

## Review Article

# Vascular Endothelial Cells as Biomarkers of Microvascular Endothelium Damage and Repair in Cardiovascular and Neurodegenerative Diseases

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Vascular endothelium damage is a significant pathophysiological component of cardiovascular and neurodegenerative diseases. Circulating putative endothelial progenitor cells (CPEPCs) and circulating Endothelial Cells (CECs) have high potential as diagnostic and prognostic clinical indicators. Changes in peripheral blood levels of both cell types are associated with detrimental vascular events. Currently, CPEPCs are considered cells of vascular repair, while CECs represent vascular damage. Regrettably, there is confusion regarding characterizations of these two cell types. The review covers definitions and fundamental biologies of CPEPCs and CECs. Importantly, methods to isolate CPEPC subsets, and subset roles and origins are discussed. Means of identifying CECs, their biological significance, and the troubling phenotypic overlap with CPEPCs are also discussed. The review also focuses on the role of CPEPCs and CECs as biomarkers in cardiovascular and neurodegenerative diseases. However, contradictory data on these cell types signifies the necessity of standardized methods for applying CPEPCs and CECs as clinical biomarkers.

**Keywords:** Endothelial progenitor cells; Circulating endothelial cells; Biomarkers; Cardiovascular disease; Neurodegenerative disease**Abbreviations**

ACLDL: Acetylated Low Density Lipoprotein; ACS: Acute Coronary Syndrome; AD: Alzheimer's Disease; AF: Atrial Fibrillation; ALS: Amyotrophic Lateral Sclerosis; AMI: Acute Myocardial Infarction; AS: Atherothrombotic Stroke; BBB/BSCB: Blood-Brain Barrier/Blood Spinal Cord Barrier; CAC: Circulating Angiogenic Cell; CAD: Coronary Artery Disease; CEC: Circulating Endothelial Cell; CFU-EC: Colony Forming Unit-Endothelial Cell; CFU-EPC: Colony Forming Unit- Endothelial Progenitor Cell; CFU-Hill: Colony Forming Unit-Hill; CNS: Central Nervous System; COMT: Catechol-O-Methyltransferase; CPEPC: Circulating Putative Endothelial Progenitor Cell; CRP: C-Reactive Protein; CVD: Cardio Vascular Disease; EC: Endothelial Cell; ECFC: Endothelial Colony Forming Cell; EOC: Endothelial Outgrowth Cell; EPC: Endothelial Progenitor Cell; FACS: Fluorescence Activated Cell Sorting; HAEC: Human Aortic Endothelial Cell; HSC: Hematopoietic Stem Cell; HUVEC: Human Umbilical Vein Endothelial Cell; IFN- $\gamma$ : Interferon- $\gamma$ ; IS: Ischemic Stroke; KDR: Kinase Insert Domain Receptor; MACE: Major Cardiovascular Endpoints; MN: Motor Neuron; NSTEMI: Non-ST-Elevation Myocardial Infarction; NVE: New Vascular Event; PBMC: Peripheral Blood Mononuclear Cell; PD: Parkinson's Disease; PFC: Polychromatic Flow Cytometry; RF: Cardiovascular Risk Factor; STEMI: ST-Elevation Myocardial Infarction; TNF: Tumor Necrosis Factor; UA: Unstable Angina; UEA-1: Ulexeuropaeus agglutinin 1; VEGF: Vascular Endothelial Growth Factor; VEGFR2: Vascular Endothelial Growth Factor Receptor 2; VWF: Von Willebrand Factor

**Introduction**

Vascular endothelial cell dysfunction is associated with various disorders such as cardiovascular disease, ischemic stroke, and neurodegenerative diseases [1-3]. Determination of biomarkers for endothelial cell dysfunction is necessary not only for their diagnostic and/or prognostic value, but also to gain insight into the vascular pathology associated with these disorders. Two potential systemic biomarkers of alterations to the vascular endothelium are Circulating Endothelial Cells (CECs) and Circulating Putative Endothelial Progenitor Cells (CPEPCs), which reflect endothelial damage and vascular repair processes, respectively [4-6].

Asahara *et al* [7]. Showed that CPEPCs isolated from human peripheral blood are capable of differentiating into Endothelial Cells (ECs) *in vitro*. Further *in vivo* experiments demonstrated that bone marrow-derived Endothelial Progenitor Cells (EPCs) enter the systemic circulation, mobilize to injured vessels and contribute to new blood vessel formation [8]. Thus, CPEPC levels may be indicative of the body's potential for vascular endothelial repair [1]. When damage occurs to the vasculature, the endothelium is compromised, resulting in detached ECs that enter the blood stream and become CECs [6]. Although specific cellular markers have been established for the identification of CECs and EPCs, distinguishing between the two cell types is still difficult due to overlapping marker expression [9].

CECs and CPEPCs may be important biomarkers of endothelium status in cardiovascular disease and neurodegenerative disorders. As biomarkers of endothelial damage, elevated CECs are indicative of recent acute myocardial infarction or acute ischemic stroke [10,11].

Similarly, CPEPCs are elevated in the blood of patients with acute myocardial infarction or ischemic stroke [12,13]. Uniquely, CPEPCs are presumed to mobilize from the bone marrow in response to vascular injury and are important for regeneration and repair of blood vessels [13]. Both CECs and CPEPCs serve as predictors of disease outcome in ischemic vascular disease [10,14-16].

CECs and CPEPCs are also potential biomarkers of endothelial damage or repair in neurodegenerative disorders such as Amyotrophic Lateral Sclerosis (ALS), Alzheimer's Disease (AD), and Parkinson's Disease (PD) [17-21]. These diseases can be classified as neurovascular disorders due to disruption of the blood-brain/spinal cord barriers [22-24], and therefore identification of associated vascular endothelial biomarkers may become important for diagnosis and prognosis.

In this review, current evidence for CECs and CPEPCs as potential biomarkers of vascular endothelial damage and repair in cardiovascular and neurovascular/neurodegenerative disease is discussed. The first part of the review highlights the fundamental biology of ECs, CPEPCs, and CECs. The second part discusses the current literature on CECs and EPCs as vascular biomarkers of damage and repair in cardiovascular disease, stroke, ALS, AD, and PD.

## Circulating Putative Endothelial Progenitor Cells (CPEPCs)

### Discovery of the CPEPC

In 1997, Asahara *et al.* [7] isolated CPEPCs (i.e., angioblasts) from adult human Peripheral Blood Mononuclear Cells (PBMCs) via magnetic bead selection using cell surface markers CD34, a human hematopoietic stem cell antigen [25], and Flk-1 (also called KDR or VEGFR2), a receptor for vascular endothelial growth factor [26,27]. The study results [7] suggested that CD34+ and Flk1+ PBMCs differentiate into ECs *in vitro*, and contribute to new blood vessel formation in animal models of hindlimb ischemia *in vivo*. This seminal study [7] led to a paradigm shift in vascular biology with regard to the mechanisms by which new blood vessels might be formed in the adult. As noted by Asahara *et al.* in a later study [28], it was originally thought that new blood vessels formed in the adult exclusively by a process called "angiogenesis", which also occurs in the embryo. However, after the discovery of CPEPCs that differentiate into ECs and integrate into the vasculature [7], a second mechanism of new blood vessel formation, similar to embryonic "vasculogenesis," was recognized in the adult. As reviewed by Risau [29], vasculogenesis occurs in the embryo when "the early vascular plexus forms from mesoderm by differentiation of angioblasts (vascular endothelial cells that have not yet formed a lumen), which subsequently generate primitive blood vessels". The author noted that angiogenesis occurs "after the primary vascular plexus is formed" when "more endothelial cells are generated, which can form new capillaries by sprouting or by splitting from their vessel of origin". To account for the evidence that bone marrow-derived CPEPCs might mobilize to and integrate into sites of new blood vessel formation where they differentiate into ECs, Asahara and his colleagues [7,8] utilize the term "postnatal vasculogenesis." However, this term seems controversial, likely since vasculogenesis traditionally refers to a specific embryological process requiring the formation of blood islands from the mesoderm, a

formation which does not occur in adults [30-32].

