Circulating Endothelial Progenitor Cells in Patients with Heart Failure with Preserved versus Reduced Ejection Fraction

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ABSTRACT **Background:** Heart failure with preserved ejection fraction (HFpEF) is a common clinical entity, with a mechanism that appears to involve endothelial dysfunction of the cardiac microcirculation. Endothelial progenitor cells (EPC) are bone marrow derived cells that are able to differentiate into functional endothelial cells and participate in endothelial surface repair.

Objectives: To compare the level and function of EPCs in patients with HFpEF compared with heart failure with reduced ejection fraction (HFrEF) and control subjects.

Methods: We enrolled 21 patients with HFpEF (LVEF ≥ 50%, age 74.5 ± 9.9 years, 43% men, 48% diabetes), 20 patients with HFrEF (LVEF < 40%, age 70 ± 11.5 years, 90% men, 60% diabetes), and 11 control subjects with cardiovascular risk factors (age 53.3 ± 6.1years, 90% men, 64% diabetes). Circulating EPC levels were evaluated by expression of vascular endothelial growth factor receptor-2 (VEGFR-2), CD34, and CD133 by flow-cytometry. EPCs colony forming units (CFUs) were quantified after 7 days in culture.

Results: The proportion of cells that co-expressed VEGFR-2 and CD34 or VEGFR-2 and CD133 was similar among the HFpEF and HFrEF groups, and significantly lower than in the control group. The number of EPC-CFUs was also similar among the two heart failure groups and significantly lower than the control group.

Conclusions: Patients with HFpEF, like HFrEF, have significant reduction in EPC level and function.

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Heart failure with preserved ejection fraction (HFpEF) is a common clinical entity causing significant morbidity, mortality, and frequent hospitalizations. The entity is defined by the European Society of Cardiology by the following criteria: signs and symptoms of heart failure in the presence of normal left ventricular ejection fraction (≥ 50%) and evidence of diastolic dysfunction or relevant structural heart disease [1]. To date HFpEF remains a condition for which no treatment has been shown to reduce morbidity and mortality [2]. The mechanism of HFpEF is multifactorial, involving several pathways. Endothelial dysfunction of the cardiac microcirculation, mediated by a pro-inflammatory state [2,3], appears to be a pivotal part in HFpEF pathogenesis.

Endothelial progenitor cells (EPCs) are bone marrow-derived circulating cells able to proliferate and differentiate into functional mature endothelial cells [4]. EPCs are mobilized into the circulation in response to tissue or vessel injury and incorporate into the sites of injury. They participate in the process of vascular repair, endothelial surface repair, and neovascularization [5-7]. Impairment of EPCs is related to endothelial dysfunction [8,9]. In patients with cardiovascular diseases or with cardiovascular risk factors, there is reduction in the level of circulating EPCs [8,10,11], which in turn is associated with an increased risk for the development of future cardiovascular events and mortality [11]. Circulating EPCs can be evaluated by measuring the expression of CD133, CD34, and vascular endothelial growth factor receptor-2 (VEGFR-2) antigens on their surface [5,12,13].

The characteristics of EPCs have been thoroughly studied in patients with heart failure with reduced ejection fraction (HFrEF). In patients with severe HFrEF, the level of EPCs, as well as their function, is reduced, suggesting impaired EPC recruitment in this setting [14]. Moreover, the level of circulating EPCs is an independent predictor of survival over time among patients with HFrEF [15]. The mobilization of EPCs in patients with chronic heart failure can be enhanced by statin treatment [16] as well as exercise programs [17]. Despite the abundant data on EPCs in the setting of HFrEF, there is limited information about EPCs in patients with HFpEF.

Given the potential role of the microvascular endothelium in the pathogenesis of HFpEF, we hypothesized that the level and function of EPCs might be attenuated in this condition as well. Accordingly, in the current study we compared the level and function of EPCs in patients with HFpEF, those with HFrEF, and control subjects.

