**Original Article**

**Number of Endothelial Progenitor Cells in Peripheral Artery Disease as a Marker of Severity and Association with Pentraxin-3, Malondialdehyde-Modified Low-Density Lipoprotein and Membrane Type-1 Matrix Metalloproteinase**

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**Aims:** Circulating endothelial progenitor cells (EPCs) were mobilized in cardiac ischemia, heart failure and vascular injuries associated with endothelial damage. Despite the occurrence of endothelial dysfunction in peripheral artery disease (PAD), few data are available on EPC mobilization in this setting.

**Methods:** We investigated the correlations between EPC and disease severity and also other biomarkers in PAD. EPCs assessed as CD34⁻ cells co-expressing CD45dim, CD133 and vascular endothelial growth factor receptor-2 were studied in PAD (n=48) and non-PAD (n=22) patients. Membrane type-1 matrix metalloproteinase (MT1-MMP) on peripheral blood mononuclear cells, serum malondialdehyde-modified low-density lipoprotein (MDA-LDL) and plasma pentraxin-3 were also measured.

**Results:** The EPC level changed in the Fontaine and Trans-Atlantic Inter-Society Consensus (TASC) classification. EPC was increased in Fontaine class IIa as compared with class IV and non-PAD patients (p<0.05). EPCs and pentraxin-3 were increased in TASC II type A/B as compared with type C/D and non-PAD patients (p<0.05), whereas the expression of MT1-MMP on peripheral blood mononuclear cells was significantly decreased in TASC II type A/B (both p<0.05 versus type C/D and non-PAD patients). The EPC level showed a positive association with pentraxin-3 (r=0.31; p<0.05). There was an inverse association between the EPC level and MT1-MMP (r=−0.54; p<0.01). The cardiovascular events was associated with reduced EPC and increased MDA-LDL (p<0.05).

**Conclusion:** EPC changed according to the Fontaine and TASC II classification and decreased in the advanced phases, and was associated with novel biomarkers and related to the severity of PAD.


**Key words:** Endothelial progenitor cells, Peripheral artery disease, Membrane type-1 matrix metalloproteinase, Pentraxin-3

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**Introduction**

Circulating endothelial progenitor cells (EPCs), which have the ability to differentiate into mature endothelial cells, can home to organs undergoing atherosclerotic endothelial dysfunction, where they induce angiogenesis¹, vasculogenesis, and vessel repair². Circulating EPC levels are altered in cardiac ischemia³, heart failure⁴, and vascular injuries⁵, ⁶ caused by endothelial damage.

Inflammation⁷ and oxidative stress⁸ are involved in EPC mobilization⁹. Pentraxin-3, which is produced predominantly by macrophages and vascular endothelial cells, is considered to reflect inflammation of the
endothelium\textsuperscript{10,11}, more directly than levels of C-reactive protein\textsuperscript{12}; however, the relationship between EPCs and pentraxin-3 remains unclear. Malondialdehyde-modified low-density lipoprotein (MDA-LDL) is an oxidized lipoprotein, which is increased in patients with coronary artery disease\textsuperscript{13} and associated with stent restenosis in patients with diabetes mellitus\textsuperscript{14}. Oxidized low-density lipoprotein decreased the survival of EPCs and impaired their function through decreased endothelial nitric oxide synthetase expression\textsuperscript{15}.

EPC mobilization occurs also through matrix metalloproteinase-mediated mechanisms\textsuperscript{16,17}. Matrix metalloproteinase species activate local protease activity and weaken stromal cell-EPC interactions in the bone marrow. Membrane type-1 matrix metalloproteinase (MT1-MMP), which contributes to vascular remodeling and atherosclerotic plaque disruption\textsuperscript{18}, was shown to regulate the mobilization of CD34\textsuperscript{+} progenitor cells from the bone marrow in a mouse model\textsuperscript{19}.

In spite of the occurrence of endothelial dysfunction in peripheral artery disease (PAD), little information is available on EPC mobilization in these patients according to clinical status. In this study, we aimed to investigate the patterns of EPC mobilization in patients with PAD and to evaluate the association of EPCs with inflammation and oxidative stress markers.

