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ENDOTHELIAL PROGENITOR CELLS AS BIOMARKERS FOR CARDIOVASCULAR RISK

Master dissertation in Applied Pharmacology, supervised by Professor Doctor Sónia Alexandra Silva Santos and Professor Doctor Carlos Alberto Fontes Ribeiro and submitted to the Faculty of Pharmacy of the University of Coimbra

September 2014

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UNIVERSIDADE DE COIMBRA

Acknowledgements

I would like to express my gratitude to all those who gave me the possibility to complete this dissertation.

To my supervisors, Professor Doctor Sónia Alexandra Silva Santos and Professor Doctor Carlos Alberto Fontes Ribeiro, for the continuous support and availability.

To Professor Bárbara Oliveiros, of the Faculty of Medicine of the University of Coimbra, for the help and collaboration.

To my family for the patience, comprehension and support.

To my friends for their encouragement and support.

To Drª Capitolina Pinho and all my work colleagues,
for the availability and effort, and
for the time they granted me.

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Resumo

As células progenitoras endoteliais (EPCs) foram isoladas a partir de sangue periférico humano, pela primeira vez, em 1997. Estas células demonstraram capacidade de se diferenciarem em células endoteliais maduras. Além disso, demonstraram capacidade de se mobilizarem a partir de medula óssea e de se incorporarem em locais de isquemia para promover neovascularização. A formação de novos vasos sanguíneos pode ocorrer através de angiogénese, durante a qual são formados novos vasos sanguíneos a partir de vasos já existentes, processo este que depende da proliferação de células endoteliais. No entanto, a formação de novos vasos sanguíneos não ocorre apenas através de angiogénese, mas também através da vasculogénese, que consiste na formação de novos vasos sanguíneos a partir de EPCs, que se diferenciam em células endoteliais maduras, contribuindo para a homeostase e reparação do endotélio. A vasculogénese contribui para a neovascularização de tumores em desenvolvimento, a cicatrização de feridas, isquemia grave dos membros posteriores, e a isquemia do miocárdio, bem como a neovascularização fisiológica. Existe controvérsia sobre a identificação de EPCs no sangue periférico e quais os marcadores de superfície que melhor caracterizam estas células. O número de EPCs assim como o seu potencial proliferativo podem ser alterados, sob condições patológicas, incluindo doenças cardiovasculares. Para além disso, existem fatores de risco que afetam a concentração de EPCs no sangue periférico.

Este estudo de revisão teve como objetivo descrever o papel das EPCs como biomarcadores de risco cardiovascular e de doença cardiovascular. Foi realizada uma pesquisa eletrónica na base de dados MEDLINE, através da PubMed, para obter dados de artigos científicos, de forma a concretizar este objetivo.

Estas células podem ser identificadas e caracterizadas através de citometria de fluxo ou através da cultura de células sanguíneas mononucleares. De acordo com a informação obtida a partir dos estudos que resultaram da pesquisa eletrónica, foi demonstrado que a citometria de fluxo é o método mais utilizado para definir EPCs. Quanto às combinações de marcadores de superfície mais utilizados para identificar estas células no sangue periférico, foi demonstrado que a combinação do cluster de diferenciação (CD) CD34 e do domínio de inserção da cinase (KDR), isto é, CD34+KDR+, é a combinação mais utilizada para identificar EPCs em doenças cardiovasculares. Verificou-se que o aumento dos níveis de EPCs está geralmente associado a fases agudas de doença cardiovascular, mas as fases avançadas de doença estão associadas à diminuição dos níveis de EPCs.

Esta dissertação demonstra que as EPCs estão envolvidas na doença cardiovascular e que também estão relacionadas com as complicações vasculares da diabetes e risco cardiovascular em doentes renais. Desta forma, a monitorização dos níveis circulantes de EPCs como biomarcadores de risco cardiovascular pode ser vantajosa para identificar indivíduos com risco elevado de desenvolver doença cardiovascular. Esta monitorização poderia resultar na deteção precoce de doenças cardiovasculares e na possibilidade de iniciar a terapêutica adequada o mais precocemente possível.

Palavras-chave: células progenitoras endoteliais/EPC, disfunção endotelial, marcadores biológicos, antigénios/antigénios de superfície, terapêutica, diagnóstico, doenças cardiovasculares, risco cardiovascular.

Abstract

Endothelial progenitor cells (EPCs) were first isolated from peripheral blood (PB) by Asahara and colleagues in 1997. These cells were capable of differentiating into mature endothelial cells (ECs). Moreover, they were capable of mobilisation from bone marrow (BM) and incorporation into sites of ischaemia to promote neovascularisation. BM-derived EPCs home and incorporate into sites of neovascularisation where differentiation into ECs is completed. Neovasculogenesis contributes to endogenous neovascularisation of developing tumours, wound healing, severe hindlimb ischaemia, and myocardial ischaemia, as well as physiological neovascularisation. There is controversy regarding the identification of EPCs in PB and which are the surface markers that best characterize these cells. The number of EPCs as well as their proliferative potential may change under pathological conditions, including cardiovascular diseases. Besides, there are risk factors affecting the concentration of EPCs in PB.

The objective of this review study was to describe the role of EPCs as biomarkers of cardiovascular risk and cardiovascular disease. An electronic search was performed within the MEDLINE database, assessed through PubMed, in order to accomplish the objective of this work.

It was found that increased levels of EPCs are generally associated with acute phases of cardiovascular disease, but advanced stages of disease are associated with decreased EPC levels. Besides, it was demonstrated that flow cytometry is the most used method to define EPCs and that cluster of differentiation (CD) 34+ and kinase insert domain (KDR) +, namely, CD34+KDR+ is the most used combination of markers to identify EPCs in cardiovascular diseases. This dissertation demonstrated that EPCs are involved in cardiovascular disease and that are also related to vascular complications of diabetes and cardiovascular risk in renal patients. Therefore, monitoring the levels of circulating EPCs as biomarkers of cardiovascular risk could be specifically useful to identify subjects at low or high risk of developing cardiovascular disease. This could result in the early detection of cardiovascular disease and in the possibility of initiating appropriate therapy as early as possible and subsequent monitoring.

Keywords: endothelial progenitor cells/EPC, endothelial dysfunction, biological markers, antigens/surface antigens, therapeutics, diagnosis, cardiovascular diseases, cardiovascular risk.

List of abbreviations

AGEs	Advanced glycation end products
ACE	Angiotensin-converting enzyme
acLDL	Acetylated low density lipoprotein
ACS	Acute coronary syndrome
ADMA	Asymmetric dimethyl-arginine
AG	Acylated ghrelin
ALDH	Aldehyde dehydrogenase
ALDHbr	Aldehyde dehydrogenase bright activity
ARWMC	Age-related white matter changes
BM	Bone marrow
BMI	Body mass index
BNP	B-type natriuretic peptide
C3a	Complement C3 a-fragment
C3b	Complement C3 b-fragment
CAD	Coronary artery disease
CADASIL	Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy
CD	Cluster of differentiation
CECs	Circulating endothelial cells
CFU	Colony forming unit
CL	Collagen
CPC	Circulating progenitor cell
CPR	Cardiopulmonary resuscitation
CRP	C-reactive protein
CTA	Computed tomography angiography
CXCR4	C-X-C chemokine receptor type 4
DiI-acLDL	1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate-acetylated low-density lipoprotein
EC	Endothelial cell
ECFC	Endothelial colony-forming cell
ECG	Electrocardiographic
EGM	Endothelial cell growth medium

EL	Elastin
EMPs	Endothelial microparticles
eNOS	Endothelial nitric oxide synthase
EPC	Endothelial progenitor cell
EPO	Erythropoietin
Fib	Fibrillin
Flt-1	Fms-like tyrosine kinase-1 receptor
FMD	Flow-mediated dilatation
G-CSF	Granulocyte colony-stimulating factor
GFR	Glomerular filtration rate
GIP	Glucose-dependent insulinotropic polypeptide
GLP-1	Glucagon like peptide-1
GM-CSF	Granulocyte monocyte colony-stimulating factor
hCG	Human chorionic gonadotropin
HDL	High density lipoprotein
HIF-1	Hypoxia-inducible factor-1
HIF-1α	Hypoxia-inducible factor-1 alpha subunit
HIF-1β	Hypoxia-inducible factor-1 beta subunit
HIV	Human immunodeficiency virus
HMG-CoA	hydroxymethylglutaryl–coenzyme A
hs-CRP	High-sensitivity C-reactive protein
ICAM-1	Intercellular cell adhesion molecule-1
IFN-I	Type I interferon
IL-6	Interleukin 6
IMT	Intima-media thickness
KDR	Kinase insert domain receptor
LDL	Low density lipoprotein
LV	Left ventricular
LVEF	Left ventricular ejection fraction
LVH	Left ventricular hypertrophy
MDA-LDL	Malondialdehyde-modified low-density lipoprotein
MDCT	Multidetector computed tomography
miRNA	Micro-ribonucleic acid
mkitL	Membrane-bound Kit Ligand

MMP	Matrix metalloproteinase
MMP-2	Matrix metalloproteinase 2
MMP-9	Matrix metalloproteinase 9
MNC	Mononuclear cell
MPs	Microparticles
ms	Milliseconds
MT1-MMP	Membrane type-1 matrix metalloproteinase
NO	Nitric oxide
NT-proBNP	N-terminal pro-B-type natriuretic peptide
NYHA	New York Heart Association
PAD	Peripheral arterial disease
PB	Peripheral blood
PCI	Percutaneous coronary intervention
PECAM-1	Platelet-endothelial cell adhesion molecule-1
PI3K	Phosphatidylinositol-3 kinase or phosphoinositide-3 kinase
PKB	Protein kinase B, also known as Akt
PlGF	Placental growth factor
PPARγ	Peroxisome proliferator-activated receptor gamma
PWV	Pulse wave velocity
ROS	Reactive oxygen species
SDF-1	Stromal cell-derived factor-1
SDF-1α	Stromal cell-derived factor-1 alpha
sICAM-1	Soluble intercellular cell adhesion molecule-1
skitL	Soluble Kit Ligand
SMC	Smooth muscle cell
STEMI	ST-elevation myocardial infarction
sVCAM-1	Soluble vascular cell adhesion molecule-1
T1DM	Type 1 diabetes *mellitus*
T2DM	Type 2 diabetes *mellitus*
TASC II	Trans-Atlantic Inter-Society Consensus II
TIMP-1	Inhibitor of metalloproteinases-1
TNF-α	Tumour necrosis factor-alpha
TSP-1	Thrombospondin-1
UAG	Unacylated ghrelin

VCAM-1	Vascular cell adhesion molecule-1
VE-cadherin	Vascular endothelial cadherin, also known as CD144
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
VEGFR2	Vascular endothelial growth factor receptor 2
vWF	von Willebrand Factor

Introduction

Endothelial progenitor cells (EPCs) were first isolated from peripheral blood (PB) by Asahara and colleagues in 1997. These cells were capable of differentiating into mature endothelial cells (ECs). Moreover, they were capable of mobilisation from bone marrow (BM) and incorporation into sites of ischaemia to promote neovascularisation. The concept that vasculogenesis only occurs in the embryo changed and studies demonstrated that BM-derived EPCs home and incorporate into sites of neovascularisation where differentiation into ECs is completed, consistent with neovasculogenesis. Therefore, neovasculogenesis contributes to endogenous neovascularisation of developing tumours, wound healing, severe hindlimb ischaemia, and myocardial ischaemia, as well as physiological neovascularisation.

Since EPCs were first identified in PB, many authors studied the characteristics and effects of these cells. However, there is still controversy regarding the identification of EPCs in PB and which are the surface markers that best characterize these cells.

Nowadays, it is known that EPCs play a significant role in the re-endothelialisation and neovascularisation of injured endothelium. The number of EPCs as well as their proliferative potential may change under pathological conditions, including cardiovascular diseases, in which endothelial dysfunction is also present. Besides, there are risk factors affecting the concentration of EPCs in PB. Drugs, lifestyles, age and gender may influence circulating levels of EPCs.

A review study was conducted to describe the role of EPCs as biomarkers of cardiovascular risk and cardiovascular disease.

Objectives

The main objective of this dissertation is to describe the role of EPCs as biomarkers of cardiovascular risk and cardiovascular disease. In detail, the objectives are:

- Describe the relationship between EPCs, vasculogenesis and angiogenesis, as well as EPC mobilisation from the BM, in order to contextualise the subject;
- Identify, explain and compare the methods and markers currently used by researchers to identify and characterise EPCs;
- Evaluate which method is most used by researchers to identify EPCs;
- Evaluate which markers are mostly used by researchers to identify EPCs;
- Describe the relationship between EPCs and cardiovascular diseases or cardiovascular risk factors;
- Describe the relationship between EPCs, diabetes and cardiovascular disease;
- Describe the relationship between EPCs, renal function and cardiovascular disease;
- Refer the relationship between EPCs, cardiovascular risk and other diseases (found in the research);
- Describe the relationship between EPCs, family history of cardiovascular disease and genetic regulation;
- Describe the relationship between EPCs, age, gender and lifestyles;
- Describe the relationship between EPCs and drugs;
- Conclude the results.

Methods

An electronic search using certain keywords was performed within the MEDLINE database, assessed through PubMed. The keywords used were endothelial progenitor cells/EPC, endothelial dysfunction, biological markers, antigens/surface antigens, therapeutics, diagnosis, cardiovascular diseases, cardiovascular risk. With these keywords, the author created two search phrases (research 1 and research 2), with the help of some tools from PubMed, like Medical Subject Headings of the U.S. National Library of Medicine (NLM), known as MeSH terms. According to National Institute for Health and Care Excellence (NICE) guidelines, MEDLINE contains a much better developed collection of scope notes for its subject heading (MeSH) terms, which can assist development of the search strategy (1). The NLM Medical Subject Headings controlled vocabulary of biomedical terms is used to describe the subject of each journal article in MEDLINE.

The search phrases were:

Research 1: ((endothelial dysfunction) AND (endothelial progenitor cells) AND ("Biological Markers"[Mesh] OR "Antigens, Surface"[Mesh]) AND (("Therapeutics"[Mesh]) OR "Diagnosis"[Mesh])) AND "Cardiovascular Diseases"[Mesh]

Research 2: ("endothelial progenitor cells" OR "EPC") AND "Cardiovascular Diseases"[Mesh] AND "cardiovascular risk"

The author did not create a time window for research. First, because the available data about EPCs are relatively recent. Moreover, the first study identifying EPCs in PB dates from 1997. Second, to not exclude important studies in the context of the topic. However, the author retreated a bit in time to contextualise some relevant issues, like vasculogenesis, for example. The last update of the research was made July 31, 2014. Some studies were excluded due to the full text was not available. The author selected only the studies in the English, Spanish or Portuguese languages and the research was limited to articles that relate human's research. It is important to note that some studies that resulted from the two surveys include tests in animals (*in vivo*) because these are mixed studies with results in humans and animals. The separation between human studies from animal studies was made manually and not using the PubMed database tools, since the PubMed filter for human studies still resulted in a research with studies in animals, perhaps because these studies were associated with the keyword 'human' and maybe because the results of such studies could be applied to humans. However, the motives are just speculation.

According to the author, the second research provided better information agreeing to the theme of the thesis than the first research. This occurred because the first search resulted in

many articles with information about other cell types other than EPCs, while the second search was more specific. It is important to note that the search phrases resulted in many review articles. Since this dissertation is also a review study, preference was given to original studies and only some of the review studies were included, especially to clarify some issues.

Manual searches of bibliographies were also conducted to identify additional pertinent studies. All of the references were then organised and analysed.

Results

1. Vasculogenesis and Angiogenesis

The first step of blood vessel formation is the differentiation of vascular endothelial cells (ECs), which later cover the entire inner surface of all blood vessels (2). As soon as the early mesoderm has formed via the process of gastrulation, a subset of the primitive mesodermal cells is committed to differentiate into ECs that in turn give rise to the vascular primordia of the embryo. These cells are called angioblasts. The differentiation of angioblasts from mesoderm and the formation of primitive blood vessels from angioblasts at or near the site of their origin are the two distinct steps during the onset of vascularisation that are defined as vasculogenesis. Blood islands (aggregates of cells) are the earliest discernible vascular structures that give rise to a primitive vascular network in the yolk sac. The peripheral cells are the precursors of ECs called angioblasts, whereas the cells in the centre of a blood island are haematopoietic precursor cells. Angioblasts are defined as a cell type that has certain markers characteristic of an EC (but not yet all markers) and has not yet formed a lumen. The close association of haematopoietic and endothelial precursor cells has led to the assumption that ECs and haematopoietic cells may have a common precursor called the hemangioblast (2).

After the primary vascular plexus is formed, more ECs are generated, which can form new capillaries by sprouting or by splitting from their vessel of origin in a process termed angiogenesis (3). There are at least two different types of angiogenesis: true sprouting of capillaries from pre-existing vessels, and non-sprouting angiogenesis or intussusception. Sprouting angiogenesis occurs both in the yolk sac and in the embryo (most frequently during later organogenesis, particularly in the brain). Proteolytic degradation of the extracellular matrix is followed by chemotactic migration and proliferation of ECs, formation of a lumen and functional maturation of the endothelium. Almost all known angiogenesis-activating factors induce one or more of these activities in ECs *in vitro*, but is unclear which factors act *in vivo*. One may be vascular endothelial growth factor (VEGF), as it is an endothelial-specific growth and chemotactic factor. VEGF exerts its biological function through high-affinity tyrosine kinase receptors, the vascular endothelial growth factor receptors (VEGFRs), on the cellular membrane, namely, the kinase insert domain receptor (KDR) or vascular endothelial growth factor 2 (VEGFR2) and the fms-like tyrosine kinase-1 receptor (Flt-1) (4). Non-sprouting angiogenesis is a process of splitting pre-existing vessels by trans-capillary pillars or posts of extracellular matrix, first described in the embryonic lung. Concurrent sprouting and non-

sprouting angiogenesis are involved in the vascularisation of organs or tissues such as the lung, heart and yolk sac during development. *In vivo*, non-sprouting angiogenesis can occur by proliferation of ECs inside a vessel, producing a wide lumen that can be split by trans-capillary pillars, or fusion and splitting of capillaries. The type of angiogenesis in a given organ or tissue may depend on the number of vessels already present when the organ starts to grow rapidly (3). After angiogenesis other processes occur to stabilise the nascent vessels. During 'vascular myogenesis', mural cells stabilise nascent vessels by inhibiting endothelial proliferation and migration, and by stimulating production of extracellular matrix. They thereby provide haemostatic control and protect new endothelium-lined vessels against rupture or regression. Indeed, vessels regress more easily as long as they are not covered by smooth muscle cells (SMCs). During the subsequent arteriogenesis, vessels become covered by a muscular coat, thereby endowing blood vessels with viscoelastic and vasomotor properties, necessary to accommodate the changing needs in tissue perfusion (5). The processes described above are represented schematically in Figure 1.

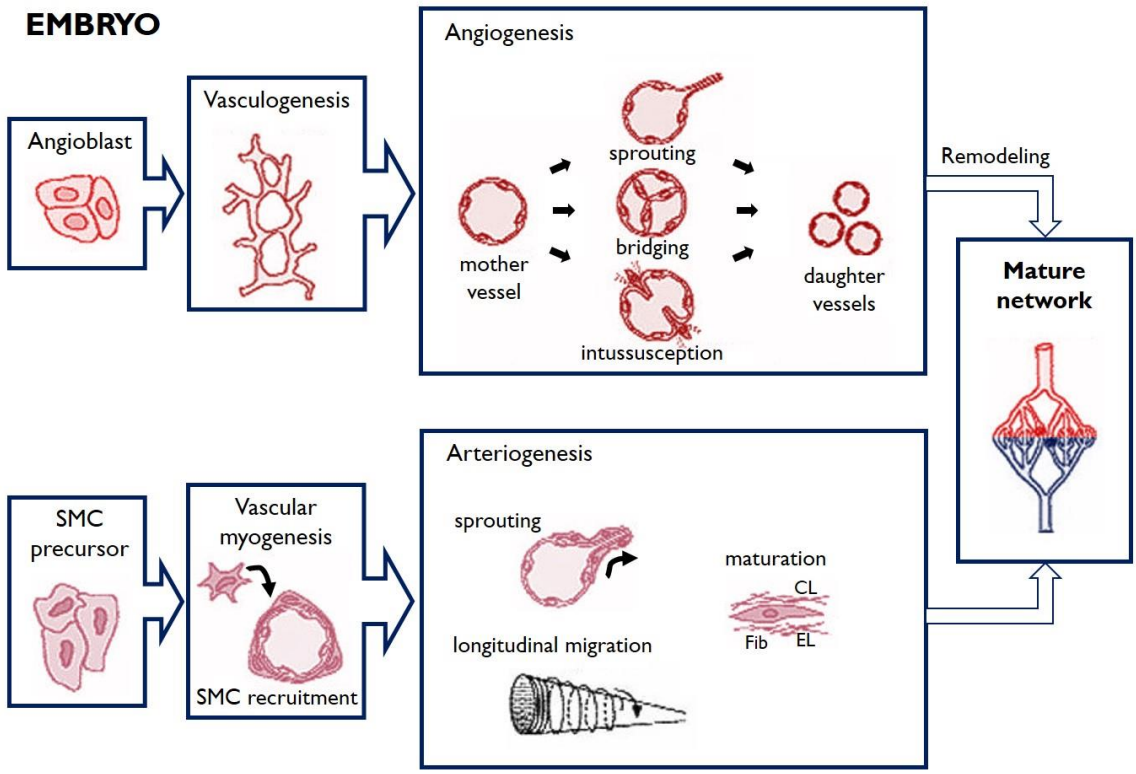

Figure 1 - Endothelial precursors (angioblasts) in the embryo assemble in a primitive network (vasculogenesis) that expands and remodels (angiogenesis). SMCs cover ECs during vascular myogenesis, and stabilise vessels during arteriogenesis. CL: collagen; EL: elastin; Fib: fibrillin (Fib). Adapted from Carmeliet and colleagues (5).

New vessels in the adult arise mainly through angiogenesis, although vasculogenesis also may occur (5). In 1994, the hypothesis that graft endothelialisation can arise from cells in the circulation was tested in an experimental model of impervious vascular grafts in the dog. The authors observed scattered islands of ECs on flow surfaces in each implant site (6). In 1995, other study has demonstrated that neoendothelialisation can take place on a porous synthetic arterial prosthesis implanted in a human being (7). Given these findings, in 1997, Asahara and colleagues investigated the hypothesis that PB contains cells than can differentiate into ECs (8). This was one of the studies that has challenged the classical theory about vasculogenesis. The authors of the study recognised EPCs as cluster of differentiation (CD) 34 positive (CD34+) mononuclear cells (MNCs) and identified their presence in the PB (8). These cells isolated from PB were capable of differentiating into mature ECs *in vivo*. Moreover, they were capable to mobilise and incorporate into sites of ischaemia and promote neovascularisation *in vivo* (8). This was the first step in identification of the EPC and confirmed that adult vasculogenesis was different from embryonic vasculogenesis in that it did not rely exclusively on the division of native ECs in the blood vessels (8, 9). In 1999, Asahara and colleagues planned to determine the origin and role of EPCs contributing to neovasculogenesis, also called postnatal vasculogenesis, using two murine models of BM transplantation (10). The findings of the study underscore the notion that neovasculogenesis is not synonymous with angiogenesis, at least as the latter has been classically defined, to consist of sprouts that originate as the result of proliferation and migration of differentiated ECs from parent vessels (10). The series of BM transplantation experiments established proof of the concept that neovasculogenesis contributes to endogenous neovascularisation of developing tumours, wound healing, severe hindlimb ischaemia, and myocardial ischaemia, as well as physiological neovascularisation (10). The authors concluded that BM-derived EPCs home and incorporate into sites of neovascularisation where differentiation into ECs is completed, which is consistent with neovasculogenesis (10). As a consequence, augmented or retarded neovascularisation, whether endogenous or iatrogenic, likely includes enhancement or impairment of vasculogenesis (10).

2. EPCs, from bone marrow to vascular endothelium: mobilisation, homing and incorporation

The odyssey of EPCs from BM to vascular endothelium can be divided into three stages (Figure 2). First, EPCs are mobilised or released from the marrow. A number of cytokines and growth factors appear to promote this step, as it will be described in the next chapter. Subsequently, the cells move through the circulation and appear to home preferentially to sites of tissue injury. Finally, some EPCs are incorporated into new blood vessels formed by the extension of existing vessels (angiogenesis) or formed *in situ* (vasculogenesis) (11), like previously described. These processes may have important clinical implications. First, genetic or acquired defects in any of these steps may undermine the formation of new blood vessels and could therefore contribute to an impaired healing response in some patients. Second, EPCs may provide an opportunity for therapeutic intervention, on those same patients, either through enhancement of the mobilisation, migration, or incorporation of endogenous EPCs or through transplantation of exogenous cell populations that have been expanded *ex vivo* (11).

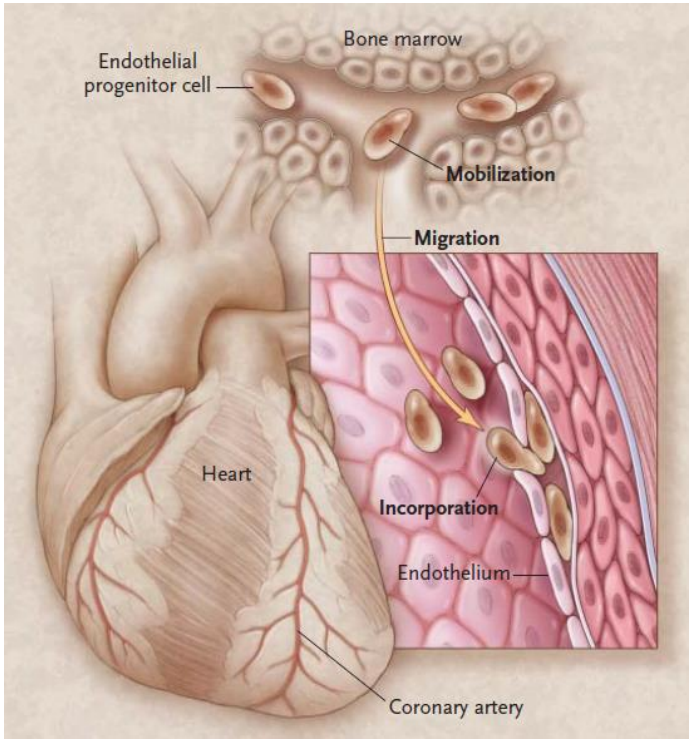

Figure 2 - Three stages in the movement of EPCs from BM to vascular endothelium.

From Rosenzweig 2003 (11).

3. EPC mobilisation

The local BM microenvironment, the so-called stem cell niche consisting of fibroblasts, osteoblasts, and ECs, governs the maintenance and mobilisation of BM stem cells. Mechanistically, cytokines inducing mobilisation interfere with the interactions between stem cells and BM stromal cells, which allow stem cells to disengage the BM, and to pass through the sinusoidal endothelium to enter the blood stream. Stem cell mobilisation is mediated by proteinases such as elastase, cathepsin G, and matrix metalloproteinases (MMPs). A cytokine clinically used for the mobilisation of CD34+ cells in patients is the granulocyte colony-stimulating factor (G-CSF), which releases the proteinases elastase and cathepsin G from neutrophils. These proteinases induce cleavage of adhesive bonds on stromal cells, which interact with integrins on haematopoietic stem cells. Moreover, these proteinases cleave the cytokine stromal cell-derived factor-1 (SDF-1), which is released by stromal cells, and its receptor C-X-C chemokine receptor type 4 (CXCR4) on stem and progenitor cells (12). The cross-talk between CXCR4+ EPC and SDF-1-expressing stromal cells may be partly responsible for the progenitor cell retention within the BM niche. SDF upregulation during hypoxia is facilitated by the transcription factor hypoxia-inducible factor 1 (HIF-1). Activation of HIF-1 alpha subunit (HIF-1α) requires inhibition of hydroxylase activity by low molecular oxygen concentrations leading to HIF-α binding transcriptional co-activators and translocating to the nucleus, where it binds HIF-1 beta subunit (HIF-1β). The resulting heterodimer interacts with the promoter and enhancer of the target gene. SDF-1 gene expression may be mediated by HIF-1, resulting in a proportionate selective expression of SDF-1 in ischaemic tissues. Exposure to known HIF-1 activators in gene transfection studies indicate increased adhesion, and migration of CXCR4+ EPC to ischaemic tissue. The degree of SDF-1 elevation in the circulation appears tightly regulated and may be the primary molecular thermostat regulating mobilisation of EPC (13). Stem cell mobilisation as a result of high levels of circulating SDF-1 appears to reverse the SDF-1 gradient across the BM barrier, forcing CXCR4+ cells to exit the BM. However, VEGF, SDF-1, and placental growth factor (PlGF)-induced stem cell mobilisation was shown to rely on matrix metalloproteinase 9 (MMP-9) (12), an extracellular proteinase expressed by BM stromal cells (13). On entering the BM microenvironment, SDF-1 activates MMP-9. MMP-9 is dependent on nitric oxide (NO), a potent mediator of vasodilation that further assists in mobilisation by allowing egress of the liberated EPC into the circulation through the BM sinusoids. NO mostly derives from endothelial nitric oxide synthase (eNOS). MMP-9 causes the release of soluble kit Ligand (skitL) from membrane-

bound kit Ligand (mkitL). MkitL is the ligand for c-kit (also known as CD117), a tyrosine kinase receptor expressed by EPC. After ligand binding, the receptor undergoes autophosphorylation, a prerequisite for EPC efflux from the BM. SkitL is capable of signalling the release of more SDF-1, facilitating egress of CXCR4+ and c-kit cells into the plasma. Proteinases breach the adhesive bonds between the EPC and stromal cells, already compromised by SDF-1 cleavage, allowing the progenitor cell to exit the stem cell niche. VEGF is upregulated during ischaemia and is known to promote EPC mobilisation and facilitate their role in angiogenesis. Simultaneous activation of phospholipase C also occurs, resulting in elevated cytoplasmic calcium levels capable of enhancing phosphorylated eNOS activity. eNOS activation by protein kinase B (PKB or Akt) signaling occurs in response to several stimuli, for example, VEGF (13). The integrity of the eNOS pathway seems to be of pivotal importance for EPC mobilisation, proliferation and differentiation. Both upstream (phosphatidylinositol-3 kinase or phosphoinositide-3 kinase (PI3K), PKB/Akt) and downstream effectors of the eNOS pathway have key roles in the mobilisation, migration and vessel formation by EPCs (14). The Akt family consists of genes Akt 1, 2 and 3, which code for enzymes that are members of the serine/threonine-specific protein kinase family. Akt 1 is involved in cellular survival by inhibiting apoptosis, and in stimulating endothelial NO synthesis. Akt possesses a protein domain that binds phosphoinositides, which are themselves phosphorylated by members of the PI3K family. PI3K may be activated by G protein-coupled receptors such as CXCR4. Activation of Akt appears to promote phosphorylation of eNOS, increasing endothelial NO production and hence cell growth and migration. Oxidative stress, characterised by increased generation of reactive oxygen species (ROS), that are capable of causing oxidative damage to biological structures may influence EPC mobilisation. Free radicals exert a direct cytotoxic effect on vascular endothelium and can inactivate NO, depleting the supply available for maintenance of the local environment including assisting in EPC function (13). Erythropoietin (EPO) is also an important physiological determinant of EPC mobilisation. EPO elicits a similar potency for the improvement of EPC mobilisation as VEGF. Additional cytokines mobilizing EPCs and haematopoietic progenitor cells include G-CSF, mentioned above, and granulocyte monocyte colony-stimulating factor (GM-CSF) with the first being used for BM transplantation in the clinical setting for years (12). Figure 3 schematically represents EPC mobilisation and the involvement of PI3K/Akt/eNOS pathway.

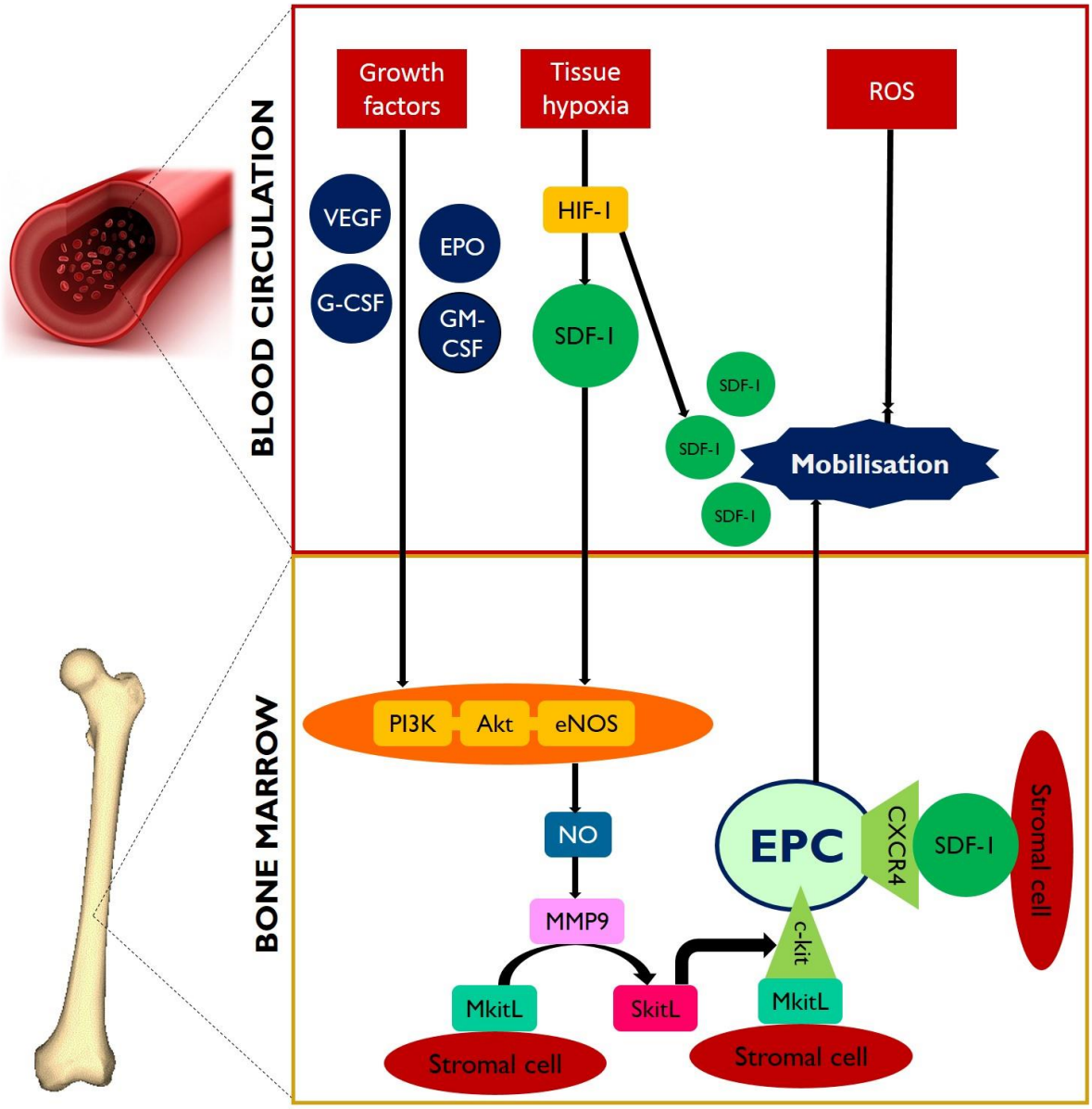

Figure 3 - Schematic representation of EPC mobilisation and the involvement of the PI3K/Akt/eNOS pathway. Tissue hypoxia or vascular trauma results in the elevation of growth factors and chemokines that activate vascular stromal endothelial cells in the BM, resulting in induction of the PI3K/Akt/eNOS pathway. This increases NO concentrations in the BM, leading to elevated MMP-9 activity. MMP-9 converts MkitL into SkitL, which competes with MkitL for adhesion to the c-kit receptor on EPCs. In concert with increased local protease activity and circulating SDF-1 levels, stromal cell-EPC interactions are weakened and EPCs are mobilised out of the BM along a concentration gradient of SDF-1. Free radical formation deplete local NO bioavailability and impair EPC mobilisation out of the BM (14). Adapted from Tilling and colleagues (13) and Everaert and colleagues (14).

4. Methods to study EPCs

There are a few methods described in the literature that allow investigators to determine or study EPCs. They are flow cytometry and cell culture assays. Some authors use more than one method in the same study to define and characterise EPCs, as it will be mentioned later.

4.1. Flow cytometry

Cell count by flow cytometry is based on immunolabelling cells with antibodies directed against surface or intracellular antigens and it is currently the best method to obtain pure quantitative data on putative EPCs (15). Flow cytometry analysis allows the simultaneous determination of both cell differentiation and cellular morphology. In this technique the shift of cellular light scatter properties represents a very sensitive indicator for changes in cell size and complexity (16). When count of PB EPCs is conceived as a disease biomarker, flow cytometry should be considered the gold standard (17) since it has been extensively used in the clinical setting, and has proved to be a sensitive, reproducible, and reliable technique (18, 19). Among. However, this method has two important limitations. The first limitation is related with the antigenic profile of EPCs. The antigens currently used to define EPCs may also be used to define other cell lineages. Thus, the precise antigenic phenotype of EPCs is still not known. According to this, EPCs should be defined using has many antigens as possible to ensure the specificity of the identified cells. However, a second limitation arises and it is contradictory, in some way, to what was just mentioned. In fact, when performing flow cytometry on fresh PB, the rarity of circulating EPCs imposes the use of a very limited number of surface antigens. Therefore, assuring that a phenotype based on 2–3 antigens definitively identifies a cell population with a complex function is virtually impossible (15, 17). Accordingly, EPCs should be referred as "putative EPCs", since their definitive antigenic profile remains to be discovered (15). The antigens used to define EPCs and suggested phenotypes will be described later in this work.

