Circulating Progenitor Cells Identify Peripheral Arterial Disease in Patients With Coronary Artery Disease


Rationale: Peripheral arterial disease (PAD) is a clinical manifestation of extracoronary atherosclerosis. Despite sharing the same risk factors, only 20% to 30% of patients with coronary artery disease (CAD) develop PAD. Decline in the number of bone marrow–derived circulating progenitor cells (PCs) is thought to contribute to the pathogenesis of atherosclerosis. Whether specific changes in PCs differentiate patients with both PAD and CAD from those with CAD alone is unknown.

Objective: Determine whether differences exist in PCs counts of CAD patients with and without known PAD.

Methods and Results: 1497 patients (mean age: 65 years; 62% men) with known CAD were identified in the Emory Cardiovascular Biobank. Presence of PAD (n=308) was determined by history, review of medical records, or imaging and was classified as carotid (53%), lower extremity (41%), upper extremity (3%), and aortic disease (33%). Circulating PCs were enumerated by flow cytometry. Patients with CAD and PAD had significantly lower PC counts compared with those with only CAD. In multivariable analysis, a 50% decrease in cluster of differentiation 34 (CD34+) or CD34+/vascular endothelial growth factor receptor-2 (VEGFR2+) counts was associated with a 31% (P=0.032) and 183% (P=0.002) increase in the odds of having PAD, respectively. CD34+ and CD34+/VEGFR2+ counts significantly improved risk prediction metrics for prevalent PAD. Low CD34+/VEGFR2+ counts were associated with a 1.40-fold (95% confidence interval, 1.03–1.91) and a 1.64-fold (95% confidence interval, 1.07–2.50) increases in the risk of mortality and PAD-related events, respectively.

Conclusions: PAD is associated with low CD34+ and CD34+/VEGFR2+ PC counts. Whether low PC counts are useful in screening for PAD needs to be investigated. (Circ Res. 2016;119:564-571. DOI: 10.1161/CIRCRESAHA.116.308802.)

Key Words: aortic aneurysm ■ atherosclerosis ■ coronary artery disease ■ peripheral arterial disease ■ stem cells

Peripheral arterial disease (PAD) is a clinical manifestation of atherosclerosis that leads to obstruction of blood flow by embolism, thrombosis, or narrowing of peripheral arteries. It may involve one or multiple vascular beds including the cerebrovascular, aorta, renal, or the upper and lower extremities.1 An accurate estimate of the incidence of PAD is difficult to ascertain because it is often asymptomatic, but it is thought to be present in 10% to 20% of the population aged >60 years.1-5 Clinical syndromes of PAD share common risk factors, such as older age, diabetes mellitus, smoking, hypertension, and hyperlipidemia.6 These factors, in addition to endothelial dysfunction and inflammation, only partially explain the pathogenesis of atherosclerosis. Moreover, despite sharing the same etiologic risk factors, only 20% to 30% of patients with coronary artery disease (CAD) develop PAD.7-9 Why some patients are predisposed to CAD, others to PAD, and some to both, despite similar predisposing factors, remains unknown.

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marrow–derived mononuclear cells expressing cluster of differentiation 34 (CD34) are enriched for PCs that can differentiate into hematopoietic, endothelial, and other lineages.\textsuperscript{11,12,16–18} CD34-expressing mononuclear cells include hematopoietic, endothelial, and nonhematopoietic (mesenchymal, lacking CD45 expression) PCs.\textsuperscript{19} CD133 is a 5-transmembrane antigen of primitive stem cells that is lost during maturation, and dual expression of these markers (CD34+/CD133+) identifies a PC-enriched subpopulation.\textsuperscript{17,20} Whereas coexpression of vascular endothelial growth factor receptor-2 (VEGFR2) seems to identify a rarer subpopulation of PCs further enriched for endothelial progenitors.\textsuperscript{21–23} Finally, coexpression of chemokine (C-X-C motif) receptor 4 (CXCR4), which promotes homing of PC to stromal derived factor–rich hypoxic environments, may further characterize CD34+/PC with capacity for tissue repair.\textsuperscript{24}

Lower circulating PC counts and impaired PC activity, measured by colony-forming and migration assays, have been reported in subjects with cardiovascular risk factors or CAD in some but not all studies.\textsuperscript{25–27} We and others have also shown that lower circulating PC levels in patients with CAD is associated with an increased risk of adverse CAD events.\textsuperscript{28,29} Previous studies investigating the role of PCs in diabetics with PAD found significantly decreased CD34+/VEGFR2+ cell counts and proliferation compared with healthy or diabetic subjects without PAD.\textsuperscript{30–32} It remains unclear whether the observed impairment in PC counts is specific for PAD or whether it occurs in all individuals with atherosclerosis including those with CAD. To address this, we investigated whether circulating PC counts in patients with both CAD and PAD differed from those with only CAD but no known PAD. We hypothesized that abnormalities in endogenous regenerative capacity, enumerated as differences in circulating PC numbers, would contribute to the development of extensive atherosclerosis and be lower in patients with more extensive disease (PAD plus CAD) compared with patients with CAD and no known PAD.

