Research Paper

Pattern of endothelial progenitor cells and apoptotic endothelial cell-derived microparticles in chronic heart failure patients with preserved and reduced left ventricular ejection fraction

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Abstract

Background: Chronic heart failure (HF) remains a leading cause of cardiovascular (CV) mortality and morbidity worldwide. The aim of the study was to investigate whether the pattern of angiogenic endothelial progenitor cells (EPCs) and apoptotic endothelial cell-derived microparticles (EMPs) would be able to differentiate HF with reduced (HFrEF) and preserved (HFpEF) ejection fraction.

Methods: One hundred sixty four chronic HF subjects met inclusion criteria. Patients with global left ventricular ejection fraction ≥50% were categorized as the HFpEF group (n = 79) and those with ≤45% as the HFrEF group (n = 85). Therefore, to compare the circulating levels of biological markers 35 control subjects without HF were included in the study. All control individuals were age- and sex-matched chronic HF patients. The serum level of biomarkers was measured at baseline. The flow cytometric technique was used for predictably distinguishing circulating cell subsets depending on expression of CD45, CD34, CD14, Tie-2, and CD309 antigens and determining endothelial cell-derived microparticles. CD31+/annexin V+ was defined as apoptotic endothelial cell-derived MPs, MPs labeled for CD105+ or CD62E+ were determined as MPs produced due to activation of endothelial cells.

Results: In multivariate logistic regression model T2DM (R² = 0.26; P = 0.001), obesity (R² = 0.22; P = 0.001), previous MI (R² = 0.17; P = 0.012), galectin-3 (R² = 0.67; P = 0.012), CD31+/annexin V+ EMPs (R² = 0.11; P = 0.001), NT-proBNP (R² = 0.11; P = 0.046), CD14+ CD309+ cells (R² = 0.058; P = 0.001), and CD14+ CD309+ Tie-2+ cells (R² = 0.044; P = 0.028) were found as independent predictors of HFpEF. Using multivariate Cox-regression analysis adjusted etiology (previous myocardial infarction), cardiovascular risk factors (obesity, type 2 diabetes mellitus) we found that NT-proBNP (OR 1.08; 95% CI = 1.03 – 1.12; P = 0.001) and CD31+/annexin V+ EMPs to CD14+CD309+ cell ratio (OR 1.06; 95% CI = 1.02 – 1.11; P = 0.02) were independent predictors for HFpEF.

Conclusion: We found that CD31+/annexin V+ EMPs to CD14+CD309+ cell ratio added to NT-proBNP, clinical data, and cardiovascular risk factors has exhibited the best discriminate value and higher reliability to predict HFpEF compared with NT-proBNP and clinical data/cardiovascular risk factors alone.

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Keywords: Chronic heart failure, Preserved left ventricular function, Biomarkers, Endothelial progenitor cells, Endothelial cell-derived microvesicles

1. Introduction

Chronic heart failure (HF) remains a leading cause of cardiovascular (CV) mortality and morbidity worldwide (Go et al., 2014). Although over the last decades the incidence of newly HF in developed countries have been substantially declined particularly for HF with reduced ejection fraction (HFrEF) (Gerber et al., 2015), there is marked increase in hospital admissions, CV and non-CV death rate predominance of HF with preserved ejection fraction (HFpEF) (Dunlay et al., 2015; Jorge et al., 2015). As expected, the routine use of biomarkers on diagnosis of HFrEF and HFpEF might help stratify the patients at higher risk of death and clinical outcomes. In fact, both 2012 European Society of Cardiology (ESC) Guidelines for the Diagnosis and Treatment of Acute and Chronic Heart Failure and 2013 American College of Cardiology Foundation/ American Heart Association (ACCF/AHA) Guideline for the Management
of Heart Failure are well accepted by many clinicians regarding HFrEF diagnosis. Indeed, the HFrEF is that one that really needs improvement of biomarkers for diagnosis and prognosis (McMurray et al., 2012; Yancy et al., 2013). In this context, many biological markers, which reflect several faces of pathogenesis of HF, have been investigated in detail, but by now natriuretic peptides, soluble ST2, galectin-3, and high-sensitive cardiac specific troponins were validated only. However, there was not a large body of evidence regarding perspectives that may provide clinically useful prognostic information both concerning the future risk of HFrEF/HFpEF manifestation in asymptomatic subjects, the risk of fatal events and primary/re-admissions in the hospital in individuals for those already established symptomatic acute, acutely decompensated/advanced, and chronic stable HF related to ischemic and non-ischemic causes (D’Elia et al., 2015). It is suggested that multimorbidity in HF may limit the diagnostic and predictive utility of biomarkers (Chamberlain et al., 2015).

