Long-term Prediction of Cardiovascular Outcomes by Circulating $CD34^+$ and $CD34^+CD133^+$ Stem Cells in Patients With Type 2 Diabetes

Gian Paolo Fadini, Mauro Rigato, Roberta Cappellari, Benedetta Maria Bonora, and Angelo Avogaro

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OBJECTIVE

Cardiovascular risk varies substantially in the population with diabetes, and biomarkers can improve risk stratification. Circulating stem cells predict future cardiovascular events and death, but data for the population with diabetes are scant. In this study we evaluated the ability of circulating stem cell levels to predict future cardiovascular outcomes and improve risk discrimination in patients with type 2 diabetes.

RESEARCH DESIGN AND METHODS

A cohort of 187 patients with type 2 diabetes was monitored for a median of 6.1 years. The primary outcome was time to a first cardiovascular event, defined as 3-point major adverse cardiovascular event (cardiovascular death, nonfatal myocardial infarction, or nonfatal stroke) plus hospitalization for cardiovascular causes. At baseline, we measured six stem/progenitor cell phenotypes in peripheral blood based on expression of CD34, CD133, and KDR.

RESULTS

The primary outcome occurred in 48 patients (4.5/100 patient-years). Patients with incident cardiovascular events had significantly lower $CD34^+$ and $CD34^+CD133^+$ cells than those without. Higher rates of cardiovascular events occurred in patients with below median levels of $CD34^+$ and $CD34^+CD133^+$. In Cox proportional hazards regression analyses, a reduced $CD34^+$ (hazard ratio 2.21 [95% CI 1.14–4.29]) and $CD34^+CD133^+$ (2.98 [1.46–6.08]) cell count independently predicted future events. Addition of the $CD34^+$ cell count to the reference model or the UK Prospective Diabetes Study risk engine improved C statistics, continuous net reclassification improvement, and/or integrated discrimination index.

CONCLUSIONS

In patients with type 2 diabetes, a reduced baseline level of circulating $CD34^+$ stem cells predicts adverse cardiovascular outcomes up to 6 years later and improves risk stratification.

Department of Medicine, University of Padova, Padova, Italy

Corresponding author: Gian Paolo Fadini, gianpaolo.fadini@unipd.it or gianpaolofadini@hotmail.com.

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Patients with diabetes experience a high rate of cardiovascular disease, but the risk varies considerably, even within the population with diabetes (1,2). This notion provides a compelling rationale for using biomarkers to improve individual risk prediction.

More than 10 years ago, we demonstrated that patients with diabetes with vascular disease have lower levels of circulating $CD34^+$ stem cells (3), a finding that others have confirmed (4,5). The peripheral blood $CD34^+$ cell population is mainly composed of hematopoietic stem cells (HSCs), and putative endothelial progenitor cells (EPCs) represent a minor subset. Quite interestingly, $CD34^+$ cells have been successfully used in cell therapies for cardiac and limb ischemia (6,7), which lend support to the cardiovascular properties of such cells. Coexpression of the HSC marker CD133 was initially suggested to enrich for functional EPCs (8,9), but this is still controversial (10,11). Human $CD34^+$ cells are provided with vascular regenerative capacity and proangiogenic potential in vivo (12), and their pauperization is now considered a significant contributor to the impaired cardiovascular homeostasis in diabetes. However, circulating $CD34^+$ cells are so rare in the circulation ($\sim$3 cells/μL), that the clinical and biological meaning of a further reduction in their level was unclear. The findings that blood stem cells aid cardiovascular repair in experimental models were sometimes considered not sufficiently conclusive to support that a reduction in $CD34^+$ cells from 3 to 2/μL has any relevant clinical implication.

To address this issue, several authors have reported that patients with low levels of circulating stem or progenitor cells were at a significantly higher risk for future cardiovascular disease than were patients with higher cell levels (13–15). A pooled analysis of such longitudinal studies with an average duration of 2 years shows that a low level compared with a high level of stem/progenitor cells is independently associated with a twofold risk for future cardiovascular events and death (16). Some studies also reported that stem cell levels improved stratification of patients into the correct risk category (13,14,17), but whether this is true also in patients who already show low levels of stem cells at baseline was unknown. Furthermore, the ability of stem cell levels to predict long-term cardiovascular outcomes term is poorly explored.