\textbf{Methods}

\textbf{Study Design and Subjects}

We compared PC counts in patients with CAD and no known PAD with counts in those with both CAD and PAD in at least one site (upper or lower extremity PAD, carotid stenosis, and thoracic or abdominal aortic aneurysms). We identified 1497 subjects with CAD who had PC counts measured and were enrolled in the Emory Cardiovascular Biobank, a prospective registry of adult patients undergoing cardiac catheterization at 3 Emory Healthcare sites in Atlanta, Georgia (Online Figure I; Table 1).\textsuperscript{7} Subjects presenting with acute myocardial infarction were excluded. PC counts were measured at the time of enrollment from blood samples obtained at the time of catheterization. CAD was defined by the presence of atherosclerotic plaque on the coronary angiogram and obstructive CAD as the presence of ≥50% stenosis in at least one major coronary artery. Demographic characteristics, medical history, medication use, and behavioral habits were documented as previously described.\textsuperscript{28} Patients were followed up for outcomes. The study was approved by the Institutional Review Board at Emory University (Atlanta, GA). All subjects provided written informed consent.

\textbf{Defining Peripheral Arterial Disease}

We extensively reviewed patients’ self-reported and physician-documented medical history and imaging reports to identify the presence of PAD. PAD was defined as a history of symptomatic or asymptomatic noncoronary atherosclerotic disease in at least one of the following arteries: carotid, thoracic, or abdominal aorta, and subclavian, brachial, iliac, femoral, or popliteal arteries. No routine testing was performed to screen for asymptomatic PAD as part of this study. PAD of the lower extremities was diagnosed when at least one of the following were present: documented ankle-brachial index <0.90; lower limb revascularization; atherosclerotic plaques or stenosis on imaging (computed tomography, ultrasound, or fluoroscopy) in the iliac, femoral, or popliteal arteries; and history of amputation for critical limb ischemia. PAD of the carotid artery was diagnosed if there was ≥20% stenosis in any carotid artery on imaging (ultrasound, computed tomography, or magnetic resonance angiography). Aortic disease was diagnosed when there was a history of abdominal or thoracic aneurysms (excluding subjects with aortic root aneurysm associated with bicuspid aortic valves) or evidence of atherosclerotic plaques of the aorta or renal arteries on computed tomography imaging.

\begin{table}
\centering
\caption{Characteristics of Patients With CAD With and Without Peripheral Vascular Disease}
\begin{tabular}{|l|l|l|l|}
\hline
Variables & Without Peripheral Vascular Disease (n=1189) & Peripheral Vascular Disease (n=308) & P Value \\
\hline
Age, y & 65 (13) & 71 (11) & <0.001 \\
Men, n (%) & 725 (61%) & 199 (65%) & 0.264 \\
Black, n (%) & 254 (21%) & 58 (19%) & 0.346 \\
Body mass index, kg/m\(^2\) & 30 (6) & 28 (6) & <0.001 \\
Clinical characteristics & & & \\
Smoking history, n (%) & 773 (65%) & 225 (73%) & 0.008 \\
Hypertension, n (%) & 1063 (90%) & 298 (97%) & <0.001 \\
Diabetes mellitus, n (%) & 489 (42%) & 148 (50%) & 0.026 \\
Hyperlipidemia, n (%) & 899 (76%) & 245 (80%) & 0.268 \\
Statin use, n (%) & 400 (34%) & 138 (45%) & <0.001 \\
Low-density lipoprotein, mg/dL & 91 (39) & 86 (35) & 0.054 \\
High-density lipoprotein, mg/dL & 45 (15) & 45 (13) & 0.404 \\
Heart failure, n (%) & 270 (23%) & 101 (33%) & <0.001 \\
Ejection fraction, % & 53 (13) & 51 (14) & 0.017 \\
Obstructive coronary artery disease, n (%) & 722 (61%) & 229 (74%) & <0.001 \\
ACEI/ARB use, n (%) & 323 (27%) & 111 (36%) & 0.003 \\
Estimated glomerular filtration rate, mL/min/1.73 m\(^2\) & 70 (26) & 60 (25) & <0.001 \\
\hline
\end{tabular}
\end{table}

