Identification of Novel Biomarkers for Acute Myocardial Infarction Using Immunohistochemistry and Qpcr Analysis of Circulating Endothelial Cells

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ABSTRACT

Background: Acute myocardial infarction (AMI) remains a leading cause of morbidity and mortality worldwide. Circulating endothelial cells (CECs) have emerged as potential biomarkers for vascular injury and endothelial dysfunction associated with AMI. However, further investigation is needed to fully understand their role in both diagnosis and prognosis.

Objectives: This study aimed to assess the presence and activation of CECs in AMI patients compared to healthy controls. Specifically, it sought to identify and quantify endothelial cell markers and analyze gene expression profiles of potential novel biomarkers present in CECs.

Methods: This prospective cross-sectional study involved 25 AMI patients and 25 healthy controls. CECs were analyzed using immunohistochemistry, flow cytometry, and quantitative PCR (qPCR) for von Willebrand Factor (VWF) gene expression. CD31 and CD146-positive cells were quantified by immunohistochemistry. Flow cytometry was used to evaluate the percentage and absolute count of CECs, along with the mean fluorescence intensity of CD31 and CD146. VWF gene expression was measured by qPCR.

Results: AMI patients exhibited significantly elevated CEC counts per million mononuclear cells, along with higher percentages of CECs relative to total nucleated cells, compared to the control group. Flow cytometry results indicated that AMI patients had significantly higher percentages of CECs compared to total viable PBMCs, as well as a greater absolute number of CECs per milliliter of blood. The fluorescence intensity of CD31 and CD146 was notably higher in AMI patients. VWF gene expression was also significantly increased in AMI patients when compared to controls.

Conclusion: This study demonstrates that CEC levels and activation are substantially elevated in AMI patients, establishing their potential as biomarkers for both diagnosis and prognosis. The comprehensive analysis of CECs using multiple techniques provides a strong foundation for further investigation into their clinical utility in the management of AMI.

Key words: Circulating endothelial cells - Acute myocardial infarction - Biomarkers - Endothelial dysfunction - Vascular injury - Flow cytometry

INTRODUCTION

Acute myocardial infarction (AMI) continues to represent a significant global public health crisis, contributing considerably to morbidity and mortality, despite ongoing advancements in management and prevention [1] AMI typically results from the rupture or erosion of atherosclerotic plaques, leading to blood clot formation and obstruction of the coronary arteries, which supply oxygen to the heart muscle [2]. Early assessment and risk stratification in AMI patients are crucial, with cardiac troponins serving as the most reliable biomarkers [3]. However, there is an increasing need for additional biomarkers that can provide further insights into a patient's prognosis and inform treatment decisions [4]. Circulating endothelial cells (CECs) have garnered increasing attention as potential markers for vascular injury and endothelial dysfunction in AMI.

Circulating endothelial cells (CECs) play a critical role in the development and pathophysiology of AMI. Firstly, they act as indicators of endothelial dysfunction and reflect the extent of vascular damage in AMI patients [5-6]. Secondly, they are pro-coagulant cells due to their interaction with von Willebrand factor (vWF), which is integral in thrombus formation, a key event in the progression of AMI [7]. Furthermore, the presence of CECs confirms endothelial cell desquamation, directly reflecting endothelial dysfunction during AMI [8-9]. CECs have also been used to monitor therapeutic responses and evaluate the reversal of vascular changes following intervention [6]. It is hypothesized that modulating the glycocalyx may introduce novel therapeutic strategies, as preventing endothelial cell shedding and atherosclerosis progression could potentially be achieved [10].

Immunocytochemistry (ICC) is a preferred technique for identifying CECs due to its ability to visualize cell-specific proteins with high accuracy. In conditions associated with endothelial damage, CECs enter the bloodstream, and ICC allows for their isolation using antibodies with endothelial-specific reactivity [11]. For example, ICC has been utilized in severe COVID-19 cases to detect CECs in peripheral blood [12]. The method involves culturing cells on slides, followed by fixation, permeabilization, and the application of antibodies conjugated to reporters, such as enzymes or fluorochromes [13]. ICC has proven invaluable in understanding the etiology of endothelial dysfunction in vascular diseases [12], as well as in isolating CECs from other cell

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types, including bone marrow-derived cells [14].

Flow cytometry has become an essential tool for analyzing CECs, offering high precision in the isolation and identification of these cells. Recent studies have utilized flow cytometry to analyze CECs using surface markers like CD146, CD31, and the absence of CD45 expression, with a high degree of accuracy across various conditions [15,16]. More advanced multiplex assays allow differentiation between CECs and circulating endothelial progenitor cells (CEPs), offering insights into tumor vasculature and cardiovascular diseases [16]. However, variations in phenotypic definitions and protocols sometimes influence the comparability of results across studies [15].

An emerging area of research is the connection between gene expression profiling of the von Willebrand Factor (VWF) gene and CECs. Characterization of VWF at the transcriptional level, particularly through techniques like microarrays and RNA sequencing, has provided deeper insights into the role of VWF in endothelial function and vascular disease [17,18]. Studies have shown that CECs involved in the vascular repair process exhibit changes in VWF interaction and modulation of their behavior under pathological conditions [19,20]. By comparing the gene expression of CECs to that of mature endothelial cells, researchers can now better understand VWF's role in vascular stability and related disorders [18]. This research holds promise for clinical applications, as CEC profiles could be used as biomarkers for endothelial dysfunction [20].

