 ± 0.64 to 56.8 ± 1.14%) were quite similar among all subgroups. We then arbitrarily defined the HA as <70% of the mean echo level. The % HA in the CSA of both nerves in patients without DN was greater than in controls and further enlarged associated with DN severity. The MTNF of both nerves in patients without DN was also greater than that in controls, and increased depending on DN severity (Figure 2e,f).

The neurophysiological tests in patients without DN were not significantly different from those in controls, with deterioration relating to DN severity (Table 2).

Compared with controls, the CNFD, CNFL, CNBD and BF in patients without DN were reduced, and TG was increased (Table 2). The CNFD, CNFL, CNBD and CNBL were further reduced in patients at stage IV+V.

Table 1 | Clinical characteristics of control subjects and type 2 diabetic patients with or without stratified severity of diabetic neuropathy

	Control subjects	Stage of diabetic neuropathy			
		Stage I	Stage II	Stage III	Stage IV+V
n (M/F)	40 (27/13)	47 (30/17)	72 (45/27)	48 (33/15)	33 (22/11)
Age (years)	53.6 ± 2.0	53.4 ± 1.1	55.5 ± 0.9	55.8 ± 1.3	56.8 ± 1.2
BMI (kg/m^2)	22.9 ± 0.5	24.4 ± 0.5	25.6 ± 0.5**	25.6 ± 0.6*	25.0 ± 0.9
SBP (mmHg)	140.1 ± 2.5	140.5 ± 2.7	144.8 ± 2.2	144.9 ± 3.2	160.4 ± 4.6***,###,&&,$$
DBP (mmHg)	83.7 ± 1.3	83.4 ± 1.3	84.9 ± 1.1	83.1 ± 1.4	90.7 ± 2.2*,##,&,$$
No. treated with ARB/ACEI (%)	4 (10.0)	16 (34.0)**	28 (38.9)***	17 (35.4)**	15 (45.5)***
HbA1c, % (NGSP)	5.7 ± 0.05	7.3 ± 0.20***	7.7 ± 0.17***	8.4 ± 0.27***,##	8.8 ± 0.39***,###,&&
LDL-C (mmol/L)	3.04 ± 0.10	3.43 ± 0.13	3.30 ± 0.10	3.69 ± 0.14**	3.25 ± 0.20
No. treated with statins (%)	2 (5.0)	7 (14.9)	13 (18.1)	7 (14.6)	4(12.1)
HDL-C (mmol/L)	1.81 ± 0.081	1.47 ± 0.059**	1.51 ± 0.047**	1.55 ± 0.065	1.53 ± 0.092
Triglycerides (mmol/L)	1.80 ± 0.23	1.89 ± 0.22	1.89 ± 0.14	2.25 ± 0.20	1.90 ± 0.21
ACR (mg/gCr)	10.2 ± 2.2	29.2 ± 12.0	59.3 ± 31.2	58.0 ± 16.1	234.6 ± 60.9***,###,&&&,$$$
eGFR (mL/min)	77.2 ± 1.9	82.2 ± 3.4	79.5 ± 1.8	84.8 ± 2.4	82.6 ± 4.5
Duration of diabetes (years)		10.5 ± 1.2	11.3 ± 0.8	11.6 ± 1.3	14.3 ± 1.4

Data are the mean ± standard error of the mean in control subjects and type 2 diabetic patients with or without diabetic neuropathy stratified by the criteria of Diabetic Neuropathy Study Group in Japan[10]. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with control subjects, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with patients with stage I neuropathy, $^{\&}P < 0.05$, $^{\&\&}P < 0.01$, $^{\&\&\&}P < 0.001$ compared with patients with stage II neuropathy, $^{\$\$}P < 0.01$, $^{\$\$\$}P < 0.001$ compared with patients with stage III neuropathy. Statistical analyses were carried out with analysis of variance for continuous variables, and with χ2-test with Bonferroni correction for categorical variables. To standardize glycated hemoglobin (HbA1c) values to National Glycohemoglobin Standardization Program (NGSP) units, 0.4% was added to the measured HbA1c values[15]. ACEI, angiotensin-converting enzyme inhibitor; ACR, urinary albumin/creatinine ratio; ARB, angiotensin receptor blocker; BMI, body mass index; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate, HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; SBP, systolic blood pressure.

