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Immune Phenotype of Circulating Endothelial-derived Microparticlesin Elderly Patients with Metabolic Syndrome and Diabetes Mellitus

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Abstract

Type two diabetes mellitus (T2DM) remains a leading contributor to cardiovascular mortality worldwide. This study was conducted to investigate the pattern of circulating EMPs in adults and elderly patients with T2DM and metabolic syndrome (MetS).

Methods: The study retrospectively evolved 76 elderly patients (43 subjects with T2DM and 33 patients with MetS) and 101 adult subjects (54 subjects with T2DM and 47 patients with MetS) with metabolic disorders. 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 adult subjects and elderly patients enrolled in the study regarding CD31+/annexin V+ EMPs to CD62E+ EMPs ratio and CD144+/CD31+/annexin V+ EMPs to CD62E+ EMPs ratio, which reflects impaired phenotype of EMPs. Therefore, CD31+/annexin V+ EMPs to CD62E+ EMPs ratio and CD144+/CD31+/annexin V+ EMPs to CD62E+ EMPs ratio were found to be higher in the T2DM elderly patients compared to MetS elderly patients. 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. Therefore, T2DM (r=0.42, P=0.003), OPG (r=0.34, P=0.001), and adiponectin (r=0.32, P=0.001) predicted increased CD144+/CD31+/annexin V+ EMPs to CD62E+ EMPs ratio.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 and by 9.1% for increased CD144+/CD31+/annexin V+ EMPs to CD62E+ EMPs ratio.

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 and CD144+/CD31+/annexin V+ to CD62E+ EMPs ratio are indicator of impaired immune phenotype of EMPs, which allows determining pattern of EMPs 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] especially in elderly patient population [3,4]. Recent studies have shown that T2DM duration predicts incident cardiovascular events and death, differently from prior myocardial infarction (MI) or stroke at any age [5,6]. Moreover, history of T2DM and a prior MI confer similar risk for subsequent fatal coronary heart disease [7]. Indeed, men with T2DM only have a cardiovascular risk intermediate between men with angina and men with prior MI [8,9]. Whether metabolic syndrome (MetS) is a pre-morbid factor contributed negative effect of T2DM on survival among elderly persons is still understood [10]. However, both clinical conditions T2DM and MetS may contribute in cardiovascular outcomes through interaction of similar pathogenesis' mechanisms [11]. In fact, hyperglycemia, insulin resistance (IR), coagulation, activated immunity and cytokine production, oxidative stress that are 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 [15-20]. 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 [21,22]. 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 [23-25]. Although elevated levels of EMPs, mostly defined as CD144+/CD31+, CD144+/annexin V+, CD31+/Annexin-V+, CD62E+ microparticles, have been found in various cardiovascular diseases, the potential relevance of dysmetabolic diseases to different immune phenotypes of circulating EMPs in elderly patients is still not clear. The aim of the study was to investigate whether pattern of circulating EMPs associates with dysmetabolic disorders in elderly patients.

Methods

The study retrospectively evolved 76 elderly patients (43 subjects with T2DM and 33 patients with MetS) and 101 adult subjects (54 subjects with T2DM and 47 patients with MetS) who were examined in three our centers between February 2013 and November 2014. The main inclusion criterion for the study was documented dysmetabolic disorder defined as T2DM or MetS. We enrolled both adult and elderly dysmetabolic disorder subjects without typical anginal sings and symptoms, without myocardial infarction and any revascularization procedures, as well as without exiting asymptomatic coronary artery disease documented by contrast-enhanced multispiral tomography angiography 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 [26]. 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 [27]. 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 16 (34.0%) MetS adult patients and in 11 (33.3%) MetS elderly subjects.

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) [28].

Calculation of glomerular filtration rate

Glomerular filtration rate (GFR) was calculated with CKD-EPI formula [29].

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) [30] 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 enzymatic method. Concentration of cholesterol of low-density lipoproteins (LDL-C) was calculated according to the Friedewald formula (1972) [31].