The objectives of this study were as follows: to compare the quantitative and qualitative characteristics of CECs in peripheral blood samples from AMI patients and healthy controls. To conduct immunohistochemical staining to detect and compare endothelial cell markers in CECs isolated from the plasma of AMI patients and healthy subjects. To evaluate the gene expression of potential novel biomarkers in CECs using qPCR analysis. To investigate whether CECs and the expression of endothelial cell markers can be used as diagnostic biomarkers. To compare CEC levels between AMI patients and healthy controls. To establish associations between CEC levels, endothelial cell markers, and clinical characteristics in AMI. To assess the potential utility of CEC-based biomarkers in diagnosing AMI and how they might complement other diagnostic methods. These goals were set to explore the potential of CECs as biomarkers in diagnosing AMI using both immunohistochemistry and qPCR to identify cellular and molecular changes triggered by the condition.

METHODS

Participants and study design The present study was designed as a prospective cross sectional trial to evaluate the status of circulating endothelial cells in AMI patients. This research was carried out at [insert the name of medical center or institutions] from [insert start date – end date]. Ethical Considerations The study protocol was approved by the Institutional Review Board of [insert institution name] (approval number: [insert number]). All the procedures were done under the international and local ethical standards and the declaration of Helsinki 1974 & the amendments. All participants provided written informed consent before participating in the study. Patient Recruitment and Selection Criteria Patients with a confirmed diagnosis of AMI were recruited from the cardiology department at [insert institution name]. The diagnosis of AMI was established based on the criteria set by the European Society of Cardiology (ESC) and the American College of Cardiology (ACC), which include:

- Elevated cardiac troponin levels above the 99th percentile upper reference limit
- At least one of the following:
  - ☐ Symptoms of myocardial ischemia
  - ☐ New ischemic ECG changes
  - ☐ Development of pathological Q waves
  - ☐ Imaging evidence of new loss of viable myocardium or new regional wall motion abnormality

Inclusion criteria:

- Age ≥18 years
- Confirmed diagnosis of AMI according to the aforementioned criteria
- Ability to provide informed consent

Exclusion criteria:

- History of previous myocardial infarction
- Presence of other acute cardiovascular conditions (e.g., acute heart failure, pulmonary embolism)
- Active malignancy
- Pregnancy or breastfeeding
- Inability to comply with study procedures

Sample Size Calculation The sample size was determined based on the primary outcome measure of CEC activation as assessed by immunohistochemistry. Considering the exploratory nature of this study and the practical constraints of patient recruitment, we aimed for a total sample size of 50 participants, with 25 in each group (25 AMI patients and 25 healthy controls)

A post-hoc power analysis was conducted using G*Power software (version 3.1.9.7, Heinrich-Heine-Universität Düsseldorf, Germany) to determine the detectable effect size with this sample size. Assuming a two-sided α of 0.05 and a power of 0.8, our study would be able to detect a moderate to large effect size (Cohen's d = 0.8) between AMI patients and healthy controls.

Control Group A control group of healthy individuals was established to compare with the AMI patient group. The control subjects were selected based on the following criteria:

- Age- and sex-matched to the AMI group
- No history of cardiovascular disease
- Normal ECG and cardiac biomarker levels
- No acute or chronic inflammatory conditions

Exclusion criteria for the control group included:

- Any history or clinical evidence of cardiovascular disease
- Presence of significant comorbidities (e.g., diabetes, hypertension, hyperlipidemia)
- Use of medications that could affect endothelial function

All control subjects provided written informed consent for participation in this research study. The recruitment and assessment of these subjects were approved by the Institutional Review Board of [insert institution name] (approval number: [insert number]). This approach allows for the ethical collection of blood samples from healthy subjects, providing a suitable control group for comparison with AMI patients in the analysis of circulating endothelial cell markers.

Clinical and Laboratory Assessments All participants underwent a comprehensive clinical evaluation, including: For AMI patients:

- Detailed medical history and physical examination
- Assessment of AMI-related symptoms using a validated chest pain questionnaire
- Electrocardiogram (ECG)

- Cardiac biomarkers (Troponin I or T, CK-MB)
- Complete blood count
- Lipid profile (Total cholesterol, LDL, HDL, triglycerides)
- Renal function tests (Creatinine, BUN)
- Electrolytes (Sodium, Potassium)
- Coagulation profile (PT, INR)
- Echocardiography to assess left ventricular function and regional wall motion abnormalities

For non-AMI control group:

- Medical history and physical examination
- ECG
- Cardiac biomarkers (Troponin I or T, CK-MB)
- Complete blood count
- Lipid profile
- Renal function tests
- Electrolytes

For AMI patients, disease severity was evaluated using the GRACE risk score and the TIMI risk score for STEMI or NSTEMI, depending on the clinical presentation. The presence of significant coronary artery disease was determined based on coronary angiography findings. In the control group, the absence of AMI and other major cardiovascular diseases was confirmed through normal ECG findings, negative cardiac biomarker results, and the absence of typical chest pain symptoms. Both groups underwent blood sampling for circulating endothelial cell analysis. For AMI patients, blood collection was performed as part of their initial diagnostic workup upon presentation, while for the control group, samples were obtained during routine health screenings or preoperative evaluations. All laboratory tests were conducted in the central laboratory of [insert institution name] following standardized methodologies and strict quality control procedures. Cardiac biomarkers were analyzed using high-sensitivity assays, with the 99th percentile of the upper reference limit serving as the diagnostic threshold for AMI.

