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The Effect of Eplerenone on Circulating Microparticle Numbers in Patients with Chronic Heart Failure

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Abstract

The study aim was to evaluate whether the Mineralocorticoid Receptors (MR) antagonist eplerenone, effects circulating microparticles originated from apoptotic and activated endothelial cells (EMP) in Chronic Heart Failure (CHF) patients.

Methods: The study population consisted of 152 consecutive patients with CHF who underwent angiography or PCI between April 2010 and June 2014, and post-myocardial infarction subjects. All subjects enrolled in the study were divided into two cohorts: eplerenone treated (n=45) or placebo (n=107) for 52 weeks. Patients were treated with optimal dose of eplerenone (25-50 mg daily) adjusted to plasma potassium. All biomarkers were determined at the beginning (baseline) and at the termination of the study. Endothelial-derived Microparticles (EMPs) were phenotyped by flow cytometry using a High-Definition Fluorescence Activated Cell Sorter.

Results: At baseline, there was not difference in EMPs between groups. Eplerenone treatment reduced EMPs (CD144+/ CD31+/annexin V+ and CD31+/annexin V+) when compared with placebo. But activated CD62E+ EMP numbers were significantly increased by 24.3%.

Conclusion: Eplerenone reduces circulating EMPs in CHF patients, suggesting reduced endothelial damage. Circulating EMPs could provide a novel marker for organ damage in CHF patients.

Abbreviations: BMI: Body Mass Index; BMP: Brain Natriuretic Peptide; CI: Confidence Interval; CHF: Chronic Heart Failure; EMPs: Endothelial-Derived Microparticles; GFR: Glomerular Filtration Rate; LVEF: Left Ventricular Ejection Fraction; NYHA: New York Heart Association

Keywords: Chronic Heart Failure; Eplerenone; Microparticles

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Introduction

Chronic Heart Failure (CHF) remains a leading cause of cardiovascular morbidity and mortality worldwide [1]. Although systolic and diastolic cardiac dysfunction are risk factors for

poor clinical outcomes [2], patients with systolic CHF die more frequently from progressive heart failure or sudden cardiac death [3, 4]. However, the mode of death in subjects with diastolic CHF is not clearly defined disease [4, 5]. It has been suggested that there are several unique molecular mechanisms that accompany negative effects of risk factors, comorbidities, age- and CHF-related factors on heart failure development which can modulate prognosis, and recently, activation and over expression of the Mineralocorticoid Receptors (MRs) on the myocardium has been identified [6]. MR activation mediates inflammation, fibrosis and tissue remodeling which leads to negative consequences for cardiac function, vascular tone, and clinical outcomes following cardiac events [7, 8], thus chronic blockade of the MRs (using spironolactone or eplerenone) has significant beneficial effects on cardiovascular remodeling, reducing infarct size, oxidative stress, improving clinical outcomes, and long-term-prognosis in CHF patients [9-12].

Endothelial-derived Microparticles (EMPs) are a novel biological marker for endothelial injury and vasomotion disorders, which are associated with CHF [13]. EMPs are defined a heterogeneous population of vesicles (diameter 100-1000 nm) that are released by cellular vesiculation and fission of the membrane [14]. EMPs arise from activated or apoptotic endothelial cells and appear to play a pivotal role in vascular remodeling and reparation [15-17]. As inflammation via activation of MRs could cause endothelial injury, which in turn can stimulate EMP release, it is unknown if blocking MRs can affect EMP pattern. Thus, this study was aimed at evaluating whether the MR antagonist eplerenone, can alter the EMP pattern in CHF patients.

Methods

2.1 Study population

A total of 1427 heart failure patients were screened and 152 met the selection criteria that are given in Table 1. All these subjects (n=152) with documented ischemia induced CHF (128 subjects with old myocardial infarction, 24 patients with asymptomatic atherosclerosis) were enrolled in the study. The study protocol was approved by the Zaporozhye State Medical University Ethnics committee and complied with the Declaration of Helsinki. Voluntary informed written consent was obtained from all patients. Study population characteristic is presented in Table 2. All subjects enrolled in the study were given a contemporary treatment for CHF and then divided into two cohorts: eplerenone treated group (n=45) and control group (n=107) for 52 weeks. Treatment strategy in CHF patients enrolled in the study is given I Table 3.