Recent studies showed that endothelium injury is common for HF onset and development beyond etiology (Fujisue et al., 2015). Endothelial dysfunction closely associates with activation and/or apoptosis of endothelial cells lead to release of newly detectable circulating biomarkers related to endothelial dysfunction called endothelial cell-derived microparticles (EMPs) (Dignat-George and Boulanger, 2011; Burger and Touyz, 2012). Human CD34+ primitive progenitors and CD14+ CD309+ (VEGFR2) mononcytic progenitors have exhibited pro-angiogenic capacities mediated through increased sensitivity to vascular endothelial growth factor and cell-to-cell cooperation via secretion of endothelial cell-derived microparticles (Awad et al., 2006; Burger and Touyz, 2012).

Therefore, endothelial progenitor cells (EPCs) labeled as CD133+CD34+CD14− (VEGFR2) and CD14+CD309+ (VEGFR2) Tie-2+ cells were found a marker of endothelial dysfunction and reparation ability (Dignat-George and Boulanger, 2011). It has been suggested that imbalance between EPCs with angiogenic capacity and apoptotic EMPs contributed in cell injury and endothelial dysfunction may reflect impaired reparative phenotype that is suitable for several CV diseases including HF (Berezin, 2015a; Berezin and Kremzer, 2015a,b; Berezin et al., 2015a). Indeed, endogenous deficiency of angiopoietic stimuli mediated by secretion of pro-inflammatory cytokines, neuro-hormones, growth factors, might lead to worsening endothelium reparation and HF progression (Singh et al., 2012; Berezin et al., 2015b). Recently we have reported that apoptotic EMP to EPC ratio might independently predict clinical outcomes in advanced chronic HF patients (Berezin et al., 2015c). However, whether impaired reparative phenotype might reflect a development of HFrEF and HFrEF is still not clear. The aim of the study was to investigate whether the pattern of endothelial progenitor cells with angiogenic capacity and apoptotic endothelial cell-derived microparticles would be able to associate with HFrEF and HFrEF phenotypes.

2. Methods

A total of 228 subjects suspected chronic HF were selected in this study after reviewing discharge reports. All these persons were treated in Zaporozhye Regional Hospital, City Hospital #6, City Hospital #10, Zaporozhye Regional Center of Cardiovascular Diseases from April 2010 to June 2015 with primary diagnosis chronic HF.

Chronic HF was defined according to contemporary criteria provided by actual clinical recommendation (McMurray et al., 2012). HFrEF (LVEF ≤ 45%) and HFrEF (LVEF ≤ 50%) were determined accordingly this recommendation. T2DM was diagnosed with revised criteria provided by American Diabetes Association when source documents were reviewed (Executive summary, 2013). When one or more of the following components were found: glycated hemoglobin [HbA1c] ≥ 6.5%; fasting plasma glucose ≥ 7 mmol/L; 2-h plasma glucose ≥ 11.1 mmol/L during an oral glucose tolerance test; a random plasma glucose ≥ 11.1 mmol/L; exposure of insulin or oral anti-diabetic drugs; a previous diagnosis of T2DM) T2DM was determined. Dyslipidemia was checked and determined according to NCEP Adult Treatment Panel III (National Cholesterol Education Program) (National Cholesterol Education Program Expert Panel on Detection, Evaluation, and Treatment of High Blood Cholesterol in Adults, 2002).

Including criteria for selection of the HF patients in the study were LVEF < 59%, ratio of mitral peak velocity of early filling (E) to early diastolic mitral annular velocity (′E′) [E/′E′] ratio > 15 units, elevated level of serum NT-proBNP > 220 pg/mL, and clinical presentation of chronic HF. Excluding criteria were severe kidney and liver diseases; malignancy; creatinine plasma level above 440 µmol/L; estimated GFR index < 35 ml/min/m2; brain injury within 3 months before the enrollment; valvular heart disease; thryotoxicosis; ischemic stroke; intracranial hemorrhage; acute infections; surgery; trauma; pregnancy; implanted pacemaker/defibrillator/cardioverter.

The flow chart representing patient in the study is reported in Fig. 1. Among these 228 prescreened subjects, only 164 chronic HF subjects were included in the study accordingly inclusion/exclusion criteria. Patients with global left ventricular ejection fraction > 55% were categorized as the HFrEF group (n = 79) and those with ≥45% as the HFrEF group (n = 85). Therefore, to compare the circulating levels of biological markers 35 control subjects without HF were included in the study. To compare EPCs and microparticles between healthy subjects, HFrEF and HFrEF individuals control group was made. Control subjects are defined as individuals with normal global cardiac function (LVEF > 55%, E/′E′ ratio < 8 units) assessed by transthoracic echocardiography and Tissue Doppler Imaging, serum NT-proBNP level < 125 pg/mL, and without any signs and symptoms of symptomatic HF (Fig. 2).

2.1. Ethical Statement

The study protocol was approved by the local Ethics Committee Review Board (IRB # 3/2010), State Medical University of Zaporozhye (Ukraine) prior to the study initiation. The study complied with the Declaration of Helsinki and voluntary informed written consent was obtained from all patients included in this study. All individuals included in the study have given voluntary informed written consent.

