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Research Article

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Test-Retest Reliability of Flow-Cytometric Quantification of Circulating Endothelial Cells and Endothelial Progenitor Cells in Cardiovascular Diseases

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Abstract

Circulating Endothelial Cells (CECs) and Endothelial Progenitor Cells (EPCs) are gaining importance as quantifiable surrogate biomarkers of Endothelial Dysfunction (ED). Lack of a common definition and, consequently, a standardized quantification method, has limited clinical applicability of these biomarkers. A reliable, reproducible, and practicable method is required to achieve the full potential of these parameters. The objective of this study was to evaluate the test- retest reliability over a short period of time (~7 days) of flow cytometric quantification of CECs and EPCs in human whole blood from patients with cardiovascular disease. 100 patients (mean age, 65 ± 10 years, 30 women) were enrolled into a prospective study consisting of 4 patients groups: heart failure with reduced ejection fraction (HFrEF; n=25), heart failure with preserved ejection fraction (HFpEF; n=26), diabetic nephropathy (DN; n=25), and hypertension (HTN; n=24). In addition, 11 healthy volunteers were included as a control group. At 2 study visits, a blood sample was drawn, which underwent an identical sequence of preparation and analysis. CECs (DNA+, CD45dim, CD31+, and CD146+) and EPCs (CD45dim, CD34br, CD133+, and CD31+, FSClow–medium, SSClow) were counted via flow cytometry. To assess short-term test-retest reliability, correlation (intraclass correlation) and agreement (Bland-Altman plot) of the measurements obtained at the 2 study visits were evaluated. Across all patients, median CECs/mL and EPCs/mL were 12 (5th/95th percentile: 6/22) and 679 (447/1281) at visit 1 and were 11 (6/24) and 736 (510/1105) at visit 2, respectively; intraclass correlation (ICC) was poor for CEC count (0.106; ICC- 95% CI -0.08–0.29) and good for EPC count (0.9; 0.86–0.93). In patients with HFpEF, ICC was poor for CEC count (0.294; 95% CI -0.08–0.6) and moderate in strength for EPC count (0.694; 0.43–0.85). In patients with HFrEF, ICC was poor for CEC count (0.076; -0.32–0.45) and excellent in strength for EPC count (0.946; 0.88–0.98). In patients with DN, ICC was poor for CEC count (-0.031; -0.44– 0.37) and excellent in strength for EPC count (0.946; 0.88–0.98). In patients with HTN, ICC was poor for CEC count (0.143; -0.27–0.51) and moderate in strength for EPC count (0.668; 0.37–0.84). In healthy controls, ICC was poor for CEC count (0.378; -0.26–0.78) and good in strength for EPC count (0.846; 0.59–0.96). A Bland-Altman plot showed a positive correlation of variations of differences and increasing median CEC counts; there were no distinct trends for median EPC counts. Our analyses indicate that flow cytometric quantification of EPC concentrations is reliable in patients with HFpEF, HFrEF, DN, and HTN. Quantification of CEC concentrations showed poor test-retest reliability across all patient groups. Further research is necessary to elucidate the nature of this finding, which could be due to higher biological variability in patients with severe ED. Clinical Trial Registration Identifier: NCT02299960.

Keywords

Circulating endothelial cells; Endothelial progenitor cells; Cardiovascular disease; Endothelial dysfunction; Heart failure; Diabetic nephropathy; Hypertension; Test-retest reliability.

Abbreviations

CEC: Circulating Endothelial Cell; CI: Confidence Interval; CTRL: Control; DN: Diabetic Nephropathy; ED: Endothelial Dysfunction; EPC: Endothelial Progenitor Cell; HFpEF: Heart Failure with Preserved Ejection Fraction; HFrEF: Heart Failure with Reduced Ejection Fraction; HTN: Hypertension; ICC: Interclass Correlation; LoA95: 95% Limits of Agreement; PMNC: Peripheral Mononuclear Cell; UACR: Urine Albumin to Creatinine Ratio.

Introduction

The endothelium has a pivotal role not just in establishing the functional integrity of the vasculature, but also in the regulation of blood pressure, coagulation, and leukocyte migration. Among a myriad of different conditions, a dysfunction of the endothelial vessel layer is associated with Cardiovascular Diseases (CVD) as well as their pathophysiologic drivers such as hypertension, diabetes mellitus, and atherosclerosis[1-3]. Endothelial Dysfunction (ED) has been shown to be integral for both the pathogenesis and the progression of CVD[4]. A measurable ED often proceeds the onset of symptoms of these diseases and its complications, such as congestive heart failure, myocardial infarction, stroke, kidney disease, and pulmonary hypertension[1,5,6]. Therefore, reliable and accurate assessment and quantification of ED has considerable potential as a diagnostic, therapeutic, and a prognostic parameter.

Circulating Endothelial Cells (CECs) and circulating Endothelial Progenitor Cells (EPCs) have been described as promising biological markers of ED [1,5,7,8]. CECs are desquamated endothelial cells that detach in response to either mechanical vessel injury or as a result of impaired cellular adhesion [8]. EPCs, on the other hand, are derived from the bone marrow and play an important role in the maintenance of endothelial integrity and hemostasis as they proliferate at the site of vessel injury and can differentiate into mature endothelial cells. While CECs reflect endothelial injury, EPCs indicate endothelial recovery; thus, the two types of cells are believed to represent different aspects of the spectrum of ED [2]. EPCs can be further differentiated into early and late EPCs based on their maturation stage.

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The two most widely used methods for measurement of CECs and EPCs are immunomagnetic separation and flow cytometry, both of which rely on the expression of different cell surface markers [9]. Polychromatic flow cytometry is currently considered the most sensitive method for quantification of CECs [10].

The reliable identification of the cell lines is challenging, as they share some of their cell surface antigens among each other as well as with many other circulating cells and the expressed antigens vary throughout the life cycle of these cells [11,12]. Typically, a combination of cell surface markers is used for their definition because no single cell surface marker is fully specific. Although various marker profiles have been proposed for standardization, detection methods and reported counts vary greatly across different laboratories and exact definitions remain controversial [13-15].