### Three Culture-Derived CPEPC Subsets

After Asahara *et al.* [7] discovery of CPEPCs, new cellular subsets were identified via specific culture assays. Some of these cell populations were mistakenly termed "endothelial progenitor cells" or "EPCs". Discussed below are three common culture methods each corresponding to one unique "EPC"-related subtype? The three subtypes include Circulating Angiogenic Cells (CACs), Colony Forming Unit-Hill (CFU-Hill) cells, and Endothelial Colony Forming Cells (ECFCs). In this review, the term CPEPC includes these three culture-derived cell groups. The term CPEPC also applies to EPCs, which might be isolated by flow cytometry.

A reported culture method establishes a cell population referred to as Circulating Angiogenic Cells (CACs) [33-35]. Vasa *et al.* [36] plated PBMCs at low density on fibronectin and gelatin coated dishes containing endothelial growth factors and fetal calf serum in media. Non-adherent cells were removed after 4 days in culture, while the adherent cells that displayed acetylated Low Density Lipoprotein (acLDL) uptake and *Ulexeuropaeus Agglutinin 1* (UEA-1) lectin binding were interpreted as EPCs according to criteria originally used by Asahara *et al.* [7]. However, later studies showed that this cell population cannot be considered true EPCs for two reasons. First, Prokopi *et al.* [37] discovered that this method for isolating putative EPCs is unreliable due to platelet protein contamination. Platelets degrade into micro particles, which might interact with the isolated mononuclear cell population and confer endothelial characteristics [37] thus creating "false positive" [33] cells. Secondly, monocytes may contaminate this putative EPC population. Monocytes isolated through attachment to fibronectin-coated dishes [38] might also express endothelial cell surface proteins when cultured with VEGF [39]. The term CACs seems to be an appropriate name for this CPEPC subset because it is a group of circulating hematopoietic cells that contribute to angiogenic blood vessel formation via secretion of growth factors [40].

Another *in vitro* method is a colony forming assay that generates colony forming unit-Hill (CFU-Hill) [41] cells, otherwise known as CFU-EC or CFU-EPC [33-35]. PBMCs are pre-plated on fibronectin-coated dishes for 48 hours and the adherent cells removed to eliminate mature CECs from the culture. The non-adherent cells are then re-plated, and 7 days later the CFU-Hill cells emerge as colonies consisting of thin flat cells surrounding a central cluster of rounded cells [41]. Like CACs, CFU-Hill cells stain positive for lectin and acLDL [41], but also contain hematopoietic cells (e.g., myeloid progenitor cells, monocytes, and T lymphocytes [42,43]). Therefore, CFU-Hill cells are also likely not true EPCs.

Additionally, the colony forming assay likely identifies a population of true EPCs, termed Endothelial Colony Forming Cells (ECFCs), otherwise known as Endothelial Outgrowth Cells (EOCs) [28,33-35]. ECFCs are produced by culturing adult peripheral blood cells in type 1 collagen-coated wells. Between 5-22 days of culture, ECFC colonies appear as monolayers of cobblestone-looking cells with individual cells displaying a hierarchy of clonal proliferative potential from low to high [44,45]. According to multiple reviews [33-35,46]. ECFCs are the only subset that follows the criteria for a true EPC: 1) ECFCs exhibit clonal proliferative potential and differentiation

dedicated only to the endothelial lineage [44,45]; 2) ECFCs form lumenized capillary-like structures (i.e., undergo tubulogenesis) *in vitro* [45,47,48]; 3) ECFCs integrate into host vasculature and form stable *de novo* human blood vessels *in vivo* [45,49].

### Flow cytometry assay

As noted in multiple reviews [1,33,50], flow cytometry via Fluorescence Activated Cell Sorting (FACS) of human peripheral blood cells is a common but flawed technique for defining CPEPCs. The human putative EPC phenotype became defined as the cell surface expressions of CD34, AC133, and VEGFR2 [7,51]. However, Case *et al.* [52] eventually discovered that these markers are also expressed by hematopoietic progenitor cells with no vessel forming capacity, and therefore do not identify a true EPC population. Most flow cytometry-based biomarker studies identify CPEPCs using at least one marker of stemness (CD34 or CD133 [25,53]) and one marker of endothelial differentiation (typically Flk1, KDR or VEGFR2 [26,27]). However, the use of VEGFR2 is problematic because this endothelial marker is also expressed by hematopoietic stem cells [54,55].

Although no specific antigenic signature exists yet for the true EPC, Mund *et al.* [56] isolated ECFCs from human umbilical cord blood via Polychromatic Flow Cytometry (PFC) that sorted for C D34+, CD146+,CD31+, CD105+, CD45-, and CD133- cells. However, these authors also isolated CECs using the same markers. These studies highlight the troubling phenotypic overlap between hematopoietic CFU-Hill and CACs, immature ECFCs, and mature CECs. Hirschi *et al.* [34]. Reviewed in detail current cell surface antigen phenotypes of CFU-Hill, CACs, and ECFCs.

### Lineage and tissue sources of CACs, CFU-Hill, and ECFCs

There is a close association of Hematopoietic Stem Cells (HSCs) and EPCs (i.e. angioblasts) in the embryonic blood islands that form the yolk sac capillary network [30]. Although controversial, the close spatial relationship and sharing of certain antigenic markers, such as Flk-1 [57], suggest the hemangioblast is a common cell precursor for HSCs and EPCs [58]. As reviewed by Risau [29], the embryonic hemangioblast gives rise to two separate lineages: one seeded by the HSC for subsequent hematopoiesis, and the other by the EPC for vasculogenesis. However, this may be a simplification as evidence exists for hemogenic endothelium [59] where HSCs are generated from special ECs at a specific developmental time point. Nevertheless, with regard to the adult condition, a true EPC should only differentiate into an EC involved in re-establishing vascularity.

It has been shown via gene expression analysis that CACs and CFU-Hill cells are closely related to hematopoietic cells, such as T lymphocytes and monocytes, and they are likely unrelated to ECs [60,61]. As stated in reviews [33,34], although CACs and CFU-Hill cells contribute to angiogenesis via paracrine signaling [40,62], these cells might not be true EPCs with properties to differentiate into ECs and incorporate into the vasculature [62,63]. As hematopoietic cells, CFU-Hill cells and CACs originate in the bone marrow and mobilize into the blood [64]. In contrast, the tissue origin of ECFCs is uncertain [35,50,65]. As previously discussed, the specific marker expression for ECFCs and CECs is identical [56]. Since CECs represent mature endothelial cells originating from the vessel wall [66], it is possible that ECFCs are vessel wall-derived rather than released from the bone marrow. An *in vitro* study [67] showed that a hierarchy of

ECFCs exists in cultures of Human Umbilical Vein Endothelial Cells (HUVECs) and Human Aortic Endothelial Cells (HAECs). Although this observation is suggestive in regards of a vessel wall origin for ECFCs, it does not rule out bone marrow as a potential ECFC source.

## Endothelial Cells

The vascular endothelium is comprised of ECs that form the inner lining of all blood vessels from arteries to capillaries to veins [68]. Throughout the vascular circulatory system, this endothelial barrier regulates the selective transport of nutrients between tissues and the systemic compartment [3]. Specific to the Central Nervous System (CNS) endothelium, the Blood-Brain/Blood Spinal Cord Barrier (BBB/BSCB) is essential for the maintenance of constant cerebral homeostasis [69]. ECs in the CNS capillaries overlap by tight junctions, which anchor two adjacent cells and prevent various molecules from passing between the cells [70]. ECs of the BBB/BSCB are also characterized by the presence of specific membrane transport systems, and the absence of fenestrae [71], which are transcellular pores useful for increased filtration or transendothelial transport [72]. Interestingly, non-fenestrated endothelium is also found in the heart [72]. In addition to their function as a barrier between the blood and the tissues, ECs contribute to vascular homeostasis through regulation of vascular tone, coagulation, solute permeability, leukocyte trafficking, and vessel growth [73].

