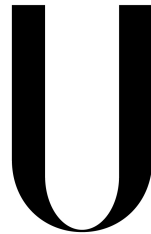


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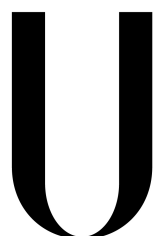
**THE ROLE OF UCX<sup>®</sup> CELLS IN THE  
TREATMENT OF PERIPHERAL ARTERIAL DISEASE  
BY PROMOTING THERAPEUTIC ANGIOGENESIS**

**Ana Rita Duarte Simões Pereira**

Dissertação  
Mestrado em Bioquímica  
Especialização em Bioquímica

**2014**

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Orientadores: Professora Doutora Susana Constantino  
Professora Doutora Margarida Meireles

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## ABBREVIATIONS

aFGF	Acidic Fibroblast Growth Factor
Ang-1	Angiopoietin 1
Ang-2	Angiopoietin 2
bFGF	Basic Fibroblast Growth Factor
CLI	Critical Limb Ischemia
ECs	Endothelial Cells
ECM	Extracellular matrix
EPCs	Endothelial Progenitor Cells
FDA	Food and Drug Administration
FGF	Fibroblast Growth Factor
FGFRs	Tyrosine kinase FGF receptors
HIFs	Hypoxia-inducible Transcription Factor
IL	Interleukin
IGF	Insulin Growth Factor
MMPs	Matrix Metalloproteinases
MSC	Mesenchymal Stem Cells
PAD	Peripheral arterial disease
PDGF	Platelet-derived Growth Factor
PIGF	Placental Growth Factor
PIK-3	Phosphatidylinositide 3-Kinase
qRT-PCR	Quantitative real-time PCR
SMCs	Smooth Muscle Cells
T $\beta$ R-I	Serine-threonine kinase receptor I
T $\beta$ R-II	Serine-threonine kinase receptor II
TGF $\beta$	Transforming Growth Factor $\beta$
Tie	Tyrosine kinase with immunoglobulin and EGF homology domains
UCX <sup>®</sup>	Umbilical Cord Tissue Mesenchymal Stem Cells
VEGF A	Vascular Endothelial Growth Factor A
VEGF B	Vascular Endothelial Growth Factor B
VEGF C	Vascular Endothelial Growth Factor C
VEGF D	Vascular Endothelial Growth Factor D
VEGFR1	Vascular Endothelial Growth Factor Receptor 1
VEGFR2	Vascular Endothelial Growth Factor Receptor 2

# GENERAL INTRODUCTION

## 1. Blood vessels: an overview

Vertebrates require an efficient circulatory system able to carry out vital functions ensured by two main networks: the blood vascular and the lymphatic system, both formed by endothelial cells (ECs)<sup>1,2</sup>.

The adult vascular system is composed of an arterial, a venous and a lymphatic compartment. These different compartments respectively provide oxygen and nutrients to peripheral organs and tissues; are able to remove carbon dioxide and other metabolic waste products which have to be transmitted to the excretory organs and maintain an immune barrier to defend the host against foreign organism<sup>1</sup>.

Blood, which is the carrier of oxygen, carbon dioxide and metabolic products, is pumped from the heart through the arterial system into the tissue capillary bed, where changes occur. The blood is then channeled through the venous system back into the heart. The lymphatic system drains extravasated fluid, the lymph, from the extracellular space and returns it into the venous circulation. The lymphatic vasculature is also essential for the immune defense, as lymph and any foreign material present in it, such as microbial antigens, are filtered through the chain of lymph nodes<sup>1</sup> (Figure 1).

Blood vessels are divided into three main groups: arteries, veins and capillaries. Arteries and veins are divided according to caliber into large, medium and small blood vessels. These vessels are thickest and their walls more complex in the immediate vicinity of the heart, and decrease in caliber when their wall becomes thinner and less complex. Arteries form a high-pressure system, enabling the transportation of blood to capillaries, whereas veins face low-pressure gradients. The differences in hemodynamic load are reflected in their structures since arteries are supported by layers of vascular smooth muscle cells (SMCs) and a specialized matrix whereas veins are thinner and surrounded by fewer SMCs<sup>3</sup>.

Histologically, the capillary bed, which comprises the largest surface of the vascular system, is composed only by ECs that support tissue growth and repair, occasionally associated with external pericytes<sup>1</sup>.

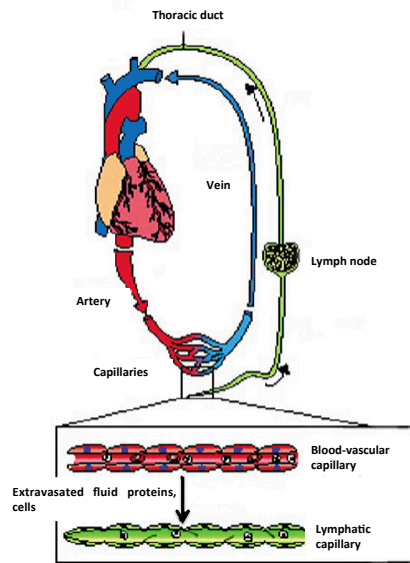


Figure 1. Schematic representation of the circulatory system. Blood is pumped from the heart through arteries, arterioles and capillaries to the tissues, where exchanges occur. Blood is returned to the heart via the venous circulation. Adapted from Eichman et al., 2005 <sup>1</sup>.

Heterogeneity in vessel wall composition is evident and varies between vessels of different sizes and between arterial and venous vessels (Figure 2). Large vessel vascular walls are composed of multiple layers of cellular and extracellular materials such as the *tunica intima*, the *tunica media* and *tunica adventia*<sup>4</sup>. The first one consists of the endothelium, basal membrane and internal elastic layer, the second one consists of a thick layer of smooth muscle with reticular fibers (collagen III), elastin and proteoglycans and the latter consists of connective tissue with both elastic and collagenous fibers containing vasa vasorum and nerves<sup>1</sup>. Veins are different from arteries in several aspects. Given that veins conduct blood back to the heart, the pressure exerted by the heartbeat on them is much less strong than in the arteries. In turn, arteries have strong elastic vessel walls for withstanding the high blood pressure downstream of the heart<sup>4</sup>. Moreover, veins have semi-lunar valves, which prevent backflow of blood.

Small blood vessels consist of ECs forming the inner lining of the vessel wall and surrounded by a basal lamina covered by pericytes that envelop the surface of the vascular tube<sup>5</sup>. Pericytes also referred to vascular SMCs because of their contractile fibers or mural cells, do not serve only as scaffolding, but also communicate with ECs by direct physical contact and paracrine signaling pathways<sup>5</sup>. Gap junctions provide direct connections between the cytoplasm of pericytes and ECs enabling the exchange of ions and small molecules. Besides these functions, they have been associated mainly with stabilization and hemodynamic processes of blood vessels, they can sense angiogenic stimuli, guide sprouting tubes, elicit endothelial survival functions, and even exhibit

macrophages like activities<sup>5</sup>. When vessels lose pericytes, they become hemorrhagic and hyperdilated leading to conditions such as edema, diabetic retinopathy and even embryonic lethality<sup>6</sup>.

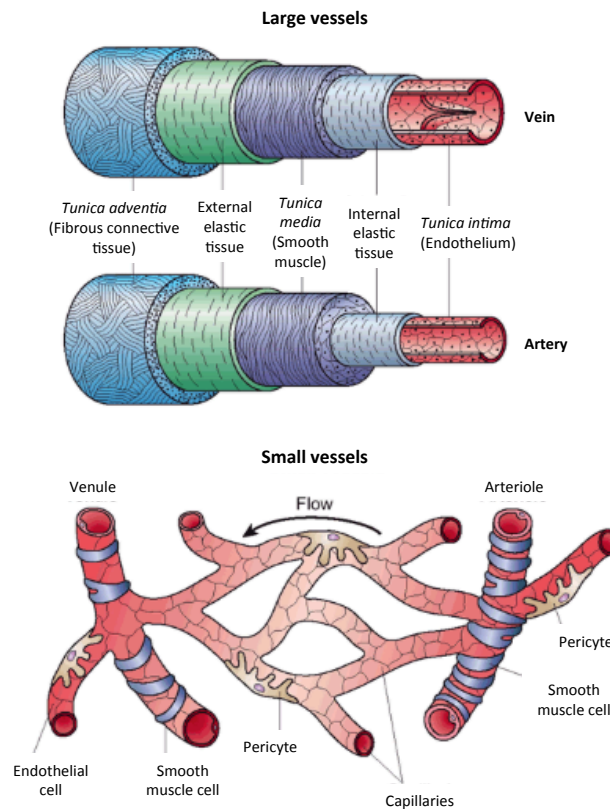


Figure 2. Morphology of mural cells associated with large blood vessels (top) and capillaries (bottom). Adapted from Cleaver and Melton, 2003<sup>4</sup>.

