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Cutaneous microangiopathy in patients with type 2 diabetes: Impaired vascular endothelial growth factor expression and its correlation with neuropathy, retinopathy and nephropathy

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Keywords
Microangiopathy, Skin, Vascular endothelial growth factor

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ABSTRACT

Aims/Introduction: To examine the three-dimensional morphology and vascular endothelial growth factor (VEGF) expression of skin microvasculature in patients with type 2 diabetes in relation to neuropathy, retinopathy and nephropathy.

Materials and Methods: The present study enrolled 17 individuals with type 2 diabetes and 16 without. Skin sections were double-immunostained for type IV collagen and VEGF-A or protein gene product 9.5. Projected images from confocal microscopy served to quantify the occupancy rate of subepidermal type IV collagen-immunoreactive microvascular basement membrane area (OR-T4MBM), subepidermal VEGF-A-immunoreactive area and the VEGF/T4MBM ratio, as well as the protein gene product 9.5-immunoreactive intraepidermal nerve fiber density. Reduced intraepidermal nerve fiber density was applied for the diagnosis of neuropathy, fundic ophthalmoscopy and fluorescein angiography for retinopathy, and microalbuminuria or persistent proteinuria for nephropathy.

Results: A total of 12 patients with diabetes had neuropathy, 10 had retinopathy and eight had nephropathy. Regardless of the presence or absence of neuropathy, retinopathy or nephropathy, OR-T4MBM was significantly increased in patients with diabetes compared with individuals without diabetes. In contrast, VEGF/T4MBM ratio was significantly decreased in those with neuropathy and retinopathy, as well as in those with and without nephropathy, whereas a trend toward a decreased VEGF/T4MBM ratio was seen in patients without retinopathy, as compared with individuals without diabetes.

Conclusions: The present study is the first report to show that cutaneous microangiopathy, as indicated by subepidermal microvascular proliferation and impaired VEGF expression, appears to occur before the development of overt clinical neuropathy, retinopathy or nephropathy in patients with type 2 diabetes.

INTRODUCTION

Thickening or hypertrophy of the microvascular basement membrane represents a cardinal pathological hallmark of diabetic microangiopathy in humans, and is typically found in neuropathy[1], retinopathy[2] and nephropathy[3]. However, microvascular changes in diabetes patients are not confined to just those findings, and are generalizable to a wide variety of organs, including the skin[4], muscle[5], heart[6], brain[7] and gingiva[8]. Patients with type 2 diabetes with retinopathy and nephropathy reportedly show pronounced skin microangiopathy[4]. In contrast, Malik *et al*[1]. reported that in patients with neuropathy, pathological alterations of the microvessels appeared more severe in the peripheral nerves than in skin and muscles. Given the paucity of available information, further exploration of the

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relationship between systemic microvascular changes and representative complications of diabetes would lead to a better understanding of the pathophysiology underlying diabetic microangiopathy.

The pathogenesis of diabetic microangiopathy remains poorly understood. Hyperglycemia-induced metabolic alterations, such as sorbitol formation, accumulation of advanced glycation end-products, enhanced signaling of the receptor for advanced glycation end-products, altered protein kinase C activity and oxidative stress, have all been postulated to contribute to the development of diabetic microangiopathy[9]. Another leading hypothesis involves the impairment of microcirculation in response to various vasoactive substances[10], with vascular endothelial growth factor (VEGF) representing an attractive candidate[11]. Previous studies have indicated the involvement of VEGF in the pathophysiology of diabetic neuropathy[12], retinopathy[13] and nephropathy[14], showing differential VEGF expression in different target tissues of diabetes. Quattrini *et al.*[15] reported that VEGF expression in the epidermis and on the dermal microvessels was reduced in patients with type 1 and type 2 diabetes with severe neuropathy, when compared with control participants. However, no direct comparisons among various organs were made for VEGF expression in a systematic manner. The aim of the current study was thus to assess the three-dimensional morphology and VEGF expression of the skin microvasculature with the use of confocal immunofluorescence microscopy in patients with type 2 diabetes in the presence or absence of neuropathy, retinopathy or nephropathy, and to compare these results with those in participants without diabetes.

MATERIALS

Study participants

All analyses were carried out with the approval of the ethics committees of Hirosaki University Graduate School of Medicine, Hirosaki, Japan (approval no: 2008-038), and Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima, Japan (approval no: 19-84). Between March 2005 and July 2010, we enrolled one male outpatient with type 2 diabetes who visited and 13 male inpatients with type 2 diabetes admitted to Hirosaki University Hospital, as well as three male inpatients with type 2 diabetes who were admitted to Kagoshima University Hospital. Patients who had a history of psychiatric disorder, liver cirrhosis, chronic renal failure or active malignant neoplasm, or who had neuropathy, retinal or renal diseases attributable to causes other than diabetes were excluded. To obtain reference values for the assessment of skin biopsy samples as described below, we recruited 16 age-matched male volunteers without diabetes who showed documented fasting plasma glucose levels <5.6 mmol/L and hemoglobin A1c (HbA1c) levels ≤5.6% (38 mmol/mol) in annual health checkup records enrolled in the Department of Pathology and Molecular Medicine, Hirosaki University Graduate School of Medicine between October 2010 and January 2011. All participants provided written informed consent for study participation before enrolment. Fasting blood samples were obtained to quantify levels of plasma glucose, HbA1c and serum lipids. At the beginning of hospitalization, we measured fasting serum C-peptide and average 24-h urinary excretion of albumin (UEA) in two to three consecutive collections, although UEA was not evaluated in one outpatient and two inpatients with persistent proteinuria.

Definition of chronic microvascular complications

Positive neuropathic symptoms, such as prickling, tingling, burning or aching pain in toes, feet or legs, were recorded. Ankle reflexes were scored as abnormal if they were unequivocally decreased or absent despite reinforcement. Vibratory perception was assessed with a C128-Hz tuning fork and scored as reduced if the average time for which the patient sensed vibration over bilateral medial malleoli was ≤10 s[16]. Symmetric distal reduction of touch perception was determined using a 10-g monofilament applied to the plantar surfaces of the great toes and feet. In addition to these clinical profiles, we applied skin biopsy to intraepidermal nerve fiber (IENF) quantification for diagnosis of diabetic neuropathy, particularly small fiber neuropathy[17]. Patients with and without neuropathic symptoms or signs (sensory symptoms or abnormal ankle reflexes or reduced vibration or touch perception) and reduced IENF density (IENFD), as defined below, were diagnosed as having confirmed clinical and subclinical diabetic neuropathy, respectively[18]. During hospitalization or at the time of skin biopsy, nerve conduction studies were carried out on the sural sensory and tibial motor nerves using an electromyography system with surface electrodes for stimulation and recording as described elsewhere[19,20]. Diabetic retinopathy in the form of retinal changes was detected by ophthalmoscopy and fluorescein angiography. Diabetic nephropathy was defined by the presence of microalbuminuria (UEA 30–300 mg/day) or proteinuria (dipstick ≥1+).

Surrogate markers for atherosclerosis

During the hospital stay, bilateral brachial-ankle pulse wave velocity (PWV) and ankle brachial index (ABI) in a supine position were measured using a Form PWV/ABI (Omron Colin, Tokyo, Japan). Maximum intima-media thickness of the carotid arteries was determined bilaterally by ultrasonographic examination according to a previously described method[21]. Values for the two sides were averaged for use in statistical analysis. Intima-media thickness data were missing for two inpatients.

