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IMPAIRED IMMUNE PHENOTYPE OF CIRCULATING ENDOTHELIAL-DERIVED MICROPARTICLES IN NONE-DIABETIC PATIENTS WITH CHRONIC HEART FAILURE: IMPACT ON INSULIN RESISTANCE

Alexander E. Berezin[1†] --- Alexander A. Kremzer[2] --- Tatyana A Samura[3] --- Tatyana A Berezina[4]

[1]*Internal Medicine Department, State Medical University, Zaporozhye, Ukraine*

[2,3]*Clinical Pharmacology Department, State Medical University, Zaporozhye, Ukraine*

[4]*Private Medical Center "Vita-Center", Zaporozhye, Ukraine*

ABSTRACT

Background: The causality role of different immune phenotype in IR developing among chronic heart failure (CHF) subjects has not determined obviously. The aim of the study was to assess relationship between IR and immune phenotype of circulating endothelial-derived microparticles (EMPs) in patients with CHF. Methods: The study retrospectively involved 300 CHF patients aged 48 to 62 years. All the patients have given written informed consent for participation in the study. Biomarkers were measured at baseline of the study. Results: These were not significant differences between both cohort patients in EMPs labeled as CD144+/CD31+, CD144+/annexin V+, and CD62E+ microparticles. Higher concentrations of CD144+/CD31+/annexin V+ EMPs and CD31+/annexin V+ EMPs were found in IR subjects when compared with none IR patients. Using multivariate logistic regression analyses, we found that HOMA-IR (OR = 1.14, 95% CI=1.08-1.21, P = 0.001), NT-proBNP (OR = 1.07, 95% CI=1.04-1.10, P = 0.001), hs-CRP (OR = 1.04, 95% CI=1.02-1.07, P = 0.001), and NYHA class (OR = 1.03, 95% CI=1.01-1.05, P = 0.001) were predictors for increased CD31+/annexin V+ EMPs. Therefore, HOMA-IR (OR = 1.10, 95% CI=1.05-1.17, P = 0.001), NT-proBNP (OR = 1.08, 95% CI=1.04-1.12, P = 0.001), and NYHA class (OR = 1.05, 95% CI=1.02-1.09, P = 0.001) significantly predicted elevation of CD144+/CD31+/annexin V+ EMPs. Conclusion: we found that IR remains statistically significant predictor for increased apoptotic-derived EMPs labelled as CD144+/CD31+/annexin V+ and CD31+/annexin V+ EMPsin none-diabetic patients with CHF patients and that these findings reflect exiting impaired phenotype of circulating EMPs in this patient population.

Keywords: Chronic heart failure, Insulin resistance, Endothelial-derived microparticles, Immune phenotype, Apoptotic-derived microparticles, Activated endothelial cell-derived microparticles.

† Corresponding author

Contribution/ Originality

The paper's primary contribution is finding that insulin resistance may predict being of impaired phenotype of circulating endothelial-derived microparticles among none-diabetic patients with chronic heart failure.

1. INTRODUCTION

Chronic heart failure (CHF) is an increasingly common condition that is characterized raised prevalence worldwide and associated with cardiovascular morbidity and mortality [1]. The results of few population-based and epidemiological investigations show that multiple risk factors and various metabolic comorbidities presented in CHF patients are able to affect nature evolution of cardiac failure [2-5]. Therefore, exiting differences in the prevalence of risk factors and comorbidities in patients with CHF may not completely explain sufficient distinguishes in survival in CHF patient population [6-8]. Recently, increasing attention has been paid to insulin resistance (IR) as a distinct cause of cardiac dysfunction and CHF in diabetic and non-diabetic patients [9, 10]. IR mediates excessive or inadequate proliferation of the extracellular matrix accelerates apoptosis via increased oxidative stress, neurohumoral and inflammatory activation that negatively effect on cardiac remodeling, vasomotion, and endothelial function [11-14]. Despite IR is considered a main component of metabolic syndrome and type two diabetes mellitus (T2DM), individuals with CHF may present IR prior to other dysmetabolic conditions [15, 16]. However, the causality association of IR with none T2DM patients with CHF is unclear and the underlying mechanisms of advance CHF affected IR have not been fully elucidated.