2.2. Anthropometric Measurements

Anthropometric measurements were made using standard procedures.

2.3. Echocardiography and Doppler Imaging

Transthoracic B-mode echocardiography and Tissue Doppler Imaging were performed according to a conventional procedure on ACUSON scanner (Siemens, Germany) using phased probe with modulated frequency of 2.5–5 MHz. Left ventricular end-diastolic and end-systolic volumes, and LVEF were measured by modified Simpson’s method (Quiñones et al., 2003). E/′E′ ratio was measured using pulsed wave Tissue Doppler Imaging according contemporary protocol (Paulus et al., 2007).

2.4. Glomerular Filtration Rate Measurement

Calculation of glomerular filtration rate (GFR) was calculated by CKD-EPI formula (Levey et al., 2009).

2.5. Blood Sampling

After an overnight fast 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 immediately centrifuged upon permanent cooling at 6000 rpm for 10 min. Then, plasma was refrigerated immediately to be stored at a temperature −70 °C. All laboratory tests were performed using standard methods to measure the serum fasting plasma glucose, fasting lipid profiles and other biomarkers.
N-terminal pro-brain natriuretic peptide (NT-pro-BNP) level was measured by immunoelectrochemoluminescent assay using sets by R&D Systems (USA) on Elecsys 1010 analyzer (Roche, Mannheim, Germany). High-sensitive C-reactive protein (hs-CRP) was measured by commercially available standard kit (R&D Systems GmbH, Wiesbaden-Nordenstadt, Germany). Galectin-3 was measured using an ELISA kit (BG Medicine, Germany). Concentrations of total cholesterol (TC), cholesterol of high-density lipoproteins (LDL-C), and cholesterol of high-density lipoproteins (HDL-C) were measured by enzymatic colorimetric method according to standardized methodology on Beckman Synchron LX20 chemistry analyzer.

2.6. Assay of Circulating Endothelial Progenitor Cell Subsets

The flow cytometric technique (FCT) was used for predictably distinguishing circulating cell subsets, which depend on expression of CD45, CD34, CD14, Tie-2, and CD309 (VEGFR2), using High-definition Fluorescence Activated Cell Sorter (HD-FACS) methodology (Tung et al., 2004). Accordingly, the cells were labeled on the basis of their forward scatter characteristic (FSC) and side scatter characteristic (SSC) profiles, and standardize and calibrate instruments, fluorescence and light scatter resolution, and sensitivity were determined according to standard protocol (Hoffman, 2005).

The cells were directly stained and analyzed for phenotypic expression of surface proteins using anti-human monoclonal antibodies, including anti-CD45 FITC (BD Biosciences, San Jose, CA, USA), anti-CD34 FITC (BD Biosciences), anti-VEGFR-2 known as anti-CD309 (BD Biosciences), anti-Tie-2 (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 the samples were washed twice with PBS and fixed immediately. Double- or triple-positive events were determined using Boolean principles (“and”, “not”, “or”, etc.).
2.7. Determination of Circulating Endothelial Progenitor Cells

Circulating EPCs were defined as CD34+/CD309 (VEGFR2) positive cells with lack of CD45 expression. From each tube 500,000 events were analyzed. For CD14+ populations, co-expression with Tie-2- and/or VEGFR-2- was determined using quadrant analysis. Standardized cell counts were presented as a percentage of the total of the white blood cell count, identified as the total number of all CD45+ cells. The FITC-labeled isotype control was analyzed with the same gate and window settings. Pro-angiogenic phenotype for EPCs was determined as CD14+CD309+ (VEGFR2) Tie-2+ antigen presentation. The reproducibility of EPC measurements using the standard protocol was 3.5%.

2.8. Assay of Circulating Microparticles

Circulating MPs were isolated from 5 mL of venous citrated blood drawn from the fistula-free arm. To prevent contamination of samples platelet-free plasma (PFP) was separated from whole blood. PFP was centrifuged at 20,500 × rpm for 90 min. MP pellets were washed with DMEM (supplemented with 10 μg/mL polymyxin B, 100 U of streptomycin, and 100 U/mL penicillin) and centrifuged again (20,500 rpm for 60 min). The obtained supernatant was extracted, and MP pellets were re-suspended into the remaining 200 μL of supernatant. PFP, MPs, and supernatant were diluted five-, ten-, and five-fold in PBS, respectively. Only 100 μL of supernatant was prepared for further analysis through incubation with different fluorochrome-labeled antibodies or their respective isotypic immunoglobulins (Beckman Coulter).

