

Circulating endothelial progenitor cells and clinical outcome in patients with congestive heart failure

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Background: Circulating endothelial progenitor cells (EPCs) are increased in conditions associated with ischaemia and can potentially support angiogenesis and vasculogenesis. EPC levels were also shown to predict outcome in patients with atherosclerotic vascular disease. We tested the hypothesis that circulating EPC can predict outcome in patients with congestive heart failure (CHF).

Methods: EPC-colony-forming units were measured in the peripheral blood of 107 consecutive patients with CHF with New York Heart Association (NYHA) functional class II–IV. Serum levels of vascular endothelial growth factor (VEGF), N-terminal pro-B-type natriuretic peptide (NT-pro-BNP) and high-sensitivity C-reactive protein (hsCRP) were also measured. End points were defined as CHF-related hospital admissions and all-cause mortality.

Results: Age ($p=0.01$), diabetes mellitus ($p=0.002$) and EPC levels ($p=0.02$) were found to be independent predictors of all-cause mortality. EPC levels did not predict CHF-related hospitalisations. EPC levels correlated positively with NYHA ($p=0.05$, $r=0.19$), but did not correlate with VEGF, NT-pro-BNP or hsCRP. EPC levels did not differ by the aetiology of CHF.

Conclusions: EPC levels are independent predictors of all-cause mortality among patients with CHF.

Recent population-based studies on patients with heart failure (HF)^{1,2} report an improved survival and a trend towards a plateau or a decline in hospital admissions. These encouraging reports are attributed to improved understanding of disease mechanisms, and to the development of new pharmacological and mechanical treatment modalities. However, despite improved outcome, HF continues to be a prevalent disease with substantial morbidity and mortality.

In an attempt to improve patient care, clinicians try to define clinical and/or laboratory markers of an adverse outcome. Clinical factors include high New York Heart Association (NYHA) functional class, low left ventricular ejection fraction (LVEF), advanced age, cachexia and related comorbidities including renal disease, anaemia and diabetes mellitus.^{1,3,4} Laboratory markers include mediators of the neurohormonal and inflammatory axes that are activated in these patients. In this respect, B-type natriuretic peptide (BNP),⁵ and to a lesser extent C-reactive protein,⁶ were shown to predict clinical outcome in patients with congestive heart failure (CHF). None of these clinical or laboratory markers is either sensitive or specific enough, and development of additional predictors is needed.

Endothelial progenitor cells (EPCs)⁷ reside in the bone marrow and are found to some extent in the peripheral blood. EPCs participate in vasculogenesis through their capacity to proliferate, migrate and differentiate, and serve as paracrine factories for proangiogenic cytokines.⁷ Conditions that induce tissue ischaemia or endothelial damage—that is, vascular trauma,⁸ acute myocardial infarction⁹ and unstable angina¹⁰—are associated with a release of angiogenic factors, including vascular endothelial growth factor (VEGF) and erythropoietin, which promote mobilisation of EPC from the bone marrow to the peripheral circulation. A decrease in EPC levels was shown to correlate inversely with atherosclerotic risk factors¹¹ and the occurrence of ischaemic stroke,¹² and to correlate with endothelial dysfunction,^{11,13} accelerated atherosclerosis¹⁴ and in-stent restenosis.¹⁵

CHF is characterised by tissue ischaemia and endothelial dysfunction.³ Indeed, it has recently been demonstrated that patients with CHF have elevated circulating EPC levels¹⁶ compared with healthy controls. However, whether this observation has prognostic clinical implications has not yet been resolved.

In this study, we have made a quantitative assessment of circulating EPC in patients with CHF and studied their predictive value on clinical outcome including mortality and CHF-related hospital admissions.

