

Decreased Reactivity of Skin Microcirculation in Response to L-Arginine in Later-Onset Type 1 Diabetes

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OBJECTIVE—The aim of our study was to evaluate the vasodilatory effect of L-arginine infusion on the skin microcirculation and to assess the relationship between this effect and the presence of microangiopathy in patients with type 1 diabetes.

RESEARCH DESIGN AND METHODS—Capillaroscopy was performed before and after L-arginine infusion in 48 diabetic patients (26 women and 22 men; age, 39.8 ± 6.3 years) and 24 volunteers free of any chronic disease (13 women and 11 men; age, 38.0 ± 6.7 years). The skin microcirculation reactivity, as expressed by the percentage of area covered by capillaries (coverage) and the distance between capillaries (distance), and the relationship between microcirculation reactivity and the presence of microangiopathic complications were assessed.

RESULTS—The distance before L-arginine infusion was significantly lower in patients than in controls (221 [153–311] vs. 240 [185–356] μm ; $P = 0.02$) and did not differ after L-arginine infusion (223.5 [127–318] vs. 242.5 [181–341] μm ; $P = 0.27$). The difference between the coverage values obtained before and after L-arginine infusion (Δ coverage) was significantly different from zero in the control group but not in the diabetes group. Patients with later onset of diabetes were characterized by decreased skin microcirculation reactivity when compared with patients with earlier onset of diabetes ($-1.18 [-5.07 \text{ to } 11.60]$ vs. $1.36 [-6.00 \text{ to } 8.06]$; $P = 0.02$) despite the higher prevalence of retinopathy in patients with earlier onset of diabetes (64% vs. 26%; $P = 0.02$).

CONCLUSIONS—Skin microvascular reactivity is impaired in patients with later onset of type 1 diabetes. Capillaroscopy with L-arginine infusion is useful for the identification of skin microangiopathy in type 1 diabetes.

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The impairment of endothelial function leads to microvascular complications in type 1 diabetes, such as retinopathy, neuropathy, or nephropathy (1). Some studies also have shown that endothelial dysfunction of the skin vessels in type 1 diabetes is associated with the presence of diabetic neuropathy or retinopathy (2–4). However, the relationship between the microcirculation of the skin and the clinical course of type 1

diabetes has not been definitively established (5–12).

The L-arginine infusion serves as a selective stimulus for the nitric oxide (NO)-dependent vasodilatory function of endothelium as a substrate for NO synthase (13). However, L-arginine infusion has not been previously used to determine the reactivity of the skin microcirculation in patients with type 1 diabetes, despite the fact that it has been recognized as a safe and effective

approach to studying retinal and cerebrovascular vasomotor reactivity (14,15).

Capillaroscopy is a widely recognized method for the assessment of skin microcirculation (16,17). It is routinely used in the diagnosis of connective tissue diseases coexisting with primary or secondary Raynaud phenomenon, hypertension, and other microangiopathies (18). The capillaroscopy studies focused on patients with type 1 diabetes used non-selective stimuli such as postocclusive reactive hyperemia (6,12,19). Selective stimulators of endothelial vasodilatory function of skin microcirculation with use of L-arginine infusion has not been reported previously.

Therefore, the aim of our study was to evaluate the vasodilatory effect of L-arginine infusion on the skin microcirculation and to assess the relationship between this effect and the presence of microangiopathy in patients with type 1 diabetes.

RESEARCH DESIGN AND METHODS

The study group consisted of 48 patients with type 1 diabetes (26 women and 22 men; age, 39.8 ± 6.3 years) recruited from the Regional Diabetological Centre of the Medical University of Gdansk and 24 volunteers who were free of any chronic diseases (13 women and 11 men; age, 38.0 ± 6.7 years) (see Table 2). We included patients with a minimum diabetes duration of 4 years and who were free of focal neurologic deficits and renal insufficiency. We excluded patients with previously diagnosed rheumatologic diseases. Additional exclusion criteria were pregnancy and pathological obesity ($\text{BMI} > 35 \text{ kg/m}^2$). All examinations were performed at the same time of day (between 8:00 A.M. and 1:00 P.M.). All study subjects were asked to stop drinking coffee and to stop smoking tobacco on examination day and to avoid sleep deprivation on previous days. The study protocol included medical history, neurologic examination, funduscopy, and laboratory testing. The study

