

Vascular endothelial growth factor (VEGF) – key factor in normal and pathological angiogenesis

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Abstract

Vascular endothelial growth factor (VEGF) represents a growth factor with important pro-angiogenic activity, having a mitogenic and an anti-apoptotic effect on endothelial cells, increasing the vascular permeability, promoting cell migration, etc. Due to these effects, it actively contributes in regulating the normal and pathological angiogenic processes. In humans, the VEGF family is composed of several members: VEGF-A (which has different isoforms), VEGF-B, VEGF-C, VEGF-D, VEGF-E (viral VEGF), VEGF-F (snake venom VEGF), placenta growth factor (PlGF), and, recently, to this family has been added endocrine gland-derived vascular endothelial growth factor (EG-VEGF). VEGF binds to tyrosine kinase cell receptors (VEGFRs): VEGFR-1 [Fms-like tyrosine kinase 1 (Flt-1)], VEGFR-2 [kinase insert domain receptor (KDR) in human; fetal liver kinase 1 (Flk-1) in mouse] and VEGFR-3 [Fms-like tyrosine kinase 4 (Flt-4)]. While VEGFR-1 and VEGFR-2 are expressed predominantly on vascular endothelial cells, VEGFR-3 is expressed especially on lymphatic endothelial cells. VEGFR-2 has the strongest pro-angiogenic activity and a higher tyrosine kinase activity than VEGFR-1. Endothelial cells also express co-receptors, such as neuropilin-1 (NP-1) and neuropilin-2 (NP-2), which modulate tyrosine kinase receptor activity. Both VEGF and VEGFRs are expressed not only on endothelial cells, but also on non-endothelial cells. This article aims to highlight the most recent data referring to the VEGF family and its receptors, as well as its implications in the angiogenesis process. At present, blocking angiogenesis in cancer or in other pathological processes, using anti-VEGF and anti-VEGFRs therapies, is considered to be extremely important.

Keywords: VEGF receptors, VEGF, isoforms, angiogenesis, anti-angiogenic therapy.

Introduction

Angiogenesis is an extremely complex process, influenced by multiple factors, some of them acting as pro-angiogenic agents, others as inhibitors of angiogenesis [1].

An extremely potent pro-angiogenic factor is vascular endothelial growth factor (VEGF) and, for this reason, there are numerous studies that demonstrated its implication in angiogenesis.

During the embryonic period, the formation of new vessels occurs by the differentiation of endothelial cells from hemangioblasts (vasculogenesis) [2–5]. Later, after birth, in certain physiological processes (menstrual cycle, pregnancy, wound healing and repair, etc.), new vascular networks are formed by angiogenesis, based on preexisting vessels (neoangiogenesis) [1–3, 6–9].

At the same time, data suggests that VEGF plays an important role in pathological angiogenesis, inducing the development and progression of certain pathological conditions in the postnatal period, such as: tumor growth and metastasis, macular degeneration, diabetic retinopathy, inflammatory processes (e.g., rheumatoid arthritis), ischemic processes (myocardial ischemia), preeclampsia, etc. [2, 4, 7–11].

At present, increased attention is focused on the

process of formation and development of certain new lymphatic vessels (lymphangiogenesis) [2].

The human *VEGF* gene, located on the 6p21.3 chromosome, is part of the *VEGF*/platelet-derived growth factor (*PDGF*) gene family, also called the cystine-knot superfamily of growth factors [2, 12, 13]. From a structural point of view, VEGF is a 40-kDa heterodimeric glycoprotein, which contains the cystine-knot motif, characterized by the disposition of certain disulfidic bridges in the protein structure [2, 12, 14]. Alongside VEGF, there are additional growth factors from the cystine-knot motif family: PDGF, nerve growth factor (NGF) and transforming growth factor-beta (TGF- β) [14, 15].

In humans, the VEGF family includes several members that perform various functions: VEGF-A (which presents several isoforms), VEGF-B, VEGF-C, VEGF-D, VEGF-E (viral VEGF, in parapoxvirus 1), VEGF-F (snake venom VEGF) and the placenta growth factor (PlGF) [2, 16, 17]. More recently, a new member has been added to this family, named the endocrine gland-derived vascular endothelial growth factor (EG-VEGF) [17, 18].

Short history

In 1983, Senger *et al.* described a protein called

vascular permeability factor (VPF), secreted by animal tumor cells (hamster, guinea, pig), which is responsible for the increased permeability of the tumor blood vessels and the development of ascites associated with certain abdominal tumors [19].

In 1989, Ferrara *et al.*, from Genentech, independently, isolated and described the VEGF protein, demonstrating its role in angiogenesis [20]. Subsequently, the two proteins, VPF and VEGF, turned out to have a similar structure [21].

Knowing that during carcinogenesis an important role is played by tumor angiogenesis, the need to find certain angiogenesis inhibitors has become more stringent. Hence, in 1993, Ferrara *et al.* obtained an arrest in tumor growth by blocking the angiogenesis with monoclonal anti-VEGF antibodies [20, 22, 23]. Nowadays, anti-VEGF targeted therapies are successfully used in cancer treatment. These results confirm the reports made since 1971 by Judah Folkman, who advanced the idea that tumor progression and growth depend on the tumor angiogenesis [20, 22, 23].

☐ VEGFR receptors

VEGF binds to tyrosine kinase receptors, which present three domains: an extracellular domain for VEGF binding, a transmembrane domain and an intracellular domain with tyrosine kinase activity [2, 24, 25, 26]. VEGF, binding to the extracellular receptor domain, promotes the activation of tyrosine kinase enzyme in the intracellular receptor domain, which phosphorylates the tyrosine residues, thus activating several intracellular signaling pathways [25].

There are three types of VEGF receptors: VEGFR-1, VEGFR-2 and VEGFR-3 [2, 3, 12, 25]. Members of the VEGF family can also interact with other proteins, such as neuropilins [3, 25, 27–29], integrins [27–29], cadherins [27, 28], or heparan sulphate proteoglycans [3, 25, 27–29]. Neuropilin-1 (NP-1) and neuropilin-2 (NP-2) co-receptors are non-tyrosine kinase receptors and they selectively attach to certain VEGF subtypes or isoforms (such as VEGF-A₁₆₅) [3, 25]. Neuropilins enhance the VEGFR-2 and VEGFR-3 functions, guiding the endothelial cells migration in angiogenesis [3].

VEGFR-1 and VEGFR-2 receptors are expressed predominantly on vascular endothelial cells [2, 25, 26], but can also be found on non-endothelial cells [16, 25, 26], while VEGFR-3 is expressed especially on endothelial lymphatic cells [30].

