

## METHODOLOGICAL ARTICLE

# Circulating endothelial cells as biomarker for cardiovascular diseases

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### Abstract

**Background:** Endothelial dysfunction is involved in several cardiovascular diseases. Elevated levels of circulating endothelial cells (CECs) and low levels of endothelial progenitor cells (EPCs) have been described in different cardiovascular conditions, suggesting their potential use as diagnostic biomarkers for endothelial dysfunction. Compared to typical peripheral blood leukocyte subsets, CECs and EPCs occur at very low frequency. The reliable identification and characterization of CECs and EPCs is a prerequisite for their clinical use, however, a validated method to this purpose is still missing but a key for rare cell events.

**Objectives:** To establish a validated flow cytometric procedure in order to quantify CECs and EPCs in human whole blood.

**Methods:** In the establishment phase, the assay sensitivity, robustness, and the sample storage conditions were optimized as prerequisite for clinical use. In a second phase, CECs and EPCs were analyzed in heart failure with preserved (HFpEF) and reduced (HFrEF) ejection fraction, in arterial hypertension (aHT), and in diabetic nephropathy (DN) in comparison to age-matched healthy controls.

**Results:** The quantification procedure for CECs and EPCs showed high sensitivity and reproducibility. CEC values resulted significantly increased in patients with DN and HFpEF in comparison to healthy controls. CEC quantification showed a diagnostic sensitivity of 90% and a sensitivity of 68.0%, 70.4%, and 66.7% for DN, HFpEF, and aHT, respectively.

**Conclusion:** A robust and precise assay to quantify CECs and EPCs in pre-clinical and clinical studies has been established. CEC counts resulted to be a good diagnostic biomarker for DN and HFpEF.

### KEYWORDS

biomarkers, cardiovascular diseases, endothelial cells, endothelial progenitor cells, flow cytometry

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## Essentials

- CEC and EPC levels are potential biomarkers of cardiovascular diseases.
- A robust and precise method for the quantification of CECs and EPCs was established.
- CECs and EPCs were quantified in HFpEF, HFrEF, DN, aHT, and healthy controls.
- CEC counts resulted to be a reliable diagnostic biomarker for DN and HFpEF.

## 1 | INTRODUCTION

Cardiovascular diseases are still the major cause of death worldwide.<sup>1</sup> The endothelium does not only form a physical barrier between blood and tissue but has important functional roles in regulation of trafficking, coagulation, and regulation of blood pressure.<sup>2</sup> Impaired endothelial function has been described in diverse disease conditions like diabetes, chronic kidney disease, and hypertension.<sup>3–6</sup> Endothelial dysfunction precedes the full manifestation of many chronic diseases<sup>7,8</sup> and may therefore be a very valuable diagnostic parameter useful for early treatment or prevention of cardiovascular diseases.

The common readout for endothelial function is the endothelium depending vasodilatation driven by the release of endothelium-derived relaxing factors (EDRFs), mainly nitric oxide (NO). The endothelium in its tissue surrounding is difficult to analyze but circulating endothelial cells (CECs)<sup>9–11</sup> may now offer a possibility to assess the integrity and function of the endothelium in order to confirm a diagnosis, predict the course of disease, or support treatment decisions. CECs have been microscopically described already decades ago<sup>12</sup> and their identity was confirmed by specific staining with endothelium-specific antibodies.<sup>13,14</sup> The origin of CECs, their detection methods, and the association with cardiovascular diseases have been reviewed.<sup>9,15–19</sup> Already during vascular damage, CECs are released into the bloodstream suggesting that their increase precede that of established tissue-damage markers like troponins or creatine kinase. Presently, CD146 is the most widely used surface marker for the detection of CECs, the specificity of the detection is sometimes enhanced by addition of other markers, eg, CD31, lack of CD45, or staining with UEA-1.<sup>20</sup>

Mature CECs have to be discriminated from circulating endothelial progenitor cells (EPCs). The progenitor cells are responsible for repair and renewing of damaged endothelium because mature endothelial cells are believed to have only limited regenerative potential.<sup>21</sup> The progenitor cells are bone marrow-derived cells expressing CD34, CD133, and VEGFR2. Also vWF, CD117, and CD144 have been used as EPC markers but may be less specific.<sup>22,23</sup> As with CECs, the exact definition and use of surface markers for EPCs is still under debate.<sup>24</sup>

Although the protocols and surface markers used for enumeration of CECs and EPCs are quite diverse and also the reported baseline values for CECs in healthy volunteers vary significantly from 0 to 7900 cells per ml blood,<sup>15</sup> clear trends emerged from these studies: Increased levels of CECs can be found in hypertension, diabetes, pre-eclampsia, and chronic kidney failure. In contrast, EPCs are reported to be reduced in subjects with cardiovascular risk factors and/or

established atherosclerosis.<sup>9–11</sup> The clear association of CEC and EPC counts with cardiovascular disease biologically validates their potential to estimate the balance between endothelial damage and repair capacity,<sup>9</sup> but some efforts are still required to establish truly validated robust assays for the clinical use of these rare cell populations.

In this work, we established two multicolor flow cytometry panels to count and characterize CECs and EPCs in human whole blood. In order to ensure high quality data, the detection methods were validated based on sensitivity and reproducibility. As CECs counts are very low in healthy individuals the validation experiments for their detection were performed using whole blood samples from healthy donors spiked with human umbilical vein endothelial cells (HUVEC) and lung microvascular endothelial cells (L-HMVEC) at increasing concentrations. The experimental procedure was complemented with the use of transfix tubes in order to allow delayed analysis in multicenter studies. Finally, CECs and EPCs were quantified in patients with diabetic nephropathy (DN), heart failure with preserved ejection fraction (HFpEF), heart failure with reduced ejection fraction (HFrEF), arterial hypertension (aHT), and age-matched healthy donors.