2.9. Determination of Endothelial Cell-derived Microparticles

MPs were labeled and characterized by flow cytometry technique per HD-FACS (High-definition Fluorescence Activated Cell Sorter) methodology independently after supernatant diluted without freeze (Orozco and Lewis, 2010). Two size gates were defined based on forward angle light scattering from polystyrene microsphere (0.5–0.9 μm) according to standard protocol (Shah et al., 2008). Accordingly, MPs’ gate was defined less than a 0.4 μm polystyrene microsphere extending down to the noise threshold level that is equivalent to cell-derived MPs < 1 μm diameter (Lacroix et al., 2010).

CD31 antigen was determined as essential marker for endothelial cells. CD31+/annexin V was defined as apoptotic endothelial cell-derived MPs, MPs labeled for CD105+ or CD62E+ were determined as MPs produced due to activation of endothelial cells (Lacroix et al., 2013). We used anti-CD31 (platelet endothelial cell adhesion molecule [PECAM]-1)-phycoerythrin (PE; 20 μL/test), anti-CD62E [E-selectin]-FITC (20 μL/test) antibodies obtained from Beckman Coulter. MPs that expressed phosphatidylserine were labeled using fluorescein-conjugated Annexin V solution (20 μL/test; BD Biosciences, USA) in the presence of CaCl2 (5 mM) according to the recommendation of the supplier.

The samples were incubated in the dark for 15 min at room temperature according to the manufacturer’s instructions. The analysis of area, height, and width forward scatter (FSC) and side scatter (SSC) parameters was performed as well as side scatter width (SSC-W). The gate for MPs was defined by size, using 0.5 and 1.0 μm beads (Sigma, St Louis, MO, USA). For each sample, 500 thousand events have been analyzed. Compensation tubes were used with similar reagents as were used in the sample tubes. Data were constructed as numerous of MPs depending on marker presentation (positive or negative) and determination of MP populations.

Calculation of the number of MPs per liter plasma was based upon the particle count per unit time, the flow rate of the flow cytometer, and the net dilution during sample preparation of the analyzed MP suspension. MP-exposed antigen concentrations were calculated in each sample by multiplying the total concentration of positive MPs by the mean fluorescence intensity of the antigen exposure of the total positive MP population. The reproducibility of EPCs using standard protocol was 4.5%.

2.10. Statistical Analysis

Data were analyzed using SPSS 20.0 (SPSS, IBM Corporation, NY, USA) and Prism v.6 (GraphPad Software Inc., La Jolla, CA, USA). Quantitative variables were expressed as mean (M) and standard deviation (± SD), median and interquartile range (IQR), estimated marginal mean (95% confidence interval [CI]) or number (percentage). An independent group t-test was used to compare all the interval parameters matching the criteria of normality and homogeneity of variance. For interval parameters that fail to match these criteria, the non-parametric Mann–Whitney test was used to compare variables. Categorical variables and frequencies were compared using Chi² test and Fisher exact test of independence. Comparisons of control group with a combined population of both HF groups are done using ANOVA. The potential factors that may be associated with HFrEF were identified first with the univariate analysis (ANOVA), and then the independent predictors of HFrEF were searched with the multivariate one-step backward logistic regression analysis, initially including variables for which a P value of <0.1 was achieved from the univariate analysis. R², B-coefficient were calculated for all regression models. The odds ratio (OR) and 95% CI were calculated for factors independently predicted HFrEF vs HFpEF in Cox-regression model. For each model that is able to differentiate HFpEF from HFrEF OR (95% CI) and AUC [Area Under Curve] (95% CI) were calculated. A calculated difference of P < 0.05 was considered significant.

3. Results

The study population consisted of 164 HF subjects (86 males and 78 females), with mean age of 52.13 ± 7.80 years, and 35 healthy volunteers as control for biomarker examination. All control individuals were age- and sex-matched chronic HF patients. The baseline data of eligible individuals are listed in Table 1. We did not find any significant difference between both HF cohorts in age, sex, NYHA class representation, hypertension, adherence to smoke, body mass index (BMI), systolic and diastolic blood pressure (BP) and heart rate. Previous myocardial infarction (MI), dilated cardiomyopathy, dyslipidemia were determined frequently in subjects with HFpEF compared with persons with HFrEF. Contrary, obesity, and type 2 diabetes mellitus (T2DM) patients were found frequently in patients with HFpEF then in HFrEF patient cohort.

Subjects with HFpEF compared with HFrEF have demonstrated higher levels of creatinine, total cholesterol, high-density lipoproteins, uric acid, NT-proBNP, galectin-3, and lower estimated GFR and hemoglobin (Table 2). However, level of circulating biomarkers have sufficiently differentiated between healthy volunteers and HF group patients apart from hemoglobin. Therefore, numerous of CD14+CD309+ cells were significantly higher (P = 0.001) in HFpEF patient cohort than in HFrEF patient cohort. Contrarily, HFpEF patients have exhibited lower levels of CD31+/annexin V+ EMPS compared with HFpEF patients. However, healthy volunteers have exhibited a lower level of CD31+/annexin V+ EMPS compared with entire HF patient cohort and both HFpEF and HFrEF individuals. There were not found significant changes between HF cohorts in numerous of CD14+CD309+ Tie-2+ cells and CD62E+ EMPS, while in the control healthy subjects the level of both biomarkers was significantly higher.