4.2. Cell culture assays

Ex vivo analysis of blood cells by flow cytometry can only count EPCs and quantify the expression of a limited number of surface or intracellular antigens. To gather qualitative data, investigators are compelled to isolate EPCs from the bloodstream and, given their very low frequency, to expand them in culture (15). So far, EPCs can be established in culture from

embryonic stem cells, umbilical cord blood, BM and PB (20). With respect to cell culture assays, several approaches and protocols are described in the literature. In the original protocol by Asahara and colleagues (8), CD34+ MNCs isolated from PB, when plated on fibronectin and endothelial medium, promptly attached and became spindle-shaped within 3 days. Besides, the number of attaching cells in culture increased with time. These cells displayed typical functional properties of endothelial cells, such as uptake of acetylated low density lipoproteins (acLDL) and binding of Ulex lectin, in addition to the expression of other endothelial lineage markers (such as CD31, Tie-2, KDR) (8). Subsequently, a number of variants of this protocol have been proposed, for example employing different culture media, gelatine or collagen in place of fibronectin, etc. (15). Briefly, this method of EPC evaluation involves *ex vivo* cultivation of MNCs or pre-selected CD34+ cells on fibronectin or gelatine-coated dishes in the presence of a specific medium (21). Importantly, in most of the studies, unfractionated MNCs are used instead of cells preselected on the basis of the expression of key EPC markers, as in the original protocol (15). To overcome this lack of specificity, some authors conceived a pre-plating procedure claiming to avoid contamination of early-adherent cells of mesenchymal origin or mature ECs (15, 19, 22). This method was described by Hill and colleagues (22). In their study, samples of PB MNCs were isolated by Ficoll density-gradient centrifugation. Recovered cells were washed twice with phosphate-buffered saline and placed in growth medium. Isolated cells were subsequently resuspended in growth medium and plated on dishes coated with human fibronectin. To eliminate the possibility of contaminating the assay with mature circulating endothelial cells, an initial pre-plating step in a fibronectin-coated six-well plate was performed. After 48 hours, the non-adherent cells were collected and re-plated onto fibronectin-coated 24-well plates for a final assessment of the number of colonies. Growth medium was changed every three days, and the numbers of colonies were counted seven days after plating. A colony of EPCs consisted of multiple thin, flat cells emanating from a central cluster of rounded cells (Figure 4). A central cluster alone without associated emerging cells was not counted as a colony. These have been termed EPC colony forming units (CFUs). Colonies were counted manually in a minimum of four wells. This assay was termed as 'CFU assay'. Confirmation of EC lineage was performed as previously described by Asahara and colleagues (8). Indirect immunostaining was performed with the use of endothelial-specific antibodies directed against VEGFR2, and CD31 or 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate–acetylated low-density lipoprotein (DiI-acLDL) and co-staining with lectin. PB MNCs formed distinct colonies on fibronectin-coated dishes (22). This method is found in many studies, with some of them exhibiting only slight

modifications (19, 23-42). It is then suggested that the ability to clonally expand and to create colonies in an endothelial-specific medium is a key functional feature of EPCs (22).

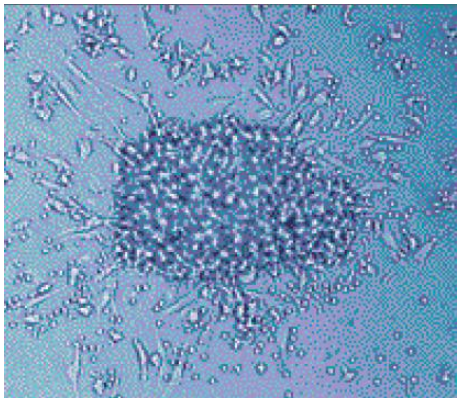

Figure 4 - Phase-contrast micrograph of an EPC colony characterised by a central cluster of rounded cells surrounded by radiating thin, flat cells (x200). From Hill and colleagues (22).

According to the literature, EPCs can be distinguished phenotypically in two major cell types, early EPCs and late EPCs. Hur and colleagues described the identification of these two cell kinds according to their time-dependent appearance (43). MNCs isolated from other components of PB were suspended in endothelial cell growth medium (EGM), consisting of endothelial basal medium, with other components, including VEGF in this case. Then, cells were seeded on gelatine culture media and incubated in a carbon dioxide incubator at 37 °C. First media change was performed approximately 6 days after plating. Subsequently, media were changed every 3 days and each cluster or colony was followed-up every day. The initially seeded cells were round. After 3 to 5 days, attached cells appeared and appeared to be clusters. They were elongated and had a spindle shape similar to that of the EPC that Asahara and colleagues (8) first reported. These cells were called early EPCs. Their number increased for 2 weeks. Thereafter, they did not replicate *in vitro* and gradually disappeared in 4 weeks after plating. Another population of cells with different morphology and growth pattern was observed. These cells appeared in 2 to 4 weeks after plating, with more smooth cytoplasmic outline and were firmly attached to the plate, showing a cobblestone appearance. They were called late EPCs. These cells rapidly replicated from several cells to a colony, became monolayer with almost full confluence, and showed multiple population doublings without senescence. Both types of EPC took-up DiI-acLDL and showed lectin binding affinity. They showed different morphology, proliferation rate, and survival features. Freshly isolated peripheral MNCs expressed Flt-1, eNOS, and von Willebrand Factor (vWF) (43). Flt-1 is able to mediate the biological functions of VEGF (4). vWF is synthetised and stored in by endothelial

cells, and, when released, appears to mediate platelet aggregation and adhesion (44). On day 10 after plating, the endothelial differentiation of early EPC could be assumed with the additional expression of vascular endothelial cadherin (VE-cadherin) and KDR, although weak. VE-cadherin, also known as CD144 (45), is a protein expressed at endothelial cell-cell junctions that holds together endothelial cells and plays a crucial role in the maintenance and restoration of endothelium integrity and in the regulation of vessel permeability (46). Also, the level of Flt-1 expression was elevated. These EC-specific gene expressions decreased at 3 weeks after plating. Late EPC, however, exhibited strong expression of all endothelial genes such as VE-cadherin, Flt-1, KDR, eNOS and vWF. Peripheral MNCs on day 0 expressed CD31 and CD45. The expression of pan-leukocyte maker CD45 gradually decreased from MNCs to late EPCs, whereas the expression of endothelial-specific markers, such as KDR and VE-cadherin, gradually increased. CD31, whose expression is shared by monocytes and endothelial cells, showed biphasic pattern; that is, it showed strong expression in MNCs and late EPCs, whereas it showed weak expression in early EPCs. Such differences in gene expression profiles lead to the functional differences between the two types of EPCs. NO formation in response to VEGF was confirmed in both types of EPCs. However, it was found that late EPC was brighter than early EPC when the fluorescence intensity was compared, suggesting that late EPCs have more competent endothelial function in NO production (43).

The literature suggest the existence of another type of EPCs, named endothelial colony-forming cells (ECFCs) (47). The approach that identifies these cells was described by Ingram and colleagues 2004 (47). MNCs were isolated and washed with endothelial basal medium supplemented with other components, termed complete EGM. After resuspension in complete EGM, cells were seeded onto separate wells of a tissue culture plate pre-coated with type I rat tail collagen and incubated in a humidified carbon dioxide incubator at 37 °C. After 24 hours of culture, non-adherent cells and debris were aspirated, adherent cells were washed once with complete EGM, and complete EGM was added to each well. Medium was changed daily for 7 days and then every other day until the first passage. Colonies of endothelial cells appeared between 5 and 22 days of culture and were identified as well-circumscribed monolayers of cobblestone appearing cells. Endothelial cell colonies were enumerated by visual inspection using an inverted microscope. Endothelial cells derived from the endothelial cell colonies were released from the original tissue culture plates, resuspended in complete EGM, and plated onto tissue culture flasks coated with type I rat tail collagen for further passage. The uptake of DiI-acLDL was studied. Additionally, immunophenotyping revealed that cord blood and adult cells expressed the endothelial cell-surface antigens CD31, CD141,

CD105, CD146, CD144, vWF, and KDR. Importantly, neither cell population expressed the hematopoietic cell-specific surface antigens CD45 and CD14. However, a small percentage of cord blood and adult cells did express CD34, CD133, and CD117 (47), cell-surface antigens previously identified on endothelial and hematopoietic progenitor cells (48). Nevertheless, plating the EPC-derived endothelial cells that expressed CD34, CD133, or CD117 failed to give rise to hematopoietic colony-forming cells in methylcellulose assays, demonstrating that these cells do not have hematopoietic activity. The study demonstrated that there are different types of cord and adult EPC-derived endothelial cell colonies that can be distinguished by their proliferative and clonogenic potential and that EPCs display a hierarchy of proliferative potentials similar to the hematopoietic progenitor cell hierarchy (47).

A similar methodology was used by Teofili and colleagues 2010 to assess ECFCs. Only a few changes were applied. MNCs were suspended in EGM on human fibronectin coated dishes. At day 2 non-adherent cells were discharged and residual adherent cells were grown in EGM for 28 days, with medium replacement every 3 days. Well circumscribed monolayers of cobblestone-appearing cells growing from day 9 to day 28 were counted as ECFCs (40).

In another study, ECFCs were cultivated for up to 10 passages and examined from passages 2 to 10 to investigate changes in their phenotypes, purity, activation potential and NO secretion. Expression of CD31 and CD144 markers was increased in early passages. Expressions of KDR, and c-kit, a marker of EPCs, as mentioned before, and hematopoietic stem cells, were also higher in early than in late passages. Therefore, in early passages, ECFCs exhibited higher endothelial phenotype expression. Besides, they also showed better biological functions, in terms of NO secretion and tubular formation, but lower activation potentials compared with later passages. It was suggested that the purity of ECFCs is acceptable for their use in early passages. ECFCs exhibited well-preserved biological functions in early passages as suggested by good NO-secreting and angiogenesis capacity (49).

ECFCs display properties of an EPC, this means, cells that can clonally give rise to progeny that spontaneously form human blood vessels *in vivo* (neovasculogenesis) and become a part of a circulatory system (50).

4.3. Flow cytometry *versus* culture methods

The methods commonly used to study EPCs have been described above. These cells can be measured from PB either by direct enumeration using flow cytometry and particular markers or after cultivation of MNCs. The advantages of flow cytometry include its ability to enumerate

untouched, naive cells with a defined phenotype and the identification of specific cell populations in multicolour fluorescence-activated cell sorting analysis. On the other hand, the definition of cells based on surface markers is under heavy debate, while surface markers do not necessarily correlate with cell function. With respect to culture methods, reproducibility and repeatability severely depend on the method used (51). The literature shows different methodologies, some of them with only minor differences when compared to others. However, small changes can make a difference and the measure of EPCs using culture methods becomes less specific.

A current misunderstanding is that the number of putative EPCs that can be isolated in culture corresponds to the number of circulating EPCs (52). Usually, researchers plate a given number of PB MNCs per well, then count the number of EPCs/well at the end of the culture period, and finally deduce how many EPCs (cells with the potential to differentiate into endothelial cells) were present in the original blood sample. It is apparent that this equation is an oversimplification, because a myriad of steps divide the blood sample from the end of the culture. Actually, the appearance of endothelial-like cells (putative EPCs) from a culture of PB MNCs depends upon a complex set of events, including adhesion, proliferation and differentiation of the original cells. Any variation in each of these passages will influence the number of putative EPCs at the end of the culture. The obvious consequence is that the culture method ought to globally explore the function of EPCs and provide qualitative, not quantitative, data. What should be acknowledged is that flow cytometry EPCs probably have little in common with cultured EPCs. Two profoundly different techniques yield profoundly different biological information, even if they usually show consistent variation in many studies. Therefore, when EPCs are used as a surrogate marker of cardiovascular risk or vascular damage, pure quantitative data should be obtained with flow cytometry of fresh blood samples (15).

It is suggested that clonogenic assay of CFUs allows a functional evaluation of the endothelial potential and improves the reliability of the information that could be obtained by flow cytometry (53). However, other authors suggested that CFU is a cell-based phenotype distinct from CD34+ and CD34+/KDR+ with respect to both character and function (54, 55). Despite these controversial opinions, if investigators intend to use culture methods, they should prolong cultures beyond two weeks, avoid trypsin use, and perform parallel immunofluorescence, to have definitive data on EPC characteristics (56). Another way to overcome the discrepancy between flow cytometry and culture methods is to pre-sort cells

according to EPC markers before culturing them. Isolated cells will probably have some characteristics in common with circulating EPCs counted by flow cytometry (15).

Given the differences of opinion about the best method to study EPCs, a circle graph was prepared to assess which method is most used by the authors, based on the studies that resulted from the two search phrases mentioned above (Methods Section) (Figure 5).

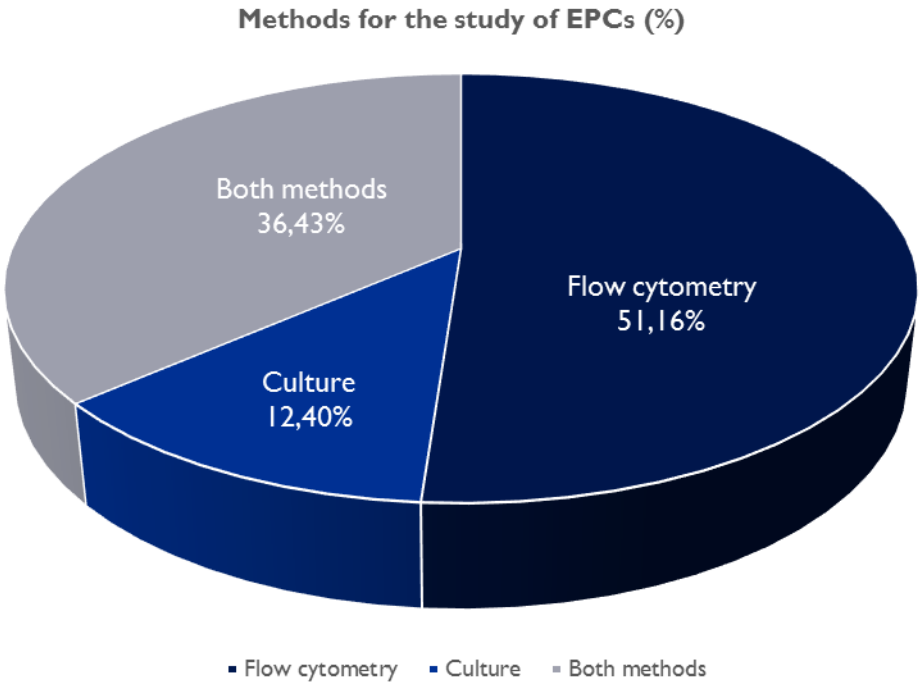

Figure 5 – Methods used for the study of EPCs.

Through the analysis of the graph (Figure 5), it can be concluded that flow cytometry is the method most commonly used to define EPCs, although some authors have used both methods (flow cytometry and culture). This is in agreement with the suggestion that flow cytometry is the gold standard for EPC identification (17).

The graph was constructed from the information of 129 studies included, that resulted from the two research phrases (Methods Section).

5. EPCs and markers

In this chapter, the markers found in the studies from the two researches used for EPCs identification and characterisation are described. Some of them have already been described in this document. Starting with the markers used to define EPCs by flow cytometry, they were CD34, CD133, KDR, CD45, CD144, CD31, CD14, CD117, CD146 and aldehyde dehydrogenase (ALDH). CD34 is an adhesion molecule expressed on hematopoietic stem cells and is typically considered as a marker of immaturity. CD133 is a surface antigen of unknown function that identifies more immature progenitor cells than CD34 alone. KDR, or kinase insert domain receptor, represents type 2 VEGFR and indicates early endothelial differentiation (57). CD45 is generally considered a specific pan-leukocyte marker (58). It is suggested that it might not be a reliable watershed between the hematopoietic and endothelial lineage. Expression of CD45 is used to distinguish monocytes (CD45+) from endothelial cell types (CD45-) (59). CD144, known as VE-cadherin, is a protein expressed at endothelial cell-cell junctions (46), as already described in this document. It is considered as one of the best and most specific markers for mature endothelial cells, despite the fact that it can be detected on subpopulations of haematopoietic cells in the BM (58). CD31, also known as platelet-endothelial cell adhesion molecule-1 (PECAM-1), is a marker of endothelial lineage, as mentioned before. It is also expressed by platelets, monocytes, neutrophils and T cells (60). In addition, cells expressing CD31, a surface antigen that is present in monocytes and endothelial cells, were isolated from PB and BM, and these cells showed a high proangiogenic and vasculogenic activity (61). Some studies used CD31, vWF, or CD144 as markers for the endothelial commitment. Although there is no comparative analysis to recommend the use of KDR instead of other markers, it should be noted that CD31, vWF, and particularly CD144 may identify cells in a more advanced stage of maturation along the endothelial differentiation process (62). CD14 is a typical myeloid marker (58). A study that examined the lineage relationship between early EPCs and late outgrowth endothelial cells in culture found that the vast majority of EPCs arose from a CD14+ subpopulation of PB MNCs but outgrowth endothelial cells developed exclusively from the CD14 negative fraction (63). CD117, also known as c-kit, is a marker of immaturity (58) and, as already cited in this text, it is also used as a marker of hematopoietic stem cells (49) and EPCs (13). With respect to CD146, it is a marker of endothelial cell lineage (64), also expressed by activated T cells, trophoblasts and melanoma cells (60). Finally, ALDH activity is present at high levels in progenitor precursors of a variety of mature cell lines and may play an important role in maintaining the progenitor

cell phenotype. It was shown that cells expressing it at high intensity (ALDHbright) are enriched in EPCs and have clinical correlates (65).

Through the analysis of the included studies (129 studies) that resulted from the two search phrases (Methods Section), the authors of this document found 164 uses of markers or combinations of markers in flow cytometry. Markers or combinations of markers that were observed only once, were resumed in 'Others' (Figure 6).

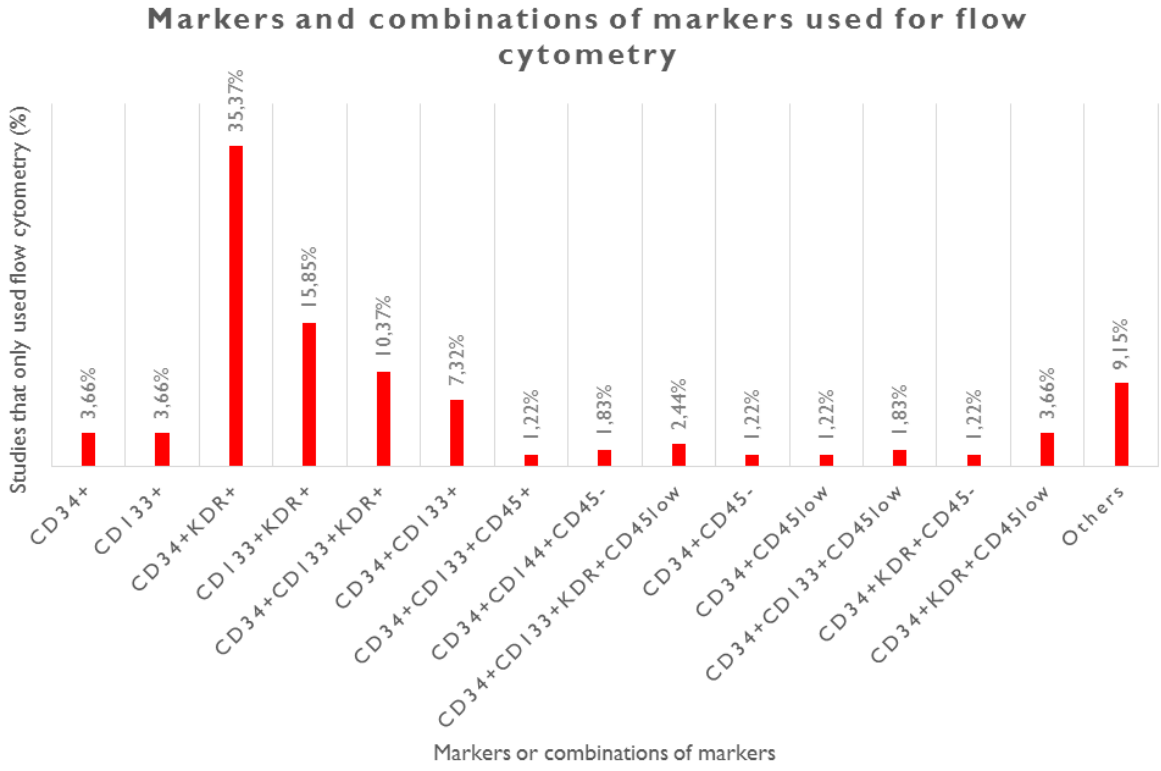

Figure 6 – Markers and combinations of markers used to identify EPCs by flow cytometry.

Through the analysis of the graph (Figure 6), it can be concluded that the mostly used markers or combinations of markers were CD34+KDR+, CD133+KDR+, CD34+CD133+KDR+ and CD34+CD133+. The utilisation of this four combinations of markers was subjected to a small statistical analysis. The chi-square adjustment test indicated that there is a difference statistically significant in the distribution of these four combinations of markers ($p < 0.001$). As compared with the expected values (if the markers were indifferent), it was found that CD34+KDR+ is used more often than would be expected, whereas the frequency of selecting each of the remaining three is lower than expected.

CD34+CD133+KDR+ and CD133+KDR+ cells may be considered more immature EPCs (66) such as those recently mobilised from BM (67). Once in the PB, EPCs progressively loose CD133, and CD34+KDR+ cells thereafter become the main constituent of the circulating EPC pool (68). Consistently, in steady-state conditions (namely, without any stimulus for BM mobilisation), PB CD34+KDR+ cells are 3-fold and 10-fold more frequent than CD133+KDR+ and CD34+CD133+KDR+ cells, respectively. Progenitor cells lacking KDR expression (CD34+, CD133+, and CD34+CD133+ cells) should be considered undifferentiated and do not strictly correspond to EPCs (57). With the consideration that EPCs should express at least one marker of immaturity and one additional marker reflecting endothelial commitment, three main antigenic profiles have been proposed: CD34+KDR+, CD133+KDR+ and CD34+CD133+KDR+ (66, 68, 69). Even if it has no data directly indicating that CD34+KDR+ cells functionally correspond to EPCs, it is suggested that this exact phenotype may be preferred in future studies in which EPC count is intended as a cardiovascular biomarker (57). The results presented in the graph of figure 6 demonstrate that CD34+KDR+ was the mostly used combination of markers. This is in agreement with the suggestion referred.

Some of the described markers are used in culture assays to confirm EPC or EC lineages. Flow cytometry may also be used after culture to perform this confirmation. The markers used in the studies, found from the two researches, after culture assays were: DiI-acLDL or acLDL, lectin, CD34, CD133, KDR, CD31, CD144, vWF, Tie-2, CD14, CD45, eNOS, CXCR4, Factor VIII, CD105, CD18, CD90, CD115, CD146 and CD117. Tie-2, eNOS, CXCR4, Factor VIII, CD105, CD18, CD90 and CD115 were not described before in this chapter. Tie-2 is a tyrosine kinase receptor that is used as an endothelial cell marker (70). Besides, it was shown that Tie-2+ cells can be recruited to sites of vascular damage and may take part in re-endothelialisation during vascular injury (71). eNOS was used as an endothelial marker in one study of the research (31). Moreover, it was shown before in this document that eNOS plays an important role in EPCs mobilisation. CXCR4 and CD18 were used in a study to confirm the endothelial phenotype of EPCs after culture (72). Besides, as mentioned above, CXCR4 is the receptor for SDF-1 in progenitor cells (12). Factor VIII was also used in a study to confirm endothelial cell lineage (23). CD105, or endoglin, is expressed in endothelial cells, activated monocytes, tissue macrophages and erythroid precursors (60). CD90 is also known as Thy-1. Thy-1 is a receptor on EC, involved in arrest and firm adhesion of leukocytes to the endothelium. In humans, Thy-1 expression is restricted to activated EC, fibroblasts, neuronal cells, and a subset of peripheral CD34+ cells (73). Finally, CD115, also known as colony-stimulating factor-1

receptor (74), is a macrophage antigen. Cells comprising CFUs were founded to be positive to CD115 (37) in only one study of the research.

Through the analysis of the included studies (129 studies) that resulted from the two search phrases (Methods Section), the authors of this document found 162 uses of markers that are used to confirm EPC or EC lineages. Again, markers that were observed only once, were resumed in 'Others' (Figure 7).

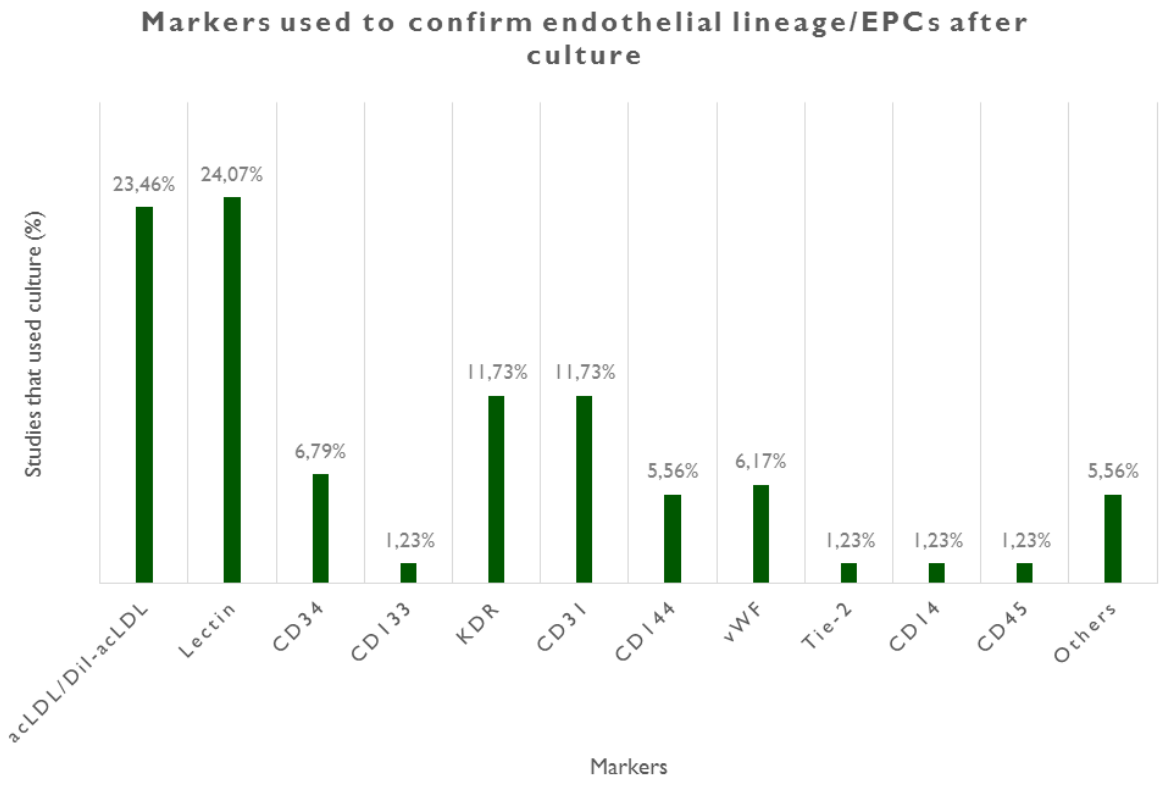

Figure 7 – Markers used to confirm endothelial lineage/EPCs after culture.

Through the analysis of this graph (Figure 7), it can be concluded that acLDL/DiI-acLDL, lectin, KDR and CD31 were the mostly used markers to confirm EPC or EC lineage after culture.

6. EPCs and cardiovascular disease or cardiovascular risk

In adult life, revascularisation is essential for the survival of growing, injured, and ischaemic tissue. New vessels in the adult arise mainly through angiogenesis (5), but this is not the only process responsible for development of new vascular networks in the setting of growth or tissue ischaemia. The challenging study of Asahara and colleagues showed that neovasculogenesis really occurs and EPCs are involved (10). It contributes to endogenous neovascularisation of developing tumours, wound healing, ischaemia, as well as vascular homeostasis, as mentioned above (Figure 8). Consequently, many authors studied EPCs in the last years within the context of cardiovascular disease and cardiovascular risk.

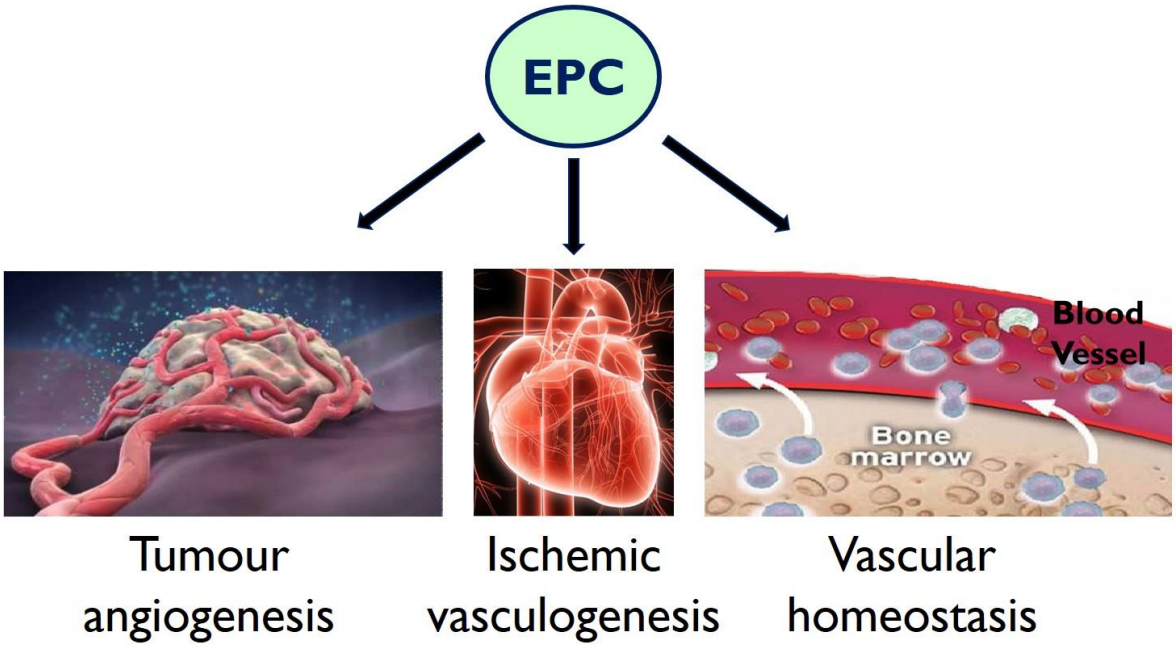

Figure 8 - Experimental evidence suggests these cells can participate in tumour angiogenesis and vasculogenesis within ischaemic tissues, as well as in the maintenance of vascular homeostasis. Adapted from Khakoo and colleagues (75).

Through the results of the studies obtained from the two search phrases, the relationship between EPCs and cardiovascular disease or cardiovascular risk factors is described below.
Circulating EPCs are involved in the pathogenesis of endothelial dysfunction, atherosclerosis and coronary artery disease (CAD). This enables novel insights into the cellular and molecular determinants of these diseases which account for most cases of death in the western world. Endothelial function closely correlates with the number of circulating EPC providing new mechanistic insights and options for risk assessment in patients with coronary heart disease (26). Endothelial cell injury and endothelial dysfunction are predictors of the risk of vascular

events, providing stimuli for the development of atherosclerotic plaque (76). It was suggested that EPCs (CD34+KDR+) contribute to the restoration of the endothelial monolayer (19). EPCs are responsible for the maintenance of the complex network of vessels as a component of the cardiovascular system in health and disease. Several factors are known to be involved in pathologic conditions in the regulation of endothelial compartment, as it will be described later, while very limited information exists on its physiological homeostasis. In order to define the burden of angiogenesis in physiological conditions, the frequency of PB endothelial colonies and their relation with other factors possibly involved in their function such as high-sensitivity C-reactive protein (hs-CRP), VEGF and tissue inhibitor of metalloproteinases-1 (TIMP-1) were assessed in a highly selected healthy population, without known cardiovascular risk factors. The serum level of hs-CRP, VEGF, TIMP-1, the frequency of PB endothelial CFUs by clonogenic assay, and the number of early EPCs (KDR+CD144-CD45-) and late EPCs (CD146+CD31+CD45-) by flow cytometry analysis were evaluated. The differences in the frequency of colony formation between genders were not statistically significant. Importantly, high levels of hs-CRP confirmed a low cardiovascular risk profile. The subjects with PB endothelial colonies (CFUs) were characterised by higher values of hs-CRP, when compared with those not forming colonies, and of VEGF. No significant differences were found in TIMP-1 values. In this study, the frequency of PB endothelial colonies and the endothelial colonies cell-specific mitogen VEGF level seem to be unrelated to age and gender. When attempting to characterise the population in respect of colony forming capacity, associated with higher VEGF values, the authors found higher hs-CRP values in colony formers supporting the concept that hs-CRP is a mediator involved in ECs function and not solely a biomarker of cardiovascular risk. In fact, the low levels of hs-CRP, formerly not associated with an increased cardiovascular risk, suggest that hs-CRP could contribute to normal EC homeostasis in physiological conditions causing minimal stress to the endothelium or following subclinical damages requiring minimal tissue repair. Even in normal subjects, defined according to several selective criteria, quite an elevated number of subjects who formed PB endothelial colonies were found suggesting an active homeostasis involving the endothelium, possibly associated to local low grade inflammation processes (denoted as microinflammation) revealed by higher hs-CRP levels. Therefore, the determination of microinflammation in apparently healthy individuals might have prognostic significance in terms of future vascular events and accelerated atherothrombotic disease. The EPC clonogenic potential seems to be related to hs-CRP and VEGF levels even in healthy population supporting the concept that these mediators are involved in physiological function of endothelial colonies (53).

6.1. Physical forces as initial triggers of vessel remodelling

Initial triggers of vessel remodelling are physical forces such as blood pressure and fluid shear stress. Cold pressor test has often been used in the literature for the diagnosis of cardiovascular reactivity in normotensive and hypertensive subjects. It was investigated whether or not a sudden physiologic stimulus on vascular vessels, applied by the cold pressor test, could stimulate the mobilisation of EPCs from the BM, to test the hypothesis that endothelial cells damaged by hemodynamic stress are thus replaced. Patients with essential hypertension, patients with chronic kidney disease and healthy subjects underwent cold pressor test, by dipping their hands in icy water for 4 minutes. EPCs (CD133+VEGRF2+) were quantified immediately before cold pressor test and after 4 and 60 minutes. Adhesion soluble molecules (soluble intercellular cell adhesion molecule-1, known as sICAM-1, soluble vascular cell adhesion molecule-1, known as sVCAM-1, and soluble E-selectin, known as endothelial-leukocyte adhesion molecule 1) were also measured, as markers of endothelial activation. In chronic kidney disease patients, EPO treatment was suspended in order to obviate any interference in the proliferation and differentiation of EPCs. It was confirmed that immediate vascular stress causes a sudden and marked variation in the number of EPCs and that a parallel variation also occurs in adhesion soluble molecules, indicating endothelial activation. The pattern in patients with diseases is characterised by endothelial dysfunction and is different from that in healthy subjects. At the beginning of the test, the percentage of EPCs in chronic kidney disease subjects was significantly higher than that in controls and hypertensive patients. The percentage of EPCs increased significantly with respect to baseline in each group just after cold pressor test. This increase was significantly greater in chronic kidney disease patients than in hypertensive and healthy subjects. In the healthy and hypertensive group, 60 min after cold pressor test, EPCs returned to basal levels. On the other hand, in chronic kidney disease patients the number of EPCs maintained an increasing trend, reaching twice the level at baseline, and showed a significant difference with respect to the level 4 minutes after cold pressure test. The findings of the study confirm the hypothesis that BM responds to injury by releasing a greater number of progenitor cells into the circulation as an immediate response to endothelial mechanical stress. The arterial wall is influenced by pressure-related forces such as longitudinal, circumferential, and radial wall stresses. Cold pressor test might induce hemodynamic stress whose severity and efficacy are limited in time. This might explain why the increase in progenitor cells disappeared within 60 min in the hypertensive patients and controls. In chronic kidney disease patients, on the other hand, an increasing trend was

observed, and this continued after 60 min. The concentrations of the three main endothelial-expressed cell adhesion molecules associated with leukocyte activation were measured. The adhesion of monocytic lineage cells on the vascular wall, which depends on their interaction with endothelial cells, is mediated by cell adhesion molecules, including intercellular cell adhesion molecule-1 (ICAM-1), E-selectin, and vascular cell adhesion molecule-1 (VCAM-1). It was confirmed that high basal levels of endothelial adhesion molecules are present in hypertensive and chronic kidney disease patients and that cold pressor test induces a rapid increase in these molecules in all categories. Summarily, adhesion molecule levels may be considered a marker of endothelial activation in response to haemodynamic trauma altering the vascular surface. EPCs and adhesion molecule levels increase rapidly after sudden haemodynamic stress from cold pressor test. There is close contact between BM and vessels mediated by swift cytokine release in the blood circulation. Moreover, cold pressor test has a marked effect on the vessels of patients with diseases causing endothelial dysfunction, such as hypertension and chronic kidney disease. The recruitment of EPCs and the release of adhesion molecules are severely impaired in chronic kidney disease patients with respect to other groups, thus suggesting new mechanisms underlying the increased cardiovascular risk in this population (77).