Assay of circulating endothelial-derived microparticles

EMPs were quantified as described previously [32]. Circulating EMPs were isolated from 5 ml of venous citrated blood drawn from the fistula-free arm. The platelet-rich plasma was further centrifuged for 2 min at 13,000× g to obtain platelet-free plasma (PFP). 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), andannexin 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. 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). Microbeads from a FACS Size Calibration Kit (Sigma, St Louis, MO, USA) were used for size calibration [32]. 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 [33].

	Entire cohort of adult patients (n=101)	Entire cohort of elderly patients (n=76)	P value
Age, years	48.34 ± 7.80	66.12 ± 5.20	0.001
males, n (%)	64 (63.3%)	51 (67.1%)	0.92
BMI, kg/m2	28.7 (25-75% IQR=16.5–32.4)	29.3 (25-75% IQR=17.8–32.9)	0.76
Waist circumference, sm	91 (25-75% IQR=71–103)	93 (25-75% IQR=73–106)	0.78
Hypertension, n (%)	68 (67.3%)	72 (94.7%)	0.001
Dyslipidemia, n (%)	59 (58.4%)	59 (77.6%)	0.001
T2DM, n (%)	54 (53.5%)	43 (56.5%)	0.82
MetS, n (%)	47 (46.5%)	33 (43.2%)	0.81
Adherence to smoking, n (%)	31 (30.7%)	22 (29.0%)	0.82
Framingham risk score	8.12 ± 2.88	8.43 ± 2.90	0.88
Systolic BP, mm Hg	136 ± 6	148 ± 5	0.001
Diastolic BP, mm Hg	86 ± 6	88 ± 4	0.001
Heart rate, beats per 1 min.	72 ± 7	68 ± 5	0.74
GFR, mL/min/1.73 m^2	93.1 (95% CI=79.5–109.7)	89.6 (95% CI=75.3–102.1)	0.012
HbA1c, %	7.0 (95% CI=4.3-9.2)	7.2 (95% CI=4.5-9.1)	0.21
fasting blood glucose, mmol/L	5.40 (95% CI=3.4-9.1)	5.62 (95% CI=3.9-9.3)	0.36
Insulin, μU/mL	15.15 (25-75%IQR=13.69-16.62)	15.46 (25-75% IQR=14.10-17.55)	0.22
HOMA-IR, mmol/L × μU/mL	3.83 (25-75%IQR=3.47-4.20)	3.98 (25-75%IQR=3.50-4.40)	0.16
creatinine, μmol/L	70.5 (95% CI=59.6–88.3)	96.7 (95% CI=73.3–118.6)	0.001
Total cholesterol, mmol/L	5.3 (95% CI=4.6-6.0)	5.6 (95% CI=4.9-6.1)	0.001
LDL-C, mmol/L	3.60 (95% CI=3.20–4.18)	3.80 (95% CI=3.50–4.20)	0.046
HDL-C, mmol/L	0.94 (95% CI=0.92–1.06)	0.92 (95% CI=0.88–1.00)	0.048
TG, mmol/L	1.68 (95% CI=1.44–1.98)	1.92 (95% CI=1.74–2.17)	0.044
hs-CRP, mg / L	7.96 (25-75%IQR=4.72–9.34)	8.15 (25-75%IQR=4.35–11.20)	0.012
sRANKL, pg / mL	25.80 (25-75%IQR=15.2-46.5)	36.50 (25-75%IQR=21.0-55.6)	0.042
Osteoprotegerin, pg / mL	725.9 (25-75%IQR=579.9-871.9)	793.7 (25-75%IQR=591.5-889.1)	0.001
Adiponectin, mg / L	13.65 (25-75%IQR=10.12-24.93)	17.84 (25-75%IQR=13.32-26.50)	0.001
CD144+/CD31+ EMPs, n/mL	0.91 (25-75% IQR=0.36-1.35)	0.95 (25-75% IQR=0.35-1.47)	0.066
CD144+/annexin V+ EMPs, n/mL	1.15 (25-75% IQR=0.13-2.41)	1.23 (25-75% IQR=0.16-2.56)	0.042
CD144+/CD31+/ annexin V+ EMPs, n/mL	1.01 (25-75% IQR=0.39-1.70)	1.12 (25-75% IQR=0.44-1.96)	0.048
CD31+/annexin V+ EMPs, n/mL	0.296 (25-75% IQR=0.261-0.339)	0.331 (25-75% IQR=0.294-0.388)	0.001
CD62E+ EMPs, n/mL	1.03 (25-75% IQR=0.86-1.13)	0.98 (25-75% IQR=0.80-1.02)	0.001