Values are represented as mean (SD) or n (%) as noted. Obstructive coronary artery disease denotes the presence of at least 50% obstruction in any of the coronary arteries on angiogram. ACEI indicates angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; and CAD, coronary artery disease.
PC Assays
Venous blood was collected in EDTA tubes and incubated with fluorochrome-labeled monoclonal antihuman mouse antibodies within 4 hours. Cell populations enumerated for circulating PCs were enumerated using flow cytometry as CD45\textsuperscript{hi} cells coexpressing CD34\textsuperscript{hi}, CD133\textsuperscript{hi}, VEGFR2\textsuperscript{hi}, or CXCR4\textsuperscript{hi}. We incubated 300 µL of peripheral blood with 7 µL of fluorescent isothiocyanate–CD34 (BD Biosciences), PerCP–CD45 (BD Biosciences), PE–VEGFR2 (R&D system), and 5 µL APC–CD133 (Miltenyi), and 3 µL PE–Cy7–conjugated anti-CXCR4 (EBioscience, clone 12G5) in the dark for 15 minutes. Then, 1.5 mL ammonium chloride lysing buffer was added to lyse red blood cells. 1.5 mL staining medium (PBS with 3% heat-inactivated serum and 0.1% sodium azide) was added to stop the lysing reaction. Before flow cytometry, 100 µL of AccuCheck Counting Beads (Invitrogen, catalog number: PCB100) were added to act as an internal standard for direct estimation of the concentration of target cell subsets. At least 2.5 million events were acquired from the cytometer. Flow data were analyzed with FlowJo software (Treestar, Inc; Online Figure II). Absolute mononuclear cell count was estimated as the sum of lymphocytes and monocytes using a Coulter ACT/Diff cell counter (Beckman Coulter). PC populations are reported as cell counts/mL. In 20 samples that were repeatedly analyzed on 2 occasions by the same technician, the coefficients of variation were ≤7.\% for CD34\textsuperscript{hi}, 2.9\%; CD34\textsuperscript{hi}/CD133\textsuperscript{hi}, 4.8\%; CD34\textsuperscript{hi}/CXCR4\textsuperscript{hi}, 6.5\%; CD34\textsuperscript{hi}/CD133\textsuperscript{hi}/CXCR4\textsuperscript{hi}, 7.5\%; and CD34\textsuperscript{hi}/VEGFR2\textsuperscript{hi} cells, 21.6\%. There were significant correlations between the PC subtypes, with moderate-to-strong correlations between CD34\textsuperscript{hi}, CD34\textsuperscript{hi}/CD133\textsuperscript{hi}, and CD34\textsuperscript{hi}/CXCR4\textsuperscript{hi} (r, 0.75–0.91; P<0.001) and weak correlations (r, 0.12–0.34; P<0.001) between CD34\textsuperscript{hi}/VEGFR2\textsuperscript{hi} subtypes and the aforementioned PCs (Online Table I).

Follow-Up and Outcomes
We conducted follow-up as previously described to identify prespecified incident adverse cardiovascular outcomes including death and myocardial infarction.29 In brief, follow-up and adjudication were conducted by personnel blinded to the PC data by phone, electronic medical record review, and social security death index and state record. PAD-related events such as peripheral revascularization and amputation were identified using standard current procedure terminology codes for vascular procedures.37 We examined the association between PC counts and death, PAD-related events, and a composite outcome of death, myocardial infarction, and PAD-related events.

Statistical Analysis
Subject characteristics were reported as descriptive statistics with means, medians, SDs, and ranges. Differences between groups were assessed using t tests for continuous variables and χ\textsuperscript{2} or Fisher exact tests for categorical variables where appropriate. For non-normally distributed variables such as circulating PC counts, the Mann–Whitney test was used to compare groups in unadjusted analyses. For multivariable analyses adjusting for aforementioned clinical covariates and analyzing each PC subset separately, CD34\textsuperscript{hi} and CD34\textsuperscript{hi}/VEGFR2\textsuperscript{hi} cell counts (models 2 and 5), but not CD34\textsuperscript{hi}/CD133\textsuperscript{hi} (model 3) or CD34\textsuperscript{hi}/CXCR4\textsuperscript{hi} (model 4) counts, were independently associated with the presence of PAD (Table 2). Thus, a 50\% decrease in CD34\textsuperscript{hi} or CD34\textsuperscript{hi}/VEGFR2\textsuperscript{hi} cell counts was associated with a 31\% odds ratio [OR], 1.31; \(P=0.032\) and 183\% (OR, 2.83; \(P=0.002\)) increase in the odds of having PAD, respectively.

Results
Characteristics of the 1189 subjects with CAD and 308 with both CAD and PAD are shown in Table 1. Patients with both CAD and PAD were more likely to be older, smokers, hypertensives, diabetics, with heart failure, with lower body mass index, and with higher incidence of obstructive CAD (Table 1). Among patients with PAD, 53\% had carotid disease, 41\% had lower extremity PAD, 3\% had upper extremity PAD, and 33\% had either abdominal or thoracic aortic disease. Most patients (74\%) had only 1 site of documented PAD, 69 (22\%) had 2, and 10 (3\%) had at least 3. In multivariable analyses, age, lower body mass index, a history of smoking, statin use, heart failure, and lower estimated glomerular filtration rate were independently associated with PAD (Table 2).

Relationship Between PCs and PAD
In unadjusted analyses, cell populations enriched for hematopoietic progenitors (CD34\textsuperscript{hi}, CD34\textsuperscript{hi}/CD133\textsuperscript{hi}, and CD34\textsuperscript{hi}/CXCR4\textsuperscript{hi}) and those enriched for endothelial progenitors (CD34\textsuperscript{hi}/VEGFR2\textsuperscript{hi} cells) were lower in patients with PAD compared with those without PAD (Table 3). Notably, CD34\textsuperscript{hi}/VEGFR2\textsuperscript{hi} cells were close to 2-fold lower in number in those with PAD compared with those without (Table 3). There were no significant differences in PC counts among patients with PAD according to the location of disease (Table 3). On multivariable analyses adjusting for aforementioned clinical covariates and analyzing each PC subset separately, CD34\textsuperscript{hi} and CD34\textsuperscript{hi}/VEGFR2\textsuperscript{hi} cell counts (models 2 and 5), but not CD34\textsuperscript{hi}/CD133\textsuperscript{hi} (model 3) or CD34\textsuperscript{hi}/CXCR4\textsuperscript{hi} (model 4) counts, were independently associated with the presence of PAD (Table 2). Thus, a 50\% decrease in CD34\textsuperscript{hi} or CD34\textsuperscript{hi}/VEGFR2\textsuperscript{hi} cell counts was associated with a 31\% odds ratio [OR], 1.31; \(P=0.032\) and 183\% (OR, 2.83; \(P=0.002\)) increase in the odds of having PAD, respectively.

