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CIRCULATING PROGENITOR CELLS: A NOVEL BIOMARKER OF MICROVASCULAR AND MACROVASCULAR DISEASE IN TYPE 2 DIABETIC PATIENTS

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ABSTRACT

Objective. We evaluated the ability of circulating stem cell levels to predict future micro and macrovascular complications in patients with type 2 diabetes. We further investigate the prognostic value of stem cells in a wide and heterogeneous cohort of patients, using a meta-analytic approach.

Research design and methods. A cohort of 187 patients with type 2 diabetes was followed-up for a median of 3.3 years and 6.1 years for the evaluation of microvascular and macrovascular outcomes, respectively. The primary outcomes were onset or progression of any microangiopathy, and time to a first cardiovascular event. In addition, we meta-analysed all studies reporting the prognostic role of the CPC/EPC measure on cardiovascular outcomes and death in a heterogeneous population of 4451 patients at high cardiovascular risk.

Results. New onset or progression of microangiopathy occurred in 70 patients (9.5% per year). After controlling the false discovery rate (FDR), baseline CD34$^+$ CPCs and EPCs were significantly lower in patients with onset/progression of microalbuminuria and any microangiopathy. Patients with baseline CD34$^+$ CPC or CD133$^+$KDR$^+$ EPC levels below median were more likely to experience worsening microangiopathy than those with high cell levels. In FDR-fully-adjusted analysis, CD34$^+$ cells predicted onset/progression of microalbuminuria, retinopathy, and any microangiopathy. A first cardiovascular event occurred in 48 patients (4.5% per year). Patients with incident cardiovascular events had significantly lower CD34$^+$ and CD34$^+$CD133$^+$ cells than those without. Patients with below median levels of CD34$^+$ and CD34$^+$CD133$^+$ cells experienced higher rates of cardiovascular events. In Cox proportional hazard regression analyses, a reduced CD34$^+$ and CD34$^+$CD133$^+$ cell count independently predicted future events. Addition of the CD34$^+$ cell count to the UKPDS risk engine model improved C-statistics, continuous NRI and/or IDI. In the meta-analysis, reduced CPC/EPC levels were associated with a ~2 fold increased risk of future cardiovascular events and cardiovascular death and the most predictive phenotypes were CD34$^+$ and CD34$^+$CD133$^+$.

Conclusions. In patients with type 2 diabetes, a reduced baseline level of circulating CD34$^+$ stem cells predicts worsening of microangiopathy and cardiovascular events up to 6 years later, and improves risk stratification. The meta-analysis suggests that prognostic impact of reduced stem cell levels was similar in diabetic and non-diabetic patients.

INTRODUCTION

Diabetes is a major risk factor for the development of both cardiovascular and microvascular disease and confers a two to fourfold increase in the risk of coronary heart disease, stroke and peripheral artery disease. Even if cardiovascular events remain the leading cause of mortality, microvascular disease represents an independent predictor of vascular damage and contributes significantly to reduce the life expectation of diabetic patients. However, the risk of both macro and microvascular events varies considerable even within the diabetic population, providing a rationale for improving individual risk prediction using biomarkers.[1,2]

The pathogenesis of vascular abnormalities in diabetic complications is generally focused on Brownlee's unifying hypothesis, whereby intracellular damage pathways are triggered by overproduction of oxygen reactive species from mitochondria. In contrast, vascular repair processes have been long neglected. However, vascular cell turnover plays a crucial role in maintaining the structural and functional integrity of endothelium and defects in such mechanism can be accelerated in patients with vascular noxae. Twenty years ago, Asahara et al. has isolated endothelial progenitor cells (EPCs) in peripheral blood for the first time.[3] Since then, mounting studies have shown that circulating progenitor cells (CPCs) and endothelial progenitor cells (EPCs) are immature cells derived from bone marrow (BM), which have been involved both in angiogenesis and vascular repair processes.[4] These cells are mobilized into the peripheral circulation in response to tissue damage and ischaemia.[5] Once in the bloodstream, CPCs can differentiate into different phenotypes including endothelium, smooth muscle and cardiomyocytes, according to the additional antigenic phenotype they acquire (e.g. CD34+KDR+ for endothelial, CD34+ a-actin+ for smooth muscle and CD34+ c-met+ for potential cardiomyocyte progenitors).[6-8] Yeh et al. demonstrated that human CD34+ injected into immunodeficient mice were able to differentiate into endothelium, smooth muscle and cardiomyocytes.[9] In another study, human CD34+ were shown to contribute to neovascularization and were potent regulator of the host angiogenic and pro-inflammatory response

in a nude mouse model.[10] Therefore, these evidences confirm that bone marrow CD34+ cells migrate into the circulation, undergo multi-lineage differentiation and are involved in vascular and tissue repair.

In clinical studies, flow cytometry allows the identification and characterization of CPCs and EPCs through the analysis of differential expression of specific surface markers.[7] Circulating progenitor cells (CPCs) are typically defined by the surface expression of the hematopoietic stem cell markers CD34 and/or CD133. Endothelial progenitor cells (EPCs) should be considered as a specific phenotype of CPCs with vascular endothelial commitment and are characterized by the co-expression of CD34/CD133 and endothelial markers (most frequently the type 2 VEGF receptor KDR).

In humans, the levels of CPCs and EPCs are reduced in patients with traditional cardiovascular risk factors,[11-17] established cardiovascular disease[18] or microangiopathy.[19-22] Several studies have reported that CPCs and EPCs are reduced in number and are dysfunctional in patients with type 1 and 2 diabetes. The reduction of $CD34^+$ occurs early in the natural history of type 2 diabetes and can already be observed in subjects with impaired glucose tolerance.[23] The depletion of progenitor cells persists over time and is significantly worse in patients with established vascular complications.[18,24] Indeed, previous evidences by Fadini et al. showed that the levels of $CD34^+KDR^+$ negatively correlated to the severity of peripheral vascular damage in type 2 diabetic patients.[25] Furthermore, the link between hyperglycaemia and EPCs dysfunction is supported by several studies on type 1 diabetes. The number of circulating CD34+KDR+ EPCs is reduced in young type 1 diabetic patients compared with controls, particularly in those with suboptimal glucose control,[26] a longer diabetes duration, microangiopathy[19,20] and initial surrogates signs of macroangiopathy.[27,28] These studies suggest that levels of progenitor cells follow the natural history of atherosclerosis, from its subclinical stage to later complications of the plaque.[29] However, if the relation between reduction of CPCs/EPCs and cardiovascular disease is well established, only few

studies investigated the effect on microangiopathy, suggesting conflicting results. In patients with chronic renal failure, Choi et al. demonstrated a significant impairment of EPCs, both in culture and circulation.[30] Reinhard et al. found no alteration in early EPC cultures obtained from type 1 diabetic patients with nephropathy compared to those without.[31] Dessapt et al. found lower levels of CD34^{+} and CD34^{+}CD133+ in type 1 diabetic patients with microalbuminuria compared to those without,[19] and Makino et al. reported that CD34^{+} was an independent predictor of albuminuria progression in type 2 diabetic patients. As EPCs are characterized by pro-angiogenic activity, they may have opposite effects in the different stages of retinopathy. Several reports have shown increased levels of circulating (CD34^{+}KDR^{+}) and cultured EPCs in patients with proliferative retinopathy, suggesting a potential excess of EPCs may contribute to aberrant retinal angiogenesis, as demonstrated in animal models.[32,33] However, studies are needed to support this hypothesis in humans. It must also be noted that no clinical study has so far evaluated the relationship between progenitor cells and diabetic neuropathy. This should be an area of great interest, in view of experimental studies showing that EPC-based approach may be a promising therapy for diabetic neuropathy.[34,35]

The putative mechanisms for progenitor cell reduction include deranged differentiation, decreased survival, increased homing and impaired mobilization. An aberrant differentiation of blood-derived progenitor cells into endothelial cells has been observed in vitro models after exposure to hyperglycemia, and has been attributed to overactivity of the p38 MAP kinase.[36,37] However, there is no evidence that this occurs in vivo. In vitro studies showed that blood-derived progenitor cells undergo apoptosis when exposed to high glucose, and this is generally attributed to an increased oxidative stress, or downregulation of the PI3-K /Akt signaling.[38] However, this is true in vitro but no study has confirmed a reduced survival of diabetic progenitor cells in vivo, and a study by Fadini et al. showed that CD34+ cells from type 2 diabetic patients did not exhibit an increased rate of early apoptosis, evaluated by Annexin-V staining.[24] The reduction of circulating progenitor cells

may also be the consequence of increased homing at the site of vascular injury. However, this mechanism cannot account for low progenitor cells in diabetes because it has been demonstrated that experimental type 1 and type 2 diabetes impaired homing and migration of EPCs to damaged tissue.[39,40] Several studies have shown a defective mobilization of progenitor cells, suggesting that diabetes strongly affect bone marrow structure and function. Fadini et al. demonstrated that mobilization of EPCs after inducing ischemia was completely impaired in streptozotocin (type-1) diabetic rats when compared to healthy controls, and this defect was partially restored by insulin treatment. In the same study, the mobilization of EPCs was significantly blunted in diabetic rats after administration of granulocyte colony-stimulating factor (G-CSF).[41] Diabetic patients are also unresponsive to the effects of G-CSF, which fails to mobilize CD34+ cells and EPCs.[42,43] The impairment in progenitor cell mobilization may be a consequence of the deep remodeling induced by diabetes in bone marrow environment. In fact, both in mice[44] and in humans,[45] diabetic bone marrow is characterized by microangiopathy and alterations of the stem cell niche, that are similar to those observed in diabetic retinopathy.[46] These evidences not only provide a mechanistic explanation for the impaired vascular repair by bone marrow-derived cells, but also identify bone marrow as a new potential site of diabetic microangiopathy. In this perspective, bone marrow dysfunction may represent the common soil for development of distant end-organ diabetic complications that together contribute to reduce the life expectancy of such patients.

We herein evaluated the ability of CPCs/EPCs levels to predict future micro and macrovascular events in a cohort of 187 type 2 diabetic patients. Furthermore, we performed a meta-analysis of studies reporting the association between baseline progenitor cell levels and CVE or death in a heterogeneous population of 4551 patients.

MATERIALS AND METHODS

Patient selection

This pseudo-prospective study was approved by local institutions and ethical committee, and conducted in accordance to the principles of the Declaration of Helsinki. All patients provided informed consent. Patients included in the analysis were retrospectively selected from those regularly attending the Diabetes Outpatient Clinic of the University Hospital of Padova at 6-months intervals, over a period of ten years (2004-2014). Inclusion criteria were: T2D, age 30-80, both genders, at least 6 months observation, live status at follow-up, and availability of a baseline CPC / EPC determination. Exclusion criteria were: acute disease or infection at baseline; surgery, trauma or cardiovascular event in the 3 months prior to CPC/EPC determination; immune disorders or organ transplantation; cancer; baseline advanced liver (cirrhosis) or kidney (uremia) disease; pregnancy or lactation; inability to provide informed consent. We collected the following baseline data: age, sex, BMI, diabetes duration, HbA1c, urinary albumin/creatinine ratio (UACR), serum creatinine, concomitant risk factors, complications, and medications. The estimated glomerular filtration rate was calculated according to the CKD-EPI formula [47]. Hypertension was defined as a systolic blood pressure ≥140 mm Hg or a diastolic blood pressure ≥90 mm Hg, or the use of anti-hypertensive medications. Dyslipidemia was defined in the presence of a total cholesterol ≥200 mg/dl, or LDL cholesterol ≥130 mg/dl, or triglycerides ≥150 mg/dl, or the use of statins/fibrates. Smoking was defined as habitual active smoking of 1 or more cigarettes per day. Retinopathy was defined on the basis of standardized digital retinal fundus images, examined and scored remotely by an experienced ophthalmologist, according to the ETDR classification [48]. Somatic peripheral neuropathy was diagnosed, after exclusion of non-diabetic causes, in the presence of typical sensory or motor symptoms (numbness, tingling, or pain in the toes, feet, legs, hands, arms, and fingers, or wasting of the muscles of the feet or hands), confirmed by clinical examination (ankle reflexes, vibratory perception threshold, pinprick, and 10-g monofilament sensitivity) and eventual

determination of neural conduction velocity, in a minority of unclear cases [49]. Autonomic neuropathy was screened annually using 4 routine cardiovascular autonomic function tests: deep breathing, lying-to-standing, Valsalva manoeuvre and orthostatic hypotension. Coronary artery disease (CAD) was defined as a history of myocardial infarction or angina, or evidence of significant coronary artery disease at coronary angiography. Peripheral arterial disease (PAD) was defined as a history of claudication or rest pain, or significant stenosis in leg arteries. Asymptomatic atherosclerosis was defined as the presence of carotid artery plaques (stenosis >15%) at routine ultrasound examination. Macroangiopathy was defined as the presence of CAD, PAD or asymptomatic atherosclerosis.

Progenitor cell analysis

Circulating CPCs and EPCs were quantified by flow cytometry on whole blood samples.[50] Briefly, after red blood cell lysis, cells were stained with anti-CD34 (Becton Dickinson), CD133 (Miltenyi Biotec), and KDR (R&D System) monoclonal antibodies. After gating $CD34^+$ or $CD133^+$ cells in the mononuclear cell population, cells were scored for dual or triple expression of KDR. For technical reasons, CD133 staining was not available in 18 patients. CPC were defined as $CD34^+$, $CD133^+$ and $CD34^+CD133^+$ cells, whereas EPC were defined as $CD34^+KDR^+$, $CD133^+KDR^+$ and $CD34^+CD133^+KDR^+$ cells. Baseline progenitor cell levels were quantified by the same two trained operators using the same method and materials throughout the study, though lots of antibodies for KDR have changed. Reproducibility of this method has been reported previously, with CV ranging from 6.3% for $CD34^+$ CPCs to 15-16% for EPCs [50]. A representative example of the gating strategy is illustrated in Figure 1. We considered both relative (cells / 10^6 white blood cells, WBC), and absolute cell counts. Absolute levels were obtained by multiplying relative levels to WBC (/mL).

