

# Relationship Between Serum RANKL/Osteoprotegerin Complex and Endothelial Progenitor Cells in Ischemic Chronic Heart Failure

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## ABSTRACT

**AIM:** The objective of this study was to assess an interrelationship serum RANKL/OPG complex with counts of circulating proangiogenic endothelial progenitor cells (EPCs) labeled as CD14<sup>+</sup>CD309<sup>+</sup>, and CD14<sup>+</sup>CD309<sup>+</sup>Tie<sup>2+</sup> in patients with ischemic chronic heart failure (CHF).

**METHODS:** The study retrospectively evolved 153 patients (86 males) aged 48 to 62 years with angiographically documented stable coronary artery disease (CAD). One hundred nine patients (71.2%) had systolic or diastolic CHF. The control group consisted of 25 healthy volunteers with matching age, sex, and body mass index. Serum RANKL (sRANKL) and OPG were measured by high-sensitive ELISA at baseline. EPC populations were phenotyped by flow cytometry per High-Definition Fluorescence Activated Cell Sorter methodology.

**RESULTS:** Numerous of EPCs with phenotypes of CD14<sup>+</sup>CD309<sup>+</sup> and CD14<sup>+</sup>CD309<sup>+</sup>Tie<sup>2+</sup> were significantly lower in CAD patients when compared with healthy subjects. There is a significant trend to decrease of EPC numerous depending presence of CHF. The sRANKL level, 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<sup>+</sup>CD309<sup>+</sup> and CD14<sup>+</sup>CD309<sup>+</sup>Tie<sup>2+</sup>. Using reclassification methods, we found

that the addition of sRANKL/OPG ratio to the ABC standard model (CHF) improved the relative integrated discrimination indices by 12.5% for CD14<sup>+</sup>CD309<sup>+</sup> depletion, by 17.3% for CD14<sup>+</sup>CD309<sup>+</sup>Tie<sup>2+</sup> depletion.

**CONCLUSION:** We found that sRANKL/OPG ratio remained statistically significant predictor for depletion of proangiogenic EPCs in CAD patients.

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**Key words:** Chronic heart failure; RANKL; Osteoprotegerin; Endothelial progenitor cells

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## INTRODUCTION

Chronic heart failure (CHF) remains one of the major causes of mortality worldwide<sup>[1,2]</sup>. Recent evidence suggests that CHF is associated with factors that may contribute to deteriorate vascular integrity and endothelial function, worse angiogenesis, modulate coagulation and inflammation<sup>[2,3]</sup>. The effects mentioned above lead tissue repair disorder that is represented a crucial event in the development of CHF<sup>[4]</sup>. Recent studies suggested that circulating level of endothelial progenitor cells (EPCs) with proangiogenic capacities is key player in the pathogenesis of cardiac failure<sup>[5,6]</sup>. EPCs ensure reparative processes including endothelialization of vascular lesion fragments, as well as remodeling of extracellular matrix and neovascularization<sup>[6]</sup>. Circulating EPCs, which co-expressed CD34+ antigen and VEGFR-2+ vascular growth ligands (Vascular Endothelial Growth Factor Receptor-2), CD133+, CD14+, and Tie2+ (tyrosine kinase ligand), were tested as predictors of nature evolution of ischemic CHF<sup>[7,8]</sup>. The main cytokines that regulate maturation and differentiation of EPCs are the receptor activator of nuclear factor- $\kappa$ B ligand (RANKL) and its decoy

receptor, osteoprotegerin (OPG)<sup>[9]</sup>. Recent data have linked RANKL and OPG to cardiovascular disease, including CHF, immunity, vascular calcification, osteoporosis, and bone remodeling as well<sup>[10-13]</sup>. However, the potential role of serum RANKL/OPG complex in modulation of the circulating proangiogenic 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 proangiogenic endothelial progenitor cells labeled as CD14<sup>+</sup>CD309<sup>+</sup>, and CD14<sup>+</sup>CD309<sup>+</sup>Tie<sup>2+</sup> in patients with ischemic CHF.