Table 1:general characteristic of patients participating in the study.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 is a comparison of mean or median variables between both cohorts (ANOVA test).Abbreviations: CI:Confidence Interval; IQR: Inter Quartile Range; T2DM: TypeTwo 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-κBLigand.

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 ($\chi 2$) 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 adults and elderly patients with dysmetabolic disorder was 48.34 and 66.12 years (P=0.001). Therefore 67.1% of elderly patients and 63.3% of adults were men (P=0.92). Except hypertension and dyslipidemia there was not a significant difference between adults and elderly patients with dysmetabolic disorder in BMI, waist circumference, cardiovascular risk factors (adherence to smoking), HOMA-IR, HbA1c, fasting blood glucose, insulin, and Framingham risk score. Therefore, lipid abnormalities, GFR, creatinine, hs-CRP, sRANKL, osteoprotegerin, and adiponectin were higher in elderly patient cohort when compared with adult patient cohort. We also found that numerous of EMPs labeled CD31+/annexin V+ CD144+/CD31+, CD144+/annexin V+, and CD144+/CD31+/annexin V+ was elevated in elderly patient cohort. In contrary, CD62E+ EMPs were elevated in adult persons when compared with dysmetabolic disorder elderly patients (P=0.001). Numerous of both circulating CD31+/annexin V+ EMPs and CD144+/CD31+/annexin V + EMPs reflect more exactly apoptosis of endothelial cells, but CD62E + EMPs are secreted due to activation of endothelial cells. In this context, CD31+/annexin V+ EMPs to CD62E+ EMPs ratio (immune pattern 1 [IM1]) and CD144+/CD31+/annexin V+ EMPs to CD62E+ EMPs ratio (immune pattern 2 [IM2]) were calculated for both cohorts and presented in Figure 1. There is a significant difference between adult subjects and elderly patients enrolled in the study regarding IM1 (Figure 1A) and IM2 (Figure 1B), which reflects impaired phenotype of EMPs with surpassed apoptotic labeled microparticles.

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 IM1 and by 9.1% for increased IM2 (Table 3).

Thus, we suggest that inflammatory biomarkers (hs-CRP, OPG and adiponectin) remain statisticallysignificant predictors for increased IM1 and IM2 in T2DM patients, which reflect impaired phenotype of circulating EMPs.

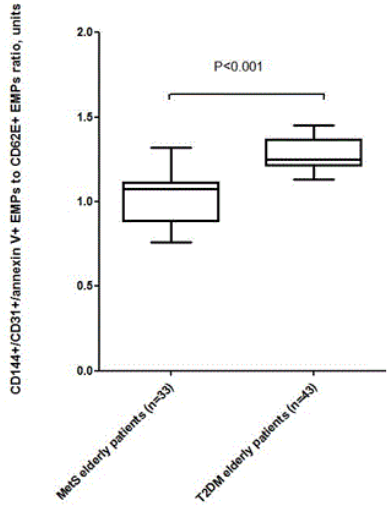

Figure 2: CD31+/annexin V+ EMPs to CD62E+ EMPs ratio (Figure 2A) and CD144+/CD31+/annexin V+ EMPs to CD62E+ EMPs ratio (Figure 2B) in elderly patients with MetS and T2DM. Values are reported as median and IQR, and were compared using ANOVA.The line within the box represents the median value; the top 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; MetS:Metabolic Syndrome; T2DM:Type Two Diabetes Mellitus; ANOVA: Analysis of Variance; IQR:Interquartile Range.

