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CLINICAL REVIEW
Vascular biology and medicine

Circulating stem cells and cardiovascular outcomes: from basic science to the clinic

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The cardiovascular and haematopoietic systems have fundamental inter-relationships during development, as well as in health and disease of the adult organism. Although haematopoietic stem cells (HSCs) emerge from a specialized haemogenic endothelium in the embryo, persistence of haemangioblasts in adulthood is debated. Rather, the vast majority of circulating stem cells (CSCs) is composed of bone marrow-derived HSCs and the downstream haematopoietic stem/progenitors (HSPCs). A fraction of these cells, known as endothelial progenitor cells (EPCs), has endothelial specification and vascular tropism. In general, the levels of HSCs, HSPCs, and EPCs are considered indicative of the endogenous regenerative capacity of the organism as a whole and, particularly, of the cardiovascular system. In the last two decades, the research on CSCs has focused on their physiologic role in tissue/organ homoeostasis, their potential application in cell therapies, and their use as clinical biomarkers. In this review, we provide background information on the biology of CSCs and discuss in detail the clinical implications of changing CSC levels in patients with cardiovascular risk factors or established cardiovascular disease. Of particular interest is the mounting evidence available in the literature on the close relationships between reduced levels of CSCs and adverse cardiovascular outcomes in different cohorts of patients. We also discuss potential mechanisms that explain this association. Beyond CSCs' ability to participate in cardiovascular repair, levels of CSCs need to be interpreted in the context of the broader connections between haematopoiesis and cardiovascular function, including the role of clonal haematopoiesis and inflammatory myelopoiesis.

Keywords Stem cells • Outcomes • Biomarkers • Regeneration • Inflammation • Bone marrow • Haematopoiesis • Myelopoiesis

Biology of circulating stem/progenitor cells

Circulating stem cells (CSCs) are a heterogeneous cellular population within peripheral blood (PB) with different anatomical and developmental origins. Haematopoietic stem and progenitor cells (HSCs/HSPCs) constitute the most abundant and best-characterized CSC type. Haematopoietic stem cells generate all cells of blood lineage while retaining the ability to divide and self-maintain.[1] According to the most recent theories of haematopoiesis, a heterogeneous population of HSCs remains flexible to give rise to lineage-restricted progenitors (HSPCs) through a continuum of undifferentiated states.[2] Murine HSCs emerge in the aorta/gonad/mesonephron region of the embryo. Specialized haemogenic endothelial cells in the ventral wall of the dorsal aorta undergo endothelial-to-haematopoietic transition and detach into the circulation, reaching the liver. There, HSCs proliferate and expand before finally colonizing the bone marrow (BM) and installing haematopoiesis within a dedicated and specialized niche.[3]

Haematopoietic stem cells/HSPCs retain such migratory activity during adulthood, where they freely circulate in the blood and can be found in various organs (e.g. the thymus, intestine, liver, lungs, kidneys, skin).[4–7] Although some of the mechanisms governing HSPC migration are known, the reasons why they are released from the BM, and their biological role in PB remain largely unknown. It has been hypothesized that HSPC traffic allows their better relocation at preferred BM niches, thereby helping normal haematopoiesis. Indeed, the migration of HSPCs out of the BM follows a circadian rhythm,[8,9] and the continuous trafficking of HSPCs between the PB

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and BM is a mechanism to redistribute and replenish the depleted BM niches.[6] Further, circulating HSPCs have patrolling functions in tissues where they tend to stay longer and help fight infections and promote tissue repair when exposed to noxious stimuli.[4] Haematopoietic stem cells unexposed to such stimuli return to PB via the lymph.[4] Finally, HSPCs can contribute to peripheral tissue homoeostasis, for example, by regulating vascular repair and regeneration.[4,10,11] This hypothesis, which is relevant to the relationship between CSCs and cardiovascular outcomes, is supported by the aforementioned ontologic overlap between the haematopoietic and vascular systems.[12] The existence of haemangioblasts in post-natal life has long been debated.[13] Since the first ground-breaking description of endothelial progenitor cell (EPC) isolation from the adult PB,[14] there has been a debate on the persistence of these ancestral progenitors in adulthood (Supplementary material online, *Discussion S1*).[15]

Some investigators have hypothesized that, upstream of HSCs in the stem cell hierarchy, embryonic-like cells persist in the adult organism.[16] Such very small embryonic-like (VSEL) cells would be able to differentiate into cells of the three germ layers, thereby providing great regenerative potential. However, VSELs are extremely rare in the circulation and their phenotype in humans is still elusive.[17] Residing in the $CD45^{neg}CD34^{bright}$ population, VSELs probably overlap at least in part with the EPC progeny.[18]

Vascular smooth muscle cell (SMC) progenitors among CSCs has been hypothesized, but such cells have been then redefined as 'SMC-like macrophages'.[19] Biological implications of circulating SMC progenitors have not been fully elucidated, being involved in either reparative (e.g. angiogenesis) or pathologic (e.g. atherosclerosis and fibrosis) processes in the cardiovascular system.

Several authors reported the ability of blood cell subtypes to act as osteoprogenitors. The identity and origin of circulating osteoprogenitors (COP) are described in Supplementary material online, *Discussion S2*. Circulating osteoprogenitor may assume a pro-reparative function (e.g. by differentiation into bone, fat, and cartilage) or a detrimental effect (e.g. by inducing ectopic calcification). In the cardiovascular system, COPs may be involved in the so-called bone-vascular axis, by representing one of the links between bone disease and vascular calcification.[20]

In summary, it should be assumed that most CSCs are haematopoietic in nature and based on some but not all data available, HSPCs retain an overlap with or give rise to EPCs in adulthood as the haemangioblast does in the embryo. However, endothelial potential of adult HSPCs is incomplete, whereas circulating cells with true endothelial differentiation capacity likely derive from the vasculature itself (*Figure 1*). Other cells of haematopoietic origin, like monocytes-macrophages, are endowed with great plasticity, being able to assume endothelial-like, SMC-like, or pro-calcific potential. Such cells are devoid of stem cell features but they nonetheless can be important actors in cardiovascular homoeostasis.

