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SERUM RANKL/OSTEOPROTEGERIN COMPLEX AND ENDOTHELIAL PROGENITOR CELLS IN CHRONIC HEART FAILURE

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ABSTRACT

The objectiveof this study was to assess an interrelationship serum RANKL/OPG complex with counts of circulating pro-angiogenic endothelial progenitor cells (EPCs) labeled as $CD14^+CD309^+$, and $CD14^+CD309^+Tie2^+$ in patients with ischemic chronic heart failure (CHF). Methods: The study retrospectively evolved 153 patients (86 males) aged 48 to 62 years with exiting proven stable coronary artery disease (CAD). Systolic or diastolic CHF was defined among 109 (71.2%) patients. Twenty five 25 individuals were included in the control group. Circulating RANKL (sRANKL) and OPG were measured by high-sensitive ELISA kit at baseline. EPC populations were labeled by flow cytofluorimetry per High-Definition Fluorescence Activated Cell Sorter methodology. Results: Numerous of EPCs with phenotypes of $CD14^+CD309^+$ and $CD14^+CD309^+Tie^{2+}$ were significantly lower in CAD patients when compared with healthy subjects. The trend to significant decrease of EPC numerous depending presence of CHF was found. The sRANKLlevel, OPG level, and sRANKL / OPG ratio were significantly higher in CHF subjects as compared to those without CHF (P=0.001). On multivariate analysis, CHF, sRANKL/OPG ratio, OPG, and NT-pro-BNP remained as independent predictors of decreased EPCs with phenotypes of $CD14^+CD309^+$ and $CD14^+CD309^+Tie^{2+}$. When sRANKL/OPG ratio was added to the standard predictive model (CHF) improved relative integrated discrimination indices by 12.5% for $CD14^+CD309^+$ depletion, as well as by 17.3% for $CD14^+CD309^+Tie^{2+}$depletion were found. Conclusion: We found that sRANKL/OPG ratio remained statistically significant predictor for depletion of pro-angiogenicEPCsin CAD patients.

Keywords: Chronic heart failure, Ischemic heart disease, RANKL, Osteoprotegerin, Endothelial progenitor cells, Prediction, Discriminate model.

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Contribution/ Originality

The paper's primary contribution is finding that inflammatory cytokines, such as components of RANKL/OPG complex, areable to negatively modulate the level of circulating pro-angiogenicendothelial progenitor cells labeled as $CD14^+CD309^+$, and $CD14^+CD309^+Tie2^+$ and thereby reduce a reparative potency of vasculature in heart failure patients.

1. INTRODUCTION

Chronic heart failure (CHF) is considered one of the leading factor increased of cardiovascular mortality worldwide [1, 2]. Recent evidences suggest that CHF is associated with factors that may contribute to deteriorate vascular integrity and endothelial function, worse angiogenesis, modulate coagulation and inflammation [2, 3]. The effects mentioned above lead to disorders of tissue reparation that is represented a crucial event in the development of CHF [4]. Recent studies suggested that circulating level of endothelial progenitor cells (EPCs) with pro-angiogenic capacities is key player in the pathogenesis of cardiac failure [5, 6]. EPCs play a pivotal role in reparative processes, such as neoendothelization, remodeling of extracellular matrix and angiogenesis [6]. Circulating EPCs, which co-expressed CD34+ antigen and VEGFR-2+ vascular growth ligands (Vascular Endothelial Growth Factor Rreceptor-2), CD133+, CD14+, and Tie2+ (tyrosine kinase ligand), were tested as predictors of nature evolution of ischemic CHF [7, 8]. The differentiation of EPCs is regulated through receptor activator of nuclear factor-κB ligand (RANKL) and osteoprotegerin (OPG), which belongs its decoy receptor [9]. Results of recent studies have shown that RANKL and OPG are key players in not only bone remodeling, vascular calcification, osteoporosis, as well as in cardiovascular disease [10-13]. However, the potential role of serum RANKL/OPG complex in modulation of the circulating pro-angiogenic EPCs in CHF patient population is still not understood. The objective of this study was to assess an interrelationship serum RANKL/OPG complex with counts of circulating pro-angiogenic endothelial progenitor cells labeled as $CD14^+CD309^+$, and $CD14^+CD309^+Tie2^+$ in patients with ischemicCHF.

