Immune Phenotypes of Endothelial-Derived Microparticles in Dysmetabolic Patients

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Abstract

Type two diabetes mellitus remains a leading contributor to cardiovascular mortality worldwide. This study was conducted to investigate the pattern of circulating endothelial-derived microparticles in diabetes patients in comparison with metabolic syndrome subjects.

Methods: The study retrospectively evolved 101 patients (54 subjects with type two diabetes mellitus and 47 patients with metabolic syndrome) and 35 healthy volunteers. All the patients have given written informed consent for participation in the study. Biomarkers were measured at baseline of the study.

Results: There is a significant difference between healthy subjects and patients regarding CD31+/annexin V+ to CD62E+ ratio of endothelial-derived microparticle, which reflects impaired phenotype of microparticles. Therefore, CD31+/annexin V+ to CD62E+ ratio was found to be higher in the type two diabetes mellitus patients compared to metabolic syndrome patients. Using multivariate linear regression analyses, independent impact of type two diabetes mellitus (r=0.40, P=0.003), OPG (r=0.37, P=0.001), hs-CRP (r=0.347, P=0.001), and adiponectin (r=0.33, P=0.001) on increased CD31+/annexin V+ to CD62E+ ratio of endothelial-derived microparticles was determined. Using C-statistics we found that inflammatory biomarkers (hs-C reactive protein, osteoprotegerin and adiponectin) added to the based model (type two diabetes mellitus) improved the relative integrated discrimination indices by 12.6% for increased CD31+/annexin V+ to CD62E+ ratio.

In conclusion, we found that patients with type two diabetes mellitus and metabolic syndrome may distinguish predominantly appeared phenotypes of circulating endothelial-derived microparticles associated with pro-inflammatory cytokine over production. Elevated CD31+/annexin V+ to CD62E+ ratio is indicator of impaired immune phenotype of endothelial-derived microparticles, which allows determining pattern of microparticles in dysmetabolic disorder patients.

Keywords: Diabetes mellitus; Metabolic syndrome; Circulating endothelial-derived microparticles; Cardiovascular risk factors

Introduction

Type two diabetes mellitus (T2DM) remains to be increased metabolic disease achieved worldwide epidemic [1,2], although quality assurance in care of pre diabetes states including metabolic syndrome (MetS) is continuously arised in the development countries [3]. Recent studies have emerged that genetic, early-life-depended, age-related, and sociodemographic factors, as well as dietary particularities, exiting comorbidities are discussed leading causes for current prevalence of T2DM in general population [4-7]. However, both clinical conditions T2DM and MetS are considered major risk factors that contribute in cardiovascular outcomes through interaction of similar pathogenesis' mechanisms [8, 9]. Moreover, hyperglycemia, insulin resistance (IR), coagulation, activated immunity and cytokine production, oxidative stress that is suitable for T2DM and MetS may realize their effect on development of cardiovascular complication through inducing endothelial dysfunction [10,11]. There is evidence that systemic pro-inflammatory response induced by T2DM and MetS is cause of microvascular endothelial cell inflammation [12], which affects cell-to-cell cooperation, negatively effects tissue reparation, and may mediates by endothelial-derived microparticles [13].

Extracellular microparticles are microvesicles with sizes ranging between 50 and 1000 nm released from plasma membrane of wide variety of cells, including endothelial cells, by specific (cytokine stimulation, apoptotic agents, mononuclear cooperation, coagulation,

etc) and non-specific (shear stress) stimuli [14]. Circulating endothelial-derived microparticles (EMPs) depending on their origin (apoptotic-derived or activated endothelial cell production) are capable of transferring biological information (regulating peptides, hormones) or even genetic material (micro-RNA, mRNA, and DNA), as well as proteins, lipid components, from one cell to another without direct cell-to-cell contact to maintain cell homeostasis [15,16]. Additionally, circulating EMPs derived from activated endothelial cells did not contain nuclear components and they have also been shown to have pro-angiogenic and cardio-protective properties [17-19]. In opposite, apoptotic EMPs may originate from damaged endothelial cells that concentrate immune mediators, generating powerful signaling by the simultaneous receptor interaction and they are discussed a marker of endothelial cell injury and vascular aging [20]. However, the potential

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relevance of different phenotypes of circulating EMP among T2DM patients is still not understood. The aim of the study: to investigate the pattern of circulating EMPs in T2DM patients in comparison with MetS subjects.

