CHAPTER 1

GENERAL INTRODUCTION
1. **ANGIOGENESIS AND TUMOR ANGIOGENESIS**

Angiogenesis, the formation of new blood vessels from pre-existing vessels [1], is an important natural process occurring in the body, both as physiological process and as ‘repair mechanism’ in damaged tissues. Vasculogenesis, is the formation of vascular structures from circulating or tissue-resident endothelial stem or progenitor cells, which differentiate and proliferate into a *de novo* endothelial cell tube like structure (bloodvessel). The latter particularly relates to the early embryonic development of the vascular system [2], but this type of blood vessel formation was recently introduced as a new concept in pathological situations, such as during tumor neo-vascularisation in adult life.

In a normal healthy body, a perfect balance is maintained in the vasculature between positive and negative angiogenesis modulators. In such case, angiogenesis remains suppressed by the presence of more anti-angiogenic than pro-angiogenic factors, and endothelial cells rarely divide. However, during growth and tissue repair and in pathological conditions endothelial cells become activated, highly proliferative and involved in neovascularization. Examples of physiological angiogenesis include embryonic vasculogenesis and angiogenesis, angiogenesis in the female reproductive system and wound healing [3]. Angiogenesis associated with pathological conditions includes arthritis, pulmonary diseases, diabetes, atherosclerosis and cancer progression [4]. In 1986 Dvorak *et al.* proposed that tumors are never healing wounds and suggested that tumors can promote the activation of the wound-healing response [5], which underlines the direct interaction between physiological and pathological angiogenesis. However, specific features of angiogenesis in tumors have also been mentioned. Differences between tumor angiogenesis and physiological angiogenesis include the development of aberrant vascular structures and excessive vascular sprouting, altered endothelial-cell-pericyte interactions, abnormal blood flow, increased permeability and delayed maturation of vessels [6] (Figure 1).

Clinical research on angiogenesis can be envisioned into two major fields: anti-angiogenic therapies and pro-angiogenic therapies (therapeutic angiogenesis). Whereas angiogenesis inhibitors are being investigated to fight cancer, pro-angiogenic therapies are being explored for cardiovascular disease.

In the following sections we shall survey four important aspects of tumor angiogenesis and anti-angiogenic treatment: 1) Regulation of tumor angiogenesis and the involvement of circulating endothelial cells, 2) Hypoxia, tumor progression and angiogenesis, 3) Anti-angiogenic treatment, and 4) Biomarker to predict response to anti-angiogenic treatment. Finally, this chapter will end with the aim and outline of this thesis.
1. Tumor cells produce and release angiogenic growth factors (proteins) that diffuse into the nearby tissues. 2. The angiogenic growth factors bind to specific receptors such as vascular endothelial growth factor (VEGF) receptors located on the endothelial cells (EC) of nearby pre-existing blood vessels. 3. Once growth factors bind to their receptors, the endothelial cells become activated. Signals are sent from the cell's surface to the nucleus. 4. The endothelial cell's machinery begins to produce new molecules including enzymes. These enzymes dissolve tiny holes in the sheath-like covering (basement membrane) surrounding all existing blood vessels. 5. The endothelial cells begin to divide (proliferate) and migrate out through the dissolved holes of the existing vessel towards the tumor. 6. Specialized adhesion molecules called integrins (αvβ3, αvβ5) serve as grappling hooks to help pull the sprouting new blood vessel sprout forward. 7. Additional enzymes (matrix metalloproteinases or MMP) are produced to dissolve the tissue in front of the sprouting vessel tip in order to accommodate it. As the vessel extends, the tissue is remolded around the vessel. 8. Sprouting endothelial cells roll up to form a blood vessel tube. 9. Individual blood vessel tubes connect to form blood vessel loops that can circulate blood. 10. Finally, newly formed blood vessel tubes are stabilized by specialized pericytes that provide structural support. Blood flow then begins.

*From The Angiogenesis Foundation website.*
1.1 Regulation of Tumor Angiogenesis

The healthy body controls angiogenesis by maintaining a perfect balance between pro-angiogenic and anti-angiogenic factors. In case of tumor angiogenesis this balance is disturbed in favor of pro-angiogenic factors, termed the ‘angiogenic switch’ [7]. Like normal tissues, tumors require an adequate supply of oxygen, nutrients and an effective way to remove waste products via the vasculature [8]. Beyond a certain size (typically occurring before the tumor reaches a size of 2-3 mm$^3$) a tumor demands more oxygen and nutrients than available by the local supply and becomes hypoxic [9]. As a response to hypoxia, tumor cells will produce and secrete several factors that stimulate novel blood vessel formation (pro-angiogenic factors), like vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and interleukin-8 (IL-8). Among these stimulating factors, the most dominant and extensively studied is vascular endothelial growth factor-A (VEGF-A), which was first known as vascular permeability factor discovered by Senger et al. [10]. A VEGF gene family exists, which includes VEGF-A, -B, -C, -D, and placental growth factor (PIGF, also known as PGF) [11]. VEGF-A (further referred to as VEGF), the best characterized molecule of the VEGF family, is commonly overexpressed in several solid tumors and interacts primarily with two tyrosine kinase receptors; VEGF receptor-1 (VEGFR-1, Flt-1) and VEGFR-2 (KDR, Flk-2) [12]. VEGFR-2 expression is restricted primarily to the endothelial cells and is the major positive mediator in tumor angiogenesis. Mechanistically, VEGF promotes tumor angiogenesis through numerous processes contributing to changes within the tumor vasculature, including endothelial cell proliferation, migration, invasion, survival, chemotaxis of bone marrow derived progenitor cells, vascular permeability and vasodilatation [13, 14]. VEGF and VEGF receptors are current targets for anti-tumor treatment.