Sensitivity Analyses
We performed sensitivity analyses to determine whether the association between PCs and PAD differed according to conventional PAD risk factors: age, sex, diabetes mellitus and smoking status, and presence of obstructive CAD (Figure 2). We found a significant interaction between CD34\textsuperscript{hi} and smoking status in the prediction of PAD (interaction \(P=0.019\)). Patients with a history of smoking and low CD34\textsuperscript{hi} (≤1652 point of death, and myocardial infarction and PAD-related events, adjusting for the aforementioned variables including PAD. Sensitivity analyses explored whether the association with outcomes differed between patients with and without known PAD. Two-tailed \(P\) value ≤0.05 were considered statistically significant. Analyses were performed using IBM SPSS Statistics version 22 (Armonk, NY).
There were otherwise no cells did not (OR, 0.90; \( P = 0.003 \)), whereas nonsmokers with low CD34+ (OR, 1.69; \( P = 0.003 \)) had significantly higher odds of having PAD compared with those without known PAD at enrollment. When dichotomized by median, patients with low CD34+ counts (≤1652 cells/mL, log-rank \( P = 0.012 \)) or low CD34+/VEGFR2+ counts (≤33 cells/mL, log-rank \( P = 0.002 \)) had greater mortality compared with those with higher counts. Only subjects with low CD34+/VEGFR2+ counts experienced a higher rate of PAD-related events (log-rank \( P = 0.001 \)). In Cox regression analyses adjusting for the aforementioned covariates and PAD history, a low CD34+/VEGFR2+ cell count was associated with a 1.43-fold increase in risk of death, a 1.65-fold increased risk of PAD-related events, and a 1.64-fold increased risk of the composite event rate of death, myocardial infarction, and PAD events (Table 5). There was no interaction with PAD status, suggesting that this cell type was predictive of interactions between the remainder of the characteristics and CD34+ or CD34+/VEGFR2+ cell counts.

**Prediction Performance**

To determine the potential of PCs as biomarkers of PAD, we compared the likelihood, c-statistic, net reclassification improvement, and integrated discrimination improvement between the model with traditional risk factors only (model 1) and 3 models incorporating PC counts (models 2, 3, and 4) in addition to demographics and risk factors (Table 4). Addition of either CD34+ counts (model 2) or CD34+/VEGFR2+ counts (model 3) to the risk factor model was associated with a significant improvement in the likelihood ratio, net reclassification improvement, and integrated discrimination improvement (Table 4). The largest improvement was noted when both CD34+ and CD34+/VEGFR2+ PC counts were added to the clinical model together (model 4), with a net reclassification improvement of 0.390 (95% confidence interval, 0.234–0.543) and an integrated discrimination improvement of 0.027 (95% confidence interval, 0.017–0.036). The improvement in c-statistic with addition of both cells counts to the clinical model was not statistically significant (estimated change=0.010; 95% confidence interval, –0.001 to 0.020).

**PC Counts and Outcomes**

Lastly, we examined the association between CD34+ and CD34+/VEGFR2+ cell counts and incident adverse cardiovascular outcomes (Table 5). There were 217 deaths (14%), 67 myocardial infarctions (4%), and 142 PAD-related events (9%) during a median follow-up period of 2 years (1.2–2.9). Patients with PAD were more likely to die (21% versus 12%; \( P < 0.001 \)), have a myocardial infarction (8% versus 3%; \( P < 0.001 \)), or undergo a vascular procedure (27% versus 4%; \( P < 0.001 \)) compared with those without known PAD at enrollment. When dichotomized by median, patients with low CD34+ counts (≤1652 cells/mL, log-rank \( P = 0.012 \)) or low CD34+/VEGFR2+ counts (≤33 cells/mL, log-rank \( P = 0.002 \)) had greater mortality compared with those with higher counts. Only subjects with low CD34+/VEGFR2+ counts experienced a higher rate of PAD-related events (log-rank \( P < 0.001 \)). In Cox regression analyses adjusting for the aforementioned covariates and PAD history, a low CD34+/VEGFR2+ cell count was associated with a 1.43-fold increase in risk of death, a 1.64-fold increased risk of PAD-related events, and a 1.65-fold increased risk of the composite event rate of death, myocardial infarction, and PAD events (Table 5). There was no interaction with PAD status, suggesting that this cell type was predictive of interactions between the remainder of the characteristics and CD34+ or CD34+/VEGFR2+ cell counts.