The CSAs of both nerves were negatively associated with female gender (Table 3), and the CSA in women was smaller than in men (MN, 9.88 ± 0.22 vs 11.53 ± 0.18; PTN, 6.33 ± 0.17 vs 7.09 ± 0.11 mm^2, $P < 0.0001$). HbA1c levels were a positive predictor for the CSA, % HA and MTNF of both nerves. HbA1c levels were negatively associated with MCV ($\beta = -0.269$, $P < 0.0001$) and SCV ($\beta = -0.300$, $P < 0.0001$) but not with their amplitudes ($\beta = -0.043$, $P = 0.557$, $\beta = -0.127$, $P = 0.075$). The DN severity was closely associated with all morphological parameters of both nerves. The CSAs of both nerves were directly associated with their HA and MTNF. Between the MN and PTN, the CSA ($\beta = 0.476$, $P < 0.0001$), HA ($\beta = 0.313$, $P < 0.0001$) and MTNF ($\beta = 0.646$, $P < 0.0001$) were closely associated. The association between the pathology of corneal C fiber and morphological parameters of peripheral nerves were poor.

All morphological parameters of both nerves had good negative associations with MCV and SCV, but not with their amplitudes, except for the CSA. The 2,000-Hz CPT was positively associated with all the morphological parameters of both nerves. The 250-Hz CPT was significantly related with their MTNF. In the analysis of correlations between the morphological parameters of peripheral nerves and predictors, the VIF ranged 1.04–1.27 between clinical factors, 1.02–1.18 between neurophysiological examinations and 1.07–1.20 between corneal C fiber pathology.

DISCUSSION

In the diagnosis of DN, neurophysiological tests have a confirmatory role and provide detailed information about the dysfunction of affected nerves[16]; however, they offer no insight into the pathological changes. Therefore, HRU of peripheral nerves can be used to show pathological changes with relative ease and excellent resolution. This complementary role of nerve ultrasonography is increasingly accepted in mononeuropathies, but is lagging in diagnosing polyneuropathies, including DN[17]. The CSA[7–9,11,18,19], HA[9] and MTNF[8] have been reported for transverse sections of peripheral nerves in DN. However, an accurate evaluation of these morphological parameters of peripheral nerves requires HRU using HFLP (>15 MHz)[17]. Although the CSA of peripheral nerves can be calculated by a formula, the measurement by direct tracing is accurate. Previous investigations have not simultaneously determined these three morphological parameters of peripheral nerves in diabetic patients. Additionally, these studies incorporated relatively small numbers of patients (n = 25–100) to analyze the relationship between the morphological parameters of peripheral nerves and neurophysiological tests or other predictors. Furthermore, previous studies have not fully related these morphological parameters with neurophysiological tests or corneal C fiber pathology.

Herein, we assessed the CSA by direct tracing, and the HA and MTNF of the MN and PTN using HRU and HFLP. We

