ASSOCIATION OF CAROTID ATHEROSCLEROSIS AND PERIPHERAL ARTERY DISEASE IN PATIENTS WITH TYPE 2 DIABETES: RISK FACTORS AND BIOMARKERS

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BACKGROUND: Carotid atherosclerosis (CA) and lower extremity peripheral artery disease (PAD) is a common and potentially life-threatening comorbidity in diabetes.
AIM: to determine risk factors and biomarkers of the association of CA and PAD in patients with type 2 diabetes.
MATERIALS AND METHODS: A single-center cross-sectional comparative study was carried out. Three hundred ninety one patients with type 2 diabetes were included. Duplex ultrasound of carotid and low limb arteries, screening/monitoring of diabetic complications and associated diseases, and assessment of glycemic control, biochemical and coagulation parameters were performed. Factors involved in vascular wall remodeling, including calponin-1, relaxin, L-citrulline, matrix metalloproteinase-2 and -3, were measured in blood serum by ELISA.
RESULTS: The signs of CA and PAD were observed in 330 and 187 patients respectively. In 178 patients, both CA and PAD were revealed. The risk of combined involvement of carotid and lower extremity arteries was higher in patients with diabetic retinopathy (OR=2.57, p<0.001), chronic kidney disease (OR=4.48, p<0.001), history of myocardial infarction (OR=5.09, p<0.001), coronary revascularization (OR=4.31, p<0.001) or cerebrovascular accident (OR=3.07, p<0.001). In ROC-analysis, age ≥65.5 years (OR=3.43, p<0.001), waist-to-hip ratio ≥0.967 (OR=3.01, p=0.001), diabetes duration ≥12.5 years (OR=3.7, p<0.001), duration of insulin therapy ≥4.5 years (OR=3.05, p<0.001), duration of arterial hypertension ≥16.5 years (OR=1.98, p=0.002), serum L-citrulline ≥68 μmol/l (OR=3.82, p=0.003), and mean amplitude of glucose excursions ≥3.72 mmol/l (OR=1.79, p=0.006) were the risk factors for atherosclerosis of two vascular beds. In multivariate logistic regression analysis, age, diabetes duration and waist-to-hip ratio were independent risk factors for association of CA and PAD (p=0.005, p=0.0003, and p=0.004 respectively).
CONCLUSION: In subjects with type 2 diabetes, carotid and lower extremity atherosclerotic disease is associated with age, diabetes duration, abdominal obesity, microvascular and macrovascular complications, glucose variability, and high serum levels of L-citrulline.

KEYWORDS: type 2 diabetes; carotid atherosclerosis; peripheral artery disease; risk factor; biomarker

СОЧЕТАННОЕ АТЕРОСКЛЕРОТИЧЕСКОЕ ПОРАЖЕНИЕ СОННЫХ АРТЕРИЙ И АРТЕРИЙ НИЖНИХ КОНЕЧНОСТЕЙ У БОЛЬНЫХ САХАРНЫМ ДИАБЕТОМ 2 ТИПА: ФАКТОРЫ РИСКА И БИОМАРКЕРЫ

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ОБОСНОВАНИЕ. Атеросклероз сонных артерий (СА) и артерий нижних конечностей (АНК) — распространенные и потенциально жизнеугрожающие коморбидные состояния при сахарном диабете (СД).
ЦЕЛЬ. Определить факторы риска и биомаркеры сочетанного атеросклеротического поражения СА и АНК у больных СД 2 типа (СД2).
МАТЕРИАЛЫ И МЕТОДЫ. Проведено одноцентровое поперечное сравнительное исследование. В исследование включен 391 больной. Всем пациентам выполнены ультразвуковое дуплексное сканирование СА и АНК, скрининг/мониторинг осложнений диабета и ассоциированных состояний, исследованы параметры гликемического контроля, биохимические параметры, показатели гемостаза. В сыворотке крови с помощью иммуноферментного анализа определяли факторы, вовлеченные в ремоделирование сосудистой стенки: кальпонин-1, релаксин, L-цитруллин, матриксные металлопротеиназы-2 и -3.
РЕЗУЛЬТАТЫ. Признаки атеросклероза СА и АНК выявлены у 330 и 187 больных соответственно. У 178 пациентов выявлено сочетание атеросклероза СА и АНК. Риск сочетанного поражения СА и АНК был выше у больных с диабетической ретинопатией (ОШ=2,57; p<0,001), хронической болезнью почек (ОШ=4,48; p<0,001), инфарктом миокарда (ОШ=5,09; p<0,001), коронарной реваскуляризацией (ОШ=4,31; p<0,001) или острым нарушением мозгового кровообращения (ОШ=3,07; p<0,001) в анамнезе. В ROC-анализе факторами риска сочетанного атеросклероза СА и АНК являлись: возраст ≥65,5 года (ОШ=3,43; p<0,001), соотношение окружность талии/окружность бедер (ОТ/ОБ) ≥0,967 (ОШ=3,01; p=0,001), длительность СД ≥12,5 года (ОШ=3,7; p<0,001), длительность инсулинотерапии ≥4,5 года

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(ОШ=3,05; p<0,001), длительность артериальной гипертензии ≥16,5 года (ОШ=1,98; p=0,002), уровень L-цитруллина сыворотки ≥68 мкмоль/л (ОШ=3,82; p=0,003), средняя амплитуда колебаний гликемии (MAGE) ≥3,72 ммоль/л (ОШ=1,79; p=0,006). В многофакторном логистическом регрессионном анализе независимыми факторами риска атеросклероза СА и АНК были возраст (p=0,005), длительность СД (p=0,0003) и ОТ/ОБ (p=0,004).

