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The Pattern of Circulating Microparticles in Diabetes Mellitus Patients with Known Subclinical Atherosclerosis

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Abstract

Background: Accelerating atherosclerosis in Type 2 diabetes mellitus (T2DM) patients may relate with imbalance between pattern of microparticles (MPs), which frequently involved in repair of vasculature, tissue injury, inflammation and thrombosis. The aim of the study: to investigate the pattern of circulating MPs in T2DM patients with asymptomatic atherosclerosis.

Methods: The study retrospectively evolved a total of 103 patients with T2DM (54 subjects without documented coronary atherosclerosis and 49 patients with angiographic evidence of asymptomatic coronary atherosclerosis) who were undergone a contrast-enhanced multispiral tomography angiography prior study entry and 35 healthy volunteers. To determine circulating biomarkers, blood samples were collected at baseline. MPs were labeled and characterized by flow cytometry.

Results: There were no significant differences between healthy volunteers and T2DM patients in circulating numbers of MPs labeled as CD41a+, CD64+, CD144+, CD144+/CD31+, Annexin V+, CD144+/annexin V+, and CD144+/CD31+/annexin V+. However, lower number of MPs with immune phenotypes CD62E+, CD105E+ and higher numbers of CD31+/annexin V+ MPs were reported in T2DM patients when compared with healthy volunteers. Therefore, we found an increased level of circulating CD41a+ MPs, CD144+/CD31+ MPs, CD31+/annexin V+ MPs, and decreased level of CD62E+ MPs in T2DM patients with asymptomatic coronary atherosclerosis in comparison with those who had no asymptomatic atherosclerosis. Using multivariate Cox regression analysis, BMI (odds ratio [OR]=1.04, P=0.001), LDL-C (OR=1.05, P=0.046), hs-CRP (OR=1.07, P=0.044), osteoprotegerin (OR=1.07, P=0.026), CD62E+ MPs (OR=1.07, P=0.001) and CD31+/annexin V+ MPs (OR=1.12, P=0.003) were determined independent predictive factors of asymptomatic atherosclerosis in T2DM patients.

Conclusion: circulating levels of MP originated apoptotic endothelial cell-derived were significantly increased in diabetic patients as compared with normal subjects, but level of activated endothelial cell-derived MPs was lower than in healthy volunteers. Among T2DM patients only an increased level of CD31+/annexin V+ MPs and decreased CD62E+ MPs were significantly associated with asymptomatic atherosclerosis.

Keywords: Diabetes mellitus; Circulating microparticles; Cardiovascular risk factors; Asymptomatic atherosclerosis

Introduction

Type 2 diabetes mellitus (T2DM) remains a major public health problem worldwide [1]. Recent epidemiology studies have demonstrated sufficient associations between T2DM and the risk of cardiovascular (CV) diseases and events [2-4]. It is well known that accelerated atherosclerosis is mainly attributable to T2DM [5] and that diabetes-induced endothelial dysfunction and dyslipidemia might have direct adverse impact of atherosclerosis development and CV clinical outcomes [6-8]. Although symptomatic CV diseases contributes to a high mortality rate in patients with T2DM, the asymptomatic atherosclerosis in this patient population is validated a surrogate marker of new CV events [9].

Accelerating atherosclerosis among T2DM patients may realize through release of microparticles (MPs), which frequently involved in controversial processes, i.e., repair of vasculature, as well as tissue injury, inflammation and thrombosis [10]. Extracellular MP are microvesicles with sizes ranging between 50 and 1000 nm released from plasma membrane of wide variety of cells by specific (cytokine stimulation, apoptotic agents, mononuclear cooperation, coagulation, etc) and non-specific (shear stress) stimuli [11]. In fact, patients with T2DM exhibited a significantly elevated level of circulating MP derived from platelets, endothelial and mononuclear cells [12,13]. However, the significance of MPs in T2DM patients as an inductor of development and progression of atherosclerosis remains controversial. An example of this controversy is that it is still unknown if circulating MPs found in peripheral blood cause an injury of endothelium and worsening of atherosclerosis whether they are the result of disease progression in response to endothelial dysfunction and vascular dysintegrity [14,15]. We hypothesized that imbalance between numerous of circulating MP originated from various cells due to activation and / or apoptosis might be a surrogate marker of the risk of asymptomatic atherosclerosis in T2DM subjects. The aim of the study: to investigate the pattern of circulating MPs in T2DM patients with asymptomatic atherosclerosis.