## Circulating Endothelial Cells (CECs)

### Identification of circulating endothelial cells

Circulating Endothelial Cells (CECs) are typically acquired from blood samples using the immunomagnetic bead isolation technique combined with fluorescence microscopy [56,74,75] or flow cytometry [56,76,77]. Similarly to EPCs, there is uncertainty regarding the precise antigenic profile of CECs [1,78]. Schmidt *et al.* [78] have defined CECs as positive for CD34, CD146, CD31, CD105, UEA-1 lectin, and von Willebrand Factor (vWF), and negative for CD45 and CD133 expressions in various marker combinations. The most common marker used to isolate CECs is CD146 [1,78,79], a mediator of endothelial cell-to-cell cohesion [80] and a participant in endothelial cell signalin [81].

Due to overlap in cell surface protein expressions, it is challenging to distinguish CECs from EPC subsets. As mentioned above, Mund *et al.* [56] used Polychromatic Flow Cytometry (PFC) to isolate CD34+/CD146+/CD31+/CD105+/CD45-/CD133- cells from human umbilical cord blood, which surprisingly contained ECFCs with high proliferative potential and CECs with limited or no clonogenic potential. Additionally, the stem cell marker CD133 [82] has commonly been used to discriminate between mature CD133-CECs and immature CD133+ EPCs [77], but may fail to discriminate between CECs and ECFCs as implied by Mund *et al.* [56]. Furthermore, a previous study confirmed the presence of putative EPCs in a population of CD146+ cord blood cells which eliminated CECs by an adhesion step [83].

Two other characteristics that distinguish EPCs from CECs are colony forming ability and cell size. As reviewed [84], CECs are not able to form cell colonies with high proliferative potential [66]. Cell size is another important characteristic that distinguishes CECs from EPCs [1]. CECs range from 10-50  $\mu\text{m}$  [74,85] in diameter, while EPCs are less than 15  $\mu\text{m}$  as reviewed [86].

## Significance of CECs

CECs, as mature ECs, might be detectable in the blood after vascular damage potentially due to their detachment from the endothelium. To determine if CECs originate from blood vessel walls or the bone marrow, Lin *et al.* [66] performed fluorescence *in situ* hybridization analysis of blood samples from bone marrow transplant recipients who had received gender-mismatched transplants. After 5-20 months, 95% of CECs in the recipient peripheral blood exhibited the recipient genotype, indicating that CECs were not originating from the donor bone marrow. This suggests that CECs are primarily derived from blood vessels [66]. There are multiple mechanisms by which ECs possibly detach from the vascular wall and release into the circulated blood as CECs. Mechanical disruption, such as percutaneous coronary intervention, is one mechanism [74]. Vascular inflammation can also cause CEC detachment. In Wegener's granulomatosis, a disorder characterized by blood vessel inflammation, Ballieux *et al.* [87] showed that release of protease 3 by polymorphonuclear leukocyte degranulation caused EC detachment. Furthermore, Ruegg *et al.* [88] provide evidence that inflammatory cytokines such as Tumor Necrosis Factor (TNF) and  $\gamma$ -interferon (IFN- $\gamma$ ) impede the activation of a specific integrin receptor, resulting in decreased EC adhesion and increased EC detachment and apoptosis. Reactive oxygen species are another factor that may contribute to endothelial dysfunction and CEC detachment [89].

Some reviews [6,78] also implicate necrosis and apoptosis as contributors to EC detachment and CEC appearance. However, more evidence is needed to clarify these cellular processes. A study [90] on ANCA-associated small-vessel vasculitis showed that CD146+ CECs stained positively for annexin and propidium iodide, markers of necrosis, but were negative for the TUNEL. This indicates necrotic rather than an apoptotic phenotype for these CECs. Furthermore, in acute myocardial infarction, only 10% of CECs showed DNA signs of apoptosis [11]. Thus necrosis may be a more significant contributor to CEC release than apoptosis. Nevertheless, necrotic [90] and apoptotic [91] CECs have pro-coagulant properties that may contribute to thrombosis and cardiovascular or neurovascular events.

## CPEPCs and CECs as Vascular Disease Biomarkers

### Cardiovascular disease

Coronary Artery Disease (CAD) is a *chronic* condition that occurs due to atherosclerotic plaque accumulation and inflammation in the coronary arteries resulting in decreased blood flow to the heart [92]. Vasa *et al.* [93] isolated CACs and CD34+/KDR+ CPEPCs, and found that both cell populations were decreased in the peripheral blood of CAD patients compared to healthy controls. Wang *et al.* [94] showed that the CD133+/KDR+ cell level was lowest in stable CAD patients with multiple vessel disease versus those with single vessel disease or normal coronary arteries, suggesting that the level of CPEPCs is a measure of coronary stenosis severity. Paradoxically, Guven *et al.* [95] reported a trend toward higher numbers of CACs in patients with "significant CAD" defined as greater than or equal to 70% diameter vessel stenosis. In contrast to EPC biomarker studies, CD146+ CEC levels are approximately the same in stable CAD patients and healthy controls, and therefore stable CAD subjects are often grouped as a "disease control" for comparison to Acute Coronary Syndrome (ACS) groups [11,14,96].

When an atherosclerotic plaque ruptures, a thrombotic clot can cause an Acute Coronary Syndrome (ACS), such as Unstable Angina (UA), Non-ST-Elevation Myocardial Infarction (NSTEMI), or the more severe ST-Elevation Myocardial Infarction (STEMI) [97-99]. Shintani *et al.* [100] demonstrated that cultured CPEPC colonies, similar to CFU-Hill, were significantly increased in the peripheral blood of patients 7 days after Acute Myocardial Infarction (AMI) compared to day 1 post-AMI. The results suggest that CPEPCs mobilize into the peripheral blood after an acute ischemic event [100]. Supporting the concept of injury-induced EPC mobilization, Massa *et al.* [12] showed via flow cytometry that circulating CD34+/VEGFR2+ and CD34+/CD133+/VEGFR2+ CPEPCs are increased in AMI patients upon admission, relative to controls, with residual changes to EPC levels detectable up to 2 months [12]. In ACS, DAPI+/CD146+/vWF+/CD45- CECs [101] and CD146+/UEA-1+ CECs 10-50  $\mu$ m in diameter [96,102] were increased compared to controls, with the highest levels found in STEMI followed by NSTEMI followed by UA.

With regard to prognostic value, Werner *et al.* [16] studied 519 CAD patients and found that low baseline levels of circulating CD34+/KDR+CPEPCs correlated with an increased incidence of death from cardiovascular causes. Additionally, Werner *et al.* [16] demonstrated a significant association between increased levels of CFU-Hill cells and decreased risk of first major cardiovascular events (e.g. myocardial infarction, revascularization, and hospitalization). Regarding CEC prognostic value, Lee *et al.* [14] demonstrated that elevated CD146+ CECs collected from the blood 48 hours post-ACS were the strongest predictor of Major Cardiovascular Endpoints (MACE) upon 30-day and 1-year follow-ups compared to IL-6 and vWF. Cardiovascular death and non-fatal MI are the main components of MACE [14].

As reviewed by Fadini *et al.* [50], ECFCs are not efficiently obtained from patients with CVD. Meneveau *et al.* [103] showed that ECFCs were detectable in only 45.5% of patients within 12 hours of the first AMI, and that ECFC detection was limited to a few days post-AMI. Mund *et al.* [56] also found that ECFCs were barely detectable, if at all, by polychromatic flow cytometry in the peripheral blood of normal adults.