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Table 1—Subgroup analysis in patients with type 1 diabetes

	n	Δ Coverage (%)			Δ Distance (μ m)		
		Present	Absent	P	Present	Absent	P
Male	22	-0.05 (-6.00 to 11.60)	1.44 (-0.05 to 0.08)	0.9	13.5 (-66.8 to 40.8)	-4.2 (-56.5 to 50.0)	0.07
Age at onset of diabetes							
<19, years	25	-1.18 (-5.07 to 11.60)	1.36 (-6.00 to 8.06)	0.02	14.5 (-66.8 to 50)	-5.1 (-56.5 to 40.8)	0.02
Duration of diabetes							
<19.7, years	24	1.27 (-6.00 to 11.60)	-0.99 (-5.07 to 3.95)	0.83	-3.4 (-66.8 to 50.0)	9.7 (-29.9 to 41.6)	0.31
Age >37.9, years	24	-0.98 (-5.07 to 3.95)	1.27 (-6.00 to 11.60)	0.04	9.7 (-29.9 to 41.6)	-3.4 (-66.8 to 50.0)	0.13
HbA _{1c} >7.0%	38	-0.36 (-6.00 to 11.66)	2.00 (-3.69 to 5.87)	0.12	9.7 (-66.8 to 41.6)	-0.7 (-46.3 to 50.0)	0.51
Hypertension	8	0.60 (-4.79 to 8.06)	-0.16 (-6.00 to 11.60)	0.8	2.2 (-56.5 to 16.3)	9.5 (-66.8 to 50.0)	0.91
Hyperlipidemia	40	0.03 (-6.00 to 11.60)	0.36 (-3.92 to 2.05)	0.14	6.2 (-66.8 to 50.0)	15.6 (-13.4 to 40.8)	0.15
Neuropathy	13	-0.56 (-5.07 to 8.06)	0.06 (-6.00 to 11.60)	0.6	-2.5 (-56.5 to 41.6)	9.5 (-66.8 to 50.0)	0.79
Retinopathy	22	-0.15 (-6.00 to 11.60)	0.03 (-5.07 to 4.38)	0.9	7.6 (-66.8 to 40.8)	9.5 (-52.9 to 50.0)	0.95
Overt nephropathy	8	-0.24 (-5.07 to 8.06)	0.03 (-6.00 to 11.60)	0.9	-3.9 (-56.5 to 41.6)	9.7 (-66.8 to 50.0)	0.95
Microalbuminuria	5	-0.33 (-2.37 to 2.66)	0.06 (-6.00 to 11.60)	0.9	5.4 (-15.2 to 12.5)	9.5 (-66.8 to 50.0)	1
ACE inhibitor, ARB, or β -blocker treatment	8	0.60 (-4.79 to 8.06)	-0.16 (-6.00 to 11.60)	0.8	2.2 (-56.5 to 16.3)	9.5 (-66.8 to 50.0)	0.64
Statin treatment	5	-1.66 (-4.79 to -0.71)	0.38 (-6.00 to 11.60)	0.02	16.3 (-5.1 to 39.4)	8.4 (-66.8 to 50.0)	0.14
Smoking	10	-1.33 (-6.00 to 11.60)	0.08 (-4.79 to 8.06)	0.79	-3.9 (-66.8 to 41.6)	9.3 (-56.5 to 50.0)	0.19

Median values are given first, followed by the range of values in parentheses. ARB, angiotensin receptor blocker.

protocol was approved by the Medical Ethics Committee of the Medical University of Gdansk (NKEBN/335/2008, NKEBN/335-60/2009, NKEBN/204/2010). On entry to the study, each participant gave informed consent.

Subject characteristics

Patient histories were obtained, including information on past and current disorders as well as comorbid conditions and cigarette smoking. Weight and height were measured and BMI was calculated. Obesity was defined as BMI \geq 30 kg/m², and overweight was defined as BMI in the range of 25–30 kg/m². Blood pressure control in all subjects was monitored with conventional sphygmomanometry.