**Methods**

**Study Subjects and Design**

Patients were recruited from the Department of Cardiovascular Medicine of the University of Fukui Hospital. Seventy patients were enrolled in this study. Ethics committee approval and informed consent from all patients were obtained. The patients were classified into 2 groups: patients with PAD (n = 48) and patients without PAD (n = 22), who were used as gender- and age-matched controls. The diagnosis of PAD was assessed by patient complaints (claudication, numbness, and resting pain), physical pulse examination, an ankle-brachial index lower than 0.90, and duplex sonography. Patients with a history of neoplastic, hepatic, infectious, or autoimmune diseases were excluded from this study. Chronic kidney disease was defined as estimated glomerular filtration rate of less than 60 mL/min/1.73m\textsuperscript{2}.

All PAD patients received catheter angiography and/or computed tomography angiography. The Fontaine clinical classification and the Trans-Atlantic Inter-Society Consensus (TASC) II classification\textsuperscript{20} were made on the basis of clinical symptoms and the results of angiography.

Circulating EPC levels, which were assessed as CD34\textsuperscript{+} cells co-expressing CD45\textsuperscript{dim}, CD133 and vascular endothelial growth factor receptor-2, were studied in 70 PAD and non-PAD patients. The expression of MT1-MMP on peripheral blood mononuclear cells, the plasma levels of pentraxin-3, the serum levels of MDA-LDL and the plasma levels of stromal cell-derived factor-1 (SDF-1) were measured in 39, 42, 37 and 45 patients, respectively. All patients were treated with ordinary regimens, and all drugs were continued in all patients before blood collection.

We observed the vascular events that occurred during the follow-up period [mean (SD), 8.4 (4.8) months], and examined the possibility of a correlation between the events and EPC levels, MT1-MMP levels, pentraxin-3 levels, MDA-LDL levels and SDF-1 levels. During the follow-up period, vascular events defined as cardiovascular death (including sudden deaths and deaths from myocardial infarction or heart failure), cardiovascular revascularization (percutaneous coronary and peripheral intervention or bypass surgery), stent thrombosis, stent restenosis, non-healing ulcers, and amputation were counted.

**Pentraxin-3, MDA-LDL and SDF-1 Measurements**

Pentraxin-3, MDA-LDL and SDF-1 levels were measured by commercially available sandwich ELISA for pentraxin-3 (Perseus Proteomics Inc., Tokyo, Japan) MDA-LDL (Sekisui Medical Co., Inc., Tokyo, Japan) and SDF-1 (R&D Systems Inc., Minneapolis, MN), respectively.

**Flow Cytometry Analysis**

EPCs and MT1-MMP levels were quantified in collected blood samples by flow cytometry. EPCs were analyzed for the expression of CD34, CD45, CD133, vascular endothelial growth factor receptor-2 with four-color flow cytometry (FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA). Two milliliters of whole blood was obtained. Red cells were lysed by the addition of ammonium chloride-based lysing reagent. The samples were washed in 0.2% phosphate-buffered saline with bovine serum albumin. FcR-blocking reagent (1% human gamma-globulin; Sigma-Aldrich Inc., St. Louis, MO, USA) was added and incubated for 15 minutes at room temperature in the dark. The samples were incubated with anti-CD34 FITC (Beckman Coulter Inc., Marseille, France), anti-CD45-PerCP (BD Biosciences), anti-CD133/2 (293C3)-APC (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), and anti-vascular endothelial growth factor receptor-2-phycoerythrin Conjugated (R&D Systems)
for 40 minutes at 4°C, followed by erythrolysis by the
addition of lysing reagent, and were washed once with
0.2% phosphate-buffered saline with bovine serum al-
bumin. The cells were resuspended in 0.2% phos-
phate-buffered saline with bovine serum albumin for
flowcytometric analysis. As a control for analysis, cells
in a separate tube were treated with PE-labeled mouse
IgG1 antibody. CD34⁺ cells were analyzed using se-
quential gating strategies. CD45 versus side scatter dot
plot was set to include all CD45⁺ events (Fig. 1A).
CD45⁺ events were established to include all nucleat-
ed white blood cells and to exclude red blood cells,
nucleated red blood cells, platelets, and other cellular
debris, which do not express CD45. CD45⁺ cells were
gated on a forward scatter versus side scatter dot plot
to confirm mononuclear cell fraction (R1). Mononuclear
cells formed a cluster with low side scatter and low to intermediate forward scatter. We gated
CD34⁺ and CD45⁺⁺ cells in the mononuclear cell fraction (Fig. 1C, R2) and
gated on a forward scatter versus side scatter dot plot to obtain a cluster of true
CD45⁺⁺ CD34⁺⁺ cells (Fig. 1D, R3). True CD45⁺⁺ and CD34⁺⁺ events were displayed on a CD133 versus
vascular endothelial growth factor receptor-2⁺ dot plot
and then the resulting population was examined for
the dual expression of vascular endothelial growth fac-
tor receptor-2 and CD133. CD45⁺⁺⁺CD34⁺⁺⁺CD133⁺ vascular endothelial growth factor receptor-2⁺ cells
were enumerated in the upper right quadrant of plot
(Fig. 1E). At least 2,000,000 events were measured in
the CD45⁺ gate. The same trained operator, who was
blind to the patient’s clinical status, performed the en-
tire test throughout the study. Data were analyzed us-
ing CELLQuest (BD Biosciences).

