Enrichment of Circulating Endothelial Cells by CD34 Microbeads Followed by Enumeration Using Flow Cytometry

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Abstract

Background  Circulating endothelial cells (CECs) are a potential biomarker of angiogenesis. CECs increase in numbers after vessel injury. Higher CEC numbers are reported in cancer patients. Most methods for CEC detection and enumeration rely on flow cytometry (FCM); however, there is no agreement on CEC phenotype and the detection method to be used. This leads to uncertainty about the clinical applicability and variation between studies on CEC numbers reported.

Objective  To develop a selective and accurate method for CEC enumeration in peripheral blood by enrichment, followed by FCM in healthy volunteers (HV) and cancer patients.

Methods  Samples were enriched using CD34 microbeads, stained with nuclear dye and anti-CD14, CD15, CD45, CD34 and CD146 antibodies. Putative CECs were examined for Weibel–Palade bodies (WPBs) using anti–von Willebrand factor (vWF) antibody and fluorescence microscopy. Linear range of detection ($R^2$), recovery and precision (coefficient of variation percentage [CV%]) were defined in three experiments by spiking a known number (range 12–12,800 CECs/4 mL) of surrogate endothelial cells in peripheral blood. Sample storage was determined at –80°C for up to 2 months.

Results  Sorted CECs showed vWF in the WPBs. The relationship between spiked and detected surrogate cells was $R^2 = 1.0$, recovery of 94.0 to 101.4% and CV% of 1.0 to 18.4%. Recovery ± standard deviation (within-run days 1, 2 and 3) were, respectively, 102.5% ± 8.2, 97.8% ± 4.6, 99.1% ± 7.7, and after 2 months 94.3% ± 15.3. The median CECs/mL in patients was 24.1 versus 14.4 in HVs.

Conclusion  This method for selective, sensitive and reliable CEC analysis by FCM allows for investigation of CECs as a biomarker in clinical research.

Keywords

► circulating endothelial cells
► enrichment
► CD34 magnetic microbeads
► flow cytometry
► angiogenesis

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Introduction

Angiogenesis contributes to the process of tumour development, proliferation and metastasis by formation of new vessels. Inhibition of angiogenesis could therefore result in growth stabilization and ultimately regression of tumours. This has led to the development of anti-angiogenic agents that inhibit the vascular endothelial growth factor (VEGF), for instance bevacizumab, or its receptor (VEGFR) such as sorafenib and sunitinib. The availability of these new anti-angiogenic agents has driven the search for biomarkers that reflect the extent of disease activity and the effect of therapy. However, no adequate, easily applicable, predictive and prognostic biomarker has been identified at present. The absence of a biomarker for angiogenesis also impedes selection of patients who might benefit most from these agents.

A candidate biomarker for angiogenesis are circulating endothelial cells (CECs).1–6 CECs are mature endothelial cells that have shed from the vessel wall, for instance as a response to injury. Due to poor vessel formation, CECs are more commonly seen in cancer patients.1,7–9 Their role in angiogenesis is unclear; however, it is hypothesized that they may contribute to tumour vessel formation by stimulating pro-angiogenic factors.10 The estimated median CEC numbers reported vary from 8 to 18 CECs/mL in healthy subjects to 11,900 CECs/mL in cancer patients.7,11 However, reported results are conflicting about the relationship between CECs and their prognostic and predictive value as a biomarker. Important factors contributing to discrepancies between studies evaluating CECs as a biomarker include the variety of applied methods, different CEC phenotype definitions and analysis of activated or resting CEC subsets.1,12,13 Numerous methods for the CEC enumeration rely on immunomagnetic bead isolation, followed by microscopy like the fully automated CellSearch method. Others have used flow cytometry (FCM) with a sample enrichment step using immunomagnetic beads.7,14–18

The advantages of the use of FCM for CEC analysis include the opportunity of simultaneous analysis of a wide spectrum of antigens increasing the selectivity while decreasing the analysis time. The commonly used antigens defining CECs include CD31, CD34, CD105, CD144, CD146, von Willebrand factor (vWF), Ulex Europaeus Lentin 1. However, an incorrectly designed FCM-based CEC analysis method may lead to overestimation of the CEC count as result of the phenotypic overlap with haematopoietic cells, thrombocytes and apoptotic or dead cells, and their resulting microparticles.16,19 The selectivity of a method can be improved by addition of the pan-haematopoietic antigen CD45, and a nuclear and/or viability dye that can exclude thrombocytes, dead cells and microparticles.8,20 Moreover, false-positive counts caused by the non-specific binding of antibodies to the Fc-receptors on haematopoietic cells can be prevented by using a blocking buffer.

The advantages of using immunomagnetic beads for enrichment for analysis of rare cells in the blood circulation are as follows: depletion of redundant blood cells, improved sample purity and sample volume reduction. Important disadvantages are as follows: a possible low yield of recovered cells, time consuming and high cost. The success of using immunomagnetic beads for enrichment depends on the size of the rare cell population to be isolated, the negative or positive selection approach chosen and the level of expression of the target antigen.

