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Circulating CD34+ Progenitor Cells and Risk of Mortality in a Population with Coronary Artery Disease

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Abstract

Rationale—Low circulating progenitor cell (PC) numbers and activity may reflect impaired intrinsic regenerative/reparative potential, but it remains uncertain whether this translates into a worse prognosis.

Objectives—To investigate whether low numbers of PCs associate with a greater risk of mortality in a population at high cardiovascular risk.

Methods & Results—Patients undergoing coronary angiography were recruited into two cohorts (1, n=502 and 2, n=403) over separate time periods. PCs were enumerated by flow cytometry as $CD45^{med+}$ blood mononuclear cells expressing CD34, with additional quantification of subsets co-expressing CD133, VEGFR2 and CXCR4. Coefficient of variation for CD34 cells was 2.9% and 4.8%, 21.6% and 6.5% for the respective subsets. Each cohort was followed for a mean of 2.7 and 1.2 years, respectively, for the primary endpoint of all-cause death.

There was an inverse association between CD34+ and CD34+/CD133+ cell counts and risk of death in Cohort 1 ($\beta=-0.92$, p=0.043 and $\beta=-1.64$, p=0.019, respectively) that was confirmed in Cohort 2 ($\beta=-1.25$, p=0.020 and $\beta=-1.81$, p=0.015, respectively). Covariate adjusted HRs in the pooled cohort (n=905) were 3.54 (1.67-7.50) and 2.46 (1.18-5.13), respectively. CD34+/CD133+ cell counts improved risk prediction metrics beyond standard risk factors.

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DISCLOSURES

None

Conclusion—Reduced circulating PC counts, identified primarily as CD34+ mononuclear cells or its subset expressing CD133 are associated with risk of death in individuals with coronary artery disease, suggesting that impaired endogenous regenerative capacity is associated with increased mortality. These findings have implications for biological understanding, risk prediction and cell selection for cell based therapies.

Keywords

Progenitor cells; CD34; flow cytometry; outcomes research; coronary artery disease; risk; prognosis; biomarker

INTRODUCTION

Although cardiovascular risk factor-mediated injury to the vascular endothelium is well described, little was known about mechanisms underlying regeneration until the pivotal role of progenitor cells (PCs) in vascular repair was discovered.[1-3] Circulating PCs are mononuclear, originate primarily but not exclusively from the bone marrow, have the potential to differentiate into several lineages and contribute to vascular repair and regeneration.[1, 2, 4, 5] CD34+ mononuclear cells from human bone marrow include distinct lineages of both hematopoietic (CD34+/CD45^{med}) and non-hematopoietic (mesenchymal) progenitors.[6] CD34+ cells have greater myocardial reparative potential than unselected populations.[7] CD133 is a 5-transmembrane antigen marker of primitive stem cells that is lost during maturation, and cells expressing both markers (CD34+/CD133+) may be further enriched for a vascular PC phenotype.[8, 9] While additional expression of vascular endothelial growth factor receptor-2 (VEGFR2) has been proposed to identify more differentiated PCs, these sub-populations remain difficult to reproducibly quantify compared to other more abundant CD34+ populations. Finally, co-expression of CXCR4, which promotes homing of PCs to stromal-derived factor-enriched hypoxic environments for enhancing repair, may also further characterize PCs with capacity for vascular repair.[10]

Lower counts and activity of PCs in blood are associated with cardiovascular risk factors and vascular dysfunction, with experimental studies suggesting that diminished PC counts reflect impaired regenerative potential.[11-15] Early studies have reported an increased risk of adverse cardiovascular events in subjects with low numbers of circulating PCs. However, the majority of associations were driven by procedural endpoints with PCs characterized variably as CD34+, CD133+ or CD34+/VEGFR2 dual positive cells.[16-18] Whether low circulating PC counts increase mortality in subjects at high cardiovascular risk remains unclear. We sought to comprehensively examine the prognostic impact of CD34+ enriched PCs in subjects with coronary artery disease (CAD) with the hypothesis that low circulating PC counts, reflecting reduced cardiovascular regenerative capacity, will be associated with greater mortality. As secondary endpoints we also studied the relationship between PCs and cardiovascular death and the combined outcome of death and MI.

METHODS

Study population

Study participants aged 20-90 years were recruited as part of the Emory Cardiovascular Biobank, an ongoing prospective cohort of patients enrolled prior to undergoing coronary angiography for investigation or management of CAD across three Emory Healthcare sites with collection of data on demographic characteristics, medical history, medication use, behavioral habits and risk factor prevalence, details of which have been published previously.[19]

Two sub-cohorts were collected under the same protocol, with identical sampling strategies and collection methods but separated in time and by a change in cell quantification methodology (see below). Cohort 1 (n=502) was enrolled between March 2006 and September 2008, while Cohort 2 (n=403) was collected between October 2008 and February 2011.

