Circulating endothelial cells, microparticles and progenitors: key players towards the definition of vascular competence

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Abstract

The balance between lesion and regeneration of the endothelium is critical for the maintenance of vessel integrity. Exposure to cardiovascular risk factors (CRF) alters the regulatory functions of the endothelium that progresses from a quiescent state to activation, apoptosis and death. In the last 10 years, identification of circulating endothelial cells (CEC) and endothelial-derived microparticles (EMP) in the circulation has raised considerable interest as non-invasive markers of vascular dysfunction. Indeed, these endothelial-derived biomarkers were associated with most of the CRFs, were indicative of a poor clinical outcome in atherothrombotic disorders and correlated with established parameters of endothelial dysfunction. CEC and EMP also behave as potential pathogenic vectors able to accelerate endothelial dysfunction and promote disease progression. The endothelial response to injury has been enlarged by the discovery of a powerful physiological repair process based on the recruitment of circulating endothelial progenitor cells (EPC) from the bone marrow. Recent studies indicate that reduction of EPC number and function by CRF plays a critical role in the progression of cardiovascular diseases. This EPC-mediated repair to injury response can be integrated into a clinical endothelial phenotype defining the 'vascular competence' of each individual. In the future, provided that standardization of available methodologies could be achieved, multimarker strategies combining CEC, EMP and EPC levels as integrative markers of 'vascular competence' may offer new perspectives to assess vascular risk and to monitor treatment efficacy.

Keywords: circulating endothelial cells ● endothelial microparticles ● endothelial progenitor cells ● endothelial injury/repair ● endothelial biomarkers ● vascular competence

Introduction

Strategically located between blood and tissues, the endothelium is exposed to blood-borne mediators, biomechanical stimuli and cells from circulating blood and neighbouring tissues, which determines a switch from a quiescent to an activated phenotype. Such activated phenotype, resulting from the physiological adaptation of a given region, can also occur pathophysiologically.

It is now well recognized that disruption of endothelial integrity and physiological functions represent a pivotal mechanism in the initiation and development of cardiovascular disorders (CVD) [1]. Consequently, exploration of endothelium has raised growing interest and has led to the development of a range of methods that can be divided into functional testing and surrogate biomarkers.

The former includes endothelium-dependent vasorelaxation, arterial stiffness and pulse wave propagation, and has recently been reviewed [2]. A second category is defined by established soluble molecules reflecting alterations of the main regulatory

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functions of the endothelium such as inflammation, haemostasis or permeability. However, these markers have been disappointing by their lack of specificity and clinical relevance at the individual level. The capacity of the adherent endothelium to undergo cellular alterations has revisited our vision of the endothelium as a dynamic tissue in equilibrium with a circulating compartment reflecting both lesion and regeneration of the vascular tree. This endothelial-derived compartment has led to delineate a third group of cellular markers, corresponding to circulating endothelial cells (CEC) and endothelial-derived microparticles (EMP) released from the injured vessels and progenitors endothelial cells (EPC) involved in vascular regeneration.

Since CEC, EMP and EPC have been individually reviewed [3–8], the present review aims to summarize the novel insight provided by these emerging cellular biomarkers (*i*) in the current understanding of the endothelial dynamics between injury and repair, (*ii*) in the evaluation of vascular integrity in a specific and non-invasive way and (*ii*) in the assessment of vascular risk. This review will also discuss the promises of multimarker strategies combining CEC, and EMP and EPC as integrative markers of vascular competence, an emerging concept for vascular risk stratification and therapeutic options.

Dynamics between endothelial injury and repair: the response to injury theory 'revisited'

Initially viewed as a single-cell lining of the vascular system, the endothelium has emerged as a dynamically mutable interface locally responsive to environmental stimuli. As a consequence, reprogramming of the endothelium generates an impressive repertoire of biological responses playing a key role in the control of vascular homeostasis such as vasomotion, permeability, haemostasis, inflammation or angiogenesis [9]. According to the response to injury theory [10], mechanical damage or chronic exposure to cardiovascular risk factors (CRF) alters the regulatory functions of the endothelium, which progress towards pro-inflammatory activation, senescence and apoptosis (Fig. 1). As a consequence, the endothelium not only displays altered functions, but also loses its integrity. Microparticles released from activated or apoptotic endothelial cells [11] and endothelial cells detached from injured vessels [12] constitute a hallmark of these deleterious responses affecting the vessel wall. The response to injury theory has been enlarged by the discovery of a powerful physiological repair process based on EPC. In response to injury, regenerative mechanisms are triggered to restore endothelium integrity. In the past, endothelial repair was solely attributed to angiogenesis, involving adjacent mature EC able to replicate locally and replace the lost cells. Since the pioneering works by Asahara [13], it has become obvious that the recruitment of EPC represents an additional mechanism for endothelial repair. Recruited from the bone marrow, these cells are able to differentiate into mature cells and restore endothelial integrity at sites of vascular injury. The demonstration that patients with high EPC levels have preserved endothelial function, despite exposure to CRF, underlines their critical role in the ongoing maintenance of vascular integrity [14].