In this article, we describe a validated method for the enumeration of CECs from peripheral blood by FCM. The aim of this study was to develop a selective and accurate method for enumeration of CECs in both fresh and cryopreserved (at −80°C) peripheral blood of healthy volunteers and cancer patients. This method uses sample enrichment by CD34 magnetic microbeads, subsequent staining for endothelial antigens (CD34, CD146) and haematopoietic antigens (CD14, CD15, CD45) antigens, followed by a nuclear staining using Hoechst33258. To ensure that exclusively CECs were enumerated, cells that were expected to be CECs based on the predefined phenotype were isolated using a cell sorting system followed by intracellular staining of vWF, which is stored in the Weibel–Palade bodies (WPBs), a specific cell organelle for CECs. Sorted cells were then morphologically examined under a microscope to confirm their CEC phenotype. The recovery and coefficient of variation of the method were assessed in peripheral blood samples spiked with the surrogate human umbilical vein endothelial cells (HUVECs). The stability of the endogenous CECs was assessed in peripheral blood samples cryopreserved at −80°C for up to 2 months.

Methods

Patients and Volunteers

CEC numbers were studied in two clinical protocols. In the first protocol, registered in the Netherlands Trial Register as NTR3632, daily oral administration of paclitaxel co-administered with ritonavir was investigated. In this trial, CECs were collected prior to start and at day 2, 8, 22, 43 and every 6 weeks thereafter. The second study was designed to evaluate CEC numbers in both cancer patients and healthy volunteers (HVs). From patients included in a clinical phase I trial, samples were drawn prior to start and weekly thereafter for up to five times. The treatments administered are presented in Table 1. HVs were asked to provide five samples at weekly intervals. Both studies were approved by the ethics committee of the Netherlands Cancer Institute and both patients and HVs were fully informed and had to provide written informed consent prior to sample collection. Patients with a WHO (World Health Organization) performance score of 0 to 2 were included in the clinical studies.

Blood Sample Collection

Peripheral blood was drawn in 4 mL CTAD (citrate, theophylline, adenosine and dipyridamole) tubes (Becton Dickinson B.V., Vianen, the Netherlands), in triplicate from HVs and in duplicate or triplicate from cancer patients. The blood was transferred into a 50-mL tube and 46 mL of erythrocyte lysis buffer (ELB; 0.15-M ammonium chloride) was added for 15 minutes at room temperature (RT), centrifuged at 500 g for 7 minutes at 4°C. Thereafter, lysis buffer was removed and the cell pellet was resuspended in 50 mL of phosphate buffered saline (PBS; Gibco Life Technologies, Fisher Scientific, Landsmeer, the Netherlands) supplemented with 0.5% BSA and 2-mM EDTA (ethylenediaminetetraacetic acid;
Table 1 Characteristics of the patients and healthy volunteers

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Patients</th>
<th>Healthy volunteers</th>
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</thead>
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<tr>
<td>Total number</td>
<td>39</td>
<td>11</td>
</tr>
<tr>
<td>Male: Female</td>
<td>21 (54%)</td>
<td>5 (45%)</td>
</tr>
<tr>
<td>Mean age (range), y</td>
<td>59 (38–74)</td>
<td>34 (26–49)</td>
</tr>
</tbody>
</table>

Note: Percentages have been rounded off.

Gibco Life Technologies, Fisher Scientific, Landsmeer, the Netherlands; hereafter washing buffer), washed by centrifugation at 500 g for 7 minutes at 4°C. Pelleted cells were resuspended in 1 mL of 4% formaldehyde and incubated for 15 minutes at RT. The cell suspension was then washed in 49 mL of washing buffer and centrifuged at 500 g for 7 minutes at 4°C. Pelleted peripheral blood mononuclear cells (PBMCs) were enriched using the CD34 MicroBead kit (Miltenyi Biotec B.V., Leiden, the Netherlands) and the magnetic-activated cell sorting (MACS) method, or the PBMC pellet was resuspended in 1.5 mL of freezing medium, which consisted of RPMI 1640 supplemented with 20% FBS and 10% DMSO. The specimens were kept on ice. Tumour type was colorectal (49%, 12/25 of the total number of patients), lung (21%, 5/25), and miscellaneous (24%, 6/25). Prior treatment included systemic therapy (79%, 31/39 of the total number of patients), radiotherapy (38%, 15/39), surgery (44%, 17/39), and treatment in study (78%, 31/39). Human Umbilical Vein Endothelial Cell Isolation and Purification