6.2. Atherosclerosis

Disruption of the endothelial layer is the first step for atherosclerosis development. The conceptual revolution in the field of progenitor cells implies that EPC depletion represents at the same time a pathogenic step of atherogenesis and a biomarker of cardiovascular risk. It was found that the level of circulating CD34+KDR+ EPCs is an independent determinant of the extent of early subclinical atherosclerosis, measured by carotid-intima media thickness (IMT) using ultrasound, in a sample representative of the healthy general population. A high carotid IMT was associated with a worse cardiovascular profile, as shown by a strong correlation with the Framingham risk score (used to estimate the 10 year cardiovascular risk of an individual). The lack of correlation found between carotid IMT and CD34+, CD133+, and CD34+CD133+ cells suggested that undifferentiated progenitors are distinct from EPCs and do not reflect endothelial homeostasis in healthy subjects. Furthermore, the lack of correlation found between IMT and CD133+KDR+ and CD34+CD133+KDR+ cells suggested that those immature cells are not very relevant in steady-state conditions, as they provide a limited contribution to the circulating EPC pool, which is made up mainly of CD34+KDR+ cells. EPC depletion is a determinant of the anatomic remodelling of the common carotid

artery wall, which is a strong indicator of generalised atherosclerosis, and they provide proof of principle for the role of EPCs in vascular homeostasis. In summary, the level of circulating CD34+KDR+ EPCs is a determinant of early atherosclerosis in the general population. The emerging concept is that EPC depletion reduces the ability to repair the endothelium, thus triggering subsequent steps in the development of the atherosclerotic plaque (57). Other authors found similar results. The number of CD34+KDR+ EPCs decreased with the presence and extent of preclinical atherosclerosis. Accordingly, this reinforced the possibility that circulating EPCs may play a role at the early stage of atherosclerotic disease, as referred above. Lower numbers of CD34+KDR+ EPCs were found in the presence than in the absence of aging, and in the presence than in the absence of hypertension, hypercholesterolemia, smoking and family history of coronary heart disease. The CD34+KDR+ EPCs number also decreased with increasing level of Framingham risk score (78). Contrarily to the previous study (57), a lack of relationship between CD34+KDR+ EPCs and common carotid IMT was found. In this case, atherosclerotic plaque was detected by ultrasound in carotid, abdominal aortic and femoral sites and the number of sites affected by plaque among these three sites was counted. IMT was measured by computerised ultrasound imaging of both common carotid segments. According to the latter authors, the lack of correlation between EPCs and IMT is interesting to remark because IMT, measured in the common carotid segment free from atherosclerosis, is not a specific marker of atherosclerosis and may represent also medial hypertrophy that is a non-atherosclerotic process. Therefore, the lack of association between EPCs and IMT, contrasting with the relationship of EPCs and carotid plaque, suggests that the reduction of circulating EPCs is clearly a phenomenon specifically linked to atherosclerosis. The carotid atherogenic effect of multiple risk factors burden was found to be considerably attenuated by high level of CD34+KDR+ EPCs while worsened by low level of these cells. Summarizing the findings of the mentioned study, the number of circulating EPCs is involved in the pathogenesis of early atherosclerosis in asymptomatic subjects, and provides additional information to that of traditional risk factors as regards the status of the carotid artery that is as a sentinel vessel for assessing global atherosclerotic risk (78).

Despite the differences between these two studies with respect to IMT, the results remain similar, atherosclerosis is associated with low levels of EPCs.

Using a culture method to define EPCs, the association of EPCs with atherosclerosis and possible ethnic differences in EPCs were evaluated. Using coronary artery calcification and both carotid and femoral IMT as measures of atherosclerosis, it was shown that acLDL and lectin positive EPCs are reduced in male individuals with coronary and lower limb

atherosclerosis and that this is independent of other risk factors. The EPCs did not differ between Europeans and south Asian subjects and, according to the authors, it is doubtful that differences in EPCs explain possible ethnic differences in cardiovascular risk (79).

The association between EPC numbers (defined as double-positive stained cells for DiI-acLDL and lectin on day 5 of culture), EPC CFUs, cardiovascular risk factors and life-style behaviours was assessed in a large population-based study. The potential relation of EPC characteristics with atherosclerosis as well as levels of cytokines and growth factors previously implicated in EPC mobilisation, homing and differentiation was evaluated. According to the authors, this study lends further support to previous observations that EPC numbers decline with advancing age and are influenced by standard cardiovascular drugs; refutes the concept that the circulating EPC pool is exhausted and repair capacity impaired by vascular risk factors and an unfavourable lifestyle; and reports important findings like the significant association between EPC numbers and cytokine levels as well as carotid artery IMT. Besides, the number of cultured EPCs, but not EPC CFUs, was associated with some cardiovascular risk factors, Framingham risk score and common carotid artery atherosclerosis. The number of cultured EPCs include total EPCs, with lower or higher proliferative potential, whereas EPC CFU assay assesses a proportion of EPCs with higher proliferative ability and clonogenic capacity. Therefore, one possible explanation for such difference between the number of cultured EPCs and EPC CFU assay is that they represent different functional subpopulation of circulating EPCs. A modest but significant decline in the pool of circulating EPC emerged across the age range from 55 to 94 years. Moreover, it was demonstrated that the EPC numbers are significantly lower in (predominantly postmenopausal) females than in males of equal age and higher among subjects reporting regular (predominantly low-to-moderate) alcohol consumption. In addition, subjects with statin, hormone replacement and angiotensin converting enzyme (ACE) inhibitor/angiotensin-receptor blocker therapy exhibited higher EPC levels. Positive associations of EPC numbers with a number of cardiovascular risk factors and with the Framingham risk score were found. The finding persisted after adjusting for statin, hormone replacement and ACE inhibitor/angiotensin receptor blocker therapy. Speculatively, the positive association between EPC numbers and the Framingham risk score may reflect a protective compensatory response to the individual vascular risk burden. An inverse association was found between EPC numbers and common carotid artery IMT as well as the carotid artery atherosclerosis score, both of which representing surrogates for the severity of systemic vessel pathology. However, the associations obtained were only of moderate

strength and did not extent to femoral artery atherosclerosis. A highly significant inverse relation was found between plasma SDF-1α levels and both EPC number and EPC CFUs and a significant positive relation between MMP-9 level and EPC numbers. Against expectations, EPC numbers did not significantly differ between subjects with and without a history of previous cardiovascular disease. On interpreting these findings, however, we have to considered that most events had occurred already years before conducting this study and that patients had been subject to extensive life-style modifications and drug therapy. Furthermore, many patients had again entered a clinically inactive stage of vessel disease. According to the authors, the data from this study implicated that the changes of the number of EPCs are loosely associated with certain risk factors for the cardiovascular disease and may not directly associated with the disease development. Additionally, the findings are unfavourable to the traditional view that the EPC number is negatively affected by cardiovascular risk factors, indicating that the role of EPC is more complex than assumed previously. The findings also implicated that the capacity to mobilise EPCs in acute disease is more important than EPC baseline levels (80).

Increasing evidence indicates a link between bone and the vasculature. BM and circulating osteogenic cells have been identified by staining for the osteoblastic marker, osteocalcin. EPCs contribute to vascular repair, but repair of vascular injury may result in calcification. A higher number and percentage of circulating cell populations containing EPCs (CD34+KDR+) co-stain for osteocalcin in patients with early or late coronary atherosclerosis compared with control subjects with normal endothelial function and no structural CAD were demonstrated. The data suggested that the activation of an osteogenic program by EPCs may play a role in the response to vascular injury and contribute to vascular calcification, as opposed to non-calcifying vascular repair. Additionally, the same pro-inflammatory factors involved in the pathogenesis of osteoporosis may lead to the expression of an osteogenic phenotype by endothelial lineage cells, providing a potential mechanism for the link between osteoporosis and vascular calcifications. In this context, the findings of the study suggest that the addition of osteocalcin as a biomarker to EPCs may be of utility in predicting cardiovascular outcomes. Finally, the finding that the number of EPCs positive for osteocalcin can predict coronary atherosclerosis suggests that these cell populations may hold promise as novel markers for vascular disease (81).

Advanced glycation end products (AGEs) contribute to the pathogenesis of atherosclerosis. It was investigated whether serum level of AGEs is associated with EPC number and functions in apparently healthy subjects, independent of traditional cardiovascular risk factors. Apparently healthy volunteers who were not on any medications underwent a complete history and physical examination and determination of AGEs, as well as determination of the number, differentiation and migratory activity of circulating EPCs (CD34+KDR+, CD133+KDR+ and culture). All the subjects were normotensive and normoglycaemic, and no one suffered from hypertension or diabetes. It was demonstrated that the serum level of AGEs was one of the independent correlates of decreased cell number and impaired migratory activity of circulating EPCs in apparently healthy subjects. Given the protective role of circulating EPCs against atherosclerosis, it was suggested that, even in young healthy subjects, serum level of AGEs could be a biomarker that could predict the progression of atherosclerosis and future cardiovascular events. It was also suggested that circulating AGE levels and traditional risk factors are interrelated with each other and that AGEs could partly explain the link between cardiovascular risk factors and reduced EPC number and function in humans. AGEs could directly act on EPCs *in vivo*, thus leading to the reduction of the EPC number and functions in apparently healthy subjects (82).

6.2.1. Coronary arterial calcification

In a study investigating whether low EPC numbers were associated with measures of coronary and aortic arterial calcification, reduced CFU quantity was associated with greater subclinical coronary and aortic calcification, whereas neither CD34+/KDR+ EPCs nor CD34+ EPCs variation was associated with significant differences in coronary or aortic calcification. The association between CFUs and subclinical coronary and aortic arterial calcification was present even after adjustment for cardiovascular risk factors. These findings are consistent with the hypothesis that reduced angiogenic potential could contribute to the development of atherosclerosis. It is important to note that this investigation focused on ambulatory individuals without acute illnesses that could lead to mobilisation of proangiogenic cells. The apparently discordant findings of this study are consistent with the hypothesis that CFU is a cell-based phenotype distinct from CD34+ and CD34+/KDR+ cells with respect to both characteristics and function, as mentioned before in this dissertation. The dependence of CFU formation on monocyte populations may have particular relevance for their observed association with coronary and aortic calcification. Thus, because of their affiliation with the monocytic lineage, it is plausible that the CFU phenotype reflects cellular activities that directly affect the

progression or regression of atherosclerotic lesions and, in particular, calcified lesions in the setting of advanced subclinical disease (38). CD34+ and KDR+ phenotypes may lack an observed association with arterial calcification for several reasons. First, CD34+ and CD34+/KDR+ cells may not be physiologically active in processes related to vascular calcification. Because arterial calcification denotes the presence of advanced atherosclerotic plaque, it is possible that CD34+ and CD34+/KDR+ quantity reflects a capacity for endothelial repair that is associated with earlier rather than later manifestations of atherosclerosis or alterations in vascular integrity that do not result in calcification. Second, CD34 and KDR cell surface markers, even when used together, are not highly specific for progenitor cells with endothelial repair and regenerative capacity (54). Finally, the number of circulating cells positive for both CD34 and KDR is very low in ambulatory individuals (17), which may limit statistical power for detecting significant effects. This could also account for differences between the study results (38).

6.3. Coronary artery disease

The level of circulating CD34+KDR+ EPCs predicts the occurrence of cardiovascular events and death from cardiovascular causes and may help to identify patients at increased cardiovascular risk. A single measurement of CD34+KDR+ EPCs may be considered as a useful tool to predict cardiovascular outcomes in patients with CAD. The occurrence of a first major cardiovascular event, like acute myocardial infarction, hospitalisation, revascularisation, or death from cardiovascular causes, was associated with reduced EPC levels, in patients with CAD. However, EPC levels were not predictive of acute myocardial infarction or stroke, but in fact EPCs that were mobilised after acute myocardial infarction were functionally impaired. In order to determine the functional properties of formerly circulating EPCs, the number of endothelial colony-forming units was determined in a subgroup of patients with CAD. The results confirmed and extended the findings of Hill and colleagues (22), demonstrating that functional properties of EPCs influence cardiovascular outcomes. Finally, the results of these authors suggest that circulating EPCs in patients with CAD can be used to identify patients at high risk for major adverse cardiac events. This finding supports the notion that immature cells play an important part in the pathogenesis of atherosclerotic disease and that the measurement of EPCs may improve risk stratification (19).

The possible correlation between levels of circulating EPCs and atherosclerotic disease progression was investigated to establish a clinically meaningful role of ongoing endogenous endothelial repair mediated by circulating EPCs. Patients suffering from cardiovascular events showed significantly lower numbers of EPCs. Therefore, it was suggested that the levels of circulating EPCs may be a surrogate marker of vascular function and cumulative cardiovascular risk. Reduced levels of circulating EPCs were founded to be an independently predictor of atherosclerotic disease progression. Clinical evidence was then provided for the hypothesis that circulating EPCs contribute to ongoing vascular repair. In summary, reduced levels of circulating EPCs independently predict future cardiovascular events, thus supporting an important role for endogenous vascular repair to modulate the clinical course of CAD. Monitoring the levels of circulating EPCs as a surrogate biological marker might be specifically useful for identifying novel therapeutic approaches targeted to enhance endogenous vascular repair capacity and thereby modify the progression of cardiovascular disease (18).

The potential association between the number of EPCs in PB and the presence and severity of CAD was evaluated in patients undergoing coronary angiography. EPCs were identified by the formation of discrete colonies of endothelial cells on days 14 to 28 of culture. Cells in these colonies stained with lectin and took up acLDL. These cells expressed high levels of the endothelial antigens CD34, CD31, CD105, and CD144 but were largely negative for the leukocyte antigens CD45 and CD14. It was demonstrated that, among patients referred for coronary angiography, the number of EPCs in the blood is increased in association with angiographically significant CAD (45).

In a study evaluating whether numbers and activity of circulating EPCs (CD133+KDR+ cells, defined using flow cytometry) correlate with severity of coronary stenosis, as well as cardiovascular risk factors in patients with stable CAD, the number of EPCs was lower in patients with a single diseased coronary artery or multiple diseased arteries compared to those with normal coronary arteries. Besides, fluorescent chemical detection of EPCs was performed on attached MNCs after 7 days in culture and cells demonstrating double-positive fluorescence for DiI-acLDL and lectin were identified as differentiating EPCs. EPCs were inversely correlated with conventional cardiovascular risk factors such as age, hypertension and family history of CAD. The number of EPCs was also inversely correlated with C-reactive protein (CRP) suggesting that CRP may reduce the number of functional EPCs in the bloodstream, thereby leading to increased risk of cardiovascular diseases. The authors of the

referred study speculated that either traditional risk factors or CRP may reduce the number of functional EPCs in the blood stream, thereby leading to endothelial dysfunction and the acceleration of atherosclerosis. Also, the migratory capacity of EPCs was compromised in patients with a single diseased artery and those with multiple diseased arteries as compared with patients with angiographically normal coronary arteries. The ability of EPCs to adhere to fibronectin was significantly lower in patients with CAD. Concluding the results of the study, in patients with stable CAD, reduction in the number and impairment in the function of circulating EPCs were correlated with the severity of coronary stenosis. CRP may play an important role in reducing the number of EPCs and accelerating atherosclerosis. Given the important role of EPCs in neovascularisation of ischaemic tissue, a decrease in the number and activity of EPCs may contribute to impaired vascularisation and reduced endothelial repair in patients with CAD. Because of the strong relationship between the severity of CAD and EPCs in the blood stream, it was speculated that one cause of CAD might be an increasing inability of these EPCs to keep up with the endothelial damage (83).

In another study, reduced levels of putative EPCs, defined as CD34+CD31+, CD34+CD117+ or CD34+KDR+, were found in CAD patients, and compared to a group of healthy controls. Not only levels of putative EPCs, but also the EC marker KDR was approximately two-fold lower in CAD patients. It was also noted a two-fold reduction in CXCR4+ cells, evaluated because of its possible implication in EPCs mobilisation from BM to PB. The EPCs' capacity of increasing in response to the level of vascular injury incurred was determined and it became apparent that levels of the majority of cells examined were increased in a temporal manner to a greater extent in angioplasty patients compared to patients that received angiogram alone. Accordingly, this suggested that even though CAD patients present a generalised deficit in circulating EPCs, the processes that regulate the mobilisation and/or recruitment of these cells would appear to still maintain the capacity to respond to acute ischaemia. Levels of putative EPCs were also shown to be negatively correlated with the number of risk factors in CAD patients. When clinical characteristics were separated into major (age, hypertension, total cholesterol, high density lipoprotein (HDL)-cholesterol, smoking, diabetes) and minor cardiovascular risk factors (family history of CAD, gender, obesity, triacylglycerol and CRP), it was noted that the number of CD34+CD31+ and CD34+KDR+ cells were highly associated with major cardiovascular risk factors, but not with minor cardiovascular risk factors. In addition, a significant negative association was observed between the presence of diabetes and the three putative EPC populations examined. Also, patients receiving statin treatment

revealed increased levels of putative EPCs. Summarizing it was shown that baseline levels of putative EPCs are differentially increased depending upon the severity of vascular injury incurred, regardless of a significant deficit in baseline levels in CAD patients. Furthermore, baseline levels of putative EPCs were predominantly dependent upon age and the presence of diabetes in CAD patients (84).

It was suggested that different subpopulations of EPCs have a discordant behaviour in CAD patients with ischaemic left ventricular (LV) dysfunction, with CD34+KDR+CD45- and CD133+KDR+CD45- being increased and CD45+CD14+ cells, that promote vasculogenesis and microvascular development, being significantly reduced (85). In this particularly study, CD45+CD14+ were mentioned as EPCs, but it is not clear if this phenotype can be used to define true EPCs. The findings of increased CD34+KDR+CD45- and CD133+KDR+CD45- cells, regarded as EPCs, compared to reduced CD45+CD14+, may suggest that these phenotypes are characteristic of completely different cells.

In a study using ECFCs, in high-risk patients, ECFCs were already activated before inflammatory stimulation. ECFCs from patients with CAD also demonstrated impaired eNOS expression, which was improved by *ex vivo* pretreatment with atorvastatin. This data raise the possibility that without additional modifications, transplantation of dysfunctional ECFCs may lead to impairment of endothelial functional recovery or may even cause atherosclerosis (49).

A study was performed in order to identify possible predictors of circulating EPC (CD34+KDR+) levels in PB and to evaluate their correlation with direct and indirect cardiovascular risk factors in patients referred for non-invasive assessment of CAD using multidetector computed tomography (MDCT) angiography (a method of computed tomography angiography (CTA)). According to the authors of this study, in patients without known CAD referred for MDCT, circulating EPC counts in PB cannot be significantly predicted from baseline population characteristics, anthropometric measures, adiposity measurements, clinical context, risk factors or even CTA. Some correlations were found between cardiovascular risk factors and circulating EPC levels but those relations did not appear to be sufficiently strong to be used for predicting EPC levels in a clinical context. In this study's low-to-intermediate risk population no significant correlation between the Framingham risk score and EPCs was found. Concerning cardiovascular risk factors, smoking appears to be the best predictor for lower EPC levels; in the study, it was the only risk factor significantly associated with lower EPC counts. EPC levels were significantly lower in diabetic patients and inversely related to glycated haemoglobin. Hypertension was not significantly associated with EPC levels in PB. Interestingly, low density lipoprotein (LDL) cholesterol was found to correlate positively

with circulating EPC levels, while patients with higher triglyceride levels tended to have lower levels. In this study's population, patients under chronic therapy with statins were found to have lower levels of circulating EPCs. Interpretation of these findings is difficult, since there is significant collinearity and interaction between the parameters. For instance, patients on statins are more likely to have a history of dyslipidaemia and hence greater exposure to vascular damage. This study's population didn't include patients with known CAD and did not include patients with acute coronary syndromes or post-coronary artery bypass grafting. In this stable population, the authors were able to confirm the positive correlation of circulating EPCs with CRP expression. This study aimed to investigate whether EPC counts could be predicted with any degree of certainty on the basis of patients' baseline characteristics. The results showed that, at least in this population of patients with low-to intermediate cardiovascular risk without known CAD, only very limited prediction is possible: all the possible associations were found to be weak or even non-significant. Also, no correlation was detected between EPCs and body mass index (BMI) and no significant differences were found between obese and non-obese patients. Similarly, no significant relation was found between EPC levels in PB and abdominal circumference, abdominal fat or epicardial fat. No significant correlation was shown between EPC counts and calcium score (as a marker of atherosclerotic burden). No correlation was found in the present study between the presence or severity of CAD and the number of circulating EPCs. Only patients without known CAD referred for MDCT were included in this single-center exploratory analysis, and so this sample may not represent the general population. The potential value of quantifying circulating EPCs for cardiovascular risk prediction was not tested, since there was no follow-up of events. Furthermore, only the number of circulating EPCs in PB, and not EPC activity or mobility, was analysed in the study. According to the authors, this should be taken in consideration when comparing the results with studies in which these parameters were measured. Although some correlations were found between circulating EPC levels and cardiovascular risk factors, namely diabetes and smoking (inverse relation) and CRP (direct relation), those relations were found to be too weak for EPC prediction in a clinical context. Therefore, the authors concluded that in patients without known CAD referred for MDCT, EPC levels in PB cannot be significantly predicted from knowledge of patient anamnesis, risk factors, visceral fat, coronary artery calcification or CTA (86).

EPCs (CD34+KDR+) were evaluated in relation to classical adverse outcome predictors for cardiovascular disease (plasma N-terminal pro-B-type natriuretic peptide (NT-proBNP) levels,

impaired LV relaxation pattern and exercise-induced ischaemia) in a relatively homogenous group of non-diabetic men with stable angina, one-vessel CAD and preserved LV function. The authors studied non-diabetic men with one-vessel CAD, LV ejection fraction (LVEF) ≥ 60% and normal LV diastolic function or impaired LV relaxation, and non-CAD controls matched for risk profile and medication. It was demonstrated that stable angina patients with preserved LVEF and one-vessel obstructive CAD exhibited higher plasma NT-proBNP levels, especially at impaired LV relaxation but also at normal LV diastolic function. Additionally, a significantly lower EPC blood count was observed in the presence of impaired relaxation associated with a more pronounced ischaemic burden. Importantly, CAD patients with abnormal LV relaxation exhibited a more pronounced ischemic response to exercise. Finally, Duke treadmill score, a prognostic parameter reflecting ischaemic burden at stress testing, was a stronger correlate of both augmented NT-proBNP levels and depressed EPC numbers than indices of diastolic function which were eliminated by stepwise multiple regression. Additionally, adjustment for the treadmill score abolished the relationship between NT-proBNP and EPCs. Accordingly, a factor yet unidentified, possibly related to different clinical characteristics or ischaemia by itself, might have mediated the coincidence of a high NT-proBNP and EPCs deficiency in symptomatic stable CAD with impaired LV relaxation. The authors were not able to fully exclude potential alternative mechanisms that might have been responsible for the observed relationships. It was suggested that a common factor, probably repetitive ischaemic burden, can be accountable for the coincidence of an elevated NT-proBNP concentration and a depressed EPC count in stable angina patients exhibiting features of impaired LV relaxation. Summarily, propensity to symptomatic exertional ischaemia may underlie the coincidence between moderately elevated NT-proBNP and EPC deficiency in patients with stable CAD. Additionally, chronic subclinical ischaemia can also be involved in these associations. These might result from B-type natriuretic peptide (BNP) overexpression in the ischemic myocardium and a hypothetical exhaustion of the BM capacity to mobilise EPC in response to multiple ischemic episodes, thus contributing to the prognostic effect of B-type natriuretic peptides irrespective of hemodynamic factors (87).

More recently, another group of investigators investigated the relationship between coronary collateral formation and circulating EPCs (CD34+CD13+ and CD34+KDR+) in patients undergoing coronary angiography for suspicion of CAD. Patients were subdivided in three groups: patients with normal coronary vessels, patients with critical coronary stenosis and poor collaterals and patients with critical coronary stenosis and good collateral formation.

Circulating EPCs were higher among patients with normal coronary vessels compared to patients with CAD despite the presence of similar cardiovascular risk factors. When patients were subdivided into poor and good collateral groups, patients with good collaterals had higher EPC counts than poor collateral group. As the collateral grade increases, the number and percentage of EPCs rose significantly. It was shown that only CD34+/KDR+ EPCs were independent predictors for good collateral development. However, circulating EPCs were similar between normal coronary arteries and CAD with good collateral development groups despite similar cardiovascular risk factor burden. Patients with CAD were also evaluated according to the extent of significant coronary stenosis. Circulating EPCs were similar in patients with CAD when grouped into either 1 vessel, 2 vessel and 3 vessel disease or extent of CAD. Also, the findings revealed that EPC is an independent predictor for coronary collateral formation after adjustment for other cardiovascular risk factors and extent of CAD. The expression of the KDR receptor was not reduced in EPCs derived from patients with CAD and good collateral development compared to normal coronary vessels. Moreover, both patients with poor and good collaterals have similar atherosclerotic burden. This may imply that an intact response of the endothelium and BM is also necessary for collateral development in addition to lesion severity. The authors thought that EPCs may create the difference between patients in their capacity to develop sufficient collateral circulation. The mechanisms underlying the variation in collateral formation between patients, even with similar patterns of CAD, are unclear. Despite the similarities between cardiovascular risk factors, there is significant heterogeneity regarding collateral development in the study population. As a result, the reduction in EPC count and their functional impairment might contribute to reduced collateral formation in patients with CAD despite myocardial ischaemia. Therefore, variation in collateral development cannot be simply explained by lesion severity or myocardial ischaemia as in the study population. In conclusion, circulating EPCs are increased in patients with good collateral grade compared to poor collateral formation among patient with critical coronary stenosis. However the study results only indicate an association between collateral grade and circulating EPCs derived from PB not a causal relationship. These findings may contribute to the hypothesis aiming to enhance circulating EPC numbers strategies to collateral formation as a therapeutic option (88).

6.4. Arterial stiffness

To elucidate the mechanisms underlying the association between plasma cholesterol levels, endothelial injury and arterial stiffness, subjects with a wide range of plasma cholesterol levels,

from normo-cholesterolaemia to frank hypercholesterolaemia were recruited. Plasma lipid levels, aortic pulse wave velocity (PWV, a measure of arterial stiffness), CD31+/CD42- endothelial microparticles (EMPs), EPCs (CD34+KDR+), and the ratio of circulating EMPs to EPCs, as an index of endothelium injury and repair ability, were measured. It was found that hypercholesterolaemic patients have higher circulating EMPs and lower EPCs than normo-cholesterolaemic subjects and also that the ratio of EMPs to EPCs is directly associated with aortic PWV. Therefore, it was suggested that hypercholesterolemia may contribute to large artery stiffness both by increasing the release of microparticles that are mainly of endothelial origin and by reducing the number of circulating EPCs. It was found that microparticles, besides injuring mature endothelial cells, may also play a role in reducing the vitality of EPCs. In fact, it was observed that microparticles from hypercholesterolaemic patients caused a significant *in vitro* EPCs apoptosis and reduced their colony forming capacity which suggest a novel mechanism of human EPCs incompetence. The finding of a lower number of EPCs in hypercholesterolaemic patients compared with normo-cholesterolaemic subjects, and the inverse association between the number of circulating EPCs and aortic PWV, further suggested that, also in the case of hypercholesterolaemia, the integrity of the endothelium appears essential for the preservation of a proper aortic distensibility. The demonstration of low plasma EPCs levels is therefore important because it reflects an impaired potential of EPCs to participate in the repair of injured endothelium. According to the authors, the findings of this study suggested that both increased endothelium injury and impaired endothelial repair may contribute to reduce aortic distensibility, and the balance between endothelium injury and repair is critical for the maintenance of the vascular homeostasis (89).

It was investigated whether microparticles shedding from EPCs (CD34+KDR+) are detectable in cultures of EPCs and in the circulation of subjects with various degrees of cardiovascular risk. The relationship of EPCs-derived microparticles (defined as CD34+KDR+ particles) to cardiovascular risk factors and aortic stiffness, a marker of cardiovascular risk and impaired vascular repair by EPCs, was also investigated. The authors sought to investigate *ex vivo* whether cultured EPCs may undergo apoptosis and release microparticles, and *in vivo* whether the number of EPCs-derived MPs correlates with the number of circulating EPCs, the total burden of estimated cardiovascular risk and with aortic stiffness. With regard to the *ex vivo* study, it was demonstrated that cultured EPCs undergo extensive apoptosis and in turn release a significant amount of MPs when exposed to a range of concentrations of the pro-apoptotic hydrogen peroxide. EPCs apoptosis and microparticle vesiculation was also found after

exposure of EPCs cultures to sera from hypercholesterolaemic patients. These MPs express KDR and CD34 surface antigens, indicating their EPCs derivation, and the majority of them stain with annexin-V, a marker of death cells, as a consequence of their apoptotic origin. A lower number of apoptotic EPCs and annexin-V positive EPCs-derived MPs were also found in cultures which had not been exposed to hydrogen-peroxide or sera from hypercholesterolaemic patients. According to the authors, this suggests that EPCs apoptosis and fragmentation into MPs may probably happen at a lesser extent without exposure to these pro-apoptotic stimuli. In the *in vivo* study, CD34+/KDR+ MPs have also been found in human blood, possibly indicating that EPCs may be injured in the circulation and may consequently release MPs. The authors provided an *in vivo* observation of the presence of circulating CD34+/KDR+ MPs without a *in vivo* demonstration of the mechanism of MPs formation; however, *ex vivo* demonstration of EPCs-derived MPs formation and the negative association the authors found *in vivo* between the number of CD34+/KDR+ MPs and that of EPCs supports the conclusion that CD34+/KDR+ MPs originate from circulating EPCs. The negative association between EPCs-derived MPs and EPCs suggests that the higher the degree of EPCs fragmentation into MPs, and the lower the number of circulating EPCs. This assumption may be added to other intriguing theories explaining the loss of circulating EPCs and their reparative incompetence. A positive correlation between Framingham risk score and EPCs count was also showed. However, the additional finding of a negative association between Framingham risk score and the number of EPCs-derived MPs suggests a novel mechanism of risk factor-induced EPCs loss. The authors of this study suggested the hypothesis that the greater the burden of cardiovascular risk factors, the greater the apoptotic fragmentation of circulating EPCs into MPs. The results of the present study also suggest that the more EPCs fragment into microparticles, as documented by an increased number of circulating EPCs-derived MPs and a reduced EPCs count, the greater the stiffness of large arteries. The concurrent presence of high MPs and low EPCs levels was associated with the greatest increase in aortic stiffness. Subjects with increased levels of EPCs-derived MPs might have stiffer arteries just because they also have a greater burden of cardiovascular risk factors and lower EPCs levels, which are both significant positive correlates of arterial stiffness. However, a direct pro-atherogenic role of EPCs-derived MPs may be supposed because the association between EPCs-derived MPs and aortic stiffness was independent of both Framingham risk and EPCs count. According to the authors, explanations of the mechanisms responsible for increased aortic stiffness in subjects with reduced EPCs and increased EPCs-derived MPs levels cannot be inferred from the present study. Given that the bioavailability of nitric oxide relies

upon endothelial integrity and is considered critical in maintaining arterial distensibility, reduced endothelial renovation by fragmented EPCs might also impair arterial distensibility. Summarily, the balance between EPCs production and death is critical for the maintenance of arterial integrity. Further research is needed in this field to offer new diagnostic tools for the detection of vascular dysfunction (90).

Asymmetric dimethyl-arginine (ADMA) is an endogenous eNOS inhibitor. Therefore, inhibiting eNOS activity in EPCs, ADMA affects mobilisation, differentiation, and function of EPCs. This contributes to the cardiovascular risk in patients with high ADMA levels and may explain low numbers and function of EPCs in patients with CAD (33).
Central arterial stiffness represents a well-established predictor of cardiovascular disease. The relationship between cardiovascular risk factors and arterial stiffness, with emphasis placed on risk factors, such as ADMA and EPCs (CD34+KDR+), among subjects with pre-diabetes was investigated. According to the findings of this study, LDL-cholesterol levels, ADMA levels and physical exercise are strongly associated with PWV among pre-diabetic subjects. In contrast, the number of EPCs did not correlate with arterial stiffness. Cardiovascular disease is so closely linked to increased PWV, so that identification of the predisposing factors of the latter will improve not only treatment efficacy, focusing on specific de-stiffening drugs, but also the quality of life of pre-diabetic individuals (91).

6.5. Acute coronary syndrome

Early mobilisation of EPCs (CD34+CD31+ EPC CFUs) into the peripheral circulation after percutaneous coronary intervention (PCI, a defined vascular manipulation), was studied in patients with coronary artery lesions. EPCs were quantified before and 12 hours after PCI. They were also quantified in a control group of patients with angiographically normal coronary arteries. All patients had one or more cardiovascular risk factors. PCI was performed in acute coronary syndrome (ACS) and non-ACS patients (in this case, elective PCI). The authors were able to compare EPC levels before, and early after the manipulation, at reproducible time points, avoiding possible non-specific perturbations. In addition, the vascular prosthesis presented a relatively limited, standardised intervention, and the procedure did not introduce other major vascular perturbations such as surgical wounds or cardiopulmonary bypass, aside from the catheterisation itself. It was found that the majority of patients receiving PCI on an elective basis showed a significant early rise in circulating EPCs. While the average EPC CFU

count increased by only 40%, the second sample was drawn only 12 hours after intervention, an early time point chosen to avoid bias against patients stable enough for early discharge from the hospital. In addition, all patients in this group possessed multiple cardiac risk factors and by definition had CAD. Thus, EPC recruitment after PCI may not have been as robust as in normal individuals, although PCI in this latter group cannot be studied. In contrast to patients receiving elective PCI, the group of patients suffering from ACS failed to recruit EPC into their PB within 12 hours after PCI. In addition, the initial pre-PCI EPC CFU counts, obtained within 24 h of an acute clinical syndrome, were not different from either the elective PCI or control groups, which itself suggests a failure to respond to a presumed fresh thrombotic focus. Besides being presented with a foreign surface (PCI), this group differed from the elective PCI group insofar as myocardial ischaemia was present. However, ischaemic myocardium itself is not likely to account for the lack of EPC mobilisation in this group. The findings of this study are easily reconciled with the hypothesis that acute coronary events during ACS at least in part result from a failure of EPC recruitment to intravascular thrombotic niduses. The precise mechanisms mediating PCI-induced EPC recruitment in humans remains to be determined. VEGF and GM-CSF are known to mobilise progenitor cells from the BM, as referred early in this dissertation. However, the data from this study suggest that EPC mobilisation early after PCI may not be due to either cytokine. The presenting levels of VEGF in stable CAD patients referred for an elective PCI were higher than in patients with ACS. Interestingly, VEGF levels did not increase after an elective PCI, despite an observed rise in EPC levels. In contrast, a robust increase in VEGF levels following PCI was seen in the ACS patients without a corresponding rise in EPC counts. The discordance between VEGF and EPC kinetics suggests that the acute rise in circulating EPC counts is not well explained by changes in VEGF levels. One may therefore speculate that VEGF independent endothelial cell mobilisation might contribute to the observed rapid increase in the number of peripherally circulating EPC, early after a vascular injury. Viewed in light of recent advances in the pathogenesis of the vulnerable plaque, these findings suggest that humans respond to vascular injury with a rapid early mobilisation of EPC into the peripheral circulation. Further, patients with deficient re-endothelialisation of sites of coronary endothelial injury may have an increased risk of plaque rupture and ensuing ACS. The lack of EPCs response in ACS patients may also partly explain the higher risk for stent thrombosis in this patient subset compared with recipients of elective PCI. Endothelial coverage of implanted stent surfaces, which may be impeded by vascular stem cell exhaustion or, in theory, cytostatic drug elution, may represent a logical target for novel therapeutic strategies. According to the authors, the results should be interpreted

conservatively because they only assessed a few of the many markers affected by PCI. However, the statistically consistent differences between EPC recruitment between ACS and the other patients suggests a pathogenic link (34).

The potential biomarker usefulness of EPCs (CD31+CD133+CD34brCD45-/dim) was evaluated on ACS patients and healthy subjects. A proteomic characterisation of EPCs was also conducted. The number of EPCs was extremely low in both groups. No differences statistically significant were found in the percentage of EPC population between ACS patients and control healthy subjects. With respect to proteomic characterisation, EPCs presented a high number of proteins related with DNA repair and metabolism, mRNA splicing and processing factors suggesting a potential "regenerative" role in vessel maintenance. In addition, differences in the identified proteins and pathways were found between control subjects and ACS patients in EPCs, suggesting a potential active role of cardiovascular events in the expression profile of these cells. According to the authors, the results also suggest a specific cell response to cardiovascular events presenting a pro-thrombotic state (92).

The platelet-bound SDF-1 expression and its correlation with platelet activation, plasma levels of SDF-1, and number of EPCs in patients with coronary artery disease and/or reduced left ventricular (LV) function were evaluated. It was found that the platelet-surface expression of SDF-1 is elevated in patients with ACS compared with patients with stable angina pectoris. Also, it is increased in patients with reduced LV function compared with patients with normal LVEF. Besides, in a subgroup of patients, platelet-bound SDF-1 was correlated with the number of circulating EPCs (CD34+CD133+). These findings imply that increased surface expression of platelet-bound SDF-1 takes place in cases of acute or chronic tissue ischaemia, such as ACS or reduced systolic ventricular function, and in diseases or pathophysiological phenomena characterised by enhanced platelet activation such as stroke, sepsis, and inflammation. Therefore, in diseases where tissue regeneration is demanded, platelet-bound SDF-1 may be increased. In addition, platelet-surface-bound SDF-1 slightly correlates with plasma SDF-1 and with the number of circulating EPCs in patients with ACS, a phenomenon which could potentially or partially explain the reported mobilisation of EPCs in those patients. Platelet-bound SDF-1 may play an important role in peripheral homing of circulating progenitor cells thus in tissue regeneration (93).
It was shown that platelets derived from healthy subjects are a source of soluble components that increase the number and improve the function of *ex vivo* cultivated EPC derived from

patients with cardiovascular risk factors. Co-incubation of platelets and PB MNCs, both from healthy volunteers, dose-dependently increased the number of adherent EPC. In contrast, patient-derived platelets failed to augment the number of adherent and migrating healthy and patient-derived EPC. However, co-incubation of platelets from healthy donors with MNCs from patients with cardiovascular risk factors significantly enhanced the number of EPC, indicating that platelets from healthy volunteers are able to partially rescue the impairment of patient-derived EPC formation. Likewise, healthy donor-derived platelets augmented the impaired migration and clonal capacity of patient-derived EPC. Analysis of individual cardiovascular risk factors of platelet donors revealed that only DM inversely correlated with EPC number, colony formation and migration. The platelet supernatants from healthy volunteers that significantly increased EPC number contained SDF-1. The release of SDF-1 by patient-derived platelets was increased, thus, indicating that these soluble factors are not mediating the effect of platelet supernatants. The identification of the distinct factors released by platelets that mediate the effects observed may help to develop novel therapeutic options to improve the severe impairment of EPC function in patients with cardiovascular risk factors, in particular with DM (94).

