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Preliminary report

Lack of evidence of the correlation between plasma Asymmetrical Dimethylarginine correlation and IMT in type 2 diabetic patients with chronic vascular complication

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Introduction. Patients with type 2 diabetes represent 50% of all sudden cardiac deaths. Disseminated arteriosclerotic lesions are the cause of vascular incidents that cause permanent disability resulting from lower limb amputations. Objectives. Our study was designed to investigate the relationship between asymmetrical dimethylarginine (ADMA), symmetric dimethylarginine (SDMA) plasma concentration and intima–media thickness (IMT) in subjects with diabetes mellitus without vascular complications (group A) and a group of diabetic patients diagnosed with diabetes micro- and macroangiopathy (group B). Patients and Method. The experimental groups included 42 diabetic patients. Group A – 22 patients (9 W and 13 M), free from vascular complications (mean age 55.83±7.37 years), group B – 20 patients (6 W, 14 M) with accompanying micro- and macropathic changes (mean age 63.80±8.79 years). Group C (n=22), the control group, consisted of healthy volunteers (12 W and 10 M), between the ages of 40 to 60 (mean age 51.16±6.39), selected in reference to the age and sex of the research group. The carotid artery intima-media complex thickness (IMT) was evaluated with the use of a duplex ultrasound. Conclusions. There was no correlation between ADMA and the maximal or mean IMT of the common carotid artery (CCA) and internal carotid artery (ICA). We demonstrated a correlation between SDMA concentration and CCA IMT. The results suggest that ICA IMT may serve as a marker of vascular complication among patients with diabetes.

Keywords: carotid artery, diabetes, IMT, macroangiopathy, microangiopathy

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Abbreviations: ADMA, asymmetrical dimethylarginine; CCA, common carotid artery; ICA, internal carotid artery; IMT, intima–media thickness; SDMA, symmetric dimethylarginine

INTRODUCTION

Diabetes is a group of metabolic diseases of various etiologies characterized by hyperglycemia. Experts predict that the number of diabetics will increase to 629 million people in 2045, which constitutes a 48% increase in the number of patients. It is reported that approximately 3.5–5 million people annually die from diabetes complications, which equals one death per 8 seconds. Acute complications associated with diabetes include hypoglycemia, hyperglycemia, ketoacidosis, coma, loss of consciousness and common infections. Chronic complications include microangiopathy and macroangiopathy associated with end stage renal failure, stroke, myocardial infarction, and diabetic foot syndrome which entails a high risk of amputation.

The endothelium is an integral part of the vessel wall and the largest organ of the body. Endothelial dysfunction includes morphological and functional changes, such as increased IMT (intima media thickness) or vasoconstriction and is thought to be the first initial step of atherosclerosis. High ADMA (asymmetric dimethylarginine) plasma concentration is associated with endothelium dysfunction. ADMA is an endogenous inhibitor of nitric oxide synthases (NOS) and causes a decrease of NO* availability – one of the most common vasodilating factors. ADMA concentration correlates positively with dyslipidemia, age, high blood pressure, chronic renal insufficiency, and diabetes. The association with IMT thickness has also been described (Doroszko *et al.,* 2008). Symmetric dimethylarginine (SDMA) is another product of post-translational methylation of arginine. SDMA does not inhibit NOS, however, it is stated that it could decrease NO* availability by competitive binding to NO* synthases and blocking enzyme active center for arginine according to Bode-Böger and others (Bode-Böger *et al.,* 2006). SDMA could be a useful marker of early-stage renal failure and a determinant of cardiovascular risk.

Molecular background changes in the ADMA and SDMA levels are associated with the activity of PRMTs. Protein arginine methyltransferases (PRMTs) mediate the methylation of a number of protein substrates of arginine residues and serve critical functions in many cellular responses, including cancer development, progression, aggressiveness, T-lymphocyte activation, and hepatic gluconeogenesis. There are nine members of the PRMT family, divided into 4 types (types I–IV) (Ji Hye Kim *et al.,* 2016).

Objectives

Our study was designed to investigate the relationship between ADMA, SDMA plasma concentration and intima–media thickness (IMT) in subjects with diabetes mellitus without vascular complications (group A) and a group of diabetic patients with established diabetes micro- and macroangiopathy (group B).