Table 1. Clinical characteristics

	Controls (n=11)	HFpEF (n=21)	HFrEF (n=20)	*P* value
Age, years	53.3 ± 6.1	74.5 ± 9.9	70.0 ± 11.5	0.0001
Male	10 (91%)	9 (43%)	18 (90%)	0.0005
Current smoker	2 (18%)	1 (5%)	6 (30%)	0.1
Type 2 diabetes mellitus	7 (64%)	10 (48%)	12 (60%)	0.5
Hypertension	5 (45%)	17 (81%)	13 (65%)	0.2
Ischemic heart disease	0	6 (29%)	16 (80%)	0.0001
Atrial fibrillation	0	12 (57%)	4 (20%)	0.0001
Hyperlipidemia	6 (55%)	12 (57%)	16 (80%)	0.3
NYHA FC 1-2	0	17 (81%)	15 (75%)	0.0001
NYHA FC 3-4	0	4 (19%)	5 (25%)	0.0001
International Classification of Diseases	0	0	15 (75%)	0.0001
LDL-C, mg/dl	84.6 ± 54.1	62.0 ± 22.2	68.4 ± 24.3	0.22
Creatinine, mg/dl	0.9 ± 0.1	1.0 ± 0.32	1.2 ± 0.4	0.15
GFR 30-59	8 (38%)	0	5 (25%)	0.06
GFR 15-29	0	0	1 (5%)	0.5
BMI, kg/m2	29.1 ± 2.8	32.3 ± 5.4	28 ± 4.1	0.1
COPD	0	2 (10%)	1 (5%)	0.5
ACEI/ARB	4 (36%)	17 (81%)	14 (70%)	0.1
Loop diuretics	0	5 (24%)	9 (45%)	0.03
MRIs	1 (9%)	5 (24%)	9 (45%)	0.05
Beta blockers	2 (18%)	14 (67%)	17 (85%)	0.001
CCB	2 (18%)	7 (33%)	0	0.02
Statins	5 (45%)	15 (71%)	17 (85%)	0.1
Insulin	2 (18%)	0	1 (5%)	0.1
SGLT2	1 (9%)	3 (14%)	3 (15%)	0.9
NOAC	1 (9%)	11 (52%)	4 (20%)	0.02
Aspirin	4 (36%)	4 (19%)	12 (60%)	0.03
LVEF	60 ± 3%	58 ± 4%	29 ± 10%	0.0001
DD-grade I	0	1 (4.8%)	3 (33%)	0.3
DD-grade II	0	14 (67%)	3 (33%)	0.0001
DD-grade III	0	2 (9.5%)	3 (33%)	0.4
LA-diam.	34.8 ± 14.9	43.3 ± 5.0	45.2 ± 4.7	0.0005
Pulmonary blood pressure, mmHg	28.0 ± 12.1	45.5 ± 20.8	47.6 ± 21.0	0.3

ACEI/ARB = angiotensin-converting enzyme inhibitors / angiotensin receptor blockers, BMI = body mass index, CCB = calcium channel blockers, COPD = chronic obstructive pulmonary disease, GFR = glomerular filtration rate, HFpEF = heart failure with preserved ejection fraction, HFrEF = heart failure with reduced ejection fraction, LDL-C = low-density lipoprotein cholesterol, MRI = magnetic resonance imaging, NOAC = novel oral anticoagulants, NYHA = New York Heart Association, SGLT2 = sodium-glucose cotransporter-2

PATIENTS AND METHODS

STUDY POPULATION

The study included three groups of patients: 21 patients with HFpEF (with an LVEF ≥ 50%), 20 patients with HFrEF (with an LVEF < 40%), and 11 control subjects with cardiovascular risk factors. All were enrolled between February 2019 and February 2020. Patients with HFpEF and HFrEF were recruited from the cardiology department at the Assuta Ashdod Medical Center, Israel. Control subjects were recruited from the cardiology preventive clinic at Assuta Ashdod Medical Center, according to the following criteria: age > 18 years, matching to the heart failure groups according to diabetes mellitus status and statin therapy. Patients were excluded from the study if they had an acute coronary syndrome in the previous 3 months, had renal insufficiency (estimated glomerular filtration rate < 30 ml/min/1.73 m^2 according to the MDRD formula), anemia (Hg < 10 gm/dl), immunological disorders on steroids treatment or hepatic dysfunction (alanine aminotransferase / aspartate aminotransferase ≥ 2.5 times the upper limit of normal).