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Methods

The study retrospectively evolved a total of 103 patients with T2DM (54 subjects without documented coronary atherosclerosis and 49 patients with angiographic evidence of asymptomatic coronary atherosclerosis) who were undergone a contrast-enhanced multispiral tomography angiography prior study entry and 35 healthy volunteers who were examined in three our centers (City Hospital #6, Regional Center of Cardiovascular Diseases, and Regional Zaporozhye Hospital located in Zaporozhye, Ukraine) between February 2013 and November 2014. Patients with typical anginal signs and symptoms, subjects with clinical evidences of pre-existing coronary artery disease, i.e., myocardial infarction/acute coronary syndrome, heart failure, atrial fibrillation, any atherothrombotic events, as well as patients with declined glomerular filtration rate <60 mL/min/1.73 m^2, and candidates for insulin therapy were excluded from the study.

All the patients have given their voluntary informed written consent for participation in the study. The study was approved by the local ethics committee of State Medical University, Zaporozhye, Ukraine. The study was performed in conformity with the Declaration of Helsinki.

Study design: Retrospective cohort study.

T2DM was diagnosed with revised criteria provided by American Diabetes Association when source documents were reviewed [16]. When one or more of the following components were found (glycated hemoglobin [HbA1c] ≥ 6.5%; fasting plasma glucose ≥ 7 mmol/L; 2-h plasma glucose ≥ 11.1 mmol/L during an oral glucose tolerance test; a random plasma glucose ≥ 11.1 mmol/L; exposure of insulin or oral antidiabetic drugs; a previous diagnosis of T2DM) T2DM was determined.

Current smoking was defined as consumption of one cigarette daily for three months. Anthropometric measurements were made using standard procedures.

No untreated subjects were enrolled. Patients with T2DM were treated with life-style modification, diet and orally taken antidiabetic drugs except sulfonylurea derivates and glitazones. Metformin in monotherapy or in combination with glinides and/or gliptines was given in individually optimized daily doses to be achieving full or partly full control for T2DM. Therefore, insulin was not used in enrolled patients.

Methods for visualization of coronary arteries

Contrast-enhanced multispiral computed tomography angiography has been performed for all the patients with T2DM prior to their inclusion in the study on Optima CT660 scanner (GE Healthcare, USA) using non-ionic contrast Omnipaque (Amersham Health, Ireland) [17]. Obtained results were interpreted by cardiologist and one of sub-investigator independently each other before study entry. Atherosclerosis was determined when plaques in at least of one coronary artery were visualized.

Cardiovascular risk calculation

A 10-year cardiovascular risk for study patients was calculated using the Framingham General Cardiovascular Risk Score (2008) by on-line calculator and interpreted using contemporary approaches [18].

Calculation of glomerular filtration rate

Glomerular filtration rate (GFR) was calculated with CKD-EPI formula [19].

Measurement of circulating biomarkers

To determine circulating biomarkers, blood samples were collected at baseline in the morning (at 7-8 AM) into cooled silicone test tubes wherein 2 mL of 5% Trilon B solution were added. Then they were centrifuged upon permanent cooling at 6,000 rpm for 3 minutes. Plasma was collected and refrigerated immediately to be stored at a temperature -70°C. Serum adiponectin, RANKL and osteoprotegerin (OPG) were measured by high-sensitive enzyme-linked immunosorbent assays using commercial kits (R&D Systems GmbH, Wiesbaden-Nordenstadt, Germany) according to the manufacturers' recommendations. The inter-assay coefficients of variation were as follows: adiponectin: 5%, RANKL: 7.0%; OPG: 8.2%.