ЗАКЛЮЧЕНИЕ. Сочетанное поражение СА и АНК у больных СД2 ассоциировано с возрастом, длительностью СД, абдоминальным ожирением, микрососудистыми и макрососудистыми осложнениями, вариабельностью гликемии, высоким уровнем L-цитруллина.

КЛЮЧЕВЫЕ СЛОВА: сахарный диабет 2 типа; атеросклероз сонных артерий; макроангиопатия нижних конечностей; фактор риска; биомаркер

BACKGROUND

Peripheral artery disease (PAD) and carotid artery disease are common and dangerous manifestations of atherosclerosis in patients with diabetes mellitus (DM). PAD is a risk factor for diabetic foot and lower limb amputations, which are complications that have a significant impact on the quality of life and disability of patients with diabetes. Five-year mortality rate in patients with critical lower limb ischemia exceeds the mortality rate associated with breast or colorectal cancer [1]. Carotid atherosclerosis (CA) is associated with the risk of cerebrovascular accidents [2]. Diabetic patients with hemodynamically significant carotid stenosis are at extremely high risk of cardiovascular death [3].

The incidence of atherosclerosis in diabetic patients is known to be 2-4 times higher than that in general population [1]. This higher risk of atherosclerosis in patients with type 2 diabetes mellitus (T2DM) is related to a large number of abnormalities including accumulation and changes in the collagen and elastin structure, changes in the contractility of the vascular wall, an increased concentration of the advanced glycation end products [4], overexpression of adhesion molecules, oxidative stress, activation of inflammatory signaling pathways, dyslipidemia, hypertension, and increased rigidity of the arterial wall [5].

Despite the fact that the classical risk factors for atherosclerosis such as hypertension, smoking, dyslipidemia and others are well known, they cannot explain predominant involvement of one or another vascular territory in a specific diabetic patient. Therefore, it is necessary to continue studying the mechanisms of development, to search for mediators and biomarkers of vascular lesions of different location. It is also important to identify the risk factors and biomarkers of combined atherosclerotic lesions.

AIM OF THE STUDY

The aim of the study was to determine risk factors and biomarkers of the association of CA and PAD in patients with T2DM.

MATERIALS AND METHODS

Study sites, start and end dates

Patients were recruited to the study in Research Institute of Clinical and Experimental Lymphology — Branch of the Institute of Cytology and Genetics, Siberian Branch of Russian Academy of Sciences (RICEL — Branch of IC&G SB RAS; Novosibirsk, Russia) from January 2016 to December 2019.

Studied populations

The principal group included male and female patients >30 years of age with T2DM diagnosed at least one year earlier and receiving glucose-lowering therapy. The control group comprised persons without atherosclerotic lesions and with normal blood glucose levels.

Non-inclusion criteria included diabetic ketoacidosis or hyperosmolar hyperglycemic state at the enrollment to the study, history of malignancies, major connective tissue disorders, end-stage renal disease (ESRD) or renal replacement therapy.

Sample selection from the study population (or several samples from several study population)

Continuous sampling from the patients admitted to the clinic to receive specialized or high-tech medical care was used.

Study design

A single-center comparative cross-sectional study was performed. Clinical examination of the patients was conducted in accordance with a current version of national guidelines for diabetes care [6] and included screening/monitoring for diabetic complications and associated conditions, assessment of of glycemic control, blood biochemistry and blood coagulation parameters. Three hundred ninety one patients with T2DM were included.

Duplex ultrasound of carotid and low limb arteries was performed in all patients. Based on the results of these investigations, the patients were allocated to three groups. Group 1 included patients with no signs of atherosclerotic lesions in the evaluated arteries (CA-, PAD-). Group 2 included patients with CA (CA+, PAD-). Group 3 included patients with signs of CA and PAD (CA+, PAD+).

Age, sex, smoking status, diabetes duration, body mass index (BMI), waist-to-hip ratio (WHR), diabetes complications and associated conditions, characteristics of glucose-lowering, lipid-lowering and antihypertensive therapy, glycemic control and glycemic variability parameters, lipid profile, kidney function, albuminuria, complete blood count and blood coagulation parameters were assessed as potential risk factors for combined involvement of the carotid arteries and lower limb arteries in models of multivariate logistic regression analysis and ROC analysis.

A substudy of biomarkers included 152 patients with DM (at least 40 patients per group) and 30 patients without DM and cardiovascular disorders (control group). The control group comprised 6 male and 24 female patients aged 41 to 65 years (median age 46 years). There were 6 active smokers and 24 non-smokers in this group. The following factors involved in the vascular wall remodeling were

measured in blood serum: calponin-1, relaxin, L-citrulline, matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-3 (MMP-3). Among them, calponin-1 is a marker of smooth muscle integrity [7], relaxin is an important regulator of the vascular tone [8], L-citrulline is involved in the nitric oxide (NO) production and nitrosamine stress [9], while MMP-2 and MMP-3 play an important role in atherogenesis and instability of atherosclerotic plaques [10].