VEGFR-1

VEGFR-1 [Fms-like tyrosine kinase 1 (Flt-1)] is a member of the receptor tyrosine kinases family (RTKs), with a molecular weight of 180 kDa [12] and with high receptiveness for VEGF-A [3, 4, 12, 13], VEGF-B [3, 4, 12, 13], PlGF [3, 4, 12, 13] and VEGF-F [3, 13]. Besides endothelial cells, there are other cells that can express VEGFR-1: inflammatory cells, monocytes/macrophage cells [3, 17, 25, 26], bone marrow-derived hematopoietic progenitor cells [17, 31], trophoblastic cells [25], mesangial renal cells [25], tumor cells [25], vascular smooth muscle cells (VSMCs) [17]. VEGFR-1 receptors have also been

identified on myofibroblasts located in the connective tissue of mouse myocardium, in the infarction areas [16].

VEGFR-1 plays an important role in migration of endothelial cells [12, 25], monocytes, macrophages, and hematopoietic stem cells [3, 12, 17, 27, 32], thus being mainly involved in pathological angiogenesis in adult life (tumors, inflammation, ischemia, preeclampsia, etc.) [12, 17, 32].

VEGFR-1 has a 10 times higher affinity for VEGF than VEGFR-2 and a lower tyrosine kinase activity [27, 29, 33].

Recent data on the biological role of VEGFR-1 in vasculogenesis, during the embryonic development, is contradictory [33]; most authors state that VEGFR-1 plays a role in endothelial cells differentiation and migration, but not in their proliferation [12, 25, 27].

The inactivation of murine gene that encodes VEGFR-1 (*flt-1* null mutant mice or *flt-1*^{-/-} mice), lead to embryonic death on days 8 or 9 of gestation, because, despite the fact that the endothelial cells undergo differentiation, they formed anarchic vascular channels; thus, the development and organization of a functional and viable vascular system did not occur [4, 24, 25, 27, 32].

Consequently, according to these studies, VEGFR-1 is involved only in the differentiation of endothelial cells and is not actively involved in early stages of angiogenesis during embryogenesis [4, 24, 25, 27, 32]. Molecular mechanisms referring to the involvement of the *VEGFR-1* gene in vasculogenesis are not completely understood and there still are aspects that need to be clarified [25]. It seems that VEGFR-1 inhibits the pro-angiogenic signals in the early development stage [26], preventing the binding of VEGF to VEGFR-2, which is expressed on the new formed endothelial cells (knowing that VEGFR-2 has a stimulating effect on endothelial cells proliferation) [3, 4, 26, 29].

Some experiments have tried to determine which of the three domains of the VEGFR-1 receptors is involved in vasculogenesis or in pathological angiogenesis.

Experimentally, in mice, the mutation of the *VEGFR-1* gene segment which encoded the intracellular domain with tyrosine kinase activity (*flt-1* *TK*^{-/-} mice) has enabled the differentiation of endothelial cells [4, 24, 26, 29], but not the migration of macrophages in pathological conditions [4, 24, 29]. During all this time, the extracellular domain and the transmembrane domain of the receptor have remained unmodified [26, 34]. Hence, the effect of VEGFR-1 on vasculogenesis is influenced by the extracellular and transmembrane domain of the VEGFR-1 [24, 26, 29].

According to the data presented above, the tyrosine kinase activity of the receptor does not influence the differentiation of endothelial cells during embryogenesis, but plays an important role in pathological angiogenesis [26, 29]. In the given context, experiments on mutant mice that do not contain the tyrosine kinase region (*flt-1* *TK*^{-/-} mice) have highlighted a lower rate of invasion and tumor metastasis and also a lower degree of inflammation (e.g., in rheumatoid arthritis) compared with wild-type mice [4].

A soluble form of VEGFR-1 (sVEGFR-1 or sFlt-1), was obtained by alternative splicing of the *VEGFR-1*

gene [3, 17, 27]. sVEGFR-1 does not contain neither the transmembrane, nor the intracellular domain of the VEGFR-1 receptor. Even though, it still shows affinity for VEGF, preventing its binding to VEGFR-2 [3, 17], thus having an anti-angiogenic effect [4, 35]. The soluble form of sVEGFR-1 (sFlt-1), with its negative role in angiogenesis, is expressed during the embryonic development [27, 36]. Its presence in the corneal epithelium explains the absence of corneal vascularization [26, 35, 37]. sVEGFR-1 has affinity for VEGF-A and PlGF and, in adults, increased serum levels of sVEGFR-1 are observed in pathological angiogenesis (e.g., preeclampsia) [12, 26, 35, 36].

Postnatally, in pathological conditions, the interaction between VEGFR-1 and VEGFR-2 can be modified, VEGFR-1 having the same binding interface for VEGF and PlGF [4]. While PlGF binds only to VEGFR-1 [4, 17], VEGF binds to both VEGFR-1 and VEGFR-2, still having a 10 times higher affinity for VEGFR-1 [12]. According to some authors, binding of PlGF to VEGFR-1 stimulates endothelial cells to release VEGF, which will bind to VEGFR-2. Consequently, activation of VEGFR-2 stimulates angiogenesis, migration and proliferation of endothelial cells [3, 4, 38].

Hence, in pathological conditions, VEGFR-1 and PlGF act synergistically, representing “key factors” in pathological angiogenesis, PlGF modulating the interaction between VEGFR-1 and VEGFR-2 in this context [4, 17].

In non-endothelial cells [monocytes, macrophages, polymorphonuclears (PMN) leukocytes, hematopoietic stem cells, VSMCs] [27, 33], the activation of VEGFR-1 in pathological conditions (tumors, inflammation, ischemia, etc.) has an important role in chemotaxis and cell migration [27, 39, 40], due to the activation of different signaling pathways: phosphoinositide-3-kinase (PI3K)/protein kinase B (PKB/Akt) [4, 29, 41], mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway (p38-MAPK/ERK1/2) [4, 29, 33, 41]. The main activating factor of VEGFR-1 in inflammatory cells seems to be PI3K [29, 41], and activating of other proteins (e.g., Akt, p38, ERK1/2, etc.) also depends on PI3K [29, 41].

In pathological angiogenesis, the activation of tyrosine kinase domain of VEGFR-1 promotes the inflammatory cells migration and secretion of inflammatory cytokines [27, 39, 40], such as: tumor necrosis factor- α (TNF- α) [42, 43], interleukin-1 β (IL-1 β) [42, 43], IL-6 [43], IL-8 [42, 43], monocyte chemoattractant protein-1 (MCP-1) [42, 43], macrophage inflammatory protein-1 β (MIP-1 β) [42, 44].

Hence, VEGFR-1 plays an important role in pathological angiogenesis [4, 8]. It has been observed that in certain types of solid tumors (breast cancer, lung, hepatic carcinoma), activation of VEGFR-1 induced the secretion of proteolytic enzymes into the extracellular matrix (ECM) [e.g., matrix metalloproteinase-9 (MMP-9)] [4, 45], which favors tumor dissemination.