In a cohort of 187 patients with type 2 diabetes, we found that low $CD34^+$ and $CD34^+CD133^+$ cell levels significantly and independently predicted the development or worsening of microangiopathy (18). In this study, we have extended the follow-up of this cohort to examine the association between stem/progenitor cell levels and cardiovascular outcomes up to 12 years after the baseline examination.

RESEARCH DESIGN AND METHODS

The study was approved by local institutions and ethical committees and was conducted in accordance with the principles of the Declaration of Helsinki. All patients provided informed consent.

Study Design

As described previously (18), this was a pseudoprospective study. Baseline data were recorded at the time of stem/progenitor cell analysis, and follow-up data were collected retrospectively from August 2016 to baseline by accessing patients' electronic files. The routine follow-up of patients at 6-month intervals and standardization of electronic medical records allowed simulating a prospective design.

Study Patients

This study cohort has been described previously (18). Briefly, patients were selected from those regularly attending the Diabetes Outpatient Clinic of the University Hospital of Padova at 6-month intervals during a 10-year period (2004–2014). Inclusion criteria were type 2 diabetes, age 30–80, both sexes, $>$6 months' observation, and availability of a baseline determination of stem/progenitor cells. Exclusion criteria were acute disease or infection; recent ($\leq$3 months) surgery, trauma, or a cardiovascular event at baseline; immune disorders or organ transplantation; cancer; advanced liver (cirrhosis) or kidney (uremia) disease; pregnancy or lactation; and inability to provide informed consent. Detailed methods for characterization of this patients' cohort can be found elsewhere (18).The analysis of progenitor cells by flow cytometry has been described previously (18). The gating strategy and representative examples are illustrated in Supplementary Fig. 1. We considered both relative (cells/10^6 white blood cells) and absolute cell counts. Absolute levels were obtained by multiplying relative levels to white blood cells (/mL).

Study Outcomes

The primary outcome was time to a first cardiovascular event (all events). Secondary outcomes were 3-point and 4-point major adverse cardiovascular events (MACEs). The 3-point MACEs was a composite of death from cardiovascular causes, nonfatal myocardial infarction, or nonfatal stroke. The 4-point MACEs was a composite of the 3-point MACEs or hospitalization for heart failure or unstable angina. All events included the 3-point MACEs and hospitalization for any cardiovascular cause. The cause of death was determined by the principal condition and was considered to be cardiovascular in case of sudden death, death occurring up to 14 days after an acute myocardial infarction, death occurring in the context of clinically worsening symptoms and/or signs of heart failure, death occurring up to 30 days after a stroke, or death from another documented cardiovascular cause (e.g., dysrhythmia, pulmonary embolism, or intervention). Any deaths not attributed to a noncardiovascular cause were presumed to be cardiovascular.

Nonfatal myocardial infarction was defined in the presence of at least two of the following three criteria: cardiac biomarker elevation, electrocardiogram changes consistent with new ischemia, or imaging evidence of new nonviable myocardium or new wall motion abnormalities. Nonfatal stroke was defined as the rapid onset of a focal/global neurological deficit (change in level of consciousness, hemiplegia, hemiparesis, numbness or sensory loss affecting one side of the body; dysphasia/aphasia; hemianopia, other new neurological sign/symptom), with a duration of $\geq$24 h ($<$24 h if the event was associated with pharmacological treatment, or in the presence of available brain imaging showing new hemorrhage or infarct, or resulting in death [fatal stroke]), and confirmed by a neurology specialist or by brain imaging. Unstable angina was defined as resting, new-onset, or worsening angina in the absence of an elevation in

cardiac biomarkers and in the presence of new or worsening ST-T changes on electrocardiogram, or evidence of ischemia by cardiac imaging, or angiographic evidence of ≥70% stenosis in an epicardial coronary artery. Heart failure was defined in the presence of typical clinical manifestations or their worsening (dyspnea, orthopnea, paroxysmal nocturnal dyspnea, edema, pulmonary basilar crackles, jugular venous distension, third heart sound or gallop rhythm, radiological evidence of worsening heart failure), needing new therapy or uptitration of doses (diuretics, inotropes, vasodilators), eventually supported by changes in biomarkers (e.g., brain natriuretic peptides). Other cardiovascular events considered included unplanned coronary, peripheral, or carotid revascularization and arrhythmia requiring hospitalization.