Methods

Examination of the carotid arteries and lower limb arteries

Duplex ultrasound of the carotid arteries was performed by a certified specialist with the use of expert ultrasound diagnostic systems Vivid 7™ Dimension (*GE Healthcare*, USA) and RS85-RUS (*Samsung Medison*, Korea) with wideband ultrasonic transducers (7-10 MHz). Mannheim Carotid Intima-Media Thickness Consensus guidelines were applied for the results interpretation [11]. The investigation was conducted with examination of both common carotid, internal carotid and external carotid arteries along their entire length available for scanning. In the distal portion of the common carotid artery, the maximum intima-media thickness (IMT) was measured. The values of IMT ≥1.1 mm were considered as increased [12]. Focal increase in the IMT >50% compared to the adjacent vascular wall or a local area with the IMT >1.5 mm and protrusion into the vascular lumen was considered an atherosclerotic plaque.

Duplex ultrasound of the lower limb arteries was performed by a certified specialist using a high-resolution Vivid 7™ Dimension (*GE Healthcare,* USA) machine and a linear transducer (7-12 MHz). The examination was started from the branching of the common femoral artery; in case of blood flow acceleration >100 ms, the common and external iliac arteries were also examined. Then, examination of the common, deep and external femoral arteries, popliteal artery, tibioperoneal trunk, posterior and anterior tibial, peroneal arteries and the dorsalis pedis artery was performed. The intima-media complex was assessed along the entire length of these arteries, and the IMT was measured at the level of the femoral and popliteal arteries. IMT >50% compared to the adjacent vascular wall was considered abnormal. Local increase in the IMT >2 mm was considered an atherosclerotic plaque. The severity of stenosis was assessed by the ratio of the peak systolic velocity in the area of stenosis and prestenotic arterial segment; the ratio >2 was regarded as a sign of hemodynamically significant stenosis [13].

Laboratory tests

The level of glycated hemoglobin (HbA1c) was measured by turbidimetry using a biochemical analyzer AU480 (*Beckman Coulter,* USA). Glycemic variability (GV) was assessed based on three daily measurements of fasting blood glucose and three daily measurements of blood glucose 2 h after meals over a period of 5 days. Measurements were carried out using a blood glucose meter *One Touch Verio®* (*Johnson & Johnson / LifeScan,* USA). Using an *EasyGV* calculator (version 9.0.R2) [14], the mean glucose level, MAGE (*Mean Amplitude of Glycemic Excursions*) and LBGI (*Low Blood Glucose Index*) were calculated.

Blood biochemistry parameters, including glucose, total cholesterol, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, triglycerides, creatinine and uric acid, as well as urine creatinine, were measured using an AU480 biochemical analyzer (*Beckman Coulter*, USA). Estimated glomerular filtration rate (eGFR) was calculated by the CKD-EPI formula (2009). Urine albumin was determined by immunoturbidimetry using a biochemical analyzer AU480 (*Beckman Coulter,* USA). Plasma concentrations of fibrinogen, soluble fibrin monomer complex (SFMC), and D-dimer were determined using an automated hemostasis analyzer *ACL Elite Pro* (*Instrumentation Laboratory,* USA). Complete blood count tests were performed using a hematology analyzer BC-5300 (*Mindray Medical International Limited,* China).

The concentrations of factors involved in the vascular remodeling were assessed in blood serum by ELISA. The level of calponin-1 was determined using *CUSABIO Technology* test kit (USA), the level of relaxin and L-citrulline — using *Immundiagnostik AG test kit* (Germany), MMP-2 — using *Abcam* test kit (UK), MMP-3 — using *BCM Diagnostics* test kit (USA).

Statistical analysis

The normality of data distribution was assessed using the Kolmogorov-Smirnov test. To compare two or several groups, the Student's test or the analysis of variance (ANOVA) were used, respectively, if the quantitative parameters had normal distribution; otherwise, the nonparametric Mann-Whitney U–test or the Kruskal-Wallis H–test were used. The differences between the categorical variables were assessed using the χ^2 (chi-square) test. The differences were considered significant if $P<0.05$. Spearman's Rank correlation coefficient was used to examine the relationship between variables. For most statistical procedures, Statistica 13.0 software package (Dell, USA) was applied. The sample size was calculated with a preset type I error rate of $\alpha = 0.05$ and a power of $1–\beta = 90\%$. Quantitative variables are presented as medians and interquartile intervals.

To assess the risk factors of atherosclerosis, the Receiver Operating Characteristic (ROC) Curve Analysis was conducted using IBM SPSS Statistics 26.0 (IBM, USA) software package. The area under the ROC curve (AUC), 95% confidence intervals (CI) and *p*-values were calculated. The results were considered significant if the AUC with a lower limit of 95% CI was >0.5, and the *p* value was <0.05. The cut-off values were selected with the sensitivity (Se) and specificity (Sp) >0.55. The evaluation of the laboratory parameters with other than normal distribution (calponin-1, relaxin, L-citrulline and MMR-2) was carried out using the respective logarithmic values (decimal logarithm, Lg).