Table 1: Selection criteria for the study

Inclusion criteria	Exclusion criteria
Men and women	Q-wave and non-Q-wave MI within 3 months before study entry
Age > 18 years old	Malignancy
Documented ischemic induced CHF II-IV NYHA class	Creatinin plasma level above 440 μmol/L and / or estimated GFR < 35 ml/min/м^2
Old MI or documented atherosclerotic lesion>50% at least one coronary artery	Severe kidney and liver diseases
	Body mass index above 30 kg/m^2
	Pulmonary edema
	Valvular heart disease
	Acute stroke or intracranial hemorrhage
	Acute infections
	Implanted pacemaker
	Pregnancy
	Any disorder that may discontinue patient's participation in the study according to investigators

Note: MI – Myocardial Infarction

Table 2: The characteristics of participants

	Entire CHF patient cohort (n=152)	Eplerenone treated group (n=45)	Control group (n=107)	P value for comparison
Age, years	58.34±9.60	57.81±6.99	58.53±7.13	0.88
Male, n (%)	81 (53.2%)	24 (53.3%)	57 (53.2%)	0.98
II NYHA class, n (%)	51 (33.6%)	16 (35.5%)	35 (32.7%)	0.72
III NYHA class, n (%)	53 (34.9%)	15 (33.3%)	38 (35.5%)	0.82
IV NYHA class, n (%)	48 (31.6%)	14 (31.1%)	34(31.8%)	0.98
Hypertension, n (%)	84 (55.2%)	22 (48.9%)	62 (57.9%)	0.48
Dyslipidemia, n (%)	114 (75.0%)	36 (80.0%)	78 (72.9%)	0.62
Type two diabetes mellitus, n (%)	56 (36.8%)	17 (37.7%)	39 (36.4%)	0.88
Obesity, n (%)	72 (47.4%)	24 (53.3%)	48 (44.9%)	0.22
Old myocardial infarction, n (%)	128 (84.2%)	38 (84.4%)	90 (84.1%)	0.96
One coronary artery lesion, n (%)	37 (24.3%)	11 (24.4%)	26 (24.3%)	0.96
Two coronary artery lesions, n (%)	69 (45.4%)	21 (46.6%)	48 (44.9%)	0.82
Three and more coronary artery lesions, n (%)	46 (30.3%)	13 (28.9%)	33 (30.8%)	0.80
Adherence to smoke, n (%)	26 (17.1%)	8 (17.7%)	18 (16.8%)	0.87
BMI, kg/m^2	24.1 (21.6 – 28.7)	23.9 (20.7–25.9)	24.5 (21.4–25.8)	0.68
Systolic BP, mm Hg	131±8	130±5	131±5	0.84
Diastolic BP, mm Hg	78±5	78±6	77±5	0.92
Heart rate, beat per min.	70.52±3.34	73.20±4.6	69.10±6.2	0.48
LVEF, %	43.80±6.76	43.20±5.10	43.20±6.18	0.76
GFR, 1.73 ml/ min/ m^2	82.3 (68.7 – 102.6)	82.0 (71.3–94.7)	81.9 (77.1–102.6)	0.78
Creatinine, µmol/L	72.3 (58.7 – 92.6)	73.1 (60.9–83.5)	71.7 (59.1 – 88.1)	0.86
Fasting glucose, mmol/L	5.20 (3.6-8.7)	5.17(3.8-7.4)	5.22 (3.8-8.1)	0.68
HbA1c, %	6.8 (4.1-9.5)	6.9 (4.3-9.2)	6.7 (4.6-8.5)	0.66
Hemoglobin, g/L	135.4 (128.5 – 140.1)	134.1 (126.2–143.4)	136.1 (125.1 – 144.8)	0.46
Total cholesterol, mmol/L	5.1 (3.9 – 6.1)	5.1 (4.6-6.0)	5.0 (4.5 – 5.9)	0.72
Cholesterol HDL, mmol/L	0.91 (0.89 – 1.12)	0.93 (0.93–1.05)	0.90 (0.86 – 1.01)	0.74
Cholesterol LDL, mmol/L	3.68 (3.11 – 4.40)	3.70 (3.50–4.20)	3.65 (3.11–3.97)	0.66
Uric acid, mmol/L	33.5 (25.3 – 40.1)	34.3 (25.3 – 39.4)	33.1 (22.7 – 37.5)	0.76
Potassium, mmol/L	3.98 (3.45 – 4.30)	4.10 (3.50–4.46)	3.95 (3.46–4.82)	0.90
NT-pro-BNP, pg/mL	1977.2 (984.7 – 2993.2)	1976.5 (1001.3 – 2677.5)	1980.6 (994.1 – 2568.1)	0.92
hs-CRP, mg/L	7.34 (6.77-7.95)	7.35 (6.78-7.90)	7.33 (6.83-7.83)	0.86