Statistical Analysis

Data are expressed as mean ± SE or as percentage, where appropriate. Normality was checked with the Kolmogorov-Smirnov test. Nonnormal variables were log-transformed for statistical analysis. Comparisons between two groups were performed using the Student *t* test for continuous variables or χ^2 for binary variables. The Benjamini-Hochberg (BH) procedure was used to correct for multiple testing and inflation of the type I error. This method was used because it has greater power than the Bonferroni correction and provides a hierarchical scaling of significance testing, which well applies to hierarchically organized subtypes of stem/progenitor cells. Receiver operating characteristic curves were used to assess the ability of stem/progenitor cell levels to discriminate patients with adverse outcomes. The best cutoffs were chosen as those that optimized the product of sensitivity and specificity. With 187 patients, the probability was 80% that the study detected a difference at a two-sided 5% significance level, if the true hazard ratio was 1.60, based on the assumption that the accrual period was 10 years, the follow-up period was 6 years, and the median event-free survival time was 4 years. Stem/progenitor cell levels were dichotomized as below/above the median value to divide the patients into equal groups. The Cox proportional hazards regression model was used to evaluate the predictive capacity of a low (below median) versus a high (above median) stem/progenitor cell level, independently from confounders. Potential confounders were variables associated with the outcome in the univariate logistic analysis at $P < 0.10$. Discrimination improvement was assessed using C statistics applied to time-to-event data, the integrated discrimination index (IDI), and the continuous net reclassification improvement (NRI) (19). Statistical significance was accepted at $P < 0.05$. SPSS 22.0 software and Microsoft Excel 2003 software were used.

RESULTS

Study Patients and Outcomes

Baseline characteristics of the study population have been described previously (18) and can be found in Table 1. Patients had an average age of 63 years, with 10-year diabetes duration and HbA_{1c} of 7.9% (63 mmol/mol). Approximately 50% had microangiopathy and 59% had macroangiopathy, suggesting this was a population at high cardiovascular risk.

During a median follow-up period of 6.1 years (interquartile range 3.4–7.4 years), 48 cardiovascular events were registered, equal to an annual rate of 4.5%. The breakdown of all events was 3 cardiovascular deaths, 5 nonfatal strokes, 10 nonfatal acute myocardial infarctions, 16 hospitalizations for heart failure, 6 hospitalizations for unstable angina, and 8 hospitalizations for other cardiovascular causes. The rates of 3-point and 4-point MACEs are comparable to those reported in recent cardiovascular outcome trials in which similar populations of patients were enrolled (20).

Progenitor Cell Levels According to Cardiovascular Outcomes

We first divided patients into those with or without adverse cardiovascular outcomes at follow-up (Table 1). Patients who experienced a cardiovascular event (primary end point) during observation had significantly lower relative and absolute levels of $CD34^+$ cells, relative levels of $CD133^+$ cells, and relative and absolute levels of $CD34^+CD133^+$ cells than patients without an event at follow-up. After correction for multiple testing with the BH procedure, relative $CD34^+$ and $CD34^+CD133^+$ cell counts remained significantly lower in patients with events. Owing to the smaller number of events, trend associations were detected with the 3-point and 4-point MACEs, which were nonsignificant before or after BH correction (Fig. 1*A* and Supplementary Fig. 2*A*). No significant differences were noted for KDR-expressing phenotypes.

Rates of Cardiovascular Events According to Progenitor Cell Status

We then divided patients into equal groups based on the median value for each progenitor cell phenotype and calculated the annual rate of incident cardiovascular outcomes (Fig. 1*B*). The rate of all events (primary end point) was significantly higher in patients with low than in those with high relative levels of $CD34^+$ and $CD34^+CD133^+$ cells, even after BH correction. The associations between low relative levels of $CD34^+$ or $CD133^+$ cells and a higher rate of the 3-point or 4-point MACEs did not survive after the BH correction nor did the associations between absolute levels of $CD34^+$ cells and the rates of all events and 3-point MACEs (Supplementary Fig. 2*B*). No significant differences were noted for KDR-expressing phenotypes.