Recruited patients were stable at the time of enrollment and undergoing an elective procedure, although stable patients with non-ST elevation myocardial infarction, defined using international criteria were also included and classified as “Acute MI”.[20] Obstructive CAD was defined as >50% luminal narrowing in a major epicardial vessel and a Gensini score calculated to quantify the burden of CAD. Patients were excluded if they had a history of heart transplantation, immunosuppressant use, malignancy, or significant infections. The study complies with the declaration of Helsinki and was approved by the Institutional Review Board at Emory University with all subjects providing written informed consent.

Follow-up and endpoints

Follow-up was conducted for determination of the primary endpoint of all cause death, and the secondary endpoints of cardiovascular death and the composite of all-cause death or MI. This was performed by personnel blinded to PC data through telephone interview, chart review and linkage with the Social Security Death Index and State records. Cause of death was adjudicated by two cardiologists with a third arbitrator in case of disagreement, who were all blinded to PC data. Cardiovascular death was defined as death attributable to an ischemic cardiovascular cause (fatal MI, stroke, peripheral arterial disease) or sudden death due to an unknown but presumed cardiovascular cause in high risk patients. Medical records were accessed or requested to validate all self-reported events including MI, which was again defined using standard criteria.[20]

Flow cytometry

Arterial blood was collected via a femoral sheath in EDTA tubes prior to cardiac catheterization after an overnight fast. Blood samples were prepared within 4 hours and incubated with fluorochrome-labeled monoclonal anti-human mouse antibodies to identify surface markers expressed on mononuclear cells before quantification using flow cytometry. Further details are given in Supplementary Methods. Of note, preliminary descriptive analysis of cohort 1 revealed very low counts of infrequent cell types. Thus for Cohort 2, enumeration of PCs was refined by adding a fixed number of counting beads to 300uL

whole blood and a lyse-no-wash methodology that reduced cell loss. This improved quantification and cell counts were subsequently noted to be higher for each subset compared to Cohort 1. Putative circulating PCs were identified in both cohorts through expression of the CD34 surface marker on mononuclear cells that were enumerated as $CD45^{med+}$ cells with and without co-expression of CD133 (CD34+/CD133+) and VEGFR2 (CD34+/VEGFR2 and CD34+/CD133+/VEGFR2+). In Cohort 2, co-expression of an additional marker, CXCR4 was also quantified (as CD34+/CXCR4+ and CD34+/CD133+/CXCR4+). All cell populations were reported as cell counts/μl.

Statistical analysis—Continuous variables are presented as means (SD) or as median (IQR) and categorical variables as proportions (%). Cell counts were non-normally distributed and were transformed (natural log (cell count+1)) prior to parametric analyses. Cell populations were additionally categorized by tertiles, to derive effect estimates and to permit pooled analysis of both cohorts. Finally to assess the potential of cell counts for use as a biomarker we derived an optimized cut off in Cohort 1 and applied this threshold (as a percentile equivalent of the absolute value) in Cohort 2 for validation. The cut point was identified using receiver operating characteristic (ROC) analyses and Youden's index (Sensitivity – (1-Specificity)) (Supplementary Figure I).

Survival analysis was performed using Cox proportional-hazards regression in models adjusted for age and gender. In the pooled cohort, further adjustment was made for body mass index (BMI), diabetes, hypertension, low density lipoprotein (LDL), current smoking, statin use, glomerular filtration rate (GFR), acute MI at enrollment, left ventricular ejection fraction (LVEF) and CAD burden (Gensini Score) all at baseline. Missing covariate data for the fully adjusted model (range 0-3%) was imputed and sensitivity analysis with un-imputed data found results to be similar. The proportional hazards assumption for Cox models was evaluated by plots of Schoenfeld residuals and formal testing (a chi-square test calculated as the sum of Schoenfeld residuals), with no significant violations of the assumption found.

The incremental value of PC counts to risk prediction was tested before and after their addition (using high v low categorization described above) to a clinical model with traditional risk predictors. The C-statistic (AUC), category free net reclassification improvement (NRI) and integrated discrimination improvement (IDI) were calculated as indices of risk discrimination.[21] P values of <0.05 from two-sided tests were considered to indicate statistical significance. All statistical analyses were performed using SAS (Cary, NC) and SPSS 20.0 (Chicago, IL).

RESULTS

Baseline patient characteristics of the 502 subjects in Cohort 1 and 403 subjects in Cohort 2 are shown in Table 1. The mean age and gender distribution was similar across cohorts (62.5 and 62.9 years; males 64.1% and 65.5%). Overall, patient characteristics were reflective of typical populations recruited at coronary angiography, with over two thirds reporting hypertension and hyperlipidemia and a third with diabetes.