## 2 | MATERIAL AND METHODS

### 2.1 | Subjects and sample collection

Healthy donors were recruited at Clinical Research Services Berlin GmbH, Berlin, Germany, in August 2015 with informed consent and approval of the local ethical committee. The patients with DN, HFpEF, HFrEF, or aHT were enrolled between September 2014 and September 2015 at the Charité Center for Cardiovascular Diseases (ClinicalTrials.gov identifier: NCT02299960). The protocol and informed consent forms were approved by the local ethical committee and all donors provided written consent prior to participation in the trial. Characteristics of patients and healthy donors are listed in Table S1.

Blood samples from patients and controls were drawn in Transfix/EDTA Blood collection tubes (Cytomark, UK). The first 3 mL of blood (potentially contaminated with endothelial cells from the venipuncture) were discarded. Samples were stored and shipped at 4°C and processed within 72 hours of blood collection. For assay validation experiments blood samples were also collected in tubes with Li-Heparin or EDTA for comparison.

### 2.2 | Flow cytometry

Circulating endothelial cells and EPCs were detected by flow cytometry using a panel of monoclonal antibodies and the nuclear staining Syto16 as listed in Tables S2 and S3. CECs were defined as DNA<sup>+</sup>, CD45<sup>dim</sup>,

CD31<sup>+</sup>, and CD146<sup>+</sup>.<sup>25</sup> Microvascular CECs (mvCECs) were identified as CD36<sup>+</sup> CEC.<sup>18,26,27</sup> EPCs were defined as CD45<sup>dim</sup>, CD34<sup>br</sup>, CD133<sup>+</sup>, and CD31<sup>+</sup>, FSC<sup>low-medium</sup>, SSC<sup>low</sup>.<sup>28</sup> The optimal working antibody concentrations were determined by titration experiments using whole blood samples spiked with HUVEC or HMVEC for CEC detection and whole blood alone for EPC detection; for data analysis both the percentage of positive cells and the signal to noise ratio of five serial antibody dilutions were evaluated. In the optimized staining procedure whole blood samples (500  $\mu$ L and 300  $\mu$ L for CECs and EPCs, respectively) were incubated with FcR blocking Reagent (Miltenyi, Bergisch Gladbach, Germany) for 15 minutes at 4°C, and then with the respective antibody mixtures for 40 minutes at 4°C. After red cell lysis with High-Yield Lyse (Life Technologies, Carlsbad, CA, USA) for 15 minutes at RT, the samples were centrifuged and resuspended in FACS buffer (PBS+ 0.5% BSA+ 0.5 mmol L<sup>-1</sup> EDTA+ 0.05% NaN<sub>3</sub>). Due to high variability associated to the detection of cell populations with low frequency, samples for both CECs and EPCs quantification were stained and measured in triplicate. Acquisition was done using a LSR II Flow Cytometer (BD Biosciences, Heidelberg, Germany), equipped with 488-, 633-, and 405-nm lasers. Flow cytometer setup and calibration were performed using CS&T beads (BD Biosciences).<sup>29</sup> For sample acquisition, the mononuclear cells (PMNCs) were set as stopping gate and at least  $5 \times 10^5$  and  $3 \times 10^5$  PMNCs were acquired for CEC and EPC detection, respectively. Data were acquired using FACSDiva 6.0 Software (BD Biosciences) and analysis was performed by using Flowjo 10 (Ashland, OR, USA). The workflow from sample preparation to analysis is summarized in Figure S1. An example of the flow cytometric gating strategy is depicted in Figure 1. Fluorescence compensation was performed by using the BD CompBeads Set Anti-Mouse Ig. CECs and EPCs levels were first calculated as percentage of PMNC. Absolute counts (cells mL<sup>-1</sup>) were then determined by multiplying the CEC or EPC percentage of the PMNC by the absolute PMNC count obtained in separate tubes by using Flow-Count Fluorospheres (Beckmann Coulter, Brea, CA, USA).

### 2.3 | Cell culture and cell spiking

The endothelial cell lines HUVEC (human umbilical vein endothelial cells), L-HMVEC (lung human microvascular endothelial cells), HPAEC (human pulmonary arterial endothelial cells), and HAEC were obtained from Lonza (Basel, Switzerland). HUVEC, HPAEC, and HAEC were cultured in Medium200 supplemented with LSGS kit (Life Technologies), whereas Lung-HMVEC (L-HMVEC) with endothelial cell basal medium (EGM-2; Lonza) containing EGM-2 MV Bulletkit supplements (Lonza). Cell were maintained in culture for no more than six culture passages. For spiking experiments cells were harvested using Accutase (Sigma-Aldrich, St.Luis, MO, USA), and resuspended in phosphate-buffered saline without Ca<sup>2+</sup>Mg<sup>2+</sup>. After determining the cell concentration in a hemocytometer, the cell suspension was added to blood samples to achieve the theoretical cell concentrations of 100, 1000, and 10 000 cells mL<sup>-1</sup> blood. To test the Transfix collection tubes blood samples were collected in EDTA-tubes, mixed with the HUVEC or HMVEC and then added of Transfix solution at ratio 1:5.

### 2.4 | Assay validation

To test the sensitivity of CEC quantification, blood samples from healthy donors were spiked with HUVECs or HMVECs at increasing concentrations in range 100-10 000 cells mL<sup>-1</sup> as described in the previous section. The recovery rate of endothelial cells in spiked whole blood samples was calculated as: (detected ECs concentration)/(spiked EC concentration)  $\times$  100. Assay precision was determined using whole blood samples for EPC detection and whole blood samples spiked with HUVECs or HMVEC for CEC detection. Intra-assay variability was determined by calculating cells counts in four aliquots of the same blood samples. Inter-assay variability and sample stability were determined by measuring the cell counts in duplicate in four independent assays on four different days (0 hour, 24 hours, 48 hours, and 72 hours after blood sampling). Coefficient of variation (CV) was calculated as standard deviation (SD)/average value  $\times$  100.

