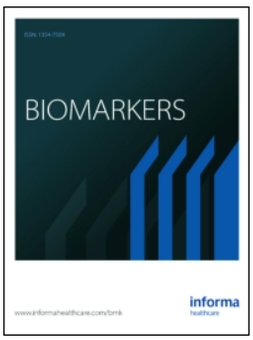

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ORIGINAL ARTICLE

Longitudinal profile of circulating endothelial cells in post-acute coronary syndrome patients

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ABSTRACT

Introduction: Patients who have experienced an acute coronary syndrome (ACS) are at risk of a recurrent event, but their level of risk varies. Because of their close temporal relationship with vascular injury, longitudinal measurements of circulating endothelial cells (CECs) carry potential to improve individual risk assessment.

Methods: We conducted an explorative nested case-control study within our multicenter, prospective, observational biomarker study (BIOMArCS) of 844 ACS patients. Following an index ACS, high-frequency blood sampling was performed during 1-year follow-up. CECs were identified using flow cytometric analyses in 15 cases with recurrent event, and 30 matched controls.

Results: Cases and controls had a median (25^{th}-75^{th}percentile) age of 64.1 (58.1-75.1) years and 80% were men. During the months preceding the endpoint, the mean (95%CI) CEC concentration in cases was persistently higher than in controls (12.8 [8.2-20.0] *versus* 10.0 [7.0-14.4] cells/ml), although this difference was non-significant ($P=0.339$). In controls, the mean cell concentration was significantly ($P=0.030$) lower in post 30-day samples compared to samples collected within one day after index ACS: 10.1 (7.5-13.6) *versus* 17.0 (10.8-26.6) cells/ml. Similar results were observed for CEC subsets co-expressing CD133 and CD309 (VEGFR-2) or CD106 (VCAM-1).

Conclusion: Despite their close relation to vascular damage, no increase in cell concentrations were found prior to the occurrence of a secondary adverse cardiac event.

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KEYWORDS
Circulating endothelial cells; acute coronary syndrome; repeated measurements; atherosclerosis; vascular injury; cardiovascular disease

Clinical significance

- In the current study, we investigated the longitudinal trajectory of circulating endothelial cells using repeated blood sampling over a one-year follow-up in patients admitted for an acute coronary syndrome (ACS) and correlated these trajectories with the occurrence of a repeat cardiac adverse event.
- Circulating endothelial cell concentrations were higher directly after index ACS than in the clinically stable post-ACS phase.
- In the clinically stable post-ACS period, repeated measures of the circulating endothelial cell concentration appeared not predictive of a repeat cardiac adverse event.

Introduction

Patients who have experienced an acute coronary syndrome (ACS) are at risk of a recurrent event, but their level of risk varies (Virani *et al.* 2020). Hence, risk assessment tools for individualised prediction of impending ACS are urgently needed.

Circulating endothelial cells (CEC) are present in the peripheral blood and represent mature endothelial cells, that have detached from vessel walls at sites of vascular injury, often as result of ischaemic disease (Kachamakova-Trojanowska *et al.* 2015). Because of their close relationship with vascular injury (Kachamakova-Trojanowska *et al.* 2015), CECs may provide a window into the pathophysiologic, vulnerable state preceding an ACS event and carry potential to improve individual risk assessment (Schmidt *et al.* 2015). To date, two prospective studies in patients with ACS have demonstrated associations between baseline CEC count and incident cardiovascular events (Lee *et al.* 2005, Boos *et al.* 2008). Nonetheless, distinguishing patients at different levels of risk of adverse events based on a single baseline measurement is challenging. Longitudinal profiles of CECs may improve personalised risk assessment and could ultimately aid in the timing of

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treatment. However, studies on repeated CEC measurements and the temporal evolution of CEC preceding a recurrent event are lacking.

Therefore, we conducted an explorative study and investigated the longitudinal profile of CECs in peripheral blood of patients diagnosed with an acute coronary syndrome.