The EPC values were defined as the percentage
of CD34⁺⁺, CD45⁺⁺⁺, CD133⁺⁺, and vascular endothe-

dicial growth factor receptor-2⁺ cells per CD34⁺⁺⁺CD45⁺⁺⁺
cells fraction. The surface expression of MT1-MMP
was estimated by the frequency of CD14-positive cells
expressing MT1-MMP at the single cell level, as
described previously.²¹

Fig. 1. Flow cytometry quantification of EPC following the multi-gating strategy (see Methods section)
(A) CD45 versus side scatter dot plot included all CD45⁺ events. (B) CD45⁺ cells were gated on a for-
ward scatter versus side scatter dot plot to confirm mononuclear cell fraction (R1). (C) CD34⁺ and
CD45⁺⁺ cells in the mononuclear cell fraction (R2). (D) A cluster of true CD45⁺⁺⁺CD34⁺⁺ cells was
gated on a forward scatter versus side scatter dot plot (R3). (E) CD45⁺⁺⁺CD34⁺⁺⁺CD133⁺vascular endo-
thelial growth factor receptor-2⁺ cells were enumerated in upper right quadrant of plot.
Statistical Analysis

Data are expressed as the mean (SD). Fisher’s exact test was used to compare the categorical data. Comparisons between 2 or more groups of continuous measurements were performed by an unpaired Student’s t test or ANOVA followed by multiple comparison tests using Fisher’s PLSD test for 3 groups and Scheffe’s test for more than 4 groups, respectively. Correlations between 2 variables were assessed by Pearson’s coefficient (r). Statistical significance was accepted at \( p < 0.05 \).

Results

Patient Characteristics

The clinical characteristics of the patients are summarized in Table 1. There was a high frequency of coronary artery stenosis, stroke, and other cardiovascular risk factors in both groups. The patient backgrounds were matched between the PAD and non-PAD patient groups, except for the prevalence of hypertension and stroke. The TASC II and Fontaine classification results are also shown in the table.

The study subjects were divided into 4 groups (PAD+coronary artery disease (CAD), PAD alone, CAD alone and non-vascular patients) to compare the EPC levels among groups. EPC levels in PAD+CAD patients [1.3 (1.3) %/CD34+/CD45(dim) cells] were higher than those in non-vascular patients [0.4 (0.5) %/CD34+/CD45(dim) cells, \( p = 0.035 \)] and CAD-alone patients [0.6 (0.6) %/CD34+/CD45(dim) cells, \( p = 0.035 \)]. No significant difference was found between the EPC levels in PAD+CAD patients and those in PAD patients [1.1 (1.2) %/CD34+/CD45(dim) cells, \( p = 0.381 \)].

We screened the prevalence of CAD in each Fontaine I/II and III/IV group. The prevalence of CAD showed no significant difference between the Fontaine I/II group [20/30 (66.7%) and Fontaine III/IV group [8/18 (44.4%), \( p = 0.147 \)].

Circulating EPCs in Patients with PAD

The distribution of EPCs according to the Fontaine classification is delineated in Fig. 2A. EPC levels in Fontaine class IIa patients [2.2 (1.6)%/CD34+/CD45(dim) cells] were higher than those in class IV [0.5 (0.4)%/CD34+/CD45(dim) cells, \( p = 0.041 \)] and non-PAD patients [0.6 (0.6)%/CD34+/CD45(dim) cells, \( p = 0.0061 \)]. Additionally, EPC levels in class IV were non-significantly decreased compared with those in non-PAD patients.