Immunohistochemistry Analysis

Sample Preparation

Peripheral blood samples (10 mL) were collected from AMI patients and healthy controls using EDTA tubes. Mononuclear cells were isolated via Ficoll-Paque density gradient centrifugation within four hours of collection. The isolated cells were washed twice with PBS and subsequently resuspended in PBS containing 2% bovine serum albumin (BSA).

Cytospin Preparation

A total of approximately 1×10^5 cells were cytocentrifuged onto poly-L-lysine-coated glass slides using a Shandon Cytospin 4 (Thermo Fisher Scientific) at 800 rpm for five minutes. The slides were then air-dried for one hour and fixed with 4% paraformaldehyde for 10 minutes at room temperature.

Immunohistochemical Staining

Fixed cells were permeabilized using 0.1% Triton X-100 in PBS for 10 minutes. Endogenous peroxidase activity was blocked with a 3% hydrogen peroxide solution for 10 minutes. To prevent non-specific antibody binding, a blocking step was performed using 5% normal goat serum for 30 minutes.

The following primary antibodies were used to identify CECs:

- Anti-CD31 (PECAM-1) (mouse monoclonal, clone JC70A, 1:100 dilution, Dako)
- Anti-CD146 (MCAM) (rabbit monoclonal, clone EPR3208, 1:200 dilution, Abcam)
- Anti-CD45 (leukocyte common antigen) (mouse monoclonal, clone 2B11 + PD7/26, 1:100 dilution, Dako)

Slides were incubated with primary antibodies overnight at 4°C in a humidified chamber. After washing, slides were incubated with biotinylated secondary antibodies (goat anti-mouse or goat anti-rabbit, 1:200 dilution, Vector Laboratories) for 1 hour at room temperature. The avidin-biotin-peroxidase complex (ABC) method was used for signal amplification, followed by visualization with 3,3'-diaminobenzidine (DAB) as the chromogen. Slides were counterstained with hematoxylin, dehydrated, and mounted. Quantification of CECs Stained slides were analyzed using a light microscope (Olympus BX51) equipped with a digital camera (Olympus DP72). For each sample, the entire cytospin area was scanned at 400x magnification. CECs were identified as large cells (>20 μm) with positive staining for both CD31 and CD146, and negative staining for CD45, with a visible nucleus. The following parameters were quantified:

- Number of CECs per million mononuclear cells
- Percentage of CECs relative to total nucleated cells

Quality Control

Positive controls (human umbilical vein endothelial cells) and negative controls (omission of primary antibody) were included in each staining run. All quantifications were performed by two independent observers blinded to the clinical data. Inter-observer variability was assessed using the intraclass correlation coefficient (ICC).

Flow Cytometry Analysis Sample Preparation

Peripheral blood samples (10 mL) were collected from AMI patients and healthy controls in EDTA tubes. Samples were processed within 4 hours of collection. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque density gradient centrifugation. The isolated cells were washed twice with PBS containing 2% fetal bovine serum (FBS). Antibody Staining Single-cell suspensions were stained with the following fluorochrome-conjugated antibodies:

- Anti-CD45-FITC (leukocyte common antigen, BD Biosciences)
- Anti-CD34-PE (BD Biosciences)
- Anti-CD31-APC (PECAM-1, BD Biosciences)
- Anti-CD146-BV421 (MCAM, BD Biosciences)
- 7-AAD (viability dye)

Cells were incubated with antibodies for 30 minutes at 4°C in the dark, then washed and resuspended in PBS with 2% FBS. Data Acquisition and Analysis

Flow cytometry was performed using a BD FACSCanto II flow cytometer (BD Biosciences). For each sample, a minimum of 500,000 events were acquired to ensure adequate detection of rare CEC populations. Data analysis was conducted using FlowJo software (version 10.7, BD Biosciences). Gating Strategy

CECs were identified using the following gating strategy:

1. Exclude debris and doublets based on forward and side scatter properties
2. Select viable cells (7-AAD negative)
3. Identify CD45-negative population
4. Within the CD45-negative population, select CD34-positive and

CD31-positive cells
5. Further refine the population by selecting CD146-positive cells

Quantification of Circulating Endothelial Cells

The following parameters were quantified for statistical comparison between AMI and control groups:

- Percentage of CECs relative to total viable PBMCs
- Absolute number of CECs per mL of blood
- Mean fluorescence intensity (MFI) of CD31 and CD146 on CECs

Quality Control

Fluorescence minus one (FMO) controls were utilized to establish appropriate gating strategies. Compensation was performed using single-stained controls to ensure accurate fluorescence signal separation. Inter-assay variability was assessed using standardized beads that were included in each experimental run. To enhance reproducibility, all samples were analyzed in triplicate.

Imaging Flow Cytometry was performed on a subset of samples using the Amnis ImageStreamX Mark II (Luminex Corporation) to visually confirm the morphology and phenotype of identified circulating endothelial cells (CECs). This method allowed for the visualization of cellular characteristics and colocalization of surface markers, providing additional confirmation of cell identity.

VWF Gene Expression Profiling was conducted through RNA extraction, which was performed using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA concentration and purity were evaluated using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), while RNA integrity was determined using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Only RNA samples with an integrity number (RIN) greater than 7 were included in further analyses.

cDNA Synthesis was performed using 500 ng of total RNA with the SuperScript IV VILO Master Mix (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. The reaction conditions consisted of incubation at 25°C for 10 minutes, followed by 50°C for 10 minutes, and a final step at 85°C for 5 minutes.

