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Correlation between sudomotor function, sweat gland duct size and corneal nerve fiber pathology in patients with type 2 diabetes mellitus

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ABSTRACT

Aims/Introduction: To study the correlation between sudomotor function, sweat gland duct size and corneal nerve fiber pathology in type 2 diabetes.

Materials and Methods: Sudomotor function was quantified by Neuropad test, and sweat gland duct and corneal nerve fibers were visualized by confocal microscopy in 78 patients with type 2 diabetes stratified by diabetic neuropathy and 28 control participants.

Results: In patients with diabetic neuropathy, sudomotor function, as judged by the time required for complete color change of a Neuropad, was impaired compared with that of controls ($P < 0.0001$), thereby showing deterioration was related to the severity of diabetic neuropathy ($P < 0.0001$). Sweat gland ducts were smaller in patients without neuropathy than in controls ($P < 0.0001$), and further shrinking was seen in patients with severe diabetic neuropathy ($P < 0.05$). Patients without diabetic neuropathy showed reduced density and length ($P < 0.001$) of corneal nerve fibers and beading frequency ($P < 0.0001$), and increased tortuosity ($P < 0.0001$) compared with controls, and these changes deteriorated in patients with severe diabetic neuropathy. Sudomotor function was negatively associated with corneal nerve fibers ($P < 0.002$) and branches ($P < 0.01$), and influenced by the severity of diabetic neuropathy ($P < 0.0001$); sweat gland duct size correlated with serum triglycerides ($P < 0.02$), uric acid ($P < 0.01$), corneal nerve branch ($P < 0.03$), sudomotor function ($P < 0.03$) and severity of neuropathy ($P < 0.03$).

Conclusions: Type 2 diabetic patients had sudomotor dysfunction and smaller sweat gland ducts compared with controls. The stage of diabetic neuropathy and corneal nerve fiber pathology were independent predictors of sudomotor dysfunction, and serum triglycerides, uric acid, corneal nerve branch, stage of diabetic neuropathy and sudomotor function were predictors of sweat gland duct size.

INTRODUCTION

Sympathetic sudomotor function is commonly impaired in diabetic patients with the early occurrence of abnormalities[1]. Loss of sweating in the feet is a recognized risk for foot ulceration in patients with diabetes[2,3]. Recently, the Neuropad, an adhesive indicator patch that detects sweating through color change, has been proposed as an easy practical test for assessing sudomotor function in the feet[4]. Previous studies[5,6] showed that the Neuropad demonstrated good sensitivity and modest specificity for assessing sudomotor dysfunction in patients with diabetes. If damage to small somatic nerve fibers occurs concomitantly with that to sympathetic fibers in the lower limbs, sudomotor dysfunction in the lower limbs might be strongly related to the

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degeneration of somatic small unmyelinated fibers. However, the association between intra-epidermal nerve fiber density (IENFD) and the graded, but not quantified, Neuropad response was reported to be modest[6].

Confocal microscopy (CM) is a non-invasive method for assessing the morphology of corneal unmyelinated C fibers, and the morphological degeneration of the corneal nerve has been reported to be proportional to the severity of diabetic neuropathy (DN)[7,8]. However, the correlation between sudomotor dysfunction quantified by Neuropad test and C fiber anomalies in corneal nerve fibers (NFs) in a cohort composed entirely of type 2 diabetic patients has never been studied, although the relationship between the qualified (normal, intermediate and abnormal response) Neuropad test and corneal C fiber pathology in a mixed population with type 1 and type 2 diabetes was reported only in one abstract[9]. CM can visualize not only corneal NFs, but also all tissues within 0.2 mm beneath the body surface, including the sweat gland ducts[10].

The present study aimed to determine the correlation between sudomotor dysfunction quantified by Neuropad test, corneal C fiber pathology and the cross-sectional area (CSA) of sweat gland ducts in patients with type 2 diabetes with stratified DN, and to compare these variables with those of age-matched non-diabetic control participants.

MATERIALS AND METHODS

The present study included 78 patients with type 2 diabetes and 28 age-matched non-diabetic control participants. Patients were recruited from the Ishibashi Clinic, Hiroshima, Japan. Exclusion criteria were as follows: age <30 or >65 years, peripheral arterial disease, allergy to metals, skin diseases (neurodermatitis, psoriasis, Raynaud's syndrome and hyperhydrosis), drug therapy (corticosteroids, β-blocker, antihistaminic and psychoactive drugs, which may affect sweating), chronic alcohol abuse, thyroid disease and lumbar spine disorders or any other causes of peripheral neuropathy. Written informed consent was obtained from all participants. The protocol of the present research was approved by the ethics committee of Ishibashi Clinic.

Assessment of DN

DN was diagnosed in patients with type 2 diabetes based on the presence of two of the following three factors recommended in the simplified diagnostic criteria proposed by the Diabetic Neuropathy Study Group in Japan (DNSGJ)[11]: (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. Patients with diabetes were classified into the following stages of DN according to the DNSGJ criteria[12]. Stage I is no neuropathy, which includes patients who do not meet the diagnostic criteria for DN. Stage II is asymptomatic DN, in which patients have no subjective symptoms, but meet the diagnostic criteria for DN. At stage III, subjective symptoms are positive, but either ankle reflex or vibration sense is within the normal range. At stage IV, patients show clinically manifested autonomic neuropathy, such as orthostatic hypotension. Motor neuropathy appears at stage V. Because small numbers of patients were classified into stage IV and V, we combined stage IV with stage V (IV + V).