Materials and methods

The current study is embedded in the 'BIOMarker study to identify the Acute risk of a Coronary Syndrome' (BIOMArCS), which has been described in detail elsewhere (Oemrawsingh *et al.* 2016, Boersma *et al.* 2019, Oemrawsingh *et al.* 2019). Briefly, BIOMArCS is a multicenter, observational study with a high-frequency sampling design to assess the temporal pattern of blood biomarkers in patients following an acute coronary syndrome in anticipation of an imminent repeat coronary event. Patients were eligible for inclusion if they were aged 40 years or above, were admitted with an acute coronary syndrome, including unstable angina pectoris, non-ST-elevation MI and ST-elevation MI (STEMI), and had one or more cardiovascular risk factors. Exclusion criteria were ischaemia precipitated by a condition other than atherosclerotic coronary artery syndrome, a left ventricular ejection fraction <30%, end-stage congestive heart failure (New York Heart Association class ≥3), severe chronic kidney disease with measured or calculated glomerular filtration rate (Cockroft-Gault or Modification of Diet in Renal Disease-4 formula) of <30 mL/min per 1.73 m^2, or a coexistent condition with life expectancy <1 year. Patients underwent regular blood sampling after the initial admission for ACS according to a strict schedule. Venepuncture was performed at admission, at hospital discharge, and subsequently every fortnight during the first half year, followed by monthly blood sample collection until 1 year. Ultimately, a median of 17 repeated blood samples were obtained during 1-year follow-up in 844 patients admitted for ACS at inclusion. All patients were treated according to prevailing guidelines and at the discretion of the treating physician. The study protocol was approved by the institutional review boards of the participating hospitals, and all study subjects gave written informed consent.

Primary study endpoint

A clinical event committee, blinded to the CEC assay results, adjudicated the study endpoints. The primary endpoint comprised the composite of cardiac death, non-fatal myocardial infarction or unstable angina requiring urgent coronary revascularization during one year of follow-up after the index acute coronary syndrome.

Case–control design

Within BIOMArCS, we performed an explorative nested case-control study with a limited patient sample (patient flow diagram is shown in Figure 1). Namely, 15 patients who experience the primary endpoint >30 days after the index ACS (cases) and 30 matched controls (two per case) were selected. Cases and controls were matched by age (± 5-year range) and sex. We studied differences in the temporal evolution of CECs between cases and controls starting 30 days after the index ACS, a phase in which patients were clinically stable (van den Berg *et al.* 2020). For reasons of efficiency, we selected the first blood sample drawn in this period and the last two samples drawn prior to the occurrence of the study endpoint (in cases), or the last two samples drawn during the 1-year follow-up (in controls). We further aimed to study a potential washout of CECs directly after an ACS event. For that purpose, in controls only, we selected the

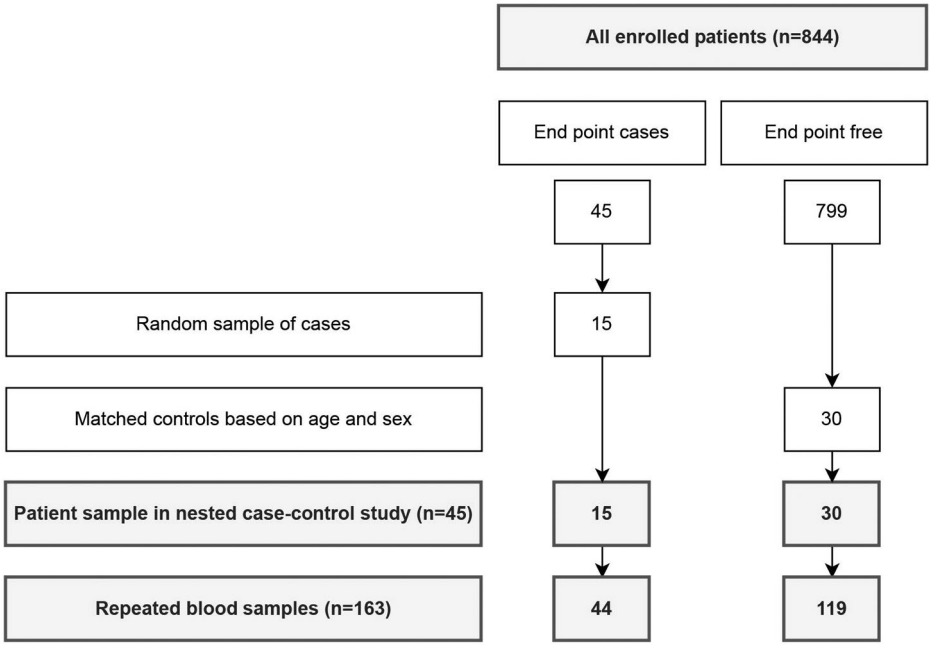

Figure 1. Patient flow diagram.

first sample within one day after the index ACS. In total, 163 repeated blood samples were available for the current study.

Circulating endothelial cells

Standardised protocols for sample collection, handling, and long-term storage were applied. Within a median of 82 minutes (25th–75th percentile 58–117) after withdrawal, blood samples were processed, and whole blood was stored on dimethyl sulfoxide at −80 °C until (batch) flow cytometric analyses.