## 2. Vasculogenesis and Angiogenesis during embryonic development

Two distinct molecular mechanisms account for the genesis of blood vessels, vasculogenesis and angiogenesis. Vasculogenesis is the process of *de novo* blood vessel formation driven by the recruitment and differentiation of mesodermal cells into the endothelial lineage and the *de novo* assembly of such cells into blood vessels. Angiogenesis is the generation of new blood vessels from pre-existing ones, a process driven by EC proliferation.

### 2.1 Early blood vascular development

From the earliest stages, the embryo develops in the absence of blood vessel vascularization, receiving its nutrition by diffusion. However, in an orderly and sequential manner, the embryo rapidly transforms into a highly vascular organism, survival being dependent on a functional, complex network of capillary plexuses and blood vessels<sup>7</sup>. The first evidence of blood vessel



development appears on the extra-embryonic yolk sac and intraembryonic tissue, where groups of splanchnic mesoderm cells, specified to become hemangioblasts, aggregate and condense, forming what is known as blood islands. Here, hemangioblasts, the precursors of blood cells and ECs, start to differentiate. Cells localized at the perimeter of the blood islands become angioblasts, the precursors of all blood cells<sup>7</sup>.

In the initial events, angioblasts migrate to distant locations, multiply and differentiate *in situ* and assemble into solid endothelial cords, forming a primary capillary plexus. This process, where blood vessels are created *de novo* from endothelial progenitor cells (EPCs), is known as vasculogenesis and it is driven by the recruitment and differentiation of mesodermal cells into the endothelial lineage and the *de novo* assembly of such cells into blood cells (Figure 3).

This primitive network then differentiates and new blood vessels sprout and branch from pre-existing capillaries. The subsequent growth, expansion and remodeling of these primitive vessels into a mature vascular network is referred to as angiogenesis, a process driven by ECs proliferation.

This process is characterized by a combination of sprouting of new vessels from sides and ends of pre-existing ones, or by longitudinal division of existing vessels with peri-endothelial cells, either of which may then split and branch into precapillary arterioles and capillaries. Depending on the ultimate fate with respect to the type of vessel (artery, vein, capillary) and vascular bed, activated ECs that are migrating and proliferating to form new vessels forming anastomotic connections with each other, become variably surrounded by layers of peri-endothelial cells (pericytes for small vessels and smooth muscle cells for large vessels).

Once the peri-endothelial cells are recruited, they continue to migrate along sprouting vessels or pre-existing vasculature<sup>7</sup>.

Further functional modifications of larger arteries occur during collateral development also termed as arteriogenesis and thick muscular coat is added, concomitant with acquisition of viscoelastic and vasomotor properties<sup>7</sup>.

Vascular endothelial growth factor (VEGF) and its receptor-2 (VEGFR2) are the most absolute critical drivers of embryonic vessel formation, since the first one regulates endothelial proliferation, permeability and survival and studies with knock-out mice for either of both molecules result in embryonic lethality.

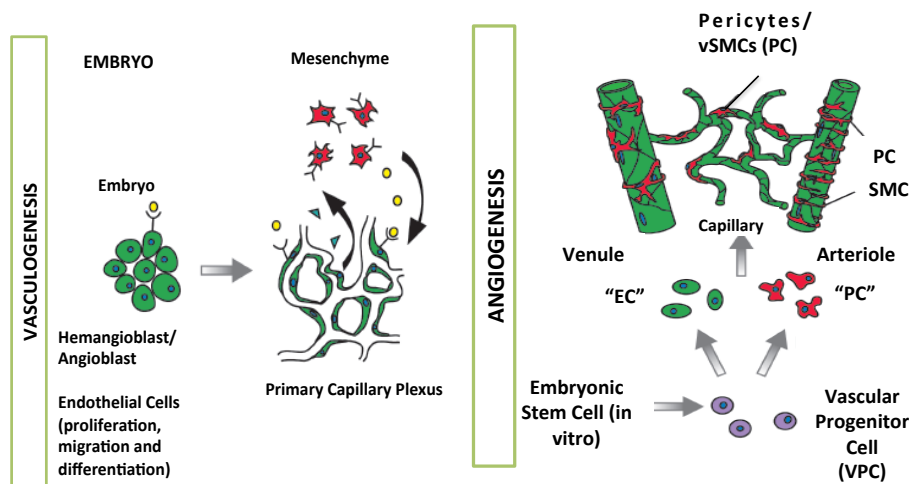


Figure 3. Vasculogenesis and angiogenesis in embryo. Endothelial cells (ECs) and pericytes/vSMCs (PC) arise from different precursor cells. ECs develop from angioblasts or hemangioblasts in the embryo, while pericytes/vSMCs are derived from mesenchymal stem cells. *In vitro* data indicate that exists a common vascular progenitor derived from embryonic stem cells that can give rise to EC and to PC. In the embryo, ECs first assemble into a simple capillary network. Vessels, then sprout and prune (angiogenesis), become stabilized by pericytes/vSMCs that are recruited by PDGF-B secreting ECs, and secrete into different vessel types. Arterioles exhibit a high density of circumferentially oriented SMCs and thicker EC walls to withstand the blood pressure. Venules, like capillaries, have irregularly arranged pericytes with multiple cytoplasmic processes and are composed of thinner EC walls with valves to prevent blood backflow. Adapted from Bergers & Song, 2005 <sup>5</sup>.

## 2.2 Maturation of blood vessels

Vascular cells are equipped with a set of molecules that allow them to perform their functions. In quiescent vessels, vascular endothelial cadherin in adherent junctions and claudins, as well as occludin in tight junctions, provide mechanical strength and tightness, and can establish a permeability barrier. These molecules do not only serve as "mechanical zippers", but also transmit crucial signals for endothelial survival and other functions. When ECs migrate during vessel sprouting, these contacts are transiently dissolved but later re-established, once ECs assemble a new sprout<sup>8</sup>.

The endothelial cell matrix (ECM) provides necessary contacts between ECs and the surrounding tissue, preventing vessels from collapsing. A basement membrane of collagen IV, laminin and other components encases vascular cells allowing pericytes and ECs to be embedded in the same basement membrane<sup>8</sup>. An interstitial matrix of collagen I and elastin between vascular cells further provides visco-elasticity and strength to the vessel wall. Moreover, ECM also regulates the formation of new vessel sprouts. When vascular cells migrate to form new sprouts, this matrix network is not only proteolytically broken down, but its composition is also altered. A provisional matrix of fibronectin, fibrin and other components provides a support scaffold, guiding ECs to their target.

Integrins are cell surface receptors of specific ECM molecules that assist vascular cells to build new vessels in coordination with their surroundings by bidirectionally transmitting information between the outside and inside of vascular cells<sup>8</sup>.

When considering the critical role of ECM in the vessel growth and maintenance, it is conceivable that proteolytic remodeling of the ECM must occur in a balanced manner. Insufficient breakdown prevents vascular cells from leaving their original position, but excessive breakdown removes critical support and guidance for ECs and, in fact, inhibits angiogenesis.

The establishment of a functional vascular network further requires that nascent vessels mature into durable vessels and the association of pericytes and SMCs with newly formed vessels in order to regulate EC proliferation, survival, migration, differentiation, vascular branching, blood flow and vascular permeability<sup>8</sup>.

### **3. Postnatal neovascularization**

Until 1997, it was generally accepted that vasculogenesis could only occur during embryogenesis. More recently, the existence of a postnatal vasculogenesis has been supported by the evidence that EPCs circulate postnatally in the peripheral blood, and that they may be recruited from the bone marrow and incorporated into sites of active neovascularization. Although vasculogenesis may occur during adulthood, often associated with pathological conditions, new vessels in the adult arise mainly through angiogenesis.

#### **3.1 Physiological angiogenesis**

Angiogenesis, as defined before, is the formation of new capillary blood vessels from pre-existent vessels and involves differential recruitment of associated supporting cells to different segments of vasculature.

