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The Inflammatory Biomarkers as Predictors of Decreased Number of Mononuclear Progenitor Cells in Patients with Metabolic Syndrome and Type 2 Diabetes Mellitus

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Abstract

Introduction: Metabolic disorders remain a leading contributor to cardiovascular mortality worldwide. Circulating endothelial progenitor cells (EPCs) are decreased in metabolic disorders, thus identifying the different populations of EPCs could assist in prognosis. This study was conducted to investigate the population of circulating endothelial progenitor cells (EPCs) in patients with type two diabetes mellitus (T2DM) or metabolic syndrome (MetS).

Materials and Methods: The study retrospectively involved 101 patients (54 subjects with T2DM and 47 patients with MetS) and 35 healthy volunteers. Flow cytometry was used for detecting EPCs using CD45, CD34, CD14, Tie-2, and VEGFR2 (CD309) markers, which were measured at the beginning of the study.

Results: There is a significant difference between the median total number and frequency of CD14/CD309+ and CD14/CD309/Tie2+ in patients with dysmetabolic disorders vs control. CD14/CD309+ and CD14/CD309/Tie2+ EPCs were 19% and 14% higher among MetS subjects in comparison with T2DM patients. Osteoprotegerin (OPG) and hs-C-reactive protein (hs-CRP), significantly improved the predictive model based on T2DM + number of multiple cardiovascular risk factors (MCRFs)>3 for both subsets of EPCs. Among patient study population for category-free NRI, 5% of events and 11% of non-events were correctly reclassified by the addition of hs-CRP and OPG to the base model for decreased absolute number of circulating EPCs labeled CD14+CD309+. Therefore, 6% of events and 14% of non-events were correctly reclassified using category-free NRI for depleted CD14+CD309+Tie2+ EPCs

Conclusions: In conclusion, we suggest that inflammatory biomarkers (hs-CRP, OPG) could be a predictor for decreased CD14+CD309+ and CD14+CD309+Tie2+ EPCs among dysmetabolic patients, without preexisting atherosclerotic lesions of coronary arteries.

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Introduction

Metabolic disorders, such as type two diabetes mellitus (T2DM), metabolic syndrome (MetS) and obesity are increasing worldwide to an epidemic level [1-3]. It has been known that both T2DM and MetS contribute in development of endothelial dysfunction (ED), accelerating atherosclerosis, inducing oxidative stress and inflammation [4-7]. Consequently, they are considered powerful causal factors for cardiovascular diseases (CVD) and cardiovascular outcomes [8]. It is well established that ED associates with poor clinical outcomes in patients with established CVD [9], thus pathogenic factors that induce endothelial dysfunction in the earlier stages of dysmetabolic diseases might further stimulate CVD progression [5].

Endothelial progenitor cells (EPCs) are a population of cells that expresses endothelial and progenitor markers i.e. CD34+, VEGFR-2+, $CD133^+$, as well as $CD14^+$, and $Tie2^+$ [10-12]. EPCs may play a pivotal role in tissue repair, maturation of endothelial cells, angiogenesis, and revascularization [13]. EPCs are mobilized from bone marrow and possibly from peripheral tissues upon injury as a result of growth factors and inflammatory cytokines [14], and they are increased in ED [15]. As well, increased production of reactive oxygen species, insulin resistance, reduced nitric oxide bioavailability have been associated with tissue injury and ED [16,17]. The one major type of EPCs is so called "early" EPCs, which might produce endothelial-like cells within few days of in vitro culture [18]. Although they have been exhibited to be predominantly of monocytic origin, they contribute to angiogenesis, modulate activity of the tissue resident cells, and participate in development of cardiovascular disease [19, 20]. Given the monocytic origin of these cells they express specific monocytes' antigens (CD14 and Tie2) additionally to classic endothelial antigens (CD133, CD309) [21]. However, "none classical" phenotypes of EPCs labeled as $CD14/CD309^+$ and CD14/CD309/Tie2+ can be assessed by flow cytometry from fresh blood samples.

T2DM is associated with a depletion of circulating classical EPCs resulting in severely reduced angiogenic capacity in vivo [22], which could lead to increased frequency of CVD events [23,24]. However, the role of different subsets of EPCs (including "none classical"

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phenotypes) in development and progression of T2DM / MetS is still not understood and requires to be carefully investigated. Thus, the aim of this study was to investigate the pattern of circulating EMPs in T2DM patients in comparison with MetS subjects and healthy control.