Methods

The study retrospectively evolved 101 patients (54 subjects with T2DM and 47 patients with MetS) and 35 healthy volunteers who were examined in three our centers between February 2013 and November 2013. We enrolled dysmetabolic disorder subjects without typical anginal symptoms and without exiting coronary artery disease who have not angiographic evidence of atherosclerosis obtained by contrast-enhanced multispiral tomography angiography provided prior study entry. All the patients have given their informed written consent for participation in the study. T2DM was diagnosed with revised criteria provided by American Diabetes Association [21]. 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. MetS was diagnosed based on the National Cholesterol Education Program Adult Treatment Panel III criteria [22]. Patients were enrolled in the MetS cohort when at least three of the following components were defined: waist circumference ≥ 90 cm or ≥ 80 cm in men and women respectively; high density lipoprotein (HDL) cholesterol <1.03 mmol/l or <1.3 mmol/l in men and women respectively; triglycerides ≥ 1.7 mmol/l; blood pressure ≥ 130/85 mmHg or current exposure of antihypertensive drugs; fasting plasma glucose ≥ 5.6 mmol/L or previously defined as T2DM or treatment with oral antidiabetic agents or insulin. Current smoking was defined as consumption of one cigarette daily for three months. Anthropometric measurements were made using standard procedures. 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. Subjects with MetS were treated with life-style modification and diet, therefore metformin was given in 12 patients.

Methods for Visualization of Coronary Arteries

Contrast-enhanced multispiral computed tomography angiography has been performed for all the patients with dysmetabolic disorder prior to their inclusion in the study on Optima CT660 scanner (GE Healthcare, USA) using non-ionic contrast Omnipaque (Amersham Health, Ireland) [23].

Calculation of glomerular filtration rate

Glomerular filtration rate (GFR) was calculated with CKD-EPI formula [24].

Measurement of circulating biomarkers

To determine circulating biomarkers, blood samples were collected at baseline in the morning (at 7-8 a.m.) 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%.

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) [25] using the following formula:

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

Insulin resistance was defined when estimated HOMA-IR value was over 2.77 mmol/L × μU/mL.

Concentrations of total cholesterol (TC) and cholesterol of high-density lipoproteins (HDL-C) were measured by fermentation method. Concentration of cholesterol of low-density lipoproteins (LDL-C) was calculated according to the Friedewald formula (1972) [26].

Assay of circulating endothelial-derived microparticles

Circulating EMPs were isolated from 5 ml of venous citrated blood drawn from the fistula-free arm. Platelet-free plasma (PFP) was separated from whole blood and then was centrifugated at 20,500 × rpm for 30 min. EMPs 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 pellets were re-suspended into the remaining 200 μl of supernatant. PFP, EMPs, pellet, and supernatant were diluted five-, 10-, and five-fold in PBS, respectively.

Endothelial-derived apoptotic and activated microparticles were phenotyped by flow cytometry by phycoerythrin (PE)-conjugated monoclonal antibody against CD31 (platelet endothelial cell adhesion molecule [PECAM]-1), 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 [27]. The samples were incubated in the dark for 15 min at room temperature according to the manufacturer's instructions. For each sample, 500 thousand events have been analyzed. EMPs gate was defined by size, using 0.8 and 1.0 mm beads (Sigma, St Louis, MO, USA). CD31+/annexin V+ and CD144+/CD31+/annexin V+ microparticles were defined as apoptotic EMPs, EMPs positively labeled for CD62E+ were determined as EMPs produced due to activation of endothelial cells [28].