As a whole, this EPC-mediated repair constitutes the third step of the response to injury initiated by chronic activation of the endothelium in response to CRF. This spectrum of endothelial responses can be integrated in a dynamic triad 'activation/injury/repair' which has critically transformed our understanding of vascular biology (Fig. 1).
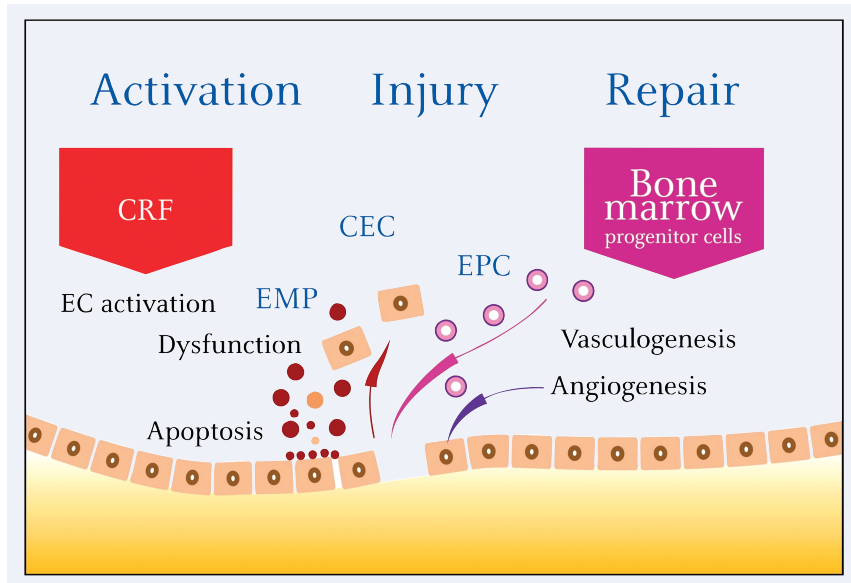
Emerging biomarkers reflecting the dynamics between endothelial injury and repair: from pathophysiology to clinical testing

Appreciation of the multifaceted biology of endothelial cells has been the basis of new assays providing the opportunity to measure endothelium integrity in a non-invasive and specific manner. Whereas the determination of CEC and EMP levels have raised considerable interest to appreciate the status of activated/damaged endothelium, EPC levels have been used to evaluate the endogenous repair potential. The comparative characteristics of CEC, EMP and EPC have been summarized in Table 1.
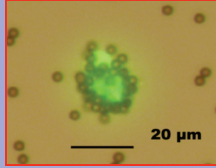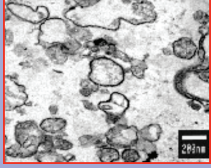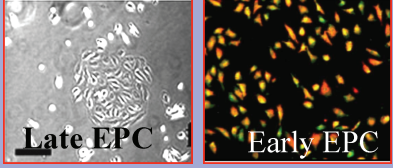
Circulating endothelial cells

Current consensus supports the view that CEC are mature cells shed from the vessel wall in response to injury [15]. These cells present a heterogeneous size (from 10 to 50 micron), express endothelial markers (von Willebrand factor [vWF], CD31, CD144) but are negative for leucocyte markers. In contrast to EPC, they do not express immature markers such as CD133, and do not give rise to cell colonies with a high proliferative potential. Due to the extreme scarcity of CEC in peripheral blood, an important step in their identification has been the development of sensitive technologies for the detection of rare events based on the immunolabelling with monoclonal antibodies to novel endothelial antigens [16]. In 1992, an antibody recognizing the CD146 antigen combined with an immuno-magnetic separation assay (IMS) allowed the immunological characterization of CEC in the peripheral blood of patients submitted to coronary angioplasty chosen as a model of vascular injury. The marked elevation of CEC number found after angioplasty, confirmed that they result from endothelial trauma triggered by the catheter procedure itself [12].

IMS assay has become the consensus protocol for CEC enumeration [17]. This method combines a first step of enrichment using CD146 coated magnetic microbeads and a second step of

Activation Injury Repair

Table 1 Comparative characteristics of CEC, EMP and EPC

	CEC	EMP	EPC
Origin	Blood vessel wall	Blood vessel wall CEC ? EPC ?	Bone marrow, other niches
Morphology	Mature cells of diameter 15–50 μm	Endothelial derived vesicles of diameter 0.1–1 μm	Immature cells of diameter less than 15 μm
Phenotype	CD31$^+$, CD34$^+$, CD105$^+$, CD146$^+$	CD144$^+$, CD146$^+$, CD62E$^+$, CD51$^+$	CD133$^+$, CD34$^+$, KDR$^+$, CD117+
	Ulex Europaeus lectin$^+$, vWF$^+$	CD31$^+$/CD42$^-$	CD146 +/−
	eNOS+	CD31$^+$/CD51$^+$	CD45 +/−
	CD45$^-$, CD133$^-$		
Detection Methods	Density centrifugation	Flow cytometry	Flow cytometry
	CD146 IMS and fluorescence microscopy	ELISA	Clonogenic assays :
	CD146 IMS and flow cytometry	Solid phase capture	- CFU-EC or Culture assay ('early EPC')
	CD146 IMS and image analysis		- HPP-ECFC ('late EPC')
	Flow cytometry		
Pathophysiopathology	Endothelial damage	Endothelial activation / apoptosis	Neovascularisation, repair

Abbreviations : CEC, Circulating endothelial cells, EMP, endothelial microparticles, EPC, endothelial progenitor cells, IMS, immuno-magnetic separation, CFU-EC, colony formit unit-endothelial cells, HPP-ECFC, high proliferation potential-endothelial colony forming cells.

cell numeration, according selected criteria, using fluorescent microscopy after cell staining with acridin orange or with the endothelial specific Ulex europaeus agglutinin 1 lectin. The rationale for cell enrichment comes from the very low number of CEC since, with IMS normal values lower than 10 cells/ml have been consensually reported [15]. Alternative methodologies for CEC counting include flow cytometry, which has been largely used to detect CEC in cancer patients [18]. However, despite an attempt of standardization recently proposed by Duda [19], normal values using flow cytometry are very heterogeneous and about 100–1000-fold higher compared to those from IMS. More recently, hybrid methods combining immunomagnetic enrichment using paramagnetic particles coated with CD146 mab followed by multiparametric identification of CEC by flow cytometry or by image analysis have been proposed [20, 21]. With these approaches CEC counts in healthy individuals are in the same order of magnitude than those reported by IMS.

Although it is clear that CEC represent mature cells, the potential mechanisms of endothelial cell detachment are probably multiple and not exclusive. Different experimental models have documented that denudation of the vessels can be triggered by mechanical injury, protease- or cytokine-mediated detachment, or activation of apoptosis. A potential mechanism is represented by anoikis, a cell death mechanism induced by lack of anchorage leading to a disruption of the interaction between cells and extracellular matrix. This can result from integrin disengagement that impairs their association with focal adhesion kinase in cells and inhibits survival signals [22]. In addition, endothelial cell detachment can also result from pericellular proteolysis and defective adhesion of cells to the extracellular matrix and to neighbouring cells that involves loss of VE-cadherin-mediated homotypic interactions [23].