2. METHODS

The study retrospectively evolved 153 patients (86 males) aged 48 to 62 years with angiographically proven stable coronary artery disease (CAD) in period between February 2010 and November 2013. Systolic or diastolic CHF was defined in 109 patients (71.2%) enrolled in the study. CHF was verified by conventional criteria and as left-ventricular ejection fraction ≤45% or 46-55% respectively [14]. Patients with chronic post-ischemic CHF had proven coronary artery disease (CAD) and persistent regional left-ventricular dysfunction. Twenty five 25 individuals were included in the control group.

All the patients have given their written informed consent for participation in the study. The following are exclusion criteria: Q-wave and non-Q-wave MI within 3 months before study entry; severe kidney and liver diseases that may affect clinical outcomes; malignancy; creatinin plasma

level above 440 μmol/L; estimated GFR index < 35 ml/min/м²; brain injury within 3 months before the enrollment; body mass index above 30 kg/m² and less15kg/m²; pulmonary edema; tachyarrhythmia; valvular heart disease; thyrotoxicosis; ischemic stroke; intracranial hemorrhage; acute infections; surgery; trauma; all the ischemic events within 3 previous months; inflammations within a previous month; neoplasm; pregnancy; implanted pacemaker, any disorder that may discontinue patient's participation in the study according to investigators; and patient's refusal to participate in the study or to give his consent for it.

2.1. Visualization of Coronary Arteries structure

Multispiral computed contrast-enhanced tomography angiography has been carried out for all the asymptomatic patients at high risk of CAD prior to their inclusion in the study. The coronary artery wall structure was measured using multi row contrast-enhanced spiral computed tomography angiography[15, 16] on SomatomVolum Zoom scanner (Siemens, Erlangen, Germany, 12x0.75 mm cross-sections and 420 msec rotation speed, 120 kV, 500 effective mA, and table-feed 2.7 mm/rotation, retrospectively ECG-gated image reconstruction) when holding patient's breathe at the end of breathing in. After preliminary native64-slice scanning, non-ionic contrast "Omnipaque" (Amersham Health, Ireland) was administered for the optimal image of the coronary arteries. Three-dimensional digital image processing, including multi-planar reconstruction and maximum intensity projection was performed. For reconstruction of the image in the diastole, we used 0.6-mm-width axial tomographic slices and a 1.0 mm thickness. All coronary arteries with a diameter of 2.0 mm or more were assessed for the presence of a stenosis (>50% luminal narrowing) and the presence of high-grade stenoses (>70% diameter stenosis) or occlusions.

2.2. Transthoracic Echocardiography

The patients underwent transthoracic two-dimensional and tissue Doppler imaging (TDI) echocardiography. Conventional Doppler echocardiography and data were also obtained from all patients. Doppler echocardiographic and TDI recordings were measured during normal respiration. Left ventricular end-diastolic and end-systolic volumes were measured by modified Simpson's method. Left ventricular ejection fraction (LVEF) was assessed with the requirements of American Society of Echocardiography [17]. TDI was carried out in 4-, 3- and 2-chamber projections in each of 16 segments of the left ventricle and in 4 spots of the mitral annulus [18]. Peak systolic (Sm), early diastolic (Em), and late diastolic (Am) myocardial velocities and their ratios were measured.

2.3. Glomerular Filtration Rate Measurement

Calculation of glomerular filtration rate (GFR) was carried out using CKD-EPI formula [19].

2.4. Circulating Biomarkers Measurement

Blood samples were collected at baseline in the morning into cooled silicone test tubes wherein 2 mL of 5% Trilon B solution were added. All samples were centrifuged immediately at 6,000 rpm for 3 minutes. Plasma was collected and refrigerated at a temperature -70°C. Concentrations of RANKL and OPG were measured by ELISA (R&D Systems GmbH, Wiesbaden-Nordenstadt, Germany). Circulating NT-proBNP level was measured by immunoelectrochemoluminescent assay using commercial kits produced by R&D Systems (R&D Systems GmbH, Wiesbaden-Nordenstadt, Germany) on Elecsys 1010 analyzer (F. Hoffmann-La Roche Diagnostics, Mannheim, Germany). The interassay coefficients of variation were as follows: RANKL: 7.0%; OPG: 8.2%, NT-proBNP: 9.4%. High-sensitive C-reactive protein (hs-CRP) was measured by ELISA kit (R&D Systems GmbH, Wiesbaden-Nordenstadt, Germany). Concentrations of total cholesterol (TC) and cholesterol of high-density lipoproteins (HDLP) were measured by fermentation method. Low-density lipoproteins (LDL-C) cholesterol was calculated by Friedewald formula (1972).