Neuropad Test

Participants were allowed a 10-min acclimatization period in constant temperature (25°C) after they removed their socks and shoes. Indicator test patches (Neuropad; Trigocare, GmbH, Wiehl-Drabenderhohe, Germany) were applied to both soles at the level of the first through second metatarsal heads. The time to complete color change (CCC) was defined as the time in seconds required until CCC of the indicator test from blue to a uniform smooth pink, and was recorded with the exactitude of 10 s. For each participant, we observed the time to CCC for each foot, and recorded the longer of these two values as more representative of neuropathy status[13]. The reproducibility of the Neuropad test was evaluated in six healthy volunteers by repeating the Neuropad test five times and calculating the coefficient of variation (CV).

Corneal CM

Participants were examined using Heidelberg Retina Tomograph-3 equipped with a Rostock Cornea Module (Heidelberg Engineering, Heidelberg, Germany) as reported previously[14]. In brief, each examined eye was anesthetized with a drop of 0.4% benoxinate hydrochloride (Santen Pharmaceutical Co., Osaka, Japan). A drop of Comfort Gel (Dr. Mann Pharma, Berlin, Germany) was applied to the tip of 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. Collected images of the sub-basal nerve plexus were used to quantify the following parameters for defining corneal NF changes: (i) corneal NF density/mm^2 (CNFD); (ii) corneal NF 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 nerve trunk; (v) tortuosity; and (vi) frequency/0.1 mm of beading. All measurements except for tortuosity were determined using Image J (Texelcraft, Tokyo, Japan); tortuosity was determined according to the grading system proposed by Oliveira-Soto and Efron[15].

CM of Sweat Gland Ducts

Before examination, participants were allowed a 10-min acclimatization period in constant room temperature (25°C). After applying Comfort Gel to the Tomocap, the tip of the index finger of the right hand was attached to the Tomocap. Next, 15–20 images of the sweat gland duct were recorded. The CSA of an individual sweat gland duct was assessed by counting the number of pixels in an image of the duct using Photoshop Elements 8.0 (Adobe Systems Inc., San Jose, CA, USA) after

smoothing and enlarging the images to fivefold using PhotoZoom Pro4 (BenVista Ltd., Houston, TX, USA).

Assessment of Nerve Function

Large Fiber Functions

Current perception threshold (CPT) and vibration perception threshold (VPT) were measured as reported previously[14] using a neurometer (Neurotron, Baltimore, MD, USA) and a biothesiometer (Bio-Medical Instrument, Newbury, OH, USA), respectively. The lowest stimulus perceivable by the participant was defined as the CPT for that current frequency in each individual. To determine the VPT, eight readings were obtained and averaged. Nerve conduction studies were carried out by conventional procedures with an electromyography machine (Neuropak S1, NIHON KOHDEN, Tokyo, Japan). A motor nerve study was carried out on the median nerve, and a sensory nerve study was carried out on the ulnar nerve.

Small Fiber Functions

Warm and cold perception thresholds (PTs) were determined using a thermal stimulator that was controlled by a Peltier element and a push-button switch (Intercross-200; Intercross Co., Tokyo, Japan). Warm and cold PTs were measured at the thenar eminence and cheek, respectively. The surface temperature of the stimulation probe was automatically set at the skin temperature of the target body region. The measurement was repeated five times and averaged. To assess the cardiovagal function of the autonomic nervous system, $CV_{R\text{-}R}$ was calculated from the R-R intervals of 200 samples on the electrocardiogram:

$$CV_{R-R}(\%) = \text{standard deviation/average of R-R intervals} \times 100$$

Laboratory Data

Glycated hemoglobin (HbA1c) levels (converted to National Glycohemoglobin Standardization Program units by adding 0.4% to the measured values[16]), serum blood urea nitrogen (BUN), creatinine, uric acid, urinary albumin creatinine ratio (ACR) and lipid levels were determined.

Statistical Analyses

All statistical analyses were carried out using the SPSS medical package (SPSS, Chicago, IL, USA). Data are presented as the mean ± standard error of the mean. Analysis of variance (ANOVA) was used to compare the control participants and patients with type 2 diabetes with or without DN graded by DNSGJ staging[12]. Multivariate regression analysis was used to determine the independent relationship between the time to CCC in the Neuropad test or CSA of the sweat gland ducts, and clinical factors, neurological examinations or morphological parameters of corneal NFs. Receiver operating characteristic (ROC) curve analysis established cut-off levels of the time to CCC in the