**Table 2. Independent Predictors of Peripheral Vascular Disease**

<table>
<thead>
<tr>
<th>Variables</th>
<th>( \beta ), ( P ) Value</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, per 10 y</td>
<td>1.41, &lt;0.001</td>
<td>1.24–1.604</td>
</tr>
<tr>
<td>Men</td>
<td>1.10, 0.523</td>
<td>0.82–1.48</td>
</tr>
<tr>
<td>Black</td>
<td>1.00, 0.982</td>
<td>0.69–1.46</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>0.95, &lt;0.001</td>
<td>0.93–0.98</td>
</tr>
<tr>
<td>Smoking history</td>
<td>1.43, 0.021</td>
<td>1.06–1.95</td>
</tr>
<tr>
<td>Hypertension</td>
<td>2.33, 0.026</td>
<td>1.11–4.90</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>1.30, 0.070</td>
<td>0.98–1.74</td>
</tr>
<tr>
<td>Hyperlipidemia</td>
<td>0.83, 0.300</td>
<td>0.58–1.18</td>
</tr>
<tr>
<td>Statin use</td>
<td>1.47, 0.020</td>
<td>1.06–2.03</td>
</tr>
<tr>
<td>Heart failure</td>
<td>1.30, 0.085</td>
<td>0.96–1.76</td>
</tr>
<tr>
<td>Obstructive coronary artery disease</td>
<td>1.65, 0.002</td>
<td>1.19–2.27</td>
</tr>
<tr>
<td>ACEI/ARB use</td>
<td>1.18, 0.325</td>
<td>0.85–1.66</td>
</tr>
<tr>
<td>eGFR, per mL/min/1.73 m²</td>
<td>0.99, 0.001</td>
<td>0.98–0.99</td>
</tr>
</tbody>
</table>

**Table 3. Circulating Progenitor Cell Counts Stratified by Peripheral Vascular Disease**

<table>
<thead>
<tr>
<th>Variables, cells/mL</th>
<th>Without Peripheral Vascular Disease (n=1189)</th>
<th>Peripheral Vascular Disease (n=308)</th>
<th>( P ) Value†</th>
<th>Carotid Disease (n=162)</th>
<th>Lower Extremity Disease (n=127)</th>
<th>Aortic Disease (n=100)</th>
<th>( P ) Value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD34+</td>
<td>1696 (1080, 2622)</td>
<td>1456 (867, 2253)</td>
<td>&lt;0.001</td>
<td>1495 (850, 2194)</td>
<td>1417 (889, 2286)</td>
<td>1260 (843, 2202)</td>
<td>0.795</td>
</tr>
<tr>
<td>CD34+/CD133+</td>
<td>786 (474, 1251)</td>
<td>671 (398, 1138)</td>
<td>0.004</td>
<td>682 (0392, 1070)</td>
<td>665 (383, 1109)</td>
<td>649 (384, 1191)</td>
<td>0.928</td>
</tr>
<tr>
<td>CD34+/CXCR4+</td>
<td>829 (501, 1370)</td>
<td>725 (417, 1231)</td>
<td>0.005</td>
<td>697 (394, 0829)</td>
<td>726 (450, 1268)</td>
<td>713 (400, 1117)</td>
<td>0.105</td>
</tr>
<tr>
<td>CD34+/VEGFR2+</td>
<td>39 (11, 125)</td>
<td>22 (8, 85)</td>
<td>&lt;0.001</td>
<td>23 (8, 77)</td>
<td>33 (8, 121)</td>
<td>25 (7, 86)</td>
<td>0.601</td>
</tr>
</tbody>
</table>

*Progenitor cell counts are reported as median (25th, 75th percentiles). CD34 indicates cluster of differentiation 34; and VEGFR2, vascular endothelial growth factor receptor-2.

†P value for ANOVA comparing progenitor cell counts among patients with various types of peripheral vascular disease. Of note, patient overlap exists between carotid, lower extremity, and aortic disease columns.
events in both subjects with and without PAD. We did not find an association between CD34+ cell counts, dichotomized by median value, and future PAD-related events.

Discussion

In the large study in patients with known CAD to date, we have identified an association between low CD34+ and CD34+/VEGFR2+ PC counts and the presence of PAD. Subjects with both CAD and PAD had a 2-fold lower CD34+/VEGFR2+ cell count compared with subjects with only CAD and no known PAD. After adjusting for known risk factors for PAD, low CD34+ ($\leq 1652$ cells/mL) and CD34+/VEGFR2+ ($\leq 33$ cells/mL) cell counts were associated with a 41% and 55% increase in the odds of having PAD, respectively. Moreover, subjects with both low CD34+ and low CD34+/VEGFR2+ cell counts had a 65% increase in the odds of PAD and improved risk discrimination metrics when added to a model with traditional risk factors. Most importantly, low CD34+/VEGFR2+ cell counts were associated with increased mortality and risk of incident PAD-related events. These findings build on the growing body of evidence indicating an important role for circulating PCs in the pathogenesis of atherosclerosis and may explain why, despite similar risk factors, certain patients develop isolated CAD while others have more widespread atherosclerosis of the peripheral circulation.