Because the endothelial lining is regularly renewed, levels of CECs are very low (~10 CECs/mL) in healthy individuals, making the release of these cells a rare event [1] In CVD, CEC count is increased significantly (often exceeding 100 CECs/mL) [1]. EPCs, in contrast, are typically decreased in advanced CVD such as coronary artery disease and severe congestive heart failure, reflecting an impaired ability of vascular recovery [6,16]. In mild congestive heart failure, on the other hand, EPCs are increased [17] presumably reflecting increased recruitment of progenitor cells from the bone marrow [18]

Though numerous studies have been published that praise the potential of CECs as a biomarker of endothelial injury, the lack of common definitions and the difficulty of quantifying CECs and EPCs reliably has so far limited their application in clinical practice [10]. A prerequisite for the transfer of this method from the laboratory to clinics is a reproducible, reliable, and practicable approach for identifying and quantifying endothelial dysfunction based on these cell counts.

Recently, our group reported a novel, robust, reproducible flow cytometric approach of quantifying CECs (DNA+, CD45dim, CD31+, and CD146+) and EPCs (CD45dim, CD34br, CD133+, and CD31+, FSClow–medium, SSClow) from whole blood in healthy volunteers and in patients with cardiovascular diseases. We found significantly increased CEC counts in patients with cardiovascular diseases compared to healthy volunteers, while EPC counts were similar to healthy individuals [19].

To the best of our knowledge, no data have been reported concerning the test- retest reliability of flow cytometric enumeration of CECs or EPCs in adult patients with CVD. In addition, there are no available studies about the biological variability of CEC and EPC counts, which impacts test-retest reliability, in patients with CVD. Lastly, limited data are available about disease-specific CEC/EPC counts in general.

We are now reporting the results of the first test-retest reliability study of flow cytometric quantification of CECs/EPCs in four separate patient groups over a short (~7 days) time span: heart failure with preserved Ejection Fraction (HFpEF), Heart Failure with Reduced Ejection Fraction (HFrEF), Diabetic Nephropathy (DN), and hypertension without structural cardiovascular damage (HTN). All of these conditions have been shown to be associated with endothelial dysfunction[20-23]. To assess test-retest reliability, we evaluated intraclass correlation as well as agreement. We hypothesized this method would demonstrate a high short-term test-retest reliability, indicating that CEC/EPC count could serve as a stable biomarker of endothelial dysfunction.

Methods

Study design

We performed a prospective, single-center, investigator-initiated test-retest study in 100 patients with HFpEF, HFrEF, DN, and HTN and 11 age-matched healthy volunteers. The study protocol and amendments were approved by the Medical Ethics Committee of Charité – University Medicine Berlin. All subjects provided written informed consent prior to participation in the trial. All procedures related to the trial conformed to the principles outlined in the Helsinki Declaration and ICH-GCP. This trial is registered at clinicaltrials.gov under clinical trials number NCT02299960.

Patient selection

Patients were recruited using the database of the study center, from the Charité Center for Cardiovascular Diseases, and from outpatient clinics between August 2014 and September 2015. A partially different subset of the patient cohort has been described previously[19,24]. In summary, inclusion criteria were age 35–80 years and absence of clinical symptoms for 4 weeks (HFpEF, DN, and HTN) and 7 days (HFrEF) prior to screening. Exclusion criteria were symptomatic coronary artery disease, recent (<3 months) myocardial infarction or stroke, complex congenital heart disease, valvular defects, cardiac arrhythmia, active myocarditis, significant respiratory disease, change of medications during the course of the study, recent implementation of cardiac resynchronization therapy or a cardiac pacemaker, uncontrolled hypertension (systolic blood pressure>180 mm Hg; diastolic blood pressure>95 mm Hg), current participation in a rehabilitation program, significant anemia (hemoglobin concentration<10 mg/dL), untreated symptomatic thyroid disease, and known malignancy or any other disease with a life expectancy of less than one year. Detailed inclusion and exclusion criteria can be found under the registered trial number.

171 potentially eligible patients were screened based on the criteria described above, of which 71 were excluded and 101 were included in the study. One patient dropped out of the study after the baseline screening assessment, leaving 100 patients, who were subsequently divided into four study groups based on additional criteria:

1. 26 patients with HFpEF based on the modified criteria suggested by Paulus et al. [25] with symptomatic heart failure NYHA I-III at least three months prior to screening, sinus rhythm, and capability to perform spirometry.
2. 25 patients with HFrEF based on LVEF<45% as measured through echocardiography. Patients showed symptoms of heart failure (NYHA I-III) at least 30 days prior to screening. Underlying causes of heart failure included ischemic and nonischemic etiologies.
3. 25 patients with DN based on the presence of type 2 diabetes mellitus and intake of oral antidiabetic medication or insulin. Further criteria were GFR<90mL/min/1,73m² (CKD-EPI) [4], macroalbuminuria (Urine Albumin to Creatinine Ratio (UACR)>300mg/g creatinine) or microalbuminuria (UACR 30–300 mg/g creatinine).
4. 24 patients with HTN without clinical or echocardiographic evidence of structural heart disease (ejection fraction > 55%, no clinical signs for coronary heart disease or HFpEF, stable

antihypertensive medication, and systolic blood pressure during screening visits<140 mmHg). In addition, 11 healthy volunteers were enrolled at Clinical Research Services Berlin GmbH.

Each patient underwent a baseline screening assessment and two study visits: visit 1 (V1, 0–7 days after baseline assessment) and visit 2 (V2, 4–14 days after visit 1). On study visit days, patients were instructed to take their home medication as usual. V1 and V2 each included a physical examination, vital sign measurement, and taking peripheral venous blood samples under the same conditions. The median time between the two measurements was 7 days. Baseline clinical characteristics, medications, and laboratory parameters of patients at the baseline screening assessment are shown in Table 1.

CEC/EPC identification and quantification

CECs and EPCs were assessed in each sample according to the recently published method by Farinacci et al. [19] which showed robust and reproducible cell count results in healthy volunteers as well as in patients with HFpEF, HFrEF, DN, and HTN. In summary, multicolor flow cytometry panels were used to determine the cell counts from whole blood samples using monoclonal antibodies directed at CD31, CD 36, CD45, and CD146 and nuclear staining SYTO16. Following previously described protocols, CECs were defined as DNA+, CD45dim, CD31+, and CD146+,12 and EPCs were defined as CD45dim, CD34br, CD133+, and CD31+, FSClow–medium, SSClow[26]. Cell counts were counted in each sample in triplicate to determine a mean count. Counts were then assessed in relation to peripheral mononuclear cells (PMNCs) as CECs/EPCs per PMNC. To calculate absolute CEC/EPC counts (cells/mL), the initial results were multiplied by the absolute PMNC count, which, in turn, was determined through flow-count fluorospheres.