After birth, angiogenesis still contributes to organ growth, although during adulthood most of the blood vessels remain quiescent. However, physiological angiogenesis can occur in response to some particularly situations, since ECs keep their capacity to divide in response to some stimuli. These stimuli may be hypoxia, wound healing and female reproductive organs that are undergoing physiological growth injured tissue.

##### **3.1.1 The angiogenic switch**

Angiogenesis is a highly functional process that requires a precise coordination spatially and temporally of multiple steps regulated by an exact balance of inducers (pro-angiogenic) and inhibitors (anti-angiogenic) factors. Physiologically, the body controls angiogenesis through a series of “on” and “off” regulatory switches. The main “on” switches are known as angiogenesis growth

factors (cytokines) and the main “off” switches are termed as endogenous angiogenesis inhibitors<sup>9</sup>. When angiogenic growth factors are produced in excess of angiogenesis inhibitors, the balance is tipped in favor of blood vessel growth. In cases where inhibitors are present in excess of stimulators, angiogenesis is “turned off”. Both situations lead to an angiogenic switch and uncontrolled angiogenesis process.

### 3.1.2 Angiogenic regulators

As it was previously referred, angiogenesis requires a precise coordination of multiple steps (Figure 4) regulated by a delicate balance between pro- and antiangiogenic factors.

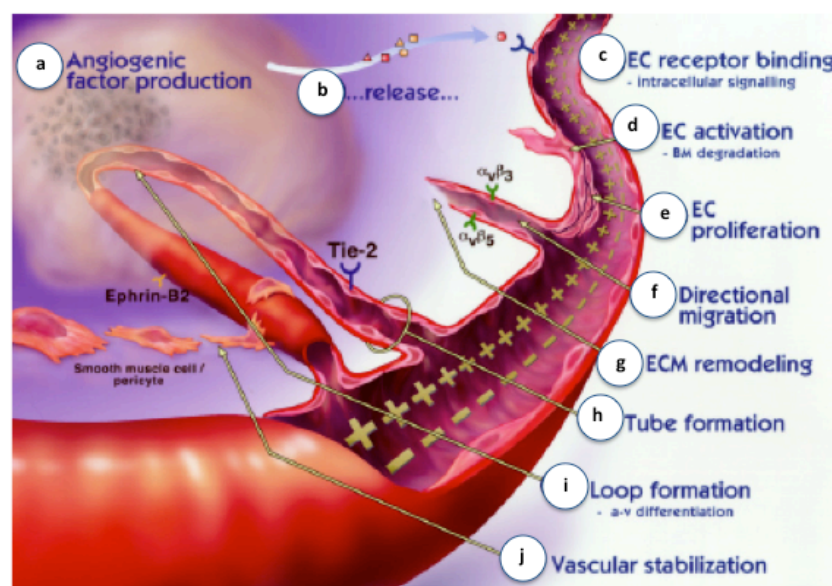


Figure 4. The process of angiogenesis. Diseased or injured tissues produce and release angiogenic growth factors that diffuse into nearby tissues (a, b); the angiogenic growth factors bind to specific receptors located on the ECs and nearby preexistent blood vessels (c); once growth factors bind to their EC receptors, a cascade of intracellular signaling is activated (c); pericytes detach and blood vessels dilate before the basement membrane and extracellular matrix is degraded by ECs produced enzymes (d); the ECs begin to divide (e) and they migrate out through the dissolved holes of the existing vessel towards the growth factor producer tissue through the action of integrins ( $\alpha_v\beta_3$ ,  $\alpha_v\beta_5$ ) that serve as grappling hooks to help pull the sprouting new blood vessel to sprout forward (f); additional enzymes are produced to dissolve and remodel the ECM around the vessel (g); sprouting ECs roll up to form a blood vessel tube (h); individual blood vessel tubes differentiate in arterial-venous systems and connect to form blood vessel loops that can circulate blood (i); newly formed blood vessel tubes are stabilized by SMCs and pericytes that provide structural support. Blood flow then begins. Adapted Pandya et al., 2006<sup>9</sup>.

### 3.1.2.1 Vascular endothelial growth factor (VEGF)

VEGF was initially termed vascular permeability factor (VPF) because of its ability to induce vascular leakage. Native VEGF is a heparin-binding homodimeric glycoprotein of 45 kDa<sup>10</sup>. VEGF is a mitogen for vascular ECs derived from arteries, veins and lymphatics, defined as a unique potent angiogenic factor, that is able to stimulate capillary formation *in vivo* and has direct mitogenic actions that are restricted to ECs<sup>9</sup>.

The family currently comprises six members: VEGF-A (which denotes the originally VEGF), placenta growth factor (PlGF), VEGF-B, VEGF-C, VEGF-D and the parapox virus VEGF, referred to as VEGF-E. The human VEGF gene is located in the short arm of chromosome 6 and organized as eight exons separated by seven introns<sup>9,11</sup>. Alternative exon splicing of the gene encoding VEGF-A was shown to result in the generation of five isoforms (VEGF-A<sub>121</sub>, VEGF-A<sub>145</sub>, VEGF-A<sub>165</sub>, VEGF-A<sub>189</sub>, VEGF-A<sub>206</sub>) having respectively 121, 145, 165, 189 and 206 aminoacids, after the signal sequence of cleavage<sup>12</sup>. VEGF-A<sub>165</sub> is the predominant isoform and is over-expressed in a variety of solid tumors. VEGF-A<sub>189</sub> is thought to be the most potent for vascularization in various cancers.

VEGF expression is transcriptionally regulated by hypoxia, which occurs during tumor expansion and ischemia<sup>13</sup>. More specifically, oxygen tension has a key role in regulating expression of a variety of genes. VEGF mRNA expression is induced by exposure to low oxygen tension under a variety of pathophysiological circumstances. Hypoxia is an important stimulus for expansion of the vascular bed that sprouts and matures into a system of stable vessels. Initially, cells are oxygenated by simple diffusion of oxygen, but when tissues grow beyond the limit of oxygen diffusion, hypoxia triggers vessel growth by signaling through hypoxia-inducible transcription factors (HIFs). These factors upregulate many angiogenic genes, although the induction of VEGFs is perhaps the most remarkable<sup>8</sup>.

Other via for VEGF gene expression regulation is through growth factors and oncogenes<sup>14</sup>. Several major growth factors, including epidermal growth factor, transforming growth factor- $\alpha$  and - $\beta$  (TGF- $\alpha$  and TGF- $\beta$ ), keratinocyte growth factor, insulin-like growth factor-1, fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF), all upregulate VEGF mRNA expression, suggesting that the paracrine or autocrine release of such factors cooperates with local hypoxia in the regulation of VEGF release in the microenvironment. Moreover, inflammatory cytokines such as IL- $\alpha$  and IL-6, induce the VEGF expression in several cell types, including synovial fibroblasts, which is in agreement with the hypothesis that VEGF may be a mediator of angiogenesis and permeability in inflammatory diseases<sup>14</sup>. Specific transforming events also result in induction of VEGF gene expression. For example, oncogenic mutations or amplification of Ras lead to VEGF upregulation, indicating that

mutant Ras-dependent VEGF expression is necessary, although insufficient, for progressive tumor growth *in vivo*<sup>14</sup>.

Initially, VEGF binding sites were identified on the cell surface of vascular ECs *in vitro* and *in vivo*, but subsequently, it became apparent that receptors for VEGF also occur on bone marrow-derived cells<sup>14</sup>.

VEGF binds two related receptor tyrosine kinases (RTKs), VEGFR1 and VEGFR2<sup>14</sup>.

The importance of VEGF in vascular development is highlighted by the fact that the loss of a single VEGF-A allele results in abnormal blood vessel development and embryonic death<sup>15,16</sup>.

A well-documented *in vitro* activity of VEGF is the ability to promote growth of vascular ECs derived from arteries, veins and lymphatics<sup>14</sup>. VEGF induces a potent angiogenic response in a variety of *in vivo* models and is a survival factor for ECs, both *in vivo* and *in vitro*. As referred before, VEGF is also known as VPF, based on its ability to induce vascular leakage<sup>13,14</sup>. Such permeability-enhancing activity underlies significant roles of this molecule in inflammation and other pathological conditions. VEGF induces an increase in hydraulic conductivity of isolated microvessels, which is an effect mediated by increased calcium influx<sup>17</sup>. Consistent with a role in the regulation of vascular permeability, VEGF also induces endothelial fenestration in some vascular beds as well as vasodilatation *in vitro* in a dose-dependent fashion as a result of EC-derived nitric oxide, and produces transient tachycardia, hypotension and a decrease in cardiac output when injected intravenously in conscious, instrumented rats<sup>14</sup>.