Statistical Analysis

Statistical analysis of the results obtained was performed in SPSS system for Windows, Version 22 (SPSS Inc, Chicago, IL, USA). The data were presented as mean (M) and standard deviation (± SD) or 95% confidence interval (CI); as well as median (Me) and 25%-75%

interquartile range (IQR). To compare the main parameters of patient cohorts, two-tailed Student t-test or Shapiro–Wilk U-test were used. To compare categorical variables between groups, Chi2 test (χ2) and Fisher F exact test were used. Predictors of EMPs elevation in patients were examined in univariable and multivariable linear regression analysis. C-statistics, integrated discrimination indices (IDI) and net-reclassification improvement (NRI) were utilized for prediction performance analyses. 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 patients with dysmetabolic disorder and healthy volunteers was 48.34 years and 46.12 years (P=0.68). Therefore 63.3% of dysmetabolic disorder patients and 65.7% of healthy volunteers were men (P=0.86). As expected, there was a significant difference between healthy volunteers and entire cohort

of enrolled patients in BMI, waist circumference, cardiovascular risk factors (hypertension, dyslipidemia, adherence to smoking), HOMA-IR, lipid abnormalities, and Framingham risk score. HbA1c, fasting blood glucose, insulin, hs-CRP, TG, sRANKL, osteoprotegerin, and adiponectin were higher in patient cohort when compared with healthy volunteers. Therefore, CD31+/annexin V+ EMPs were elevated in patient cohort, while EMPs labeled as CD144+/CD31+, CD144+/ annexin V+, and CD144+/CD31+/annexin V+ did not. However, CD62E+ EMPs were elevated in healthy persons when compared with dysmetabolic disorder patients (P=0.024). CD31+/annexin V+ EMPs to CD62E+ EMPs ratio was calculated for both cohorts and presented in Figure 1A. There is a significant difference between healthy subjects and patients enrolled in the study regarding CD31+/annexin V+ EMPs to CD62E+ EMPs ratio, which reflects impaired phenotype of EMPs with surpassed apoptotic labeled microparticles.

Patients with MetS have demonstrated lower incidence of dyslipidemia, lower concentrations of HbA1c, fasting blood glucose,