Another important question is the vascular bed origin of CEC that varies according to the disease process and can be delineated using specific antigens. Besides its structural heterogeneity, the endothelium displays different functions linked to the expression of specific constitutive or inducible antigens. EphrinB2, delta-like 4 or neuropilin-1 are preferentially expressed by arterial endothelial cells whereas EphrinB4 and neuropilin-2 are markers of venous cells [24, 25]. In practice, CD36, a maker of microvessels, has been used to document the anatomical origin of CEC. In addition, E-selectin, VCAM-1 or tissue factor (TF) expression by CEC have been used to quantify pro-inflammatory and pro-coagulant activation of the vessels (Table 2). Assuming that CEC are representative of the vessel they derived from, analysis of their phenotype may provide important insights not only on their anatomical origin but also on their pathogenic involvement (see below).

Several reviews indicated that increased CEC levels have been reported in cardiovascular diseases [43, 44] such as acute coronary syndromes (ACS) [26–29] or stroke [32] and in most of their associated risk factors like diabetes [30], hypertension [32] and chronic renal failure [35]. CEC were also increased in other pathological situations associated with atherosclerotic complications including inflammatory vasculitis [39], transplantation [45] or lupus [46]. These main clinical studies indicate that CEC levels are indicative of disease activity and generally correlate with functional or plasma markers of endothelial injury (Table 2). An interesting illustration of CEC relevance, both in pathophysiology and clinical testing, has been provided by ACS. Consistent data reported by several studies indicated that myocardial infarction and unstable angina are associated with increased CEC counts compared to normal persons or patients with stable angina. In clinical testing, a multimarker strategy combining CEC and cardiac Troponin markedly improved diagnosis accuracy in patients with unstable angina [27], because CEC elevation was earlier and independent of troponin level. Besides this diagnostic interest, CEC have been proposed as a novel prognostic indicator since raised CEC levels during the first 48 hrs of ACS independently predicted both death and major adverse cardiovascular events at 30 days and 1 year [28]. Analysis of the phenotype of CEC from patients with ACS indicated that these cells are positive for TF [26], suggesting that they could disseminate a pro-coagulant potential. Consistently, high CEC levels also correlated with soluble TF, a hallmark of activation of the coagulation pathways, and with vWF, a marker of endothelial dysfunction [29]. Moreover, increased CEC were found to be associated with abnormal vascular responses, because patients with the highest CEC values presented the lowest flow-mediated dilatation [47]. Taken together these data suggest a link between endothelial detachment, vascular dysfunction and prothrombotic status. A novel insight that could change current opinion on CEC relates to their recently described capacity of apoptotic CEC to induce inflammatory signals in quiescent endothelial cells [48]. This response further support their possible pathogenic role as well as a survival mechanism by which healthy endothelium could be alerted to ongoing cell death.

Endothelial microparticles

The first description of endothelial microparticles (EMP) was done in patients with lupus anticoagulant presenting thrombotic complications [11]. They were defined as small vesicular structures with a heterogeneous diameter (from 0.1 to 1 micron), resulting from the remodelling of membrane phospholipids and expressing phosphatidylserine (PS) and antigens representative of endothelial cells. Although commonly used to define microparticles, exposure of PS, has been shown to be undetectable in a significant proportion of circulating or *in vitro* generated EMP. The lack of PS expression may either involve its unavailability for annexin V binding due to PS engagement in other molecular interactions with plasma proteins, or suggest that a vesiculation process can occur independently of membrane asymmetry loss [49].

The current knowledge on EMP composition derives mainly from experiments performed on cultured endothelial cells. Therefore caution should be made because *in vitro* studies are not necessarily representative of the tissue specific features dictated by endothelial cell localization in the vascular tree. Extensive phenotypic characterization indicated that EMP display the same molecules as their parent cells [11, 50]. For example, receptors such as TF, vascular cell adhesion molecule-1 or E-selectin were

Table 2 Changes in blood level of CEC in patients with cardiovascular and other diseases

Disease/condition	CEC changes and phenotypes			Methods	Main finding(s)	Reference
	Anatomic origin	Activation	Apoptosis			
Coronary angioplasty	↑			IMS	CEC elevation indicative of traumatic vessel wall injury	[12]
Acute coronary syndromes	↑	CD36	ICAM-1, TF+	10%	IMS	CEC elevation indicative of injury	[26]
					IMS	CEC elevation earlier and independent of troponin 1	[27]
					IMS	CEC level correlated with vWF, IL6 are predictive of MACE	[28]
Heart failure	↑				IMS	CEC correlated inversely with FMD and positively with vWF and sTF	[29]
Diabetes mellitus	↑				IMS	CEC levels independent of plasma glucose level and HbA1c	[30]
Thrombotic microangiopathy	↑	CD36			IMS	CEC level correlated with prognosis	[31]
Stroke	↑				IMS	CEC level correlated with sE-selectin and vWF	[32]
Pulmonary hypertension	↑	CD36	E sel		IMS	CEC correlated with SBP and DBP	[33]
Sickle cell anaemia	↑	CD36	ICAM-1, E sel, VCAM-1,TF+,	80%	IMS	Elevated CEC correlated with crisis	[34]
Haemodialysis	↑				IMS	CEC are predictive of cardiovascular events	[35]
Kidney transplantation	↑↓				IMS	CEC are indicative of vascular rejection	[36]
					IMS	CEC decrease 1-year post-transplant and correlated with IS treatment	[37]
HSC transplantation	↑				IMS	CEC correlated with intensity of conditioning treatment	[38]
Systemic sclerosis	↑		ICAM-1, E sel		FCM	Total and activated CEC correlated with disease activity score and the severity of pulmonary hypertension	[39]
Vasculitis	↑		TF+		IMS	CEC correlated with disease severity and with IS treatment	[40]
Systemic lupus	↑		Nitrotyrosine	100%	FCM	CEC level inversely correlated with FMD and positively with vWF and sTF	[41]
					FCM	CEC correlated with disease severity; plasma c3a	[42]