High-sensitive C-reactive protein (hs-CRP) was measured by commercially available standard kit (R&D Systems GmbH, Wiesbaden-Nordenstadt, Germany). The intra-assay and inter-assay coefficients of variation were <5%.

Serum uric acid level (SUA) was determined by enzymatic methods using a Beckman Synchron LX20 chemistry analyzer. The analytical average range for SUA was 0.5-12 mg/dL.

Fasting insulin level was measured by a double-antibody sandwich immunoassay (Elecsys 1010 analyzer, F. Hoffmann-La Roche Diagnostics, Mannheim, Germany). The intra-assay and inter-assay coefficients of variation were <5%. The lower detection limit of insulin level was 1.39 pmol/L.

Insulin resistance was assessed by the homeostasis model assessment for insulin resistance (HOMA-IR) [20] using the following formula:

HOMA-IR (mmol/L × μU/mL)=fasting glucose (mmol/L) × fasting insulin (μU/mL) / 22.5

Concentrations of total cholesterol (TC) (Catalog Number 3460-06, MaxDiscovery™ Cholesterol Enzymatic Assay Kit, Bio Scientific Corporation, USA), cholesterol of high-density lipoproteins (LDL-C) (Catalog Number DZ128A-KB1, Diazyme Europe GmbH, Germany), and cholesterol of high-density lipoproteins (HDL-C) (Catalog Number DZ129A-KY1, Diazyme Europe GmbH, Germany) were measured by enzymatic colorimetric method according standardized methodology on Beckman Synchron LX20 chemistry analyzer.

Direct Enzymatic HbA1c Assay was used for glycated hemoglobin A1C (HbA1c) measurements (Catalog Number DZ168A-K, Diazyme Europe GmbH, Germany) on Beckman Synchron LX20 chemistry analyzer.

Assay of circulating microparticles

Circulating MPs were isolated from 5 ml of venous citrated blood drawn from the fistula-free arm. To prevent contamination of samples platelet-free plasma (PFP) was separated from whole blood. PFP was centrifugated at 20,500 × rpm for 30 min. MP pellets were washed with DMEM (supplemented with 10 μg/mL polymyxin B, 100 UI of streptomycin, and 100 U/ml penicillin) and centrifuged again (20,500 rpm for 30 min). The obtained supernatant was extracted, and MP pellets were re-suspended into the remaining 200 μL of supernatant. PFP, MPs, and supernatant were diluted five-, 10-, and five-fold in PBS, respectively.

MPs were labeled and characterized by flow cytometry by phycoerythrin (PE)-conjugated monoclonal antibody against CD31 (platelet endothelial cell adhesion molecule [PECAM]-1), CD41a,

CD64, CD105, CD144 (vascular endothelial [VE]-cadherin), CD62E (E-selectin), and Annexin V (BD Biosciences, USA) followed by incubation with fluorescein isothiocyanate (FITC)-conjugated Annexin V (BD Biosciences, USA) per HD-FACS (High-Definition Fluorescence Activated Cell Sorter) methodology independently after supernatant diluted without freeze [21].

The samples were incubated in the dark for 15 min at room temperature according to the manufacturer's instructions. It was performed the analysis of area, height, and width forward scatter (FSC) and side scatter (SSC) parameters as well as side scatter width (SSC-W). The gate for MPs was defined by size, using 0.5 and 1.0 μm beads (Sigma, St Louis, MO, USA). For each sample, 500 thousand events have been analyzed. Compensation tubes were used with similar reagents as were used in the sample tubes. Data were constructed as numerous of MPs depending on marker presentation (positive or negative) and determination of MP populations.

Determination of MP populations

CD41a+ was used as a more specific marker of platelets, and CD64+ was considered a more specific marker of monocytes. CD31 antigen was determined as essential marker for endothelial cells, platelets, and leukocytes. CD144+ was used to identify a pure population of endothelial cells. CD31+/annexin V+ and CD144+/CD31+/annexin V+ microparticles were defined as apoptotic endothelial cell-derived MPs, MPs labeled for CD105+ or CD62E+ were determined as MPs produced due to activation of endothelial cells [22].