### 3.1.2.2 Angiopoietins (Ang1 and Ang2) and Tie receptors

A signaling system involved in vessel maintenance, growth and stabilization is the Tie2 receptor, which binds the angiopoietins (Ang1 and Ang2). The four identified angiopoietins (Ang1, Ang2, Ang3 and Ang4) are a family of secreted proteins that bind tyrosine kinase receptors (TKRs), Tie1 and Tie2. Expression patterns of Tie1 and Tie2 are similar to those of VEGFRs. Tie1 mRNA is highly expressed in embryonic vascular endothelium, angioblasts and endocardium, whereas it is expressed strongly in lung, capillaries but weakly in endocardium in adult tissues. Tie2 mRNA shows an analogous embryonic localization<sup>11</sup>.

Ang1 induces EC survival, capillary sprouting and pericytes recruitment. Moreover, it stabilizes blood vessels by increasing the interaction between ECs and pericytes. *In vivo* studies also suggest that Ang1 is essential for maturation and stabilization of the developing vasculature and for normal remodeling, since mice lacking Ang1 start to develop a primary vasculature which fails to stabilize or remodel leading to embryonic lethality<sup>11</sup>. Ang2 can either promote or inhibit vessel growth, depending on the presence of other growth factors, such as VEGF<sup>8,11</sup>. First, it was described that

ANGPT2 blocked Ang1 mediated Tie2 receptor activation, acting as an anti-angiogenic factor capable of promoting *in vivo* EC apoptosis and regression of blood vessels. In fact, Ang2 antagonizes Ang1 *in vivo* in a manner that may act to prevent excessive sprouting and branching of blood vessels by enhancing the decay of blood vessels. It is now known that these vessels can be prone to two fates as they can suffer regression in the absence of growth factors or they can be predisposed to angiogenic sprouting induced by available angiogenic factors. For example, in the presence of VEGF, ANGPT2 is responsible for an increase in capillary diameter, migration and proliferation of ECs and sprouting of new vessels<sup>18</sup>.

### 3.1.2.3 Platelet-derived growth factor (PDGF)

PDGF was initially purified from platelets and was then identified in fibroblast, astrocytes, keratinocytes, epithelial cells and other cell types<sup>11</sup>. PDGFs exist as heterodimers (PDGF-AB) or homodimers (PDGF-AA or PDGF-BB), composed of chains A and B. PDGF receptors are made up of complexes between  $\alpha$  and  $\beta$  subtypes. PDGF has been found to stimulate angiogenesis *in vivo*, and a role for PDGF in the recruitment of tumor pericytes, required for the development of capillaries, has been demonstrated in knock-out mice<sup>6</sup>. PDGF-BB and its receptor, PDGFR-B, have essential roles in the stabilization of nascent blood vessels by recruiting PDGF-B positive mesenchymal progenitors. Dropout or insufficient recruitment of mural cells results in EC growth, permeability, fragility, vessel enlargement, bleeding, impaired perfusion and hypoxia in embryos lacking PDGF-B.

### 3.1.2.4 Fibroblast growth factors (FGF)

Acidic and basic FGF are heparin-binding protein mitogens that play an important role in angiogenesis. *In vitro*, both of them induce processes in the ECs that are significant to angiogenesis, such as stimulate EC proliferation and migration as well as production of collagenase<sup>11</sup>.

FGFs exert their biological activities by binding to high affinity tyrosine kinase FGF receptors (FGFRs) on the surface of target cells. Activation of FGFR1 or FGFR2 by angiogenic FGFs (including FGF1, FGF2 and FGF4) leads to ECs proliferation. The first receptor stimulation leads, also, to ECs migration, protease production and tubular morphogenesis whereas the latter receptor besides ECs proliferation increases only cell motility<sup>19</sup>.

Alternative splicing of the FGFR mRNA generates receptor variants, which display a range of receptor-ligand interactions. Disruption of the genes encoding FGFR1 that is known to be required for the development and maintenance of the vasculature in the embryo or FGFR2 that is important for vascular tone and maintenance of normal blood pressure, leads to embryonic death before

gastrulation. This early lethality has made impossible to define specifically the role of these FGF receptors in the later stages of development and in angiogenesis<sup>13,20</sup>.

### 3.1.2.5 Transforming growth factor- $\beta$ (TGF- $\beta$ )

The transforming growth factors  $\beta$  represent a superfamily of highly conserved cytokines typified by TGF- $\beta$ 1. TGF- $\beta$  binds to two different types of serine-threonine kinase receptors, known as type I (T $\beta$ R-I) and type II (T $\beta$ R-II). Both receptors are interdependent, meaning that T $\beta$ R-I requires T $\beta$ R-II to bind to TGF $\beta$  and T $\beta$ R-II requires T $\beta$ R-I for signaling.

Both pro-angiogenic and anti-angiogenic properties have been attributed to TGF- $\beta$ 1, through effects on ECs and other cell types<sup>11</sup>. At low doses, TGF- $\beta$ 1 contributes to the angiogenic switch by upregulating angiogenic factors and proteinases, whereas at high doses, TGF- $\beta$ 1 inhibits EC growth, promotes basement membrane reformation and stimulates SMCs differentiation and recruitment<sup>8,11</sup>.

Gene studies in mice demonstrated that the loss of TGF- $\beta$  signaling components results in frail vessels with decreased vessel wall integrity and inactivation of the TGF- $\beta$ 1 caused lethality due to defects in the hematopoietic system and yolk sac vasculature<sup>21</sup>.

In addition, many other factors contribute to the control and influence angiogenesis, although their effects are not as extensive as the previously discussed. Besides growth factors, cytokines and chemokines, a number of membrane-bound proteins play important roles in angiogenesis. Integrins, ephrins and cadherins are membrane-bound proteins that affect many functions involved in blood vessel assembly (Figure 5).

Induction of the angiogenic switch depends on how heavily that balance tips in favour of pro-angiogenesis or anti-angiogenesis.



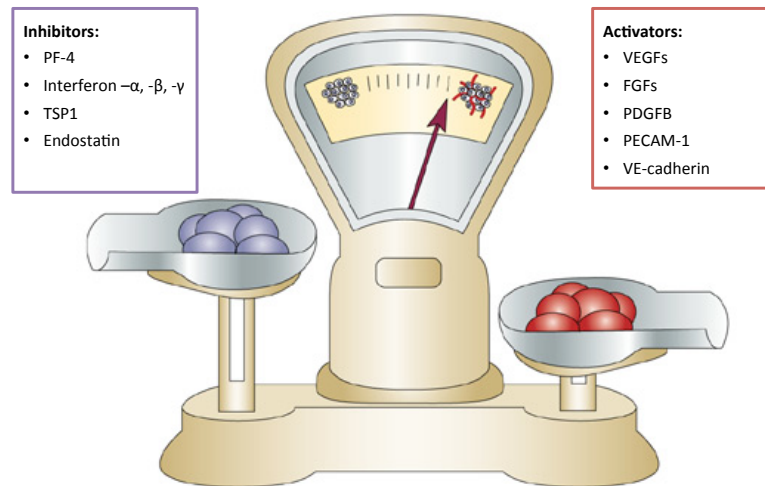


Figure 5. The angiogenic balance. Angiogenesis is orchestrated by a variety of activators and inhibitors. Activators of EC proliferation and migration are mainly receptor tyrosine kinase ligands, such as VEGF, fibroblast growth factors (FGFs), platelet-derived growth factor (PDGF); and also matrix proteins and adhesion molecules as platelet endothelial cell adhesion molecule-1 (PECAM1), which contributes to EC aggregation, tube formation and vessel stabilization and vascular endothelial cadherin (VE-cadherin) that besides decreasing EC apoptosis also contributes to vessel stabilization.

On the other hand, some examples of angiogenesis inhibitors are the platelet factor-4 (PF-4) that decreases FGF mediated angiogenesis; interferon  $-\alpha$ ,  $-\beta$ ,  $-\gamma$  that reduce FGF and matrix proteins and adhesion molecules as thrombospondin-1 (TSP1) which modulates EC proliferation and motility, enhancing EC apoptosis and endostatin that decreases EC proliferation. Adapted Bergers and Benjamin, 2003<sup>22</sup>.