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

	Control participants	Stage of diabetic neuropathy			
		Stage I	Stage II	Stage III	Stage IV+V
n (Male/female)	28 (17/11)	23 (15/8)	28 (15/13)	20 (12/8)	7 (3/4)
Age (years)	50.2 ± 1.4	48.1 ± 2.2	55.7 ± 1.5$^{\#}$	54.8 ± 2.2	58.7 ± 2.2
BMI (kg/m^2)	22.9 ± 0.6	25.1 ± 0.9	25.3 ± 0.6	25.6 ± 1.1	22.3 ± 0.9
SBP (mmHg)	134.6 ± 8.5	141.3 ± 5.2	143.7 ± 3.2	147.4 ± 5.1	159.7 ± 11.4
DBP (mmHg)	79.8 ± 3.6	83.7 ± 1.9	85.2 ± 1.1	85.9 ± 1.9	89.7 ± 4.5
No. treated with ARB/ACEI (%)	1 (3.6)	7 (30.4)	11 (39.3)*	10 (50.0)**	4 (57.1)*
HbA1c (%)	5.6 ± 0.05	7.7 ± 0.44***	7.5 ± 0.27***	8.3 ± 0.44***	8.3 ± 0.53**
Uric acid (mmol/L)	0.32 ± 0.02	0.34 ± 0.02	0.32 ± 0.01	0.29 ± 0.02	0.29 ± 0.03
LDL-C (mmol/L)	3.29 ± 0.16	3.41 ± 0.22	3.39 ± 0.15	3.31 ± 0.21	3.02 ± 0.16
No. treated with statins (%)	2 (7.1)	3 (13.0)	6 (21.4)	4 (20.0)	1 (14.3)
HDL-C (mmol/L)	1.78 ± 0.097	1.47 ± 0.10	1.38 ± 0.079*	1.43 ± 0.082	1.79 ± 0.12
Triglycerides (mmol/L)	1.49 ± 0.20	1.93 ± 0.30	2.69 ± 0.59	2.64 ± 1.16	1.53 ± 0.30
ACR (mg/gCr)	8.5 ± 2.1	39.5 ± 16.0	31.3 ± 8.4	168.6 ± 105.7	168.8 ± 100.4
eGFR (mL/min)	81.9 ± 2.4	83.0 ± 4.0	80.7 ± 2.5	86.8 ± 4.1	96.9 ± 7.5
Duration of diabetes (years)		5.8 ± 1.2	8.5 ± 1.0	9.9 ± 1.9	10.4 ± 3.7

Data are the mean ± standard error of the mean in control participants and type 2 diabetic patients with or without diabetic neuropathy stratified by the criteria of Diabetic Neuropathy Study Group in Japan[12]. *$P < 0.01$, **$P < 0.001$, ***$P < 0.0001$ compared with control participants, $^{\#}P < 0.05$ compared with type 2 diabetic patients with diabetic neuropathy stage I. Statistical analyses were carried out with analysis of variance for continuous variables, and with χ^2-test with Bonferroni correction for categorical variables. To standardize glycated hemoglobin (HbA1c) values to National Glycohemoglobin Standardization Program units, 0.4% was added to the measured HbA1c values[16]. 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.

Neuropad test and CSA of the sweat gland ducts between the control participants and patients with type 2 diabetes. Sensitivity and specificity were equally weighted. A *P*-value of <0.05 was considered statistically significant.

RESULTS

Table 1 shows the clinical and demographic data of the control participants and patients with type 2 diabetes with or without different stages of DN. Patients with stage II DN were older than patients without DN. HbA1c level in the control participants was lower than that in patients with diabetes. Angiotensin receptor blocker or angiotensin-converting enzyme inhibitor was more frequently prescribed for patients with DN than for the control participants, whereas no difference was found among subgroups divided according to DN severity. High-density lipoprotein cholesterol was higher in the control participants than in patients with stage II DN. There was no difference in BMI, SBP, DBP, triglycerides and ACR among control participants and type 2 diabetic patients stratified by diabetic neuropathy. The duration of diabetes was comparable among subgroups of diabetic patients.

The neurophysiological findings in the control participants and patients with type 2 diabetes with different stages of DN are shown in Table 2. All neurophysiological parameters, except for cold PT, in patients at stage IV+V were impaired compared with those in control subjects. MCV in stage IV+V was lower than that in stage I and II, and SCV in stage IV+V was significantly slower than that in stage I. VPT was higher in stage IV+V than in stage I. CPT of all frequencies increased, and warm PT decreased, and $CV_{R\text{-}R}$ became shorter as DN severity increased.