1.2 Circulating Endothelial Cells and Tumor Angiogenesis

There are several regulators in the process of (tumor) angiogenesis, some of which represent therapeutic targets. Endothelial cells (EC) are key players in angiogenesis, forming a thin layer that lines the interior surface of blood vessels, and form a barrier between the blood and the subendothelial extracellular matrix. A major mechanism of angiogenesis is the formation of EC vessel sprouts from an existing vessel. Tumor vasculature does not necessarily derive from sprouting angiogenesis, but instead, tumors may well acquire their vasculature by other mechanisms including cooption of existing vessels and by vasculogenesis. In the process of vasculogenesis, circulating bone marrow
(BM)-derived endothelial progenitor cells (EPCs) have been hypothesized to home to sites of neovascularization and differentiate into ECs [15].

With regard to tumor vasculature two types of circulating endothelial (progenitor) cells have been studied extensively namely, circulating endothelial cells (CECs) and endothelial progenitor cells (EPCs). The term CECs is used with two distinct definitions: First, CECs in general are endothelial cells that circulate in the blood regardless of their function and origin (they may include the endothelial cells with proliferative potential, and may contain progenitors). Second, mature CECs represent endothelial cells in circulation that have been detached from the vessel wall (endothelial lining) elsewhere, which have a limited growth capability, and bear a mature phenotype. Identification of CECs is highly complex and has been hampered by the overlapping antigenic similarities with other cell populations, which caused a lack of consensus about the definition of these endothelial cells [16]. Besides CD31 or CD146, CD34 is one of the markers used to identify ECs because (tumor) vessels are often CD34+ and it is present on endothelial progenitors [15]. CD34+ cells alone can repopulate bone marrow in vivo [17], and CD34 is also a marker for HPCs. Therefore a larger panel of markers is essential to identify ECs. The pan-hematopoietic marker CD45 has been widely used to exclude hematopoietic cells (HPCs) [18]. Accordingly, the definition of an EPC, - in circulation called a circulating endothelial progenitor cell (CEP) - includes CD34+ and CD45−. An EPC is best defined as an immature precursor cell that individually displays postnatal vasculogenic activity, that should be capable of forming new ECs and blood vessels in vivo [19].

**CONCEPT OF CIRCULATING ENDOTHELIAL CELLS**

*Mature* CECs gained interest because they are thought to be potentially surrogate markers of damage to the endothelium, for instance as a response to anti-angiogenic treatment of cancer, and may reflect vascular dysfunction [20-24]. Differently from *mature* CECs, are circulating endothelial progenitor cells (CEPs; EPCs in circulation), which were originally considered as non-leukocyte endothelial progenitors. It was hypothesized that they mobilize in response to tumor cytokines including VEGF [25], from the bone marrow into the peripheral circulation, and move to the tumor bed where they incorporate into lining of the newly formed blood vessel [14, 26]. At present the existence and the concept that CEPs home to the place were neovascularization is needed is still questioned [27], and EPCs appear to be a complex group of cells consisting mainly of myeloid cells that acquire endothelial characteristics [28]. Because of the concept that EPCs/CEPs and subtypes of pro-angiogenic HPCs are actively participating in neovascularization they are of considerable interest in the field of angiogenesis treatment (Figure 2). CECs and progenitors not only represent an attractive
novel target as potential vehicles for delivering therapeutic molecules [14], but also are potentially useful biomarkers for monitoring cancer progression, as well as for optimizing the efficacy of anti-angiogenic therapies [29, 30].

**CONTRIBUTION OF CIRCULATING ENDOTHELIAL CELLS TO TUMOR ANGIOGENESIS**

The contribution of bone marrow derived progenitors to neovascularization was first described by Asahara et al. [31]. In addition, Lyden et al. was the first that demonstrated in vivo the recruitment and incorporation of VEGFR2⁺ (progenitor) cells into vessels and the role of VEGFR1⁺ HPCs to support this process [26]. Currently, the role of EPCs/CEPs in tumor angiogenesis remains under debate. The extent to which EPCs/CEPs are incorporated into tumor vasculature has been controversial with high variation of EPCs/CEPs incorporation [26, 27, 32-37].