Skin biopsy

Three-millimeter skin punch biopsies were carried out 10-cm proximal to the lateral malleolus, and specimens were fixed in cold Zamboni's solution (2% paraformaldehyde, 0.2% picric acid in 0.1 mol/L phosphate buffer), cryoprotected with 20% sucrose and cryosectioned perpendicularly at 60 μm. Skin

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sections were double-immunostained with type IV collagen (1:1,000; Chemicon, Temecula, CA, USA) and VEGF-A (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or the pan-neuronal marker protein gene product 9.5 (PGP9.5; 1:1,000; Chemicon). Sections were cover-slipped with Prolong Gold antifade reagent (Molecular Probes, Eugene, OR, USA) to avoid photo-bleaching, and were examined using a Zeiss LSM510 confocal laser scanning microscope (Carl Zeiss, Oberkochen, Germany) at identical settings including contrast, brightness level and pinhole size. The linear density of PGP9.5-immunoreactive IENF was determined using a ×40 water immersion objective lens and appropriate filters at a resolution of 1,024 × 1,024 pixels, as described previously[22]. In brief, 16 confocal images were captured at 2-μm intervals, and image stacks were superimposed to produce a layered image for quantitation in three dimensions. Individual IENFs that penetrated the basement membrane were counted using Zeiss LSM 510 image browser software (Carl Zeiss). Participants with IENFD <7.4 fibers/mm were deemed to have neuropathy, as the value was below the 5th percentile of participants without diabetes in the present study.

Confocal microscopy of thick sections is preferable to conventional light microscopy of thinner sections, allowing us to increase sampling, obtain in-focus images, and carry out better quantitative and morphological analyses of the stained cutaneous organs in three dimensions[23]. For type IV collagen and VEGF-A, projected images of 30-μm optical sections at 2-μm increments were acquired using a ×20 objective lens from each of five to six representative areas of a thick section. A total of 50–60 confocal images per section were analyzed using Image J software (National Institutes of Health, Bethesda, MD, USA) with the aid of a binary function and a fixed threshold. The epidermis except for the keratin layer of each confocal image was assessed to obtain the mean pixel intensity value for epidermal VEGF-A immunoreactivity above the threshold set. Positive type IV collagen and VEGF-A-immunoreactive areas above the threshold set were divided by the total area examined to obtain the occupancy rate (%) to obtain the mean immunoreactive areas of subepidermal type IV collagen and VEGF-A. Subepidermal VEGF-A-immunoreactive areas were also expressed relative to subepidermal type IV collagen-immunoreactive microvascular basement membrane areas. We evaluated the subepidermal layer lying up to 200-μm beneath the epidermal basement membrane.

Statistical analysis

All statistical analyses were carried out using IBM SPSS software version 24.0 software (Advanced Analytics, Tokyo, Japan). Data are presented as mean ± standard deviation (SD) for variables with a parametric distribution, and median (interquartile range) for variables with a non-parametric distribution. The normality of data distribution was assessed using the Shapiro–Wilk test. Continuous variables were compared between individuals with and without type 2 diabetes using the parametric Student's *t*-test or the non-parametric Mann–Whitney *U*-test, as applicable. Categorical variables were compared between individuals with and without type 2 diabetes using the Pearson χ^2-test. Simple linear regression analyses were carried out to assess relationships between the clinical variables and quantitative immunofluorescence results. A trained observer (SO) carried out quantitative immunofluorescence analyses in a blinded manner. IENFD and the subepidermal occupancy rate of type IV collagen-immunoreactive microvascular basement membrane area (OR-T4MBM) were determined on two skin sections per participant and the results were averaged for statistical analysis, while epidermal VEGF-A immunoreactivity and subepidermal occupancy rate of VEGF-A-immunoreactive area (OR-VEGF) were determined on one skin section per participant. The significance of differences in median values of IENFD and mean values of epidermal VEGF-A immunoreactivity and subepidermal OR-T4MBM, and subepidermal OR-VEGF and subepidermal VEGF-A-immunoreactive area relative to type IV collagen-immunoreactive microvascular basement membrane area (VEGF/T4MBM ratio) among people with and without type 2 diabetes in the presence and absence of neuropathy, retinopathy or nephropathy was tested by the Kruskal–Wallis test and analysis of variance followed by the Tukey honest significant difference test, respectively. *P*-values <0.05 were considered significant.

RESULTS

The clinical characteristics of 16 individuals without diabetes and 17 individuals with type 2 diabetes are summarized in Table 1. Patients with diabetes included more current cigarette smokers, and showed a greater body mass index, higher HbA1c and lower serum high-density lipoprotein (HDL) cholesterol compared with participants without diabetes. Other clinical variables including age, sex, height, alcohol drinking, blood pressures, serum total cholesterol, triglycerides, and the current use of statins, fibrates and angiotensin-receptor blockers did not differ between individuals with and without diabetes. Among patients with diabetes, the mean (SD) duration of diabetes was 6.4 ± 8.2 years. On neurological examination, six patients with diabetes showed sensory symptoms, nine had abnormal ankle reflexes, four had reduced vibration perception, 10 had reduced touch perception, nine had confirmed neuropathy and three had subclinical neuropathy (see below). Nerve conduction studies showed that peripheral nerve function was generally preserved in the present patients. Three patients with diabetes had simple retinopathy, and seven had either preproliferative or proliferative retinopathy, while five had microalbuminuria and three had persistent proteinuria. Four, five and five patients with diabetes were treated with biguanides, sulfonylureas and insulin, respectively.

IENFD

The intraobserver (SO) variability for IENFD, expressed as the mean of the absolute difference between the two examinations, was 1.2 fibers/mm (SD 4.2, 95% confidence interval −0.6 to

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Table 1 | Clinical characteristics of study participants

	Participants without diabetes	Patients with diabetes	*P*-value
n	16	17	–
Age (years)	41 ± 10	44 ± 15	0.427
Sex (male/female)	16/0	17/0	–
Height (cm)	173.7 ± 5.6	171.0 ± 6.9	0.226
Body mass index (kg/m^2)	23.0 ± 2.4	26.1 ± 4.6	0.021*
Current smoking, *n* (%)	3 (18.8)	11 (64.7)	0.008*
Current alcohol drinking, *n* (%)	14 (87.5)	11 (64.7)	0.225
Diabetes duration (years)	–	11.0 ± 8.2	–
Systolic BP (mmHg)	125 ± 12	129 ± 15	0.266
Diastolic BP (mmHg)	77 ± 9	77 ± 11	0.706
HbA1c (%)	5.2 ± 0.3	9.9 ± 2.9	<0.001*
HbA1c (mmol/mol)	33 ± 3	78 ± 29	<0.001*
Total cholesterol (mmol/L)	5.0 ± 0.9	5.0 ± 1.2	0.949
Triglycerides (mmol/L)	1.0 (1.3)	1.6 (1.2)	0.288
HDL cholesterol (mmol/L)	1.7 (0.8)	1.0 (0.3)	0.004*
Fasting serum C-peptide (nmol/L)	–	0.6 ± 0.3	–
Neuropathy			
Sensory symptom, *n* (%)	0	6 (35.3)	–
Abnormal ankle reflex, *n* (%)	0	9 (52.9)	–
Reduced vibration perception, *n* (%)	0	4 (23.5)	–
Reduced touch perception, *n* (%)	0	10 (58.8)	–
IENFD <7.4 fibers/mm, *n* (%)	0	12 (70.6)	–
Confirmed neuropathy, *n* (%)	0	9 (52.9)	–
Subclinical neuropathy, *n* (%)	0	3 (17.6)	–
Tibial MNCV (m/s)	–	41.5 ± 5.5	
Tibial CMAP (mV)	–	8.2 ± 3.9	
Tibial F wave latency (ms)	–	51.9 ± 5.5	
Sural SNCV (m/s)	–	51.2 ± 9.1	
Sural SNAP (μV)	–	8.3 ± 4.4	
Retinopathy			
NDR, *n* (%)	–	7 (41.1)	–
SDR, *n* (%)	–	3 (17.6)	–
PPDR/PDR, *n* (%)	–	7 (41.1)	–
Nephropathy			
UEA (mg/day)[†]	–	18 (77)	–
Normoalbuminuria, *n* (%)	–	9 (52.9)	–
Microalbuminuria, *n* (%)	–	5 (29.4)	–
Proteinuria, *n* (%)	–	3 (17.6)	–
Brachial-ankle PWV (cm/s)[‡]	–	14,202 (397)	–
ABI[‡]	–	1.11 ± 0.10	–
Maximum IMT (mm)[§]	–	1.00 (0.65)	–
Medication			
Statins, *n* (%)	4 (25.0)	1 (5.9)	0.175
Fibrates, *n* (%)	1 (6.3)	1 (5.9)	1.000
ARBs, *n* (%)	2 (12.5)	3 (17.6)	1.000
Biguanides, *n* (%)	–	4 (23.5)	–
Sulfonylureas, *n* (%)	–	5 (29.4)	–
Insulin, *n* (%)	–	5 (29.4)	–