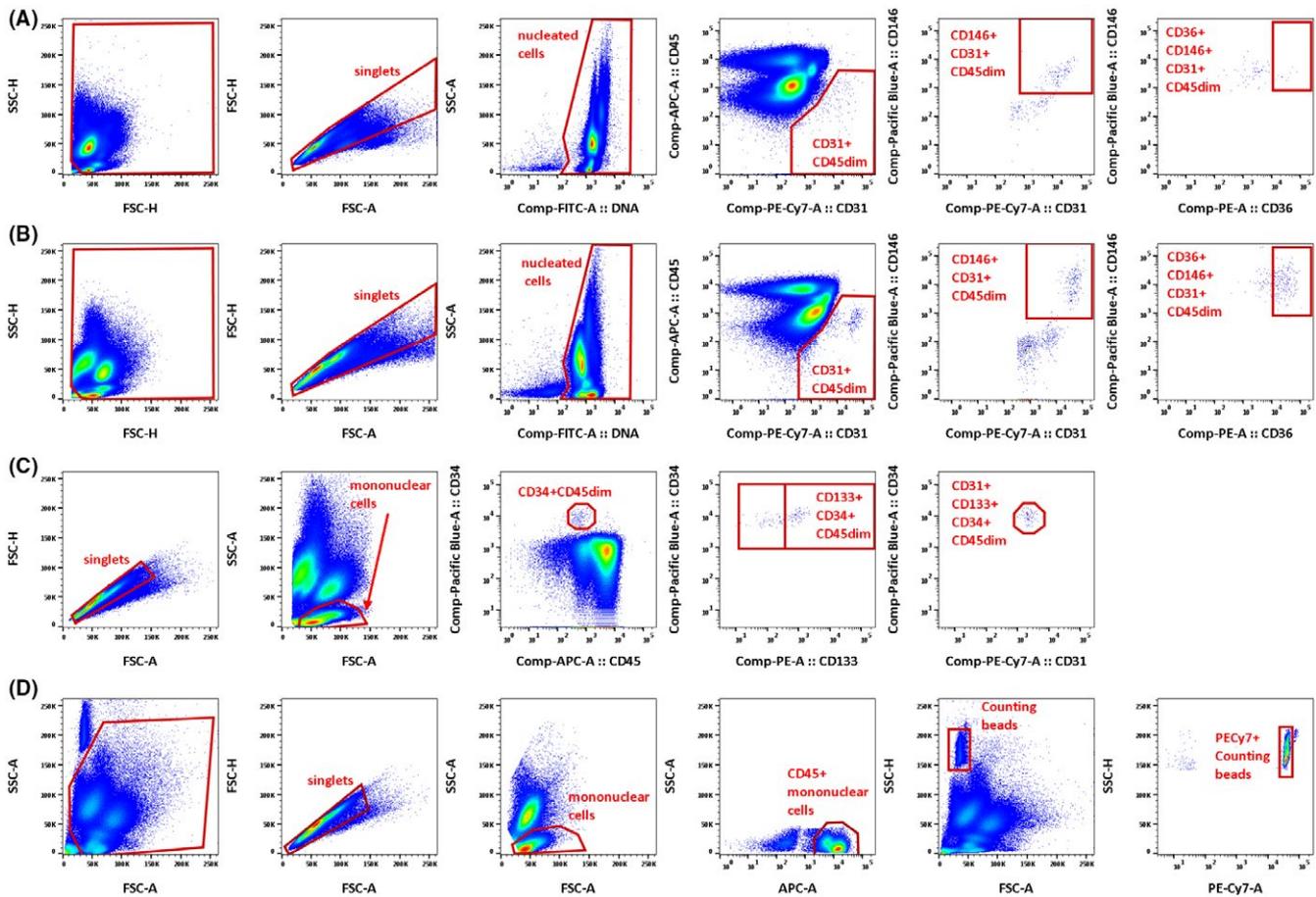
### 2.5 | Statistical analysis

Comparison between groups was performed using GraphPad software (GraphPad Software for Science Inc., San Diego, CA, USA). Cell counts for each group were summarized with medians and interquartile range (IQR). Intergroup differences were evaluated with the nonparametric methods of one-way ANOVA with a Dunnett post-test to compare each patient group to the healthy one. Statistical significance was defined as  $P < 0.05$ . Diagnostic sensitivity/specificity pairs were calculated by varying the threshold level for CEC counts over the range of (median + [0-3SD]). The specificity was calculated as ([true negative]/[true negative + false positive]), the sensitivity for each analyzed disease as ([true positive]/[true positive + false negative]). The positive predictive value was calculated as ([true positive]/[true positive + false positive]) and the negative predictive value as ([true negative]/[false negative + true negative]).