Clinical implications of circulating stem cells

Over the last two decades, CSCs have been extensively studied in three main areas of cardiovascular research: (i) physiological contribution to myocardial and vascular homoeostasis; (ii) experimental and human cell therapies for the treatment of cardiac or peripheral ischaemia; and (iii) clinical biomarkers for diagnosis and prognosis (*Figure 2*). Although the present review is mostly focused on the relationships between CSCs and cardiovascular outcomes, we will briefly review the status of research in these three areas.

The ground-breaking discovery in 2001 that extra-cardiac cells repopulate the infarcted myocardium[21] has spawned a multitude of studies on both the cardiovascular regenerative potential of BM-derived cells and their therapeutic potential. The extent to which BM-derived CSCs physically contribute to cardiovascular repair has been redefined and appears to be much more limited than previously believed (Supplementary material online, *Discussion S3*). There are, nonetheless, several proof-of-concept studies investigating the relationships between CSCs and cardiovascular repair and regeneration. Beyond their ability to physically become cardiomyocytes, CSCs exert much of their activity via paracrine signals.[22] Indeed, cell suicide-based studies have shown that BM-derived cells recruited after experimental acute myocardial infarction (AMI) need to remain physically located within the myocardium for a short period of time[23] and may no longer be required after they have exerted their paracrine effect. The physical contribution of CSCs to peripheral endothelial cell repopulation has also been debated, with different results obtained in the ischaemic[24–26] vs. the non-ischaemic[25,27] microcirculation and conductance arteries.[21,22] Yet, most pro-angiogenic effects of CSCs in peripheral ischaemia appear to be mediated by secretory activity: for example, when injected in mice with hind-limb ischaemia, exosomes from human $CD34^{+}$ CSCs mimicked the beneficial activity of their parent cells,[28] thereby making the physical contribution of CSC to the growing vasculature dispensable.

Over the years, preclinical findings with BM-derived CSCs, mainly HSPCs and EPCs, have propagated several clinical trials of BM-derived cell therapy for cardiac or peripheral arterial disease. Although a detailed review of this topic is beyond the scope of this article, in Supplementary material online, *Discussion S4*, we summarize conclusions from the most recent meta-analyses of clinical trials. Overall, positive results of cell therapy in chronic ischaemic heart and peripheral arterial diseases support the scientific claim that CSCs are involved in cardiovascular homoeostasis.

Identification and enumeration of circulating stem cells in the clinical context

The discovery that CSCs have the capacity for vasculogenic cell differentiation and angiogenesis has prompted clinical translational studies in human subjects.[14] As detailed in Supplementary material online, *Discussion S5*, there are two broad approaches for characterizing CSCs in the clinical context: (i) culture or colony forming assays of PB cells; and (ii) flow cytometric analysis of circulating mononuclear cells expressing specific surface antigens that identify populations enriched for CSCs.[29] Culture assays are expensive, time consuming, and have low throughput, but allow isolation of cells for further analyses. Flow cytometry is a relatively inexpensive, rapid, and high-throughput method for identifying and quantifying CSCs. In general, HSPCs are identified based on surface expression of haematopoietic markers CD34 and/or CD133. Further specification staining for VEGF receptor-2 (or KDR) or the chemokine receptor CXCR4 identifies