	Healthy volunteers (n=35)	Entire cohort of enrolled patients (n=101)	MetS patients (n=47)	T2DM patients (n=54)
Age, years	46.12 ± 4.22	48.34 ± 7.80	48.30 ± 3.94	48.50 ± 6.60
Males, n (%)	23 (65.7%)	64 (63.3%)	30 (63.8%)	34 (63.0%)
BMI, kg/m^2	21.5 (25-75% IQR=16.1–23.5)	28.7 (25-75% IQR=16.5–32.4)*	28.2 (25-75% IQR=16.7–31.0)	28.5 (25-75% IQR=16.8–32.1)
Waist circumference, sm	78 (25-75% IQR=63–89)	91 (25-75% IQR=71–103)*	92 (25-75% IQR=69–105)	89 (25-75% IQR=72–100)
Hypertension, n (%)	-	68 (67.3%)*	32 (68.0%)	36 (66.7%)
Dyslipidemia, n (%)	-	59 (58.4%)*	26 (55.3%)	33 (61.1%)#
T2DM, n (%)	-	54 (53.5%)*	-	-
MetS, n (%)	-	47 (46.5%)*	-	-
Adherence to smoking, n (%)	6 (17.1%)	31 (30.7%)*	16 (34.0%)	15 (27.7%)
Framingham risk score	2.55 ± 1.05	8.12 ± 2.88*	8.09 ± 2.12	8.18 ± 2.32
Systolic BP, mm Hg	122 ± 5	136 ± 6*	137 ± 4	136 ± 5
Diastolic BP, mm Hg	72 ± 4	86 ± 6*	87 ± 5	86 ± 4
Heart rate, beats per 1 min.	66 ± 6	72 ± 7*	71 ± 6	72 ± 5
GFR, mL/min/1.73 m^2	102.1 (95% CI=91.4–113.2)	93.1 (95% CI=79.5–109.7)	92.5 (95% CI=83.1–107.4)	93.8 (95% CI=80.4–106.8)
HbA1c, %	4.75 (95% CI =4.36-5.12)	7.0 (95% CI =4.3-9.2)*	6.82 (95% CI =4.61-5.37)	7.3 (95% CI =4.3-9.1)#
Fasting blood glucose, mmol/L	4.52 (95% CI=4.43-4.76)	5.40 (95% CI =3.4-9.1)*	5.46 (95% CI =4.23-4.76)	5.54 (95% CI =4.49-9.0)#
Insulin, μU/mL	4.98 (25-75% IQR =1.5–14.1)	15.15 (25-75% IQR =13.69-16.62)*	14.2 (25-75% IQR =12.5–15.7)	15.6 (25-75% IQR =12.9-16.8)#
HOMA-IR, mmol/L × μU/mL	1.01 (25-75% IQR =0.91-1.07)	3.83 (25-75% IQR =3.47-4.20)*	3.45 (25-75% IQR =3.22-3.78)	3.86 (25-75% IQR =3.41-4.10)#
Creatinine, μmol/L	62.1 (95% CI =55.7–82.4)	70.5 (95% CI =59.6–88.3)	72.3 (95% CI =56.1–86.9)	71.2 (95% CI =59.9–87.2)
Total *cholesterol*, mmol/L	4.76 (95% CI =4.21-5.05)	5.3 (95% CI =4.6-6.0)*	5.3 (95% CI =4.5-5.9)	5.4 (95% CI =4.8-5.8)
LDL-C, mmol/L	3.10 (95% CI =2.78–3.21)	3.60 (95% CI =3.20–4.18)*	3.48 (95% CI =3.30–4.07)	3.80 (95% CI =3.20–4.20)#
HDL-C, mmol/L	1.13 (95% CI=1.05–1.17)	0.94 (95% CI=0.92–1.06)*	1.01 (95% CI=0.90–1.13)	0.94 (95% CI=0.88–1.04)#
TG, mmol/L	1,18 (95% CI=1.07–1.30)	1,68 (95% CI=1.44–1.98)*	1.77 (95% CI =1.62–1.95)	1.45 (95% CI =1.42–1.51)#
hs-CRP, mg / L	4.11 (25-75% IQR=0.97 – 5.03)	7.96 (25-75% IQR=4.72 – 9.34)*	7.87 (25-75% IQR=4.92 – 9.43)	8.10 (25-75% IQR=4.80 – 9.54)
sRANKL, pg / mL	16.10 (25-75% IQR=2.1-30.1)	25.80 (25-75% IQR=15.2-46.5)*	24.10 (25-75% IQR=14.7-36.9)	26.20 (25-75% IQR=15.3-40.7)
Osteoprotegerin, pg / mL	88.3 (25-75% IQR=37.5-136.6)	725.9 (25-75% IQR=579.9-871.9)*	718.5 (25-75% IQR=572.1-846.2)	732.1 (25-75% IQR=587.5-866.3)
Adiponectin, mg / L	6.17 (25-75% IQR=3.44-10.15)	13.65 (25-75% IQR=10.12-24.93)*	13.61 (25-75% IQR=9.74-22.35)	14.12 (25-75% IQR=10.12-23.10)
CD144+/CD31+ EMPs, n/mL	0.87 (25-75% IQR=0.27-1.25)	0.91 (25-75% IQR=0.36-1.35)	0.89 (25-75% IQR=0.32-1.29)	0.93 (25-75% IQR=0.39-1.34)
CD144+/annexin V+ EMPs, n/mL	0.95 (25-75% IQR=0.11-1.78)	1.15 (25-75% IQR=0.13-2.41)	1.08 (25-75% IQR=0.13-2.39)	1.17 (25-75% IQR=0.15-2.55)
CD144+/CD31+/annexin V+ EMPs, n/mL	0.82 (25-75% IQR=0.27-1.55)	1.01 (25-75% IQR=0.39-1.70)	0.94 (25-75% IQR=0.38-1.52)	1.10(25-75% IQR=0.40-1.67)
CD31+/annexin V+ EMPs, n/mL	0.154 (25-75% IQR=0.03-0.21)	0.296 (25-75% IQR=0.261-0.339)*	0.285 (25-75% IQR=0.253-0.318)	0.319 (25-75% IQR=0.279-0.368)#
CD62E+ EMPs, n/mL	1.35 (25-75% IQR=0.95-1.68)	1.03 (25-75% IQR=0.86-1.13)*	1.05 (25-75% IQR=0.88-1.18)	0.99 (25-75% IQR=0.92-1.16)

Note: Data are presented as mean and ± SE or 95% CI; median and 25-75% IQR. Categorical variables are expressed as numerous (n) and percentages (%). P-value was used for comparison of mean or median variables between both cohorts (ANOVA test). * - significant difference (P<0.05) between healthy subjects and entire patient cohort, # - significant difference (P<0.05) between MetS and T2DM patients.