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 angina pectoris and without existing coronary artery disease (negative contrast-enhanced multispiral tomography angiography). All patients have given their informed written consent for participation in the study. T2DM was diagnosed with revised criteria provided by American Diabetes Association when source documents were reviewed [25]. 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 [26]. 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.

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 achieve control of T2DM. Therefore, insulin was not used in enrolled patients. The majority of patients with established MetS were treated with life-style modification and diet. Metformin was given 12 patients taking into consideration insulin resistance due to abdominal obesity as main indication for prescription.

Methods for visualization of coronary arteries

Contrast-enhanced multispiral computed tomography angiography was 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) [27]. Asymptomatic atherosclerosis was defined as stenosis of plaque at least in one coronary artery > 50% and / or coronary calcification quantified using Agatston scoring [28].

Methods to assess blood pressure, body mass index and waist circumference

Blood pressure, waist circumference, body mass index (BMI) was observed at baseline using standard procedures.

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.

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 -70oC. Serum adiponectin, Receptor activator of nuclear factor kappa-B ligand (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%, Receptor activator of nuclear factor kappa-B ligand: 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 25–741 µmol/L.

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 detection limits of insulin level were 0.2-1000. µU/mL.

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

Concentrations of total cholesterol (TC), low-density lipoproteins (LDL-C) and cholesterol of high-density lipoproteins (HDL-C) were measured by enzymatic method.

Hemoglobin A1c (HbA1c) were determined by high-pressure liquid chromatography method.

Blood sampling for circulating EPCs’ measurement

Blood samples were received from peripheral vein in blood collection tubes. Each sample contains 75 µL into 1mL PBS containing 5 µM EDTA (10 µL of 0.5 M stock). To prevent clotting samples were mixed immediately. Peripheral blood mononuclear cells were removed using density gradient centrifugation with Ficoll-Paque (Miltenyi Biotec Inc., Germany). After layer 35 mL of diluted cell suspension over 15 mL of Ficoll-Paque in a 50 mL conical tub all blood samples with anticoagulants (EDTA) have centrifuged at 400×g for 30–40 minutes at 20°C in a swinging-bucket rotor without brake. The upper layer leaving the mononuclear cell layer was aspirated to prevent a contamination of samples before measurement of real EPCs.

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RBCs from samples were removed from the samples using the classic LYSE-WASH protocol. Cells were washed 2-3x with FACS buffer (PBS supplemented with either 1% BSA or 5% FBS and containing 0.05% NaNO3). Fifty µL FACS buffer per each analysis on a single sample was suspended the pellet from the final wash. Then 50 µL of cell suspension to 10 µL of antibody solution were added and immediately mixed. After incubation for 30 minutes on ice cells 2-3x with FACS buffer was washed and suspended in 200-300 µL FACS buffer for analysis. The samples were centrifuged at 200× g for 15 min; then they were washed twice with PBS and fixed immediately.

Determination of circulating EPCs

For further analysis, endothelial derived and mononuclear progenitor cells were re-suspended in 100 µl of a fluorescence-activated cell-sorting buffer containing phosphate-buffered saline, 0.1 percent bovine albumin, and aprotinin (20 µL /mL). The flow cytometric technique (FCT) was used for predictably distinguishing circulating cell subsets, which depend on expression of CD45, CD34, CD14, Tie-2, and VEGFR2, using High-Definition Fluorescence Activated Cell Sorter (HD-FACS) methodology [31].

Accordingly, the cells were labeled on the basis of their forward scatter characteristic (FSC) and side scatter characteristic (SSC) profiles. The cells were directly stained and analyzed for phenotypic expression of surface proteins using anti-human monoclonal antibodies, including anti-CD45 FITS (BD Biosciences, San Jose, CA, USA), anti-CD34 FITS (BD Biosciences), anti-VEGFR-2 known as anti-CD309 (BD Biosciences), anti-Tie2 (BD Biosciences) and anti-CD14 (BD Biosciences). The fluorescence minus one technique was used to provide negative controls and establish positive stain boundaries. Double- or triple-positive events were determined using Boolean principles ("and", "not", "or", etc.). IgG2a–FITC–PE antibody (Becton Dickinson) served as a negative control.

Circulating EPCs were defined as CD34/CD309 positive cells with lack of CD45 expression. CD14 is common antigen for mononuclears and endothelial cells, while CD309 antigen is represented in endothelial cells. For mononuclear progenitor CD14+ cells' populations, co-expression with Tie-2– and/or VEGFR-2– was determined using quadrant analysis. Cell fluorescence was measured immediately after staining, and data were analyzed with the use of CellQuest software (FACSCalibur, Becton Dickinson). Units of all measured components are absolute cell counts (cells × 10^3/µL). The Fluorescence Minus One Control (FMO control) was used to properly interpret flow cytometry data.