Statistical analysis

Statistical analysis of the results obtained was performed in SPSS system for Windows, Version 22 (SPSS Inc, Chicago, IL, USA). Continuous variables were expressed as mean (M) and standard deviation (± SD) or 95% confidence interval (CI); as well as median (Me) and 25%-75% interquartile range (IQR). Categorical variables are given as number (n) and percentage (%). To compare the main parameters of patient cohorts, two-tailed Student t-test or Mann-Whitney U-test were used. To compare categorical variables between groups, Chi^2 test (χ^2) and Fisher F exact test were used. Predictors of depleted EPCs in patients were examined in multivariable regression analysis. All models were adjusted for age, sex, and glomerular filtration rate. In the logistic regression model the significance of odds ratios was tested on the basis of Wald statistics. A two-tailed probability value of <0.05 was considered as significant.

Results

General characteristic of patients participating in the study was reported in Table 1. The mean age for T2DM patients and healthy volunteers was 48.41 years and 46.12 years (P=0.68). Therefore, 64.1% of T2DM patients and 65.7% of healthy volunteers were men (P=0.86). There was a significant difference between healthy volunteers and entire cohort of T2DM patients in BMI, waist circumference, cardiovascular risk factors (hypertension, dyslipidemia, adherence to smoking), Framingham risk score, and lipid abnormalities. HOMA-IR, HbA1c, fasting blood glucose, insulin, and hs-CRP, TG, SUA, sRANKL, osteoprotegerin, and adiponectin were higher in T2DM patients when compared with healthy volunteers.

As we found T2DM patients with asymptomatic coronary atherosclerosis in comparison with those who had no coronary atherosclerotic lesions demonstrated significantly higher BMI, waist circumference, circulating levels of LDL-C, hs-CRP, osteoprotegerin, adiponectin, and lower level of HDL-C.

Numbers of MPs in participators of the study were shown in Table 2. There were no significant differences between healthy volunteers and T2DM patients in circulating numbers of MPs labeled as CD41a+, CD64+, CD144+, CD144+/CD31+, Annexin V+, CD144+/annexin V+, and CD144+/CD31+/annexin V+. However, lower number of MPs with immune phenotypes CD62E+, CD105E+ and higher numbers of CD31+/annexin V+ MPs were reported in T2DM patients when compared with healthy volunteers. Therefore, we found an increased level of circulating CD41a+ MPs, CD144+/CD31+ MPs, CD31+/annexin V+ MPs, and decreased level of CD62E+ MPs in T2DM patients with asymptomatic coronary atherosclerosis in comparison with those who had no asymptomatic atherosclerosis.

There were significant correlations between BMI and osteoprotegerin (r=0.36; P=0.001), hs-CRP (r=0.32; P=0.001), LDL-C (r=0.32; P=0.044), CD31+/annexin V+ (r=0.34; P=0.001), adiponectin (r=0.30; P=0.026), CD62E+ (r=-0.26; P=0.001). We found weak correlation between LDL-C and numbers of MPs labeled as CD41a+ (r=0.20; P=0.034), CD62E+(r=-0.15; P=0.046), and CD31+/annexin V+ (r=0.24; P=0.018). Therefore, osteoprotegerin correlated with numbers of CD31+/annexin V+ MPs (r=0.32; P=0.001), CD62E+ (r=-0.28; P=0.001). We found closely correlation hs-CRP with CD31+/annexin V+ MPs (r=0.30; P=0.044), CD144+/CD31+ MPs (r=0.21; P<0.05), CD62E+ (r=-0.23; P=0.026). Serum adiponectin correlated with LDL-C (r=0.26; P=0.001), CD31+/annexin V+ (r=0.24; P=0.001), CD144+/CD31+ MPs (r=0.20; P=0.014), and CD62E+ (r=-0.23; P=0.001).

Univariate regression analysis has shown that BMI, waist circumference, LDL-C, HDL-C, hs-CRP, osteoprotegerin, adiponectin, as well as MPs phenotyped CD41a+, CD62E+, CD31+/annexin V+ were determined as predictors for asymptomatic atherosclerosis in T2DM patients (Table 3). However, after including in multivariate regression model all variables with p value <0.2, we found that BMI, LDL-C, hs-CRP, osteoprotegerin, CD62E+ and CD31+/annexin V+ MPs remained independent predictors for asymptomatic atherosclerosis.