Abbreviations: CI: Confidence Interval; 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; EMPs: Endothelial-Derived Microparticles; 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 patients participating in the study.
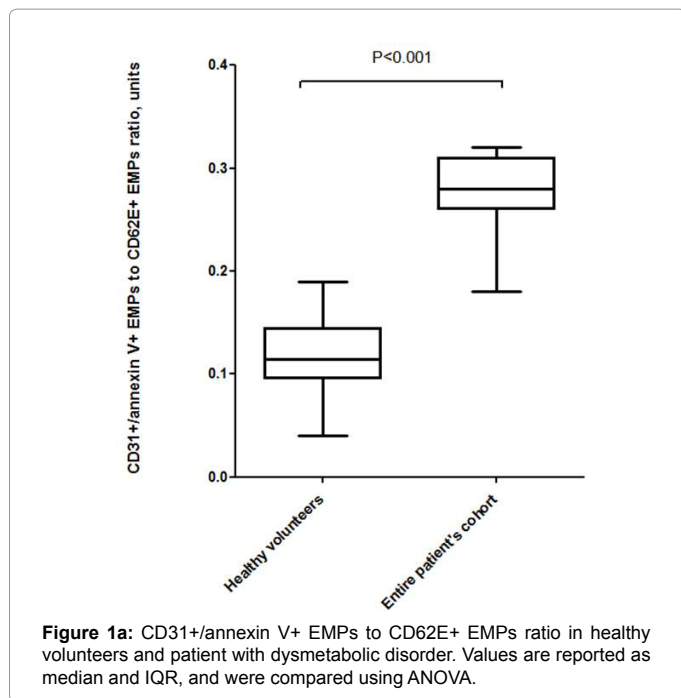
Figure 1a: CD31+/annexin V+ EMPs to CD62E+ EMPs ratio in healthy volunteers and patient with dysmetabolic disorder. Values are reported as median and IQR, and were compared using ANOVA.
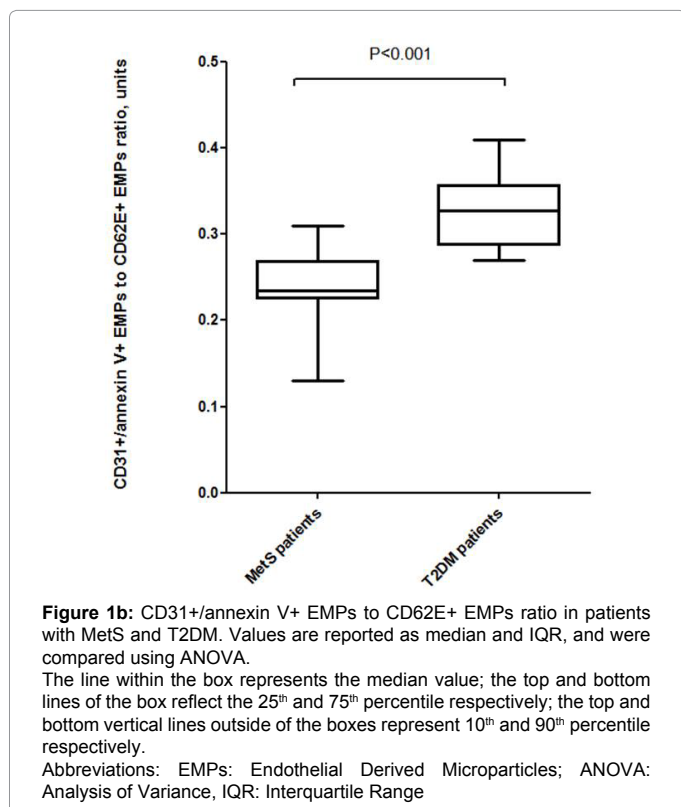

Figure 1b: CD31+/annexin V+ EMPs to CD62E+ EMPs ratio in patients with MetS and T2DM. Values are reported as median and IQR, and were compared using ANOVA.
The line within the box represents the median value; the top and bottom lines of the box reflect the 25th and 75th percentile respectively; the top and bottom vertical lines outside of the boxes represent 10th and 90th percentile respectively.
Abbreviations: EMPs: Endothelial Derived Microparticles; ANOVA: Analysis of Variance, IQR: Interquartile Range

insulin, LDL-C, and CD31+/annexin V+ EMPs when compared with T2DM subjects. Higher HDL-C and HOMA-IR were found in T2DM patients than in MetS subjects. Interestingly, similarities of circulating levels of EMPs different origin were determined in both cohorts apart from CD31+/annexin V+ EMPs. Therefore, CD31+/annexin V+ EMPs to CD62E+ EMPs ratio was found to be higher in the T2DM patients compared to MetS patients (Figure 1B).