Abbreviations : IMS, CD146 driven immunomagnetic separation, FCM, Flow cytometry, E sel, E-selectin, vWF, von Willebrand factor, sTF, soluble tissue factor, FMD, Flow mediated dilatation, ACS, acute coronary syndromes, SBP, systolic blood pressure, DBP, Diastolic blood pressure, MACE, major adverse cardiovascular events, E-sel, E-selectin; HbA1c, haemoglobin A1c, HSC, haematopoïetic stem cells. IS, immunosuppressive.

detected on MP shed from tumour necrosis factor activated human umbilical vein endothelial cells (HUVEC), suggesting that EMP represented a signature of endothelial pro-coagulant and pro-inflammatory responses. The expression of PS and TF the main activator of the extrinsic coagulation pathway indicate that EMP could provide pro-coagulant surfaces for the assembly of clotting enzymes promoting thrombin generation [51]. *In vivo*, the contribution of endothelial cells to the circulating pool of TF$^+$ MP has been demonstrated in human endotoxemia [52] and patients with intravascular coagulopathies [53]. The presence of pro-coagulant EMP has also been found in atherosclerotic plaques [54] and in the circulation of patients with ACS [55]. However, MP generated from cultured endothelial cells stimulated with activated protein C harbour functional endothelial protein C receptor, protected from metalloproteinase cleavage, and display anticoagulant ability towards factor Va inactivation [56]. Although this mechanism is

still undocumented *in vivo*, it may be indicative of potential antico-agulant properties that may counterbalance prothrombotic characteristics of EMP.

In normal persons, EMP represent a minority of total circulating MP. Flow cytometry is the most widely used method to characterize EMP, but the pro-coagulant function of EMP can also be measured using functional assays (based on coagulation activation by MP derived phospholipids and/or TF), two complementary approaches that have been reviewed in a forum [57, 58]. However, as it is the case for the other MP subpopulations, their measurement remains a technical challenge due to the lack of standardization. First, several pre-analytical variables such as blood collection, sample processing, transportation and centrifugation may have a major impact on MP measurement and have not been studied in the literature. Although flow cytometry provides useful information, some limitations regarding MP measurement need to be considered such as threshold for particle size detection, standardized instrument settings or well-characterized antibodies against cell-associated antigens that are not expressed by other cell lineage.

The mechanisms controlling EMP formation remains mostly unknown. A recent study based on a gene profiling analysis has identified an original pathway of endothelial vesiculation induced by thrombin, involving the ρ-kinase ROCK-2 in the absence of cell death [59]. A better understanding of the mechanisms controlling the endothelial vesiculation is crucial for the pharmacological control of EMP release.

Taking into account the broad distribution of endothelial cells along the vascular tree, yet another open question is the anatomical site the EMP derived from, a question that relies on the availability of tissue specific endothelial markers. The possibility that they could also originate from apoptotic CEC cannot be excluded because both EMP and CEC are elevated in a number of CVD. In addition, EMP co-expressing immaturity and endothelial markers originating from apoptotic EPC have been recently identified [60].

A survey of the literature, summarized in Table 3, indicated that elevated EMP levels have been reported in CVD and other vascular settings associated with a thrombotic propensity [6, 74]. Elevated EMP levels are also associated with most of the CRF such as obesity [75], hypertension [63], diabetes [65, 66] and appear indicative of a poor clinical outcome. *In vivo* evidence that EMP are representative of the endothelial dysfunction came from convergent data demonstrating that EMP correlated with the severity of the endothelial dysfunction determined during angiography or flow-mediated dilatation in patients with end stage renal disease [67] and coronary artery disease [76]. EMP are not only a reflection of endothelial dysfunction but also may be deleterious by inducing or aggravating pre-existing vascular dysfunction, as shown by their ability to impair nitric oxide release from vascular endothelial cells [77]. The deleterious potential of EMP opens new pharmacological perspectives. Several therapies widely used for their beneficial effect in CVD, such as antioxidants, β-blockers or statins [6] also reduce EMP concentration.

Knowledge accumulated from *in vitro* and *in vivo* experiments indicated that EMP provide a storage pool of bioactive effectors able to modulate the vascular homeostasis equation. EMP enriched in both PS and TF trigger monocytic TF-dependent pro-coagulant responses *in vitro* [78] and thrombus formation *in vivo* [54, 79]. In addition, EMP amplify endothelial dysfunction by impairing nitric oxide pathways, as shown by alteration to acetyl choline-induced relaxation using the rat aortic ring model [80].

Besides their role as pro-coagulant effectors, recent findings support the concept that MP also behave as vectors of plasminogenolytic activity due to the expression of the uPA/uPAR system, a mechanism that modulates the angiogenic response of endothelial progenitors *in vitro* [81]. MP exposes other proteases such as metalloproteinase 2 and 9 that affect vascular remodelling and angiogenesis [82]. Such data have led to the concept that EMP are key factors at the crossroads between inflammation, coagulation, proteolysis and vascular repair.