Definition of microvascular outcomes

New onset or progression of microangiopathy was assessed at last available visit as compared to baseline, by retrieving electronic chart data reporting the results of routine screening performed every 6 months. Onset of nephropathy was defined as development of a pathologic UAER (UACR≥30 mg/g) in patients with a baseline UACR<30 mg/g. Progression of UAER was defined as worsening from micro- to macroalbuminuria. We also collected data on serum creatinine levels at last visit and determined the change in CKD-EPI eGFR from baseline. Onset/progression of CKD was defined as worsening category of eGFR from ≥60 to <60 ml/min/1.73 m^2, or from 30-60 to <30 ml/min/1.73 m^2). Onset of retinopathy was defined as detection of retinopathy (any grade) in patients who were free from retinopathy at baseline. Progression of retinopathy was defined as any worsening in the ETDRS grade, or the need of photocoagulation therapy or intravitreal therapy, or onset of diabetic macular edema. Onset of neuropathy was defined as a new diagnosis of somatic or autonomic neuropathy in patients who were free from neuropathy at baseline. Progression of neuropathy was defined as the onset of autonomic neuropathy in patients with baseline somatic neuropathy, and vice-versa. Onset of microangiopathy was defined as a new diagnosis of nephropathy, retinopathy or neuropathy in patients free from all these complications at baseline, whereas progression of microangiopathy was defined as increasing number or severity of microangiopathic complications. To increase power in statistical analysis, onset and progression were always pooled together, under the term "progression".

Definition of macrovascular outcomes

The primary outcome was time to a first cardiovascular event (all events). Secondary outcomes were the 3-point and 4-point MACE. The 3-point MACE (major cardiovascular events) was a composite of death from cardiovascular causes, nonfatal myocardial infarction, or nonfatal stroke. The 4-point MACE was a composite of the 3-point MACE or hospitalization for heart failure or unstable angina. All events included the 3-point MACE 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; death due to another documented cardiovascular cause (e.g. dysrythmia, pulmonary embolism, or intervention). Any death not attributed to a non-cardiovascular cause were presumed to be cardiovascular. Nonfatal myocardial infarction was defined in the presence of at least 2 of the following 3 criteria: cardiac biomarker elevation; ECG changes consistent with new ischemia; imaging evidence of new non-viable 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 hours (<24 hours if the event was associated with pharmacologic 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 elevation in cardiac biomarkers, and in the presence of new or worsening ST-T changes on ECG, or evidence of ischemia by cardiac imaging, or angiographic evidence of $\geq$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, radiologic 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 ± standard error, or as percentage where appropriate. Normality was checked with the Kolmogorov-Smirnov test. Non-normal variables were log transformed for statistical analysis. Comparisons between 2 groups were performed using the Student's t test for continuous variables or chi-square for binary variables. As 6 progenitor cell phenotypes were always tested for each outcome, adjustment for type I error inflation due to multiple testing and false discovery rate (FDR) control were performed using the Benjamini-Hochberg (BH) procedure. ROC curves were used to assess the ability of stem/progenitor cell levels to discriminate patients with adverse outcomes. The best cut-offs were chosen as those that optimized the product of sensitivity and specificity. With n=187 patients, the probability was 80% that the study detected a difference at a 2 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 6 years and the median event-free survival time 4 years. Stem/progenitor cell levels were dichotomized as below/above the median value in order to divide the patients into equal groups. The Cox proportional hazard 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) [51]. Statistical significance was accepted at p<0.05. SPSS version 22.0 and Microsoft Excel 2003 were used.

Meta-analysis

We screened the literature for prospective observational studies reporting occurrence of cardiovascular events among patients whose levels of CPCs/EPCs were determined at baseline. Studies using enumeration of cultured endothelial colony-forming cells (CFU), often referred to as

"early EPCs", "circulating angiogenic cells" or "pro-angiogenic cells", were also considered. Eligible studies had to be reported in the English literature from 1997 to end of February 2016 and could include either patients undergoing coronary angiography for suspected coronary artery disease or acute coronary syndrome, or patients admitted for stroke, or patients without acute events but with cardiovascular risk factors. Eligible outcomes were: 1) CVE (defined as myocardial infarction, percutaneous or surgical coronary revascularisation, any acute coronary syndrome, heart failure, stroke, uncontrolled arrhythmia, cardiovascular death), 2) cardiovascular death, 3) death from any cause, 4) restenosis (defined as intra-stent luminal loss or stenosis progression accessed by angiography), 5) revascularization. We did not exclude a priori any study on the basis of methodological standards, sample size, duration of follow-up. We searched the MEDLINE database via PubMed up to 29 February 2016 using the following search terms: ("progenitor cells" or "CD34+ cells" or "stem cells") and ("cardiovascular events" or "myocardial infarction" or "stroke" or "angina" or "tia" or "failure" or "hospitalization" or "restenosis" or "death" or "mortality") and ("follow-up" or "followed-up" or "incident" or "incidence"). This strategy was complemented by hand searching in the reference lists of retrieved articles and contact with authors. As 2 of the authors (AA and GPF) of the present article are also authors of potentially eligible studies, eligibility and risk of bias for such studies were assessed independently by an author with no secondary interest (MR), as recommended by the Cochrane Collaboration guidelines.[52] We followed the MOOSE guidelines for performing and reporting results of observational studies.[53] Two reviewers (MR and GPF) independently extracted data from eligible articles (n=28) using a predefined coding protocol. Individual item disagreement between the two reviewers was resolved by consensus or consultation with a third author (AA). We extracted information on year of publication, number of patients at baseline, country, their mean age and percentage of males, the baseline prevalence of diabetes, hypertension, dyslipidemia, chronic kidney disease, smoke habit, coronary artery disease (proportion with previous coronary events), and the use of cardiovascular medications such as statins or blockers of the RAAS. We collected data on the phenotypic

characteristics and baseline levels of CPCs/EPCs, defined as low or high according to ROC curve cut-off, median value, or in relation to their subdivision into tertiles or quintiles. We extracted the reported relative risk, odds ratio or hazard ratio, and 95% confidence intervals (C.I.) from each study. For articles reporting multiple risk measures, we extracted risk measures for the pre-specified outcomes with the largest number of adjustment variables. When available, we checked the MACE breakdown to detect any significant imbalance in its composition. Studies wherein MACE contained a small percentage of hard events (myocardial infarction and stroke), were considered only for more specific outcomes (e.g. revascularization). We evaluated the quality of individual study reports according to REMARK guidelines for prognostic biomarker studies.[54] We extracted details of 16 items related to the purpose of studies, population description, biomarker measurement, confounders, outcomes and analytic choice. We compared the effect of low versus high level of CPCs / EPCs on all pre-specified outcomes. The reported comparisons included risk estimates onto a standard scale (i.e. per 1 SD, per tertile, or per quintile), according to ROC curve cut-off, or per unit of change in cell count. To allow the comparison on a same scale, we standardized the risk expressed per unit of change using the attributable risk approach. The relative risk estimates of each study and their corresponding standard error (SE) were transformed to their natural logarithms to normalize distributions. Due to heterogeneity among studies, we used the random effect meta-analysis using the inverse variance method. In this approach, the weight given to each study is the inverse of the variance of the effect estimate. In general, the larger studies (smaller SE) are given more weight than smaller studies (larger SE), leading to a reduction of the imprecision of pooled effect estimate. However, for comparison, we also report summary statistics obtained using the fixed effect model, as suggested by Sterne et al.[55] For each outcome, we performed an overall meta-analysis and up to five subgroups analysis: 1) considering only studies wherein patients without acute cardiovascular events were enrolled; 2) considering only studies reporting adjusted risk estimates onto a standard scale of effect; 3) considering only studies wherein patients with acute cardiovascular events were enrolled; 4) considering only higher-quality studies,

defined as having a REMARK score above the median value; 5) considering only studies wherein the MACE outcome was composed by >50% hard endpoints (myocardial infarction or stroke). All meta-analyses were performed using the software Revman version 5.3. Meta-regression analyses was performed under the random effect model to assess the relationship between the prognostic cardiovascular impact of CPCs / EPCs and covariates of interest. Outliers were screened and defined as previously described.[56]

RESULTS

Baseline characteristics of enrolled patients

Out of 257 total patients having a baseline progenitor cell determination initially retrieved, 187 met inclusion/exclusion criteria and had full data available. Reasons for exclusion were: age<30 (n=3) or >80 (n=15); T1D (n=12); dead status (n=18); follow-up duration <6 months (n=13) and missing data at follow-up (n=9). Baseline clinical characteristics of the study cohort are reported in Table 1. Mean age was 63 years and 67% of patients were males. The average diabetes duration was 10 years and HbA1c indicated an overall fair glycemic control. At baseline, 58.8% of patients had macroangiopathy and 46.5% had at least one microangiopathic complication.

Microvascular outcomes

During a median follow-up of 3.3 years (interquartile range 1.8-5.6 years), a total of 70 patients (37.4%) experienced any progression of microangiopathy, equal to an annual rate of 9.5%. The annual rates of UAER, CKD, retinopathy and neuropathy progression were 2.9%, 3.0%, 3.8% and 2.5%, respectively. Table 1 also shows clinical characteristics in patients divided according to progression or non-progression of microvascular complications. At baseline, progenitor cell phenotypes were generally not significantly associated with the prevalence of microangiopathy, with the exception of $CD34^+CD133^+$ CPCs, which were lower in patients with retinopathy or neuropathy than in those without. $CD34^+$ CPCs were lower in patients with asymptomatic atherosclerosis than in those without (Figure 2).

Progenitor cell levels according to microvascular outcomes. We first compared baseline progenitor cell counts in patients experiencing progression of each microangiopathy and in those without. Baseline $CD34^+$ CPC levels were significantly lower in patients with progression of UAER and any microangiopathy, whereas FDR-unadjusted significant differences in relation to CKD, retinopathy, neuropathy disappeared after applying the BH procedure. EPC phenotypes were significantly lower

in patients with progression of UAER, any microangiopathy, whereas the association with neuropathy disappeared after FDR control (Figure 3).

Rates of microvascular outcomes according to progenitor cell status. We then divided patients into equal groups based on median values of each CPC and EPC phenotype (baseline data in Table 2). In this univariate analysis, patients with low CD34$^+$ CPCs had a higher chance of UAER, CKD, and any microangiopathy progression, than those with high CD34$^+$ CPCs, whereas associations of low CD34$^+$ CPCs with retinopathy and neuropathy progression rate disappeared after FDR control. Among EPC phenotypes, low CD133$^+$KDR$^+$ cells were associated with a significantly higher rate of UAER, CKD, and any microangiopathy progression, whereas a low CD34$^+$CD133$^+$KDR$^+$ cell count remained only associated with progression of CKD after FDR control.

In a logistic multivariable analysis, adjusted for age, sex, BMI, HbA1c, diabetes duration, prevalence of hypertension, dyslipidemia, smoking habit, baseline macroangiopathy, and follow-up duration, the CD34$^+$ CPC count remained significantly associated with progression of UAER, retinopathy, neuropathy, and any microangiopathy. CD133$^+$KDR$^+$ EPCs levels remained significantly associated with progression of UAER, neuropathy, and any microangiopathy, whereas CD34$^+$CD133$^+$KDR$^+$ cells remained significantly associated with progression of UAER, and any microangiopathy (Table 3).

In patients with high CD34$^+$ CPCs, the use of ACE inhibitors or ARB since baseline was associated with a significantly lower rate of microalbuminuria progression, whereas this protection was lost in patients with low CD34$^+$ CPCs (Figure 4). The interaction between CD34$^+$ cell count and ACEi/ARB therapy remained significant in the multivariable analysis (not shown).

Macrovascular outcomes

During a median follow-up period of 6.1 years (interquartile range 3.4-7.4 years), a total of 48 cardiovascular events were registered, equal to an annual rate of 4.5%. The breakdown of all events was: 3 cardiovascular death, 5 non-fatal stroke, 10 non-fatal AMI, 16 hospitalization for hear

failure, 6 hospitalization of unstable angina, and 8 hospitalizations for other cardiovascular causes. The rate of 3-point and 4-point MACE are comparable to those reported in recent cardiovascular outcome trials wherein similar populations of patients were enrolled.[57]

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 endpoint) 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 did 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 MACE, which were non-significant before or after BH correction (Figure 5A and 6A). 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 (Figure 5B and 6B). The rate of all events (primary endpoint) 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 MACE did not survive after BH correction (Figure 5B), nor did the associations between absolute levels of $CD34^+$ cells and the rates of all events and 3-point MACE (Figure 6B). No significant differences were noted for KDR-expressing phenotypes.

According to the area under curve (AUC) from ROC curves, the discrimination capacity of $CD34^+$ cells against the primary outcome was higher than that of $CD34^+CD133^+$ cells (AUC [95% C.I.] 0.687 [0.596-0.779] versus 0.617 [0.529-0.704]). The optimal cut-off value for $CD34^+$ cell count

was 305 cells / 10^6 (sensitivity 75.4%; specificity 58.2%), or 2668 cells / ml (sensitivity 53.2%; specificity 72.9%).

<u>Analysis of event-free survival according to progenitor cell status</u>. To evaluate whether low versus high progenitor cell levels predicted adverse cardiovascular outcomes independently of confounders, we used the Cox proportional hazard model. Variables associated with cardiovascular events at follow-up with p<0.10 were BMI, HbA1c, hypertension, albumin-creatinine ratio, eGFR, macroangiopathy, and several therapies (Table 1). Though determinants of the 3-point and 4-point MACE may be slightly different, these variables were chosen as covariates in the fully-adjusted model, because definition of the primary outcome included those of the secondary outcomes. Table 4 shows hazard ratios (HR) with 95% C.I. 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 7 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 MACE were non-significant before or after BH correction. The associations of absolute CD34$^+$ or CD34$^+$CD133$^+$ cells with cardiovascular outcomes were quantitatively similar, but statistically weaker (Table 4). KDR-expressing phenotypes were not predictive of adverse outcomes or sometimes showed a direct association with future cardiovascular events, not surviving correction for multiple testing.