According to the area under curve from receiver operating characteristic curves, the discrimination capacity of $CD34^+$ cells against the primary outcome was higher than that of $CD34^+CD133^+$ cells (area under the curve 0.687 [95% CI 0.596–0.779] vs. 0.617 [0.529–0.704]). The optimal cutoff value for the $CD34^+$ cell count was 305 cells/10^6 (sensitivity 75.4%; specificity 58.2%) or 2,668 cells/mL (sensitivity 53.2%; specificity 72.9%).

Analysis of Event-Free Survival According to Progenitor Cell Status

We used the Cox proportional hazards model to evaluate whether low versus high progenitor cell levels predicted adverse cardiovascular outcomes independently of confounders. Variables associated with cardiovascular events at follow-up with $P < 0.10$ were BMI, HbA_{1c}, hypertension, albumin-to-creatinine ratio, estimated glomerular filtration rate (eGFR), macroangiopathy, and several therapies (Table 1). Although determinants of the 3-point and 4-point MACEs may be slightly different, these variables were chosen as covariates in the fully-adjusted model, because the definition of the primary outcome included those of the secondary outcomes.

Table 1—Baseline characteristics of patients in the entire cohort and divided into those with and without incident cardiovascular events

	All patients (N = 187)	Without events (n = 139)	With events (n = 48)	P value
Age, years	63.7 ± 0.7	63.3 ± 0.7	65.1 ± 1.5	0.222
Sex male, %	67	65	75	0.176
BMI, kg/m^2	29.5 ± 0.4	29.1 ± 0.4	30.7 ± 0.9	0.046
HbA_{1c}, %	7.9 ± 0.1	7.8 ± 0.1	8.3 ± 0.3	0.055
HbA_{1c}, mmol/mol	63 ± 1	62 ± 1	67 ± 2	
Duration, years	10.4 ± 0.6	10.4 ± 0.8	10.3 ± 1.1	0.925
Hypertension, %	84	80	98	0.003
Dyslipidemia, %	81	82	79	0.665
Smokers, %	13	13	15	0.776
Microangiopathy				
ACR, mg/g	76.3 ± 16.3	51.4 ± 14.9	148.0 ± 45.5	0.009
eGFR, mL/min/1.73 m^2	81.7 ± 1.6	83.9 ± 1.8	75.6 ± 3.4	0.003
Retinopathy, %	22	20	28	0.285
Neuropathy, %	13	13	15	0.789
Macroangiopathy	59	53	75	0.008
Coronary artery disease, %	15	10	29	0.001
Peripheral arterial disease, %	17	12	31	0.001
Subclinical atherosclerosis, %	48	45	58	0.102
UKPDS CHD risk, %	25.7 ± 1.1	24.2 ± 1.2	30.0 ± 2.2	0.015
Diabetes therapy				
Insulin, %	47	42	58	0.058
Secretagogues, %	42	40	48	0.314
Metformin, %	72	76	60	0.045
Thiazolidinediones, %	5	6	2	0.246
Incretins, %	9	10	4	0.059
Other medications				
Antiplatelets, %	49	45	63	0.033
Statins, %	72	71	75	0.554
ACE inhibitors, %	54	53	58	0.488
ARBs, %	24	21	33	0.082
β-Blockers, %	25	20	38	0.016
Calcium antagonists, %	27	20	48	<0.001
Stem/progenitor cells				
$CD34^+/10^6$	420 ± 16	452 ± 20	328 ± 21	<0.001
$CD34^+$/mL	3,076 ± 152	3,295 ± 189	2,443 ± 201	0.014
$CD133^+/10^6$	257 ± 12	273 ± 16	211 ± 16	0.029
$CD133^+$/mL	1,834 ± 95	1,933 ± 82	1,542 ± 142	0.072
$CD34^+CD133^+/10^6$	156 ± 9	169 ± 11	119 ± 10	0.014
$CD34^+CD133^+$/mL	1,118 ± 66	1,194 ± 82	893 ± 95	0.047
$CD34^+KDR^+/10^6$	45 ± 3	47 ± 3	41 ± 5	0.348
$CD34^+KDR^+$/mL	327 ± 22	339 ± 27	292 ± 36	0.360
$CD133^+KDR^+/10^6$	31 ± 2	32 ± 3	27 ± 3	0.248
$CD133^+KDR^+$/mL	221 ± 17	232 ± 22	188 ± 20	0.270
$CD34^+CD133^+KDR^+/10^6$	12 ± 1	14 ± 2	9 ± 2	0.141
$CD34^+CD133^+KDR^+$/mL	90 ± 10	98 ± 13	63 ± 12	0.143