6.5.1. Myocardial infarction

Because the identification of EPC, therapeutic vasculogenesis has become a real possibility for treatment of ischaemic vascular disease (95). It was shown that expanded EPC-derived progeny improve LV heart function after reperfusion in myocardial infarction. Regarding a possible treatment of acute myocardial infarction with expanded EPC-derived cells, the time frame for differentiation from CD34+ cells needs to be considered. In addition, expanded cells might be applicable to patients with chronic ischaemic disease that is present in severe CAD or peripheral occlusive disease. Whether EPC or EPC-derived progeny can still improve LVEF when transplanted a few weeks after myocardial infarction has, however, not been addressed in this series of experiments (95).

The number and potential differentiation of EPCs (CD34+, CD34+CD133+, CD34+CD133+VEGFR2+, all CD45low) were evaluated and compared with clinical parameters 6 months after myocardial infarction, in order to test the hypothesis that the angiogenic function of EPCs affects post-myocardial infarction myocardial salvage. The potential of EPCs to differentiate into endothelial cells was also confirmed by the upregulation

of CD31 and VEGFR2 after 7 days of culture. According to the proportion of EPC fraction, patients were divided into 2 groups, the differentiated EPC group and the non-differentiated EPC group. Although no difference was seen in myocardial damage between the differentiated group and non-differentiated group, the number of attached cells was greater in differentiated group than in the non-differentiated group. The study revealed diversity in the capability of differentiation to an endothelial lineage, in both the circulating fraction of EPCs and cultured EPCs. A greater area of myocardial salvage accompanied with a reduction of the ischaemic area and greater recovery of LV function were observed in patients with differentiated EPCs compared with those with non-differentiated EPCs. Regarding EPC differentiation, the authors first measured the abundance of EPC fraction defined as CD45lowCD34+CD133+KDR+ in the sample of patients with acute myocardial infarction and healthy volunteers and compared this with parameters of clinical outcome. The EPC fraction was also present in the samples of healthy subjects, although the abundance was less than that in myocardial infarction patients. Regarding the mobilisation of EPCs, the increase of CD34+ cells and CD34+CD133+ KDR+ cells at 7 days were 3.3-fold and 1.7-fold greater in myocardial infarction patients than in healthy controls, respectively. According to the authors of this study, the findings support the notion that even in a healthy condition, EPCs circulate to maintain the vascular function and that after the onset of acute myocardial infarction, these EPCs are mobilised to repair the damaged myocardium and capillary network. It is important to note that, during the follow-up period, all patients received statins and angiotensin type I receptor antagonist (both of which may affect the prognosis of MI) in addition to the anticoagulants aspirin and ticlopidine. Four cases of the differentiated group showed impaired myocardial salvage that was even less than the average in the non-differentiated group. Although each of the risk area sizes did not differ significantly from those of other patients of the same group, 3 of 4 patients resumed smoking after hospital discharge, and all of these 4 patients had at least 4 cardiovascular risk factors. In these cases, such unfavorable behavior during the recovery period may reverse the good symptoms of EPC differentiation in the acute phase. Summarily, it was shown that, in patients with primary acute myocardial infarction, the capability of EPCs to differentiate is associated with functional improvement and infarct size reduction, indicating that manipulation of EPCs could become a novel therapeutic target to salvage ischaemic damage (96).

The potential correlation between the extent of ischaemic myocardium (area at risk) or of necrotic myocardium (infarct size) and the levels of circulating EPCs (CD34+KDR+) was investigated. Peripheral EPCs were measured in ST-elevation myocardial infarction (STEMI)

patients at 24 hours after successful primary percutaneous coronary intervention. EPC levels were related to the extent of ischaemic myocardium but not with myocardial necrosis. Moreover, at 2-month follow up, EPC levels were significantly reduced compared to baseline. No correlations were observed between EPC levels, both at baseline and at follow up, and hs-CRP concentrations. A significant correlation between the extent of myocardial ischaemia and potentially reversible injury, and EPC mobilisation in STEMI patients was found. Conversely, no consistent relationship was observed between EPC mobilisation and the amount of myocardial necrosis, microvascular damage, myocardial salvage or systemic inflammation as expressed by hs-CRP levels. This observation supports the notion that myocardial ischaemia, and not myocardial necrosis, is the main stimulus for EPC mobilisation from BM in the earlier phases of a myocardial infarction. Since myocardial ischaemia and necrosis are both events occurring soon after the acute occlusion of a coronary artery, both might be stimuli for EPC mobilisation. Concluding the results, a direct relationship between circulating levels of EPC and the amount of myocardial ischaemia was reported. According to the authors, the study provided support, by use of a quantitative imaging technique, to the hypothesis that myocardial ischaemia and reversible cellular injury is the main substrate driving very early EPC mobilisation from BM during myocardial infarction (97).

It was investigated whether the levels of EPCs in PB were related to the presence of collateral blood vessels in patients with a recent non-STEMI. Patients who underwent PCI within a week of non-STEMI were divided in two groups, the group of patients without collaterals and the group of patients with collateral vessel formation. Blood samples were drawn before and after PCI and EPCs were counted using culture (EPC colonies) and also by flow cytometry (CD133+VEGFR2+). It was found that the relative number of EPCs (CD133+VEGFR2+) before PCI was higher in patients with coronary collateral vessels than in those without such vessels. It is possible that, in the study population, a stronger ischaemic trigger caused the mobilisation of more EPCs from the BM, because the group of patients that presented collateral vessel formation had larger infarctions, as evidenced by higher levels of markers of myonecrosis, than the other group. However, the differences in collateral formation between the two groups may be attributed to factors other than differences in circulating EPC levels. The two groups also differed in the severity of the culprit vessel stenosis, and coronary lesion severity is a strong and consistent predictor of collateral flow. Surprisingly, in contrast to the difference in the number of EPCs determined by flow cytometry, the number of colony-forming cells was similar in the two study groups. This discrepancy may be related to the *in*

vitro culture conditions used for EPC colony isolation and quantification. It may also be explained by differences in the 'age' of EPCs detected by the two methods. Flow cytometry, performed from the fresh blood samples, detects 'early' EPCs, whereas the colony counting method, performed after 7 days of culture, may detect both 'early' and 'late' EPCs, as described before in this document. The presence of 'late' EPCs, which usually lose the CD133 phenotype may have attenuated potential differences between the two study groups in the number of EPC colony-forming cells. Another finding was the increase in EPC colony numbers after PCI in the group of patients without collateral vessels. According to the authors, this increase may have been caused by mobilisation of EPCs from the BM in response to coronary vessel injury during the PCI and suggests that the patients in this group may have had higher BM EPC reserves than did patients in the group with collateral vessels. Alternatively, a large myocardial damage might induce the release of circulating factors that suppress EPC mobilisation (24). Because circulating EPC levels increase within a few hours after an acute myocardial infarction and then decrease gradually during the next several days (98), the one-day difference (before and after PCI) may have limited the potential differences in EPC levels between the two groups. This may have contributed to the lack of a difference in the number of EPC colony-forming cells between the two groups. Accordingly, this study supports an association between PB EPC levels and collateral formation in patients with a non-STEMI (24).

6.6. Heart failure

The levels of circulating EPCs (CD34+CD133+VEGFR2+) and endothelial CFUs were assessed in patients with heart failure and correlated with the origin and severity of the disease. Patients were divided according to the New York Heart Association (NYHA) classification and compared with a control group consisting of healthy subjects. The number of EPCs and endothelial CFUs were higher in patients than in controls. Besides, tumour necrosis factor-alpha (TNF-α, a cytokine involved in inflammation), VEGF, SDF-1, and BNP were also elevated in patients (23). BNP is one of the most objective markers for heart failure (99). Notably, EPCs and endothelial CFUs were elevated in NYHA class I and class II compared with class III and class IV. According to the authors of the study, the main finding is the peripheral recruitment of EPCs in early stages of heart failure, whereas in class NYHA IV, their peripheral mobilisation was reduced not only with respect to class II and I but also with respect to control group. Increased EPCs during heart failure may be a reflection of diffuse and severe endothelial damage. The inverse correlation between EPCs and TNF-α and related soluble receptors led

the authors to hypothesise a role of TNF-α in this biphasic response. During the early stages of the disease, when TNF-α is not yet significantly elevated, EPCs are increased as a reflection of a functional BM response to diffuse and severe endothelial damage. In advanced disease phases, an additional and significant increase of TNF-α occurs that, by exerting a possible suppressive effect on haematopoiesis, finally counteracts and overwhelms the triggers able to increase and EPC mobilisation during the early phases. VEGF and SDF-1 were elevated in patients, and both tended to be further increased in advanced NYHA classes. They might fail to effectively recruit EPCs during advanced phases of the disease, because of the contrasting effect of TNF-α on BM cell mobilisation. In conclusion, more than one mechanism could be involved in the degree of EPC mobilisation in heart failure, and these findings suggest that it could be stage dependent. An exhaustion of progenitor cells in the advanced phases of the disease could also contribute to the biphasic BM pattern of response to heart failure (23).

6.7. Left ventricular dysfunction

Since systolic LV dysfunction is associated with abnormal endothelial function, it was hypothesised that patients with systolic LV dysfunction would show enhanced endothelial cell apoptosis and no compensation via increases of EPC numbers. All patients in this study were diagnosed with significant CAD, various degrees of systolic LV dysfunction and having a similar risk profile. It was demonstrated that CAD patients with a reduced systolic LV function have increased levels of circulating CD31+annexin-V+ EMPs compared with CAD patients with a preserved or normal LV function. The number of EMPs correlated inversely with endothelial dysfunction as assessed in the brachial artery circulation. The number of circulating EPCs (CD34+VEGFR2+) were somewhat decreased in patients with LV dysfunction. This data showed that abnormal endothelial function in patients with ischaemic LV dysfunction is related to endothelial cell damage. According to the authors, this study demonstrated a correlation of LV dysfunction and the balance of circulating EPC/EMP, independent of the cardiovascular risk profile. Taken together, CAD patients with significant LV dysfunction show an increased index of endothelial cell damage. This damaging process is associated with reduced EPC numbers in the blood. Because EPCs are believed to play a role in repair, the lack of a compensatory elevation may contribute at least in part to the pathologic development of endothelial dysfunction (100).

6.7.1. Left ventricular hypertrophy

The role of circulating EPCs (CD34+CD133+CD45low and CD34+KDR+CD45low) and endothelial apoptotic MPs (CD31+/annexin-V+) in hypertensive patients with and without electrocardiographic LV hypertrophy (LVH) was investigated. Hypertensive patients with LVH had higher levels of circulating CD31+/annexin-V+ apoptotic MPs compared with those without evidence of LVH, but the statistic was not significant. It was shown that levels and adhesive function of circulating EPCs in hypertensive patients with electrocardiographic (ECG) LVH decreased compared with those without ECG evidence of LVH. These findings indicate that attenuated endothelial repair capacity may contribute to atherosclerotic disease progression and enhanced cardiovascular events in hypertensive patients with LVH. In humans, extensive endothelial cell damage caused by cardiovascular risk factors can result in endothelial cell apoptosis with subsequent loss of integrity of the endothelium. The extent of endothelial injury may represent a balance between the magnitude of injury and the capacity for repair, and predicts cardiovascular event rates. In was shown that hypertensive patients with ECG evidence of LVH had decreased circulating EPC levels and attenuated EPC adhesive function, implying attenuated endothelial repair capacity in hypertensive patients with cardiac hypertrophy. The association between cardiac hypertrophy and endothelial dysfunction resulting from impaired vascular repair capacity may contribute to the pathogenesis of the high incidence of vascular events that is well documented in hypertensive patients with LVH. According to the authors, the findings of this study may explain the pathogenic processes that link hypertensive LVH and endothelial injury in cardiovascular disease (101).

6.8. Peripheral arterial disease

The interrelationships between EPCs, peripheral arterial disease (PAD), and atherosclerotic risk factors were evaluated. Number of circulating EPCs was evaluated using two methods commonly utilised in the literature, referred early in this dissertation. In patients with PAD, systolic blood pressure, hs-CRP, fasting glucose, and triglycerides were all significantly increased compared to a control group of healthy subjects. The number of EPCs and EPC CFUs were significantly increased in PAD compared to healthy subjects and were not significantly different between patients with PAD and diabetes and patients with PAD without diabetes. Also, the EPCs number and EPC CFU were not significantly different between patients with PAD treated with statins and patients with PAD and not on statin therapy or in patients who are non-smokers and smokers. In a subgroup of patients with PAD, the CD34+

cell count was significantly decreased compared to healthy subjects as well as CD34+ CD133+ cells. The CD34+ KDR+ cell count was not significantly different between the two groups. Plasma VEGF concentration was significantly increased in patients with PAD compared to healthy. The coexistence of diabetes *mellitus* (DM) and PAD in a subgroup of patients would not appear to decrease either the EPC count or the colony-forming capacity, although these patients do show a significant increase in EPC and EPC CFU compared to healthy controls. Though the results of the study may seem at variance with other studies dealing with EPCs in PAD, there are fundamental differences in the methodology. According to the authors it is possible that the types of EPCs measured are distinct (102). True EPCs were identified by the formation of colonies of endothelial cells on days 14 to 28 of culture. These cells took up acLDL and stained with lectin. Furthermore, these cells expressed high levels of CD34, CD31, and CD144 but were negative for antigen CD45 (45). All these features are clearly represented in the cells cultivated in this investigation (102). In fact, after 15 days of culture, cells acquired a characteristic endothelial "cobblestone" morphology, together with a characteristic receptor pattern consisting of loss of anti-CD45 positivity (pan-leukocyte pattern) during the first days in culture and gained positivity for CD31, KDR, and CD144 (endothelial pattern). The authors cultured the cells of virtually all individuals, which confirmed the endothelial phenotype with the above-described methods at the end of culture. Using fluorescence-activated cell sorting (FACS) analysis (specialised type of flow cytometry), a statistically significant decrease in circulating cells positive for CD34 or CD133 in patients with PAD compared to healthy controls and no differences in the number of cells positive for CD34 and KDR were. According to the opinion of this study's authors, these results add strength to the interpretation that different results obtained by other authors may be related to methodological differences. If the data on EPCs are collected by FACS analysis, it is shown that patients with PAD had a significant reduction in these cells with respect to healthy. It seems evident that the diverse methods used can quantify different cells, and it is possible that the cells measured by FACS do not have a single destiny and that only a small fraction of these cells give rise to the cells quantified by the uptake of LDL, lectin, or CFUs. It is worthwhile noting that these latter demonstrate phenotypic characteristics of endothelial cells, including a typical receptor pattern, classic morphological features such as a cobblestone aspect, and angiogenic capacity. It was hypothesised that in patients with PAD there is an increased number of circulating cells that may give rise to endothelial cells and are capable of angiogenic activity. No relation between the severity of disease and the number of EPCs or CFU was found. Moreover, dividing the patients into subgroups based on severity of PAD did not

demonstrated significant differences with either the number of EPCs or CFU. Concerning the possible influence of drugs on the results, because hydroxymethylglutaryl–coenzyme A (HMG-CoA) reductase inhibitors are known to increase EPC number and function, it is reasonable to hypothesise that this might be related to the increase in EPCs. Despite this, the authors were not able to demonstrate a statistically significant difference in patients under statin therapy compared to those not taking statins as far as number of EPCs and CFUs were concerned. According to the opinion of the authors, the increase in the number and proliferative activity of EPCs in patients with PAD may be linked to the two major functions of EPCs, namely maintaining vasculogenesis after birth and endothelial repair. It is quite evident that in the group of patients with severe PAD both functions are highly favoured. A significant increase in the plasma concentration of VEGF in patients with PAD compared to controls was also found. Alternatively, the increased levels of EPCs may reflect EPC mobilisation in response to inflammatory signals. However, the lack of correlation between hs-CRP levels and EPCs is in contrast with this hypothesis. Concluding these results, in patients with PAD both the number and proliferative activity of circulating progenitors of endotheliocytes are increased with respect to healthy controls. In contrast, circulating cells positive for CD34 and CD133 were decreased in patients with PAD, which leaves many open questions concerning the order of physiopathologic mechanisms in the disease process. Even if in PAD comorbidities can influence the results, in this group of patients about two thirds of cases did not present with associated pathologies. Therefore, it is likely that in chronic PAD an elevated number of circulating EPCs with increased proliferative capacity might play an important role in compensating endothelial damage (102).

It was examined whether plasma levels of angiogenic factors are altered in plasma of patients with PAD and whether these factors affect EPC-induced angiogenesis. The concentrations of the circulating angiogenesis-related factors VEGF, PlGF, and thrombospondin-1 (TSP-1) in PAD patients and healthy controls were compared. It was suggested that the balance between angiogenic and anti-angiogenic factors are altered in PAD and that TSP-1 is associated with a loss of EPC angiogenic potential. Plasma concentrations of TSP-1 in PAD patients were significantly higher than in controls, whereas VEGF and PlGF levels did not significantly differ between cases and controls. The authors of this study also assessed whether common PAD risk factors affected VEGF, PlGF, and TSP-1 concentration. VEGF and PlGF significantly correlated with CRP. In addition, PlGF correlated with age, hypertension, and antiplatelet therapy. TSP-1 plasmatic levels were not associated with clinical parameters. Whereas TSP-1

levels were associated with the occurrence of PAD, no significant difference in plasma levels of VEGF, PlGF, and TSP-1 according to disease severity was found. According to the authors of the study, the increased TSP-1 plasma concentrations observed may reflect an overexpression by ischaemic tissues. Also, it was found that newly formed vessels in PAD patients who received local injections of BM-MNCs were positive for TSP-1, pointing to an autocrine *in situ* effect during neoangiogenesis. The aim was to discover an angiogenic biomarker potentially detectable in PAD patient plasma and to correlate it with the neoangiogenesis process in patients treated with a cell therapy product. One explanation is that treated patients who had to receive amputations had a neoangiogenic process inefficient to allow the vessel salvage. In this case, TSP-1 expression could represent a signal to prevent neoangiogenesis. The effect of TSP-1 on the angiogenic properties of cultured ECFCs was investigated. It was found that TSP-1 had a double-edged effect on ECFCs, enhancing ECFC adhesion probably mediated by its N-terminal part, while reducing their proliferation and differentiation in pseudotubes. Adhesive properties of TSP-1 did not allow EPCs to form viable and normal vessels in patients with critical limb ischaemia who had received BM-MNCs. Newly formed vessels showed disorganised structures. The authors found that TSP-1 inhibition enhanced the ECFC angiogenic potential and that increased differentiation in pseudotubes could be attributed to SDF-1/CXCR4 pathway upregulation. In conclusion, the increased plasma levels of TSP-1 found in PAD patients might contribute to the inadequate neovascularisation observed in this setting. TSP-1 targeting *ex vivo* or *in vivo* might have the potential to modulate angiogenesis (103).

The patterns of EPC mobilisation in patients with PAD and the possible association of EPCs with inflammation and oxidative stress markers were investigated (104). EPCs assessed as CD34+ cells co-expressing CD45dim, CD133 and VEGFR2 were studied in PAD and non-PAD patients. The study patients were divided into 4 groups (PAD+CAD, PAD, CAD, non-vascular patients) to compare the EPC levels among groups. Membrane type-1 MMP (MT1-MMP) on peripheral MNCs, serum malondialdehyde-modified low-density lipoprotein (MDA-LDL) and plasma petraxin-3 were also measured. The Fontaine clinical classification and the Trans-Atlantic Inter-Society Consensus (TASC) II classification were made on the basis of clinical symptoms and the results of angiography. The authors observed the vascular events that occurred during the follow-up period and examined the possibility of a correlation between the events and EPC levels, MT1-MMP levels, pentraxin-3 levels, MDA-LDL levels and SDF-1 levels. During the follow-up period, vascular events defined as cardiovascular death

(including sudden deaths and deaths from myocardial infarction or heart failure), cardiovascular revascularisation (percutaneous coronary and peripheral intervention or bypass surgery), stent thrombosis, stent restenosis, non-healing ulcers, and amputation were counted. EPC levels were inversely related to Fontaine and TASC II classifications. EPC levels showed a significant association with the levels of pentraxin-3 and MT1-MMP. In addition, patients with vascular events had significantly lower EPCs and higher MDA-LDL. EPC decreased as the disease progressed and, in Fontaine class IV patients, were even lower than in controls. Thus, the number of EPCs indicates the status of a patient on the continuum between EPC exhaustion and endothelial damage. Worsening of cardiovascular disorders and increases in cardiovascular risks cause endothelial dysfunction and decreasing numbers of EPCs. EPCs are upregulated and mobilised sufficiently in response to vascular damage in the mild to moderate phase, and in the early stages of endothelial dysfunction, EPCs are sufficiently mobilised and supplied from BM to the damage organ. As endothelial damage progresses, EPCs are exhausted and cannot respond to and restore endothelial dysfunction. Pentraxin-3, one of the superfamily of pentraxins, which includes CRP, is highly produced in vascular endothelial and smooth muscle cells in response to atherosclerotic change; therefore, pentraxin-3 is considered to be a more specific biomarker of vascular inflammation. This study implies that endothelial damage and vascular inflammation are closely associated with the number of peripheral EPCs in clinical settings. Increased EPC and pentraxin-3 could possibly play an atheroprotective role, thus correlating with the severity of PAD; however, the study only found that EPC and pentraxin-3 were upregulated in TASC A/B patients. Both SDF-1 and VEGF attract progenitor cells and are involved in homing, migration and mobilisation from BM to peripheral circulation, contributing to vascular regeneration. SDF-1 was upregulated in the PAD group compared to non-PAD group; however, no significant difference was found between TASC A/B and C/D groups in SDF-1 levels. These results may reflect that EPCs are upregulated and mobilised sufficiently in the mild to moderate phase in response to chemokine attraction, such as SDF-1. As disease progresses, EPCs are exhausted or no longer respond to homing signals. MMP plays a key role in EPC mobilisation and angiogenesis. MT1-MMP, a transmembrane type protease, has been recognised as an important regulator in EPC mobilisation from BM and angiogenesis. MT1-MMP cleaves CD44, a cells adhesion molecule, and reduces BM stromal cells and progenitor cell interaction. The data from this study showed that MT1-MMP expression was high in conditions of decreased and exhausted numbers of EPCs being mobilised from the BM. In conditions where sufficient EPCs were preserved in the PB pool, MT1-MMP expression was downregulated. MT1-MMP activity occurs not only in BM, but is

also involved in pericellular proteolysis at the site of angiogenesis. Thus, MT1-MMP activity may reflect activation in both BM and areas of angiogenesis, which leads to the mobilisation and recruitment of EPCs. This study contributes to elucidation of the mechanism regulating EPC mobilisation and homing. Collectively, the results of the study suggested that EPC mobilisation occurs in PAD and shows a biphasic response, with elevated EPC in the moderate phase and reduced EPC in the advanced phase. EPC levels are associated with the levels of novel circulating biomarkers and several aspects of PAD, including the severity, progression, and outcome of this disease (104).

The role of acute ischaemia on EPC (CD34+KDR+ and CD133+KDR+) mobilisation was assessed in patients with peripheral arterial occlusive disease and in healthy volunteers. The number of circulating EPCs was analysed in peripheral arterial occlusive disease patients, with exercise induced limb ischaemia for up to 72 h after a maximal treadmill test, and in healthy volunteers, who underwent a 15-min supra-systolic occlusion of one lower extremity to induce limb ischaemia. In this clinical trial, the interaction between externally induced ischaemia and presence/absence of peripheral vascular disease on the increase of EPCs was assessed. It was found that, related to healthy participants, the vasculogenic potential in patients with peripheral arterial occlusive disease is reduced as indicated by a reduced baseline EPC concentration and an impaired migratory capacity of cultured MNCs. Besides, both in peripheral arterial occlusive disease patients and in healthy participants, a single episode of peripheral limb ischaemia leads to a significant increase of peripheral EPC numbers, which is preceded by an increase in VEGF levels. The relative increase in response to the ischemic stimulus found in healthy participants seems to be reduced in patients with peripheral arterial occlusive disease. These observations imply that a single episode of reversible artificial or exercise-induced tissue ischaemia is sufficient to increase the plasma level of VEGF and the amount of circulating EPCs. This finding is consistent with the hypothesis that a single ischemic stimulus may trigger the release of EPCs from the BM in the PB. As a clinical implication, the authors have confirmed that a vigorous 4-week exercise training above the ischemic threshold, a well-established therapeutic concept in peripheral arterial occlusive disease patients, leads to a four-fold increase in circulating EPCs, thereby possibly enhancing neovascularisation, endothelial repair, and alleviation of symptoms. Nevertheless, based on investigations showing that the quality and quantity of mobilised EPCs are impaired it is tempting to assume that the increase of circulating EPCs by a single ischemic bout will be insufficient to improve the symptoms of cardiovascular diseases. A possible approach to enhance circulating EPCs in

peripheral arterial occlusive disease patients in advance to such procedures might be the use of a single maximal exercise bout to optimise quantity of EPCs. Concluding the results of the authors, this study shows that a short episode of tissue ischaemia is sufficient to induce a significant increase in the number of circulating EPCs in both patients with peripheral arterial occlusive disease and in healthy participants. The reduced response among peripheral arterial occlusive disease patients underlines their impaired regenerative capacity and illustrates that EPCs are a potential target for non-pharmacologic interventions such as exercise training (105).

6.9. Vasculopathy

Circulating EPCs were quantified in cardiac transplantation subjects with established coronary arteriopathy with and without angiographic evidence of vasculopathy. The number of EPCs in cardiac transplantation subjects with established coronary arteriopathy were compared with matched transplantation subjects without evidence of vascular disease. There was a significant reduction in the number of endothelial outgrowth colony-forming units (EPC CFU) in subjects with disease compared with subjects without angiographic evidence of disease. Human circulating EPCs were significantly decreased in subjects with transplant atherosclerosis compared with matched transplantation subjects without evidence of disease. It was also shown that recipient endothelial cells were significantly recruited to the lumen of epicardial vessels and adventitial microvessels of coronary artery segments after cardiac transplantation. According to this study, there are several potential mechanisms to explain the different levels of circulating EPCs seen in the 2 study groups. It is possible that EPCs or their putative progenitors may have been recruited into the plaque during development of transplant atherosclerosis. It is unknown why EPCs might be reduced in primary atherosclerosis or transplant atherosclerosis, but possibilities include ongoing recruitment of EPCs to sites of endothelial injury or dysfunction in the transplanted heart. The authors provided circumstantial evidence for but not proof of depletion of a circulating pool of EPCs (decreased colonies in transplant arteriopathy patients) with seeding of (most likely) circulating recipient-derived endothelial cells to areas of vascular injury (recipient CD31-positive cells in areas of transplant vasculopathy). According to the study, an alternative explanation for diminished EPCs may involve molecules released (in the context of transplant arteriopathy or chronic low-grade rejection) that alter mobilisation, migration, cell survival, or intravascular seeding functions of EPCs after transplantation. The presented findings extend to transplant

atherosclerosis a paradigm of abnormal EPC regulation in the presence of vascular disease (106).

Cardiac allograft vasculopathy is the leading cause of morbidity and mortality in heart transplantation. The relationship between circulating EPCs (CD34+KDR+; CD133+KDR+ and CD34+CD133+KDR+ cells were considered as putative EPC phenotypes) and their incorporation into allografts and coronary microvascular function in heart transplantation patients was assessed. It was demonstrated that human EPCs in the circulation and in the graft are significantly decreased in heart transplant recipients with normal angiogram and microvasculopathy, defined as a severe impairment of coronary flow reserve. It was also showed that EPCs are reduced in heart transplantation patients compared with healthy individuals matched for age and gender. The reduction of circulating EPCs in heart transplantation patients compared with controls can be explained by the effect of immunosuppressive therapy (the immunosuppression protocol consisted of cyclosporine, azathioprine, mycophenolate mofetil, or everolimus, and steroids). With this study, differential levels of circulating EPCs in individuals who have developed transplant microvasculopathy and a different engraftment of recipient endothelial cells into the donor microvasculature were shown. It is possible that EPCs are mobilised from BM of the recipient and engrafted into the donor coronary microcirculation during immunologic myocardial injury over time. It is conceivable that EPCs are recruited early and late. It can be speculated that EPCs may be continuously engrafted to areas of donor endothelial dysfunction in a cycle of continuous repair and in the context of ongoing alloimmune interactions. Cell mobilisation and engrafting after heart transplantation seems to preserve microvascular function. The authors' findings may be crucial in understanding the pathogenesis of allograft vasculopathy and in establishing new strategies for therapeutic intervention. If you could learn to control chimerism, it might be possible to delay or prevent this disease, which is the most common cause for failure of transplanted hearts (107).

6.10. Bicuspid aortic valve degeneration

In a study assessing level and function of EPCs in patients with bicuspid aortic valve associated with significant valve dysfunction, it was found that, compared to a control group of patients with bicuspid aortic valve and normal valve function, patients with at least moderate aortic regurgitation and/or stenosis had impaired functional properties of EPCs after 7 days of culture. Specifically, EPCs from patients with dysfunctional valves had decreased capacity to

form colonies and to migrate compared to patients with normal functioning valves. In addition, patients with dysfunctional bicuspid aortic valves tended to have a lower level of circulating EPCs (CD34+VEGFR2+ and CD133+VEGFR2+). This study demonstrated an association between decreased EPC function and bicuspid aortic valve dysfunction. However, it did not established any causality (30).

6.11. Refractory hypertension

It was investigated if the number and function of circulating EPCs (CD34+CD133+CD45+) are reduced in patients with refractory hypertension. Circulating EPCs were isolated from patients with refractory hypertension and normotensive controls. EPC number was also determined *in vitro* after 7 days in culture, defined as double-stained cells for both lectin and Dil-acLDL. It was shown that the concentration of circulating EPCs is significantly reduced in patients with refractory hypertension as compared to healthy subjects. It was also suggested that refractory hypertension is as an independent determinant of lower number of circulating and after culture EPCs. Moreover, the increase in EPCs after *in vitro* culture was also lower in refractory hypertension patients, suggesting a functional impairment. A relationship between impaired renal function and reduced levels of EPCs was also demonstrated. It could be speculated that this is the reason for differences in circulating EPC number between both groups. However, both factors independently predict decreased EPC number. Given these findings, one may speculate that the continuous detrimental effects of cardiovascular risk factors on circulating EPC number and function would result in an impairment of the endothelial monolayer and its regenerative capacity, leading to atherosclerotic diseases and its associated cardiovascular consequences. Refractory hypertension becomes definitively added to the list of cardiovascular risk factors that are known to negatively influence EPCs. Several underlying mechanisms could account for hypertension-induced downregulation of EPCs, such as an enhanced consumption of EPCs due to an increase in endothelial damage, an impairment in the mobilisation process from BM into PB or an increase in EPC apoptosis and/or impaired proliferation after culture, but these mechanisms were not explored. On the other hand, the authors cannot deduce from this study how secondary causes of hypertension influence EPCs. Moreover, the findings of this study apply to refractory hypertension. So, these results cannot be extended to all hypertensive patients. In summary, the number of circulating EPCs after culture is reduced in patients with refractory hypertension, which may be related to the increased rate of endothelial dysfunction, atherosclerotic disease and cardiovascular events observed in the studied population (108).

EPCs and complement C3 (the third component of complement) are involved in the pathophysiology of arterial hypertension. C3 a-fragment (C3a) is a negative regulator of progenitor cells egress during their mobilisation from BM. It was hypothesised that resistant arterial hypertension would be associated with complement activation and reduced number of circulating EPCs (CD34+CD133+KDR+). Plasma complement C3a/C3b levels were measured and their correlation with circulating EPC was evaluated in three groups of patients, resistant arterial hypertension group, controlled arterial hypertension group and control group (healthy subjects). The authors found a significant difference in blood complement C3a and C3b levels between resistant arterial hypertension subjects and normotensive subjects but not between controlled arterial hypertension and normotensive subjects. There was a significant positive correlation between activated C3 complement components and systolic blood pressure and negative correlation with circulating EPCs. These results suggest that complement activation may have some association with blood pressure regulation and endothelial regeneration. However, the precise mechanisms of these relationships are currently unknown. The authors cannot claim whether a higher systolic pressure activates C3 in resistant arterial hypertension or higher C3a/C3b contribute to blood pressure increases in the pathogenesis of resistant arterial hypertension. Another intriguing finding of the study is a negative correlation between circulating C3a and EPCs. Innate immunity has a pivotal involvement of in the mobilisation of hematopoietic stem/progenitor cells. C3a, by enhancing responsiveness of these cells to decreasing concentrations of SDF-1 in BM, prevents mobilisation and promotes their BM retention. Because resistant arterial hypertension represents an extreme phenotype of a multifaceted and complex disease, it seems reasonable that more than one, perhaps a significant number of factors, inflammatory and non-inflammatory factors may play a role in the pathogenesis of this syndrome (109).

6.12. Cardiopulmonary bypass

During cardiopulmonary bypass the endothelium is the first organ to be affected by mechanical and immunologic stimuli. A group of investigators assessed how high-risk coronary artery bypass grafting surgery patients, who are usually the targets of cell therapy strategies, respond to cardiopulmonary bypass-induced inflammatory mobilisation of intrinsic EPCs. The cardiovascular morbidity profile in the study population was quantified using the European System for Cardiac Operative Risk Evaluation (Euro-SCORE). Patients were divided in two groups, low risk and high risk, based on EuroSCORE. Pre-operatively the number of circulating

EPCs (CD34+CD133+VEGFR2+) was reduced in the high risk group with respect to the low risk group. There was a significant increase of EPCs at 1 hour after cardiopulmonary bypass in both groups, with almost complete normalisation 24 hour later. However, the high risk group showed a significantly lower number of EPC after 1 hour post-operatively compared with those with a low surgical risk. These data clearly demonstrated a significantly impaired endogenous mobilisation capacity of patients belonging to the high risk group. The pre-operative EuroSCORE correlated closely and inversely with the EPC count at 1 hour after cardiopulmonary bypass. It was found that the number of EPC mobilised into the circulation depends on the duration of cardiopulmonary bypass, but is indeed strongly influenced by the patients' pre-operative risk profile. The finding that the number of detectable circulating EPC is reduced in high-risk patients, especially those with impaired LV function, is in line with the hypothesis that cardiovascular disease and blood-borne progenitor cell function are interrelated. Even a strong traumatic stimulus such as cardiopulmonary bypass cannot completely overcome the impairment of EPC mobilisation in high-risk patients with advanced cardiovascular disease. Regarding the correlation between cytokines and EPC in post-cardiopulmonary bypass patients, it is interesting to note that not the absolute plasma concentration level but the magnitude of cytokine increase (G-CSF or VEGF) seemed to be responsible for the amount of EPC mobilisation early after surgery. Concluding the results of the authors, cardiovascular risk factors influence the mobilisation of EPC from the BM after stimulation by cardiopulmonary bypass. This could be secondary to impaired mobilisation or the result of increased EPC turnover, and may have implications for future cell therapy strategies in cardiac surgical patients (110).

6.13. PR prolongation

Epidemiological studies showed that PR prolongation (first-degree atrioventricular block, or first-degree heart block, is defined as prolongation of the PR interval on an electrocardiogram) is associated with increased risk of adverse cardiovascular outcomes. The relations of PR interval with indices of vascular function and endothelial repair, as the underlying mechanisms, were investigated. The study comprised high-risk patients with prior CAD, ischemic stroke, and/or diabetes and healthy subjects without such a history. PR interval was considered prolonged if >200 milliseconds (ms), as determined from resting 12-lead electrocardiogram. Vascular function was assessed by brachial FMD and circulating EPCs (CD133+/KDR+) were also determined. It was demonstrated that circulating EPC levels were significantly increased in patients with PR prolongation >200 ms. Adjusted for potential confounders, increased PR

interval remained independently associated with increased EPCs. Also, it was found that among high-risk patients with cardiovascular disease or equivalent risk, endothelial dysfunction occurred more frequently in patients with PR interval prolongation than those without. Furthermore, after controlling for confounding variables, PR interval remained predictive of endothelial dysfunction. It was shown that there is heightened vascular repair in patients with cardiovascular disease with PR interval prolongation versus those without, suggesting that PR interval prolongation further identifies a group with high risk for cardiovascular events over and above traditional risk factors for cardiovascular disease. Therefore, it was suggested that PR interval prolongation may be a risk marker for the continuum of endothelial dysfunction, both in healthy subjects and in those with established cardiovascular disease, and is a marker for vascular damage and repair. The raised circulating EPCs in patients with PR interval prolongation likely reflects the subclinical vascular injury sustained and the corresponding activation of repair mechanisms. Summarily, the authors conclude that PR interval prolongation in patients with cardiovascular disease or equivalent risk is associated with worsening endothelial function and raised circulating EPCs indicating activation of vascular repair mechanisms. These findings lend support to the adverse cardiovascular effects associated with PR interval prolongation (111).

6.14. Idiopathic dilated cardiomyopathy

Idiopathic dilated cardiomyopathy is a cardiac disease characterised by depressed contractility and increased ventricular chamber size in the absence of atherosclerotic CAD, valvular abnormalities or pericardial disease. Common clinical manifestations are progressive heart failure and increased risk of sudden cardiac death. It was also suggested that impaired vascularisation may be a characteristic in idiopathic dilated cardiomyopathy. It was shown that, even though patients with idiopathic dilated cardiomyopathy had abundant mobilisation of circulating EPCs (CD133+VEGFR2+ and EPC CFUs), their hearts show impaired vascularisation. It was also found that this defective vascularisation was associated with reduced expression of β-catenin (an important angiogenic regulator, which directly enhances the survival, proliferation and migration of mature endothelial cells) in myocardial vessels. Collectively, these data indicate that both vasculogenesis (the de novo vascular organisation of mobilised endothelial progenitors) and angiogenesis (by which new blood vessels are formed from pre-existing mature endothelial cells) are altered in human idiopathic dilated cardiomyopathy. The vasculogenesis switch is on in idiopathic dilated cardiomyopathy, but EPCs appear unable to restore myocardial revascularisation, likely due to multifactorial

reasons. Based on these results, cardiac revascularisation in idiopathic dilated cardiomyopathy may not be a matter of additional exogenous delivery of progenitor cells, but rather to promote a conducive microenvironment for homing and functional incorporation of the mobilised EPCs (25).