When morphological parameters of corneal NFs were compared among the control participants and patients with type 2 diabetes with or without stratified DN (Table 3), patients with

Table 2 | Comparison of neurophysiological examinations among control participants and type 2 diabetic patients with or without stratified diabetic neuropathy

	Control participants	Stage of diabetic neuropathy			
		Stage I	Stage II	Stage III	Stage IV+V
MCV (m/s)	57.8 ± 0.7$^{\#\#\#}$	56.0 ± 1.0$^{\#\#}$	53.9 ± 1.3$^{\#}$	52.5 ± 1.4	46.7 ± 3.8
SCV (m/s)	63.6 ± 0.7$^{\#\#}$	62.5 ± 0.7$^{\#}$	60.5 ± 1.0	59.4 ± 0.8	54.9 ± 2.4
VPT (μ/120c/s)	1.7 ± 0.19$^{\#}$	2.1 ± 0.31$^{\#}$	3.4 ± 0.53	3.2 ± 0.44	4.5 ± 0.61
CPT-2000 Hz	226.3 ± 12.9$^{\#\#\#}$	225.5 ± 10.8$^{\#\#\#}$	254.7 ± 16.1$^{\#\#}$	357.1 ± 33.8$^{\$\$,\&\&\&}$	426.7 ± 67.5
CPT-250 Hz	95.6 ± 6.2$^{\#\#\#}$	95.8 ± 4.5$^{\#\#\#}$	102.3 ± 7.3$^{\#\#}$	147.8 ± 12.8$^{\$\$,\&\&\&}$	166.3 ± 20.4
CPT-5 Hz	50.3 ± 3.5$^{\#}$	53.2 ± 4.9$^{\#}$	54.0 ± 5.6$^{\#}$	84.1 ± 10.0$^{\$\$,\&\&}$	89.4 ± 6.5
Warm PT (w/m^2)	−594 ± 39.8$^{\#\#\#}$	−605 ± 26.5$^{\#\#\#}$	−580 ± 30.7$^{\#\#\#}$	−659 ± 37.9$^{\#\#\#}$	−1028 ± 189
Cold PT (w/m^2)	473 ± 42.8	524 ± 26.5	563 ± 20.7	628 ± 54.2	664 ± 62.5
$CV_{R\text{-}R}$ (%)	3.32 ± 0.19$^{\#}$	3.50 ± 0.18$^{\#\#}$	3.20 ± 0.28	3.01 ± 0.25$^{\&}$	1.85 ± 0.27

Data are mean ± standard error of the mean in control participants and type 2 diabetic patients with or without diabetic neuropathy stratified by the criteria of Diabetic Neuropathy Study Group in Japan[12]. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with stage IV + V, $^{\$\$}P < 0.01$ compared with stage II, $^{\&}P < 0.05$, $^{\&\&}P < 0.01$, $^{\&\&\&}P < 0.001$ compared with stage I. CPT, current perception threshold; CV, coefficient of variation; MCV, motor neuron conduction velocity; PT, perception threshold; SCV, sensory neuron conduction velocity; VPT, vibration perception threshold.

Table 3 | Comparison of morphological parameters of corneal nerve plexus observed by corneal confocal microscopy between control participants and type 2 diabetic patients with or without stratified severity of diabetic neuropathy

	Control participants	Stage of diabetic neuropathy			
		Stage I	Stage II	Stage III	Stage IV+V
Corneal nerve fiber density (/mm^2)	33.1 ± 0.85	27.3 ± 0.78$^{**,\#\#}$	25.4 ± 1.4$^{***,\#}$	24.2 ± 1.5***	17.7 ± 2.8***
Corneal nerve fiber length (mm/mm^2)	14.2 ± 0.94	10.2 ± 0.3$^{**,\#\#}$	9.4 ± 0.4$^{***,\#}$	9.2 ± 0.43$^{***,\#}$	7.1 ± 1.1***
Corneal nerve branch density (/mm^2)	14.2 ± 0.99$^{\#\#}$	13.1 ± 1.25$^{\#}$	12.7 ± 1.1$^{\#}$	10.8 ± 1.0	6.1 ± 2.1
Corneal nerve branch length (mm/mm^2)	3.28 ± 0.34$^{\#\#}$	2.45 ± 0.20$^{\#}$	2.43 ± 0.19	2.39 ± 0.20	1.28 ± 0.42
Tortuosity grade	2.03 ± 0.08	2.66 ± 0.06***	2.59 ± 0.06***	2.80 ± 0.10***	2.50 ± 0.37**
Beading frequency (/0.1 mm)	24.9 ± 0.52	19.6 ± 0.48***	20.1 ± 0.60***	19.5 ± 0.47***	20.0 ± 1.60**

Data are mean ± standard error of the mean in control participants and type 2 diabetic patients with or without diabetic neuropathy stratified by the criteria of Diabetic Neuropathy Study Group in Japan[12]. $^{**}P < 0.001$, $^{***}P < 0.0001$ compared with control participants, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ compared with stage IV+V. Statistical analysis was carried out with analysis of variance.

type 2 diabetes without DN had lower CNFD, CNFL and beading frequency, and greater tortuosity. DN progression further reduced CNFD, CNFL, CNBD and CNBL. As glycemic control might influence the corneal nerve pathology, we analyzed the influence of HbA1c at CCM examination or annual mean HbA1c 1–3 years before CCM examination on the morphological parameters of corneal NFs (other predictors included sex, age, BMI, duration of diabetes, DBP and ACR). HbA1c did not significantly correlate with any morphological parameters of corneal NFs (P = 0.406–0.980).

Figure 1 shows a representative sweat gland duct in a control participant (Figure 1a) and a patient with type 2 diabetes (Figure 1b). Compared with the control participant, the patient with diabetes had a smaller CSA of the sweat gland duct.