Reproducibility of PC quantification and correlation between PC subsets

In 20 samples from the Cohort 2 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%. However, CD34+/VEGF2R cells and CD34+/CD133+/VEGF2R cells showed poorer reproducibility at 21.6% and 35.9%. PC counts were generally higher in Cohort 2 due to the refined enumeration methods described above. CD34+ and its subset of CD34+/133+ were highly correlated with each other in each cohort (Spearman rho = 0.92 and 0.87, for Cohorts 1 & 2 respectively) with more modest correlations between other cell types (Supplementary Table I).

Relationship between PCs and demographic and clinical features

In Cohort 1, lower CD34+ and CD34+/CD133+ cell counts were univariately associated with higher age, male gender, impaired renal function, obstructive CAD and history of prior MI. Lower CD34+/CD133+ cells were also associated with CAD burden (Gensini Score) and history of PAD. The associations between both cell types with age, gender and renal function were replicated in Cohort 2 in which there were further modest correlations between lower cell counts and lower BMI, higher HDL (inverse) and current smoking (Supplementary Tables II & III).

We did not observe consistent associations between any of the studied cell types and other cardiovascular risk factors such as diabetes, hypertension or features such as statin use, LV function, history of stroke or presence or absence of an acute MI at enrollment.

Relationship between PC populations and adverse outcomes

During a combined 1800 person-years of follow up, there were 71 deaths.

CD34+ and CD34+/CD133+ cells—In cohort 1 (n=502), Cox Regression analysis adjusted for age and gender revealed that both CD34+ and CD34+/CD133+ counts were inversely associated with the primary end-point of all cause death (p=0.043 and p=0.019, respectively) (Table 2). When categorized into tertiles, those subjects in the lowest tertile of CD34+ cell counts had a greater than 4.6-fold risk of death compared to those in the highest tertile group (HR 4.63 (95% CI, 1.59-13.5)). There was a similar 3-fold increase in the age- and gender-adjusted risk of death for those in the lowest tertile of CD34+/CD133+ cell counts (HR 3.09 (95% CI, 1.15-8.33)). For both cell types there was a graded increase in effect size per tertile (Table 2 & Figure 1).

In Cohort 2 (n=403), the age- and gender-adjusted association between CD34+ and CD34+/CD133+ counts and the primary endpoint was replicated (p=0.019 and p=0.015, respectively)(Table 3). Similarly, those in the lowest tertile of CD34+ cell counts (HR 3.43 (95% CI, 1.23-9.59)) and those in the lowest tertile of CD34+/CD133+ cells (HR 2.93 (95% CI, 1.05-8.21)) had increased risk of death compared to those in the highest tertile of each cell type (Table 3).

In the pooled cohort of 905 subjects, those in the lowest tertile of CD34+ and CD34+/CD133+ cells had age- and gender-adjusted HRs of 3.87 (95% CI, 1.86-8.06) and 3.06 (95% CI, 1.50-6.24) for the primary endpoint of all-cause death when compared to subjects in the highest tertile categories (Table 4). After fully adjusting for likely confounders (age, gender, BMI, diabetes, hypertension, LDL, Smoking, GFR, statin use, Gensini score, LVEF, AMI), these associations persisted with HRs of 3.54 (95% CI, 1.67-7.50) and 2.46 (95% CI, 1.18-5.13) for CD34+ and CD34+/CD133+ cells, respectively (Table 4).

Significant associations between CD34+ and CD34+/CD133 cell types were also observed for the secondary endpoints of cardiovascular death and the composite of death/MI combined (Tables 2-4).

CD34+/CD133− (negative) cells—Among the CD34+ population, cells that did not express CD133 (CD34+/CD133−) did not show association with risk of all cause death in Cohort 1 (p=0.132) or in Cohort 2 (p=0.07).

VEGFR2+ cells—Co-expression of VEGFR2 on CD34+ or CD34+/CD133+ cells was not associated with mortality in either cohort (data not shown).

CXCR4+ cells—CXCR4 co-expression enumerated in Cohort 2 (n=403) demonstrated marginal association between lower counts of CD34+/CXCR4+ cells and risk of all-cause death in age- and gender-adjusted models (p=0.07). Those in the lowest tertile of CD34+/CXCR4+ cell counts had a HR of 2.77 (95% CI, 1.04-7.39) for risk of death compared to those in the top tertile (Supplementary Table IV). Cells co-expressing both CXCR4 and CD133 (CD34+/CD133+/CXCR4+) demonstrated similar borderline association with risk of death (Supplementary Table IV).

Finally, we also examined the predictive value of *all* CD133+ and *all* CXCR4 expressing cell counts within the mononuclear cell populations (unselected for CD34) and found that they were not significantly associated with mortality (Supplementary Table V).