Table 1. Patient Characteristics

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>PAD</th>
<th>non-PAD</th>
<th>( p ) Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>48</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>Age [mean (SD) years]</td>
<td>74.6 (8.1)</td>
<td>70.5 (9.8)</td>
<td>0.071</td>
</tr>
<tr>
<td>Gender (n, male/female)</td>
<td>37/11</td>
<td>18/4</td>
<td>0.761</td>
</tr>
<tr>
<td>CAD, n (%)</td>
<td>28 (58.3)</td>
<td>13 (59.1)</td>
<td>&gt;0.999</td>
</tr>
<tr>
<td>Stroke, n (%)</td>
<td>16 (33.3)</td>
<td>1 (4.5)</td>
<td>0.014</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>46 (95.8)</td>
<td>15 (68.2)</td>
<td>0.003</td>
</tr>
<tr>
<td>DM, n (%)</td>
<td>30 (62.5)</td>
<td>9 (40.9)</td>
<td>0.122</td>
</tr>
<tr>
<td>Dyslipidemia, n (%)</td>
<td>28 (58.3)</td>
<td>14 (63.6)</td>
<td>0.442</td>
</tr>
<tr>
<td>Smoking, n (%)</td>
<td>34 (70.8)</td>
<td>15 (68.2)</td>
<td>&gt;0.999</td>
</tr>
<tr>
<td>CKD, n (%)</td>
<td>27 (56.3)</td>
<td>9 (40.9)</td>
<td>0.305</td>
</tr>
<tr>
<td>TASC II, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>2 (4.2)</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>B</td>
<td>6 (12.5)</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>C</td>
<td>5 (10.4)</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>D</td>
<td>35 (72.9)</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>Fontaine, n (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>4 (8.3)</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>IIa</td>
<td>10 (20.8)</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>IIb</td>
<td>16 (33.3)</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>III</td>
<td>10 (20.8)</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>IV</td>
<td>8 (16.7)</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
</tbody>
</table>

Fig. 2B presents the correlation between EPC levels and the TASC II classification. EPC levels in TASC II type A/B patients [2.1 (1.5)%/CD34+CD45dim cells] were higher than in type C/D [1.0 (1.1)%/CD34+CD45dim cells, \(p = 0.003\)] and non-PAD patients [0.6 (0.7)%/CD34+CD45dim cells, \(p = 0.0001\)].

Relationship between pentraxin-3 and EPCs; Pentraxin-3 in TASC II Classification

The correlation between EPC and pentraxin-3 levels is presented in Fig. 3A. EPC levels showed a significantly positive association with pentraxin-3 \((r = 0.31; \ p = 0.043)\). The plasma pentraxin-3 levels in patients in each TASC II stage are shown in Fig. 3B. Pentraxin-3 in TASC II type A/B cases [7.0 (6.6) ng/mL] was significantly higher than in type C/D [3.7 (2.3) ng/mL, \(p = 0.018\)] and non-PAD patients [2.7 (1.6) ng/mL, \(p = 0.002\)].

Relationship between MT1-MMP and EPCs; MT1-MMP in TASC II Classification

We observed an inverse association between EPC and MT1-MMP levels \((r = -0.54; \ p = 0.0004)\) (Fig. 4A). MT1-MMP in TASC II type A/B patients [11.2
(2.9)%] was lower than in type C/D [26.2 (11.9)%], non-PAD patients [28.0 (20.3)%], as illustrated in Fig. 4B.

**MDA-LDL and EPCs in Patients with Cardiovascular Events**

During the follow-up period, 20 patients developed 21 cardiovascular events (amputation, 3; non-healing ulcer, 1; bypass surgery, 3; percutaneous peripheral intervention, 11; and percutaneous coronary intervention, 3). Fig. 5A shows the association between EPC levels and the occurrence of vascular events. EPC in patients with vascular events [0.6 (0.6) %/CD34^+CD45^dim cells] were significantly lower than in those without vascular events [1.6 (1.4) %/CD34^+CD45^dim cells, \( p = 0.008 \)].