Statistical analysis

All analyses were conducted with R V 3.3.1. Continuous variables with normal distribution are expressed as mean and Standard Deviation (SD). Continuous variables with skewed distribution are summarized as median and 25th/75th percentile unless otherwise specified. Probability was significant at a level of <0.05. Reliability was analyzed as test-retest reliability with intraclass correlation (ICC) type

Table 1: Characteristics, medications, and laboratory parameters of enrolled patients at baseline screening assessment.

	All	HFpEF	HFrEF	DN	HTN	*CTRL*	*p*
Variable	(*n*=100)	(*n*=26)	(*n*=25)	(*n*=25)	(*n*=24)	*(n=11)*	
Age (years)	65 ± 10	69 ± 8	62 ± 11	70 ± 9	59 ± 7	56 ± 3	0.001
Female	30 (30%)	11 (42%)	5 (25%)	4 (16%)	10 (42%)	6 (55%)	0.076
BMI	29 ± 5	28 ± 4	29 ± 4	31 ± 5	29 ± 6	24 ± 4	0.233
HR (bpm)	70 ± 13	69 ± 13	69 ± 10	74 ± 16	69 ± 10	-	0.378
BPsys (mmHg)	134 ± 20	137 ± 22	127 ± 22	140 ± 19	130 ± 14	-	0.114
BPdia (mmHg)	80 ± 11	78 ± 10	78 ± 12	79 ± 12	86 ± 8	-	0.035
NYHA I	15 (15%)	6 (23%)	7 (28%)	2 (8%)	0 (0%)	-	
NYHA II	39 (39%)	16 (62%)	13 (52%)	10 (40%)	0 (0%)	-	
NYHA III	16 (16%)	4 (15%)	5 (20%)	7 (28%)	0 (0%)	-	0.001
LVEF (%)	52 ± 13	59 ± 5	35 ± 8	53 ± 11	62 ± 6	-	0.001
LAVI (mL/m²)	30 ± 11	36 ± 10	33 ± 13	29 ± 11	22 ± 4	-	0.001
LVMI (g/m²)	106 ± 30	92 ± 26	126 ± 31	117 ± 23	88 ± 21	-	0.001
E/E'	13 ± 5	13 ± 6	14 ± 5	13 ± 6	11 ± 2	-	0.054
Current smoker	17 (17%)	2 (8%)	8 (32%)	5 (20%)	2 (8%)	-	0.075
ACE inhibitor	52 (52%)	12 (46%)	15 (60%)	16 (64%)	9 (38%)	-	0.225
Acetylsalicylic acid	44 (44%)	9 (35%)	15 (60%)	17 (68%)	3 (13%)	-	0.001
Beta blocker	69 (69%)	19 (69%)	24 (96%)	19 (76%)	7 (29%)	-	0.001
Calcium inhibitor	25 (25%)	7 (27%)	2 (8%)	12 (48%)	4 (17%)	-	0.008
Statin	49 (49%)	11 (42%)	15 (60%)	20 (80%)	3 (13%)	-	0.001
Oral antidiabetic	33 (33%)	7 (27%)	2 (8%)	20 (80%)	4 (17%)	-	0.001
Diuretic	53 (53%)	12 (46%)	17 (68%)	17 (68%)	7 (29%)	-	0.015
eGFR (mL/min)	72 ± 21	77 ± 15	76 ± 18	51 ± 21	85 ± 10	-	0.001
HbA1c (%)	5.9(5.5/7.0)	5.8(5.6/6.2)	5.8(5.5/6.1)	7.2(6.5/7.8)	5.4(5.3/6.0)	-	0.001
NTproBNP (pg/mL)	187(75/444)	219(89/372)	459(224/1,306)	257(150/930)	62 (42/95)	-	0.001
TSH (µIU/mL)	1(0.7/1.7)	0.9(0.8/1.3)	0.8(0.5/1.5)	1.4(0.7/2.1)	1.4(0.9/2.1)	-	0.154
Hemoglobin (g/dL)	14 ± 2	14 ± 1	15 ± 2	14 ± 1	15 ± 1	-	0.039
Total cholesterol	182 ± 49	180 ± 44	172 ± 37	172 ± 52	206 ± 54	-	0.04
HDL cholesterol (mg/dL)	52 (41/65)	61 (45/67)	51 (43/64)	42 (31/51)	56 (51/72)	-	0.002
LDL cholesterol (mg/dL)	112 ± 42	106 ± 44	108 ± 37	101 ± 49	134 ± 28	-	0.021
TAG (mg/dL	137(92/193)	123(91/174)	134(76/196)	180(109/309)	137(105/172)	-	0.207

Values are mean ± SD, median and 25th/75th percentile, or n (%). P-values represent the analysis of variance. HFpEF, heart failure with preserved ejection fraction; HFrEF, heart failure with reduced ejection fraction; DN, diabetic nephropathy; HTN, arterial hypertension; CTRL, control; BMI, body mass index; HR, heart rate; BP, blood pressure; NYHA, New York Heart Association; LVEF, left ventricular ejection fraction; LAVI, left atrial volume index; LVMI, left ventricular mass index; E/E', ratio between early mitral inflow velocity (E) and mitral annular early diastolic velocity (E'); ACE, angiotensin-converting enzyme; eGFR, estimated glomerular filtration rate; HbA1c, glycosylated hemoglobin A1c; NTproBNP, N-terminal prohormone of brain natriuretic peptide; TSH, thyroid-stimulating hormone; HDL, high-density lipoprotein; LDL, low-density lipoprotein; TAG, triacylglycerides

II (single random raters), calculated as (MSB–MSE)/(MSB+ (nr–1)* MSE+nr* (MSJ–MSE)/nc) Sb2–Sw2/Sb2+Sw2 (where 'Sb2'=between-subject variance; 'Sw2'=within-subject variance; MSB=between mean square variance; MSE=variance and mean squared error; MSJ=mean square between judges). Confidence Intervals (CI) for ICC (ICC-CI) were calculated according to Shrout and Fleiss[27]. The mean variability was reported as mean and SD of absolute differences in V1 and V2. For a better comparison of variabilities, Coefficients of Variation (CVs) were computed based on absolute differences. CVs were calculated as SD/meanx100 and were reported in percentages. The necessary minimal sample size is based on the expected ICC of rho1=0.8 versus rho0=0.5, as the minimal ICC of clinical relevance. For interpretation of ICC, we follow the recommendations as proposed by Koo et al. [28] ICC<0.50, 0.5–0.75, 0.75–0.9, and>0.9 indicate poor, moderate, good, and excellent reliability. Data were plotted as scatterplots for V1 *vs*. V2 with the expected fit, and the estimated linear fit using the nonparametric loess (locally weighted scatterplot smoothing) regression method[29]. Furthermore, Bland-Altman plots were generated depicting the relationship between differences in V1 and V2 and mean (V1/V2)[30]. Bias was calculated as mean (V2-V1). Limits of agreement (LoA95) were calculated as bias ± 1.96*SD(V2-V1).