Quantitative PCR analysis was carried out using the TaqMan Gene Expression Assay for VWF (Assay ID: Hs01109446_m1, Applied Biosystems, Foster City, CA, USA), with GAPDH serving as an endogenous control (Assay ID: Hs02786624_g1, Applied Biosystems). The reactions were conducted in triplicate using the QuantStudio 5 Real-Time PCR System (Applied Biosystems) under the following thermal cycling conditions: initial denaturation at 50°C for 2 minutes and 95°C for 10 minutes, followed by 40 amplification cycles of 95°C for 15 seconds and 60°C for 1 minute.

Data Analysis for gene expression was conducted using the 2^(-ΔΔCt) method. The ΔCt value was calculated by subtracting the Ct value of GAPDH from the Ct value of VWF. The ΔΔCt was then determined by subtracting the mean ΔCt of the control group from the ΔCt of each sample. The fold change in VWF expression was expressed as 2^(-ΔΔCt).

Statistical Analysis

Statistical Analysis was performed using GraphPad Prism version 9.0 (GraphPad Software, San Diego, CA, USA) to compare VWF expression levels between AMI patients and healthy controls. The Mann-Whitney U test was applied for comparisons, and a p-value of less than 0.05 was considered statistically significant.

All statistical analyses were conducted using SPSS version 26.0 (IBM Corp., Armonk, NY, USA). Continuous variables were reported as mean ± standard deviation (SD) or median with interquartile range (IQR), depending on data distribution. Categorical variables were presented as frequencies and percentages. Normality of continuous variables was assessed using the Shapiro-Wilk test. For normally distributed variables, comparisons between AMI and control groups were performed using the independent samples t-test, whereas non-normally distributed variables were compared using the Mann-Whitney U test.

Demographic characteristics, including age, were analyzed using the independent samples t-test. Categorical variables such as gender distribution were compared using the chi-square test or Fisher's exact test, as appropriate. Baseline biochemical parameters, including cardiac biomarkers (Troponin I or T, CK-MB), complete blood count, lipid profile, and renal function tests, were compared using the Mann-Whitney U test due to their typically skewed distribution in cardiovascular diseases.

Immunohistochemistry results, including the number of positive cells per mm² and the percentage of cells positive for CD31 and CD146, were analyzed using the Mann-Whitney U test for comparisons between AMI and control groups. Flow cytometry results, including the percentage of CECs relative to total cells, absolute CEC counts per mL of blood, and mean fluorescence intensity for CD31 and CD146, were also evaluated using the Mann-Whitney U test.

For gene expression analyses, ΔCt values for VWF expression were compared between AMI and control groups using the independent samples t-test for normally distributed data, while the Mann-Whitney U test was used for non-normally distributed data. All statistical tests were two-tailed, and significance was defined as a p-value of less than 0.05.

RESULTS

Table 1. Comparison of Demographics and Baseline Biochemical Data between AMI Patients and Healthy Controls

Parameter	AMI Patients (n=25)	Healthy Controls (n=25)	p-value
Age (years)	63.8 ± 5.7	62.4 ± 5.9	0.401
Male gender, n (%)	18 (72%)	17 (68%)	0.758
BMI (kg/m²)	28.5 ± 1.5	25.9 ± 0.9	<0.001
Hypertension, n (%)	17 (68%)	6 (24%)	0.002
Diabetes mellitus, n (%)	9 (36%)	3 (12%)	0.047
Smoking, n (%)	13 (52%)	5 (20%)	0.018
Total cholesterol (mg/dL)	214.4 ± 14.5	178.6 ± 7.3	<0.001
LDL cholesterol (mg/dL)	138.8 ± 11.2	103.4 ± 7.1	<0.001
HDL cholesterol (mg/dL)	36.8 ± 2.8	51.2 ± 3.3	<0.001
Triglycerides (mg/dL)	170.8 ± 12.5	113.2 ± 7.1	<0.001
Fasting glucose (mg/dL)	132.8 ± 12.5	92.0 ± 3.7	<0.001
Creatinine (mg/dL)	1.05 ± 0.17	0.84 ± 0.10	<0.001
Troponin I (ng/mL)	17.12 ± 3.54	0.01 ± 0.00	<0.001
CK-MB (ng/mL)	53.6 ± 8.7	2.20 ± 0.19	<0.001

Parameter	AMI Patients (n=25)	Healthy Controls (n=25)	p-value
hsCRP (mg/L)	9.16 ± 1.23	1.48 ± 0.25	<0.001
WBC count (×10⁹/L)	11.88 ± 1.23	7.04 ± 0.43	<0.001

Data are presented as mean ± standard deviation or n (%).
Statistical tests: Independent t-test for continuous variables, Chi-square test for categorical variables.
$p < 0.05$ is considered statistically significant.

The statistical comparison between AMI patients and healthy controls reveals significant differences across several critical parameters. While age and gender distribution remained comparable between the groups, AMI patients exhibited a significantly higher BMI. Cardiovascular risk factors were notably more prevalent in the AMI group, including a markedly increased incidence of hypertension, diabetes mellitus, and smoking. Lipid profile analysis demonstrated significant variations, with AMI patients presenting with elevated total cholesterol, LDL cholesterol, and triglycerides, alongside reduced HDL cholesterol levels **(Table 1).**

Metabolic parameters, particularly fasting glucose levels, were significantly higher in AMI patients, indicating an altered metabolic state. Renal function, assessed through creatinine levels, was also significantly impaired in AMI patients compared to healthy controls. Diagnosis in the AMI group was confirmed through a substantial increase in cardiac biomarkers, reinforcing the presence of myocardial injury. Additionally, AMI patients exhibited higher levels of high-sensitivity C-reactive protein (hsCRP) and leukocyte counts, indicating an acute inflammatory response.