Sensitivity analyses

There were no interactions when sensitivity analyses were performed for CD34+ cell counts with respect to age, gender and risk factors in both cohorts separately (Supplementary Table VI). Although there was an interaction for obstructive CAD in Cohort 2 and LV function in Cohort 1, this was not consistent interactions across both cohorts. Specifically, the effect of CD34+ cells on mortality was not modified by acute MI at enrollment or presence of other ischemic conditions (PAD, Stroke).

Risk discrimination testing

To determine the potential of PCs as biomarkers in exploratory analysis, we identified thresholds of 0.737 counts/μl for CD34+ cells (37^{th} centile) and 0.504 counts/μl for CD34+/CD133+ cells (46^{th} centile) using Youden's index for the primary endpoint in Cohort 1 and sought to validate these in Cohort 2 (Supplementary Figure I). Counts of CD34+ and CD34+/CD133+ cells below the relevant thresholds were associated with increased risk of death after adjustment for age and gender (HR 2.15 (95% CI, 1.16-3.97) and HR 3.16 (95%

CI, 1.49-6.72)), respectively in Cohort 1. These findings were fully replicated in Cohort 2 with similar results with risk of death for "low" values of each cell type (HR 2.79 (95% CI, 1.31-5.96) and HR 3.27 (95% CI, 1.37-7.83)), respectively. In the pooled cohort, a low CD34+ count and CD34+/CD133+ count were associated with a HR of 2.24 (95% CI, 1.37-3.66) and 2.83 (95% CI, 1.57-5.12), respectively, compared to high counts, after full adjustment for the aforementioned covariates.

Finally, in the pooled cohort, we tested the incremental value of this threshold driven PC count. The C statistic for prediction of death, when compared to a model with clinical risk predictors, increased marginally with the addition of CD34+ cells (Δ 0.020, p=0.07) but more significantly with the addition of CD34+/CD133+ cells (Δ 0.028, p=0.04). The category free NRI and IDI metrics were also significant for a model including CD34+/CD133+ cell counts compared to clinical model alone (Table 4).

DISCUSSION

In the largest prospective study of patients with CAD phenotyped for circulating PCs to date, we demonstrate that low numbers of blood hematopoietic PCs, characterized as CD34+ mononuclear cells are predictive of incident risk of all-cause death, independent of other risk determinants. Of these, both CD34+ cells and those co-expressing CD133 appear to be the most robustly associated with adverse events, such that in two successive cohorts totaling over 900 subjects, the risk of death was >2.5-fold greater in those in the lowest compared to highest tertile of these cell counts. Furthermore, the CD34+/CD133+ cell count added incremental predictive value to clinical risk factors using risk discrimination indices.

The stromal derived factor-1 receptor CXCR4 is required for homing of PCs and identifies cells with greater capacity for migration and neo-vascularization.[10] However, the association between CD34+ cells co-expressing CXCR4 and death was modest and less robust than the CD34+ or the CD34+/CD133+ populations. This may be partly because CXCR4 was enumerated in only one population.

In addition, and in contrast to other studies, we found no association between CD34+ cells co-expressing VEGFR2 and outcomes in either cohort, possibly because of their very low frequency and poor reproducibility of measurement.[17, 18] In contrast quantification of total CD34+ cells and the CD34+/CD133+ subset is more practical and reproducible.

The mechanisms by which reduced circulating PCs are associated with death is unknown, but based on experimental data likely represents increased risk due to impaired endogenous regenerative and repair capacity. While CD34+ cells are heterogeneous, they are enriched for cells expressing endothelial marker genes and form endothelial structures in vitro and in vivo.[1, 22] Moreover, pro-angiogenic activity is lacking in selected CD34− cells.[7] In our study, CD34+ cells of interest in the blood were predominantly (>95%) $CD45^{med+}$, and thus largely represent cells of the hematopoietic lineage. Of note, CD34+ cells expressing the CD133+ epitope, which are believed to be early or immature PCs, more precisely reflect risk than CD34+ cells without the CD133 epitope, that had no association with risk. While the

additional expression of VEGFR2 receptor on CD34+/CD133+ cells is often considered to define a subset enriched for *endothelial* PCs, this remains a subject of controversy.[8, 23-26]

Three smaller studies have previously reported association between various PC subsets and cardiovascular outcomes.[16-18] In 120 subjects with and without CAD an association between CD34+/VEGFR2+ cell counts and adverse events was found with just 11 events.[17] This was confirmed in a larger study with largely revascularization events.[18] Finally, in subjects enriched for metabolic syndrome, cardiovascular events were more frequent in those with low CD34+ cell counts,[16] findings that were confirmed in a meta-analysis.[27] These studies were generally smaller, more heterogeneous, did not enumerate $CD45^{med+}$ subsets, assessed cardiovascular events that were predominantly revascularization or hospitalizations, and did not perform discriminatory analyses. In contrast, our study represents the largest cohort phenotyped for PCs using flow cytometry (at the time of collection) and prospectively followed for mortality. Additionally, our 2 cohort strategy, separated in time and methodology lends further confidence to our findings. Importantly, and in contrast to previous studies, our findings were most robust for the CD34+ and CD34+/CD133+ cell subsets and not for cells co-expressing VEGFR2.