The sensitivity and specificity of the time to CCC of the Neuropad and CSA of the sweat gland ducts were assessed by ROC curve analysis (Figure 2). For the CCC of the Neuropad and the CSA of the sweat gland ducts, the sensitivity, specificity and tentative cut-off levels between the control participants and patients with type 2 diabetes were 83.1%, 84.0%, and 815 s, respectively, for the time to CCC of the Neuropad, and 73.3%, 74.0%, and 2282 μm^2, respectively, for the sweat gland duct CSA. Intra-individual variation of the Neuropad test was established by repeating the test five times in six healthy volunteers; the average CV was 10.6%.

The time to CCC of the Neuropad in patients without DN was not significantly different from that in the control participants, whereas in patients with DN it increased with DN progression (Figure 3a). However, the CSA of the sweat gland ducts was significantly smaller in patients with diabetes irrespective of the stage of DN, and was further reduced in stage IV+V compared with that in stage II (Figure 3b).

The time to CCC of the Neuropad had a modest inverse relationship with MCV and warm PT, and a close inverse association with $CV_{R\text{-}R}$. However, the CSA of the sweat gland ducts was positively correlated with MCV (Table 4).

Multiple regression analysis showed that the time to CCC of the Neuropad prolonged and negatively correlated with CNFD, CNFL, CNBD and CNBL, and the CSA of the sweat gland ducts, and had a close direct relationship with the severity of DN. The CSA of the sweat gland ducts directly correlated with triglycerides, serum uric acid levels, CNBD and CNBL, and inversely correlated with the severity of DN (Table 5).

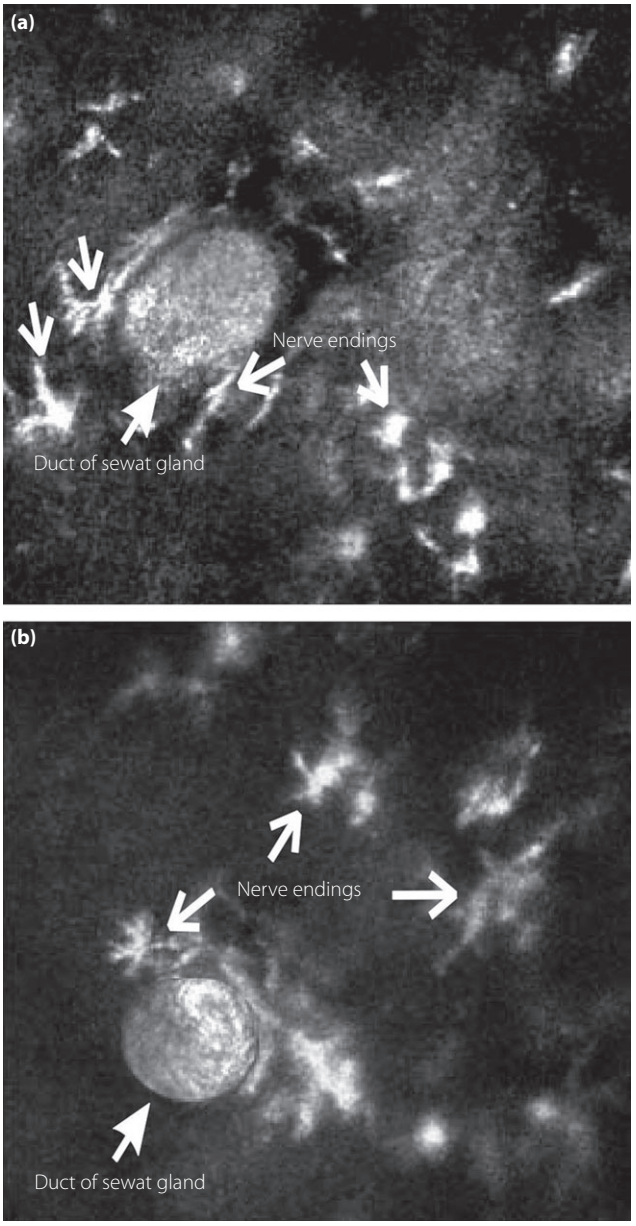

Figure 1 | (a) Confocal microscopic image of sweat gland duct in the epidermis of the finger pad in a 41-year-old healthy participant with normal neurophysiological examinations and normal parameters of the corneal nerve plexus by confocal microscopy. (b) Sweat gland duct of a 46-year-old patient with type 2 diabetes who did not have diabetic neuropathy with abnormal morphological parameters of corneal nerve plexus. A smaller sweat gland duct than the control participant was seen.

DISCUSSION

The Neuropad is a simple non-invasive indicator test for the assessment of sudomotor function and innervation by post-ganglionic cholinergic sympathetic NFs[17,18]. The present study confirmed that the quantitative Neuropad response (the time to CCC of the Neuropad) had good reproducibility, whereas the ROC curve analysis showed that there was excellent separation between the control participants and patients with diabetes for the time to CCC of the Neuropad. In type 2 diabetic patients, the sudomotor function deteriorated relative to the severity of DN[19] and the neurophysiological tests for DN, as reported previously[6,20,21].