## METHODS

The study retrospectively evolved 153 patients (86 males) aged 48 to 62 years with angiographically documented stable coronary artery disease (CAD) in period between February 2010 and November 2013. One hundred nine patients (71.2%) had systolic or diastolic CHF, that was defined as left-ventricular ejection fraction  $\leq 45\%$  or 46-55% respectively assessed by quantitative echocardiographic and other conventional criteria according to current clinical guidelines<sup>[14]</sup>. Patients with chronic postischemic CHF had angiographically documented coronary artery disease (CAD) and persistent regional left-ventricular dysfunction. The control group consisted of 25 healthy volunteers with matching age, sex, and body mass index.

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  $\mu\text{mol/L}$ ; estimated GFR index  $< 35 \text{ mL/min/m}^2$ ; brain injury within 3 months before the enrollment; body mass index above 30  $\text{kg/m}^2$  and less 15  $\text{kg/m}^2$ ; 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.

### Methods for visualization of coronary arteries

Multispiral computed tomography angiography and/or angiographic study have been carried out to verify the ischemic nature of the disease in patients. Multispiral computed tomography angiography has been carried out for all the asymptomatic patients at high risk of CAD prior to their inclusion in the study. When atherosclerotic lesions of the coronary arteries were verified, patients were subjected to conventional angiographic examination provided indications for revascularization were available. CAD was considered to be diagnosed upon availability of previous angiographic examinations carried out not later than 6 months ago provided no new cardiovascular events occurred for this period, and the procedure are available for assay.

### Multispiral computed tomography angiography

The coronary artery wall structure were measured by means of contrast spiral computed tomography angiography<sup>[15,16]</sup> on Somatom Volum Zoom scanner (Siemens, Erlangen, Germany) with two detector rows when holding patient's breathe at the end of breathing in. After preliminary native scanning, non-ionic contrast "Omnipak" (Amersham Health, Ireland) was administered for the optimal image

of the coronary arteries. To reconstruct the image, 0.6-mm-width axial tomographic slices were used.

### Transthoracic echocardiography

Cardiac hemodynamics was evaluated by transthoracic echocardiography according to a conventional procedure on ACUSON scanner (SIEMENS, Germany) in B-mode regimen and tissue Doppler regimen from parasternal, subcostal, and apical positions over the short and long axis with phased-mode transducer of 5 MHz. Left ventricular end-diastolic and end-systolic volumes were measured by modified Simpson's method. Left ventricular ejection fraction (LVEF) was assessed in compliance with the requirements of American Society of Echocardiography<sup>[17]</sup>. Tissue Doppler echocardiography 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: at the base of posterior septal, lateral, inferior, and anterior left ventricular walls<sup>[18]</sup>. Peak systolic (Sm), early diastolic (Em), and late diastolic (Am) myocardial velocities were measured in the mitral annulus area, followed by calculating velocity of early diastolic left ventricular filling (E) to Am (E/Am) ratio and to Em (E/Em) ratio.

### Calculation of glomerular filtration rate

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

### 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  $-35^\circ\text{C}$ . Serum RANKL and 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. 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 1,010 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) were measured by commercially available standard 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. Concentration of cholesterol of low-density lipoproteins (LDL-C) was calculated according to the Friedewald formula (1972).

### Identifying fractions of mononuclear and endothelial progenitor cells

Mononuclear cells populations were phenotyped by flow cytometry by means of 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 Hematology and Graft Engineering sequential (ISHAGE protocol of gating strategy)<sup>[20]</sup>. For each sample, 500 thousand events have

been analyzed. Circulating EPCs have been identified as CD45<sup>+</sup>CD34<sup>+</sup>. CD309(VEGFR2) and Tie-2 antigens were also determined to identify subpopulations of EPC coexpressing CD14 antigen. Obtained when laser beam is scattered in longitudinal and transversal directions in the flow cytometer, the scattergram results were analyzed by using Boolean principles for double or triple positive events. The pool of the cells identified was standardized with regard to CD45<sup>+</sup> circulating mononuclear count.