Notes: P value was calculated between variables for subjects enrolled in treated and control group; data were presented as mean and standard deviation (±SD), median and 25-75% interquartile range; NYHA – New York Heart Association; GFR – Glomerular Filtration Rate; BMP – Brain Natriuretic Peptide; BP – Blood Pressure; LVEF – Left Ventricular Ejection Fraction; BMI – Body Mass Index, EMPs – Endothelial-Derived Apoptotic Microparticles; HbA1c – Glycated Hemoglobin, HDL - High-Density Lipoprotein; LDL - Low-Density Lipoprotein.

Table 3: Treatment strategy in CHF patients enrolled in the study

	Entire CHF patient cohort (n=152)	Eplerenone treated group (n=45)	Control group (n=107)	P value for comparison
ACE inhibitors or ARBs, n (%)	152 (100%)	45 (100%)	107 (100%)	1.0
Aspirin, n (%)	119 (78.3%)	35 (77.7%)	84 (78.5%)	0.92
Other antiplatelet drugs, n (%)	33 (21.7%)	10 (22.3%)	23 (21.5%)	0.93
Beta-adrenoblockers, n (%)	134 (88.2%)	39 (86.6%)	95 (88.7%)	0.92
Dihydropyridine calcium channel blockers, n (%)	24 (15.8%)	7 (15.5%)	14 (13.1%)	0.88
Ivabradine, n (%)	57 (37.5%)	18 (40.0%)	39 (36.5%)	0.22
Loop diuretics, n (%)	152 (100%)	45 (100%)	107 (100%)	1.0
Statins, n (%)	114 (75.0%)	36 (80.0%)	78 (72.9%)	0.62
Metformin, n (%)	56 (36.8%)	17 (37.7%)	39 (36.4%)	0.88
Sitagliptin, n (%)	19 (12.5%)	5 (11.1%)	14 (13.1%)	0.78

Notes: P value was calculated between variables for subjects enrolled in treated and control group; data were presented as numerous and frequency; ACE – Angiotensin-Converting Enzyme; ARBs – Angiotensin-2 Receptors Blockers.

Eplerenone (25mg) was administered orally and the following parameters were monitored: plasma potassium and creatinine level, eGFR rate and myocardial ECG. Eplerenone (50mg) was administered orally only if potassium level was less than 4.1 mmol/L. Thus, patients were treated with a customized optimal dose of eplerenone (25-50 mg daily), to maintain physiological potassium plasma level.

2.2 Methods for visualization of coronary arteries

Multispiral computed tomography angiography and/or angiography were performed to verify the ischemic nature of the disease in patients when atherosclerotic lesions of the coronary arteries were confirmed, patients were subjected to conventional angiographic examination to provide indications for revascularization. CAD was diagnosed upon availability of previous angiographic examinations carried out not later than 6 months previous, provided no new cardiovascular events occurred for this period. The coronary artery wall structure was measured by contrast-enhanced spiral computed tomography angiography [18] on Somatom Volume Zoom scanner (Siemens, Erlangen, Germany) with two detector rows using non-ionic contrast Omnipaque (Amersham Health, Ireland).