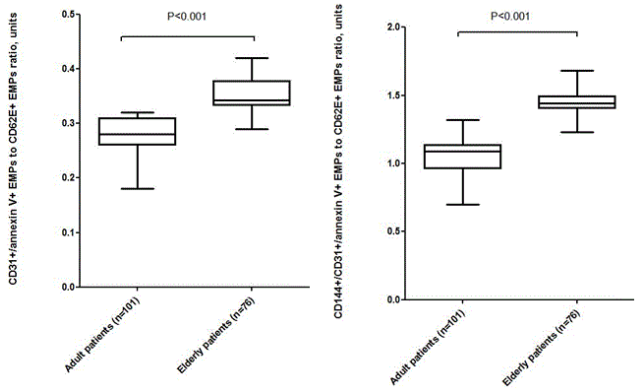

Figure 1: CD31+/annexin V+ EMPs to CD62E+ EMPs ratio (Figure 1A) and CD144+/CD31+/annexin V+ EMPs to CD62E+ EMPs ratio (Figure 1B) in adult patients and elderly patients with dysmetabolic disorder. Values are reported as median and IQR, and were compared using ANOVA.The line within the box represents 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.

Elderly patients with MetS have demonstrated lower concentrations of HbA1c, fasting blood glucose, insulin, creatinine, LDL-C, and CD144+/CD31+/annexin V+ EMPs, and CD31+/annexin V+ EMPs, when compared with T2DM elderly subjects. Lower GFR, *HDL-C,* HOMA-IR *and* CD62E+ EMPs were found in T2DM elderly patients in comparison with MetSelderly subjects. Similarities of circulating levels of EMPs labeled CD144+/CD31+ and CD144+/annexin V+ were determined in both cohorts. Therefore, IM1 (Figure 2A) and IM2 (Figure 2B) were found to be higher in the T2DM elderly patients when compared to MetS elderly patients.

The univariate linear correlation between apoptotic-derived to activated endothelial cell-derived EMP ratio, cardiovascular risk factors, hemodynamic performances, and other biomarker were evaluated. The data have shown that IM1were directly related with BMI (r=-0.545, P=0.003), OPG (r=0.526, P=0.003), adiponectin (r=0.521, P=0.001), sRANKL (r=0.508, P=0.001), hs-CRP (r=0.473, P=0.001), HOMA-IR (r=0.455, P=0.003), T2DM (r=0.412, P=0.003), eGFR (r=-0.368, P=0.001), TG (r=0.341, P=0.001), creatinine (r=-0.360, 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 IM1 with fasting plasma glucose, HbA1c, means of systolic and diastolic BP, waist circumference was found. IM2 was associated with BMI (r=-0.533, P=0.001), OPG (r=0.521, P=0.001), adiponectin (r=0.513, P=0.001), sRANKL (r=0.448, P=0.001), hs-CRP (r=0.442, P=0.001), HOMA-IR (r=0.438, P=0.003), T2DM (r=0.422, P=0.001), eGFR (r=-0.312, P=0.001), TG (r=0.321, P=0.001), creatinine (r=-0.310, P=0.001), gender (r=0.303, P<0.001 for male), Framingham risk score (r=0.302, P=0.001), dyslipidemia (r=0.287, P=0.001).

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. Moreover, T2DM (r=0.42, P=0.003), OPG (r=0.34, P=0.001), and adiponectin (r=0.32, P=0.001) predicted increased CD144+/CD31+/annexin V+ EMPs to CD62E+ EMPs ratio.