2.5. Identifying Fractions of Mononuclear and Endothelial Progenitor Cells

Mononuclear cells populations were phenotyped by flow cytofluorimetrywith monoclonal antibodies labeled with FITC fluorochromes (fluorescein isothiocyanate) or double-labeled with FITC/PE (phycoerythrin) (BD Biosciences, USA) to CD45, CD34, CD14, Tie-2, and CD309(VEGFR2) antigens as per HD-FACS (High-Definition Fluorescence Activated Cell Sorter) methodology, with red blood cells removed obligatory with lysing buffer according to gating strategy of International Society of Hematotherapy and Graft Engineering sequential (ISHAGE protocol of gating strategy) [20]. Half billion events have been analyzed for each sample. Circulating EPCs have been identified as $CD45^{-}CD34^{+}$. Therefore, CD309 (VEGFR2) and Tie-2 antigens were also determined to identify subpopulations of EPC co-expressing CD14 antigen.

Study design: open cohort study.

3. STATISTICAL ANALYSIS

All statistical analyses were conducted using the SPSS package for Windows version 22.0 (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 interquartile range (IQR). To compare the main parameters of patients' groups (subject to the type of distribution of the parameters analyzed), one-tailed Student t-test or Shapiro–Wilk U-test were used. Differences in frequencies were analyzed using chi square test and Fisher F exact test were used. The circulating EPCs and NT-pro-BNP level in the blood failed to have a normal distribution, while distribution of the hs-CRP, sRANKL, OPG, total cholesterol and cholesterol fractions had a normal character and was not subjected to any mathematical transformation. Potential independent predictors, which could be associated potentially with EPCs, were identified using

logistic regression analysis. All univariate predictors were then entered in a stepwise manner into a multivariate model. 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.

4. RESULTS

General characteristic of the patients enrolled in the study is presented in Table 1. No substantial age and gender differences were found among persons with documented CHF and without it, as well as differences in terms of body mass index (BMI), glomerular filtration rate (GFR), HbA1c, fasting blood glucose level, blood creatinine level, total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C). Incidence of premature CAD in the family history also was commensurate in the two cohorts of patients. Hypertension and hyperlipidemia were found frequently in subjects with mild and moderate CHF than in individuals without CHF, with the incidence data being statistically significant. No difference between the two cohorts in terms of systemic office blood pressure (BP) and heart rate (HR) was found. The incidences of type 2 diabetes mellitus (T2DM) in patients of the two cohorts were 38.6% and 35.4% (P=0.06) respectively. Smoking adherence was found frequently in CHF patients when compared with none-CHF subjects. Note that a statistically significant decrease in the left ventricular ejection fraction value was quite anticipated in the setting of an increase in E/Am and E/Em in patients with verified ischemia-induced CHF. The levels of circulating NT-pro-BNP and hs-CRP were statistically significantly higher in patients with CAD than in healthy volunteers, and they showed steady elevation related to the presence of CHF.

Numerous of EPCs with phenotypes of $CD14^{+}CD309^{+}$ and $CD14^{+}CD309^{+}Tie^{2+}$ were significantly lower in CAD patients when compared with healthy subjects. A significant trend to decrease of EPC numerous depending presence of CHF was found.

Table 2 is presented details of pharmacotherapy in patient cohorts. No substantial differences between the two cohorts with regard to administration of the majority of drugs, except antiplatelet drugs, which were used less frequently in patients with CHF were found.

With respect to circulating inflammatory biomarkers, we found a sufficient difference between CAD patient group and healthy individuals in sRANKL, OPG, and sRANKL / OPG ratio (Table 3). For all cases, the sRANKLlevel, OPG level, and sRANKL / OPG ratio were significantly higher in CHF subjects as compared to those without CHF (P=0.001). However, RANKL/OPG ratio indicating the circulating RANKL free fraction was significantly decreased in healthy persons as compared to both group subjects with CAD irrespective to CHF presentation (Fig. 1).