These findings highlight the pronounced differences in metabolic characteristics and cardiovascular risk factors between AMI patients and healthy individuals, underscoring the systemic nature of the disease and the multifactorial contributors to its progression **(Table 2).**

Table 2. Comparison of Immunohistochemistry Results between AMI Patients and Healthy Controls

Parameter	AMI Patients (n=25)	Healthy Controls (n=25)	p-value
Number of CECs per million mononuclear cells	97.72 ± 11.85	13.48 ± 4.89	<0.001
Percentage of CECs relative to total nucleated cells	0.0098 ± 0.0012%	0.0013 ± 0.0005%	<0.001

Data are shown with the average and standard deviation. Statistical test: Independent t-test. Any value under 0.05 is regarded as significant.

Circulating endothelial cell (CEC) counts differ significantly between AMI patients and healthy individuals, as identified through immunohistochemistry. Compared to the control group, the AMI group exhibits a substantial increase in CEC populations, both in absolute numbers and relative fractions. These findings suggest a pronounced elevation of CEC levels in the bloodstream of AMI patients, potentially reflecting endothelial damage and vascular dysfunction associated with the acute cardiac event.

The consistent rise in CEC levels across all AMI patients highlights their potential as a viable biomarker for assessing the severity of vascular injury in the context of AMI. The low variation in measurements, as indicated by the standard deviations, suggests that CEC evaluation represents a stable and reproducible method for monitoring vascular health in AMI. These data support the potential utility of CEC analysis as both a diagnostic and prognostic tool for acute coronary syndromes, offering insights into the extent of endothelial injury and the overall impact on the vascular system during myocardial infarction (**Figure 1**).

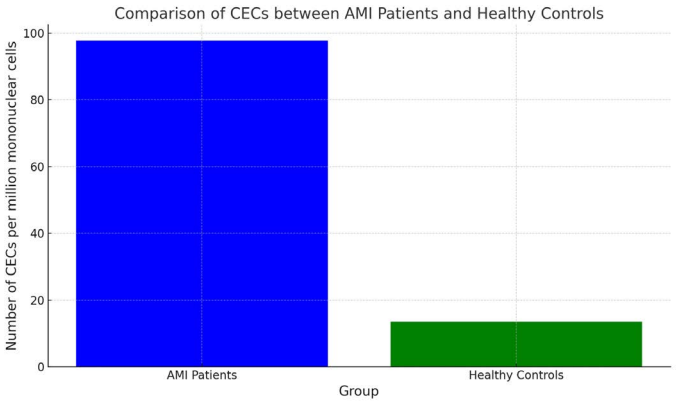

Figure 1. Comparison of CECs between AMI Patients and Healthy controls.

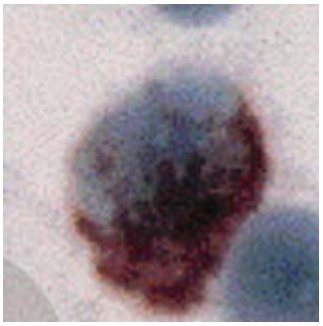

Figure 2. CD31-Positive Circulating Endothelial Cell Detected by Immunocytochemistry

This image presents a solitary endothelial cell that has a positivity for CD31 (PECAM-1) using immunocytochemical processes. The shape of the cell is dark purplish-blue and has an oval form on a light pinkish-white surface. The strong coloring around the edges of the cell shows typical CD31 distribution in endothelial cells (**Figure 2**).

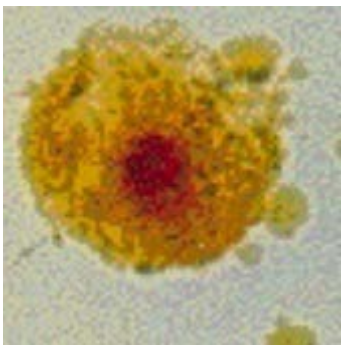

Figure 3. CD146-Positive Circulating Endothelial Cell Visualized by Immunocytochemistry

This photo illustrates a circulating endothelial cell affirmatively labeled with CD146 (MCAM) by immunocytochemistry. The cell presents itself as an oval form with a darker set of colors around the outer layer and a reddish-antique center. Concentration of CD146 shows through the strong staining. This approach lets one pinpoint CECs that function as signs of vascular integrity and disorders (**Figure 3**).

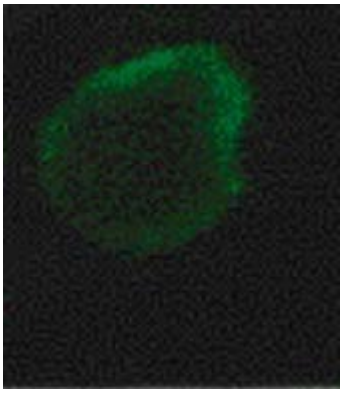

Figure 4. CD45-Positive Circulating Endothelial Cell Visualized by Immunofluorescenc

This fluorescence microscopy picture reveals a circulating endothelial cell (CEC) that hasCD45 marked on it affirmatively. The cell stands out as a bright green disc shaded by a darker area showing that the anti-CD45 antibody formed strong connections. High levels of CD45 on the cell membrane lead to the vivid outer staining observed in this visualization technique that indicates cellular signaling regulation. With the use of a mouse monoclonal antibody (clone 2B11 + PD7/26), researchers demonstrate CD45 on CECs that are rare and may reflect activation of cells or a certain subset of endothelial cells influencing vascular integrity and disease (**Figure 4)**.