Diabetic neuropathy was diagnosed using the criteria defined by the Neurologic Symptoms Score and Neurologic Disability Score based on patient symptoms and neuropathic deficits found on neurologic examination (20). Laboratory examinations in patients with type 1 diabetes included microalbuminuria, albuminuria/creatinine index, total serum cholesterol, LDL cholesterol, HDL cholesterol, triglycerides, and glycated hemoglobin (HbA_{1c}).

Hyperlipidemia was diagnosed if total cholesterol was >175 mg/dL and/or triglycerides were >150 mg/dL and/or LDL cholesterol was >100 mg/dL. Hyperlipidemia also was diagnosed if HDL level was <40 mg/dL in males or <50 mg/dL in women, or if cholesterol-lowering/triglyceride-lowering

medications were used (21). In the control group, hyperlipidemia was diagnosed if total cholesterol and/or triglycerides and/or LDL cholesterol exceeded 190, 150, and 130 mg/dL, respectively, or if HDL level was <40 mg/dL in males or <50 mg/dL in women (22).

Based on the criteria of the European Association for the Study of Diabetes, microalbuminuria was defined as the albuminuria/creatinine index in a random spot collection (>30 μ g/mg creatinine) or overnight collection (>20 μ g/min) in two out of three urine collections repeated at intervals of up to 6 months. Clinical albuminuria or "overt nephropathy" according to the American Diabetes Association and the European Association for the Study of Diabetes recommendations corresponded to protein excretion >300 mg/24 h (21).

Fundoscopy

Retinopathy was recognized on fundoscopy performed by an experienced ophthalmologist certified by the Polish Ophthalmic Society. Retinopathy was graded according to the stages of diabetic retinopathy defined by the American Academy of Ophthalmology (23). Previous therapy with photocoagulation also was recognized as a marker of diabetic retinopathy.

Capillaroscopy

Subjects participating in the study were asked not to dispatch the skin of their

fingernails at least for 2 weeks before the start of the study. During examination, patients remained in a comfortable sitting position with the hand conveniently supported and laid stably under the capillaroscope. Tags were drawn on their nail folds to establish areas for further examination during repeated measurements. The room temperature was controlled by air conditioning and was kept the same during all tests. Body temperature was controlled with the use of a contactless thermometer and was within normal range in all examined patients and controls.

The skin capillaries were examined by a stereomicroscope with spot lighting. The nail-fold capillaries were made transparent by adding a drop of immersion oil, and images were generated by a lens providing a magnification of 200 \times . Images were taken using a digital camera (5MPx; OPTA-TECH, Warsaw, Poland) attached to the capillaroscope (OPTA-TECH, CS-CREATIVE SOLUTIONS Group, Warsaw, Poland) and archived on disc. The software for image analysis was written in C++ language according to concepts formulated by the authors (24).

Offline computer analysis included the following steps: rotation of the image; selection of the part of image for further analysis; tuning process of selected area; recognition of capillaries; and final acceptance of achieved results. The first two rows of capillaries in the nail fold were evaluated. The images with less than five

capillaries in each row detected were not considered for statistical analysis. The image analysis allows determination of the mean distance between successive capillaries (distance) and the ratio between area of the capillaries and total area of determined rows (coverage).

The capillaroscopy was performed twice, before and after 30 min of L-arginine infusion (30 g 21% [wt/vol] L-arginine hydrochloride solution [B. Braun Melsungen AG, Germany] in 100 mL sodium chloride). The L-arginine infusion was performed intravenously within 30 min into intermediate cubital vein of the arm ipsilateral to the capillaroscopy. The skin microcirculation reactivity was expressed as the difference between the values of distance (Δ distance) and coverage (Δ coverage) obtained before and after infusion of L-arginine.

Reproducibility

Two-hundred fifty-two random coded images were used for the reproducibility study. The same observer performed the image analysis over 504 images. The unbiased intraclass correlation coefficient between the sets of data obtained was considered to be an acceptable measure of agreement if intraclass correlation coefficient (mean and 90% confidence interval) for distance and coverage yielded 0.64 (0.58–0.70) and 0.63 (0.56–0.70), respectively. The results achieved indicated good reproducibility for the measurement procedure.