## 3 | RESULTS

### 3.1 | Detection of CECs and EPCs by flow cytometry

Circulating endothelial cells were detected by five-color flow cytometry using the nuclear staining Syto16 and monoclonal antibodies specific for the antigens CD45, CD31, CD146, and CD36. CECs were defined as DNA<sup>+</sup>, CD45<sup>dim</sup>, CD31<sup>+</sup>, CD146<sup>+</sup>, CD36<sup>+/-</sup>, similarly to previously published studies.<sup>25,30</sup> The gating strategy for CEC or mvCECs was as following: after gating out cell debris in the FSC/SSC dot-plot and doublets in the FSC-A/FSC-H dot-plot, DNA<sup>+</sup> cells were identified on the basis of the positivity for Syto16, and then DNA<sup>+</sup> CD45<sup>low</sup> CD31<sup>+</sup> cells were subgated. Finally, CECs were identified subgating from this subpopulation the CD31<sup>+</sup> CD146<sup>+</sup> cells. Microvascular CECs were then identified as CD36<sup>+</sup> CECs. This gating strategy was set and validated using blood samples spiked with HUVECs and HMVECs, as model for respectively macrovascular and microvascular cells (Figure 1B). In particular, the gate for CD36 positivity was set

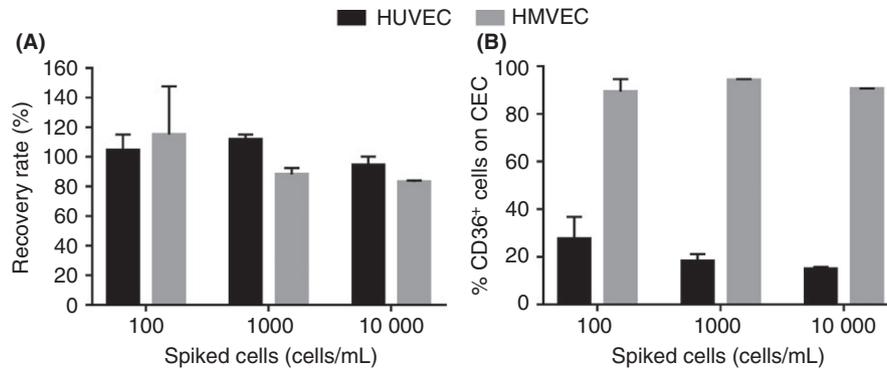


**FIGURE 1** Gating strategy for identification of CECs, EPCs, and for PMNC absolute count. The gating strategy for CEC or mvCECs (A, B) was as following: after gating out cell debris in the FSC/SSC dot-plot and doublets in the FSC-A/FSC-H dot-plot, DNA<sup>+</sup> cells were identified as Syto16<sup>+</sup>, and leukocytes were excluded in the CD45/CD31 dot-plot. Finally, CECs were identified as DNA<sup>+</sup>, CD45<sup>dim</sup>, CD31<sup>+</sup>CD146<sup>+</sup> cells. mvCECs were then identified as CD36<sup>+</sup> CECs. (A) Blood sample from a patient; (B) blood sample spiked with L-HMVEC 1000 cells mL<sup>-1</sup>. For the detection of EPCs (C), cell debris were excluded in the FSC/SSC dot-plot and doublets in the FSC-A/FSC-H dot-plot, mononuclear cells were gated in the FSC/SSC plot and then subgated to identify the CD45<sup>dim</sup> CD34<sup>+</sup> cells. From these cells, the CD45<sup>dim</sup>CD34<sup>+</sup>CD133<sup>+</sup> EPCs were then identified. CD31 expression was analysed to further confirm EPCs identity. Absolute counts of CEC and EPCs were determined multiplying the cell percentages relatively to the PMNCs by the absolute PMNC count obtained in separate tubes using Flow-Count™ Fluorospheres (D). Here, blood samples were only stained with CD45; PMNC were identified by means of size and CD45 expression, the fluorospheres by means of size and fluorescence of PE-Cy7, to exclude the PE-Cy7-blood cells. CECs, circulating endothelial cells; EPCs, endothelial progenitor cells

comparing his fluorescence intensity on HUVECs, of macrovascular origin, and HMVECs, of microvascular cells. CEC analysis in patient samples confirmed the setting correctness, as showed in Figure 1A (CEC detected in a blood sample from a patient) and Figure 1B (CEC detected in a blood sample spiked with HMVEC). Antibody panel and gating strategy for EPCs were adapted from the method of Duda et al.<sup>28</sup> (Figure 1C). EPCs were defined as CD45<sup>dim</sup>, CD34<sup>br</sup>, CD133<sup>+</sup>, and CD31<sup>+</sup>, FSC<sup>low-medium</sup>, SSC<sup>low</sup>. The gating strategy was as following: after gating out cell debris in the FSC/SSC dot-plot and doublets in the FSC-A/FSC-H dot-plot, mononuclear cells were gated on the FSC/SSC plot and then subgated to identify the CD45<sup>dim</sup> CD34<sup>+</sup> cells. From these cells, the CD45<sup>dim</sup>, CD34<sup>+</sup>, and CD133<sup>+</sup> EPCs were then identified. EPC identity was further confirmed analyzing CD31 expression. Positive/negative boundaries were set through FMO controls using matched isotype controls (Figure S2).

### 3.2 | Detection recovery of CECs in whole blood samples

Assay sensitivity was assessed through spiking experiments, where known amounts of HUVECs and L-HMVECs (100, 1000, 10 000 cells mL<sup>-1</sup>) were spiked into peripheral blood samples from healthy volunteers. The number of detected CECs in the spiked samples was corrected with the basal levels of non-spiked samples and then compared with the known number of spiked cells. As shown in Figure 2, the mean percentages of recovered HUVECs and HMVECs ranged between 83% and 115% for the three levels of spiking concentrations. The level of CD36 staining on L-HMVEC remained stable at the different spiked cell concentrations tested; the percentage of CD36<sup>+</sup> cells at 100, 1000, and 10 000 cells mL<sup>-1</sup> were, respectively, 89.3%, 94.2%, and 90.5% for the L-HMVECs. A background/not-specific staining of CD36



**FIGURE 2** Performance of CEC detection and identification of microvascular cells. The graph in (A) shows the recovery rate of HUVEC and L-HMVEC spiked in a healthy whole blood sample at 100, 1000 and 10 000 cells mL<sup>-1</sup>. The graph in (B) shows the percentage of CD36<sup>+</sup> positive cells detected on HMVEC and HUVEC at increasing spiking concentrations as in (A). The results are expressed as mean ± SD of duplicate quantification. CECs, circulating endothelial cells

was observed on a small percentage of HUVEC, which decreased at higher spike numbers; the percentage of CD36<sup>+</sup> cells at 100, 1000, and 10 000 cells mL<sup>-1</sup> were, respectively, 27.6%, 18.2%, and 14.9%. The specificity of CD36 expression to discriminate between microvascular and macrovascular cells was assessed in preliminary experiments by comparing the percentage of CD36<sup>+</sup> cells between different macrovascular endothelial cell lines (HPAEC, HAEC, HUVEC) and L-HMVEC (data not shown).