The sweat glands are innervated by small C fibers. However, the relationship between the quantitative Neuropad response and the morphology of small C fibers has never been reported in a cohort composed entirely of type 2 diabetics, because the

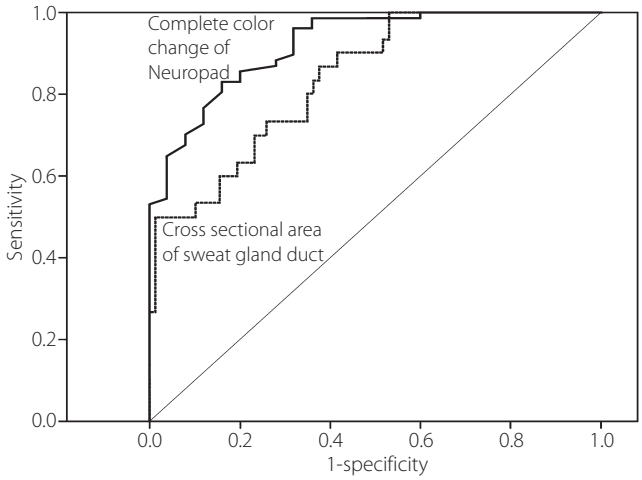

Figure 2 | Receiver operating characteristic (ROC) curves establishing cut-off levels between controls and type 2 diabetic patients for the period until complete color change of the Neuropad and the cross-sectional area of sweat gland ducts visualized by confocal microscopy of the epidermis of the finger pad. The cross-sectional area of the sweat gland duct was analyzed using Photoshop Elements 8.0 (Adobe Systems Inc., San Jose, CA, USA) and Simple PCI (Compix Inc., Cranberry Township, PA, USA) after smoothing and enlarging five times by PhotoZoom Pro4 (BenVista Ltd., Houston, TX, USA).

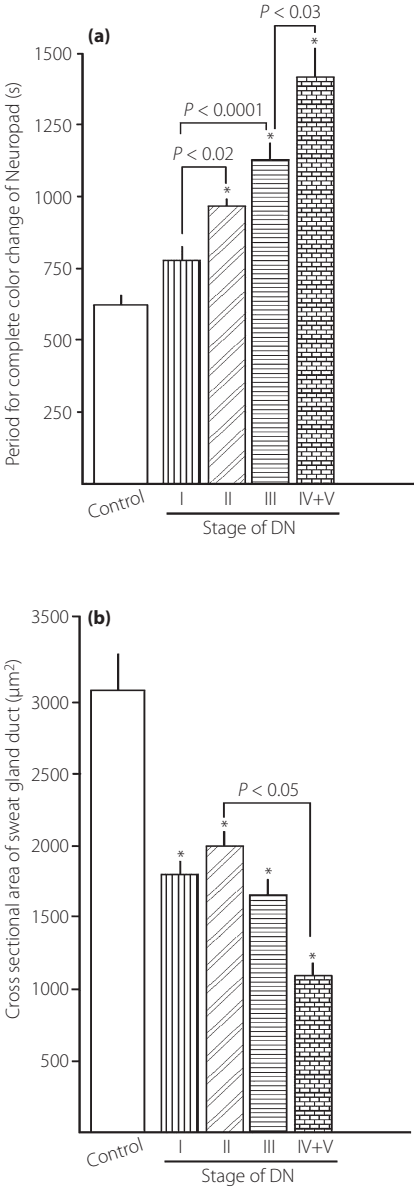

Figure 3 | Comparison of (a) period for complete color change of the Neuropad and (b) cross-sectional area of sweat gland duct in the epidermis of the finger pad among controls and type 2 diabetic patients with stratified diabetic neuropathy (DN) defined by a criteria of Diabetic Neuropathy Study Group in Japan[12]. The sweat gland ducts in the epidermis of the finger pad were visualized by confocal microscopy and analyzed by Photoshop Elements 8.0 (Adobe Systems Inc., San Jose, CA, USA) and Simple PCI (Compix Inc., Cranberry Township, PA, USA) after smoothing and enlarging five times by PhotoZoom Pro4 (BenVista Ltd., Houston, TX, USA). The significance of difference between groups was determined by analysis of variance. *$P < 0.0001$ compared with control participants.

dichotomous interpretation of Neuropad (i.e., normal and abnormal at 10 min; or normal, intermediate and abnormal at different time thresholds) was carried out previously. Papanas *et al.*[19] used the time until CCC of the Neuropad as a quantitative assessment of the sudomotor function to enable the staging of DN using a Neuropad. Quattrini *et al.*[6] compared IENFD in patients with type 1 and type 2 diabetes who were grouped according to their dichotomous Neuropad responses (normal, intermediate and abnormal). IENFD was reduced in the latter two groups compared with the control participants. However, they did not quantify the Neuropad response as the period until the CCC; therefore, they could not correlate IENFD directly with the Neuropad response. Because an invasive skin biopsy is required for the determination of IENFD, IENFD cannot be used as a routine clinical procedure to evaluate the morphology of small C fibers. Alternatively, non-invasive corneal CM can visualize corneal NFs, which mainly comprise C fibers. Only one previous study by Tavakoli *et al.*[9] correlated the Neuropad test with the corneal NF pathology. That study comprised 61% patients with type 1 diabetes and 39% patients with type 2 diabetes, and the Neuropad response was qualitatively classified as a normal response (100% pink), intermediate response (patchy pink) or abnormal response (100% blue) after 10 min. To the best of our knowledge, the present study is the first to directly correlate the quantified Neuropad response directly with the corneal NF pathology visualized by corneal CM in a population of type 2 diabetics. The quantified Neuropad responses had high inverse correlations with CNFD, CNFL, CNBD and CNBL. The morphological parameters of the corneal NFs were impaired before the development of DN (stage I), when the sudomotor function was normal; therefore,