Data represent the mean ± standard deviation, median (interquartile range) for variables with skewed distribution or number (percentage). ABI, ankle brachial index; ARBs, angiotensin-receptor blockers; BP, blood pressure; CMAP, compound muscle action potential amplitude; HbA1c, glycated hemoglobin; HDL, high-density lipoprotein; IENFD, intraepidermal nerve fiber density; IMT, intima-media thickness; MNCV, motor nerve conduction velocity; NDR, no diabetic retinopathy; PDR, proliferative diabetic retinopathy; PPDR, preproliferative diabetic retinopathy; PWV, pulse wave velocity; SDR, simple diabetic retinopathy; SNAP, sensory nerve action potential amplitude; SNCV, sensory nerve conduction velocity; UEA, urinary excretion of albumin. *Statistical significance ($P < 0.05$). [†]Three patients with persistent proteinuria were excluded from analysis. [‡]One outpatient was not included for analysis. [§]One outpatient and two inpatients were not included for analysis.

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Table 2 | Quantitative immunofluorescence results

	Participants without diabetes (n = 16)	Patients with diabetes (n = 17)	P-value
IENFD (fibers/mm)	13.9 (5.9)	5.5 (10.7)	<0.001*
Epidermal VEGF-A immunoreactivity (arbitrary units)	11.0 ± 3.5	8.3 ± 1.6	0.012*
Subepidermal OR-T4MBM (%)	2.55 ± 0.84	4.37 ± 1.35	<0.001*
Subepidermal OR-VEGF (%)	0.73 ± 0.55	0.57 ± 0.47	0.222
Subepidermal VEGF/T4MBM ratio	0.29 ± 0.20	0.13 ± 0.10	0.005*

Data represent mean ± standard deviation, or median (interquartile range) for variables with skewed distribution. *Statistical significance (P < 0.05). IENFD, intraepidermal nerve fiber density; OR-T4MBM, occupancy rate of type IV collagen-immunoreactive microvascular basement membrane area; OR-VEGF, occupancy rate of vascular endothelial growth factor-A immunoreactive area; VEGF, vascular endothelial growth factor; VEGF/T4MBM, vascular endothelial growth factor-A immunoreactive area relative to type IV collagen immunoreactive microvascular basement membrane area.

3.1 fibers/mm). IENFD was significantly decreased in patients with diabetes compared with participants without diabetes (P < 0.001; median [interquartile range] 5.5 [10.7] vs 13.9 [5.9] fibers/mm; Table 2). Among patients with diabetes, 12 showed a significant reduction of IENFD with <7.4 fibers/mm (Figure 1a), whereas none of the participants without diabetes showed reduced IENFD. In those individuals with reduced IENFD, nine patients with neuropathic symptoms or signs were diagnosed as having confirmed neuropathy and three patients without neuropathic symptoms or signs were diagnosed as having subclinical diabetic neuropathy (Table 1)[18]. The decrease in IENFD was significantly greater among patients with diabetes with retinopathy compared with participants without diabetes (P < 0.001) and patients with diabetes without retinopathy

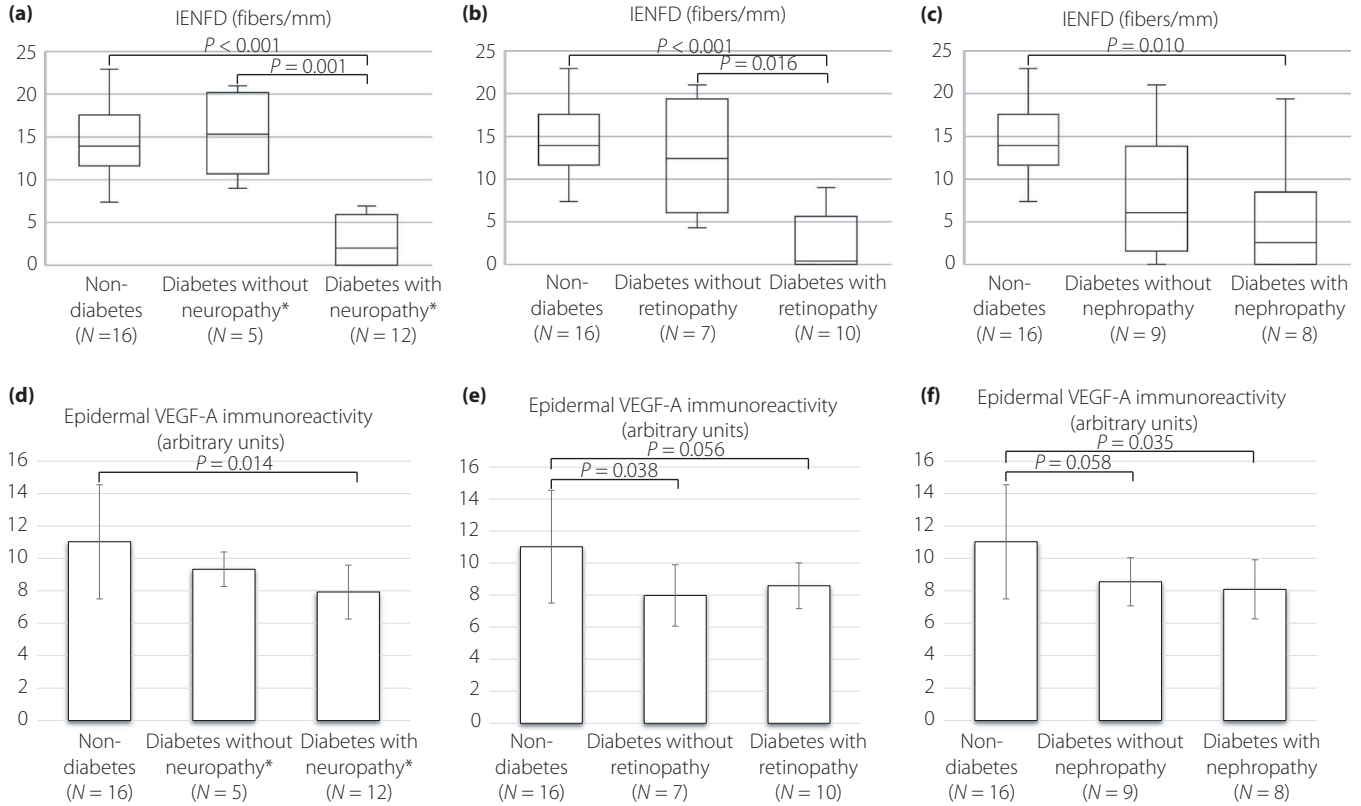

Figure 1 | Results of quantitative analyses of confocal immunofluorescence microscopy images with respect to (a–c) intraepidermal nerve fiber density (IENFD) and (d–f) epidermal vascular endothelial growth factor-A (VEGF-A) immunoreactivity in participants without diabetes and in patients with diabetes with and without (a,d) neuropathy, (b,e) retinopathy or (c,f) nephropathy. (a–c) The solid line represents the median value, the box represents the interquartile range, the upper error bar represents the 95th percentile value and the lower error bar represents the 5th percentile value. (d–f) Error bars indicate mean ± standard deviation. *Including both confirmed and subclinical neuropathy.