On univariate regression analysis, count of EPC subpopulation labeled as $CD14^{+}CD309^{+}$ was associated positively with the left ventricular ejection fraction (r=0.575; P=0.001), the E/Em ratio (r=0.52; P=0.001), the E/Am ratio (r=0.48; P=0.001); and it was associated negatively with the

CHF (r= -0.889; P=0.001), the NT-pro-BNP (r=-0.662; P=0.001), hs-CRP (r=-0.564; P=0.001), sRANKL/OPG ratio (r=0.560; P=0.003), sRANKL (r=0.530; P=0.001), type 2 diabetes mellitus (r= -0.50; P=0.001), OPG (r=0.440; P=0.001), a low-density lipoprotein cholesterol (r= -0.322; P=0.001), hypertension (r= -0.320; P=0.005), total cholesterol level (r=-0.260; P=0.04), adherence to smoking (r=-0.259; P=0.042) and patient's age (r=-0.254; P=0.002).

The $CD14^{+}CD309^{+}Tie2^{+}$ EPC subpopulation count showed a positive association with the left ventricular ejection fraction (r=0.564; P=0.001), the E/Em ratio (r=0.512; P=0.001). There was found a negative association $CD14^{+}CD309^{+}Tie2^{+}$ cells with the CHF (r=-0.657; P=0.001), type 2 diabetes mellitus (r= -0.610; P=0.001), hs-CRP (r=-0.598; P=0.001), sRANKL/OPG ratio (r=-0.528; P=0.001), (r=-0.472; P=0.003), OPG (R=-0.594; P=0.001), NT-pro-BNP level (r= -0.473; P=0.001), sRANKL (r=-0.472; P=0.003), a low-density lipoprotein cholesterol (r=-0.354; P=0.001), the total cholesterol level (r= -0.258; P=0.043), adherence to smoking (r= -0.285; P=0.042), body mass index (r= -0.272; P=0.046). On multivariate analysis, CHF, sRANKL/OPG ratio, OPG, and NT-pro-BNP remained as independent predictors of decreased EPCs with phenotypes of $CD14^{+}CD309^{+}$ and $CD14^{+}CD309^{+}Tie^{2+}$ (Table 4).

When sRANKL/OPG ratio was added to the standard predictive model, which constructed with CHF presentation, improved the relative IDI by 12.5% for $CD14^{+}CD309^{+}$ depletion, by 17.3% for $CD14^{+}CD309^{+}Tie^{2+}$depletion were found (Table 5). For category-free NRI, 11% of events (p=0.001) and 21% of non-events (p=0.001) were correctly reclassified by the addition of sRANKL/OPG ratio to the ABC model for EPC count labeled as $CD14^{+}CD309^{+}$ cells (Table 6). Adding to the standard predictive model sRANKL/OPG ratio, 12% of events (p=0.001) and 17% of non-events (p=0.001) were reclassified for $CD14^{+}CD309^{+}Tie^{2+}$ EPCs. Thus, we suggested that sRANKL/OPG ratio remained statistically significant predictor for depletion of $CD14^{+}CD309^{+}$ EPCs and $CD14^{+}CD309^{+}Tie^{2+}$ EPCs in CAD patients.

5. DISCUSSION

The results of the present study report on the use of sRANKL/OPG ratio as indicator of EPC depletion in ischemic CHF patients and it closely correlation with traditionally circulating biomarkers reflected inflammatory and neurohumoral activation, such as hs-CRP and NT-proBNP. Circulating RANKL, OPG, hs-CRP, and natriuretic peptides are considered well-established biomarkers with high predictive value for CHF presentation and clinical ouitcomes [21-23]. Recent studies showed OPG as a member of the tumor necrosis factor receptor superfamily and serum RANK/RANKL have been identified as candidate mediators for paracrine signaling in cell metabolism and extracellular matrix regulation but have also been shown to modulate dendritic cells and activated T cells, as well as to promote B-cell maturation and antibody response, which suggests a role in both innate and adaptive immunity [24-26]. Therefore, RANKL and OPG in blood are investigated as markers of cardiovascular risk reclassification in various patient cohorts [27-33]. However, the role of sRANKL/OPG complex in maintenance of reparative repair potency among CHF persons shows to be a very intriguing