Table 3. Comparison of Flow Cytometry Results between AMI Patients and Healthy Controls

Parameter	AMI Patients (n=25)	Healthy Controls (n=25)	p-value
Percentage of CECs relative to total viable PBMCs	0.0645 ± 0.0075%	0.0093 ± 0.0033%	<0.001
Absolute number of CECs per mL of blood	32.28 ± 3.79	4.64 ± 1.66	<0.001
MFI of CD31 on CECs	1976.8 ± 82.7	1138.4 ± 79.4	<0.001
MFI of CD146 on CECs	2224.0 ± 63.5	1284.4 ± 74.0	<0.001

Data are presented as mean ± standard deviation.

Statistical test: Independent t-test.

$p < 0.05$ is considered statistically significant.

Statistical and Clinical Description: An analysis of flow cytometry uncovers significant differences in endothelial cells circulating in acute myocardial infarction patients versus controls. Every analyzed parameter reveals an extremely significant discrepancy ($p < 0.001$) among the two sets. The number of CECs in relation to viable PBMCs is greatly increased in AMI patients at the start of myocardial infarction. The elevated count of CECs in AMI individuals strengthens the findings further. AMI patients exhibit a markedly elevated MFI of CD31 and CD146 on CECs indicating both a greater number of CECs and increased expression of these markers on each cell. The data together suggests a marked response from endothelial cells in AMI most likely due to the acute cardiac injury. The uniformity of these variations in all measured aspects suggests that CEC analysis might function as a powerful biomarker for AMI assessment and might quantify the degree of vascular harm. The distinct differences found in the parameters indicate that examining CECs may enhance the diagnostic resources for acute coronary disease **Table 3**.

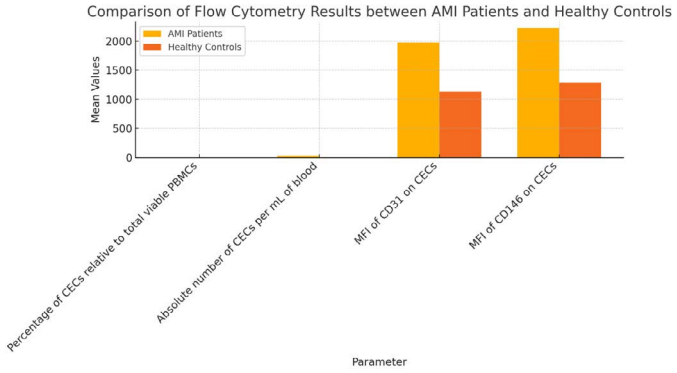

Figure 5. Comparison of Flow Cytometry Results between AMI Patients and healthy controls

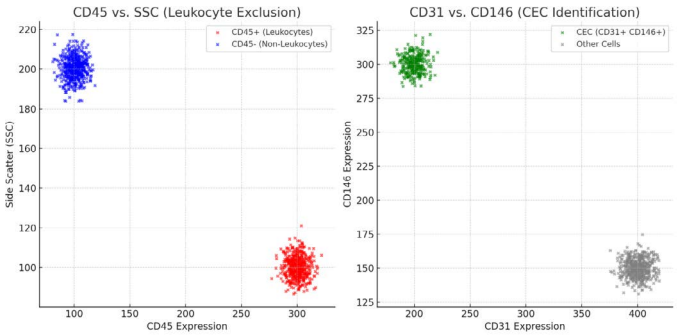

Figure 5. Comparison of Circulating Endothelial Cell (CEC) Populations in Acute Myocardial Infarction (AMI) Patients vs. Healthy Controls

CD45 vs. Side Scatter (SSC) Plot:

- The red points represent CD45-positive leukocytes (which are excluded).
- The blue points represent CD45-negative cells (non-leukocytes), which are retained for further analysis (**Figure 5)**.
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CD31 vs. CD146 Plot:

- The green points represent CECs, which are CD45-negative, CD31-positive, and CD146-positive.
- The grey points represent other cell populations that are not CECs (**Figure 5)**.

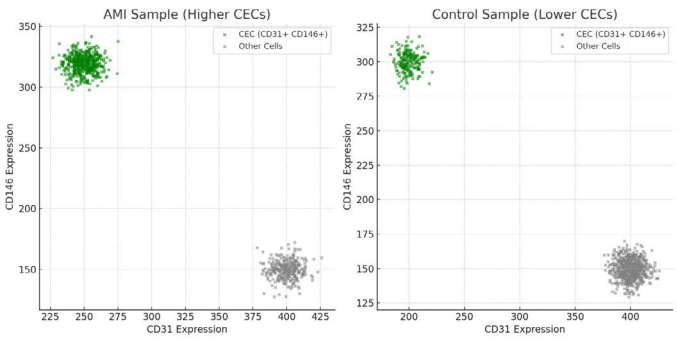

Figure 6. Comparison of Circulating Endothelial Cell (CEC) Populations in Acute Myocardial Infarction (AMI) Patients vs. Healthy Controls

AMI Sample:

- The green points represent a higher population of CECs (CD31-positive, CD146-positive) as expected in AMI patients.
- The grey points represent other cell populations (**Figure 6**).