Data are mean ± SE, unless otherwise stated. ACR, albumin-to-creatinine ratio; ARB, angiotensin receptor blocker.

Table 2 reports the hazard ratios (HR) with 95% CI for low versus high relative levels of progenitor cell phenotypes: low $CD34^+$ cells and $CD34^+CD133^+$ cells independently predicted the primary outcome, with quite similar HRs. Figure 2 shows fully adjusted Kaplan-Meier curves. The HR remained statistically significant for $CD34^+CD133^+$ cells after BH correction. The associations with the 3-point and 4-point MACEs were nonsignificant before and after BH correction. The associations of absolute $CD34^+$ or $CD34^+CD133^+$ cells with cardiovascular outcomes were quantitatively similar but statistically weaker (Table 2). KDR-expressing phenotypes were not predictive of adverse outcomes or sometimes showed a direct association with future cardiovascular events but did not survive correction for multiple testing.

Because clinical determinants of death may differ from those of cardiovascular events, we selected covariates with significance level of <0.10 in the comparison of patients who were alive and those who had died at follow-up: age, dyslipidemia, neuropathy, peripheral arterial disease, and therapy (secretagogues, β-blockers, calcium antagonists). No significant association was detected between progenitor cell levels and death from any cause (Table 2).

Discrimination Improvement by Addition of Progenitor Cell Levels

We finally compared the discrimination capacity of the model described in Table 2, with and without inclusion of relative $CD34^+$ cells, against the primary end point. C statistics improved from 0.758 to 0.799 ($P < 0.001$), the continuous NRI improved by 35% ($P = 0.038$), but IDI was not significantly improved (4.5%, $P = 0.059$). Discrimination capacity was not significantly improved by the addition of relative $CD34^+CD133^+$ cells (C statistics from 0.767 to 0.781, $P = 0.108$; NRI = 28.9%, $P = 0.069$; IDI = 4.2%, $P = 0.084$). The addition of the relative $CD34^+$ cell count to the coronary heart disease (CHD) risk provided by the UK Prospective Diabetes Study (UKPDS) risk engine significantly improved the C statistics from 0.616 to 0.704 ($P < 0.001$), continuous NDI by 46.8% ($P = 0.006$), and IDI by 7.2% ($P < 0.001$). The addition of the relative $CD34^+CD133^+$ cell count to the UKPDS risk also significantly improved the C statistics from 0.590 to 0.642 ($P < 0.001$), continuous NDI by 37.4% ($P = 0.019$), and IDI by 2.5% ($P = 0.022$).

CONCLUSIONS

Our results show that a reduced level of circulating stem cells predicts the occurrence of cardiovascular events in patients with type 2 diabetes over a period of 6 years. Addition of the stem cell measure improved risk stratification compared with reference models.

Previous studies have shown associations between circulating stem/progenitor cell levels and cardiovascular outcomes (13–15). This study validates the long-term clinical meaning of circulating stem cells defects in patients with complicated diabetes, which was described more than