As clinical determinants of death may differ from those of cardiovascular events, we selected covariates with significance level <0.10 in the comparison of patients who were alive and those who were died at follow-up: age, dyslipidemia, neuropathy, peripheral arterial disease and therapy (secretagogues, beta-blockers, calcium antagonists). No significant association was detected between progenitor cell levels and death from any cause (Table 4).

<u>Discrimination improvement by addition of progenitor cell levels</u>. We finally compared the discrimination capacity of the model described in Table 4 with and without inclusion of relative CD34$^+$ cells, against the primary endpoint. C-statistics improved from 0.758 to 0.799 (p<0.001), the

continuous NRI improved by 35% (p=0.038) whereas IDI was not significantly improved (4.5%; p=0.059). Discrimination capacity was not significantly improved by 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]). Addition of relative CD34$^+$ cell count to the CHD risk provided by the UKPDS risk engine significantly improved C-statistics (from 0.616 to 0.704; p<0.001), continuous NDI (46.8%, p=0.006), and IDI (7.2%; p<0.001). Addition of relative CD34$^+$CD133$^+$ cell count to the UKPDS risk significantly also improved C-statistics (from 0.590 to 0.642; p<0.001), continuous NDI (37.4%, p=0.019), and IDI (2.5%; p=0.022).

Meta-analysis

We identified 695 studies. One duplicate was excluded. Of the remaining studies, 666 were irrelevant to this review and were excluded on the basis of their titles and abstracts. Of the remaining, 7 studies were considered only for descriptive purpose due to missing data, and 21 were included in meta-analysis, for a total of 4,155 patients (Figure 8).

Overall characteristics of included studies. The meta-analysis included 21 studies, for a total of 4,155 patients (average pts/study = 198, median [IQR] = 154 [121-215]).[58-77] Characteristics of studies included from meta-analysis are given in Table 5. The most important reason whereby studies initially retrieved were finally excluded from meta-analysis was the lack of a poolable risk estimate and impossibility to calculate such estimate from the data provided. Pooled cumulative clinical characteristics of the meta-analysed patient population are reported in Table 6. Four of the 21 studies, (n=512 patients, 12.8% of the total population) were conducted in patients with acute coronary syndrome, acute myocardial infarction, or stroke.[62,67,69,76] For the remainders, the underlying disease or condition was elective percutaneous intervention in 7/17 studies (n=795 patients, 19.1%),[59-61,64,71,73,75] elective coronary angiography for suspected CAD in 2/17 (n=1,412 patients, 34.0%),[70,74] end-stage renal disease in 4/17 studies (n=705 patients, 17.0%),[65,66,68,77] chronic heart failure in 1/17 studies (n=156 patients, 3.8%),[58] and aortic stenosis in 1/17 study

(n=261 patients; 6.3%).[78] One study included patients with and without chronic CVD at baseline,[63] and one study included both healthy subjects and patients with chronic or acute CVD.[72] Five of 21 studies considered multiple CPCs/EPCs phenotypes at the same time. Phenotypes most frequently used were CD34$^+$ CPCs (6 studies) and CD34$^+$KDR$^+$ EPCs (12 studies). The outcome most commonly considered was the occurrence of future cardiovascular events (16 studies).

Quality of included studies. According to a 16-item evaluation, modified from the REMARK guidelines to fit the purpose of this meta-analysis,[54] the median (IQR) score was 10 (9-12). Studies had a good quality (>50% of studies) for the following items: pre-specified hypothesis, setting, inclusion/exclusion criteria, number of patients at each stage, details on manufacturers and assays for CPCs/EPCs, confounders, hierarchy of outcomes, univariate estimate and adjustment, rationale for group comparisons. Vice versa, quality was overall poor (<50% of studies) for: rationale for sample size, description of sample handling, endpoint validation and masking, handling of missing values (Figure 9).

Cardiovascular events. Two studies reporting on CVE were excluded from this analysis because the MACE breakdown indicated an excessive contamination with non-hard events.[58,69] The analysis was first conducted for each single cell phenotype and then summary statistics for all phenotypes were pooled together. The risk ratio of future cardiovascular events (random effect model) in patients with low versus high cell count was statistically significant for CD34$^+$CD133$^+$ CPCs (RR 2.61 [1.44-4.74]), CD34$^+$CD133$^+$KDR$^+$ EPCs (RR 7.91 [2.65-23.57]), and CFU (RR 1.18 [1.00-1.39]), whereas it was not significant for CD34$^+$, CD34$^+$KDR$^+$, and CD133$^+$KDR$^+$ cells. The latter showed significant heterogeneity among studies in the association with future cardiovascular events. When phenotype-specific risk estimates were pooled together, the overall risk ratio indicated that a low CPCs/EPCs count was associated with a significant 97% higher risk of future cardiovascular events (Figure 10).

When the analysis was repeated excluding studies on patients with acute CVD, who have the highest risk for future events, the overall statistics was lower as expected but still significant (RR

1.71 [1.16-2.52]). When the analysis excluded studies wherein the risk estimate had to be calculated, the overall statistics was higher (RR 2.52 [1.56-4.06]) and highly significant. Limiting the analysis to studies with a REMARK score >10 yielded a risk ratio of 1.99 (1.25-3.16) (Figure 11a). Such studies also had a more homogeneous definition of the composite cardiovascular outcome, with at least 50% composed by hard endpoints.

According to the fixed effect model, the risk ratio would be significant for $CD34^+$ (2.02 [1.43-2.85], p<0.001), $CD34^+CD133^+$ (2.61 [1.44-4.74], p=0.002), $CD34^+KDR^+$ (1.24 [1.07-1.43], p=0.003), $CD34^+CD133^+KDR^+$ (7.91 [2.65-23.57], p<0.001).

Cardiovascular and all-cause mortality. Four studies reported cardiovascular death in relation to baseline CPCs/EPCs. Altogether, they show an association between low versus high cell count and the risk for future cardiovascular death, yielding a pooled risk ratio of 1.87 (95% C.I. 1.15-3.02). The corresponding risk ratio according to the fixed-effect model was 1.45 (1.22-1.72). However, multiple studies were available only for $CD34^+KDR^+$ cells (n=3) and their association with cardiovascular death was highly heterogeneous, yielding a non-significant pooled risk ratio (Figure 12a).

Data on all-cause mortality was available for most phenotypes. The pooled risk ratio of future death in patients with low versus high cell count was statistically significant for $CD34^+$ (RR 3.40 [1.99-5.83]), $CD34^+CD133^+$ CPCs (2.56 [1.26-5.17]), and $CD34^+CD133^+KDR^+$ EPCs (RR 1.36 [1.21-1.53]). Some EPC phenotypes showed paradoxical opposite trend associations with future death from any cause, and a large heterogeneity among studies was found. As a result, the overall summary statistics for all phenotypes was marginally significant with the random-effect model (Figure 12b). According to the fixed-effect model, the pooled risk ratio for all phenotypes would be 1.37 (1.23-1.52; p<0.001). When the random-effect model analysis excluded studies wherein the risk estimate had to be calculated, the overall statistics was 1.75 (1.23-2.49; p=0.002) (Figure 11b).

Restenosis and revascularization. A few studies reported the risk of restenosis after percutaneous intervention and/or the need for future revascularization in patients with baseline acute or chronic

CVD. The association between low versus high CPCs/EPCs levels and restenosis was highly variable according the cellular phenotype, ranging from a significant protection for low CD34^{+} cells to a significant harm for low CD34^{+}CD133^{+}KDR^{+} cells or CFU. The overall statistics showed a trend increased risk of future restenosis in patients with low versus high CPCs/EPCs, with high and significant heterogeneity (Figure 13a). Similar results were obtained when the analysis excluded studies wherein the risk estimate had to be calculated, whereas limiting the analysis to higher quality studies yielded a risk ratio of RR 4.33 (4.01-4.69) for restenosis associated with low versus high cell count (Figure 11c). According to the fixed-effect model, the risk of restenosis associated with a low CPCs/EPCs cell count would be 2.97 (2.77-3.17).

Data on the risk for future revascularization was available only for 3 phenotypes: there was a non-significant trend association of reduced risk in patients with low CPCs/EPCs, but heterogeneity was high and statistically significant (Figure 13b). Excluding studies conducted in acute CVD patients or studies for which the risk estimate had to be calculated did not change the results using the random effect model (Figure 11d), though it yielded a significant risk ratio using the fixed effect model (RR 1.21 [1.02-1.43]).

Meta-regression analysis. A meta-regression was conducted to detect whether any overall characteristic of study populations consistently modulated the risk estimate. As robustness of meta-regression relies on the number of studies included, we only analyzed the risk ratio for the most commonly used progenitor cell phenotype (CD34^{+}KDR^{+} EPCs) and the most common outcome (CVE). With the random effect model, the log of risk ratio was significantly associated with the percentage of male patients (direct correlation, p=0.002), the percentage of patients with a previous AMI (direct correlation, p=0.002), and with the percentage of patients on statin (inverse association, p<0.0001) (Figure 14). No statistically significant outlier was detected in this meta-regression.

Excluded studies. Excluded studies reported data on the relation between CPCs/EPCs levels and functional outcome after acute myocardial infarction or stroke. Four studies were conducted on a total of n=309 patients with AMI, overall showing that high CD34^{+}/CD133^{+} CPCs predicted

improvements in regional or global left ventricular function,[79-81] and in coronary flow reserve.[82] One study found that mobilization of EPCs during acute ischemia was significantly lower in patients who developed restenosis,[83] whereas a small study in patients with stable angina showed that EPC levels directly correlated with the degree of restenosis.[84] In one study conducted in patients with acute ischemic stroke, a low number of baseline EPCs predicted worse functional outcomes after 6 months.[85]

Biases. The number of studies available for which a given CPC / EPC phenotype was assessed in relation to a given outcome was limited. In funnel plots showing all phenotypes simultaneously (Figure 15), there is a suggestion of missing studies on the bottom left hand side of the plot. Since most of this area contains regions of high significance, publication bias is unlikely to be the underlying cause of asymmetry. Heterogeneity likely arose from selective outcome and analysis reporting, and poor quality of some studies.[55]

DISCUSSION

We demonstrated that reduced levels of circulating progenitor cell phenotypes, including EPCs, predict future worsening of microangiopathy and cardiovascular events in patients with T2D. The CD34^{+} cell count outperformed other CPC and EPC phenotypes in prediction of microvascular outcomes, likely because of its higher reproducibility and stability [4,50]. Indeed, after applying the most stringent FDR control, adjusting simultaneously for the 6 progenitor phenotypes and 5 outcomes tested (equal to 30 multiple comparisons), CD34^{+} cells remained significantly associated with UAER, retinopathy, and microangiopathy progression in all univariate and multivariate analyses. Interestingly, we also found that reduction in CD34^{+} CPCs abolished the protective effects of ACE inhibitors/ARBs on UAER progression, though this interaction needs to be explored further.

Previous works have shown associations between circulating progenitor cell levels and cardiovascular outcomes [70,74,86]. Our study validates the clinical meaning of circulating stem cells defects in diabetic patients on long-term follow-up (up to 12 years). This is important because the majority of events occurred after 5 years of observation and we show that stem cell performance as biomarkers was not diluted over time. By analysing 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 [4], did not predict cardiovascular outcomes, despite they are believed to be vasculoregenerative [87,88]. This discrepancy is counterintuitive but has different plausible explanations.[7] First, CPCs could reflect the healthy of bone marrow better than EPCs.[89] Second, the number of circulating KDR^{+}EPCs is lower than CPCs and their enumeration has higher variability, in particular for CD34^{+}CD133^{+}KDR^{+}. Furthermore, KDR^{+} staining is not clinical-grade and inter-lots variations may occur for anti-KDR antibodies. Conversely, enumeration of CD34^{+} cells is more standardised, according to International Society

for Hematotherapy and Graft Engineering (ISHAGE) protocol, and is routinely available in most hospital laboratories.[90]

The addition of CD34$^+$ cell level to traditional UKPDS model of risk assessment significantly improves the event prediction (as determined by C-statistics, NRI and IDI) and therefore is expected to perform well as a clinical biomarker.

We detected weaker associations between low stem cell levels and the 3-point or 4-point MACE and non-significant 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 meta-analysis of longitudinal studies, including 4,155 patients followed for an average of 2 years, showed that a reduction in the levels of circulating CD34$^+$ and CD34$^+$CD133$^+$ cells was associated with a ~2 fold increased risk of future cardiovascular events and death [91]. In meta-regression analyses, we detected no correlation between the prevalence of diabetes and HRs, suggesting that the prognostic impact of reduced stem cell levels was similar in diabetic and non-diabetic patients [91]. The association between low CPCs/EPCs and reduced risk for future revascularization observed in meta-analysis is counter-intuitive. However, differences in the clinical setting (acute versus chronic CVD) and in progenitor cell phenotype may account for this discrepancy. Biologically, reduction of vasculoprotective cells may prevent successful revascularization, by inducing a more severe and occlusive atherosclerosis, less amenable to surgical or endovascular intervention.

Observational studies indicate that microangiopathy contributes to the risk of MACE in diabetes [92]. While this is most obvious for CKD [93], the reasons whereby the presence of retinopathy increases the risk of myocardial infarction [94] are less clear. In addition, about 40% of excess mortality in diabetes is attributable to non-vascular causes [95], onto which microangiopathy may play a role. A common pathogenic ground for micro- and macroangiopathy has been postulated, and we

hypothesize that CPCs are candidate mediators of multi-organ complications in diabetes [96,97]. Recently, the BM has emerged as a site of diabetic end-organ damage, and BM dysfunction accounts for the CPC reduction seen in diabetic patients [98]. Interestingly, BM remodelling includes neurovascular changes and strongly resembles microangiopathy seen in the kidney and retina [44,45,99]. The present study provides new evidence in support of a model wherein the BM acts as a central housekeeper of organismal health, whereas BM failure can link disparate end-organ complications. However, although progenitor cells have been shown to predict adverse long-term diabetic outcomes, whether CPC stimulation is able to modify the natural history of chronic complications in diabetic patients is still unknown. This is a relevant challenge that we need to further investigate considering the ancillary effects of known therapies on CPC levels. For example, dipeptidyl peptidase 4 (DPP-4) inhibitors and peroxisome proliferation activator receptor γ (PPAR-γ) agonists have shown the capacity to increase EPCs levels, acting on several pathways that are impaired in diabetic patients.[100,101] Similarly, intensive statin therapies seem to increase the levels of EPCs independently by lipid-lowering action of such drugs.[102] In a clinical prospective, these evidences highlight the need for an individualization of therapy based on individual cardiovascular risk profile.