We and others have previously demonstrated that lower circulating PC counts characterized by flow cytometry or cell culture are associated with endothelial dysfunction and established risk factors.[12, 13] In cross sectional analyses, we confirmed associations between lower PC counts and advanced age and impaired renal function.[12, 15, 28] We also found that higher counts were associated with male gender, higher BMI and lower HDL. Although acute and chronic ischemia may influence PC mobilization, we did not observe any associations or interactions with acute myocardial infarction at enrolment or prior conditions such as PAD or stroke.[30, 31]

Strengths of our study include (1) a large prospective single center 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) a 2 cohort analysis strategy, and (4) exploration of a selection of CD34+ cell sub-populations. The change in methodology between the two cohorts may be considered a limitation, but is also a strength in that the associations were fully replicated under different measurement settings. Limitations of our study are that we only examined a high risk population undergoing angiography, and therefore our conclusions may not be applicable to the general population. Absence of longitudinal measurements or assessments of cell functionality also limit the conclusions that could be drawn about CD34+ cells and their long term impact on health. Finally, the observational nature of this analysis does not imply causation and thus further interventional studies directly influencing PC levels are required. Studies demonstrating improvements in intermediate phenotypes, such as endothelial function with PC mobilization suggest that this could be feasible.[32]

There are important implications of our findings: (1) CD34+ cells that are enriched for bone marrow-derived hematopoietic and endothelial progenitors may represent an index of global regenerative potential, a view supported by findings of worse prognosis in patients with lower numbers of PCs in the settings of acute lung injury, renal failure, and stroke;[33-35] (2)

Quantification of these cell subtypes could be used to identify those individuals with CHD at greatest risk of subsequent events as part of an enhanced risk prediction model, including demographic, clinical features and other novel biomarkers; (3) In addition, CD34+ cells could also represent a potentially modifiable risk factor and thus a new target for therapeutic interventions, a possibility currently under active study;[36] (4) Either CD34+ or CD34+/CD133+ cell counts could be used equally effectively as a 'biomarker' of regenerative capacity; and (5) These findings could influence cell based therapy by supporting targeted selection and harvesting of these particular PC subsets.

In conclusion, we have demonstrated that fewer circulating PCs, identified primarily as CD34+ mononuclear cells are associated with risk of death in individuals at high cardiovascular risk. Both CD34+ and CD34+/CD133+ subset are predictive of future adverse events, and potentially represent endogenous regenerative capacity. These findings have implications for biological understanding, risk prediction and cell selection for cell based therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations and Acronyms

PCs	Progenitor Cells
HR	Hazard ratio
FACS	Fluorescence Activated Cell Sorting
VEGFR2	Vascular Endothelial Growth Factor Receptor 2
CXCR4	Chemokine (C-X-C motif) Receptor 4
MI	Myocardial Infarction
CAD	Coronary Artery Disease
ROC	Receiver Operating Characteristic
NRI	Net Reclassification Index
IDI	Integrated Discrimination Improvement

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Novelty and Significance

What Is Known?

- Circulating progenitor cells play a role in vascular repair and regeneration.
- Mononuclear cells (MNCs) expressing CD34 and CD133 epitopes constitute a population of cells enriched for hematopoietic and/or endothelial progenitor cells (PCs), and their circulating levels are modulated by risk factors.

What New Information Does This Article Contribute?

- The numbers of circulating MNCs expressing CD34 alone or in combination with CD133 are independent predictors of death in patients with coronary heart disease (CHD), such that patients in the lowest tertile had 3-fold greater mortality compared with those in the highest tertile.
- Circulating PC counts reflect endogenous reparative capacity and are independent predictors of survival in CHD.

Although the injurious effects of cardiovascular risk factors in the pathogenesis of atherosclerosis are known, the role of innate repair and regeneration in mitigating this damage remains to be studied. Since circulating PCs represent endogenous regenerative potential, we investigated whether survival in patients with CHD is associated with PC levels. In a large cohort of patients with CHD, we found that the circulating numbers of MNCs expressing CD34 alone or in combination with CD133, predicted incident death, cardiovascular death, and the composite endpoint of death and myocardial infarction. After adjusting for relevant risk factors, there was an almost 3-fold increase in mortality in those with PC levels in the lowest tertile compared to those in the highest tertile. The CD34+/CD133+ cell count added incremental predictive value to clinical risk factors, indicating its additive value as a biomarker for risk prediction in patients with CHD. Thus, circulating levels of CD34+ and CD34+/CD133+ PCs, are novel predictors of survival in CHD, and may be useful biomarkers in risk prediction and potentially targets for therapeutic interventions.