Differences between the study groups with respect to ADMA and SDMA levels have already been proved and published in another article (Fiodorenko-Dumas *et al.,* 2017).

Patients and Method

The research was approved by the Scientific Research Ethics Committee of the Wroclaw Medical University. All persons participating in the program were informed about the purpose of the studies to which they gave their consent. The study involved 42 patients with type 2 diabetes (15 W and 27 M), aged 40 to 60 (mean age 59.45±8.83 years), with the duration of diabetes ranging from 5 to 15 years (mean age 7.54±3.50 years), treated at the Department of Angiology, Hypertension and Diabetology of the Wroclaw Medical University. Two groups of patients were distinguished: **Group A** (n=22) – patients with type 2 diabetes with no vascular complications 9 W and 13 M, aged from 48 to 63 (mean 55.83±7.37 years), and the duration of diabetes ranging from 5 to 15 years (mean 5.32±1.70 years). The group included patients with negative history of chronic coronary heart disease, normal resting ECG, no symptoms of peripheral artery disease (ankle-brachial index >0.9), moderate IMT (<0.9 mm) of carotid arteries in duplex-doppler ultrasound, excretion of albumin with normal urine and free from diabetic retinopathy. **Group B** (n=20) – included diabetic patients micro- and/or macroangiopathy, 6 W and 14 M, aged from 55 to 71 years (mean 63.80±8.79 years) with the duration of diabetes ranging from 5 to 15 years (mean 10.25±7.16 years). The following were observed in this group of patients: diabetic retinopathy (early stage of the disease), confirmed by ophthalmoscopic examination or fluorescein angiography (based on ophthalmic documentation), diabetic nephropathy diagnosed on the basis of microalbuminuria or proteinuria, ischemic heart disease (diagnosed with coronary angiography), peripheral artery disease (ABI <0.9), cerebral arteriosclerosis and symptoms of transient ischemia of the CNS in medical history. **Group C** (n=22) consisted of healthy volunteers, 12 W and 10 M, aged 46 to 58 (mean 51.16±6.39), selected as a control group in reference to age and sex of the study group.

Individuals in whom any inflammation occurred within the previous 3 months or who developed vascular event (stroke or acute coronary syndrome) within the previous 6 months were excluded from the study. People taking glucocorticosteroids or non-steroidal anti-inflammatory drugs (except for 75–150 mg of acetylsalicylic acid daily), as well as people diagnosed with cancer, liver failure, renal failure or other serious comorbidities were also excluded.

Test methods

Examinations carried out on all patients qualified for the study program included a thorough analysis of the history of the disease and a thorough physical examination, determination of body mass index (BMI), repeatedly performed blood pressure measurements, as well as obtaining a resting ECG, chest X-ray, abdominal ultrasound and Doppler examination with colour coding of carotid arterial flow (Vivid 7 device). Lower limb blood pressure was measured with the use of the Doppler method with the calculation of ankle/brachial index (ABI) using the Vasodop VQ 4000 device with the ELCAT GmbH Vasolab 5000 software.

Morning venous blood samples were collected from the antecubital vein of all subjects in order to assess the peripheral blood count using the 16-parameter ABX MIKROS OT hematological analyser, lipid profile (LDL-cholesterol level was calculated using Friedewald equation), creatinine, urea, uric acid concentrations were determined using enzymatic method, and fibrinogen level was examined using the Clauss method. The percentage of HbA1c was additionally measured in patients with diabetes using the Roche Unimate set.

Highly sensitive CRP was assessed using the ELISA kit High Sensitivity C-Reactive Protein Enzyme Immunoassay (nr EIA-3954 DRG International Inc., USA). The results were given in mg/l.

Concentrations of ADMA, SDMA and L-arginine in blood plasma were determined with the use of the HPLC method with fluorescent detection. The SPE (solid phase) extraction method was applied in preparation of plasma for analysis, using Varian's SCX 50 columns. Before being dispensed onto the Water Co.'s Symmetry C18, 150×4.6 mm, 5 um chromatographic column, the obtained analytes were subjected to a process of derivatization, with an OPA reagent (o-diphtalaldehyd). Test samples and standards were eluted from the column in isocratic system, with a solution of 12% v/v acetonitrile in K-phosphate buffer (50 mM, pH 6.6), flow 1.1 mL/min and temperature of 35°C. Wavelengths of detector excitation and emission amounted to 340 and 450 mm, respectively. Varian's equipment, consisting of Pro Star 240 pomp, Pro Star 363 spectrofluorescent detector, Pro Star 410 automatic sample changer and Star Chromatography Workstation software v. 6.3, was used in HPLC analysis. The obtained values are expressed in μmol/L.