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 EPCs in patients were examined in multivariable 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 48.3 years and 46.1 years (P=0.68). Equal numbers of men were in dysmetabolic disorder group and control (63.3% vs 65.7%, 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, soluble Receptor activator of nuclear factor kappa-B ligand, osteoprotegerin, and adiponectin were higher in patient cohort when compared with healthy volunteers. No increase in circulating CD34+ subset cells (CD45/CD34+ and CD45–CD34+) in dysmetabolic patients when compared with healthy volunteers was found. A significant difference between the medians of absolute numbers and frequencies of CD14/CD309+ and CD14/CD309/Tie2+ in healthy volunteers and patients with dysmetabolic disorders respectively (P<0.01 and P=0.04).

Table 2 shows patients with MetS have lower incidence of dyslipidemia, lower concentrations of HbA1c, fasting blood glucose, insulin, and LDL-C when compared with T2DM subjects. Higher HDL-C and HOMA-IR were found in T2DM patients than in MetS subjects. Interestingly, similarities of circulating EPCs were determined in both cohorts, although absolute numbers of CD14/CD309+ and CD14/CD309/Tie2+ were higher among MetS subjects in comparison with T2DM patients (P=0.18 and P=0.012 respectively).

The univariate linear correlations between both absolute numerous of EPCs with immune phenotypes labelled CD14/CD309+ and CD14/CD309/Tie2+, cardiovascular risk factors, hemodynamic performances, and other biomarkers were evaluated. Absolute numbers of CD14/CD309+ were inversely related with T2DM (r = -0.542, P = 0.003), BMI (r = -0.54, P = 0.001), OPG (r = -0.518, P = 0.001), number of multiple cardiovascular risk factors (MCRFs) (r = -0.486, P = 0.001), hs-CRP (r = -0.478, P = 0.001), soluble Receptor activator of nuclear factor kappa-B ligand (r = -0.477, P = 0.001), adiponectin (r = -0.402, P = 0.001), eGFR (r = -0.398, P = 0.001), Framingham risk score (r = -0.394, P = 0.001), TG (r = -0.392, P = 0.001), creatinine (r = -0.387, P = 0.001), SUA (r = -0.315, P < 0.001), gender (r = -0.318, P < 0.001 for male), dyslipidemia (r = -0.313, P = 0.001), age (r = -0.275, P = 0.001), smoking (r = -0.212, P = 0.001), and positively related with HOMA-IR (r = 0.465, P = 0.001).

Therefore, absolute numbers of CD14/CD309/Tie2+ EPCs were negatively related T2DM (r = -0.55, P = 0.001), OPG (r = -0.522, P = 0.001), number of MCRFs (r = -0.492, P = 0.001), hs-CRP (r = -0.486, P = 0.001), BMI (r = -0.483, P = 0.001), adiponectin (r = -0.472, P = 0.001), soluble Receptor activator of nuclear factor kappa-B ligand (r = -0.466, P = 0.001), TG (r = -0.412, P = 0.001), eGFR (r = -0.392, P = 0.001), Framingham risk score (r = -0.39, P = 0.001), creatinine (r = -0.365, P = 0.001), dyslipidemia (r = -0.322, P = 0.001), SUA (r = -0.309, P < 0.001), gender (r = -0.303, P < 0.001 for male), age (r = -0.262, P = 0.001), smoking (r = -0.211, P = 0.001), and positively related with HOMA-IR (r = 0.482, P = 0.001). No significant association of absolute numerous of CD14/CD309+ and CD14/CD309/Tie2+ EPCs with fasting plasma glucose, HbA1c, number of MetS components, means of systolic and diastolic BP, waist circumference was found. No evidence was found to suggest possible age- and gender-related correlation between metabolic status and the presence of EPCs.