The univariate linear correlation between apoptotic-derived to activated endothelial cell-derived EMP ratio, cardiovascular risk factors, hemodynamic performances, and other biomarker was evaluated. The data have shown that CD31+/annexin V+ to CD62E+ ratio were directly related with BMI (r=-0.58, P=0.001), OPG (r=0.522, P=0.001), adiponectin (r=0.516, P=0.001), sRANKL (r=0.502, P=0.001), hs-CRP (r=0.479, P=0.001), HOMA-IR (r=0.462, P=0.003), T2DM (r=0.402, P=0.003), eGFR (r=-0.388, P=0.001), TG (r=0.342, P=0.001), creatinine (r=-0.362, P=0.001), gender (r=0.318, P < 0.001 for male), dyslipidemia (r=0.313, P=0.001), Framingham risk score (r=0.308, P=0.001), age (r=0.275, P=0.001), smoking (r=0.212, P=0.001). No significant association CD31+/annexin V+ to CD62E+ ratio with fasting plasma glucose, HbA1c, means of systolic and diastolic BP, waist circumference was found.

Using multivariate linear regression analyses, independent impact of T2DM (r=0.40, P=0.003), OPG (r=0.37, P=0.001), hs-CRP (r=0.347, P=0.001), and adiponectin (r=0.33, P=0.001) on increased CD31+/annexin V+ to CD62E+ ratio of EMPs was determined.

Using C-statistics for Models with T2DM, and circulating biomarkers (hs-CRP, OPG and adiponectin) as Continuous Variables we found that adding of combination of inflammatory biomarkers (hs-CRP, OPG and adiponectin) to the based model (T2DM) improved the relative IDI by 12.6% for increased CD31+/annexin V+ EMPs to CD62E+ EMPs ratio (Table 2).

When we used other model constructed on entering variables IDI appears to be improved up to 4% for increased CD31+/annexin V+ EMPs to CD62E+ EMPs ratio (available for three inflammatory biomarkers as continuous variables) (Table 3). Three biomarkers (hs-CRP, OPG and adiponectin) improve significantly predictive model based on T2DM for increased CD31+/annexin V+ EMPs to CD62E+ EMPs ratio. In patient study population for category-free NRI, 6% of events (p=0.001) and 14% of non-events (p=0.001) were correctly reclassified by the addition of circulating inflammatory biomarkers (hs-CRP, OPG and adiponectin) to the base model (T2DM) for increased CD31+/annexin V+ EMPs to CD62E+ EMPs ratio. Thus, we suggest that inflammatory biomarkers (hs-CRP, OPG and adiponectin) remain statistically significant predictors for increased CD31+/annexin V+ EMPs to CD62E+ EMPs ratio in T2DM patients, which reflects impaired phenotype of circulating EMPs.

Discussion

The results of the study clarified that patients with T2DM and MetS may have different predominantly appeared phenotypes of circulating EMPs. As expected the Annexin V+ subset of EMPs should be significantly higher in T2DM patients when compared with MetS, but the results of the study did not confirm this assumption. In fact, annexin V binds to molecule of phosphatidylserine expressed on surface of EMPs due to inversion of the lipid membrane during apoptosis [16]. Therefore, pro-inflammatory cytokines (hs-CRP, OPG and adiponectin) are able to stimulate apoptosis and provoke EMP vesiculation [12,13]. Although microvesicules that are phenotypically nearly identical to CD31+/annexin V+ EMPs were not elevated in dysmetabolic disorders without exiting atherosclerosis and cardiovascular complications, we suggest CD31+/annexin V+ EMPs to CD62E+ EMPs ratio might be referred as object characterized predominantly immune phenotype of circulating EMPs, because of elevated CD62E+ EMPs in healthy volunteers were found. Here we reported that patients with dysmetabolic disorders, such as T2DM and MetS, who have not angiographic evidence of atherosclerosis may