### 3.2 Pathological angiogenesis

Insufficient vessel growth and regression contribute to numerous disorders, ranging from myocardial infarction and stroke to neurodegeneration. In diseases where an ischemia occurs, angiogenic inhibitors gain weight against stimulators, resulting in EC dysfunction, vessel malformation or regression<sup>23</sup>. Conversely, uncontrolled vessel growth promotes ocular disorders such as age-related macular degeneration and tumorogenesis, characterized by an excessive angiogenesis<sup>24</sup>.

#### 3.2.1 Tumor angiogenesis

Angiogenesis is essential for the growth of most primary tumors and their subsequent metastasis. The point at which the “normal processes” differentiate from pathological angiogenesis is in the tightly related balance of pro-angiogenic and anti-angiogenic signals.

The angiogenic switch can occur at different stages of the tumor-progression pathway, depending on the tumor type and environment. It has been shown that dormant lesions and, in some instances, premalignant lesions, also initiate neovascularization, which allows them to progress (Figure 6).

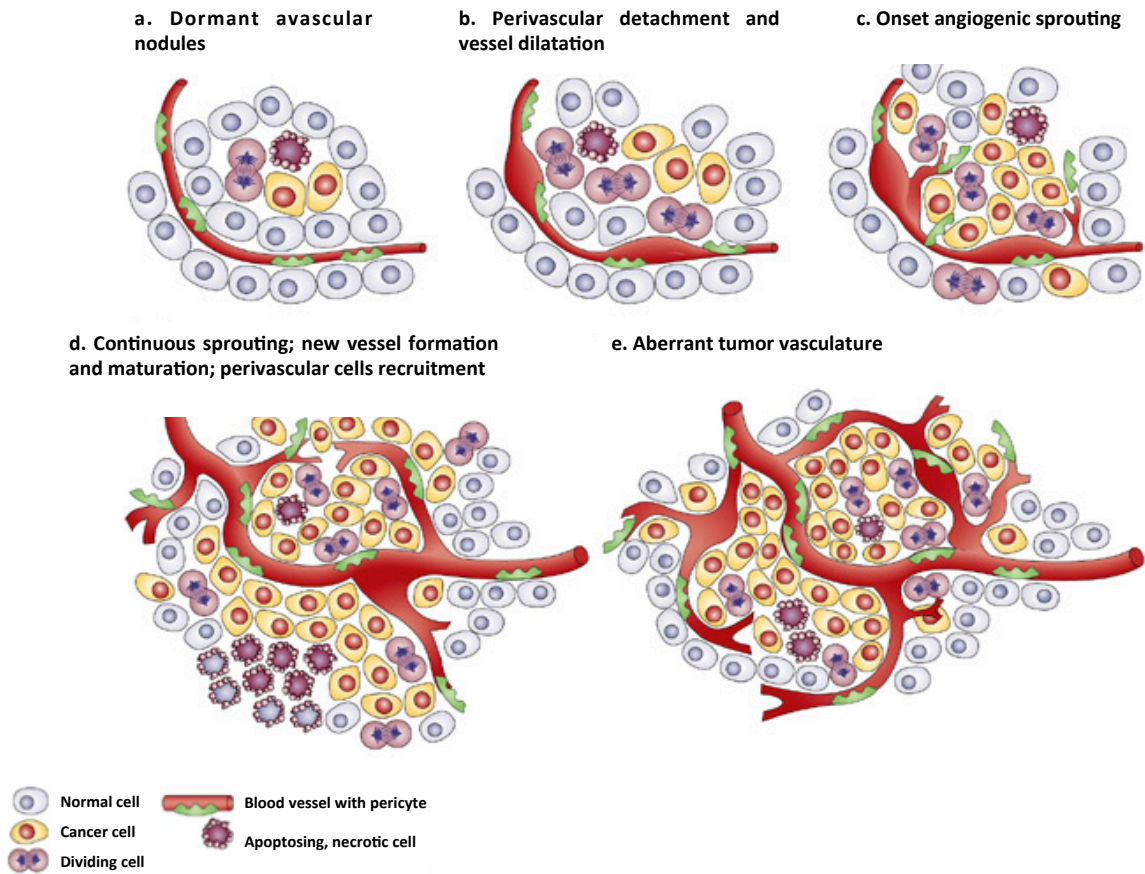


Figure 6. The classical angiogenic switch. The angiogenic switch is a step in tumor development that can occur at different stages in the tumor-progression pathway, depending on the nature of the tumor and its microenvironment. Most tumors start growing as avascular nodules (a) until they reach a steady-state level of proliferating and cells' apoptosis. The switch begins with perivascular detachment and vessel dilatation (b), followed by angiogenic expansion (c), new vessel formation and maturation and the recruitment of perivascular cells (d). Blood vessel formation will continue as long as tumor grows and the blood vessels specifically feed hypoxic and necrotic areas of the tumor providing it the essential nutrients and oxygen (e). Adapted from Bergers & Benjamin, 2003<sup>22</sup>.

The classical proof that tumors depend on angiogenesis and thus of blood supply, came from experiments whereby tumor fragments or cultured tumor cells were placed in an avascular site, the cornea of a rabbit eye<sup>25</sup>. The implants attracted new capillaries that grew in from limbus to vascularize the expanding tumor mass. Although if the capillaries were physically prevented from reaching the implant or were inhibited from undergoing angiogenesis, tumor growth was dramatically impaired, restricting the tumor nodule to a smaller diameter<sup>25</sup>. Subsequent experiments have confirmed this result and further revealed that, in the absence of access to an adequate vasculature, tumor cells become necrotic and/or apoptotic, restraining the increase in tumor volume that should result from continuous cell proliferation, the hallmark of cancer<sup>26,27</sup>.

Tumor vessels fail to become quiescent enabling the constant growth of new tumor blood vessels and, consequently, the tumor vasculature develops unique characteristics and becomes quite distinct from the normal supply system. Tumor blood vessels are architecturally different from blood vessels, as they are irregularly shaped, dilated, tortuous and can have dead ends. The vascular network that forms tumors is often leaky and haemorrhagic, partly due to the overproduction of VEGF<sup>5</sup>. Blood flows irregularly in tumor vessels, moving more slowly and sometimes even oscillating, leading to dysfunctional capillaries. Also, tumors can be quite heterogeneous in their vascular patterns and are able to overproduce their capillary networks<sup>5</sup>.

The angiogenic switch seems to result mainly from the action of mutated oncogenes (e.g. Ras family, src, EGFR and erbB-2/HER2), or loss or mutational inactivation of a variety of tumor-suppressor genes (mutant p53)<sup>22</sup>. Other causes include numerous environmental (epigenetic) factors such as hypoxia, low pH, inflammatory cytokines and growth factors<sup>28</sup>.

Factors that stimulate angiogenesis, such as VEGFA, PlGF and Ang1 have been shown to stimulate this process<sup>22</sup>. High levels of VEGFA expression alone are capable of initiating angiogenesis in a quiescent vasculature. The earliest stages are defined by vasodilatation and an increased vascular permeability of pre-existing capillaries, or post-capillary venules, in response to VEGF. This allows extravasation of plasma proteins which lay down a provisional matrix on which activated EC migrate and is accompanied by the loosening of pericyte covering, which is thought to be a function of Ang2.

### 3.2.2 Peripheral arterial disease

Peripheral arterial disease (PAD) involves the narrowing and obstruction of blood flow in arteries, most commonly affecting the circulation in the arteries of the pelvis and legs due to atherosclerosis related problems. PAD is a progressive illness and the most severe manifestation is termed “critical limb ischemia” (CLI). This disease describes patients with chronic ischemic rest pain or patients with ischemic skin lesions, ischemic ulcers or gangrene and occurs when arterial lesions impair blood flow and distal perfusion pressure to a level insufficient to satisfy the nutritive needs of the limb. The progression of PAD to these acute symptoms may be gradual or progress rapidly reflecting sudden worsening of limb perfusion and often threatening limb viability<sup>29,30</sup>.

Acute limb ischemia may also occur as the result of an embolic event or a local thrombosis in a previously asymptomatic patient. Moreover, a number of studies have allowed an analysis of the risk factors that seem to be associated with the development of CLI and they appear to be independent, therefore, probably additive<sup>29</sup>.

There is few information about the incidence of acute leg ischemia, but acute leg ischemia due to emboli has decreased over the years, possibly as a consequence of less cardiac valvular disease from better monitoring and anticoagulant management of atrial fibrillation. Meanwhile, the incidence of

thrombotic acute leg ischemia has increased, even with the extensive use of newer endovascular techniques<sup>29</sup>.