6.15. Pulmonary arterial hypertension

Impaired endothelial homeostasis underlies the pathophysiology of pulmonary arterial hypertension. It was speculated that pulmonary arterial hypertension patients are deficient in circulating EPCs in 2 well-characterised forms of the disease, potentially contributing to endothelial dysfunction and disease progression. It was found that the number of circulating EPCs (CD34+/KDR+ or CD34+/CD133+/KDR+) was markedly reduced in patients with pulmonary arterial hypertension. Elevated levels of inflammatory mediators such as TNF-α and interleukin 6 (IL-6), indexes of NO synthesis, and ADMA were found in pulmonary arterial hypertension patients and correlated directly with EPC numbers. TNF-α and IL-6 have a negative effect on the number and function of EPCs. It also found that plasma CRP was elevated and, together with the inflammatory cytokines, was negatively associated with the number of circulating EPCs. Summarily, circulating EPCs are reduced in patients with pulmonary arterial hypertension. It was found that circulating EPC numbers correlated with levels of inflammatory mediators, indexes of NO synthesis, and ADMA production (27).

6.16. Cardiopulmonary resuscitation

Ischaemia and reperfusion after cardiopulmonary resuscitation (CPR) induce endothelial activation and systemic inflammatory response, resulting in post-resuscitation disease. It was hypothesised that endothelial injury takes place during and after CPR, which in turn may contribute to post-resuscitation disease. The authors of this study detect direct markers of endothelial damage such as circulating endothelial cells (CECs, defined here as CD146+CD45- cells) and EMPs, as well as markers of endothelial repair (EPCs, defined as CD34+CD133+VEGFR2+ cells) in PB of patients after successful CPR. In all resuscitated patients, overall duration of mechanical resuscitation varied from 5 to 120 minutes. As more of the CPR patients in the EPC study population presented ventricular fibrillation or ventricular tachycardia as the initial rhythm compared with patients in the CECs and EMPs study, duration of CPR in the EPC study group was shorter, and outcome was better. Patients in the EPC study were showing higher rates of out-of-hospital cardiac arrests, and a lower incidence of

acute renal failure compared with resuscitated patients in the CECs and EMPs study. To assess vascular repair following endothelial injury in patients after CPR, circulating EPCs were measured in PB of resuscitated patients in a follow-up study. Percentage of circulating EPCs in resuscitated patients were significantly higher than in control patients. The results indicated early endothelial damage and ongoing endothelial dysfunction detected by elevated CECs and EMPs in resuscitated patients compared with control groups. Furthermore, numbers of EPCs increased on the second day after return to spontaneous circulation, which points to an early initiation of endothelial regeneration. The direct comparison with patients with stable CAD undergoing coronary intervention excluded effects possibly caused by CAD or coronary intervention (112). Catecholamines are known to induce angiogenesis in tumour tissues (113) and dopamine has been shown to mobilise EPCs from the BM during tumour growth (114). In the EPC study group, all CPR patients received vasopressors such as norepinephrine and epinephrine but none of them received dopamine. Concluding the results of the authors, this study provided evidence for an endothelial injury occurring in patients after CPR. The obtained data suggest a two-step process: the early stage during and directly after CPR is prevailed by severe endothelial damage. Within the following 24 hours, inflammation and endothelial repair are taking place (112).

6.17. Cerebrovascular disease

Circulating immature cell populations, especially CD34+ and CD133+ cells, are associated with maintenance and repair of the cerebral vasculature. It was suggested that the level of CD34+ cells serves as a marker for cerebrovascular function. Analysis of CD133+, CD117+, and CD135+ cells, which identify other populations of immature cells, demonstrated that only CD133+ cells correlated with cerebrovascular function in a manner paralleling CD34+ cells. Given the fact that EPCs may be a subpopulation of CD34+ and/or CD133+ cells, as described before, also progenitor cells of endothelial lineage may be involved in maintenance and repair of the cerebral vasculature (115).

6.17.1. Stroke

Patients with ischaemic stroke were prospectively included in a study to evaluate the prognostic value of EPCs (EPC colonies, CFU-EC) within 12 hours of symptoms onset. Remarkably, the EPC increase during the first week was associated with good outcome at 3 months. This favourable effect was supported by positive effects on the reduction of infarct

growth and neurological improvement at day 7 and 90. Therefore, it was demonstrated that circulating EPCs increase in response to cerebral ischaemia in patients after acute ischaemic stroke, and that the magnitude of this increase is directly related to a better functional outcome. According to the authors of this study, the fact that patients with good outcome showed higher CFU-EC number on day 7 and at 3 months, but not at admission, support the hypothesis that EPC can mediate processes of chronic vessel repair and neurorepair. In conclusion, a higher increase in circulating EPC during the first week after cerebral ischaemia is independently associated with a better clinical outcome in acute ischaemic stroke patients (116).

In another study, the number of CFUs, representing EPCs, were evaluated in patients with acute stroke. CFU numbers were counted after culturing them for 7 days, and outgrowth cell appearance was measured during the 2 months of culture. It was found that patients with acute stroke had lower CFUs than healthy subjects. The authors of the study suggested that CFU analysis provides an additional means of understanding stroke pathophysiology. The EPC dysfunction observed prominently in large artery atherosclerosis subgroup of patients might induce endothelial cell dysfunction and, thus, the progression of vascular disease. Besides, it was suggested that a high prevalence of vascular risk factors in patients with acute stroke might lead to further reduction on CFU levels. EPCs of patients with stroke were impaired in a paracrine function in *in vitro* angiogenesis assays. Therefore, it was proposed that stroke is not only associated with the absolute number of CFUs, but also significantly with their paracrine functions, and that this is modulated in a stroke mechanism-dependent manner. An age-dependent change in the CFU levels was found in the patients with acute stroke, resulting in much lower CFU levels in aged patients. Furthermore, indices of neurological damage were found to be significantly associated with outgrowth cell appearance. According to the authors, it is possible that immediate endothelial or neural damage by stroke might induce a compensatory overproduction of progenitor cells from BM for damage repair. However, it remains to be seen whether low CFU levels can predict stroke. On the other hand, the high output of outgrowth cells observed in acute stroke during the study suggests that circulating EPCs may provide a means of endogenous repair to counteract the effects of acute tissue injury and to replace dysfunctional or damaged endothelium. The isolation and expansion of EPCs might be particularly useful for identifying therapeutic approaches that modify the progression and recurrence of stroke (76).

In the studies described above, CFUs were used to quantify EPCs in stroke patients. Here, a study identifying EPCs also by flow cytometry is mentioned. Circulating levels of EPCs

(CD34+CD133KDR+CD45+ and cell culture assays) were quantified in subacute and acute stroke patients and in healthy controls, and the potential of EPCs to induce vasculogenesis *in vitro* was evaluated. It was shown that EPCs are mobilised in the PB during the acute phase of stroke (<24 hours). However, EPCs obtained in a delayed stroke phase (3 to 7 days) might display enhanced endothelial differentiation and greater capacity to induce vasculogenesis. Discrepancies were found in the number and differentiation capacity between the EPCs from stroke patients and those from control subjects, and in functioning between the EPCs from the acute stroke and delayed stroke patients. A significant difference found in levels of a specific EPC population was that the acute stroke group had higher levels of CD34+CD133+KDR+CD45+ EPCs as compared to the control subjects, which probably reflects a response to the ischaemic event. Supporting this data, stroke severity was positively associated to CD34+/CD133+ levels and to the appearance of outgrowth EPCs. A higher number of early EPCs was found in the cell cultures from the subacute group than in those from the control group. Moreover, endothelial-like outgrowth EPCs appeared only in the cultures from the stroke patients. In this sense, it was suggested that EPCs truly capable of inducing vasculogenesis might be more efficiently isolated from stroke patients than from controls. The results demonstrated that outgrowth EPCs from ischaemic stroke patients can perfectly form capillary-like structures *in vitro*. Furthermore, greater tubulogenic capacity was found in cells obtained from subacute stroke patients, suggesting that after stroke, EPC functioning may vary with the time following the ischaemic event. The data also indicate that ischaemic cells can secrete growth factors known to be crucial for angiogenesis such as VEGF. Clear inter-individual differences were found, which make difficult to conclude how stroke time-course modulates the growth factor secretion. Summarily, it was suggested that higher circulating levels of EPCs do not correlate to better endothelial function *in vitro*. Moreover, the data indicate that the ischaemic insult may modulate the vasculogenic capacity of EPCs, such that those obtained in the sub-acute phase of stroke would have the greatest potential. The acute increase of EPC counts in the blood stream might be explained by a rapid release from the BM activated by the ischaemic event and the inflammatory response, whereas a later decrease but enhanced functionality of EPCs could be explained by the activation of endogenous angiogenesis and be an advantage for neurorepair (117).

6.17.2. Intracranial atherosclerotic disease

Intracranial atherosclerotic disease is an important cause of ischaemic stroke and endothelial dysfunction plays a critical role in its onset and progression. Therefore, the association of EPCs

(CD34+KDR+) and angiogenic growth factors with intracranial atherosclerotic disease severity was investigated in patients who had experienced a transient ischaemic attack or ischaemic stroke attributable to symptomatic intracranial atherosclerotic disease. Clinical evaluations were conducted between 2.4 and 8.7 years after the initial cerebrovascular event. Severe intracranial atherosclerotic disease was defined as the presence of at least 1 severe intracranial stenosis, and extensive intracranial atherosclerotic disease as 3 or more intracranial stenoses. It was shown that patients with symptomatic intracranial atherosclerotic disease have modestly increased levels of circulating EPC, VEGF and fibroblast growth factor depending on intracranial atherosclerotic disease severity, and have a lower concentration of fibroblast growth factor, VEGF and platelet-derived growth factor, than controls. However, no correlation between the studied angiogenic growth factors and circulating EPC levels was found. In this study, the authors found no difference between intracranial atherosclerotic disease patients and healthy controls regarding circulating EPCs. In addition to the presence of intracranial atherosclerotic disease, several other significant factors could have led to this result, such as age, sex and drug treatments. However, no difference in EPC circulating levels was found between patients with or without treatment with statins, antiplatelets or other drugs. Regarding sex, the number of women in the control cohort was significantly higher than in the intracranial atherosclerotic disease group. Although oestrogens have been described as modulators of EPC levels and function, this boosting effect was probably minimised in female subjects, because only women without hormonal treatment at postmenopausal age were included. Interestingly, it was found a moderate increase in the EPC count, but no increase in CD34+ cells (a less specialised population of bone-marrow-derived cells) in patients with a higher number of intracranial stenoses. Although cerebral perfusion was not assessed in the patients, these results may reveal a balance between chronic ischemic status, which induces EPC mobilisation, and atherosclerotic disease, which is associated with a lower EPC count. The results suggest that EPC mobilisation may be partially preserved in chronic intracranial atherosclerotic disease in association with a higher number of intracranial stenoses. It was observed that indicators of a more severe cerebral ischemic stimulus, such as the presence of severe intracranial stenosis, were associated with higher levels of fibroblast growth factor, VEGF and platelet-derived growth factor (although this latter result was not significant). Conversely, lower levels of VEGF, fibroblast growth factors and platelet-derived growth factor were found in intracranial atherosclerotic disease patients than in healthy controls. This appears to indicate that the angiogenic response to ischaemia is partially preserved in intracranial atherosclerotic disease patients, but that a paracrine dysfunction may occur in the

atherosclerotic tissue or in EPCs themselves, leading them to produce lower amounts of angiogenic growth factors than in healthy subjects, which may ultimately cause reduced EPC migration and endothelial repair. The results also indicate a lack of correlation between circulating EPCs and angiogenic growth factors in the patients, whereas a positive correlation was found between fibroblast growth factor and EPCs in healthy controls. This could be due to the intrinsic dysfunctional response of EPCs to angiogenic growth factors in the context of atherosclerotic disease and/or angiogenic growth factors production from other sources in the vessel. Summarily, angiogenic growth factor levels are lower in symptomatic intracranial atherosclerotic disease patients than in controls, but the number of circulating EPCs is similar. In this context, EPCs and/or atherosclerotic vessels (as the principal sources of these angiogenic growth factors) seem to retain dysfunctional characteristics in intracranial atherosclerotic disease patients through a reduced capacity for proangiogenic factor production. Furthermore, patients presenting with severe forms of intracranial atherosclerotic disease in terms of the number or severity of intracranial stenoses presented higher circulating levels of EPCs and angiogenic growth factors but not of CD34+ cells, suggesting that at some levels of chronic hypoperfusion, more EPCs could be mobilised from the BM and could stimulate angiogenic growth factors production sources. These findings support the hypothesis that angiogenic growth factors play a role in the pathogenesis of intracranial atherosclerotic disease and indicate that EPC or angiogenic growth factor replacement could constitute a therapeutic approach for intracranial atherosclerotic disease in the future (118).

6.17.3. Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy

Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is a hereditary disease due to cerebral microangiopathy presenting with variable pictures, including stroke, progressive cognitive impairment, and disability. Since endothelial dysfunction may be involved, the role of EPCs (CD34+KDR+, CD133+KDR+, and CD34+CD133+KDR+) was evaluated in CADASIL. The finding that patients with CADASIL have a reduced number of circulating EPCs is consistent to the presence of endothelial dysfunction in CADASIL. However, according to the authors of the study, even if the findings support the involvement of the endothelium in the pathogenesis of the disease, differently from the experimental setting, *in vivo* studies do not allow to establish whether this is associated with a failure of angiogenesis. Moreover, it is not possible to determine whether

the endothelium is damaged and cells are not being replaced or there might be endothelium degeneration not compensated by increase in EPCs. This study may have potential confounders, both those involved in the relation between cell numbers and the disease (for example, age, sex, or drugs) and those influencing the severity of phenotype (for example, hypertension and smoking). According to the authors, they documented, for the first time, an association between EPCs and CADASIL. These data corroborate the hypothesis that endothelial dysfunction in CADASIL plays an active role and may contribute to its phenotypic expression (119).

6.17.4. Age-related white matter changes

Age-related white matter changes (ARWMC) are associated with both an increased risk of stroke and cognitive impairment. ARWMC generally correlate with vascular disease pathology, particularly in the elderly. Given that endothelial dysfunction plays a role in the development of ARWMC, the hypothesis that levels of EPC (EPC colonies) are associated with severity of ARWMC on computerised tomography imaging of the brain was tested. EPCs were found to be significantly lower in patients with severe ARWMC, independent of measured vascular risk factors. Severe ARWMC was also identified to be associated with age, hypertension, and hypertriglyceridemia. Low levels of EPC in persons with severe ARWMC are likely a manifestation of vascular risk factors, particularly age and hypertension. However, an independent relationship between EPC and ARWMC was found after adjustment for vascular risk factors and after repeat analysis excluding subjects with EPC measured within 15 days of stroke or transient ischaemic attack onset. According to the authors of the study, EPCs may reflect vascular risk that was not accounted for. A possible explanation of the independent association of EPC with severe ARWMC is that EPC themselves play a role in the development of ARWMC independent of other vascular risk factors. Patients with low levels of EPC may represent a selected group of subjects more predisposed to endothelial damage and hence the development of severe ARWMC. It was suggested that white matter damage may limit neuroplasticity and thus recovery after ischaemic stroke. EPC levels may be a reflection of ARWMC severity and possibly cumulative vascular risk. EPC levels may also be a reflection of a patient's ability to repair damaged endothelium. Summarily, EPCs were found to be significantly lower in patients with severe ARWMC. Other variables identified to be significant independent predictors of severe ARWMC were age, hypertension, and hypertriglyceridemia. However, the authors argued that this hypothesis-generating study warrants further

investigation to clarify the relationship between ARWMC, EPC, and vascular risk factors, particularly age and hypertension (28).

7. EPCs, diabetes and cardiovascular disease

Reduced numbers of colony-forming units of endothelial cells, in order to determine the functional capacity of circulating EPCs, were associated with diabetes (19).

7.1. Diabetes and stroke

In a study, whose primary objective was the validation of the significance of CFU number and outgrowth cell in acute stroke, the populations studied (patients with acute stroke, patients with chronic stroke, and age-matched healthy volunteers) were compared in relation to cardiovascular risk factors, particularly with respect to diabetes. The results of the study confirmed that diabetes impairs EPC function. This effect of diabetes on EPCs may be mediated by an increased consumption of EPCs due to their anchoring to diffusely damaged arteries or to a more central impact on BM. An alternative explanation for the observed association is that the higher HbA1c levels, characteristic of DM, are associated with lower CFU levels. In particular, HbA1c level was found to be an independent predictor of a low CFU number, which suggests that degree of glycaemic dysregulation directly affects EPC function. On the other hand, CFU levels were found to be related to DM but not to hypertension, dyslipidaemia, or smoking (76).

7.2. Vascular endothelial function in type 2 diabetic patients

The relationship between vascular endothelial function and circulating EPC (CD34+VEGFR2+CD45low) number in type 2 DM (T2DM) was investigated. Based on the changes of flow-mediated dilation (FMD, a method to assess endothelial function), it was confirmed that endothelial dysfunction was evidently impaired in newly diagnosed T2DM and metformin could markedly improve the endothelial dysfunction. The number of circulating EPC in newly diagnosed diabetic patients was significantly lower than that in healthy subjects, which was consistent with the impaired endothelial function measured by FMD. As circulating EPCs have an important role in vascular endothelial homeostasis, the result observed in the study indicated that the decrease in the circulating EPC number might contribute to endothelial dysfunction which had taken place in the early process of T2DM. The study demonstrated that the circulating EPC number was positively correlated with FMD at baseline and this relationship remained tight after metformin treatment. In addition, the close relationship between circulating EPC number and FMD demonstrated the diagnostic value of

EPCs in vascular diseases. The exact mechanism on the close relationship between EPC number and FMD in T2DM remains unclear. However, there are possible explanations. On one hand, the resulting effect of T2DM on the reduction of the circulating EPC number may be that it decreases EPCs mobilisation from BM by inhibiting nitric oxide (NO) bioavailability through a phenomenon called eNOS uncoupling. Meanwhile, FMD provides a technique to assess the integrity of the shear stress-mediated pathway of NO production. A reduction in FMD represents the reduced NO bioavailability. Therefore, it may be assumed that the decreased number of circulating EPC in T2DM implies the reduction of NO bioavailability and thus may reflect endothelial dysfunction. On the other hand, a significant reduction in the number of circulating EPCs would be expected to result in a reduced capacity for endothelial repair, consequently contributing to the unbalance of vascular homeostasis and finally leading to endothelial dysfunction. Considering the close correlation with FMD in diabetes and its physiologic function, the circulating EPC number may be proposed as a biomarker of diabetic vascular endothelial function. Furthermore, alterations in the circulating EPC number may also have an important role in the development and progression of endothelial dysfunction in diabetes. In addition, as it can be detected relatively easily and safely, measurement of the circulating EPC number may be applied as a valuable clinical test to identify T2DM with impaired endothelial function. Besides, as the number of circulating EPCs is decreased in T2DM, elevation of the circulating EPC number could be considered as a target of therapeutic interventions for diabetic vascular protection. It was shown that metformin treatment could increase the number of circulating EPCs, which represented a potential mechanism in metformin's known vasculoprotective effects. Concluding, the results of the authors demonstrated that the circulating EPC number was associated with vascular endothelial function and may be a surrogate biological marker of vascular endothelial function for T2DM. Measurement of the circulating EPC number may be applied as a valuable clinical test to identify T2DM with impaired endothelial function. The alteration of circulating EPC numbers can also be regarded as a therapeutic target for diabetic vascular protection (120).

7.3. Peripheral vascular disease

Since peripheral vascular disease is a common and severe complication of DM, it was investigated whether a reduction in EPCs (CD34+KDR+) has a putative role in peripheral vascular disease of type 2 diabetic patients. Circulating progenitor cells (CPCs), defined as CD34+ cells, were also quantified. It was shown that type 2 diabetic patients have a 40% mean reduction in PB EPC numbers. Besides, there was a significant correlation between plasma

glucose at the time of blood collection and absolute EPC numbers, as well as the EPC/CPC percent ratio (endothelial fraction of all CPCs). This finding indicates that metabolic control could influence the EPC count. Therefore, it was suggested that a lower EPC/CPC ratio may reflect a shortened peripheral survival of EPCs rather than a weak BM mobilisation, which should also involve CPCs. According to this hypothesis, preliminary data from the patients of this study confirm that rapid metabolic compensation is followed by an increase in the EPC number and EPC/CPC ratio. It was also suggested that DM is the most relevant risk factor associated with EPC reduction and that low circulating EPCs could account for both endothelial dysfunction and poor collateralisation typical of diabetics, although functional vascular studies were not performed in the patients. However, the finding of a lower EPC count in patients with metabolic syndrome (described in this document ahead) as compared with those without, together with the negative correlation between the number of risk factors and progenitor cell counts, underscores the importance of risk factor clustering in determining the reduced EPC blood content. A profound reduction in circulating EPCs was reported in patients with peripheral vascular disease. In the patients of this study, CAD was characterised by a mild and non-significant reduction in EPC levels. The ankle-brachial index was used to diagnose lower extremity vascular disease. The strong correlation between progenitor cell levels and the ankle-brachial index suggests that the reduction in vascular progenitor cells is related to the severity of peripheral vascular disease, as well as to global cardiovascular risk in both diabetic and non-diabetic patients with peripheral vascular disease. Among all patients selected for the presence of peripheral vascular disease, those with diabetes had a lower number of EPCs, and those with foot lesions complicating end-stage obstructive vascular disease had the lowest values of both CPCs and EPCs. Although the study was cross-sectional and does not establish cause-effect relationships, these data suggest that an EPC reduction may have a role in the pathogenesis of peripheral vascular disease. Thus, the authors proposed a possible pathophysiologic model. DM and clustered risk factors reduce circulating EPC levels: an EPC decrease contributes to endothelial dysfunction, accelerates atherogenic processes, and leads to vascular diseases. Furthermore, EPC depletion impairs collateralisation and favours complications, such as foot lesions. Statin therapy has been associated with an EPC increase (80, 121). At the time of the study, 42% of diabetic patients with peripheral vascular disease were taking statins: the actual severity of EPC reduction in peripheral vascular disease is unmasked when only patients not treated with statins are considered. It was actually found that circulating EPC numbers and EPC/CPC ratios were higher in peripheral vascular disease patients on statin therapy than in non-treated patients. Summarily, this study reports a

reduction in circulating EPCs in T2DM and a further, progressive EPC and CPC decrease in diabetic patients with peripheral vascular disease and distal lesions, in relation to ankle-brachial index values. These data may offer a new pathophysiologic hypothesis for the high incidence of vascular damage in patients with T2DM (122).

7.4. Diabetic retinopathy

Diabetic retinopathy is characterised by pathological retinal neovascularisation and the literature suggests that high levels of circulating EPCs are an important risk factor for pathological neovascularisation. The previous study demonstrated a reduction and dysfunction of circulating EPCs in diabetic patients. It was hypothesised that EPCs are differentially altered in the various vascular complications of DM, exhibiting distinct behaviours in terms of angiogenic response to ischaemia and growth factors and potentially playing a potent role in motivating vascular precursors to induce pathological neovascularisation. Circulating levels of EPCs (CD34+CD133+) and CFU-EPCs from diabetic retinopathy patients were analysed. The authors of the study enrolled type 2 diabetic patients with diabetic retinopathy, type 2 diabetic patients with PAD, and age and sex-matched non-diabetic healthy controls. Circulating EPCs were significantly increased in diabetic retinopathy patients, as compared with non-diabetic controls. In addition, elevated EPC levels and a high concentration of circulating neurotrophic factors (NTFs) were seen in diabetic retinopathy patients, indicating that EPC, as well as NTFs levels levels in PB are impacted by the presence of diabetic retinopathy. They found a significantly increased number of CFU-EPCs in the diabetic retinopathy patient samples than in controls. Such propensity for colony formation might represent heightened differentiation efficiency of EPCs in diabetic retinopathy (29). Besides, higher circulating EPC levels and differentiation efficiency may be related to the neovascularisation in diabetic retinopathy patients (123). NTFs are reported to be potent stimuli for new vessel growth, which may explain the unusual neovascularisation in retinal vascular complications of DM. It was found that, similar to the classic angiogenic factor VEGF, NTFs serum concentrations were higher in diabetic retinopathy groups than in PAD groups and non-diabetic controls. EPC and VEGF were shown to be significantly associated with diabetic retinopathy. Therefore, according to the authors, it is tempting to speculate that signals generated by the retina in response to ischaemia may stimulate activation of BM EPCs, using systemic NTFs as messengers released into the circulation. The study provides further evidence that diabetic retinopathy may be an aberrant neovascularisation process initially driven by NTFs released from neuronal tissue to activate EPCs. The study data provide additional evidence that NTFs may contribute to

enhanced EPC migration and differentiation in the neovascularisation that occurs in diabetic retinopathy, representing a further and previously unveiled effect of NTFs on retinal neovascularisation. The findings with regard to the neuronal control of angiogenesis suggest that inhibiting the angiogenic function of NTFs may be a new therapeutic strategy. Concluding the results of the study, the authors found increased NTFs levels in the PB of diabetic retinopathy patients, an increase that was correlated with the levels of circulating EPCs. These results support the concept that NTFs may induce neoangiogenesis, a process that to a degree may be reparative, but when excessive leads to pathological retinopathy (29).

In another study, the functional properties of CD34+ CD45- endothelial colony forming cells (ECFCs, late outgrowth EPCs) in patients with proliferative diabetic retinopathy were evaluated. The authors observed an approximately two-fold increase in levels of highly proliferative circulating ECFCs in proliferative diabetic retinopathy patients despite a higher prevalence of renal impairment and peripheral vascular disease. The subset of EPCs that have been isolated in this study may represent an additional cellular contributor to vascular complications seen in diabetic patients. One limitation of this study is the sole comparison between cells from patients with proliferative diabetic retinopathy and those from non-diabetics. Ideally, a comparison including cells from diabetics without retinopathy would allow us to definitively conclude whether these functional abnormalities are a consequence of diabetes or occur exclusively in diabetics with a propensity for progressing to proliferative diabetic retinopathy. It is also unclear whether diabetics early in their disease or diabetic patients without end-organ damage manifest similar increases in this particular progenitor cell subset. It was observed that levels of SDF-1 but not VEGF were higher in the plasma of proliferative diabetic retinopathy patients who grew ECFC colonies compared to the healthy patients who grew colonies. From migration assays, it appears that ECFCs from proliferative diabetic retinopathy patients are impaired in their ability to mobilise in response to either SDF-1 or serum from non-diabetic or diabetic patients. One surprising finding was the inability of ECFCs from both normal and proliferative diabetic retinopathy patients to generate a robust migratory response towards VEGF. Besides, both subgroups exhibited similar migratory dysfunction towards VEGF and SDF-1. The authors have shown that despite higher SDF-1 levels in the plasma and in the vitreous humour and increased circulating ECFCs, ECFCs from patients with proliferative diabetic retinopathy are impaired in their ability to form functional vessels. It could be hypothesised that down-regulation of SDF-1 receptors on ECFCs contributes to the lack of migratory response even with high levels of SDF-1 in the circulation and vitreous humour. Downstream signalling from the SDF-1 receptor may also be

disrupted in proliferative diabetic retinopathy-ECFCs for unknown reasons. Taken together, the data from this study indicate that despite upregulation of SDF-1 level, patients with proliferative diabetic retinopathy are unable to utilise their increased ECFC pools to form functional vessels. In light of the results and from work already done in the EPC field, the "diabetic paradox" might actually be explained by two synergistic vascular derangements: impairment of EPC-mediated vessel repair and aberrant vasculogenesis induced by functionally modified EPCs. This finding suggests that the microvascular and macrovascular changes seen in diabetic patients may be attributed to functionally altered ECFCs both in normal vessel repair and in abnormal neovascularisation (124).

7.5. Diabetes and arterial stiffness

The relationships between glycaemic control, levels of EPCs and arterial stiffness are unknown. Circulating EPCs (CD34+/KDR+ and CD133+/KDR+) and brachial-ankle PWV were measured in patients with T2DM and compared with a control group. A close inter-relationship between glycaemic control, circulating EPCs and arterial stiffness in patients with T2DM was demonstrated. Patients with T2DM had a lower level of circulating EPCs and increased arterial stiffness compared with normal controls. Amongst those with diabetes, a poor glycaemic control correlated with a lower number of EPCs as well as increased arterial stiffness, whilst better glycaemic control correlated with a relatively greater number of EPCs as well as a lesser degree of arterial stiffness. Furthermore, after adjusting for other cardiovascular risk factors and medications, glycaemic control and EPC counts were identified as independent risk predictors for arterial stiffness. The mechanisms by which hyperglycaemia leads to an increase in arterial stiffness remain unclear but is likely related to an imbalance between the protective versus detrimental pathways. It was confirmed that patients with DM have a blunted endothelium regenerating capacity as reflected by a decrease in number of circulating EPCs and was associated with large artery atherosclerosis as measured by PWV. Importantly, the results further demonstrated that the degree of hyperglycaemia control in those patients with T2DM was closely related to the levels of circulating EPCs as well as the arterial stiffness. In this study, DM patients who could achieve satisfactory hyperglycaemia had significantly higher circulating level of EPCs and lower arterial stiffness. This might be one of the mechanisms in which satisfactory glycaemic control in T2DM patients (HbA1c < 6.5%) had reduced cardiovascular events (125).

7.6. Type 2 diabetes and ghrelin levels

The effects of T2DM on subpopulations of EPCs in PB, as compared with the effects on ghrelin levels, which may play an important role in endothelial dysfunction associated with diabetes, were investigated. PB samples were analysed from (i) diabetic patients with a history of disease of less than 1 year and no clinical evidence of angiopathy (T2DM), (ii) diabetic patients with long-standing disease with vascular complications, and (iii) healthy donors. In T2DM with vascular complications, the most representative complication of impaired vasculogenesis was peripheral arterial occlusive disease (PAD) associated with diabetic retinopathy. The authors identified pre-EPCs as CD34+CD133+CD117+, EPCs as CD34+CD133+VEGFR2+ and late EPCs CD31+VEGFR2+CD144+. The analysis of different EPC subpopulations enabled the authors to study the effect of T2DM during the differentiation and commitment of these cells, from early precursors to mature circulating EPCs. The results suggested that diabetes did not impair the production of the very early precursor EPCs (pre-EPCs). In fact, no significant differences in the number of pre-EPCs were found among the three groups. Interestingly, subsets of the T2DM patients and T2DM patients with vascular complications showed significantly higher numbers of pre-EPCs than the healthy donors. This could represent a physiological response to the requirement for new blood vessels that is present in only some subjects: the endothelial damage that is induced by diabetes stimulates the mobilisation of precursor cells from the BM. The presence of endothelial damage was confirmed by an increase in the number of CECs. The finding that EPCs were not mobilised in many patients with diabetes could be explained by the inhibitory effects of such a long-term disease on the activity of the BM. The number of pre-EPCs correlated with that of EPCs, but it did not correlate with the population of late EPCs, which was reduced dramatically by the effects of diabetes. The authors have no direct evidence that the late EPCs were derived directly from pre-EPCs; however, according to them, the available evidence suggests that impairment of neovascularisation in diabetes could be caused, at least in part, by an alteration in the maturation/commitment and perhaps in the homing of EPCs, rather than by altered production of EPCs in the BM. In addition, they noted that some control participants showed low numbers of EPCs and late EPCs. This raised the question of whether these participants were in a pre-diabetic condition (metabolic syndrome) or were more prone to the development of atherosclerosis in a manner that was independent from diabetes. Another intriguing question is, whether the T2DM patients and T2DM patients with vascular complications with a higher number of pre-EPCs represented subgroups with a more favourable long-term outcome. The authors analysed serum levels of unacylated (UAG) and acylated ghrelin (AG) to obtain more

specific and sensitive markers for the vascular complication associated to diabetes. It was confirmed that levels of ghrelin (both AG and UAG) were decreased in patients with diabetes. Interestingly, it was found that patients in the T2DM and T2DM with vascular complications' groups showed a significant relative increase in AG with respect to UAG. This increase could be part of the ongoing complications that are associated with diabetes, and interestingly, it comes together with decreased numbers of late EPCs. Therefore, it was suggested that the pre-EPC/late EPC ratio and the inverse of UAG/AG ratio, together with the profile EPC-ghrelin are specific and sensitive markers of endothelial dysfunction in diabetes. However, it is important to point out that definitive conclusions about these markers might be drawn from further studies performed on a larger cohort of subjects. Concluding the results of the authors, the data from this study indicate that the endothelial dysfunction that is seen in diabetes is probably the result of an altered process of maturation or commitment of EPCs rather than a failure of their production or mobilisation from the BM. They suggest that EPC subpopulations (pre-EPCs and late EPCs) and ghrelin levels might be used as markers to assess endothelial damage. Furthermore, the data on the number of CECs confirm the endothelial injury as an early event in diabetes (126).

7.7. Type 1 diabetes and children

A study was performed to estimate the EPC (CD34+CD144+ and CD34+VEGFR2+) number and its relationship with vascular function and structure in children with type 1 DM (T1DM). One of the most intriguing novel findings of this study is that EPC frequencies are not reduced in T1DM children with poor metabolic control and no obvious vascular complications. In contrast, the authors found that frequencies of CD34+VE-cadherin+ cells and, to a lesser extent, CD34+VEGFR+ cells, were higher in diabetic children compared with the healthy group. However, as glucose control in studied patients was less than ideal, the results may not be generalise to all children with T1DM. Impaired endothelial function and early structural vessel changes were also confirmed, proving an ongoing atherosclerotic process in the study population. Moreover, increased levels of some inflammatory (hs-CRP) and endothelial (sICAM-1) cardiovascular disease biomarkers in diabetic children as well as lipid abnormalities were reported. In the current study, the authors chose to evaluate a very young population, below 18 years. Such a study population allows to avoid participants at advanced stages of vascular injury in whom endothelial repair mechanisms might have already been activated by arterial wall damage. The main results are contradictory to studies in adult populations. Interestingly, no correlations were found between HbA1c and hs-CRP with endothelial

function and endothelial progenitor subpopulations. According to the authors, that observation seems sobering because these measures are currently widely used in the clinic for assessment of risk of future microvascular and macrovascular diseases. Altogether, these observations suggest that other factors besides 'average' of glycaemic values (such as glucose variability) may affect circulating progenitor cell numbers and vascular reactivity at a given moment in time. Another interesting finding of the study is a positive, significant correlation between EPCs and BMI. Cardiovascular disease begins in childhood and primary prevention must be a priority for paediatricians. Interventions to enhance vascular health are likely to be most successful early in disease before sustaining irreversible vascular damage, emphasizing the importance of studies on high-risk children and adolescents. Assessment of EPCs could potentially provide a non-invasive means of assessing cardiovascular risk in clinic settings and serve as a marker of efficacy of treatments for cardiovascular disease prevention and enhance the potential for discovery of novel therapies. In conclusion, the results of this study indicate that contrary to adult population with diabetes, diabetic children demonstrated increased frequencies of EPCs (especially delineated by CD34+CD144+ phenotype) that correlated inversely with endothelial function. Whether this phenomenon could be the result of the effective mobilisation of EPCs in the young population at increased risk for atherosclerosis in order to repair damaged endothelium will need further investigation. Taken together, higher levels of EPCs in very young patients with cardiovascular risk factors might also reflect an unfavourable constellation, which has to be investigated in future studies (127).

7.8. Diabetes, myocardial dysfunction and oxidative stress

The relationship of EPCs (CD34+, CD133+, CD34+KDR+ and CD133+KDR+) and oxidative stress, as determined by superoxide dismutase, to myocardial function was investigated in patients with T2DM and no history of CAD. It was demonstrated that at least 30% of T2DM patients, with apparently normal LV dimensions and ejection fraction, have impaired myocardial function. The study further demonstrated that EPCs in patients with T2DM and no atherosclerotic disease, were independently associated with myocardial dysfunction. Therefore, it was suggested that depletion of EPCs contributes to the development of diabetic heart disease. It was found that CD34+ EPCs were most strongly correlated with myocardial dysfunction, compared with other subtypes of EPCs. Although the reason is uncertain, this could be partly explained by the ability of CD34+ EPCs to differentiate not only into hematopoietic stem cells, but also cardiomyocytes, smooth muscle and endothelial cells. The multi-lineage property of CD34+ EPCs may thus provide a more comprehensive assessment

of the pathophysiological development of diabetic heart disease. The results further demonstrate the independent role of superoxide dismutase in relation to myocardial dysfunction, thus highlighting the role of oxidative stress in the development of diabetic heart disease. The close interplay between oxidative stress and EPCs independently contributes to myocardial dysfunction. According to the authors, this study is the first to demonstrate that impaired myocardial function in patients with T2DM is independently associated with depletion of EPCs and increased oxidative stress. Summarily, it was demonstrated that patients with T2DM and no clinical evidence of macrovascular disease showed impaired myocardial function. Importantly, LV global circumferential strain was independently associated with both CD34+ EPCs and superoxide dismutase. These findings suggest that myocardial dysfunction in patients with T2DM is related to depletion of EPCs and increased oxidative stress (128).