HFpEF was defined according to the ESC criteria [1] as LVEF > 50%, elevated levels of natriuretic peptides and at least one of the following: relevant structural heart disease (LVH and/or left atrial enlargement) or diastolic dysfunction. HFrEF was defined as heart failure with an LVEF < 40%.

All patients had one venous blood sample drawn in EDTA tubes for EPC testing. Blood samples were processed within one hour of blood collection.

The study was approved by the investigational review board (ethics committee) of the Assuta Ashdod Medical Center, Israel, and all participants provided written informed consent.

CIRCULATING EPCS

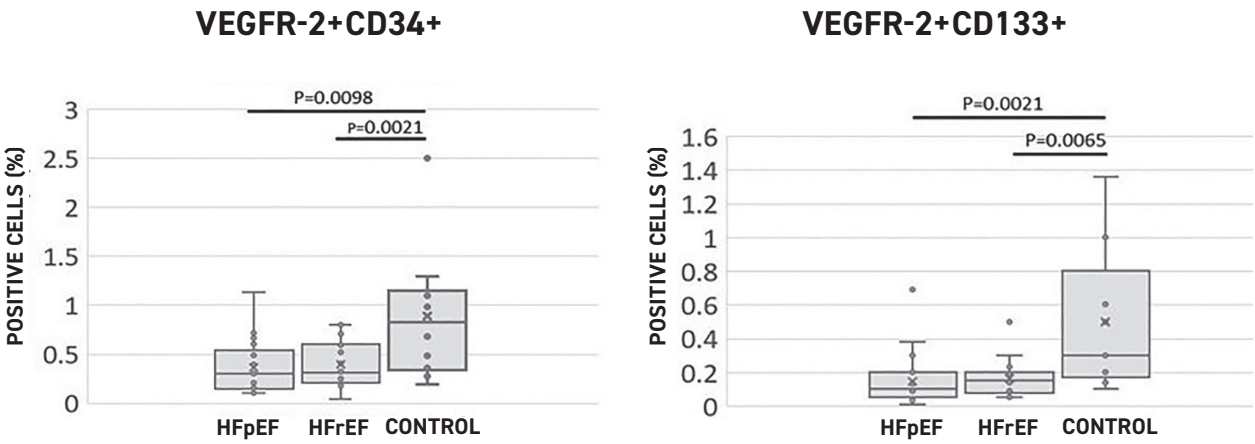
ISOLATION OF MONONUCLEAR CELLS

Peripheral mononuclear cells were fractionated using Ficoll density-gradient centrifugation and washed with phosphate buffered saline after red cell lysis.

FLOW CYTOMETRY ANALYSIS OF CIRCULATING ENDOTHELIAL PROGENITOR CELLS LEVEL

Circulating EPC levels were quantified by measurement of the surface markers VEGFR-2, CD34 and CD133 by flow cytometry. Aliquots of peripheral blood mononuclear cells (PBMCs) were incubated with monoclonal antibodies against VEGFR-2 (FITC labeled, R&D, Minneapolis, MN, USA) and either CD133 (APC-labeled, Miltenyi Biotech, Auburn, USA) or CD34 (PE-labeled, Miltenyi Biotech). Isotype-identical antibodies were used as controls. After incubation, cells were washed with phosphate-buffered saline and analyzed by flow cytometry (BD FACSCalibur™, Becton Dickinson, USA). Each analysis included 100,000 events, after exclusion of debris. Gated CD34- or CD133-positive cells were examined for the expression of VEGFR-2. Analyses were