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($P = 0.016$; Figure 1b). IENFD was also significantly decreased in patients with diabetes with nephropathy compared with participants without diabetes ($P = 0.010$; Figure 1c).

Neuropathy, retinopathy and nephropathy in relation to clinical variables

Clinical characteristics including age, body mass index, duration of diabetes, HbA1c, blood pressures, serum lipids, cigarette smoking and alcohol drinking did not differ between patients with and without neuropathy or between those with and without nephropathy. Patients with retinopathy showed longer duration of diabetes and lower levels of serum total cholesterol than those without (Table S1).

Epidermal VEGF-A

VEGF-A expression was more apparent in basal cells and less apparent in spinous cells of the epidermis, and was significantly decreased in patients with diabetes compared with participants without diabetes (mean [SD] 8.3 [1.6] vs 11.0 [3.5] arbitrary units; $P = 0.012$; Table 2). In a similar manner, VEGF-A expression was significantly decreased in patients with diabetes with neuropathy ($P = 0.014$; Figure 1d), without retinopathy ($P = 0.038$; Figure 1e) and with nephropathy ($P = 0.035$; Figure 1f), whereas it was non-significantly decreased in those with retinopathy ($P = 0.056$; Figure 1e) and without nephropathy ($P = 0.058$; Figure 1f), compared with participants without diabetes.

Subepidermal type IV collagen

The mean intraobserver (SO) difference of subepidermal OR-T4MBM was 0.22% (SD 1.29%, 95% confidence interval −0.23 to 0.68%). A significant increase in subepidermal OR-T4MBM was evident in patients with diabetes compared with participants without diabetes (mean [SD] 4.37% [1.35%] vs 2.55% [0.84%]; $P < 0.001$; Table 2; Figures 2,3a,d,g). Subepidermal OR-T4MBM was significantly increased in patients with diabetes with and without neuropathy ($P = 0.005$ and $P = 0.002$, respectively; Figure 4a), retinopathy ($P = 0.003$ and $P = 0.010$, respectively; Figure 4b) and nephropathy ($P = 0.002$ and $P = 0.003$, respectively; Figure 4c).

Subepidermal VEGF-A

No difference in subepidermal OR-VEGF was identified in patients with diabetes compared with participants without diabetes (Table 2), in the presence or absence of neuropathy (Figure 4d), retinopathy (Figure 4e) or nephropathy (Figure 4f). In contrast, the VEGF/T4MBM ratio in the subepidermis was significantly decreased ($P = 0.005$) in patients with diabetes compared with participants without diabetes (mean [SD] 0.13 [0.10] vs 0.29 [0.20]; Table 2; Figure 3). The VEGF/T4MBM ratio was significantly decreased in patients with diabetes with neuropathy ($P = 0.009$; Figure 4g), with retinopathy ($P = 0.022$; Figure 4h) and with and without nephropathy ($P = 0.023$ and $P = 0.039$, respectively; Figure 4i), whereas it was non-significantly decreased in patients without retinopathy ($P = 0.055$; Figure 4h), compared with participants without diabetes.

Quantitative immunofluorescence results in relation to clinical variables and micro- and macrovascular complications

Simple regression analyses showed that current alcohol drinking correlated positively with IENFD and negatively with subepidermal OR-T4MBM in people with diabetes. In addition, serum total cholesterol correlated negatively with subepidermal

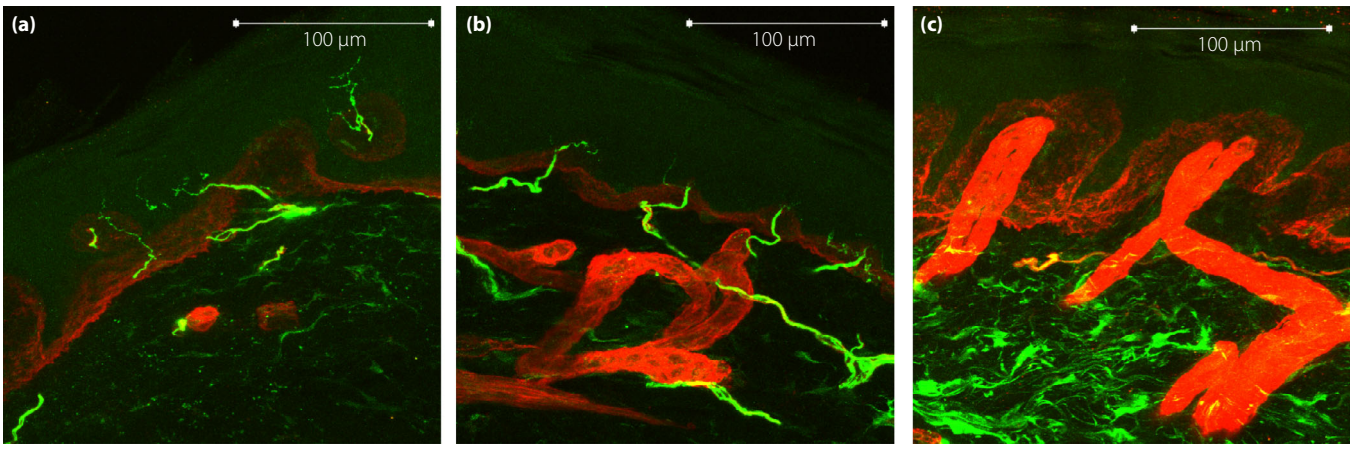

Figure 2 | Representative confocal image stacks of 30-µm thick sections double-immunostained with antibody to protein gene product 9.5 for nerves and with antibody to type IV collagen for basement membrane from (a) a 40-year-old participant without diabetes, (b) a 41-year-old patient with diabetes showing preserved intraepidermal nerve fiber density and (c) a 29-year-old patient with diabetes showing complete loss of intraepidermal nerve fibers. Nerve fibers immunoreactive for protein gene product 9.5 appear green or yellow. Epidermal basement membrane and subepidermal microvasculature immunoreactive for type IV collagen appear red. (b,c) Note the proliferation of microvessels in the subepidermis from patients with diabetes.