	Healthy volunteers (n=35)	Entire cohort of enrolled patients (n=101)	P value
Age, years	46.12±4.22	48.34±7.80	0.68
Males, n (%)	23 (65.7%)	64 (63.3%)	0.86
BMI, kg/m2	21.5 (25-75% IQR=16.1–23.5)	28.7 (25-75% IQR 16.5–32.4)	0.001
Waist circumference, sm	78 (25-75% IQR=63–89)	91 (25-75% IQR=71–103)	0.001
Hypertension, n (%)	-	68 (67.3%)	0.001
Dyslipidemia, n (%)	-	59 (58.4%)	0.001
T2DM, n (%)	-	54 (53.5%)	0.001
MetS, n (%)	-	47 (46.5%)	0.001
Adherence to smoking, n (%)	6 (17.1%)	31 (30.7%)	0.001
Framingham risk score	2.55± 1.05	8.12 ± 2.88	0.001
Systolic BP, mm Hg	122±5	136±6	0.001
Diastolic BP, mm Hg	72±4	86±6	0.001
Heart rate, beats per 1 min.	66±6	72±7	0.01
GFR, mL/min/1.73 m2	102.1 (95% CI=91.4–113.2)	93.1 (95% CI=79.5–109.7)	0.12
HbA1c, %	4.75 (95% CI =4.36-5.12)	7.0 (95% CI =4.3-9.2)	0.001
Fasting blood glucose, mmol/L	4.52 (95% CI =4.43-4.76)	5.40 (95% CI =3.4-9.1)	0.01
Insulin, µU/mL	4.98 (25-75% IQR =1.5–14.1)	15.15 (25-75% IQR =13.69-16.62)	0.001
HOMA-IR, mmol/L × µU/mL	1.01 (25-75% IQR =0.91-1.07)	3.83 (25-75% IQR =3.47-4.20)	0.001
Creatinine, µmol/L	62.1 (95% CI =55.7–82.4)	71.9 (95% CI =56.9–88.3)	0.24
Total cholesterol, mmol/L	4.76 (95% CI =4.21-5.05)	5.3 (95% CI =4.6-6.0)	0.001
LDL-C, mmol/L	3.10 (95% CI =2.78–3.21)	3.60 (95% CI =3.20–4.18)	0.001
HDL-C, mmol/L	1.13 (95% CI = 1.05–1.17)	0.94 (95% CI = 0.92–1.06)	0.001
TG, mmol/L	1,18 (95% CI = 1.07–1.30)	1,68 (95% CI = 1.44–1.98)	0.001
SUA, µmol/L	230.3 (95% CI = 150.5–339.7)	231.1 (95% CI = 159.5–345.1)	0.05
hs-CRP, mg / L	4.11 (25-75% IQR=0.97 – 5.03)	7.96 (25-75% IQR=4.72 – 9.34)	0.001
sRANKL, pg / mL	16.10 (25-75% IQR=2.1-30.1)	25.80 (25-75% IQR=15.2-46.5)	0.002
Osteoprotegerin, pg / mL	88.3 (25-75% IQR=37.5-136.6)	725.9 (25-75% IQR=579.9-871.9)	0.001
Adiponectin, mg / L	6.17 (25-75% IQR=3.44-10.15)	13.65 (25-75% IQR=10.12-24.93)	0.001
CD45+CD34+, cells × 103/µL	0.114 (25-75% IQR = 0.095–0.120)	0.112 (25-75% IQR = 0.090–0.121)	0.76
CD45−CD34+, cells × 10−1/µL	0.06 (25-75% IQR = 0.05–0.07)	0.057 (25-75% IQR = 0.053–0.067)	0.86
CD14+CD309+, cells × 10−1/µL	4.26 (25-75% IQR = 3.70–5.74)	2.96 (25-75% IQR = 2.25–4.21)	0.01
CD14+CD309+Tie2+, cells × 10−1/µL	0.465 (25-75% IQR = 0.253–0.710)	0.270 (25-75% IQR = 0.241–0.411)	0.01

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; 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.

Using multivariate age- and gender-adjusted logistic regression analysis, independent impact of T2DM (odds ratio [OR] = 1.08, P = 0.003), hs-CRP per 4.50 mg/L (OR = 1.12, P = 0.001), number of MCRFs >3 (OR = 1.15, P = 0.001), OPG per 125.5 pg / mL (OR = 1.14, P = 0.002) on decreased of CD14/CD309+ EPCs was determined (Table 3). Therefore, CD14/CD309/Tie2+ EPCs were negatively impacted by T2DM (OR = 1.10, P = 0.001), hs-CRP per 4.50 mg/L (OR = 1.12, P = 0.001), number of MCRFs >3 (OR = 1.17, P = 0.001), OPG per 125.5 pg / mL (OR = 1.11, P = 0.001), and HOMA-IR per 0.65 mmol/L × µU/mL (OR = 1.06, P = 0.001).