Table 3 is reported the concomitant study medication. As one can see, all HF patients were treated with ACE inhibitors or ARBs in combination with loop diuretics. Proportions of the beta-blocker treated patients in both HF cohorts were similar. Aspirin, ibabradine, mineralocorticoid receptor antagonists, and statins were used frequently in HFpEF subjects. Contrarily, metformin and anti-platelet drugs distinguished as aspirin were prescribed frequently in HFpEF patients.

We did not find any sufficient association between CD14+CD309+ cells, CD14+CD309+ Tie-2+ cells, CD31+/annexin V+ EMPS, CD62E+
EMPs, and age, sex, GFR, NT-pro-BNP, galectin-3 and hs-CRP in control healthy volunteers. However, there were significant associations between CD31+/annexin V+ EMPs with adherence to smoke (r = 0.27; P = 0.001), and negatively with CD14+ CD309+ cells (r = 0.28; P = 0.001). Additionally, hs-CRP positively associated with NYHA class of CHF (r = 0.36; P = 0.001), and negatively with LVEF (r = −0.28; P = 0.001), obesity (r = −0.24; P = 0.001). Furthermore, hs-CRP associated significantly with type 2 diabetes mellitus (r = 0.22; P = 0.001) in entire group of HF patients. No associations between numerous EPCs and EMPs with medications were found.

In multivariate logistic regression model type 2 diabetes mellitus (R² = 0.26; P = 0.001), obesity (R² = 0.22; P = 0.001), previous MI

Table 1
The characteristics of participants in the study.

| Variables | Healthy volunteers | Entire patient group | P value between healthy volunteers and entire HF group | Subjects with HFpEF | Subjects with HFrEF | P value between HF cohorts |
|-----------|-------------------|----------------------|-------------------------------------------------------|---------------------|---------------------|--------------------------|
| Age, years | 54.85 ± 5.20      | 56.13 ± 7.80         | 0.44                                                  | 57.50 ± 6.70        | 54.79 ± 6.62        | 0.78                     |
| Male      | 18 (51.4%)        | 86 (52.4%)           | 0.24                                                  | 49 (57.6%)          | 37 (46.8%)          | 0.24                     |
| II NYHA class | 57 (34.8%)  | –                    | –                                                     | 29 (34.1%)          | 28 (35.4%)          | 0.96                     |
| III NYHA class | 65 (39.6%) | –                    | –                                                     | 36 (42.4%)          | 29 (36.7%)          | 0.66                     |
| IV NYHA class | 42 (25.6%) | –                    | –                                                     | 20 (23.5%)          | 22 (27.8%)          | 0.84                     |
| Previous MI | 112 (62.3%)       | –                    | –                                                     | 66 (77.6%)          | 46 (58.2%)          | 0.01                     |
| Dilated cardiomyopathy | – | 21 (12.8%) | 0.23; P = 0.001) in entire group of HF patients. No associations between numerous EPCs and EMPs with medications were found.

Note: The values correspond to medians and IQR of 25%-75%. Comparisons of control group with a combined population of both HF groups are done using ANOVA. Statistical comparisons between both HF groups are made using Mann–Whitney test with significance levels of <0.05 (for 2-tailed).

Abbreviations: NYHA — New York Heart Association; T2DM — type 2 diabetes mellitus; MI — myocardial infarction; LVEF — left ventricular ejection fraction.

Table 2
The biomarkers in the patient study population.