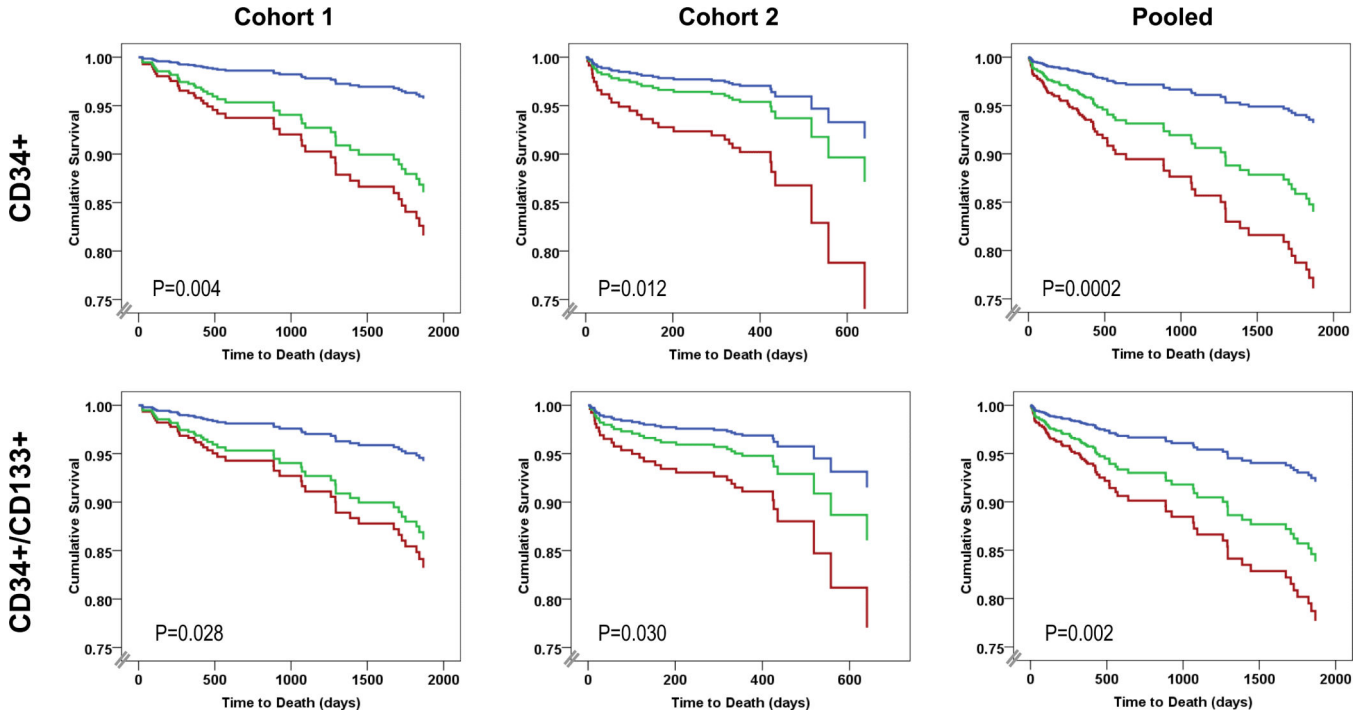

Figure 1.

Survival curves adjusted for age and gender, for the primary endpoint of death by tertiles of CD34+ (upper panel) and CD34+/133+ (lower panel) cell counts. Results are show separately for Cohorts 1 and 2 and the combined “pooled” cohort. Colored lines represent tertiles of PC counts (Blue=top tertile; Green=middle tertile; Red=bottom tertile). P values for effect estimate in Cox regression model.