The IMT (intima-media thickness) of the carotid artery was evaluated semi-automatically with the Vivid 7 Dimension ultrasound (GE Healthcare). The measurements were performed on both sides of the common and internal carotid artery. Distal wall IMT in the common carotid artery was analyzed on a 15 mm section directly below the division. The evaluation area in the internal carotid artery included the artery bulb. The average and maximum IMT thickness was automatically obtained in the analyzed section of the common and internal carotid arteries. The results were expressed as the mean value (mm) of the measurements carried out on both sides of the homonymous arteries.

Mean CCA IMT and maximum ICA IMT are both proposed as markers of subclinical cardiovascular disease by two consensus groups (Stein *et al.,* 2007; Touboul *et al.,* 2007).

Statistical analysis

The results are presented as means ±S.D. Non-parametric tests were used in the analyses, including Mann Whitney, Kruskal-Wallis, Wilcoxon, and Spearman's correlation coefficient (Aczel 2006). In each statistical test, approximate or exact (depending on the number of observations), the p-value (the minimum materiality level at which there are no grounds for rejecting the tested hypothesis) was determined, which was used in statistical reasoning, concurrently increasing the credibility of the presented results (Francuz *et al.,* 2007). The *p*-value <0.05 was considered statistically significant.

RESULTS

The summary of the demographic data of subjects from both study groups are presented in Table 1. The development of vascular complications of diabetes was associated with longer duration of the disease ($p<0.05$). Waist circumference was higher in patients with vascular complications in comparison with patients without complications ($p<0.01$). Mean BMI and the incidence of hypertension in particular groups of patients were comparable.

Table 1. Demographic data of the studied group

Parameters	Type 2 diabetes patients		Control (Group C)	Significance of differences
	Patients with no vascular complications (Group A)	Patients with the diabetic micro and macroangiopathy (Group B)		
N	22	20	22	
Women	9	6	12	
Men	13	14	10	
Age (years)	55.83±7.37	63.80±8.79	51.16±6.39	A *vs* B; p=0.7324SN A *vs* C: p=0.6727SN B *vs* C: p=0.4034SN
Duration of diabetes (years)	5.32±1.70	10.25±7.16		A *vs* B; p=0.05
BMI (kg/m^2)	30.9±5.76	32.54±3.86	27.66±4.27	A *vs* B; p=0.4268SN A *vs* C: p=0.0887SN B *vs* C: p=0.06
Waist (cm)	102.00±9.84	112.30±8.34	99.83±9.01	A *vs* B; p=0.01 A *vs* C: p=0.7437SN B *vs* C: p=0.001
Hypertension (%)	72.7% of the studied subjects	80% of the studied subjects	18%	
Microangiopathy		Non-proliferative retinopathy (90% of the studied subjects)		
		Microalbuminuria (40% of the studied subjects)		
Macroangiopathy		Ischaemic heart disease (25% of the studied subjects)		

Results have been presented as mean values ± standard deviation. SN – statistically non-significant

Table 2. ADMA and selected parameters of the studied group.

	Type 2 diabetes patients		Control (Group C)	Significance of differences
	Patients with no vascular complications (Group A)	Patients with the diabetic micro and/or macroangiopathy (Group B)		
ADMA (µmol/l)	0.794±0.050	0.887±0.074	0.532±0.046	A *vs* B; p=0.1349NS A *vs* C: p=0.001 B *vs* C: p=0.001
SDMA (µmol/l)	0.842±0.103	0.902±0.161	0.443±0.071	A *vs* B; p=0.0941 NS A *vs* C: p=0.001 B *vs* C: p=0.001
L-arginine (µmol/l)	70.620±15.413	61.874±14.331	82.760±24.901	A *vs* B; p=0.3333NS A *vs* C: p=0.2415NS B *vs* C: p=0.0659NS

Results have been presented as mean values ± standard deviation. SN – statistically non-significant

Mean concentrations of ADMA, SDMA and L-arginine in patients' plasma are presented in Table 2. We observed high ADMA and SDMA concentration in both study groups and it was significantly higher than the control group (p<0.05) (Fiodorenko-Dumas *et al.,* 2017). There were no significant differences in the assessed parameters between group A and B. There was no correlation between ADMA and CCA IMT or ICA IMT. However, SDMA concentrations correlated significantly with mean CCA IMT (correlation coefficient 0.631, p<0.05) and max CCA IMT (correlation coefficient 0.622, p<0.05) in the group of patients with diabetic microangiopathy and/or macroangiopathy (Table 3).