Statistical analysis

All the analyses were performed using STATISTICA data analysis software system, version 9.1. (StatSoft Inc., Tulsa, OK). Shapiro-Wilk tests were performed to analyze the distribution of continuous variables. Differences between groups were analyzed with the Student *t* test in the case of normally distributed variables (age) or with Mann-Whitney *U* test in the case of non-normally distributed variables (HbA_{1c}, parameters describing the skin microcirculation). Correlation was assessed by the Spearman rank correlation test for all continuous variables. The unbiased intraclass correlation coefficient was considered as an acceptable measure of agreement between the sets of repeated data.

Median age of onset of diabetes coincided with the time of puberty, and therefore was used as the threshold value to divide the whole diabetic group into subgroups (Table 1).

The χ^2 test was used to compare the proportion of genders and the presence of smoking habit, obesity or overweight, hypertension, hyperlipidemia, microalbuminuria, physical activity, contraception, microangiopathy, and treatment with statins, β -blockers, angiotensin receptor blockers, or ACE inhibitors. The level of $P < 0.05$ was regarded as statistically significant.

RESULTS—All patients and volunteers were free of diabetic foot, orthostatic hypotension, and chronic renal insufficiency, as determined by physical examination and biochemical analysis. There were no significant differences regarding age, sex, cigarette smoking, BMI and obesity, hormonal contraceptive usage, or regular physical activity between patients and control subjects. The percentage of subjects with dyslipidemia was significantly higher in type 1 diabetic patients than in controls (Table 2).

The coverage was significantly higher and the distance was significantly lower in patients with type 1 diabetes than in control subjects before L-arginine infusion; the differences between groups regarding both coverage and distance diminished after L-arginine infusion (Table 1).

The duration of the disease and patient age did not correlate with Δ distance and Δ coverage. Similarly, there were no significant correlations between age and Δ distance and Δ coverage in the controls.

In the whole studied cohort ($n = 72$), regardless of the group, there were significant correlations between age and both Δ distance and Δ coverage ($r = 0.27$, $P = 0.02$ and $r = -0.025$, $P = 0.04$, respectively).

We found no significant differences in Δ coverage and Δ distance in subgroups of patients with type 1 diabetes distinguished by the presence of diabetic retinopathy, neuropathy, overt nephropathy, microalbuminuria, hypertension, or hyperlipidemia. Additionally, no differences in Δ coverage and Δ distance were found between groups of patients distinguished on the basis of treatment with ACE inhibitors, angiotensin receptor blockers, or β -blockers, or HbA_{1c} level equal to 7%.

Within the group of patients with type 1 diabetes, the onset of diabetes correlated positively with Δ distance ($r = 0.43$; $P = 0.002$) and negatively with Δ coverage ($r = -0.39$; $P = 0.006$). When subgroups were created according to the average age of onset of diabetes (19.7 years; median, 19 years), further analysis showed a significantly lower Δ coverage and significantly higher Δ distance in patients with later

onset of type 1 diabetes (group B) in comparison with those with earlier onset of type 1 diabetes (group A) (Table 2, right panel). Patients with later onset of type 1 diabetes were characterized by similar BMI, shorter diabetes duration ($P = 0.002$), and older age ($P = 0.04$), as well as higher triglyceride ($P = 0.03$) and urine creatinine levels ($P = 0.01$) than in those with earlier onset. Neither subgroup of the type 1 diabetes group differed from the control groups according to age.

The analysis of obtained data showed no correlation between the duration of diabetes and microvascular parameters in either of the subgroups.

The group with earlier onset of diabetes had significantly greater skin microcirculation reactivity described by Δ coverage and Δ distance ($P = 0.02$; Fig. 1) and a higher incidence of retinopathy (64% for group A vs. 26% for group B; $P = 0.02$), but no neuropathy, nephropathy, or any of these microangiopathies in comparison with the diabetes group with later onset of the disease. Both variables were significantly different between the group with later but not with earlier onset of diabetes in comparison with the control group (Fig. 1).