### Stroke

Similarly to AMI, acute ischemic strokes are caused by vascular clots due to atherosclerotic large-vessel thrombosis, but can also be triggered by cardiovascular emboli, cerebral small vessel disease (lacunar infarcts), or other pathophysiological mechanisms [104-106]. Yip *et al.* [107] performed a 150-patient study analyzing CD34+/KDR+ CPEPCs in acute Ischemic Stroke (IS) patients. Similarly to the AMI a study, the level of CPEPCs was significantly higher in the blood from IS patients at acute phase versus at-risk control subjects, which suggests that EPCs are also mobilized in response to IS. Yip *et al.* [107] also demonstrated that a low level of CPEPCs 48 hours post-stroke was predictive for severe neurological impairment, while an increased level of CPEPCs at the acute phase of IS predicted an absence of 90-day combined major adverse clinical outcomes. Similarly, Sobrino *et al.* [15] showed that non-lacunar IS patients with good outcomes showed a higher CFU-Hill increment during day 7 and month 3 post-stroke compared to the poor outcome group. Cuadrado-Godia *et al.* [108] analyzed AMI and Atherothrombotic Stroke (AS) patients and showed that very low levels of CD34+/

KDR+/CD133+/CD45- CPEPCs correlated with a higher risk of a New Vascular Event (NVE), such as stroke or ACS, occurring during the first 6 months.

Regarding CECs, Nadar *et al.* [10] demonstrated that CD146+ CECs were increased in acute IS patients compared to healthy and hypertensive control subjects. Interestingly, Freestone *et al.* [109] discovered that CD146+ CECs were unchanged in chronic stable Atrial Fibrillation (AF) patients compared to healthy controls, but that patients with an acute (non-hemorrhagic) stroke complicated by AF did show increased CECs. This supports the pattern of CECs being unchanged in chronic conditions (e.g., stable CAD, stable AF, and chronic hypertension) and then elevating around acute vascular events (e.g., AMI and IS). However, the prognostic power of CECs in stroke and even ACS is questionable. Cuadrado-Godia *et al.* [108] found that CD146+/CD31+/CD45- CECs from AS and AMI patients were not predictive of a 6-month follow-up NVE in contrast to EPCs (see above).

### Rationale for using CPEPC and CEC as Potential Biomarkers in Vascular Disease

Generally, low levels of CPEPCs occur in patients with chronic cardiovascular disease, such as CAD, and such levels are also predictive of cardiovascular and neurovascular events. As discussed by Wang *et al.* [94], it is possible that CPEPCs are reduced in CAD due to deficits in their production, mobilization, and half-life, or might be continually exhausted by chronic endothelial damage. In addition, CPEPCs may undergo increased apoptosis in CAD. For instance, Wang *et al.* [94], found that CPEPC levels decreased as C-Reactive Protein (CRP) increased in CAD patients, while Verma *et al.* [110] hawed that CRP increases CPEPC apoptosis *in vitro*. Assuming the prognostic CPEPCs [16] mentioned above have pro-angiogenic properties [40], low levels of these cells should indicate a reduced capacity for new blood vessel formation and repair, likely increasing vulnerability to harmful vascular events.

In contrast with chronic CAD, acute events such as AMI and IS, are characterized by a marked increase in CPEPCs. Masa *et al.* [12] suggest that a severe ischemic event resulting in major tissue damage (rather than chronic milder ischemia) is required to mobilize CPEPCs for vascular repair. Cytokines such as VEGF are released in response to this insult [12,100] and may be responsible for CPEPC mobilization from the bone marrow [111]. A rise in CPEPC numbers may be a compensatory response to ischemia in order to induce blood vessel formation [8], reduce ischemic tissue damage [112], and improve tissue function [113]. Importantly, Fadini [50] notes that CPEPCs may decline as chronic atherosclerotic blood vessel stenosis worsens [94,114-116], until a major acute clotting/ischemic event causes their mobilization from the tissues and subsequent increased CPEPCs [12,100,107]. It is evident that atherosclerosis is a unifying pathophysiological component of vascular disease.

In chronic conditions, such as stable CAD, CEC levels remain unchanged, but are increased in association with acute vascular events. It is possible that a population of necrotic/apoptotic CECs actually contributes to the onset of acute ischemic events such as AMI and IS due to CECs' pro-coagulant characteristics, and this contribution may account for the association between elevated CECs and future

adverse cardiovascular events [14]. Conversely, CECs may not act as a biological trigger, but only serve as biomarkers of endothelial damage. Acute events in AMI and IS are likely more damaging to the vascular endothelium than preceding chronic conditions such as CAD, which may explain CECs elevation only in the presence of acute vascular episodes, and not chronic endothelial dysfunction [10,109].

### CPEPCs and CECs as Potential Biomarkers for Neurodegenerative Diseases

#### Amyotrophic Lateral Sclerosis (ALS)

ALS is characterized by Motor Neuron (MN) degeneration in the brain and spinal cord, which eventually leads to paralysis and death [117]. The Blood-Brain Barrier/Blood Spinal Cord Barrier (BBB/BSCB) is altered in ALS, mainly due to EC damage that precedes neuroinflammation and MN degeneration [118-120]. This disease has been recognized as a neurovascular disorder [23,118,119,121]. In light of this known neurovascular damage, Garbuzova-Davis *et al.* [17] characterized CD146+ CECs from peripheral blood of ALS patients and found that CECs surprisingly *decreased* during disease progression in comparison to healthy controls. The multiple endothelial layers in the brainstem and spinal cord capillaries of ALS patients and mice modeling ALS [23,118] suggest that CECs are not desquamating into the blood as a result of endothelial detachment by BBB/BSCB disruption, but rather due to an impaired endothelialization process [17]. Additionally, it is possible that CD146 is not specific enough for CECs [56,83], and that CPEPC levels are possibly affected, likely due to impaired mobilization from the bone marrow [17,41].

#### Alzheimer's Disease (AD)

AD is a form of dementia characterized pathologically by amyloid plaques, neurofibrillary tangles, vascular damage from plaque deposition, and neuronal cell death [122]. Neurovascular mechanisms and BBB dysfunction contribute to AD pathogenesis [22,123]. For instance, Wu *et al.* [124] showed that endothelial MEOX2, a homeobox gene that regulates vascular differentiation, is down regulated in AD, resulting in decreased brain angiogenesis, reduced capillary density and cerebral blood flow, and BBB disruption. Similarly to the cardiovascular diseases mentioned above, atherosclerosis is strongly associated with AD [125,126].

Lee *et al.* [20]. Found that "CFU-EPCs" (similar to CFU-Hill cells, but confusingly labeled as a "CAC" subtype by the authors) were decreased in AD patients compared to cardiovascular Risk Factor (RF) controls with no neurological issues. Furthermore, decreasing cognitive function and increasing dementia in AD patients correlated with decreasing levels of CFU-EPCs [20]. Further studies by Lee *et al.* [19] on the same cell type showed that high concentrations of amyloid  $\beta_{1-42}$  reduced "CAC" counts in culture, and that CACs from AD patients had decreased migratory capacity and increased senescence compared to RF controls. These are possible explanations for why CFU-EPC is decreased in AD. Interestingly, flow cytometry analysis of CD34+/KDR+ and CD133+ cell counts showed no changes in AD patients versus controls [20], which highlight the importance of using *in vitro* methods when analyzing "EPC" subsets as circulating biomarkers.

## Parkinson's Disease (PD)

PD is a movement disorder characterized by a reduction in nigrostriatal dopaminergic activity [127]. Like ALS and AD, BBB disruption has been identified in PD [24]. Different EPC subsets have been studied with conflicting results. Lee *et al.* [18] examined CD34+/KDR+ CPEPCs and demonstrated a decreased level of these cells in PD patients receiving chronic levodopa treatment compared to levodopa/COMT inhibitor-treated patients and healthy controls. The authors concluded that CD34+/KDR+ CPEPCs in levodopa-treated patients were reduced in response to increased endothelial damage [18], possibly resulting from hyperhomocysteinemia [128] brought about by Catechol-O-Methyltransferase (COMT) metabolism of levodopa [129]. In contrast, Pezzoli *et al.* [21] showed an increase in CD34+/KDR+/CD45+CPEPCs in levodopa-treated patients and non-treated PD patients versus healthy controls. In the non-treated PD patients, the authors suggest that low dopamine levels are coupled to high CPEPC levels because dopamine modulates EPC mobilization and negatively correlates with CPEPC levels as found in rodents [130]. However, it is not clear why the CPEPCs are increased in the levodopa-treated patients in this study. These studies reiterate the weakness of flow cytometry in EPC biology, and show how analyses of a single marker (CD45) can produce paradoxical results.