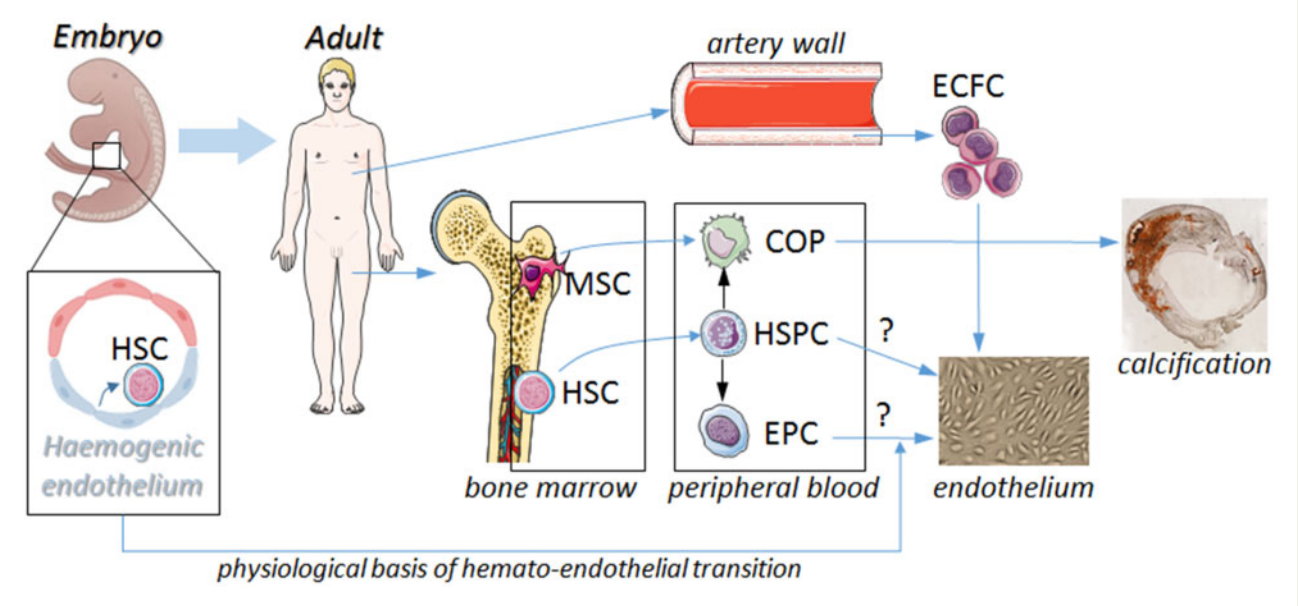

Figure 1 Origin and fate of circulating stem cells in the vascular system. In the embryo, haematopoietic stem cells arise from the haemogenic endothelium. This haemato-vascular overlap explains why haematopoietic stem cell, haematopoietic stem/progenitor cells, and endothelial progenitor cells are endowed with vascular tropism. In the adult, haematopoiesis transfers to the bone marrow, where two stem cell compartments, haematopoietic stem cells, and mesenchymal stem cells, contribute to circulating stem cells. Circulating osteoprogenitors can arise from both haematopoietic stem/progenitor cells and mesenchymal stem cells and may contribute to ectopic vascular calcification observed within advanced atherosclerotic plaques. In parallel, the arterial wall contains a hierarchy of vascular stem cells that can be isolated from peripheral blood as endothelial colony forming cells. While the endothelial differentiation capacity of endothelial colony forming cell is well-established *in vitro* and *in vivo*, it is probably less efficient for endothelial progenitor cell and haematopoietic stem/progenitor cell (question mark).

cells with vascular tropism (including EPCs) and homing capacity, respectively, but yields lower cell counts. Enumeration of more specific but rarer CSC phenotypes (such as EPCs) is intrinsically burdened by lower technical reliability,[30] with no clear benefit in terms of clinical prognostic information. To date, which CSC phenotype is most strongly associated with diagnostic and prognostic utility remains unclear (Supplementary material online, *Table S2*). In the following section, we discuss the clinical implications of changes in CSC levels with the natural history of cardiovascular health and disease (*Figure 3*).

Cardiovascular risk factors and circulating stem cells

The relationship between CSCs and cardiovascular disease (CVD) risk factors is complex.[31–42] Advancing age, one of the strongest determinants of cardiovascular risk, correlates inversely with CSC function and number.[32,33] Leucocyte telomere length, a marker of biological aging, is also correlated with CSC counts.[34] In general, this reflects the well-known age-related changes occurring in the haematopoietic system.[43] In a recent study of over 2500 subjects, an overall age-related decline in CSC counts was found, but such decline was observed only in patients with multiple cardiovascular risk factors. In contrast, there was no apparent decline of CSCs with age among healthy individuals free of cardiovascular risk factors.[31] Moreover, young subjects with risk factors had higher CSC counts compared to age-matched healthy subjects, but a similar risk factor burden in older subjects was associated with a decline in CSCs.[31] This seems to imply that risk factor exposure at a young age may stimulate the BM to release CSCs into the peripheral circulation, presumably in response to the risk factor-mediated vascular injury. After decades of sustained mobilization, the endogenous reparative potential may be exhausted, and the CSC count then declines. This model is supported by experimental data demonstrating that the atheroprotective and vascular reparative effects of CSCs declines with both aging and prolonged exposure to cardiovascular risk factors in a murine model of atherosclerosis.[44] In the clinic, where most patients have advanced age and multiple risk factors, this adverse impact on BM-derived CSCs is thought to limit effectiveness of autologous cell therapies.[45]

Among non-modifiable risk factors, biological sex and ethnicity show complex relationships with CSC counts that can offer insight into the association between demographics and cardiovascular outcomes (Supplementary material online, *Discussion S6*).

Exposure to virtually any of the known modifiable cardiovascular risk factors impacts CSC or EPC-like phenotypes. For example, accelerated CSC senescence and impaired endothelial repair capacity have been demonstrated among subjects with essential hypertension.[37,38] Cigarette smoking impairs CSC functional activity and smoking cessation increases circulating CSCs among those who smoke chronically.[39,40] Oxidized low-density lipoprotein induces human EPC senescence *in vitro* as well.[42] Diabetes is one of the traditional risk factors most strongly associated with quantitative defects and functional impairment of CSCs, including EPCs.[46–48] Several

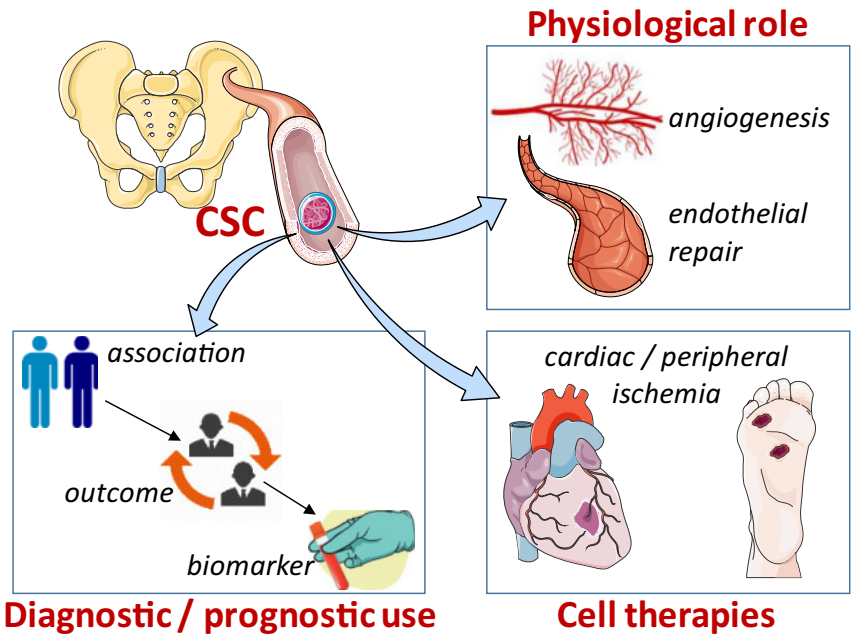

Figure 2 Major topics in circulating stem cell research. Three areas of research related to bone marrow-derived circulating stem cells include exploration of their (i) physiologic role in ischaemia, angiogenesis and vascular repair; (ii) therapeutic potential for treatment of cardiac or peripheral ischaemia; and (iii) utility as diagnostic and/or prognostic markers.

