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

Arterial Disease and Progenitor Cells in CAD

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Abstract

Rationale— Peripheral arterial disease (PAD) is a clinical manifestation of extra-coronary atherosclerosis. Despite sharing the same risk factors, only 20-30% of patients with coronary artery disease (CAD) develop PAD. Declines 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, 62% male) 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 to those with only CAD. In multivariable analysis, a 50% decrease in CD34+ or CD34+/VEGFR2+ counts were 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%CI, 1.03, 1.91) and a 1.64-fold (95%CI 1.07, 2.50) increase 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.

Non-standard Abbreviations and Acronyms

CAD: Coronary Artery Disease

CD: Cluster of Differentiation

CXCR4: Chemokine (C-X-C Motif) Receptor 4

FITC: Fluorescein isothiocyanate

PAD: Peripheral Arterial Disease

PC: Progenitor Cells

PerCP: Peridinin Chlorophyll Protein Complex

SSDI: Social Security Death Index

VEGFR2: Vascular Endothelial Growth Factor Receptor-2

Key Words

Atherosclerosis, stem cells, CD34, CD133, VEGFR2, KDR, carotid, peripheral arterial disease, aortic aneurysm

INTRODUCTION

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 10-20% of the population >60 years old.[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 20-30% 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.

Recently, a pivotal role for progenitor cells (PCs) in vascular repair and regeneration was uncovered.[10-13] Circulating PCs are mononuclear, originate primarily but not exclusively from the bone marrow, and have been described as having the potential to differentiate into hematopoietic, endothelial, and other lineages, and contribute to vascular repair and regeneration through both direct angiogenic and local paracrine mechanisms.[11, 14-16] A relatively rare population of bone marrow-derived mononuclear cells expressing cluster of differentiation 34 (CD34) are enriched for PCs that can differentiate into hematopoietic, endothelial, and other lineages.[11, 14, 16-18] CD34-expressing mononuclear cells include hematopoietic, endothelial, and non-hematopoietic (mesenchymal, lacking CD45 expression) PCs.[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,[17, 20] while co-

expression of vascular endothelial growth factor receptor-2 (VEGFR2) appears to identify a rarer subpopulation of PCs further enriched for endothelial progenitors.[21-23] Finally, co-expression 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.[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.[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.[28, 29] Previous studies investigating the role of PCs in diabetics with PAD found significantly decreased CD34+/VEGFR2+ cell counts and proliferation compared to healthy or diabetic subjects without PAD.[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. In order 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.

METHODS

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, 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 three Emory Healthcare sites in Atlanta, GA, (Online Figure I, Table 1).[29] 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.[29] 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.

Defining Peripheral Arterial Disease

We extensively reviewed patients' self-reported as well as physician-documented medical history and imaging reports to identify the presence of PAD. PAD was defined as a history of symptomatic or asymptomatic non-coronary atherosclerotic disease in at least one of the following arteries: carotid, thoracic or abdominal aorta, 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 (CT, ultrasound or fluoroscopy) in the iliac, femoral, or popliteal arteries; 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, CT or MRA). 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 CT imaging.

Progenitor Cell Assays

Venous blood was collected in EDTA tubes and incubated with fluorochrome-labeled monoclonal anti-human mouse antibodies within 4 hours. Cell populations enriched for circulating PCs were enumerated using flow cytometry as $CD45^{dim}$ cells co-expressing CD34+, CD133+, VEGFR2+, or CXCR4+.[33-36] We incubated 300 µL of peripheral blood with 7 µL of FITC-CD34 (BD Biosciences), PerCP-CD45 (BD Biosciences), PE-VEGFR2 (R&D system) and 5 µL APC-CD133 (Miltenyi), and 3ul 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. Prior to flow cytometry, 100 µL 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 analysed 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 two occasions by the same technician, the coefficients of variation of the cell types were: CD34+ 2.9%; CD34+/CD133+ 4.8%; CD34+/CXCR4+ 6.5% and CD34+/CD133+/CXCR4+ 7.5%, CD34+/VEGFR2+ cells 21.6%. There were significant correlations between the PC subtypes, with moderate-strong correlations between CD34+, CD34+/CD133+, CD34+/CXCR4+ (r range

0.75-0.91, P<0.001), and weak correlations (r range 0.12-0.34, P<0.001) between CD34+/VEGFR2+ subtypes and the aforementioned PCs (Online Table I).