Figure 1. Proportion of peripheral blood mononuclear cell (PBMC) that co-express vascular endothelial growth factor receptor-2 (VEGFR-2) and CD34. PBMCs were isolated from patients with heart failure with preserved ejection fraction (HFpEF) (n=21), patients with heart failure with reduced ejection fraction (HFrEF) (n=20), and healthy controls (n=11). Results are expressed as median (25th–75th percentile). 1×106 peripheral mononuclear cells (PMNCs) were incubated with monoclonal antibodies against VEGFR-2, FITC labeled and CD34, PE labeled (top panel) or CD133, APC labeled (lower panel) and analyzed with flow cytometer FACSARIE (BD). Isotype-identical antibodies and fluorescence minus one (FMO) were used as controls



performed in duplicates. Results are presented as the percentage of peripheral mononuclear cells (PMNCs) co-expressing either VEGFR-2 and CD133 or as VEGFR-2 and CD34.

COLONY FORMING UNIT QUANTIFICATION

Isolated PBMCs were re-suspended in M199 medium supplemented with 10% FCS and plated on 6-well plate coated with fibronectin at a concentration of 4 x 106 cells per well. EPCs colonies were counted using a microscope 7 days after plating. An EPC colony is defined as at least 100 flat cells surrounding cluster of rounded cells. EPC colony forming units (CFU) quantification is a functional assay that reflects the ability of circulating EPCs to proliferate and perform cellular interactions [8].

STATISTICAL ANALYSIS

Results are presented as median (25th–75th percentile) and mean ± SD, as indicated. Comparisons of EPC parameters (flow cytometry determined levels and number of CFUs) between the groups were performed by Wilcoxon–Mann–Whitney test, as these parameters are non-normally distributed. Comparisons of other variables were performed by unpaired Student's *t*-tests (two-tailed) or ANOVA for continuous variables, and chi-square tests for categorical variables. $P < 0.05$ was considered statistically significant. Statistical analyses were performed using Statistical Package for the Social Sciences software version 15 (SPSS Inc., Chicago, IL, USA).

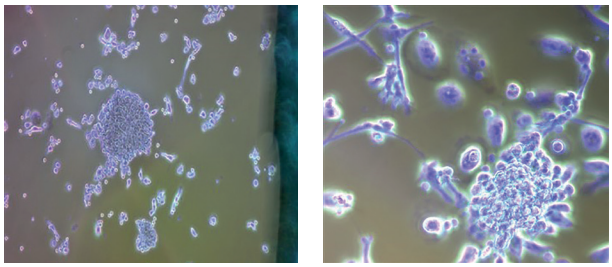
RESULTS

Twenty-one patients with HFpEF, 20 patients with HFrEF, and 11 matched control subjects were recruited for the study. Table 1 presents clinical and echocardiographic characteristics among the 3 groups. The mean age of the HFpEF, HFrEF, and the control groups was 74.5 ± 9.9, 70.0 ± 11.5, and 53.3 ± 6.1, respectively. Men were the majority of patients in the HFrEF and control groups (90, 91%), but not in the HFpEF group (43%).The proportion of patients with diabetes mellitus was similar among the groups (HFpEF 48%, HFrEF 60%, and control 64%). As expected LVEF significantly differed between the groups (HFpEF 58 ± 4%, HFrEF 29 ± 10%, and control 60 ± 3%), and left atrial diameter and pulmonary artery pressure were higher among the HF groups than the control subjects [Table 1].

