Signature of Circulating Endothelial-Derived Progenitor Cells in Patients with Metabolic Syndrome and Diabetes Mellitus

Alexander E. Berezin [1,*] and Alexander A. Kremzer [2]

[1] Internal Medicine Department State Medical University, 26, Mayakovsky av.
Zaporozhye, Postcode 69035, Ukraine
[*] Coresponding author

[2] State Medical University Clinical Pharmacology Department
Zaporozhye, Ukraine

Abstract

Type two diabetes mellitus (T2DM) remains a leading contributor to cardiovascular mortality worldwide. This study was conducted to investigate the pattern of circulating endothelial progenitor cells (EPCs) 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. All the patients have given written informed consent for participation in the study. The flow cytometry was used for predictably distinguishing cell subsets, which depend on expression of CD45, CD34, CD14, Tie-2, and VEGFR2. Biomarkers were measured at baseline of the study.
Results: There is 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. $CD14^+CD309^+$ and $CD14^+CD309^+Tie^{2+}$ subsets of circulating EPCs were determined in higher concentration among MetS subjects in comparison with T2DM patients.

Osteoprotegerin (OPG) and hs-C-reactive protein (CRP), improve significantly predictive model based on T2DM + number of multiple cardiovascular risk factors (MCRFs) >3 for decreased both angiopoetic phenotypes of circulating EPCs. 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 hs-CRP and OPG to the base model 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 $CD14^+CD309^+Tie^{2+}$.

In conclusion, we suggest that inflammatory biomarkers (hs-CRP, OPG) may consider statistically significant predictors for decreased EPCs labeled $CD14^+CD309^+$ and $CD14^+CD309^+Tie^{2+}$ among dysmetabolic patients without preexisting atherosclerotic lesions of coronary arteries.

Keywords: Diabetes mellitus; metabolic syndrome; circulating endothelial-derived progenitor cells; cardiovascular risk factors

Introduction

Dysmetabolic disorders, such as type two diabetes mellitus (T2DM), metabolic syndrome (MetS), obesity, remain to be increased worldwide achieving epidemic level [1, 2]. Both metabolic states i.e. T2DM and MetS contribute endothelial dysfunction (ED), atherogenesis, oxidative stress, inflammatory responses, and they are considered causality factors of cardiovascular diseases (CVD) [3-5]. Despite development of dysmetabolic disorders associates with ED affecting the macro- and the microvascular systems, T2DM patients demonstrated the most severe ED than MetS subjects [6]. Recent studies have shown that ED associates with poor clinical outcomes in patients with established CVD [7]. In this context, pathogenic factors that induce endothelial dysfunction at the earlier stages of dysmetabolic diseases might have a causality value for CVD progression [4].

Endothelial progenitor cells (EPCs) is a population of mononuclear cells that expresses endothelial and progenitor markers i.e. CD34+ antigen and VEGFR-2+ vascular growth ligands (Vascular Endothelial Growth Factor Rreceptor-2), CD133+, as well as CD14+, and Tie2+ (tyrosine kinase ligand) [8]. EPCs may play a pivotal role in mechanisms of tissue repair, maturation of endothelial cells, angiogenesis, and revascularization [9]. EPCs are mobilized from bone marrow and probably from peripheral tissues upon injury in result in growth factors and inflammatory cytokines over production [10]. There are evidences regarding elevated EMPs as a marker of ED [11]. Indeed, increased production of reactive oxygen species, insulin resistance, reduced NO bioavailability have been identified as mediators of tissue injury and ED [12, 13]. In contrast, T2DM has well characterized as dysmetabolic state with depletion of circulating EPCs resulting in severely reduced angiogenic capacity *in vivo* [14]. Although decreased number and impaired functions of CD34+CD133+ EPCs in T2DM patients might lead to increased frequency of CVD events [15, 16], the role of angio-

genic subsets of EPCs in T2DM and MetS is still not fully 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 when source documents were reviewed [17]. When one or more of the following components were found (glycated hemoglobin [HbA1c] $\geq 6.5\%$; 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 [18]. 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 $\geq 130/85$ 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 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) [19].

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 [20].

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, RANKL and osteoprotegerin (OPG) were measured by high-sensitive enzyme-linked immunosorbent assays using commercial kits (R&D Systems GmbH, Wiesbaden-Nordenstadt, Germany) according to the manufacturers' recommendations. The inter-assay coefficients of variation were as follows: adiponectin: 5%, RANKL: 7.0%; OPG: 8.2%.