Models	Dependent variable: CD31+/annexin V+ EMPs to CD62E+ EMPs ratio			
	AUC (95% CI)	ΔAUC	IDI (± SE)	Relative IDI (%)
Model 1 (based model: T2DM)	0.626	-	-	-
Model 1 + OPG	0.681	-	-	-
Model 1 + OPG vs Model 1	-	0.055; P<0.05	0.06 ± 0.010	10.2%
Model 1 (based model: T2DM)	0.626	-	-	-
Model 1 + hs-CRP	0.661	-	-	-
Model 1 + hs-CRP vs Model 1	-	0.035; P=0.024	0.03 ± 0.012	5.1%
Model 1 (based model: T2DM)	0.626	-	-	-
Model 1 + OPG + hs-CRP	0.683	-	-	-
Model 1 + OPG + hs-CRP vs Model 1	-	0.057; P<0.05	0.06 ± 0.009	11.1%
Model 1 (based model: T2DM)	0.626	-	-	-
Model 1 + adiponectin	0.655	-	-	-
Model 1 + adiponectin vs Model 1	-	0.045; P=0.043	0.02 ± 0.010	4.6%
Model 1 (based model: T2DM)	0.626	-	-	-
Model 1 + adiponectin + OPG	0.664	-	-	-
Model 1 + adiponectin + OPG vs Model 1	-	0.038; P<0.05	0.03 ± 0.008	7.9%
Model 1 (based model: T2DM)	0.626	-	-	-
Model 1 + hs-CRP + OPG + adiponectin	0.690	-	-	-
Model 1 + hs-CRP + OPG + adiponectin vs Model 1	-	0.064; P<0.001	0.02 ± 0.015	12.6%

Note: Relative IDI – calculated as the ratio of IDI over the discrimination slope of the model without T2DM.

Abbreviations: AUC: Area Under Curve; SE: Standard Error; T2DM: Type Two Diabetes Mellitus; OPG: Osteoprotegerin; hs-CRP: High Sensitive C-Reactive Protein

Table 2: C-statistics for Models with T2DM, hs-CRP, OPG, and adiponectin as Continuous Variables.

Model 2 vs Model 1	
Categorical NRI	0.14 (95% CI 0.10-0.19)
Percentage of events correctly reclassified	4 (p=0.14)
Percentage of non-events correctly reclassified	7 (p=0.001)
Categorical free NRI	0.29 (95% CI 0.22-0.36)
Percentage of events correctly reclassified	6% (p=0.001)
Percentage of non-events correctly reclassified	14% (p=0.001)

Note: Model 1- T2DM; Model 2 – T2DM + hs-CRP + OPG + adiponectin

Abbreviations: NRI: Net Reclassification Improvement; T2DM: Type Two Diabetes Mellitus; OPG: Osteoprotegerin; hs-CRP: High Sensitive C-Reactive Protein

Table 3: Prediction Performance Analyses for Models with T2DM and circulating inflammatory biomarkers (hs-CRP, OPG and adiponectin) for increased CD31+/annexin V+ to CD62E+ ratio.

distinguish in profile of circulating EMPs and that these differences are more much sufficient than adipocytokine profile, glucose impairment, and lipid abnormalities. Indeed, elevated apoptotic EMPs levels reflect cellular injury and appear to be a surrogate marker of vascular dysfunction [29,30]. Moreover, apoptotic-derived EMPs play a pivotal role in the development of vascular complications in T2DM for they stimulate pro-inflammatory responses in target cells and promote coagulation, thrombosis, angiogenesis, and neovascularization [31,32]. These findings support hypothesis that elevated EMPs are associated with several cardiovascular risk factors and metabolic syndrome, might consider a predictor for the presence of coronary artery lesions, and it is a more significant independent risk factor than length of diabetic disease, lipid levels or presence of hypertension [30-32]. In contrast, activated endothelial cell-derived microparticles may avoid inducing tissue injury and worsening vasomotion function via genome involved mechanisms, and they are thereby able to protect the endothelium from damage [17-19]. Although it has been continued to emphasise that apoptotic subpopulation of EMPs are elevated in metabolic disorders, we did not found significant differences in circulating EMPs labeled as CD144+/annexin V+, CD144+/CD31+/annexin V+, and CD144+/CD31+, except CD31+/annexin V+ and CD62E+ between healthy volunteers and patients with metabolic disorders without