Follow-up and Outcomes

We conducted follow-up as previously described to identify pre-specified 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 (SSDI) and state records. PAD-related events such as peripheral revascularization and amputation were identified using standard CPT 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, standard deviations and ranges. Differences between groups were assessed using t-tests for continuous variables, and chi-square or Fischer exact tests for categorical variables where appropriate. For non-normally distributed variables such as circulating PC counts, the Mann-Whitney U test was used to compare groups in unadjusted analyses. For multivariable analyses, CD34+, CD133+ and CXCR4+ cell counts were log-transformed (base 10) to a normal distribution, while CD34+/VEGFR2+ cell counts were analyzed as a dichotomous variable using the median as a cutoff. Independent predictors of PAD were identified using binary logistic regression modeling accounting for age, gender, 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. All multivariable analyses incorporated the aforementioned covariates and specific PC subsets. Sensitivity analyses was

performed to explore the interactions between clinical variables significantly associated with PAD and PC subtypes.

The incremental value of PC counts to prediction of PAD was tested by addition of individual PC subsets dichotomized to high versus low using the median as a cutoff, to a clinical model with the risk factors including the aforementioned variables. The c-statistic, continuous net reclassification improvement (NRI), and integrated discrimination improvement (IDI) were calculated to evaluate the improvement in predictive ability of the models with and without PCs.[38]

Cox regression analyses examined the association between PC counts and all-cause death, PAD-related events and the combined endpoint of death, 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, USA).

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, lower BMI and 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 one site of documented PAD, 69 (22%) had two, and 10 (3%) had at least three. In multivariable analyses, age, lower BMI, 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+, CD34+/CD133+, and CD34+/CXCR4+ cells) as well as those enriched for endothelial progenitors (CD34+/VEGFR2+ cells) were lower in patients with PAD compared to those without PAD (Table 3). Notably CD34+/VEGFR2+ cells were close to 2-fold lower in number in those with PAD compared to 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+ and CD34+/VEGFR2+ cell counts (Models 2 and 5), but not CD34+/CD133+ (Model 3) or CD34+/CXCR4+ (Model 4) counts were independently associated with the presence of PAD (Table 2). Thus, a 50% decrease in CD34+ or CD34+/VEGFR2+ cell counts was associated with a 31% (OR 1.31, P=0.032) and 183% (OR 2.83, P=0.002) increase in the odds of having PAD respectively.

Given the weak correlation between CD34+ and CD34+/VEGFR2+ cell counts (r=0.22, P<0.001), we examined their association with PAD in the same multivariable model and found them to be both associated with PAD independent of each other (OR 1.35 for CD34+ and OR 1.49 for CD34+/VEGFR2+, Table 2, Model 6). Moreover, patients with both low (≤median) CD34+ and CD34+/VEGFR2+ had a higher prevalence of PAD (28% versus 15%, P<0.001) compared to those with higher than median counts in both subsets (Figure 1), as well as higher odds (1.65, P=0.002) of having PAD (Model 7, Table 2). Subjects with low levels in only one cell subset had intermediate prevalence of PAD (Figure 1).

Sensitivity Analyses

We performed sensitivity analyses in order to determine whether the association between PCs and PAD differed according to conventional PAD risk factors; age, gender, diabetes and smoking status, and presence of obstructive CAD (Figure 2). We found a significant interaction between CD34+ and smoking status in the prediction of PAD (interaction P=0.019). Patients with a history of smoking and low CD34+ (≤1652 cells/mL) had significantly higher odds of having PAD (OR 1.69, P=0.003), while non-smokers with low CD34+ cells did not (OR 0.90, P=0.68). There were otherwise no 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 NRI and IDI between the model with traditional risk factors only (Model 1) and three 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, NRI as well as IDI (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 NRI of 0.390 (95%CI 0.234, 0.543) and an IDI of 0.027 (95%CI 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%CI -0.001, 0.020).

Progenitor Cell Counts and Outcomes

Lastly, we examined the association between CD34+, 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), suffer from a myocardial infarction (8% versus 3%, P<0.001) or undergo a vascular procedure (27% versus 4%, P<0.001) compared to 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 to 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 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+, 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 to subjects with only CAD and no known PAD. After adjusting for known risk factors for PAD, low CD34+ (≤1652 cells/mL) and CD34+/VEGFR2+ (≤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.[27, 29] While 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.[11, 39] In our study, CD34+ cells of interest were predominantly (>95%) $CD45^{dim}$, and thus largely represent cells of the hematopoietic lineage. While 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.[17, 40-43]

Our findings are consistent with prior smaller studies showing similarly lower levels of circulating CD34+/VEGFR2+ cells in patients with PAD.[30-32, 44] Shaffer et al. and Bitterli et al. noted similar findings when comparing patients with PAD to healthy subjects,[30, 44] while Fadini et al. reported decreased counts in diabetic subjects with lower extremity PAD or carotid stenosis compared to diabetics without PAD.[32] 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 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, as 33% of our subjects with PAD had aortic disease. While 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.[45-47] 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.[48, 49]

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 sub-populations enriched for both hematopoietic and endothelial 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.[31, 50,25, 26] Thus, identifying subjects at risk for PAD may allow for earlier interventions, and potentially abrogation of that risk. A low CD34+/VEGF2R+ PC cell count is indicative of worse long term prognosis, especially from vascular events. Given the significant impact of PAD on morbidity and mortality, whether a sustained decrease in PC counts precedes development of PAD is worthy of further study.