### 3.3 | Sample stability and quantification assay precision

In preliminary experiments the quantification and stability of CEC and EPC levels was assessed in blood samples collected in EDTA, Li-Heparin, or Transfix tube (Figure 3A-C). For both CEC and EPC detection the highest sample stability and assay precision were achieved when collecting blood samples with Transfix tubes. After 72 hours, the EPC levels measured (Figure 3A) in the Transfix samples were 109% of the initial value (T = 0 hour), but they decreased to 77.1% and 79.1% when the samples were collected in EDTA and Li-Heparin, respectively. Moreover, the inter-assay variation between the analyses at 0 hour, 24 hours, 48 hours, and 72 hours was much lower in Transfix (6.7%) than in EDTA and Li-heparin tubes (17.7% and 10.5%, respectively). The levels of CECs quantified in EDTA and LH tubes decreased dramatically at 24 hours and later time points (Figure 3B). In contrast, collection of blood in Transfix tubes allowed detection of endogenous CEC (Figure 3B) as well as of HUVEC and HMVEC spiked in healthy whole blood (Figure 3D) up to 72 hours. The levels of mvCEC was very low in healthy blood samples already at time 0 h (Figure 3C). However, the analysis of spiked HMVEC revealed that the staining of CD36 is not affected by Transfix fixation and sample storage (Figure 3E), allowing stable discrimination between macrovascular and microvascular cells. The intra-assay and inter-assay variations for the detection of both spiked endothelial cells and EPCs (Table 1) were lower than 20%, proving the reproducibility of the developed quantification method.

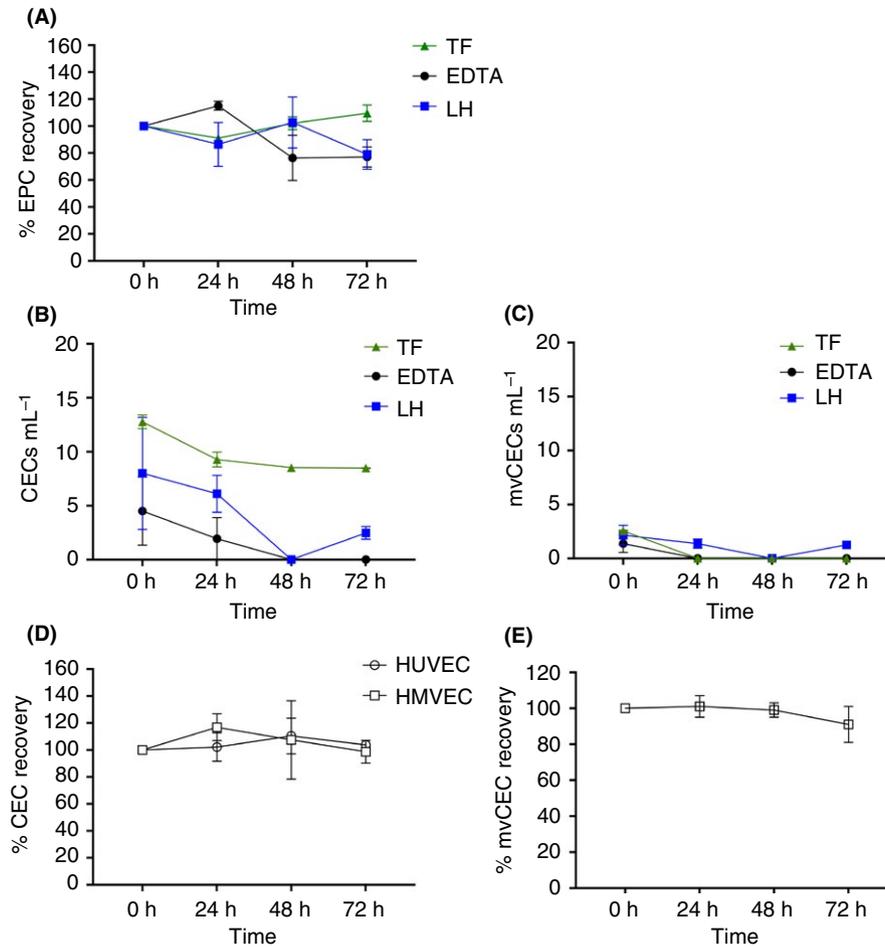
### 3.4 | CEC and EPC levels in clinical samples

Circulating endothelial cells and EPCs were quantified in patients with DN, HFpEF, HFREF, aHT, and age matched healthy controls. Patient characteristics are shown in Table S1. Blood samples were collected in Transfix-tubes and analyzed between 1 and 3 days after collection. CEC counts were significantly elevated in DN (16.7 [9.7-28.1] cells mL<sup>-1</sup>,  $P < 0.05$ ) and HFpEF (23.0 [8.1-31.7] cells mL<sup>-1</sup>,  $P < 0.05$ ) in comparison to healthy individuals (5.5 [4-7.3] cells mL<sup>-1</sup>), whereas CEC levels were not elevated in HFREF (11.7 [4.7-28.5] cells mL<sup>-1</sup>) and aHT (14.40 [6.3-21.4] cells mL<sup>-1</sup>) (Figure 4; Table S4). The proportion of microvascular cells of total CEC was significantly increased in HFpEF (66.7% [46.3-71.2];  $P < 0.01$ ), and aHT (62.2% [51.7-79.7],  $P < 0.01$ ) in comparison to healthy controls (27.5% [14.9-51.8]), but not in DN (59.4% [39.7-70.4]) and HFREF (52.2% [22.7-70.3]).