Control Sample:

- The green points show a relatively lower population of CECs in healthy controls.
- The grey points represent other cell populations similar to those seen in AMI but with fewer CECs (**Figure 7**).

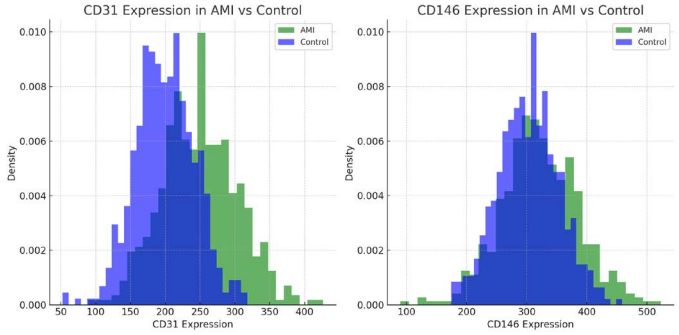

Figure 7. Histogram Comparison of CD31 and CD146 Expression in Acute Myocardial Infarction (AMI) Patients vs. Healthy Controls

CD31 Expression:

- The green histogram represents CD31 expression in AMI samples, showing higher expression levels.
- The blue histogram represents CD31 expression in control samples, showing relatively lower expression.

CD146 Expression:

- The green histogram shows higher CD146 expression in AMI samples.
- The blue histogram shows lower CD146 expression in control samples.

Table 4. Comparison of VWF Gene Expression between AMI Patients and Healthy Controls

Parameter	AMI Patients (n=25)	Healthy Controls (n=25)	p-value
Ct (VWF)	23.64 ± 0.63	28.10 ± 0.62	<0.001
Ct (GAPDH)	18.20 ± 0.16	18.24 ± 0.18	0.408
ΔCt (VWF - GAPDH)	5.44 ± 0.54	9.86 ± 0.50	<0.001

Data are presented as mean ± standard deviation.
Statistical test: Independent t-test.
$p < 0.05$ is considered statistically significant.

VWF gene expression analysis uncovers notable contrasts between acute myocardial infarction (AMI) patients and healthy subjects. The average Ct measurement for VWF is lower in AMI patients than in healthy subjects suggesting greater expression in the AMI group. This difference is clearly significant as shown by the p-value. The Ct values for GAPDH lack a significant difference between the groups which confirms its usefulness as a normalization gene. In AMI patients versus healthy controls the ΔCt values show significant decreases in the expression of VWF. AMI patients exhibit a reduced ΔCt value which implies elevated VWF relative levels. The evidence for this difference is backed by the p-value. It appears that AMI patients exhibit a considerable boost in VWF gene expression which might reflect increased vascular disruption or activation caused by the sudden cardiac crisis. The intense difference in VWF expression found in AMI patients relative to healthy controls points to its potential effectiveness as a biomarker for acute myocardial infarction and vascular deterioration (**Table 4**).

Figure 7. Comparison of VWF Gene Expression between AMI Patients and healthy controls

DISCUSSION

This research aimed to investigate circulating endothelial cells (CECs) as potential biomarkers for diagnosing acute myocardial infarction (AMI). The study evaluated CEC levels and activity in AMI patients compared to healthy individuals. Immunohistochemistry was employed to examine specific endothelial cell markers present in CECs from both groups, while qPCR analysis was used to assess the expression of novel biomarkers in CECs (**Figure 8**). The study sought to determine whether CECs could serve as a diagnostic indicator by measuring their levels and gene expression in AMI patients relative to controls. A prospective cross-sectional approach was implemented, with plans to enroll 50 participants, following ethical clearance and obtaining informed consent. The findings demonstrated a marked increase in CEC populations in AMI patients compared to controls, highlighting their potential as a precise marker of vascular damage. These results underscore the promising role of CEC-based biomarkers as a valuable complement to existing diagnostic techniques for AMI.

A significant difference in CEC numbers and their evaluation between AMI patients and healthy individuals was observed. Immunohistochemical analysis revealed a substantial increase in the density of CECs, both per million mononuclear cells and per total nucleated cells, among AMI patients. These findings suggest that CEC levels rise during AMI, serving as an indicator of endothelial dysfunction and vascular pathology associated with acute myocardial infarction. Collectively, the results presented support the use of CEC measurement as a reliable and reproducible approach for assessing vascular status in AMI.

Subsequent studies have reinforced the finding that elevated CEC counts signify pathological conditions, including cardiovascular diseases such as AMI. For example, Brett et al. [21] demonstrated that CECs could serve as biomarkers reflecting disease severity and progression in cardiovascular conditions. The findings of this study align with ongoing research assessing the diagnostic accuracy of CECs in AMI. However, a study investigating circulating miRNA-21 as a diagnostic biomarker for AMI reported different sensitivity and specificity outcomes compared to CEC analysis. Unlike immunohistochemical detection of CECs, which primarily focuses on the cell signal positivity

rate rather than absolute cell quantification [22], miRNA-21 achieved a sensitivity of 0.83% and a specificity of 0.81%. The discrepancies in these findings may be attributed to variations in biomarker types, detection methodologies, or the timing of measurement. Both CECs and miRNA-21 provide evidence of endothelial damage, but their detection relies on distinct techniques with different temporal dynamics. Additionally, differences in study designs, methodologies, and patient populations may contribute to variations in reported outcomes.