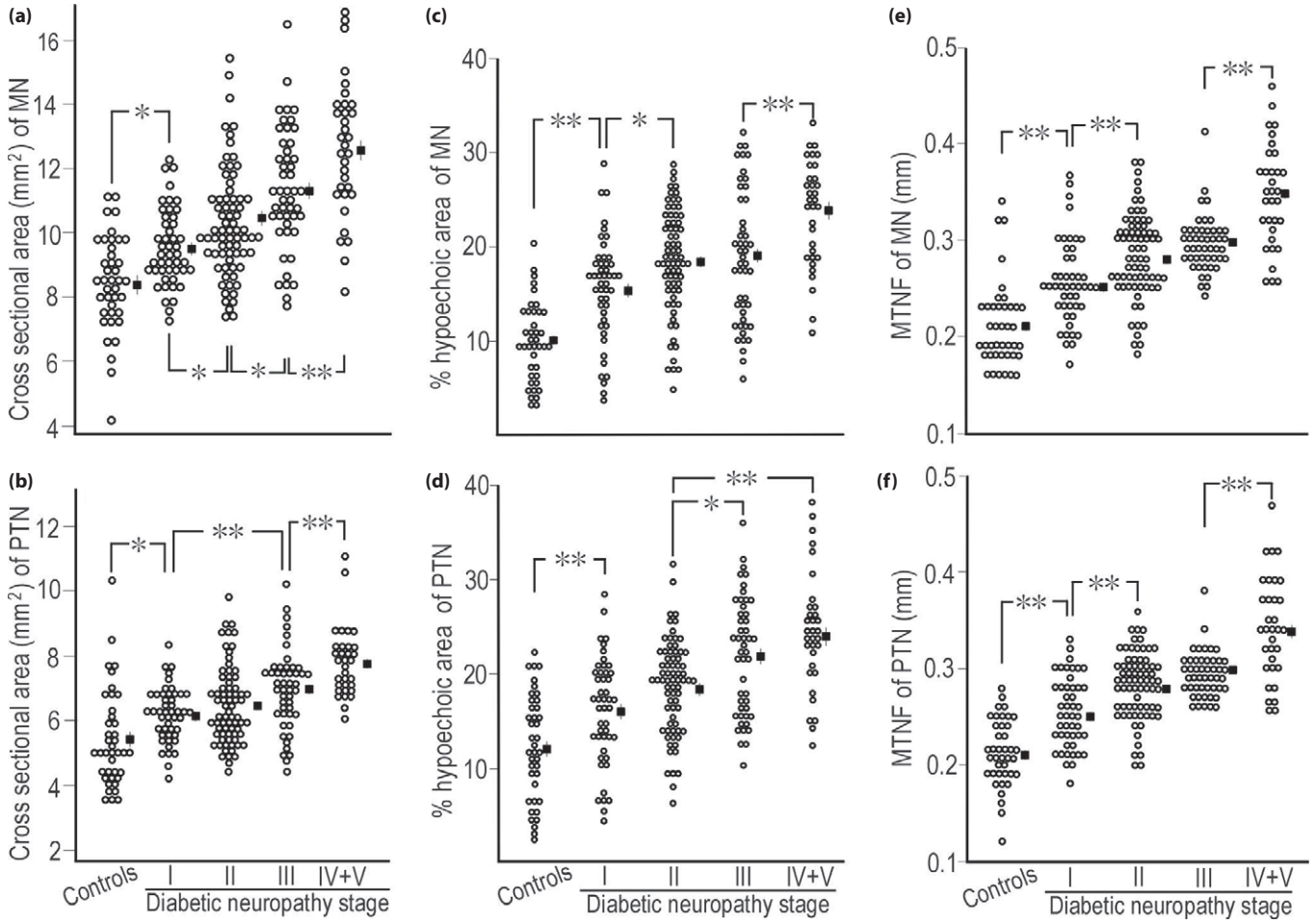

Figure 2 | Dot plot graphs comparing a cross-sectional area of (a) the median nerve and (b) the posterior tibial nerve, the % hypoechoic area of (c) the median nerve and (d) the posterior tibial nerve, and the maximum thickness of nerve fascicle of (e) the median nerve and (f) the posterior tibial nerve between control subjects and type 2 diabetic patients stratified by the severity of diabetic neuropathy. Diabetic patients were classified into one of five stages of diabetic neuropathy, according to the criteria proposed by Diabetic Neuropathy Study Group in Japan criteria[10]. The cross-sectional area of the median nerve and the posterior tibial nerve was measured by high-resolution ultrasonography using a high frequency linear probe (18.0 MHz, HIVISION Ascendus; Hitachi, Tokyo, Japan). The ultrasonographic transverse scans were obtained 5 cm proximal to the distal wrist crease for the median nerve[9] and 3 cm proximal to the cephalad border of the medial malleolus for the posterior tibial nerve[11]. The cross sectional area was evaluated by direct tracing. The pixel numbers were counted using Photoshop Elements 8.0 (Adobe Systems Inc.). The percentage of the hypoechoic area with an echo intensity <70% of the mean echo level of the cross-sectional area was determined by histogram analysis in Photoshop Elements 8.0. The MTNF[8] was measured along the short axis of the nerve fascicle using the measure tool of Photoshop 5.0 (Adobe Systems Inc.). *$P < 0.05$, **$P < 0.01$. The closed square (■) indicates the mean value and an error bar represents the standard error of the mean. MN, median nerve; MTNF, maximum thickness of nerve fascicle; PTN, posterior tibial nerve.

also carried out thorough neurophysiological tests, and determined corneal C fiber pathology by CCM in 40 age- and sex-matched controls, and 200 type 2 diabetic patients stratified by DN severity.