METHODS

Patients

The study included 107 consecutive patients with CHF attending the outpatient clinic of the Tel Aviv Sourasky Medical Center, Tel Aviv, Israel, between September 2003 and March 2004. This is a referral clinic: all patients attending which have had symptomatic HF and had undergone an echocardiographic examination. Patients with chronic HF in NYHA II–IV were recruited. The clinical diagnosis of HF was based on the history of acute pulmonary oedema or two of the following signs or symptoms not explained by other identifiable causes and improved with diuresis: exertional dyspnoea or fatigue, paroxysmal nocturnal dyspnoea, orthopnoea, pleural effusion or bilateral lower extremity oedema. Patients with LVEF >45% and echocardiographic signs of diastolic dysfunction were diagnosed as having HF with preserved systolic function. LVEF was measured echocardiographically. Echocardiographic criteria for diastolic dysfunction were mainly based on mitral valve inflow pattern as described elsewhere¹⁷ and in some patients in conjunction with pulmonary vein flow pattern and mitral annular Doppler tissue

Abbreviations: BNP, B-type natriuretic peptide; CFU, colony-forming unit; CHF, congestive heart failure; EPC, endothelial progenitor cell; HF, heart failure; hsCRP, high-sensitivity C-reactive protein; LVEF, left ventricular ejection fraction; NT-pro-BNP, N-terminal pro-B-type natriuretic peptide; NYHA, New York Heart Association; VEGF, vascular endothelial growth factor

imaging. Patients were examined, and they answered a thorough questionnaire on medical history, performance status, atherosclerotic risk factors and medications. Patients were followed every 3 months or at higher frequency as dictated by the patients' clinical status.

Follow-up

The study end points were all-cause mortality and hospital admission due to HF.

End points data were collected during follow-up visits. In addition, records from hospital admissions were reviewed to confirm whether it was CHF related. No patient was lost to follow-up that ended on January 2005.

The local ethics committee approved the study.

Isolation of EPC and colony-forming unit assay

At baseline, 20 ml of blood was withdrawn for the isolation of EPC as described previously.^{11,12} Briefly, peripheral blood mononuclear cells were isolated by Ficoll density-gradient centrifugation (Sigma, St. Louis, Maryland, USA). After washing, isolated cells were resuspended in growth medium and plated on dishes coated with human fibronectin (Chemicon, Temecula, California, USA). To eliminate initial contamination with mature circulating endothelial cells, pre-plating of peripheral blood mononuclear cells onto fibronectin-coated six well plates was performed (5×10^6 /well) for 48 h, after which non-adherent cells were collected and replated onto fibronectin-coated plates for a final evaluation of colony numbers counted at day 7.

Confirmation of colony-forming unit phenotype

Colonies were assayed for endothelial cell markers at day 7. The following antibodies were used for immunofluorescent and flow-cytometric phenotyping: rabbit polyclonal anti-Tie-2 (C-20), mouse monoclonal anti-Flk-1 (A-3) and goat polyclonal anti-CD31 (platelet endothelial cell adhesion molecule 1, M-20), all from Santa-Cruz. Endothelial-cell lineage was further confirmed by indirect immunostaining with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate-acetylated low-density lipoprotein and co-staining with banderina simplicifolia -1 lectin (both from Sigma).

ELISA for the detection of VEGF

Quantitative determination of VEGF was performed using sandwich ELISA kit according to the manufacturer's protocol (R&D Systems, Minneapolis, Minnesota, USA).

High-sensitivity C-reactive protein concentrations

High-sensitivity C-reactive protein (hsCRP) concentration was determined by the Behring BN II Nephelometer according to the manufacturer's instructions (Dade Behring, Deerfield, Illinois, USA).

Determination of N-terminal pro-BNP levels

Measurements of serum N-terminal pro-B-type natriuretic peptide (NT-pro-BNP) were done by automated immunoassay (Elecsys proBNP, Roche Diagnostics, Mannheim, Germany). The test principle included using two polyclonal antibodies directed against NT-pro-BNP—epitope 1: amino acid 1–21 and epitope 2: amino acid 39–50. The results were calibrated against a synthetic NT-pro-BNP (amino acid 1–76). The range of results was between 5 and 35 000 pg/ml.

Statistical analysis

End point analysis based on Cox proportional-hazards model was used to analyse the data. A different model was constructed for each of the following adverse events: mortality

and CHF-related hospital admissions. These models explain the independent relationship between each parameter and time to incidence, and enables us to estimate the relative hazard ratio (HR) for each risk factor. Variables generally accepted as being of prognostic value in CHF were inserted, including age, gender, NYHA, LVEF, smoking, diabetes mellitus, hypertension, hyperlipidaemia, history of ischaemic heart disease, and laboratory parameters of proBNP, hsCRP, VEGF and EPC level.

Comparison regarding EPC levels between two or more groups was performed by Student's t test and analysis of variance. Correlations were examined by Pearson's correlation test.