Using C-statistics for Models with T2DM, HOMA-IR, number of MCRFs >3, and circulating biomarkers (hs-CRP, OPG) as Continuous Variables we found that adding of combination of inflammatory biomarkers (hs-CRP, OPG) to the based model (T2DM + MCRFs >3) improved the relative IDI by 9.7% for decreased CD14/CD309+ EPCs and by 10.2% for decreased CD14/CD309/Tie2+ EPCs, but HOMA-IR did not demonstrate significant impact on improving of based predictive model (Table 4). In contrast, OPG alone, hs-CRP alone added to Model 1 have exhibited significant improvement of discriminative value of combined models vs Model 1. However,

	MetS patients (n=47)	T2DM patients (n=54)	P value
Age, years	48.30±3.94	48.50±6.60	0.88
Males, n (%)	30 (63.8%)	34 (63.0%)	0.96
BMI, kg/m2	28.2 (25-75% IQR=16.7–31.0)	28.5 (25-75% IQR=16.8–32.1)	0.92
Waist circumference, sm	92 (25-75% IQR=69–105)	89 (25-75% IQR=72–100)	0.12
Hypertension, n (%)	32 (68.0%)	36 (66.7%)	0.78
Dyslipidemia, n (%)	26 (55.3%)	33 (61.1%)	0. 026
Adherence to smoking, n (%)	16 (34.0%)	15 (27.7%)	0.44
Framingham risk score	8.09± 2.12	8.18 ± 2.32	0.78
Systolic BP, mm Hg	137±4	136±5	0.94
Diastolic BP, mm Hg	87±5	86±4	0.96
Heart rate, beats per 1 min.	71±6	72±5	0.96
GFR, mL/min/1.73 m2	92.5 (95% CI=83.1–107.4)	93.8 (95% CI=80.4–106.8)	0.92
HbA1c, %	6.82 (95% CI =4.61-5.37)	7.3 (95% CI =4.3-9.1)	0.036
Fasting blood glucose, mmol/L	4.46 (95% CI =4.23-4.76)	5.54 (95% CI =4.49-9.0)	0.042
Insulin, μU/mL	14.2 (25-75% IQR =12.5–15.7)	15.6 (25-75% IQR =12.9-16.8)	0.048
HOMA-IR, mmol/L × μU/mL	3.45 (25-75% IQR =3.22-3.78)	3.86 (25-75% IQR =3.41-4.10)	0.012
Creatinine, μmol/L	72.3 (95% CI =56.1–86.9)	71.2 (95% CI =59.9–87.2)	0.94
Total cholesterol, mmol/L	5.3 (95% CI =4.5-5.9)	5.4 (95% CI =4.8-5.8)	0.96
LDL-C, mmol/L	3.48 (95% CI =3.30–4.07)	3.80 (95% CI =3.20–4.20)	0.012
HDL-C, mmol/L	1.01 (95% CI = 0.90–1.13)	0.94 (95% CI = 0.88–1.04)	0.014
TG, mmol/L	1.77 (95% CI =1.62–1.95)	1.45 (95% CI =1.42–1.51)	0.044
SUA, μmol/L	230.9 (95% CI = 161.8–296.9)	229.6 (95% CI = 165.4–327.3)	0.86
hs-CRP, mg / L	7.87 (25-75% IQR=4.92 – 9.43)	8.10 (25-75% IQR=4.80 – 9.54)	0.24
sRANKL, pg / mL	24.10 (25-75% IQR=14.7-36.9)	26.20 (25-75% IQR=15.3-40.7)	0.26
Osteoprotegerin, pg / mL	718.5 (25-75% IQR=572.1-846.2)	732.1 (25-75% IQR=587.5-866.3)	0.38
Adiponectin, mg / L	13.61 (25-75% IQR=9.74-22.35)	14.12 (25-75% IQR=10.12-23.10)	0.88
CD45+CD34+, cells × 103/μL	0.114 (25-75% IQR = 0.094–0.122)	0.109 (25-75% IQR = 0.091–0.117)	0.56
CD45–CD34+, cells × 10–1/μL	0.060 (25-75% IQR = 0.055–0.066)	0.056 (25-75% IQR = 0.052–0.064)	0.82
CD14+CD309+, cells × 10–1/μL	3.35 (25-75% IQR = 2.57–4.18)	2.71 (25-75% IQR = 2.31–3.52)	0.018
CD14+CD309+Tie2+, cells × 10–1/μL	0.294 (25-75% IQR = 0.245–0.396)	0.253 (25-75% IQR = 0.232–0.311)	0.012

Table 2: Demographic, risk factors, blood pressure, circulating biomarkers, and endothelial-derived microparticles in MetS and T2DM 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; BMI - Body mass index, T2DM – type two diabetes mellitus, BP – blood pressure, BMI - Body mass index, GFR - glomerular filtration rate, EPCs – endothelial progenitor cells; 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-κB ligand.

combination of both biomarkers (hs-CRP, OPG) has represented more much IDI for decreased CD14/CD309+ EPCs and lowered CD14/CD309/Tie2+ EPCs compared with OPG alone, hs-CRP alone added to Model 1.