Among six previous studies[7–9,11,18,19] comparing the CSA of peripheral nerves between controls and diabetic patients with or without DN, only one[8] used HRU and HFLP while using a formula to measure the CSA of sural nerves. We found the CSA to be larger in men than in women; therefore, controls and diabetic patients with stratified DN should be sex-matched. Except for the study by Hobson *et al.*,[7] all previous investigations reported an enlarged CSA of peripheral nerves in diabetic patients with DN compared with patients without DN. As in the present study, only Liu *et al.*[8] matched sex and identified a larger CSA of sural nerves in non-neuropathic diabetic patients than in controls. Thus, to detect CSA differences between controls and non-neuropathic diabetic patients, HRU using HFLP and sex matching are mandatory. Moon *et al.*[19] divided neuropathic patients into mild and severe groups, reporting that the CSA of MN in the latter was larger. We fully stratified diabetic

Table 2 | Comparison of neurophysiological tests, and corneal nerve fiber pathology between control subjects and type 2 diabetic patients with or without stratified diabetic neuropathy

	Control subjects	Stage of diabetic neuropathy			
		Stage I	Stage II	Stage III	Stage IV+V
MCV of median nerve (m/s)	57.1 ± 0.5	56.8 ± 0.5	53.6 ± 0.5***,###	50.3 ± 0.5&&&	46.1 ± 0.8$$$
Amplitude of median nerve (mV)	6.3 ± 0.52	5.0 ± 0.44	3.4 ± 0.33#	2.9 ± 0.33##	2.7 ± 0.36##
SCV of ulnar nerve (m/s)	63.7 ± 0.6	62.6 ± 0.5	60.3 ± 0.6***	57.7 ± 0.5&&	53.4 ± 1.0$$$
Amplitude of ulnar nerve (μV)	26.9 ± 1.7	20.9 ± 2.0	17.1 ± 1.4***	15.7 ± 1.6	8.0 ± 0.7&&,$
CPT 2000 Hz	209 ± 8.1	237 ± 12.1	271 ± 8.7*	324 ± 19.3&	407 ± 31.1$$
CPT 250 Hz	84.6 ± 3.8	95.7 ± 6.1	110.4 ± 4.3	134.3 ± 15.2*	196.9 ± 26.1$$
CPT 5 Hz	48.6 ± 2.4	52.9 ± 4.0	57.0 ± 2.8	77.9 ± 11.8*	110.2 ± 20.0&&&
$CV_{R\text{-}R}$ (%)	3.84 ± 0.31	3.50 ± 0.20	3.69 ± 0.20	2.97 ± 0.19*	2.18 ± 0.26&&&
Corneal nerve fiber density (/mm^2)	31.7 ± 0.9	24.1 ± 0.7***	22.9 ± 0.6***	21.5 ± 0.9***	20.5 ± 0.9***,#
Corneal nerve fiber length (mm/mm^2)	12.2 ± 0.3	9.6 ± 0.3***	9.5 ± 0.3***	8.4 ± 0.4***	7.7 ± 0.4***,#
Corneal nerve branch density (/mm^2)	12.3 ± 0.8	9.9 ± 0.5*	9.3 ± 0.5***	9.1 ± 0.6***	7.2 ± 0.5***,#
Corneal nerve branch length (mm/mm^2)	2.51 ± 0.14	2.08 ± 0.09	2.06 ± 0.09*	2.04 ± 0.15*	1.57 ± 0.12&
Tortuosity grade	1.98 ± 0.04	2.53 ± 0.06***	2.52 ± 0.05***	2.41 ± 0.07***	2.49 ± 0.05***
Beading frequency	23.8 ± 0.23	21.9 ± 0.31***	21.4 ± 0.24***	21.7 ± 0.28***	21.4 ± 0.39***

Data are mean ± standard error of the mean in control subjects and type 2 diabetic patients subdivided by stratified diabetic neuropathy. *$P < 0.05$, ***$P < 0.001$ compared with control subjects, #$P < 0.05$, ##$P < 0.01$, ###$P < 0.001$ compared with patients with stage I neuropathy, &$P < 0.05$, &&$P < 0.01$, &&&$P < 0.001$ compared with patients with stage II neuropathy, $$P < 0.05$, $$$P < 0.01$, $$$$P < 0.001$ compared with patients with stage III neuropathy. Statistical analysis was carried out with analysis of valiance. CPT, current perception threshold; CV, coefficient of variation; MCV, motor nerve conduction velocity; NF, nerve fascicle; SCV, sensory nerve conduction velocity.

patients into four subgroups by DN severity, and found that the enlargement of the CSA precedes DN development. We identified a close association between the CSA of both nerves and the DN severity. The CSA had a tight negative association with MCV and SCV, and a positive association with 2,000-Hz CPT. The amplitudes of both nerves were negatively associated with the CSA, but not with the HA or MTNF.