Table 4 | Correlation between period of complete color change of Neuropad or cross-sectional area of sweat gland duct and neurophysiological examinations in patients with type 2 diabetes

	Period of CCC for Neuropad		CSA of sweat gland duct	
	β	*P*	β	*P*
MCV (m/s)	−0.241	0.034	0.275	0.022
SCV (m/s)	−0.227	0.060	0.087	0.485
VPT (μ/120c/s)	0.198	0.105	−0.133	0.328
CPT-2000 Hz (mV)	0.192	0.092	−0.236	0.051
CPT-250 Hz (mV)	0.158	0.163	−0.178	0.138
CPT-5 Hz (mV)	0.107	0.337	−0.079	0.504
Warm perception threshold (w/m^2)	−0.252	0.031	0.240	0.055
Cold perception threshold (w/m^2)	0.118	0.358	−0.116	0.391
$CV_{R\text{-}R}$ (%)	−0.407	<0.0001	0.100	0.428

CCC, complete color change; CPT, current perception threshold; CSA, cross-sectional area; CV, coefficient of variation; MCV, motor neuron conduction velocity; SCV, sensory neuron conduction velocity; VPT, vibration perception threshold.

Table 5 | Correlation between period until complete color change of Neuropad or cross sectional area of sweat gland duct and clinical factors or morphology of corneal nerve plexus in patients with type 2 diabetes

	Period for CCC of Neuropad		CSA of sweat gland duct	
	β	*P*	β	*P*
Sex	0.207	0.073	0.032	0.776
Age	0.104	0.401	−0.048	0.706
BMI	−0.070	0.565	0.140	0.266
Duration of diabetes	0.091	0.422	0.147	0.209
SBP	−0.019	0.924	0.151	0.208
DBP	0.203	0.173	0.076	0.631
HbA1c	0.137	0.296	−0.183	0.175
ACR	0.188	0.191	−0.051	0.679
Triglycerides	−0.107	0.354	0.294	0.015
Uric acid	−0.077	0.601	0.386	0.009
Corneal nerve fiber density	−0.422	<0.0001	0.011	0.926
Corneal nerve fiber length	−0.386	0.002	0.001	0.992
Corneal nerve branch density	−0.296	0.004	0.277	0.021
Corneal nerve branch length	−0.278	0.009	0.265	0.027
Tortuosity grade	−0.187	0.196	−0.029	0.800
Beading frequency	0.079	0.492	−0.174	0.142
Stage of diabetic neuropathy	0.541	<0.0001	−0.290	0.021
CSA of sweat gland duct	−0.266	0.028		

Stage of diabetic neuropathy was defined by the criteria of Diabetic Neuropathy Study Group in Japan[12]. ACR, albumin creatinine ratio; BMI, body mass index; CSA, cross-sectional area; DBP, diastolic blood pressure; HbA1c, glycated hemoglobin; SBP, systolic blood pressure.

C fiber damage might play a hierarchical role in the development of sudomotor dysfunction.

Skin biopsy or iontophoresis of pilocarpine have been used to evaluate the density of sweat glands[18,22]. Because these procedures are invasive, they cannot be used as routine clinical procedures to assess the morphologies of the sweat glands and their connecting ducts. CM can visualize all cells or tissues within 0.2 mm of the body surface[10], but the foot is not amenable to CM study because of the mechanical limitations of the CM apparatus. In contrast, the finger pad is readily accessible to the objective lens of the CM. The epidermis of the finger pad is thicker than that of other body surface areas, so the sweat glands in the dermis of the finger pad cannot be observed by CM. However, the connecting ducts of the sweat glands in the epidermis are clearly visible as round or oval concentric structures[10] using CM. To the best of our knowledge, the present study is the first to compare the CSA of the connecting ducts of the sweat glands between healthy participants and patients with diabetes, who were stratified according to DN. The present study showed that the CSAs of the sweat gland ducts in type 2 diabetic patients without DN were significantly smaller than those in the control participants. They were even smaller in patients with severe DN, and were inversely related to the severity of DN. Because CNBD and CNBL are directly related to the CSA of the connecting ducts, the duct size might have been affected by autonomic C fibers.