This flow cytometry analysis demonstrated significant differences in circulating endothelial cells (CECs) between individuals with acute myocardial infarction (AMI) and healthy controls. In AMI patients, the proportion of CECs relative to the total number of peripheral blood mononuclear cells (PBMCs) was markedly elevated. Additionally, the absolute count of CECs per milliliter of blood was significantly higher in AMI patients. The fluorescence intensity of antibodies targeting CD31 and CD146 on CECs was also substantially increased, reflecting elevated expression of these endothelial markers. These observations indicate a strong endothelial response in AMI, highlighting endothelial injury and dysfunction associated with the acute cardiac event. The findings suggest an active endothelial reaction during AMI, representing vascular damage and impaired function linked to the disease process.

In 2006, B. Dignat-George and colleagues validated these results, demonstrating that flow cytometry enables accurate identification of CECs and typically reveals elevated counts during events such as AMI. Their findings confirmed similar increases in CEC numbers, further supporting the role of flow cytometry in detecting endothelial injury [23]. More recently, Costa Monteiro (2023) identified discrepancies in protocols used to assess CECs across different studies, which may explain inconsistencies in reported findings. This variability underscores the challenges in standardizing flow cytometry techniques for CEC assessment and highlights the need for methodological harmonization to ensure consistency across studies[24].

The study also examined the expression of the von Willebrand Factor (VWF) gene in CECs from AMI patients in comparison to healthy controls. The findings revealed a significant increase in VWF expression in AMI patients. The mean cycle threshold (Ct) value for VWF was significantly lower in AMI patients than in controls, indicating higher gene expression. Additionally, the ΔCt values, which represent the normalized expression of VWF relative to the housekeeping gene GAPDH, were significantly lower in AMI patients, further confirming an upregulation of VWF expression. This elevated expression suggests enhanced endothelial activation or damage associated with AMI.

A study by Yin et al. (2021) supports these findings by emphasizing the role of VWF in endothelial cell function and its heightened expression under pathological conditions, including cardiovascular diseases (25). Their research demonstrated that VWF expression is elevated in disease-related endothelial dysfunction, aligning with the present study's conclusions. Conversely, a study by Marrero et al. (2023) examined endothelial colony-forming cells and found that while VWF expression varied, its correlation with disease severity was inconsistent across different cardiovascular conditions. This suggests that the significance of VWF expression may differ depending on specific endothelial cell subtypes and the disease context (26).

CONCLUSION

The study examines the behavior of circulating endothelial cells (CECs) in acute myocardial infarction (AMI) using a range of analytical techniques. The findings reveal significant differences in CEC counts between AMI patients and healthy controls, indicating their potential as biomarkers for endothelial damage and dysfunction in AMI. Immunohistochemistry and flow cytometry analyses demonstrated a substantial increase in CEC levels in AMI patients, both in absolute numbers and relative frequencies. Elevated expression of endothelial markers CD31 and CD146 further suggests heightened endothelial activation and damage associated with AMI. Additionally, the significant upregulation of von Willebrand Factor (VWF) in CECs from AMI patients provides further evidence of vascular injury at the cellular level.

The observed differences across multiple parameters distinguish AMI patients from healthy controls, highlighting the potential of CEC analysis as a diagnostic tool for acute coronary syndromes. These findings suggest that CEC levels could provide valuable insights into the severity of endothelial injury and the broader impact on the vascular system during acute coronary events. While the results are promising, further large-scale studies are necessary to validate the clinical utility of CEC-based biomarkers in diagnosing and predicting AMI outcomes. Future research should focus on the standardization of CEC detection techniques and the evaluation of CEC levels in relation to long-term cardiovascular prognosis.

Study Limitations

While this study presents promising results regarding the potential role of circulating endothelial cells (CECs) as biomarkers in acute myocardial infarction (AMI), several limitations should be considered. First, the sample size of 50 participants may limit the generalizability of the findings. A larger, more diverse cohort would provide more robust data and allow for a better understanding of the potential variability of CEC levels across different populations, including individuals with comorbidities or those from different demographic backgrounds.

Second, the study's cross-sectional design provides valuable insights into the differences between AMI patients and healthy controls, but it does not allow for the assessment of CECs over time or their role in predicting long-term outcomes. Longitudinal studies are needed to evaluate whether changes in CEC levels can be used to predict AMI recovery or cardiovascular events in the future.

Third, while immunohistochemistry and flow cytometry techniques are well-established methods for detecting CECs, variability in laboratory techniques and reagents could introduce inconsistencies in the results. Standardization of CEC detection methods across studies would improve the reproducibility of findings and enhance the comparability of data between research centers.

Additionally, while we focused on CECs and specific endothelial markers, other biomarkers or molecular pathways related to endothelial dysfunction were not explored in this study. Future research should consider a broader array of biomarkers to better understand the complexity of endothelial injury in AMI.

Finally, the lack of data on long-term clinical outcomes or follow-up in this study limits the ability to assess the prognostic value of CEC levels. Future studies with extended follow-up periods are necessary to explore the clinical implications of CEC-based biomarkers in AMI diagnosis, prognosis, and treatment.

In conclusion, although this study provides compelling evidence for the potential role of CECs in AMI, further research is required to validate these findings, standardize detection methods, and evaluate the long-term clinical utility of CEC biomarkers in cardiovascular diseases.

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