The only reliable large prospective population studies on the incidence of CLI, showed a figure of approximately between 500 and 1000 new cases per million per year<sup>29</sup>. In a large proportion of these patients, the anatomic extent and the distribution of arterial occlusive disease make patients unsuitable for operative or percutaneous revascularization, although, any kind of surgical/endovascular revascularization should be done whenever technically possible. Attempts to manipulate and normalize the microcirculatory flow pharmacologically may enhance the results of revascularization or be one option. In patients with CLI not eligible for arterial revascularization, prostanoids are vasoactive drugs with proven efficacy<sup>29,59,60</sup>. However, recent trials do not support the benefit of prostanoids in promoting amputation-free survival<sup>58</sup>.

Amputation, despite its associated morbidity, mortality rates and functional implications, is often recommended as a solution to the disabling symptoms, in particular excruciating ischemic rest pain. Indeed, a second major amputation is required in nearly 10% of such patients (Figure 7)<sup>31</sup>.

It is clear that decreasing the level of any risk factor, such as blood pressure and LDL cholesterol, can help improve prognosis. Nevertheless, it is not clear what the optimal values are in the general population and in individual disease states. Modifying several risk factors is at least as beneficial as changing only one. Combination therapy with several drugs will become inevitable; however it must be paid attention to the compliance of the patients who are faced with such therapy.

The prevalence of occlusive vascular disease of peripheral limb circulations underscores the importance of understanding how adaptive remodeling of the vasculature in ischemia is regulated<sup>32</sup>. Unlike distal capillaries, which distribute blood flow to individual cells, arteries provide bulk flow to the tissue and are therefore, of utmost importance. When an artery is occluded, its vascular territory becomes ischemic since arterial systems are often inter-connected by pre-existing collateral vessels. However, the collaterals can enlarge and salvage the ischemic region. The mechanism of angiogenesis and collateral growth differ significantly. Because of the large pressure differences between the perfusion territories, the increased shear stress activates ECs, which then recruit monocytes. These cells produce growth factors, proteinases, which enable SMCs to migrate and divide, explaining why depletion of monocytes impairs, whereas delivery of monocytes enhances collateral growth<sup>8</sup>. Cytokines that attract monocytes or prolong their life span enhance collateral growth, whereas anti-inflammatory cytokines are inhibitory. PIGF for example enhances collateral growth, not only because it recruits monocytes but also because it stimulates EC and SMCs growth. Delivery of aFGF or bFGF (together with PDGF-BB) stimulates collateral growth, in part by upregulating PDGFR expression<sup>8</sup>.

Preclinical studies have demonstrated that angiogenic growth factors can stimulate the development of collateral arteries in animal models of peripheral and myocardial ischemia. In addition, evidence suggests that new collaterals may form and undergo outward remodeling<sup>24</sup>. According to literature, flow-induced shear stress is an important biomechanical force in the initiation of arteriogenesis. In the most tissues, a small number of preexisting collaterals make interconnections between most arterioles of adjacent vascular beds. Shear stress increases within collaterals when the lumen of the conduit artery supplying one bed becomes narrowed and pressure below it is reduced. Although collateral development, also termed arteriogenesis, has greater potential for restoring blood flow to severely ischemic beds than does angiogenesis alone, less is known about the mechanisms that direct arteriogenesis<sup>24,33</sup>.

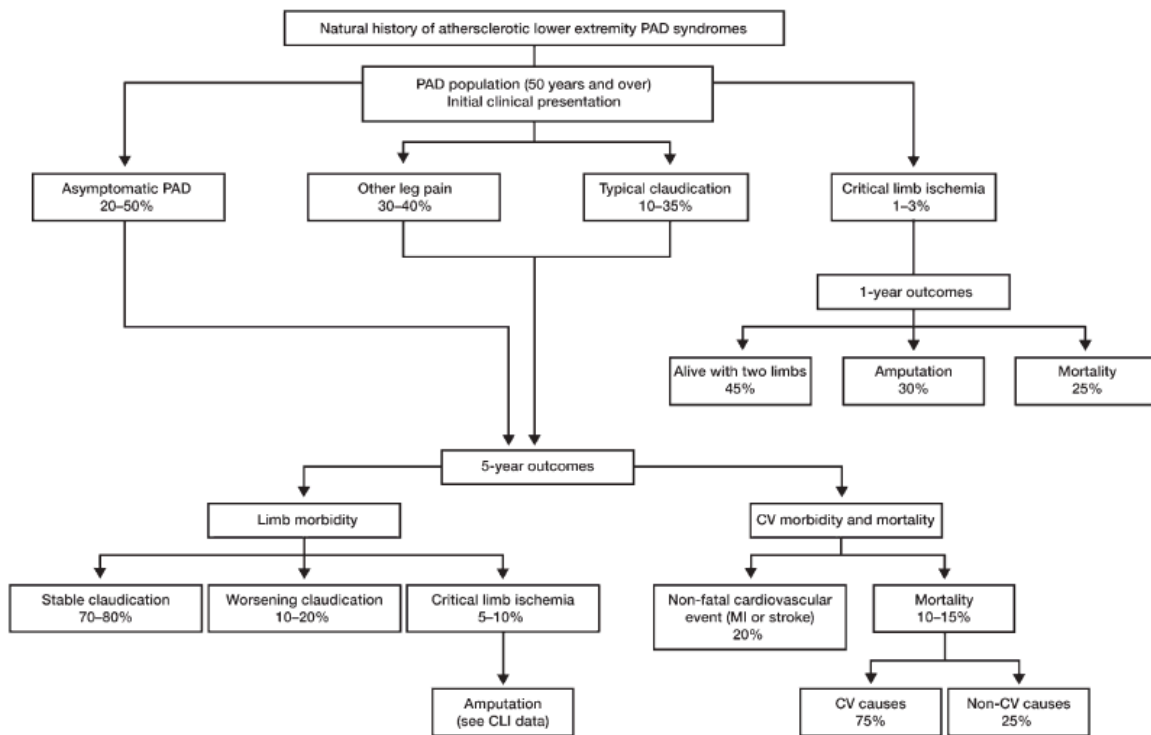


Figure 7. Schematic representation of fate of the claudicant over 5 years (adapted from ACC/AHA guidelines<sup>5</sup>). PAD – peripheral arterial disease; CLI – critical limb ischemia; CV – cardiovascular; MI – myocardial infarction. Adapted from Norgren et al.,2007<sup>29</sup>.

### 3.3 Therapeutic angiogenesis

The recognition that both vasculogenesis and angiogenesis are fundamental in normal developmental and adult physiological process, requiring the coordinated action of a variety of growth factors and cell-adhesion molecules in endothelial and mural cells, has led to the establishment of numerous *in vitro* and *in vivo* research models.

The importance of these processes sparked hopes that manipulating them could offer therapeutic opportunities. Their full understanding would give the ability to induce or inhibit blood vessels growth in a controlled manner and it would provide possible strategies for the treatment of several diseases.

### 3.3.1 Anti-angiogenic therapy

Anti-angiogenic therapy inhibits the growth of blood vessels and so it is an important process in the treatment of many diseases, such as arthritis, blinding disorders and cancer. Anti-angiogenic drugs exert their effects by i) disabling the agents that activate and promote cells growth, survival and/or migration; ii) up-regulate or deliver of endogenous inhibitors; iii) directly blocking the growth of ECs in its receptors or iv) downstream signaling<sup>24</sup>.

The aim of using anti-angiogenic agents in the treatment of cancer is to normalize vessels by pruning the immature and inefficient vessels and remodeling the remaining ones.

The fact that tumors are dependent on blood supply has inspired many researchers to find anti-angiogenic molecules and to design anti-angiogenic strategies for cancer treatment. Preventive strategies could be especially useful in patients who are at high risk of developing cancer, metastasis or recurrence of cancer. Given the requirement of angiogenesis for growth and progression of dormant lesions, it would be assumed that inhibition of the angiogenic switch could prevent progression of tumors and their metastases. Therefore, anti-angiogenic agents really represent a promising therapy for a number of reasons: i) normal ECs are quiescent in physiological conditions, whereas tumor ECs and EPCs have high rate of proliferation with an abnormal phenotype, lowering the possible toxicity of the angiogenic inhibitors; ii) in opposition to tumor cells, ECs are generically stable and iii) tumor endothelium expresses high levels of specific molecules that can be targeted by selective inhibitors<sup>22</sup>.