| Variables | Healthy volunteers | Entire patient cohort | P value between healthy volunteers and entire HF group | Subjects with HFpEF | Subjects with HFrEF | P value between HF cohorts |
|-----------|-------------------|----------------------|-------------------------------------------------------|---------------------|---------------------|--------------------------|
| GFR, ml/min/1.73 m² | 112.4 (102.2–123.4) | 82.3 (68.7–102.6) | 0.01                                                  | 79.6 (63.1–92.3) | 88.2 (71.1–102.1) | 0.046                    |
| Hemoglobin, g/L | 138.3 (129.8–151.2) | 135.4 (128.5–142.1) | 0.04                                                  | 128.1 (124.2–131.1) | 138.5 (126.2–141.8) | 0.001                    |
| Fasting glucose, mmol/L | 4.24 (3.6–4.9) | 5.17 (3.5–9.6) | 0.01                                                  | 4.98 (3.8–8.1) | 5.27 (3.6–9.3) | 0.28                     |
| HbA1c, % | 4.78 (4.2–5.5) | 6.8 (4.1–9.5) | 0.01                                                  | 6.4 (4.6–8.0) | 6.9 (4.3–9.2) | 0.22                     |
| Creatinine, μmol/L | 65.4 (58.2–81.2) | 72.3 (58.7–92.6) | 0.01                                                  | 82.1 (64.9–90.5) | 67.7 (59.1–84.1) | 0.01                     |
| Total cholesterol, mmol/L | 4.56 (3.25–4.88) | 5.1 (3.9–6.1) | 0.01                                                  | 5.3 (4.6–6.0) | 5.0 (3.5–5.9) | 0.02                     |
| HDL cholesterol, mmol/L | 1.03 (0.98–1.08) | 0.92 (0.88–1.13) | 0.01                                                  | 0.97 (0.92–1.08) | 0.88 (0.83–1.03) | 0.042                    |
| LDL cholesterol, mmol/L | 2.77 (2.33–3.10) | 3.23 (3.11–4.40) | 0.01                                                  | 3.71 (3.50–4.20) | 3.50 (3.10–3.96) | 0.05                     |
| Uric acid, μmol/L | 295 (210–367) | 345 (253–420) | 0.06                                                  | 357 (253–412) | 311 (206–369) | 0.01                     |
| NT-pro-BNP, pg/ml | 33.1 (18.3–63.6) | 233.62 (988.5–3552.8) | 0.001                                                  | 2774.5 (1520.4–3870.2) | 2130.8 (954.5–3065.2) | 0.02                     |
| hs-CRP, mg/L | 3.27 (0.5–3.3) | 7.10 (6.25–8.20) | 0.001                                                  | 7.05 (6.09–8.03) | 7.14 (6.22–8.32) | 0.46                     |
| Galectin-3, μg/L | 4.36 (0.98–0.37) | 18.82 (14.25–23.15) | 0.001                                                  | 19.03 (15.80–21.96) | 16.99 (13.77–19.20) | 0.022                    |
| CD14+ CD309+ cells/μL | 0.426 (0.370–0.574) | 0.296 (0.225–0.351) | 0.001                                                  | 0.236 (0.202–0.325) | 0.325 (0.233–0.407) | 0.001                    |
| CD14+ CD309+ Tie-2+ cells/μL | 0.0465 (0.0253–0.0710) | 0.032 (0.025–0.410) | 0.001                                                  | 0.030 (0.021–0.403) | 0.036 (0.019–0.465) | 0.26                     |
| CD31+/annexin V+ EMPs, n/mL | 0.154 (0.03–0.21) | 0.48 (0.29–0.64) | 0.001                                                  | 0.59 (0.42–0.65) | 0.32 (0.25–0.43) | 0.001                    |
| CD62E+ EMPs, n/mL | 1.35 (0.95–1.68) | 0.98 (0.87–1.12) | 0.01                                                  | 1.02 (0.81–1.25) | 0.96 (0.82–1.17) | 0.66                     |
diffatentiation of HFpEF from HFrEF. We have demonstrated that HF patients have exhibited elevated levels of EPCs with angiopoietic capacities, apoptotic-derived EMCs, as well as decreased level of CD62E- EMPs secreted by activated endothelial cells. Recently higher level of CD62- EMP secreted from activated endothelial cells in the healthy subjects versus HF patients was related to rather endothelial dysfunction than endothelial cell injury (Dignat-George and Boulanger, 2011; Burger and Touyz, 2012). In our study we did not find any significance changes between HFrEF and HFpEF patients, probably due to similar molecular mechanisms that might lead to endothelial cell activation. We have suggested that lack of sufficient difference between co-morbidities’ presentation among HFrEF and HFpEF groups might express similar finding. Interestingly, because of the number of existing CV risk factors which are variable between HF patients, simple EPC counts do not adequately describe vascular disease risk in all clinical conditions and, as such, the CV risk remains (Sen et al., 2011). However, the imbalance in pattern of circulating EMPs and EPCs might affect endothelium ability to repair and previously we have described similar changes as “impaired” phenotype (Berezin, 2015b). The concept of “impaired” phenotype as imbalance between factors originating from endothelium with innate angiogenic and/or injury capacities directly contributed in the endothelial dysfunction and requires further investigation because the molecular mechanism of their release into circulation still remains elusive.

Because biomarkers’ levels might suggest different amounts of activation of several pathophysiologic pathways between HFpEF and HFrEF development (Sanders-van Wijck et al., 2015; Kaila et al., 2012), we have hypothesized that numerous apoptotic EMPs to EPCs might be distinguished in HFpEF and HFrEF individuals. It is important to note that recent clinical studies have shown increased serum level of NT-proBNP as powerful predictive factor in both HFpEF and HFrEF, although patients with HFpEF exhibits lower NT-proBNP levels (Kang et al., 2015). Several biomarkers, i.e. soluble suppression of tumorigenicity 2 protein, galectin-3, cardiac specific troponins, provides robust prognostic information in HFrEF, but not for HFpEF, while they could improve the prognostication pattern via adding to NT-proBNP in predictive model (Friës et al., 2015). In the LUDwigshafen Risk and Cardiovascular Health (LURIC) study high-sensitive C-reactive protein was found to be an independent and strong predictor of mortality in HFpEF (Koller et al., 2014), but this biomarker even after adding to predictive model based on NT-proBNP was not able to predict HFpEF development. Overall, discriminative values of several cardiac biomarkers including NT-proBNP to stratify patients with HFpEF and HFrEF are not fully adequate.