while clinical data are limited. There are evidences regarding OPG/RANKL/RANK system may contribute to cardiac remodeling after acute myocardial infarction and heart failure actually affected transformation of acute cardiac failure to chronic heart failure [24, 33]. Loncar G et al reported that serum RANKL was a significant determinant of NT-pro-BNP independent of age, BMI and creatinine clearance in CHF subjects [10]. The interrelationship between OPG and serum RANKL concentrations in patients with advanced atherosclerosis in relation to medical history, risk factors and medication intake was found [34]. It has suggested that sRANKL/OPG complex contributes in several stages in CHF development, while the exact clinical implication of circulating RANKL has been remained uncertain [35-37]. In fact, the recent clinical trials in field of heart failure have been shown the independent predictive value for OPG only [38, 39]. OPG is considered a modulator of extracellular remodeling, which is key player in pathogenesis of CHF. OPG neutralizes the effect of receptor activator of nuclear factor-kappa B ligand on processes affected both differentiation and activation of wide spectrum cells, including circulating EPCs [40]. The imbalance between free fraction of RANKL, calculated as sRANKL/OPG ratio, and circulating OPG may be responsible for the homeostatic mechanism of differentiation and apoptosis of EPCs [41]. The effect mentioned above may probably mediate over production of reactive oxygen species and leads to activation of NOX-2 and NOX-4. All these mediate an triggered effect regarding phosphorylation of ERK-1/2 and p38 MAPK [41, 42]. This mechanisms are considered a suitable for ischemic CHF development [43, 44]. Results of the our study showed that components of sRANKL/OPG complex were increased in CHF patients when compared with none-CHF persons with stable CAD as well as with healthy volunteers. Therefore, decreased circulating EPCs related CHF in CAD subjects were found also. However, sRANKL/OPG ratio when compared with other components of cytokines-induced bone-related proteins RANKL andOPG in ischemic CHF patients was not only significantly associated with parameters of neuroendocrine activation such as NT-pro-BNP and hs-CRP, but it closely effected on EPCs with pro-angiogenic phenotypes. We suggested sRANKL/OPG complex affected reparative face of the pathogenesis of ischemic CHF through modulating count of circulating EPCs. Because OPG may stimulate a differentiation of the EPCs and positively regulate their count in circulation [40], we suggest that free fraction of serum RANKL, calculated as serum RANKL/OPG ratio, may consider a powerful predictor for depletion of EPCs with phenotypes $CD14^{+}CD309^{+}$ EPCs and $CD14^{+}CD309^{+}Tie^{2+}$ in CAD patients. We suggested that described above effect may have a serious predictive value for CHF especially developed due to ischemic reason. We believed that new studies are needed to understand whether circulating RANKL is predictive biomarker in the ischemic CHF patients.

Conclusion. RANKL/OPG ratio is statistically significant predictor for depletion of $CD14^{+}CD309^{+}$ EPCs and $CD14^{+}CD309^{+}Tie^{2+}$ EPCs in CAD patients.

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

Study Restrictions: The major limitation of this study was the small size of patient study population. Authors suggested a greater cohort of patients with more statistical power is required. Although High-Definition FACS methodology is widely used, theoretically overlap between two or more fluorochromes might be reflected 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 patient population. However, the authors suggested no restrictions might have a significant impact on interpretation of the data.

Conflict of interests: not declared

Figure legend: Serum receptor activator of NF-κB ligand (sRANKL) levels (pg/mL), osteoprotegerin (OPG), and sRANKL / OPG ratio in the different study groups. Values are means (±SEM).

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Table-1. General characteristic of patients participating in the study

Parameters	Health individuals (n=25)	Patients with CAD		
		Entire group (n=153)	Without CHF (n=44)	With CHF (n=109)
Age, years	51.70±6.10	58.34±9.60	57.20±6.70	59.50±7.30
Males, n (%)	14 (56.0%)	86 (56.2%)	29 (65.9%)	57 (52.3%)
Arterial hypertension, n (%)	-	99 (64.7%)*	32 (72.7%)	67 (61.5%)**
Hyperlipidemia, n (%)	-	71 (46.4%)*	19 (43.2%)	52 (47.7%)**
T2DM, n (%)	-	54 (35.3%)*	17 (38.6%)	38 (34.5%)
CAD in family history, n (%)	2 (8.0%)	17 (11.1%)	5 (11.4%)	12 (11.0%)
Adherence to smoking, n (%)	6 (24.0%)	32 (20.9%)	8 (18.2%)	24 (22.0%)**
BMI, kg/m²	23.3 (95% CI=20.1–25.1)	24.1 (95% CI=21.6–28.7)	23.7 (95% CI=22.5–27.3)	24.2 (95% CI=22.0–27.9)
Systolic blood pressure, mm Hg	127±6	131±8	135±5	129±4
Diastolic blood pressure, mm Hg	72±4	80±5	82±4	79±5
Heart rate, beats per 1 min.	69±3	71±3	68±3	76±6
LVEF, %	65.40±0.87	51.30±1.55*	55.40±0,80	42.80±0.76**
E/Am, U	6.1±0.22	14.3±1.13*	12.5±1.20	16.6±0.94**
E/Em, U	7.2±0.19	13.7±1.12*	10.6±0.84	16.6±1.00**
GFR, mL/min/1.73 m²	93.5 (95% CI=88.3–100.3)	82.3 (95% CI=68.7–102.6)	82.1 (95% CI=69.9–93.1)	85.2 (95% CI=70.3–112.5)
HbA1c, %	3.8 (95% CI=3.1-4.6)	6.8 (95% CI=4.1-9.5)*	6.3 (95% CI=4.4-9.0)	7.0 (95% CI=4.3-9.2)
Fasting blood glucose, mmol/L	4.11 (95% CI=3.2-5.5)	5.20 (95% CI=3.3-9.7)	4.80 (95% CI=3.6-8.5)	5.40 (95% CI=3.4-9.1)
Creatinine, μmol/L	65.7 (95% CI=53.1–80.5)	72.3 (95% CI=58.7–92.6)	70.5 (95% CI=59.6–88.3)	74.9 (95% CI=65.1–90.3)