7.9. Diabetes, cardiac ischaemia and leukotrienes

Since numerous eicosanoids including leukotrienes and hydroxyeicosatetraenoic acids have been shown to exert potent pro-inflammatory activities, their levels were evaluated in chronic diabetic patients with severe cardiac ischemia in conjunction with the level and function of EPCs. This study aimed at examining the levels of inflammatory and angiogenic eicosanoids in serum from diabetic and non-diabetic patients undergoing coronary artery bypass surgery in conjunction with the level and function of EPCs (CD34+VEGFR2+, CD133+VEGFR2+ and culture). It was reported that levels of inflammatory eicosanoids with potent angiogenic activity are significantly elevated in serum from diabetic patients with CAD as compared to their counterpart non-diabetic patients. These elevated levels corresponded with decreased EPC levels and function in the diabetic patients. Besides, the number of circulating EPCs was significantly lower in diabetic patients compared to the non-diabetic patients. The number of EPC CFUs following 7 days of culture was also lower among the diabetic patients compared with the controls. The authors did not find statistically significant correlations between the number of EPCs and the levels of HbA1c in the diabetic patients. In contrast, negative correlations between EPC CFUs and HbA1c and also between viability of the colonies and HbA1c were observed. It seems that the EPC function as measured by their ability to form viable EPC CFU colonies, but not number, is affected by hyperglycemia (measured through HbA1c levels). Concluding the results of the authors, this study demonstrates increased levels of several eicosanoids with potent inflammatory properties, reduced levels of circulating EPCs and impaired functional properties of EPC in diabetic as compared to non-diabetics patients with ischemic heart conditions. The association between plasma levels of eicosanoids and EPC

function in the diabetic patients is unclear and needs to be further investigated using a larger cohort of patients. However, the authors refer that despite the presence of inflammatory eicosanoids with potent angiogenic activity in diabetic patients, inflammation may be a contributing factor to impaired EPC function. Non-functional EPCs may reduce the vascular regenerative potential of these patients and could further contribute toward diabetes-associated vascular complications (70).

8. EPCs, kidney disease and cardiovascular disease

8.1. Renal insufficiency in stable angina

The relationship between estimated glomerular filtration rate (GFR) and EPCs (defined as a subpopulation of CD34+/KDR+) was investigated in a relatively homogenous group of men subjects with stable angina, angiographically significant CAD, normal LV systolic function and GFR ≥ 30 mL/min/1.73 m^2. An independent positive correlation between estimated GFR and blood counts of CD34+/KDR+ cells was described. The relationship was attenuated after adjustment for levels of sVCAM-1, a marker of endothelial dysfunction, and haemoglobin. This was found despite the fact that a lower GFR was associated with insignificantly higher EPO levels that correlated inversely with haemoglobin. Deficiency of CD34+/KDR+ cells in CAD men with stable angina and angiographically proven CAD was proportional to the degree of renal insufficiency, which was in part mediated by factors affecting endothelial function and haemoglobin levels irrespective of an angiographic CAD extent, inflammatory activation or homocysteine accumulation. Irrespective of mechanisms involved, CD34+/KDR+ cell depletion in mild-to-moderate renal dysfunction accompanying stable angina may exacerbate an imbalance between endothelial injury and EPC-mediated repair, thus contributing to excessive cardiovascular risk in CAD coexisting with renal insufficiency (129).

8.2. Renal function, stable angina and asymmetric dimethyl-arginine

Renal insufficiency predisposes to CAD, but also CAD and traditional risk factors accelerate renal function loss. EPCs deficiency and elevated ADMA, an endogenous NO formation inhibitor as referred before, predict adverse CAD outcomes. The changes in estimated GFR over time in relation to baseline EPCs (defined as a subpopulation of CD34+/KDR+) and ADMA levels were investigated in non-diabetic men with stable angina, who were followed up for 2 years after elective coronary angioplasty. According to the authors of this study, the salient finding consists of synergistic contributions of elevated plasma ADMA and depressed CD34+/KDR+ cell counts to renal function decline in non-diabetic men with stable angina receiving an optimal complex therapy according to current practice guidelines. Although both ADMA accumulation and CD34+/ KDR+ cells deficiency were more pronounced in CAD subjects with pre-existing mild-to-moderate renal insufficiency, their effects on further renal deterioration appeared independent of the initial renal function (130). Moreover, the effect was specific for ADMA, being unrelated to symmetrical dimethyl-L-arginine (a stereoisomer

of ADMA, whose circulating levels increase at earlier stages of renal dysfunction (129)) or sVCAM-1 levels, despite the fact that the latter biomarkers, not ADMA, were significant correlates of baseline estimated GFR and/or angiographic CAD extent. Additionally, the results provide a clinical argument in favour of the experiment-based concept of the importance of BM-derived cells for the maintenance of vascular integrity also within the kidney. Deteriorating renal function may potentiate cardiovascular risk exacerbating not only hypertension, but also dyslipidaemia and inflammatory activation, and presumably the retention of putative pro-atherogenic compounds, such as ADMA (130). It is important to note that all patients in this study were receiving low-dose aspirin, ACE inhibitors and statins. The optimal medical therapy, adequate blood pressure control and complex revascularisation might have accounted for a weak tendency to rises in eGFR in this study, instead of the expected age-related decline. In summary, elevated ADMA and EPC deficiency may synergistically contribute to accelerated renal function decline in stable angina. This could result from the impairment of the EPC-dependent endothelial renewal in the kidney, a NO-dependent process (130).

8.3. Renal functional recovery after ischaemia

In ischaemic acute kidney injury, renal blood flow is decreased. Reperfused, transplanted kidneys exhibit ischaemic injury on the vascular endothelium and preservation of peritubular capillary endothelial integrity may be critical to recovery from ischaemic injury. It was hypothesised that BM-derived EPCs (CD34+) might play an important role in renal functional recovery after ischaemia. This hypothesis was tested in recipients of cadaveric renal allografts before and for 2 weeks after transplantation. The findings of the study suggested that BM-derived circulating EPCs and ECs had migrated to the injured endothelium of the kidney, inasmuch as the recipients of cadaveric renal allografts did not manifest signs suggesting simultaneous endothelial injury in other organ systems. The finding of ECs of recipient BM origin in renal allograft tissues also supports this concept. According to the authors, the numbers of circulating CD34+ EPCs among patients showing recovery and sustained acute kidney injury, compared with controls, did not reach statistical significance, possibly because of the small number of subjects studied. Nonetheless, the median values of CD34+ EPCs on each postoperative day lead to a speculation that CD34+ EPCs disappear from the circulation more precipitously after ischaemia-reperfusion in patients with recovery of the graft function, compared with those destined to have sustained acute kidney injury. Circulating CD146+ ECs decreased significantly in cadaveric renal allograft recipients compared with controls for at

least 14 days after transplantation. Patients showing recovery of graft function had significantly lower numbers of CD146+ ECs compared with preoperative numbers. According to the authors, these findings of the study suggested that BM-derived EPCs and ECs may contribute to endothelial repair immediately after ischaemia-reperfusion and that tubular repopulation with BM-derived cells occurs later (131).

8.4. Chronic kidney disease stage V

The association between functionally active EPCs (cell culture) and traditional cardiovascular risk factors was evaluated in patients with chronic kidney disease stage V receiving haemodialysis therapy. The authors found a significant relationship between the number of EPCs and incident cardiovascular events in stable patients with chronic kidney disease stage V on maintenance haemodialysis in a prospective follow-up period of a median of 36 months. Moreover, in a stepwise logistic regression analysis including traditional cardiovascular risk factors EPCs were the only independent variable associated with incident cardiovascular events. In contrast, haematopoietic stem cells (assessed by flow cytometry as CD34+ cells) were not associated with cardiovascular events and patient survival in the follow-up, highlighting the potential role of EPCs in vascular repair and cardiovascular morbidity in chronic kidney disease patients. In contrast to individuals with normal kidney function, a significant but only weak correlation was found between EPCs and age, but not with other traditional cardiovascular risks factors such as high blood pressure or total serum cholesterol, and with cardiovascular risk factors present only in haemodialysis patients such as reduced nutritional status (low BMI and/or serum albumin), and presence of inflammation (increased hs-CRP). The latter finding is of particular important, since it supports the notion that EPCs obtained by labour intensive and time consuming cell culture methodology, thus exhibiting several functional properties of endothelial cells, are indeed related to vascular biology, and are not monocytic cells that may also be involved in inflammation. Since chronic kidney disease patients are characterised by high cardiovascular morbidity and mortality due to vascular complications, their survival could also be influenced, at least in part, by deficient vascular repair as a result of reduced EPC number and/or function. The authors did not found an association between recombinant human EPO treatment and EPCs. According to the authors, it could be speculated that patients who do not need recombinant human EPO therapy despite kidney failure, still may have enough endogenous EPO production to maintain normal EPC numbers. No differences in EPCs were found between patients treated with statins and those who did not receive these drugs. Therefore, it was assumed that other factors override the

effect of recombinant human EPO and statins on EPCs in this population. As a matter of fact, an important confounding factor may be the haemodialysis procedure by itself. The authors have studied EPCs in patients after a long haemodialysis interval in order to minimise this potential confounding factor. In conclusion, a significant relationship between EPCs and incident cardiovascular events was found, as well as patient survival in stable chronic kidney disease patients receiving haemodialysis therapy. Thus, the data from this study support a role for EPCs in vascular repair in chronic kidney disease patients (132).

8.5. Intradialytic hypertension

Intradialytic hypertension is associated with adverse outcomes. It was hypothesised that intradialytic hypertension is associated *in vivo* with endothelial cell dysfunction (assessed using the number of EPCs and FMD). To test this hypothesis, a case-control cohort study was performed, which included haemodialysis subjects without intradialytic hypertension, as controls, and subjects with intradialytic hypertension (defined as an increase in systolic blood pressure pre- to post-dialysis ≥10 mmHg during at least four of six consecutive haemodialysis sessions). Baseline characteristics and comorbidities were similar between the studied groups. It was found that maintenance haemodialysis patients with intradialytic hypertension have abnormal *in vivo* endothelial cell function. A 50% difference in the number of EPCs (CD34+CD133+ or aldehyde dehydrogenase bright activity (ALDHbr)) was found in patients with intradialytic hypertension as compared with control subjects. In addition, there was impaired endothelial-dependent vasodilation (assessed using FMD) among patients with intradialytic hypertension as compared with controls. The findings of decreased EPCs among subjects with intradialytic hypertension suggest ongoing endothelial injury with impaired regenerative capacity and may explain the higher adverse events found in this population. It was also found that subjects with intradialytic hypertension exhibit a higher blood pressure burden compared with subjects without intradialytic hypertension. In this study, more patients with intradialytic hypertension were prescribed renin-angiotensin-aldosterone system inhibitors, suggesting that renin-angiotensin-aldosterone system inhibition may not be fully effective at controlling intradialytic increases in blood pressure. Although novel associations between intradialytic hypertension and impaired endothelial cell function were identified, this study consisted of a case-control study and therefore no direct cause-effect relationship can be identified. Giving the findings, it was proposed that impaired endothelial cell function may partially explain the higher rate of adverse events among patients with intradialytic hypertension (133).

The authors of this previous study hypothesised that carvedilol, a non-selective α and β-blocker, would improve endothelial cell function and reduce the occurrence of intradialytic hypertension, performing a prospective 12-week pilot study of carvedilol titrated to 50 mg twice daily among haemodialysis participants with intradialytic hypertension (each patient served as his or her own control). Paired tests were used to analyse changes in blood pressure and endothelial cell function assessed by FMD, EPCs (ALDHbr or CD34+CD133+), ADMA, and endothelin-1, from baseline to study end. It was shown that FMD significantly improved with carvedilol. However, there was no significant change in EPCs, endothelin-1, or ADMA. The principal finding was that carvedilol administered at the maximum dose tolerated (not exceeding 50 mg twice daily) is associated with significant improvements *in vivo* in EC function in haemodialysis patients, as shown by improvement of FMD. Thus, this study supports evidence that EC dysfunction may play a primary role in intradialytic hypertension and introduces new evidence that targeted pharmacologic therapy is associated with improvements in both EC function and intradialytic hypertension. Although the authors identified improvements *in vivo* in EC function, they failed to identify significant improvements in circulating markers of potential EPC. It is plausible that although carvedilol improved *in vivo* EC function translated by improved FMD, the study follow-up was not long enough to identify BM regeneration of reparative EPCs (134).

8.6. Chronic haemodialysis

The possible association of EPC (double stained cells for both lectin and DiI-acLDL after 7 days in culture and CD34+KDR+ cells using flow cytometry) with inflammation, endothelial dysfunction and atherosclerosis was investigated in chronic haemodialysis patients and compared with healthy controls. Higher levels of inflammation and lower number of EPCs were observed in haemodialysis patients. The influence of inflammation on EPC biology was unclear for haemodialysis patients. The authors did not observe an association between EPC number and serum hs-CRP levels, but they have found that EPC counts were negatively correlated with serum IL-6 levels. A significant inverse association between EPC number and serum TNF-α levels were also observed in both haemodialysis patients and healthy controls. This study pointed out the association of increased serum TNF-α with decreased number of EPCs in patients with uraemia. Despite the relation between decreased levels of EPCs and endothelial dysfunction was previously demonstrated in this dissertation, decreased EPC number was not associated with impaired FMD in haemodialysis patients, in this particular

study. It was suggested that the impact of EPC on endothelial dysfunction in haemodialysis patients might be disappeared due to interfering uraemia related factors. Again, despite the relation between decreased levels of EPCs and atherosclerosis was previously demonstrated in this dissertation, the authors of this particular study could not find any correlation between EPC and carotid atherosclerosis in haemodialysis patients. The study population was composed of well-dialyzed patients which may explain the lack of association. Although the age difference between groups did not reach statistical significance, control group was younger than the study group. It may have influence on the differences between the groups which is a limitation of the study. In conclusion, EPC number was decreased in uraemia and was associated with inflammation. TNF-α might have specific inhibitory actions on EPC in both haemodialysis patients and healthy controls. EPCs were not related to endothelial function and/or atherosclerosis in haemodialysis patients (135).

8.7. Haemodialysis, microinflammation and endothelial damage

Another study examined whether amelioration of microinflammation using on-line haemodialfiltration had an effect on EMPs (CD31+annexin-V+) and ECPs (CD31+CD14+VEGFR2+) as biomarkers of endothelial injury and repair, respectively. Two types of haemodialysis, namely on-line haemodialfiltration and high-flux haemodialysis, were compared in patients in a sequential manner. It was shown that a significantly high number of circulating EMPs were observed in patients on high-flux haemodialysis as compared with healthy controls. The elevated number of EMPs was associated with an also remarkably high count of EPCs. Both circulating EMPs and EPCs were reduced after 4 months of on-line haemodialfiltration. A significant correlation between pro-inflammatory CD14+CD16+ cells, and both the number of EMPs as well as the number of EPCs was documented. The authors speculated that CD14+CD16+ monocyte-derived dendritic cells may have a dual effect triggering endothelial activation and injury leading to the release of EMPs and the stimulation of the BM to increase the production of circulating EPCs. They were unable to claim a direct cause-effect relationship between microinflammation, formation of EMPs, and increased numbers of circulating EPCs. Other factors such as uremic toxins, or other activated cells (e.g., platelets, leukocytes) may come into play. It is of interest that no increase in EMP was observed in post-dialysis blood samples after on-line haemodialfiltration. In all cases, high-flux haemodialysis was associated with a remarkable increase of EMPs. In both high-flux haemodialysis and on-line haemodialfiltration, EPCs decreased significantly in the post-dialysis blood samples in respect to the pre-dialysis ones. It was concluded that on-line haemofiltration

attenuates endothelial dysfunction possibly by decreasing microinflammation. This effect may be directly caused by a modulatory effect of on-line haemofiltration on pro-inflammatory cells or by a complex interaction that encompasses a wider removal of uremic toxins (136).

8.8. Renal transplant and cardiovascular risk

The risk of cardiovascular disease in renal transplant patients is higher than in the general population. It was hypothesised that the concentration of EPCs (defined as CD34+CD133+CD45+ cells by flow cytometry or, in culture, by double staining with DiI-acLDL and lectin) could be altered in renal transplant patients who are also at a high risk for cardiovascular disease. The relation of EPCs with the level of graft function and other cardiovascular risk factors in such patients was determined, as well as the proliferation of EPCs *in vitro*, as a marker of functional impairment. It was shown that the concentration of circulating EPCs is significantly reduced in renal transplant patients as compared to a gender- and body weight-matched group of healthy controls. GFR was an independent predictor of EPCs concentration in patients. Moreover, in patients with reduced graft function (GFR <30 mL/min), the EPCs concentration was lower than in those with GFR above 30 mL/min. Among other cardiovascular risk factors that were included in the analysis, HDL cholesterol and LDL cholesterol correlated in a negative and a positive direction, respectively with reduced EPCs concentration. The correlation coefficients were modest, but it should be noted that a majority of this renal transplant patients were receiving statins and other lipid-lowering agents. Although patients were older than the controls, no significant correlation was found between age and EPCs concentration. It was also not found a significant influence of gender, hypertension, DM and smoking on the concentration of EPCs in renal transplant patients. It was also considered the potential impact of treatment with immunosuppressive agents, and recombinant human EPO or its analogues on EPCs concentration. Since all the patients were treated with calcineurin inhibitors, the analysis was limited to search for differences between subjects with or without mycophenolate and corticosteroids treatment. Renal transplant patients receiving mycophenolate appeared to have higher concentration of EPCs, whereas no significance differences were found between those receiving corticosteroids or not. Renal transplant patients receiving recombinant human EPO had lower number of EPCs than those not receiving it. GFR, however, was lower in patients receiving human recombinant EPO that in those not receiving it. This finding was interpreted as an effect of the reduced GFR rather than a negative effect of human recombinant EPO on the concentration of EPCs. The findings of reduced EPCs in renal transplant patients suggest that their deficiency may play a role in

endothelial dysfunction and increased cardiovascular risk in kidney transplant recipients. This may be an acquired feature as it was more frequently seen in individuals with reduced graft function. Pre-existing endothelial dysfunction prior to transplantation could account for reduced EPCs concentration in some cases as well. To investigate EPCs function, the authors of this study obtained cells from the majority of the patients in whom they had measured their concentration *in vivo*, and studied the cells *in vitro*. Interestingly, it was found that *in vitro* proliferation was reduced in EPCs from renal transplant patients suggesting a functional impairment. In summary, this study shows that the concentration of EPCs is reduced in renal transplant recipients, particularly in those patients with reduced GFR. Moreover, EPCs obtained from renal transplant patients when studied *in vitro*, displayed reduced proliferation, as a sign of functional impairment. These alterations may be involved in the increased cardiovascular risk of renal transplant patients (137).

9. EPCs, cardiovascular risk and other diseases

The research carried out for this dissertation included studies with data on EPCs, cardiovascular risk and other diseases. Thus, this chapter is a brief description of the results of these studies.

EPCs were enumerated in the PB of patients with systemic lupus erythematosus (from here only referred to as lupus) and healthy controls, using flow cytometry (CD34+KDR+) and a colony-forming assay (EPC CFUs). It was demonstrated that serum levels of type I interferon (IFN-I) and CRP are independent predictors of EPC depletion in lupus patients. In summary, the authors have described a drastic reduction in the number of circulating EPCs in patients with lupus, an autoimmune condition with frequent cardiovascular complications, and have identified IFN-I as a novel risk factor for EPC depletion and endothelial dysfunction (36). In another study, it was demonstrated that the level of circulating mature EPCs (CD34+VEGFR2+) in the PB of patients with lupus is significantly reduced, whereas the level of early immature EPCs (CD133+VEGFR2+) is increased. It was suggested that the mobilisation of EPCs is unaffected in lupus, but the diminished number and the altered functionality of circulating CD34+/VEGFR2+ EPCs reduce the ability to repair vascular damage and thus may trigger the development of atherosclerosis in patients with lupus (138).

Primary aldosteronism is associated with a higher incidence of cardiovascular events, probably through mineralocorticoid receptor-dependent endothelial cell dysfunction, in comparison with essential hypertension. It was suggested that low EPC (CD34+KDR+ and culture)

numbers in primary aldosteronism plays a crucial role in the high incidence of arterial stiffness and in predicting residual hypertension in patients with aldosterone-producing adenoma after adrenalectomy (139).

The number of BM-derived EPCs (CD34+CD133+) was determined in patients with erectile dysfunction both with and without cardiovascular risk factors. It was demonstrated that patients with erectile dysfunction, both with and without cardiovascular risk factors, have a lower number of circulating EPCs probably as a result of a generalised endothelial dysfunction (140). In another study, the influence of EPCs (CD34+/KDR+ and CD133+) on erectile dysfunction was determined in patients with known CAD. It was demonstrated that decreased numbers of circulating EPCs are an independent risk factor for erectile dysfunction, suggesting EPCs as a possible link between cardiovascular risk factors and endothelial dysfunction in the penile arteries and the corpus cavernosum (141). The low pharmacological response to phosphodiesterase type 5 inhibitors may represent an expression of higher endothelial damage in certain categories of patients with erectile dysfunction and high cardiovascular risk. The results of this study corroborate the clinical value of the low clinical response to phosphodiesterase type 5 inhibitors in the treatment of erectile dysfunction in the patients with high cardiovascular risk profile, such as diabetics (142).

Long-term survivors of testicular cancer who received cisplatin-based chemotherapy have an increased risk of cardiovascular disease. A cross-sectional study was performed to objectively assess cardiovascular risk, subclinical atherosclerosis, and endothelial function in long-term survivors of testicular cancer. EPCs were also quantified (CD133+VEGFR2+). Long-term survivors of testicular cancer who received chemotherapy demonstrate objective evidence of endothelial injury and dysfunction, despite the fact that no difference was observed in the levels of EPCs between groups (143).

Rheumatoid arthritis is characterised by increased cardiovascular morbidity and mortality that cannot be explained solely by traditional cardiovascular risk factors. Because the quantity of EPCs in the PB is correlated inversely with cardiovascular risk, it was studied whether such abnormalities could also be observed in patients with rheumatoid arthritis. EPCs (CD34+CD133+KDR+ and EPC CFUs) were determined in patients with rheumatoid arthritis patients and in healthy referents. Patients were divided into groups characterised by active disease and low disease activity. It was shown that EPCs were significantly decreased in rheumatoid arthritis patients suffering from active disease compared with those from healthy subjects. In contrast, the frequency of circulating EPCs from patients with low disease activity was comparable to that of healthy individuals (32).

Given the essential role of EPCs in endothelial repair and neovascularisation, it is likely that insufficient angiogenesis seen in systemic sclerosis is related to EPC alterations. The number of circulating EPCs (CD34+VEGFR2+ and CD133+VEGFR2+) and their contribution into cardiovascular involvement were analysed in patients with systemic sclerosis and in healthy subjects. The data from the study demonstrated an increase of circulating EPCs in early stage of systemic sclerosis that correlated positively with the severity of disease and the presence of digital ulcers. In conclusion, while blocking endothelial cell death must be the primary goal in early systemic sclerosis, as long as large numbers of EPC are available, their replacement may be a useful therapeutic intervention in late stage disease (144).

Klinefelter's syndrome is associated with a significant reduced life expectancy including greater mortality from cardiovascular diseases. Low testosterone may have a direct effect on vascular tissue or act indirectly via metabolic effects. Since cardiovascular disease may be a cause of death within Klinefelter's syndrome patients and that EPCs are related with cardiovascular diseases, the number of circulating EPCs (CD34+CD133+VEGFR2+) was evaluated in adult men with Klinefelter's syndrome and in healthy males. It was demonstrated that all patients with Klinefelter's syndrome, independently from testosterone levels and from the presence or absence of cardiovascular risk factors, had low levels of circulating EPCs that further increase their cardiovascular risk (145).

Non-alcoholic fatty liver disease is associated with advanced atherosclerosis and a higher risk of cardiovascular disease. It was hypothesised that patients with non-alcoholic fatty liver disease might have decreased EPC levels (CD34+, CD34+KDR+, and CD34+KDR+CD133+) and attenuated EPC function (culture). It was demonstrated that patients with non-alcoholic fatty liver disease have decreased circulating EPC numbers and impaired adhesive function and migration than those without non-alcoholic fatty liver disease. Therefore, it was suggested that non-alcoholic fatty liver disease should be carefully considered as an independent risk factor for cardiovascular diseases (146).

It was hypothesised that the number of endothelial progenitor CFUs derived from maternal blood are decreased in women with pre-eclampsia compared to normal pregnancy. It was reported that the number of endothelial progenitor CFUs, derived from equivalent numbers of PB MNCs in culture, is 4-fold lower in women with preeclampsia compared to women with uncomplicated pregnancies during the third trimester (37). With respect to pregnancy and childbirth, it was demonstrated that giving birth to a low birth weight infant was associated with lower maternal EPC number (CD34+VEGFR2+ and culture) and reduced EPC migratory function *in vitro*, supporting the hypothesis that EPC pathology may represent the elusive link

between utero placental insufficiency and future risk of cardiovascular disease in the mother (147).

Cardiovascular risk is increased in premature ovarian failure. To determine the effects of premature ovarian failure on different parameters of cardiovascular health, the relationship between premature ovarian failure and circulating EPCs (CD34+CD133+ and CD34+KDR+), endothelial function, carotid IMT and LV diastolic function was investigated in female patients, and gender- and age- matched healthy controls. The findings of this study indicated that endothelial function as well as circulating EPCs, carotid IMT and diastolic function are significantly affected in young women with premature ovarian failure, which may have an adverse long-term effect on cardiovascular prognosis (148).

Haemangioma is the most common tumour of infancy and its primary cause is unknown. It was demonstrated that proliferating haemangioma contains EPCs that co-express CD133 and KDR. These findings suggest that EPCs participate in haemangioma pathogenesis (149).

Endothelial dysfunction may be one of potential mechanisms by which depression and stress might contribute to the development of CAD. The relationships between the level of circulating EPCs (CD34+KDR+ and CD133/KDR+), brachial FMD, and scores of depression and stress measured with the Depression Anxiety Stress Scales were investigated in patients with stable angina patients without major psychiatric disorders. It was demonstrated that in stable angina patients, the presence of a high depression or stress score was associated with decreased brachial FMD and depletion of circulating EPCs (150).

Since Type D personality, a joint tendency toward negative affectivity and social inhibition, is associated with poor prognosis in cardiovascular patients, it was assessed whether the number and function of EPCs might be an explanatory factor for the observed relationship between Type D personality and poor cardiovascular prognosis. It was suggested that Type D personality may be associated with a significantly impaired release and/or survival of circulating EPCs in chronic heart failure patients. The relation between Type D personality and reduced EPC numbers may be associated to TNF-α increased concentrations, increased levels of stress hormone cortisol or a disrupted autonomic balance, in patients with this disorder (151).

10. EPCs and metabolic syndrome

Metabolic syndrome is characterised by low-grade inflammation and confers an increased risk for cardiovascular disease. EPCs were enumerated (CD34+KDR+) and characterised (culture) in subjects with metabolic syndrome in comparison to healthy controls. There were no significant differences in age and gender between the 2 groups. It was demonstrated that the number of circulating progenitor cells (CD34+) and EPCs is decreased in subjects with metabolic syndrome. It needs to be emphasised that none of this study's subjects were diabetic and the majority with metabolic syndrome were females. Importantly, the study population of metabolic syndrome did not include patients with diabetes or cardiovascular disease. Using EPCs as a dependent variable, age, plasma glucose, triglycerides, and CRP were found to be variables predictive of EPC measurements. The novelty of this study is the functional characterisation of EPCs in metabolic syndrome subjects which, according to the authors, has not been reported earlier in comparison to controls. A highly significant decrease in the number of CFUs was reported in metabolic syndrome compared to controls. Multivariate analysis revealed that BMI, HDL cholesterol, and CRP were independent predictors for the reduction in the number of EPCs colonies. It was revealed that the incorporation of EPCs into EC was reduced in metabolic syndrome subjects compared to controls which suggest that EPC incorporation into damaged endothelium or neovascularisation may be impaired in metabolic syndrome. These occurrences could contribute to the deterioration in the repair of damaged endothelium or angiogenesis in metabolic syndrome. Interestingly, individual risk factors seem to differentially affect the number and functional capacity of EPCs. These data suggest that different mechanisms contribute to the impairment in functional activity compared with the reduced levels of circulating EPCs. Overall, CRP emerged as a major predictor in most of the parameters analysed in the study. Therefore, this study extends the role of CRP not only as an inflammatory biomarker but also mediator in the disease process. This data implicate CRP in dysregulation of EPCs and based on multivariate analysis, this observation is significant for calling attention to a possible effect of CRP in contributing to atherosclerosis in metabolic syndrome. This effect may result from impaired ability for endothelial repair and regeneration due to reduced EPCs number. Summarily, decreased number of EPCs along with impaired functionality were demonstrated in both male and female subjects with metabolic syndrome without diabetes, hypercholesterolemia or manifest cardiovascular disease when compared to matched controls and clearly fills a hiatus with respect to EPC functionality in metabolic syndrome. Also, these subjects were non-smokers and drug naive except for anti-

hypertensive medications. These findings may explain in part, the increased cardiovascular risk in metabolic syndrome population (39).

11. EPCs and family history of cardiovascular disease

With respect to family history of cardiovascular disease, the results of the studies are not homogeneous. Reduced numbers of CFUs of ECs, used to determine the functional capacity of circulating EPCs, were associated with family history of premature CAD (19). In another study, lower numbers of EPCs (CD34+KDR+) were found in the presence than in the absence of family history of coronary heart disease, in a sample representative of the healthy general population (78). In patients with stable CAD, EPCs (CD133+KDR+) were inversely correlated with family history of CAD (83). Contrariwise, in another study, EPC levels (CD34+CD31+ and CD34+KDR+) were not associated with family history of CAD (85).
From these results, it can be suggested that family history of cardiovascular disease may have an impact on EPC levels. However, this hypothesis requires further investigation, maybe through a specific study in a large population of healthy subjects with family history of cardiovascular disease, in order to achieve definitive conclusions.

12. EPCs and genetic regulation

It was hypothesised that EPC number and/or function may be genetically regulated and may vary in healthy adult offspring depending on parental history of CAD. Healthy parent-healthy offspring pairs and CAD parent-healthy offspring pairs were studied. The authors of this study assessed the number of EPCs (CD34+VEGFR2+, CD133+VEGFR2+ and culture) and the migration capacity of cultured EPCs towards VEGF. The primary object of this study was to ascertain whether EPC number and/or function display heritability. According to the authors, novel evidence of a significant correlation in cultured EPC number between healthy parents and their healthy adult offspring, as well as between subjects with CAD and their offspring, is presented. Despite these differences in the parental groups, consistent correlations between parental and offspring EPC numbers were seen in both comparisons, adding to the robustness of the association. Although an effect of 'shared environment' on EPC number and/or function cannot be excluded, they feel this is unlikely to be significant, as most of the offspring in this study were living separately from their parents at the time of participation. Therefore, the results suggest that EPC numbers are, at least partially, genetically regulated. The findings

further suggest that any genetic regulation is specific. Thus, the authors observed correlation between parents and offspring for the number of cultured EPCs but, for example, did not see any correlation for migration sensitivity to VEGF. Moreover, with regard to circulating EPC numbers, the findings were mixed. There was a significant positive correlation between parents with premature CAD and their offspring in the level of circulating CD133+VEGFR2+ EPCs, but a negative correlation between healthy subjects and their offspring. For CD34+VEGFR2+ EPCs, there was no correlation in either group. These findings require more cautious interpretation, as, unlike the cultured cells, for a significant proportion of both parents and offspring cohorts, levels of circulating EPCs were undetectable or very low, increasing the margin of any errors for a correlation analysis. The other interesting finding in this study was that offspring of subjects with CAD had significantly higher levels of both types of circulating EPCs than offspring of healthy parents. Although at first this may appear paradoxical, as CAD has been associated with reduced EPCs, it may suggest that although the offspring of CAD had no clinically apparent coronary disease, they could have occult vascular damage and the raised EPCs could reflect a necessary repair response. If this is the case, then the results suggest that elevated EPCs, particularly of the immature CD133+VEGFR2+ type, may represent a biological marker of future risk of CAD in healthy young adults. The lack of difference between the offspring groups in the number of cultured EPCs suggests that either the inherent ability of the offspring's cells to adhere and differentiate is not impaired by a family history of premature CAD, or that different cell types are measured by culture methods. An important observation is that the authors did not see any correlation between the level of either type of circulating EPC and the number of EPCs grown *in vitro* in any of the groups. This was the case even when subjects with no detectable levels of circulating EPCs were excluded. The lack of correlation between the level of circulating EPCs and the number of EPCs grown *in vitro* suggests that these two methods may not be identifying the same cell or group of cells and are therefore not interchangeable. Antibody-guided labelling of cells by surface marker expression is a specific method of cell identification, whereas *in vitro* culture of any precursor cell, exhibiting plasticity, in endothelial conditions could potentially result in an endothelial phenotype. Since findings from either approach are currently being widely used as an index of EPC numbers, the results from this study emphasise that care needs to be taken in the interpretation of data, using the two methods. Despite the differences between the methods of EPC identification, there does appear to be a genetic contribution to the number of EPCs and/or the differentiation capacity of a subpopulation of MNCs to endothelial lineage cells, between

parents and their offspring. In conclusion, the data from this study suggest that a degree of the heritability seen in CAD could be explained through the genetic regulation of EPCs (152).

13. EPCs and age

Age may have an impact on EPCs. Reduced numbers of EC CFUs, used to determine the functional capacity of circulating EPCs, were associated with advanced age (19).

It was investigated whether human age-related endothelial dysfunction is accompanied by quantitative alterations of the EPC pool and hypothesised that possible age-related alterations in progenitor cell number and function correlate directly with the degree of senescent endothelial dysfunction. The number and function of EPCs (CD34+VEGFR2+ and CD133+VEGFR2+) isolated from PB were determined in young healthy subjects (age 25±1) and old healthy subjects (age 61±2), without major cardiovascular risk factors. Although no quantitative differences in EPCs were observed, it was demonstrated that culture-enriched EPCs from old but otherwise healthy subjects are impaired in terms of fundamental functional features like proliferation (important for amplifying the cellular pool), migration (critical for homing of circulating EPCs), and survival. A significant correlation was found between the proliferative and migratory capacity of EPCs and FMD. Also, both functional features of progenitor cells represented independent predictors of endothelial function, indicating that abnormalities in EPC function may account for the impaired vascular regeneration and repair observed in the older subjects. Elderly individuals showed endothelium-specific dysfunction of vascular reactivity, indicated by impaired FMD with preserved endothelium-independent dilation. In order to specifically investigate the effect of aging on EPCs and endothelial function, major cardiovascular risk factors associated with endothelial dysfunction, hypertension, hyperlipidemia, DM, and cigarette smoking were excluded. Plasma glucose, LDL cholesterol, blood pressure, CRP, and BMI were not significantly different between old and young subjects and were not independent predictors of FMD. Other than EPC migration and proliferation, the only independent predictor of endothelial function was the baseline diameter of the brachial artery. Interestingly, this did not reveal a significant difference in the total number of circulating stem cells and the progenitor pool between old and young persons. Rather, the authors found an increased percentage of CD34+/KDR+ EPCs in old individuals. However, due to the decrease in the number of MNCs in the older subjects, the total number of circulating stem cells was not significantly different. Together with the data revealing a significantly higher VEGF plasma level and reduced EPC survival in the elderly, this may reflect

the attempt of the aged organism to mobilise vascular stem and progenitor cells into the PB in response to endothelial dysfunction at an early stage of atherosclerosis. It also suggests that greater stimulation is required to maintain the circulating numbers of progenitor cells in the circulation (153).

In a study whose primary objective was the validation of the significance of CFU number and outgrowth cell yield in acute stroke, it was observed an age-dependent change in the CFU levels in the patients with acute stroke, resulting in much lower CFU levels in aged patients. This finding suggests that the functional activities of EPCs induced by vascular insults decline gradually with age (76).

The number of circulating EPCs (CD34+CD133+KDR+ and CD133+KDR+) has also been evaluated in adolescents and compared with adult controls. The number of EPCs in male adolescents was lower than in adults. This observation was found for both EPC types. In adolescents and adults, levels of CD133+KDR+CD34- EPCs are higher than CD34+CD133+KDR+ EPCs. This finding might reflect the comparably healthy endothelium in adolescents. Low risk factor exposure lowers the need of endogenous repair mechanisms. The turnover is maintained at a very low level. The surprising finding about a comparable small, but very significant, increase of EPCs between adolescents and adults indicates that the interrelation between ageing and EPCs is not fully understood and more complex than a trivial decrease with advancing age. An elevation of EPCs in early life might reflect occult vascular damage and could be seen as a biological marker of subclinical atherosclerosis. Conversely, the overall reduced level of EPCs in adolescents compared to adults may reflect the lower need for endogenous repair mechanisms at that age (154).

In a highly selected healthy population, without known cardiovascular risk factors, the frequency of PB endothelial colonies and the endothelial colonies cell-specific mitogen VEGF level seemed to be unrelated to age (53). Besides, in a large population-based study, it was demonstrated that a modest but significant decline in the pool of circulating EPCs emerged across the age range from 55 to 94 years. Therefore, it was suggested that EPC numbers may decline with advancing age (80). In patients with stable CAD, EPCs were inversely correlated with age (83). In another study, baseline levels of putative EPCs (CD34+CD31+, CD34+CD117+ or CD34+KDR+) were predominantly dependent upon age in CAD patients (84). In heart transplantation patients, EPCs (CD34+KDR+; CD133+KDR+ and CD34+CD133+KDR+) were reduced compared with healthy individuals matched for age and gender (107) and an age-dependent change in the CFU levels, representing EPCs, was found in patients with acute stroke, resulting in much lower CFU levels in aged patients (76). Age

was also identified to be significant independent predictors of severe ARWMC (28), as expected, since this disease is associated with age. In patients with chronic kidney disease stage V receiving haemodialysis therapy, a significant but only weak correlation was found between EPCs and age (132). In a study evaluating the possible association of EPCs (CD34+KDR+ and culture) with inflammation, endothelial dysfunction and atherosclerosis in chronic haemodialysis patients and healthy controls, lower number of EPCs were observed in haemodialysis patients. However, although the age difference between groups did not reach statistical significance, control group was younger than the study group and this fact may have influence on the differences between the groups (135). Additionally, in renal transplant patients, no significant correlation was found between age and EPCs concentration, despite the fact that patients were older than the controls (137). Finally, in a study that examined the clinical and genetic correlates of early-outgrowth CFUs in 1799 participants of the Framingham Heart Study, a lower number of CFUs was observed in older individuals (42).