Limitations of this study need to be acknowledged. Data collection was formally retrospective, but routine follow-up of patients after the baseline CPCs/EPCs analysis allowed a pseudo-prospective design. The absolute need to analyze progenitor cells in fresh blood samples discourages truly prospective studies with adequate follow-up to detect disease progression, whereas our pseudo-prospective study is the longest ever performed using CPCs/EPCs as biomarkers. However, accrual time was very long and follow-up duration variable among patients, thus precluding time-dependent analyses and possibly increasing the risk of bias. Our findings may not be generalizable also because BM impairment and progenitor cell pauperization is a typical feature of aging and age-associated diseases [103]. As aging contributes to microangiopathy in T2D, it is unclear whether similar findings would apply to a younger population of T1D patients. In addition, the relatively

small sample size prevented separated analyses for onset and progression of microangiopathy, disease severity, and hard endpoints (such as visual loss, dialysis, or amputations). Finally, changes in antibody lots and updates in FACS system setup over time may have generated some heterogeneity in progenitor cell quantification, especially for KDR staining, whereas CD133 staining was missing in about 10% of cases.

In conclusion, we show that low CPC and EPC levels predict microvascular and macrovascular outcomes in T2D. These data support the importance of the endogenous vascular regenerative capacity, mediated by BM-derived cells, in the global burden of diabetic complications [96]. Our meta-analysis suggests that prognostic impact of reduced stem cell levels is similar in diabetic and non-diabetic patients.

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FIGURES

Figure 1. Representative FACS plots. Representative analyses of circulating stem/progenitor cell identification and enumeration from a patient without (A) and with (B) cardiovascular events at follow-up. After gating lymphocytes and monocytes in the FSC versus SSC morphologic plot (where 5×10^5 events are shown), total $CD34^+$ cells were identified and scored (a parallel analysis was performed for total $CD133^+$ cells). Gated $CD34^+$ cells were then examined for expression of CD133 and KDR. Here, $CD34^+CD133+$ cell count derive from the sum of $CD34^+CD133^+KDR+$ and $CD34^+CD133^+KDR^-$ events.
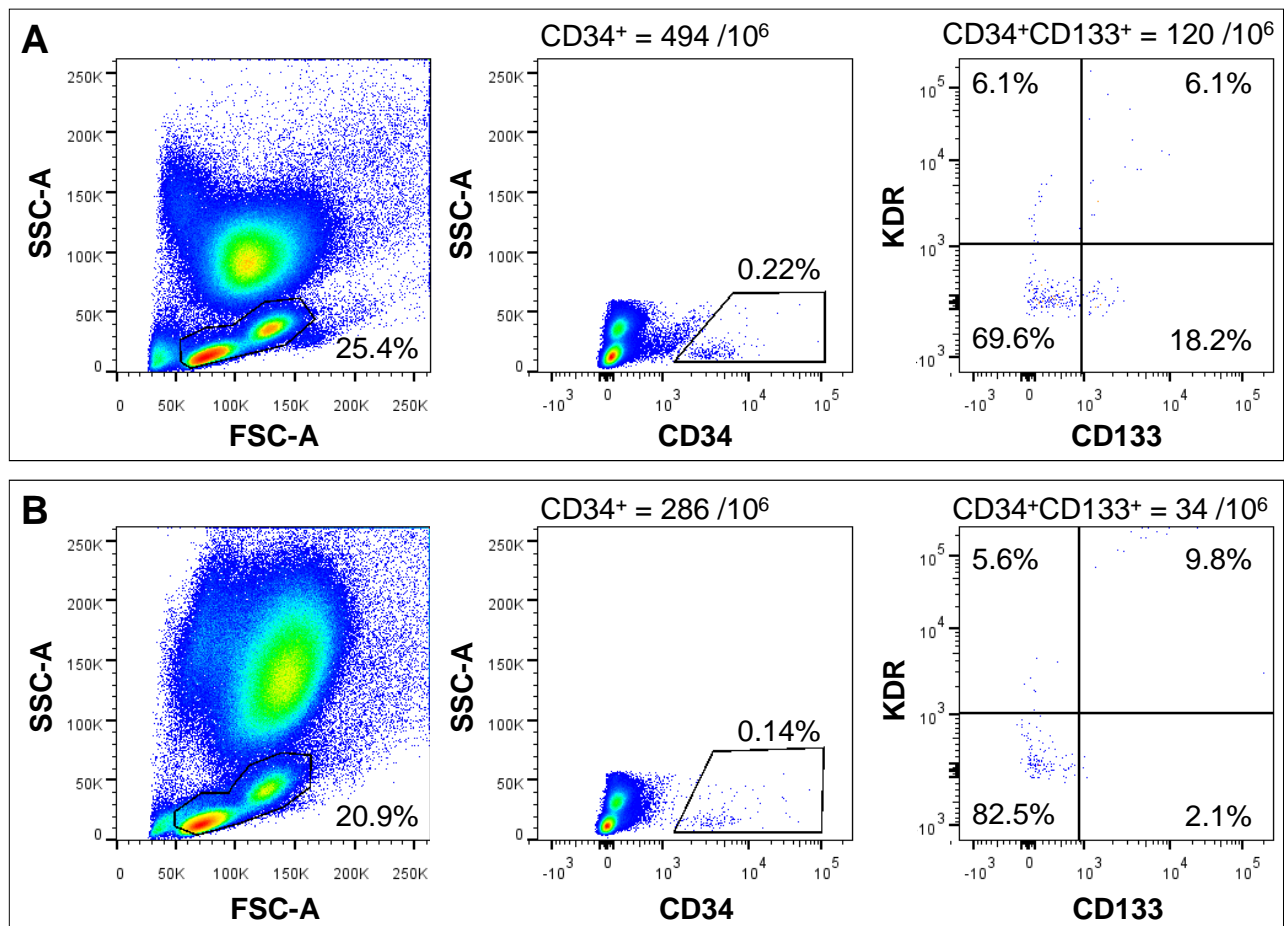
Figure 2. Association between progenitor cells levels and complications at baseline. Only significant associations are shown. *p<0.05.
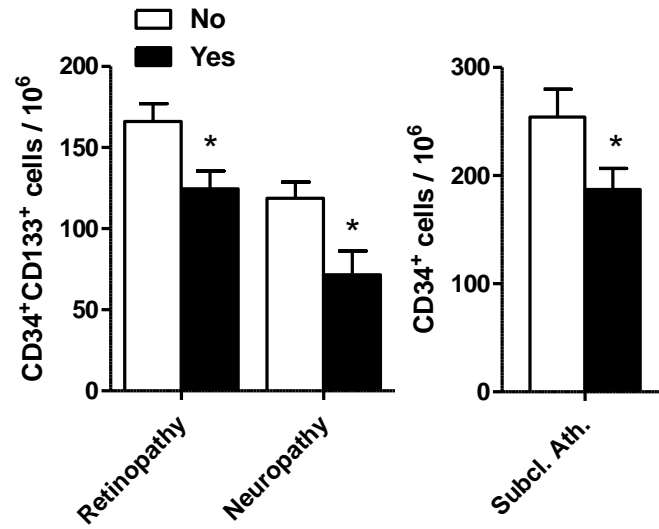
Figure 3. **Associations between progenitor cell levels and progression of microangiopathy**. The upper lane reports mean±SEM CPC and EPC levels in patients without (white) and with (black) progression of each microvascular complication during follow-up. The lower lane reports annual incidence of each microvascular disease progression in patients categorized as having high or low CPC/EPC cells, based on median levels. *p<0.05 in the indicated paired comparisons; # not significant after false discovery rate correction with the BH procedure.
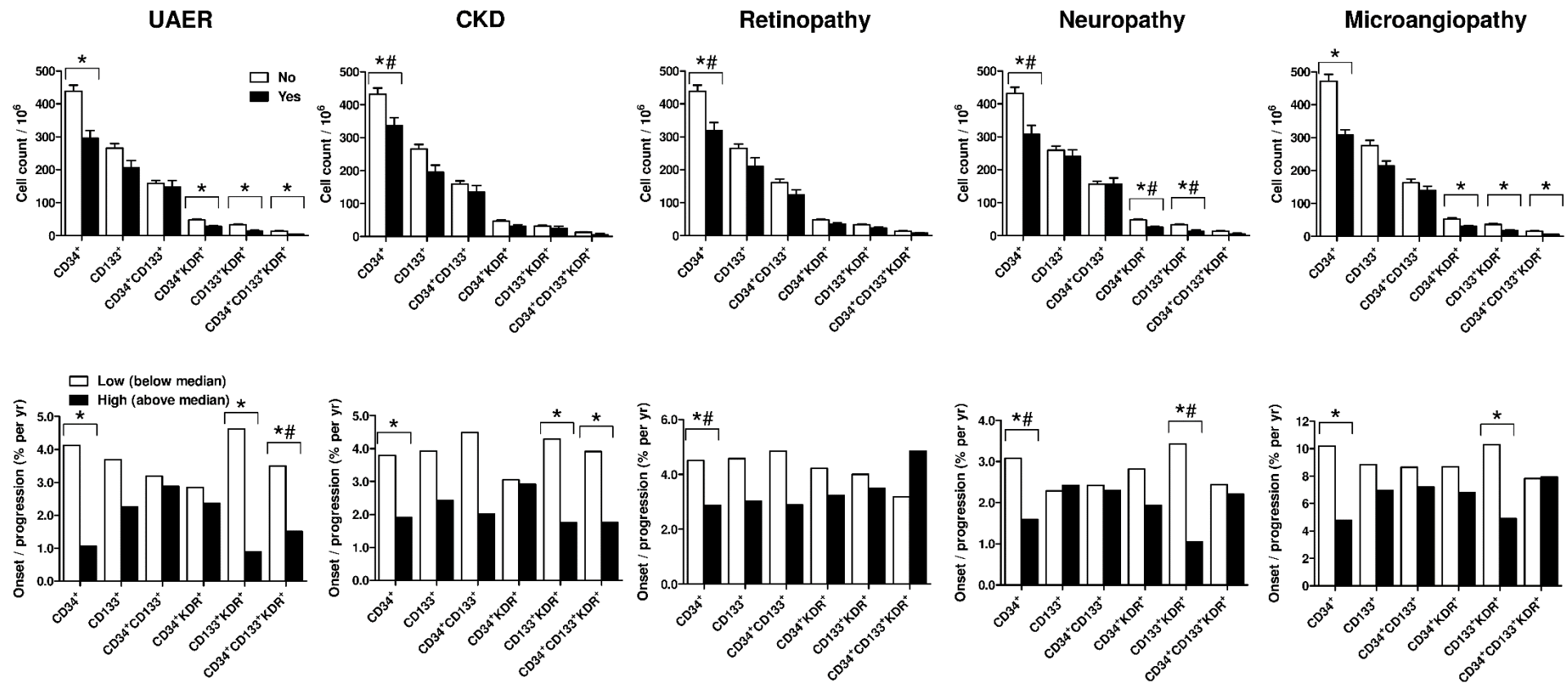
Figure 4. Univariate interaction between high (above median) versus low (below median) CD34^{+} CPC count and use of ACE inhibitors / ARBs in determining the rate of onset/progression of microalbuminuria. *p<0.05 as indicated.
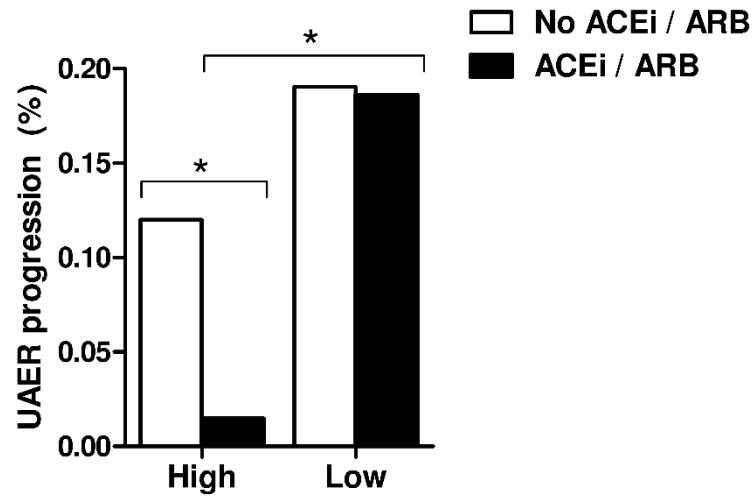
Figure 5. Relative progenitor cell levels and cardiovascular outcomes. A) Progenitor cell levels (mean ± standard error) in patients divided into those who developed or not developed an event (all events, 3-point MACE, 4-point MACE) at follow-up. B) Annual incidence of all events, 3-point MACE, and 4-point MACE in patients divided according to high or low stem/progenitor cell levels based on the median value. *p<0.05; **significant after BH correction.
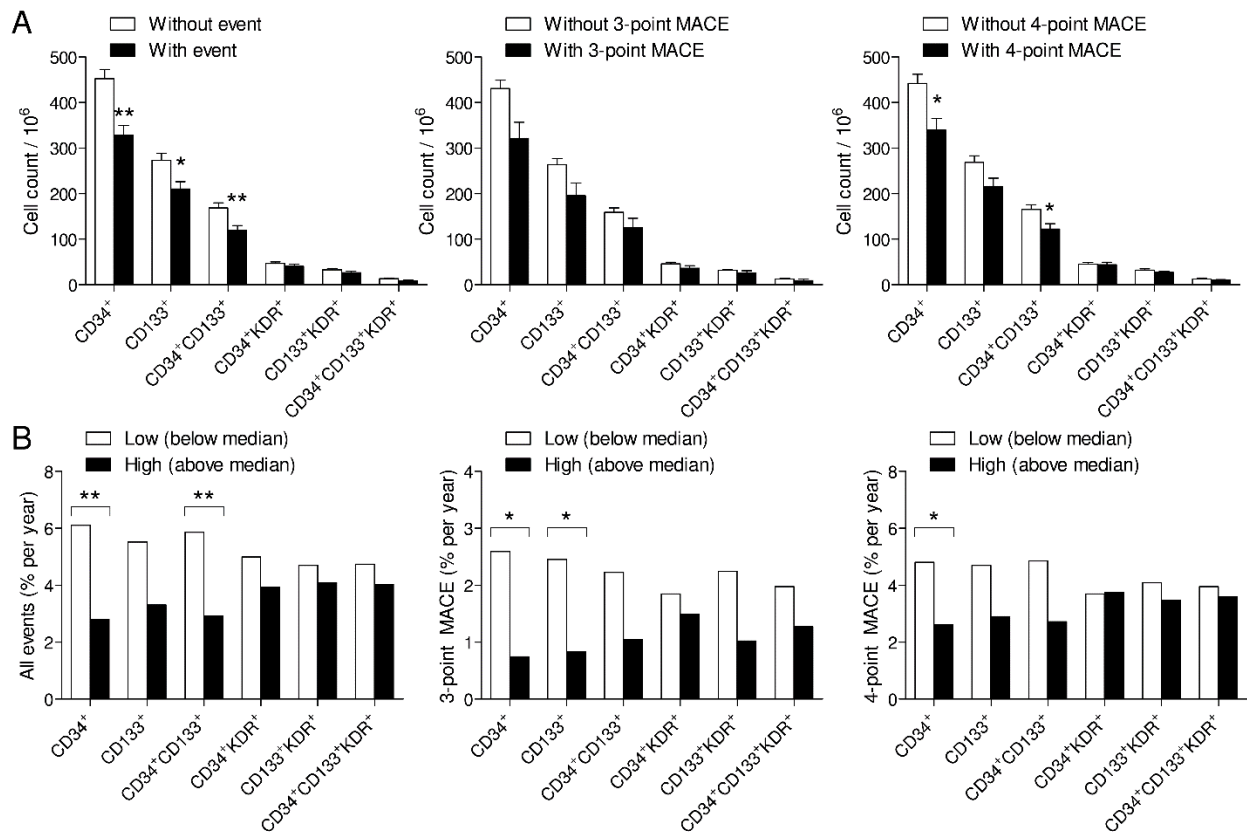
Figure 6. Absolute progenitor cell levels and cardiovascular outcomes. A) Progenitor cell levels (mean ± standard error) in patients divided into those who developed or not developed an event (all events, 3-point MACE, 4-point MACE) at follow-up. B) Annual incidence of all events, 3-point MACE, and 4-point MACE in patients divided according to high or low stem/progenitor cell levels based on the median value. *p<0.05; **significant after BH correction.
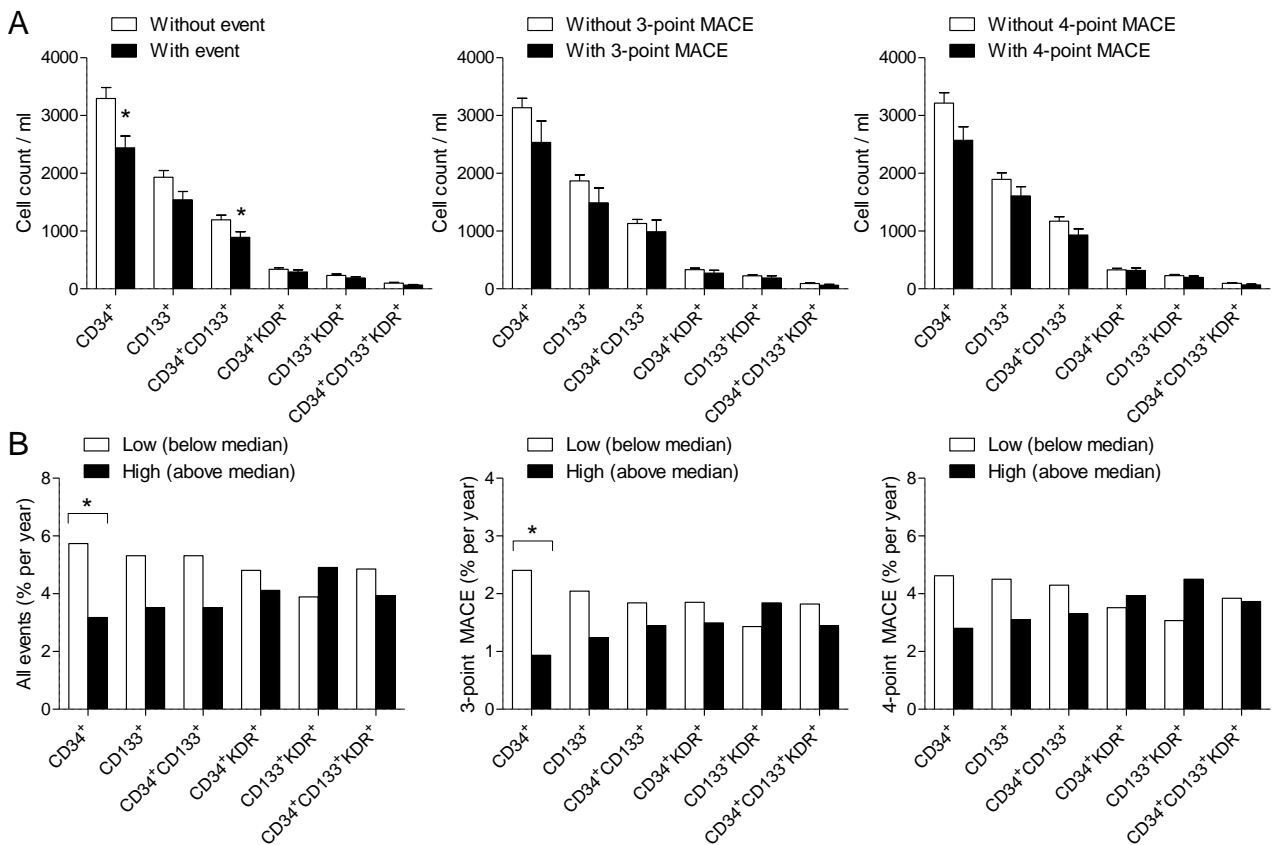
Figure 7. Kaplan-Meier curves. The panels show fully adjusted event-free survival curves for the primary outcome (all events) from Cox proportional hazard regression analyses in patients with low versus high levels of CD34$^+$ cells (left) or CD34$^+$CD133$^+$ cells (right).