Apoptotic EMPs are considered a marker of endothelial cell injury and a factor that contributed in transferring biological information, active molecules, hormones, proteins, lipid components, as well as regulating cell homeostasis and cell response (Sansone et al., 2015; Bank et al., 2015). Interestingly, apoptotic EMPs may directly injure endothelium (Montoro-García et al., 2015). In contrast, EPCs labeled

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**Table 3**

| Variables | Entire patient cohort (n = 164) | Subjects with HFrEF (n = 85) | Subjects with HFpEF (n = 79) | P value |
|-----------|--------------------------------|-----------------------------|----------------------------|---------|
| ACE inhibitors or ARBs, n (%) | 166 (100%) | 85 (100%) | 79 (100%) | 1.0 |
| Aspirin, n (%) | 128 (78.0%) | 77 (90.5%) | 51 (64.6%) | 0.022 |
| Other anti-platelet drugs, n (%) | 36 (22.0%) | 8 (9.5%) | 28 (35.4%) | 0.001 |
| Beta-adrenoblockers, n (%) | 135 (82.3%) | 71 (83.5%) | 64 (81.0%) | 0.76 |
| Dihydropyridine calcium channel blockers, n (%) | 27 (16.5%) | 15 (17.7%) | 12 (15.2%) | 0.24 |
| Ixibradine, n (%) | 48 (29.3%) | 38 (44.7%) | 10 (12.7%) | 0.001 |
| Mineralocorticoid receptor antagonists, n (%) | 57 (34.8%) | 43 (50.5%) | 14 (17.7%) | 0.001 |
| Loop diuretics, n (%) | 164 (100%) | 85 (100%) | 79 (100%) | 0.043 |
| Statins, n (%) | 116 (70.7%) | 68 (80.0%) | 48 (70.8%) | 0.01 |
| Metformin, n (%) | 38 (23.2%) | 15 (17.6%) | 23 (29.1%) | 0.01 |
| Sitagliptin, n (%) | 21 (12.8%) | 9 (10.6%) | 12 (15.2%) | 0.12 |

Notes: Data are expressed as numerals (n) and frequencies (%). Comparisons of control group with a combined population of both HF groups are done using ANOVA.

Abbreviations: T2DM — type 2 diabetes mellitus; GFR — glomerular filtration rate; EMP — endothelial cell-derived microparticles; EPCs — endothelial progenitor cells.

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**Table 4**

| Variables | Entire patient cohort (n = 164) | Subjects with HFrEF (n = 85) | Subjects with HFpEF (n = 79) | P value |
|-----------|--------------------------------|-----------------------------|----------------------------|---------|
| ACE inhibitors or ARBs, n (%) | 166 (100%) | 85 (100%) | 79 (100%) | 1.0 |
| Aspirin, n (%) | 128 (78.0%) | 77 (90.5%) | 51 (64.6%) | 0.022 |
| Other anti-platelet drugs, n (%) | 36 (22.0%) | 8 (9.5%) | 28 (35.4%) | 0.001 |
| Beta-adrenoblockers, n (%) | 135 (82.3%) | 71 (83.5%) | 64 (81.0%) | 0.76 |
| Dihydropyridine calcium channel blockers, n (%) | 27 (16.5%) | 15 (17.7%) | 12 (15.2%) | 0.24 |
| Ixibradine, n (%) | 48 (29.3%) | 38 (44.7%) | 10 (12.7%) | 0.001 |
| Mineralocorticoid receptor antagonists, n (%) | 57 (34.8%) | 43 (50.5%) | 14 (17.7%) | 0.001 |
| Loop diuretics, n (%) | 164 (100%) | 85 (100%) | 79 (100%) | 0.043 |
| Statins, n (%) | 116 (70.7%) | 68 (80.0%) | 48 (70.8%) | 0.01 |
| Metformin, n (%) | 38 (23.2%) | 15 (17.6%) | 23 (29.1%) | 0.01 |
| Sitagliptin, n (%) | 21 (12.8%) | 9 (10.6%) | 12 (15.2%) | 0.12 |

Notes: Data are expressed as numerals (n) and frequencies (%). Comparisons of control group with a combined population of both HF groups are done using ANOVA.