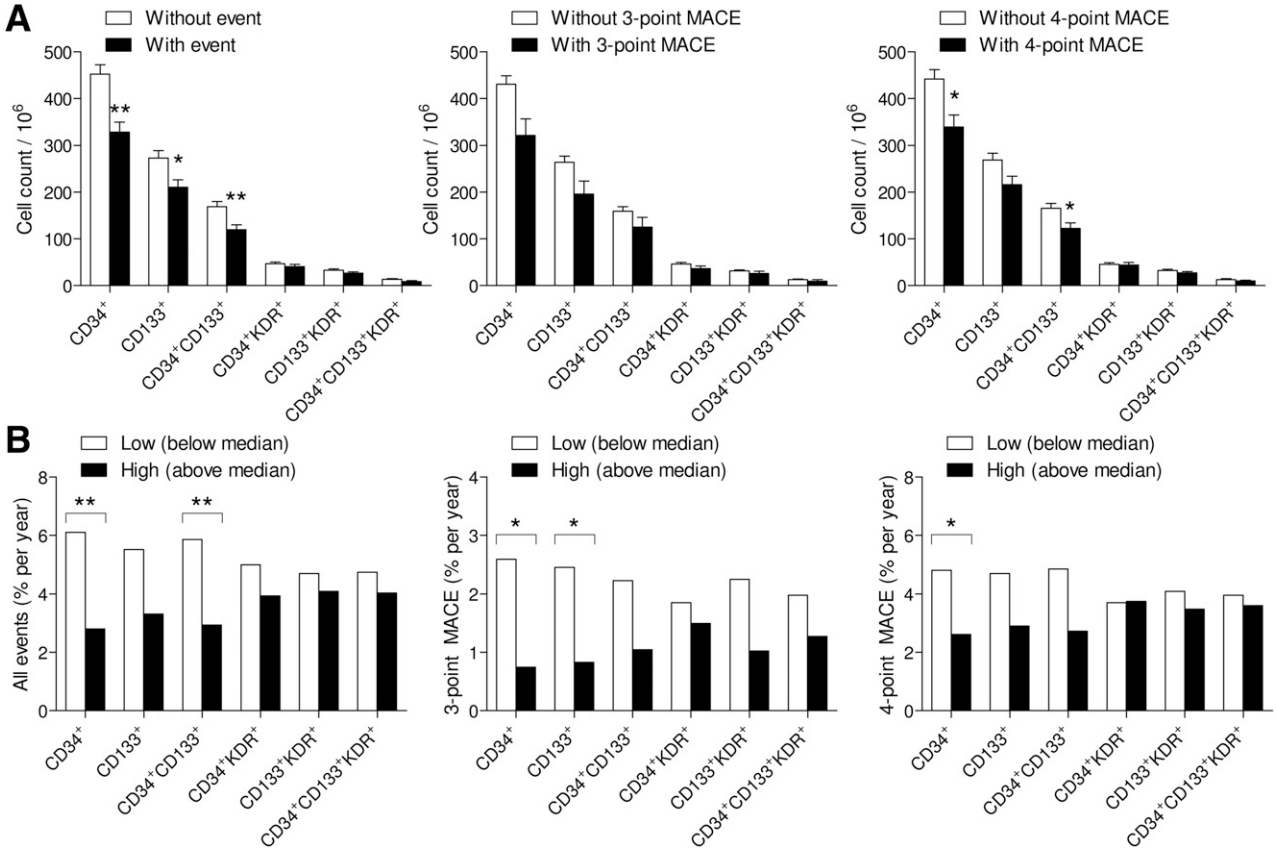

Figure 1—Relative progenitor cell levels and cardiovascular outcomes. *A*: Progenitor cell levels (mean ± SE) in patients divided into those who did or did not develop an event (all events, 3-point MACEs, 4-point MACEs) at follow-up. *B*: Annual incidence of all events, 3-point MACEs, and 4-point MACEs in patients divided according to high or low stem/progenitor cell levels based on the median value. *$P < 0.05$; **significant after BH correction.