## Conclusion

Circulating Putative Endothelial Progenitor Cells (CPEPCs) consist of three main subsets: CACs, CFU-Hill cells, and ECFCs. Both CACs and CFU-Hill cells are hematopoietic cells that contribute to angiogenic blood vessel formation via perigrine secretions. ECFCs are probably the true EPC which is capable of proliferation and differentiation into ECs and incorporation into blood vessels. All three cell subsets are possible sources of endothelial repair. In contrast, CECs are representative of endothelial vascular damage and may even trigger acute vascular events due to their pro-coagulant activity.

CACs, CFU-Hill, and CECs all have strong potential as biomarkers for the prevalence and prognosis of vascular and neurodegenerative/neurovascular diseases. However, the literature is inundated with conflicting results. Improved methods for identifying these cell types are crucial for obtaining consistent data across studies [1,50,78]. Flow cytometry or immunobead capture is likely insufficient for identifying CPEPC subsets or discriminating between CPEPCs and CECs. The phenotypic overlap between these two cell groups could have particularly negative consequences for the field considering that CPEPCs represent vascular repair and CECs reflect vascular damage. For instance, a study that isolates CECs using only CD146 may actually be isolating ECFCs [56], leading to questionable results. It is thus imperative that flow cytometry/immunobead capture be paired with previously defined *in vitro* methods. Discriminating between CPEPCs and CECs requires consideration of both cell size and ability to form colonies with proliferative potential. Importantly, the ECFC subset is special in that it may represent the true EPC population, but the ECFC may not be clinically practical as a circulating biomarker.

Future studies are needed to standardize methods characterizing CPEPCs and CECs before these potential clinical biomarkers will be truly useful for diagnosis and prognosis of various cardiovascular and neurodegenerative disorders.

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## References

- Burger D, Touyz RM. Cellular biomarkers of endothelial health: microparticles, endothelial progenitor cells, and circulating endothelial cells. *J Am Soc Hypertens.* 2012; 6: 85-99.
- Endemann DH, Schiffrin EL. Endothelial dysfunction. *J Am Soc Nephrol.* 2004; 15: 1983-1992.
- Rodrigues SF, Granger DN. Blood cells and endothelial barrier function. *Tissue Barriers.* 2015; 3: e978720.
- Erdbruegger U, Haubitz M, Woywodt A. Circulating endothelial cells: a novel marker of endothelial damage. *Clin Chim Acta.* 2006; 373: 17-26.
- Hunting CB, Noort WA, Zwaginga JJ. Circulating endothelial (progenitor) cells reflect the state of the endothelium: vascular injury, repair and neovascularization. *Vox Sang.* 2005; 88: 1-9.
- Woywodt A, Bahlmann FH, De Groot K, Haller H, Haubitz M. Circulating endothelial cells: life, death, detachment and repair of the endothelial cell layer. *Nephrol Dial Transplant.* 2002; 17: 1728-1730.
- Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, et al. Isolation of putative progenitor endothelial cells for angiogenesis. *Science.* 1997; 275: 964-967.
- Asahara T, Masuda H, Takahashi T, Kalka C, Pastore C, Silver M, et al. Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. *Circ Res.* 1999; 85: 221-228.
- Dome B, Timar J, Ladanyi A, Paku S, Renyi-Vamos F, Klepetko W, et al. Circulating endothelial cells, bone marrow-derived endothelial progenitor cells and proangiogenic hematopoietic cells in cancer: From biology to therapy. *Crit Rev Oncol Hematol.* 2009; 69: 108-124.
- Nadar SK, Lip GY, Lee KW, Blann AD. Circulating endothelial cells in acute ischaemic stroke. *Thromb Haemost.* 2005; 94: 707-712.
- Mutin M, Canavy I, Blann A, Bory M, Sampol J, Dignat-George F. Direct evidence of endothelial injury in acute myocardial infarction and unstable angina by demonstration of circulating endothelial cells. *Blood.* 1999; 93: 2951-2958.
- Massa M, Rosti V, Ferrario M, Campanelli R, Ramajoli I, Rosso R, et al. Increased circulating hematopoietic and endothelial progenitor cells in the early phase of acute myocardial infarction. *Blood.* 2005; 105: 199-206.
- Paczkowska E, GoÅ, Å...b-Janowska M, Bajer-Czajkowska A, MachaliÅ, ska A, Ustianowski P, et al. Increased circulating endothelial progenitor cells in patients with haemorrhagic and ischaemic stroke: the role of endothelin-1. *J Neurol Sci.* 2013; 325: 90-99.
- Lee KW, Lip GY, Tayebjee M, Foster W, Blann AD. Circulating endothelial cells, von Willebrand factor, interleukin-6, and prognosis in patients with acute coronary syndromes. *Blood.* 2005; 105: 526-532.
- Sobrinho T, Hurtado O, Moro MA, Rodríguez-Yáñez M, Castellanos M, Brea D, et al. The increase of circulating endothelial progenitor cells after acute ischemic stroke is associated with good outcome. *Stroke.* 2007; 38: 2759-2764.
- Werner N, Kosiol S, Schiegl T, Ahlers P, Walenta K, Link A, et al. Circulating endothelial progenitor cells and cardiovascular outcomes. *N Engl J Med.* 2005; 353: 999-1007.
- Garbuzova-Davis S, Woods RL 3<sup>rd</sup>, Louis MK, Zesiewicz TA, Kuzmin-Nichols N, Sullivan KL, et al. Reduction of circulating endothelial cells in peripheral blood of ALS patients. *PLoS One.* 2010; 5: e10614.
- Lee PH, Kim HS, Lee JE, Choi Y, Hong JY, Nam HS, et al. Comparison