**DETECTION OF CIRCULATING ENDOTHELIAL CELLS**

To monitor the effects of anti-angiogenic treatment on the number or properties of mature CECs and EPCs/CEPs in cancer patients it is important that the method of detection is easily applicable, reliable (reproducible) and in the end validated. In developing this method, there are two major problems; the very low number of mature CECs as well as EPCs/CEPs (both < 0.01% of circulating nucleated cells) and lack of unique marker proteins for the identification of both populations. As both cell populations are present in low numbers (0- several hundreds/mL blood) it requires high demands on the sensitivity and reliability of analytical techniques. Moreover, the techniques used to identify blood cells are usually based on morphological criteria or on the presence of specific surface proteins but no unique endothelial marker is known to specifically identify mature CECs and EPCs/CEPs. In general CECs can be detected and quantified by different methods such as quantitative PCR, immunomagnetic separation (IMS), flow cytometry, and cell culture techniques. The original method for measuring mature CECs is by IMS and involves an isolation step using immunomagnetic beads coated with anti-CD146 antibodies, which bind to CD146-expressing CECs and are separated by magnetic retrieval [38]. The endothelial origin of CD146-positive cells in peripheral blood (PB) is subsequently validated by staining with other endothelial markers, such as Ulex europaeus lectin-1 (UEA-1), vWf, or CD31. This method is highly sensitive for mature CEC enumeration but is not completely CEC-specific as CD146 expression is also reported on various other cells [39-41]. Flow cytometry is an alternative approach for the isolation and quantification of CECs in general [42]. With this technique, (multichannel Fluorescence-Activated Cell Sorting (FACS)), whole PB blood or mononuclear cells (MNCs) [43] can be labeled with endothelial-specific antibodies conjugated with different fluorochromes. This method has the advantage that multiple cell parameters can be
Figure 2 - Concept of circulating endothelial cells (CECs) and circulating endothelial progenitor (CEPs) cells in tumor-angiogenesis.

CEPs, that are mostly thought to derive from the bone marrow, circulate in the blood and home to tumor attracted by cytokines produced by the tumor. Mature CECs, are detached from the endothelial lining of the blood vessel due to vascular damage (for example as a response on anti-angiogenic drugs), and come into the circulation. Hematopoietic progenitor cells (HPCs) are circulating in the blood and modulate the initiation, recruitment, and formation of the new vessels via stimulation of the circulating and resident CECs. Possible targets of anti-angiogenic drugs; (1) the CEPs, or (2) the endothelial cells lining the blood vessel. ECs; endothelial cells.

measured at the same time, with the ability to detect subpopulations (such as “bright” vs “dim” or “low” expression), and the numbers of cells can be accurately quantified. Identification of endothelial progenitors, EPCs/CEPs, is at this moment based on their morphological, clonogenic and proliferative potential in cell culture because no unique marker profile for flow cytometry is known.

CHARACTERIZATION OF CIRCULATING ENDOTHELIAL CELLS
Consensus on markers to phenotypic definitively distinguish mature CECs from HPCs and EPCs/CEPs is still lacking, since truly specific markers for these cell populations are still
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A viable gate

B CD45 selection

C Gating out debris

D CEC

E CEC

F HPC
Figure 3 - Detection of hematopoietic progenitor cells (HPCs) and circulating endothelial cells (CECs) using four-color flow cytometry.

Data represent the mononuclear cell (MNC)-fraction of a patient treated with anti-angiogenic treatment. (A) viable cells are selected based on size and granularity, (B) non-leukocyte selection based on CD45, (C) extra selection of viable cells excluding debris, (D) CD45 and CD34 expression for HPCs and CECs, HPCs measured as CD45^{dim}/CD34^{bright} and CECs measured as CD45^{neg}/CD34^{bright}, (E) CD133 and VEGFR2 expression on CECs, and (F) CD133 expression on HPCs when compared with the isotype control.

not available. Currently, to identify mature CECs phenotypically by multicolour FACS, it is generally accepted that they express the markers CD31 (present on endothelial cells, platelets and monocytes), CD34 (present on hematopoietic precursors and endothelial cells) and VEGFR2 (present on endothelial cells, perhaps on certain monocytes and hematopoietic precursors) and do not express the pan-leukocyte marker CD45, monocytic marker CD14 and HPC progenitor marker CD133 (CECs;CD31^{+}/CD34^{+}/VEGFR2^{+}/CD133^{+}/CD45^{neg}/CD14^{+}) [44]. Furthermore, two distinct hematopoietic progenitor cell subsets (referred to as circulating progenitor cells; CPCs) can be detected in the peripheral blood of patients or in the mononuclear cell fraction [45], one pro-angiogenic (CD31^{+}/CD34^{bright}/CD45^{dim}/CD133^{+}/CD14^{+}) and the other non-angiogenic (CD31^{+}/CD34^{bright}/CD45^{dim}/CD133^{+}/CD14^{+}) [46]. The gating strategy to enumerate CECs and HPCs (or subpopulations of CPCs) by FACS, used in this thesis, is depicted in figure 3. The possible origin of our CEC population in terms of functional definition or role as mature or progenitor-like will be further discussed in the “Summarizing discussion & future perspectives” in section “The ‘small size EC-like cells’ population” of this thesis.