HUVECs were isolated by trypsin digestion. Briefly, the umbilical cord vein was rinsed with PBS, trypsin-EDTA (0.5%) was injected into the vein and incubated for 5 minutes at 37°C to digest. Digested content was flushed into a 50-ml tube, washed in RPMI 1640 with 10% FBS and centrifuged at 1,200 rpm for 10 minutes. Isolated HUVECs were resuspended in 40-µl human FcR blocking reagent (Miltenyi Biotec B.V., Leiden, the Netherlands) and 50-µl BSA 4% (Sigma-Aldrich Chemie N.V., Zwijndrecht, the Netherlands) in PBS and incubated for 1 hour on ice. Then mouse antihuman antibodies were added to an HUVEC sample: CD14-PE-Cy7 (clone TÜK4), CD15-PE-Cy7 (VIMC6), CD34-APC (AC136), CD45-FITC (5B1) and CD146-PE (541–10B2). To define background staining: (1) unstained cells, (2) cells stained with CD14-PECy7, CD15-PE-Cy7, CD34-APC, CD45-FITC, mouse isotype IgG1-PE (clone IS5–21F5; Miltenyi Biotec B.V., Leiden, the Netherlands), incubated for 1 hour on ice. Samples were washed in washing buffer and centrifuged at 1,000 g for 4 minutes at 4°C. HUVECs were purified from the digested content, by fluorescence-activated cell sorting (FACS) sorting on an FACS Aria II equipped with three lasers (excitation wavelength: 405, 488 and 633 nm), an 85-µm nozzle and Diva software (Becton Dickinson B.V.). HUVEC phenotype was CD14–, CD15–, CD45–, CD34+, CD456– and Hoechst33342+. HUVECs were sorted into a 2-ml Eppendorf tube containing HUVEC optimized growth medium EGM-2 BulletKit (Lonza Benelux B.V., Breda, the Netherlands) containing 2% FBS and VEGF. Collected HUVECs were directly used for further culturing or stored at −80°C as follows: HUVECs were centrifuged at 1,000 g for 4 minutes at 4°C and the pellet was resuspended in freezing medium consisting of HUVEC optimized growth medium EGM-2 supplemented with 20% FBS and 10% DMSO. Purified HUVECs were cultured in the HUVEC optimized growth medium EGM-2 BulletKit. The cells were cultured according to manufacturer's instructions in a 25 cm² flask till confluent. Confluent cells were harvested using trypsin-EDTA and seeded on glass coverslips in a 12-well plate and cultured till confluent. The medium was removed, the cells were washed in PBS and then fixed in cold (−20°C) methanol (Sigma-Aldrich Chemie N.V., Zwijndrecht, the Netherlands) for 1 minute on ice. After methanol fixation, the cells were washed in PBS and blocked in PBS supplemented with 10% FBS for 45 minutes at RT, followed by incubation for 15 minutes at RT in the dark with polyclonal sheep antihuman vWF-FITC (1:1,000; Abcam, Cambridge, United Kingdom), an immunoglobulin G (IgG) isotype-FITC antibody was used to define the background (Abcam, Cambridge, United Kingdom) and Hoechst33342 (100 µM; Sigma-Aldrich Chemie N.V., Zwijndrecht, the Netherlands). The specimen was washed in PBS, dehydrated and inversely layered on a microscopic glass with mounting medium VectaShield H–1000 in between. The background of the anti-vWF-FITC and its specificity were defined by staining PBMCs, freshly isolated and FACS-sorted HUVECs, and cultured HUVECs (supplementary data). The specimen was analysed on a Zeiss Axio Observer Z1 inverted microscope (Carl Zeiss B.V., Breda, the Netherlands) equipped with Hamamatsu ORCA AG Black and White CCD camera (Hamamatsu Photonics, Almere, the Netherlands) and analysed with ZEN lite software (Carl Zeiss B.V., Breda, the Netherlands).
Peripheral Blood Sample Enrichment and Immunostaining

The cryostored PBMC samples were quickly defrosted in a water bath at 37°C, centrifuged at 1,000 g for 4 minutes at 4°C, pelleted sample was washed in washing buffer and centrifuged at 1,000 g for 4 minutes at 4°C. Pelleted PBMCs were resuspended at 40-µL FcR blocking reagent and 50-µL BSA 4% in PBS and incubated for 1 hour on ice. Washing buffer was added to achieve a final volume of 500 µL, then 20 µL of CD34 MicroBeads was added; samples were incubated with overnight rotation for 1 hour at 4°C. Unbound CD34 MicroBeads were washed away twice by adding washing buffer, centrifuged at 300 g for 10 minutes at 4°C, and the washing buffer was discarded. The magnetically labelled cells were then immunostained with a mix of mouse antihuman antibodies: CD14-Pe-Cy7, CD15-Pe-Cy7, CD34-APC, CD45-FITC and isotype IgG1-PE. The FCM analysis of CECs was done on an FACS Fortessa (Becton Dickinson B.V., Vianen, the Netherlands) equipped with four lasers (excitation wavelength: 405, 488, 561 and 640 nm); a compensation matrix was set up using a single stained sample. The CEC number was defined by acquiring the samples to completion. The CEC phenotype was defined as CD14+, CD15+, CD45+, CD34+, CD146+ and Hoechst33258+. Haematopoietic cells were defined as CD14+, CD15+, CD45+, CD34+, CD146+ and Hoechst33258+. The gating strategy is shown in Fig. 1.

CEC Sorting, vWF Staining and Morphology Analysis

PBMC samples were prepared, enriched and immunostained as described earlier; however, CD45-FITC was substituted by CD45-PerCP (Becton Dickinson B.V., Vianen, the Netherlands) to enable the use of polyclonal sheep antihuman vWF-FITC. The putative CECs were sorted into a 0.8-mL tube, washed with PBS supplemented with 4% FBS, centrifuged at 1,000 g for 4 minutes at 4°C. The cell pellet was resuspended in Perm/Wash (1X) (Becton Dickinson B.V., Vianen, the Netherlands) in washing buffer supplemented with 4% FBS, incubated 1 hour at RT to block for non-specific antibody binding, incubated 15 minutes at RT in the dark with vWF-FTC (1:1,000). The putative CECs were then washed and centrifuged twice at 1,000 g for 4 minutes at 4°C in Perm/Wash (1X) in washing buffer with 4% FBS, transferred onto a glass slide, dehydrated and protected by mounting medium VectaShield H-1000 (Vector Laboratories, Inc., Burlingame, United States) and a coverslip. The fluorescent labelled cells were viewed as described.