Results

Median, CV, ICC, and ICC confidence interval (ICC-CI) of investigated parameters at V1 and V2 overall as well as for the individual patient groups are displayed in Table 2.

Across all patients (n=100), median CEC and EPC counts were 12 (5th/95th percentile: 6/22) CECs/mL and 679 (447/1281) EPCs/mL at V1 and 11 (6/24) CECs/mL and 736 (510/1105) EPCs/mL at V2, respectively. Coefficients of variations for CECs/mL and EPCs/mL were 111% and 112%, respectively. Correlations between paired measurements (V1 *vs* V2) overall were poor for CEC count (ICC 0.106; 95% confidence interval: -0.08–0.29) and good in strength for EPC count (ICC 0.9; 0.86–0.93).

In patients with HFpEF, median CEC and EPC counts were 16 (9/24) CECs/mL and 612 (427/906) EPCs/mL at V1 and 26 (9/37) CECs/mL and 719 (537/883) EPCs/mL at V2, respectively. Coefficients of variations for CECs/mL and EPCs/mL were 100% and 121%, respectively. Correlations between paired measurements (V1 *vs* V2) in this group was poor for CEC count (ICC 0.294; - 0.08–0.6) and moderate in strength for EPC count (ICC 0.694; 0.43–0.85).

In patients with HFrEF, median CEC and EPC counts were 8 (4/24) CECs/mL and 810 (499/1514) EPC/mL at V1 and 10 (4/17) CECs/mL and 783 (524/1537) EPCs/mL at V2, respectively. Coefficients of variations for CECs/mL and EPCs/mL were 118% and 138%, respectively. Correlations between paired measurements (V1 *vs* V2) in this group were poor for CEC count (ICC 0.076; -0.32–0.45) and excellent in strength for EPC count (0.946; 0.88–0.98).

In patients with DN, median CEC and EPC counts were 14 (7/33) CECs/mL and 674 (464/1306) EPCs/mL at V1 and 14 (7/22) CECs/mL and 711 (407/1194) EPCs/mL at V2, respectively. Coefficients of variations for CECs/mL and EPCs/mL were 123% and 59%, respectively. Correlations between paired measurements (V1 *vs* V2) in this group were poor for CEC count (-0.031; -0.44– 0.37) and excellent in strength for EPC count (0.946; 0.88–0.98).

In patients with HTN, median CEC and EPC counts were 10 (5/17) CECs/mL and 699 (437/1253) EPCs/mL at V1 and 13 (7/22) CECs/mL and 779 (497/1114) EPCs/mL at V2, respectively. Coefficients of variations for CECs/mL and EPCs/mL were 120% and 119%, respectively. Correlations between paired measurements (V1 *vs* V2) in this group were poor for CEC count (0.143; -0.27–0.51) and moderate in strength for EPC counts (0.668; 0.37–0.84).

In healthy controls, CEC and EPC counts were 7 (5/11) CECs/mL and 609 (408/1169) EPCs/mL at V1 and 3 (1/7) CECs/mL and 841 (519/894) EPCs/mL at V2, respectively. Coefficients of variations for CECs/mL and EPCs/mL were 60% and 103%, respectively. Correlations between paired measurements (V1 *vs* V2) in this group

Table 2: Correlations between paired measurements (V1 vs V2) for all patients, individual patient groups, and control group.

Group	Value	Visit 1 (median; 5th/95th percentile)	Visit 2 (median; 5th/95th percentile)	Average CV (%)	ICC	ICC- 95%CI
All patients (n=100)						
	CECs/mL	12 (6/22)	11 (6/24)	111	0.106	-0.08–0.29
	EPCs/mL	679 (447/1,281)	736 (510/1,105)	112	0.9	0.86–0.93
Heart failure with preserved ejection fraction (n=26)						
	CECs/mL	16 (9/24)	26 (9/37)	100	0.294	-0.08–0.6
	EPCs/mL	612 (427/906)	719 (537/883)	121	0.694	0.43–0.85
Heart failure with reduced ejection fraction (n=25)						
	CECs/mL	8 (4/24)	10 (4/17)	118	0.076	-0.32–0.45
	EPCs/mL	810 (499/1,514)	783 (524/1,537)	138	0.946	0.88–0.98
Diabetic nephropathy (n=25)						
	CECs/mL	14 (7/33)	14 (7/22)	123	-0.031	-0.44–0.37
	EPCs/mL	674 (464/1,306)	711 (407/1,194)	59	0.946	0.88–0.98
Arterial hypertension (n=24)						
	CECs/mL	10 (5/17)	13 (7/22)	120	0.143	-0.27–0.51
	EPCs/mL	699 (437/1,253)	779 (497/1,114)	119	0.668	0.37–0.84
Control group (n=11)						
	CECs/mL	7 (5/11)	3.1 (1/7)	60	0.378	-0.26–0.78
	EPCs/mL	609 (408/1,169)	841 (519/894)	103	0.864	0.59–0.96

V1, visit 1; V2, visit 2; ICC, interclass-correlation; CI, confidence interval; CEC, circulating endothelial cell; CV; coefficient of variability; EPC, endothelial progenitor cell

were poor for CEC count (0.378; -0.26–0.78) and good in strength for EPC counts (0.864; 0.59–0.96).

Test-retest raw data for CECs/mL and EPCs/mL (Figures 1and 2), respectively. Scatterplots of CECs/mL and EPCs/mL at V1 in relation to V2 (Figures 3 and 4), respectively. For a better overview, the scale of the graphs was reduced and does not show the outliers for CECs and EPCs (Figures 1 and 2). Bland-Altman plots were conducted for further examination of the differences. In CECs/mL (Figure 5), visual inspection of the plot showed increased variations of differences as the average CEC count increased. The mean difference between V1 and V2 CEC count was 0 ± 20 CECs/mL overall and the width of LoA95 was -38 to 39 CECs/mL; 94 (94%) patients fell within the 95% limits of agreement. In EPCs/mL (Figure 6), the Bland Altman plot showed no obvious systemic trend. The mean difference between V1 and V2 CEC count was 25 ± 360 EPCs/mL overall and the width of LoA95 was -680 to 731 EPCs/mL; 89 (89%) patients fell within the 95% limits of agreement.