### Statistical Analysis

Statistical analysis of the results obtained was carried out in SPSS system for Windows, Version 22 (SPSS Inc, Chicago, IL, USA). The data were presented as mean (M) and standard deviation ( $\pm$ 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. To compare categorical variables between groups, Chi2 test ( $\chi^2$ ) 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 (estimated by means of Kolmogorov-Smirnov test) and was not subjected to any mathematical transformation. The factors, which could be

associated potentially with EPCs, were determined by logistic regression analysis. Predictors of EPCs declining in CHF subjects were examined in univariable and multivariable linear regression analysis. C-statistics, integrated discrimination indices (IDI) and net-reclassification improvement (NRI) were utilized for prediction performance analyses. A two-tailed probability value of  $<0.05$  was considered as significant.

## RESULTS

Table 1 shows a general characteristic of the patients included in the study. As one can see from 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. Arterial hypertension and hyperlipidemia, however, were found more often in individuals with mild and moderate CHF than in individuals without documented CHF (72.7% versus 61.0% and 43.2% versus 32.9% respectively), with the incidence data being statistically significant. At the same time, no difference was found between the two cohorts

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

Parameters	Health individuals (n=25)	Entire group (n=153)	Without CHF (n=44)	With CHF (n=109)
Age, years	51.70 $\pm$ 6.10	58.34 $\pm$ 9.60	57.20 $\pm$ 6.70	59.50 $\pm$ 7.30
Males, n (%)	14 (56.0%)	86 (56.2%)	29 (65.9%)	57 (52.3%)
Hypertension, n (%)	-	99 (64.7%)*	32 (72.7%)	67 (61.5%)**
Dyslipidemia, 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/m <sup>2</sup>	23.3 (IQR=20.1–25.1)	24.1 (IQR=21.6–27.7)	23.7 (IQR=22.5–27.3)	24.2 (IQR=22.0–27.9)
Systolic blood pressure, mm Hg	127 $\pm$ 6	131 $\pm$ 8	135 $\pm$ 5	129 $\pm$ 4
Diastolic blood pressure, mm Hg	72 $\pm$ 4	80 $\pm$ 5	82 $\pm$ 4	79 $\pm$ 5
Heart rate, beats per 1 min.	69 $\pm$ 3	71 $\pm$ 3	68 $\pm$ 3	76 $\pm$ 6
LVEF, %	65.40 $\pm$ 8.54	45.50 $\pm$ 11.7*	55.40 $\pm$ 6.70	42.80 $\pm$ 8.8**
E/Am, U	6.1 $\pm$ 0.22	14.3 $\pm$ 1.13*	12.5 $\pm$ 1.20	16.6 $\pm$ 0.94**
E/Em, U	7.2 $\pm$ 0.19	13.7 $\pm$ 1.12*	10.6 $\pm$ 0.84	16.6 $\pm$ 1.00**
GFR, mL/min/1.73 m <sup>2</sup>	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, $\mu$ mol/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 <sup>+</sup> CD309 <sup>+</sup> $\times 10^4$ , %	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 <sup>+</sup> CD309 <sup>+</sup> Tie2 <sup>+</sup> $\times 10^4$ , %	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)**
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)**
ACEI/ARAs, n (%)	-	153 (100%)	44 (100%)	109 (100%)
Acetylsalicylic acid, n (%)	-	139 (90.8%)	38 (86.4%)	101 (92.7%)
Other antiplatelet drugs, n (%)	-	14 (9.2%)	6 (13.6%)	8 (7.3%)*
Statins, n (%)	-	80 (74.5%)	34 (77.3%)	80 (73.4%)
Metformin, n (%)	-	50 (32.7%)	12 (27.3%)	38 (34.9%)
Diuretics, n (%)	-	129 (84.3%)	38 (86.4%)	91 (83.5%)
Mineralocorticoid receptors antagonists, n (%)	-	41 (26.8%)	11 (25.0%)	30 (27.5%)