Watanabe *et al.*[9] reported that the HA of MN increased in patients with slow MCV compared with those in patients with normal MCV, but they found no difference in the HA of tibial nerves between slow and normal tibial MCV subgroups. In the present study, as with an enlarged CSA, the increased % HA of the MN and PTN preceded DN, and further increases were associated with the decreases in MCV and SCV. They defined the HA as an echo intensity less than the mean echo level[9]. In the present study, when the same cut-off level for the HA was used, there was no difference in % HA between controls and diabetic patients with stratified DN. We arbitrarily defined the HA as the echo intensity <70% of the mean echo level, but there was no rationale for our criterion of the HA. Furthermore, the intrarater variability in the determination of the HA of both nerves was greater than those for the CSA and MTNF. Therefore, the interpretation of the present results regarding the HA must await a rational definition of the HA and the appearance of an innovative method for assessing the HA in peripheral nerves.

As nerve fascicular size is one of determinants of the HA in the CSA of peripheral nerves[20,21], we measured the MTNF. The MTNF in non-neuropathic patients increased compared with controls, and a tight association between the MTNF of both nerves and the DN severity, MCV or SCV was found. Therefore, the increased MTNF might be a surrogate morphological marker for DN severity. Because the amplitude of both nerves had no significant association with the MTNF, the increased MTNF might be related mainly to the demyelination, but not to axon loss of peripheral nerves. In Charcot–Marie–Tooth disease 1A, a predominantly demyelinating neuropathy, the CSA and fascicle diameter of the MN were significantly increased compared with controls, and tended to be larger than axonal Charcot–Marie–Tooth disease 2A, although the difference was not statistically significant[22]. However, there were substantial differences in the MTNF of controls. The MTNF in the present study was 0.211 ± 0.007 (MN) and 0.213 ± 0.005 mm (PTN), similar to that of the sural nerve (0.33 ± 0.04 mm)[8], whereas Schreiber *et al.*[22] reported that the MTNF of MN was 0.60 ± 0.21~0.63 ± 0.13 mm. The diversity in the MTNF measurement might be due to the frequency of linear probe, whether the measurement was made along the short axis, or racial differences among subjects. Recently, pathological changes in the non-compressed posterior interosseous nerve lying close to the MN in diabetic or non-diabetic patients with CTS have been reported[23]. Compared with non-diabetic subjects, the fascicular area was increased in the diabetic group, but not significantly. However, the myelinated nerve fiber density, fiber and axon area were significantly reduced, whereas the unmyelinated axon density increased with a significant reduction in the axon diameter. The morphological changes detected by HRU in the present study might represent these histological findings.

Table 3 | Correlation between morphological parameters of peripheral nerves assessed by high-resolution ultrasonography and clinical factors, corneal nerve fiber pathology or neurophysiological tests in type 2 diabetic patients