Linearity, Recovery and Precision using HUVECs

Purified HUVECs were resuspended in 40-µL FcR blocking reagent and 50-µL BSA 4% in PBS and incubated for 1 hour on ice, then immunostained with CD146-PerCP (Becton Dickinson B.V., Vianen, the Netherlands) for 1 hour on ice, washed in washing buffer and centrifuged at 1,000 g for 4 minutes at 4°C. According to parameters used previously for CEC sorting and morphology examination, a homogenous population of

![Fig. 1](image-url) Five-colour flow cytometry analysis of circulating endothelial cells (CECs). CECs were analysed using FACS Diva software and sequential gating strategy. The black arrow depicts the gating direction. Doubles were excluded (FSGA vs. FSCH, plot not shown) prior to gate A. (A) Gate A selects for mononuclear cells, and excludes debris and platelets. (B) Gate B (dump channel) is derived from gate A and excludes possible contamination by monocytes, macrophages and neutrophils. (C) Gate C is derived from gate B and shows the gate with CD34+/nucleated Hoechst33258+ population. (D) Gate D is derived from gate C and selects nucleated, CD45 CD34+/CD146+ circulating endothelial cells. (E-H) An enriched control sample stained with an isotype IgG1-PE. FACS, fluorescence-activated cell sorting; FSQA, forward scatter area; FSCH, forward scatter height, SSQA, side scatter area.
CD146-PerCP pre-labelled HUVECs were FACS sorted to select those HUVECs that resemble the endogenous CECs in terms of size and marker expression. Then pre-labelled HUVECs were spiked by the FACS sorter at the theoretical range of 12 to 12,800 HUVECs into three 4-mL CTAD tubes with peripheral blood and processed as described earlier, and into three 0.8-mL tubes containing washing buffer to prepare reference samples. The theoretical input is the number of cells planned to be spiked into a sample with the FACS machine. However, as some less viable HUVECs might crush under the pressure of the FACS sorter or more than one cell at a time might have been sorted, the actual number of spiked HUVECs might differ from the theoretical input. The linear relationship \( R^2 \) was defined at 12, 50, 200, 800, 1,600 and 12,800 HUVECs spiked per 4 mL, and the recovery and coefficient of variation (precision) were evaluated at 12, 50 and 200 HUVECs spiked per 4 mL.

**Sample Stability in Storage**
The stability of the processed peripheral blood in freezing medium in storage at \(-80^\circ\)C was defined in samples of HVs and cancer patients. Briefly, peripheral blood was collected from four HVs in nine 4-mL CTAD tubes and from four cancer patients in six 4-mL CTAD tubes. Three samples of each donor were directly processed as described earlier, after fixation of the samples were stored in freezing medium at \(-80^\circ\)C for 1 and 2 months, and were enriched, immuno-stained and analysed by FCM after the planned storage time.

**Effect of ELB on CEC Recovery and Comparative Analysis with CD34 and CD146 Beads, and without Sample Enrichment**
The effect of ELB on CEC recovery, the recovery using CD146 beads and CEC analysis without enrichment, was tested according to the methods described in the supplementary data.

**Statistical Analysis**
The FCM data were analysed using FACS Diva software by Becton Dickinson B.V.. The regression analysis and the Mann–Whitney U test were performed using GraphPad Prism 6 (GraphPad Software, Inc., United States).

**Results**
**Morphology Analysis of HUVECs and Putative CECs**
The putative CECs were evaluated under a fluorescence microscope for the presence of the WPBs and vWF. As positive control served HUVECs cultured on glass coverslips and as an additional negative control, the CD45 positive haematopoietic and lymphoid cells were sorted and stained with vWF antibody. The microscopic evaluation of putative CECs showed presence of WPBs, which confirmed the endothelial origin of sorted CECs as the WPBs are elongated organelles distributed throughout the cytoplasm of CECs as shown in \(-\text{Fig. 2}\). The varying degree of the vWF staining and nuclear disintegration implicates that these CECs were in different viability stages including apoptotic and necrotic cells. In addition, the sorted control cells stained weakly/non-specifically positive for vWF (\(-\text{Supplementary Fig. 1}\)). These cells had a segmented or rounded nucleus and WPBs could not be identified, implicating that these cells were not of endothelial origin, but more likely to be haematopoietic cells.

**Assay Linearity**
The regression analysis of the relationship between the actual number of spiked HUVECs \((x)\) and detected HUVECs \((y)\) was \( R^2 = 1.0 \) (\(-\text{Fig. 3}\)). The overall within-run (including all ranges) recovery \pm standard deviation was 96.9% \pm 5.6 (range 94.0–101.4) and coefficient of variation percentage (CV%) of 5.8% (1.0–18.4; \(-\text{Table 2, –Fig. 4}\)).

**Within-Run and between-Run Recovery and Reproducibility**
The assay recovery and reproducibility was defined in three independent experiments, at 3 days by seeding CD146-PerCP pre-labelled HUVECs using an FACS sorter into fresh peripheral blood and washing buffer at the theoretical input of 12, 50 and 200 HUVECs per 4 mL in triplicate. The actual number of spiked HUVECs in the reference samples was defined by FCM analysis. At day 1, the overall within-run recovery was 102.5% \pm 8.2 and CV% was 8.0%; on day 2, the overall within-run recovery and CV% were 97.8% \pm 4.6 and 4.7%, respectively; and the overall within-run recovery and CV% on day 3 were 99.1% \pm 7.7 and 7.6%, respectively (\(-\text{Table 3, –Fig. 5}\)). The between-run recovery was 99.8% \pm 7.0 and CV% was 7.0%.