The proportion of circulating PMNCs that co-expressed VEGFR-2 and CD34 or VEGFR-2 and CD133 is presented in Figure 1. Both patients with HFpEF and HFrEF had a significantly lower proportion of cells that were VEGFR-2+ and CD34+ or VEGFR-2+ and CD133+ then the control subjects, reflecting a lower level of circulating EPCs. The median proportion of cells expressing VEGFR-2 and CD34 in control subjects was 0.83% (0.19–2.5%), and in patients with HFpEF and HFrEF the median proportion was 0.30% (0.1–1.13%), and 0.31% (0.04–0.8%), respectively, $P < 0.01$ for both comparisons (Figure 1). The median proportion of cells expressing VEGFR-2 and CD133 in control subjects was 0.3% (0.–1.36%), and in patients with HFpEF and HFrEF the median proportion was 0.1% (0.01–0.069%) and 0.15% (0.05–0.5%), respectively, $P \leq 0.01$ for both comparisons [Figure 1].

The median number of CFU was compared among the 3 groups and presented in Figure 2 and Figure 3. The median CFU number obtained in patients with HFpEF: 9.3 (0.5–71) colonies per 106 cells and HFrEF: 12.3 (1.2–53) colonies per 106 cells was significantly lower than the median CFU num-

Figure 2. Representative endothelial progenitor cells (EPCs) colonies (magnification ×20)



ber obtained in the control subjects: 35.9 (11–89) colonies per 106 cells] ($P = 0.001$ and 0.004, respectively).

DISCUSSION

The present study demonstrates an attenuated profile of EPCs in patients with HFpEF, which was similar to that of patients with HFrEF. Patients with both types of heart failure had both markedly low levels of circulating EPCs and reduced EPC function, compared with the control subjects. Notably, the attenuated EPCs profile in both groups of heart failure patients was observed despite a relatively stable stage of their disease and treatment with guideline-based medical therapy.

Heart failure with preserved ejection fraction is a common condition and, currently, gains a significant share of the entire heart failure population. HFpEF, as HFrEF, is a multifactorial disease and is a common pathway for many cardiac disorders. However, these manifestations of heart failure do not share the same response to medical regimes despite similarities in their clinical presentation [18].

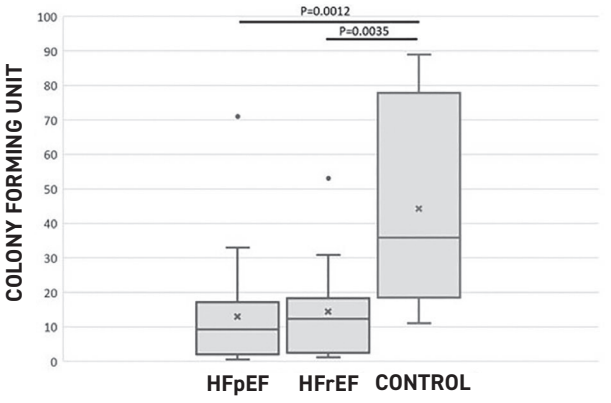
Coronary microvascular dysfunction mediated by inflammation is regarded as a pivotal part in the pathogenesis of HFpEF, and it is the basis of left ventricular remodeling, dysfunction, and eventually, clinical heart failure [3]. However, not only local but also systemic endothelial dysfunction contributes to the evolution of HFpEF [19]. The endothelial dysfunction is associated with a reduction of nitric oxide signaling in the blood vessels and the cardiomyocytes. It results in a lower cGMP concentration, increased calcium sensitivity, and subsequent impaired relaxation [20]. Endothelial dysfunction is also manifested in HFpEF by impaired flow-mediated vasodilation of the extra-cardiac vasculature, which contributes to the dyspnea and fatigue that characterizes this clinical condition [21].

EPCs dysfunction possesses a key role in the failure of the vascular endothelium to overcome the various insults associated with the co-morbidities that lead to heart failure [22]. It is still debatable whether the main cause of EPCs dysfunction is the direct effect of cardiovascular risk factors on EPCs mobilization and half-life. An alternative explanation may be an extensive and continuous endothelial damage that exhausts the EPCs reserve. This study does not provide an answer, although the attenuation in EPCs level and function despite a stable clinical status under guideline based medical therapy including statin therapy, may support the latter theory in our heart failure cohort.