10 years ago (3). In a meta-analysis of longitudinal studies, including 4,155 patients monitored for an average of 2 years, we found that a reduction in the levels of circulating $CD34^+$ and $CD34^+CD133^+$ cells was associated with an approximate twofold increased risk of future cardiovascular events and death (16). In meta-regression analyses, we detected no correlation between the prevalence of diabetes and HRs, suggesting that the prognostic effect of reduced stem cell levels was similar in patients with and without diabetes (16). Our study shows that the predictive capacity of low compared with high stem cell levels is preserved in patients with diabetes who already display stem cell defects compared with control subjects without diabetes (4) and extends long-term. By analyzing only $CD34^+$ cells, Makino et al. (21) reported data consistent to ours in Japanese patients with type 2 diabetes, showing an association with cardiovascular events during a 4.6-year follow-up. By analyzing multiple phenotypes, we confirm that the $CD34^+$ and $CD34^+CD133^+$ phenotypes are those provided with the strongest prognostic power, whereas KDR-expressing phenotypes, sometimes referred to as EPCs (22), did not predict cardiovascular outcomes, even though they are believed to be vasculoregenerative (9,23). There are technical reasons for this apparent paradox, because reliable KDR staining is challenging and updated flow cytometry protocols provide different results, showing that EPCs may be rarer than expected (10,24). Nonetheless, in a cohort of 1,497 patients with coronary artery disease, Hayek et al. (13) recently found that low $CD34^+KDR^+$, but not $CD34^+$, cells predicted the risk of mortality and PAD-related events. These data leave open the possibility that the most predictive phenotype is population- and/or disease-specific.

The primary outcome of this study was a composite of the traditional 3-point MACEs plus hospitalization for any cardiovascular cause. We detected weaker associations between low stem cell levels and the 3-point or 4-point MACEs and nonsignificant trend associations with death, because of the small number of events. It is also important to note that, owing to the large number of cell phenotypes tested, we had to correct for the false discovery rate. Focusing on associations that survived after BH correction allows for more robust conclusions from a statistical perspective.

The sample size in our study was smaller than in recent works from the Emory Cardiovascular Biobank (13,14), but we report data on long-term follow-up of up to 12 years. This is important, because most events occurred after 5 years of observation, and we show that stem cell performance as biomarkers was not diluted over time. Reporting data on specific populations and on long-term prediction is required for blood stem cell levels to be leveraged to a clinical-grade cardiovascular risk biomarker. Although several technical issues need to be fixed before a widespread diffusion takes place, this study, along with the study by Makino et al. (21), supports the effect of stem cell levels beyond

Table 2—Cardiovascular events in patients with low versus high relative or absolute levels of the six stem/progenitor cell phenotypes

Cell type (low vs. high level)	All events	3-point MACEs	4-point MACEs	Death
Relative $CD34^+$	2.21 (1.14–4.29)	2.70 (0.75–9.67)	1.78 (0.88–3.56)	1.41 (0.48–4.09)
P	0.018	0.127	0.108	0.532
Absolute $CD34^+$	1.89 (1.00–3.55)	2.14 (0.65–7.05)	1.65 (0.84–3.24)	1.53 (0.49–4.75)
P	0.049	0.210	0.148	0.466
Relative $CD133^+$	1.74 (0.90–3.36)	1.88 (0.60–5.89)	1.07 (0.99–1.15)	1.83 (0.61–5.51)
P	0.100	0.277	0.113	0.283
Absolute $CD133^+$	1.97 (0.99–3.93)	1.27 (0.44–3.65)	1.64 (0.79–3.39)	0.65 (0.22–1.92)
P	0.054	0.660	0.186	0.435
Relative $CD34^+CD133^+$	2.98 (1.46–6.08)	2.35 (0.76–7.32)	2.37 (1.13–4.98)	3.44 (0.96–12.38)
P	0.003*	0.140	0.023	0.058
Absolute $CD34^+CD133^+$	1.99 (1.01–3.93)	1.21 (0.42–3.53)	1.58 (0.78–3.20)	0.79 (0.25–2.55)
P	0.048	0.722	0.209	0.696
Relative $CD34^+KDR^+$	1.06 (0.56–2.00)	1.31 (0.45–3.79)	0.78 (0.40–1.55)	1.12 (0.40–3.12)
P	0.855	0.620	0.479	0.829
Absolute $CD34^+KDR^+$	1.03 (0.53–2.00)	0.73 (0.24–2.23)	0.73 (0.36–1.50)	0.93 (0.33–2.60)
P	0.924	0.582	0.388	0.882
Relative $CD133^+KDR^+$	0.82 (0.40–1.65)	2.54 (0.70–9.18)	0.70 (0.33–1.48)	1.53 (0.47–5.18)
P	0.570	0.155	0.356	0.474
Absolute $CD133^+KDR^+$	0.50 (0.25–1.03)	0.62 (0.18–2.10)	0.34 (0.16–0.73)	1.80 (0.59–5.49)
P	0.059	0.442	0.009	0.300
Relative $CD34^+CD133^+KDR^+$	0.62 (0.31–1.23)	0.99 (0.31–3.15)	0.44 (0.21–0.93)	0.53 (0.17–1.67)
P	0.172	0.985	0.031	0.281
Absolute $CD34^+CD133^+KDR^+$	0.56 (0.28–1.13)	0.60 (0.18–1.99)	0.33 (0.15–0.72)	0.33 (0.10–1.07)
P	0.105	0.401	0.008	0.065