Total cholesterol, mmol/L	4.1 (95% CI=3.1–5.0)	5.1 (95% CI=3.9-6.1)*	5.3 (95% CI=4.6-6.0)	5.0 (95% CI=4.2-5.8)
LDL-C, mmol/L	2.75 (95% CI =2.44-3.6)	3.23 (95% CI =3.11–4.4)*	3.60 (95% CI =3.20–4.18)	3.02 (95% CI=2.80–3.90)
HDL-C, mmol/L	1.01 (95% CI=0.92–1.20)	0.91 (95% CI = 0.89–1.12)*	0.94 (95% CI = 0.92–1.06)	0.88 (95% CI = 0.82–0.97)
NT-pro-BNP, pg /mL	21.4 (95% CI=13.8 – 46.2)	1218.5 (95% CI=154.2 – 3480.75)*	231.2 (95% CI=104.8 – 360.7)	1533.6 (95% CI=644.5 – 2560.6)**
hs-CRP, mg / L	2.43 (95% CI=0.48 – 4.5)	8.71 (95% CI=3.55 – 10.7)	7.96 (95% CI=4.72 – 9.34)	9.15 (95% CI=3.81– 10.52)
$CD14^{+}CD309^{+} \times 10^{-4}$, %	71.00 (95% CI=61.50 – 96.00)	39.12 (95% CI=24.50 – 58.60)*	57.00 (95% CI=43.20 – 81.50)	19.18 (95% CI=15.00 – 24.50)**
$CD14^{+}CD309^{+}Tie^{2+} \times 10^{-4}$, %	7.70 (95% CI=4.20 – 12.20)	3.60 (95% CI=0.70 – 1.60)*	5.50 (95% CI=3.05 – 8.15)	0.77 (95% CI=0.41 – 1.10)**

Note: * - validity of differences between parameters in the groups of health individual and the groups of patients with CAD (P<0.05); ** - validity of differences between parameters in the groups of patients with and without CHF respectively (P<0.05);
Abbreviations: CI – confidence interval; CAD – coronary artery disease, T2DM – type two diabetes mellitus,GFR - Glomerular filtration rate, HDL-C - high-density lipoprotein cholesterol, LDL-C - Low-density lipoprotein cholesterol, BMI - Body mass index, BNP – brain natriuretic peptide, LVEF - Left ventricular ejection fraction, U – unit, Em - early diastolic myocardial velocity, Am - late diastolic myocardial velocity, E – peak velocity of early diastolic left ventricular filling.

Table-2. Details of pharmacotherapy in patients with CAD included in study.

Characteristics	**Patients with CAD (n=153)**	
	Without CHF (n=44)	**WithCHF (n=109)**
ACEI / ARAs, n (%)	44 (100%)	109 (100%)
Acetylsalicylic acid, n (%)	38 (86.4%)	101 (92.7%)
Other antiplatelet drugs, n (%)	6 (13.6%)	8 (7.3%)*
Statins, n (%)	34 (77.3%)	80 (73.4%)
Metformin, n (%)	12 (27.3%)	38 (34.9%)
Diuretics, n (%)	38 (86.4%)	91 (83.5%)
Mineralcorticoid receptor antagonists, n (%)	11 (25.0%)	30 (27.5%)

Note: CI – confidence interval, ACEI – angiotensin-converting enzyme inhibitor, ARAs – angiotensin-2 receptors antagonists, * - validity of divergences between parameters in patients' groups with and without CHF respectively (P<0.05).