There was no evidence suggesting an association between CD34+ cells expressing the CD133 or CXCR4 epitopes and the co-occurrence of PAD and CAD in this population. We and others have previously shown these cells to be predictive of outcomes in patients with CAD. Although peripheral blood CD34+ cells are heterogeneous, they are enriched for cells with endothelial lineage potential, express endothelial marker genes, and form endothelial structures in vitro and in vivo. In our study, CD34+ cells of interest were predominantly (>95%) CD45dim and thus largely represent cells of the hematopoietic lineage. Although the additional expression of VEGFR2 receptor on CD34+ cells is often considered to define a subset enriched for endothelial PCs, this remains a subject of controversy.

Our findings are consistent with previous smaller studies showing similarly lower levels of circulating CD34+/VEGFR2+ cells in patients with PAD. Shaffer et al and Bitterli et al noted similar findings when comparing patients with PAD to healthy subjects, whereas Fadini et al reported decreased counts in diabetic subjects with lower extremity PAD or carotid stenosis compared with diabetics without PAD. These studies were limited by small sample size and most importantly the inability to account for the presence or absence of CAD. Our study examined the association between PCs and PAD in a much larger cohort of patients with CAD, with and without diabetes mellitus or obstructive CAD. Moreover, we demonstrated that the association between lower CD34+/VEGFR2+ PC counts extends to forms of PAD beyond diabetic vasculopathy, lower extremity PAD, and carotid stenosis.

![Figure 1. Prevalence of peripheral arterial disease stratified by cluster of differentiation 34 (CD34+) and CD34+/vascular endothelial growth factor receptor-2 (VEGFR2+) cell counts. Three-dimensional bar plot depicting the prevalence of peripheral vascular disease (y axis) stratified by the median counts of CD34+ and CD34+/VEGFR2+ cells.](image1)

![Figure 2. Sensitivity analyses. Forest plot of interaction with traditional risk factors and median cluster of differentiation 34 (CD34+) cell counts (A) and median CD34+/vascular endothelial growth factor receptor-2 (VEGFR2+) cell counts (B) for predicting the presence of peripheral arterial disease. There was a significant interaction between smoking and CD34+ cell counts (highlighted in box, A). BMI indicates body mass index; CAD, coronary artery disease; and CI, confidence interval.](image2)
because 33% of our subjects with PAD had aortic disease. Although the association between PC counts and risk of death and myocardial infarction has been previously described,28,29 our findings that low CD34+/VEGFR2+ PC counts are predictive of incident PAD-related events are novel. Experimental studies have shown that disruption of the bone marrow is a major contributor to the pathogenesis of atherosclerosis.44–46 In humans with critical limb ischemia, examination of the bone marrow demonstrated profound changes including microvascular disruption and reduced CD34+ cells, indicating that changes in peripheral blood we described are likely associated with similar disruption of PCs in the bone marrow in PAD.47,48

Strengths of our study include (1) a large cohort study design to limit heterogeneity, (2) use of commonly used high-throughput technology (flow cytometry) for quantification of PCs by the same technical team, (3) exploration of several CD34+ cell subpopulations enriched for both hematopoietic and endothelial

### Table 4. Risk Prediction Metrics

<table>
<thead>
<tr>
<th>Model</th>
<th>Likelihood Ratio Test (P-value)</th>
<th>C-statistic (95% CI)</th>
<th>ΔC-statistic (95% CI)</th>
<th>Continuous NRI (95% CI)</th>
<th>IDI (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model 1: RF only</td>
<td>…</td>
<td>0.717 (0.685 to 0.749)</td>
<td>…</td>
<td>…</td>
<td>…</td>
</tr>
<tr>
<td>Model 2: RF and CD34+ cells</td>
<td>&lt;0.001</td>
<td>0.721 (0.689 to 0.753)</td>
<td>0.004 (–0.004 to 0.011)</td>
<td>0.256 (0.129 to 0.382)</td>
<td>0.005 (0.001 to 0.009)</td>
</tr>
<tr>
<td>Model 3: RF and CD34+/VEGFR2+ cells</td>
<td>&lt;0.001</td>
<td>0.722 (0.691 to 0.754)</td>
<td>0.006 (–0.003 to 0.014)</td>
<td>0.255 (0.128 to 0.382)</td>
<td>0.005 (0.001 to 0.010)</td>
</tr>
<tr>
<td>Model 4: RF and CD34+ and CD34+/VEGFR2+ cells</td>
<td>&lt;0.001</td>
<td>0.727 (0.695 to 0.758)</td>
<td>0.010 (–0.001 to 0.020)</td>
<td>0.390 (0.234 to 0.546)</td>
<td>0.027 (0.017 to 0.036)</td>
</tr>
</tbody>
</table>

Model 1 includes age, sex, race, body mass index, smoking history, hypertension, diabetes, hyperlipidemia, history of heart failure, statin use, angiotensin pathway antagonist use, estimated glomerular filtration rate at enrollment, and obstructive CAD. Model 2 include aforementioned risk factors in model 1 in addition to CD34+ cell counts. Model 3 includes RF and CD34+/VEGFR2+ cell counts. Lastly, model 4 includes RF, CD34+, and CD34+/VEGFR2+ cells. CAD indicates coronary artery disease; CD34, cluster of differentiation 34; CI, confidence interval; IDI, integrated discrimination improvement; NRI, net reclassification index; RF, risk factor; and VEGFR2, vascular endothelial growth factor receptor-2.