The significance of clinical and laboratory parameters was assessed using logistic regression analysis with purposeful selection of variables. The values of biomarkers and basic laboratory parameters as possible risk factors for atherosclerosis of the brachiocephalic and lower limb arteries were evaluated with adjustment for demographic factors and clinical parameters. Models with fewer uncorrelated variables, lower p values and the highest AUC, Se and Sp were selected. For the parameters included in the models, uncorrected and adjusted odds ratios (OR), 95% CI and *p*-values were calculated. The characteristics of the models (the value of the free term, the value of p, Se, Sp) and the logistic regression cut-off value (Lp) are provided.

Ethical review

The study was approved by the Ethics Committeel of RICEL — Branch of IC&G SB RAS (Protocol No.115, December 24, 2015). All patients gave their written informed consent to participate in the study.

RESULTS

General characteristics of patients included in the study

The study included 391 patients with T2DM (113 male and 287 female patients) aged 36 to 88 years (median: 65 years). Mean duration of the disease since the time of the diagnosis was 13 years (range: 1 year to 38 years), mean HbA1c — 8.5% (range: 4.9% — 15.8%). Patients were receiving combine glucose-lowering therapy that included metformin (n=321), sulfonylurea (n=128), sodium-glucose co-transporter 2 inhibitors (SGLT2i; n=53), dipeptidyl peptidase-4 inhibitors (DPP4i; n=50), and insulin (n=293). Most patients had chronic complications of diabetes and associated conditions: peripheral neuropathy (n=336; 88%), autonomic neuropathy (n=270; 69%), diabetic retinopathy (DR, n=197; 52%), CKD (n=132, 32%), hypertension (HP, n=377, 92%), coronary artery disease (CAD, n=151, 37%). Diabetic foot syndrome was diagnosed in 27 patients (7%), among them, 10 and 17 individuals respectively had neuropathic and neuroischemic form of the syndrome. Dyslipidemia was revealed in 356 examined patients (91%); 173 patients (44%) received regular lipid-lowering therapy: 154 patients were receiving statins; 19 patients — statins and fenofibrate. 179 and 154 patients reported having relatives with diabetes or cardiovascular diseases, respectively.

Characteristics of patients with T2DM, CA and PAD

Fifty-two (13.3%) patients did not demonstrate atherosclerotic lesions in the carotid and lower limb arteries; these patients comprise Group 1 (CA- PAD-). Sonographic signs of CA were revealed in 330 patients (84%), including 131 -ones with an increased IMT and 199 patients with atherosclerotic plaques. The number of patients with carotid artery stenosis was 134 (29.5% of all patients included in the study or 40% of patients with CA). 39 patients had hemodynamically significant arterial stenosis. In 152 (40%) patients, CA was not accompanied by PAD; these patients comprised Group 2 (CA+ PAD-). PAD was diagnosed in 187 (47.8%) of the examined patients; 58 of them had stenosis of at least one artery (which was hemodynamically significant in 7 patients), and 64 patients had occlusion of at least one artery. Chronic lower limb ischemia was diagnosed in 82 patients; 8 patients had a history or amputations (including 7 people with a history of minor amputations [one or several toes), and one patient had a history of amputation at the level of the upper third of the lower leg due to phlegmon of his foot. There were no patients with critical lower limb ischemia in our study group. In 9 patients, PAD was not accompanied by CA. Due to a small number of observations, these patients were not included in the subsequent analysis. Patients with CA in combination with PAD (n=178; 46.5%) were included in Group 3 (CA+ PAD+).

Clinical characteristics of patient groups are provided in Table 1. Patients with lesions in two vascular territories were older and had a longer duration of DM and a lower BMI, compared to those with CA and no PAD or to patients without any signs of atherosclerosis. Both groups of patients with atherosclerosis had a lower BMI compared to patients without CA and PAD, but did not differ from each other in terms of this parameter. The WHR was higher in CA+PAD+ group - compared to CA+PAD- group. Moreover, patients with involvement of two vascular territories had longer duration of hypertension, were more likely to have diabetic retinopathy and coronary artery disease, as well as a history of myocardial infarction, coronary revascularization and cerebrovascular events. Patients with combined atherosclerosis had higher values of the carotid artery IMT.

Patients in the CA+PAD+ group more often received insulin therapy compared to the patients examined (see Table 2). The duration of insulin therapy was also longer in this group -. The frequency of use of other groups of glucose-lowering therapy did not differ between the groups. Patients with combined involvement of the carotid and lower limb arteries more often received beta-blockers, statins and aspirin.

Median HbA1c value was 0.9% higher in patients with isolated and combined carotid atherosclerosis compared with patients without atherosclerosis, although the differences between the groups were not statistically significant (Table 3). Furthermore, MAGE and LBGI were significantly higher in patients with involvement of two vascular territories. The levels of total cholesterol and LDL cholesterol were lower in the group of patients with combined atherosclerosis. There were no significant differences in the level of triglycerides, uric acid and albuminuria between the groups. eGRR was lower in patients with involvement of two vascular territories. SFMC and fibrinogen concentrations in groups of patients with atherosclerosis were significantly higher than in patients without atherosclerosis, and the fibrinogen level was significantly higher in patients with combined atherosclerotic lesions compared to other patient groups. There were no differences in terms of D-dimer levels (see Table 3) or complete blood count parameters (data not shown).