The statistical significance level was set to 0.05 and SPSS V.12.0 was used for the analysis.

RESULTS

Table 1 shows the baseline clinical characteristics of our cohort. The aetiology for CHF was ischaemic heart disease in 74 (69.1%) patients, idiopathic dilated cardiomyopathy in 12 (11.2%), valvular disease in 11 (10.2%) and hypertensive disease in 10 (9.3%) patients. In all, 78 (72.8%) patients had systolic HF and 29 (27.1%) had HF with preserved systolic function. NYHA distribution was as follows: 38 (35.5%) patients in class II, 61 (57%) in class III and 8 (7.4%) in class IV. Patients were followed for a minimum of 10 months and for a maximum of up to 17 months, mean 13.8 (1.96). During follow up, there were 21 (19.6%) mortality events, 2 of them were non-cardiac deaths, and 26 (24.2%) patients were hospitalised due to exacerbation of CHF. Table 2 presents the levels of the different tested parameters in patients with and without the event. As it shows, EPC levels were highest among patients with mortality events.

A representative example of EPC-colony-forming unit (CFU) and its phenotype confirmation is depicted in fig 1.

There were no statistically significant differences in the levels of EPC-CFU between patients with systolic as compared with diastolic HF (78.4 (60.9) and, 87.4 (82.1)/well, respectively, $p = 0.71$). EPC-CFU levels did not correlate with the aetiology of CHF. EPC-CFU numbers in ischaemic HF, idiopathic dilated

Table 1 Clinical characteristics of the outpatient cohort with congestive heart failure (n = 107)

Age, years*	71.3 (10.1)
Males (%)	84 (78.5)
HF duration, years†	3.2 (1.7)
NYHA*	2.6 (0.5)
LVEF*	35.3% (14.1%)
Hyperlipidaemia	65 (60.7)
Smoking	10 (9.3)
Past smoker	64 (59.8)
Hypertension	73 (68)
Diabetes mellitus	47 (43)
Ischaemic heart disease	79 (73.8)
Chronic atrial fibrillation	12 (11.2)
Transient ischaemic attacks/cerebrovascular accidents	8 (7.4)
Percutaneous transluminal coronary angioplasty	64 (59.8)
Coronary artery bypass surgery	45 (42)
ACE inhibitors/ARBs	81 (75.7)
Spirolactone	53 (49.3)
β blockers	66 (61.6)
Statins	65 (60.7)
Digoxin	21 (19.6)
Diuretics	104 (97)
Anticoagulants	32 (29.9)

ARB, angiotensinogen receptor blocker; HF, heart failure; LVEF, left ventricular ejection fraction; NYHA, New York Heart Association.

*Values are given as mean (SD).

†All other values are mean (%).

†Disease duration before enrolment.

Table 2 Levels of the different tested parameters in patients with and without the event

Event*	All patients n = 107	Mortality n = 21	Hospital admission n = 26	No events n = 70
Variable				
EPC-CFU	81.08 (67.5)	98.48 (81.84)	71.15 (58.55)	77.29 (62.46)
NT-pro-BNP, pg/ml	1942 (2626)	3622 (7333)	3784 (6587)	1732 (2994)
hsCRP, μ g/ml	10.66 (26.59)	14.95 (24.89)	13.65 (19.52)	8.68 (27.99)
VEGF, pg/ml	69.53 (71.18)	66.35 (41.65)	77 (17.8)	67.7 (50)

EPC-CFU, endothelial progenitor cells-colony-forming units; hsCRP, high-sensitivity C-reactive protein; NT-pro-BNP, N-terminal pro-B-type natriuretic peptide; VEGF, vascular endothelial growth factor.
*10 patients had CHF-related hospital admission prior to mortality event.
Values are given as mean (SD).

cardiomyopathy, and valvular and hypertension related HF were 79.3 (69.9), 79.6 (61.4), 102.2 (63.9) and 71 (67.8)/well, respectively ($p = \text{NS}$ for all comparisons). There was no correlation between EPC-CFU levels and circulating VEGF ($p = 0.31$), proBNP ($p = 0.92$) or hsCRP ($p = 0.13$). However, a statistically significant correlation was found with NYHA ($p = 0.05$, $r = 0.188$). EPC levels did not correlate with disease duration.