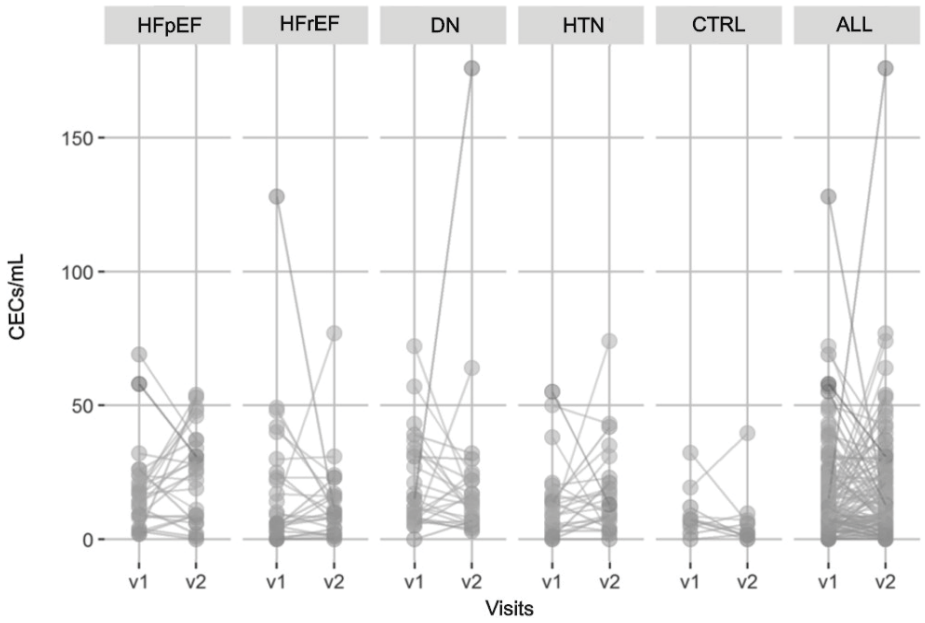

Figure 1: Test-retest raw data for circulating endothelial cells measurements (CECs/mL) at visit 1 and visit 2. (v1, visit 1; v2, visit 2; HFpEF, heart failure with preserved ejection fraction; HFrEF, heart failure with reduced ejection fraction; DN, diabetic nephropathy; HTN, arterial hypertension; CTRL, control; ALL, all participants).

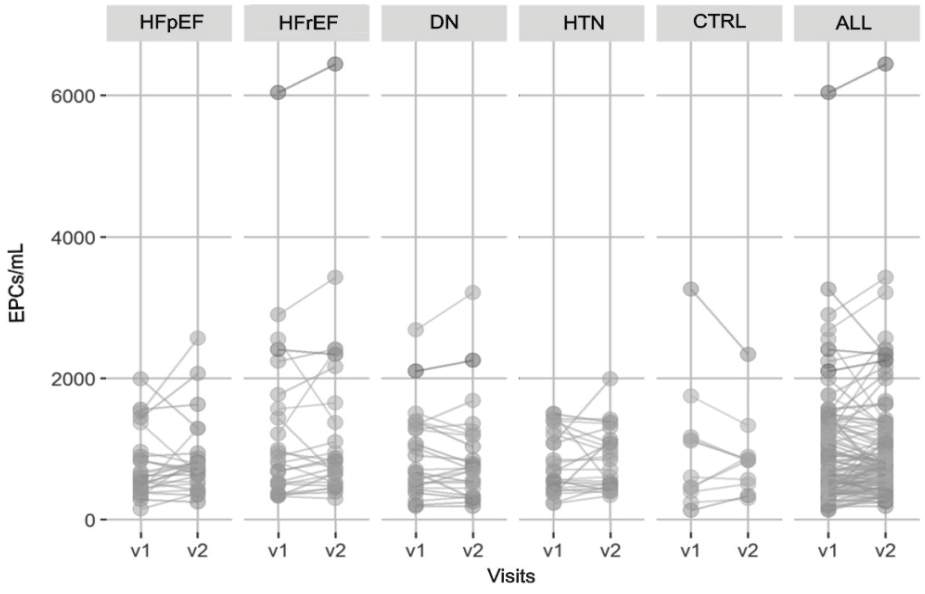

Figure 2: Test-retest raw data for endothelial progenitor cell measurements (EPCs/mL) at visit 1 and visit 2. (v1, visit 1; v2, visit 2; HFpEF, heart failure with preserved ejection fraction; HFrEF, heart failure with reduced ejection fraction; DN, diabetic nephropathy; HTN, arterial hypertension; CTRL, control; ALL, all participants).

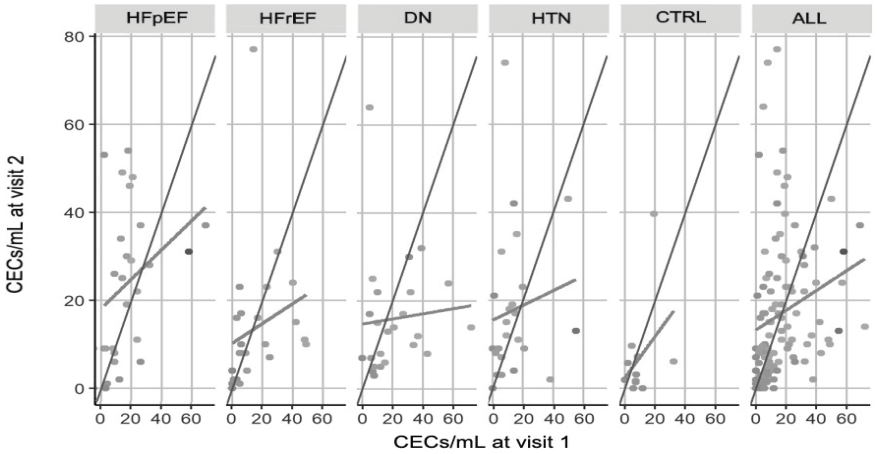

Figure 3: Concentration of circulating endothelial cells (CECs/mL) at visit 1 and visit 2 with expected fit (thin line) and estimated linear fit (bold line). (HFpEF, heart failure with preserved ejection fraction; HFrEF, heart failure with reduced ejection fraction; DN, diabetic nephropathy; HTN, arterial hypertension; CTRL, control; ALL, all participants).