2.3 Echocardiography and tissue Doppler imaging

Transthoracic B-mode echocardiography and tissue Doppler imaging were performed according to a conventional procedure

on ACUSON scanner (SIEMENS, Germany) using phased transducer of 5 MHz. Left ventricular end-diastolic and end-systolic volumes, and ejection fraction (LVEF) were measured by modified Simpson's planimetric method [19, 20]. Inter- and intra-observer variability coefficients for LVEF were 3.2% and 1.1% respectively

2.4 Glomerular filtration rate measurement

Calculation of Glomerular Filtration Rate (GFR) was calculated by CKD-EPI formula [21].

2.5 Biomarker determination

All biomarkers were determined at baseline and at the termination of the study. To measure biological markers, blood samples were collected between 7.00 and 8.00 am) into cooled silicone test tubes and processed according to the recommendations of the manufacturer of the analytical technique used. They were centrifuged upon permanent cooling at 6,000 rpm for 3 minutes, then, plasma was refrigerated immediately and stored at -70^{o}C.

Circulating NT-pro-BNP was measured by immunoelectrochemoluminescent assay using sets produced by R&D Systems (USA) on Elecsys 1010 analyzer (Roche, Mannheim, Germany). High-sensitivity C-reactive protein (hs-CRP) levels were measured by using nephelometric technique on AU640 analyzer (Diagnostic Systems Group, Japan).

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) [22].

Serum samples (100µl) were assayed in parallel to known standard concentrations for each biological marker. The mean intra-assay coefficients of variation were <10% for all cases.

Endothelial-derived microparticles determination

Endothelial-derived microparticles were phenotyped by flow cytofluorimetry by phycoerythrin (PE)-conjugated monoclonal antibody against CD31, CD144, CD62E, 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. The samples were incubated in the dark for 15 min at room temperature according to the manufacturers' instructions. The samples were then analyzed on a FC500 flow cytometer (Beckman Coulter). For determination of annexin V+ EMPs, 400 μL of annexin-V binding buffer was added. For each sample, 500 thousand events were analyzed. EMPs gate was defined by size, using 0.8 and 1.1 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+ as well were identified as EMPs produced due to activation of endothelial cells. Therefore, double-positive EMPs (CD31 and CD144) and triple-positive (CD144+/CD31+/annexin V+) were defined as most specific EMPs [23, 24].

2.6 Statistical Analysis

Statistical analysis of the results obtained was carried out in SPSS system for Windows, Version 22 (SPSS Inc, Chicago, IL, USA) and GraphPad Prism for Windows, Version 5 (GraphPad Software Inc, La Jolla, CA, USA). The data were presented as Mean (M) and standard deviation (±SD) or 95% Confidence Interval (CI); Median (Me) and Interquartile Range (IQR), as well as numerous (n) and frequencies (%) for categorical variables. To compare the main parameters of patients' groups (subject to the type of distribution of the parameters analyzed), two-tailed Student t-test or Mann-Whitney U-test were used. To compare categorical variables between groups, Chi^2 test (χ2) and Fisher F exact test were used. A calculated difference of $P<0.05$ was considered significant.

Results

3.1 Study patient population

The characteristics of the patients enrolled in the study are depicted in Table 2. At baseline, mean age in both sexes was 58.3 years. The prevalence of II (33.6%) and III (34.9%) NYHA class were determined. At least 55.2% of the subjects who were

eligible in the study were hypertensive. There were no significant differences between eplerenone treated group and placebo) regarding the demographic, risk factors, hemodynamic performances, biomarkers mentioned above.

Figure 1 presents numerous of EMPs originated from activated and apoptotic endothelial cells in both patient cohorts before treatment. There was no significant difference between EMP counts between patient groups at baseline.