Concentration of factors involved in vascular remodeling in T2DM patients with atherosclerosis of the carotid arteries and lower limb arteries

As a whole group, patients with diabetes had higher levels of calponin-1 and tended to have lower relaxin levels compared to non-diabetic subjects: 7.7 (4.4 — 12.9) and 6.5 (5.6 — 8.2) ng/mL; p=0.03 and 90 (26 — 182) and 146 (57 — 166) pg/mL; p=0.13, respectively. In diabetic patients, the levels of L-citrulline were similar to those in the comparison group: 34.5 (0 — 153) and 20.2 (15 — 48) μmol/L; p=0.48, and this parameter was significantly higher in patients with atherosclerosis of the carotid and lower limb arteries compared to other patient groups (Table 3). The study revealed correlations between the concentration of L-citrulline and the number of atherosclerotic plaques in the carotid arteries (r=0.22, p=0.005), hemoglobin level (r=-0.5, p<0.0001) and the urine albumin-creatinine ratio (r=0.57, p<0.0001).

Serum MMP-2 levels tended to be higher in diabetic patients compared to the control group: 314.4 (208.4 — 429.6) and 254 (167.8 — 341.3) ng/ml; p=0.06, however, the differences between the groups of patients were not significant. MMP-3 levels were similar in the diabetic group and non-diabetic subjects: 14.8 (10.5 — 24.5) and 13.5 (8.9 — 20.2) ng/mL; p=0.63. However, MMP-3

Table 1. Clinical characteristics of patient groups

Parameter	Patient groups		
	CA-PAD- (n=52)	CA+PAD- (n=152)	CA+PAD+ (n=178)
Sex, M/F, n (%)	9/43 (21/79)	49/103 (32/68)	52/126 (29/71)
Age, years	56 (50 — 62)	64 (57 — 69)**	68 (62.5 — 71)***###
Duration of DM, years	7 (4 — 13)	10 (6 — 15)	15.5 (10 — 22)***###
BMI, kg/m^2	37.7 (32.7 — 42.8)	32.8 (29.4 — 36.8)*	32.1 (28.9 — 37.2)**
WHR	0.97 (0.9 — 1.04)	0.94 (0.91 — 0.99)	0.98 (0.93 — 1.07)###
Smoking, n (%)	6 (11.5)	18 (11.8)	19 (10.7)
Diabetic retinopathy, n (%)	20 (38.5)	64 (43)	113 (63)**##
CKD, n (%)	12 (23.1)	45 (30)	66 (37)
Arterial hypertension, n (%)	48 (92.3)	147 (97)	177 (99.4)
Duration ofarterial hypertension, years	11 (7 — 17)	15 (6 — 22)	18 (10 — 28)**##
Coronary artery disease, n (%)	8 (15.4)	36 (23.4)	105 (58.7)***###
Myocardial infarctiion inmedical history, n (%)	1 (1.9)	9 (7)	37 (21)***###
Coronary revascularization in medical history, n (%)	3 (5.8)	8 (6)	36 (21)*###
Cerebrovascular event in medical history, n (%)	0	8 (6)	27 (16)** ##
CA IMT on the left, mm	0.7 (0.6 — 0.8)	1.4 (1.2 — 1.9)***	1.7 (1.2 — 2.5)***#
CA IMT on the right, mm	0.7 (0.6 — 0.8)	1.4 (1.1 — 2.2)***	1.8 (1.2 — 2.8)***###

Comments. Here and in Table 2,3: the data are shown as medians and interquartile intervals.
* p<0.05, ** p<0.01, *** p<0.001 versus Group CA-PAD-; # p<0.05, ## p<0.01, ### p<0.001 versus Group CA+PAD-.

Table 2. Therapy in patients with T2DM

Parameter	Patient groups		
	CA- PAD- (n=52)	CA+ PAD- (n=152)	CA+ PAD+ (n=178)
Metformin, n (%)	36 (69.2)	128 (84.2)	155 (86.3)
Sulfonylurea, n (%)	19 (36.5)	52 (34.2)	55 (30.9)
DPP-4i, n (%)	6 (11.5)	22 (14.5)	21 (11.8)
SGLT2i, n (%)	4 (7.7)	23 (15.1)	26 (14.8)
Insulin, n (%)	30 (57.7)	108 (71)	154 (87)***###
Duration of insulin therapy, years	2 (0.1; 6)	4 (1; 7)	8 (3; 12)***###
Insulin dose, IUs/day	37 (22; 76)	50 (32; 64)	50 (32; 68)
Insulin dose, IU/kg/day	0.47 (0.26; 0.7)	0.54 (0.32; 0.71)	0.58 (0.36; 0.8)
ACEi, n (%)	16 (30.8)	58 (37.7)	66 (36.9)
ARB, n (%)	21 (40.4)	60 (39)	86 (48)
Diuretics, n (%)	19 (36.5)	68 (44.2)	95 (53.1)
Calcium channel inhibitors, n (%)	17 (32.7)	51 (33.1)	69 (38.5)
Beta-blockers, n (%)	19 (36.5)	60 (39)	96 (53.6)**##
Centrally-acting antiadrenergic agents, n (%)	6 (11.5)	16 (10.4)	31 (17.9)
Statins, n (%)	17 (32.7)	47 (32)	109 (61)***###
Aspirin, n (%)	12 (23)	80 (52.6)***	129 (72.5)***###