Data are presented as HRs (95% CI). All analyses of time to event were adjusted for BMI, HbA_{1c}, hypertension, albumin-to-creatinine ratio, eGFR, macroangiopathy, and therapy (insulin, metformin, incretins, antiplatelet agents, angiotensin receptor blockers, β-blockers, calcium antagonists). The analyses of time to death were adjusted for age, dyslipidemia, neuropathy, peripheral arterial disease, and therapy (secretagogues, β-blockers, calcium antagonists). *Significant after BH correction.

traditional clinical assessment in diabetes. To demonstrate an improvement in risk stratification, we used C statistics, NRI, and IDI, metrics specifically designed to address this issue (19). The reference model was initially built on clinical variables that were associated with the outcome at a <10% type I error. Because this model may be overfitted to the population under investigation and therefore not generalizable, we then used the UKPDS risk engine, which was specifically developed to test CHD risk in patients with diabetes (25). In both cases, addition of the $CD34^+$ cell level was able to improve event prediction and is therefore expected to perform well as a clinical biomarker.

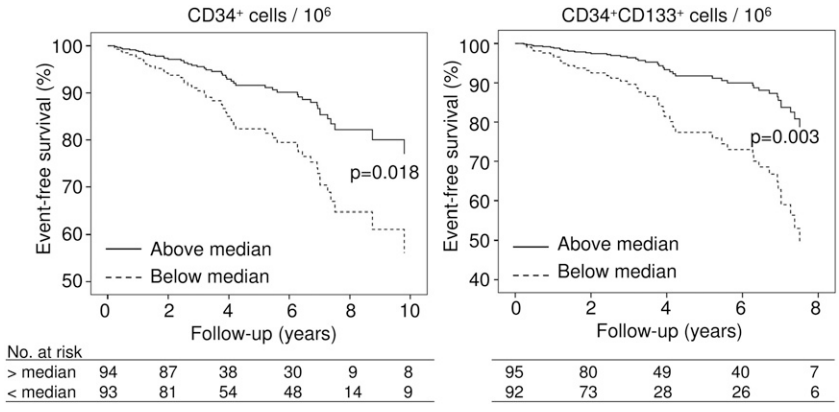

Figure 2—Fully adjusted Kaplan-Meier event-free survival curves are shown for the primary outcome (all events) from Cox proportional hazards regression analyses in patients with low vs. high levels of $CD34^+$ cells (left) or $CD34^+CD133^+$ cells (right).

Stem cell levels fluctuate on a circadian basis (26) and change in relation to disease states and therapies (27). Even if we did not detect any significant association with glucose-lowering medications, several confounders can affect baseline cell levels. Importantly, circulating stem cells are amenable to pharmacological modulation, for instance via CXCR4 antagonism (24,28), but whether these changes reset cardiovascular risk to the new stem cell level achieved remains to be elucidated. It is also intriguing that bone marrow cells, in addition to mirroring the overall risk of complications, can actively be used for the treatment of type 1 and type 2 diabetes (29,30).

Mechanistically, we still do not know whether a reduction of blood stem cells causes cardiovascular events per se or whether it represents a bystander of inflammation, hematopoietic expansion,

and bone marrow abnormalities, which in turn promote atherosclerosis (31). Mounting evidence suggests that circulating stem cells reflect the endogenous regenerative capacity, which is affected by biological aging (32). In this regards, it is interesting to note that diabetes shortens life expectancy and is considered a condition of accelerated aging (33). The availability of a clinical-grade biomarker reflecting healthy versus unhealthy aging may aid an improved tailoring of cardiovascular prevention.

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