Endothelial progenitor cell levels were similar between the different groups analyzed, with values of 831 (556.8-1138.0) cells mL<sup>-1</sup> in healthy volunteers, 661.0 (466.0-1267.0) cells mL<sup>-1</sup> in DN, 716.0 (527.0-904.0) cells mL<sup>-1</sup> in HFpEF, 853.0 (514.5-1889) cells mL<sup>-1</sup> in HFREF, and 801.5 (477.5-1186) cells mL<sup>-1</sup> in aHT.

### 3.5 | Diagnostic values of CEC count for DN and HFpEF

Comparing the cohorts of DN, HFpEF, HFREF, and aHT with the healthy group we set the optimal CEC cutoff at 10.5 cells mL<sup>-1</sup> (= median value + 1 SD in healthy controls), as shown in Figure 5C. For the diagnosis of DN this cut-off gave a sensitivity of 68% (95% CI 46.5%-85.1%), and a PPV of 94.4% (72.7-99.9). For HFpEF the sensitivity was of 70.4% (49.8%-86.3%) and the PPV was 95% (75.1%-99.9%). The sensitivity for aHT detection was of 66.7% (44.7%-84.4%) and the PPV of 94.1% (71.3%-99.9%); the sensitivity for HFREF was 52% (31.3%-72.2%), and the PPV 92.8% (66.1%-99.8%). The CEC counts showed a diagnostic specificity of 90% (55.5%-99.8%), and a NPV of 52.9% (27.8%-77%) for all diseases



**FIGURE 3** Stability of EPCs (A), CECs (B, D), and mvCECs (C, E) in blood samples collected with different anticoagulants. Analyses were performed on fresh blood samples, 0 h, and after 24 h, 48 h and 72 h of storage at 4°C. EPC (A) and CEC (B, C) were quantified on whole blood samples collected from healthy donors in Transfix, EDTA or Lithium Heparin. Recovery of CECs and mvCECs (D, E) in Transfix tubes was assessed on whole blood samples spiked with HUVEC or L-HMVEC at 100 cells mL<sup>-1</sup> and expressed in percentage of time=0 h. Results are expressed as mean ± SD of two or three independent experiments. TF, transfix; LH, Lithium Heparin; CECs, circulating endothelial cells; EPCs, endothelial progenitor cells

**TABLE 1** Assay precision. The intra assay variations were measured as the CV value of four replicates. The inter assay variations were calculated on three independent cell quantifications on the same blood samples

Cell population	Inter-assay CV, %	Intra-assay CV, %
CECs mL <sup>-1</sup>	13.0	15.1
mvCECs mL <sup>-1</sup>	26.1	11.2
EPCs mL <sup>-1</sup>	6.7	9.9

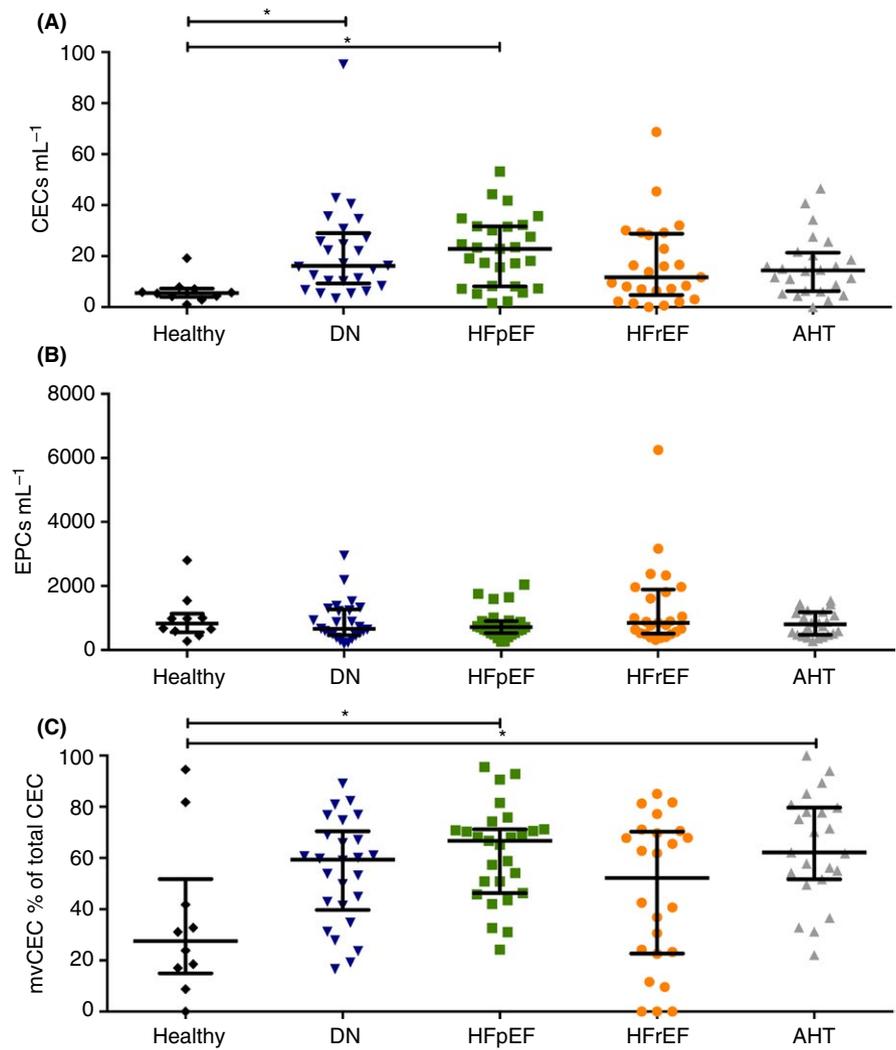
CECs, circulating endothelial cells; CV, coefficient of variation; EPCs, endothelial progenitor cells; mvCECs, microvascular circulating endothelial cells.

except for HFREF where NPV was of 42.9% (21.8%-65.9%). The values of diagnostic sensitivity and specificity are showed in Figure 5A,B.