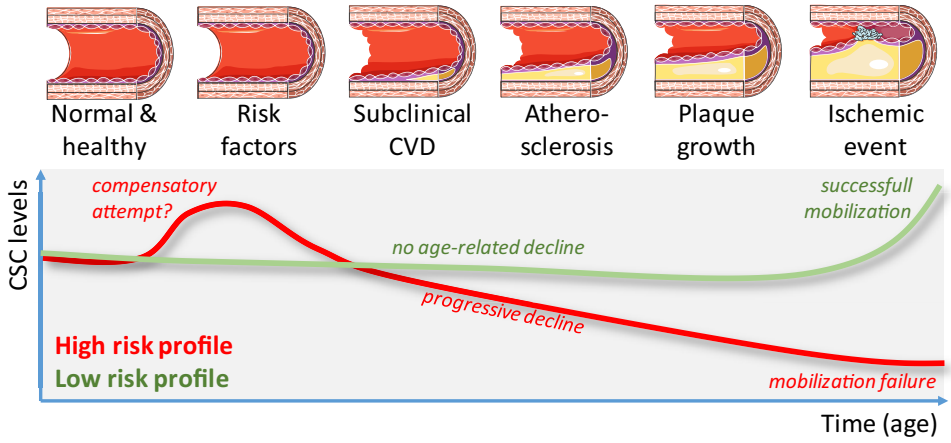

Figure 3 Circulating stem cells and the natural history of atherosclerosis. Changes in circulating stem cells throughout the lifespan with either healthy aging or aging with cardiovascular risk factors or cardiovascular disease. The green line shows no appreciable age-related decline in circulating stem cell in individuals free of cardiovascular risk factors, with an ability to mobilize circulating stem cells during injury. The red line shows age-related trends in circulating stem cells in individuals exposed to cardiovascular risk factors or cardiovascular disease, characterized by a higher circulating stem cell level when young due potentially to risk factor-mediated compensatory mobilization, followed by progressive age-related decline due to exhaustion, and an impaired mobilization response to injury. CVD, cardiovascular disease.

studies have consistently reported a reduction of $CD34^+$ CSCs and other progenitor cell phenotypes in the PB of patients with Type 1 or 2 diabetes vs. controls.[49] This alteration occurs early in the natural history of Type 2 diabetes, is only partially reversible with glucose control,[50] and becomes more profound in long-standing complicated diabetes.[51] Paradoxically, in overweight/obese individuals, an increase in CSC counts was shown to predict worsening insulin resistance.[52] Again, this suggests that a transient early phase of BM stimulation to release CSCs is associated with worse outcomes and then followed by a diseased state of CSC pauperization.

Owing to the different methods and populations investigated in each study, it is hard to establish a hierarchy of risk factors in terms of their negative impact on CSCs. However, it is important to remember that multiple cardiovascular risk factors cluster together in the same patients. The effects of combined risk factors on CSCs have been examined in the setting of the metabolic syndrome: increasing number of metabolic syndrome components was linearly related to a progressive decline in $CD34^+$ CSCs,[35] which may be related to systemic inflammation.[35,36,41] Thus, combined rather than individual effects of risk factors can restrict the number of CSCs and drive the subsequent development or progression of CVD.

Circulating stem cells among patients with subclinical and established cardiovascular disease

Exposure to cardiovascular risk factors leads to subclinical CVD, such as endothelial dysfunction, estimated as impaired arterial flow-mediated dilation, increased carotid intima-media thickness (CIMT), and development of coronary artery calcifications. Higher numbers of CSC-colony forming units were associated with better endothelial function,[53] and circulating counts of $CD34^+VEGFR2^+$ cells were inversely correlated with CIMT,[54,55] both well-established markers of increased cardiovascular risk.[56,57]

Subclinical CVD progresses to symptomatic CVD over time and manifests as coronary artery disease (CAD), heart failure (HF), peripheral artery disease (PAD), or cerebrovascular disease. In general terms, CSC counts and function continue to decline with this progression.[35,58] The higher the degree of atherosclerotic plaque stenosis in the peripheral and cerebrovascular district, the lower the levels of circulating CSCs/EPCs (*Figure 3*).[59] Yet, many acute events are caused by unstable plaques that are not necessarily the most stenotic. A double-edged role has been theoretically proposed for CSCs in plaque stability, being either protective by promoting endothelial integrity or detrimental by inducing intra-plaque angiogenesis and abrupt plaque growth.[60] The observation that EPC injection can exacerbate unstable plaque features in mice is not substantiated by data from cell therapy trials in humans.[61] Although some differences in CSC, such as higher CXCR4 expression, is indeed associated with plaque instability also in humans,[62] the exact pattern of CSC alterations in the presence of unstable plaques is unknown.