**Sample Stability in Storage at \(-80^\circ\)C**
The stability of endogenous CECs was investigated in samples stored at \(-80^\circ\)C for up to 2 months. The number of CECs detected in freshly processed and analysed samples (time 0) was compared with the number of CECs detected after 1 month (time 1) and 2 months (time 2) of storage. This was done for samples of cancer patients \((n = 4)\) and HVs \((n = 4)\). The mean recovery of CECs after 1 month of storage varied from 83.8% \pm 15.1 to 89.3% \pm 2.5 and CV% from 2.8 to 18% (\(-\text{Table 4, –Fig. 6}\)). The within-run mean recovery was 86.8% \pm 7.8 and CV% was 9.0%. After 2 months of storage, the mean recovery varied from 87.0% \pm 5.4 and 104% \pm 9.0, with a CV% of 6.3 to 27.4%; the within-run recovery was 94.3% \pm 15.3 and CV% was 16.2%.

**CEC Levels in Cancer Patients and HVs**
The number of CECs was investigated in 39 patients with metastatic cancer and in 11 HVs who served as a reference group to assess CEC numbers in healthy individuals (\(-\text{Supplementary Table 1}\)). The number of CECs in patients varied from 2.1 to 361 CECs/mL whole blood with a mean of 27.2 \pm 17.8 CECs/mL (median 24.1), whereas in HVs it varied from 5.8 to 26.7 CECs/mL with a mean 14.3 \pm 2.3 CECs/mL (median 14.4; \(-\text{Figs. 7 and 8}\)). The median number of CECs at baseline in cancer patients was significantly different from that in HVs (24.1 vs. 14.4 CECs/mL, respectively; Mann–Whitney U test, \( p = 0.0386; -\text{Fig. 8}\) and \(-\text{Supplementary Tables 2 and 3}\)). The median CEC number of 9.2 CECs/mL at baseline in patients with lung
Fig. 2 Morphology analysis of putative circulating endothelial cells (CECs) and HUVEC. (A) FACS-sorted endogenous CECs from a healthy volunteer’s peripheral blood. (B) HUVECs (control sample) isolated from an umbilical cord and FACS sorted. CECs and HUVECs were stained with an FITC-labelled anti-vWF antibody (green) and nuclear DNA stain Hoechst33258 (blue). FACS, fluorescence-activated cell sorting; HUVECs, human umbilical vein endothelial cells; vWF, von Willebrand factor.

Fig. 3 Linear regression analysis. The relationship ($R^2$) between the reference samples spiked with HUVECs (x) versus detected numbers in peripheral blood of spiked HUVECs (y) at the range of 12, 50, 200, 800, 1,600 and 12,800 in 4 mL of peripheral blood. (A) The relationship $R^2$ at the full range of spiked HUVECs. (B) The lower four ranges of spiked HUVECs. HUVECs, human umbilical vein endothelial cells.
cancer \( n = 8 \) was not significantly different from that in HVs \( p > 0.05 \). The median CEC number in HV was significantly different from that in patients with colorectal cancer (CRC; median 22.2, \( p = 0.0348, n = 11 \)) and in patients with miscellaneous tumours (median 29.5, \( p = 0.0047, n = 20 \); ► Fig. 8).

**Effect of ELB on CECs Recovery**

The effect of ELB on endogenous CEC recovery was tested in Ficoll Paque and cell preparation tube (CPT) Hypaque isolated CECs samples from HV whole blood. The CEC number was compared with the CEC number of samples drawn in CTAD tubes followed by ELB (as described in our method).

The numbers of CECs isolated using CPT and CTAD tubes (► Supplementary Table 4) in the sample of HV1 were 1.0 \pm 1.4 (\pm SD) CECs/4 mL and 40.0 \pm 5.7 CECs/4 mL, respectively, and HV2 contained 2.5 \pm 2.1 CECs/4 mL and 32.0 \pm 7.1 CECs/4 mL, respectively.

Whole blood of 56 mL was separated using Ficoll Paque into a PBMC fraction and a pass-through fraction (► Supplementary Fig. 2). The PBMC fraction of HV1 contained 2.7 CECs/4 mL, after ELB treatment 3.0 CECs/4 mL and the pass-through fraction contained 35.1 CECs/4 mL. The PBMC fraction of HV2 contained 4.0 CECs/4 mL, after ELB treatment 4.0 CECs/4 mL and the pass-through fraction had 23.6 CECs/4 mL. In total, 56 mL of whole blood sample of HV1 contained 40.8 CECs/4 mL and HV2 contained 31.6 CECs/4mL (► Supplementary Table 5).

**Comparative Analysis with CD34 and CD146 Beads, and without Enrichment**

The samples for the comparative CEC analysis were drawn in CTAD tubes and prepared according to our method. The CD34 bead–enriched samples (► Supplementary Table 6) of HV1 contained 27.3 CECs/mL (109.3 \pm 4.2 CECs/4 mL). CD146 bead–enriched samples had 12.4 CECs/mL (49.7 \pm 9.7 CECs/4 mL) and in samples without enrichment it was 30.0 CECs/mL. In HV2, CD34 bead–enriched samples contained 19.0 CECs/mL (76.0 \pm 2.6 CECs/4/mL), CD146 bead–enriched samples had 6.75 CECs/mL (27.0 \pm 3.6 CECs/mL) and in samples without enrichment it was 15.0 CECs/mL.

**Discussion**

Here, we presented the results of a polychromatic FCM method for enumeration of the absolute number of CECs in peripheral blood of patients with metastatic cancer and HVs. The method includes a CD34 bead sample enrichment. CECs were phenotypically defined as nucleated cells (Hoechst positive), positive for CD34 and CD146, and negative for CD14,
CD15 and CD45. The specificity and reproducibility of the assay were defined by spiking surrogates— the HUVECs— of the CECs into peripheral blood and by morphologic examination using fluorescence microscopy.