Using multivariate logistic regression analysis, BMI (odds ratio [OR]=1.04, P=0.001), LDL-C (OR=1.05, P=0.046), hs-CRP (OR=1.07, P=0.044), osteoprotegerin (OR=1.07, P=0.026), CD62E+ MPs (OR=1.07, P=0.001) and CD31+/annexin V+ MPs (OR=1.12, P=0.003) were determined independent predictive factors of asymptomatic atherosclerosis in T2DM patients (Table 4). CD62E+ MPs and CD31+/annexin V+ MPs were similar in predictive value for asymptomatic atherosclerosis. Therefore, lower effect on atherosclerotic lesion was determined for osteoprotegerin, hs-CRP, BMI and LDL-C.

Discussion

The results of our study demonstrate that there are at least three groups of variables produced similar effect on risk of asymptomatic atherosclerosis in T2DM patients, i.e., circulating levels of inflammatory cytokines (hs-CRP, osteoprotegerin), angiogenic MPs (CD62E+ MPs and CD31+/annexin V+ MPs), as well as T2DM-associated factors (BMI and LDL-C concentration). Because of accelerated atherosclerosis is considered a the major cause of mortality in diabetic patients and increased oxidative stress, inflammation, endothelial dysfunction play an important role in its development, we suggest that imbalance between MPs realized due to activation of endothelial cells (CD62E+ MPs) and apoptosis (CD31+/annexin V+ MPs) might be conductor of insufficient vascular tissue reparation and probably mediates vascular dysintegrity and dysfunction. Indeed, various spectrums of serum MPs levels, particularly those of endothelial origin, reflect cellular injury

	Healthy volunteers (n=35)	Entire cohort of T2DM patients (n=103)	T2DM patients without asymptomatic coronary atherosclerosis (n=54)	T2DM patients with asymptomatic coronary atherosclerosis (n=49)
Age, years	46.12 ± 4.22	48.41 ± 7.50	47.55 ± 6.90	48.74 ± 7.80
Males, n (%)	23 (65.7%)	66 (64.1%)	35 (64.8%)	31 (63.3%)
BMI, kg/m^2	21.5 (16.1-23.5)	28.7 (16.8-32.2)*	26.2 (17.0-31.0)	29.9 (17.6-33.5) #
Waist circumference, sm	78 (63-89)	92 (71-104)*	90 (68-101)	94 (74-106) #
Hypertension, n (%)	-	71 (68.9%)*	37 (68.5%)	34 (69.4%)
Dyslipidemia, n (%)	-	61 (59.2%)*	32 (59.3%)	29 (59.2%)
Adherence to smoking, n (%)	6 (17.1%)	31 (30.1%)*	16 (29.6%)	15 (30.1%)
Framingham risk score, %	2.55 ± 1.05	8.12 ± 2.88*	8.03 ± 2.65	9.04 ± 2.43
Systolic BP, mm Hg	122 ± 5	137 ± 6*	137 ± 4	138 ± 5
Diastolic BP, mm Hg	72 ± 4	86 ± 6*	85 ± 5	86 ± 7
Heart rate, beats per 1 min.	66 ± 6	74 ± 7*	75 ± 4	72 ± 6
GFR, mL/min/1.73 m^2	102.1 (91.4-113.2)	93.1 (79.5–112.2)	95.9 (82.1–118.3)	91.8 (77.6-104.8)
HbA1c, %	4.75 (4.36-5.12)	7.0 (4.3-9.5)*	7.1 (4.2-9.3)	7.0 (4.3-9.7)
Fasting blood glucose, mmol/L	4.52 (4.43-4.76)	5.40 (3.40-9.57)*	5.37 (3.38-9.12)	5.87 (3.41-9.68)
Insulin, μU/mL	4.98 (1.5-14.1)	15.15 (13.69-17.84)*	14.92 (13.50-16.50)	15.23 (13.60-17.90)
HOMA-IR, mmol/L × μU/mL	1.01 (0.91-1.07)	3.83 (3.47-4.50)*	3.60 (3.35-4.12)	3.98 (3.42-4.90)
Creatinine, μmol/L	62.1 (55.7-82.4)	70.5 (59.6-88.1)	69.2 (58.9-83.8)	72.4 (59.1-89.6)
Total *cholesterol,* mmol/L	4.76 (4.21-5.05)	5.30 (4.61-6.04)*	5.25 (4.58-6.11)	5.39 (4.66-6.15)
LDL-C, mmol/L	3.10 (2.78-3.21)	3.60 (3.20-4.18)*	3.46 (3.04-3.92)	3.65 (3.11-4.15) #
HDL-C, mmol/L	1.13 (1.05-1.17)	0.94 (0.92-1.06)*	0.96 (0.93-1.11)	0.91 (0.88-1.02) #
TG, mmol/L	1.18 (1.07-1.30)	1.68 (1.44-1.97)*	1.61 (1.40-1.91)	1.72 (1.51-2.09)
SUA, mmol/L	17.1 (9.1-25.7)	23.8 (15.8-31.3)*	23.4 (15.3-30.1)	24.1 (15.5-33.4)
hs-CRP, mg / L	4.11 (0.97-5.03)	7.96 (4.72 -9.34)*	5.66 (3.65 - 7.82)	9.14 (5.19 -12.82) #
sRANKL, pg / mL	16.10 (2.1-30.1)	25.80 (15.20-46.53)*	23.20 (11.60-36.12)	28.20 (14.30-47.13)
Osteoprotegerin, pg / mL	88.3 (37.5-136.6)	725.9 (579.9-871.9)*	693.2 (550.2-831.5)	745.6 (602.4-885.3) #
Adiponectin, mg / L	6.17 (3.44-10.15)	17.65 (10.12-24.93)*	15.18 (9.78-19.55)	19.94 (14.87-26.33) #