Endothelial progenitor cells

Since the pioneer's work of Asahara [83] 10 years ago, increasing data have extended our knowledge on EPC biology. Two functional criteria are recognized to distinguish between EPC and CEC. *In vitro*, EPC display the ability to form adherent colonies that proliferate, and differentiate into endothelial lineage as assessed by expression of various specific endothelial antigens including KDR, UEA-1, vWF, CD146 and CD144. *In vivo*, they contribute to neoangiogenesis within ischemic sites or tumours or to vascular repair after vessel wall injury [13, 84]. Nevertheless, accumulating data demonstrate that these properties identify a heterogeneous pool of circulating cells related to their origin differentiation and functional characteristics. Within bone marrow, various stem cell populations may differentiate into endothelial progenitors including haemangioblast, a common precursor of haematopoietic stem cells and endothelial cells, multipotent adult progenitor cells or mesenchymal stem cells. Alternative sources of EPC may also include parenchymatous organ and blood vessels [85]. This heterogeneity is also demonstrated by the recognition of two distinct cell populations derived by culturing peripheral blood mononuclear cells. These two different types are usually named by the generic term of EPC, despite highly differing characteristics. According to their development in culture within 4 to 7 days, a first type is called early outgrowth colonies and exhibits spindle-shaped morphology emanating from a central cluster of cells. These cells can be maintained in culture for 2 to 3 weeks but cannot be expanded by further passaging. They express endothelial markers but also myeloid markers like CD45, and CD14 attesting for a partial endothelial differentiation and a probable monocytic origin. Recently, Yoder *et al.* confirmed that this cell type displaying macrophage/monocyte characteristics is clonally related to the haematopoietic lineage [86]. Paracrine mechanisms mainly contribute to the capacity of these cells to facilitate new vessel formation *in vivo*. In addition these cells are not able to differentiate into functional endothelium. Based on these considerations, it has been proposed that these cells should not be considered as a

Table 3 Changes in blood level of EMP in patients with cardiovascular and other diseases

Disease/condition	EMP changes and phenotypes		Methods	Main finding(s)	Ref
Acute Coronary syndrome	↑	CD146$^+$, CD31$^+$	SPC	Elevated EMP levels with a pro-coagulant activity	[55]
	↑	CD31$^+$	FCM	EMP levels correlated with high-risk lesions	[61]
Stroke	↑	CD105$^+$, CD144$^+$	FCM	Link between EMP, severity lesions and clinical outcome	[62]
Hypertension	↑	CD31$^+$/CD42$^-$	FCM	EMP levels correlated with both SBP and DPB	[63]
Pulmonaryhypertension	↑	CD31$^+$/CD41$^-$, CD144	FCM	Levels of EMP predict haemodynamic severity of pulmonary hypertension	[64]
Type 2 diabetes	↑	CD144$^+$	FCM	EMP levels correlated with coronary artery disease	[65]
Type 1 diabetes	↑	CD51$^+$	FCM	EMP levels correlated with HbA1c	[66]
End-stage renal disease	↑	CD144$^+$, CD31$^+$/CD41$^-$	FCM	High levels of EMP correlated with impaired vascular function	[67]
Antiphospholipidsyndrome	↑	CD51$^+$	FCM	EMP correlated with Lupus anticoagulant	[68]
Obesity	↑	CD31$^+$/CD42$^-$,	FCM	High levels of EMP correlated with altered FMD	[69]
Post-prandial hypertriglyceridemia	↑	CD31$^+$/CD42$^-$	FCM	EMP correlated with high fat meal	[70]
TTP	↑	CD31$^+$, CD51$^+$, CD54+, CD62E$^+$, CD105$^+$, CD106+	FCM	Elevated EMP correlated with endothelial activation	[71]
Paroxysmal nocturnal haemoglobinuria	↑	CD105$^+$, CD144$^+$	FCM	Elevated EMP reflected the inflammatorystatus of endothelial cells	[72]
Pulmonary or venous embolism	↑	CD62E$^+$, CD31$^+$/CD42$^-$	FCM		[73]
Sickle cell disease	↑	CD144$^+$	FCM	EMP levels correlated with crisis	[53]

Abbreviations: TTP, thrombotic thrombocytopenic purpura, SPC, solid phase capture, SBP, systolic blood pressure, DPB, diastolic blood pressure, FMD, flow mediated dilation.

'progenitor' population. The second cell type, called late out-growth colonies, do not appear in culture until 2 to 3 weeks, exhibits a 'classic endothelial' phenotype and an exponential growth. Late EPC appear far more capable of *in vitro* and *in vivo* morphogenesis into capillary structures. On this basis, the progenitor cells that give rise to this endothelial progeny may best refer to EPC. Their nature remains to be precisely defined although it has been proposed that such cells derive from a non-haematopoietic progenitor expressing CD34 [86, 87].

Despite the lack of consensual definition and phenotypic characterization, EPC detection has been intensively developed to appreciate cardiovascular risk, based on various methodologies. Consistent with the original description of Asahara, enumeration of putative EPC in clinical situation is most often based on flow cytometric analysis of circulating mononuclear cells expressing CD34 as immaturity marker, and KDR as marker of endothelial lineage, although this phenotype may overlap in part with that of mature endothelial cells [88, 89]. Association of CD133 to the previous markers, while strengthening the stemness phenotype,

yields lower cell count hardly detectable in steady state conditions. In addition, it has been suggested that CD133$^+$ cells include haematopoietic rather than endothelial progenitors. EPC can also be evaluated using *ex vivo* culture assays based on their clonogenic potential. A variety of culture protocols have been reported and referred as colony forming unit–endothelial cells including the commercially available Endocult™. However, most of such tests evaluate early EPC that are increasingly considered as manifestation of plasticity of the monocyte/macrophage lineage, prominently related to inflammation rather than endothelial biology. By contrast, quantification of late EPC reflecting probable 'true angioblasts' requires prolonged culture protocols, large volume of peripheral blood and is hardly used in large clinical studies [90]. These approaches are not correlated and may contribute to discrepant results [91]. So despite extensive research activities there is no accepted standard method to quantitate EPC. Such standardization may progress from an exact and consensual definition of EPC, including discrimination of cells with functionally heterogeneous pro-angiogenic activities.

Important insights from experimental models were to identify factors that regulate EPC-mediated endothelial repair [92]. EPC release from bone marrow, homing and recruitment are triggered by increased availability of angiogenic growth factors like VEFG or SDF1 produced by hypoxic areas or in response to vessel damage. Consistently, although present in the peripheral blood of healthy individuals at very low levels, this population can dramatically increase at times of acute pathophysiological stress such as myocardial infarction [93] or vascular injury (Table 4, panel A). Mobilization of EPC is also associated with atheroprotective strategies including regular physical training, and pharmacological substances like statins, estrogens or erythropoietin [115]. By contrast, classical CRF such as diabetes, age or uraemia negatively affect EPC.