When we used other model constructed on entering variables IDI appears to be improved up to 4% for increased IM1 and up to 5% for increased IM2 (available for three inflammatory biomarkers as continuous variables) (Table 4). Three biomarkers (hs-CRP, OPG and adiponectin) improve significantly predictive model based on T2DM for increased IM1. 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 IM1. For increased IM2 we obtained data clarified that for category-free NRI, 5% of events (p=0.001) and 9% 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).

	MetS elderly patients (n=33)	T2DM elderly patients (n=43)	P value
Age, years	65.12 ± 4.90	66.31 ± 5.10	0.72
males, n (%)	23 (69.7%)	28 (61.5%)	0.86
BMI, kg/m^2	29.2 (25-75% IQR=18.7–31.0)	29.5 (25-75% IQR=18.8–32.1)	0.90
Waist circumference, sm	92 (25-75% IQR=74–104)	94 (25-75% IQR=73–105)	0.92
Hypertension, n (%)	32 (97.0%)	40 (93.0%)	0.66
Dyslipidemia, n (%)	25 (75.6%)	34 (79.0%)	0.46
Adherence to smoking, n (%)	7 (21.2%)	15 (34.9%)	0.44
Framingham risk score	8.39 ± 2.3	8.48 ± 2.2	0.28
Systolic BP, mm Hg	147 ± 4	149 ± 5	0.74
Diastolic BP, mm Hg	87 ± 5	89 ± 6	0.76
Heart rate, beats per 1 min.	67 ± 6	68 ± 5	0.76
GFR, mL/min/1.73 m^2	90.5 (95% CI=83.1–102.4)	87.8 (95% CI=80.4–100.8)	0.062
HbA1c, %	6.82 (95% CI=4.61-5.37)	7.33 (95% CI=4.3-9.1)	0.036
fasting blood glucose, mmol/L	5.46 (95% CI=4.23-4.76)	6.14 (95% CI=4.51-9.2)	0.042
Insulin, μU/mL	15.2 (25-75% IQR=14.5–15.7)	15.6 (25-75%IQR=14.9-16.9)	0.044
HOMA-IR, mmol/L × μU/mL	4.16 (25-75%IQR=3.70-4.20)	3.85 (25-75%IQR=3.60-4.08)	0.012
creatinine, μmol/L	92.5 (95% CI=76.2–106.9)	101.1 (95% CI=79.1–117.5)	0.044
Total cholesterol, mmol/L	5.5 (95% CI=4.9-5.7)	5.6 (95% CI=5.0-5.8)	0.86
LDL-C, mmol/L	3.78 (95% CI=3.53–4.17)	3.82 (95% CI=3.62–4.19)	0.012

HDL-C, mmol/L	0.92 (95% CI=0.87–1.03)	0.91 (95% CI=0.88–1.00)	0.014
TG, mmol/L	1.85 (95% CI=1.62–1.95)	1.95 (95% CI=1.90–2.01)	0.046
hs-CRP, mg / L	8.12 (25-75%IQR=3.92–10.43)	8.20 (25-75%IQR=4.40–10.74)	0.44
sRANKL, pg / mL	37.10 (25-75%IQR=22.7-56.5)	36.10 (25-75%IQR=25.3-49.9)	0.46
Osteoprotegerin, pg / mL	785.8 (25-75%IQR=592.1-886.2)	799.1 (25-75%IQR=597.5-886.3)	0.68
Adiponectin, mg / L	17.61 (25-75%IQR=13.14-22.85)	18.10 (25-75%IQR=13.12-23.10)	0.48
CD144+/CD31+ EMPs, n/mL	0.92 (25-75% IQR=0.36-1.39)	0.97 (25-75% IQR=0.35-1.47)	0.22
CD144+/annexin V+ EMPs, n/mL	1.20 (25-75% IQR=0.18-2.44)	1.27 (25-75% IQR=0.16-2.56)	0.18
CD144+/CD31+/annexin V+ EMPs, n/mL	1.08 (25-75% IQR=0.48-1.62)	1.19 (25-75% IQR=0.46-1.95)	0.048
CD31+/annexin V+ EMPs, n/mL	0.319 (25-75% IQR=0.299-0.365)	0.346 (25-75% IQR=0.307-0.381)	0.022
CD62E+ EMPs, n/mL	0.99 (25-75% IQR=0.83-1.07)	0.95 (25-75% IQR=0.80-1.01)	0.034