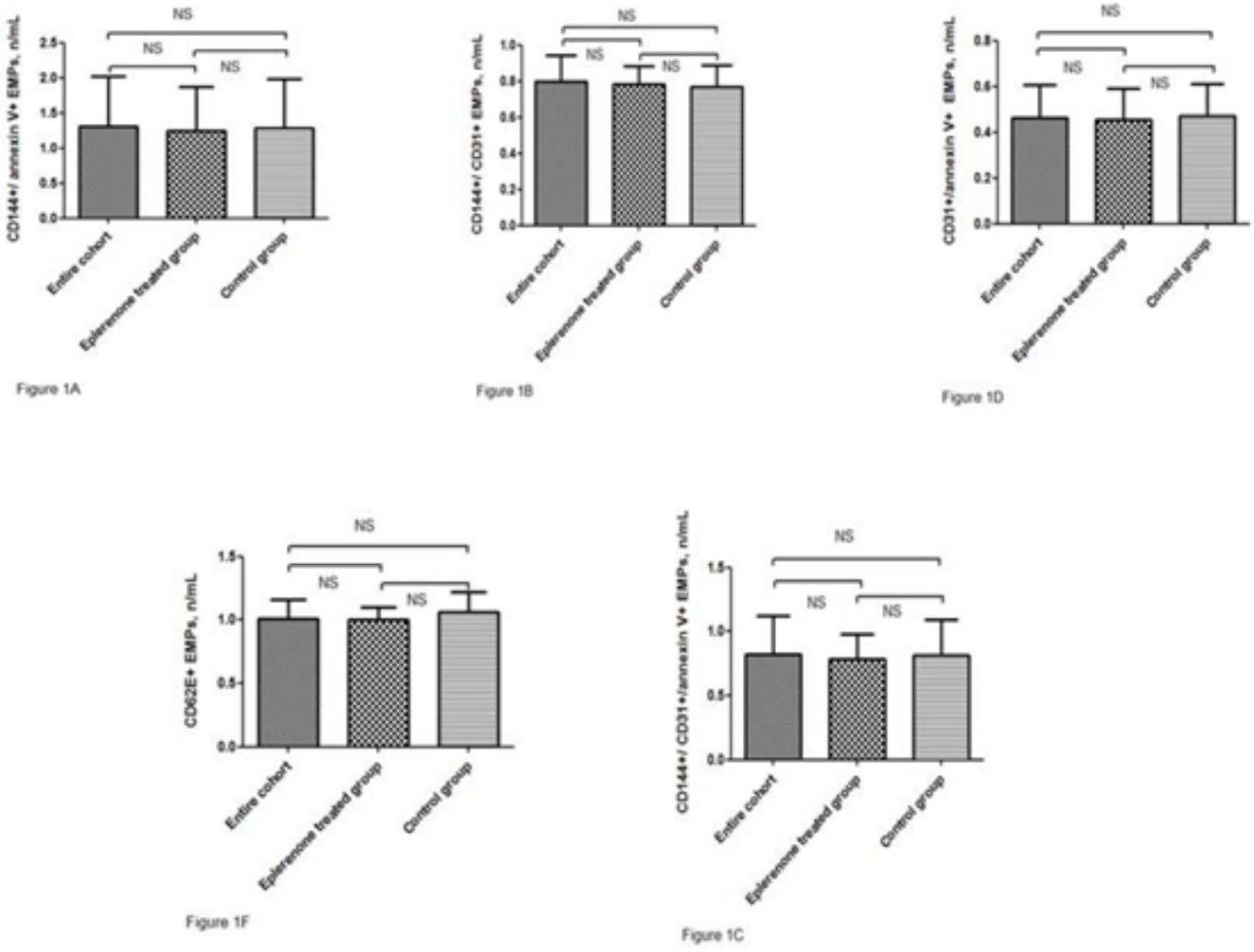

Figure 1: Comparison of the profile of circulating endothelial-derived microparticles in CHF patients at baseline. Values for all figures are mean and SD (standard deviation) and were compared using Student's paired t-test. Figure 1 (A) shows CD144+/ annexin V+ EMP numbers isolated from peripheral blood from CHF patients. (B) Shows circulating CD144 +/ CD31+ EMPs in both cohort patients. (C) Presents CD144+/ CD31+/annexin V+ EMP numbers isolated from circulation from CHF patients. (D) Shows CD31+/annexin V+ EMP numbers isolated from peripheral blood from CHF patients. (F) Shows CD62E+EMP numbers isolated from peripheral blood from CHF patients.