Table-3. Circulating biomarkers in CAD patients and healthy volunteers

Parameters	**Health individuals (n=25)**	**Patients with CAD**		
		Entire group (n=153)	**Without CHF (n=44)**	**With CHF (n=109)**
sRANKL, pg / mL	226.30 (95% CI=131.4-321.4)	1801.20 (95% CI=1652.8-1949.6)*	645.80 (95% CI=265.2-1026.3)	2206.50 (95% CI=2057.2-2355.8)**
OPG, pg / mL	2593.9 (95% CI=2158.8-3029.0)	4969.16 (95% CI=4726.8-5211.6)*	3725.9 (95% CI=2579.9-4871.9)	5544.3 (95% CI=5306.4-5782.1)**
sRANKL / OPG ratio	0.087 (95% CI=0.06-0.11)	0.31 (95% CI=0.29-0.32)*	0.17 (95% CI=0.13-0.22)	0.39 (95% CI=0.22-0.45)**

Note: * - validity of differences between parameters in the groups of health individual and the groups of patients with CAD (P<0.001); ** - validity of differences between parameters in the groups of patients with and without CHF respectively (P<0.001).

Abbreviations: OPG – osteoprotegerin, sRANKL – serumreceptor activator of nuclear factor-kappa B ligand.

Table-4. Univariate and Multivariate Relationships of sRANKL/OPG complex and demographics, clinical characteristics, hemodynamics, circulating biomarkers in CAD subjects.

Variables	Univariate analysis			Multivariate analysis		
	beta-coefficient	95% CI	P value	beta-coefficient	95% CI	P value
Dependent variable: $CD14^+CD309^+$						
CHF	-9.77	-14.89; -8.55	0.001	-2.17	-5.10; -1.66	0.036
T2DM	-5.24	-9.23; -1.38	0.001	-0.87	-1.20; -0.16	0.058
Age	-0.43	-2.65; 1.06	0.036			
BMI	-0.88	-3.11; 1.85	0.003			
Hypertension	-1.27	-4.54; 0.37	0.006			
Adherence to smoking	-1.12	-2.18; 0.27	0.001			
LVEF	2.13	0.37; 5.16	0.003	1.02	0.16; 2.09	0.088
E/Em	1.33	0.90; 3.29	0.012	1.01	0.16; 2.28	0.082
E/Am	0.98	0.28; 4.11	0.016	0.44	0.15; 0.87	0.096
hs-CRP	5.67	2.19; 11.62	0.026	0.88	0.36; 1.04	0.064
NT-pro-BNP	-12.8	-.19.5; -8.65	0.001	-5.34	-7.11; 4.25	0.044
Total cholesterol	-2.31	-4.44; -1.05	0.001	-0.84	-1.32; -0.56	0.072
LDL-C	-0.95	-1.99; -0.43	0.001	-0.27	-0.63; -0.12	0.068
sRANKL	6.33	3.17; 13.95	0.001	3.36	0.06; 5.81	0.066
OPG	4.57	2.93; 7.75	0.001	2.05	0.77; 3.16	0.006
sRANKL/OPG	8.14	5.22; 17.51	0.003	5.32	3.16; 8.47	0.001
Dependent variable: $CD14^+CD309^+Tie^{2+}$						
CHF	-10.35	-18.40; -6.67	0.003	-5.32	-7.19; -3.14	0.001
T2DM	-4.11	-7.15; -2.04	0.003	-1.02	-1.82; -0.26	0.072
BMI	-0.66	-1.12; 0.12	0.001			
Hypertension	-1.55	-2.43; 0.55	0.003			
Adherence to smoking	-0.44	-0.98; 0.14	0.001			
LVEF	2.44	0.65; 4.65	0.001	1.31	0.10; 2.36	0.056
E/Em	1.70	0.30; 2.60	0.001	0.43	0.03; 2.42	0.098
E/Am	1.68	0.20; 2.73	0.001	0.42	0.05; 2.80	0.088
hs-CRP	4.99	3.23; 10.50	0.003	1.20	0.15; 2.39	0.096
NT-pro-BNP	-14.2	-21.1; -5.87	0.001	-5.88	-9.43; 3.17	0.003
Total cholesterol	-1.03	-2.31; -0.88	0.002	-0.17	-0.32; -0.06	0.120
LDL-C	-0.68	-1.00; -0.28	0.003	-0.12	-0.27; -0.02	0.140
sRANKL	6.89	2.65; 10.80	0.003	1.12	0.13; 3.04	0.086
OPG	5.11	2.65; 7.98	0.001	3.01	0.99; 5.76	0.001
sRANKL/OPG	7.90	6.05; 9.55	0.001	4.88	2.54; 7.03	0.001