The correlation analysis between Δ distance and Δ coverage with the duration of the disease in subgroups with earlier and later onset of diabetes did not reach statistical significance.

CONCLUSIONS—The influence of L-arginine on NO-dependent vasodilatation has been the subject of several studies regarding diabetes (25–28). However, the regional differences in the vascular response to L-arginine are not fully determined. To our best knowledge, the relationship between microangiopathy and the skin microcirculation reactivity, as revealed by capillaroscopy, in response to L-arginine infusion in long-duration type 1 diabetes has not yet been described in the literature.

Our results showed a lack of capillary response to L-arginine in patients with type 1 diabetes in contrast to control subjects when an effect was observed. The L-arginine-related Δ coverage in the control group was significantly greater than zero ($P = 0.02$), whereas for patients with type 1 diabetes there was no change.

Our findings are in line with the results of Tibiriçá et al. (12), who demonstrated maximal rest recruitment of the skin microcirculation in response to post-occlusive hyperemia in diabetic patients. It is noteworthy that similar conclusions

Table 2—Baseline characteristics and the comparison of the skin capillary data between patients with type 1 diabetes and control subjects

Characteristics	Diabetic patients N = 48		Control group N = 24		P for between-group comparison	Earlier onset of diabetes (group A) N = 25		Later onset of diabetes (group B) N = 23		P for between- group comparison
	Mean ± SD and median (range)		Mean ± SD and median (range)			Mean ± SD and median (range)		Mean ± SD and median (range)		
Age, years	39.8 ± 6.3		38 ± 6.7		0.7	37.6 ± 4.6		42.3 ± 7		0.04
Males, %	45.8		45.8		1					
Age at onset of diabetes, years	19.7 ± 7.3		—		NA	14 ± 3.8		25.8 ± 4.7		By definition
Duration of diabetes, years	20.2 ± 6.6		—		NA	23.6 ± 5.2		16.5 ± 6.3		0.002
Circadian insulin dose, units/24 h	56.7 ± 16.99		—		NA	55.4 ± 17.6		58.3 ± 16.6		0.56
HbA _{1c} , %	7.84 (5.95–11.79)		5.5 (5.14–6.10)		<0.001	7.99 (6.15–11.79)		7.66 (5.95–9.09)		0.38
HbA _{1c} <7%, %	20.8		0		<0.001	24		17.4		0.84
Smokers, %	21		6.3		0.58	12		30.4		0.22
BMI, kg/m ²	25.03 (19.02–32.61)		23.58 (20.08–34.72)		0.5	24.82 (19.02–32.60)		25.25 (21.31–32.61)		0.45
BMI >25 kg/m ² , %	50		37.5		0.45					0.53
Hypertension, %	16.7		0		0.8	20		13		0.80
ACE inhibitors, ARB, and β-blockers, %	16.7		0		0.08	20		13		0.80
Dyslipidemia, %	83		41.7		<0.001	84		82.6		0.80
Statin treatment, %	10.4		0		0.3	16		17.4		0.80
Hormonal										
contraception, %	4.2		4.2		0.5	4		4.3		0.51
Regular physical activity, %	25		29.2		0.9	20		30.4		0.62
Microangiopathic complications, %										
Neuropathy	58					72		43		0.09
Retinopathy	27					32		22		0.64
Microalbuminuria	46					64		26		0.02
Overt nephropathy	10.5					8		9		0.92
Coverage, %	16.7					20		13		0.8
Before L-arginine infusion	20.4 (13.7–30)		18.1 (13–24)		0.02	19.6 (14–30)		21 (17–28)		0.07
After L-arginine infusion	20.6 (13.4–32.6)		19.9 (15–32)*		0.3	21.1 (13–27)		20.3 (14–33)		0.71
Distance, μm										
Before L-arginine infusion	221 (153–311)		240 (185–356)		0.02	232 (197–311)		209 (153–280)		0.02
After L-arginine infusion	223.5 (127–318)		242.5 (181–341)		0.27	225.6 (201–318)		223.1 (127–298)*		0.5

NA, not applicable; ARB, angiotensin receptor blocker. *P < 0.05 for comparison with before L-arginine test value.