In conclusion, VEGFR-1 is not actively involved in vasculogenesis during the embryonic period, since it does not have a “mitogenic” effect on endothelial cells [4, 26]. It has a “negative role” in vasculogenesis, acting more like a “trap or decoy receptor” for VEGF [4], thus making VEGF less accessible for VEGFR-2 [3, 4, 26].

In exchange, the activation of VEGFR-1 is important in pathological angiogenesis (tumor growth and progression, amplification of the inflammatory process, preeclampsia, ischemia, atherosclerosis, etc.), by various mechanisms: chemotaxis of inflammatory cells [4, 17, 27], secretion of inflammatory cytokines [4, 17, 27], the recruitment of medullary progenitor cells at the injury site [27, 31], secretion of growth factors [e.g., hepatocyte growth factor (HGF), in the liver endothelial cells] [46, 47], interaction with PlGF [4, 17, 27], and activation of proteolytic enzymes (e.g., MMP-9) [27, 45].

VEGFR-2

VEGFR-2 [kinase insert domain receptor (KDR) in human; fetal liver kinase 1 (Flk-1) in mouse], the predominant receptor [8], is also a member of the tyrosine kinase receptor family, with a molecular weight of 200–230 kDa [12]. It shows greater affinity towards VEGF-A and VEGF-E, and lower affinity for VEGF-C and VEGF-D [3, 13].

VEGFR-2 is expressed especially on endothelial cells of blood and lymphatic vessels [12], but it has also a weak expression in: hematopoietic cells [12, 25, 26], megakaryocytes [12, 25, 26], retinal progenitor cells [25, 26], neurons [26], osteoblasts [26] pancreatic ductal cells [26], tumor cells [25, 33].

VEGFR-2 is expressed in early embryonic life, at day 7.5 of gestation, on hemangioblasts with mesodermic origin, hence influencing their migration, differentiation into endothelial cells and the formation of vascular islands in the Yolk sac, with the initiation of vasculogenesis [3, 26].

The inactivation of the murine gene that encodes VEGFR-2 in homozygotic animals (*VEGFR-2^{-/-}*) leads to embryonic death on days 8–9, due to a failure of vascular island formation [25]. In this case, the differentiation of endothelial cells has not occurred, thus blocking the development and organization of the vascular system [3, 25, 34]. In fact, several studies reported that VEGFR-2 could also interfere with lymphangiogenesis by binding VEGF-C and VEGF-D, although the mechanisms involved in this process are still being clarified [3]. Therefore, VEGFR-2 is essential for the normal course of vasculogenesis during the embryonic development [4, 17, 34].

VEGFR-2 presents the same domains as the other receptors in this family. Binding VEGF to the extracellular domain of VEGFR-2 causes the autophosphorylation of tyrosine residues and the activation of certain signaling pathways (Figure 1), such as: phospholipase-C γ (PLC γ)/protein kinase C (PKC) [4, 24, 29, 33] and Ras/Raf/ERK/MAPK pathways [4, 12, 29], these signaling pathways being involved in proliferation of endothelial cells. Moreover, by the activation of the PI3K/Akt pathway [4, 12, 33], VEGFR-2 plays a role in endothelial cell survival, playing an anti-apoptotic role. Also, it activates certain integrins, which disrupt cell to cell cohesion and initiate cellular migration [3, 4, 12, 33]. In this context, it has been concluded that, by activating PI3K-kinase and p38 MAPK pathways [27], adhesion molecules such as: cadherins [vascular endothelial (VE)-cadherin] [27, 48, 49], β -catenin [27, 50], occludins [27, 51] and connexin 43 [27], could form a complex with VEGFR-2, that weakens

the intercellular junctions, destabilize the cytoskeleton of endothelial cells and induces the formation of endothelial fenestrae [27]. Thus, the vascular permeability increases, favoring cell migration [3, 27]. Moreover, by the activation

of Akt protein kinase, the formation of endothelial nitric oxide synthase (eNOS) and the production of nitric oxide (NO) is stimulated in endothelial cells, inducing vasodilation and increased vascular permeability [27].