Recent studies have shown the role of circulating endothelial-derived microparticles (EMPs) in nature evolution of CHF with possibility predictive value [17-20]. Extracellular EMPs are defined as microvesicles with sizes ranging between 50 and 1000 nm released from plasma membrane of endothelial cells due to apoptosis or cell activation by specific (cytokine stimulation, mononuclear cooperation, coagulation, etc) and non-specific (shear stress) stimuli [21]. Apoptotic-derived or activated endothelial cell-derived EMPs are capable of transferring biological information, regulating peptides, hormones, proteins, lipid components without direct cell-to-cell contact to maintain cell homeostasis [22, 23]. Interestingly, circulating EMPs derived from activated endothelial cells did not contain nuclear components and they have also been shown to have pro-angiogenic and cardio-protective properties [24, 25]. In opposite, apoptotic-derived EMPs consist immune mediators generated powerful signaling by the simultaneous receptor interaction and they are discussed a marker of endothelial cell injury and vascular aging [26, 27].

However, the role of different immune phenotype in developing IR among CHF patients has not determined obviously. The aim of the study was to assess relationship between IR and immune phenotype of circulating EMPs in patients with CHF.

2. METHODS

The study involved 300 CHF patients aged 48 to 62 years who were undergone multispiral computed tomography angiography or coronary angiography in our centers between February 2011 and November 2013. Sample size is calculated by using single population proportion formula by considering the following assumptions; 50% prevalence assumption, 95% confidence level of significance alpha 0.05 = 1.96, and 5% margin of error, which results in the sample size of 299.

Enrolled subjects presented atherosclerotic stenosis > 50% of at least one coronary artery or they reported previously defined myocardial infarction. We excluded patients with acute infections; active inflammation; pulmonary edema; tachyarrhythmia; valvular heart disease; thyrotoxicosis; ischemic stroke; intracranial hemorrhage; surgery; trauma, autoimmune disease, malignancy, and acute coronary syndrome within 3 months prior to the study entry. All participants gave full written informed written consent.

2.1. Methods for Visualization of Coronary Arteries

Contrast-enhanced multispiral computed tomography angiography has been performed for all the patients with dysmetabolic disorder prior to their inclusion in the study on Optima CT660 scanner (GE Healthcare, USA) using non-ionic contrast Omnipaque (Amersham Health, Ireland) [28].

2.2. Echocardiography and Doppler Examination

Transthoracic echocardiography and Tissue Doppler Imaging was performed according to a conventional procedure on ACUSON ultrasound system (SIEMENS, Germany) using 2.5 - 5 MHz phased probe. The left ventricular ejection fraction (LVEF) was measured by modified Simpson's method [29]. Tissue Doppler echocardiography was carried out in 4-, 3-, and 2-chamber views in each of 16 segments of the left ventricle and in 4 spots of the mitral annulus [30].

2.3. Insulin Resistance Assessment

Insulin resistance was assessed by the homeostasis model assessment for insulin resistance (HOMA-IR) [31] using the following formula:

HOMA-IR (mmol/L × μU/mL) = fasting glucose (mmol/L) × fasting insulin (μU/mL) / 22.5

Insulin resistance was defined when estimated HOMA-IR value was over 2.77 mmol/L × μU/mL.

2.4. Calculation of Glomerular Filtration Rate

Glomerular filtration rate (GFR) was calculated with CKD-EPI formula [32].

2.5. Blood Sampling

Blood samples were drawn 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. Then, plasma was refrigerated immediately to be stored at a temperature not higher than -35°C. N-terminal pro-brain natriuretic peptide (NT-pro-BNP) level was measured by immunoelectrochemoluminescent assay using sets by R&D Systems (USA). High-sensitivity C-RP levels were measured by ELISA technique. Concentrations of total cholesterol (TC) and cholesterol of high-density lipoproteins (HDLP) were measured by enzymatic method. The concentration of cholesterol of low-density lipoproteins (LDL-C) was calculated according to the Friedewald, et al. [33].