3.2 Concomitant medication in the patient population

The CHF patients enrolled into two groups were treated with ACE inhibitors or ARBs, beta-adrenoblockers, loop diuretics, current (I) blocker ivabradine, and antiplatelet drugs (Table 3). Dihydropyridine (calcium channel blocker) was used when it was needed for hypertension treatment. Metformin and / or

sitagliptin were used in type two diabetes patients as a component of contemporary treatment for CHF. No significant differences in medications between eplerenone treated group and placebo were found.

3.3 Microparticles in CHF patients

Total CD144+/annexin V+ phenotyped Endothelial-derived Microparticles (EMPs) were not changed between groups of the patients enrolled in the study (Table 4). However, CD144+ / CD31+ EMPs were significantly reduced by 19% ($p<0.05$) in patients treated with eplerenone compared to control group. The comparison of EMP numerous before and after treatments in both group patient was presented in Figure 2. In both groups declined CD144+/CD31+/annexin V+ EMPs (by 13.4% and 6.3% respectively) and CD31+/annexin V+ EMPs (by 7.2% and 3.2% respectively) were found ($p<0.05$). In contrast, activated CD62E+ EMP numbers were significantly increased in the eplerenone treated group by 24.3% ($p<0.05$) that was associated with increased CD144+/CD31+/annexin V+ EMPs ratio by 35.5% in eplerenone group($p<0.05$) versus by 8.57% in control group ($p=0.46$).

Table 4: The changes in total numbers of EMPs originated from apoptotic and activated endothelial cells in CHF patients

Phenotype of EMPs	Treatment groups	At baseline	At the study end	Δ%	P value for comparison
CD144+/annexin V+ EMPs, n /mL	Eplerenone treated group	1.16 (95% CI=0.19-2.37)	1.10 (95% CI=0.25-2.11)	-5.2	0.44
	Control group	1.14 (95% CI=0.14-2.56)	1.15 (95% CI=0.10-2.62)	-0.8	0.68
CD144+/CD31+ EMPs, n /mL	Eplerenone treated group	0.79 (95% CI=0.61-0.95)	0.64 (95% CI=0.47-0.89)	-19.0	<0.05
	Control group	0.84 (95% CI=0.53-0.93)	0.82 (95% CI=0.50-0.95)	-2.3	0.72
CD144+/CD31+/annexin V+ EMPs, n /mL	Eplerenone treated group	0.82 (95% CI=0.44-1.10)	0.71 (95% CI=0.30-0.96)	-13.4	<0.05
	Control group	0.85 (95% CI=0.32-1.28)	0.80 (95% CI=0.33-1.08)	-6.3	<0.05
CD31+/annexin V+ EMPs, n /mL	Eplerenone treated group	0.456 (95% CI=0.221-0.689)	0.423 (95% CI=0.188-0.615)	-7.2	<0.05
	Control group	0.501 (95% CI=0.213-0.695)	0.485 (95% CI=0.203-0.637)	-3.2	<0.05
CD62E+ EMPs, n /mL	Eplerenone treated group	0.998 (95% CI=0.845-1.170)	1.318 (95% CI=0.885-1.680)	+24.3	<0.05
	Control group	1.104 (95% CI=0.776-1.315)	1.12 (95% CI=0.79-1.332)	+1.43	0.24
CD62E+ EMPs to CD144+/CD31+/annexin V+ EMPs ratio	Eplerenone treated group	1.20 (95% CI=1.07-1.85)	1.86 (95% CI=1.12-2.93)	+35.5	<0.05
	Control group	1.28 (95% CI=0.83-2.15)	1.40 (95% CI=1.18-2.23)	+8.57	0.46

Notes: P value was calculated between variables for subjects at baseline and at the study end; data were presented as median and 95 Confidence Interval (CI); EMPs – Endothelial-Derived Apoptotic Microparticles.