For the current analyses, samples were thawed and prepared in a lyse-stain wash procedure with minimal sample handling. To induce red blood cell lysis, the thawed peripheral blood was transferred to a 50 ml tube and 45 ml of ammonium chloride 0.15 M lysing solution was added. After 15 minutes of lysis at room temperature, the suspension was centrifuged for 5 min at 1000 x g. Next, the supernatant was removed from the sample tube without disturbing the pellet. The pellet was carefully homogenised in 50 µL of CD32 monoclonal antibody (clone IV.3; Stemcell Technologies, Vancouver, BC, Canada; 100 µg/mL in phosphate-buffered saline [PBS]), to block the Fc receptor and reduce Fc receptor-mediated antibody binding, and 50 µl DNA dye 5-bis[2-(di-methylamino) ethyl]amino-4, 8-dihydroxyanthracene-9,10-dione (DRAQ5; Biostatus Ltd, Shepshed, UK) using a 200 µl pipette. After 10 minutes of incubation at room temperature, the cells were stained using 155 µl of a mix of the following monoclonal antibodies: fluorescein isothiocyanate (FITC) conjugated CD34 (clone 8G12; BD Biosciences, San Jose, CA); peridinin chlorophyll protein (PerCP) conjugated CD45 (clone 2D1; BD Biosciences); R-phycoerythrin (PE) conjugated VEGFR-2 (clone 89106; R&D Systems, Minneapolis, MN); allophycocyanin (APC) conjugated CD146 (clone 541-10B2; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany); Phycoerythrin-Vio 770 (PE-Vio 770) conjugated CD133 (clone 293C3; Miltenyi Biotec), and Brilliant Violet 711 (BV711) conjugated CD106 (clone 51-10C9; BD Biosciences). All reagents were diluted in PBS/1% BSA based on titration (i.e. absence of non-specific staining on negative populations and optimal discriminatory power between negative and positive populations). After 15 minutes of incubation at room temperature, 20 ml of PBS was added to the cell suspension and the suspension was transferred onto a sterile 70 µm EASYstrainer™ filter (Greiner Bio-One GmbH, Frickenhausen, Germany), to remove erythrocytes and/or granulocyte aggregates. Additionally, the 50 ml tube was rinsed with 25 ml of PBS and any remaining cells were also transferred to the filter to assure optimal recovery of cells. The filtered suspension was collected in a new 50 ml tube and was centrifuged for 5 minutes at 1000 × g. After the removal of the supernatant, cells were resuspended in 350 µL of PBS and transferred to a standard 5-mL flow cytometry tube. The 50-mL tube was rinsed with 350 µL of PBS, and any remaining cells were also transferred to the cytometry tube. Samples were immediately acquired on a BD LSRFortessa flow cytometer with FACSDiva v8.0.1 software (BD Biosciences). Data acquisition was started by collecting ungated data of 50 000 nucleated cells (DRAQ5+) at a low flow rate (12 µL/min) Data acquisition was completed by acquiring the remainder of the sample at a high flow rate (60 µL/min) with a threshold (live-gate) on CD34+ events in a separate file. A specific gate strategy (as previously described in detail (Kraan *et al.* 2012)) was used to enumerate CECs, which are defined as nucleated cells staining positively with the DNA-specific nuclear dye DRAQ5, that express the endothelial markers CD34 and CD146, and lack the expression of the pan-leukocyte marker CD45. We identified the CEC subsets that co-expressed CD133 and CD309 (a.k.a. vascular endothelial growth factor receptor-2 [VEGFR-2])(markers for identification of endothelial progenitor cells) or CD106 (a.k.a. vascular cell adhesion molecule-1 [VCAM-1]; a marker for endothelial activation). CEC counts are presented as number of cells/ml of peripheral blood.

For each sample, data acquisition of the CD34+ absolute count tube was performed first, prior to the CEC analysis tube described above. Absolute counts were obtained by multiplying the CEC percentage within the CD34+ population in 4 mL of blood by the simultaneously obtained absolute CD34 counts – obtained on the same blood sample – using the single platform FCM assay and analysis strategy according to ISHAGE guidelines (Sutherland *et al.* 1996). In brief, 200 µL of blood was incubated with CD34-FITC (clone 8G12), CD45-PErCP (clone 2D1) and DRAQ5. After 15 minutes of incubation at room temperature, 2 mL ammonium chloride 0.15 M lysing solution was added. After 10 minutes of lysis at room temperature, 100 µL Flow Count fluorospheres (Beckman Coulter, Brea, CA) were added using reverse pipetting. Data was acquired on a BD LSRFortessa flow cytometer for 10 minutes at medium flow rate (35 µL/min).