2.6. Identifying Immune Phenotype of EMPs

Circulating EMPs were isolated from 5 ml of venous citrated blood drawn from the fistula-free arm. Platelet-free plasma (PFP) was separated from whole blood and then was centrifugated at 20,500 × rpm for 30 min. EMPs pellets were washed with DMEM (supplemented with 10 μg/ml polymyxin B, 100 UI of streptomycin, and 100 U/ml penicillin) and centrifuged again (20,500 rpm for 30 min). The obtained supernatant was extracted, and pellets were re-suspended into the remaining 200 μl of supernatant. PFP, EMPs, pellet, and supernatant were diluted five-, 10-, and five-fold in PBS, respectively [34].

Endothelial-derived apoptotic and activated microparticles were phenotyped by flow cytometry by phycoerythrin (PE)-conjugated monoclonal antibody against CD31 (platelet endothelial cell adhesion molecule [PECAM]-1), CD144 (vascular endothelial [VE]-cadherin), CD62E (E-selectin), and annexin V (BD Biosciences, USA) followed by incubation with fluorescein isothiocyanate (FITC)-conjugated annexin V (BD Biosciences, USA) per HD-FACS (High-Definition Fluorescence Activated Cell Sorter) methodology independently after supernatant diluted without freeze [35]. The samples were incubated in the dark for 15 min at room temperature according to the manufacturer's instructions. For each sample, 500 thousand events have been analyzed. EMPs gate was defined by size, using 0.8 and 1.0 mm beads (Sigma, St Louis, MO, USA). CD31+/annexin V+ and CD144+/CD31+/annexin V+ microparticles were defined as apoptotic EMPs, EMPs positively labeled for CD62E+ were determined as EMPs produced due to activation of endothelial cells [36].

2.7. Statistical Analysis

Statistical analysis of the results obtained was carried out in SPSS system for Windows, Version 20 (SPSS Inc, Chicago, IL, USA). The data were presented as mean (M) and standard deviation (±SD) or a 95% confidence interval (95% CI); the median (Me) and the 25%-75% interquartile range (IQR). The hypothesis of normal distribution of the parameters analyzed was checked by means of Shapiro–Wilk test and Kolmogorov-Smirnov test. 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. The two-tailed version of Wilcoxon test was used for paired comparison of parameter values inside the group. To compare categorical variables between groups, Chi² test ($\chi 2$) and Fisher F exact test were used. The factors, which could be associated potentially with circulating EMPs, were determined by means of univariate analysis of variance (ANOVA). Finally, we used logistic regression to calculate the odds ratio (OR) and a 95% CI for all the independent predictors of elevated circulating EMPs. The calculated difference of $P<0.05$ was considered statistically significant and all tests are reported two-tailed.

3. RESULTS

General characteristic of the patients included in the study is reported in Table 1. Three hundred none diabetes patients with CHF (62.0% males) with mean age for 59.50±7.30 years were divided into two cohorts depended on calculated value of HOMA-IR. Subjects with HOMA-IR > 2.77 mmol/L × μU/mL were defined as patients with IR (n=171). However, patients with HOMA-IR < 2.77 mmol/L × μU/mL were classified as none IR subjects (n=129). The patients of both cohorts were age- and sex-matched. Cardiovascular risk factors (adherence to smoking, hypertension, dyslipidemia) were found in similar proportion in both cohort patients. There was no significant difference in GFR, creatinine, serum total ***cholesterol***, high-density lipoprotein cholesterol between both patient cohorts. However, obesity was appeared more frequent in IR patients. Therefore, IR patients had a significantly higher BMI, HbA1c, fasting blood glucose, insulin, hs-CRP, serum low-density lipoprotein cholesterol, NT-pro-BNP. Systolic and diastolic blood pressures, heart rate in both patient cohorts were comparable.