Figure 8. **Meta-analysis flow-chart.**


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┌─────────────────────────────┐
│  695 records identified      │
│  through database searching  │
└─────────────┬───────────────┘
              │
              ▼
┌─────────────────────────────┐
│  694 records after           │
│  duplicate remove            │
└─────────────┬───────────────┘
              │
              ▼
┌─────────────────────────────┐      ┌──────────────────────────┐
│  694 records screened        │─────▶│  666 records excluded     │
└─────────────┬───────────────┘      └──────────────────────────┘
              │
              ▼
┌─────────────────────────────┐      ┌──────────────────────────┐
│  28 full-text articles assesed│────▶│  7 considered only for    │
│  for eligibility             │      │  descriptive purpouse     │
└─────────────┬───────────────┘      └──────────────────────────┘
              │
              ▼
┌─────────────────────────────┐
│  21 studies included in the  │
│  quantitative synthesis      │
└─────────────────────────────┘
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Figure 9. Quality of studies included in the meta-analysis. The percentage of studies satisfying each of the 16 items modified from the REMARK guidelines is reported. Studies excluded from meta-analysis (n=7) are not shown.
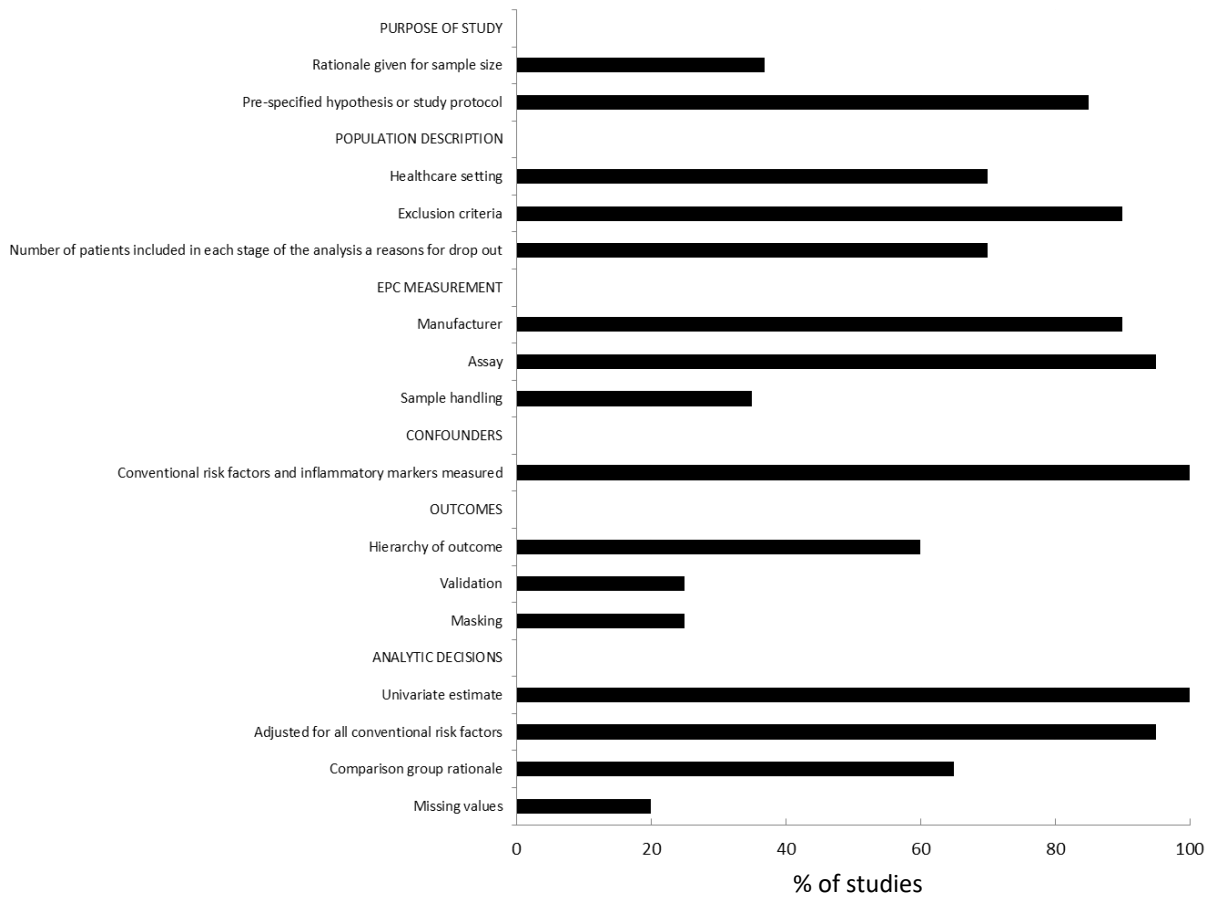
Figure 10. **Forest plot of the risk for future cardiovascular events associated with a low versus a high CPC / EPC count**. Separated results are shown for each different phenotype of progenitor cells. Therefore, studies reporting risk estimates associated with the levels of different phenotypes are reported more than once. The group called "Overall" reports subtotal risk estimates for each phenotype and a pooled risk ratio. Weights of each study, and individual risk ratios with 95% C.I. are shown, along with tests for heterogeneity and overall effect. CFU, colony forming cells.