However, in the setting of an acute ischaemic event, such as AMI or stroke, CSCs are mobilized from the BM to PB.[9,63–67] Ischaemia and tissue injury trigger an acute inflammatory response with hypoxia-dependent up-regulation of hypoxia inducible factor-1 alpha (HIF-1α) that in turn stimulates expression of SDF-1α/CXCL12, a homing signal for recruitment of CSCs to ischaemic tissues.[68,69] HIF-1α activation also promotes synthesis and release of VEGF that circulates in higher concentrations and stimulates nitric oxide-dependent increase in matrix metallopeptidase 9 in the BM, triggering release of CSCs into the circulation.[70–72]This increased supply of CSCs to the ischaemic tissues should enhance vascular and tissue repair.[73]

Accordingly, in patients presenting with AMI, HSPCs have been shown to be ~25% higher and EPCs were more than doubled compared to those with stable CAD,[74] findings substantiated in other studies.[63–65] Mobilization of CSCs starts within a few minutes after AMI, peaks after several days and normalizes within 60 days.[64] Repetitive episodes of transient myocardial ischaemia are associated with adaptive processes that include increased collateral formation,[75,76] a phenomenon that involves recruitment of local cells and BM-derived CSCs for vascular regeneration.[14,77] In a clinical investigation of patients with CAD with and without myocardial ischaemia during stress testing, patients without ischaemia had a 15% increase in $CD34^+CXCR4^+$ CSC count after exercise, whereas patients with myocardial ischaemia had an 18% reduction post-exercise, and this CSC decrease was proportional to the magnitude of ischaemia and to the change in circulating SDF-1α level.[78] Thus, impaired availability of BM-derived CSCs after acute ischaemia may well result in worse outcomes, as discussed later.

Circulating stem cells in peripheral arterial disease and heart failure

In patients with diabetes, who are particularly at risk for PAD,[79] a strong progressive reduction of CSCs and EPCs was found with increasing severity of atherosclerosis obliterans and carotid atherosclerosis.[35] Peripheral blood levels of $CD34^+$ HSPCs and $CD34^+VEGFR2^+$ EPCs were found to be reduced in patients with evidence of both CAD and PAD compared to subjects with CAD alone,[80] findings that were confirmed by other studies.[35,81,82] Subjects with low levels of both $CD34^+$ and $CD34^+VEGFR2^+$ counts had a 65% higher odds of having PAD and CAD compared to those with CAD, indicating that reduction in EPC populations, in particular, is associated with more extensive multi-site atherosclerosis.[80]

There have been conflicting reports on the relationships between CSC numbers and presence of HF or its severity in small populations.[83–87] However, in a study involving over 1500 patients, compared to patients without HF, those with HF had significantly lower circulating levels of $CD34^+CXCR4^+$ CSCs and their levels correlated with the severity of HF estimated as New York Heart Association functional class, presence of diastolic dysfunction, left atrial size, pulmonary hypertension, and brain-derived natriuretic peptide (BNP) levels.[88] Patients with non-ischaemic cardiomyopathy had the lowest levels of $CD34^+CXCR4^+$ CSC counts, possibly due to the lack of CSC mobilization in response to ischaemic episodes. Similar level of CSC reduction was observed in patients with preserved or reduced ejection fraction.[88]

Circulating stem cells and outcomes in patients with cardiovascular disease

Several investigators have studied the prognostic significance of CSC counts among patient with cardiovascular risk factors, CAD, or other cardiovascular conditions. These have been summarized in a recent meta-analysis.[89] An updated summary of studies with sample size >100 subjects is provided in Supplementary material online, *Table S1*. Notwithstanding the differences in the assays employed, studies have uniformly reported that CSC depletion is independently associated with poor outcomes among patients with established CAD or those at high risk for CVD. In one of the largest studies to date, including over 900 patients undergoing coronary angiography for suspected or confirmed CAD, low

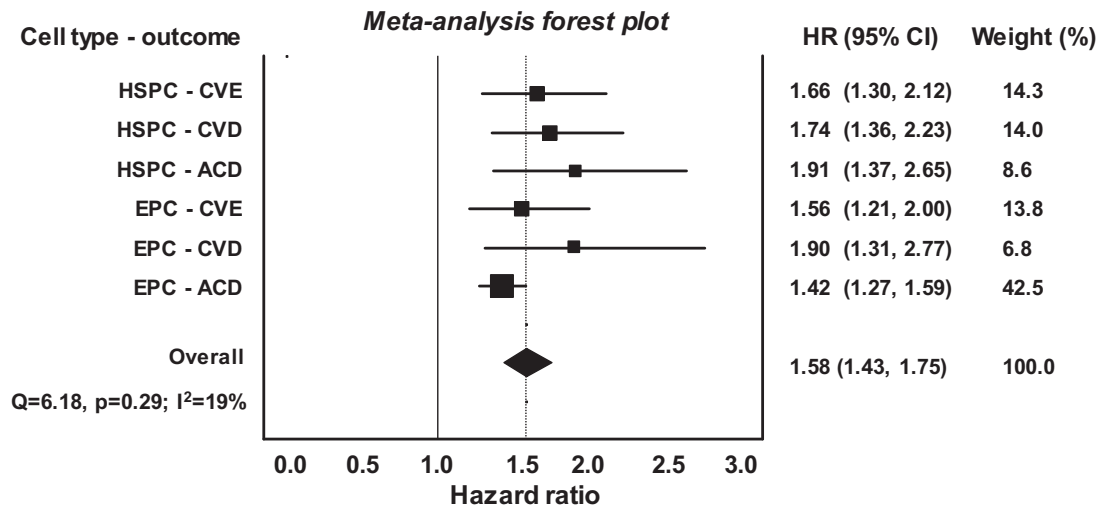

Figure 4 Prognostic role of circulating stem cells: an updated meta-analysis. The forest plot shows pooled hazard ratios derived from individual studies investigating the association between low levels (below specific cut-offs) of circulating haematopoietic stem/progenitor cells and endothelial progenitor cells and cardiovascular events (mostly defined as atherosclerotic events or cardiovascular death), cardiovascular death, or all-cause death. Pooled hazard ratio, their 95% confidence intervals and attributed weights, calculated using the random effect model are shown. Tests for heterogeneity among summary statistics (Q and I^2) were not statistically significant. An expanded version of the figure is provided in the Supplementary material online, *Appendix*.