In order to define the clinical relevance of EPC levels to future outcomes, a Cox regression model was used. Table 3 provides multiple adjusted HRs of the clinical and laboratory parameters that were examined as predictors of mortality. Advanced age, diabetes mellitus and levels of EPC-CFU were the only independent variables predictive of mortality in our study cohort. Severely compromised LVEF trended as an independent predictor. Examining the same model on cardiac death alone yielded the same results (p values: age, 0.01; diabetes mellitus, 0.003; EPC, 0.02; and LVEF, 0.058). Table 4

provides data on CHF-related hospital admissions. Only female gender and NT-pro-BNP levels were independent predictors of CHF-related hospital admissions. Table 5 gives data regarding mortality prediction among patients with systolic HF. None of the parameters tested were statistically significant among patients with HF and preserved systolic function.

DISCUSSION

The present study is the first to show that levels of circulating EPC are an independent predictor of all-cause mortality in patients with CHF. In fact, in our cohort, three independent variables were found to independently predict mortality, including advanced age, diabetes mellitus and EPC-CFU levels.

The study cohort included patients with advanced CHF selected from the outpatient clinic who were all controlled and were not admitted to hospital during the past 6 months so as not to introduce bias from potential exacerbation of their primary disorder. It should be noted that EPC levels failed to

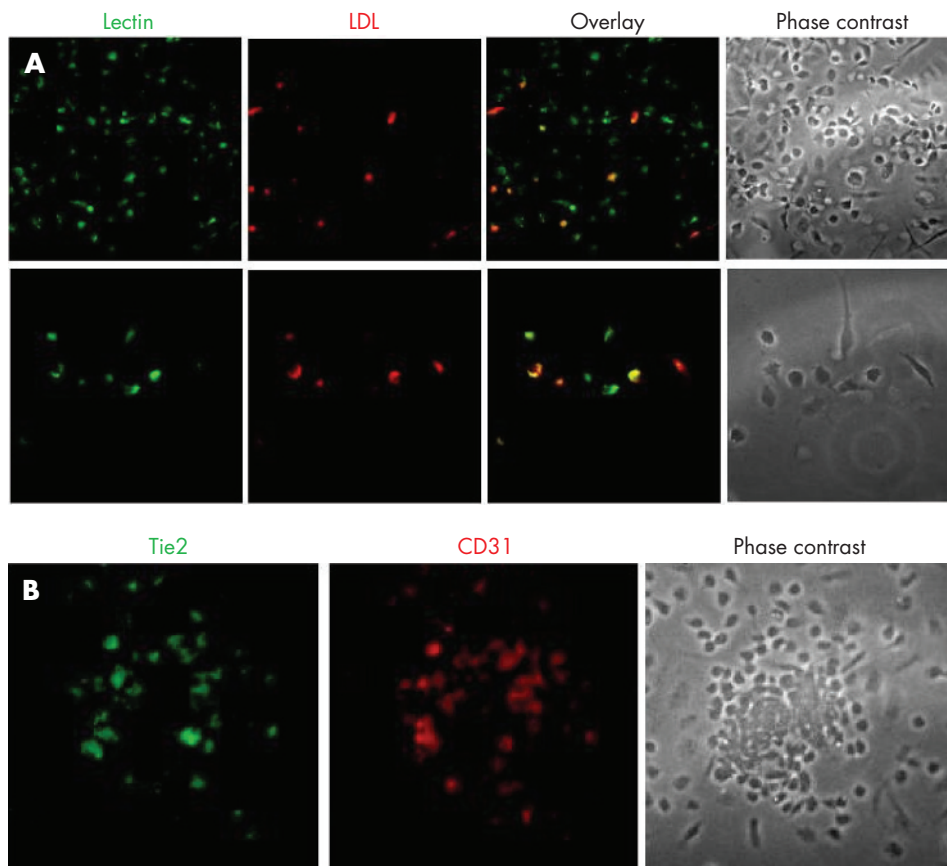


Figure 1 Confirmation of endothelial progenitor cell (EPC) phenotype by immunohistochemistry. To validate the identity of EPC, we employed two methods as described in Methods. (A) Confirmation of adherent EPC by costaining with anti-acetylated low-density lipoprotein (LDL) and anti-bandeiraea simplicifolia -1. (B) Endothelial marker expression of the colony-forming unit counted by antibodies to Tie-2 and CD-31.