Sample size

Since there are no studies that investigated the association between the longitudinal profile of circulating endothelial cells and cardiovascular risk, we performed a sample size calculation on a well-known cardiovascular marker: LDL cholesterol. Based on 500 repeated simulations, using input parameters from a pilot study with up to five repeated measurements in patients with ACS, we learned that 15 cases and 30 non-cases with three repeated samples per person would be sufficient to determine a difference between cases and non-cases in mean LDL level of 0.45 mmol/L, and a mean difference of 0.13 mmol/L/month change in LDL level during 1 year follow-up (power 80%, two-sided alpha error 0.05). These differences are small in clinical terms in view of normal LDL concentrations ranging from 1.09–5.56 mmol/L (Balder *et al.*, 2017). Therefore, we assumed that differences and changes in circulating endothelial cells of (at least) similar magnitude could be identified.

Statistical analysis

Descriptive statistics were used to present baseline characteristics. Linear or logistic regression analyses, when appropriate, were performed to compare baseline characteristics between cases and controls, whereas a generalised estimating

equation (GEE) approach was used to correct for the dependency of observations within one matched case-control pair.

Differences in CEC concentrations between cases and controls, as well as differences in patient-specific CEC trajectories, were studied using linear mixed effect (LME) models with nested random effects to account for repeated sampling and for case-control matching. To allow the patient-specific trajectories to differ at baseline and over time, both intercepts and slopes were included in the repeated measurement random-effect part of the model. In the matched case-control pair random-effect part of the model, only an intercept was included. In the fixed effect part of the model, repeated CEC measurements were used as outcome (dependent variable). Time from index event to moment of sampling during follow-up or time from moment of sampling to primary endpoint, a group variable (case/control status) and an interaction term (group*time) were entered in the model as independent variables. Using an interaction term allowed us to evaluate and display the CEC trajectories in cases and controls separately. Model assumptions were checked by visual examination of the residuals

Since CEC concentrations in controls remained stable during follow-up (see Results), to increase statistical power, samples were simply labelled as ≤1 or ≥30 days, thus ignoring the exact timing. Again, LME models with nested random effects were applied.

CEC concentrations were log2-transformed for all analyses. Results were back-transformed to present mean differences (95% confidence intervals [CI]) between cases and controls on the linear scale. All statistical analyses were performed using R v.4.0.3.

Results

Baseline characteristics of the studied patients are presented in Table 1. Median (25th–75th percentile) age was 64.1 (58.1–75.1) years and 80% were men. Cases and controls showed similar cardiovascular risk profiles, with the exception that cases significantly more often had a history of percutaneous coronary interventions (PCI) and were more frequently on ACE inhibitors than controls.

Details on blood sample measurements are described in Supplemental Table1. Based on the LME model, cases and controls had similar mean concentration of CECs at 30 days after the index ACS: 11.2 (95%CI 6.8-18.3) and 11.4 (95%CI 8.0–16.5) cells/ml, respectively. During the months thereafter, the mean concentration slightly decreased in controls and increased in cases (Figure 2A), but this difference in temporal evolution was statistically non-significant ($P = 0.417$). From another perspective, during the months preceding the endpoint, the mean CEC concentration in cases (12.8 [95%CI 8.2–20.0] cells/ml) was persistently higher than in controls

Table 1. Clinical characteristics of study population.