Table 2: Demographic, risk factors, blood pressure, circulating biomarkers, and in MetS and T2DM elderly patients.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 is a comparison of mean or median variables between both cohorts (ANOVA test).Abbreviations: CI: Confidence Interval; IQR:Inter Quartile Range; T2DM: Type Two Diabetes Mellitus;BP:Blood Pressure; BMI: Body Mass Index; GFR: Glomerular Filtration Rate; EMPs:Endothelial-derived Microparticles; TG: Triglycerides; 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-κBLigand (Table 2).

Models	**Dependent variable: CD31+/annexin V+ EMPs to CD62E+ EMPs ratio**				**Dependent variable: CD144+/CD31+/annexin V+ EMPs to CD62E+ EMPs ratio**			
	AUC (95% CI)	ΔAUC	IDI (± SE)	Relative IDI (%)	AUC (95% CI)	ΔAUC	IDI (± SE)	Relative IDI (%)
Model 1 (based model: T2DM)	0.626	-	-	-	0.622	-	-	-
Model 1 + OPG	0.681	-	-	-	0.686	-	-	-
Model 1 + OPG vs. Model 1	-	0.055; P<0.05	0.06 ± 0.010	10.2%	-	0.06; P<0.05	0.05 ± 0.007	8.8%
Model 1 (based model: T2DM)	0.626	-	-	-	0.622	-	-	-
Model 1 + hs-CRP	0.661	-	-	-	0.660	-	-	-
Model 1 + hs-CRP vs. Model 1	-	0.035; P=0.024	0.03 ± 0.012	5.1%	-	0.038; P<0.05	0.04 ± 0.010	5.5%
Model 1 (based model: T2DM)	0.626	-	-	-	0.622	-	-	-
Model 1 + OPG + hs-CRP	0.683	-	-	-	0.679	-	-	-
Model 1 + OPG + hs-CRP vs. Model 1	-	0.057; P<0.05	0.06 ± 0.009	11.1%	-	0.055; P<0.05	0.05 ± 0.011	8.7%
Model 1 (based model: T2DM)	0.626	-	-	-	0.622	-	-	-
Model 1 + adiponectin	0.655	-	-	-	0.660	-	-	-
Model 1 + adiponectin vs. Model 1	-	0.045; P=0.043	0.02 ± 0.010	4.6%	-	0.048; P<0.05	0.03 ± 0.005	5.4%
Model 1 (based model: T2DM)	0.626	-	-	-	0.622	-	-	-

Model 1 + adiponectin + OPG	0.664	-	-	-	0.662	-	-	-
Model 1 + adiponectin + OPG vs. Model 1	-	0.038; P<0.05	0.03 ± 0.008	7.9%	-	0.036; P<0.05	0.03 ± 0.010	6.6%
Model 1 (based model: T2DM)	0.626	-	-	-	0.622	-	-	-
Model 1 + hs-CRP + OPG + adiponectin	0.690	-	-	-	0.685	-	-	-
Model 1 + hs-CRP + OPG + adiponectin vs. Model 1	-	0.064; P<0.001	0.02 ± 0.015	12.6%	-	0.063; P<0.05	0.04 ± 0.008	9.1%

Table 3: C-statistics for Models with T2DM, hs-CRP, OPG, and adiponectinas Continuous Variables.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.