\* 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 ( $P<0.05$ ); 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; ACEI: angiotensin-converting enzyme inhibitor; ARAs: angiotensin-2 receptors antagonists; OPG: osteoprotegerin; sRANKL: serum receptor activator of nuclear factor-kappa B ligand.

in terms of systemic office blood pressure (BP) and heart rate (HR). Documented incidence of type 2 diabetes mellitus in patients of the two cohorts was 38.6% and 35.4% ( $P=0.06$ ). Adherence to smoking was found more often in individuals with documented CHF. 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 inpatients 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<sup>+</sup>CD309<sup>+</sup> and CD14<sup>+</sup>CD309<sup>+</sup>Tie<sup>2+</sup> were significantly lower in CAD patients when compared with healthy subjects. Therefore, there is a significant trend to decrease of EPC numerous depending presence of CHF.

With respect to circulating inflammatory biomarkers, we did find any difference between the CAD patient group and healthy

individuals in terms of sRANKL, OPG, and sRANKL/OPG ratio (Table 3). For all cases, the sRANKL level, 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.

When analyzing details of pharmacotherapy, no substantial differences were found between the two cohorts with regard to administration of the majority of drugs, except antiplatelet drugs other than acetylsalicylic acid, which were used less frequently in patients with CHF.

Results of univariate and multivariate regression analysis were presented in table 2. No any significant association EPC count with study medications was found. However, CHF, sRANKL/OPG ratio, OPG, and NT-pro-BNP remained as independent

**Table 2** 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 <sup>+</sup> CD309 <sup>+</sup>						
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
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
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 <sup>+</sup> CD309 <sup>+</sup> Tie <sup>2+</sup>						
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
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
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

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 3** 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 <sup>+</sup> CD309 <sup>+</sup> 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 <sup>+</sup> CD309 <sup>+</sup> Tie <sup>2+</sup>				
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%

Relative IDI: calculated as the ratio of IDI over the discrimination slope of the model without CHF; 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.

predictors of decreased EPCs with phenotypes of CD14<sup>+</sup>CD309<sup>+</sup> and CD14<sup>+</sup>CD309<sup>+</sup>Tie2<sup>+</sup>.

Using reclassification methods, however, we found that the addition of sRANKL/OPG ratio to the ABC model (CHF) improved the relative IDI by 12.5% for CD14<sup>+</sup>CD309<sup>+</sup> depletion, by 17.3% for CD14<sup>+</sup>CD309<sup>+</sup>Tie2<sup>+</sup> depletion (Table 3). 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<sup>+</sup>CD309<sup>+</sup> cells (Table 4). When we added to the ABC model sRANKL/OPG ratio, 12% of events ( $p=0.001$ ) and 17% of non-events ( $p=0.001$ ) were reclassified for CD14<sup>+</sup>CD309<sup>+</sup>Tie2<sup>+</sup> EPCs. Thus, we suggested that sRANKL/OPG ratio remained statistically significant predictor for depletion of CD14<sup>+</sup>CD309<sup>+</sup> EPCs and CD14<sup>+</sup>CD309<sup>+</sup>Tie2<sup>+</sup> EPCs in CAD patients.

**Table 4** 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+Tie2+ 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% ( $p=0.001$ )

Model 1: CHF; Model 2: CHF + sRANKL/OPG ratio; NRI: net reclassification improvement; BNP: brain natriuretic peptide; sRANKL: serum receptor activator of NF- $\kappa$ B ligand; OPG: osteoprotegerin; CHF: chronic heart failure.