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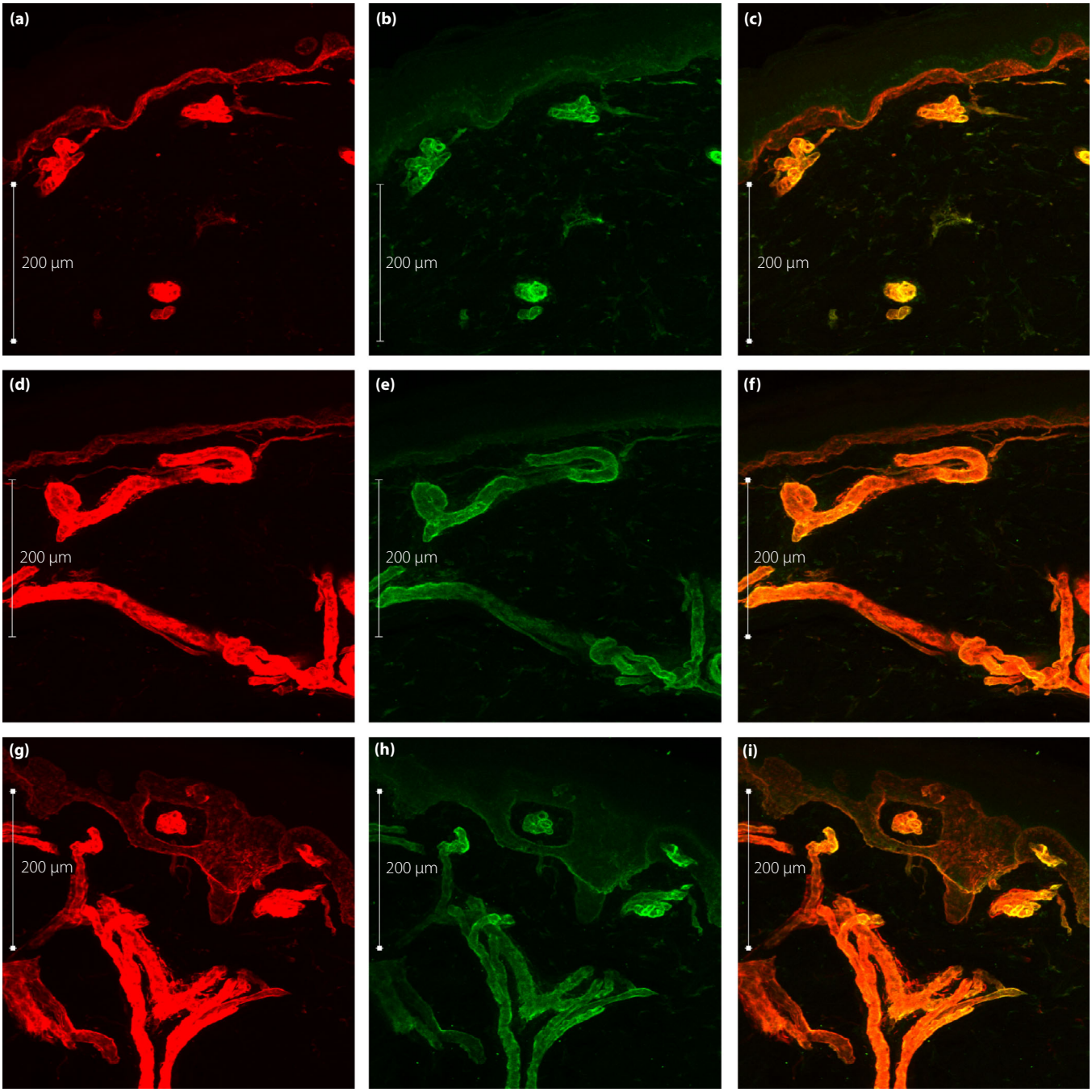

Figure 3 | Representative confocal image stacks of 30-µm thick sections double-immunostained with antibodies to type IV collagen and vascular endothelial growth factor-A (VEGF-A) from (a–c) a 34-year-old participant without diabetes, (d–f) a 55-year-old patient with diabetes without reduced intraepidermal nerve fiber density, retinopathy or nephropathy and (g–i) a 58-year-old patient with diabetes with loss of intraepidermal nerve fibers, proliferative retinopathy and persistent proteinuria. (a,d,g) The epidermal basement membrane and subepidermal microvasculature immunoreactive for type IV collagen appear red. (b,e,h) Epidermal keratinocytes and subepidermal microvasculature and fibroblast immunoreactive for VEGF-A appear green. (c,f,i) Yellow-orange coloration in merged images indicates colocalization of type IV collagen and VEGF-A. Note that the most intense VEGF-A immunoreactivity is observed in endothelial cells, less intense immunoreactivity in vascular wall cells (pericytes or smooth muscle cells) and keratinocytes (mainly basal cells), and faint immunoreactivity in fibroblasts. VEGF-A immunoreactivity in these cells appears less intense in (e,h) both patients with diabetes than in (b) the participant without diabetes.

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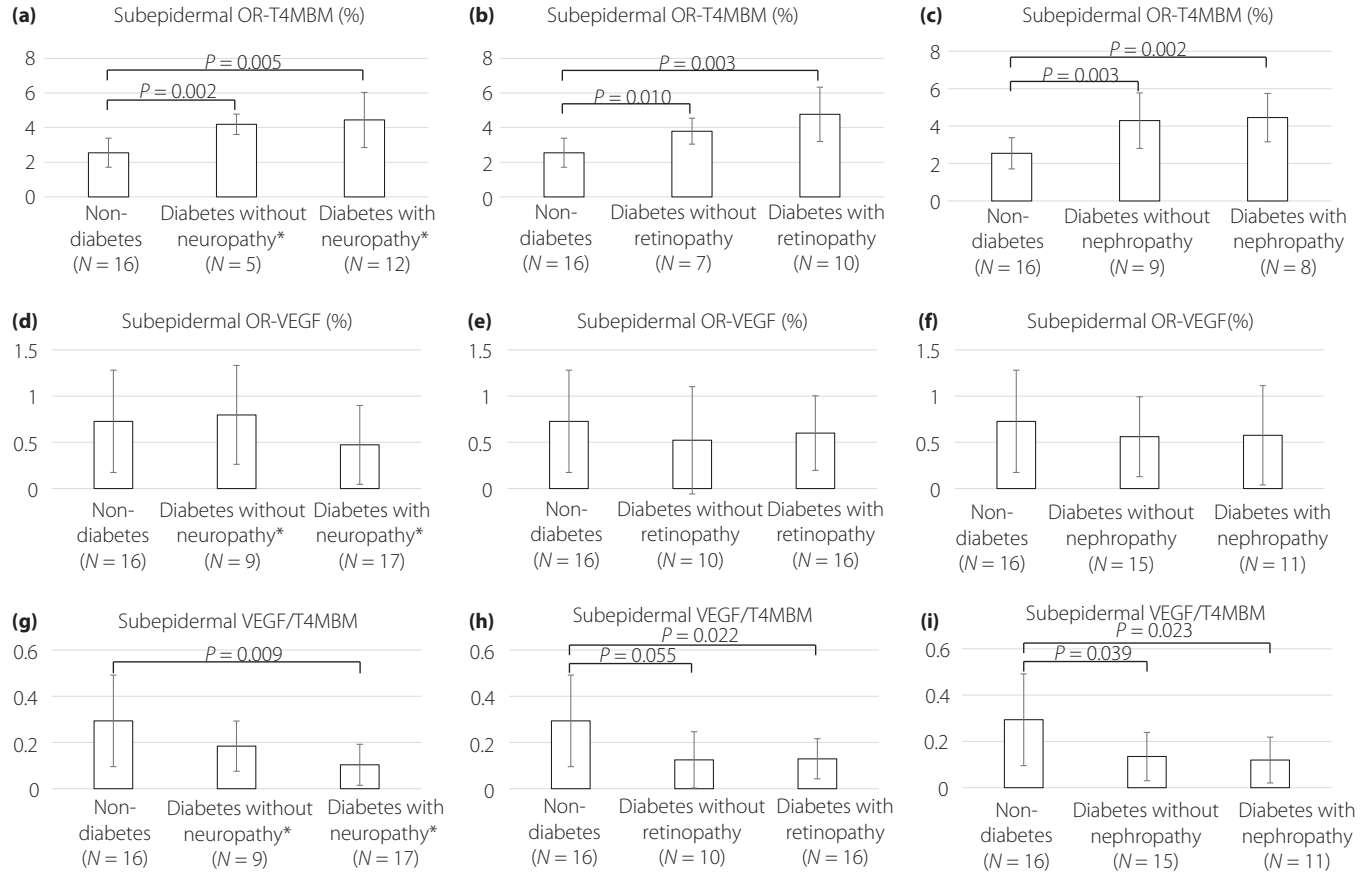

Figure 4 | Results of quantitative analyses of confocal immunofluorescence microscopy images with respect to (a–c) subepidermal occupancy rate of type IV collagen-immunoreactive microvascular basement membrane area (OR-T4MBM), (d–f) subepidermal occupancy rate of VEGF-A immunoreactive area (OR-VEGF) and (g–i) subepidermal VEGF-A-immunoreactive area relative to subepidermal type IV collagen-immunoreactive microvascular basement membrane area (VEGF/T4MBM ratio) in participants without diabetes and patients with diabetes with and without (a,d,g) neuropathy, (b,e,h) retinopathy or (c,f,i) nephropathy. (a–c) The solid line represents the median value, the box represents the interquartile range, the upper error bar represents the 95th percentile value and lower error bar represents the 5th percentile value. (d–i) Error bars indicate mean ± standard deviation. *Including both confirmed and subclinical neuropathy.