Cardiovascular events

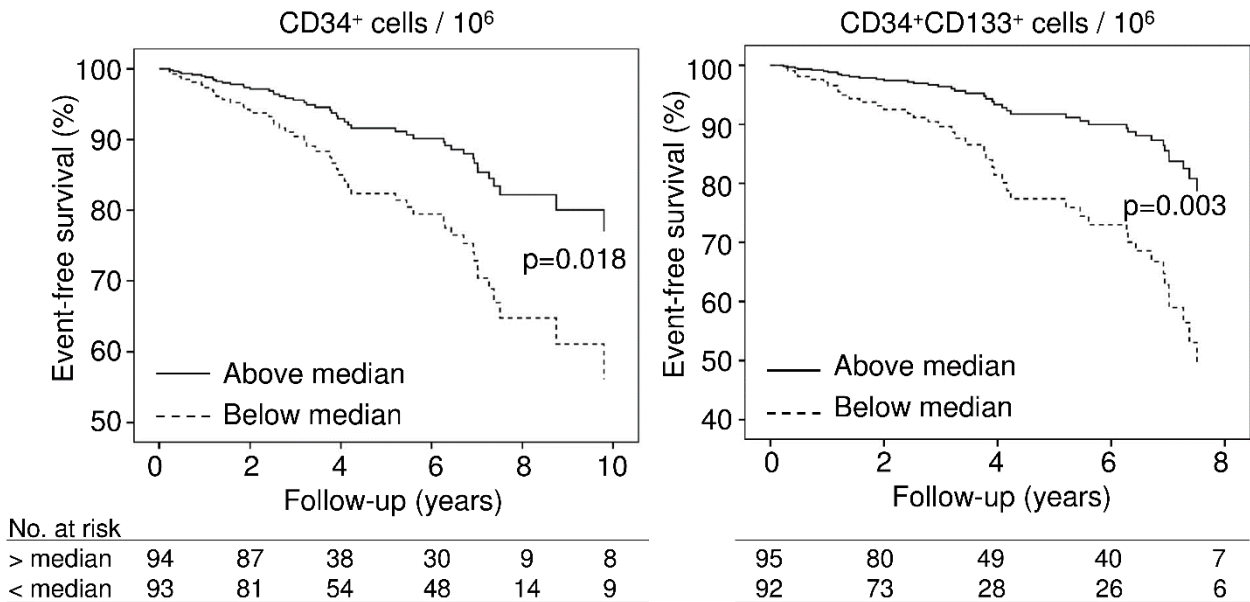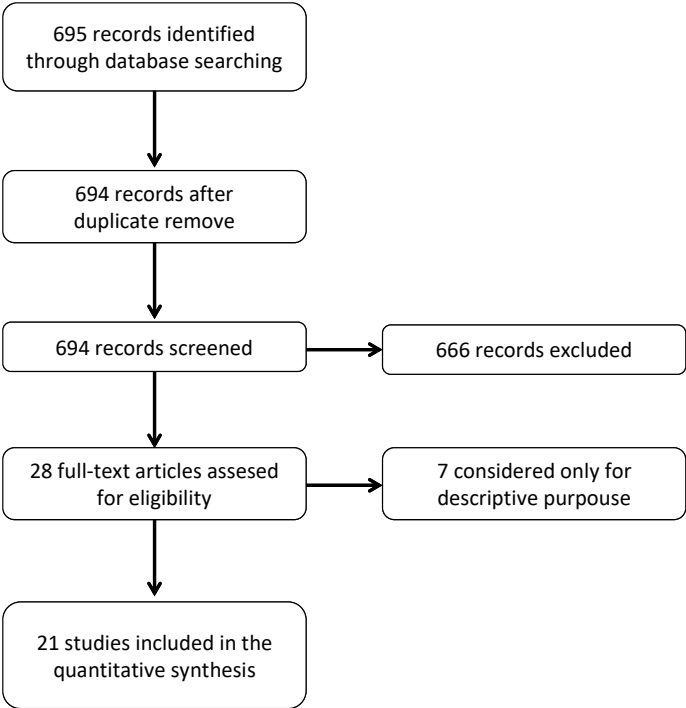| Study or Subgroup	Weight	Risk Ratio IV, Random, 95% CI
1.1.1 CD34$^+$		
Yu et al. 2013	16.8%	0.34 [0.10, 1.14]
Fadini et al. 2009	28.7%	1.90 [1.05, 3.41]
Maruyama et al. 2008	25.8%	2.23 [1.08, 4.60]
Patel et al. 2015	28.7%	3.06 [1.70, 5.52]
Subtotal (95% CI)	**100.0%**	**1.70 [0.86, 3.34]**
Heterogeneity: Tau^2 = 0.32; Chi^2 = 10.32, df = 3 (P = 0.02); I^2 = 71%		
Test for overall effect: Z = 1.54 (P = 0.12)		
1.1.2 CD133$^+$		
Yu et al. 2013	88.8%	8.08 [0.68, 95.54]
Fadini et al. 2009	11.2%	11.47 [0.01, 12062.69]
Subtotal (95% CI)	**100.0%**	**8.41 [0.82, 86.18]**
Heterogeneity: Tau^2 = 0.00; Chi^2 = 0.01, df = 1 (P = 0.93); I^2 = 0%		
Test for overall effect: Z = 1.79 (P = 0.07)		
1.1.3 CD34$^+$CD133$^+$		
Patel et al. 2015	96.0%	2.53 [1.38, 4.65]
Fadini et al. 2009	4.0%	5.47 [0.28, 105.59]
Subtotal (95% CI)	**100.0%**	**2.61 [1.44, 4.74]**
Heterogeneity: Tau^2 = 0.00; Chi^2 = 0.25, df = 1 (P = 0.62); I^2 = 0%		
Test for overall effect: Z = 3.17 (P = 0.002)		
1.1.4 CD34$^+$KDR$^+$		
Pelliccia et al. 2013	16.0%	0.37 [0.23, 0.60]
Patel et al. 2015	15.3%	0.88 [0.51, 1.52]
Fadini et al. 2009	17.0%	1.23 [0.83, 1.83]
Werner et al. 2005	18.8%	1.35 [1.13, 1.61]
Lee et al. 2015	13.0%	2.75 [1.30, 5.78]
Chiang et al. 2014	12.0%	3.13 [1.35, 7.26]
Schimdt–Lucke et al. 2005	7.9%	3.90 [1.07, 14.20]
Subtotal (95% CI)	**100.0%**	**1.33 [0.83, 2.14]**
Heterogeneity: Tau^2 = 0.30; Chi^2 = 38.04, df = 6 (P < 0.00001); I^2 = 84%		
Test for overall effect: Z = 1.19 (P = 0.23)		
1.1.5 CD133$^+$KDR$^+$		
Pelliccia et al. 2013	58.5%	0.44 [0.27, 0.73]
Fadini et al. 2009	41.5%	7.69 [0.73, 80.80]
Subtotal (95% CI)	**100.0%**	**1.45 [0.09, 22.78]**
Heterogeneity: Tau^2 = 3.31; Chi^2 = 5.41, df = 1 (P = 0.02); I^2 = 82%		
Test for overall effect: Z = 0.27 (P = 0.79)		
1.1.6 CD34$^+$CD133$^+$KDR$^+$		
Marti–Fabrega et al. 2015	71.3%	7.10 [1.95, 25.88]
Fadini et al. 2009	2.6%	9.87 [0.01, 9230.55]
Cuadrado et al. 2015	26.1%	10.38 [1.23, 87.91]
Subtotal (95% CI)	**100.0%**	**7.91 [2.65, 23.57]**
Heterogeneity: Tau^2 = 0.00; Chi^2 = 0.09, df = 2 (P = 0.95); I^2 = 0%		
Test for overall effect: Z = 3.71 (P = 0.0002)		
1.1.7 CFU		
Briguori et al. 2010	30.8%	1.05 [0.78, 1.41]
Lorenzen et al. 2010	69.2%	1.25 [1.02, 1.52]
Subtotal (95% CI)	**100.0%**	**1.18 [1.00, 1.39]**
Heterogeneity: Tau^2 = 0.00; Chi^2 = 0.89, df = 1 (P = 0.35); I^2 = 0%		
Test for overall effect: Z = 2.02 (P = 0.04)		
1.1.8 Overall		
Subtotal CFU	26.4%	1.18 [1.00, 1.39]
Subtotal CD34+KDR+	21.2%	1.33 [0.83, 2.13]
Subtotal CD133+KDR+	2.5%	1.45 [0.09, 23.36]
Subtotal CD34+	17.1%	1.70 [0.86, 3.36]
Subtotal CD34+CD133+	18.7%	2.61 [1.44, 4.73]
Subtotal CD34+CD133+KDR+	10.7%	7.91 [2.65, 23.61]
Subtotal CD133+	3.4%	8.41 [0.82, 86.25]
Subtotal (95% CI)	**100.0%**	**1.97 [1.24, 3.12]**
Heterogeneity: Tau^2 = 0.20; Chi^2 = 20.07, df = 6 (P = 0.003); I^2 = 70%		
Test for overall effect: Z = 2.89 (P = 0.004)		