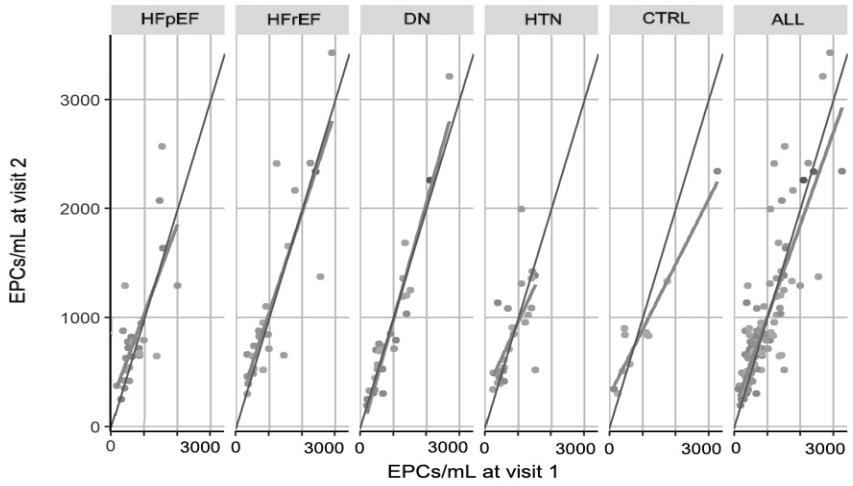

Figure 4: Concentration of endothelial progenitor cells (EPCs/mL) at visit 1 and visit 2 with expected fit (thin line) and estimated linear fit (bold line). (HFpEF, heart failure with preserved ejection fraction; HFrEF, heart failure with reduced ejection fraction; DN, diabetic nephropathy; HTN, arterial hypertension; CTRL, control; ALL, all participants).

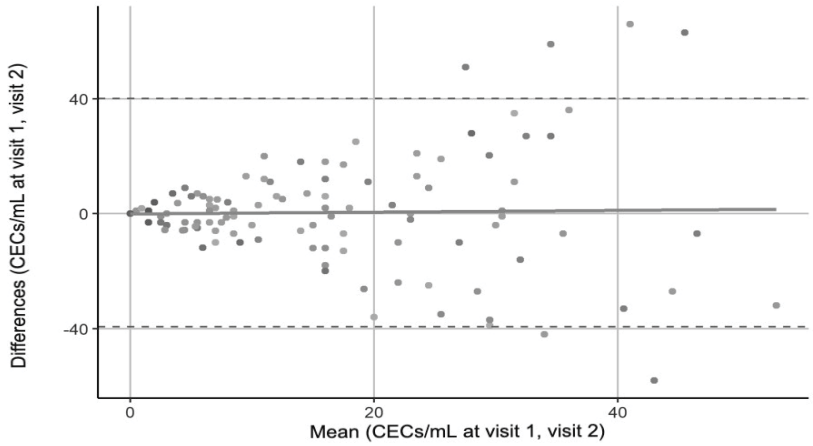

Figure 5: Bland-Altman plot showing the relationship between CECs/mL differences at visit 1 and visit 2 and mean (visit 1/visit 2); also showing the regression lines between delta and mean (bold solid line) to explore potential dependencies, as well as the limits of agreement (dashed lines). No formal test of significance is included.

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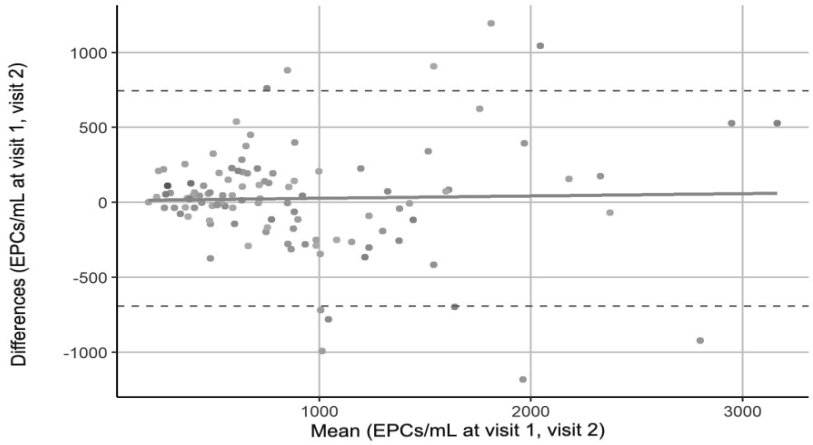

Figure 6: Bland-Altman plot showing the relationship between EPCs/mL differences at visit 1 and visit 2 and mean (visit 1/visit 2); also showing the regression lines between delta and mean (bold solid line) to explore potential dependencies, as well as the limits of agreement (dashed lines). No formal test of significance is included.

Discussion

In this manuscript, we evaluate the test-retest reliability of flow cytometric quantification of CECs and EPCs in patients with HFpEF, HFrEF, DN and HTN for the first time. We carried out a prospective, single-center, investigator-initiated study in which we quantified cell counts with a multicolor flow cytometric protocol from whole blood samples taken at separate points in time and following an identical protocol. We subsequently determined the correlation (ICC) and agreement (Bland-Altman plot) to evaluate short-term test-retest reliability.

If measured reliably and accurately, CECs and EPCs can serve as biomarkers of ED. CECs and EPCs have been described as independent predictors of cardiac events [31,32]. As these cell counts change even before the onset of clinical symptoms of cardiovascular events, timely quantification could help predict the morbidity and mortality of cardiovascular diseases, monitor the response to pharmacotherapy, and prevent progression of these conditions [3,33,34].

Lanuti et al. [10] recently reported their results from a standardized multicenter study in which the median CEC counts in healthy patients was 9.31 CECs/mL (5th/95th percentile:1.85/35.40 CECs/mL) for females and 11.55 CECs/mL (2.53/32.04 CECs/mL) for males, but they defined CECs differently (alive/nucleated/CD45-/CD34bright/CD146+). While they found that CEC counts in healthy patients remained stable over time (3 months), they described high intrasubject and intersubject variability of CEC counts. Ryder et al. [35] reported longitudinally reproducible CEC counts in a cohort of healthy children and adolescents using immunobeads, but similarly found a high intrasubject variability.