OR-T4MBM in people both with and without diabetes, whereas serum HDL cholesterol correlated negatively with subepidermal OR-T4MBM in people with diabetes, but not in those without diabetes (Table 3). In terms of microvascular complications and their key parameters in patients with diabetes (Table 4), neuropathy, nerve conduction parameters and retinopathy, but not nephropathy or UEA, correlated significantly with IENFD. Sural sensory nerve conduction velocity and sensory nerve action potential correlated positively with epidermal VEGF-A and with subepidermal VEGF/T4MBM ratio, respectively. Among the surrogate markers for atherosclerosis in patients with diabetes (Table 5), only ABI correlated negatively with subepidermal OR-T4MBM.

DISCUSSION

The present study provided the first direct evidence that cutaneous microangiopathy, as shown by the increase in subepidermal OR-T4MBM, occurred before the development of overt clinical evidence of neuropathy, retinopathy and nephropathy. Among these, diabetic neuropathy might often be the earliest to appear and even precede the onset of type 2 diabetes[24,25]. The present study used IENFD as an index of diabetic neuropathy, as this measure has been reported to offer a sensitive marker of early diabetic neuropathy[26] and was reduced in participants with small-fiber neuropathy associated with prediabetes[27]. The present study found obvious microvascular basement membrane changes in non-neuropathic patients with diabetes with normal IENFD, while IENFD reduction was detected concurrently in patients with diabetes with retinopathy and without nephropathy, suggesting early development of cutaneous microangiopathy associated with type 2 diabetes. As cutaneous microangiopathy appeared to progress after the development of neuropathy, retinopathy and nephropathy, causal relationships might exist between cutaneous

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Table 3 | Simple regression analyses of quantitative immunofluorescence results in relation to clinical variables of participants with and without diabetes

	Coefficient (95% CI) *P*-value					
	Participants	IENFD (fibers/mm)	Epidermal VEGF-A immunoreactivity (arbitrary units)	Subepidermal OR-T4MBM (%)	Subepidermal OR-VEGF (%)	Subepidermal VEGF/T4MBM
Age (years)	Without diabetes	0.130 (−0.103 to 0.363) 0.252	−0.125 (−0.315 to 0.065) 0.179	−0.010 (−0.57 to 0.038) 0.676	0.011 (−0.020 to 0.043) 0.444	0.003 (−0.008 to 0.014) 0.548
	With diabetes	0.002 (−0.255 to 0.251) 0.985	−0.011 (−0.070 to 0.048) 0.699	−0.24 (−0.072 to 0.024) 0.298	0.003 (−0.014 to 0.020) 0.741	0.001 (−0.003 to 0.004) 0.694
Height (cm)	Without diabetes	−0.347 (−0.737 to 0.042) 0.076	0.039 (−0.323 to 0.401) 0.820	0.030 (−0.055 to 0.114) 0.462	−0.007 (−0.064 to 0.050) 0.800	−0.004 (−0.024 to −0.017) 0.697
	With diabetes	−0.069 (−0.619 to 0.481) 0.793	−0.006 (−0.136 to 0.124) 0.922	0.019 (−0.086 to 0.127) 0.716	0.017 (−0.020 to 0.053) 0.343	0.003 (−0.004 to 0.011) 0.382
Body mass index (kg/m^2)	Without diabetes	0.144 (−0.873 to 1.162) 0.766	−0.426 (−1.237 to 0.385) 0.279	−0.068 (−0.265 to 0.129) 0.473	−0.011 (−0.144 to 0.121) 0.855	0.007 (−0.040 to 0.055) 0.752
	With diabetes	0.163 (−0.654 to 0.981) 0.676	−0.027 (−0.220 to 0.166) 0.770	−0.117 (−0.266 to 0.031) 0.113	−0.002 (−0.058 to 0.054) 0.950	0.003 (−0.009 to 0.015) 0.571
Current smoking, *n* (%)	Without diabetes	−2.705 (−8.544 to 3.135) 0.337	0.664 (−4.333 to 5.661) 0.780	−0.654 (−1.782 to 0.473) 0.234	−0.475 (−1.213 to 0.263) 0.189	−0.169 (−0.433 to 0.096) 0.193
	With diabetes	6.600 (−0.167 to 13.367) 0.055	0.708 (−1.057 to 2.473) 0.406	−0.284 (−1.789 to 1.222) 0.693	0.124 (−0.394 to 0.643) 0.617	0.025 (−0.084 to 0.135) 0.628
Current alcohol drinking, *n* (%)	Without diabetes	1.916 (−5.129 to 8.962) 0.569	−0.646 (−5.257 to 6.550) 0.818	−0.413 (−1.795 to 0.969) 0.532	0.219 (−0.701 to 1.139) 0.618	0.047 (−0.284 to 0.379) 0.764
	With diabetes	7.398 (0.886 to 13.911) 0.029*	0.171 (−1.635 to 1.976) 0.843	−1.511 (−2.776 to −0.247) 0.022*	0.040 (−0.482 to 0.563) 0.872	0.039 (−0.070 to 0.147) 0.461
Diabetes duration (years)	Without diabetes	–	–	–	–	–
	With diabetes	−0.338 (−0.828 to 0.152) 0.160	0.031 (−0.092 to 0.154) 0.600	0.042 (−0.051 to 0.136) 0.349	0.010 (−0.025 to 0.044) 0.603	0.001 (−0.006 to 0.009) 0.700
Systolic BP (mmHg)	Without diabetes	0.102 (0.087 to 0.291) 0.266	−0.052 (−0.214 to 0.109) 0.498	0.000 (−0.039 to 0.039) 0.994	−0.004 (−0.029 to 0.022) 0.768	−0.001 (−0.010 to 0.008) 0.834
	With diabetes	−0.016 (−0.262 to 0.230) 0.891	0.004 (−0.054 to 0.062) 0.878	−0.016 (−0.064 to 0.032) 0.488	0.005 (−0.012 to 0.021) 0.548	0.002 (−0.002 to 0.005) 0.303
Diastolic BP (mmHg)	Without diabetes	−0.009 (−0.271 to 0.254) 0.944	−0.066 (−0.280 to 0.148) 0.521	−0.008 (−0.059 to 0.044) 0.748	−0.018 (−0.050 to 0.015) 0.258	−0.005 (−0.017 to 0.007) 0.350
	With diabetes	0.195 (−0.138 to 0.529) 0.231	0.013 (−0.069 to 0.095) 0.737	−0.012 (−0.081 to 0.056) 0.704	0.008 (−0.016 to 0.031) 0.507	0.002 (−0.002 to 0.007) 0.309
HbA1c (mmol/mol)	Without diabetes	0.624 (−0.375 to 1.622) 0.183	−0.784 (−1.571 to 0.003) 0.051	0.003 (−0.255 to 0.261) 0.979	−0.095 (−0.251 to 0.060) 0.191	−0.028 (−0.086 to 0.030) 0.285
	With diabetes	−0.042 (−0.170 to 0.086) 0.491	−0.023 (−0.051 to 0.005) 0.106	−0.015 (−0.009 to 0.010) 0.216	−0.003 (−0.012 to 0.005) 0.426	−0.001 (−0.002 to 0.001) 0.546
Total cholesterol (mmol/L)	Without diabetes	−2.428 (−6.086 to 1.230) 0.168	1.353 (−1.636 to 4.341) 0.333	−0.741 (−1.358 to −0.124) 0.024*	−0.172 (−0.637 to 0.292) 0.423	0.000 (−0.004 to 0.005) 0.951
	With diabetes	1.064 (−1.759 to 3.887) 0.433	−0.554 (−1.113 to 0.006) 0.052	−0.648 (−1.098 to −0.198) 0.008*	−0.068 (−0.257 to 0.121) 0.452	0.000 (−0.001 to 0.001) 0.951