Table 3 Cox regression model: variables as predictors of mortality

Variable	HR (95% CI)	p Value
Age, years	1.097 (1.019 to 1.182)	0.01
Female gender	0.599 (0.158 to 2.265)	0.45
NYHA	1.035 (0.295 to 3.64)	0.95
LVEF	0.966 (0.929 to 1.005)	0.08
Hyperlipidaemia	0.593 (0.189 to 1.857)	0.37
Smoking	0.662 (0.203 to 2.162)	0.49
Hypertension	0.457 (0.127 to 1.651)	0.23
DM	6.276 (1.972 to 19.969)	0.002
IHD	1.135 (0.263 to 4.888)	0.86
EPC	1.008 (1.001 to 1.015)	0.02
NT-pro-BNP	1.043 (0.952 to 1.143)	0.36
hsCRP	1.006 (0.991 to 1.02)	0.45
VEGF	1.001 (0.988 to 1.014)	0.87

DM, diabetes mellitus; EPC, endothelial progenitor cell; hsCRP, high-sensitivity C-reactive protein; IHD, ischaemic heart disease; LVEF, left ventricular ejection fraction; NT-pro-BNP, N-terminal pro-B-type natriuretic peptide; NYHA, New York Heart Association; VEGF, vascular endothelial growth factor.

predict CHF-related hospital admissions. This can be explained partially by the relatively high mortality of this cohort of patients.

Subgroup analysis demonstrated that the value of EPC levels as mortality predictors was limited to patients with systolic HF. Whether this is related to the smaller cohort of patients with preserved systolic function, or reflects inferiority of the predictive value among this patients' population needs further investigation.

There are several potential explanations for the predictive power of circulating EPCs regarding mortality. HF is characterised by peripheral and myocardial tissue ischaemia. This in turn results in systemic release of angiogenic factors and activation of multiple neurohormonal axes that may promote the peripheral mobilisation of EPCs from the bone marrow niche in an attempt to facilitate a process of angiogenesis and vasculogenesis.⁷ The more severe the ischaemia, the higher the driving force for the release of EPCs. Additionally, patients with CHF are known to exhibit endothelial dysfunction that is correlated with disease severity.³ Several studies have demonstrated that circulating EPCs serve as a pool of progenitor cells^{11–18} that participate in endothelial repair processes; thus, a larger pool may reflect a higher need for progenitor cells due to more intense endothelial damage.

Studies on animals have suggested that bone-marrow-derived progenitor cells may transdifferentiate into myocardial and vascular cells after myocardial injury, as an attempt to limit the extent of myocardial damage.^{19–21} It is tempting to speculate that these mechanisms are also operative in patients with HF, and that EPC mobilisation is a compensatory response intended to attenuate cardiac remodelling.

Valgimigli *et al*¹⁶ recently tested EPC levels in patients with CHF. They found that as a group, patients with CHF had higher EPC levels than healthy controls. However, they reported a bimodal behaviour of EPC levels, with higher levels in early stages of CHF and lower levels in advanced stages of CHF. They proposed a mechanism of bone marrow exhaustion by cytokines such as tumour necrosis factor α that are increased in advanced CHF. These results are in contrast with our findings of a correlation between EPC levels and NYHA. The difference may be partially explained by the different patient population studied—that is, in our cohort, 27% of patients had HF with preserved systolic function, and we included patients with NYHA II–IV, with only a minority from NYHA IV, whereas in the cohort studied by Valgimigli *et al*¹⁶ all patients had systolic HF and were evenly distributed from functional class I

Table 4 Cox regression model: variables as predictors of CHF-related hospital admissions

Variable	HR (95% CI)	p Value
Age, years	0.981 (0.937 to 1.027)	0.41
Female gender	3.433 (1.153 to 10.222)	0.02
NYHA	1.629 (0.658 to 4.029)	0.29
LVEF	0.978 (0.946 to 1.01)	0.17
Hyperlipidaemia	1.097 (0.436 to 2.76)	0.84
Smoking	0.825 (0.29 to 2.353)	0.72
Hypertension	1.629 (0.593 to 4.472)	0.34
DM	1.027 (0.426 to 2.476)	0.95
IHD	1.055 (0.372 to 2.995)	0.91
EPC	0.996 (0.989 to 1.004)	0.31
NT-pro-BNP	1.069 (1.004 to 1.139)	0.03
hsCRP	1 (0.987 to 1.014)	0.94
VEGF	1.001 (0.995 to 1.007)	0.79