CECs are rare cells in blood circulation; enrichment allows analysis of a large sample volume by reducing its original volume and overcomes long sample acquisition and large data files. However, enrichment may introduce bias into the analysis by selecting cells that are more abundant and/or highly express the target marker, as compared with the cells of interest. CECs were previously successfully isolated by positive selection using CD146 magnetic beads, and negative selection using CD15 magnetic microbeads.\(^7,14,17\) In a single experiment, we have tested if CD146 magnetic microbeads qualify as an alternative for CD34 bead enrichment of endothelial cells with low CD34 expression. We were able to detect CECs in HVs after CD146 bead enrichment; however, their number was significantly lower compared with samples enriched using CD34 beads and samples prepared without enrichment. A lower number of detected CECs in CD146-enriched samples may be explained by the predominant presence of CD45\(^+\)/CD146\(^-\) cells in the analysed samples, which are likely activated T cells. Furthermore, the widely used CD146 surface marker is expressed on the apical side and at the cell–cell junctions; its loss is related to detachment of endothelial cells.\(^21,22\) Hence, CD146 expression level could have led to an incomplete absolute enrichment, especially in the presence of competitor cells, and underestimation of CEC number.\(^20\) In the FCM analysis, we have seen that this marker is more heterogeneous and weaker than CD34. The results of the number of CECs in CD34 bead–enriched versus non-enriched samples were similar, showing that CD34 beads have not introduced a bias. Therefore, CD34 beads are a better alternative for versatile enrichment of endothelial cells of different origins.

### Table 3 Within-run and between-run recovery and coefficient of variation

<table>
<thead>
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<th>Spiked HUVECs/4 mL</th>
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<th>Recovery %</th>
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<td>49</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>201</td>
<td>201</td>
<td>202</td>
<td>190</td>
</tr>
<tr>
<td>Overall within run</td>
<td>99.1</td>
<td></td>
<td>7.7</td>
<td></td>
<td>7.6</td>
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<tr>
<td>Between run</td>
<td>99.8</td>
<td></td>
<td>7.0</td>
<td></td>
<td>7.0</td>
</tr>
</tbody>
</table>

Abbreviations: CV, coefficient of variation; HUVEC, human umbilical vein endothelial cells; SD, standard deviation; WR, within run.

Notes: The actual number of spiked and recovered HUVECs/4 mL performed in triplicate in peripheral blood of healthy volunteers in three independent runs of the assay. The overall within-run recovery of each day is the mean recovery percentage calculated at all levels of spiked HUVECs together, and accompanying standard deviation and CV%. The between-run recovery is the mean of all within-run means recovery percentages, and accompanying standard deviations and CV%.

*Missing due to technical problems during measurement acquisition.

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Fig. 5 Recovery and reproducibility of spiked HUVECs/4 mL in peripheral blood performed on three independent days. Overall within-run (WR) and between-run recovery and coefficient of variation percentages (CV%) of HUVECs spiked in 4-mL peripheral blood performed on three independent days, plot accompanying Table 3. The bars represent the WR recovery percentage and accompanying standard deviation and CV% of each day. The between-run recovery is calculated as the mean recovery for all WR means recovery percentages, and accompanying standard deviations and CV%.
**Table 4** Stability test: measurements of the CECs in the samples of cancer patients and healthy volunteers stored at –80°C for 1 and 2 months

<table>
<thead>
<tr>
<th>Donor</th>
<th>Detected time 0</th>
<th>Detected 1 mo</th>
<th>Recovery % 1 mo</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>HV1</td>
<td>56</td>
<td>72</td>
<td>64</td>
</tr>
<tr>
<td>HV2</td>
<td>55</td>
<td>59</td>
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</tr>
<tr>
<td>HV3</td>
<td>61</td>
<td>56</td>
<td>50</td>
</tr>
<tr>
<td>HV4</td>
<td>48</td>
<td>39</td>
<td>34</td>
</tr>
<tr>
<td>Overall within run</td>
<td>86.6</td>
<td>7.8</td>
<td>9.0</td>
</tr>
<tr>
<td>P1</td>
<td>113</td>
<td>135</td>
<td>124.0</td>
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<tr>
<td>P2</td>
<td>716</td>
<td>726</td>
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<td>P3</td>
<td>195</td>
<td>166</td>
<td>181.0</td>
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<tr>
<td>P4</td>
<td>47</td>
<td>62</td>
<td>55.0</td>
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<tr>
<td>Overall within run</td>
<td>102.5</td>
<td>17.3</td>
<td>16.7</td>
</tr>
<tr>
<td>Donor</td>
<td>Detected time 0</td>
<td>Detected 2 mo</td>
<td>Recovery % 2 mo</td>
</tr>
<tr>
<td>-------</td>
<td>-----------------</td>
<td>---------------</td>
<td>----------------</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>HV1</td>
<td>74</td>
<td>69</td>
<td>69</td>
</tr>
<tr>
<td>HV2</td>
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</tr>
<tr>
<td>HV4</td>
<td>52</td>
<td>40</td>
<td>36</td>
</tr>
<tr>
<td>Overall within run</td>
<td>94.3</td>
<td>15.3</td>
<td>16.2</td>
</tr>
</tbody>
</table>

Abbreviations: CECs, circulating endothelial cells; CV, coefficient of variation; HV, healthy volunteers; P, patients; WR, within run.

Notes: Recovery percentage and CV percentage were calculated relative to the samples processed and analysed the same day (time 0). The overall within-run recovery is the mean recovery percentage calculated at all samples together, and accompanying standard deviation and CV percentage.