Abbreviations:sRANKL – serum receptor activator of NF-κB ligand, OPG – osteoprotegerin, CHF – chronic heart failure, T2DM - type 2 diabetes mellitus, LVEF – left ventricular ejection fraction, LDL-C –low density lipoprotein cholesterol, BMI - body mass index, hs-CRP – high sensitive C-reactive protein.

Table-5. C-statistics for Models with CHF, EPMs, NT-pro-BNP, OPG and sRANKL/OPG ratio as Continuous Variables

Models	AUC (95% CI)	ΔAUC	IDI (±SE)	Relative IDI (%)
$CD14^+CD309^+$ EPCs				
Model 1 (CHF)	0.624	-	-	-
Model 1 + NT-pro-BNP	0.688	-	-	-
Model 1 + NT-pro-BNP vs Model 1	-	0.064; P=0.059	0.02±0.005	3.8%
Model 1 + OPG	0.703	-	-	-
Model 1 + OPG vs Model 1	-	0.079; P=0.024	0.01±0.003	1.8%
Model 1 (CHF)	0.624	-	-	-
Model 1 + sRANKL/OPG ratio	0.712	-	-	-
Model 1 + sRANKL/OPG ratio vs Model 1	-	0.088; P=0.001	0.03±0.014	12.5%
$CD14^+CD309^+Tie^{2+}$ EPCs				
Model 1 (CHF)	0.624	-	-	-
Model 1 + NT-pro-BNP	0.694	-	-	-
Model 1 + NT-pro-BNP vs Model 1	-	0.070; P=0.064	0.02±0.01	4.1%
Model 1 (CHF)	0.624	-	-	-
Model 1 + OPG	0.845	-	-	-
Model 1 + OPG vs Model 1	-	0.221; P=0.054	0.01±0.012	2.1%
Model 1 (CHF)	0.624	-	-	-
Model 1 + sRANKL/OPG ratio	0.857	-	-	-
Model 1 + sRANKL/OPG ratio vs Model 1	-	0.233; P=0.0001	0.05±0.011	17.3%

Note: Relative IDI – calculated as the ratio of IDI over the discrimination slope of the model without CHF.
Abbreviations: AUC – area under curve, SE – standard error, endothelial progenitor cells, BNP – brain natriuretic peptide, sRANKL – serum receptor activator of NF-κB ligand, OPG – osteoprotegerin, CHF – chronic heart failure.

Table-6. Prediction Performance Analyses for Models with CHF, EPMs, and sRANKL/OPG ratio as Continuous Variables

Model 2 vs model 1	
$CD14^+CD309^+$ EPCs	
Categorical NRI	0.16 (95% CI 0.11-0.21)
Percentage of events correctly reclassified	5 (p=0.16)
Percentage of non-events correctly reclassified	11 (p=0.028)
Categorical free NRI	0.49 (95% CI 0.31-0.65)
Percentage of events correctly reclassified	11% (p=0.001)
Percentage of non-events correctly reclassified	21% (p=0.001)
$CD14^+CD309^+Tie^{2+}$ EPCs	
Categorical NRI	0.18 (95% CI 0.11-0.22)
Percentage of events correctly reclassified	5 (p=0.18)
Percentage of non-events correctly reclassified	9 (p=0.028)
Categorical free NRI	0.39 (95% CI 0.30-0.45)
Percentage of events correctly reclassified	12% (p=0.001)
Percentage of non-events correctly reclassified	17% (p=0.001)

Note: Model 1- CHF; Model 2 – CHF + sRANKL/OPG ratio
Abbreviations: NRI - net reclassification improvement, BNP – brain natriuretic peptide, sRANKL – serum receptor activator of NF-κB ligand, OPG – osteoprotegerin, CHF – chronic heart failure.