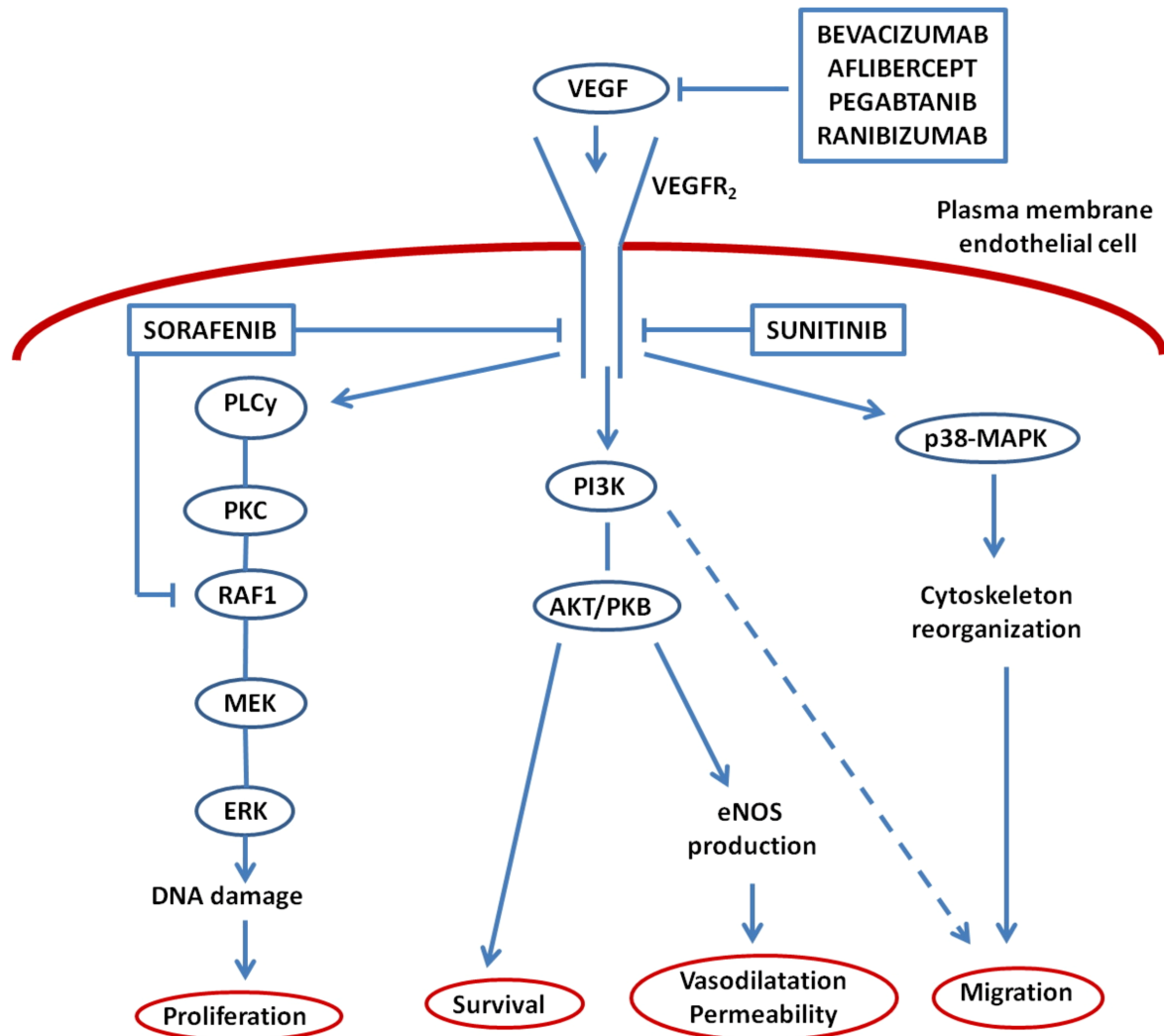


Figure 1 – VEGF-A/VEGFR-2 signaling pathways. Targets of anti-angiogenic agents. VEGF binding on VEGFR-2 lead to downstream signaling of the PLCγ/PKC, PI3K and MAPK signaling. VEGF(R): Vascular endothelial growth factor (receptor); PLCγ: Phospholipase-Cγ; PKC: Protein kinase C; MEK: Mitogen-activated protein kinase; ERK: Extracellular signal-regulated kinase; DNA: Deoxyribonucleic acid; PI3K: Phosphoinositide-3-kinase; AKT/PKB: Protein kinase B; eNOS: Endothelial nitric oxide synthase; MAPK: Mitogen-activated protein kinase.

During tumor neoangiogenesis, there are numerous paracrine interactions between endothelial cells and tumor cells [33, 52]. Binding VEGF to VEGFR-2 stimulates the secretion of von Willebrand factor (vWF) by endothelial cells [33, 52]; the activation of endothelial cells is an essential event for tumor progression [52].

To conclude, VEGFR-2 is involved in vasculogenesis, normal and pathological angiogenesis, acting through different mechanisms, such as: migration of the hemangioblasts towards the Yolk sac [25] and differentiation into endothelial cells [4, 12, 25], formation of vascular tubes (tubulogenesis) [29], proliferation of endothelial cells (mitogen effect) [4, 12], increase of vascular permeability [4, 12], migration of endothelial cells [4, 12], transmission of signals which promote the endothelial cells survival, preventing their apoptosis [3, 8, 12, 53] and formation of endothelial fenestrae [1, 12, 54]. In pathological processes, VEGFR-2 is often involved in tumoral angiogenesis [33].

It is considered that VEGFR-2 has the strongest pro-angiogenic activity, thus blocking VEGFR-2 may have useful clinical implications. VEGFR-2 has a stronger tyrosine kinase activity than VEGFR-1, but a weaker affinity for VEGF (VEGF-A) [4, 20, 26].

Through the alternative splicing of *VEGFR-2* gene, a soluble form of VEGFR-2 was obtained (sVEGFR-2), which has an affinity for VEGF-C, preventing its binding to VEGFR-3, thus impeding the proliferation of lymphatic endothelial cells and blocking lymphangiogenesis [33, 55].

VEGFR-3

VEGFR-3 [Fms-like tyrosine kinase 4 (Flt-4)] [2] also belongs to the tyrosine kinase receptor family, having a molecular weight of 195 kDa [12]. It plays an important role in the morphogenesis of the lymphatic vessel network during embryonic development, also being involved in formation of new lymphatic vessels in the adult life [12].

In this situation, lymphangiogenesis, or *de novo* formation of lymph vessels from the pre-existing postcapillary venules (high endothelial venules), could occur in certain pathological conditions, most frequently in inflammation or tumors [3, 33, 56]. VEGFR-3 has an affinity for VEGF-C and VEGF-D [3, 13, 33].

VEGFR-3 is expressed in the lymphatic endothelium or in high endothelial venules [30], influencing the differentiation of lymphatic endothelial cells, tubulogenesis, proliferation (mitogen effect), migration and survival of lymphatic endothelial cells [3, 33]. Expression of VEGFR-3 has been observed in other cells, such as osteoblasts [57], macrophages [58], neural progenitors [59], while its presence in tumor cells remains controversial [60].

Signaling pathways which activate lymphogenesis, especially during the embryonic development, are the following: activation of MAPK extracellular signal-regulated kinases (ERK1/2) through the PKC and Ras pathways [24] (important pathways in cell proliferation), as well as the PI3K–Akt/PKB pathway (involved especially in survival of lymphatic endothelial cells) [3, 61].

Expression of *VEGFR-3* murine gene begins on day 8.5 of early intrauterine development, determining the differentiation of lymphatic endothelial cells and formation of structures similar to lymph sacs, which will later undergo remodeling and extension, reorganizing themselves into a functional network of lymphatic vessels [3, 61]. The inactivation of this gene leads to mouse embryo death due to the absence of lymphatic vessels development and massive edema [3, 62]. It is considered that, in adults, the onset of human hereditary primary lymphedema is linked to VEGFR-3 activity [62].

Binding of VEGF-C to VEGFR-3 is responsible for most of the biological effects of VEGFR-3 [3]. The discovery of a soluble form of VEGFR-3 (sVEGFR-3) and experiments on transgenic mice expressing this gene led to the conclusion that sVEGFR-3 inhibits the development of lymphatic vessels and induces edema, inhibiting the signals mediated by VEGF-C and VEGF-D [62].

It has been observed that tumors with lymph nodes metastasis expressed high levels of VEGF-C or VEGF-D, possibly by involvement of VEGFR-3 in migration of tumor cells through lymphatic vessels [24].

Neuropilins

Neuropilins, NP-1 and NP-2, are transmembrane receptors located on endothelial cells, which function as co-receptors, modulating the activity of RTKs. Neuropilins selectively link to certain subtypes or isoforms of VEGF [2, 3, 63]. They have a low molecular weight of 120–135 kDa [33], and were initially identified as receptors for different types of semaphorins (class 3 semaphorins) [3, 25, 63].

NP-1 is expressed on endothelial cells in arteries [3, 25, 64] and has affinity especially for VEGF-A₁₆₅ [3, 25, 33], but not for VEGF-A₁₂₁ [3, 4, 25]. Some authors also describe affinity for PlGF-2 [25] or VEGF-B₁₆₇ [33]. NP-1 enhances the activity of VEGFR-2 up to six times [3, 33], influencing angiogenesis and the migration of endothelial cells [25, 33]. The convergent effect of NP-1

and VEGFR-2 leads to the intensifying of platelet-activating factor (PAF) secretion by endothelial cells, promoting inflammation, increasing vascular permeability and migration of endothelial cells [33]. NP-1 can be also present in other cells, such as neurons [3], smooth muscle cells [33] or tumor cells [3, 25, 65], being detected on the surface of tumor cells in breast cancer [3, 25], prostate, lung, pancreas or colon cancers [65], as well as in astrocytomas, glioblastomas, melanomas [65].