Note: Data are presented as mean and ± SD or median and 25-75% IQR. Categorical variables are expressed as numerous (n) and percentages (%). P-value is a comparison of mean or median variables between both cohorts (ANOVA test). * - significant difference between healthy subjects and entire cohort of T2DM patients; # - significant difference between T2DM patients with and without asymptomatic atherosclerosis

Abbreviations: IQR – inter quartile range; BMI - Body mass index, T2DM – type two diabetes mellitus, *TG – triglycerides, BP – blood pressure,* BMI - Body mass index, GFR - glomerular filtration rate, *HDL-C - high-density lipoprotein cholesterol,* LDL-C - *Low-density lipoprotein cholesterol,* hs-CRP – high sensitive C reactive protein, sRANKL – serum receptor activator of NF-κB ligand.

Table 1: General characteristic of subjects participating in the study.

Immune phenotype of MPs	Healthy volunteers (n=35)	Entire cohort of enrolled T2DM patients (n=103)	T2DM patients without asymptomatic coronary atherosclerosis (n=54)	T2DM patients with asymptomatic coronary atherosclerosis (n=49)
CD41a+ MPs, n/μL	23 (19-28)	25 (16-33)	23 (17-30)	29 (19-38) #
CD64+ MPs, n/μL	3.9 (3.5-4.6)	4.2 (3.2-5.1)	4.1 (3.3-4.9)	4.4 (3.4-5.0)
CD144+ MPs, n/μL	0.29 (0.22-0.36)	0.33 (0.24-0.39)	0.30 (0.25-0.38)	0.35 (0.26-0.42)
CD144+/CD31+ MPs, n/μL	0.87 (0.27-1.25)	0.92 (0.36-1.32)	0.88 (0.39-1.28)	0.96 (0.35-1.41) #
CD62E+ MPs, n/μL	1.35 (0.95-1.68)	1.03 (0.86-1.13)*	1.10 (0.89-1.17)	0.98 (0.79-1.10) #
CD105E+ MPs, n/μL	2.32 (1.92-2.56)	2.24 (1.85-2.41)*	2.29 (1.92-2.60)	2.18 (1.86-2.50)
Annexin V+ MPs, n/μL	4655 (3724-6237)	5495 (3988-6957)	5371 (3855-6792)	5673 (3952-7099)
CD144+/annexin V+ MPs, n/μL	0.95 (0.11-1.78)	1.15 (0.13-2.31)	1.11 (0.12-2.23)	1.19 (0.16-2.37)
CD144+/CD31+/annexin V+ MPs, n/μL	0.82 (0.27-1.55)	1.01 (0.39-1.70)	0.95 (0.37-1.56)	1.12 (0.39-1.81)
CD31+/annexin V+ MPs, n/μL	0.154 (0.03-0.21)	0.316 (0.261-0.374)*	0.298 (0.255-0.341)	0.337 (0.280-0.395) #