	No primary endpoint (controls)	Primary endpoint (cases)	
	n = 30	*n = 15*	*P*-value
Presentation and initial treatment			
Age (median [IQR])	64.17 [58.55, 75.03]	63.88 [57.56, 72.68]	0.776
Sex (% male)	24 (80.0)	12 (80.0)	0.801
Body mass index, kg/m2 (mean (SD))	27.1 (3.9)	28.0 (3.1)	0.406
Systolic blood pressure, mmHg (mean (SD))	129.8 (22.0)	144.4 (27.8)	0.068
Admission diagnosis (%)			
STEMI or LBBB	18 (60.0)	5 (33.3)	0.086
NSTEMI	11 (36.7)	8 (53.3)	0.247
UAP	1 (3.3)	2 (13.3)	0.241
CAG performed (%)	29 (96.7)	15 (100.0)	n.a.*
PCI performed (%)	29 (100.0)	13 (92.9)	n.a.*
Cardiovascular risk factors			
Current smoker (%)	15 (50.0)	5 (33.3)	0.206
Diabetes mellitus (%)	6 (20.0)	5 (33.3)	0.358
Hypertension (%)	12 (40.0)	6 (40.0)	0.946
Hypercholesterolemia (%)	12 (40.0)	6 (40.0)	0.946
Cardiovascular history			
Myocardial infarction (%)	7 (23.3)	7 (46.7)	0.114
PCI (%)	5 (16.7)	8 (53.3)	0.017
CABG (%)	3 (10.0)	3 (20.0)	0.371
Peripheral arterial disease (%)	4 (13.3)	3 (20.0)	0.634
Stroke (%)	3 (10.0)	5 (33.3)	0.070
Valvular heart disease (%)	1 (3.3)	2 (13.3)	0.709
Heart Failure (%)	0 (0.0)	0 (0.0)	n.a.*
Medication at first blood sample moment >7 after the index ACS			
Aspirin (%)	29 (96.7)	15 (100.0)	n.a.*
P2Y12 inhibitor (%)	30 (100.0)	15 (100.0)	n.a.*
Vitamin K antagonist (%)	1 (3.3)	2 (13.3)	0.241
Statin (%)	30 (100.0)	14 (93.3)	n.a.*
Beta-blocker (%)	26 (86.7)	14 (93.3)	0.459
ACE inhibitor (%)	18 (60.0)	14 (93.3)	0.046
ARB (%)	3 (10.0)	2 (13.3)	0.869

*P-value was not calculated due to complete separation. Abbreviations: ACE, angiotensin-converting enzyme; ACS, acute coronary syndrome; ARB, angiotensin receptor blockers; CABG, coronary artery bypass grafting; CAG, coronary artery angiography; LBBB, left bundle branch block; NSTEMI, non-ST-elevation myocardial infarction; PCI, percutaneous coronary intervention; STEMI, ST-elevation myocardial infarction; UAP, unstable angina pectoris.

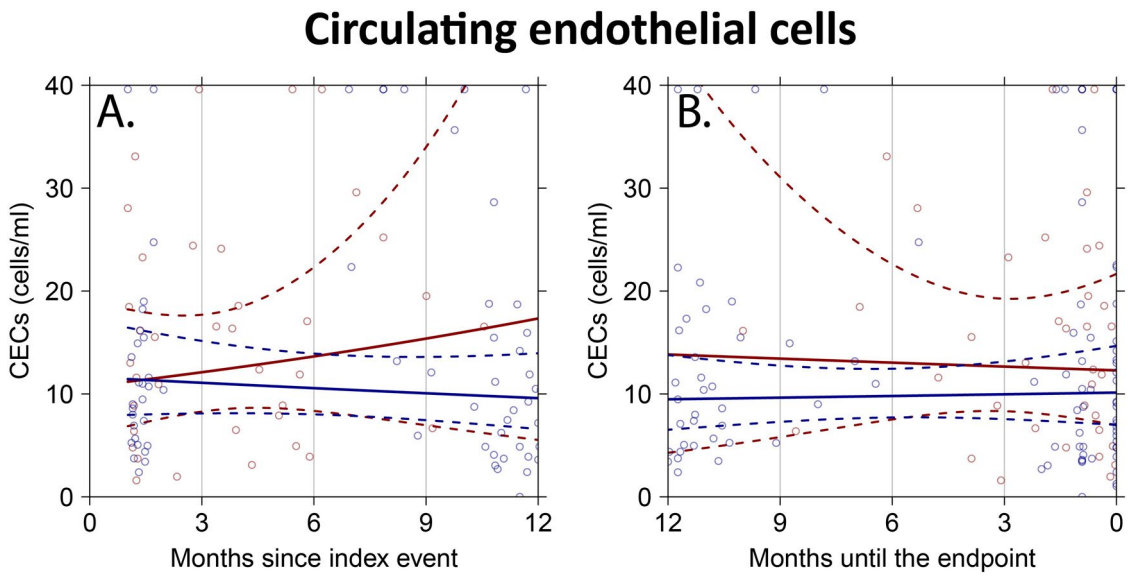

Figure 2. Longitudinal evolution of circulating endothelial cells preceding a coronary event. (A) The average evolution of circulating endothelial cells (CECs) during follow-up is depicted for patients who reached the study endpoint (cases) and patients who remained endpoint-free (controls). (B) The average evolution of CECs is depicted during the twelve months preceding a primary endpoint. The mean(95%CI) CEC concentrations were 12.8 (8.2–20.0) cells/ml in cases and 10.0 (7.0–14.40) cells/ml in controls. 'Time zero' is defined as the occurrence of the endpoint in cases and last sample moment in controls and is depicted on the right side of the x-axis; inherently to this representation, baseline sampling preceded this 'time zero'. In both panel A and panel B, the solid red line depicts the average evolution of CECs in cases and the solid blue line depicts the average evolutions in controls. The dashed lines represent the 95% confidence interval. Red and blue dots represent the absolute cell concentrations in cases and controls, respectively. Measurements exceeding the upper limit of the y-axis of the graph are shifted towards this upper limit.