Table 3. Laboratory parameters in patients with T2DM with and without CA and PAD

Parameter	Patient groups		
	CA-PAD- (n=52)	CA+PAD- (n=152)	CA+PAD+ (n=178)
HbA1c, %	7.4 (6.2 — 10.3)	8.3 (7.1 — 9.5)	8.3 (7.1 — 10.1)
MAGE, mmol/L	3.0 (1.8 — 4.0)	3.5 (2.5 — 4.6)	4.0 (2.8 — 5.2)*#
LBGI	0 (0 — 0.22)	0.06 (0 — 0.65)*	0.31 (0 — 1.28)*****#
Total cholesterol, mmol/L	5.1 (4.4 — 6.0)	5.3 (4.6 — 6.3)	4.8 (4.0 — 5.9)###
LDL cholesterol, mmol/L	3.3 (2.9 — 3.9)	3.4 (2.7 — 4.1)	3.0 (2.4 — 3.9)##
HDL cholesterol, mmol/L	1.2 (1.0 — 1.4)	1.2 (1.0 — 1.4)	1.1 (1.0 — 1.4)
Triglycerides, mmol/L	2.5 (1.6 — 3.1)	2.1 (1.4 — 3.2)	1.8 (1.2 — 2.4)##
Uric acid, μmol/L	340 (263 — 389)	331 (282 — 393)	313 (251 — 395)
eGFR, mL/min/1.73 m^2	74 (63 — 90)	68 (56 — 82)	64 (54 — 76)*#
Urinary albumin-to-creatinine ratio, mg/mmol	1 (0.5 — 2.5)	1 (0.4 — 4.3)	1.4 (0.5 — 4.8)
Fibrinogen, mmol/L	3.9 (3.3 — 4.4)	4.3 (3.6 — 4.9)*	4.4 (3.6 — 5.3)****#
SFMC, mg/dL	7.25 (3.5 — 14)	11 (4.75 — 17)**	12 (4.5 — 19)**
D-dimer, ng/mL	265 (229 — 336)	276 (237 — 321)	273 (244 — 330)
Calponin-1, ng/mL	6.4 (5.7 — 7.8)	6.3 (5.7 — 7)	6.7 (5.6 — 8.7)
Relaxin, pg/mL	140 (59 — 159)	152 (93 — 167)	136 (44 — 167)
L-Citrulline, μmol/L	48 (0 — 153)	9 (0 — 50)*	134 (18 — 345)###
MMP-2, ng/mL	319 (220 — 414)	353 (207 — 469)	318 (203 — 400)
MMP-3, ng/mL	9.7 (7.9 — 14.5)	15.9 (7.4 — 22.8)*	15.3 (9.9 — 21.8)**

concentrations in the CA+PAD- and CA+PAD+ groups were higher compared to the CA-PAD- group (see Table 3). MMP-3 levels were similar in patients with CA and patients with combined lesions (CA and PAD).

There were no significant differences in the levels of biomarkers in patients with hemodynamically insignificant and hemodynamically significant stenosis (p>0.05 in all comparisons).

Risk factors for combination of CA and PAD in patients with T2DM

The risk of concomitant involvement of the coronary arteries and lower limb arteries turned out to be higher in patients with a history of diabetic retinopathy, CKD, myocardial infarction, coronary revascularization or cerebrovascular events (Fig. 1).

In ROC analysis the risk factors of combined involvement of the carotid arteries and lower limb arterieswere identified: age ≥65.5 years, WHR ≥0.967, diabetes duration ≥12.5 years, duration of insulin therapy ≥4.5 years, duration of hypertension ≥16.5 years, serum L-citrulline level ≥68 μmol/L, MAGE ≥3.72 mmol/L (Table 4).

In the multivariate logistic regression (Table 5), combination of CA and PAD was associated with age (+8% for each year), diabetes duration (+11% for each year of illness) and WHR (+6% for every 0.01 unit ratio).

Adverse events

No adverse events were reported during the study.

DISCUSSION

Our study was aimed at evaluating risk factors and searching for potential biomarkers of combined involvement of the carotid arteries and lower limb arteries in patients with T2DM.

Sample representativeness

A large number of included patients (n=391), continuous selection of patients, a minimum set of –non-inclusion criteria allow us to assume that the sample is generally representative for a population of patients with T2DM receiving specialized and high-tech medical care in inpatient settings.

Comparison with other publications

The proportion of patients with CA in our sample was quite high (84%). This may partly be due to the fact that the study was conducted in a sample of inpatients with long duration of DM. The proportion of patients with PAD was almost one half of that value (48%). It is noteworthy that almost all patients with PAD had signs of carotid atherosclerosis. Differences in the prevalence of vascular lesions in the two locations can be explained by discrepancy in diagnostic criteria or earlier development of abnormalities in the carotid arteries compared to lower limbones.