Fig. 6 Stability of samples in cryostorage. Samples of cancer patients (n = 4) and healthy volunteers (n = 4) were stored at –80°C for 1 and 2 months. Mean recovery percentages and CV% were calculated relative to samples processed and analysed the same day (time 0). The bars represent the mean recovery percentage of a triplicate (for HV, slashed bars) and duplicate (patients, black bars) measurement and accompanying standard deviation of the mean recovery, and CV% (checkered bars). HV, healthy volunteers; CV%, coefficient of variation percentage.

CD34 is a surface marker expressed on normal and tumour vascular cells. It is present on micro- and macro-endothelial vessels, for example, coronary artery endothelial cells, lung microvascular endothelial cells and liver endothelial cells. In addition, the CD34 cell population in peripheral blood is small; CD34 beads are therefore more suitable to select the CECs from between redundant blood cells and the technical selection process using these microbeads is very practical. Moreover, previously, CD34 beads were used reliably for the isolation of HUVECs, endothelial colony-forming cells and for the detection of CECs in blood of patients after myocardial infarction, yet they have not previously been applied for CEC enumeration. In our laboratory, the utility of magnetic microbeads and MACS sorting for enrichment was also applied in the detection and enumeration of circulating tumour cells. Furthermore, enrichment reduced the sample volume, which results in more manageable samples for FCM analysis, as larger data files result in machine’s software limitations. We confirmed the usefulness of the enrichment method for the selection of CECs by morphological analysis after FACS sorting CECs, nuclear DNA staining and intracellular staining of the vWF in the WPBs. Isolated CECs had a large nucleus and the vWF was clearly present in the WPBs, a characteristic specific for endothelial cells. Therefore, the presence of other cells expressing these markers, like mesenchymal or haematopoietic cells, can be excluded. Furthermore, fluorescence microscopy images by others are comparable with the CEC images we have made, which further emphasizes the specificity of our method.

The performance of the method in terms of recovery, linearity, reproducibility and sensitivity was further explored in fresh peripheral blood samples spiked with...
HUVECs, and long-term stability in cryostored samples at –80°C. The regression analysis demonstrated that the method is capable of detecting 12 to 12,800 CECs/4mL with high and reproducible recovery (overall recovery of 96.9% and CV% of 20%). The high assay recovery and reproducibility were further underscored in three subsequent experiments with spiked HUVECs (overall recovery of 99.8% and CV% of 7.0%). In several experiments, we have observed recoveries higher than 100%. A probable cause of recoveries exceeding 100% was the variation within the number of spiked HUVECs in the reference samples. The reference samples used for the regression analysis were subject to variation ranging from 1.8 to 18.4%. In addition, one cell difference accounts for 8.3% change in recovery at the level of 12 HUVECs/mL; hence, the samples spiked with HUVECs at the lower range are subject to the greatest variation. In comparison with studies employing enrichment followed by FCM, this method was found to be more stable over a wide range of spiked HUVECs without indications for significant cell loss or overestimation of CEC numbers.3,9,17 A previous study showed that CECs could be cryostored and enumerated after 14 days. With our method, we were able to store samples at –80°C for 1 and 2 months with a high yield of CECs (102.5% after 1 month and 94.3% after 2 months). The coefficient of variation remained below the predefined 20%, which further emphasizes the robustness of this method. Storage allows for simultaneous analyses of samples drawn at different time points from the same patient within 2 months and increases comparability and reproducibility between measurements. The variation in reproducibility of the FCM measurements was higher in fresh than in cryostored samples, possibly due to sample handling. Nevertheless, delayed sample analysis for up to 2 months did not lead to significant cell loss, allowing more flexible use of this method. A remark could be made on the sensitivity of our method that was defined using selected HUVECs, the cell type that most closely resemble CECs. These surrogate cells do express the same surface markers as CECs, though the expression can be different from endogenous CECs. Furthermore, HUVEC are generally larger than CECs. Another remark is on the use of ELB that showed in one study to influence CEC number in samples treated with ELB. CECs in the circulation generally have a low viability; ELB might therefore disrupt these cells, resulting in underestimation of CEC levels.

Fig. 7 The range of the enumerated CECs/mL in individual healthy volunteers and cancer patients. The dotted lines represent the lower range of 5.8 CECs/mL and the upper range of 26.7 CECs/mL in HV (HV1–HV11). The CECs in cancer patients (1–39) varied from 2.1 to 361.2 CECs/mL.

CECs, circulating endothelial cells; HV, healthy volunteers.