DM, diabetes mellitus; EPC, endothelial progenitor cell; hsCRP, high-sensitivity C-reactive protein; IHD, ischaemic heart disease; LVEF, left ventricular ejection fraction; NT-pro-BNP, N-terminal pro-B-type natriuretic peptide; NYHA, New York Heart Association; VEGF, vascular endothelial growth factor.

to IV. Our patients had more advanced CHF, but all were well controlled in a dedicated HF clinic. Moreover, statins, that were shown to influence EPC levels²² were discontinued only in that study. It should be noted that the study by Valgimigli *et al*¹⁶ was observational and was not designed to test the prognostic value of EPC.

In accordance with Valgimigli *et al*,¹⁶ we found that EPC levels were not influenced by the aetiology of CHF and did not correlate with VEGF, hsCRP or NT-pro-BNP. The finding in our study that EPC levels correlated with NYHA may imply that the clinical state of the patient is the dominant factor that influences EPC levels, although the exact mechanism is probably complex and multifactorial.

Similar to previous reports, diabetes mellitus was found to be an independent predictor of mortality.³ EPC in patients with diabetes has impaired functions including proliferation, adhesion and tubulisation.²³ These functions are needed for the amplification of EPC, the attachment to the area of damaged endothelium and the creation of new blood vessels. EPC malfunction may thus contribute to the adverse prognosis of patients with diabetes. Future studies regarding EPC function in CHF and its relationship to adverse outcome are needed.

There are several limitations to this study. The study cohort is relatively small and non-homogeneous, most patients studied

Table 5 Cox regression model: variables as predictors of mortality among patients with systolic heart failure (n = 78)

Variable	HR (95% CI)	p Value
Age	1.17 (1.023 to 1.338)	0.02
Female gender	0.674 (0.104 to 4.366)	0.67
NYHA	0.233 (0.035 to 1.573)	0.13
LVEF	0.844 (0.754 to 0.944)	0.003
Hyperlipidemia	1.176 (0.184 to 7.521)	0.86
Smoking	3.033 (0.356 to 25.83)	0.31
Hypertension	1.623 (0.221 to 11.939)	0.63
DM	12.647 (1.778 to 89.966)	0.01
IHD	0.457 (0.063 to 3.297)	0.43
EPC	1.019 (1.002 to 1.036)	0.02
NT-pro-BNP	1.16 (1.042 to 1.291)	0.006
hsCRP	0.995 (0.968 to 1.023)	0.71
VEGF	1.015 (0.998 to 1.033)	0.07

DM, diabetes mellitus; EPC, endothelial progenitor cell; hsCRP, high-sensitivity C-reactive protein; IHD, ischaemic heart disease; LVEF, left ventricular ejection fraction; NT-pro-BNP, N-terminal pro-B-type natriuretic peptide; NYHA, New York Heart Association; VEGF, vascular endothelial growth factor.

were in NYHA II–III and only a minority were in class IV. Moreover, data that may affect patients' prognosis, such as changes during follow-up in drugs or other treatments, was not collected, yet the patients were all well controlled and treated by a small number of HF specialists.

An additional controversial issue is the method we used for assessment of EPC levels. The CFU assay has been used by several authors, including in our previous studies.^{10 11 15 24–26} It is criticised by some authorities as it may be indicative of the proliferative capacity of EPC rather than their mere numbers. We have recently performed a comparative analysis of the currently used methods for EPC assessment in a cross-sectional study of healthy volunteers and found that these methods are not correlated.²⁷ We have therefore called for standardisation of the assay and, until this is done, we believe that the CFU method is a valid and reproducible method of assessing EPC levels, provided appropriate measures are taken to verify the identity of the colonies by immunohistochemical staining with relevant endothelial markers (fig 1).

In conclusion, we found that EPC levels are an independent predictor of mortality in patients with CHF. EPC levels are probably not influenced by the aetiology of HF, but rather correlate with the patient's clinical status reflected by the NYHA score. If these observations are supported by additional studies, EPC level determination could serve as an additional surrogate marker for the clinical follow-up of patients with CHF.

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