Due to the importance of VEGFA and its receptor VEGFR2 in several strategies to inhibit VEGF pathway have been developed. Bevacizumab (Avastin®), a monoclonal anti-VEGF antibody, and selective TKR inhibitors sunitinib (Sutent®) and sorafenib (Nexavar®), have conquered special attention in the treatment of metastatic colorectal cancer, advance renal cell carcinoma and hepatocellular carcinoma, respectively<sup>34,35,36</sup>. Other VEGF receptor tyrosine kinase pathway inhibitors such as AG-013736 (Axitinib®)<sup>37</sup> and PTK787/ZK22584 (Vatalanib®)<sup>38</sup> have also shown promising results in metastatic renal cell carcinoma and colorectal cancer patients, respectively<sup>35</sup>. VEGF-targeted therapy inhibits neovessel growth by blocking the activation of intracellular signaling pathways, and promotes EC apoptosis, since VEGF is also a mediator of different cell pro-survival pathways. Besides, VEGF also influences the chemotaxis of EPCs and the direct growth of tumor cells

being, for that reason, expected a partial reduction in the incorporation of EPCs in the vasculature and a reduction of tumor growth<sup>34</sup>.

However, clinical experience has revealed that VEGF-targeted therapy often prolongs overall survival of cancer patients only by months, being metastasis the principal cause of death<sup>39</sup>.

Consistent with the clinical observations<sup>40,41</sup>, many studies have shown that VEGF-targeted therapies efficiently inhibited primary tumor growth in different biological models (reviewed in Ferrara & Kerbel, 2005<sup>35</sup>). However, Ebos et al. showed that pre-treatment of healthy animals with VEGF inhibitors prior to the injection of tumor cells lead to the development of more aggressive metastasis, as well as in orthotopic tumor models lead to vaso-invasion phenotype<sup>42</sup>. These, apparently inconsistent, results might be explained by different hypothesis: i) Replacement of VEGFA effects by other angiogenic pathways or other members of the VEGF family; ii) hypoxia, caused by exacerbated disruption of vessels, which can contribute to the selection of more resistant cancer cells; iii) Recruitment, by the tumors, of EPCs from the bone marrow; iv) co-option of existing vasculature; v) production of pro-inflammatory (angiogenic) molecules due to the VEGF inhibitors induction of a chronic “inflamed” state; and vi) facilitated intravasation of tumor cells in the destabilized vessels<sup>39,43</sup>.

Since VEGF-targeting therapy might not be enough to promote tumor inhibition, other strategies such as drugs blocking matrix break-down or the target of other angiogenic factors/receptors have also to be considered (e.g. cetuximab (Erbix<sup>®</sup>), a monoclonal EGFR-antibody; interferon- $\alpha$  (Roferon<sup>®</sup>), a cytokine responsible for the inhibition of FGF2, interleukin 8 (IL8) and matrix metalloproteinases (MMPs)<sup>23,24,44</sup>.

### 3.3.2 Pro-angiogenic therapy

Despite some initial setbacks, potential therapeutic approaches to induce or improve angiogenesis are already in clinical trials, including the i) direct use of angiogenic growth factors that predominantly act on ECs to promote their proliferation, migration and tube-forming activity; ii) gene therapy to better perfusion; iii) inhibition of anti-angiogenic factors and iv) mobilization of regenerative stem cell populations.

Regarding growth factors, most of them render the ECs less sensitive for apoptosis induction. Among the first growth factors identified to improve angiogenesis were members of the VEGF and FGF families.

Hepatocyte growth factor (HGF) is another potent multifunctional protein that exerts high proangiogenic activity<sup>45</sup>. It activates its receptor *c-met*, which is expressed on hematopoietic stem cells and on a variety of other cells including ECs, exclusively stimulating their growth without replication of vascular SMCs<sup>31</sup>.

Ang1, neurotrophin nerve growth factor, erythropoietin and insulin-like growth factor (IGF) are additional candidates for therapeutic angiogenesis. Some of the growth factors may enhance tissue regeneration not solely via the proangiogenic activity but also via promotion of stem/progenitor cell mobilization. For example VEGF, Ang1 and erythropoietin mobilized endothelial progenitors from the bone marrow. Transplantation of EPCs, derived from the bone marrow or peripheral blood, have demonstrated to be an alternative to angiogenic factors contributing to postnatal neovascularization in ischemic limbs and myocardium<sup>35</sup>.

It is possible that several proangiogenic factors may act indirectly via the upregulation of VEGF and possibly other growth factors. This has been shown for the members of the hedgehog (Hh) protein family which are morphogens regulating epithelial-mesenchymal signaling during several crucial processes of embryonic development<sup>45</sup>. A second class of candidate genes represents monocyte chemoattractant protein-1 and granulocyte-macrophage colony-stimulating factor, which both act on monocytic cells to promote angiogenesis.

Other proangiogenic factors activate integrin-dependent pathways to promote angiogenesis. These integrin-activatory proteins include the extracellular matrix protein Del-1, which coordinates integrin expression by homeobox genes. Additionally, regulators of wnt/frizzled pathway, as shown for the secreted frizzled-related protein FrzA, promote adult angiogenesis. This effect was shown to be independent of VEGF. Finally, Akt/protein kinase B may also represent an attractive therapeutic option, not only for augmenting tissue perfusion but also via more recently appeared to restore tissue integrity in jeopardized myocardium, emphasizing the potential potency of exploiting signaling pathways<sup>45</sup>.

Successful pro-angiogenic therapy still raises some questions regarding long-term effects. One of the most important questions is whether these therapies can indirectly contribute to trigger dormant tumors and lesions.

#### 3.3.2.1 Cell therapy: Mesenchymal stem cells

Mesenchymal stem cells (MSCs) hold great promise as a therapy for peripheral arterial disease due to their pro-angiogenic/vasculogenic paracrine activity, transdifferentiation, immunosuppressive capacity and low immunogenicity.

PAD remains a leading cause of limb loss and mortality worldwide and cell-based therapies were conceived initially as an innovative way to improve blood flow recovery to prevent amputation. Many potential candidate cells have been evaluated as promoters of blood circulation including whole bone marrow cells, bone marrow mononuclear cells, hematopoietic stem cells, EPCs and hemangiocytes. Of the various types of stem and progenitor cells, MSCs are being the most actively



pursued at both preclinical and clinical levels, owing to their ease of isolation and capacity for *ex vivo* expansion<sup>46</sup>.

No unique cell surface marker unequivocally distinguishes MSCs from other stem cells, which makes a uniform definition difficult<sup>47</sup>. The International Society for Cellular Therapy proposed criteria that comprise (1) adherence to plastic in standard culture conditions; (2) expression of the surface molecules CD73, CD90, and CD105 in the absence of CD34, CD45, HLA-DR, CD14 or CD11b, CD79a, or CD19 surface molecules as assessed by fluorescence-activated cell sorter analysis; and (3) a capacity for differentiation to osteoblasts, adipocytes, and chondrocytes *in vitro*. These criteria were established to standardize human MSC isolation but may not apply uniformly to other species. For example, murine MSCs have been shown to differ in marker expression and behavior compared with human MSCs<sup>48</sup>. MSCs were first collected from bone marrow and since then they have also been isolated from a variety of other sources: peripheral blood, cord blood/tissue, adipose tissue, lung, heart, placenta, periodontal ligament tissue, along with fetal and amniotic membranes. MSCs isolated from various adult and neonatal tissue sources have significantly different morphology and express a distinct pattern of cell surface markers<sup>49</sup>. Moreover, they exhibit different *ex vivo* differentiation capacity and pro-angiogenic properties<sup>46</sup>. These observations suggest that MSCs from different tissues may not be biologically equivalent and exhibit variable self-renewal and multipotential capabilities.

MSCs derived from each of these sources (Figure 8) have also been shown in experimental models to be effective in the treatment of hindlimb ischemia<sup>46</sup>. MSCs can home to and survive in an ischemic environment. Through paracrine effects, they aid in the promotion of arteriogenesis and angiogenesis by releasing various angiogenic growth factors including VEGF, bFGF, and stromal cell derived factor-1 $\alpha$ . Analysis of the MSC secretome using a protein array screen revealed a large fraction of chemotactic cytokines, or chemokines. Genetic modification of MSCs has been shown to further increase the range of paracrine effects. As a part of their multiple differentiation potential, MSCs can be induced to adopt endothelial properties in the presence of VEGF and/or basic FGF, and to express endothelial-specific markers. These features enable MSCs to promote post-ischemic neovascularization and blood flow recovery in ischemic diseases secondary to peripheral arterial occlusive disease, although the specific mechanisms by which they do so still need to be fully characterized.