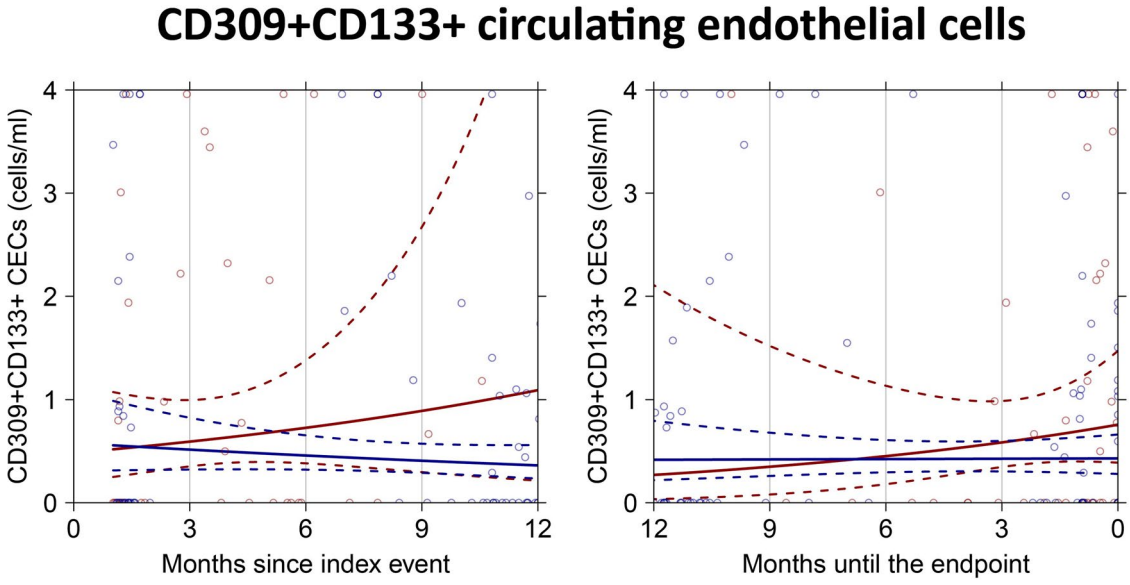

Figure 3. Longitudinal evolution of circulating endothelial cells co-expressing CD309 and CD133 preceding a coronary event. (A) The average evolution of the subset of CD309 + CD133+ cells during follow-up is depicted for patients who reached the study endpoint (cases) and patients who remained endpoint-free (controls). (B) The average evolution of the subset of CD309 + CD133+ cells is depicted during the twelve months preceding a primary endpoint.

(10.0 [95%CI 7.0–14.4] cells/ml) (Figure 2B), although, again this difference was non-significant ($P = 0.339$). The longitudinal, post 30-day trajectories of CEC subsets expressing CD309 and CD133, or CD106 are depicted in Figures 3–4 and show no significant differences between cases and controls.

As regards the washout of CECs after the index ACS in controls, based on the LME model, the mean cell concentration was significantly ($P = 0.030$) lower in the post 30-day samples compared to the samples collected within one day after the index ACS: 10.1 (95%CI 7.5–13.6) versus 17.0 (10.8–26.6) cells/ml (Figure 5A). Similar results were observed for the subset of cells expressing CD309 and CD133 (mean 0.4 versus 1.0 cells/ml, $P = 0.011$, Figure 5B) or CD106 (mean 1.3 versus 0.9 cells/ml, $P = 0.306$, Figure 5C).

Discussion

In the clinically stable post-ACS period, CEC concentrations appeared not predictive of a repeat ACS. In particular, CEC concentrations were higher directly after index ACS than in