Concomitant medications in CHF patients included in the study are summarized in Table 2. Proportion of the patients included in both cohorts who were treated with ACE inhibitors or ARBs, mineralocorticoid receptor antagonists, loop diuretics, acetylsalicylic acid, and statins were similar. Beta-blockers and i/f blocker ivabradine were prescribed statistically much more in CHF subjects with IR when compared with none IR subjects (P=0.016). Other antiplatelet drugs were used frequently in none IR subjects (9.3%) than in IR patients (5.8%; P=0.046).

Immune phenotypes of EMPs in CHF patients were presented in Table 3. These were not significant differences between both cohort patients in EMPs labeled as CD144+/CD31+, CD144+/annexin V+, and CD62E+ microparticles. In opposite, higher concentrations of CD144+/CD31+/annexin V+ EMPs and CD31+/annexin V+ EMPs were found in IR subjects when compared with none IR patients.

The univariate linear correlations between apoptotic-derived EMPs, cardiovascular risk factors, hemodynamic performances, and other biomarker were evaluated. The data have shown that numerous of CD144+/CD31+/annexin V+ EMPs was associated with NYHA class (r= 0.59; P=0.001), HOMA-IR (r=0.46; P=0.001), NT-pro-BNP (r=0.42; P=0.001), LVEF (r=0.37; P=0.001), low-density lipoprotein cholesterol (r=0.32; P=0.001), hs-CRP (r=0.31; P=0.005), and TG (r = 0.28, P = 0.001). The numerous of CD31+/annexin V+ EMPs was directly related with

NYHA class (r= 0.58; P=0.001), HOMA-IR (r = 0.462, P = 0.003), BMI (r = -0.38, P = 0.001), NT-proBNP (r = 0.522, P = 0.001), hs-CRP (r = 0.423, P = 0.001), GFR (r = -0.388, P = 0.001), TG (r = 0.342, P = 0.001), creatinine (r = -0.362, 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), hypertension (r = 0.23, P = 0.003), and smoking (r = 0.212, P = 0.001).

Using multivariate logistic regression analyses, we identified independent predictors for elevation of apoptotic-derived EMPs labelled as CD144+/CD31+/annexin V+ and CD31+/annexin V+ microparticles. The results have shown that HOMA-IR (OR = 1.14, 95% CI=1.08-1.21, P = 0.001), NT-proBNP(OR = 1.07, 95% CI=1.04-1.10, P = 0.001), hs-CRP (OR = 1.04, 95% CI=1.02-1.07, P = 0.001), and NYHA class (OR = 1.03, 95% CI=1.01-1.05, P = 0.001) were determined as predictors for increased CD31+/annexin V+ EMPs. Therefore, HOMA-IR (OR = 1.10, 95% CI=1.05-1.17, P = 0.001), NT-proBNP (OR = 1.08, 95% CI=1.04-1.12, P = 0.001), and NYHA class (OR = 1.05, 95% CI=1.02-1.09, P = 0.001) significantly predicted elevation of CD144+/CD31+/annexin V+ EMPs.

Using C-statistics for Models with HOMA-IR, NYHA class, and circulating biomarkers (hs-CRP, NT-pro BNP) as Continuous Variables we found that adding of these biomarkers (NYHA class,hs-CRP, andNT-proBNP) to the based model constructed with HOMA-IR did not improve the relative IDI for increased CD144+/CD31+/annexin V+ and CD31+/annexin V+ microparticles (Table 4).

When we used other model constructed on entering variables, IDI avoids to be improved for increased CD144+/CD31+/annexin V+ and CD31+/annexin V+ microparticles (available for NYHA class and circulating hs-CRPand NT-proBNP as continuous variables) (Table 5). Thus, we found that IR remained a statistically significant predictor for increased apoptotic-derived EMPs labelled as CD144+/CD31+/annexin V+ and CD31+/annexin V+ EMPsin none-diabetic patients with CHF patients and that these findings reflect exiting impaired phenotype of circulating EMPs in this patient population.