From these results, age may has an impact on EPC levels, with older individuals presenting lower EPC levels. However, this relationship doesn't appear to be linear, insofar as adolescents present reduce levels of EPCs. Reduced level of EPCs in younger individuals compared to older ones may reflect a lower need for endogenous repair mechanisms at early age (154).

14. EPCs and gender

EPC number and function were investigated with respect to cardiovascular risk, gender, and reproductive state, in a sample representative of a healthy middle-aged general population. EPCs were defined as CD34+KDR+ cells using flow cytometry. EPC isolation and culture were also performed. After recruitment, women were retrospectively divided into fertile and postmenopausal. The best random selection of age-matched men represented the control group for postmenopausal women. It was found that gender differences in EPC number and function correlate with surrogate indexes of cardiovascular risk and EPC gender gradient is present at birth and fluctuates during lifetime. Also, worsening of the cardiovascular risk profile after menopause is associated with EPC decline and EPCs are regulated by 17β-estradiol *in vitro*. Fertile women included in this study had a healthier risk profile than men and postmenopausal women. Parallely, FMD and carotid IMT indicated a better vascular homeostasis in females than in males, and fertile women had higher levels of circulating CD34+KDR+ EPCs than men. This difference was likely related to gender *per se* rather than to the effects of concomitant risk factors, as shown by the independent association between

gender and EPCs in multivariate analysis. The impact of risk factors was stronger in women, and the EPC gender gradient was tapered in the presence of at least 2 cardiovascular risk factors. After menopause, there were no gender differences in the risk profile, FMD, carotid IMT, and CD34+KDR+ EPCs. Given that EPCs are actively involved in endothelial healing and reflect the global cardiovascular health, the gender-related difference in EPCs represents a plausible explanation for the difference in endothelial function and carotid IMT. After menopause, EPC reduction, attributable to aging, to the change in the reproductive state, and to the worsened risk profile, may cause endothelial dysfunction and predispose to atherosclerosis. Studying ovulatory women, the authors found that EPCs varies in phase with menstrual/hormonal cycle. Therefore, it was suggested that EPCs are mobilised to the peripheral circulation of fertile women on a monthly basis. In this view, the higher steady-state levels of circulating EPCs in fertile women than in men may reflect their cyclic mobilisation, possibly related to the vascular regeneration and remodeling taking place in the endometrium. Endometrial homing is likely to be directed by the local production of growth factors and chemokines (such as VEGF and SDF-1) that favors recovery after menstrual discharge, and governs vascular proliferation and remodeling. It was also found that female blood cells gave rise to a higher number of endothelial colonies than male cells, suggesting a gender difference in EPC generation. The quantitative and functional differences in EPCs between young men and women, together with the observation that EPCs are mobilised during the hormonal cycle, strongly indicated that EPCs are influenced by female sex hormones. It was confirmed that 17β-estradiol has a potent ability to stimulate human EPCs. Besides, it was reported that KDR expression on CD34+ cells was higher in fertile women than in men, although it was similar in postmenopausal women and in age-matched men. This may indicate that 17β-estradiol promotes differentiation of circulating CD34+ progenitor cells into CD34+KDR+ EPCs *in vivo*, but the authors failed to confirm this *in vitro*, as 17β-estradiol did not modify the expression of endothelial markers during EPC culture. It was demonstrated that 17β-estradiol dose-dependently enhances EPC generation and adhesion *in vitro*. Interestingly, male EPCs were more responsive to the mitogenic effects of 17β-estradiol, whereas female EPCs seemed to be maximally stimulated in basal conditions. To explore possible reasons for this different responsiveness to 17β-estradiol, gene expression of oestrogen receptor isoforms was quantified. Male EPCs expressed larger amounts of oestrogen receptors than female EPCs, with a prevalence of the vasculoprotective α isoform, which may mediate the potent functional stimulation induced by 17β-estradiol, perhaps through upregulation of VEGF. Nonselective and α-selective oestrogen receptor inhibition prevented the ability of 17β-estradiol to

promote generation and adhesion of EPCs, confirming that these effects are mediated by classical oestrogen receptor pathways. Interestingly, oestrogen receptor inhibition abolished the higher generation of female EPCs in both basal conditions and 17β-estradiol-stimulation, suggesting a sort of "constitutive" activation of oestrogen receptors in female cells, as pharmacological inhibition downregulates both ligand-dependent and -independent oestrogen activity. As sex hormone concentrations vary not only during the fertile cycle in women, but also with age, EPC gender differences throughout human ages were investigated. A marked difference in EPC levels in the cord blood of newborns in favour of females was reported. The predominance of oestrogen receptor β over oestrogen receptor α in male cord blood EPCs may be responsible for the lower levels of circulating EPCs: in fact, the 2 receptor isoforms act as antagonists and their relative expression drives downstream events that determine the net effect of 17β-estradiol. This may represent a sort of prenatal imprinting of the endogenous vascular regenerative potential. In prepuberal children, EPC gender difference was inverted, in favour of males, suggesting that female EPCs are more sensitive to the fall in sex hormone concentrations during the prepuberal phase. Finally, there was no EPC gender gradient in the elderly. Therefore, it was confirmed that age is one major determinant of EPC level in the multivariate analysis, but EPC level tended to be relatively stable over time in males, whereas it widely fluctuated in females. This trend, apparently inconsistent with the observation that 17β-estradiol influenced male EPCs more than female EPCs *in vitro*, could be attributed to a differential expression of oestrogen receptor isoforms, and to the different effects of continuous versus cyclic hormonal stimulation in men *versus* women. Summarily, the authors provide a series of data indicating that EPCs are regulated by sex hormones in humans. Cyclic EPC mobilisation may be related to endometrial regeneration; the resulting gender gradients in EPC number and function reflect the cardiovascular protection of the fertile female population. After menopause, on cessation of both ovarian and endometrial function, female EPCs decrease to the levels of coeval males, thus hampering vascular homeostasis and increasing cardiovascular risk (72).

Another study was conducted to evaluate which are the main clinical determinants of EPC levels (CD133+KDR+ and CD34+CD133+KDR+) in a population of healthy subjects with normal glucose tolerance. It was shown that the male gender is associated with reduced EPC number compared to age-matched premenopausal females in a population of young healthy non-diabetic adults. Males displayed lower EPC number when compared to age-matched premenopausal females and this observation was also confirmed in young healthy adults. Since the authors studied only premenopausal women, this might offer a point for speculating about

the different impact of insulin resistance and the associated cardiovascular risk factors on EPC levels in males compared to females. Giving the findings, it was suggested that the reduction in EPC levels in males might contribute to explaining the higher cardiovascular risk in males compared to premenopausal age-matched females (155), as mentioned in the previous study.
In a highly selected healthy population, without known cardiovascular risk factors, the frequency of PB endothelial colonies and the endothelial colonies cell-specific mitogen VEGF level seemed to be unrelated to gender (53). In a large population-based study, it was demonstrated that the EPC numbers are significantly lower in (predominantly postmenopausal) females than in males of equal age (80). In heart transplantation patients, EPCs (CD34+KDR+; CD133+KDR+ and CD34+CD133+KDR+) were reduced compared with healthy individuals matched for age and gender (107). Additionally, in renal transplant patients, no significant correlation was found between gender and EPCs concentration (137). Finally, in a study that examined the clinical and genetic correlates of early-outgrowth CFUs in 1799 participants of the Framingham Heart Study, a lower number of CFUs was observed in women. The women in this study were almost entirely postmenopausal, suggesting the possibility of a sex hormone effect (42).
The fact that some studies didn't find significant correlations between EPCs and gender doesn't mean that this relationship doesn't occur. It was suggested that gender differences may be masked by the underlying diseases, cardiovascular risk or advanced age (72). The possibility of studying EPC levels in healthy subjects allows investigators to obtain results without interfering factors. Besides, the first study mentioned in this topic (EPCs and gender) clearly demonstrates the difference between EPC levels in males and females.

15. EPCs and lifestyles

15.1. Smoking

The effects of chronic smoking and of smoking cessation on EPC levels (CD34+CD133+VEGFR2+CD45low and culture) were investigated in apparently healthy men. Non-smokers were recruited from healthy volunteers and smokers were recruited from a group of people who wanted to quit smoking. Subjects were not using any medications such as statin, antidiabetic drugs, or antihypertensive drugs. Smokers quit smoking on their own with or without nicotine patches. Unexpectedly, all smokers were only able to quit smoking for 1 month. The authors cultured attaching EPCs from PB from non-smokers, light smokers, and heavy smokers and compared the EPC count among these 3 groups. It was shown that the number of circulating EPCs was significantly lowered as the number of cigarettes consumed increased. In the smokers group, EPCs increased rapidly after smoking cessation and, then after smoking was resumed, again decreased to the level similar to that before smoking cessation. Finally, the magnitude of increase in EPC counts after smoking cessation was slightly greater in the nicotine patch users than in the nonusers, but this difference was not statistically significant. Chronic smokers had fewer circulating EPCs than did non-smokers at the beginning of the study. However, attaching EPCs from heavy smokers died during early phase of culture. The reason for this discrepancy is unknown. In contrast, the measurement of EPCs by cell surface antigen is not affected by culture conditions. Furthermore, identification of EPCs using flow cytometry allowed to assess the direct effect of smoking on circulating EPCs. Using CD34+CD133+VEGFR2+CD45low markers, the study further revealed that smoking cessation rapidly increased the number of circulating EPCs. This quick recovery of EPCs after quitting smoking was one of the most surprising findings in the study. The possible mechanisms for these findings are as follows. First, smoking may affect BM environment, and EPCs mobilisation from the BM could be decreased by smoking. In fact, smoking is a factor inhibiting the release of physiological amounts of NO produced by eNOS, and it was already suggested in this dissertation that eNOS is important for EPC mobilisation from BM. Second, change in EPC levels associated with smoking status is possibly related to the fact that chronic smokers have endothelial dysfunction. Because smoking cessation rapidly improves endothelial function, the changes in EPC levels and endothelial function would be parallel. Besides, EPCs may accelerated re-endothelialisation of injured endothelium. Taken together, one possible explanation is that injured vessels in smokers may use EPCs to maintain endothelial function, and that the increase in circulating EPCs after smoking cessation may be the result of a

decreased number of injured vessels after cessation. These 2 hypotheses could account for the phenomenon that resuming smoking substantially reduced EPC levels in PB in this study. There are 2 additional findings in this study. First, the magnitude of the increase in EPCs after smoking cessation was slightly higher among nicotine patch users than nonusers. However, this difference was not statistically significant. Therefore, the effect of nicotine may account for the small increase in EPC levels in the nicotine patch users. Second, the magnitude of the increase in EPCs after smoking cessation was smaller in heavy smokers than in light smokers. The latter finding suggests that light smokers may have a better chance of recovery in terms of circulating EPCs after smoking cessation. This fact also indicates that BM or endothelium in heavy smokers is irreversibly damaged, at least in part. In summary, "on-and-off" cigarette smoking markedly influenced the number of circulating EPCs in apparently healthy chronic smokers. Because the decreased number of EPCs in peripheral circulation is a strong predictor for cardiovascular risk, these findings provide further evidence for the consensus that smoking cessation is to be highly recommended for the prevention of cardiovascular diseases, and the measurement of EPCs in PB is a new tool to monitor smoking-related cardiovascular burden in chronic smokers (156).

In a another study, smoking was associated with high baseline levels of EPCs CD34+KDR+ and reduced numbers of colony-forming units of endothelial cells, used in order to determine the functional capacity of circulating EPCs, in CAD patients (19). The results of this study are in contrast with the previous one, with respect do EPCs measured by flow cytometry. However, it has to be taken into consideration that, in the first study, EPCs were quantified in apparently healthy subjects, unlike the latter study in which cell levels were measured in CAD patients.

Another study tested the hypothesis that cigarette smoking is associated with EPC dysfunction. The direct effect of smoking on EPCs was studied in healthy subjects that had no symptoms associated with atherosclerosis and did not present any other conventional cardiovascular risk factors. The number of EPCs (characterised as dual staining cells positive for both DiI-acLDL and lectin) that could be isolated from the PB of chronic smokers was reduced by more than 50% compared to a control group of non-smoking healthy subjects. Low levels of circulating EPCs in smokers could be caused by a number of mechanisms. In this study, it was found that ROS formation was significantly increased in EPCs isolated from smokers. Although this was not associated with an increased cellular death in culture, high levels of oxidative stress in smokers could potentially influence the mobilisation and/or the survival of EPCs *in vivo*. Decreased plasma antioxidant levels and EPC counts in smokers were correlated with reduced

availability of NO. Therefore, lower NO levels could contribute to the reduction of peripheral EPCs in smokers. Another possibility for the reduced number of EPCs is that continuous endothelial damage or dysfunction caused by cigarette smoking eventually leads to exhaustion of EPC supply. It seems logical to believe that reduced EPC levels in smokers is the consequence of decreased supply in the setting of increased progenitor cell utilisation for vascular repair. It was also demonstrated that cigarette smoking does not only influence the absolute number of EPCs, but also significantly modulates their functional activities. Cellular proliferation and migration in response to VEGF were significantly impaired in EPCs isolated from smokers. Moreover, EPCs from smokers were found to exhibit a reduced adherent ability. These processes are obviously important for vascular healing and re-endothelialisation following injury, but are also essential in the setting of neovascularisation in response to ischaemia. In summary it was demonstrated that cigarette smoking is associated with a reduced number of EPCs together with an important impairment of EPC functional activities. Potential mechanisms responsible for the negative effect of smoking on EPCs include increased oxidative stress, decrease NO availability and impaired EPC differentiation towards an endothelial phenotype. The authors proposed that EPC dysfunction associated with smoking impairs vascular healing following injury and therefore contributes to the pathogenesis of atherosclerotic vascular diseases. Moreover, EPC dysfunction in smokers might significantly impair neovascularisation in response to ischaemia and limit the efficacy of cellular therapies aiming at blood flow restoration (157).

Owing to the fact that polycyclic aromatic hydrocarbons, such as benzo(α)pyrene, constitute major components of tobacco smoke, a study was designed to analyse the effects of these chemicals on the development of human EPC cultures from PB MNCs The data from this study bring a molecular support to the deleterious effects of smoking on the number and function of EPCs, described before. Indeed, treatment by polycyclic aromatic hydrocarbons such as benzo(α)pyrene, which constitute major components of cigarette extract, was shown to markedly impair EPC number and EPC colonies in cultures established from PB MNCs. This effect of BP was shown to be dose-dependent. Besides, relatively low concentration of benzo(α)pyrene is sufficient to exert toxicity towards EPCs. This suggests that smoking allows to reach *in vivo* polycyclic aromatic hydrocarbons concentrations required to affect EPCs. The fact that PB MNCs of heavy smokers, which therefore have been *in vivo* exposed to polycyclic aromatic hydrocarbons, failed to *in vitro* generate EPCs and became swollen and perished in cultures (156), fully supports this hypothesis. In summary, *in vitro* treatment by polycyclic aromatic hydrocarbons was shown to markedly impair the development of EPC cultures from

human PB MNCs. This effect likely accounts for the alteration in the number and function of EPCs, occurring in smokers. Owing to the protective role of EPCs towards cardiovascular diseases, these data highlight a new mechanism, namely EPC targeting, by which polycyclic aromatic hydrocarbons may affect the cardiovascular system (35).

The changes in the number and function of EPCs isolated from individuals who successfully completed a 5-week smoking cessation programme were investigated. Interestingly, flow cytometry studies revealed unchanged numbers of circulating EPC, defined as CD34+VEGFR2+ or CD34+CD133+ cells. The apparent discrepancy between previous findings and the results of the present study may be related to differences in the baseline clinical characteristics of both study collectives. For example, the authors did not select 'healthy' smokers. Instead, over half of the study population consisted of individuals with additional cardiovascular risk factors (or known cardiovascular disease), and 24% of the study participants took anti-diabetic, anti-hypertensive, or lipid-lowering medication throughout the study. However, comparison of findings in 'healthy' subjects with those persons with cardiovascular risk factors or disease, or in light (<20 cigarettes per day) *versus* heavy (≥20 cigarettes per day) smokers, respectively, revealed similar findings and no differences between the groups. Although the present study focused on the number of circulating CD34+ cells before and after smoking cessation in the same individual rather than on possible differences between smokers and non-smokers, studies comparing circulating EPC levels in smokers and non-smokers also reported contradicting findings. Of note, the number of circulating EPC is not only determined by EPC release from the BM, but also by their recruitment into damaged tissues or loss due to increased consumption in the periphery, which means, by parameters most likely to be altered in smokers. In addition, early outgrowth EPCs were isolated from the PB MNC fraction and their adhesive properties were examined. These studies revealed a reduction in the number of acLDL and lectin positive cells which could be expanded from PB monocytes in culture after smoking cessation. Smoking cessation also reduced EPC adhesion and attenuated their incorporation into endothelial cell networks. The aim of this study was not to compare EPC from smokers and non-smokers, but from the same individual before and after a smoking cessation intervention, and findings from those earlier studies can thus not be directly compared to the ones of the present analysis. Although this study's analyses revealed unchanged HDL and reduced LDL cholesterol levels, we cannot exclude the possibility that the observed functional impairment of EPC after smoking cessation may be the consequence of the observed significant increase in body weight, although no correlation was found to exist between the number of acLDL+, lectin+ cells and the BMI. According to the authors, this

study's results are in agreement with the hypothesis that cigarette smoke and the associated oxidative stress may enhance the adhesive interactions between EPCs and endothelial cells. In this regard, the EPC analysed in this study are derived from myeloid lineage cells and share both monocytic and endothelial cell features, which may render them susceptible to inflammatory changes associated with cigarette smoking. Smoking cessation may withdraw such 'activating' stimuli. Importantly, nicotine replacement therapy, which was utilised by approximately 70% of this study's participants, did not affect the number of acLDL+, lectin+ progenitor cells in culture. It was found that smoking cessation was associated with decreased circulating levels of inflammation-sensitive proteins such as fibrinogen and with reduced plasma ADMA levels and intracellular ROS formation. It may be speculated that these effects contributed to the reduction of EPC adhesiveness observed in the study. The findings of this study indicate that the impact of smoking and smoking cessation on the balance between mobilisation of EPC from the BM and adhesion to matrix proteins and activated cells of the injured vessel wall is far too complex to be simply expressed as changes in the numbers of circulating or cultivatable progenitor cells. Instead, they also show that the contribution of EPC to the beneficial effects of smoking cessation on cardiovascular end point and the improvement of endothelial cell function and repair requires further analysis. In particular, future studies should focus on clarifying how systemic inflammation and local vascular injury regulate homing of EPC, and how these processes are affected by withdrawal of 'activating' stimuli such as cigarette smoking (158).

Finally, a study evaluated whether the menopausal transition has impact on cardiovascular risk factors in a population-based group of middle-aged women. It was demonstrated that reproductive status strongly modified the effect of smoking on carotid arteries, circulating EPCs, reverse cholesterol transport, and fluctuation of free testosterone. The differences of risk factors under study between smoking and non-smoking women were detected almost uniformly only in women undergoing menopausal transition. Summarily, in a population-based group of middle-aged women, the atherogenic impact of smoking appeared to be significantly modified by reproductive status. It was found that menopausal transition especially in the presence of smoking could be more sensitive period than premenopause or menopause for atherosclerosis process in carotid arteries. This impact could be mediated through several mechanisms including impaired vascular protection, impaired reverse cholesterol transport, and impaired balance of sex hormones (159).

15.2. Exercise

Regular physical exercise improves endothelial dysfunction and promotes cardiovascular health (160). Mobilisation of BM-derived EPCs might explain the exercise-induced improvement of endothelial function (161). The effect of training on angiogenesis was investigated by measuring the number of circulating EPCs and the level of EPC-mobilizing growth factors, in patients with CAD and cardiovascular risk factors. Vascular function was also assessed using FMD. In addition, degradation products of the NO pathway were determined in order to evaluate NO production. Patients with documented CAD and/or cardiovascular risk factors joined a 12-week supervised running training. Circulating EPCs (CD34+CD133+KDR+) were measured at baseline and after exercise training. EPCs circulating in PB significantly increased in participants after 12 weeks of exercise training. In control subjects, the number of EPCs did not change during follow-up. In addition, the number of EPC was comparable between volunteers with cardiovascular risk factors and CAD patients at baseline and after training. The increase in circulating EPCs was positively correlated with both, the change of FMD and the increase of degradation products of the NO pathway. Plasma VEGF and EPO did not change in response to exercise. However, the authors observed a positive correlation between the number of EPCs and EPO at baseline and after training. Summarily, regular exercise training augments the number of circulating EPCs in patients with cardiovascular risk factors and CAD and is associated with improved vascular function and NO synthesis (160).

Another study was designed in order to assess whether a single bout of exercise can alter the number of circulating EPCs (CD34+KDR+) in the absence of vascular disease and whether this effect is related to the subject's lipid profile. It was presented that a single maximal exercise bout in healthy subjects elicited a larger increase in CD34+/KDR+ EPCs than in CD34+ cells, suggesting a shift in circulating CD34+ cells toward CD34+/KDR+ EPCs. Although still within normal limits, higher LDL and higher total cholesterol/HDL levels favoured the exercise-induced increase in EPCs. Contrary to circulating CD34+/KDR+ EPCs, the number of CFUs remained unchanged after exercise, and no relation was seen with lipid levels. An increase in VEGF concentration was identified, with a strong trend toward significance. The observed increase in VEGF levels underscores its possible role as an EPC-mobilizing factor. However, there is little data on the time course of serum VEGF regulation following a single bout of exercise. Changes in VEGF levels over a larger period of time, particularly in relation to changes in circulating EPC, could provide arguments for the possible paracrine mechanism exerted by EPCs and should be studied in future experiments. With respect to the role of

eNOS upregulation and NO dependency in mediating exercise training-induced EPC liberation, the study failed to report an increase in NO bioavailability. Possible explanations include the fact that NO levels are assessed indirectly through NO metabolites and that the assay is limited by various interfering factors. Besides, the effect of acute exercise might be short lived, and interference with NO metabolism could well be a paracrine and local endothelial phenomenon. It was also suggested that in healthy individuals, higher lipid levels, conveying a pro-oxidant vascular environment, provide a stronger EPC-mobilising stimulus. Although it seems contradictory at first glance, it could be hypothesised that exercise in these individuals, characterised by an endothelium "under stress," might stimulate the generation of ROS, thereby triggering repair mechanisms. Summarily, this study demonstrates that a single bout of exercise induces a significant shift in circulating CD34+ cells toward CD34+/KDR+ EPCs in healthy subjects. This response appeared to be larger in subjects with a less favourable lipid profile, which could be a physiological explanation for the well-known benefit of exercise in patients who are prone to develop or already manifest with atherosclerotic disease (161).

Exercise can play a vital role in primary and secondary prevention of CAD. It was hypothesised that a higher habitual physical activity is associated with an increased circulating EPCs, which improve vascular endothelial function in patients with CAD. Therefore, the relationships between habitual physical activity, brachial FMD and circulating EPC numbers (CD34+KDR+ and CD133+KDR+) were investigated in patients with stable CAD. Habitual physical activity level was assessed by using a validated International Physical Activity Questionnaire. In order to better delineate the relationship between habitual physical activity level and different clinical characteristics, the study population was grouped into three tertiles of physical activity level according to the patients' total weekly energy expenditure. Accordingly, as the energy expenditure per week is higher, higher is the tertile. It was shown that higher habitual physical activity in patients with stable CAD was associated with higher FMD and EPC count. Physical training increased EPC numbers and migratory activity, which may be mediated through upregulation of NO and VEGF as well as a reduction in EPC apoptosis. In CAD patients, exercise exerts shear stress on the vascular wall, and induces Akt-dependent eNOS phosphorylation, resulting in augmented NO synthesis and, consequently, mobilisation of EPCs from BM. Although higher habitual physical activity in patients with stable CAD was associated with higher FMD and EPC count, it was shown that FMD only significantly correlated with increased physical activity level but not EPC count. This suggests that higher habitual physical activity level may improve endothelial function through mechanisms other than increasing the

EPC count. This study showed that higher habitual physical activity increased CD133+KDR+ but not CD34+KDR+ EPCs. It is possible that higher habitual physical activity level enhances the mobilisation of the more immature EPCs, like CD133+KDR+ cells, which might have a more prominent effect on angiogenesis than endothelial repair compared with more mature EPCs, like CD34+KDR+ cells. The study also showed that increasing tertiles of physical activity level were associated with a significant reduction of diastolic blood pressure, after adjusting for age, sex, and antihypertensive use. However, there were no changes in other variables such as systolic blood pressure, total, HDL-C and LDLC, and hs-CRP. Nevertheless, there were no significant relationships between diastolic blood pressure with FMD and EPC count. These findings suggest that increased habitual physical activity did not alter the lipid profile nor attenuate inflammation, and at the same time it improved FMD and EPC levels independent of its antihypertensive effect. In this study, both FMD and EPC count showed similar increasing trends with higher tertiles of physical activity level. However, significant differences in FMD and EPC count were only observed in the highest tertile of physical activity, suggesting a possible threshold level of habitual physical activity for its vascular protective effects. The effect of habitual physical activity level on FMD was adjusted for age, sex, hypertension, DM, hypercholesterolemia, smoking status, and use of medications including β-blockers, angiotensin-converting enzyme inhibitors or angiotensin receptor blockers and statins, as these factors affect FMD. It is also worthwhile to point out that only the total volume of habitual physical activity was related to the difference in FMD and EPC levels, but the amount of vigorous physical activity alone was not predictive of changes in FMD and EPC count. Similarly, the amounts of moderate activity or walking exercise also did not determine such parameters, indicating that higher habitual physical activity modulates FMD and EPC count independent of exercise intensity. As endothelial dysfunction is an independent predictor for future cardiovascular events, a higher habitual physical activity level in these high-risk patient populations may have a beneficial effect in reducing cardiovascular morbidity and mortality (162).

The effect of exercise training on endothelial function and exercise capacity was evaluated in patients with CAD, in another study. A randomised, controlled trial was conducted to determine the effects of an 8-week exercise training programme versus controls on brachial FMD in patients with stable CAD. The involvement of EPCs (CD34+KDR+) was also assessed. It was demonstrated that the 8-week exercise training programme improved endothelial function and exercise capacity in patients with stable CAD. The changes in FMD and exercise capacity are inter-related, and more pronounced in those with baseline impaired exercise

capacity. Furthermore, exercise training also increased HDL cholesterol level and reduced resting heart rate and diastolic blood pressure compared with controls. Nevertheless, there were no significant changes in inflammation, oxidative stress or circulating EPC count after exercise training. These findings suggest that improving endothelial function and altering conventional cardiovascular risk factors such as lipid profile, blood pressure, and heart rate by exercise intervention might contribute to its cardiovascular protective effect in patients with established CAD. Summarily, exercise training improved FMD and exercise capacity in stable CAD patients independent of the changes in inflammation, oxidative stress, or EPCs (163).

The impact of exercise training on numbers of EPCs (CD34+KDR+) was studied in patients with chronic heart failure. Sedentary subjects with chronic heart failure underwent 6-month exercise training and were compared to a non-trained control group and healthy age-matched subjects. The impact of an acute maximal exercise bout was also assessed. Exercise training significantly increased the number of circulating EPCs. The levels of SDF-1α remained unchanged following exercise training, whereas an increase could be demonstrated in the non-trained control group. The training-induced increase in EPCs could have minimised the need for a further compensatory rise in SDF-1α. With respect to the impact of an acute maximal exercise bout, despite a rapid increase in SDF-1α levels following acute exercise, numbers of EPCs remained unchanged. According to the authors, the findings of this study demonstrate for the first time that exercise training in chronic heart failure patients increases levels of EPCs, which coincides with a beneficial influence on peripheral endothelial function (164).
The effect of a short-term (3 weeks) exercise training program on the number of circulating EPCs (CD34+KDR+) was studied in chronic heart failure patients as well as on serum capacity to foster EC CFUs *in vitro*. Effectiveness of training was assessed by the 6-minute walking test. PB and serum were obtained from patients with chronic heart failure due to CAD before and after an inpatient aerobic exercise training program. Patients were included in the study if they had LVEF < 40%, with NYHA class II, and clinical stability without hospital admission for heart failure in the previous 3 months. The number and function of EC CFU colonies were evaluated in cultures performed with serum obtained before and after training. According to the authors, this is the first study to use serum from heart failure patient, instead of foetal bovine serum as usual, to set-up EC CFU cultures and to report increased proliferation in cultures with serum obtained after a structured exercise training program. Exercise training resulted in a significant increase in circulating EPCs and in EC CFU number, the latter being important for vascular homeostasis and regeneration. The study indicates that exercise training could exert beneficial

effects in patients with chronic heart failure, by improving EC CFUs growth through the modification of serum composition. The authors found an inverse correlation between TNF-α levels and EPCs in PB of NYHA class II chronic heart failure patients, a finding that would be consistent with the hypothesis that TNF-α could be one of the factors that dampen EPCs in PB of patients with chronic heart failure. Although in this study exercise training decreased serum TNF-α levels, the authors did not find any significant difference in EC CFU number or adhesion capacity in cultures tested with and without an anti-TNF-α neutralizing antibody. This result could suggest that in this experimental model TNF-α does not influence the number of colonies or their adherence ability *in vitro*. Summarily, this study suggests that among the many beneficial effects of exercise training in chronic heart failure patients, improvement of serum ability to support viability of EPCs (in terms of CFU-EC colonies and circulating CD34+KDR+ cells) should also be considered (31).

The role of acute ischaemia on EPC (CD34+KDR+ and CD133+KDR+) mobilisation was assessed in patients with peripheral arterial occlusive disease exposed to exercise induced limb ischaemia. It was demonstrated that a single episode of exercise-induced tissue ischaemia is sufficient to increase the plasma level of VEGF and the amount of circulating EPCs. As a clinical implication, the authors have confirmed that a vigorous 4-week exercise training above the ischemic threshold, a well-established therapeutic concept in peripheral arterial occlusive disease patients, leads to a four-fold increase in circulating EPCs, thereby possibly enhancing neovascularisation, endothelial repair, and alleviation of symptoms. A possible approach to enhance circulating EPCs in peripheral arterial occlusive disease patients in advance to such procedures might be the use of a single maximal exercise bout to optimise quantity of EPCs. The findings of this study suggested that EPCs are a potential target for non-pharmacologic interventions such as exercise training (105).

Supervised exercise training is recommended as initial treatment to improve walking capacity in PAD patients with intermittent claudication. A prospective randomised controlled trial was designed to study the impact of supervised exercise training on markers of angiogenesis and endothelial function in PAD patients. Forty PAD patients were randomised to supervised exercise training on top of best medical treatment (antithrombotic therapy, antihypertensive therapy and statins, in this study population) for 6 months versus best medical treatment only. EPCs were assessed (CD34+CD133+KDR+ and culture) at baseline, 3, 6 and 12-months after inclusion. Changes of plasma levels of ADMA, VEGF, SDF-1 and maximum walking distance were determined. In this randomised controlled trial, a significant increase of maximum walking distance was found in PAD patients receiving a twice-weekly supervised exercise

training compared to best medical treatment only. Remarkably, increased walking capacity increased further six months after supervised exercise training cessation suggesting an ongoing benefit of the intervention. Importantly, it was found that a feasible exercise program, which is suitable for PAD patients in "real world" and might easily be implemented in the daily routine, significantly increases circulating EPC measured by various techniques. No significant changes were observed for VEGF and SDF-1 plasma levels in time course. Interestingly, the exercise induced increase of EPC counts did not sustain 6 months after training cessation, while ADMA levels were still decreased. The decrease of EPC counts suggests that daily physical activity without controlled training lacks the necessary intensity to increase shear stress and trigger ischemic stimuli as a prerequisite for EPC mobilisation. However, the sustained decrease of ADMA, which is a marker of endothelial dysfunction, might indicate persistent improvement of vascular function beyond supervised exercise sessions. In addition, a more pronounced increase of maximum walking distance was found at 12 months compared to the results at the end of the training program. The missing correlation between EPCs measurements and maximum walking distance as well as the differing time response of EPCs increase and improvement of walking capacity could indicate that the observed EPCs mobilisation upon training plays only a limited role for improvement of walking ability but rather reflect overall cardiovascular benefits. Summarily, supervised exercise training as it can be provided to PAD patients in clinical routine, increases EPC counts and decreases ADMA levels suggesting enhanced angiogenesis and improved endothelial function. By this mechanism, exercise training might contribute to cardiovascular risk reduction in PAD patients (41).

To determine the effects of physical training on circulating EPCs (CD34+KDR+CD45dim), angiogenesis and inflammation, the data obtained from chronic heart failure patients before and after 3 months of aerobic exercise training was compared to those from non-trained chronic heart failure patients. It was demonstrated that even a shorter training period of 3 months, performed at lower intensity levels compared to a longer protocol (164), is able to induce mobilisation of EPCs, together with improving endothelial dysfunction and exercise aerobic capacity in chronic heart failure patients. In the series of chronic heart failure patients, a statistically significant improvement in endothelial dysfunction, both in terms of increased FMD of the brachial artery and of blood EPC count, was found. SDF-1α plasma levels remained stable in the training group, while they slightly increased in the control group. This paradoxical finding may be due to different and alternative roles played by SDF-1: progenitor cell mobilisation, chaemoattraction for lymphocytes, increase of B cell proliferation, immune

surveillance and, finally, homing of lymphocytes. All these functions are related to systemic inflammation. A trend to increase of inflammatory markers was observed in the chronic heart failure non-trained group, in line with the increase of SDF-1α levels. Though the authors do not have a specific explanation for this finding, it seems to give more reliability to the opposite trend of the same pro-inflammatory markers in the chronic heart failure trained group. In the series of trained chronic heart failure patients, the authors detected a trend to decrease of IL-6, but without reaching statistical significance. They did not find any significant variation at follow-up versus baseline in terms of VEGF levels, either in the chronic heart failure non-trained group or trained group. While VEGF receptor expression is up-regulated by exercise, exercise may not be translated into elevated VEGF protein levels. This might explain the finding of stable serum levels of VEGF in the trained chronic heart failure population, without excluding an activation of proangiogenic molecules. The increased number of EPCs might not necessarily be directly related to the increased proangiogenic stimulus (this observation would be confirmed by the trend observed of SDF-1α), even though it might contribute to the functional improvement observed after the training program. In conclusion, these preliminary results bring data in support of an interrelationship between endothelial repair and the activation of proangiogenic mechanisms occurring even after a moderate and time-limited physical training program, and they prompt the authors to better evaluate the functional and biologic consequences of longer and stronger periods of training in a wider population of stable chronic heart failure patients (165).

Circulating EPCs might limit endothelial dysfunction in patients with microvascular angina. Exercise-induced ECFC mobilisation and platelet reactivity were evaluated in patients with microvascular angina or with obstructive CAD. Exercise stress test was performed in patients with microvascular angina, CAD patients and controls. EPCs were assessed as ECFCs, which display the phenotype CD34+VEGFR2+CD45-. ECFC number was measured before and 24 hours after exercise stress test. The main objectives of this study were to evaluate whether EPC mobilisation by exercise could display some favourable pattern in patients with microvascular angina, compared to CAD patients, and whether any relationship existed in these patients between EPC and platelet responses to exercise. However, no potentially protective behaviour of EPCs in patients with microvascular angina was identified. At the same time, no significant relationship was found in patients with microvascular angina between platelet and EPC responses to exercise stress test. The authors failed to find any significant difference at rest in the number of circulating EPCs in patients with microvascular angina

patients compared to controls and CAD patients, although EPC level tended to be reduced in the latter group. The reasons for these discordant results are not clear, but differences in patient selection, drug therapy, method used for EPC assessment and type of EPC assessed might have played a role. Of note, ECFC mobilisation in response to exercise stress test in patients with microvascular angina patients in this study also did not show any favourable behaviour compared to the other groups. The data from this study, in fact, showed a lower increase of ECFCs after exercise in patients with microvascular angina patients, compared to both healthy controls and CAD patients, although this result should be taken with caution, due to the lack of statistically significant changes in ECFC level within groups, likely related to the low number of patients. Finally, it should be underscored that, in agreement with the different individual behaviour, the authors failed to find any correlation between platelet and ECFC responses to exercise in patients with microvascular angina patients, thus suggesting that the two findings are regulated by different mechanisms. Summarily, it was shown that the CD34+KDR+CD45- ECFCs response to exercise in patients with microvascular angina patients does not contribute to a protective role of the endothelial system in these patients (166).

A sedentary workforce may be at increased risk for future cardiovascular disease. Exercise at the work site has been advocated, but effects on endothelium as a biomarker of risk and relation to weight loss, lipid changes, or circulating EPCs have not been reported. Seventy-two office and laboratory employees completed 3 months of participation in the National Heart, Lung, and Blood Institute's Keep the Beat program, with the determination of vital signs and laboratory data during treadmill exercise. Brachial artery FMD was inversely associated with Framingham risk score at baseline. It was determined that level of fitness and brachial artery endothelial function were inversely associated with Framingham risk score in this cohort of largely overweight or obese employees with sedentary occupations. Besides, it was found that 15 to 20 minutes of exercise daily, using facilities provided at the work site, can improve endothelial function in a relatively brief period of 3 months. Although obesity at baseline was associated with diminished exercise fitness and higher Framingham risk score, benefits of program participation to exercise fitness, lipids, blood pressure, and endothelial function were independent of body mass at baseline as well as weight loss during program participation, which was minimal for the entire group. Increases in the number of EPCs measured by colony assay and, in a subset, by flow cytometry, were found. The increase in EPC colonies made a small but statistically significant contribution to improvement in

endothelial function in the study participants. In conclusion, daily exercise achievable at their work sites by employees with sedentary occupations improves endothelial function, even with the absence of weight loss, which may decrease cardiovascular risk, if sustained (167).