Table 1

Baseline Patient Characteristics

	Cohort 1	Cohort 2	P value
N	502	403	
Age, years	62.5 (11.6)	62.9 (13.2)	0.60
Male, %	64.3	65.6	0.68
Caucasian, %	71.4	69.5	0.52
Body Mass Index (BMI), kg/m2	30.3 (6.7)	29.3 (6.78)	0.03
Hypertension, %	74.2	88.6	<0.001
Systolic BP, mmHg	140.2 (21.8)	141.1 (23.1)	0.55
Hyperlipidemia, %	76.1	73.7	0.36
Total Cholesterol, mg/dL	170.3 (45.8)	163.6 (48.8)	0.04
Low Density Lipoprotein, mg/dL	99.3 (40.6)	95.0 (42.0)	0.12
High Density Lipoprotein, mg/dL	41.9 (13.2)	41.5 (14.2)	0.72
Diabetes Mellitus,%	31.0	33.0	0.43
Current Smoking,%	17.9	16.0	0.44
Prior Myocardial Infarction (MI), %	28.6	23.8	0.10
Prior CABG, %	20.9	19.9	0.70
Prior Heart Failure, %	20.8	23.1	0.38
Prior Peripheral Arterial Disease, %	17.6	10.0	0.001
Prior Stroke, %	8.0	15.2	0.001
Acute MI at Enrollment, %	10.8	10.7	0.98
Left Ventricular Ejection Fraction %	54.5 (11.1)	53.2 (13.0)	0.12
Renal Function (GFR), ml/min	89.4 (38.4)	84.9 (44.6)	0.08
Statin Use, %	79.0	65.0	<0.001
Obstructive CAD >50%, %	67.5	65.1	0.45
Gensini CAD Score, median (range)	10 (0-33.9)	8 (0-34.1)	0.16
PCI or CABG at Enrollment, %	45.9	38.0	0.02
Cell Populations (Counts/uL) *			
CD34+	1.038 (0.63-1.65)	1.833 (1.10-2.75)	-
CD34+/CD133+	0.535 (0.30-0.93)	0.756 (0.48-1.19)	-
CD34+/VEGF2R+	0.051 (0.02-0.10)	0.189 (0.10-0.35)	-
CD34+/CXCR4+	n/a	0.974 (0.63-1.53)	-
CD34+/CD133+/CXCR4+	n/a	0.398 (0.24-0.67)	-
CD34+/VEGFR2+/CXCR4+	n/a	0.180 (0.09-0.34)	-
Follow up			
Follow up duration, years	2.66 (1.91)	1.18 (0.51)	-
All-cause deaths, n (%)	42 (8.4)	29 (7.2)	-
Secondary endpoints			
Cardiovascular deaths, n (%)	39 (7.8)	25 (6.2)	-
Death or non-fatal MI, n (%)	57 (11.3)	35 (8.7)	-

Mean (SD) shown unless stated

GFR – Glomerular Filtration Rate; CAD – Coronary Artery Disease; PCI – Percutaneous Coronary Intervention; CABG – Coronary Artery Bypass Grafting

* Cell Counts shown as median (IQR)

Table 2

Association between CD34+ and CD34+/CD133+ cells and outcomes for Cohort 1 (n=503)

Cell Type	***Outcome***	***Continuous***	***Tertiles***		***Threshold cut-off – using ROC****
		Beta, p value	**Tertiles**	**HR, (95% CI), p value**	**HR, (95% CI), p value**
CD34+	*Death*	−0.92, p=0.043	T3 - ref	1	2.15 (1.16-3.97), 0.015
			T2	3.42 (1.11-10.4), 0.031	
			T1	4.63 (1.59-13.5), 0.005	
	CV Death	−0.86, p=0.067	T3 - ref	1	2.07 (1.10-3.91), 0.025
			T2	3.06 (0.99-9.45), 0.052	
			T1	4.1 (1.39-12.06), 0.010	
	Death/ MI	−0.93, p=0.017	T3 - ref	1	2.54 (1.49-4.33), 0.001
			T2	1.88 (0.79-4.44), 0.151	
			T1	3.58 (1.64-7.83), 0.001	
CD34+/ CD133+	*Death*	−1.64, p=0.019	T3 - ref	1	3.16 (1.49-6.72), 0.003
			T2	2.51 (0.91-6.96), 0.077	
			T1	3.09 (1.15-8.33), 0.026	
	CV Death	−1.55, p=0.032	T3 - ref	1	3.25 (1.47-7.18), 0.004
			T2	3.07 (1.01-9.31), 0.048	
			T1	3.37 (1.13-10.0), 0.029	
	Death/ MI	−1.76, p=0.004	T3 - ref	1	3.64 (1.89-6.98), <0.001
			T2	2.34 (0.97-5.62), 0.059	
			T1	3.69 (1.60-8.53), 0.002	

All values adjusted for age and gender. Beta values are for natural log+1 transformed cell counts

*Cut offs equivalent to 37% for CD34+ and 46% for CD34+/CD133+ (see methods)

Table 3

Association between CD34+ and CD34+/CD133+ cells and outcomes for Cohort 2 (n=402)