Fig. 8 CEC numbers enumerated in healthy volunteers (n = 11) and in patients (n = 39) with metastatic cancer. CEC number in a sample drawn at baseline prior to start of treatment. The median numbers of CECs were significantly different in CRC patients (p = 0.0348) and patients with miscellaneous tumour types (p = 0.0047; Mann–Whitney U test, p < 0.05). The solid lines represent the median CECs number in each tested group of patients and HV, and dotted lines represent the lower and the upper range of the enumerated CECs in HV. CECs, circulating endothelial cells; CRC, colorectal cancer; HV, healthy volunteers.
In general, non-viable cells change morphologically: membrane integrity and cytosolic volume decreases and the cell density increases due to cell shrinkage.\(^{36,37}\) To investigate CECs (resting, active and apoptotic CECs) in relation to cancer, several studies applied Ficoll Paque, CPT or ELB to isolate them from whole blood.\(^{1,3,38–40}\) CPT also contain Ficoll Hypaque to separate PBMCs from granulocytes, erythrocytes, platelets and dead cells. They are easy to handle and deliver reproducible results, whereas Ficoll Paque and ELB methods are more laborious, deliver different cell purity, cell quality and recovery.\(^{41,42}\) In addition, isolating rare cells using Ficoll Paque requires experience, unlike CPT and ELB methods. ELB was widely used in other methods and seems to be harmless for freshly spiked HUVECs but might influence the viability of CECs.\(^{14}\) To define the effect of the ELB on CECs, we have isolated CECs from whole blood using Ficoll Paque. We observed no difference in CEC counts between ELB-treated and untreated samples. The number of detected CECs was low and comparable with the samples prepared using CPT. In contrast, higher numbers of CECs were detected in the parallel drawn CTAD samples of the same volunteers, prepared according to our method. Additionally, we collected and analysed the pass through of the Ficoll Paque separation and found relatively high numbers of CEC below Ficoll Paque. The sum of CEC numbers from below and above Ficoll Paque was comparable with the number of CECs in CTAD, the sample prepared according to our method. This provides further prove that ELB does not affect CEC numbers. The presence of CECs below Ficoll Paque indicates that if the non-viable CECs are the subject of investigation the Ficoll Paque or CPT methods may be useless.

The different phenotype definitions, poor validation, sample preparation approaches and lack of sensitivity of FCM are the factors limiting comparability of CEC numbers between studies.\(^{43}\) However, enrichment using CD34 beads may overcome the questionable correctness of methods quantifying the absolute number of CECs as their percentage of \(\geq 100,000\) events within only 100 to 200 \(\mu\)L of the sample (CECs \(<\times\) white blood cell count)/100).\(^{1,7,12,44,45}\) We observed that acquisition of an equivalent of 1 mL of whole blood sample of an HV to completion (prepared by ELB of 4 mL of whole blood without enrichment) produced 10 files of \(\geq 500,000\) total events and resulted in only 0 to 6 CEC events per file. However, a CD34 bead enrichment reduced sample volume and produced a data file of less than 100,000 total events. Still, the significantly higher CEC level in our patients is in agreement with previous reports employing FCM and CellSearch methods.\(^{1,7–9,35,46}\) The CEC levels were more variable in patients than in HVs, and generally CEC numbers increased during treatment, suggesting treatment-induced vascular damage or disease progression. Furthermore, the investigated patient group was heterogeneous, patients had multiple tumour types and had participated in multiple trials with differing experimental cancer therapies. The systemic therapy that they received before this study may have impaired the normal and the tumour vasculature. Consequently, these patients might have been more prone to vascular damage caused by the current therapy and, consequently, were more likely to have increased CEC numbers. In this regard, to answer the question whether CECs are a potential predictive and/or prognostic biomarker, the CEC level should be assessed in the context of a randomized study, in a pre-specified tumour type. Additionally, implementation of the cancer-associated endothelial markers CD133 and CD276 in our method might provide specific information on the tumour vasculature, for example, in relation to antiangiogenic therapy.\(^{37–49}\)

**Conclusion**

In conclusion, we have developed a highly selective, sensitive, robust and reliable method for enumeration of CECs in whole blood samples of cancer patients that can be stored for up to 2 months. To our knowledge, this is the first method that shows a highly reproducible and reliable method for CEC enumeration by a sample enrichment using CD34 magnetic microbeads, followed by polychromatic FCM. This method can be implemented to study the role of CECs as a biomarker in clinical trials, efforts to further study the usefulness of CEC enumeration employing this method are currently ongoing.

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**What is known about this topic?**

- Circulating endothelial cells (CEC) are a potential biomarker of vascular damage and are under investigation in metastatic cancer patients receiving an antiangiogenic therapy. CEC numbers are known to be higher in cancer patients, and might vary as a response to treatment.
- CECs in human blood are being enumerated using various approaches including polychromatic flow cytometry (FCM) and microscopy. CD146 is the best validated marker for the detection by FCM and the intracellular marker von Willebrand factor (vWF) is being used in microscopic analysis. However, microscopic analysis misses sensitivity and, due to expression of endothelial markers by different haematopoietic cells and thrombocytes, FCM may lead to incorrect results.

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**What does this paper add?**

- Enrichment of whole blood samples using CD34-conjugated magnetic microbeads and magnetic sorting is a suitable method to specifically isolate the CECs. Enrichment yielded good precision between samples and high recovery of spiked surrogate CECs—human umbilical vein endothelial cells—in whole blood.
- Microscopic examination of the morphology of sorted cells confirmed cells to be CECs as these cells expressed the intracellular marker vWF.
- Sample storage in storage buffer at \(-80^\circ\)C allows for delayed sample analysis that in a clinical setting enables analysis of samples drawn at different time points.
Conflict of Interest
The authors declare that they have no conflicts of interest in the research.

References
19 Strijbos MH, Kraan J, den Bakker MA, Lambrecht BN, Sleijfer S, Gratama JW. Cells meeting our immunophenotypic criteria of endothelial cells are large platelets. Cytometry B Clin Cytom 2007;72(02):86–93
27 Filali EE, Hirallal JK, van Veen HA, Stolz DB, Seppen J. Human liver endothelial cells, but not macrovascular or microvascular endothelial cells, engraft in the mouse liver. Cell Transplant 2013;22(10):1801–1811
33 Chong P-P, Selvaratnam L, Abbas AA, Kamarul T. Human peripheral blood derived mesenchymal stem cells demonstrate similar characteristics and chondrogenic differentiation potential to bone marrow derived mesenchymal stem cells. J Orthop Res 2012;30(04):634–642