Note: Data are presented as median and 25-75% IQR. P-value is a comparison of mean or median variables between both cohorts (ANOVA test). * - significant difference between healthy subjects and entire cohort of T2DM patients; # - significant difference between T2DM patients with and without asymptomatic atherosclerosis

Abbreviations: IQR – inter quartile range; MPs –*microparticles.*

Table 2: Numbers of microparticles in participators of the study.

and appear now as a surrogate marker of vascular dysfunction [23]. Although platelet-derived and mononuclear-derived MPs could also be involved in the development of vascular dysfunction and complications in T2DM for they stimulate pro-inflammatory responses in target cells and promote thrombosis and angiogenesis, we found that endothelial cell-derived MPs with immune phenotypes CD62E+ and CD31+/annexin V+ are probably the most optimal surrogate non-invasive markers for asymptomatic atherosclerosis in T2DM.

Recent studies have shown that elevated circulating level of endothelial MPs is result in endotheliopathy that may precede insulin resistance, diabetes and its complications [24,25]. In fact, MPs originated from activated endothelial cells may produce favorable angiopoetic effect and mediate tissue repair and endothelial integrity [26]. Contrary, apoptotic endothelial cell-derived MPs directly effect on tissue injury through several mechanisms including pro-coagulation, inflammation, oxidative stress inducing, and worse

Variable	Univariable analysis		Multivariable analysis	
	B coefficient	P value	B coefficient	P value
BMI	0.23	0.034	0.18	0.022
Waist circumference	0.14	0.038	0.06	0.09
LDL-C	0.28	0.046	0.23	0.042
HDL-C	-0.15	0.022	-0.11	0.078
hs-CRP	0.28	0.001	0.21	0.044
osteoprotegerin	0.36	0.001	0.32	0.01
adiponectin	0.25	0.022	0.11	0.06
CD41a+ MPs	0.011	0.042	0.010	0.078
CD144+/CD31+ MPs	0.013	0.54	0.009	0.64
CD62E+ MPs	-0.38	0.001	-0.26	0.001
CD31+/annexin V+ MPs	0.32	0.026	0.19	0.014

Notes: The multivariate regression model included all variables with p value <0.2.

Abbreviations: BMI – body mass index; hs-CRP – high sensitive C-reactive protein; *HDL-C - high-density lipoprotein cholesterol,* LDL-C - *Low-density lipoprotein cholesterol;* MPs *–microparticles*

Table 3: Univariate and multivariate logistic regression analysis for asymptomatic atherosclerosis.

Variables	OR	95% CI	Wald coefficients	P value
BMI per 5.0 kg/m^2	1.04	1.01 - 1.09	9.26	0.048
LDL-C per 1.0 mmol/L	1.05	1.01 - 1.08	8.11	0.046
hs-CRP per 4.50 mg/L	1.07	1.03 - 1.12	10.4	0.044
Osteoprotegerin per 125.5 pg / mL	1.07	1.02 - 1.12	11.3	0.026
CD62E+ MPs per 0.5 n/μL	1.06	1.03 -1.10	12.1	0.001
CD31+/annexin V+ MPs per 0.1 n/μL	1.12	1.04 - 1.19	12.9	0.003

Notes: ORs were calculated per unit increase apart from CD62E+ MPs, for which OR was done as per unit decrease.