Currently, two approaches are used to define EPC/CEPs; FACS analysis and colony formation in cell culture. Many flow cytometric protocols have been described to measure a variety of cell populations, which are named “EPCs” but in fact are mainly HPC or monocytic-like populations with prominent pro-angiogenic properties but no capacity to differentiate into bona fide ECs [21, 23, 40, 47, 48]. Peichev et al. [49] reasoned that the phenotype (defined by FACS) of more primitive EPCs would express CD34, VEGFR2, and CD133 while CD133 and perhaps CD34 would be lost as the endothelial cells matured. EPCs based on this marker combination have subsequently been commonly used for a number of years to enumerate EPCs in humans [19] and numbers of circulating EPCs (CEPs) have been correlated with the occurrence of several human cancers [21, 23, 47]. However, when the above mentioned EPCs (expressing CD34, VEGFR2, and CD133) were examined functionally in umbilical cord blood or mobilized adult peripheral blood, they appeared to be HPCs but not EPCs. They were enriched for hematopoietic colony forming
cells and they did *not* form endothelial cells *in vitro* or *in vivo* [50, 51]. Taken together, CD34, VEGFR2, and CD133 expression on circulating human peripheral blood cells failed to uniquely identify a circulating EPC. Thus currently no unique marker or combination of markers is known to detect EPCs/CEPs by FACS analysis. In the second approach, by culture assays, two types of EPCs have been described: ‘early EPCs’ and ‘late-outgrowth EPCs’ [28]. In literature, late-outgrowth EPCs are also called blood outgrowth endothelial cells (BOECs) [52], endothelial outgrowth cells (EOCs) [19] or – nowadays preferred - endothelial colony forming cells (ECFCs) [53]. The so-called ‘early EPC’ increase angiogenesis mainly by paracrine secretion of angiogenic factors whereas ‘late-outgrowth EPCs’ can directly participate in tubulogenesis and neovascularization [54, 55]. ECFCs display a cobblestone pattern typical of ECs, can be associated with proliferative capacity [56], and can be phenotypically identified to reside in a circulating population of cells by a combination of markers: CD31⁺/CD34bright⁺/CD133⁻/CD45⁻/CD14⁻ [57]. The protocol used in this thesis to obtain ECFC colonies and subcultured ECFCs is depicted in figure 4. Briefly, ECFCs were isolated from mononuclear cells that were obtained from the cord blood, using Ficoll Paque density gradient separation medium. The mononuclear cells were cultured in a concentration of at least 2.5 x 10⁶ cells per cm² in EBM-2 medium supplemented with 0.1% pen-strep, 10% FCS and EGM-2 SingleQuotes without hydrocortisone and gentamycin/ampicillin-B (commercially available). When the ECFC colonies started to appear after several medium refreshments (8-16 days), ECFC colonies were trypsinized, and subcultured into flasks.

2. **HYPOXIA, TUMOR PROGRESSION AND ANGIOGENESIS**

Hypoxia, a term reflecting an area of low oxygen concentration, is a common feature of many cancers. When tumors expand, the distance to the existing vasculature increases, and diffusion of oxygen will be limited resulting in a hypoxic region within the tumor [58].

**HIF REGULATORS DURING HYPOXIA AND TUMOR PROGRESSION**

A master regulator of the hypoxic response is the transcription factor hypoxia-inducible factor-1 (HIF-1) [59, 60], which can affect the transcription of numerous hypoxia-inducible genes. HIFs are heterodimeric transcription factors that consist of α and β subunits. Unlike the oxygen regulated HIF-1α subunits, HIF-1β is constitutively expressed and not responsive to oxygen. Of the three HIF-α subunits (HIF-1α, HIF-2α, and HIF-3α), HIF 1-α is the most abundantly expressed and functions as the key regulator of the response to hypoxia [61] in many tissues. Under normoxic conditions, oxygen dependent proline hydroxylation, subsequent ubiquitination and proteasomal degradation of HIF-1α
Figure 4 - Culture protocol for ECFC colony formation and subculture.

After isolation of the cord blood-mononuclear cells (CB-MNCs) ECFC colonies appear between day 6 and day 18 (A). By trypsinizing and subculture of the ECFC- cobblestone monolayer arises after 2-3 weeks and cells can be further expanded (B).

results in low levels of HIF heterodimers [62, 63]. However, when oxygen is low, the α-subunits are stabilized and form heterodimers with HIF-1β. This HIF-complex is transcriptionally active, and can activate hypoxia-inducible genes via binding to the HIF responsive element (HRE) in promoters of many genes, such as the VEGF gene. It has become increasingly clear that HIFs have a crucial role in cancer progression [64-66].

HIF-1α is overexpressed in a broad range of human malignancies [66], and HIF-1α accumulation has been associated with poor patient survival in several cancer types [67-73]. HIF activates many genes involved in glycolysis, erythropoiesis, cell migration and angiogenesis [66]. HIF directly activates VEGF and VEGFR1 transcription by HRE binding, and plays significant role during normal development and tumor formation [74, 75]. In human cancers increased tumor expression of HIF-1 and VEGF have been correlated with more aggressive and malignant lesions [71, 76]. Whereas HIF-1α is expressed universally, HIF-2α and HIF-3α are mainly found in a more restricted number of tissues, and significant associations between HIF-2α overexpression and increased patient mortality have been reported [77-80]. An association between HIF-3α and patient prognosis has not been examined.
Hypoxic Regulation of Angiogenesis

Hypoxia is an important phenomenon in the tumor microenvironment. Hypoxic tumors are more aggressive and resistant to anti-angiogenic treatments. Tumor hypoxia can occur as a result of increased metabolic activity and oxygen consumption by rapidly proliferating tumor cells leading to alterations of local pH and resulting oxidative stress in the surrounding microenvironment [81]. Part of the response to hypoxia is improving oxygenation by the induction of pro-angiogenic factors and subsequent angiogenesis and the increased production of erythropoietin. The hypoxic regulation of angiogenesis is primarily by HIF regulators with their effects on VEGF, nitric oxide synthases (NOS) and other pro-angiogenic factors, but also involve HIF-independent pathways like the binding site for the transcription factor NF-kappa B or the oncogene K-ras, both influencing VEGF that enables tumors to make the angiogenic switch [82].