Previous studies have shown that the development of carotid atherosclerosis in patients with T2DM is dependent on a combination of general characteristics (age, hypertension, dyslipidemia, smoking, etc.), as well as

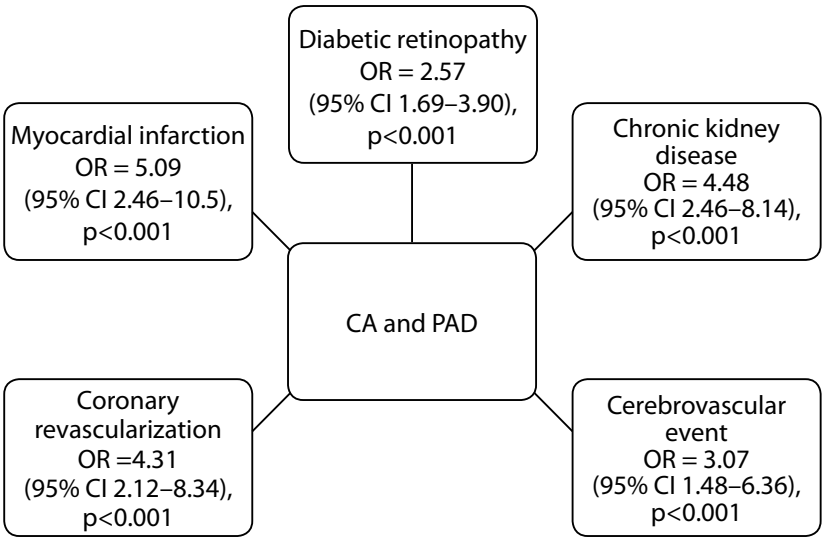

Figure 1. Associations of CA and PAD with other vascular complications in patients with T2DM.
CA, coronary atherosclerosis; PAD, peripheral artery disease

Table 4. Risk factors for CA and PAD in patients with T2DM. The results of ROC analysis

Factor	Cut-off point	AUC±SE, 95% CI, p-value	Se, %	Sp, %	OR, 95% CI, p-value
Age	≥65.5 years	0.69±0.03 (0.63 — 0.74), p<0.001	62.8	67.0	3.43 (2.24 — 5.24), p<0.001
WHR	≥0.967	0.64±0.05 (0.55 — 0.73), p=0.004	62.8	64.1	3.01 (1.54 — 5.89), p=0.001
Duration of DM	≥12.5 years	0.70±0.03 (0.64 — 0.75), p<0.001	66.1	65.5	3.70 (2.41 — 5.67), p<0.001
Duration of insulin therapy	≥4.5 years	0.68±0.03 (0.61 — 0.74), p<0.001	67.3	59.7	3.05 (1.84 — 5.04), p<0.001
Duration of arterial hypertension	≥16.5 years	0.61±0.03 (0.55 — 0.67). p<0.001	58.4	58.5	1.98 (1.29 — 3.05), p=0.002
Serum L-citrulline	≥68 μmol/L	0.68±0.06 (0.56 — 0.80), p=0.004	65.6	66.7	3.82 (1.57 — 9.31). p=0.003
MAGE	≥ 3.7 mmol/L	0.58±0.03 (0.52 — 0.64), p=0.006	56.7	57.7	1.79 (1.19 — 2.69), p=0.006

Table 5. Risk factors for CA and PAD in patiets with T2DM . The results of multivariate regression analysis

Parameter	Corrected OR	95% CI	P-value
Age (years)	1.08	1.02 — 1.14	0.005
Duration of DM, years	1.11	1.05 — 1.17	0.0003
WHR, 0.01 U	1.06	1.02 — 1.10	0.004

Model parameters: Free term = -11.9, p-value for KS tests < 0.001, area under the ROC curve = 0.78, Se = 69.9%, Sp = 70.3% for L_p = 0.61.

diabetes-specific risk factors (hyperglycemia, insulin resistance) [15-18]. Our results are generally consistent with these data. In this study we established the cut-off values of the following risk factors of combined involvement of the carotid and lower limb arteries: age ≥65.5 years, WHR ≥0.967, diabetes duration ≥12.5 years, duration of insulin therapy ≥4.5 years, and duration of hypertension ≥16.5 years. Age, diabetes duration and the WHR were identified to be ndependent risk factors in the model of multivariate stepwise logistic analysis .

The significance of some widely discussed risk factors were not confirmed in our study. In particular, we showed no correlation between male sex and the risk of atherosclerosis. This may be due to the predominance of female patients in our sample, that is a feature of T2DM population in Russia [19]. Furthermore, women with diabetes were slightly older than men*, which could eliminate the influence of gender as a risk

* Mean age of female and male patients was 66 (60-70) and 61.5 (55-68) years, respectively, p=0.0004.

factor in the analysis. In our study, patients with carotid atherosclerosis and PAD had lower levels of LDL-cholesterol and triglycerides compared to other groups of patients, which can probably be explained by the higher frequency of the use of lipid-lowering therapy in this group. Our study also did not demonstrate the role of smoking as a risk factor. The overall number of smokers in the study population was rather small (44 individuals, 11.3%). However, we didn't take into account ex-smoking status and smoking intensity.