0.1 0.2 0.5 1 2 5 10

Risk ratio of low vs high cell count

Figure 11. **Sub-analyses**. For cardiovascular events (a), all-cause mortality (b), restenosis (c), and revascularization (d), up to 5 subanalyses are reported. Subanalysis 1 was performed excluding studies conducted only in patients with acute CVD. Subanalysis 2 was performed excluding studies for which the risk estimate had to be calculated. Subanalysis 3 was performed only including studies conducted only in patients with acute CVD. Subanalysis 4 was performed only with higher quality studies (>10 REMARK items). Subanalysis 5 was performed only with studies having a homogeneous definition of the composite cardiovascular endpoint (>50% hard events).
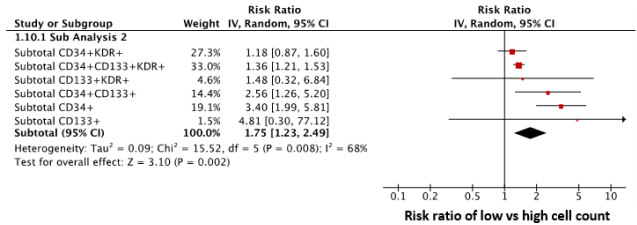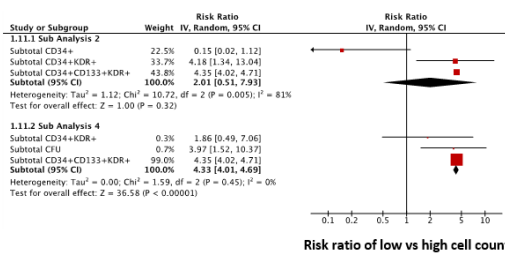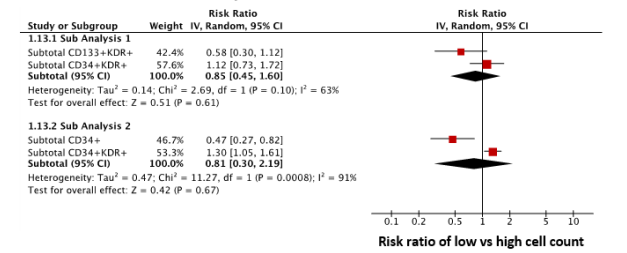
Figure 12. **Forest plot of cardiovascular (a) and all-cause (b) mortality risk associated with a low versus a high CPC / EPC count**. Separated results are shown for each different phenotype of progenitor cells. Weights of each study, and individual risk ratios with 95% C.I. are shown, along with tests for heterogeneity and overall effect.
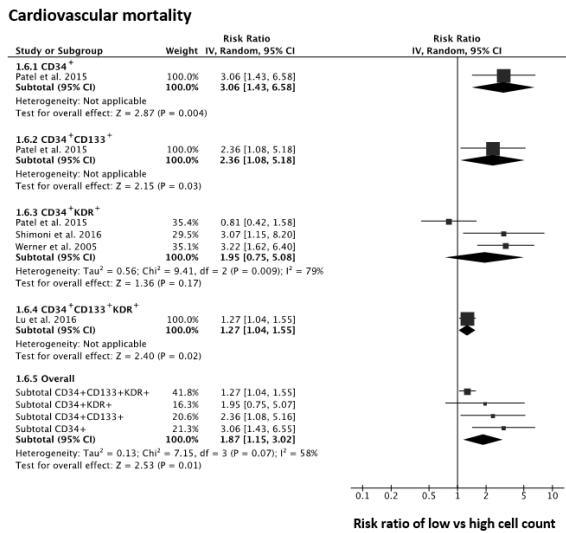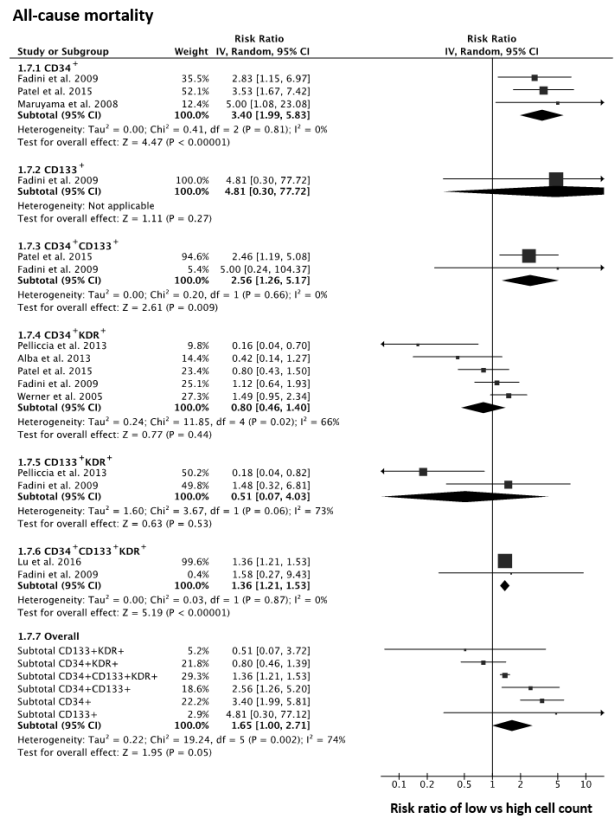
Figure 13. **Forest plot of the risk of restenosis (a) and revascularization (b) associated with a low versus a high CPC / EPC count**. Separated results are shown for each different phenotype of progenitor cells. Weights of each study, and individual risk ratios with 95% C.I. are shown, along with tests for heterogeneity and overall effect. CFU, colony forming cells.
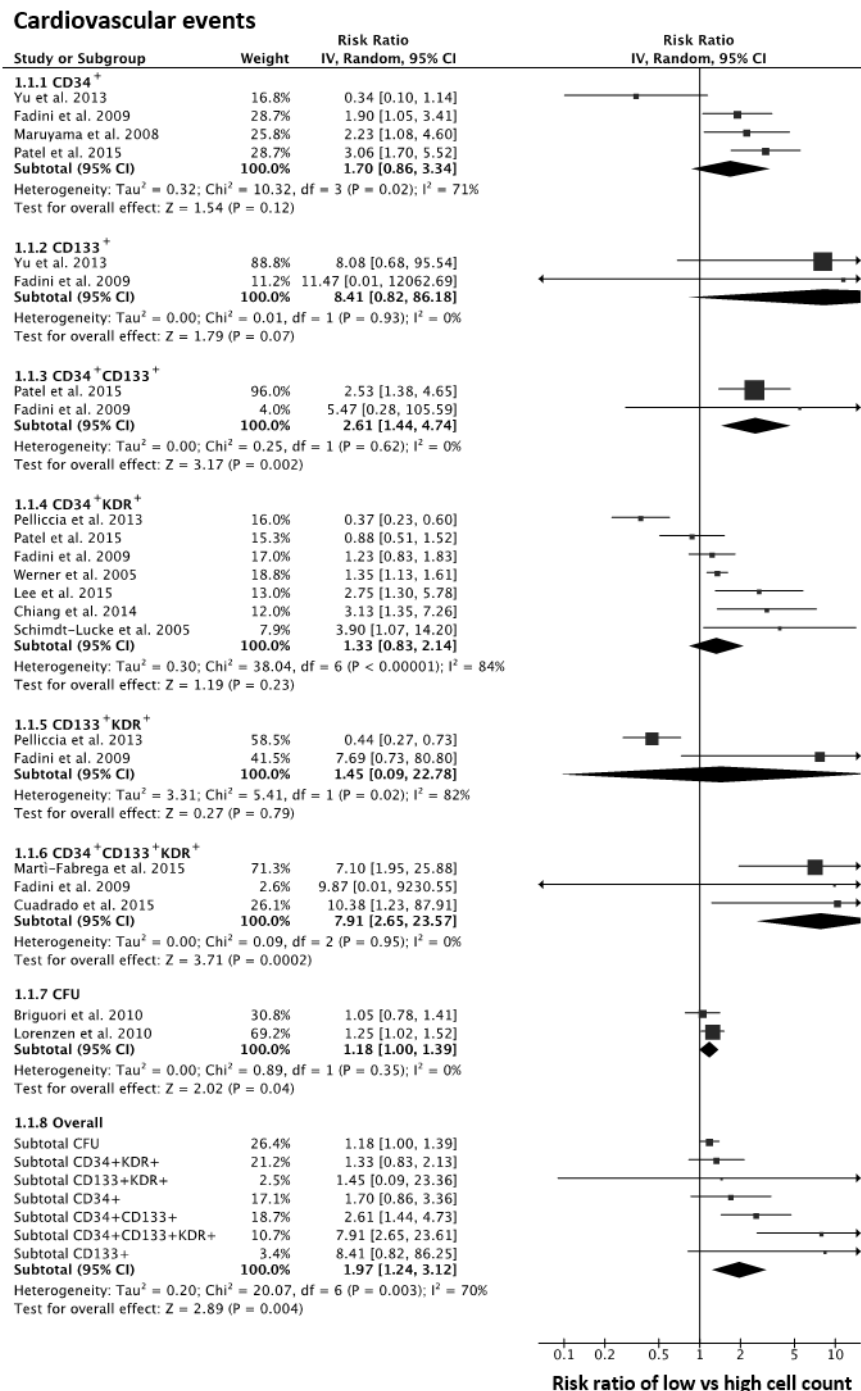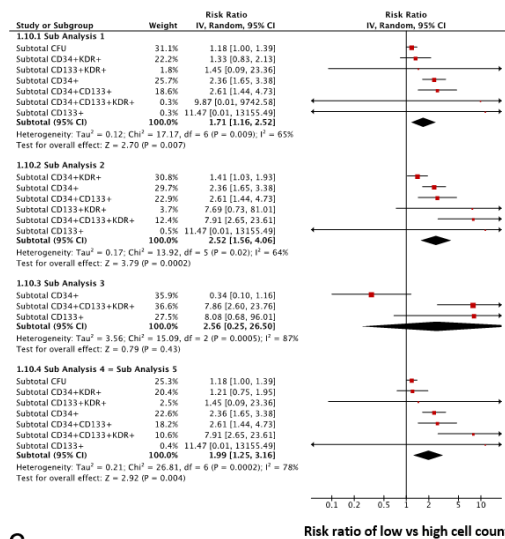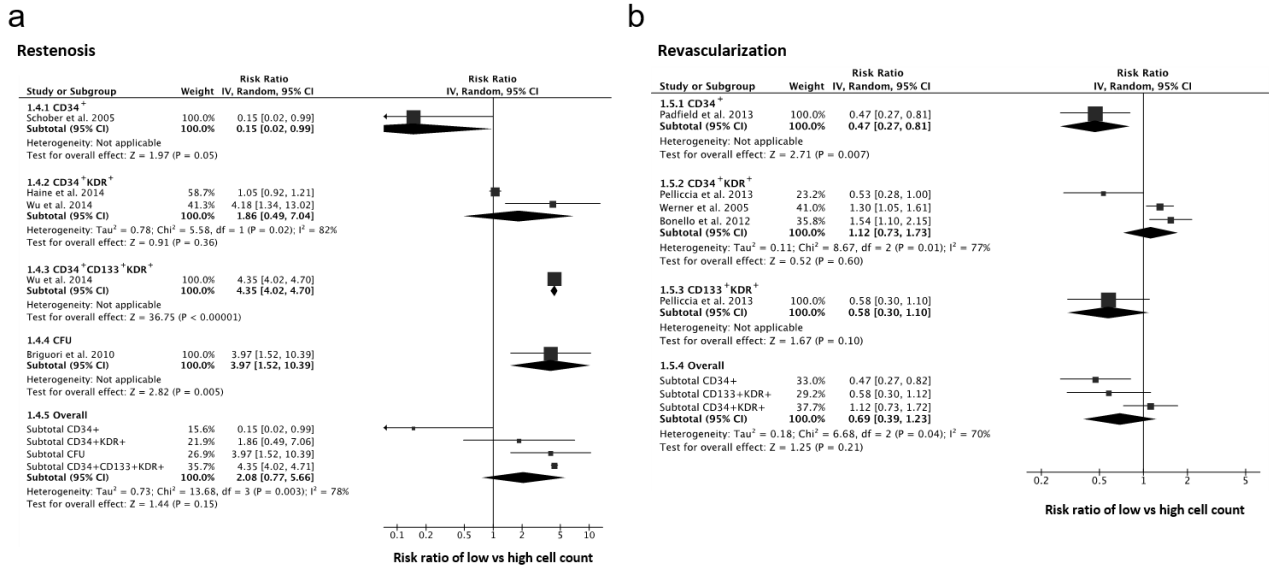
Figure 14. Meta-regression analysis. The natural log of the risk ratio for cardiovascular events is plotted against various characteristics of each study. Size of the bubbles is proportional to the weight of each study according to the random effect model. The regression lines and p-values are shown.
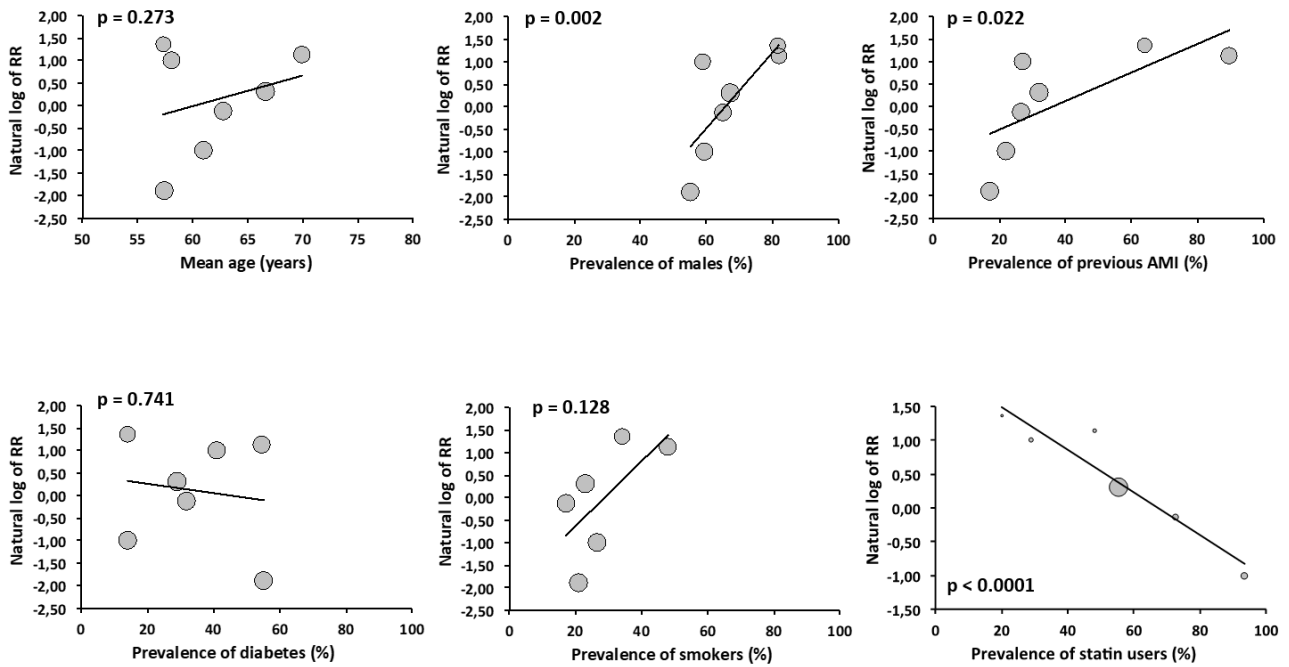
Figure 15. Funnel plots. Funnel plots have been constructed for all phenotypes versus cardiovascular events and all-cause mortality.
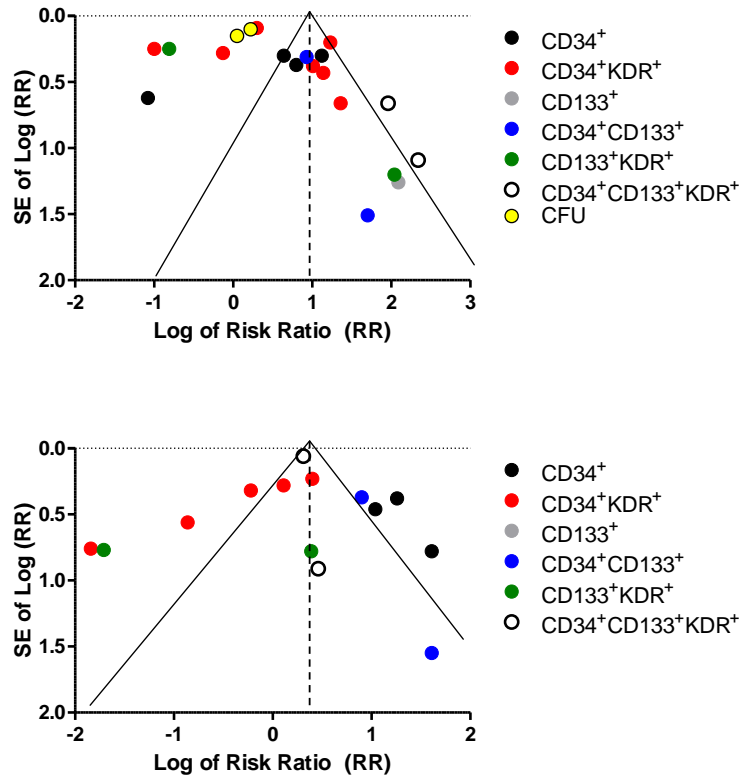
TABLES

Table 1. Baseline clinical characteristics of study patients. *p<0.05 in patients with progression versus non-progression of microvascular disease.

** p<0.05 in patients with versus without cardiovascular events.

Variable	All	UAER development / progression		Retinopathy development / progression		Neuropathy development / progression		Cardiovascular events	
		No	Yes	No	Yes	No	Yes	No	Yes
Number	187	166	21	159	28	169	18	139	48
Age, years	63.7±0.7	63.8±0.7	63.1±2.5	63.4±0.8	65.5±1.2	64.0±0.7	60.9±1.4	63.3±0.7	65.1±1.5
Sex male, %	67	67	67	67	71	67	72	65	75
BMI, kg/m²	29.5±0.4	29.5±0.4	29.0±1.0	29.4±0.4	30.0±1.1	29.3±0.4	31.6±1.3	29.1±0.4	30.7±0.9
HbA1c, %	7.9±0.1	7.9±0.1	7.7±0.3	7.8±0.1	8.4±0.3*	7.9±0.1	8.2±0.4	7.8±0.1 (62±1)	8.3±0.3 (67±2)
Duration, years	10.4±0.6	10.5±0.7	9.6±2.2	10.2±0.7	11.7±1.7	10.5±0.7	9.5±1.3	10.4±0.8	10.3±1.1
Hypertension, %	84	85	81	83	93	83	94	80	98**
Dyslipidemia, %	81	83	71	82	75	81	83	82	79
Smoke, %	13	11	29*	14	7	12	22	13	15
Microangiopathy									
ACR, mg/g	76.3±16.3	79.4±18.2	47.9±16.1	66.8±16.1	127.6±58.0	55.9±10.8	263.0±129.7*	51.4±14.9	148.0±45.5**
eGFR, ml/min	81.7±1.6	82.2±1.7	77.8±4.5	81.5±1.7	83.2±4.8	81.7±1.7	82.1±4.5	83.9±1.8	75.6±3.4**
Retinopathy, %	22	22	24	21	26	22	22	20	28
Neuropathy, %	13	13	14	12	22	14	11	13	15
Macroangiopathy								53	75**
CAD, %	15	14	19	14	18	15	11	10	29
PAD, %	17	17	14	16	21	15	28	12	31
Subclinical atherosclerosis, %	48	47	57	47	57	48	50	45	58

Variable	All	UAER development / progression		Retinopathy development / progression		Neuropathy development / progression		Cardiovascular events	
		No	Yes	No	Yes	No	Yes	No	Yes
Diabetes therapy									
Insulin, %	47	46	48	44	61	46	56	42	58
Secretagogues, %	42	44	24*	38	61*	41	44	40	48
Metformin, %	72	73	62	72	71	73	56	76	60
TZD, %	5	5	5	5	7	5	11	6	2
Incretins, %	9	8	10	10	0*	9	0*	10	4
Other medications									
Anti-platelet, %	49	49	48	47	64	49	50	45	63
Statins, %	72	72	67	72	71	73	56	71	75
ACE-inhibitors, %	54	54	52	55	50	54	50	53	58
ARBs, %	24	25	19	23	32	24	28	21	33
Beta-blockers, %	25	26	14	26	14	27	6*	20	38**
Calcium-antagonists, %	27	27	33	25	43	27	33	20	48**

Table 2. Baseline clinical characteristics of study patients divided according to the median value of each CPC (a) and EPC (b) level. *p<0.05 in paired comparisons