Abbreviations: T2DM — type 2 diabetes mellitus; GFR — glomerular filtration rate; EMP — endothelial cell-derived microparticles; EPCs — endothelial progenitor cells.
Table 5

Predictive value of biomarkers on dependent variable: HFpEF vs HFrEF. The Cox-regression analysis adjusted etiology (previous myocardial infarction), cardiovascular risk factors (obesity, type 2 diabetes mellitus).

| Variables                                      | Univariate Cox regression | Multivariate Cox regression |
|-----------------------------------------------|---------------------------|-----------------------------|
|                                              | OR | 95% CI | P value | OR | 95% CI | P value |
| NT-proBNP                                    | 1.12 | 1.06–1.27 | 0.001 | 1.08 | 1.03–1.12 | 0.001 |
| Galectin-3                                   | 1.08 | 1.03–1.12 | 0.002 | 1.04 | 1.00–1.09 | 0.12 |
| CD31+/annexin V+ EMPs to CD14+CD309+ cell ratio | 1.09 | 1.04–1.16 | 0.001 | 1.06 | 1.02–1.11 | 0.02 |
| CD31+/annexin V+ EMPs                        | 1.04 | 1.01–1.09 | 0.024 | 1.02 | 0.98–1.04 | 0.26 |
| CD31+/annexin V+ EMPs to CD14+CD309+ Tie-2+ cell ratio | 1.05 | 1.02–1.09 | 0.002 | 1.04 | 1.00–1.09 | 0.064 |
| CD31+/CD309+ cells                           | 1.04 | 1.01–1.06 | 0.044 | 1.02 | 0.99–1.05 | 0.34 |
| CD31+/CD309+ Tie-2+ cells                    | 1.03 | 1.00–1.05 | 0.12 | –   | –   | –   |

Table 6

Comparison of predictive value of models expressed HFpEF vs HFrEF.

| Models                           | OR  | 95% CI | P value | AUC  | 95% CI | P value |
|---------------------------------|-----|--------|---------|------|--------|---------|
| Model 1 (standard model)        | 1.05 | 1.02–1.08 | 0.001 | 0.62 | 0.59–0.66 | 0.044 |
| Model 2: NT-proBNP              | 1.08 | 1.03–1.12 | 0.001 | 0.68 | 0.61–0.74 | 0.012 |
| Model 3: standard model + NT-proBNP | 1.11 | 1.06–1.17 | 0.001 | 0.71 | 0.65–0.79 | 0.001 |
| Model 4: CD31+/annexin V+ EMPs to CD14+CD309+ cell ratio | 1.06 | 1.02–1.11 | 0.02 | 0.69 | 0.63–0.76 | 0.016 |
| Model 5: CD31+/annexin V+ EMPs to CD14+CD309+ cell ratio | 1.12 | 1.05–1.21 | 0.001 | 0.73 | 0.64–0.81 | 0.001 |
| Model 7: NT-proBNP + CD31+/annexin V+ EMPs to CD14+CD309+ cell ratio | 1.10 | 1.04–1.17 | 0.001 | 0.70 | 0.63–0.76 | 0.001 |
| Model 8: standard model + NT-proBNP + CD31+/annexin V+ EMPs to CD14+CD309+ cell ratio | 1.17 | 1.10–1.25 | 0.001 | 0.81 | 0.69–0.93 | 0.001 |

Note: Standard model — previous myocardial infarction, obesity, type 2 diabetes mellitus.

Abbreviations: OR — odds ratio; AUC — area under curve; CI — confidence interval; BMP — brain natriuretic peptide; EMPs — endothelial cell-derived microparticles.
patients. The next limitation is a small cohort of patients included in the study. Therefore, we used regression models for adjusted clinical data, CV risk, previous MI, metabolic co-morbidities, to analyze the role of novel biomarker to differentiate both phenotypes of chronic HF are required. The authors suppose that these restrictions might have no significant impact on the study data interpretation.

**Abbreviations**

ACEI angiotensin-converting enzyme inhibitors
ARBs angiotensin receptor blockers,
AUC area under curve
BMI body mass index
BNP brain natriuretic peptide
CHF chronic heart failure
CV cardiovascular
EMPs endothelial cell-derived microparticles
HF heart failure
HFpEF chronic HF with preserved ejection fraction
HFrEF chronic HF with reduced ejection fraction
GFR glomerular filtration rate
hs-CRP high-sensitive C-reactive protein
HDLC high-density lipoprotein cholesterol
LDLC low-density lipoprotein cholesterol
LVEF left ventricular ejection fraction

**Author contributions**

All initiated the hypothesis and designed the study protocol, contributed to the collection, analysis and interpretation of the data, performed statistical analysis, wrote the manuscript and approved the final version of the paper. AK enrolled the patients; collected and analyzed the data and reviewed the source documents. TB contributed in the cytometry and interpreted the obtained results. EG collected blood samples, contributed in the cytometry assay of endothelial cell-derived microparticles and endothelial progenitor cells, performed biomarker assay, and interpreted the obtained results. All authors read the manuscript before submitting and agree with the final version of the paper.

**Conflicts of Interest**

None of the authors has any conflict of interest related to the content of this study. This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

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