Compared to control, the values of the IMT complex in the internal carotid artery (ICA), both mean and maximum, were higher in diabetic patients, but only between the group with microangiopathy and/or macroangiopathy and the control group, the differences were statistically significant (p<0.05) (Table 4).

CCA IMT was thicker in patients with diabetes compared to the control, particularly with reference to maximal CCA, however, differences between diabetics with and without vascular complication did not reach statistical significance. ICA IMT, both mean and maximum, were higher in diabetic patients, and the marked differences between the patients with vascular complications (group B) and the control group were statistically significant (p=0.0032 and p=0.005, respectively) (Fig. 1, Fig. 2).

The process of arterial wall remodeling, expressed mainly through the thickening of the common and internal carotid intima media complex, remains dependent on high HbA1c percentage (correlation coefficients: 0.766 and 0.851, p=0.01) which was observed in group B. Studies confirmed inflammatory response stimulation

Table 3. Correlations of ADMA with the proliferative lesions of the carotid artery wall

	Type 2 diabetes patients							
	Patients with no vascular complications (Group A)				Patients with the diabetic micro and/or macroangiopathy (Group B)			
Parameter	CCA average	CCA max	ICA mean	ICA max	CCA mean	CCA max	ICA mean	ICA max
ADMA (μmol/l)	–0.063	–0.028	0.172	0.217	0.134	0.080	0.176	0.127
SDMA (μmol/l)	–0.098	0.140	–0.210	–0.112	0.631	0.622	0.231	0.255
L-arginine (μmol/l)	0.021	–0.126	–0.018	–0.077	–0.247	–0.215	–0.182	–0.158

Parameter correlation coefficient p=0.05

Table 4. Evaluation of the IMT complex of the common carotid artery (CCA) and IMT complex of internal carotid artery (ICA) in the examined patients

	Type 2 diabetes patients			
Parameter	Patients with no vascular complications (Group A)	Patients with the diabetic micro and/or macroangiopathy (Group B)	Control (Group C)	Significance of differences
CCA-IMT mean (mm)	0.68±0.11	0.81±0.21	0.68±0.16	A *vs* B; p=0.3730SN A *vs* C: p=0.7014SN B *vs* C; p=0.2615SN
CCA-IMT max (mm)	0.91±0.32	0.97±0.27	0.84±0.16	A *vs* B; p=0.9245SN A *vs* C: p=0.7447SN B *vs* C; p=0.6636SN
ICA-IMT mean (mm)	0.80±0.23	1.01±0.31	0.69±0.17	A *vs* B; p=0.0854SN A *vs* C: p=0.2468SN B *vs* C; p=0.0032
ICA-IMT max (mm)	1.04±0.23	1.32±0.37	0.88±0.22	A *vs* B; p=0.1509SN A *vs* C: p=0.08049SN B *vs* C; p=0.0054

Results have been presented as mean values ± standard deviation. SN – statistically non-significant