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Conflict of Interest Disclosures

None of the authors have conflicts of interest to disclose.

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Table 1. Characteristics of Patients with CAD with and without Peripheral Vascular Disease

Variables	Without Peripheral Vascular Disease (n=1189)	Peripheral Vascular Disease (n=308)	P-value
Age, years	65 (13)	71 (11)	**<0.001**
Male, n (%)	725 (61%)	199 (65%)	0.264
African American, 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**

Values are mean (SD), n (%) as noted. Obstructive coronary artery disease denotes the presence of at least 50% obstruction in any of the coronary arteries on angiogram.

Table 2. Independent Predictors of Peripheral Vascular Disease

Variables	β, P-value	95% CI
Model 1: Risk Factors		
Age, per 10 years	**1.41, <0.001**	**1.24, 1.604**
Male	1.10, 0.523	0.82, 1.48
African American	1.00, 0.982	0.69, 1.46
Body Mass Index, per kg/m^2	**0.95, <0.001**	**0.93, 0.98**
Smoking History	**1.43, 0.021**	**1.06, 1.95**
Hypertension	**2.33, 0.026**	**1.11, 4.90**
Diabetes Mellitus	1.30, 0.070	0.98, 1.74
Hyperlipidemia	0.83, 0.300	0.58, 1.18
Statin use	**1.47, 0.020**	**1.06, 2.03**
Heart Failure	1.30, 0.085	0.96, 1.76
Obstructive Coronary Artery Disease	**1.65, 0.002**	**1.19, 2.27**
ACEi/ARB use	1.18, 0.325	0.85, 1.66
eGFR, per mL/min/1.73 m^2	**0.99, 0.001**	**0.98, 0.99**
Model 2-5: Risk Factors + Individual PC subtypes		
CD34+, ≤1652 cells/mL	**1.41, 0.015**	**1.07, 1.87**
CD34+/CD133+, ≤762 cells/mL	1.15, 0.329	0.87, 1.53
CD34+/CXCR4+, ≤799 cells/mL	1.13, 0.376	0.59, 1.50
CD34+/VEGFR2+ ≤33 cells/mL	**1.55, 0.005**	**1.14, 2.10**
Model 6: Risk Factors and CD34+, CD34+/VEGFR2+		
CD34+ ≤1652 cells/mL	**1.35, 0.037**	**1.02, 1.79**
CD34+/VEGFR2+ ≤33 cells/mL	**1.49, 0.012**	**1.09, 2.03**
Model 7: RF and Low CD34+, CD34+/VEGFR2+		
CD34+ ≤1652 cells/mL and CD34+/VEGFR2+ ≤33 cells/mL	**1.65, 0.002**	**1.19, 2.29**

Progenitor cell subtypes were each entered into separate models incorporating demographics and risk factors. The odds ratio and confidence intervals reported for the demographics and clinical characteristics are derived from the model not incorporating any PCs.

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

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

Progenitor cell counts are reported as median (25th, 75th percentiles). # denotes P-value for comparison between patients with and without peripheral vascular disease. † denotes 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.

Table 4. Risk Prediction Metrics

Model	Likelihood ratio test (P-value)	C-statistic (95% CI)	ΔC-statistic (95% CI)	Continuous NRI (95% CI)	IDI (95%CI)
Model 1: Risk Factors Only		0.717 (0.685;0.749)	-	-	-
Model 2: RF and CD34+ cells	<0.001	0.721 (0.689;0.753)	0.004 (-0.004, 0.011)	0.256 (0.129, 0.382)	0.005 (0.001; 0.009)
Model 3: RF and CD34+/VEGFR2+ cells	<0.001	0.722 (0.691;0.754)	0.006 (-0.003, 0.014)	0.255 (0.128, 0.382)	0.005 (0.001, 0.010)
Model 4: RF and CD34+, CD34+/VEGFR2+ cells	<0.001	0.727 (0.695;0.758)	0.010 (-0.001, 0.020)	0.390 (0.234, 0.546)	0.027 (0.017, 0.036)

Model 1 includes age, gender, 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 (RF) 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. CI = confidence interval, NRI = net reclassification index.