## 4 | DISCUSSION

We have developed and validated a flow cytometric assay for enumeration of CECs and EPCs and shown that CEC counts are significantly elevated in cardiovascular diseases like DN, HFpEF, and aHT.

The CEC counts observed in healthy individuals (5.5 [4-7.3] cells mL<sup>-1</sup>) using our method were very similar to those obtained by Jacques et al.<sup>30</sup> (6.5 [0-15 cells mL<sup>-1</sup>]), where CECs were defined as CD31<sup>+</sup>CD146<sup>+</sup>CD45<sup>-</sup>7AAD<sup>-</sup> (7-amino-actinomycin-D). Moreover, our results are comparable to those obtained by other detection techniques like IHC,<sup>31</sup> IMS,<sup>20,32</sup> and CellSearch<sup>33</sup> (3.8 [0.75-16.75] cells mL<sup>-1</sup>). In contrast very high CEC counts (140 ± 171 cells mL<sup>-1</sup>) were detected by Mancuso et al.,<sup>25</sup> which used a CECs definition similar to our (CECs: Syto16<sup>+</sup>CD45<sup>-</sup>CD31<sup>+</sup>CD146<sup>+</sup>). But, however, as mentioned in Strijbos et al.,<sup>34</sup> the methods published by Mancuso et al.<sup>25,35</sup> yield much higher CEC numbers than typically reported in literature (ie, 20 cells mL<sup>-1</sup>) and should therefore be considered as unspecific.



**FIGURE 4** CECs, EPCs and mvCECs in blood samples from patients with DN, HFpEF, HFREF, aHT, and healthy individuals. Graphs of CECs (A), EPCs (B), and mvCECs (C) show median and interquartile range (grey bars). Statistical analysis was performed by ANOVA with Dunnett post-test to compare each patient group to the healthy one. \* $P < 0.05$ . CECs, circulating endothelial cells; EPCs, endothelial progenitor cells; DN, diabetic nephropathy; HFpEF, heart failure with preserved ejection fraction; HFREF, heart failure with reduced ejection fraction; aHT, arterial hypertension

In comparison to the already established methods for CEC and EPC quantification, our protocol has the following distinctive features that ensure high precision, sensitivity and robustness: (a) cell counts measured in each sample in triplicate; (b) acquisition settings setup with the BD CS&T beads, which allows for consistent MFI and gating over time; and (c) stable CEC and EPC levels in Transfix tubes.

Circulating endothelial cells have been described as a marker for damaged endothelium in several cardiovascular conditions such as chronic kidney disease, coronary heart disease, and peripheral arterial disease (described elsewhere<sup>36</sup>).

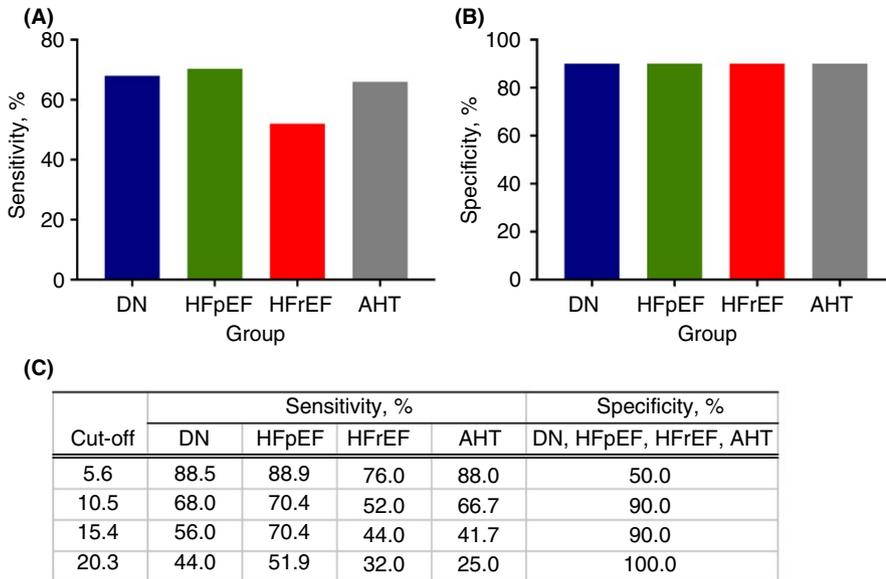
After optimizing the detection protocol, we tested CEC and EPC counts in patients with DN, HFpEF, HFREF, or aHT, which may be caused or associated with endothelial dysfunction.

In patient samples from all tested indication, elevated CEC levels were detected, and these changes were highly significant in HFpEF and in DN. In patients with HFpEF and aHT there was also a significant increase in the proportion of microvascular CECs, as assessed by CD36 staining, in comparison to healthy individuals. A similar finding was also reported in patient with Sickle cell anemia<sup>31</sup> and with pulmonary hypertension.<sup>32</sup> Although the distinction between micro- and macrovascular cells assessed on the basis

of CD36 may not be perfect (as some positive signal in HUVEC and previous literature<sup>26</sup> show) the elevated levels of microvascular CECs are in line with the endothelial dysfunction as systemic disease.