### Table 5. Progenitor Cells and Incident Adverse Cardiovascular Outcomes

<table>
<thead>
<tr>
<th>Variables</th>
<th>Death</th>
<th>PAD-Related Events</th>
<th>Death, Myocardial Infarction, and PAD-Related Events</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, per 10 y</td>
<td>1.14, 0.036</td>
<td>1.01–1.29</td>
<td>0.85, 0.050</td>
</tr>
<tr>
<td>Men</td>
<td>0.92, 0.589</td>
<td>0.69–1.24</td>
<td>1.09, 0.654</td>
</tr>
<tr>
<td>Black race</td>
<td>0.97, 0.867</td>
<td>0.67–1.40</td>
<td>0.54, 0.015</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>0.96, 0.003</td>
<td>0.93–0.99</td>
<td>0.99, 0.418</td>
</tr>
<tr>
<td>Smoking history</td>
<td>1.36, 0.061</td>
<td>0.99–1.88</td>
<td>0.93, 0.699</td>
</tr>
<tr>
<td>Hypertension</td>
<td>1.02, 0.936</td>
<td>0.58–1.80</td>
<td>1.32, 0.491</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>1.18, 0.270</td>
<td>0.88–1.60</td>
<td>1.34, 0.116</td>
</tr>
<tr>
<td>Hyperlipidemia</td>
<td>1.01, 0.961</td>
<td>0.71–1.44</td>
<td>0.92, 0.703</td>
</tr>
<tr>
<td>Statin use</td>
<td>0.73, 0.044</td>
<td>0.53–0.99</td>
<td>1.43, 0.157</td>
</tr>
<tr>
<td>Heart failure</td>
<td>1.78, &lt;0.001</td>
<td>1.33–2.38</td>
<td>1.06, 0.780</td>
</tr>
<tr>
<td>Obstructive coronary artery disease</td>
<td>0.47, 0.043</td>
<td>0.23–0.98</td>
<td>1.01, 0.988</td>
</tr>
<tr>
<td>ACEi/ARB use</td>
<td>0.72, 0.029</td>
<td>0.54–0.97</td>
<td>1.12, 0.573</td>
</tr>
<tr>
<td>eGFR, per mL/min/1.73 m²</td>
<td>0.99, 0.001</td>
<td>0.99–1.00</td>
<td>1.00, 0.441</td>
</tr>
<tr>
<td>Peripheral vascular disease</td>
<td>1.59, 0.004</td>
<td>1.16–2.19</td>
<td>9.11, &lt;0.001</td>
</tr>
</tbody>
</table>

Model 2: risk factors+individual PC subtypes

| CD34+, ≤1652 cells/mL | 1.22, 0.171 | 0.92–1.64 | 1.29, 0.179 | 0.89–1.86 | 1.03, 0.810 | 0.82–1.29 |
| CD34+/VEGFR2+≤33 cells/mL | 1.43, 0.022 | 1.05–1.94 | 1.64, 0.023 | 1.07–2.50 | 1.65, <0.001 | 1.28–2.13 |

Sensitivity analysis

Peripheral vascular disease×CD34+/VEGFR2+  P=0.580  P=0.191  P=0.918

PC subtypes were each entered into separate models incorporating demographics and risk factors. The odds ratio and CI reported for the demographics and clinical characteristics are derived from the model not incorporating any PC. ACEi indicates angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker; CD34, cluster of differentiation 34; CI, confidence interval; eGFR, estimated glomerular filtration rate; HR, hazard ratio; PC, progenitor cell; and VEGFR2, vascular endothelial growth factor receptor-2.
PCs, and (4) the association with incident cardiac and vascular events. Limitations include the lack of systematic screening for PAD. Thus, it is possible that some patients with undiagnosed or asymptomatic PAD are unaccounted for and may be included with the group of CAD-only patients. Nevertheless, our findings suggest that PC counts could help identify a subset of patients with CAD at high risk for underlying PAD. Although our findings imply that depletion of circulating PC pool may be associated with more extensive atherosclerosis, and in particular, PAD, the cohort design prevents us from establishing causation.

Clinical Implications
Measuring PC counts may be useful as a screening test in subjects without known PAD. Several measures have been found to increase mobilization of PCs, such as lifestyle modification, intensifying statin therapy, cilostazol, and exercise.25,26,31,49 To increase mobilization of PCs, such as lifestyle modification, cilostazol, and exercise.25,26,31,49

Acknowledgments
We would like to thank all members of the Emory Atlanta Clinical and Translational Science Institute for their support in performing this study. We are grateful to Ernestine Maher, Hilary Rosenthal, and Wayne Harris for their assistance with flow cytometry.

Sources of Funding

Disclosures
None.