Model 2 vs. Model 1	**CD31+/annexin V+ EMPs to CD62E+ EMPs ratio**	**CD144+/CD31+/annexin V+ EMPs to CD62E+ EMPs ratio**
Categorical NRI	0.14 (95% CI 0.10-0.19)	0.16 (95% CI 0.11-0.22)
Percentage of events correctly reclassified	4 (p=0.014)	5 (p=0.014)
Percentage of non-events correctly reclassified	7 (p=0.001)	6 (p=0.02)
Categorical free NRI	0.29 (95% CI 0.22-0.36)	0.27 (95% CI 0.23-0.30)
Percentage of events correctly reclassified	6% (p=0.001)	5% (p=0.001)
Percentage of non-events correctly reclassified	14% (p=0.001)	9% (p=0.001)

Table 4: Prediction Performance Analyses for Models with T2DMand circulating inflammatory biomarkers (hs-CRP, OPG and adiponectin) as Continuous Variables for increased CD31+/annexin V+ EMPs to CD62E+ EMPsratio.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.

Discussion

The results of the study clarified that elderly patients with T2DM and MetS may have different 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]. Something like these, pro-inflammatory cytokines (hs-CRP, OPG and adiponectin) are able to stimulate apoptosis and provoke EMP vesiculation [12,13]. Indeed, microvesicules that are phenotypically nearly identical to CD31+/annexin V+ EMPs and CD144+/CD31+/annexin V+ EMPs were elevated in elderly patients with dysmetabolic disorders without exiting atherosclerosis and cardiovascular complications when compared with adult persons. Overall we suggested CD31+/annexin V + EMPs to CD62E+ EMPs ratio and CD144+/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 adults were found. Here we reported that elderly patients with dysmetabolic disorders, such as T2DM and MetS, who have not angiographic evidence of atherosclerosis may 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 [34,35]. 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 [36,37]. 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 [38-40]. 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 found significant differences in circulating EMPs labeled as CD144+/annexin V+, CD31+/annexin V+, CD144+/CD31+/annexin V+, and CD62E+ (except CD144+/CD31+) between adult persons and elderly patients with metabolic disorders without exiting atherosclerosis. Moreover, sufficient changes in majority subpopulations of apoptotic EMPs and activated endothelial cell-derived microparticles in T2DM and MetS elderly persons were determined. The results of our investigation has

shown that exaggerated elevation of EMPs labeled CD31+/annexin V+ and CD144+/CD31+/annexin V+ with significant changes in CD62E+ EMPs may construct a specific phenotype distinguished adult persons with metabolic disorders. In fact, increased CD31+/annexin V+ EMPs to CD62E+ EMPs ratio and CD144+/CD31+/annexin V+ to CD62E+ EMPs ratio were reported in dysmetabolic elderly persons. There was a significant association between impaired phenotype of EMPs and circulating level of pro-inflammatory cytokines that are suitable for both T2DM and MetS (hs-CRP, OPG and adiponectin). Surprisingly, independent association of impaired phenotype of EMPs 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 over production of inflammatory cytokines exiting dysmetabolic disorders or opposite increased cytokine production is leading cause of impaired EMP phenotype in T2DM and MetS. Remarkably, 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 [41]. Obviously patients with different types of dysmetabolic disorders might have different EMP patterns [42], which contribute to the development of cardiovascular complications through mediating of adipocytokine production [43]. In fact, adiponectin promotes peripheral insulin sensitivity and shows several favorable properties as an antiatherogenic and anti-inflammatory agent [44]. The decreased adiponectin plasma level associated with impaired immune phenotype of EMPs may take in attention as explanation regarding the role of dysregulation affected cell-to-cell communication in progression of metabolic diseases. Indeed, pattern of EMPs shows a closely association with inflammatory markers, which is known to be the molecular link between IR, obesity, MetS and T2DM. 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 [45,46]. 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 [47]. 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.

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. Something like these, 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.

Conclusion

Wefound that patients with T2DM and MetS may distinguishpredominantly appeared phenotypes of circulating EMPs associated with over production of pro-inflammatory cytokines (hs-CRP, OPG and adiponectin). Elevated CD31+/annexin V+ EMPs to CD62E+ EMPs ratio and CD144+/CD31+/annexin V+ to CD62E+ EMPs ratio are 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.

Conflict of Interests

Not declared

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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