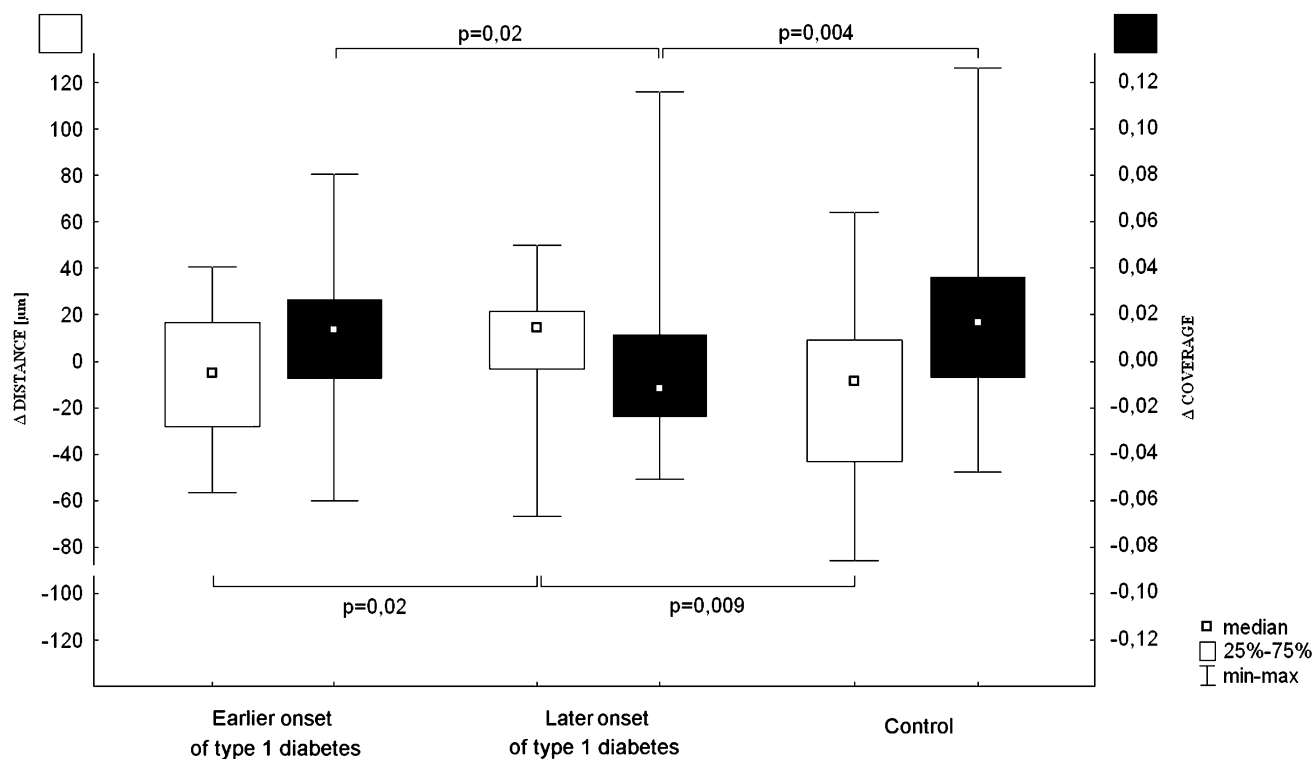


Figure 1—Comparison of changes in skin microvascular reactivity parameters attributable to L-arginine infusion between studied groups.

were obtained, even though there were essential differences in the mechanism of action between the two testing methods. The L-arginine test describes endothelial reactivity dependent on NO action solely, whereas the main mechanisms involved in the ischemic test are shear stress and its consequences (29).