References
Novelty and Significance

What Is Known?
- Despite sharing risk factors, peripheral arterial disease (PAD) and coronary artery disease (CAD) co-occur in only 20% to 30% of patients.
- Circulating progenitor cells (PCs) are thought to be involved in vascular repair and regeneration. Mononuclear cells expressing CD34 and VEGFR2 are enriched for endothelial PCs.
- In comparison with healthy patients, patients with PAD have been shown to have lower circulating levels of CD34+/VEGFR2+ and progenitor cells.

What New Information Does This Article Contribute?
- Compared with patients with CAD alone, those with both PAD and CAD have at least 30% lower CD34+/VEGFR2+ PC counts, independent of demographics and clinical characteristics.
- Addition of CD34+/VEGFR2+ PC counts to a traditional risk factor model significantly improved risk prediction metrics for PAD.
- Patients with low CD34+/VEGFR2+ PCs were at a higher risk of cardiovascular outcomes including death, myocardial infarction, and PAD-related events.

Although atherosclerosis is one of the most studied human diseases, there is still much we do not understand of its pathogenesis. Although both PAD and CAD share similar risk factors, only 20% to 30% of patients with CAD develop PAD. Why some patients are predisposed to CAD, others to PAD, and some to both, despite similar risk factors, is unknown. Circulating PCs are thought to be involved in vascular repair and are decreased in patients with PAD compared with healthy counterparts. We investigated whether PC counts could distinguish between patients with PAD and CAD and those with CAD alone. We found that patients with PAD had significantly lower CD34+ and CD34+/VEGFR2+ PCs, independent of demographics and clinical characteristics. CD34+/VEGFR2+ counts added incremental value to traditional risk factors in predicting PAD. Moreover, we found that low levels of CD34+/VEGFR2+ cells were associated with worse cardiovascular outcomes and PAD-related events. These findings imply a disruption in endogenous regenerative potential, which may underlie the pathogenesis of PAD, and suggest that PC counts could be used to identify patients with CAD who are at high risk of PAD, provide prognostic information, and potentially guide early interventions.
Circulating Progenitor Cells Identify Peripheral Arterial Disease in Patients With Coronary Artery Disease


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Supplemental Material

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Supplementary Methods

Progenitor Cell Quantification by Flow Cytometry

We incubated 300µl of peripheral blood with 7µl of the same fluorochrome-labeled monoclonal anti-human mouse antibodies but with the addition of 3µl PE-Cy7-conjugated anti-CXCR4 (EBioscience, clone 12G5). Prior to flow cytometry, 100µl of AccuCheck Counting Beads (Invitrogen, Cat#: PCB100) were added to act as an internal standard for direct estimation of the concentration of target cell subsets. At least 2.5 million events were acquired from the Cytometer. Flow data were analyzed with Flowjo software (Treestar, Inc.). Absolute mononuclear cell count was estimated as the sum of lymphocytes and monocytes using a Coulter ACT/Diff cell counter (Beckman Coulter). CD45med cells, also referred to as CD45dim cells exclude CD45bright and CD45-(negative) cells. By excluding CD45- we exclude non-hematopoietic progenitors. By excluding the rare CD45bright cells we exclude lymphoblasts.

Figure Legends

Online Figure I. Flow diagram describing subject selection for analysis.

Online Figure II. Flow cytometric analysis of human peripheral blood. Panel A: forward scatter and side scatter gates following lyse-no wash of blood and the addition of fluorescent counting beads (left upper corner in plot). Panel B: gating of CD34+, low side scatter cells from blood leukocytes shown in panel A. Panel C: histograms of CD45 expression in the CD34+ low side scatter cells (red histogram) shown in panel B. Panel C: the pattern of co-expression of CD34 and CD45dim on blood progenitors gated. Panel D: the co-expression of CD133 and VEGFR2 on CD34+CD45dim blood progenitors.
## Online Table I. Correlations between Progenitor Cell Subtypes

<table>
<thead>
<tr>
<th></th>
<th>CD34+</th>
<th>CD34+/CD133+</th>
<th>CD34+/CXCR4+</th>
<th>CD34+/VEGFR2+</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD34+</td>
<td>0.908</td>
<td>0.848</td>
<td>0.217</td>
<td></td>
</tr>
<tr>
<td>CD34+/CD133+</td>
<td>0.908</td>
<td>0.745</td>
<td>0.124</td>
<td>0.217</td>
</tr>
<tr>
<td>CD34+/CXCR4+</td>
<td>0.848</td>
<td>0.745</td>
<td>0.341</td>
<td></td>
</tr>
<tr>
<td>CD34+/VEGFR2+</td>
<td>0.217</td>
<td>0.124</td>
<td>0.341</td>
<td></td>
</tr>
</tbody>
</table>

All correlations were statistically significant at P<0.001
Online Figure I. Flow Diagram

Subjects undergoing left heart catheterization enrolled in the Emory Cardiovascular Biobank (n=6464)

Consecutive subjects with FACS-measured progenitor cell counts (n=2202)

Subjects without FACS measurements (n=4262)

Patients without known coronary artery disease or evidence on angiogram (n=705)

Subjects with coronary artery disease with and without peripheral vascular disease (n=1497)
Online Figure II. Flow Cytometry Analysis Of Blood Progenitor Cells