Inflammatory biomarkers (hs-CRP, OPG) significantly improved the predictive model based on T2DM + number of MCRFs >3 for decreased both angiopoetic phenotypes of circulating EPCs (Table 5). Among patient study population for category-free NRI, 5% of events (p=0.001) and 11% of non-events (p=0.001) were correctly reclassified by the addition of circulating inflammatory biomarkers (hs-CRP, OPG) to the base model (T2DM + number of MCRFs >3) for decreased absolute number of circulating EPCs labeled CD14/CD309+. Therefore, 6% of events (p=0.001) and 14% of non-events (p=0.002) were correctly reclassified using category-free NRI for depleted absolute number of circulating EPCs labeled CD14/CD309/Tie2+.

Discussion

The results of our study demonstrate that depletion of circulating CD14/CD309+ and CD14/CD309/Tie2+ EPCs was observed in dysmetabolic patients. Moreover, circulating levels of CD14/CD309+ and CD14/CD309/Tie2+ EPCs were significantly lower in T2DM subjects when compared with MetS. Also, inflammatory cytokines i.e. OPG and hs-CRP were significant association with reduced CD14/CD309+ and CD14/CD309/Tie2+ EPC subsets among dysmetabolic

Factors	CD14+CD309+ EPCs		CD14+CD309+Tie2+ EPCs	
	OR (95% CI)	P Value	OR (95% CI)	P Value
Hypertension (present vs abcent)	0.99 (0.87–1.04)	0.18	1.05 (0.97–1.11)	0.16
Dyslipidemia (present vs abcent)	1.04 (0.92–1.22)	0.24	1.02 (0.96–1.15)	0.34
T2DM (present vs abcent)	1.08 (1.04–1.12)	0.003	1.10 (1.02–1.20)	0.001
BMI per 5.0 kg/m^2	1.05 (0.97–1.18)	0.16	1.05 (0.95–1.07)	0.12
hs-CRP per 4.50 mg/L	1.12 (1.03–1.20)	0.001	1.12 (1.06–1.24)	0.001
Number of MCRFs >3	1.15 (1.07–1.30)	0.001	1.17 (1.11–1.22)	0.001
TC per 0.65 mmol/L	1.03 (0.88–1.10)	0.12	1.01 (0.98–1.10)	0.11
SUA per 4.5 mmol/L	1.02 (0.94–1.14)	0.10	1.04 (0.96–1.10)	0.12
OPG per 125.5 pg / mL	1.14 (1.07–1.26)	0.002	1.11 (1.08–1.21)	0.001
Adiponectin, per 9.5 mg/L	1.02 (0.99–1.04)	0.08	1.01 (0.98–1.05)	0.09
HOMA-IR per 0.65 mmol/L × µU/mL	1.04 (1.00–1.06)	0.10	1.06 (1.02–1.07)	0.001
sRANKL per 12.5 pg / mL	1.03 (0.98–1.07)	0.12	1.02 (0.99–1.04)	0.12

Table 3: The independent predictors of depletion of circulating EPCs. The results of age- and gender-adjusted multivariate logistic regression analysis

Abbreviations: CI – confidence interval; IQR – inter quartile range; BMI - Body mass index, T2DM – type two diabetes mellitus, BMI - Body mass index, EPCs - endothelial progenitor cells; TG – triglycerides, hs-CRP – high sensitive C reactive protein, sRANKL – serum receptor activator of NF-κB ligand, MCRFs—multiple cardiovascular risk factors.