$CD34^+$, and $CD34^+CD133^+$ CSCs were independently associated with a two- to three-fold higher mortality risk over a 22-month follow-up.[90] In patients presenting with an acute coronary syndrome, low $CD34^+$, $CD34^+CD133^+$, and $CD34^+CXCR4^+$ CSCs, but not $CD34^+VEGFR2^+$ EPCs, had similar predictive value for recurrent AMI or death.[74] The magnitude of $CD34^+CD133^+VEGFR2^+$ EPCs mobilization during ischaemic injury or infarction appears to correlate with the magnitude of tissue recovery including recovery of left ventricular function and mortality after AMI, or improvement of neurological function after stroke.[66,67,74,91–93]

As noted above, a low $CD34^+VEGFR2^+$ EPC count was an independent predictor of PAD prevalence, and also predicted future adverse cardiovascular and limb events.[80] In HF, studies have reported that low CSC counts are associated with all-cause and cardiovascular mortality[88,94]: low $CD34^+CXCR4^+$ CSC counts in HF patients emerged as independent predictors of premature mortality; low $CD34^+VEGFR2^+$ EPC levels predicted adverse outcomes in the heart failure with preserved ejection fraction (HFpEF) but not in the heart failure with reduced ejection fraction (HFrEF) population.

The prognostic value of CSCs against the occurrence of cardiovascular events has also been studied in patients with aortic stenosis, diabetes, metabolic syndrome, and end-stage renal disease (Supplementary material online, *Table S1*). Low $CD34^+VEGFR2^+$ EPCs portended adverse cardiovascular outcomes in patients with aortic stenosis.[95] Additionally, low $CD34^+$, $CD133^+$, and $CD34^+CD133^+$ HSPCs are independently associated with a nearly two-fold risk of cardiovascular events in diabetic individuals,[96] and CSC depletion was associated with adverse cardiovascular outcomes among Asian patients on haemodialysis.[97,98] *Figure 4* shows the forest plot of an updated meta-analysis of longitudinal studies involving 100+ patients (from Supplementary material online, *Table S1*) and reporting poolable estimates of the hazard ratio for cardiovascular events, cardiovascular death, and all-cause death associated with low CSC levels.

To evaluate to what extent CSC levels improve cardiovascular risk stratification, an early analysis of pooled patient-level data from five longitudinal studies reported that baseline CSC count, when added to a standard risk assessment model, improved discrimination of patients who will undergo a future cardiovascular event.[99] To this end, specific metrics were used to estimate discrimination of patients who experienced adverse cardiovascular outcomes compared to those who did not, demonstrating that CSC measures helps in risk stratification. These include C-statistics applied to longitudinal data, net reclassification improvement (NRI) and the integrated discrimination improvement index.[100] Based on NRI, addition of CSC count to a fully adjusted risk model including hsCRP allowed a better reclassification of up to 20% of patients into the appropriate risk category.[99] This finding, confirmed by others,[90] supports the potential use of CSCs in improving risk prediction. Although further large, prospective, long-term studies are needed to confirm usefulness of CSCs to improve risk stratification, we envisage a future when CSCs will be incorporated into clinical practice for more precisely predicting individual risk of adverse cardiovascular outcomes.

Impact of cardiovascular pharmacotherapies and lifestyle interventions on circulating stem cells

Pharmacologic intervention on cardiovascular risk factors is able to modulate CSC counts and activity.[101] For example, antihypertensive agents, including angiotensin-II receptor blockers,[102,103] angiotensin

converting enzyme inhibitors,[104] and calcium channel blockers,[105] were all able to increase CSC counts and activity. Atorvastatin treatment in patients with stable CAD augmented CSC number and function, underlining another pleiotropic effect of statins.[106] Notably, statins may exert a wider variety of effects on cellular stemness properties, not limited to CSCs.[107,108] Optimization of glycaemic control in diabetes can restore CSC levels, especially EPCs, towards levels seen in non-diabetic individuals. This effect is independent from the glucose lowering strategy used, examples being insulin or SGLT-2 inhibitor use,[109,110] but glucose control takes up to 6 months to translate into an improvement in EPC levels. In contrast, DPP-4 inhibitors can increase EPC levels in just a few days by raising PB concentrations of SDF-1α and generating an SDF-1α gradient from the BM to PB that allows CSCs to be readily mobilized.[16,111,112]

Among lifestyle interventions, smoking cessation seems to be able to revert CSC reduction observed in cigarette smokers.[39] Physical activity has the potential to increase CSC levels,[113] especially in patients with CVD.[114] The mechanisms involved are largely unknown, but the different effects exerted by different exercise types and intensity suggest that ischaemia is needed to achieve cell mobilization from the BM.[115] Finally, weight loss augments the lower CSC count and function observed in obese subjects.[116,117]

The opportunity to rescue CSC levels in patients with or at risk for CVD suggests that the cardiovascular protection exerted by risk factor control is at least in part mediated by CSCs. We wish to underline that causality in human studies linking CSCs to adverse events is not yet established. Beyond animal experiments, causality assessment in clinical research can be derived from Mendelian randomization studies or specific therapeutic approaches. Genetic determinants of CSC levels are largely unknown, prohibiting Mendelian randomization. Also, there is no firm demonstration of whether and how therapeutic strategies aimed at increasing CSCs have direct cardiovascular protective effects. Alternatively, by measuring CSC levels at baseline, during and after pharmacologic and non-pharmacologic interventions against cardiovascular risk, one could verify whether patients showing a positive CSC response are those most protected from adverse outcomes.