The impact of CRF in the reduction of EPC availability is still poorly defined. However, several mechanisms have been put forward such as (*i*) exhaustion of the pool of stem/progenitor cells in the bone marrow, (*ii*) defective mobilization due to altered stem cell niche involving reduced nitric oxide bioavailabilty, (*iii*) impaired homing caused by reduction of chemoattractive factor synthesis or disturbance of their receptor-mediated signalling pathways and (*iv*) increased apoptosis and/or deregulated differentiation. This latter effect may involve intrinsic EPC defect or change in the local environment that determines cell fate either towards endothelial lineage or towards inflammatory cells or smooth muscle cells promoting atherosclerosis [116].

Early clinical studies, summarized in Table 4 (panel B), have reported reduced EPC levels and/or function in patients with chronic cardiovascular diseases, such as stable coronary artery disease, congestive heart failure, cerebrovascular disease, transplant vasculopathy or in stent restenosis [147, 148]. EPC defects have also been linked to situations that strengthen cardiovascular risk [149], such as diabetes mellitus [131, 132], hypertension [150], chronic renal failure [139], aging [151] or smoking [143]. The relationship between EPC reduction and CRF has been convincingly established in patients at various stages of atherosclerosis [152–154]. Decreased EPC levels and migratory activity were observed in CAD patients compared to controls and were found to inversely correlate with the number of CRF [117]. Together with significant correlation with the Framingham score reported in subclinical atherosclerosis, these data indicate that EPC may constitute surrogate marker of cumulative cardiovascular risk.

A further important step in the understanding of EPC role in cardiovascular disease was the demonstration that altered EPC numbers and functional capacities are major determinants in endothelial dysfunction [155]. In the absence of established atherosclerotic disease, as illustrated by aging or rheumatoid arthritis, EPC activity correlates with endothelial function evaluated by flow-mediated dilatation. Furthermore, in both healthy population and CAD patients, EPC levels have been recently shown to be the strongest predictor of endothelial dysfunction compared to traditional risk factors [156, 157]. Such data emphasize that EPC participate in the ongoing maintenance of endothelial integrity and that insufficient endothelial cell repair by EPC may play a causal

role in vascular disease progression. Clinical evidence of this concept was provided by a recent study that investigated EPC-associated prognostic values. In more than 500 patients with CAD, followed for 12 months, the cumulative event-free survival rate increased with baseline levels of EPC, considering both the first major cardiovascular event or death from cardiovascular causes [158]. Thus, EPC-mediated vascular repair appeared as a protective mechanism able to counterbalance the impact of CRF, to modulate the clinical course of atherosclerosis and finally to determine cardiovascular outcome.

Endothelial lesion *versus* regeneration: towards the definition of 'vascular competence'

Current evidence suggests that regenerative *versus* degenerative endothelial responses can be integrated in a clinical endothelial phenotype, reflecting the net result between damage from risk factors and endogenous repair capacity. This endothelial phenotype characterizes a vascular status that could define the 'vascular competence' of each individual. Because CEC, EMP and EPC are key players of the endothelial homeostatic balance, their combined measurement offers a non-invasive and original way to estimate vascular competence at the individual level (Fig. 2). The interest in such multimarker strategies to identify patients with high vascular risk and their response to treatment has emerged from the recent literature.

If baseline levels of CEC, EMP and EPC represent the physiological equilibrium of endothelium in healthy situations, any deviation of this balance presumably indicates the existence of a deleterious process affecting endothelium integrity. Both increase in CEC and EMP may correlate with the extent of vascular injury both in acute or chronic endothelial suffering contexts. In addition, phenotypic analysis of these markers may provide insights into disease mechanism or aetiology. Indeed, EMP surface markers have been shown to depend on their production mode, $CD62E^+$ EMP being mainly released during pro-inflammatory activation whereas $CD144^+$ EMP is generated during apoptosis or structural damage [50, 65]. However, cautions are needed because these notions only rely on *in vitro* observations and have not been validated yet *in vivo*. Similarly, phenotype of elevated CEC gives clues on the pathogenic causes of detachment. CEC express inducible cell adhesion molecules in inflammatory pathologies associated with cytokine-induced detachment as documented in sickle cell anaemia models [159], or demonstrated apoptosis or necrosis of the endothelium as reported in lupus patients (Table 2). As a direct response to acute endothelial injury, like ischemia reperfusion injury, or pro-inflammatory stimulation, elevation of EMP and CEC is associated with increased number of circulating EPC (Table 4, panel A). Mobilization of functionally competent EPC indicates the existence of an endogenous compensatory repair mechanism that

Table 4 Clinical studies illustrating changes in blood levels of EPC in cardiovascular situations

Disease/condition	Methods for EPC detection		Main finding(s)	Ref
	FCM	**Culture assay**		
Panel A : Disease/condition associated with increased EPC levels				
Acute ischemia				
Myocardial infarction	CD34$^+$/CD117+, CD34$^+$/KDR$^+$		EPC correlated plasma VEGF levels and CPK values	[94]
	CD133$^+$/KDR$^+$	+	EPC correlated with collateral vessel formation	[95]
	CD34$^+$/CD133$^+$/KDR$^+$	+	EPC differentiation is associated with myocardial salvage	[96]
	CD34$^+$		EPC mobilization correlated with favourable post-AMI remodelling	[97]
Unstable angina		+	Correlation with serum CRP levels	[98]
Stroke		+	EPC increment in the first week is associated with good outcome	[99]
	CD31$^+$/CD34$^+$, CD34$^+$/KDR$^+$		EPC mobilization predicted improvement of neurological outcome	[100]
Liver transplantation	CD34$^+$/CD133$^+$, CD34$^+$/KDR$^+$		Host derived cells, associated with VEGF, SCF, G-CSF increase	[101]
Coroanary artery bypass grafting		+	EPC mobilization correlated with G-CSF levels	[102]
	CD34$^+$/CD133$^+$		EPC increase is associated with cytochemokine release and showed a negative age dependency	[103]
Vascular injury				
Coronary stent implantation	CD34$^+$	+	EPC increase is more marked in patients developing restenosis	[104]
	CD34$^+$/CD45low	+	EPC mobilization inversely correlated with vascular injury assessed by CEC count	[105]
Drugs/Life style modifications				
Statins therapy	CD34$^+$/CD133$^+$/KDR$^+$	+	EPC increase in independent of VEGF	[106]
	CD45$^-$/KDR$^+$	+ (early and late EPC)	EPC increase is associated with enhanced EPC function in relation to IL-8 production by monocytes	[107]
		+	In patients with chronic heart failure, EPC increase is associated with improvement of endothelial function (FMD)	[108]
	CD34$^+$/KDR$^+$	+ (late EPC)	Long-term treatment in CAD patients	[109]
Erythropoïetin	CD34$^+$	+	Standard EPO dose in patients with renal anaemia, increased EPC number is associated with increased EPC survival and function	[110]
	CD34$^+$/CD45$^-$	+	One bolus of EPO in patients with acute myocardial infarction	[111]
Physical training	CD34$^+$/CD133$^+$/KDR$^+$		EPC increase correlated with FMD change	[112]
		+	EPC increase is associated with intensified school sports in children	[113]
	CD34$^+$/KDR$^+$	+	EPC increase is associated with reduced EPC apoptosis in CAD patients	[114]