NP-2 is expressed especially on endothelial cells in lymphatic vessels and veins [3, 64], enhancing the effects of VEGFR-3 [3], because of binding VEGF-C [2].

Neuropilins role in vasculogenesis has been experimentally demonstrated on NP-1 null mice (*Neuropilin-1*^{−/−}), which led to death of mouse embryos through anomalies of the vascular system [3].

Types of VEGF

VEGF-A

VEGF-A, also called VEGF, is the most important and potent stimulator of angiogenesis, described for the first time by Senger *et al.* as the VPF [19, 33].

VEGF-A plays an important role in vasculogenesis and neoangiogenesis, causing cell proliferation, apoptosis inhibition, increased vascular permeability, vasodilatation, recruitment of inflammatory cells to the injury site, etc. [4, 12, 16, 25, 29].

VEGF is secreted not only by endothelial cells [1, 2, 12, 16], but also by other cells, in response to oxygen deprivation: tumor cells [1, 16], macrophages [1, 12, 16], platelets [16], keratinocytes [1, 16], kidney mesangial cells [1, 16], activated T-cells [1, 12, 16], leukocytes [2], dendritic cells [66], retinal pigmentary epithelial cells [67], Müller cells in the retina [68], astrocytes [1], osteoblasts [1], bronchial and alveolar epithelial cells [69], pericytes [70], VSMCs [71]. More recently, it has been found that VEGF is expressed in the myofibroblasts located in the myocardium, suggesting its implication in post-infarction tissue repair and remodeling [16].

Human VEGF-A contains eight exons separated by seven introns [4] and, by alternative VEGF messenger ribonucleic acid (mRNA) splicing, creates different lengths isoforms: VEGF₁₂₁, VEGF₁₄₅, VEGF₁₄₈, VEGF₁₆₂, VEGF₁₆₅, VEGF_{165b}, VEGF₁₈₃, VEGF₁₈₉ and VEGF₂₀₆ [2, 10, 12, 17, 25, 72]. These isoforms have different biological properties [17], depending on the structure and number of amino acids contained, but also depending on their affinity for heparin and heparan-sulfate proteoglycans (HSPGs) of the ECM [12, 32, 25]. Each isoform has a specific role in the differentiation and development of the vascular system [12].

All the isoforms have a common area, encoded by exons 1–5 [2]. It is supposed that exons 6 and 7 (which may be absent in some isoforms) are responsible for heparin affinity, while exon 8 (present in all isoforms) ensures endothelial cells proliferation [72].

The most expressed VEGF-A proteins are the isoforms: VEGF₁₂₁, VEGF₁₆₅ and VEGF₁₈₉ [12, 32, 72]. Of these, VEGF₁₆₅ is the predominant isoform [12] and the most active in vasculogenesis [10]. It does not contain amino acids encoded by exon 6, therefore having moderate

affinity for heparin and HSPGs [10, 12, 13, 25, 32]. For this reason, most of VEGF₁₆₅ remains bound to the cell surface [12, 13, 17, 25].

VEGF₁₂₁, without amino acids encoded by exons 6 and 7, has no affinity for heparin or HSPGs, existing in a free form [4, 10, 17, 25].

VEGF₁₈₉ and VEGF₂₀₆ are the longest isoforms, with a strong affinity for heparin, being totally bound to ECM structures and less on cell surface [12, 24, 25, 32]. It is considered that, for this reason, VEGF₁₈₉ and VEGF₂₀₆ are less active than VEGF₁₂₁ and VEGF₁₆₅ [25].

Proteolytic enzymes (*e.g.*, plasmin, urokinase) can cleave the bond between VEGF and ECM elements, consequently VEGF being released in a free, soluble form, very active in the ECM [4, 12, 17, 25].

VEGF₁₄₅, VEGF₁₈₃, VEGF₁₆₂ and VEGF_{165b} isoforms are much less common; in particular, VEGF_{165b} was found to have an anti-angiogenic effect, inhibiting VEGF-A₁₆₅ [2, 4, 10, 12]. Other isoforms of the type VEGF-A_{XXXb} have been later described, *e.g.*, VEGF-A_{121b} [60], VEGF-A_{183b} [60], VEGF-A_{143b} [60] and VEGF-A_{189b} [66]; *in vivo* and *in vitro* studies have evidenced their anti-angiogenic role, especially for VEGF-A_{165b} and VEGF-A_{121b} [10].

The strong pro-angiogenic effect of VEGF-A on embryonic vasculogenesis has been revealed by experiments with heterozygotic *VEGF-A* gene knockout mice (*VEGF-A*^{+/-} mice) who died on days 10 or 11 of gestation, due to insufficient development of the vascular system [1, 24].

VEGF-A binds to VEGFR-1 and VEGFR-2 [1, 2, 24], having a 10 times higher affinity for VEGFR-1 [12]. VEGF₁₆₅ binds to NP-1, NP-2 [2, 12, 24], as well as HSPGs of ECM [2, 24], while VEGF₁₄₅ binds to NP-2 [12].

VEGF-B

VEGF-B, discovered in 1995, is expressed in early embryonic life; in adults, it is found in various tissues, mainly in the myocardium, skeletal muscle and pancreas [12, 26, 33].

Alternative gene splicing gives rise to two isoforms:

- VEGF-B₁₆₇, the predominant isoform, has a molecular weight of 21 kDa and binds to the cell surface or ECM elements. VEGF-B₁₆₇ has affinity for VEGFR-1 [24, 26, 33] and interacts easily with NP-1 [26, 33].

- VEGF-B₁₈₆, with a molecular weight of 32 kDa, is found in a free form, having affinity for VEGFR-1 [24, 26, 33], and only if it undergoes proteolytic cleavage, it could interact with NP-1 [26, 33].

VEGF-B contributes to the development of the cardiovascular system and the formation of the myocardium in embryonic stages [12]. The role of VEGF-B in vasculogenesis is not essential, *VEGF-B*^{-/-} homozygotic mice being viable at birth, with only moderate defects of the cardiovascular system [24].

Current information on the role of VEGF-B as angiogenic factors in adult diseases is extremely controversial. At present, it is considered that, in adults, VEGF-B is more implicated in the survival of certain cell types, such as: smooth muscle cells, endothelial cells, pericytes, neurons (motor neurons in the spinal cord, cortex or retina), cardiomyocytes, rather than in angiogenesis [24, 73]. Several experiments have revealed that the obstruction of

the median cerebral artery in mice, followed by VEGF-B administration, inhibited cortical neurons apoptosis and minimized the area of cerebral infarction [73].