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

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

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

Insulin resistance was assessed by the homeostasis model assessment for insulin resistance (HOMA-IR) [21] 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) 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) [22].

Determination of circulating EPCs

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 [23]. Accordingly, the cells in question 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 expres-

sion 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. After lysis of erythrocytes with UTILIZE wash solution, the samples were centrifuged at 200× g for 15 min; then they were washed twice with PBS and fixed immediately. Double- or triple-positive events were determined using Boolean principles ("and", "not", "or", etc.). Circulating EPCs were defined as CD34/VEGFR2 positive cells with lack of CD45 expression. From each tube 500,000 events were analyzed. For CD14+ populations, coexpression with Tie-2− and/or VEGFR-2− was determined using quadrant analysis. Mononuclear cells were cultured for functional analysis (CFUs) after FCT. Standardized cell counts were presented as a percentage of the total of the white blood cells count, identified as the total number of all CD45+ cells.

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 depleted 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 48.34 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, no significant increase in circulating CD34+ subset cells (CD45+CD34+ and CD45−CD34+) in dysmetabolic patients when compared with healthy volunteers was found. The authors suppose that there are decreased CD14+ subsets of EPCs in dysmetabolic

subjects. Indeed, there is 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.

As one can see Table 2, patients with MetS have demonstrated 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 levels of EPCs different origin were determined in both cohorts apart from absolute numerous of $CD14^+CD309^+$ and $CD14^+CD309^+Tie^{2+}$ EPCs. $CD14^+CD309^+$ and $CD14^+CD309^+Tie^{2+}$ subsets of circulating EPCs were determined in higher concentration 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 $CD14^+CD309^+Tie^{2+}$, cardiovascular risk factors, hemodynamic performances, and other biomarkers were evaluated. The data have shown that absolute numerous 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), sRANKL (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 numerous of $CD14^+CD309^+Tie^{2+}$ 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), sRANKL (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 $CD14^+CD309^+Tie^{2+}$ EPCs with fasting plasma glucose, HbA1c, number of MetS components, means of systolic and diastolic BP, waist circumference was found. We did not find possible age- and gender-related correlation between metabolic status and the presence of EPCs.

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), OPG per 125.5 pg / mL (OR = 1.14, P = 0.001), number of MCRFs >3 (OR = 1.15, P = 0.001), HOMA-IR per 0.65 mmol/L × μU/mL (OR = 1.04, P = 0.001) on decreased of $CD14^+CD309^+$ EPCs was determined (Table 3). Therefore, $CD14^+CD309^+Tie^{2+}$ EPCs were prone negatively impact 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 $CD14^+CD309^+Tie^{2+}$ EPCs, but HOMA-IR has not demonstrated significant impact on improving of based predictive model (Table 4).

Inflammatory biomarkers (hs-CRP, OPG) improve significantly 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 $CD14^+CD309^+Tie^{2+}$.

Discussion

The results of our study demonstrate that depletion of circulating EPC number with angiopoetic capacity labeled $CD14^+CD309^+$ and $CD14^+CD309^+Tie^{2+}$ was defined among dysmetabolic patients. Moreover, circulating level of $CD14^+CD309^+$ and $CD14^+CD309^+Tie^{2+}$ EPCs was significant lower in T2DM persons when compared with MetS patients. Therefore, inflammatory cytokines i.e. OPG and hs-CRP appeared significant association with decreased $CD14^+CD309^+$ and $CD14^+CD309^+Tie^{2+}$ EPC subsets among dysmetabolic subjects. We suggest that low-intensity inflammation that is common in dysmetabolic person population may impact negatively on CVD development through worse of endothelial reparation process that is realized by recruitment of EPCs with angiopoetic activity.

Recent studies have shown a closely association of serum levels of OPG and hs-CRP with T2DM [24, 25]. Indeed, OPG is considered a candidate for biomarker, which modulating vascular remodeling effects and CVD development and progression [26]. In fact, OPG is over expressed on endothelial and smooth muscle cells in vasculature in dysmetabolic states [27]. Moreover, epidemiological studies have examined the relations of OPG to CVD events and mortality [28-30]. Strong associations of hs-CRP with components of MetS, T2DM, other inflammatory cytokine levels, as well as CVD events were found in several investigations [31-33]. Higher level of OPG and hs-CRP in MetS and T2DM reflected chronic low grade inflammation possibly mediates the development of diabetic complications and clinical CVD outcomes through ED

[33]. ED may be realized as result in altered endothelial cell activity and endothelial integrity due to dysregulation of vascular wall repair processes [34] mediatied angiogenic EPCs [35]. Indeed, there are evidences that decreased number and / or functionally impaired EPCs are one of the major risk factors for the development of micro vascular complications in dysmetabolic patients [36].