Models	Dependent variable: CD14+CD309+ EPCs				Dependent variable: CD14+CD309+Tie2+ EPCs			
	AUC (95% CI)	ΔAUC	IDI (±SE)	Relative IDI (%)	AUC (95% CI)	ΔAUC	IDI (±SE)	Relative IDI (%)
Model 1 (based model: T2DM + number of MCRFs >3)	0.644 (0.626-0.667)	-	-	-	0.652 (0.632-0.671)	-	-	-
Model 1 + OPG	0.689 (0.644-0.718)	-	-	-	0.688 (0.645-0.697)	-	-	-
Model 1 + OPG vs Model 1	-	0.045; P<0.05	0.03±0.004	7.5%	-	0.036; P<0.05	0.02±0.004	6.1%
Model 1 + HOMA-IR	0.677 (0.624-0.706)	-	-	-	0.676 (0.625-0.710)	-	-	-
Model 1 + HOMA-IR vs Model 1	-	0.033; P=0.058	0.02±0.009	3.2%	-	0.024; P=0.062	0.02±0.007	4.0%
Model 1 + hs-CRP	0.682 (0.649-0.709)	-	-	-	0.681 (0.633-0.715)	-	-	-
Model 1 + hs-CRP vs Model 1	-	0.038; P<0.05	0.03±0.005	6.3%	-	0.029; P=0.054	0.03±0.006	4.7%
Model 1 + OPG + hs-CRP	0.694 (0.653-0.721)	-	-	-	0.693 (0.652-0.727)	-	-	-
Model 1 + OPG + hs-CRP vs Model 1	-	0.050; P<0.05	0.06±0.007	9.7%	-	0.041; P<0.05	0.05±0.005	10.2%

Table 4: C-statistics for Models with T2DM, HOMA-IR, number of MCRFs >3, and circulating biomarkers (hs-CRP, OPG) as Continuous Variables.

Note: Relative IDI – calculated as the ratio of IDI over the discrimination slope of the model without T2DM + number of MCRFs >3.
Abbreviations: AUC – area under curve, SE – standard error, T2DM – type two diabetes mellitus, OPG – osteoprotegerin, hs-CRP – high sensitive C-reactive protein, MCRFs - multiple cardiovascular risk factors.

Model 2 versus Model 1	Decreased absolute number of circulating EPCs labeled CD14+CD309+	Decreased absolute number of circulating EPCs labeled CD14+CD309+Tie2+
Categorical NRI	0.12 (95% CI = 0.10-0.15)	0.15 (95% CI = 0.10-0.15)
Percentage of events correctly reclassified	5 (p=0.19)	6 (p=0.14)
Percentage of non-events correctly reclassified	9 (p=0.044)	8 (p=0.046)
Categorical free NRI	0.22 (95% CI = 0.20-0.27)	0.27 (95% CI = 0.21-0.35)
Percentage of events correctly reclassified	5% (p=0.001)	6% (p=0.001)
Percentage of non-events correctly reclassified	11% (p=0.001)	14% (p=0.002)

Table 5: Prediction Performance Analyses for Models with T2DM, number of MCRFs, and circulating inflammatory biomarkers (hs-CRP, OPG) as Continuous Variables for decreased absolute number of CD14+CD309+and CD14+CD309+Tie2+ EPCs.

Note: Model 1- T2DM + number of MCRFs >3; Model 2 – T2DM + number of MCRFs >3 + hs-CRP + OPG.
Abbreviations: NRI - net reclassification improvement, T2DM – type two diabetes mellitus, OPG – osteoprotegerin, hs-CRP – high sensitive C-reactive protein, MCRFs - multiple cardiovascular risk factors.

subjects. However, adiponectin level was higher in patient cohort when compared with healthy volunteers, but adiponectin was not an independent predictor for decreased angiopoetic phenotypes of circulating EPCs among T2DM patients.

Theoretically, adipokines liberated from visceral adipose tissues play a pivotal role in modulating endothelial function and might have beneficial effects on endothelial cells. Probably, adiponectin might negatively correlate with low number of circulating EPCs in MetS adult patients, while the data obtained from results provided by differenced authors are controversial [32-34]. An ability of adiponectin to reduce viability of EPCs in T2DM patients compared with non-diabetic patients was previously found [34]. It is suggested that there is an inadequate heme oxygenase-adiponectin axis response, which could compromise the compensatory antioxidant and anti-inflammatory effects consequently contributing toward EPC dysfunction in T2DM patients [35]. Additionally, this effect of adiponectin might have a clinical significance in dysmetabolic patients with previously defined coronary artery disease [35]. We did not confirm the role of adiponectin as predictor of lowed EPCs n dysmetabolic individuals without known atherosclerosis and coronary artery disease.

We propose that low-intensity inflammation associated with elevated hs-CRP and OPG that was commonly appeared in dysmetabolic persons may stimulate CVD development through impaired endothelial reparation process due to low recruitment of EPCs with angiopoetic activity.