Hypoxia Induced Recruitment of Bone Marrow-Derived Cells

Low oxygen conditions in tumors, acting in part through induced increases in HIF-1α and its targets stromal cell-derived factor-1α (SDF-1α) and VEGF, can attract a heterogeneous population of bone marrow-derived cells consisting of vascular progenitors and pro-angiogenic monocytic cells. Endothelial and pericyte progenitors are incorporated as components of new vessels to directly build new blood vessels, and pro-angiogenic monocytes fuel the tumors with pro-angiogenic cytokines, growth factors and proteases, all of which facilitate neovascularization [83].

3. Anti-Angiogenic Treatment

The original idea to use angiogenesis inhibitors to treat cancer was proposed in an article by Judah Folkman in 1971 [84]. The concept introduced in that paper was that all tumors depend on the constant growth of new blood vessels, angiogenesis, and that blocking this process should keep the tumor in a dormant state. The hypothesis that tumor progression can be arrested by anti-angiogenic treatment [84] has been confirmed experimentally in a large scale of studies. The most promising target of all pro-angiogenic growth factors and their receptors is VEGF since it is probably the most important factor in sustaining tumor angiogenesis. Additional features that make VEGF an attractive target for anti-angiogenic cancer treatment is that it may act as a survival factor for tumor cells [85, 86], it may enhance tumor metastasis [87], lymphangiogenesis [88] and it may inhibit the anti tumor immune response [89].
TARGETING NEOANGIOGENESIS; VEGF INHIBITION

Various approaches to inhibit VEGF-mediated tumor neovascularization are being investigated (Figure 5). Monoclonal antibodies bind to VEGF, thereby competing with binding at its receptor and inhibition of activation of the receptor. An example is bevacizumab (Avastin®), which is approved by the Food and Drug Administration (FDA) for the treatment of advanced colorectal cancer [23, 90], non-small-cell lung cancer (NSCLC) [91], breast [92] and glioblastoma, all in combination with chemotherapy. Currently, bevacizumab as monotherapy is under investigation for renal cell cancer [93]. Alternatively, VEGF can be targeted by small molecule tyrosine kinase inhibitors (TKIs) that bind in the ATP binding pocket of the receptor kinase. Sunitinib is an oral TKI against VEGFR-family, PDGFR-β, c-Kit, Flt3 and sorafenib against Raf, c-Kit, Flt3, PDGFR-β and the VEGF receptor family (VEGFR1, VEGFR-2 and VEGFR-3). Sunitinib and sorafenib are two examples of TKIs with proven activity in multiple advanced cancers such as metastatic colorectal cancer (mCRC), metastatic non-small-cell lung cancer (mNSCLC), metastatic breast cancer, metastatic renal cell carcinoma (mRCC), hepatocellular carcinoma (HCC) and gastrointestinal stromal tumors (GISTs) [94-97].

Another approach is the neutralization of VEGF(A) by soluble VEGF receptors. An example is VEGF-Trap, which can prevent the binding to and the activation of its target receptors, and is also a promising approach to achieve inhibition of angiogenesis [98, 99]. Anti-tumor activity can be achieved by these anti-angiogenic drugs either as monotherapy or in combination with chemotherapy or with other targeted agents. The first survival benefit of angiogenesis inhibitors was observed in a randomized phase III trial of first-line treatment of metastatic colorectal cancer; in that trial bevacizumab was combined with conventional chemotherapy [90]. Moreover, bevacizumab also increased overall survival in the first-line treatment of advanced NSCLC when used in combination with standard chemotherapy [100]. Currently, bevacizumab is being tested in combination with other targeted therapies (like the anti-EGFR inhibitor erlotinib) or combined with radiotherapy. Whereas bevacizumab was administered in combination with chemotherapy, sunitinib and sorafenib showed anti-angiogenic activity when used as monotherapy. The TKI sunitinib has shown its single-agent activity in RCC patients [97], and the TKI sorafenib in RCC as well as HCC [95, 96].

MECHANISMS OF VEGF-TARGETED TREATMENT

The mechanism by which anti-angiogenic drug inhibits neovascularization may be, besides direct inhibition of VEGF or VEGFR on ECs, through several ways. VEGF-targeted treatment can affect numerous cell types within the tumor microenvironment not limited
Figure 5 - Approaches to target neoangiogenesis.
Strategies to target the newly formed blood vessel include 1. anti-VEGF antibodies; bevacizumab, 2. small-molecule VEGFR TK inhibitors; sunitinib and sorafenib, 3. soluble VEGF receptors; VEGF Trap and specific anti-VEGFR-1 or anti-VEGFR2 antibodies directed against the receptor.