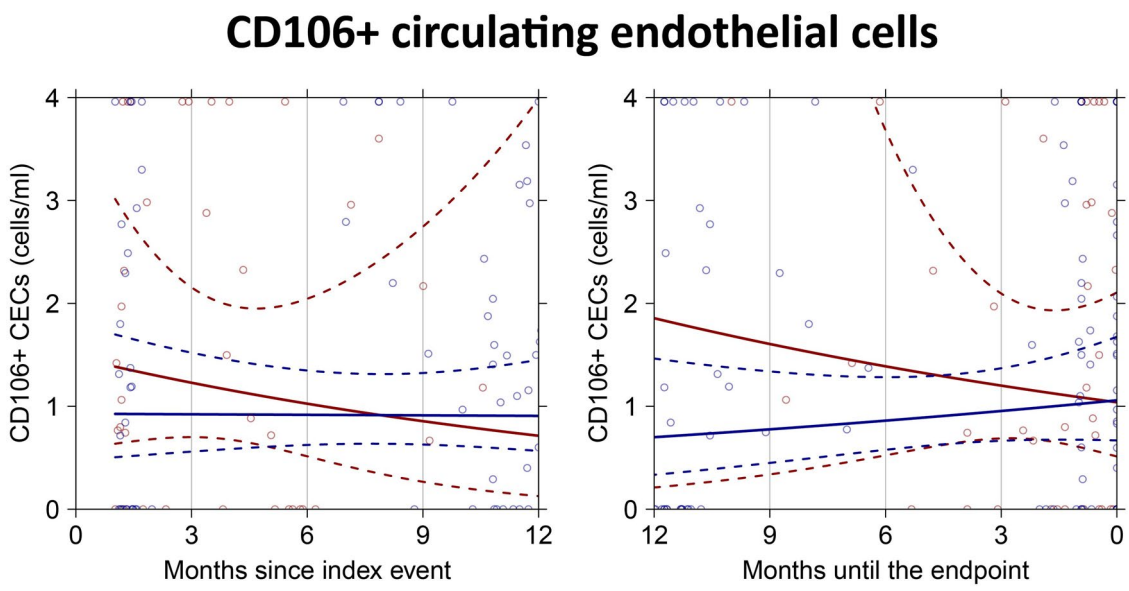

Figure 4. Longitudinal evolution of circulating endothelial cells co-expressing CD106 preceding a coronary event. (A) The average evolution of the subset of CD106+ cells during follow-up is depicted for patients who reached the study endpoint (cases) and patients who remained endpoint-free (controls). (B) The average evolution of the subset of CD106+ cells is depicted during the twelve months preceding a primary endpoint.

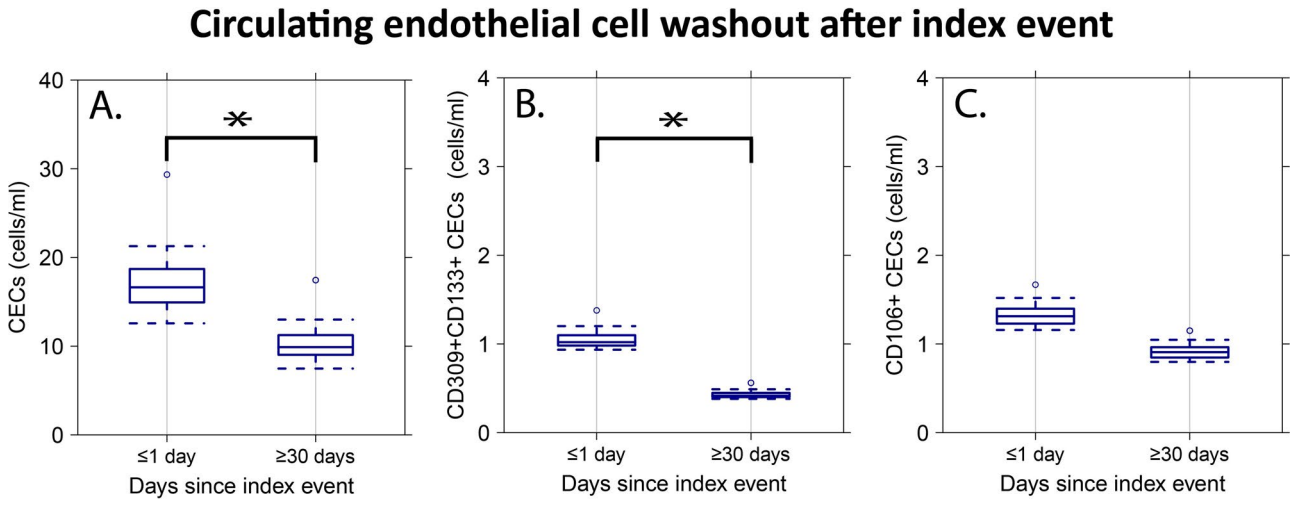

Figure 5. In controls, the estimated cell concentrations directly after index ACS and the estimated cell concentrations in samples drawn ≥30 days after the index ACS are depicted for (A) circulating endothelial cells, (B) the subset of CD309 + CD133+ cells and (C) the subset of CD106+ cells. *P-value < 0.05.

the clinically stable phase, but did not increase in the weeks or days prior to the repeat event.