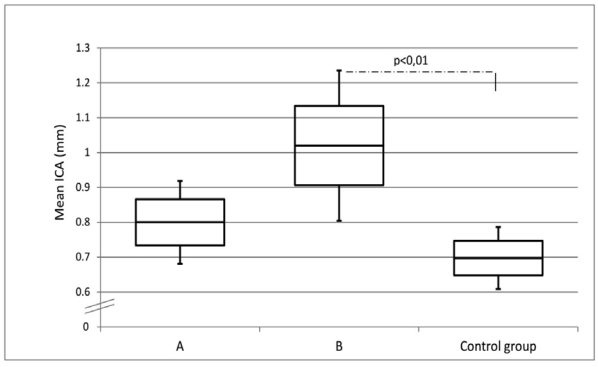

Figure 1. Mean value of ICA IMT in studied groups

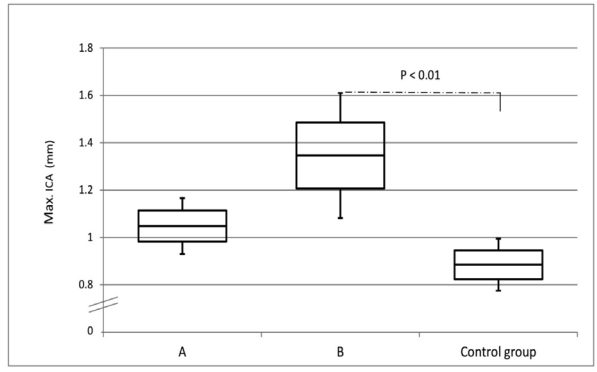

Figure 2. Maximum value of ICA IMT in studied groups

by carbohydrate metabolism disorders (correlation with average ICA IMT and maximal ICA IMT with hsCRP were 0.636, p=0.05 and 0.673, p=0.05, respectively) in the same group of patients.

DISCUSSION

The endothelium is one of the biggest paracrine organs. Its dysfunction is considered as the first stage of atherogenesis. Reduced NO bioavailability plays a crucial role in the process. ADMA is a strong inhibitor of eNOS (endothelial nitric oxide synthase) (Landim *et al.,* 2009).

According to the literature, plasma ADMA concentration remains in strong positive correlation with subject's age (Mizayaki *et al.,* 1999), high cholesterol level (Päivä *et al.,* 2003), hypertension (Moroszko *et al.,* 2008) and diabetes (Konya, 2015).

In our study, we observed high ADMA and SDMA plasma levels in both groups of patients with diabetes, regardless of the presence or absence of vascular complications. In patients with diabetic vascular complications levels of ADMA and SDMA are slightly higher, but the differences do not reach the level of statistical significance; ICA IMT was, however, significantly increased in this group. These findings contrast with Celik and oth-

ers (Celik *et al.,* 2014), who demonstrated higher ADMA concentration in diabetic patients with vascular complications compared to those without CV complications.

One of the possible explanations of the obtained results could be concomitant treatment. All group A and group B patients received antidiabetic medication, especially metformin. Group B also received ASA (acetylsalicylic acid), statins, AT I inhibitors (angiotensin II receptor type I inhibitor), ACE-I (Angiotensin-converting-enzyme inhibitors) which could affect ADMA concentration.

It has been reported that metformin reduces ADMA level (Maas, 2005), however, there is no consent regarding whether it actually was metformin or better glycemic control effect. Ojima and others (Ojima *et al.,* 2013) reported that GLP-1 RA diminished ADMA development. Several DPP-IV inhibitors could reduce ADMA level in diabetic patients' plasma (Kubota *et al.,* 2012; Cakirca *et al.,* 2014).

A randomized clinical studies proved that ADMA plasma level was diminished after treatment with enalapril or eprosartan (Delles *et al.,* 2002), and the effect was observed independently of blood pressure reduction. Statin, especially rosuvastatin, has also been reported to reduce ADMA plasma concentration (Lu *et al.,* 2004). A similar effect of fibrates (Yang *et al.,* 2004), niacin (Westphal *et al.,* 2006), estrogens (Post *et al.,* 2003) has also been reported. Conflicting results of the impact on ADMA level are described for polyunsaturated fatty acids (Eid *et al.,* 2006), L-arginine (Watanabe *et al.,* 2000), and ASA (Bode-Böger *et al.,* 2005). These drugs are the treatment options with anti-atherosclerotic effects in high CV risk subjects. On the other hand, high concentration of ADMA could be a marker of high CV risk, especially in connection with diabetes, hypercholesterolemia, and hypertension (Gać *et al.,* 2020).

Some studies demonstrate benefits of physical training on ADMA level (Gomes *et al.,* 2008).

High IMT was assessed in CCA max measurements in patients with concomitant vascular complications (group A 0.84±0.16; group B 0.97±0.27). Significantly greater differences were observed for the IMT complex of the ICA. Both mean and maximum values in group B were higher than those in group A, and high statistical significance was observed (mean ICA IMT group A 0.69±0.17; group B 1.01±0.31; max ICA IMT group A 0.88±0.22; group B 1.32±0.37; p=0.0032 and p=0.005 respectively). Studies suggest greater clinical usefulness of ICA wall evaluation as a marker which can extract group of diabetic patients with vascular complications. Moreover, thickening of the mean and maximum IMT complex of the internal carotid artery was dependent on HbA1c concentration; correlation coefficients 0.766 at p=0.01 and 0.851 at p=0.01, respectively.