to endothelial cells but also hematopoietic progenitor cells, dendritic cells (DC) and tumor cells [101]. Accumulating evidence suggests that VEGF has an important role on the immune function in cancer patients by blocking DC differentiation [102, 103] which influences the presentation of tumor antigens to various immune effector populations. Inefficient presentation of tumor antigens can result in immune tolerance and immune privilege of tumor cells in the host. Angiogenesis inhibitors might also directly affect tumor cells since expression of (all) VEGFRs has been detected on tumor cells [104]. In tumors VEGF accumulation is high which can further be influenced by factors inducing cellular stress like hypoxia, low pH, nutrient deprivation, and genotoxic stress induced by chemotherapy and radiation therapy. Therefore not only an effect of VEGF-targeted treatment can be expected on tumor vasculature, in addition it may have a direct effect on tumor cells to impair tumor growth and/or metastasis. Alternatively, Jain et al. has hypothesized that VEGF-targeted treatment can ‘normalize’ the vasculature of tumors
It is hypothesized that vascular normalization mediates multiple effects: reduced peritumoral edema, reduced contrast enhancement of the tumor, improved oxygen and drugs delivery, and decreased tumor interstitial pressure [105, 106]. In this way chemotherapeutic agents may be better delivered to the tumor.

Finally, VEGF-targeted treatment might be effective in blocking chemotaxis of bone marrow-derived progenitor cells by cytotoxic agents. Upon administration of cytotoxic drugs (vascular disrupting agents; VDA), bone marrow-derived EPCs support the tumor repopulation by homing to sites of angiogenesis and promote tumor growth, which than would be inhibited by VEGF-targeted agents.

**MODIFIED CIRCULATING ENDOTHELIAL CELLS AS ANTI-ANGIOGENIC CANCER TREATMENT**

New ways to inhibit tumor growth might be, rather than targeting circulating endothelial cell populations, to explore their potential use as carrier for cancer therapy. Transplantation of genetically modified circulating endothelial cell populations or using them as cellular vehicles for delivering suicide genes, toxins or anti-angiogenic molecules might be a promising tool for anti-angiogenic cancer therapy [107]. *Ex vivo* manipulation of these cells and subsequent transplantation into a mice model showed reduced cancer progression. For example, human EPCs were transduced with a retroviral vector expressing the herpes simplex virus thymidine kinase transgene [108] or a truncated soluble VEGFR2 retrovirus [109] and injected into sublethally irradiated mice bearing a tumor, both resulting in significant tumor regression. In addition, ex vivo expanded EPCs genetically modified with a suicide gene were able to specifically and efficiently eradicate hypoxic lung metastasis [110]. Similar results were found for genetically modified differentiated endothelial cells and pro-angiogenic HPCs as vehicles in anti-cancer therapy [34, 111, 112].

**RESISTANCE TO ANTI-ANGIOGENIC TREATMENT**

Data support that two modes of unconventional resistance underlie the restoration of tumor growth and progression in the course of anti-angiogenesis treatment; an adaptation to circumvent the specific angiogenic blockade, and intrinsic or pre-existing indifference [83, 113] Adaptive resistance is largely indirect and the current experimental evidence suggests that there are at least four distinct mechanisms that manifest adaptive resistance to anti-angiogenic therapies. First, activation and/or upregulation of alternative pro-angiogenic signalling pathways can occur within the tumor which can partly circumvent the anti-angiogenic treatment. Second, several studies showed that low oxygen tension in tumors, acting in part through induced increases in HIF-1 and its targets SDF-1α and VEGF, triggers the recruitment of bone marrow-derived cells (BMDCs) [114-117] and thereby enables tumors to overcome hypoxia. Shaked *et al.* demonstrated
that VDAs can trigger a transient accumulation of EPCs at the tumor-rim to facilitate revascularization, while untreated transplanted tumors did not contain BMDCs, suggesting an adaptive response potentially enabling resistance to anti-angiogenic treatment [118]. Third, increased pericyte coverage may protect tumor blood vessels to survive anti-angiogenic treatment. Although inhibition of VEGF signaling pathways can lead to vessel regression, a few ‘normal-appearing’ slim and functional vessels remain; these vessels are densely and tightly covered with pericytes, and are markedly distinct from the vessels that are seen in tumors of untreated animals, which are typically dilated, tortuous and irregularly shaped, and variably covered with less closely associated pericytes. Such coating by pericytes arguably helps the tumor endothelium to survive and function, and thereby enables tumors to grow during the course of an anti-angiogenic therapeutic regimen [119]. Fourth, activation and enhancement of invasion and metastasis to provide access to normal tissue vasculature without obligate neovascularization. The second mode of resistance to anti-angiogenic treatment is experimentally less defined; intrinsic, pre-existing non-responsiveness of a tumor. Suggested mechanisms include, pre-existing multiplicity of redundant pro-angiogenic signals that enables the tumor continuing angiogenesis. For example the prominent expression of fibroblast growth factor-2 (FGF2) and other pro-angiogenic factors can enable continuing angiogenesis, even when treating the patient with bevacizumab, so that inhibition of VEGF signaling does not affect angiogenesis. In addition, intrinsic resistance can result from pre-existing inflammatory cell-mediated vascular protection by expressing pro-angiogenic factors [120], alternatively by hypovascularity and indifference toward angiogenesis inhibitors, and by invasive co-option of normal vessels without requisite angiogenesis [83].