4. DISCUSSION

The results of our investigations shown that IR in none-diabetic population of CHF patients may consider a predictor of impaired phenotype of circulating EMPs, which reflects surpass of apoptotic-derived microparticles in circulation association with probably relatively deficiency of activated endothelial cell-derived microparticles in circulation. Recent studies shown that IR may frequently associate with CHF [37, 38] and even it is able appeared to be prior to clinical manifestation of CHF [39, 40]. In fact, we did not know whether impaired phenotype of circulating EMPs appears to be prior IR or after dysmetabolic disorders. However, IR emerges at early stage of CHF and probably it associates with other cardiovascular risk factors [41]. Contrary to expectation, no independent association has been verified between the circulating EMPs and such cardiovascular risk factors as smoking, obesity and hypertension. However, the causality relation IR with impaired immune phenotype in none-diabetic CHF patients are

required detail explanation, while the innate exact molecular mechanisms affected abovementioned phenomenon is still under recognized. Probably, oxidative stress and inflammation may elicit or exacerbate IR in CHF subjects [42, 43], although our results did not confirm independent causality role of these mechanisms in impaired phenotype of EMPs. We suggested that IR affects wide spectrum cells including endothelial cells, which avoid being able to secret pro-angiogenicmicroparticles. Therefore, IR did not allow endothelial cells to be resistant to inflammatory stimuli and direct injury. All these processes may lead to increased apoptotic-derived EMPs that are able to mediate endothelial inflammation and decrease ability to endothelial repair. Thus, impaired apoptotic phenotype in CHF patients reflects a limiting capacity of endothelial cell to maintain cardiac function in the face of co-morbidities such as IR. More studies are underway to evaluate the role of IR in impaired phenotype of circulating EMPs among CHF subjects.

5. CONCLUSION

We found that IR remained a statistically significant predictor for increased apoptotic-derived EMPs labelled as CD144+/CD31+/annexin V+ and CD31+/annexin V+ EMPs in none-diabetic patients with CHF.

5.1. Limitations of the Study

This study has some restrictions. Our study is limited by its retrospective nature. Therefore, there were several technical-related difficulties in the measurement of EMPs. In fact, lack of standard protocol for isolating and detecting circulating EMPs obtained from the plasma. According opinion of the majority experts, centrifugation is the main factor mediated reliability of the EMP determination in samples and contributed in biological variability of EMP count. Although HD-FACS methodology is widely used, theoretically overlap between two or more fluorochromes might reflect some obstacles for further interpretation of obtained results. Another limitation of the present study is that a specific role of EMPs is also possible and has not been characterized in depth in T2DM patients. However, the authors suppose that these restrictions might have no significant impact on the study data interpretation. Additionally, retrospective, relative small sample size may limit the significance of the present study. However, this was not a randomized and controlled study. The authors believe that a greater cohort of patients with more incidences detected is desirable to improve the credibility of the study.

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Conflict of interests: not declared

Ethical principles: All the patients have given their written informed consent for participation in the study. The investigators followed strictly all the requirements to clinical trials in conformity with the World Medical Association Declaration of Helsinki, 1964, Good Clinical Practice proveided by International Conference on Harmonization, Council of Europe Convention for the Protection of Human Rights and Dignity of the Human Being in view of using achievements in biology and medicine, Convention on Human Rights and Biomedicine, including Additional Protocol to the Convention on Human Rights and Biomedicine, concerning Biomedical Research, and legislation of Ukraine.