Abbreviations: OR – odds ratio; BMI – body mass index; hs-CRP – high sensitive C-reactive protein; *HDL-C - high-density lipoprotein cholesterol,* LDL-C - *Low-density lipoprotein cholesterol*; MPs *–microparticles.*

Table 4: Multivariate logistic regression analysis for T2DM patients: known asymptomatic atherosclerosis versus no atherosclerotic lesions.

cell-to-cell cooperation [27]. It has been postulated that even beyond markers of activated cells, endothelial derived MPs may have potential paracrine functions and influence atherosclerosis. The results of the study support our assumption that imbalance between different subsets of endothelial MPs may have more much value for tissue injury than elevated level one of MP types. Probably this impaired phenotype may be universal mechanism for several disease progressions. Moreover, deficiency of activated endothelial cell-derived MPs with immune phenotypes CD62E associated with elevated apoptotic endothelial cell-derived MPs labeled CD31+/annexin V+ was found in various diseases including coronary atherosclerosis, heart failure, diabetes, renal kidney disease, and malignancy [28-33]. Thus, endothelial cell-derived MPs may be involved in pathophysiology of endothelial injury and repair, as well as predominantly elevated level of apoptotic endothelial- cell-derived MPs might probably contribute to the increased risk of early staging vascular complications of T2DM, i.e., endothelial dysfunction and asymptomatic atherosclerosis.

In the study we found that traditional cardiovascular risk factors (BMI, LDL-C) may positively relate to circulating level of apoptotic endothelial-cell-derived MPs and they are able to negatively associate with numerous of activated endothelial cell-derived MPs, which have pro-angiogenic potency. Moreover, cardiovascular risk factors and circulating level of endothelial cell-derived MPs cross relate to inflammation biomarkers (hs-CRP, osteoprotegerin, and adiponectin). Because CD62E+ MPs and CD31+/annexin V+ MPs were similar in predictive value for asymptomatic atherosclerosis and it was superior than in osteoprotegerin, hs-CRP, BMI and LDL-C, we suggest that impaired immune phenotype affected secretion of different subsets of MPs in T2DM might consider a surrogate biomarker of asymptomatic atherosclerosis at early stage. The results of recent clinical studies reported about being association between BMI, LDL-C and circulation subsets of MPs different origin [29,34,35]. Probably these facts may explain the pathogenetic role of obese in vascular disease development and atherosclerosis in T2DM. Probably, impaired immune phenotype of cell-derived MPs in T2DM may indicate the different pathophysiological changes in vessels affected development of atherosclerosis and enhance the microvascular inflammation.

Although current approach toward prevention of target-organ damages is declared a main principle of contemporary treatment of diabetes, discovers of advanced high sensitive non-invasive methods for detection of early stage of asymptomatic tissue injury are widely discussed [33]. Cytometric bead assay for MPs and investigation of possible prognostic value of MPs are attractive for future studies. The predictive role of impaired phenotype of MPs probably modulated through inflammatory response in diabetic patients requires more investigations in large sample population.

In conclusion, circulating levels of MP originated apoptotic endothelial cell-derived were significantly increased in diabetic patients as compared with normal subjects, but level of activated endothelial cell-derived MPs was lower than in healthy volunteers. Among T2DM patients only an increased level of CD31+/annexin V+ MPs and decreased CD62E+ MPs were significantly associated with asymptomatic atherosclerosis.

Study Limitations

This study has some limitations. It is necessary to note that a large pool of nanoparticles might be produced after blood sampling due to destruction of platelets and blood cells. Therefore, preparation of isolates of microparticles in samples is the most sophisticated step for further examination. Venous citrated blood drawn from the fistula-free arm was performed obligatorily. We believe that these risks are systemic, and to minimize them, we refused to freeze the blood samples before measurement of microparticles. Additionally, retrospective, relative small sample size may limit the significance of the present study. The authors believe that a greater cohort of patients with more incidences detected is desirable to improve the credibility of the study.

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