Results of the presented study have shown that increased circulating level of inflammatory cytokines in T2DM patients may lead to more much deficiency of angiogenic EPCs when compared with MetS, while frequencies and absolute numbers of other immune phenotype EPCs may be comparable. Probably, OPG and hs-CRP seems to be determinant for depleted circulating EPCs levels and relates a progress from MetS and prediabetes to T2DM. Interestingly, that glycemic control, BMI, insulin resistance were not defined as independent predictors for decreased $CD14^+CD309^+$ and $CD14^+CD309^+Tie^{2+}$ EPCs in dysmetabolic patients, but T2DM, numbers of CVD risk factors, OPG and hs-CRP were determined. These data sort with opinion provided by investigators regarding EPCs as a potential novel marker of vascular integrity, homeostasis process, metabolic abnormalities, and cardiovascular risk [37, 38]. As it has been postulated, the reduction and dysfunction of EPCs associates with inflammatory-dependent impairment of vascular function and relates to progress from MetS to T2DM. Overall, these results 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.

Study limitations

This study has some limitations. All subjects with T2DM ad MetS were comparable to age and sex because of design of the study was retrospective. This is limitation appears to be serious for determination of possible age- and sex-related associations regarding numerous of circulating microparticles. The results of our study do not allow to produce any issues regarding this. 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 EPCs. In fact, lack of standard protocol for isolating and detecting circulating EPCs obtained from the plasma. According opinion of the majority experts, centrifugation is became the main factor mediated reliability of the EPCs determination in samples and contributed in biological variability of EPC 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

EPCs is also possible and has not been characterized in depth in T2DM and MetS 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. Finally, taken together these data are very promising, and they are required new investigations with higher statistical power and increased sample size to be overcome the internal limitations of the study.

In conclusion, we suggest that inflammatory biomarkers (hs-CRP, OPG) may consider statistically significant predictors for decreased both angiopoetic phenotypes of circulating EPCs labeled $CD14^+CD309^+$ and $CD14^+CD309^+Tie^{2+}$ among dysmetabolic patients without preexisting atherosclerotic lesions of coronary arteries.

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 performed 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.

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Table 1 General characteristic of patients participating in the study

	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/m^2	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 m^2	102.1 (95% CI=91.4–113.2)	93.1 (95% CI=79.5–109.7)	0.12

Table 1 (Continued): General characteristic of patients participating in the study

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)	70.5 (95% CI =59.6–88.3)	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, mmol/L	17.1 (95% CI = 9.1–25.7)	23.8 (95% CI = 15.8–31.3)	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
$CD45^+CD34^+$, %	1.90 (25-75% IQR = 1.49–2.10)	2.10 (25-75% IQR = 1.70–2.35)	0.66

Table 1 (Continued): General characteristic of patients participating in the study

$CD45^+CD34^+$, cells $\times 10^3/\mu L$	0.114 (25-75% IQR = 0.095–0.120)	0.112 (25-75% IQR = 0.090–0.121)	0.76
$CD45^-CD34^+ \times 10^{-4}$, %	1.00 (25-75% IQR = 0.69–1.35)	1.02 (25-75% IQR = 0.86–1.28)	0.84
$CD45^-CD34^+$, cells $\times 10^{-1}/\mu L$	0.06 (25-75% IQR = 0.05–0.07)	0.057 (25-75% IQR = 0.053–0.067)	0.86
$CD14^+CD309^+ \times 10^{-4}$, %	71.00 (25-75% IQR = 61.50–96.00)	57.00 (25-75% IQR = 43.20–81.50)	0.02
$CD14^+CD309^+$, cells $\times 10^{-1}/\mu L$	4.26 (25-75% IQR = 3.70–5.74)	2.96 (25-75% IQR = 2.25–4.21)	0.01
$CD14^+CD309^+Tie^{2+} \times 10^{-4}$, %	7.70 (25-75% IQR = 4.20–12.20)	5.50 (25-75% IQR = 3.05–8.15)	0.04
$CD14^+CD309^+Tie^{2+}$, cells $\times 10^{-1}/\mu L$	0.465 (25-75% IQR = 0.253–0.710)	0.270 (25-75% IQR = 0.241–0.411)	0.01

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.