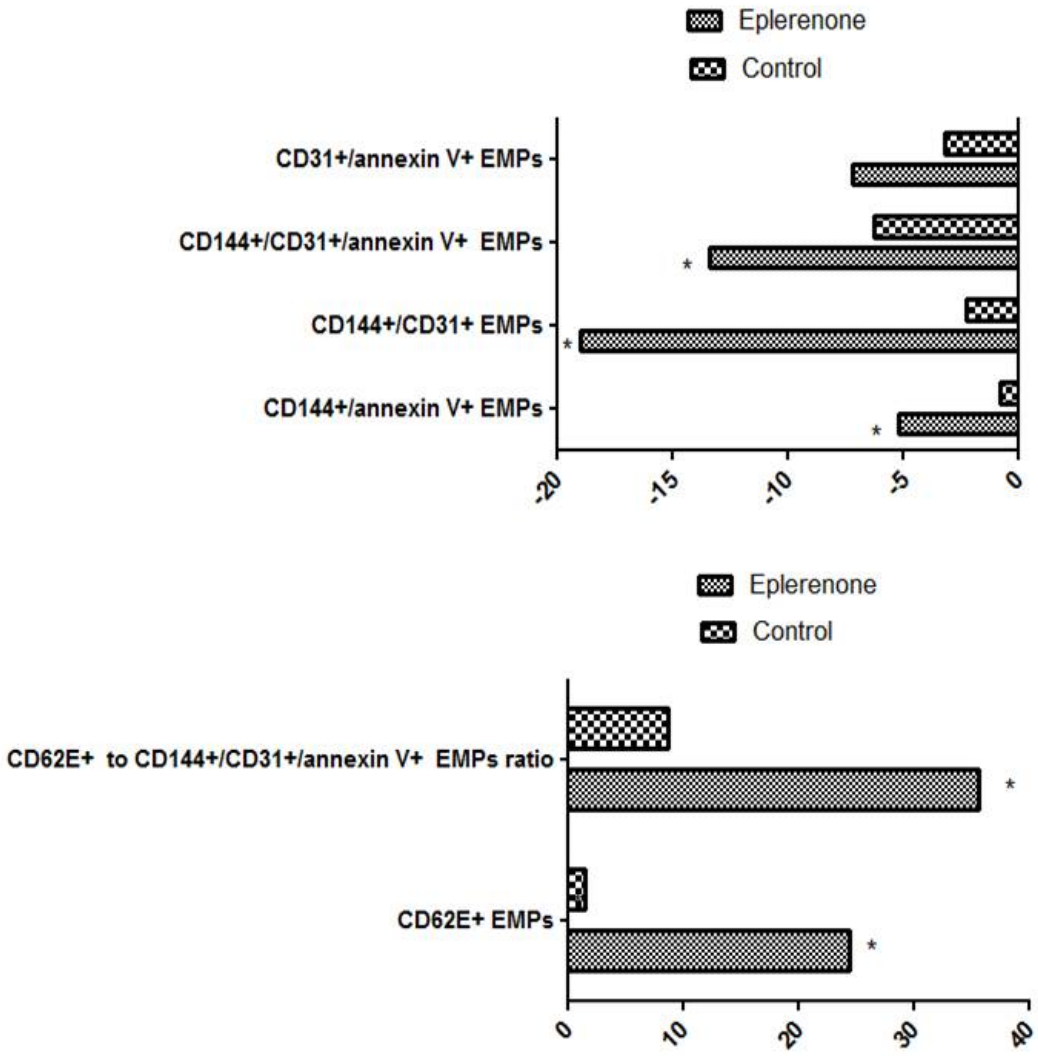

Figure 2: Comparison of the profile of circulating endothelial-derived microparticles in CHF patients. Values reported as percent of changes between baseline and end of treatment in both group of the patients for each EMP pattern.