4. **Biomarkers to Predict Response to Anti-Angiogenic Treatment**

The number of patients treated with angiogenesis inhibitors (as single treatment or as part of combination treatment) is growing rapidly. Therefore it becomes increasingly important to measure early biological effects of the agents used, and to determine whether there is a relation with the anti-tumor effect. Unlike cytotoxic cancer therapies, angiogenesis inhibitors, may achieve therapeutic levels at doses where no major toxicities arise thus making the traditional choice of dosing up to maximum tolerated doses (MTD) not applicable [121]. This further emphasizes the importance to find biomarkers that accurately reflect the effect of drugs and predict response of targeted therapies. Angiogenesis inhibitors are now being introduced as part of cancer treatment but validated surrogate markers reflecting clinical end points are still lacking. In this research
area a clinical challenge is the finding of biological markers that will help to select patients that are more likely to respond to a given anti-angiogenic treatment. In addition, biomarkers may be used to detect early clinical benefit, possible unwanted side-effects, resistance to anti-angiogenic agents and to decide whether to continue or stop given treatment. The various types of biomarkers are described in box 1.

<table>
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<th>Box 1 Definitions of various types of biomarkers.</th>
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<tr>
<td><strong>Biomarker:</strong> A distinctive biological or biologically derived indicator (as a biochemical metabolite in the body) of a process, event, or condition (as aging, disease, or exposure to a toxic substance) (Webster Medical Dictionary).</td>
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<td><strong>Prognostic biomarkers:</strong> Biomarkers that provide information about the patients overall cancer outcome, regardless of therapy [122].</td>
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<td><strong>Predictive biomarkers:</strong> Biomarkers that can be used in advance of therapy to estimate response or survival of a specific patient on a specific treatment compared with another treatment [123].</td>
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<td><strong>Pharmacodynamic biomarkers:</strong> Biomarker whose changes after treatment are associated with target modulation by a specific agent.</td>
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<td><strong>Surrogate marker:</strong> A biomarker intended to substitute for a clinical end point.</td>
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In summary, predictive surrogate markers are needed to demonstrate the activity and efficacy of anti-angiogenic agents in clinical trials and may be used for future monitoring of anti-angiogenic treatments in the clinic.

**Potential biomarkers**

Currently, several potential predictive biomarkers for anti-angiogenic treatment are being evaluated (Figure 6). The biomarkers used to measure the efficacy of therapeutic drugs of any type can be classified as either direct or surrogate (indirect) in nature. In anti-angiogenic treatment, the direct biomarker is the actual capillary network in the tumor, which is in general difficult to depict and quantify [125]. Therefore, most of the potential strategies have the objective to find reliable surrogate (indirect) biomarkers to give an indication of the effect of anti-angiogenic treatment [126, 127]. Angiogenesis can be visualized by several techniques [128]. Tumor biopsies before and after (or during) therapy would allow the measurement of highly relevant parameters (like gene and phospho-protein expression, microvascular density, perivascular cell coverage, interstitial fluid pressure, intratumor oxygen tension and drug uptake) but this biomarker type is labor intensive and highly-invasive (difficult to obtain or to access). Moreover, this technique is challenged by heterogeneity within the tumor and sample preparation and the samples of the tumor, at time of biopsy, might not accurately reflect the features of recurrent or metastatic disease and further standardization is required.
Figure 6 - Potential assays for measuring surrogate markers of anti-angiogenic treatment.

Proposed novel assays for measuring surrogate markers of tumour angiogenesis activity. The complex biology of angiogenesis inhibitors has accentuated the need for developing technologies that can be used to assess the effects of biological markers. A compilation of data from multiple assays including measuring angiogenic factors in serum, plasma, and urine; tumour biopsy analysis; radiologic imaging; and, recently, ex vivo analyses of isolated peripheral blood cells (labelled circulating endothelial cells) may facilitate defining the optimal biological dose for subsequent clinical studies of angiogenesis inhibitors. Adapted from Davis et al. [124].

Non-invasive techniques to detect early effects on the tumor vasculature include measurements of contrast enhancement, blood pressure, blood volume and oxygen saturation with computed tomography (CT), magnetic resonance imaging (MRI), positron emission tomography (PET) and single photon emission computed tomography (SPECT) [125]. Minimal-invasive biomarkers that have been studied include urine and/or blood parameters. The latter can be divided in measurements of plasma/ serum angiogenic cytokines, like the target-protein VEGF and in cellular enumerations. Since most anti-angiogenic compounds currently registered for clinical use target the VEGF pathway, it is reasonable to assume that some of those biomarkers reflecting the levels and activity of VEGF signaling might provide the most relevant pharmacodynamic information.
Nowadays, VEGF is the most frequently measured pro-angiogenic growth factor in patients. It is very hard to include these measures as a biomarker because of the VEGF contents in platelets [129]. In addition, markers for endothelium damage like von Willebrand Factor (vWF) and/or soluble growth factor receptor levels can be measured as potential biomarkers. Alternatively, enumeration of circulating endothelial cell populations are proposed to give an indication of the responsiveness of patients treated with anti-angiogenic drugs [130].