exiting atherosclerosis. Moreover, no sufficient changes in majority subpopulations of apoptotic EMPs and activated endothelial cell-derived microparticles in T2DM and MetS persons were determined. The results of our investigation has shown that exaggerated elevation of CD31+/annexin V+ EMPs with significant changes in CD62E+ EMPs may construct a specific phenotype distinguished healthy persons. In fact, increased CD31+/annexin V+ EMPs to CD62E+ EMPs ratio was reported in dysmetabolic persons especially in T2DM. Therefore, there was a significant association between CD31+/annexin V+ EMPs to CD62E+ EMPs ratio and circulating level of pro-inflammatory cytokines that are suitable for both T2DM and MetS (hs-CRP, OPG and adiponectin). Surprisingly, independent association of CD31+/annexin V+ EMPs to CD62E+ EMPs ratio with cardiovascular risk factor was not found. In this context, it is not clear whether these facts are a confirmation that impaired phenotype of EMPs cause hyper-production of inflammatory cytokines exiting dysmetabolic disorders or opposite increased cytokine production is leading cause of impaired EMP phenotype in T2DM and MetS. There are evidences regarding being of paracrine and endocrine regulation of lipid storage and cell size of white adipocytes by specific micro-RNAs derived by EMPs in metabolic diseases, such as T2DM, obesity and metabolic syndrome [33]. Obviously patients with different types of dysmetabolic disorders might have different EMP patterns [34], which contribute to the development of cardiovascular complications [35]. Collectively, there are raised reports regarding that the presence and number of single EMP population is not obligatory object reflected cardiovascular risk, while predominant immune phenotype is [36-38]. Inclusion of the EMP level into a conventional risk factor model is able to be useful for reclassification of the patients with high probability of cardiovascular disease when personalized immune phenotype of EMPs was used [39-41]. Overall, determination of predominantly immune phenotype of EMPs appears to be attractive for risk classification models and probably creating individualized prediction score in dysmetabolic disorder patients, because of circulating level of pro-inflammatory cytokines demonstrates a high biological variability. On the other hand, EMP determination is not easy for use and analytical errors are frequently

appeared. However, taken together these data are very promising, and they are required new investigation with higher statistical power and increased sample size to be overcome the internal limitations of the study.

In conclusion, we found that patients with T2DM and MetS may distinguish predominantly appeared phenotypes of circulating EMPs associated with pro-inflammatory cytokine over production. Elevated CD31+/annexin V+ EMPs to CD62E+ EMPs ratio is indicator of impaired immune phenotype of EMPs, which allows determining pattern of EMPs in dysmetabolic disorder patients.

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Ethical Principles

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

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. Therefore, there were several technical-related difficulties in the measurement of EMPs. In fact, lack of standard protocol for isolating and detecting circulating EMPs obtained from the plasma. According opinion of the majority experts, centrifugation is became the main factor mediated reliability of the EMP determination in samples and contributed in biological variability of EMP count. Although HD-FACS methodology is widely used, theoretically overlap between two or more fluorochromes might reflect some obstacles for further interpretation of obtained results. Another limitation of the present study is that a specific role of EMPs is also possible and has not been characterized in depth in T2DM patients. However, the authors suppose that these restrictions might have no significant impact on the study data interpretation. Additionally, retrospective, relative small sample size may limit the significance of the present study. However, this was not a randomized and controlled study. The authors believe that a greater cohort of patients with more incidences detected is desirable to improve the credibility of the study.

Authors Contributions

Alexander E Berezin initiated the hypothesis and designed the study protocol, contributed to collect, analyze and interpret the data, performed statistical analysis, and wrote the manuscript. Alexander A. Kremzer contributed to enroll the patients; collected and analyzed the data reviewed the source documents. Tatyana A. Samura preformed visualization procedures and analyzed the results of examinations. Tatyana A. Berezina contributed to enroll the patients in the study and collect the data. Peter Kruzliak contributed in interpretation of the obtained results.

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