The prevalence of vascular events was higher in Fontaine III/IV patients [12/18 (66.7%)] than in Fontaine I/II patients [5/30 (16.7%), \( p = 0.001 \). The study subjects were divided into 2 groups (Fontaine I/II and III/IV) to compare EPC levels between patients with and without events. As a result, in both groups, EPC were nonsignificantly lower in patients with than without events [Fontaine I/II: 0.9 (0.9) vs. 1.6 (1.3) %/CD34^+CD45^dim cells, \( p = 0.267 \), Fontaine III/IV: 0.5 (0.4) vs. 1.5 (1.5) %/CD34^+CD45^dim cells, \( p = 0.051 \).

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**Fig. 4.**

(A) Correlation between EPC and MT1-MMP levels. EPCs were negatively correlated with MT1-MMP levels. (B) MT1-MMP levels in PAD and non-PAD patients classified according to TASC II classification. MT1-MMP in TASC II type A/B patients was lower than in type C/D and non-PAD patients.

**Fig. 5.** EPC and MDA-LDL levels in patients with cardiovascular events and without events in the PAD patient group

(A) EPC numbers in patients with events were lower than in patients without events. (B) MDA-LDL in patients with events was higher than in patients without events.
MDA-LDL distribution did not change according to the Fontaine and TASC II classifications, whereas MDA-LDL in patients with vascular events [128.9 (60.2) U/L] was significantly increased compared to those without vascular events [88.8 (35.2) U/L, \( p = 0.046 \)] (Fig. 5B). We could not identify any association between MDA-LDL values and the levels of other biomarkers, EPC (\( r = -0.18; p = 0.30 \)), pentraxin-3 (\( r = -0.19; p = 0.26 \)), or MT1-MMP levels (\( r = -0.04; p = 0.87 \)).

SDF-1 in PAD cases [2862.7 (739.2) pg/mL] was significantly higher than in non-PAD cases [2192.0 (554.3) pg/mL, \( p = 0.028 \)]; however, we could not find any association between SDF-1 and the levels of other biomarkers, EPC (\( r = 0.15; p = 0.34 \)), pentraxin-3 (\( r = 0.16; p = 0.37 \)), MT1-MMP (\( r = 0.15; p = 0.47 \)) and MDA-LDL (\( r = 0.06; p = 0.75 \)). pentraxin-3, MT1-MMP and SDF-1 levels in patients with vascular events were not significantly different from those without [pentraxin-3; 4.1 (3.9) vs. 3.4 (1.8) ng/mL, \( p = 0.56 \), MT-1MMP; 25.5 (17.2) % vs. 22.1 (8.4) %, \( p = 0.54 \), SDF-1; 2831.5 (756.9) vs. 2612.0 (737.9) pg/mL, \( p = 0.36 \), respectively]

**Discussion**

On the basis of our data, we reached the following conclusions. EPC levels were inversely related to Fontaine and TASC II classifications. EPC levels showed a significant association with the levels of pentraxin-3 and MT1-MMP. In addition, patients with vascular events had significantly lower EPCs and higher MDA-LDL.

EPC decreased as the disease progressed and, in Fontaine class IV patients, were even lower than in controls. These findings are in accordance with known EPC changes in heart failure\(^4\) and diabetes mellitus\(^5\). Thus, the number of EPCs indicates the status of a patient on the continuum between EPC exhaustion and endothelial damage. Worsening of cardiovascular disorders and increases in cardiovascular risks cause endothelial dysfunction and decreasing numbers of EPCs. EPCs are closely related to endothelial functions, such as flow-mediated dilatation\(^22\) and asymmetric dimethylarginine\(^23\). EPCs are upregulated and mobilized sufficiently in response to vascular damage in the mild to moderate phase, and in the early stages of endothelial dysfunction, EPCs are sufficiently mobilized and supplied from bone marrow to the damaged organ. As endothelial damage progresses, EPCs are exhausted and cannot respond to and restore endothelial dysfunction.

We also evaluated the potential associations between EPCs and inflammation and oxidative stress. The inflammatory and most studied biomarker, C-reactive protein, is already known to correlate with EPC levels\(^24\), but C-reactive protein is also upregulated in non-vascular inflammation. Pentraxin-3, one of the superfamily of pentraxins, which includes C-reactive protein, is highly produced in vascular endothelial and smooth muscle cells in response to atherosclerotic change\(^25\); therefore, pentraxin-3 is considered to be a more specific biomarker of vascular inflammation. Our study implies that endothelial damage and vascular inflammation are closely associated with the number of peripheral EPCs in clinical settings.