16. EPCs and weight

Being overweight or obese is associated with an increased risk for the development of DM, hypertension, and cardiovascular disease. Dyslipidaemia of obesity is characterised by elevated fasting triglycerides and decreased high-density lipoprotein-cholesterol concentrations. Endothelial damage and dysfunction is considered to be a major underlying mechanism for the elevated cardiovascular risk associated with increased adiposity. The effect of a low calorie diet in combination with oral supplementation by vitamins, minerals, probiotics and human chorionic gonadotropin (hCG) on the body composition, lipid profile and circulating EPCs (CD34+CD45-) was investigated. In this study, the authors analysed the effect of weight loss on the improvement of lipid profile in plasma and the increase of the level of circulating EPCs. Weight loss was accompanied by a significant improvement in the number of circulating EPCs. It was demonstrated that a combination of a very low calorie diet with hCG treatments, and supplements, decreases overall mass and body fat while improving lipid profiles. These benefits are accompanied by increases in circulating EPCs. In conclusion, the weight loss program analysed in this study resulted in the improvement of the number of EPCs in circulation and the decrease of the values of cardiovascular risk factors. According to this study, the circulating EPC number can be improved by diet and weight loss (168).

As described before in this document, the number of circulating EPCs (CD34+CD133+KDR+ and CD133+KDR+) has been evaluated in male adolescents and compared with adult controls. The analysis of EPC numbers in conjunction with cardiovascular risk factors revealed a higher number of CD133+KDR+ EPCs in obese teenagers. Levels of CD133+KDR+ EPCs were higher than CD34+CD133+KDR+ EPCs in adolescents with overweight and normal weight adolescents, respectively. Therefore, it was suggested that overweight has a significant impact on the number of CD133+KDR+ EPCs, since overweight adolescents have more EPCs than thinner adolescents. The authors did not found a significant correlation between EPCs and VEGF, although there was a significant difference between adolescents with overweight and normal weight adolescents. This may be explained by the fact that VEGF is also expressed in adipocytes, the plasma VEGF concentration was revealed to be dependent on visceral fat accumulation. In accordance to that, VEGF plasma levels are elevated in obese adults.

Therefore, in this study, where no ischaemia was applied to the subjects, it was suggested that VEGF might also be applicable as a parameter to evaluate body fat and its consequences on the molecular level. The VEGF release is insulin dependent and the ability to stimulate VEGF release by adipocytes is discussed to be of importance for some of the deleterious effects of high levels of circulating insulin seen in obesity. The regulation of SDF-1 seems to be dependent on overweight due to a significant difference between adolescents with overweight and normal weight adolescents, favouring the normal weight group for higher SDF-1 concentrations. This may be understood by the fact that SDF-1 is expressed at lower levels with increasing blood sugar and peripheral insulin resistance. Higher blood sugar (HbA1c elevated) and beginning peripheral insulin resistance (indicated by significant elevated adiponectin and waist circumference) were found in the adolescents with overweight. Summarily, it was suggested that overweight has a significant impact on the number of CD133+KDR+ EPCs in adolescents (154). In another study, which examined the effect of social and behavioral variables on the body weight in adolescents, it was suggested that overweight may lead to the development of endothelial dysfunction and alterations of circulating EPCs in adolescents (169).

17. EPCs and drugs

17.1. Statins

It was already mentioned that high levels of ADMA, an endogenous inhibitor of mobilisation, differentiation, and function of EPCs, contribute to cardiovascular risk and may explain low numbers and function of EPCs in patients with CAD. Although it is revealed in the literature that statins increase the number of circulating EPCs (170), this was not observed in a group of patients with stable angina and/or CAD and a healthy control. In stepwise multivariate regression analysis, it was found that ADMA and CAD, independently related to the number of circulating EPCs (CD34+CD133+) and factors like age and drug treatment, including statins, were not significant predictors. In a similar analysis, it was found that only ADMA significantly predicted endothelial CFUs. Therefore, the authors undertook further *in vitro* studies. Cultured EPCs from patients and controls were pre-treated with ADMA, as well as ADMA plus rosuvastatin. It was demonstrated that ADMA reduced the amount of cultured EPCs. The HMG-CoA reductase inhibitor rosuvastatin normalised eNOS activity in ADMA-treated EPCs, and reversed the inhibitory effects of ADMA on EPC differentiation and function.

In this study more patients with the diagnosis of CAD were being treated with statins at the time of admission to the hospital when compared with patients without CAD. Although statins increase the number of circulating EPCs, this was not observed in this study cohort, as mentioned above. There are two possible reasons. First, dose-dependent effects of statin treatment regarding the amount of mobilised EPCs are currently not known. Higher doses of certain statins might result in stronger improvement of EPC mobilisation and function. It is likely that if patients with severe CAD were not being treated with statins, EPC levels could be even lower. Second, the inhibitory effects of ADMA may be far stronger compared with statin effects, and thus the development of novel drugs that effectively reduce ADMA levels could lead to a novel therapeutic concept in the prevention and treatment of atherosclerosis based on improved mobilisation and function of EPCs. However, exposure to rosuvastatin normalised EPC differentiation and function in *in vitro* assays. Therefore, it was suggested that, because patients suffering from CAD have reduced numbers, colony-forming capacity, and migratory response of EPCs, rosuvastatin may be an interesting drug for both quantitative and qualitative improvement of EPCs in patients with cardiovascular disease, but further studies are needed to check this possibility (33).

In another study, *ex vivo* pre-treatment with atorvastatin improved the eNOS expression of ECFCs from CAD patients, although not up to the baseline level of controls, and attenuated the activation levels of ECFCs (49).

The hypothesis that statins, beyond lipids-decreased effect, might have different effects in different doses of statins on circulating EPCs (culture, dual staining for both lectin and DiI-acLDL) and circulating EMPs (CD31+/CD42b- (CD42b is a platelet surface membrane glycoprotein)) was investigated in patients with ischaemic cardiomyopathy and healthy controls. Patients were randomly divided into 2 groups, according to the dose of atorvastatin (10 mg versus 40 mg), and were followed for 1 year. At the beginning of this study, there were no significant differences in age, sex, lipids, hs-CRP, circulating EMPs, and circulating EPCs between the 2 study groups. At the end of the study, the serum levels of total cholesterol, cholesterol LDL, oxidised LDL, hs-CRP and circulating EMPs significantly decreased, but the levels of circulating EPCs significantly increased in the 40 mg atorvastatin group in contrast to the 10 mg atorvastatin group. The multivariate linear regression analysis indicated that only subjects receiving 40 mg of atorvastatin had significant effect on the levels of circulating EPCs, which suggested that 40 mg atorvastatin might contribute to a stronger effect on blood vessel formation ability and recovery of endothelial cells in patients with ischaemic cardiomyopathy in contrast to 10 mg atorvastatin. But the exact effect of statins on peripheral circulating EPCs and EMPs in patients with ischaemic cardiomyopathy remains to be confirmed. Although the significantly increasing number of circulating EPCs in the 40 mg atorvastatin group might be partly due to lower suppressing effects on EPCs by oxidation and inflammation, the exact mechanism was unclear. Therefore, these findings suggested that 40 mg of atorvastatin could significantly decrease the levels of serum oxidised LDL and alleviate the levels of total cholesterol oxidation as well as decrease the levels of total cholesterol and cholesterol LDL, in contrast to 10 mg of atorvastatin. In addition, at the end of this study, the levels of serum hs-CRP significantly decreased in the 40 mg atorvastatin group. Therefore, it was suggested that 40 mg of atorvastatin could significantly alleviate the levels of systemic inflammation, in contrast to 10 mg of atorvastatin. Summarily, in comparison with 10-mg atorvastatin, 40-mg atorvastatin was found to significantly decrease the levels of circulating EMPs and increase the number of circulating EPCs, possibly in part because of its stronger effect on inflammation and oxidation. The study findings might provide support for the use of statins in patients with ischaemic cardiomyopathy (121). It was demonstrated, in another study, that the application of atorvastatin directly to cultured cells affected micro-ribonucleic acid (miRNA) expression in EPCs. Besides, dysregulation of angiogenic miRNAs was detected in EPCs from CAD

patients. Therefore, the data from this study suggested that miRNAs may play an important role in regulating EPC function. Modulation of miRNA activities, thereby restoring the regenerative potential of EPCs, may provide a therapeutic target for the prevention or treatment of cardiovascular disorders (171).

In a study evaluating the association between EPC numbers, EPC CFUs, cardiovascular risk factors and life-style behaviours in a large population-based study, subjects with statin therapy exhibited higher EPC levels (80).

In another study, reduced levels of putative EPCs, defined as CD34+CD31+, CD34+CD117+ or CD34+KDR+, were found in CAD patients, and compared to a group of healthy controls. Patients receiving statin treatment revealed increased levels of putative EPCs and levels of CD34+KDR+ EPCs presented the greatest difference (84).

However, in a study performed to identify possible predictors of circulating EPC (CD34+KDR+) levels in PB, patients referred for non-invasive assessment of CAD under chronic therapy with statins were found to have lower levels of circulating EPCs. Interpretation of these findings is difficult, since there is significant collinearity and interaction between the parameters. For instance, patients on statins are more likely to have a history of dyslipidaemia and hence greater exposure to vascular damage (86). Also, in a study evaluating the interrelationships between EPCs, PAD, and atherosclerotic risk factors, the EPC numbers and EPC CFUs were not significantly different between patients with PAD treated with statins and patients with PAD and not on statin therapy (102). Again, in patients who had experienced a transient ischaemic attack or ischaemic stroke attributable to symptomatic intracranial atherosclerotic disease, no difference in EPC circulating levels was found between patients with or without treatment with statins, antiplatelets or other drugs (118). In patients with chronic kidney disease stage V receiving haemodialysis therapy, no differences in EPCs were found between patients treated with statins and those who did not receive these drugs (132). Conversely, it was found that circulating EPC numbers and EPC/CPC ratios were higher in peripheral vascular disease patients on statin therapy than in non-treated patients (122). Besides, in a study examined the clinical and genetic correlates of early-outgrowth CFUs in 1799 participants of the Framingham Heart Study, CFU number was positively related to statin therapy in stepwise multivariable analyses (42).

17.2. Pioglitazone

The effect of the peroxisome proliferator-activated receptor gamma (PPARγ) agonist pioglitazone, used for the treatment of diabetes, on EPCs was examined. A prospective, randomised, double-blind study was performed in patients with documented stable CAD and normal glucose tolerance. Patients with normal glucose tolerance were randomised to 30-day treatment with pioglitazone (45 mg) or placebo in addition to optimal medical therapy. It was demonstrated that treatment with pioglitazone increased the number and the function of circulating EPCs (CD34+KDR+ and culture). The effects of pioglitazone on EPCs occurred on top of the treatment with aspirin, β-blockers, and inhibitors of the renin-angiotensin system and, importantly, in the presence of statin treatment. To evaluate the compliance to the study medication, serum adiponectin concentrations were measured. Adiponectin levels at least doubled in all of the pioglitazone-treated individuals, but the patients who received placebo didn't exhibit a similar increase. These findings suggest a very good compliance. To test whether adiponectin contributes to the regulation of EPCs by pioglitazone, cultured human EPCs were exposed to adiponectin at concentrations similar to those observed in the patients taking pioglitazone. Adiponectin potently increased both EPC numbers and function, suggesting that adiponectin contributes as a mediator to the effects of pioglitazone on EPCs. Pioglitazone treatment significantly lowered the established marker of vascular inflammation hs-CRP. It was shown that this effect can be observed in normoglycaemic patients with CAD after 30 days of treatment. All the three methods used for EPC quantification (flow cytometry, culture and double-staining for both DiI-acLDL and lectin, and EPC CFUs) showed a robust increase of EPC numbers in patients taking the PPARγ agonist. Besides, cell culture experiments showed that pioglitazone treatment reduces basal and phorbol myristate acetate-stimulated nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) oxidase activity in EPCs, pinpointing a mechanism by which pioglitazone treatment reduces EPC apoptosis and increases EPC numbers. Of the several sources within vascular cells, NADPH oxidase is a predominant contributor of endothelial superoxide free radicals. Two widely studied functional characteristics of EPCs are their potential to migrate and their capacity to replicate. It was shown that pioglitazone treatment increased both the migratory and the colony forming capacity per number of EPCs in patients with normal glucose tolerance, suggesting that the biological effect of pioglitazone on EPCs may be significantly greater than the extent of the increase of EPC numbers. These effects are mediated by PPARγ because GW9662, a PPARγ antagonist, reversed the effects of pioglitazone on EPC numbers and migration. The authors speculated that patients with diabetes may benefit from PPARγ agonists in addition to insulin

sensitisation by upregulation of EPCs. Summarily, the PPARγ agonist pioglitazone increases the number and function of EPCs in patients with CAD. The effect represents a potential regenerative mechanism in atherosclerosis and is observed in normoglycaemic individuals with stable CAD (172).

17.3. Dipeptidyl peptidase-4 inhibitors

Stem or progenitor cells migrate from the BM to the peripheral circulation following SDF-1α gradients, which is a natural substrate of dipeptidyl peptidase-4. Dipeptidyl peptidase-4 inhibitors represent a class of anti-diabetic drugs that act by increasing the bioavailability of incretin hormones, including glucagon like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), which in turn stimulates insulin release from β pancreatic cells. On the other hand, dipeptidyl peptidase-4 has several other non-incretinic substrates, including cytokines, growth factors, and neuropeptides. Therefore, DPP-4 inhibitors might have effects on renal hemodynamic and on the cardiovascular system in general, well beyond the incretin system. It was shown that the dipeptidyl peptidase-4 inhibitor sitagliptin increases CD34+KDR+ cells level and plasma SDF-1α concentrations in type 2 diabetic patients. Interestingly, although dipeptidyl peptidase-4 activity is increased in diabetes, improved glucose control is insufficient to lower dipeptidyl peptidase-4 activity in type 2 diabetic patients, while metformin treatment is associated with reduced dipeptidyl peptidase-4 activity, revealing that the regulation of this protease goes beyond hyperglycemia. A recent work has also shown that G-CSF is an unexpected substrate of DPP-4, thus creating a novel hematological feedback loop with important consequences for stem cell mobilization and engraftment (173).

17.4. Angiotensin-converting enzyme inhibitors

ACE inhibitors are used as antihypertensive agents, but also prevent renal and cardiovascular events in diabetes. In one study, the ACE inhibitor olmesartan mobilized CD34+ stem cells and endothelial progenitors in type 2 diabetic patients. Interestingly, the effect of ACE inhibitors on progenitor cell levels seemed to rely upon the modulation of CD26/DPP-4 system, since inhibition of DPP-4 activity dampened the mobilizing effects of enalapril on progenitors (173).

In a study evaluating the association between EPC numbers, EPC CFUs, cardiovascular risk factors and life-style behaviours in a large population-based study, subjects on ACE inhibitor therapy exhibited higher EPC levels (80).

17.5. Barnidipine versus hydrochlorothiazide

Patients with mild essential hypertension were randomised to receive barnidipine, a calcium channel blocker, up to 20 mg or the diuretic hydrochlorothiazide, up to 25 mg. hydrochlorothiazide up to 25 mg. Circulating EPCs (culture) were isolated from PB at baseline and after 3 and 6 months of treatment. EPC number at baseline did not differ in the two subgroups of patients receiving either barnidipine or hydrochlorothiazide. Considering the two treated group together, EPC number resulted increased after 3 months and 6 months of treatment compared with baseline. The difference between groups in terms of changes in EPCs persisted even after correction for baseline blood pressure, cholesterol, glucose and age. However, when patients were subdivided according to drug treatment, the EPC number significantly increased in patients treated with barnidipine after 3 and 6 months of treatment, compared with baseline. No significant difference was observed in patients treated with hydrochlorothiazide after 3 and 6 months compared with baseline. The increase of EPC number with barnidipine observed in this study's patients could be a result of either EPC mobilisation from the BM or reduced EPC senescence through an improvement of endothelial function and oxidative stress. Summarily, EPC number may represent a potential drug target in hypertension. In this regard, the calcium channel blocker barnidipine may be considered a useful therapeutic option (174).

17.6. Clopidogrel

The pharmacokinetic interactions between atorvastatin and clopidogrel and their effects, alone or combined, on EPCs and EMPs were evaluated in patients with stable coronary disease. Drugs were given daily for 3 weeks and counts of EPCs (CD34+KDR+, CD34+CD133+ and CD133+KDR+) and EMPs, and pharmacokinetic parameters, over 24 h, were assessed at each visit. At visit 1, after a 7-day washout of the previous statin therapy, atorvastatin 80 mg daily was started and aspirin was discontinued. At visit 2, 1 week after visit 1, clopidogrel 75 mg daily was added to atorvastatin 80 mg. At visit 3, 2 weeks after visit 2, atorvastatin was discontinued. At visit 4, 1 week after visit 3, patients receive clopidogrel 75 mg alone. At all these visits, biochemistry and flow cytometry for quantification of EPCs, endothelial

microparticles, and platelet microparticles were performed. Participants were predominantly middle-aged overweight men, and all subjects had stable coronary disease. All patients had diagnosis of hypertension, and one-third of participants had DM. Clopidogrel, but not atorvastatin, plasma levels were correlated with the CD133+/KDR+ subpopulation of EPC, which is considered an immature lineage of EPC, and a marker of disease severity. This effect of clopidogrel, not seen with atorvastatin, may have been related to chronic and effective use of statins in the study subjects. The authors selected patients with relatively low levels of cholesterol, which may have attenuated the effects of the homing of EPC, thus decreasing EPC mobilization. But, even with significant changes to lipid profile, either by use of high-dose or by abrupt withdrawal of atorvastatin, no changes in the amount of circulating EPC or microparticles were observed (175).

17.7. Corticosteroids

The effects of corticosteroid therapy on EPC and EMP levels were evaluated in patients with polymyalgia rheumatica. Polymyalgia rheumatica is a rheumatic disease that is characterized by intense activation of systemic inflammation. Systemic inflammation may be associated with an imbalance between endothelial injury and repair, defined by an increased number of circulating EMPs and a reduced number of EPCs. Circulating EPCs (CD34+/KDR+) and EMPs (CD31+/CD42-) were quantified by flow cytometry. Patients with polymyalgia rheumatica participated in a 1-month intervention open-label study with corticosteroid therapy. It was found that suppression of inflammation induced by corticosteroid therapy may be effective in reducing endothelial fragmentation into microparticles and in increasing the availability of circulating EPCs. These findings are, therefore, of clinical interest, although conclusions must be interpreted with caution because of the small numbers of subjects. Corticosteroid treatment significantly reduced plasma CRP levels and led to a consistent decline in the number of EMPs and a concomitant increase in the number of EPCs, with a significant association between CRP levels and the reduction in EMP/EPC ratio. Therefore, it was confirmed that systemic inflammation disturbs the balance between endothelial injury and repair and suggest that short-term anti-inflammatory treatment with a corticosteroid may be helpful in controlling the deleterious effects of inflammation on the vascular system. However, the possibility that longer corticosteroid therapy might have adverse effects on vascular health cannot be ruled out (176).

17.8. Erythropoietin and darbopoetin

Independent of its hematopoietic effect, EPO may be protective against ischemic injury in various organs, including the heart. Considering an established safety profile of EPO in clinical practice, together with the results of experimental studies in cardiac ischaemia, a pilot clinical study with EPO treatment was performed in patients with acute myocardial infarction. The primary objective of the study was to evaluate the safety and tolerability of long-acting EPO analogue darbepoetin alpha treatment in non-anaemic patients with acute STEMI treated with primary coronary angioplasty. The authors have also studied the effect of the long-acting EPO analogue darbepoetin alpha on the levels of EPCs (CD34+CD45-), that is the interesting point for this thesis. It was demonstrated that a single bolus of long-acting EPO analogue darbepoetin alpha in patients with first acute STEMI appears to be safe and well tolerated. No hypertensive, thromboembolic or other serious adverse events were observed during the 30-day long follow-up. Darbepoetin treatment led to a significant elevation of EPCs, 72 hours after the drug administration. The mechanism behind the effect of EPO on neovascularisation remains largely unknown. Both stimulation of *in situ* endothelial cells proliferation or mobilisation of EPCs derived from the bone-marrow may play a role. Administration of darbepoetin led to almost a 3-fold increase in the number of circulating EPCs, measured 72 hours after myocardial infarction, demonstrating that high-dose darbepoetin treatment after myocardial infarction stimulates EPCs mobilisation (177).

EPO enhances re-endothelialisation. The effect of low-dose EPO was investigated in patients with a first acute STEMI undergoing PCI who were randomly assigned to EPO or placebo treatment. It was demonstrated that short-term low-dose EPO to PCI-treated AMI patients did not prevent neointimal hyperplasia but rather improved cardiac function and infarct size without any clinical adverse effects. The number of CD34+ and CD34+/CD133+/CD45dim EPCs was evaluated during 4 days after low dose EPO injection. However, there were no significant increases in the numbers of EPCs and the change was not significant between EPO and control groups (178).

It was investigated whether the erythropoietin analogue darbepoetin improves FMD and whether this is influenced by preceding ischaemia/reperfusion. Patients with stable CAD were randomized to receive a single dose of darbepoetin (300 μ) or saline placebo (study 1). CD34+CD133+ and CD34+VEGFR2+ circulating EPCs were enumerated. Measurements were made immediately before darbepoetin or placebo and at 24 hours, 72 hours and 7 days. A further group of patients was studied according to the same protocol, all receiving darbepoetin, with omission of forearm ischaemia/reperfusion at 24 hours (study 2). This study

investigated the ability of darbepoetin to improve endothelium-dependent NO dependent vasomotor function as assessed by FMD in patients with established atherosclerotic disease. No significant effect of darbepoetin to augment FMD at 24 hours was found, when levels of immunoreactive EPO were maximal, nor any significant effect of darbepoetin to protect against endothelial dysfunction induced by immediate ischaemia/reperfusion injury. However, at 72 hours, 48 hours after ischaemia/reperfusion, darbepoetin resulted in a marked improvement in FMD compared with the placebo group. The fact that this was not simply the result of a time-course effect with a delay in actions of darbepoetin was confirmed by the lack of effect of darbepoetin at 72 hours in subjects who did not undergo forearm ischaemia/reperfusion injury. Although the increase in FMD seen in the study was modest in absolute terms, it represents an approximate doubling of the response to eNOS-derived NO, an effect that could be of potential clinical significance in terms of limiting ischaemia, endothelial cell dysfunction and apoptosis. Besides, there was a trend towards an increase in EPCs in the treated group which just reached significance when results from the two studies were combined. However, unlike FMD, the increase in EPCs did not differ significantly in the presence or absence of upper-limb ischaemia/reperfusion. Thus, effects of darbepoetin to enhance FMD after ischaemia/reperfusion were likely to be mediated by a direct effect on eNOS rather than by EPCs (179).

In a study evaluating the association between functionally active EPCs (cell culture) and traditional cardiovascular risk factors in patients with chronic kidney disease stage V receiving haemodialysis therapy, the authors did not found an association between recombinant human EPO treatment and EPCs. According to the authors, it could be speculated that patients who do not need recombinant human EPO therapy despite kidney failure, still may have enough endogenous EPO production to maintain normal EPC numbers (132).

17.9. Immunosupressive therapy

It was demonstrated that EPCs are reduced in heart transplantation patients compared with healthy individuals matched for age and gender. The authors of this study suggested that the reduction of circulating EPCs in heart transplantation patients compared with controls can be explained by the effect of immunosuppressive therapy (the immunosuppression protocol consisted of cyclosporine, azathioprine, mycophenolate mofetil, or everolimus, and steroids) (107).

17.10. Antiretroviral therapy

Human immunodeficiency virus (HIV) alters the EPC trafficking by infecting and depleting progenitors belonging to the hematopoietic lineage (EC CFUs, according to the authors), without affecting the 'true' endothelial progenitor pool (ECFCs, according to the authors). These observations, carried out in combined antiretroviral therapy-naive individuals, constitute the evidence for elucidating whether antiviral drugs are able to restore an adequate compartment of circulating EC CFUs (40). In another study, higher EPC frequencies were observed in HIV-positive subjects as compared to HIV-negative subjects irrespective of the presence of antiretroviral therapy, or subsequent change in carotid artery IMT. With regards to the impact of antiretroviral therapy on EPCs, the lack of a difference between treated and untreated groups is limited by the smaller number of subjects on antiretroviral therapy, as the study was focused on *a priori* selected subjects based on prospective carotid artery IMT change rather than balanced for treated versus untreated subjects at baseline (180).
In another study, the number EPCs and its relationship to carotid IMT were investigated in HIV-infected patients. It was demonstrated that antiretroviral therapy exposure was the main predictor of decreased number of EPCs in HIV-infected patients, after controlling by traditional cardiovascular risk factors and HIV parameters (181).

17.11. Hormone replacement therapy

It was suggested that EPCs are influenced by female sex hormones and it was confirmed that 17β-estradiol has a potent ability to stimulate human EPCs (72). In a study examined the clinical and genetic correlates of early-outgrowth CFUs in 1799 participants of the Framingham Heart Study, CFU number was positively related to hormone replacement therapy in stepwise multivariable analyses (42).
In a study evaluating the association between EPC numbers, EPC CFUs, cardiovascular risk factors and life-style behaviours in a large population-based study, subjects on hormone replacement exhibited higher EPC levels (80).

17.12. Other drugs

In addition to the drugs described, there are more drugs that may have an effect on EPCs. This information was found in a review study that resulted from the research and it was

considered important to refer in this dissertation. These drugs are aspirin, that in high dose, presents a negative correlation with EPCs, and in physiologic dose, presents a positive correlation with EPCs; and NO donors and enhancers that are responsible for increased levels of EPCs (14).

18. Transplantation of EPCs

With respect to cell transplantation, it was suggested that, considering the small number of applied multipotent cells and the fact that cell death affects a considerable number of cells, cell expansion could be a way to improve the transplantation results. The study by Ott and colleagues (95), already referred, provided a clinically applicable expansion method to generate large numbers of cells cultured from CD34+ EPC that are capable to form vessels *in vivo* and are suitable for autologous cell transplantation. In this study, immunohistological analysis did not reveal apoptosis of transplanted cells. Moreover, they expressed proliferation markers and contributed to an increase in vessel density. These results suggest survival of the injected cells *in vivo* (95).

Another study selected seven candidates for heart transplantation in whom all treatment alternatives were exhausted. The goal of the study was to analyse the effectiveness of the intracoronary infusion of CD133+ (a particular set of EPCs) in patients with moderate to severe post-infarct heart failure to improve heart function and quality of life, and to contribute with additional data on the safety of the procedure. These subjects had a symptomatic New York Heart Association (NYHA) scale of at least II and ejection fractions below 35%. CD133+ cells were obtained by stimulation with G-CSF, apheresis, and separation with magnetic beads. Stem cells were implanted in the infarcted zone via intracoronary percutaneous angiography. Evaluations (NYHA scale classification, plasma concentration of proBNP and the risk of sudden death, echocardiography, cardiac magnetic resonance, and others) were performed at baseline and at 3, 6, 12, and 24 months after cell infusion. The CD133+ isolation procedure was very efficient. Efficacy analyses demonstrated significant recovery of the quality of life associated with improvements in heart function. The NYHA scale demonstrated significant improvements in the heart functional class 3 months after therapy that were sustained along the 2-year observation period. The cardiac functional analyses showed improved myocardium contraction in the treated patients. The EPC infusion did not demonstrate any impact in the remodelling of the heart after the infarct, since ventricular volumes remained similar

throughout the study. The proBNP plasma concentrations showed no differences through the study, but at the 24-month observation point, a significant reduction was registered. It was shown that the treatment is safe and associated with minor adverse events, such as myalgias associated with the use of G-CSF and thrombocytopenia due to the apheresis procedure. Causes of death in the two deceased patients were not directly associated with the therapy, but it is difficult to discard the catheterism and any nosocomial-related infection as contributory factors in the case of the patient with pneumonia and cholangitis. In the second case, a cerebrovascular event was possibly related to a chronic systemic atherosclerotic disease. Plasma concentrations of proBNP, one of the most objective markers for heart failure (99), were not modified during the first year after treatment, but significantly decreased in the 24-month time-point period, suggesting that this therapy positively modifies the evolution of the heart failure in the long-term. According to the authors, this study suggested that a single intracoronary infusion of CD133+ into the infracted area may improve heart function for at least 2 years after treatment and results in remarkable improvement in quality of life in the treated patients. The intracoronary infusion of the EPCs appeared to be as a minimal invasive, safe, and less expensive method to treat patients with limited therapeutic options other than surgical procedures (182).

Conclusions

In this dissertation, it was explained that vasculogenesis and angiogenesis are two different processes that contribute to blood vessels formation and that neovasculogenesis is not synonymous with angiogenesis, at least as the latter has been classically defined. Besides, it was evidenced that vasculogenesis don't occurs only during embryologic development of the circulatory system but also contributes to endogenous neovascularization of developing tumours, wound healing, severe hindlimb ischaemia, and myocardial ischaemia, as well as physiological neovascularisation. Moreover, angiogenesis designates the formation of new blood vessels from pre-existing ones, whereas vasculogenesis resembles the formation of new blood vessels when there are no pre-existing ones. The challenging study of Asahara and colleagues showed that neovasculogenesis really occurs and EPCs are involved. BM-derived EPCs home and incorporate into sites of neovascularisation where differentiation into ECs is completed, which is consistent with neovasculogenesis. EPCs are mobilised or released from the marrow and, subsequently, the cells move through the circulation and appear to home preferentially to sites of tissue injury. EPC mobilisation from BM involves cytokines, growth factors and other substances, like VEGF, SDF-1, HIF-1 and NO. EPCs were first described by Asahara and colleagues and since then many authors have studied EPCs through various methods like flow cytometry and different culture assays. A range of different markers are used in the identification of EPCs in PB. It was evidenced that EPCs can be distinguished phenotypically in early EPCs and late EPCs and that EPC CFUs, EC CFUs and ECFCs may also represent EPCs, despite the controversy regard to CFUs. The analysis of the studies from the research used to perform this document allowed the authors to accomplish that flow cytometry is the method most used to the identification of EPCs and regarded as the gold standard. The literature suggests that EPCs should express at least one marker of immaturity and one marker of endothelial lineage and the most widely used combination of markers is CD34+KDR+, which is in agreement with what has been suggested. Despite the controversy about the best combination of markers to define EPCs, the literature suggests that this exact phenotype may be preferred in future studies in which EPC count is intended as a cardiovascular biomarker. However, to date, there is no certainty about the phenotype of EPCs. With respect to markers that are used to confirm endothelial lineage after culture, DiI-acLDL, lectin, KDR and CD31 are the most used markers.

Many authors studied EPCs in the last years within the context of cardiovascular disease and cardiovascular risk. Through the results of the studies obtained from the research, the relationship between EPCs and cardiovascular disease or cardiovascular risk factors was

described. Circulating EPCs are involved in the pathogenesis of endothelial dysfunction, atherosclerosis and CAD, and also in ischaemic events. It was evidenced that EPC depletion reduces the ability to repair the endothelium, thus triggering subsequent steps in the development of atherosclerosis. Besides, the number of EPCs decrease with the presence and extent of atherosclerosis. Because of the strong relationship between the severity of CAD and EPCs in the blood stream, it was speculated that one cause of CAD might be an increasing inability of these EPCs to keep up with the endothelial damage. Reduced levels of circulating EPCs independently predict future cardiovascular events, thus supporting an important role for endogenous vascular repair to modulate the clinical course of CAD and also supporting the use of specific EPCs clusters levels as biomarkers of cardiovascular risk.

In patients with PAD both the number and proliferative activity of circulating EPCs are increased. EPC mobilisation occurs and shows a biphasic response, with elevated EPCs in the moderate phase and reduced EPCs in the advanced phase. Besides, EPC levels are associated with the severity, progression, and outcome of PAD. It was evidenced that a short episode of tissue ischaemia is sufficient to induce a significant increase in the number of circulating EPCs. In patients with primary acute myocardial infarction, the capability of EPCs to differentiate is associated with functional improvement and infarct size reduction, indicating that manipulation of EPCs could become a novel therapeutic target to salvage ischaemic damage.

With regard to heart failure, more than one mechanism could be involved in the degree of EPC mobilisation and findings suggest that it could be stage dependent. An exhaustion of progenitor cells in the advanced phases of the disease could also contribute to the biphasic BM pattern of response to heart failure.

Because EPCs are believed to play a role in repair, the lack of compensatory elevations in damaging processes like CAD with LV dysfunction may contribute at least in part to the pathologic development of endothelial dysfunction.

The extent of endothelial injury may represent a balance between the magnitude of injury and the capacity for repair, and predicts cardiovascular event rates.

With respect to cerebrovascular disease, it was suggested that ischaemic insult may modulate the vasculogenic capacity of EPCs, such that those obtained in the sub-acute phase of stroke would have the greatest potential. The acute increase of EPC counts in the blood stream might be explained by a rapid release from the BM activated by the ischaemic event and the inflammatory response, whereas a later decrease but enhanced functionality of EPCs could be explained by the activation of endogenous angiogenesis and be an advantage for neurorepair. The high output of outgrowth cells observed in acute stroke suggests that circulating EPCs

may provide a means of endogenous repair to counteract the effects of acute tissue injury and to replace dysfunctional or damaged endothelium. Besides, patients presenting with severe forms of intracranial atherosclerotic disease in terms of the number or severity of intracranial stenoses presented higher circulating levels of EPCs and angiogenic growth factors, suggesting that at some levels of chronic hypoperfusion, more EPCs could be mobilised from the BM and could stimulate angiogenic growth factors production sources. The isolation and expansion of EPCs might be particularly useful for identifying therapeutic approaches that modify the progression and recurrence of stroke.

EPCs are also associated with vascular complications of diabetes. Diabetic patients with peripheral vascular disease present a progressive EPC decrease. Measurement of the circulating EPC number may be applied as a valuable clinical test to identify T2DM with impaired endothelial function. The alteration of circulating EPC numbers can also be regarded as a therapeutic target for diabetic vascular protection. EPCs are also associated with diabetic retinopathy. It was speculated that signals generated by the retina in response to ischaemia may stimulate activation of BM EPCs, using systemic NTFs as messengers released into the circulation and that diabetic retinopathy may be an aberrant neovascularisation process initially driven by NTFs released from neuronal tissue to activate EPCs. Besides, it was suggested that the microvascular and macrovascular changes seen in diabetic patients may be attributed to functionally altered EPCs both in normal vessel repair and in abnormal neovascularisation.

With respect to kidney disease and cardiovascular risk, EPCs depletion in mild-to-moderate renal dysfunction accompanying stable angina may exacerbate an imbalance between endothelial injury and EPC-mediated repair, thus contributing to excessive cardiovascular risk in CAD coexisting with renal insufficiency. In ischaemic acute kidney injury, it is suggested that BM-derived EPCs may contribute to endothelial repair immediately after ischaemia-reperfusion. Elevated ADMA and EPC deficiency may synergistically contribute to accelerated renal function decline in stable angina. This could result from the impairment of the EPC-dependent endothelial renewal in the kidney, a NO-dependent process. The findings of the studies support a role for EPCs in vascular repair also in chronic kidney disease patients. The concentration of EPCs is reduced in renal transplant recipients and EPCs obtained from renal transplant patients when studied *in vitro*, display reduced proliferation, as a sign of functional impairment. These alterations may be involved in the increased cardiovascular risk of renal transplant patients.

These pathological situations reflect the association between cardiovascular disease and circulating levels of EPCs. Increased levels of EPCs are generally associated with acute phases

of cardiovascular disease, but advanced stages of disease are associated with decreased EPC levels. This could be explain by the fact that there is an increased mobilisation of EPCs from BM in acute events. However, when the disease progresses, the pool of EPCs is exhausted, and cells are unable to mobilise from BM niches.

There are another factors that may influence circulating levels of EPCs. Family history of cardiovascular disease, age, gender and life styles such as smoking, exercise and obesity may have an impact on EPC levels, albeit these relationships don't appear to be linear. For example reduced numbers of EC CFUs, used to determine the functional capacity of circulating EPCs, were associated with advanced age. However, adolescents present reduce levels of EPCs. Reduced level of EPCs in younger individuals compared to older ones may reflect a lower need for endogenous repair mechanisms at early age and it was suggested that an elevation of EPCs in early life might reflect occult vascular damage and could be seen as a biological marker of subclinical atherosclerosis. Moreover, male gender is associated with reduced EPC number compared to age-matched premenopausal females. However, EPC numbers are significantly lower in predominantly postmenopausal females than in males of equal age.

Therefore, premenopausal women have a lower cardiovascular risk profile than men of the same age.

Finally, the literature also suggests a relationship between some drugs and EPCs. It was suggested that statins improve circulating EPC levels, beyond their lipids-decreased effect. Antidiabetics like the PPARγ agonists and dipeptidyl peptidase-4 inhibitors may increase the number of EPCs and, therefore, improve endothelial dysfunction in patients with cardiovascular disease. ACE inhibitors and calcium channel blocker barnidipine are also associated with higher levels of EPCs. It was also suggested that clopidogrel was correlated with higher levels of EPCs. Similarly, corticosteroid therapy was associated with increased EPC levels and darbepoetin, an EPO analogue, also increases EPC levels. In contrary, immunosuppressive therapy and antiretroviral therapy may have a deleterious effect on EPCs. It is important to refer that the possibility of studying EPC levels in healthy subjects would allow the investigators to obtain results without interfering factors.

This dissertation demonstrated that EPCs are involved in cardiovascular disease and that are also related to vascular complications of diabetes and cardiovascular risk in renal patients. Besides, there are drugs that could help to improve EPC levels and promote improvements on cardiovascular risk profile. Therefore, monitoring the levels of circulating EPCs as biomarkers of cardiovascular risk could be specifically useful to identify subjects at low or high risk of developing cardiovascular disease. This could result in the early detection of

cardiovascular disease and in the possibility of initiating appropriate therapy as early as possible and subsequent monitoring.

Future perspectives

The research conducted to perform this dissertation resulted in many data, including markers or combinations of markers used to define EPCs and different relationships between EPCs and cardiovascular disease and cardiovascular risk factors. In order to continue this work, the authors intend to perform a meta-analysis using the same research or a better one, to evaluate if there is a relationship between markers and disease and identify which specific markers or clusters of markers for EPCs should be used for monitoring each cardiovascular disease or cardiovascular risk factor.

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