Mechanisms linking circulating stem cell with cardiovascular outcomes

The links between reduced levels of CSCs and poor cardiovascular outcomes could be easily explained by the wealth of observations that BM-derived CSCs can contribute to cardiac and vascular homoeostasis either by trans-differentiation or through secretion of factors that regulate local tissue responses to damage. However, a general scepticism has developed around the possibility that BM-derived cells truly participate in cardiovascular repair. Thus, in the next paragraphs, we discuss the role of CSCs within a more complex interplay between the haematopoietic and cardiovascular systems.

Stem cells, myelopoiesis, and cardiovascular disease progression

Atherosclerosis is associated with elevations in circulating neutrophils and inflammatory monocytes.[118–120] Mounting evidence suggests that enhanced haematopoiesis with a myeloid bias contributes to the development and progression of CVD by increasing the number of inflammatory leucocytes,[121] involving activation and mobilization of HSCs/HSPCs (*Figure 5*). In hypercholesterolaemia, increased accumulation of cholesterol in BM-HSC membrane enhances sensitivity to IL-3 and GM-CSF, stimulating myelopoiesis.[122] Hypercholesterolaemia also increases G-CSF levels, allowing greater HSC mobilization and extra-medullary haematopoiesis, especially in the spleen.[123] This state in mice has its striking parallel in the transient increase of CSC levels observed in patients exposed to risk factors at a relatively young age. Quite interestingly, unstable atherosclerotic plaques that eventually rupture and cause acute events may further fuel myelopoiesis in a feed forward cycle.[124] In addition to hypercholesterolaemia, other risk factors contribute to CVD via mechanisms involving myelopoiesis and HSC mobilization. Hypertension is associated with enhanced haematopoiesis and elevated leucocyte counts.[125–127] Imbalances between the 'pro-inflammatory sympathetic' and 'anti-inflammatory parasympathetic' arms of the autonomic nervous system in spontaneously hypertensive rats[128–130] drives HSC mobilization.[131] Also, neutrophils in the BM express β-adrenergic receptors, whose stimulation results in secretion of various proteases that allow HSC mobilization and subsequent leucocytosis.[131] Studies also implicate the renin–angiotensin system (RAS) in the regulation of myelopoiesis.[132,133]

Supplementary material online, *Discussion S7* illustrates how activation of the sympathetic nervous system in various clinical conditions, including stress and sleep deprivation, influences myelopoiesis and HSPC mobilization, and how this can affect cardiovascular risk.

Diabetes mellitus is associated with higher number of leucocytes including neutrophils and monocytes with increased tendencies to enter atherosclerotic plaques and drive disease progression.[134–137] Hyperglycaemia sustains leucocytosis via proliferation of BM myeloid progenitors, [135] driven by damage associated molecular patterns released from activated neutrophils. In Type 2 diabetes and obesity, myelopoiesis is rather driven by inflammatory cytokines (e.g. IL-1β) released by adipose tissue macrophages.[138]

Inflammation, myelopoiesis, and circulating stem cell defects

The mechanisms driving reduction of CSCs in patients at risk for or with CVD have not been clearly dissected. However, in the setting of diabetes, the extremely consistent reduction in CSCs appears to emerge from an impaired mobilization from the BM to PB.[139] Diabetes induces an extensive remodelling of the BM-HSC niche, with microangiopathy, sympathectomy, and fat infiltration.[47] These alterations could be sufficient to impair HSPC traffic, which relies on the specialized BM microvasculature and sympathetic innervation. Diabetes does not lead to a pauperization of intra-marrow HSPCs, which can be readily mobilized by blocking CXCR4.[140] Yet, in experimental and human diabetes, physiologic CSC mobilization by tissue ischaemia or growth factors (like G-CSF) is inhibited.[139] Recently, it has been found that hyperglycaemia-induced myelopoiesis drives the expansion of BM macrophages, which produce excess amounts of oncostatin M, acting against HSPC mobilization. Blocking myelopoiesis, oncostatin M production or signalling was able to restore normal mobilization in experimental diabetes.[141] These new findings provide