Recent studies have shown a close association of serum levels of OPG and hs-CRP with T2DM [23,36,37]. Indeed, OPG is considered a biomarker candidate, which modulates vascular remodeling effects and CVD development and progression [38]. In fact, OPG is over expressed on endothelial and smooth muscle cells in vasculature in dysmetabolic states [39]. Moreover, epidemiological studies have examined the relationship of OPG to CVD events and mortality [40-42]. Strong associations of hs-CRP with components of MetS, T2DM, other inflammatory cytokine levels, as well as CVD events were found in several investigations [43-45]. Higher level of OPG and hs-CRP in MetS and T2DM reflected chronic low grade inflammation which could mediate the development of diabetic complications and clinical CVD outcomes through ED [8].

There is a limited body of evidence regarding the relation between inflammation and EPG reparative dysfunction. Indeed, ED could be caused by altered endothelial cell activity and integrity due to dysregulation of vascular wall repair processes [12,46] mediated via angiogenic EPCs [12,47]. In metabolic disorders the infiltration of the vascular subintima by low-density lipoproteins cause a production of free radicals, oxidation of cytoskeleton and membrane vesiculation of precursors of endothelial cells and decrease their ability to restore of pool of mature endothelial cells [46, 47]. Consequently, the oxidative-driven repair dysfunction of EPCs may relate to low intensity inflammation in vasculature, which associates with overproduction of cytokines i.e. hs-CRP and adiponectin. Indeed, decreased number and / or functionally impaired EPCs are one of the major factor for the development of metabolic memory that leads to vascular complications in dysmetabolic patients [48, 49].

Results of the presented study have shown that increased circulating level of inflammatory cytokines in T2DM patients is associated with deficiency of angiogenic EPCs when compared with MetS, while frequencies and absolute numbers of other immune phenotype EPCs remain similar. It is possible that OPG and hs-CRP determine circulating progenitor cells mononuclear and endothelial origin and relates a progress from MetS and prediabetes to T2DM. Interestingly, glycemic control, BMI, insulin resistance were not defined as independent predictors for decreased CD14/CD309+ and CD14/CD309/Tie2+ cells in dysmetabolic patients, but T2DM, numbers of CVD risk factors, OPG and hs-CRP were positive. These data suggest that EPCs as a potential novel marker of vascular integrity, homeostasis process, metabolic abnormalities, and cardiovascular risk, and are supported by others [50,51]. It is proposed that a reduction and dysfunction of EPCs associates with inflammatory-dependent impairment of vascular function and relates to progress from MetS to T2DM. Overall, the results presented in this study suggest that vascular reparative dysfunction associated with OPG and hs-CRP over production among T2DM subjects without preexisting atherosclerotic lesions of coronary arteries, is superior to MetS patients. Therefore, the results of the study may impact on future diagnostic and therapeutic strategies that appear to be improved clinical outcomes of dysmetabolic disorders.

Study Limitations

All subjects with T2DM ad MetS were comparable to age and sex as study design was retrospective. Thus, we cannot confirm such associations with EPCs. It is necessary to note that a large pool of mononuclear cells might be produced after blood sampling due to destruction of platelets and blood cells, which can interfere with FACS. Venous citrated blood drawn from the fistula-free arm was obligatorily performed and never frozen. As there is no standard protocol for isolating and detecting circulating EPCs obtained from the plasma, the opinion of the majority experts is that centrifugation of samples is the main factor mediating variability of the EPCs counts. We used the classic approach LYSE-WASH protocol on fixed cells instead NO LYSE-NO WASH protocol on fresh samples. While classic protocol approach is valid, theoretically overlap between two or more fluorochromes might reflect some obstacles for further interpretation of obtained results. Additionally, retrospective, relative small sample size may limit the significance of the present study and this was not a randomized and controlled study, thus a greater cohort of patients will further support these findings.

Conclusion

In conclusion, we suggest that inflammatory biomarkers (hs-CRP, OPG) are a significant predictor for decreased angiopoetic phenotypes of circulating progenitor cells labeled as CD14/CD309+ and CD14/CD309/Tie2+ among dysmetabolic patients without preexisting atherosclerotic lesions of coronary arteries.

Competing Interests

The authors declare that they have no competing interests in this work.

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Ethical Approval

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 performed in conformity with the Declaration of Helsinki.

Competing Interests

The authors declare that they have no competing interests in this work.

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