Placenta growth factor

Placenta growth factor (PlGF) is also a growth factor of the VEGF family and was firstly identified in human placental tissues [2, 17, 74–77]; it is implicated in the trophoblast growth and differentiation, trophoblast invasion and blastocyst implantation [2, 75–78]. Subsequently, PlGF was found in the uterine mucosa: in maternal stromal decidual cells [74], uterine glandular and luminal epithelium [75], glandular secretions, predecidual stromal cells in the secretory phase of the uterine cycle [75], as well as in the heart [2, 76], lungs [2, 76], skin (keratinocytes, dermal vessels endothelium) [76, 79].

By alternative splicing of *PlGF* gene, four isoforms result: PlGF-1 (PlGF₁₃₁), PlGF-2 (PlGF₁₅₂), PlGF-3 (PlGF₂₀₃) and PlGF-4 (PlGF₂₂₄) [2, 76], which differ in molecular structure and biological properties. All isoforms have affinity for VEGFR-1 [2, 17, 24], but PlGF-2 also binds to NP-1, NP-2 and heparin in the ECM [2, 12, 17, 24, 76]. It has no direct mitogenic effect and does not increase vascular permeability [2], but, in pathological conditions, it binds to VEGFR-1, displaces VEGF-A from VEGFR-1 and allows the binding of VEGF-A to VEGFR-2, indirectly enhancing the effects of VEGF-A (increased vascular permeability, cell migration and proliferation, etc.) [2, 4, 17, 78, 80].

To conclude, VEGFR-1 activation by binding PlGF induces the indirect activation of VEGFR-2 [38, 78].

PlGF does not play an essential role in embryonic vasculogenesis, intervening rather in pathological angiogenesis (ischemia, inflammation, cancer), by a synergism with VEGF-A [2, 12, 24, 78].

VEGF-C

VEGF-C is abundantly expressed in the embryonic tissues, where the development of lymphatic vessels is initiated (the jugular, perimetanephric, axillary areas) [33], while in adults it is expressed in the heart, ovary, placenta, intestine, thyroid, etc. [33].

It has a high affinity for VEGFR-3, which is expressed on endothelial lymphatic cells, promoting lymphangiogenesis [24]. Certain authors also described a weak affinity for VEGFR-2, which explains its poor implication in angiogenesis [2, 24]. Experimentally, in homozygotic *VEGF-C*^{-/-} mice, the development of lymphatic vessels is altered since initial phases, the consequence being the accumulation of interstitial fluid in the tissues, which may be sometimes lethal [24].

Overexpression of VEGF-C correlates with a well-developed network of lymphatic vessels, and the genic transfer of human VEGF-C (phVEGF-C) could represent a new therapeutic strategy for the patients with lymphedema [81].

Recent data suggests that VEGF-C also binds to NP-2, which acts as co-receptor for VEGFR-3, enhancing its activity [2, 82].

VEGF-D

VEGF-D presents similar properties to VEGF-C [33], also having a central role in lymphangiogenesis, but not

an essential role in angiogenesis [2, 24, 33]. In the embryo, it has high levels in the lung, where it is involved in the development of lymphatic vessels [33]; in adults, it is found in the heart, lungs, skeletal muscles, small intestine [33]. It has affinity for VEGFR-3 and also for NP-2 [82]. Experimentally, the *VEGF-D* gene inactivation produces only a moderate atrophy of the lymphatic circulation, without other significant changes [2].

VEGF-E (viral VEGF)

Orf virus is a parapoxvirus causing infections in goats and sheep, which may be transmitted to humans, producing skin lesions of pustulous dermatitis type [24, 83], characterized by local edema, vasodilatation, keratinocytes and endothelial cells proliferation, as well as abundant inflammatory infiltrate [33].

VEGF-E contains viral proteins from different strains of the Orf virus: VEGF-E_{NZ-2} (viral strain NZ-2) [12, 84], VEGF-E_{NZ-7} (viral strain NZ-7) [12, 85], VEGF-E_{NZ-10} (viral strain NZ-10) [12, 86], VEGF-E_{D1701} (viral strain D1701) [12, 87] and also VEGF-E_{VR 634} (viral strain VR 634 of pseudocowpox virus) [12, 86].

The gene encoding VEGF-E protein (from viral strains D1701, NZ-2 and NZ-7) is not found in the human genome [83], but after viral infection, it could be incorporated in the genome of the affected individuals, acting like a pro-angiogenic factor [24].

VEGF-E significantly increases vascular permeability, similar to VEGF-A₁₆₅, and also has mitogenic effect on endothelial cells [85]. It has specific affinity for VEGFR-2, not for VEGFR-1 and VEGFR-3 [2, 12, 33, 83].

EG-VEGF

EG-VEGF, also called prokineticin 1 (PK1) [88], was described and characterized by LeCouter *et al.* [89]. It is located in the steroid hormone-producing endocrine glands and placental tissues, where it is involved in physiological and pathological angiogenesis [18].

EG-VEGF, expressed in the testis, adrenal gland, ovary, placental tissues [18, 88–91], induces proliferation, growth, migration and survival of endothelial cells, tubulogenesis, increased vascular permeability and enables paracellular transport [18, 88–91].

Interestingly, the angiogenic effect of EG-VEGF is exerted only on the endothelial cells located in the mentioned glands [89], with no effect on the endothelial cells with other locations, for example in cerebral vessels, aorta and cornea [90, 91].

In the placenta, it is present in the fetal capillaries inside the chorionic villi [human placental microvascular endothelial cells (HPECs)], and is intensely expressed in the first trimester of pregnancy (weeks 8–10) [91], indicating its role in placental vessels development, but also in materno–fetal exchanges, by increasing vascular permeability and promoting paracellular transport [88, 91].

The increased vascular permeability is because EG-VEGF induces the formation of fenestrae between fetal endothelial cells (HPECs); endothelial cells being normally joined by occlusive junctions or adherens junctions [88, 91].

EG-VEGF was also found in endothelial cells that line the umbilical blood vessels [macrovascular human

umbilical vein endothelial cells (HUVECs)], as well as in syncytiotrophoblasts, regulating trophoblast invasion and formation of the maternal lacunae, during the placental growth [88, 91].

Very high serum levels of EG-VEGF were reported in preeclampsia, being associated with early pregnancy loss [88, 90, 92].

EG-VEGF binds to two receptor types, called prokineticin receptor 1 (PROKR1) and 2 (PROKR2), which are intensely expressed in the first trimester of pregnancy [88, 91]. Several studies demonstrated that EG-VEGF, when binding to PROKR1 mediated the proangiogenic effects, while PROKR2 mediates the increase in vascular permeability [88].