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Table 3 (*Continued*)

		Coefficient (95% CI) *P*-value					
	Participants	IENFD (fibers/mm)	Epidermal VEGF-A immunoreactivity (arbitrary units)	Subepidermal OR-T4MBM (%)	Subepidermal OR-VEGF (%)	Subepidermal VEGF/T4MBM
Triglycerides (mmol/L)	Without diabetes	−1.102 (−4.874 to 2.667) 0.536	−1.064 (−4.071 to 1.942) 0.455	−0.509 (−1.148 to 0.130) 0.108	−0.084 (−0.588 to 0.421) 0.724	0.000 (−0.002 to 0.002) 0.982
	With diabetes	0.697 (−3.628 to 5.022) 0.736	−0.414 (−1.410 to 0.582) 0.390	−0.221 (−1.068 to 0.626) 0.587	0.026 (−0.269 to 0.321) 0.853	0.000 (−0.001 to 0.001) 0.645
HDL cholesterol (mmol/L)	Without diabetes	−1.186 (−7.238 to 4.865) 0.668	2.624 (−3.821 to 9.068) 0.381	0.500 (−0.899 to 1.899) 0.440	−0.403 (−1.351 to 0.546) 0.362	−0.005 (−0.014 to 0.003) 0.193
	With diabetes	−0.785 (−9.937 to 8.367) 0.857	−1.820 (−3.730 to 0.090) 0.060	−1.701 (−3.245 to −0.157) 0.033*	−0.277 (−0.882 to 0.329) 0.345	−0.001 (−0.004 to 0.003) 0.687

BP, blood pressure; CI, confidence interval; HDL, high-density lipoprotein; IENFD, intraepidermal nerve fiber density; OR-T4MBM, occupancy rate of type IV collagen-immunoreactive microvascular basement membrane area; OR-VEGF, occupancy rate of vascular endothelial growth factor-A immunoreactive area; VEGF, vascular endothelial growth factor; VEGF/T4MBM, vascular endothelial growth factor-A immunoreactive area relative to type IV collagen immunoreactive microvascular basement membrane area. *Statistical significance ($P < 0.05$).

microangiopathy and chronic microvascular complications. In addition, the observed relationship between ABI and subepidermal OR-T4MBM might support concurrent micro- and macroangiopathy in type 2 diabetes patients.

Quattrini *et al.*[15] found that dermal blood vessel density was increased only in patients with moderate neuropathy, not in those with no, mild or severe neuropathy. That study used thin (5 μm) cross-sections to assess blood vessel density for a short distance of tissue under conventional light microscopy. In the present study, confocal microscopy scanned type IV collagen-stained thick (60 μm) sections at 2-μm increments for three-dimensional imaging of the tissue. The projected confocal z-series enabled us to follow microvessels throughout the 30-μm optical sections and detect proliferative changes to the microvessels in the subepidermis in patients without overt clinical neuropathy (Figures 2b and 4a), retinopathy (Figure 4b) or nephropathy (Figure 4c). Another difference between that study and the present study was that Quattrini *et al.* used markers for endothelial cells, such as CD31 and von Willebrand factor, to localize microvessels. Endothelial cell degeneration[9] and apoptosis[28] are reportedly increased in patients with cutaneous diabetic microangiopathy. Given the progressive nature of endothelial dysfunction and vascular basement membrane thickening in diabetic microangiopathy, the use of markers for vascular basement membrane, such as type IV collagen, instead of endothelial cells might be suitable for assessing dermal microvessel density, proliferation or both.

Nailfold capillary hypertension, as detected using capillary cannulation, has been shown to develop before the onset of diabetic nephropathy in patients with type 1 diabetes[29]. In contrast, other studies using laser Doppler techniques have shown that decreased skin microvascular blood flow coexisting with retinopathy and proteinuria, but not with clinical neuropathy[30,31]. More recent studies using semiquantitative videocapillaroscopy have shown that nailfold capillary abnormalities were associated with neuropathy[32,33], retinopathy[33–35] and nephropathy[33]. Such inconsistent findings with respect to cutaneous microangiopathy in relation to chronic microvascular complications of diabetes might be due to the different techniques used in different studies, suggesting the need for histopathological confirmation. In contrast to the aforementioned methods used for evaluating capillary changes in the nailfold, our three-dimensional approach to the skin for viewing the course of subdermal microvasculature offers a more accurate and sensitive quantification of basement membrane changes.

VEGF-A can exert both angiogenic and neurotrophic/neuroprotective properties[36]. In the present study, significant relationships were apparent between sural sensory nerve conduction deficits and reductions in epidermal VEGF-A expression and the subepidermal VEGF/T4MBM ratio. We observed that epidermal VEGF-A (Figure 1d) and the subepidermal VEGF/T4MBM ratio (Figure 4g) were particularly decreased in patients with neuropathy showing IENF loss and subepidermal

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Table 4 | Simple regression analyses of quantitative immunofluorescence results in relation to microvascular complications and their surrogate markers in patients with diabetes

	Coefficient (95% CI) *P*-value				
	IENFD (fibers/mm)	Epidermal VEGF-A immunoreactivity (arbitrary units)	Subepidermal OR-T4MBM (%)	Subepidermal OR-VEGF (%)	Subepidermal VEGF/T4MBM
Neuropathy[†]	−12.678 (−16.703 to −8.653) <0.001*	−1.409 (−3.139 to 0.321) 0.103	0.247 (−1.335 to 1.828) 0.744	−0.324 (−0.843 to 0.195) 0.203	0.247 (−1.335 to 1.828) 0.744
Tibial MNCV (m/s)	0.775 (0.219 to 1.331) 0.010*	0.031 (−0.134 to 0.195) 0.697	−0.113 (−0.236 to 0.010) 0.070	0.008 (−0.040 to 0.055) 0.733	0.005 (−0.005 to 0.014) 0.329
Tibial CMAP (mV)	1.130 (0.431 to 1.828) 0.004*	0.074 (−0.143 to 0.290) 0.480	−0.057 (−0.239 to 0.124) 0.511	−009 (−0.054 to 0.073) 0.760	0.005 (−0.008 to 0.018) 0.456
Tibial F wave latency (ms)	−0.746 (−1.248 to −0.245) 0.006*	−0.094 (−0.238 to 0.049) 0.182	0.040 (−0.085 to 0.166) 0.504	−0.016 (−0.059 to 0.027) 0.441	−0.005 (−0.014 to 0.004) 0.237
Sural SNCV (m/s)	0.579 (0.273 to 0.886) 0.002*	0.123 (0.028 to 0.219) 0.016*	0.012 (−0.063 to 0.086) 0.729	0.030 (0.000 to 0.060) 0.051	0.006 (0.000 to 0.013) 0.053
Sural SNAP (μV)	1.141 (0.815 to 1.466) <0.001*	0.073 (−0.089 to 0.234) 0.352	−0.072 (−0.206 to 0.061) 0.267	0.036 (0.008 to 0.080) 0.104	0.009 (0.000 to 0.018) 0.043*
Retinopathy	−9.740 (−14.925 to −4.555) 0.001*	0.603 (−1.121 to 2.327) 0.468	0.975 (−0.392 to 2.344) 0.149	0.077 (−0.429 to 0.583) 0.751	0.976 (−0.392 to 2.344) 0.149
Nephropathy	−2.683 (−9.887 to 4.520) 0.440	−0.462 (−2.174 to 1.250) 0.574	0.161 (−1.285 to 1.607) 0.816	0.015 (−0.488 to 0.516) 0.949	0.161 (−1.285 to 1.607) 0.816
UEA (mg/day)[‡]	−0.034 (−0.113 to 0.046) 0.372	−0.004 (−0.022 to 0.014) 0.640	0.000 (−0.015 to 0.016) 0.983	0.001 (−0.005 to 0.006) 0.779	0.000 (−0.001 to 0.001) 0.818