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Table-1. General characteristic of patients participating in study

Parameters	Entire cohort patients (n=300)	None IR subjects(n=129)	IR subjects (n=171)	P value
Age, years	59.50±7.30	57.90±8.10	60.30±6.33	0.26
Males, n (%)	186 (62.0%)	77 (59.7%)	109 (63.7)	0.23
Adherence to smoking, n (%)	66 (22.0%)	28 (21.7%)	38 (22.2%)	0.56
Hypertension, n (%)	184 (61.3%)	82 (63.6%)	102 (59.6%)	0.44
NYHA class I, n (%)	76 (25.3%)	34 (26.4%)	42 (24.5%)	0.62
NYHA class II, n (%)	74 (24.7%)	32 (24.8%)	42 (24.6%)	0.63
NYHA class III, n (%)	98 (32.7%)	45 (34.9%)	53 (31.0%)	0.60
NYHA class IV, n (%)	52 (17.3%)	18 (13.9%)	34 (19.9%)	0.12
Dyslipidemia, n (%)	143 (47.7%)	58 (45.0%)	85 (49.7%)	0.36
Obesity, n (%)	122 (40.7%)	44 (34.1%)	78 (45.6%)	0.042
BMI, kg/m^2, M; 95% CI	24.2 (22.0–27.9)	23.07 (22.3–25.7)	25.99(23.5–28.6)	0.054
GFR, mL/min/1.73 m^2, M; 95% CI	85.2 (70.3–112.5)	82.8 (71.5–103.1)	87.4 (73.5–110.1)	0.24
HbA1c, %, M; 95% CI	5.8 (4.3-6.3)	5.5 (4.7-6.1)	6.1 (5.4-6.5)	0.012
Fasting blood glucose, mmol/L, M; 95% CI	5.10 (3.4-6.1)	4.97 (4.87-5.07)	5.47 (5.14-6.0)	0.001
Insulin, μU/mL, M; 95% CI	13.12 (12.22-14.01)	10.41 (9.92-10.91)	15.15 (13.69-16.62)	0.016
Creatinine, μmol/L, M; 95% CI	74.9 (65.1–90.3)	72.6 (69.31–88.1)	78.6 (70.2–89.1)	0.52
Total ***cholesterol***, mmol/L, M; 95% CI	5.0 (4.2-5.8)	4.9 (4.1-5.3)	5.2 (4.5-5.7)	0.21
LDL-C, mmol/L, M; 95% CI	3.02 (2.80–3.90)	3.00 (2.82–3.75)	3.11 (2.86–3.82)	0.044
HDL-C, mmol/L, M; 95% CI	0.88 (0.82–0.97)	0.91 (0.86–0.95)	0.86 (0.83–0.92)	0.24
NT-pro-BNP, pg /mL, M; 95% CI	1533.6 (644.5 – 2560.6)	1066.9 (910.3 – 1223.6)	1480.5 (1310.4 – 1650.7)	0.001
hs-CRP, mg/L, M; 95% CI	7.34 (6.77-7.95)	7.11 (6.38-7.84)	7.51 (6.68-8.33)	0.016
Systolic BP, mm Hg, M±SD	129±4	131±6	129±5	0.52
Systolic BP, mm Hg,	77±5	78±4	77±6	0.48

Note: Categorical variables are expressed as numerous (n) and percentages (%).

Abbreviations: M – mean value; CI – confidence interval; BP – blood pressure, NYHA – New York Heart Association, T2DM – type two diabetes mellitus, GFR - glomerular filtration rate, HbA1c- glycated hemoglobin, ***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.

Table-2. Concomitant medications in CHF patients included in the study

Parameters	Entire cohort patients (n=300)	None IR subjects(n=129)	IR subjects (n=171)	P value
ACEI or ARAs, n (%)	300 (100%)	129 (100%)	171 (100%)	1.0
Mineralocorticoid receptor antagonists, n (%)	83 (27.7%)	33 (25.6%)	50 (29.2%)	0.14
Beta-blockers, n (%)	237 (79.0%)	88 (68.21%)	149 (87.1)	0.016
Acetylsalicylicacid, n (%)	278 (92.7%)	117 (90.7%)	161 (94.2%)	0.23
Other antiplatelet drugs, n (%)	22 (7.3%)	12 (9.3%)	10 (5.8%)	0.046
Ivabradine, n (%)	89 (29.7%)	26 (20.2%)	63 (36.8%)	0.026
Loop diuretics, n (%)	251 (83.7%)	109 (84.5%)	142 (83.0%)	0.48
Statins, n (%)	143 (47.7%)	58 (45.0%)	85 (49.7%)	0.36