## 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 its close correlation with traditionally circulating biomarkers reflected proinflammatory and neurohumoral activation, such as hs-CRP and NT-proBNP. Actually, serum RANKL, OPG, hs-CRP, and natriuretic peptides are well-established mutual-related biomarkers of heart failure reflecting several faces of the pathogenesis of CHF<sup>[21-23]</sup>. 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<sup>[24-26]</sup>. Therefore, serum RANKL and OPG as a RANKL's inhibitor is increasingly being investigated as markers of cardiovascular risk, subclinical atherosclerosis, and cardiac remodeling, vascular calcification<sup>[27-33]</sup>. 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 data OPG/RANKL/RANK system contributes to cardiac remodeling and left ventricular dilation after acute myocardial infarction in acute phase of cardiac failure as well as in chronic phase of heart failure development, while not so profoundly<sup>[24,33]</sup>. 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<sup>[10]</sup>. There is interrelationship

between OPG and serum RANKL concentrations in patients with advanced atherosclerosis in relation to medical history, risk factors and medication intake<sup>[34]</sup>. On the one hand, sRANKL/OPG complex contributes different stages in ischemic CHF development, whereas the clinical implication of RANKL seems uncertain<sup>[35-37]</sup>. On the other hand, the independent predictive value was determined for OPG only<sup>[38,39]</sup>. OPG is suggested to be a modulator rather than a marker of extracellular remodeling that may play critical role in CHF pathogenesis by neutralizing the effect of receptor activator of nuclear factor-kappa B ligand on differentiation and activation of wide spectrum cells, including circulating EPCs<sup>[40]</sup>. 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<sup>[41]</sup>. This effect may mediate overproduction of reactive oxygen species and oxidized lipoproteins through OPG-related activation of NOX-2 and NOX-4 and triggered phosphorylation of ERK-1/2 and p38 MAPK<sup>[41,42]</sup>. All these mechanisms are suitable for ischemic CHF development<sup>[43,44]</sup>. Results of 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 and OPG 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 proangiogenic phenotypes. We suggested that sRANKL/OPG complex affected reparative face of the pathogenesis of ischemic CHF through modulating count of circulating EPCs. Because OPG may stimulate differentiation of the EPCs and positively regulate their count in circulation<sup>[40]</sup>, we suggest that free fraction of serum RANKL, calculated as serum RANKL/OPG ratio, consider powerful predictor for depletion of CD14<sup>+</sup>CD309<sup>+</sup> EPCs and CD14<sup>+</sup>CD309<sup>+</sup>Tie2<sup>+</sup> EPCs in CAD patients. Probably, this effect may have a prognostic value for subjects with CHF due to ischemic reason. Further studies are needed to elucidate the potential role of free fraction of serum RANKL in the complex pathogenesis of ischemic CHF.

## CONCLUSION

Serum RANKL/OPG ratio remained statistically significant predictor for depletion of CD14<sup>+</sup>CD309<sup>+</sup> EPCs and CD14<sup>+</sup>CD309<sup>+</sup>Tie2<sup>+</sup> EPCs in CAD patients.

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

**Study Limitations:** This study has some limitations. The major limitation of this study was the small population size. However, this was not a randomized and controlled study. Authors believe that a

greater cohort of patients with more incidences detected is desirable to improve the power of the study. Therefore, BMI and T2DM may effect on EPCs in patient included in the study. As we have been proposed, this influence may be controversial when compared with severity of CHF. It is needed to take into consideration that for minimizing such interrelationship we withdrawn in enrollment in the study all subjects with body mass deficiency, high and extremely high obesity, and uncontrolled T2DM. It is necessary to note that large pool of nanoparticles might be produced after blood sampling due to destroy of platelets and blood cells. Venous citrated blood drawn from the fistula-free arm was performed obligatory. We believe that these risks are systemic, and to minimize them, we refused to freeze the blood samples before measurement of EPCs. 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 suppose that these restrictions might have no significant impact on the study data interpretation.

## CONFLICT OF INTERESTS

There are no conflicts of interest with regard to the present study.

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