CI, confidence interval; CMAP, compound muscle action potential amplitude; IENFD, intraepidermal nerve fiber density; MNCV, motor nerve conduction velocity; OR-T4MBM, occupancy rate of type IV collagen-immunoreactive microvascular basement membrane area; OR-VEGF, occupancy rate of vascular endothelial growth factor-A immunoreactive area; SNAP, sensory nerve action potential amplitude; SNCV, sensory nerve conduction velocity; UEA, urinary excretion of albumin; VEGF, vascular endothelial growth factor; VEGF/T4MBM, vascular endothelial growth factor-A immunoreactive area relative to type IV collagen immunoreactive microvascular basement membrane area. *Statistical significance ($P < 0.05$). [†]Both confirmed and subclinical neuropathy were included. [‡]Three patients with persistent proteinuria were excluded from analysis.

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Table 5 | Simple regression analyses of quantitative immunofluorescence results in relation to surrogate markers for atherosclerosis of patients with diabetes

	Coefficient (95% CI) *P*-value				
	IENFD (fibers/mm)	Epidermal VEGF-A immunoreactivity (arbitrary units)	Subepidermal OR-T4MBM (%)	Subepidermal OR-VEGF (%)	Subepidermal VEGF/T4MBM
Brachial-ankle PWV (cm/s)[†]	−0.006 (−0.014 to 0.002) 0.152	0.000 (−0.002 to 0.002) 0.908	0.001 (−0.001 to 0.002) 0.469	0.000 (−0.001 to 0.001) 0.786	0.000 (0.000 to 0.000) 0.768
ABI[†]	13.697 (−23.203 to 50.596) 0.439	−6.501 (−14.035 to 1.033) 0.085	−7.894 (−14.029 to −1.759) 0.015*	−0.682 (−3.173 to 1.810) 0.567	0.052 (−0.478 to 0.581) 0.837
Maximum IMT (mm)[‡]	−6.347 (−14.067 to 1.373) 0.098	−0.252 (−2.239 to 1.735) 0.787	0.750 (−0.975 to 2.474) 0.362	−0.149 (−0.771 to 0.474) 0.612	−0.047 (−0.175 to 0.080) 0.432

ABI, ankle brachial index; CI, confidence interval; IENFD, intraepidermal nerve fiber density; IMT, intima-media thickness; OR-T4MBM, occupancy rate of type IV collagen-immunoreactive microvascular basement membrane area; OR-VEGF, occupancy rate of vascular endothelial growth factor-A immunoreactive area; PWV, pulse wave velocity; VEGF, vascular endothelial growth factor; VEGF/T4MBM, vascular endothelial growth factor-A immunoreactive area relative to type IV collagen immunoreactive microvascular basement membrane area. *Statistical significance ($P < 0.05$). [†]One outpatient was not included for analysis. [‡]One outpatient and two inpatients were not included for analysis.

microvascular proliferation (Figures 1a,4a), suggesting neurotrophic/neuroprotective effects for sensory fibers rather than angiogenic effects from cutaneous VEGF-A. These findings were consistent with the results described by Quattrini *et al.*[15], showing reduced expression of VEGF-A in the epidermis among patients with severe neuropathy. Although they showed the immunolocalization of VEGF-A in basal and spinous cells of the epidermis, as well as in fibroblasts, pericytes, endothelium and smooth muscle cells of the vascular wall in the dermis[15], we found the most intense VEGF-A immunoreactivity in endothelial cells, less intense immunoreactivity in vascular wall cells (pericytes or smooth muscle cells) and keratinocytes (mainly basal cells), and faint immunoreactivity in fibroblasts (Figure 3b,e,h). Cutaneous VEGF-A thus seems likely to be produced mainly by the dermal microvasculature, and its impaired expression might be associated with sensory fiber loss in human type 2 diabetes.

The present study also showed retained VEGF-A expression in the subepidermis as a whole, as indicated by the lack of alteration in subepidermal OR-VEGF among patients with diabetes. However, double immunostaining showed decreased expression of VEGF-A per unit area of the subepidermal microvasculature, as indicated by the decrease in the VEGF/T4MBM ratio in the subepidermis, thus providing evidence for impaired microvascular expression of VEGF-A in these patients.

The present findings appear to contradict the view that VEGF plays a role in mediating active neovascularization in the process of ocular complications. Likewise in the human diabetic kidney, findings were inconsistent in terms of VEGF expression, ranging from upregulation particularly early in the course of diabetes[37] to downregulation as a reflection of progressive podocyte loss in diabetic nephropathy[38]. In human diabetic peripheral nerves, morphological abnormalities of the microvessels have been reported, similar to those observed in diabetic retina and glomerulus[39]. Whether endoneurial hypoxia can induce VEGF overexpression in the peripheral nerves remains unclear, but intramuscular plasmid VEGF gene transfer improved neuropathic symptoms and sensory loss in patients with diabetic neuropathy[12]. Clearly, further studies are required to elucidate the mechanisms underlying the tissue- and temporal-specific regulation of VEGF expression in diabetes patients.

The present study found no significant differences in clinical variables between patients with and without microvascular complications, except for a longer duration of diabetes and lower levels of serum total cholesterol observed only among patients with retinopathy compared with those without retinopathy. Linear regression analyses showed no relationships between duration of diabetes and any of the quantitative immunofluorescence results in patients with diabetes. However, serum total cholesterol correlated negatively with subepidermal OR-T4MBM in participants both with and without diabetes. In addition, serum HDL cholesterol correlated negatively with

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subepidermal OR-T4MBM, while current alcohol consumption correlated positively with IENFD and negatively with subepidermal OR-T4MBM among patients with diabetes. Although these unexpected findings warrant further investigation in a larger study, it might be worth noting that HDL can augment ischemia-induced physiological angiogenesis and inhibit inflammation-driven pathological angiogenesis through its receptor[40], and can regulate key angiogenic mediators including VEGF, thereby modulating angiogenesis in inflammatory diseases such as type 2 diabetes[41].

Various potential limitations to the present study should be considered. The statistical power of this study might have been limited by the small sample size. However, taking into consideration the difficulty of recruiting participants into studies that involve a biopsy, the current data with the use of confocal microscopy of thick-skin sections would still be of value in terms of providing direct pathological evidence and early detection of cutaneous microangiopathy in patients with diabetes. The patients with diabetes were more obese, included more current smokers, and had higher HbA1c and lower serum HDL cholesterol compared with participants without diabetes. The current findings might therefore be affected by these differences and should be validated in future studies with larger sample sizes. Another weakness of this study was the cross-sectional design, which did not allow us to assess or confirm the direction of causality for the observed findings.

In conclusion, the current protocols allowed us to detect early cutaneous diabetic microangiopathy associated with impaired VEGF-A expression in patients with type 2 diabetes. Cutaneous microangiopathy might represent early features of systemic microangiopathy preceding the onset of chronic microvascular complications in these patients. Further investigation is warranted to determine whether basement membrane changes and impaired VEGF-A expression in skin microvasculature offer novel therapeutic targets for the prevention of late complications.

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DISCLOSURE

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1| Clinical backgrounds in patients with diabetes with or without neuropathy, retinopathy, or nephropathy.

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