Continued...

Table 4 Continued

Disease/condition	Methods for EPC detection		Main finding(s)	Ref
	FCM	Culture assay		
Panel B: Disease/condition associated with decreased EPC levels				
Cardiovascular diseases				
Stable CAD	CD34$^+$/KDR$^+$		EPC number inversely correlates with the presence of CVRF	[117]
	CD34$^+$/KDR$^+$		Low levels of EPC independently predict poor prognosis	[118]
	CD133$^+$/KDR$^+$	+	EPC number and migratory activity inversely correlate with the severity of coronary stenosis and CRP	[119]
Cerebrovascular disease	CD34$^+$, CD133$^+$		EPC level correlates with regional blood flow	[120]
Pulmonary hypertension	CD133$^+$/KDR$^+$	+	EPC reduction correlates with IL-6, vWF and BNP levels and is associated with impaired migration and adhesion to fibronectin	[121]
	CD34$^+$/C133$^+$/KDR$^+$ CD34$^+$, CD34$^+$/KDR$^+$, CD34$^+$/CD133$^+$		EPC reduction is associated with raised inflammatory markers and meliorated by phosphodiesterase inhibitor Sildenafil	[122]
Heart failure	CD34$^+$		EPC inversely correlate with disease severity	[123]
	CD34$^+$ CD133$^+$/KDR$^+$	+	EPC reduction is associated with functional exhaustion of EPC within bone marrow	[124]
		+	EPC reduction is associated with the advances phases of the disease	[125]
In-stent restenos		+	Low EPC number and function is associated with diffuse restenosis	[126]
		+	Increased EPC senescence is associated with in-stent restenosis	[127]
Cardiovascular risk factors				
Type 2 Diabetes	CD34$^+$/CD117+,		EPC number negatively correlated with disease severity, and individually predict microvascular complications,	[128]
	CD34$^+$/CD133$^+$/KDR	+	EPC reduction is associated with EPC dysfunction involving eNOS	[129]
		+	EPC number is related to HbA1c levels in untreated patients and increased by pharmacological glycemic control	[130]
	CD34$^+$/KDR$^+$		EPC reduction is more marked in patients with peripheral vascular disease, EPC number correlates with ABI	[131]
Type 1 Diabetes		+	Low EPC number is associated with EPC dysfunction	[132]
Hypertension	CD34$^+$/KDR$^+$		EPC inversely correlates with systolic blood pressure	[133]
	CD34$^+$/CD133$^+$/CD45		Refractory hypertension independantly determines EPC number	[134]
End stage renal disease	CD34$^+$/KDR$^+$	+	EPC reduction and altered function are related to serum fetuin A levels, haematocrite and reticulocytes	[135]
	CD34$^+$/CD144$^+$	+	EPC number and migration inversely correlates with uraemia and systolic blood pressure and is restored by nocturnal haemodialysis	[136]
	CD34$^+$/KDR$^+$	+	Uremic serum impaired normal EPC outgrown *in vitro*	[137]
	CD34$^+$/CD45+	+	EPC number correlated with renal function and is normalized after renal transplantation	[138]
		+	EPC Inversely correlates with framingham score and dose of dialysis	[139]

Table 4 Continued

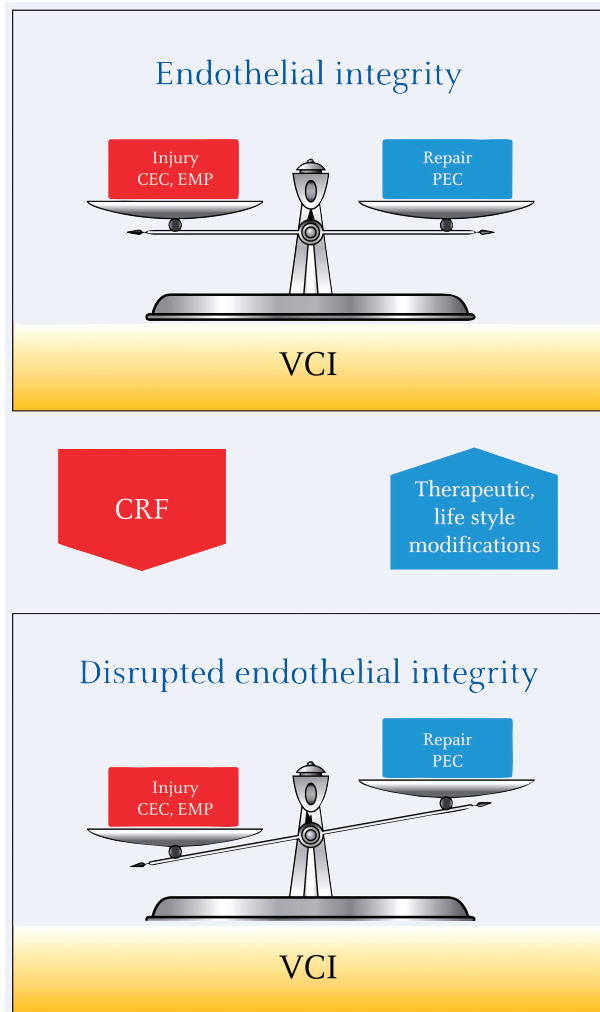| Disease/condition	Methods for EPC detection		Main finding(s)	Ref
	FCM	**Culture assay**		
Dyslipidemia		+	EPC number and impaired functionality Inversely correlated with total and LDL cholesterol	[140]
	CD34$^+$/KDR$^+$		EMP/EPC Ratio correlates with LDL cholesterol and arterial stiffness	[141]
Smoking		+	Impaired EPC function related to increased oxidative stress	[142]
	CD34$^+$/CD133$^+$/KDR$^+$	+	EPC levels increased after 2 week smoking cessation	[143]
aging	CD34$^+$ CD34$^+$/KDR$^+$	+	EPC reduction correlated with decreased arterial elasticity	[144]
			Increased EPC levels is childhood, inverse relation with age in healthy individuals	[145]
Hyperhomocysteinemia	CD133$^+$/KDR$^+$	+	EPC levels inversely correlated with Homocystein levels	[146]