Discussion

This study clearly demonstrates that circulating EMPs from CHF patients are modulated by eplerenone. The numbers of apoptotic EMPs in CHF patients were decreased by eplerenone treatment yet a subgroup of EMPs which also co-express CD62E (a marker for activated endothelial cells) were increased. This effect of eplerenone led to increased CD62E+ EMPs to CD144+/CD31+/annexin V+ EMPs ratio mainly due to the increase of the prevalence of activated EMPs and it was generally regarded as a positive. Recent investigations showed that various stimuli, such as shear stress, inflammation, and vascular damage may raise circulating EMPs via secretion from activated endothelial cells and via induction of endothelial cell apoptosis [25-27], but the apoptotic profile of EMPs (CD144+/CD31+/annexin V+ EMPs and CD31+/annexin V+ EMPs) predominate [24]. These EMPs can induce specific cellular mechanisms that leads to activation of membrane-associated receptors on the surface of wide spectrum of target cells, including endothelial cells, monocytes, etc. [28]. Apoptotic-derived EMPs contribute to vascular damage through stimulation of tissue factor-dependent procoagulant activity, increased free-radical production and inflammation, leukocyte adhesion and altered vasomotion [29, 30]. However, EMPs

which also co-express activation markers can protect tissues from further damage [31]. Indeed, recently clinical studies have reported that increased apoptotic EMPs appear to have anegative prognosis for CHF patient population [32, 33], indicating that EMP profile could be considered a useful marker for determining poor prognosis in CHF patients [34, 35]. Because activation-derived EMPs play a pivotal role in tissue repair, transcellular exchange of information and neoangiogenesis [36], the results of this study support the hypothesis about the pivotal role of chronic MR blockade in improving vascular remodeling among CHF patients. Taken together, the data presented here proposes that the overall role of secreted EMPs may relate to the balance between circulating activated-derived and apoptotic-derived microparticles.

Although apoptotic phenotype of EMPs are not specific for CHF only and may determine in other cardiovascular and metabolic diseases [37], the results presented in this study suggests that elevated apoptotic EMPs can influence endothelial cell phenotype modification that worsens endothelial function leading to poor clinical outcomes. Importantly, a positive feedback loop might be driving the endothelial dysfunction, in that endothelial dysfunction could increase apoptotic phenotype of EMPs leading to further disfunction [36-39].

It is well established that reducing the detrimental effects of mineralcorticoids using antagonists in CHF patients via attenuating inflammation and myocardial fibrosis is associated with improved outcomes, the ability of the drug to alter EMPs is novel and the mechanisms that lead to this effect requires further investigations. Our data support the hypothesis that eplerenone could reduce target-organ damage in CHF patients through modulating EMPs It is interesting to speculate that eplerenone could reduce inflammatory mediators, such as inducible nitric oxide synthase derived nitric oxide and NADPH oxidase activity [40, 41].

In conclusion, we show that the MR antagonist eplerenone can modulate EMP population in CHF patients, leading to an overall reduction in circulating microparticles but an increase in activated endothelial microparticle ratio. Whether or not this effect is causal remains to be validated.

Acknowledgment

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Ethical principles: All 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 conformed with the Declaration of Helsinki.

Study limitations

A large pool of nanoparticles might be produced after blood sampling due to destruction of platelets and blood cells. Therefore, preparation of isolates of microparticles in samples is the step for further examination. Venous citrated blood drawn from the fistula-free arm was always performed. Blood samples were never frozen before measurement of microparticles. Although HD-FACS methodology is widely used, theoretically overlap between two or more fluorochromes might complicate further interpretation of results. The relative small sample size may limit the significance of the present study, thus a larger randomized study is necessary to establish the role of EMPs in CHF.

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