Stages of angiogenesis

Angiogenesis, either in its early stages (vasculogenesis), or when it starts from pre-existing vessels (neoangiogenesis), takes place under the synergistic activity of growth factors, VEGF playing a very important role [5].

During development, VEGF induces the migration of hemangioblasts into the blood islands and their differentiation into endothelial cells [4, 12, 25, 53]. After birth, it ensures the proliferation and migration of endothelial cells [12, 25], induces tubulogenesis, increases vascular permeability [4, 12] and promotes endothelial cells survival (inhibiting apoptosis), etc. [5, 12, 16, 25, 53]. VEGF also induces the appearance of fenestrations between the endothelial cells of capillaries and venules [1, 12, 90], by altering proteins in the intercellular junctions (occludin, VE-cadherin/ β -catenin), therefore increasing vascular permeability and facilitating endothelial cells migration, vascular extravasation and metastasis [12, 25]. This process is also enabled by the secretion of matrix metalloproteinases (MMP-9, MMP-3, MMP-2), which decompose the ECM and enhance cell migration [16, 33].

In pathological angiogenesis, VEGF promotes the mobilization of inflammatory cells (macrophages, granulocytes, etc.) to the injury site, maintaining the local inflammatory process and inducing the synthesis of pro-angiogenic factors by endothelial cells [5, 93], platelets [93, 94, 95], smooth muscle cells [71, 93, 96, 97], inflammatory cells [39–43, 98], fibroblasts [5, 93, 99] and tumor cells [95, 100].

The major trigger inducing angiogenesis is hypoxia, but other factors may also be responsible: hypoglycemia, hypertension, low pH, mechanical stress, chronic inflammation, etc. [1, 8]. Hypoxic tissues release the hypoxia-inducible factor-1 (HIF-1) [4, 99, 101, 102], which activates the transcription of pro-angiogenic factors, such as: VEGF [8, 99, 101, 103], basic fibroblast growth factor (bFGF, FGF-2) [5, 95, 99, 103], PDGF- β [5, 93, 95], angiopoietin-1 (Ang-1), angiopoietin-2 (Ang-2) [1, 8], TGF- β [5], TNF- α , etc. [5, 95].

bFGF or FGF-2 was the first pro-angiogenic factor described, with an important role in pathological angiogenesis, having a mitogenic effect on endothelial cells, increasing vascular permeability, being involved in tubulogenesis, proteolytic decomposition of ECM [5, 104], etc. It also plays a role in fibroblast proliferation, thus favoring granulation tissue formation and wound healing [5, 104]. VEGF expression on endothelial cells

could be stimulated by bFGF, both growth factors having a synergistic pro-angiogenic effect [5, 103, 104].

Angiopoietins (Ang-1 and Ang-2) are pro-angiogenic and vascular remodeling growth factors [5], both binding to the same receptor, the Tie-2 receptor [5]. In normal adult tissues, Ang-1 maintains the vessels integrity, increasing endothelial cells survival (antiapoptotic effect) and also inhibits Ang-2 expression [5, 100]. Ang-2 may have a pro-angiogenic or anti-angiogenic effect, depending on the presence of VEGF [5, 100, 103]. In the absence of VEGF, Ang-2 promotes endothelial cells apoptosis, vessel regression and inhibits angiogenesis [100]. On the other hand, in the presence of VEGF and HIF-1, Ang-2 acts as antagonist of Ang-1, destabilizes the interaction between endothelial cells and the supporting cells, promoting vessel instability and formation of disorganized and immature new blood vessels [5, 100]; it also induces the proliferation and migration of endothelial cells, thus favoring tissue neovascularization and pathological angiogenesis [5, 100, 103].

Under hypoxic conditions, the injured tissues and endothelial cells will release NO, which promotes vasodilatation [70].

Increased VEGF secretion is also induced by other growth factors through paracrine mechanism [33, 104].

The integrity of blood vessels depends on the interaction between endothelial cells and other cells in the vascular wall, called supporting or mural cells (*e.g.*, pericytes, VSMCs) [11, 99, 105].

Pericytes (adventitial or perivascular cells) are associated with endothelial cells in small blood vessels [70], both cell types being included in the same basal membrane [101]. Normally, pericytes ensure the support for the endothelial cells, stabilize small vessels structure and monitor endothelial cells survival [70, 99, 101, 105]. Pericytes communicate with endothelial cells through gap junctions [105], their activation promoting the proliferation of endothelial cells. Activated pericytes exhibit a great plasticity; they can differentiate into smooth muscle cells, fibroblasts and adipocytes [70].

The blood vessels formation includes several stages.

The initial stage starts with vasodilatation and alteration of the vascular structure, as a result of the pro-angiogenic factors secreted by injured tissues (*e.g.*, NO, VEGF, bFGF or FGF-2, PDGF- β , Ang-2, etc.), which act by both paracrine and autocrine mechanisms on the endothelial and vascular mural cells [70, 103].

The PDGF- β /PDGFR- β signaling pathway is very important in pericyte recruitment and new blood vessel formation and stabilization. PDGF- β is secreted by endothelial cells and PDGFR- β is expressed on vascular mural cells and endothelial cells.

Under the action of these pro-angiogenic factors, endothelial cells (ECs) and mural cells are activated [70, 99], the effect being the proliferation of endothelial cells and their migration towards the ECM [70], where tubulogenesis will be initiated [8, 70]. VEGF released from the injured cells (pericytes, endothelial cells) will increase vascular permeability and allow extravasation of plasma proteins into the ECM; where these proteins will serve as provisional matrix for the migration of ECs and pericytes, initiation of tubulogenesis and formation of new blood

vessels [8, 70, 101]. Initially, the newly formed vascular tubes do not have a lumen [8, 70].

The process is facilitated by the decomposition of ECM, due to secretion and activation of matrix metalloproteinases (MMP-2, MMP-3, MMP-9) [16, 33] and inhibition of tissue inhibitor of metalloproteinases-2 (TIMP-2), induced by Ang-1 [8].

An important role in angiogenesis is played by membrane type-1 matrix metalloproteinase (MT1-MMP), a more recently described membrane protein group of metalloproteinases category, anchored on the cells surface, with a lytic effect on the ECM molecules; it regulates the expression of pro-angiogenic factors (*e.g.*, VEGF) and controls cell migration [70, 106]. MT1-MMP may be expressed on endothelial cells or mural cells (pericytes, VSMCs) [70, 107, 108], being involved in cell migration, tubulogenesis and cellular invasion [108]. At a more advanced stage of neoangiogenesis, MT1-MMP may activate the PDGF- β /PDGFR- β pathway, controlling the migration of pericytes and VSMCs and stabilization of the newly formed vessels [106].

Subsequently, during physiological angiogenesis, in order to ensure stability of the new vessels, supporting cells are recruited (pericytes in small vessels, VSMCs in large vessels) and cell fusion occurs. In a final stage, a new basal membrane will be formed around the new vessels [8].