Table 2a

Variable	All	CD34+ cells		CD133+ cells		CD34+CD133+ cells	
		Low	High	Low	High	Low	High
Number	187	93	94	82	89	84	87
Age, years	63.7±0.7	64.5±0.9	63.0±1.0	64.3±1.0	62.9±1.0	65.0±0.9	62.2±1.1*
Sex male, %	67	66	69	66	72	64	74
BMI, kg/m^2	29.5±0.4	29.2±0.5	29.8±0.5	29.0±0.6	29.7±0.5	29.2±0.5	29.5±0.5
HbA1c, %	7.9±0.1	7.9±0.1	7.9±0.1	8.0±0.2	7.9±0.1	8.0±0.2	8.0±0.1
Duration, years	10.4±0.6	10.2±0.9	10.6±1.0	11.1±1.0	10.1±0.9	11.5±1.0	9.7±0.9
Hypertension, %	84	91	78*	87	82	83	85
Dyslipidemia, %	81	75	87	80	81	82	79
Smoke, %	13	16	11	17	9	15	10
Microangiopathy							
ACR, mg/g	76.3±16.3	84.5±22.6	68.3±23.6	60.9±21.1	89.7±27.1	82.7±24.7	69.5±23.4
eGFR, ml/min/1.73 m^2	81.7±1.6	80.1±2.4	83.3±2.1	81.2±2.5	84.0±2.3	78.9±2.7	86.2±2.0*
Retinopathy, %	22	22	22	26	20	25	21
Neuropathy, %	13	14	13	17	11	18	10
Macroangiopathy							
CAD, %	15	15	15	20	12	20	11
PAD, %	17	19	14	18	15	24	9*
Subclinical atherosclerosis, %	48	53	44	51	48	51	48
Diabetes therapy							
Insulin, %	47	52	41	56	42	55	43
Secretagogues, %	42	45	38	43	42	42	43
Metformin, %	72	72	71	76	74	73	77
TZD, %	5	3	7	1	8*	2	7
Incretins, %	9	6	11	12	7	11	8
Diet alone, %	8	13	3*	6	6	5	7
Other medications							
Anti-platelet, %	49	53	46	54	51	52	52
Statins, %	72	69	74	73	72	76	69
ACE-inhibitors, %	54	56	52	60	54	54	60
ARBs, %	24	22	27	17	28	20	25
Beta-blockers, %	25	19	30	23	28	26	25

Table 2b

Variable	All	CD34^{+}KDR cells		CD133^{+}KDR cells		CD34^{+}CD133^{+}KDR cells	
		Low	High	Low	High	Low	High
Number	187	91	96	85	86	88	83
Age, years	63.7±0.7	63.2±1.0	64.2±0.9	64.0±1.0	63.2±1.0	63.2±1.0	64.0±1.0
Sex male, %	67	68	67	66	72	73	65
BMI, kg/m^2	29.5±0.4	29.5±0.5	29.4±0.5	29.5±0.5	29.2±0.5	29.4±0.5	29.3±06
HbA1c, %	7.9±0.1	8.1±0.1	7.7±0.2	8.2±0.2	7.7±0.1*	8.1±0.9	7.8±0.1
Duration, years	10.4±0.6	8.7±0.8	12.0±0.9*	9.9±1.0	11.4±1.0	8.8±0.9	12.5±1.0*
Hypertension, %	84	89	80	88	80	85	83
Dyslipidemia, %	81	73	90*	75	86	73	89*
Smoke, %	13	10	17	18	8	11	14
Microangiopathy							
ACR, mg/g	76.3±16.3	60.5±11.7	91.5±30.0	87.4±24.6	64.5±23.5	76.0±23.4	76.0±24.7
eGFR, ml/min/1.73 m^2	81.7±1.6	83.7±2.0	79.9±2.5	80.0±2.0	85.2±2.7	81.7±1.9	83.6±2.8
Retinopathy, %	22	23	21	29	17	24	22
Neuropathy, %	13	17	10	16	12	16	12
Macroangiopathy							
CAD, %	15	15	15	15	16	17	14
PAD, %	17	18	16	19	14	22	11*
Subclinical atherosclerosis, %	48	53	44	52	48	49	51
Diabetes therapy							
Insulin, %	47	53	41	55	42	55	42
Secretagogues, %	42	48	35	44	41	48	36
Metformin, %	72	76	68	72	78	72	78
TZD, %	5	8	3	2	7	3	6
Incretins, %	9	5	11	7	12	9	10
Diet alone, %	8	7	9	8	3	7	5
Other medications							
Anti-platelet, %	49	54	45	52	52	52	52
Statins, %	72	65	78*	66	79	63	83*
ACE-inhibitors, %	54	55	53	62	51	58	55
ARBs, %	24	30	19	20	26	20	25
Beta-blockers, %	25	22	27	20	31	23	29

Table 3. Logistic multiple regression analysis describing the association between CPC/EPC phenotypes and microvascular outcomes. All models are adjusted for age, sex, BMI, HbA1c, diabetes duration, hypertension, dyslipidemia, smoking, macroangiopathy, and duration of follow-up. *not significant after FDR control with the BH procedure.

	CD34+ (for 1 SD decrease)		CD133+KDR+ (for 1 SD decrease)		CD34+CD133+KDR+ (for 1 SD decrease)	
	OR (95% C.I.)	**p**	**OR (95% C.I.)**	**p**	**OR (95% C.I.)**	p
UAER	2.34 (1.36-3.32)	0.008	2.56 (1.36-3.72)	0.011	3.79 (1.75-5.58)	0.009
CKD	1.56 (0.84-2.28)	0.124	0.94 (0.30-1.57)	0.859	1.39 (0.33-2.38)	0.466
Retinopathy	1.89 (1.14-2.64)	0.020	1.28 (0.62-1.93)	0.402	1.22 (0.44-1.96)	0.571
Neuropathy	1.97 (1.03-2.90)	0.042*	2.55 (1.27-3.78)	0.018	2.28 (0.79-3.65)	0.091
Microangiopathy	2.54 (1.88-3.19)	<0.001	1.53 (1.04-2.01)	0.034*	1.90 (1.21-2.56)	0.011

Table 4. Hazard ratios (95% C.I.) for cardiovascular events in patients with low versus high relative or absolute levels of the 6 stem/progenitor cell phenotypes. All analyses of time to event were adjusted for BMI, HbA1c, hypertension, albumin/creatinine ratio, eGFR, macroangiopathy, therapy (insulin, metformin, incretins, anti-platelet agents, angiotensin receptor blockers, beta-blockers, calcium-antagonists). The analyses of time to death were adjusted for age, dyslipidemia, neuropathy, peripheral arterial disease and therapy (secretagogues, beta-blockers, calcium antagonists). *significant after BH correction.

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

Table 5. Summary of the characteristics studies included in the meta-analysis. *studies with acute CV events (excluded in subgroup analysis 1 and included in subgroup analysis 3). †studies where standardized HR was not directly provided (excluded in subgroup analysis 2, see Table S2 for statistical details). ‡studies with high risk of bias (excluded in subgroup analysis 4). §studies with high heterogeneity in CVE composite outcome (excluded in subgroup analysis 5). AMI: Acute myocardial infarction; ACS: Acute Coronary Syndrome; ESRD: End Stage Renal Disease; HD: Hemodialysis; HF: Heart Failure; MS: Metabolic Syndrome; PTCA: Percutaneous transluminal coronary angioplasty; REV: Revascularization; RES: Restenosis; CVE: Cardiovascular Events; RESHPF: High Power Field.

	Q % (1/16)	Country	Follow-up (months)	Sample size	Age (years) (Mean±SD)	Male (%)	Smoke (%)	CVD (%)	Dyslipidemia (%)	Diabetes (%)	Hypertension (%)	CKD (%)	Statins (%)	Ace-i/ARB (%)	Population	Phenotype	Outcomes (event rate/yr)
Chiang et al. 2014 †	93.3	Taiwan	48	77	68.5±14.5	81.8	48	89.6	59.7	54.5	76.6	59.7	48.5	36.3	Elective PTCA	CD34$^+$KDR$^+$	CVE (8.7%)
Pelliccia et al. 2013 †	68.7	Italy	60	155	61.1±10	59.3	26.4	21.9	45.1	14.1	47	n.a.	93.5	41.2	Elective PTCA	CD34$^+$KDR$^+$ CD133$^+$KDR$^+$	CVE (8.4%) All death (2.1%) REV (5.2%)
Briguori et al. 2010 †	81.2	Italy	24	136	63.5±13.9	74.2	30.8	36	54.4	42.6	81.6	39.7	88.2	n.a.	Elective PTCA	CFU	RES (18.1%) CVE (21.6%)
Bonello et al. 2012 †	68.7	France	6	156	64.7±11.4	75.6	29.5	n.a.	58.3	21.8	57.1	n.a.	69.9	81.4	Elective PTCA	CD34$^+$KDR$^+$	REV (34.6%)
Schober et al. 2005 ‡	50	Germany	8	17	66±8	n.a.	47	47	82.3	29.4	76.4	n.a.	82.3	64.7	Elective PTCA	CD34$^+$	RES (61.7%)
Wu et al. 2014	62.5	Taiwan	12	130	66±13	36	11	20	18	37	58	100	16	20	PTCA of HD access	CD34$^+$KDR$^+$ CD34$^+$CD133$^+$KDR$^+$	RES (28%)
Haine et al. 2014†	62.5	Belgium	6	124	61±3.5	77	26	11.2	82	n.a.	54	n.a.	n.a.	n.a.	Elective Stenting	CD34$^+$KDR$^+$	RES (n.a.)
Patel et al. 2015	68.7	UK	22.4	905	62.6±12.4	63.4	79	26.2	75	31.8	80.6		72.7	n.a.	Elective coronary angiography	CD34$^+$ CD34$^+$CD133$^+$ CD34$^+$KDR$^+$	CVE (5.4%) CV death (4.4%) All death (4.2%)
Lee et al. 2015	81.2	Korea	20	70	58.1±12.9	59	n.a.	27	31	41	87	100	29	n.a.	ESRD on HD	CD34$^+$KDR$^+$	CVE (16.2%)
Maruyama et al. 2008	75	Japan	23	216	65±11	56.4	29.6	n.a.	n.a.	48.6	72.7	100	12.5	40.3	ESRD on HD	CD34$^+$	CVE (10.3%) All death (3.1%)
Lorenzen et al. 2010 †	75	Germany	36	265	66±15	55.4	9.8	84.5	n.a.	34.3	81.8	100	56.6	29	ESRD on HD	CFU	CVE (13.7%)

	Q % (1/16)	Country	Follow-up (months)	Sample size	Age (years) (Mean±SD)	Male (%)	Smoke (%)	CVD (%)	Dyslipidemia (%)	Diabetes (%)	Hypertension (%)	CKD (%)	Statins (%)	Ace-i/ARB (%)	Population	Phenotype	Outcomes (event rate/yr)
Lu et al. 2016	75	Taiwan	50	154	69±15.3	46.7	16.2	n.a	n.a.	55.8	68.8	100	n.a.	n.a.	ESRD on HD	CD34⁺CD133⁺KDR⁺	CV death (6,3%) All death (12.2%)
Werner et al. 2005	75	Germany	12	507	66.6±10.8	67.1	22.9	39	79.3	29	85.2	17.9	55.2	55.4	Elective coronary angiography	CD34⁺KDR⁺	CVE (42.2%) CV death (4.5%) All death (8.4%) REV (32.1%)
Alba et al. 2013 †	62.5	Canada	7	156	55±11	77	n.a.	7	59	24	42	100	54	92	Chronic HF	CD34⁺KDR⁺	CVE (70.3%) All death (13.1%)
Fadini et al. 2009	68.7	Italy	34	214	56.3±13	55.1	21	34.5	44.3	36.9	52.3	9.8	n.a.	n.a.	MS+Healthy	CD34⁺ CD133⁺ CD34⁺CD133⁺ CD34⁺KDR⁺ CD133⁺KDR⁺ CD34⁺CD133⁺KDR⁺	CVE (6.1%) All death (3.4%)
Schmidt-Lucke et al. 2005 ‡§	50	Germany	10	120	57.2±12.6	81.6	34.1	64.1	n.a.	14	50.8	n.a.	20	54.1	CHD+Healthy+ACS	CD34⁺KDR⁺	CVE (11%)
Shimoni et al. 2016	75	Israel	20	241	77±10	39.4	14.1	43.9	73.4	34.8	80.5	n.a.	70.1	51	Aortic Stenosis	CD34⁺KDR⁺	CV Death (9.9%)
Cuadrado et al. 2015 *	62.5	Spain	6	150	57,3±9.8	84,6	68	4	27	16	40.6	n.a.	0	n.a.	AMI+Acute Stroke	CD34⁺CD133⁺KDR⁺	CVE (25.3%)
Yu et al. 2013 *†‡§	43.7	Korea	48	40	59±6.8	50	55	n.a.	45	30	45	n.a.	100	n.a.	AMI	CD34⁺ CD133⁺	CVE (7.5%)
Padfield et al. 2013 *	62.5	UK	36	201	61±11	84	66	31.8	75	15	52	n.a.	n.a	n.a.	PTCA for ACS	CD34⁺	REV (11.6%)
Martì-Fabrega et al. 2014 *	68.7	Spain	29	121	70.1±12.6	65.3	22.2	19.8	35.5	28.1	76	n.a.	30.6	n.a.	Acute Stroke	CD34⁺CD133⁺KDR⁺	CVE (6.1%)

Table 6. Summary of characteristics of all the patients (n=4,155) included in the meta-analysis.

Follow-up months (weight mean±SD)	22.4±9.7
Age, years (weight mean±SD)	63.8±11.6
Male sex (%)	63.7
Smoke (%)	39.9
Previous CVD (%)	34.5
Dyslipidemia (%)	62.8
Diabetes (%)	31.5
Hypertension (%)	69.7
Statins (%)	58.4
Ace-i/ARB (%)	50.5