In our cohort, the duration of diabetes ≥12.5 years was an independent risk factor of atherosclerosis of the carotid arteries and lower limb arteries. However, there was no association with the HbA1c level. It is possible that the duration of exposure to hyperglycemia is more important for the development of atherosclerosis thanglucose level. According to the results of the ROC analysis, high glycemic variability (estimated as MAGE values ≥3.72 mmol/L) was associated with combined atherosclerotic lesions. Earlier studies described the relationship between the glycemic variability and CA IMT and carotid artery stenosis in patients with T2DM [18, 20]. There is growing evidence of the association between the glycemic variability and the cardiovascular risk [21]. The effect of glycemic variability on the vascular wall remodeling can be mediated by oxidative stress, non-enzymatic glycation, chronic inflammation, endothelial dysfunction, platelet activation, angiogenesis disorders and renal fibrosis [22].

The study has demonstrated a higher frequency of other microvascular complications (diabetic retinopathy, CKD) and macrovascular lesions (coronary artery disease, cerebrovascular accidents) in DM patients with both CA and PAD. The close relationship of vascular complications can be explained both by the common nature of risk factors and by their mutually aggravating course. In particular, CKD is considered a trigger for atherosclerosis progression [16, 17]. In our study, eGFR was statistically significantly lower in patients with combined atherosclerosis compared to those with isolated CA. However, we could not demonstrate the role of CKD as a risk factor in the ROC analysis and multivariate analysis, which could be due to a small number of patients with renal impairment in this cohort.

In addition to the traditional risk factors, we evaluated the significance of some molecules involved in the vascular remodeling and atherogenesis (calponin-1, relaxin, L-citrulline, MMP-2, MMP-3) as potential biomarkers of atherosclerosis. The selection of these regulatory factors was based on their physiological effects. Calponin-1, a thin filament-associated protein, is expressed in smooth muscle cells and plays a role in smooth muscle contractility [7]. Relaxin is a peptide hormone that regulates vascular tone [8]. L-Citrulline is an essential alpha amino acid with antioxidant and vasodilator properties [9]. Matrix metalloproteinases (MMP-2, MMP-3) are involved in the collagen metabolism, vascular remodeling, repair and angiogenesis [10]. Patients with T2DM had higher levels of calponin-1 and MMP-3; L-citrulline levels were associated with the development of CA and PAD. Earlier studies demonstrated the association between the serum level of L-citrulline and carotid artery stenosis in patients with T2DM [18]. L-citrulline has been shown to be a cardiovascular risk factor in people without cardiovascular diseases [23]. It has been shown that the L-arginine/NO/L-citrulline pathway are related to the inflammation and oxidative stress in diabetic patients [24].

Clinical significance of the study results

The data obtained indicate more frequent development of CA compared to PAD in patients with T2DM. This indicated the need for ultrasound examinations of the carotid arteries in all patients with T2DM and PAD. The cut-off values for the risk factors estimated in our study using the ROC analysis can be used in clinical practice.

Study limitations

The limitations of the study include recruitment of patients in one clinical center and a cross-sectional design, which does not prove causality. The imbalance of the sample in a number of parameters could contribute to the appearance of a type 2 error, when the significance of a number of risk factors remained unaccounted for (smoking could be an exemple). The presence of stenosis/occlusions was not evaluated specifically in this study. Non-diabetic subjects included in the control group were generally younger than patients with diabetes because all screened patients over 65 had either cardiovascular disorders or asymptomatic CA.

Directions for further research

Further cost-effectiveness studies are required to evaluate the inclusion of Duplex ultrasound of the carotid arteries in the standards of medical care for diabetic patients. Programs for secondary and tertiary prevention of CA and PAD in diabetic patients should also be developed. Further studies are needed to evaluate the role of L-citrulline metabolism disorders in the pathogenesis and prognosis of diabetes mellitus.

CONCLUSION

The study has demonstrated a high prevalence of asymptomatic carotid artery disease in subjects with T2DM. Almost in one half of cases, the signs of carotid atherosclerosis are associated with with PAD. Carotid and lower limb atherosclerosis is associated with microvascular (diabetic retinopathy, CKD) and macrovascular (coronary artery disease, myocardial infarction, cerebrovascular event) complications. The risk of multifocal atherosclerosis in patients with T2DM increases with age and diabetes duration, it isprobably associated with insulin resistance and glycemic variability. Serum levels of L-citrulline may be considered a promising marker of atherosclerosis.

ADDITIONAL INFORMATION

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Contribution of the authors. Koroleva E.A. — collection of clinical materials, statistical data analysis, manuscript preparation; Khapaev R.S. — duplex ultrasound, manuscript preparation; Lykov A.P. — special laboratory tests, manuscript preparation; Korbut A.I. — statistical data analysis, manuscript preparation; Klimontov V.V. — study concept and design, analysis of the results, manuscript preparation. All of the authors approved the final version of the manuscript before publication, agreed to be responsible for all aspects of the work, implying proper examination and resolution of issues relating to the accuracy or integrity of any part of the study.

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