We observed poor test-retest reliability of CEC counts in all patient groups as well as in the control group as demonstrated by the low ICCs. Given that absolute numbers of CECs are so low even in patients with CVD, little variations obviously have a great effect on correlation and agreement of repeated tests, which limits the accuracy of our analyses. In addition, in patients with CVD, significant CEC elevations are often transient in nature and CEC counts are highest in patients with acute conditions (e.g., acute coronary syndrome, acute heart failure) [21,36]. Such individuals with acute illness were excluded from this study. We found a relatively wide range of CEC concentrations (0–176 CECs/mL), but with median values of only 12 CECs/mL (V1) and 11 CECs/mL (V2) and only 5% of values being above 22 CECs/mL, many of the enrolled patients had CEC counts that would be considered within the normal range according to Lanuti et al. [10] Our control group of healthy volunteers, in contrast, had lower median CEC counts of 7 CECs/mL (V1) and 3 CECs/mL (V2), which is comparable to what has been described by other authors [37]. Interestingly, the variability of measured CECs/mL appeared to be proportional to the average CEC count, which suggests the biological variability of CECs is higher in patients with elevated CEC counts. The absolute level of CECs has been found to correlate with the degree of endothelial dysfunction [1,36,38]. Thus, the positive correlation between the absolute count of CECs and their biological variability could be explained by the more widespread endothelial damage found in patients with higher absolute CEC counts, involving multiple vascular sites across the body.

Although we attempted to control for variables known to affect endothelial function (e.g., room temperature, exercise, circadian variations, recent intake of alcohol, caffeine, or tobacco, recent changes in medications), we cannot rule out that these factors have impacted the CEC count.

In contrast to the poor test-retest reliability in CECs, we demonstrated moderate test-retest reliability of EPCs in patients with HFpEF and HTN and excellent test- retest reliability in patients with HFrEF and DN. This confirms the reliability of flow cytometric assessment of EPC counts. Moreover, it supports the applicability of this parameter as a surrogate biomarker of endothelial dysfunction in these conditions.

The relatively small sample size of 24–26 patients per patient group, which was determined based on a comparison against the expected threshold ICC of rho1=0.8, limits the generalizability of our findings and the comparison of differences in reliability across the patient groups or in relation to the control group. The limited size of the groups contributes to the wide range of ICC-CIs and high CVs. In addition, we have only evaluated the short-term test-retest reliability (median time between measurements: 7 days) in these patients. Therefore, we cannot draw any conclusions about the long-term test-retest reliability of flow cytometric CEC/EPC quantification.

Further studies should assess the test-retest reliability of flow-cytometric CEC quantification in a larger sample and a broader range of CVDs to investigate the reasons for poor test-retest reliability in more detail. In addition, because CECs are considered as biomarkers for detection of endothelial damage even before it manifests clinically, the test-retest reliability of flow cytometric CEC quantification should further be assessed in a group of asymptomatic individuals with proven early endothelial dysfunction compared to a group of asymptomatic individuals without endothelial dysfunction. Such a study would help to determine the usability of flow-cytometric CEC quantification as a method to detect preclinical vascular injury.

Furthermore, future methods could allow differentiating the phenotype of CECs based on the endothelial bed from which the cells are derived, which would be crucial for a more precise assessment of endothelial function. Considering the functional and antigenic heterogeneity of endothelial cells across different organs and even within an organ [39] a more differentiated evaluation of CECs might be feasible.

Conclusion

In summary, we evaluated, for the first time, the test-retest reliability of flow-cytometric quantification of CEC and EPC counts. The study showed moderate to excellent test-retest reliability of flow-cytometric quantification of EPCs in patients with HFpEF, HFrEF, DN, and HTN, supporting the use of this biomarker in the context of both research and clinical settings. Reliability of CEC enumeration was poor across these patient groups, possibly owing to the rarity of this cellular event even in patients with CVD. Higher biological variability in patients with elevated CEC counts, possibly reflecting more extensive endothelial dysfunction across the vascular system, might also contribute to the poor test- retest reliability of CECs. Reliable enumeration of these cells is the requirement for clinical application of this method of noninvasive assessment of ED. A significant degree of intra-individual variability would limit the clinical applicability of this diagnostic measure in these patient cohorts.

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J. Wagner conducted the clinical study, collected the patient samples, supported the laboratory experiments, discussed the data, and wrote the manuscript. F. Weisrock, M. Fritschka. S. Beckmann, and S. Litmeier supported the clinical study. M. Farinacci carried out the laboratory experiments. E. Tahirovic conceived the study. S. Radenovic contributed to writing the manuscript. A. Busjahn performed the statistical analyses. T. Krahn contributed to writing the manuscript, W. Dinh, and H. Düngen conceived the study and contributed to writing the manuscript.

Authors' contributions

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The trial was supported by an unrestricted grant by Bayer Pharma AG. The study was sponsored by the Charité University Medicine Berlin. The authors report that conduction of the trial and analysis of the data were not impacted by the sponsoring. M. Farinacci has had a research collaboration contract supported by Bayer AG. T. Krahn is an employee and shareholder of Bayer AG. H.-D. Düngen reports travel grants and personal fees from Bayer AG during the conduct of the study. W. Dinh is employed at Bayer AG. All other authors claim no conflicts of interest.