Moreover, several studies show that MSCs have unique immunological characteristics that allow their persistence in a xenogeneic environment and confirmed their immunomodulatory properties<sup>50</sup>. They can induce immunosuppressive activity for a broad range of immune cells, including T

lymphocytes, B lymphocytes, natural killer cells and dendritic cells. Thus, this immunosuppressive effect may give MSCs unique advantages as a transplanted cell therapy<sup>46</sup>.

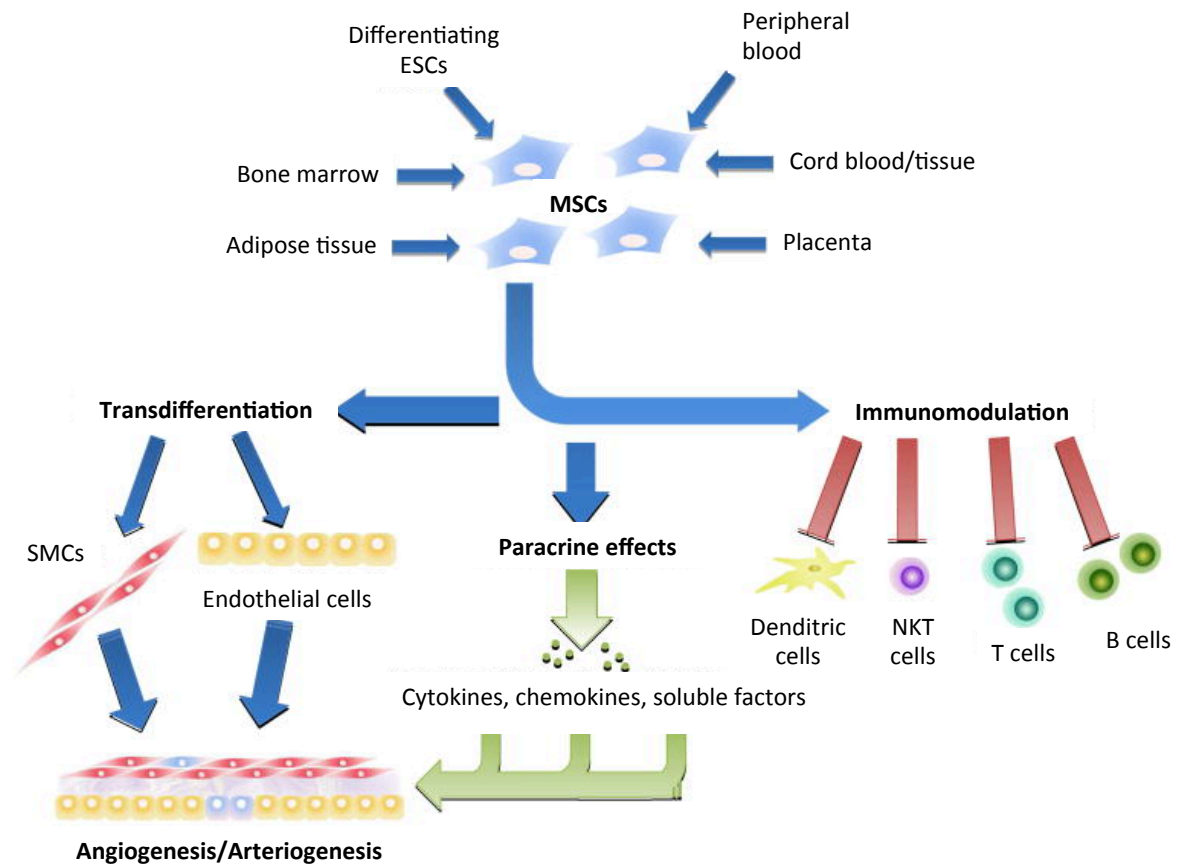


Figura 8. Biological sources and activity of MSCs. MSCs can be isolated from multiple sources (adipose tissue, bone marrow, cord blood/tissue, placenta) and exert therapeutic effects on multiple systems to contribute to peripheral arterial disease therapeutic. Adapted from Yan et al., 2013<sup>46</sup>.

### 3.3.2.1.1 Umbilical cord tissue-derived mesenchymal stromal cells (UCX<sup>®</sup>)

MSCs derived from umbilical cord tissue (UC-MSCs) have the similar surface phenotype, plastic adherence and multipotency as those of MSCs derived from other sources. Compared with the counterparts of other origins, UC-MSCs have attractive advantages: i) a noninvasive collection procedure for autologous or allogeneic use and ethically acceptable; ii) a lower risk of infection; iii) a low risk of teratoma; iv) easy to obtain a substantial number of UC-MSCs after several passages and extensive *ex vivo* expansion; v) faster self-renewal when compared to bone marrow-derived MSCs (BM-MSCs); vi) low immunogenicity with a good immunosuppressive ability<sup>51</sup>.

ECBio, a biotechnology company, has developed proprietary technology to consistently isolate, expand and cryopreserve a well-characterized population of human stem cells derived from umbilical cord tissue (Wharton's jelly) named as UCX<sup>®</sup> cells<sup>52</sup>. The technology used for UCX<sup>®</sup> isolation is different from other UC-MSC isolation methods. Very briefly: i) the introduction of a three-stage strategy for cell recovery was introduced in order to make the method 100% reliable ii) the isolation begins with peeling off the amniotic membrane which reduces the frequency of microbial contamination and augments the purity of the resulting UCX<sup>®</sup> population by eliminating epithelial progenitors iii) tissue incisions and/or tissue mincing/ crushing steps are avoided since they could hinder its application in GMP settings and jeopardize stem cell phenotype due to excessive mechanical manipulation and iv) the use of an optimized ratio between tissue mass, digestion enzyme activity units, overall solution volume and void volume allowing UCX<sup>®</sup> cell-specific release and intact cord vessels that will reduce contamination with endothelial and sub-endothelial cells from umbilical arteries and vein<sup>52</sup>.

Very recently, ECBio adapted a method to consistently isolate, expand and cryopreserve UCX<sup>®</sup> towards the production of an advanced therapy medicinal product.

UCX<sup>®</sup> cells are a homogeneous population of stem cells that comply with the current definition of MSC as established by International Society for Cellular Therapy (ISCT) namely: i) cells that adhere to a plastic surface ii) at least, 95% of UCX<sup>®</sup> cells in the population are consistently positive for the cells surface markers CD44, CD73, CD90 and CD105 and less than 2% positive for CD14, CD19, CD31, CD34, CD45 and HLA-DR iii) cells that are capable of undergoing tri-lineage differentiation into adipocytes, chondrocytes and osteoblasts<sup>52,53</sup>

Importantly, it was shown that UCX<sup>®</sup> cells may be an effective and promising new approach for treating both local and systemic manifestations of inflammatory arthritis<sup>52</sup>. It was observed that UCX<sup>®</sup> cells have the capacity to inhibit human T-cell proliferation and concomitantly promote the expansion of Tregs more efficiently than BM-MSCs. Accordingly, xenogeneic UCX<sup>®</sup> administration in both acute carrageenan-induced arthritis and chronic adjuvant-induced arthritis models for arthritic inflammation, can reduce paw edema *in vivo* more efficiently than BM-MSCs and showed faster remission of local and systemic arthritic manifestations, respectively. These data strongly suggest that UCX<sup>®</sup> are immunosuppressive and have low immunogenicity, relevant for allogeneic applications<sup>52</sup>.

Furthermore, it was shown that UCX<sup>®</sup> preserve cardiac function and attenuate adverse tissue remodeling after intramyocardial transplantation in a murine myocardial infarction model<sup>54</sup>. The cardioprotective effect of UCX<sup>®</sup> is exerted through paracrine mechanisms that appear to enhance

angiogenesis, limit the extent of the apoptosis in the heart, increase proliferation and activate the cardiomyogenic gene-expression program in a pool of myocardial resident cardiac progenitor cells<sup>54</sup>.

In this particular work it was shown that UCX<sup>®</sup> increase capillary density in the infarcted myocardium since CD31-expressing cells were more abundant within the infarcted wall of UCX<sup>®</sup>-transplanted hearts when compared to vehicle-injected hearts<sup>54</sup>.

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