	Cross sectional area				Hypoechoic area				Maximum thickness of nerve fascicle			
	Median nerve		PTN		Median nerve		PTN		Median nerve		PTN	
	β	*P*	β	*P*	β	*P*	β	*P*	β	*P*	β	*P*
Sex	−0.402	<0.0001	−0.278	<0.0001	−0.146	0.052	−0.064	0.383	0.097	0.190	−0.088	0.235
Age	0.083	0.230	0.082	0.263	0.067	0.381	0.020	0.793	0.148	0.051	0.074	0.328
BMI	−0.022	0.759	−0.050	0.505	−0.055	0.486	−0.100	0.200	0.133	0.088	0.007	0.931
Diabetes duration	0.047	0.471	0.013	0.854	0.034	0.635	−0.001	0.986	0.021	0.772	−0.080	0.267
DBP	0.033	0.639	−0.008	0.912	0.020	0.793	−0.085	0.271	0.136	0.079	0.125	0.087
HbA1c	0.216	0.002	0.183	0.013	0.177	0.025	0.197	0.010	0.191	0.020	0.198	0.011
LDL-C	−0.008	0.907	−0.053	0.475	0.011	0.885	−0.111	0.148	0.017	0.828	−0.030	0.691
HDL-C	0.044	0.543	0.015	0.843	0.009	0.908	0.099	0.207	0.034	0.661	0.019	0.813
Triglycerides	−0.013	0.859	0.016	0.835	−0.033	0.678	0.049	0.535	−0.083	0.294	−0.097	0.219
Stage of DN	0.481	<0.0001	0.427	<0.0001	0.356	<0.0001	0.462	<0.0001	0.546	<0.0001	0.599	<0.0001
CSA of MN					0.211	0.003			0.320	<0.0001		
CSA of PTN							0.182	0.014			0.253	0.001
CNFD	−0.038	0.567	0.010	0.884	0.047	0.524	−0.066	0.367	−0.084	0.251	−0.086	0.240
CNFL	−0.016	0.814	0.041	0.559	0.079	0.280	−0.069	0.339	0.065	0.371	−0.021	0.769
CNBD	−0.058	0.398	0.122	0.097	0.010	0.895	−0.180	0.017	−0.180	0.017	−0.060	0.433
CNBL	−0.021	0.759	0.122	0.087	0.119	0.122	−0.119	0.120	−0.085	0.266	−0.033	0.664
TG	−0.079	0.239	−0.017	0.814	−0.121	0.102	−0.108	0.142	−0.121	0.092	−0.008	0.912
BF	0.063	0.336	−0.032	0.643	−0.136	0.059	0.012	0.862	−0.102	0.155	−0.135	0.060
MCV of MN	−0.629	<0.0001	−0.454	<0.0001	−0.193	0.010	−0.211	0.004	−0.407	<0.0001	−0.426	<0.0001
Amp of MN	−0.175	0.010	−0.210	0.003	0.040	0.601	−0.117	0.117	−0.048	0.521	−0.029	0.701
SCV of UN	−0.480	<0.0001	−0.266	<0.0001	−0.201	0.009	−0.204	0.007	−0.365	<0.0001	−0.309	<0.0001
Amp of UN	−0.207	0.002	−0.163	0.022	−0.132	0.068	−0.114	0.213	−0.093	0.205	−0.121	0.094
CPT 2000 Hz	0.202	0.002	0.192	0.006	0.188	0.009	0.194	0.005	0.212	0.003	0.248	0.001
CPT 250 Hz	0.104	0.110	0.114	0.136	0.129	0.073	0.075	0.294	0.256	<0.0001	0.303	<0.0001
CPT 5 Hz	0.060	0.358	0.112	0.102	0.132	0.066	0.026	0.715	0.131	0.069	0.124	0.088
CV_{R-R}	−0.123	0.077	−0.102	0.154	−0.004	0.953	−0.060	0.412	−0.078	0.291	−0.070	0.341

Multivariate regression analysis was used to determine the independent relationship between the cross-sectional area (CSA), hypoechoic area (HA) or maximum thickness of the nerve fascicle of peripheral nerves and clinical factors, stage of diabetic neuropathy, morphological parameters of corneal nerve fibers or neurophysiological examinations. Amp, amplitude; BF, beading frequency; BMI, body mass index; CNBD, corneal nerve branch density; CNBL, corneal nerve branch length; CNFD, corneal nerve fiber density; CNFL, corneal nerve fiber length; CPT, current perception threshold; CV, coefficient of variation; DBP, diastolic blood pressure; DN, diabetic neuropathy; HbA1c, glycated hemoglobin; HDL-C, high-density lipoprotein-cholesterol; LDL-C, low-density lipoprotein-cholesterol; MCV, motor nerve conduction velocity; MN, median nerve; PTN, posterior tibial nerve; SCV, sensory nerve conduction velocity; TG, tortuosity grade; UN, ulnar nerve.

The imaging by magnetic resonance neurography[24] has been developed for visualizing the entrapment-related and spontaneously occurring neuropathies. Compared with HRU, more reliable lesion imaging at the level of the nerve fascicle is feasible with magnetic resonance, although magnetic resonance is more expensive and time-consuming. Therefore, HRU seems to be practical for the morphological analysis of peripheral nerves of DN in a large cohort.