References

1. Boos CJ, Lip GY, Blann AD (2006) Circulating endothelial cells in cardiovascular disease. J Am Coll Cardiol 48:1538-1547.
2. Endemann DH, Schiffrin EL (2004) Endothelial dysfunction. J Am Soc Nephrol 15:1983-1992.
3. Poredos P (2002) Endothelial dysfunction and cardiovascular disease. Pathophysiol Haemost Thromb 32: 274-277.
4. Bonetti PO, Lerman LO, Lerman A (2003) Endothelial dysfunction: a marker of atherosclerotic risk. Arterioscler Thromb Vasc Biol 23:168-175.
5. Burger D, Touyz RM (2012) Cellular biomarkers of endothelial health: microparticles, endothelial progenitor cells, and circulating endothelial cells. J Am Soc Hypertens 6:85-99.
6. Schmidt DE, Manca M, Hoefer IE (2015) Circulating endothelial cells in coronary artery disease and acute coronary syndrome. Trends Cardiovasc Med 25:578- 587.
7. Blann AD, Woywodt A, Bertolini F, Bull TM, Buyon JP, et al. (2005) Circulating endothelial cells. Biomarker of vascular disease. Thromb Haemost 93:228-235.
8. Hill JM, Zalos G, Halcox JP, Schenke WH, Waclawiw WA, et al. (2003) Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. N Engl J Med 348:593-600.
9. Goon PK, Boos CJ, Stonelake PS, Blann AD, Lip GY, et al. (2006) Detection and quantification of mature circulating endothelial cells using flow cytometry and immunomagnetic beads: a methodological comparison. Thromb Haemost 96:45-52.
10. Lanuti P, Simeone P, Rotta G, Almici C, Avvisati G, et al. (2018) A standardized flow cytometry network study for the assessment of circulating endothelial cell physiological ranges. Sci Rep 8:5823.
11. Rafii S (2000) Circulating endothelial precursors: mystery, reality, and promise. J Clin Invest 105:17-19.
12. Mancuso P, Antoniotti P, Quarna J, Calleri A, Rabascio C, et al. (2009) Validation of a standardized method for enumerating circulating endothelial cells and progenitors: flow cytometry and molecular and ultrastructural analyses. Clin Cancer Res 15:267-273.
13. Kachamakova-Trojanowska N, Bukowska-Strakova K, Zukowska M, Dulak J, Jozkowicz A, et al. (2015) The real face of endothelial progenitor cells - Circulating angiogenic cells as endothelial prognostic marker?. Pharmacol Rep 67:793-802.
14. Woywodt A, Goldberg C, Scheer J, Regelsberger H, Haller H, et al. (2004) An improved assay for enumeration of circulating endothelial cells. Ann Hematol 83:491-494.
15. Lanuti P, Rotta G, Almici C, Avvisati G, Budillon A, et al. (2016) Endothelial progenitor cells, defined by the simultaneous surface expression of VEGFR2 and CD133, are not detectable in healthy peripheral and cord blood. Cytometry A 89:259-270.
16. Shantsila E, Watson T, Lip GY (2007) Endothelial progenitor cells in cardiovascular disorders. J Am Coll Cardiol 49:741-752.
17. Valgimigli M, Rigolin GM, Fucili A, Porta MD, Soukhomovskaia O, et al. (2004) CD34+ and endothelial progenitor cells in patients with various degrees of congestive heart failure. Circulation 110: 209-1212.
18. Nonaka-Sarukawa M, Yamamoto K, Aoki H, Nishimura Y, Tomizawa H, et al. (2007) Circulating endothelial progenitor cells in congestive heart failure. Int J Cardiol 119:344-348.
19. Farinacci M, Krahn T, Dinh W, Volk HD, Düngen HD, et al. (2019) Circulating endothelial cells as biomarker for cardiovascular diseases. Res Pract Thromb Haemost 3:49-58.
20. Gevaert AB, Lemmens K, Vrints CJ, Craenenbroeck (2017) Targeting Endothelial Function to Treat Heart Failure with Preserved Ejection Fraction: The Promise of Exercise Training. Oxid Med Cell Longev 2017:4865756.
21. Martinez-Sales V, Sanchez-Lazaro I, Vila V, Almenar L, Contreras T, et al. (2011) Circulating endothelial cells in patients with heart failure and left ventricular dysfunction. Disease markers 31:75-82.
22. Eleftheriadis T, Antoniadi G, Pissas G, Liakopoulos V, Stefanidis I, et al. (2013) The renal endothelium in diabetic nephropathy. Ren Fail 35:592-599.
23. Dharmashankar K, Widlansky ME (2010) Vascular endothelial function and hypertension: insights and directions. Curr Hypertens Rep 12:448-455.
24. Weisrock F, Fritschka M, Beckmann S, Litmeier S, Wagner J, et al. (2017) Reliability of peripheral arterial tonometry in patients with heart failure, diabetic nephropathy and arterial hypertension. Vasc Med 22:292-300.

25. Paulus WJ, Tschope C, Sanderson JE, Rusconi C, Flachskampf FA, et al. (2007) How to diagnose diastolic heart failure: a consensus statement on the diagnosis of heart failure with normal left ventricular ejection fraction by the Heart Failure and Echocardiography Associations of the European Society of Cardiology. Eur Heart J 28:2539-2550.

26. Duda DG, Cohen KS, Scadden DT, et al. (2007) A protocol for phenotypic detection and enumeration of circulating endothelial cells and circulating progenitor cells in human blood. Nat Protoc 2:805-810.

27. Shrout PE, Fleiss JL (1979) Intraclass correlations: uses in assessing rater reliability. Psychol Bull 86:420-428.

28. Koo TK, Li MY (2016) A Guideline of Selecting and Reporting Intraclass Correlation Coefficients for Reliability Research. J Chiropr Med 15:155-163.

29. Cleveland WS (1979) Robust locally weighted regression and smoothing scatterplots. J Am Stat Assoc 74:829-836.

30. Bland JM, Altman DG (1986) Statistical methods for assessing agreement between two methods of clinical measurement. Lancet 1:307-310.

31. Koc M, Richards HB, Bihorac A, Ross EA, Schold JD, et al. (2005) Circulating endothelial cells are associated with future vascular events in hemodialysis patients. Kidney Int 67:1078-1083.

32. Schmidt-Lucke C, Rossig L, Fichtlscherer S, Vasa M, Britten M,et al. (2005) Reduced number of circulating endothelial progenitor cells predicts future cardiovascular events: proof of concept for the clinical importance of endogenous vascular repair. Circulation 111:2981-2987.

33. Werner N, Wassmann S, Ahlers P, Schiegl T, Kosiol S, et al. (2007) Endothelial progenitor cells correlate with endothelial function in patients with coronary artery disease. Basic Res Cardiol 102:565-571.

34. Michowitz Y, Goldstein E, Wexler D, Sheps D, Keren G, et al. (2007) Circulating endothelial progenitor cells and clinical outcome in patients with congestive heart failure. Heart 93:1046-1050.

35. Ryder JR, O'Connell MJ, Rudser KD, Fox CK, Solovey AN, et al. (2016) Reproducibility of circulating endothelial cell enumeration and activation in children and adolescents. Biomark Med 10:463-471.

36. Lee KW, Lip GY, Tayebjee M, Foster W, Blann AD, et al. (2005) Circulating endothelial cells, von Willebrand factor, interleukin-6, and prognosis in patients with acute coronary syndromes. Blood 105:526-532.

37. Jacques N, Vimond N, Conforti R, Griscelli F, Lecluse Y, et al. (2008) Quantification of circulating mature endothelial cells using a whole blood four-color flow cytometric assay. J Immunol Methods 337:132-143.

38. Makin AJ, Blann AD, Chung NA, Silverman SH, Lip GY, et al. (2004) Assessment of endothelial damage in atherosclerotic vascular disease by quantification of circulating endothelial cells. relationship with von willebrand factor and tissue factor. Eur Heart J 25:371-376.

39. Aird WC (2007) Phenotypic heterogeneity of the endothelium: I. Structure, function, and mechanisms. Circ Res 100:158-173.

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