Research by Dalla and others (Dalla *et al.,* 2007) has shown that IMT increases with duration of diabetes, higher concentrations of cholesterol, triglycerides, oxidized LDL (oxLDL) in blood serum, and higher daily insulin intake. The DCCT (Diabetes Control and Complication Trial) and their continuation, EDIC (Epidemiology of Diabetes Interventions and Complications) studies, indicated that unfavorable lipidogram was more frequently observed in patients with microangiopathy complications than in patients with no vascular lesions (Lyons *et al.,* 2006). The results of our study did not confirm a simple dependence of the IMT carotid artery complex thickness on the concentrations of total cholesterol, HDL-cholesterol, LDL-cholesterol and triglycerides in the serum of the analyzed patients.

It is recommended to use mean CCA and maximum ICA IMT measurements for cardiovascular risk assessment (Stein *et al.,* 2008; Touboul *et al.,* 2007). The association between risk factors in ICA and CCA IMT was studied by Polak and others (Polak *et al.,* 2010), who suggested that max ICA IMT might add value to mean CCA IMT for CV risk assessments.

Epidemiological investigations have linked ADMA plasma level with IMT. At first, Miyazaki and others (Miyazaki *et al.,* 1999) observed a strong positive correlation between ADMA and IMT in healthy, young population. Subsequently, other researchers reported similar findings in the case of chronic kidney disease (Zoccali *et al.,* 2002). Zsuga and others (Zsuga *et al.,* 2007) observed a strong negative correlation between ADMA concentration and IMT. The study involved a group of young patients (<55 years) with mild carotid stenosis (at least 30%). In 2012, Bai and others provided a meta-analysis of 22 studies concerning the ADMA and IMT association (Bai *et al.,* 2013). They indicated that ADMA plasma concentration was a novel marker of preclinical atherosclerosis and the association between ADMA and IMT was stronger in patient with chronic kidney disease. A large prospective study conducted by Furuki and others (Furuki *et al.,* 2008) showed that plasma level of ADMA was the only predictor of IMT progression.

We believe that the reason for the lack of correlation between ADMA concentration and IMT could be the effect of the small size of the studied groups and their heterogeneity, with high percentage of patients suffering from microangiopathic complications. For this reason further tests involving larger groups of patients are necessary.

Currently, we know that ADMA is not only a marker of vascular endothelial damage, but also a substance that is actively involved in the pathway of action of many classical risk factors for atherosclerosis (Cooke 2004). According to Yasuda and others (Yasuda *et al.,* 2006), improved glycemic control provides antiatherogenic effect through diminishing ADMA concentration in diabetic patients.

The results of authors' own research demonstrated a significant dependence of the CCA IMT mean value (correlation coefficient 0.631) and the maximum CCA IMT value (correlation coefficient 0.622) on the plasma SDMA concentration in patients with diabetic microangiopathy and macroangiopathy. SDMA is believed to be a marker of early-stage renal dysfunction.

According to a meta-analysis, (Schlesinger *et al.,* 2016), ADMA and SDMA are independent risk markers of all-cause mortality and CVD across different populations and methodological approaches.

Our findings could constitute a link between high SDMA concentrations and CVD in patients with diabetic macroangiopathy. Small size of the study, which makes it impossible to demonstrate the possible effect of the applied groups of medication on the study groups, constitutes a limitation of the study. The heterogeneity of group B, with prevalence of patients with cardiomyopathy, is also important.

CONCLUSIONS

1. We observed high ADMA and SDMA plasma level in both groups of diabetic patients, independently of the presence or absence of vascular complications. ADMA and SDMA levels are slightly higher in patients with

diabetic vascular complications, however, the differences did not reach statistical significance.

2. There was no correlation between ADMA and the mean or maximum IMT of CCA and ICA.

3. The study suggest clinical usefulness assessment of the ICA IMT as a marker, which can extract group of diabetic patients with vascular complication.

4. Our study is the first one to describe positive correlation between SDMA concentration and CCA IMT.

5. It is only a preliminary study, further tests involving larger groups of patients are necessary.

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