**Circulating Endothelial Cells as Potential Biomarkers of Anti-Angiogenic Treatment**

As anti-angiogenesis therapies for cancer become increasingly integrated into anti-cancer treatment, there is an urgent need for the proper selection of the patients most likely to benefit from these treatments. In this regard quantification of CECs and EPCs/CEPs might not only evaluate the efficacy of anti-angiogenic treatments, but also define the optimal biological dose ranges as well. *Mature* CECs are believed to be endothelial cells that have become detached from the vascular intimal monolayer in response to endothelial injury, for instance as a response to anti-angiogenic treatment of cancer. Current angiogenesis inhibitors are aimed to target the vasculature, and more precisely the ECs, lining the blood vessel. After all VEGF is also a survival factor for ECs [131]. *Mature* CECs are of interest because they are thought to be surrogate markers of severe damage to the endothelium, and may reflect vascular dysfunction [24]. Therefore the effect of anti-angiogenic treatment might be reflected by the number of (apoptotic) ECs in circulation. Several studies showed, with their own definition of CECs, that the numbers of CECs were increased in many pathological conditions, including cancer [132]. Mancuso *et al.* reported for the first time significantly increased numbers of both resting and activated CECs in breast cancer and lymphoma patients [133], but later these cells were identified as large platelets. Many other studies further explored CECs as potential biomarkers including our own studies as described in chapter 2, 3 and 6 of this thesis, but still there is a considerable variation and no consensus in detection methods to enumerate CECs. EPCs/CEPs provide both instructive (release of pro-angiogenic cytokines) and structural (vessel incorporation and stabilization) functions that contribute to the initiation of tumor neo-angiogenesis. Therefore enumeration of EPCs/CEPs is an promising biomarker for anti-angiogenic cancer therapy. EPCs/CEPs may be used as useful surrogate marker for monitoring cancer progression, as well as for optimizing the efficacy of anti-angiogenic therapies (VEGFR2 antibody treatment). Recently reported data indicate that also EPCs/CEPs circulate in increased numbers in the PB of patients with various types of cancer including lung [134, 135], hepatocellular [136, 137], breast [138-140], colorectal cancer [23] and others [141-145]. Recently, it became clear that this type of “EPC/CEP”
measured in almost every study are a fraction of HPCs/myeloid progenitors and is not capable of forming endothelial cells lining vessels *in vitro* or *in vivo* [50, 51].

5. **AIM AND OUTLINE OF THIS THESIS**

Endothelial cells, can be released from the lining of blood vessels, and provide information about the state of the vessels. A high number of CECs per millilitre of blood is an indicator of vascular damage. In contrast, high number of EPCs/CEPs, derived from the bone marrow or elsewhere, however, may constitute a “positive” signal: it is assumed that EPCs/CEPs play an important role in vascular repair in the body. Measuring numbers of CECs and progenitors can play an important role in assessing and monitoring the 'condition' of the vascular endothelium, including the effects of anti-angiogenic regiments. One possible application in oncology is the measurement of (early) effects of anti-angiogenic anti-cancer treatment.

**AIM OF THIS THESIS**

**The primary aim of this thesis** was to investigate whether circulating endothelial and progenitor cells might serve as potential biomarkers for monitoring the effect and predict the response of patients treated with angiogenesis inhibitors.

**In addition to biomarker evaluation,** because endothelial colony forming cells (ECFCs) might be functioning in (tumor) angiogenesis as pro-angiogenic progenitors homing to hypoxic regions, this thesis also addressed the initial response of ECFCs to 1% oxygen (hypoxia).

**OUTLINE OF THIS THESIS**

In **chapter 2** we determined the feasibility and reproducibility of the enumeration of VEGFR2⁺ cell populations in the blood of cancer patients by flow cytometry. The flow cytometry technique is introduced to show how this technique can be used to discriminate circulating endothelial and progenitor cell populations based on cell surface markers, and four populations of cells were analyzed and quantified in volunteers and cancer patients.
Based on the defined circulating endothelial and progenitor cells described in chapter 2, the kinetics (changes in frequencies) of these cell populations where investigated in advanced renal cell cancer (RCC) patients during the first treatment cycle with the angiogenesis inhibitor sunitinib as described in chapter 3.

In addition to the enumerated circulating endothelial and progenitor cells in RCC patients, hemoglobin levels in RCC patients treated with sunitinib were measured. In chapter 4 we report that hemoglobin changes were related to the dosing schedule of the patients treated with sunitinib.

To further investigate the specificity and potential for measurement of circulating endothelial and progenitor cells as a biomarker during anti-angiogenic treatment, this type of analysis was extended to a group of non-small cell lung cancer (NSCLC) patients treated with different angiogenesis inhibitors. In chapter 5 we investigated the effects of the anti-angiogenic agent sorafenib plus EGFR-inhibitor erlotinib in NSCLC patients on circulating endothelial and progenitor cells, and determined the specificity of these changes by comparing them with these in a control group of patients treated with another angiogenesis inhibitor (bevacizumab plus erlotinib) and in a group with erlotinib alone. Furthermore, changes of the potential biomarkers were correlated with treatment response.

In addition to obtaining data from flow cytometric enumerations of circulating endothelial progenitor cells it is important to define and quantify them by biological assays. Given the importance of the microenvironmental conditions in the outgrowth of endothelial colony forming cells (ECFCs), chapter 6 describes that the outgrowth properties of ECFCs freshly isolated from human cord blood is influenced under hypoxic (1%) conditions.

Chapter 7 summarizes all of these investigations, the results are discussed and put in perspective.
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