Although both EPC and pentraxin-3 have been described as markers for cardiovascular events, they also have atheroprotective functions\(^2, 26\). EPC is already known to promote vascular repair and vasculogenesis, and recent observations support the possibility that pentraxin-3 also possesses atheroprotective\(^26\) and cardioprotective effects\(^27\), in contrast to other pentraxins, such as C-reactive protein and serum amyloid P. Therefore, increased EPC and pentraxin-3 could possibly play a atheroprotective role, thus correlating with the severity of PAD; however, our study only found that EPC and pentraxin-3 were upregulated in TASC A/B patients, and further studies might be required to reveal the detailed atheroprotective role in PAD, including the various other factors affecting PAD\(^28, 29\).

Both SDF-1 and vascular endothelial growth factor attract progenitor cells and are involved in homing, migration and mobilization from bone marrow to peripheral circulation\(^30, 31\), contributing to vascular regeneration. SDF-1 was upregulated in the PAD group compared with the non-PAD group; however, no significant difference was found between TASC A/B and C/D groups in SDF-1 levels. These results may reflect that EPCs are upregulated and mobilized sufficiently in the mild to moderate phase in response to chemokine attraction, such as SDF-1. As disease progresses, EPCs are exhausted or no longer respond to homing signals.

As a surrogate marker of oxidative stress affecting EPC mobilization, we measured MDA-LDL; however, we could not find a relationship between MDA-LDL and disease severity. MDA-LDL is related to intima-media thickness, but in other settings, including coronary arterial disease and heart failure, a relationship between disease severity and MDA-LDL levels was not found. Additionally, MDA-LDL showed no relationship with the EPC number, but oxidative stress does correlate with EPCs. This mismatch is presumed to be due to the character of MDA-LDL.
previous reports\textsuperscript{32}, MDA-LDL was higher in patients with acute coronary syndrome. In contrast, MDA-LDL in patients with stable angina was similar to control subjects; therefore, we speculate that MDA-LDL levels reflect the existence of unstable plaques, but not disease severity.

Matrix metalloproteinase (MMP) plays a key role in EPC mobilization and angiogenesis\textsuperscript{16}. MT1-MMP, a transmembrane type protease, has been recognized as an important regulator in EPC mobilization from bone marrow and angiogenesis\textsuperscript{17}. MT1-MMP cleaves CD44, a cell adhesion molecule, and reduces bone marrow stromal cells and progenitor cell interaction. Our data showed that MT1-MMP expression was high in conditions of decreased and exhausted numbers of EPCs being mobilized from the bone marrow. In conditions where sufficient EPCs were preserved in the peripheral blood pool, MT1-MMP expression was downregulated. Experimental findings in mice\textsuperscript{17} support this finding. MT1-MMP activity occurs not only in bone marrow, but is also involved in pericellular proteolysis at the site of angiogenesis\textsuperscript{18}. Thus, MT1-MMP activity may reflect activation in both bone marrow and areas of angiogenesis, which leads to the mobilization and recruitment of EPCs.

Clinical trials using concepts of vasculogenesis and angiogenesis by EPCs are already being applied for cardiac ischemia, critical limb ischemia, and vasculopathy. The benefit and safety of autologous cell transplant therapy has already been reported\textsuperscript{33,34}, but the mechanisms that will ameliorate a patient’s condition are still unclear. Our study contributes to elucidation of the mechanism regulating EPC mobilization and homing.

The limitations of our study must be considered. The present study consisted of observational research in patients with known classical risk factors, and hence, it did not establish a cause-effect relationship of interventional therapy and did not examine subjects without any cardiovascular risk. EPC numbers change in various clinical settings, and EPCs and endothelial function are also impaired. We did not evaluate EPC function or clinical endothelial functions, such as adhesion, proliferation and migratory ability, and flow-mediated dilatation. In other studies\textsuperscript{4,5,22,36}, EPC function and endothelial function were also impaired in patients with cardiovascular disease, and these functions exhibited changes in a similar pattern with respect to EPC number\textsuperscript{17}. We assume that EPC and endothelial functions were also impaired in our patient study groups.

Collectively, the results of our studies suggest that EPC mobilization occurs in PAD and shows a biphasic response, with elevated EPC in the moderate phase and reduced EPC in the advanced phase. EPC levels are associated with the levels of novel circulating biomarkers and several aspects of PAD, including the severity, progression, and outcome of this disease.

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**Conflicts of Interest**

none declared.

**References**


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