Elevated levels of CECs in heart failure have been described<sup>37</sup> with levels doubling in stable angina and approximately four-fold increase in acute heart failure but without discrimination between HFpEF and HFREF. Our study is to our knowledge the first work that compared CEC counts between HFpEF and HFREF.

Heart failure with preserved ejection fraction is an increasingly common condition which is difficult to diagnose.<sup>38</sup> This is an issue as some of the common treatments used for HFREF, eg,  $\beta$ -blockers, have not improved prognosis of HFpEF in clinical trials.<sup>39</sup> Better diagnostic tools are required for early differential diagnosis between HFpEF and HFREF. Based on our finding, CECs may be further developed as early diagnostic marker for HFpEF. Our finding that CEC counts are higher in HFpEF compared to HFREF are in line with endothelial dysfunction as underlying disease mechanism (reviewed elsewhere<sup>40</sup>): Endothelial dysfunction is associated with HFpEF as shown by Akiyama et al.<sup>41</sup> and has prognostic value.<sup>42</sup> Mohammed and colleagues also established that myocardial hypertrophy,



**FIGURE 5** Diagnostic sensitivity and specificity. Diagnostic sensitivity (A) and specificity (B) of CEC counts calculated for DN, HFpEF, HFReEF and aHT considering a cutoff at 10.5 cells mL<sup>-1</sup>. The table in (C) shows the levels of sensitivity and specificity obtained with cut-off values higher or lower than 10.5. CEC, circulating endothelial cell; DN, diabetic nephropathy; HFpEF, heart failure with preserved ejection fraction; HFReEF, heart failure with reduced ejection fraction; aHT, arterial hypertension

fibrosis, and imbalance of coronary microvascular destruction and regeneration are associated with HFpEF.

The mechanistic link between endothelial dysfunction and HFpEF is recently emerging as reviewed by Borlaugh and Paulus<sup>43</sup> and by Gevaert et al.<sup>44</sup>: myocardial proliferation and stiffness are major reasons for the reduced diastolic ventricular filling. Collagen deposition and intrinsic cardiomyocyte stiffness are major factors, caused by reduced MMP and elevated TIMP levels on one hand and on expression of specific isoforms of Titin and their phosphorylation status on the other hand. The common denominator of these changes is the endothelial dysfunction, which leads to lower levels of nitric oxide signaling in the blood vessels and also in the cardiomyocytes. The resulting lower cGMP concentrations increase calcium sensitivity and therefore prevent diastolic relaxation and lower PKG activity, which is required for Titin phosphorylation. Therefore, endothelial dysfunction is a mechanism contributing to HFpEF.

The CEC counts showed to be a highly specific marker for diagnosis of DN and HFpEF, but with limited sensitivity. There have been two previous studies that have investigated the diagnostic value of CECs in acute coronary syndrome (ACS).<sup>16,45</sup> In both these investigations, CEC counts showed, in accordance with our results, high specificity but moderate sensitivity for ACS and for myocardial infarction (NSTEMI and STEMI).

With the improved sensitivity, our validated assay opens the perspective to a future use as additional diagnostic tool for early detection of cardiovascular diseases associated with endothelial damage.

In addition to CECs, EPCs were tested in our study. EPCs are usually quantified and analyzed by flow cytometry on fresh samples because of the high sensitivity and specific of this methodology.<sup>46</sup>

In our study we quantified EPCs by flow cytometry adapting an antibody panel established by Duda et al.<sup>28</sup> EPC counts in healthy individuals were similar to those described by Duda et al.<sup>28</sup> EPC detected in our control group were around 0.03% (0.02-0.06) of

mononuclear blood cells, in comparison to 0.01%-0.2% of mononuclear blood cells reported by Duda et al.

Endothelial progenitor cell levels did not significantly change in patients in comparison to healthy individuals. We believe that the patients included in our study still were in relatively good condition as shown by their low NYHA status (Table 1), and that EPC counts are only reduced in the advanced disease stages.

Therefore, we conclude that the CEC level is a more sensitive marker for vascular damage compared to EPC level, which might be increased secondarily as a repair mechanism due to more severe vascular damage.

Future studies should concentrate on analyzing the diagnostic value of CEC counts in a broader range of cardiovascular diseases. Determination of CEC counts during and after therapy will be crucial to understand their potential use for diagnosis and therapy monitoring.<sup>47,48</sup>

Elevated CEC counts may even serve as early diagnostic tool for endothelial damage in pre-symptomatic disease. As CECs are the more sensitive biomarker for endothelial damage, further efforts should concentrate on improving the sensitivity of CEC detection in order to increase diagnostic sensitivity.

The reliable isolation of CECs may also give rise to their functional characterization helping to increase the understanding of the underlying disease mechanisms.

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## RELATIONSHIP DISCLOSURES

M. Farinacci reports to have been employed with a research collaboration contract supported by Bayer AG. T. Krahn is an employee and

shareholder of Bayer AG. H.-D. Dungen reports grants and personal fees from Bayer AG during the conduct of the pre-clinical study. O. van Ahsen and W. Dinh are employees at Bayer AG. T. Konen worked as paid student, during which the work contributed was done. All other authors claim no conflicts of interest.

## AUTHOR CONTRIBUTIONS

M. Farinacci designed and carried out experiments, analyzed and discussed the data, and wrote the manuscript. Oliver von Ahsen designed the experiments, discussed the data, and wrote the manuscript. T. Konen and J. Wagner helped by collecting and performing the analysis on the patient samples. T. Krahn and H.-D. Volk conceived the project and discussed the data. T. Krahn, W. Dinh, and H. Dungen conceived and directed the preclinical study with the patients.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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