In studied patients with type 1 diabetes, the skin microcirculation after L-arginine infusion did not differ between patients with and without microangiopathy. The skin microcirculation reactivity also was independent of the metabolic state described by glycemia and HbA_{1c} level or hyperlipidemia. The analysis revealed a significantly negative correlation between the microcirculation reactivity and the onset of diabetes ($r = -0.39$; $P < 0.05$), whereas no correlation with the duration of disease was found. The average onset of disease in study subjects was 19.7 years (median, 19 years). The group with an age of onset of disease after 19.7 years (group B, later onset of type 1 diabetes) was characterized by lower skin micro-reactivity despite shorter duration of disease. This finding was in contrast to that of patients with an onset of disease before the age of 19.7 years (group A, earlier onset of type 1 diabetes) in which a higher prevalence of retinopathy was observed. Moreover, our results showed no significant

differences between controls and group A regarding skin microcirculation parameters. The same comparison for group B showed substantially lower significance levels than for all cohorts of patients with diabetes.

The association between the prevalence of retinopathy and the onset of diabetes before puberty is well known. Donaghue et al. (30) reported that prepubertal diabetes duration is related to the prevalence of retinopathy in adult patients. This conclusion is consistent with our results, which show significantly more retinopathy in the group with the earlier onset of type 1 diabetes. However, the relationship between the nail-fold microcirculation and microangiopathy in type 1 diabetes is still a matter of debate in the literature. Meyer et al. (9) demonstrated a correlation between the presence of diabetic microangiopathy and pathological capillaroscopic images. Forst et al. (31) did not observe effects of the presence of diabetic neuropathy on capillary blood flow increase attributable to neurovascular stimulation.

Our conclusions are in contrast to others who have pointed out that endothelial dysfunction is an important predictor of microangiopathic complications (1,32,33). This could be partially explained by the fact that these studies were performed in younger cohorts with a shorter duration of

diabetes (12) and used the postocclusive reactive hyperemia method or venous congestion to achieve maximal recruitment of capillaries (5,10). Moreover, in our study we used a quantitative approach in contrast to qualitative assessment of nail-fold circulation as the cited authors did.

In our study group, the reactivity of the skin microvasculature did not correlate significantly with BMI as was found in the study by Tibirić et al. (12). It is noteworthy that the patients with pathological obesity (BMI >35 kg/m²) were excluded by protocol.

The limitation of our study is the fact that hyperlipidemia was present in 42% of the subjects in the healthy group. This is two-times the estimated percentages for the Polish low global risk population, which are 19% and 27% for men and women, respectively (34). It should be also noted that because of the relatively small number of studied subjects, our study was not able to confirm unambiguously the findings already established in the literature, such as the association between skin microcirculation parameters and the subgroup parameters (e.g., duration of diabetes and HbA_{1c}).

We used L-arginine infusion as the selective stimulator of endothelial vasodilatory function of skin microcirculation. We observed a physiological response to

L-arginine infusion in the control group and no response in the diabetic group. These data reflect the impairment of reactivity of skin microcirculation in response to L-arginine in type 1 diabetes. The response to L-arginine differed in subgroups of type 1 diabetes. No Δ coverage and significant decrease in distance suggest paradoxical deterioration after L-arginine infusion in the subgroup with later onset of type 1 diabetes. The lack of significant response after L-arginine infusion regarding coverage and distance in patients with earlier onset of type 1 diabetes, and no difference with the control group allows for the hypothesis that these patients may benefit from supplementation with L-arginine as a substrate for NO.

The analysis we performed shows that the relationship between skin capillary reactivity and retinopathy presence (Table 1) cannot be clearly characterized by parameters such as age, duration of the disease, metabolic status, treatment, or presence of concomitant diseases.

Based on the performed analysis, we can speculate that these two vascular beds may be affected asynchronously and some other factors are in play. Therefore, further studies of the relationship between skin microcirculation and retinopathy presence need a specifically designed protocol with repeated assessments of both vascular beds.

Based on our results, we postulate that regular assessment of microvascular reactivity by capillaroscopy may be a useful method for identification of microangiopathic complications in patients with later onset of type 1 diabetes.

Based on our results, we postulate that regular assessment of skin microvascular reactivity by capillaroscopy and L-arginine infusion may extend the spectrum of methods used in microangiopathy studies in type 1 diabetes.

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J.N.-G. and G.M.K. partly designed the study and wrote the manuscript. J.N.-G. examined patients and performed analysis. B.W., S.S., and W.M.N. contributed to the discussion. B.W. recruited patients. S.S. edited the manuscript. L.B. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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