Table 5. Progenitor cells and incident adverse cardiovascular outcomes

Variables	Death		PAD-related Events		Death, myocardial infarction and PAD-related events	
	HR, P-value	**95% CI**	**HR, P-value**	**95% CI**	**HR, P-value**	**95% CI**
Model 1: Risk Factors						
Age, per 10 years	**1.14, 0.036**	**1.01, 1.29**	**0.85, 0.050**	**0.72, 1.00**	1.01, 0.830	0.91, 1.12
Male	0.92, 0.589	0.69, 1.24	1.09, 0.654	0.74, 1.61	1.04, 0.739	0.82, 1.32
African American race	0.97, 0.867	0.67, 1.40	**0.54, 0.015**	**0.33, 0.89**	0.85, 0.268	0.63, 1.14
Body Mass Index, per kg/m^2	**0.96, 0.003**	**0.93, 0.99**	0.99, 0.418	0.95, 1.02	**0.97, 0.006**	**0.95, 0.99**
Smoking History	1.36, 0.061	0.99, 1.88	0.93, 0.699	0.63, 1.37	1.12, 0.394	0.87, 1.43
Hypertension	1.02, 0.936	0.58, 1.80	1.32, 0.491	0.60, 2.87	1.48, 0.125	0.90, 2.45
Diabetes Mellitus	1.18, 0.270	0.88, 1.60	1.34, 0.116	0.93, 1.94	1.14, 0.279	0.90, 1.44
Hyperlipidemia	1.01, 0.961	0.71, 1.44	0.92, 0.703	0.58, 1.45	0.97, 0.834	0.73, 1.29
Statin use	**0.73, 0.044**	**0.53, 0.99**	1.43, 0.157	0.87, 2.36	1.07, 0.613	0.82, 1.41
Heart Failure	**1.78, <0.001**	**1.33, 2.38**	1.06, 0.780	0.71, 1.57	**1.36, 0.011**	**1.07, 1.73**
Obstructive Coronary Artery Disease	**0.47, 0.043**	**0.23, 0.98**	1.01, 0.988	0.31, 3.31	0.76, 0.473	0.35, 1.62
ACEi/ARB use	**0.72, 0.029**	**0.54, 0.97**	1.12, 0.573	0.76, 1.64	0.93, 0.518	0.73, 1.17
eGFR, per mL/min/1.73 m^2	**0.99, 0.001**	**0.99, 1.00**	1.00, 0.441	1.00, 1.01	1.00, 0.065	0.99, 1.00
Peripheral Vascular Disease	**1.59, 0.004**	**1.16, 2.19**	**9.11, <0.001**	**6.16, 13.48**	**2.94, <0.001**	**2.31, 3.75**
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	

Progenitor cell subtypes were each entered into separate models incorporating demographics and risk factors. The odds ratio and confidence intervals reported for the demographics and clinical characteristics are derived from the model not incorporating any PCs.

Figure Legends

Figure 1. Prevalence of Peripheral Arterial Disease Stratified by CD34+ and CD34+/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.

Figure 2. Sensitivity Analyses

Forest plot of interaction with traditional risk factors and median CD34+ cell counts (panel A), and median CD34+/VEGFR2+ cell counts for predicting the presence of PAD. There was a significant interaction between smoking and CD34+ cell counts (highlighted in box, panel A).

NOVELTY AND SIGNIFICANCE

What Is Known?

- Peripheral arterial disease (PAD) and coronary artery disease (CAD) co-occur in 20-30% of patients despite sharing risk factors.
- Circulating progenitor cells (PC) are thought to be involved in vascular repair and regeneration. Mononuclear cells expressing CD34 and VEGFR2 are enriched for endothelial PCs.
- Patients with PAD have been shown to have lower CD34+/VEGFR2+ PC counts compared to healthy subjects.

What New Information Does This Article Contribute?

- Patients with both PAD and CAD, compared to those with CAD alone, have at least 30% lower CD34+/VEGFR2+ PC counts, independently of demographics and clinical characteristics.
- Addition of CD34+/VEGFR2+ PC counts to a traditional risk factor model significantly improved risk prediction metrics for the presence of 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. While both PAD and CAD share similar risk factors, only 20-30% 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 to healthy counterparts. We investigated whether differences in PC counts could distinguish between patients with PAD and CAD and those with CAD alone. We found that patients with PAD (carotid, abdominal, lower or upper extremity) had significantly lower CD34+, and CD34+/VEGFR2+ PCs, independently of demographics and clinical characteristics. CD34+/VEGR2+ counts added incremental value to traditional risk factors in predicting the presence of PAD. Moreover, we found that low CD34+/VEGFR2+ was associated with worse cardiovascular outcomes, and notably PAD-related events such as peripheral revascularizations or amputations. These findings imply a disruption in endogenous regenerative potential may underlie the pathogenesis of PAD, and suggests that PC counts could be used to identify patients with CAD who are at high risk of PAD, provide prognostic information, and potentially allow for early interventions.