Abbreviations : CAD, coronary artery disease, FMD, flow mediated dilatation, EPO, erythropïetin, CPK, creatine phosphokinase, AMI, acute myocardial infarction, CRP, C reactive protein, IL-8 : interleukin-8, VEGF, vascular endothelial growth factor, SCF, stem cell factor, G-SCF, granulocyte-stem cell factor, IL-6: interleukin-6, vWF, von Willebrant factor, BNP, type B natriuretic peptide; eNOs, endothelial Nitric oxide synthase, ABI, ankle brachial index, LDL, low-density lipoprotein.

may efficiently contribute to restoration of endothelial integrity. Consequently a drive towards normalized levels of endothelial biomarkers, once repair has been effected, can be expected. Such endothelial biomarker profile, characterized by transient increase in EMP and EPC, has been recently described in healthy individuals exposed to brief second-hand smoke [160]. Insufficiency of this repair response is consistent with altered vascular competence and would argue for the development of vascular complications. In the first study reporting simultaneous measurement of CEC and EPC in patients with stable coronary artery disease who underwent percutaneous coronary intervention [105], both increased CEC and EPC mobilizations were observed after the procedure. Surprisingly, the extent of EPC mobilization was inversely related to the extent of injury reflected by CEC, suggesting that marked endothelial damage associated with poor endothelial repair could be useful to identify patients at risk for post-procedural complications.

By contrast to acute conditions, chronic exposure to CRF is associated with both endothelial-damaging process and depletion of cells efficient for repair. In that case, increased levels of EMP and/or CEC associated with reduced EPC levels or impaired EPC activity demonstrate the lack of compensatory responses that importantly contribute to the development and progression of atherosclerosis. Therefore, vascular index combining measurement of EPC and injury markers may offer better appreciation of vascular risk by evaluating both pathological processes involved in endothelial dysfunction as recently illustrated in patients with CAD patients

with ischemic left ventricular dysfunction [161]. Also, calculation of EMP to EPC ratio has been proposed as a possible index reflecting the imbalance between endothelial damage and repair capacity. In comparison to normal persons, this ratio increased in hypercholesterolaemic patients, as a result of both increased EMP and decreased EPC. Moreover, this ratio was highly correlated with low-density lipoprotein (LDL) cholesterol and pulse wave velocity, a measure of aortic stiffness [141]. Altogether, these data suggest that both increased endothelial injury and impaired repair may contribute to reduce aortic distensibility.

Despite great promises, integration of multimarker strategies based on CEC, EMP and EPC in the clinical practice is still in its infancy. Basically, a better knowledge of endothelial damage and repair processes is expected to define more precisely the relationship between CEC, EMP and EPC in physiological or pathological situations. Other limitations rely on the poor standardization of assays. Obviously, methodological issues and confusion delivered by the various definitions currently in use remain a major cause of apparent conflicting results notably regarding EPC. In addition, their physiological variations according to parameters such as age, gender or circadian rhythms has to be defined in large cohorts of healthy people. Standardization of methodologies available is therefore a crucial step for the full definition of the clinical significance of these markers. This standardization is one of the goals of the Scientific and Standardization Committee (SSC) on vascular biology of the International Society for Thrombosis and Haemostasis (ISTH).

Fig. 2 Endothelial integrity is viewed as a balance between endothelial injury reflected by CEC and EMP, and endogenous capacity for repair attested by EPC. The net result of this equilibrium can be integrated into a clinical endothelial phenotype defining vascular competence. Multimarker strategies combining CEC, EMP and EPC to define Vascular Competence Index are promising to evaluate at the individual level the impact of cardiovascular risk factors on disease progression. Such multimarker approach may also provide relevant tools for delineation and monitoring of therapeutic strategies or life style modifications aimed to improve endothelial function by limiting damage and/or reinforce regenerative mechanisms. CEC: circulating endothelial cells, EMP: endothelial microparticles, EPC: endothelial progenitor cells, VCI: vascular competence index.

Conclusion

The ability to explore the endothelium non-invasively has put in light a novel pathogenic understanding of CVD, defined by an imbalance between endothelial damage and repair capacity. In the future, multimarker approaches combining CEC, EMP and EPC measurement will be helpful as integrative markers of 'vascular competence'. In clinical testing strategies, these cellular markers will certainly provide novel insights to detect endothelial dysfunction at early pre-clinical stages, to assess vascular risk in the later stages of atherothrombotic disorders and to delineate therapeutic options. Indeed, therapeutic strategies that are efficient to improve endothelial function could target the selective reduction of endothelial injury or the promotion of regenerative mechanisms. At the individual level, such therapeutic option may integrate, not only the predominant mechanism, but also the genetic predisposition and environmental risk factors. Important insights into disease monitoring and potential new therapeutic options will certainly emerge from this rapid developing field.

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