- of endothelial progenitor cells in Parkinson's disease patients treated with levodopa and levodopa/COMT inhibitor. *PLoS One*. 2011; 6: e21536.
19. Lee ST, Chu K, Jung KH, Jeon D, Bahn JJ, Kim JH, et al. Dysfunctional characteristics of circulating angiogenic cells in Alzheimer's disease. *J Alzheimers Dis*. 2010; 19: 1231-1240.
  20. Lee ST, Chu K, Jung KH, Park HK, Kim DH, Bahn JJ, et al. Reduced circulating angiogenic cells in Alzheimer disease. *Neurology*. 2009; 72: 1858-1863.
  21. Pezzoli G, Cavanna F, Cassani E, Barichella M, Pinelli G, Iorio L, et al. Endothelial progenitor cells: Cardiovascular protection in Parkinson's disease? *Int J Cardiol*. 2015; 197: 200-202.
  22. Bell RD, Zlokovic BV. Neurovascular mechanisms and blood-brain barrier disorder in Alzheimer's disease. *Acta Neuropathol*. 2009; 118: 103-113.
  23. Garbuzova-Davis S, Hernandez-Ontiveros DG, Rodrigues MC, Haller E, Frisina-Deyo A, Mirtyl S, et al. Impaired blood-brain/spinal cord barrier in ALS patients. *Brain Res*. 2012; 1469: 114-128.
  24. Lee H1, Pienaar IS. Disruption of the blood-brain barrier in Parkinson's disease: curse or route to a cure? *Front Biosci (Landmark Ed)*. 2014; 19: 272-280.
  25. Satterthwaite AB, Burn TC, Le Beau MM, Tenen DG. Structure of the gene encoding CD34, a human hematopoietic stem cell antigen. *Genomics*. 1992; 12: 788-794.
  26. Cleaver O, Krieg PA. VEGF mediates angioblast migration during development of the dorsal aorta in *Xenopus*. *Development*. 1998; 125: 3905-3914.
  27. Terman BI, Dougher-Vermazen M, Carrion ME, Dimitrov D, Armellino DC, Gospodarowicz D, et al. Identification of the KDR tyrosine kinase as a receptor for vascular endothelial cell growth factor. *Biochem Biophys Res Commun*. 1992; 187: 1579-1586.
  28. Asahara T, Kawamoto A, Masuda H. Concise review: Circulating endothelial progenitor cells for vascular medicine. *Stem Cells*. 2011; 29: 1650-1655.
  29. Risau W. Mechanisms of angiogenesis. *Nature*. 1997; 386: 671-674.
  30. Ferguson JE 3<sup>rd</sup>, Kelley RW, Patterson C. Mechanisms of endothelial differentiation in embryonic vasculogenesis. *Arterioscler Thromb Vasc Biol*. 2005; 25: 2246-2254.
  31. Kovacic JC, Moore J, Herbert A, Ma D, Boehm M, Graham RM. Endothelial progenitor cells, angioblasts, and angiogenesis--old terms reconsidered from a current perspective. *Trends Cardiovasc Med*. 2008; 18: 45-51.
  32. Risau W, Flamme I. Vasculogenesis. *Annu Rev Cell Dev Biol*. 1995; 11: 73-91.
  33. Basile DP, Yoder MC. Circulating and tissue resident endothelial progenitor cells. *J Cell Physiol*. 2014; 229: 10-16.
  34. Hirschi KK, Ingram DA, Yoder MC. Assessing identity, phenotype, and fate of endothelial progenitor cells. *Arterioscler Thromb Vasc Biol*. 2008; 28: 1584-1595.
  35. Kachamakova-Trojanowska N, Bukowska-Strakova K, Zukowska M, Dulak J, Jozkowicz A. The real face of endothelial progenitor cells - Circulating angiogenic cells as endothelial prognostic marker? *Pharmacol Rep*. 2015; 67: 793-802.
  36. Vasa M, Fichtlscherer S, Adler K, Aicher A, Martin H, Zeiher AM, et al. Increase in circulating endothelial progenitor cells by statin therapy in patients with stable coronary artery disease. *Circulation*. 2001; 103: 2885-2890.
  37. Prokopi M, Pula G, Mayr U, Devue C, Gallagher J, Xiao Q, et al. Proteomic analysis reveals presence of platelet microparticles in endothelial progenitor cell cultures. *Blood*. 2009; 114: 723-732.
  38. Hassan NF, Campbell DE, Douglas SD. Purification of human monocytes on gelatin-coated surfaces. *J Immunol Methods*. 1986; 95: 273-276.
  39. Schmeisser A, Garlachs CD, Zhang H, Eskafi S, Graffy C, Ludwig J, et al. Monocytes coexpress endothelial and macrophagocytic lineage markers and form cord-like structures in Matrigel under angiogenic conditions. *Cardiovasc Res*. 2001; 49: 671-680.
  40. Rehman J, Li J, Orschell CM, March KL. Peripheral blood "endothelial progenitor cells" are derived from monocyte/macrophages and secrete angiogenic growth factors. *Circulation*. 2003; 107: 1164-1169.
  41. Hill JM, Zalos G, Halcox JP, Schenke WH, Waclawiw MA, Quyyumi AA, et al. Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. *N Engl J Med*. 2003; 348: 593-600.
  42. Rohde E, Bartmann C, Schallmoser K, Reinisch A, Lanzer G, Linkesch W, et al. Immune cells mimic the morphology of endothelial progenitor colonies *in vitro*. *Stem Cells*. 2007; 25: 1746-1752.
  43. Rohde E, Malischnik C, Thaler D, Maierhofer T, Linkesch W, Lanzer G, et al. Blood monocytes mimic endothelial progenitor cells. *Stem Cells*. 2006; 24: 357-367.
  44. Ingram DA, Mead LE, Tanaka H, Meade V, Fenoglio A, Mortell K, et al. Identification of a novel hierarchy of endothelial progenitor cells using human peripheral and umbilical cord blood. *Blood*. 2004; 104: 2752-2760.
  45. Yoder MC, Mead LE, Prater D, Krier TR, Mroueh KN, Li F, et al. Redefining endothelial progenitor cells via clonal analysis and hematopoietic stem/progenitor cell principals. *Blood*. 2007; 109: 1801-1809.
  46. Yoder MC. Human endothelial progenitor cells. *Cold Spring Harb Perspect Med*. 2012; 2: a006692.
  47. Whittington CF, Yoder MC, Voytik-Harbin SL. Collagen-polymer guidance of vessel network formation and stabilization by endothelial colony forming cells *in vitro*. *Macromol Biosci*. 2013; 13: 1135-1149.
  48. Sieveking DP, Buckle A, Celermajer DS, Ng MK. Strikingly different angiogenic properties of endothelial progenitor cell subpopulations: insights from a novel human angiogenesis assay. *J Am Coll Cardiol*. 2008; 51: 660-668.
  49. Critser PJ, Kreger ST, Voytik-Harbin SL, Yoder MC. Collagen matrix physical properties modulate endothelial colony forming cell-derived vessels *in vivo*. *Microvasc Res*. 2010; 80: 23-30.
  50. Fadini GP, Losordo D, Dimmeler S. Critical reevaluation of endothelial progenitor cell phenotypes for therapeutic and diagnostic use. *Circ Res*. 2012; 110: 624-637.
  51. Peichev M, Naiyer AJ, Pereira D, Zhu Z, Lane WJ, Williams M, et al. Expression of VEGFR-2 and AC133 by circulating human CD34(+) cells identifies a population of functional endothelial precursors. *Blood*. 2000; 95: 952-958.
  52. Case J, Mead LE, Bessler WK, Prater D, White HA, Saadatizadeh MR, et al. Human CD34+AC133+VEGFR-2+ cells are not endothelial progenitor cells but distinct, primitive hematopoietic progenitors. *Exp Hematol*. 2007; 35: 1109-1118.
  53. Timmermans F, Van Hauwermeiren F, De Smedt M, Raedt R, Plasschaert F, De Buyzere ML, Gillebert TC. Endothelial outgrowth cells are not derived from CD133+ cells or CD45+ hematopoietic precursors. *Arterioscler Thromb Vasc Biol*. 2007; 27: 1572-1579.
  54. Hattori K, Dias S, Heissig B, Hackett NR, Lyden D, Tateno M, et al. Vascular endothelial growth factor and angiopoietin-1 stimulate postnatal hematopoiesis by recruitment of vasculogenic and hematopoietic stem cells. *The Journal of experimental medicine*. 2001; 193: 1005-1014.
  55. Rafii S, Heissig B, Hattori K. Efficient mobilization and recruitment of marrow-derived endothelial and hematopoietic stem cells by adenoviral vectors expressing angiogenic factors. *Gene Ther*. 2002; 9: 631-641.
  56. Mund JA, Estes ML, Yoder MC, Ingram DA Jr, Case J. Flow cytometric identification and functional characterization of immature and mature circulating endothelial cells. *Arterioscler Thromb Vasc Biol*. 2012; 32: 1045-1053.
  57. Shalaby F, Rossant J, Yamaguchi TP, Gertsenstein M, Wu XF, Breitman ML, et al. Failure of blood-island formation and vasculogenesis in *Fli-1*-deficient mice. *Nature*. 1995; 376: 62-66.