Among clinical factors, HbA1c levels were positively associated with the CSA, HA and MTNF of both nerves. The CSA, HA and MTNF in non-neuropathic patients were larger than those in controls and were enlarged, depending on the DN severity. Furthermore, the CSA of both nerves was directly correlated with their HA and MTNF. Thus, the swelling of peripheral nerves precedes DN and is a result of the enlargement of neuronal fascicle (and resultant increase in the HA) in diabetic patients. The higher HbA1c levels might be a causative factor of peripheral nerve swelling. HbA1c levels had a close inverse association with the MCV and SCV, but not with their amplitudes. The morphological changes of peripheral nerves were associated with the MCV and SCV, but not with the amplitudes. These results could indicate a metabolic basis for the link between the morphological changes of peripheral nerves and conduction slowing. The acute glycemic control in diabetic patients improves the conduction slowing, but does not alter the amplitudes[25], suggesting that the reversible metabolic factors related to hyperglycemia (decreased Na^+/K^+-adenosine triphosphatase activity and altered polyol

pathway) could play a role in conduction slowing. Dunnigan *et al.*[26] classified DN as axonal, conduction slowing and combined type by electrodiagnostic tests, and found that the conduction slowing (demyelination) in type 1 diabetic patients is associated with suboptimal glycemic control. This result might be relevant in explaining the lack of association between corneal C fiber degeneration and changes in peripheral nerves detected by HRU.

Watanabe *et al.*[9] did not observe a significant relationship between HbA1c levels and the CSA or % HA of the MN, which might be as a result of the small numbers of patients ($n = 30$).

Because increases in the CSA, HA and MTNF of the MN and PTN in diabetic patients precede DN, these changes (as detected by HRU) might play a hierarchical role in DN of peripheral nerves. Recently, the sensitivity of electrophysiological assessment in the diagnosis of DN was evaluated in 132 diabetic patients with or without DN[27]. Just 3% of the patients without DN had sensory or motor nerve conduction slowing. However, a prospective study is required to determine whether the morphological changes detected by HRU predict the changes in the neurophysiological tests. Although we excluded subjects with symptoms suggestive of CTS, the prevalences of clinical CTS in diabetic patients with and without DN were 30 and 14%, respectively[28]. Furthermore, the electrodiagnostic criteria cannot completely distinguish between those with or without clinical CTS[28]. Therefore, we could not rule out the concomitant presence of CTS by conventional neurophysiological tests. However, because the CSA of the bilateral MN in controls and diabetic patients were similar, and the CSA of the MN and PTN in diabetic patients had a tight association, few patients with CTS were involved.

The corneal C fiber changes detected by CCM in the present study are similar to those of a recent investigation[29], and the corneal C fiber quantification provides an objective and reproducible means to detect DN[29]. The BF was reduced in patients without DN, but did not show further deterioration related to the DN severity because the BF is affected by the concurrent hyperglycemia[12], whereas long-standing hyperglycemia did not reduce BF any further[12]. The association between the morphological changes of peripheral nerves detected by ultrasonography and the corneal C fiber pathology in diabetic patients has never been studied. In the present study, corneal C fiber degeneration had occurred extensively before developing DN, and further deterioration was limited in patients with severe neuropathy, whereas the increases in the CSA, HA and MTNF progressed in relation to the DN severity. Except for the nerve branch, the morphological parameters of corneal C fibers did not correlate with the morphology of peripheral nerves. The earliest nerve fiber damage in DN is to the small C fibers in patients with type 2 diabetes[30]. Therefore, before the changes in neurophysiological tests and the morphology of peripheral nerves, the degeneration of corneal C fibers might occur.

We acknowledge many limitations to the interpretation of the present results. First, we measured the CSA, HA and MTNF by HRU without a histological examination. Therefore, we could not determine the robust histological etiologies of peripheral nerve swelling. Second, because we arbitrarily defined the HA as <70% of the mean echo level, the contribution of the HA in nerve swelling should be carefully interpreted. Third, as the present study was cross-sectional, the influence of long-standing hyperglycemia on the morphology of peripheral nerves must be considered, although the concurrent HbA1c levels were associated with them.

In conclusion, patients with type 2 diabetes had increased CSA, HA and MTNF of peripheral nerves before the onset of DN. After the development of DN, these morphological changes progressed in relation to increasing neuropathic severity. These changes were not correlated with the pathology of corneal C fibers, however, because the extensive corneal C fiber degeneration occurs before the onset of DN.

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