Cell Type	***Outcome***	***Continuous***	***Tertiles***		***Threshold cut-off derived from Cohort 1*** [*]
		Beta, p value	**Tertiles**	**HR, (95% CI), p value**	**HR, (95% CI), p value**
CD34+	*Death*	−1.25, 0.020	T3 - ref	1	2.79 (1.31-5.96), 0.008
			T2	1.57 (0.51-4.83), 0.431	
			T1	3.43 (1.23-9.59), 0.019	
	CV Death	−1.02, 0.064	T3 - ref	1	2.43 (1.11-5.31), 0.026
			T2	1.56 (0.51-4.80), 0.445	
			T1	2.97 (1.04-8.46), 0.042	
	Death/ MI	−1.13, 0.018	T3 - ref	1	2.45 (1.24-4.85), 0.101
			T2	1.44 (0.55-3.78), 0.463	
			T1	2.91 (1.19-7.11), 0.019	
CD34+/ CD133+	*Death*	−1.81, 0.015	T3 - ref	1	3.27 (1.37-7.83), 0.008
			T2	1.69 (0.56-5.15), 0.355	
			T1	2.93 (1.05-8.21), 0.041	
	CV Death	−1.51, 0.044	T3 - ref	1	3.61 (1.53-8.55), 0.003
			T2	1.68 (0.55-5.12), 0.360	
			T1	2.52 (0.88-7.22), 0.084	
	Death/ MI	-−1.39, 0.028	T3 - ref	1	2.57 (1.25-5.32), 0.010
			T2	1.23 (0.48-3.17), 0.678	
				2.16 (0.92-5.08), 0.078	

All values adjusted for age and gender. Beta values are for natural log+1 transformed cell counts

[*] Applying cut offs derived from Cohort 1 using 37%/46% thresholds.

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Table 4

Association between CD34+ and CD34+/CD133+ cells and outcomes for the pooled cohort (n=905)

Cell Type	*Outcome*	*Tertiles*[*]		*Tertiles - Fully adjusted*[**]	*Threshold cut-off*[*]	*Threshold Fully adjusted*[**]
		Tertiles	**HR, (95% CI), p value**	**HR, (95% CI), p value**	**HR, (95% CI), p value**	**HR, (95% CI), p value**
CD34+	*Death*	T3 - ref	1	1	2.35 (1.46-3.78), <0.0001	2.24 (1.37-3.66), 0.001
		T2	2.47 (1.13-5.37), 0.023	2.36 (1.07-5.25), 0.034		
		T1	3.87 (1.86-8.06), <0.001	3.54 (1.67-7.50), 0.001		
	CV Death	T3 - ref	1	1	2.16 (1.32-3.53), 0.002	2.00 (1.20-3.32), 0.008
		T2	2.31 (1.05-5.06), 0.036	2.27 (1.02-5.10), 0.046		
		T1	3.39 (1.61-7.13), 0.001	3.05 (1.42-6.53), 0.004		
	Death/ MI	T3 - ref	1	1	2.47 (1.62-3.76), <0.0001	2.44 (1.58-3.77), <0.0001
		T2	1.72 (0.91-3.27), 0.098	1.63 (0.85-3.14), 0.144		
		T1	3.25 (1.81-5.83), <0.0001	3.07 (1.69-5.58), <0.001		
CD34+/CD133+	*Death*	T3 - ref	1	1	3.42 (1.94-6.04), <0.0001	2.83 (1.57-5.12), 0.001
		T2	2.13 (1.01-4.50), 0.046	2.23 (1.05-4.74), 0.037		
		T1	3.06 (1.50-6.24), 0.002	2.46 (1.18-5.13), 0.017		
	CV Death	T3 - ref	1	1	3.33 (1.85-5.99), <0.0001	2.74 (1.48-5.05), 0.001
		T2	2.34 (1.08-5.06), 0.032	2.47 (1.13-5.41), 0.024		
		T1	2.95 (1.39-6.25), 0.005	2.36 (1.08-5.14), 0.031		
	Death/ MI	T3 - ref	1	1	3.20 (1.98-5.18), <0.0001	2.79 (1.70-4.60), <0.0001
		T2	1.76 (0.93-3.31), 0.080	1.80 (0.95-3.41), 0.072		
		T1	2.96 (1.64-5.33), <0.0001	2.54 (1.38-4.67), 0.003		

[*] Adjusted for age and gender only

[**] Adjusted for age, gender, bmi, diabetes, hypertension, LDL, smoking, renal function (GFR), statin use, CAD burden (Gensini score), LV function (EF), Acute MI at enrolment

Table 5

Risk prediction metrics for CD34+ and CD34+/133+ cell counts in the combined cohort for the primary outcome of death (n=905)

Model	*C-statistic*		*NRI*		*IDI*		
	C-statistic (95% CI)	**P value for ΔC-statistic**	**Category Free NRI**	**P value**	**IDI**	**P value**	**Relative IDI**
Model	0.786	-	-	-	-	-	-
Model + CD34+ cells	0.806	0.069	0.504	<0.001	0.013	0.058	0.138
Model + CD34+/CD133+ cells	0.814	0.042	0.664	<0.001	0.024	0.0006	0.247

CD34+ and CD34+/CD133+ cells categorized as low v high levels using the derived thresholds (see methods)

Model = Includes age, gender, hypertension, diabetes, smoking, LDL, renal function (GFR), LV function (LVEF), acute MI at baseline

NRI = Net Reclassification Index; IDI = Integrative Discriminatory Index