58. Flamme I, Risau W. Induction of vasculogenesis and hematopoiesis *in vitro*. *Development*. 1992; 116: 435-439.
59. Antas VI, Al-Drees MA, Prudence AJ, Sugiyama D, Fraser ST. Hemogenic endothelium: a vessel for blood production. *Int J Biochem Cell Biol*. 2013; 45: 692-695.
60. Desai A, Glaser A, Liu D, Raghavachari N, Blum A, Zalos G, et al. Microarray-based characterization of a colony assay used to investigate endothelial progenitor cells and relevance to endothelial function in humans. *Arterioscler Thromb Vasc Biol*. 2009; 29: 121-127.
61. Medina RJ, O'Neill CL, Sweeney M, Guduric-Fuchs J, Gardiner TA, Simpson DA, et al. Molecular analysis of endothelial progenitor cell (EPC) subtypes reveals two distinct cell populations with different identities. *BMC Med Genomics*. 2010; 3: 18.
62. O'Neill TJ, Wamhoff BR, Owens GK, Skalak TC. Mobilization of bone marrow-derived cells enhances the angiogenic response to hypoxia without transdifferentiation into endothelial cells. *Circ Res*. 2005; 97: 1027-1035.
63. Zentilin L, Tafuro S, Zacchigna S, Arsic N, Pattarini L, Sinigaglia M, et al. Bone marrow mononuclear cells are recruited to the sites of VEGF-induced neovascularization but are not incorporated into the newly formed vessels. *Blood*. 2006; 107: 3546-3554.
64. Civin CI, Gore SD. Antigenic analysis of hematopoiesis: a review. *J Hematother*. 1993; 2: 137-144.
65. Yoder MC. Is endothelium the origin of endothelial progenitor cells? *Arterioscler Thromb Vasc Biol*. 2010; 30: 1094-1103.
66. Lin Y, Weisdorf DJ, Solovey A, Heibel RP. Origins of circulating endothelial cells and endothelial outgrowth from blood. *J Clin Invest*. 2000; 105: 71-77.
67. Ingram DA, Mead LE, Moore DB, Woodard W, Fenoglio A, Yoder MC. Vessel wall-derived endothelial cells rapidly proliferate because they contain a complete hierarchy of endothelial progenitor cells. *Blood*. 2005; 105: 2783-2786.
68. Bruce Alberts AJ, Julian Lewis, Martin Raff, Keith Roberts, Peter Walter. *Blood Vessels and Endothelial Cells*. Molecular biology of the cell 4<sup>th</sup> edition. New York: Garland Science. 2002; 1259-1313.
69. Abbott NJ, Patabendige AA, Dolman DE, Yusof SR, Begley DJ. Structure and function of the blood-brain barrier. *Neurobiol Dis*. 2010; 37: 13-25.
70. Brightman MW, Reese TS. Junctions between intimately apposed cell membranes in the vertebrate brain. *J Cell Biol*. 1969; 40: 648-677.
71. Tontsch U, Bauer HC. Glial cells and neurons induce blood-brain barrier related enzymes in cultured cerebral endothelial cells. *Brain Res*. 1991; 539: 247-253.
72. Aird WC. Phenotypic heterogeneity of the endothelium: I. Structure, function, and mechanisms. *Circ Res*. 2007; 100: 158-173.
73. Pearson JD. Endothelial cell biology. *Radiology*. 1991; 179: 9-14.
74. Boos CJ, Balakrishnan B, Jessani S, Blann AD, Lip GYH. Effects of percutaneous coronary intervention on peripheral venous blood circulating endothelial cells and plasma indices of endothelial damage/dysfunction. *Chest*. 2007; 132: 1920-1926.
75. Woywodt A, Blann AD, Kirsch T, Erdbruegger U, Banzet N, Haubitz M, et al. Isolation and enumeration of circulating endothelial cells by immunomagnetic isolation: proposal of a definition and a consensus protocol. *J Thromb Haemost*. 2006; 4: 671-677.
76. Lampka M, GrÅ...bczewska Z, Jendryczka-MaÅ±kiewicz E, HoÅ,yÅ, skawian I, Sukiennik A, et al. Circulating endothelial cells in coronary artery disease. *Kardiol Pol*. 2010; 68: 1100-1105.
77. Mourino-Alvarez L, Calvo E, Moreu J, Padial LR, Lopez JA, Barderas MG, et al. Proteomic characterization of EPCs and CECs "*in vivo*" from acute coronary syndrome patients and control subjects. *Biochim Biophys Acta*. 2013; 1830: 3030-3053.
78. Schmidt DE, Manca M, Hoefer IE. Circulating endothelial cells in coronary artery disease and acute coronary syndrome. *Trends Cardiovasc Med*. 2015; 25: 578-587.
79. Erdbruegger U, Haubitz M, Woywodt A. Circulating endothelial cells: a novel marker of endothelial damage. *Clin Chim Acta*. 2006; 373: 17-26.
80. Bardin N, Anfosso F, Massé JM, Cramer E, Sabatier F, Le Bivic A, et al. Identification of CD146 as a component of the endothelial junction involved in the control of cell-cell cohesion. *Blood*. 2001; 98: 3677-3684.
81. Anfosso F, Bardin N, Francès V, Vivier E, Camoin-Jau L, Sampol J, et al. Activation of human endothelial cells via S-endo-1 antigen (CD146) stimulates the tyrosine phosphorylation of focal adhesion kinase p125(FAK). *J Biol Chem*. 1998; 273: 26852-26856.
82. Jiang S, Pei L, Yang ZL, Liu G. Prognostic Value of the Stem Cell Markers Epcam and CD133 Expression Of Gallbladder Adenocarcinoma. *Hepatogastroenterology*. 2014; 61: 574-579.
83. Delorme B, Basire A, Gentile C, Sabatier F, Monsonis F, Desouches C, et al. Presence of endothelial progenitor cells, distinct from mature endothelial cells, within human CD146+ blood cells. *Thromb Haemost*. 2005; 94: 1270-1279.
84. Blann AD, Woywodt A, Bertolini F, Bull TM, Buyon JP, Clancy RM, et al. Circulating endothelial cells. Biomarker of vascular disease. *Thromb Haemost*. 2005; 93: 228-235.
85. Quilici J, Banzet N, Paule P, Meynard JB, Mutin M, Bonnet JL, et al. Circulating endothelial cell count as a diagnostic marker for non-ST-elevation acute coronary syndromes. *Circulation*. 2004; 110: 1586-1591.
86. Sabatier F, Camoin-Jau L, Anfosso F, Sampol J, Dignat-George F. Circulating endothelial cells, microparticles and progenitors: key players towards the definition of vascular competence. *J Cell Mol Med*. 2009; 13: 454-471.
87. Ballieux BE, Hiemstra PS, Klar-Mohamad N, Hagen EC, van Es LA, van der Woude FJ, et al. Detachment and cytolysis of human endothelial cells by proteinase 3. *Eur J Immunol*. 1994; 24: 3211-3215.
88. Rüegg C, Yilmaz A, Bieler G, Bamat J, Chaubert P, Lejeune FJ. Evidence for the involvement of endothelial cell integrin alphaVbeta3 in the disruption of the tumor vasculature induced by TNF and IFN-gamma. *Nat Med*. 1998; 4: 408-414.
89. Wassmann S, Laufs U, Baumer AT, Muller K, Ahlborn K, Linz W, et al. HMG-CoA reductase inhibitors improve endothelial dysfunction in normocholesterolemic hypertension via reduced production of reactive oxygen species. *Hypertension*. 2001; 37: 1450-1457.
90. Woywodt A, Streiber F, de Groot K, Regelsberger H, Haller H, Haubitz M. Circulating endothelial cells as markers for ANCA-associated small-vessel vasculitis. *Lancet*. 2003; 361: 206-210.
91. Bombeli T, Karsan A, Tait JF, Harlan JM. Apoptotic vascular endothelial cells become procoagulant. *Blood*. 1997; 89: 2429-2442.
92. Rimmerman CM. *Coronary Artery Disease: The Cleveland Clinic Foundation*. 2013.
93. Vasa M, Fichtlscherer S, Aicher A, Adler K, Urbich C, Martin H, et al. Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease. *Circ Res*. 2001; 89: E1-7.
94. Wang HY, Gao PJ, Ji KD, Shen WF, Fan CL, Lu L, et al. Circulating endothelial progenitor cells, C-reactive protein and severity of coronary stenosis in Chinese patients with coronary artery disease. *Hypertens Res*. 2007; 30: 133-141.
95. Güven H, Shepherd RM, Bach RG, Capoccia BJ, Link DC. The number of endothelial progenitor cell colonies in the blood is increased in patients with angiographically significant coronary artery disease. *J Am Coll Cardiol*. 2006; 48: 1579-1587.
96. Boos CJ, Balakrishnan B, Blann AD, Lip GY. The relationship of circulating endothelial cells to plasma indices of endothelial damage/dysfunction and apoptosis in acute coronary syndromes: implications for prognosis. *J Thromb Haemost*. 2008; 6: 1841-1850.
97. Bertrand ME, Simoons ML, Fox KA, Wallentin LC, Hamm CW, McFadden