Abbreviations: IR – insulin resistance

Table-3. Immune phenotypes of EMPs in CHF patients

Immune phenotypes	Entire cohort patients (n=300)	None IR subjects(n=129)	IR subjects (n=171)	P value
CD144+/CD31+ EMPs, n/mL	0.91 (0.36-1.35)	0.90 (0.34-1.27)	0.93 (0.41-1.32)	0.62
CD144+/annexin V+ EMPs, n/mL	1.15 (0.13-2.41)	1.13 (0.10-2.22)	1.17 (0.16-2.35)	0.24
CD144+/CD31+/annexin V+ EMPs, n/mL	1.01 (0.39-1.70)	0.98 (0.35-1.53)	1.05 (0.44-1.63)	0.044
CD31+/annexin V+ EMPs, n/mL	0.296 (0.261-0.339)	0.278 (0.243-0.310)	0.315 (0.289-0.327)	0.001
CD62E+ EMPs, n/mL	1.03 (0.86-1.13)	1.05 (0.94-1.11)	1.02 (0.81-1.10)	0.73

Abbreviations: IR – insulin resistance, EMPs – endothelial-derived microparticles

Note: The values are presented as the median and 25-75% interquartile range, the differences validity values obtained by two-tailed Mann-Whitney test.

Table-4. C-statistics for Models with HOMA-IR, NYHA class, hs-CRP, NT-proBNP as Continuous Variables

Models	Dependent variable: CD144+/CD31+/annexin V+ EMPs				Dependent variable: CD31+/annexin V+ EMPs			
	AUC (95% CI)	ΔAUC	IDI (±SE)	Relative IDI (%)	AUC (95% CI)	ΔAUC	IDI (±SE)	Relative IDI (%)
Model 1 (based model: HOMA-IR >2.77 mmol/L × μU/mL)	0.669	-	-	-	0.664	-	-	-

Model 1 + NYHA class + biomarkers (hs-CRP, NT-proBNP)	0.681	-	-	-	0.685	-	-	-
Model 1 + NYHA class + biomarkers (hs-CRP, NT-proBNP) versus Model 1	-	0.012; P=0.64	0.02±0.015	1.8%	-	0.021; P=0.12	0.03±0.012	2.2%

Note: Relative IDI – calculated as the ratio of IDI over the discrimination slope of the model without IR.

Abbreviations: AUC – area under curve, SE – standard error, IR – insulin resistance, BNP – brain natriuretic peptide, hs-CRP – high sensitive C-reactive protein.

Table-5. Prediction Performance Analyses for Models with HOMA-IR NYHA class, hs-CRP, NT-proBNPas Continuous Variables for increased CD144+/CD31+/annexin V+ and CD31+/annexin V+ EMPs.

Model 2 vs Model 1	**Dependent variable: CD144+/CD31+/annexin V+ EMPs**	**Dependent variable: CD31+/annexin V+ EMPs**
Categorical NRI	0.11 (95% CI=0.07-0.16)	0.08 (95% CI=0.06-0.11)
Percentage of events correctly reclassified	3 (p=0.64)	2 (p=0.72)
Percentage of non-events correctly reclassified	4 (p=0.26)	2 (p=0.81)
Categorical free NRI	0.13 (95% CI=0.09-0.15)	0.11 (95% CI=0.06-0.14)
Percentage of events correctly reclassified	2% (p=0.44)	1% (p=0.88)
Percentage of non-events correctly reclassified	4% (p=0.48)	4% (p=0.62)

Note: Model 1- HOMA-IR > 2.77 mmol/L × μU/mL; Model 2 – NYHA class, hs-CRP, NT-proBNP.

Abbreviations: NRI - net reclassification improvement, IR – insulin resistance, BNP – brain natriuretic peptide, hs-CRP – high sensitive C-reactive protein.