However, we did not evaluate the density of the sweat gland ducts by CM. The sweat gland and duct density can be determined by counting the openings of the ducts. The volar surface of the finger has the highest density ($530/cm^2$ = one duct/0.19 mm^2) of sweat gland duct openings over the body surface[23]. Each frame of a CM image measures 0.4×0.4 mm = 0.16 mm^2, therefore CM cannot visualize multiple ducts on the finger pad in one frame, which means that the density of ducts cannot be determined non-invasively by CM.

Furthermore, we did not detect any morphological differences (pathological changes) in the ducts compared with those of the control participants. We compared the CV of the duct size in control participants with that in type 2 diabetic patients who had staged DN severity. The CV of the control participants (45.0%) was greater than diabetic patients, whereas the CV was smaller in patients with stratified DN (stage I 28.3%; stage II 28.3%; stage III 26.9%; and stage VI+V 21.2%) according to the severity of DN. This could suggest that the reduction in size and variability of the sweat gland ducts was related to the severity of DN.

Because the quantitative Neuropad response was inversely correlated with the CSA of the sweat gland ducts, the smaller connecting ducts of the sweat glands could have been reflected in the reduced sweating. However, we assessed sweating on the sole of the foot and determined the duct size on the finger pad. There have been no comparisons of the Neuropad responses between the foot and hand. SUDOSCAN[24] is used to assess the

sudomotor function non-invasively by measuring the local conductance on the basis of the electrochemical reaction between the sweat chloride and a nickel electrode. It showed that the degree of sweating on the sole of the foot and palm of the hand in patients with type 2 diabetes with (48.49 ± 21.12 vs 48.40 ± 20.9 μS) or without DN (58.32 ± 19.86 vs 58.77 ± 19.84 μS) were quite similar, whereas sweating was reduced in patients with DN. Therefore, the significant correlation between the sudomotor function assessment using the Neuropad on the foot and the CSA of the sweat gland ducts on the finger pad might suggest a possible structure–function relationship between sweating and the sweat gland duct size. However, diabetes or DN did not have equal effects on sweating in the upper and lower extremities when sweating was stimulated by acetylcholine using iontophoresis[25]. Therefore, the structure–function relationship between the sweat gland duct and sweating might not be straightforward, and confirmation is required based on further quantitative evaluations of sweating and the C fiber morphology in the foot.

Papanas *et al.*[26] reported that patients with type 2 diabetes with sudomotor dysfunction assessed by the Neuropad test had a significantly higher serum uric acid level (8.2 ± 1.4 mg/dL) compared with those without (5.8 ± 1.1 mg/dL). In addition, they had a significant direct correlation, which highlights the potential role of serum uric acid in sudomotor dysfunction. In the present study, the serum uric acid levels in patients with stratified DN (0.29 ± 0.02 to 0.34 ± 0.02 mmol/L) were within the normal range and similar to those in the control participants (0.32 ± 0.02 mmol/L). There was no correlation between the serum uric acid level and the time to CCC of the Neuropad. In contrast, the CSA of the sweat gland ducts was related directly to the serum uric acid levels. In physiological conditions, the level of uric acid in the sweat is minimal[27] (6.3% of that in the serum), and the uric acid in sweat fluid is low, even in uremic conditions with a high serum uric acid level[28]. Therefore, we could not determine why the size of the sweat gland ducts was related to the serum uric acid level. Human eccrine sweat contains small amounts of triglycerides (fatty acids)[29]. However, the effects of the serum triglycerides levels on the size of sweat glands or their ducts have never been reported. Therefore, the relationship between the triglycerides levels and the CSA of sweat gland ducts remains to be elucidated.

In conclusion, the present study showed that patients with type 2 diabetes with DN had a sudomotor dysfunction, which was related to the severity of DN. In addition, the corneal NF pathology was closely related to the sudomotor dysfunction. Furthermore, the CSA of the sweat gland ducts visualized by CM was smaller than that of the control participants, even in patients without DN, and it was positively related to the corneal NF branch, triglycerides levels and serum uric acid level. However, clarification of the latter relationship requires further studies.

We acknowledge that there were many limitations in the interpretation of the present results. First, the sudomotor function (quantitative Neuropad response) was assessed in a lower extremity, whereas the corneal NF pathology and CSA of the sweat gland ducts were determined in the upper extremities. Because the development of DN depends on the lengths of the NFs, all examinations should be carried out in the lower extremities. Second, the evaluations of DN were carried out in both the upper (MCV, SCV, warm and cold perception threshold, and $CV_{R\text{-}R}$) and lower (VPT, CPT) extremities. However, the Neuropad response was not related to the neurological function of the lower extremities, and only MCV had a significant correlation with the CSA of the sweat gland duct. Therefore, the hierarchical control of the nerve function on sudomotor function and sweat gland duct size should be considered tentative. Third, we demonstrated the smaller size of the CSA of the connecting duct, but we did not determine the density or pathological changes in the sweat gland ducts. Further characterization of the morphological changes in the sweat gland ducts, other than CSA, caused by diabetes or DN, demands the development of new non-invasive methods. Finally, the present study was cross-sectional. A longitudinal study would provide more vigorous data about the relationships between the morphological anomalies of corneal NFs, sweat gland ducts and the sudomotor function.

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