In pathological angiogenesis, the cells involved (ECs, macrophages, T-lymphocytes, platelets, VSMCs, etc.) increase the release of proinflammatory cytokines [93, 105]. The new vascular networks are anarchic, with tortuous, dilated, abnormally structured vessels, with aberrant ECs and weakly attached or even absent pericytes [8].

➤ Anti-VEGF therapies – therapeutic implications

Nowadays, a large number of studies aim to elucidate the role of anti-VEGF therapies in various diseases, especially cancer, ischemia, inflammation (*e.g.*, rheumatoid arthritis) or degenerative diseases.

The purpose of anti-VEGF medications is to inhibit angiogenesis, either by blocking VEGF itself or its receptors (VEGFRs).

A series of anti-VEGF agents have been approved by *United States Food and Drug Administration* (US FDA) and are currently used in the treatment of certain tumor types or eye diseases.

Since 2004, Bevacizumab/Avastin has been approved by the FDA and used as anti-VEGF therapy in renal [24], lung [109, 110, 111], colon [109, 112, 113], esophago-gastric [114, 115], breast [3, 24, 116], cervical [117], and ovarian cancer [118, 119], usually associated with chemotherapy. Other therapeutic strategies attempt to block VEGFRs using drugs such as: Sorafenib or Sunitinib, tested in phase III clinical trials as monotherapies or in association with chemotherapy for renal cancer [3, 24, 109], gastrointestinal stromal tumors [3, 109], lung tumors (non-small cell type) [109], breast tumors [109], colon [109], esophago-gastric adenocarcinoma [120] or hepatocellular carcinoma [24, 109].

Anti-angiogenic therapy has become very important in eye diseases, drugs such as Ranibizumab, Pegaptanib/

Macugen or Aflibercept being approved by the FDA and successfully used for age-related macular degeneration [3], diabetic retinopathy (DR) and diabetic macular edema (DME) [121, 122].

In ischemic processes, studies in large clinical trials attempt to stimulate angiogenesis by gene transfer of pro-angiogenic molecules [3, 123].

Even so, the use of anti-VEGF or anti-VEGFRs therapy in clinical trials did not always yield the expected results. Patients treated with anti-VEGF or anti-VEGFRs agents, alone or associated with chemotherapy showed an impro-

vement in overall survival (OS) and progression-free survival (PFS), but these results were not always statistically significant (Table 1). This is probably because ECs have different locations and respond differently to these therapies, depending on the local microenvironment [11]. Additionally, long-term administration of anti-VEGF therapy induced a decrease in efficacy and led to onset of therapy resistance [24]. Moreover, various negative side effects have been described, including: hypertension, proteinuria, renal dysfunctions, hemorrhage, thrombosis, arrhythmia, etc. [24, 108–120, 124].

Table 1 – Clinical trials on anti-VEGF agents in cancer therapy

Reference	Cancer type	No. of patients (n)	Intervention	Median PFS [months]	P-value	Median OS [months]	P-value
Zhao <i>et al.</i> [112]	Stage IV colorectal cancer	122	Irinotecan (FOLFIRI) + Bevacizumab + Erlotinib	PFS: 7.1	Not reported	OS: 13.5	Not reported
Yamasaki <i>et al.</i> [110]	Stage IIIB/IV non-small cell lung cancer	33	Carboplatin + Paclitaxel + Bevacizumab	PFS: 8.4	Not reported	OS: 22.2	Not reported
Tiseo <i>et al.</i> [111]	Extensive small-cell lung cancer	204	Arm A (n=103) Cisplatin + Etoposide	Arm A PFS: 5.7	0.03	Arm A OS: 8.9	0.011
			Arm B (n=101) Cisplatin + Etoposide + Bevacizumab	Arm B PFS: 6.7		Arm B OS: 9.8	
Tewari <i>et al.</i> [117]	Advanced cervical cancer	452	Group I Cisplatin + Paclitaxel or Topotecan + Paclitaxel	Not reported	Not reported	Group I OS: 13.3	0.004
			Group II The same therapy + Bevacizumab			Group II OS: 17	
Cao <i>et al.</i> [113]	Metastatic colorectal cancer	142	FOLFIRI (Irinotecan + 5-Fluorouracil + Leucovorin)	FOLFIRI PFS: 5.1	Not reported	FOLFIRI OS: 11.3	Not reported
			FOLFIRI B (FOLFIRI + Bevacizumab)	FOLFIRI B PFS: 8.5		FOLFIRI B OS: 15.2	
Ohtsu <i>et al.</i> [114]	Advanced gastric cancer	774	Group 1 Bevacizumab + Fluoropyrimidine–Cisplatin	Group 1 PFS: 6.7	0.037	Group 1 OS: 12.1	0.1
			Group 2 Placebo + Fluoropyrimidine–Cisplatin	Group 2 PFS: 5.3		Group 2 OS: 10.1	
Oza <i>et al.</i> [118]	Newly diagnosed ovarian cancer	1528	Group 1 (n=764) Carboplatin + Paclitaxel	Not reported	Not reported	Group 1 OS: 44.6	0.85
			Group 2 (n=764) Chemotherapy + Bevacizumab			Group 2 OS: 45.5	
Perren <i>et al.</i> [119]	Ovarian cancer (70% stage IIIC or IV)	1528	Group 1 Carboplatin + Paclitaxel	Group 1 PFS: 22.4	0.04	Group 1 OS: 28.8	Not reported
			Group 2 Chemotherapy + Bevacizumab	Group 2 PFS: 24.1		Group 2 OS: 36.6	
von Minckwitz <i>et al.</i> [116]	Metastatic breast cancer	494	Group 1 (n=247) Single-agent chemotherapy	Group 1 PFS: 4.2	0.0068	Not reported	Not reported
			Group 2 (n=247) Single-agent chemotherapy + Bevacizumab	Group 2 PFS: 6.3			
Moehler <i>et al.</i> [120]	Advanced esophago-gastric cancer	91	Group 1 FOLFIRI + Placebo	Group 1 PFS: 3.3	0.66	OS: 8.9	0.21
			Group 2 FOLFIRI + Sunitinib	Group 2 PFS: 3.5		OS: 10.4	

PFS: Progression-free survival; OS: Overall survival; FOLFIRI: Chemotherapy regimen – Folinic acid (Leucovorin) + 5-Fluorouracil (5-FU) + Irinotecan (Camptosar).

☐ Conclusions

The data presented in this article aimed to emphasize several important aspects regarding VEGF and its implications in the complex process of angiogenesis; but these data are not exhaustive. Taking into account that angio-

genesis is involved in the progression of pathological conditions, in the future more complex studies will be conducted, in large clinical trials, in order to identify targeted anti-angiogenic therapies with higher efficiency, long-term administration and minimal toxicity.

Conflict of interests

The authors declare that they have no conflict of interests.

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