Over twenty years ago, Mutin et al. were one of the first to describe CEC concentrations in relation to coronary artery disease (Mutin *et al.* 1999). They reported higher cell counts in peripheral blood of patients with acute MI or unstable angina than in that of patients with stable angina and healthy controls. These findings have subsequently been confirmed in multiple studies (Schmidt *et al.* 2015). Likewise, we found higher CEC concentrations early after the index ACS than later in the clinically stable phase. Furthermore, other studies have described a relation between CEC concentrations and (estimated) cardiovascular risk in ACS patients, (Boos *et al.* 2007b) as well as with adverse cardiovascular events during follow-up (Lee *et al.* 2005, Boos *et al.* 2008). In view of this evidence, we hypothesised that serial measurements of CECs may help to identify a pathophysiologic, vulnerable state preceding an ACS. Even though our explorative study failed to produce supportive evidence, it should be noted that the last sample prior to the recurrent ACS was collected at a median of 12 days before the event. Hence, it cannot be excluded that CEC concentrations increased in the days or hours prior to the endpoint ACS.

Endothelial cells expressing VCAM-1 (CD106) have been identified as activated endothelial cells, an indicator for atherosclerosis (Radecke *et al.* 2015). A recent study on VCAM-1 expressing endothelial cells reported that VCAM-1 expression was absent on cells in the peripheral circulation among patients with MI, but an increased density was found on coronary artery endothelial cells (from plaque site) (Radecke *et al.* 2015). In contrast, VCAM-1 expressing CECs were detected in the current study, but no differences were observed between cases and controls. Even so, the vascular integrity of an individual is not only reflected by markers of vascular injury or

dysfunction, but by the balance between endothelial injury and endothelial regeneration. Circulating cells co-expressing VEGFR-2 (CD309) and CD133 have been identified as endothelial progenitor cells, hallmarking regeneration (Samman Tahhan *et al.* 2018). Previous studies demonstrated that levels of circulating progenitor cells are significantly higher in acute MI patients than those with stable coronary artery disease. Among patients with ACS, a lower number of CD133+, but not CD309+, circulating progenitor cells was associated with a higher mortality (Samman Tahhan *et al.* 2018). In the current study, increased progenitor cell concentrations were detected after index ACS, but no differences were found between cases and controls during follow-up.

Three main caveats have to be mentioned. First, reported CEC levels vary greatly in published literature. This variability mainly results from the use of highly variable isolation methods and immunophenotypic definitions of CECs. Consequently, between-study comparison of CEC concentrations should be approached with caution, while repeated measurements within an individual or the comparison of groups of individuals within one study provide appropriate alternatives. The endothelial origin of the cells identified in the current study has been previously confirmed by morphological, immunohistochemical and gene analyses, and a validated assay (Kraan *et al.* 2012). Moreover, it has been demonstrated that the reproducibility of circulating endothelial cell enumeration is acceptable after thawing of frozen samples (Mancuso *et al.* 2008, Bogoslovsky *et al.* 2013). Since our samples are stored for a relatively long period (i.e. 5–11 years), we cannot exclude the possibility that the cell recovery or reproducibility is reduced in our cohort. Nonetheless, we did not observe an association between circulating endothelial cell count and storage duration in the current study ($p < 0.225$). Second, previous studies reported that PCI leads to an increase in CECs within 15 minutes after the intervention and that concentrations remain elevated 24 hours thereafter (Boos *et al.* 2007a). Hence, local endothelial damage following PCI may initiate widespread systemic endothelial activation or injury, resulting in increased levels of CECs in the peripheral circulation. Most (98%) of our patients underwent PCI shortly after the index ACS. Nonetheless, median CEC concentration did not differ between blood samples collected before PCI and samples collected after PCI (12 vs. 13 samples, respectively; data not shown). Lastly, although the sample size calculation that we performed in advance demonstrated that a total of 15 event cases and 30 non-events controls would be sufficient to determine meaningful differences in cardiovascular biomarkers, we cannot completely eliminate the possibility of a type II error in the current study. Moreover, our study was not sufficiently powered to evaluate the effect of established cardiovascular risk factors (e.g. diabetes mellitus, GRACE risk score) or disease severity (i.e. unstable angina, NSTEMI or STEMI) on the cell concentrations and trajectories observed in the current study.

In conclusion, despite their close relation to vascular damage, no increase in cell concentrations were found in this explorative study in the weeks prior to the occurrence of a secondary adverse cardiac event.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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Data availability statement

The data that support the findings of this study are available from the corresponding author, EB, upon reasonable request.

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