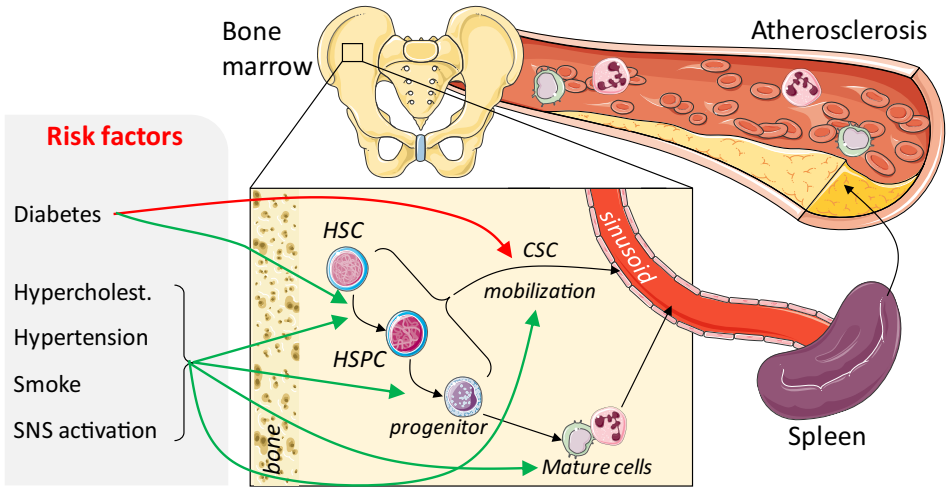

Figure 5 Interplay among risk factors, stem cells, myelopoiesis, and atherosclerosis. Most traditional risk factors for atherosclerotic cardiovascular disease have been associated with leucocytosis due to excess myelopoiesis in the bone marrow. With the exception of diabetes, risk factors also result in an enhanced mobilization of circulating stem cells from the bone marrow to peripheral blood. Diabetes instead causes an impaired ability to mobilize stem cells and a depletion of circulating stem cells. Circulating stem cells can be buffered in the spleen, where haematopoietic stem cells and haematopoietic stem/progenitor cells establish extra-medullary haematopoiesis, providing a peripheral reservoir of inflammatory cells. Thus, the extent to which risk factors stimulate haematopoietic stem/progenitor cell mobilization may not be related to circulating stem cell levels in the circulation. Mature inflammatory cells generated in the bone marrow and/or the spleen can reach the vessel wall and contribute to atherosclerosis development or progression. HSC, haematopoietic stem cells; HSPC, haematopoietic stem and progenitor cells; SNS, sympathetic nervous system. Green arrows indicate stimulatory effects of risk factors, whereas red arrows indicate inhibitory effects.

a link between chronic low-grade inflammation typically observed in diabetes and CSC defects. Thus, the underlying inflammatory state, along with low CSC levels, would altogether be responsible for accelerating atherosclerosis and cardiovascular events. Although this pathway has been demonstrated in the setting of overt hyperglycaemia, it is possible that inflammation elicited by other triggers commonly present in patients with cardiovascular risk factors exert similar effects on CSCs.

Clonal haemopoiesis, circulating stem cells, and cardiovascular disease

With biological aging, DNA of the ever-replicating HSPCs undergoes somatic mutations in hotspot genes (typically DNMT3A, TET2, ASXL1, and JAK2), resulting in the so-called clonal haematopoiesis of indeterminate potential (CHIP). Most patients with CHIP will never develop cancer, but display a two- to four-fold higher risk of CAD, stroke, and CVD death.[142] The mechanisms linking CHIP with CVD are still being investigated, but recent evidence in mouse models suggest that Tet2 mutation drives inflammatory myelopoiesis that in turn propagates to atherosclerotic plaques.[143] Interestingly, not only CHIP but also epigenetic modulation of stem cells, including those provided by microRNAs, can modify their differentiation trajectories to pathways linked with cardiovascular disease.[144,145] No study has so far explored the interplay among CHIP, epigenetic changes, CSC levels, and cardiovascular outcomes, but we speculate that myelopoiesis resulting from CHIP reduces CSCs with mechanisms similar to those demonstrated in diabetes.[146] Thus, excess myelopoiesis emerges as a possible common denominator of the link between CSCs and adverse cardiovascular outcomes. Of note, myeloid bias, i.e. preferential differentiation of HSPC towards the myeloid vs. the lymphoid lineage, is a typical feature of aging.[147] Thus, CHIP, myelopoiesis, and reduced CSCs may be biologically interconnected, thereby contributing to adverse cardiovascular outcomes by common and independent mechanisms.

Next steps to leverage circulating stem cells as a cardiovascular risk biomarker

Several studies have shown that measuring CSC levels can yield information useful for cardiovascular risk stratification. There are some reasons why CSCs have not yet been incorporated into clinical practice as a risk biomarker (Supplementary material online, *Table S2*). First, CSCs need to be quantified in fresh blood samples using expensive flow cytometry instruments that are not readily available in all clinical centres. In addition, an agreement on which is the CSC phenotype provided with the greatest prognostic power is lacking. Although inter-laboratory standardization exists for the quantification of $CD34^+$ HSPCs,[148] it has never been applied in the study of cardiovascular outcomes on a large scale. These technical issues differentiate CSCs from biomarkers that can be easily measured in frozen plasma/serum samples with inexpensive assays, like hsCRP. Even detecting CHIP, although still expensive, can be performed in frozen blood samples at core laboratories. Availability of newer fixation reagents for delayed analysis[149–151] and simplified cytometry

instruments[152] will allow collaborative efforts to collect CSC data from large multicentre and multinational cohorts of diversified patient populations. Ideally, development of rapid point-of-care diagnostics for measuring $CD34^+$ cell quantification would really make CSCs a clinical-grade biomarker.

Conclusions and future directions

The intimate ontological relationships between the haematopoietic and vascular systems leaves a legacy in the adult organism, where BM-derived cells play a major role in regulating cardiac and vascular pathology. Among such cells, CSCs have been studied in physiological, therapeutic, and prognostic settings. Supplementary material online, *Table S2* provides a summary of what we believe will be future topics on the study of CSCs in the cardiovascular system. We acknowledge that the concepts of cardiovascular regeneration and the contribution of CSCs have evolved over the last two decades. Technological developments have illustrated drawbacks in previous research findings, warranting rigorous scrutiny. Substantial technical and conceptual challenges remain and, once addressed, will further clarify the complex interplay between CSCs and cardiovascular disease. Investment in this field has the potential for transformative advancements in basic and clinical knowledge. In addition to understanding the biological contribution of CSCs to cardiovascular homoeostasis and repair, measurement of CSCs in the clinic may improve cardiovascular risk stratification in humans throughout their lifespan.

Supplementary material

Supplementary material is available at *European Heart Journal* online.

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Conflict of interest: G.P.F. is the major inventor of a patent, hold by the University of Padova, to inhibit Oncostatin M to restore stem cell mobilization. The other authors have no conflict of interest to declare.

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