- E, et al. Management of acute coronary syndromes in patients presenting without persistent ST-segment elevation. *Eur Heart J*. 2002; 23: 1809-1840.
98. Davies MJ. Acute coronary thrombosis—the role of plaque disruption and its initiation and prevention. *Eur Heart J*. 1995; 16 Suppl L: 3-7.
99. Van de Werf F, Ardissino D, Betriu A, Cokkinos DV, Falk E, et al. Management of acute myocardial infarction in patients presenting with ST-segment elevation. The Task Force on the Management of Acute Myocardial Infarction of the European Society of Cardiology. *Eur Heart J*. 2003; 24: 28-66.
100. Shintani S, Murohara T, Ikeda H, Ueno T, Honma T, Katoh A, et al. Mobilization of endothelial progenitor cells in patients with acute myocardial infarction. *Circulation*. 2001; 103: 2776-2779.
101. Bethel K, Lutgen MS, Damani S, Kolatkar A, Lamy R, Sabouri-Ghomi M, et al. Fluid phase biopsy for detection and characterization of circulating endothelial cells in myocardial infarction. *Phys Biol*. 2014; 11: 016002.
102. Boos CJ, Soor SK, Kang D, Lip GY. Relationship between circulating endothelial cells and the predicted risk of cardiovascular events in acute coronary syndromes. *Eur Heart J*. 2007; 28: 1092-1101.
103. Meneveau N, Deschaseaux F, Seronde MF, Chopard R, Schiele F, Jehl J, et al. Presence of endothelial colony-forming cells is associated with reduced microvascular obstruction limiting infarct size and left ventricular remodelling in patients with acute myocardial infarction. *Basic Res Cardiol*. 2011; 106: 1397-1410.
104. Arenillas JF. Intracranial atherosclerosis: current concepts. *Stroke*. 2011; 42: S20-23.
105. Albertson M, Sharma J. Stroke: current concepts. *S D Med*. 2014; 67: 455-461,463-465.
106. Pantoni L. Cerebral small vessel disease: from pathogenesis and clinical characteristics to therapeutic challenges. *Lancet Neurol*. 2010; 9: 689-701.
107. Yip HK, Chang LT, Chang WN, Lu CH, Liou CW, Lan MY, et al. Level and value of circulating endothelial progenitor cells in patients after acute ischemic stroke. *Stroke*. 2008; 39: 69-74.
108. Cuadrado-Godia E, Regueiro A, Nunez J, Diaz-Ricard M, Novella S, Oliveras A, et al. Endothelial Progenitor Cells Predict Cardiovascular Events after Atherothrombotic Stroke and Acute Myocardial Infarction. A PROCELL Substudy. *PLoS One*. 2015; 10: e0132415.
109. Freestone B, Lip GY, Chong AY, Nadar S, Lee KW, Blann AD. Circulating endothelial cells in atrial fibrillation with and without acute cardiovascular disease. *Thromb Haemost*. 2005; 94: 702-706.
110. Verma S, Kuliszewski MA, Li SH, Szmítko PE, Zucco L, Wang CH, et al. C-reactive protein attenuates endothelial progenitor cell survival, differentiation, and function: further evidence of a mechanistic link between C-reactive protein and cardiovascular disease. *Circulation*. 2004; 109: 2058-2067.
111. Asahara T, Takahashi T, Masuda H, Kalka C, Chen D, Iwaguro H, et al. VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells. *EMBO J*. 1999; 18: 3964-3972.
112. Bogoslovsky T, Chaudhry A, Latour L, Maric D, Luby M, Spatz M, et al. Endothelial progenitor cells correlate with lesion volume and growth in acute stroke. *Neurology*. 2010; 75: 2059-2062.
113. Kuliczowski W, Derzhko R, Prais I, Podolak-Dawidziak M, Serebruany VL. Endothelial progenitor cells and left ventricle function in patients with acute myocardial infarction: potential therapeutic considerations. *Am J Ther*. 2012; 19: 44-50.
114. Kunz GA, Liang G, Cuculi F, Gregg D, Vata KC, Shaw LK, et al. Circulating endothelial progenitor cells predict coronary artery disease severity. *Am Heart J*. 2006; 152: 190-195.
115. Chironi G, Walch L, Pernollet MG, Gariépy J, Levenson J, Rendu F, et al. Decreased number of circulating CD34+KDR+ cells in asymptomatic subjects with preclinical atherosclerosis. *Atherosclerosis*. 2007; 191: 115-120.
116. Lau KK, Chan YH, Yiu KH, Li SW, Tam S, Lau CP, et al. Burden of carotid atherosclerosis in patients with stroke: relationships with circulating endothelial progenitor cells and hypertension. *J Hum Hypertens*. 2007; 21: 445-451.
117. Rowland LP, Shneider NA. Amyotrophic lateral sclerosis. *N Engl J Med*. 2001; 344: 1688-1700.
118. Garbuzova-Davis S, Haller E, Saporta S, Kolomey I, Nicosia SV, Sanberg PR. Ultrastructure of blood-brain barrier and blood-spinal cord barrier in SOD1 mice modeling ALS. *Brain Res*. 2007; 1157: 126-137.
119. Garbuzova-Davis S, Saporta S, Haller E, Kolomey I, Bennett SP, Potter H, et al. Evidence of compromised blood-spinal cord barrier in early and late symptomatic SOD1 mice modeling ALS. *PLoS One*. 2007; 2: e1205.
120. Zhong Z, Deane R, Ali Z, Parisi M, Shapovalov Y, O'Banion MK, et al. ALS-causing SOD1 mutants generate vascular changes prior to motor neuron degeneration. *Nat Neurosci*. 2008; 11: 420-422.
121. Garbuzova-Davis S, Rodrigues MC, Hernandez-Ontiveros DG, Louis MK, Willing AE, Borlongan CV, et al. Amyotrophic lateral sclerosis: a neurovascular disease. *Brain Res*. 2011; 1398: 113-125.
122. Hardy JA, Higgins GA. Alzheimer's disease: the amyloid cascade hypothesis. *Science*. 1992; 256: 184-185.
123. de la Torre JC. Alzheimer disease as a vascular disorder: nosological evidence. *Stroke*. 2002; 33: 1152-1162.
124. Wu Z, Guo H, Chow N, Sallstrom J, Bell RD, Deane R, et al. Role of the MEOX2 homeobox gene in neurovascular dysfunction in Alzheimer disease. *Nat Med*. 2005; 11: 959-965.
125. Breteler MM. Vascular risk factors for Alzheimer's disease: an epidemiologic perspective. *Neurobiol Aging*. 2000; 21: 153-160.
126. Casserly I, Topol E. Convergence of atherosclerosis and Alzheimer's disease: inflammation, cholesterol, and misfolded proteins. *Lancet*. 2004; 363: 1139-1146.
127. Comi C, Magistrelli L, Oggioni GD, Carecchio M, Fleetwood T, Cantello R, et al. Peripheral nervous system involvement in Parkinson's disease: evidence and controversies. *Parkinsonism Relat Disord*. 2014; 20: 1329-1334.
128. Rolland PH, Friggi A, Barlatier A, Piquet P, Latrille V, Faye MM, et al. Hyperhomocysteinemia-induced vascular damage in the minipig. Captopril-hydrochlorothiazide combination prevents elastic alterations. *Circulation*. 1995; 91: 1161-1174.
129. Blandini F, Fancellu R, Martignoni E, Mangiagalli A, Pacchetti C, Samuele A, et al. Plasma homocysteine and l-dopa metabolism in patients with Parkinson disease. *Clin Chem*. 2001; 47: 1102-1104.
130. Chakraborty D, Chowdhury UR, Sarkar C, Baral R, Dasgupta PS, Basu S. Dopamine regulates endothelial progenitor cell mobilization from mouse bone marrow in tumor vascularization. *J Clin Invest*. 2008; 118: 1380-1389.