Table 2: Demographic, risk factors, blood pressure, circulating biomarkers, and endothelial-derived microparticles in MetS and T2DM patients

	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/m^2	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 m^2	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	5.46 (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

Table 2: (Continued): Demographic, risk factors, blood pressure, circulating biomarkers, and endothelial-derived microparticles in MetS and T2DM patients

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, mmol/L	23.1 (95% CI = 16.2–29.7)	23.9 (95% CI = 15.9–30.1)	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
$CD45^+CD34^+$, %	2.11 (25-75% IQR = 1.72–2.28)	2.06 (25-75% IQR = 1.73–2.25)	0.86
$CD45^+CD34^+$, cells × 10^3/µL	0.114 (25-75% IQR = 0.094–0.122)	0.109 (25-75% IQR = 0.091–0.117)	0.56

Table 2: (Continued): Demographic, risk factors, blood pressure, circulating biomarkers, and endothelial-derived microparticles in MetS and T2DM patients

$CD45^-CD34^+ \times 10^{-4}$, %	1.02 (25-75% IQR = 0.89–1.25)	1.01 (25-75% IQR = 0.88–1.22)	0.88
$CD45^-CD34^+$, cells $\times 10^{-1}$/µL	0.060 (25-75% IQR = 0.055–0.066)	0.056 (25-75% IQR = 0.052–0.064)	0.82
$CD14^+CD309^+ \times 10^{-4}$, %	59.12 (25-75% IQR = 48.10–82.30)	52.37 (25-75% IQR = 44.60–76.20)	0.054
$CD14^+CD309^+$, cells $\times 10^{-1}$/µL	3.35 (25-75% IQR = 2.57–4.18)	2.71 (25-75% IQR = 2.31–3.52)	0.018
$CD14^+CD309^+Tie^{2+} \times 10^{-4}$, %	6.03 (25-75% IQR = 3.42–8.05)	4.95 (25-75% IQR = 3.14–7.44)	0.052
$CD14^+CD309^+Tie^{2+}$, cells $\times 10^{-1}$/µL	0.294 (25-75% IQR = 0.245–0.396)	0.253 (25-75% IQR = 0.232–0.311)	0.012

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.

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

Factors	CD14$^+$CD309$^+$ EPCs		CD14$^+$CD309$^+$Tie^{2+} EPCs	
	OR (95% CI)	P Value	OR (95% CI)	P Value
Hypertension (present vs abcent)	0.99 (0.87–1.04)	0.001	1.05 (0.97–1.11)	0.001
Dyslipidemia (present vs abcent)	1.04 (0.92–1.22)	0.001	1.02 (0.96–1.15)	0.001
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)	0.001	1.05 (0.95–1.07)	0.002
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)	0.002	1.01 (0.98–1.10)	0.001
SUA per 4.5 mmol/L	1.02 (0.94–1.14)	0.001	1.04 (0.96–1.10)	0.002
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)	0.002	1.01 (0.98–1.05)	0.001
HOMA-IR per 0.65 mmol/L × μU/mL	1.04 (1.00–1.06)	0.001	1.06 (1.02–1.07)	0.001
sRANKL per 12.5 pg / mL	1.03 (0.98–1.07)	0.001	1.02 (0.99–1.04)	0.002

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.

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

Models	Dependent variable: CD14$^+$CD309$^+$ EPCs				Dependent variable: CD14$^+$CD309$^+$Tie^{2+} 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	-	-	-	0.652	-	-	-
Model 1 + OPG	0.689	-	-	-	0.688	-	-	-
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 (based model)	0.644	-	-	-	0.652	-	-	-
Model 1 + HOMA-IR	0.677	-	-	-	0.676	-	-	-
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 (based model)	0.644	-	-	-	0.652	-	-	-
Model 1 + hs-CRP	0.682	-	-	-	0.681	-	-	-
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 (based model)	0.644	-	-	-	0.652	-	-	-
Model 1 + OPG + hs-CRP	0.694	-	-	-	0.693	-	-	-
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%

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.

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$^+$Tie^{2+} EPCs

Model 2 versus Model 1	Decreased absolute number of circulating EPCs labeled CD14$^+$CD309$^+$	Decreased absolute number of circulating EPCs labeled CD14$^+$CD309$^+$Tie^{2+}
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)

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.