The presence of an abnormal EPCs profile in HFpEF patients has been previously reported in two relatively small studies. Chiang et al. [23] reported a decrease in circulating EPCs in a cohort of both HFpEF and HFrEF patients. The study included symptomatic HFpEF patients with various cardiovascular risk factors, each known as a EPCs modifier. The control group included asymptomatic patients with LVEF > 50% that were matched with age, gender, and some of the cardiovascular risk factors. This study evaluated only the amount of circulating EPCs and not their function. In addition, the study was underpowered to confirm the role of EPCs in the pathogenesis of HFpEF, and may have reflected the effect of the functional class and\or the severity of the background cardiovascular risk factors. Gevaert et al. [24] examined 26 HFpEF patients compared to a healthy cardiovascular risk factor-free control group. They showed a concomitant decrease in both circulating EPC count as well as in the number of circulating angiogenic T cells, which have a role in the normal proliferation and maturation of EPCs. They also identified abnormal endothelial function through reduced reactive hyperemia index in the HFpEF patients at rest.

The current study showed a similar EPCs' profile in both HFpEF and HFrEF. Both groups consisted of patients at a similar age, with similar rates of diabetes, hypertension and statin treatment. The patients in the two heart failure groups also had similar left atrial size and pulmonary artery pressure. However these two groups differed in LVEF and the etiology of heart failure, mainly in rates of ischemic heart disease. Thus, the similar EPC profile in the two groups is quite intriguing. The differences include dif-

Figure 3: Quantification of endothelial progenitor cells (EPCs) colony forming units. peripheral mononuclear cells (PMNCs) were isolated from patients with heart failure with preserved ejection fraction (HFpEF) (n=21), heart failure with reduced ejection fraction (HFrEF) (n=20), and from matched healthy controls (n=11).Results expressed as median (25th–75th percentile) of CFU per 106 cells plated. EPC colonies were counted using light microscope



ferent etiologies of the heart failure, especially rates of ischemic heart disease. This observation may reflect the persistence of EPCs abnormality in heart failure patients regardless of left ventricular systolic function and despite the effect of guideline-based medical therapy for heart failure. An alternative explanation for this observation can be that the abnormal EPC profile is an early marker of decompensation that precedes the symptoms. The interpretation of the abnormal EPCs profile in heart failure patients and its impact on their medical management merits further study.

LIMITATIONS

This single-center study included patients under the surveillance of a tertiary medical center cardiology clinic. Hence, the study was prone to selection bias. The main limitation of the study is the difference in clinical characteristics, mainly age and gender, between the two heart failure groups and the control group. The difference in age (lower average age in the control subjects) may have potentially contributed to the differences in EPC levels between the heart failure groups and the control group. Other factors and co-morbidities which are potentially associated with EPC function, such as the presence of atrial fibrillation, and the proportion of patients treated with mineralocorticoid receptor blockers, known to affect EPC recruitment [25], differed between the groups as well. However, it should be emphasized that the main factors, known to influence and modify EPC function, such as diabetes, hypertension, renal function and especially statin treatment were well-matched among the three groups. An additional limitation is that the etiologies of both HFpEF and HFrEF varied. In particular, the proportion of patients with ischemic heart disease was higher in the HFrEF group. Nevertheless, despite the differences in co-morbidities and medical treatment between the two heart failure groups, the findings very consistently demonstrate reduced EPC levels and function among both heart failure groups compared with the control subjects. Finally, the study did not include data regarding the adherence of the patients to their medical therapy, or long-term clinical follow-up.

CONCLUSIONS

Both HFpEF and HFrEF are clinical conditions associated with significant attenuation in EPC level and function. Further research is required to assess whether monitoring or possibly modifying the EPC properties in these patients may have clinical benefits.

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