Oligonucleotide-based interventions targeting VEGF in eye neovascularization: potential synergy between present and future molecules

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Abstract

Roughly ten years ago the FDA approved most of the presently used anti-VEGF drugs for the treatment of neovascular AMD and other eye pathologies characterized by ocular neoangiogenesis. However, the recent findings on the physiologic activities of VEGF isoforms impose to reconsider the inhibitory effects of pan-VEGF antagonists and the concept that to face pathological alterations at ocular level is possible only through the full block of all VEGF isoforms. In fact, although pan-VEGF agents rapidly and effectively contrast ocular neovascularization, vascular leakage, and other pathological changes, in the long-term the inhibition of all VEGF isoforms likely may result in the loss of the physiologic effects exerted by $VEGF_{121}$ and the anti-angiogenic $VEGF_{165}b$. Notably, selective inhibitors of $VEGF_{165}a$, such as pegaptanib, spare these targets. Moreover, preclinical and clinical evidence suggest that also systemic side effects, secondary to intraocular treatment with

non-selective anti-VEGF drugs, may be reinterpreted in light of these recent findings, which may be useful to clinicians for the choice of the most appropriate anti-VEGF agent.

Another aspect that should be considered is the involvement of VEGF-independent pathways in ocular neovascularization, therefore a combined therapy can represent a more effective pharmacological approach that might help also to counteract tachyphylaxis, an important issue in anti-VEGF treatment.

This complex picture and the recent findings on current anti-VEGF drugs should be therefore taken into account to guide the development of novel agents targeting VEGF and/or other key factors involved in the pathogenesis of neovascular ocular diseases along the signaling pathways stimulated by the various isoforms. Accordingly, this review also reports on novel pharmacological molecules targeting VEGF at ocular level and currently under development, with a special attention to oligonucleotide-based interventions.

Keywords VEGF, ocular angiogenesis, neovascular AMD, pegaptanib, ranibizumab, bevacizumab

List of abbreviations

AMD = Age-related Macular Degeneration

DR = Diabetic Retinopathy

EC = Endothelial Cell

VEGF = Vascular Endothelial Growth Factor

PLGF = Placental Growth Factor

DME = Diabetic Macular Edema

RVO = Retinal Vein Occlusion

PRN = *pro re nata*

VEGFR = VEGF Receptor

BRVO = Branch Retinal Vein Occlusion

NRP1 = Neuropilin-1

RPE = Retinal Pigment Epithelium

I/R = Ischemic/Reperfusion

iNOS = inducible Nitric Oxide Synthase

NO = Nitric Oxide

RGC = Retinal Ganglion Cell

OIR = Oxygen-Induced Retinopathy

SELEX = Systematic Evolution of Ligands by Exponential Enrichment

HUVEC = Human Umbilical Vein Endothelial Cell

HBD = Heparin-Binding Domain

PDGF = Platelet-Derived Growth Factor

FGF = Fibroblast Growth Factor

PEG = Polyethylene Glycol

HMVEC = Human Microvascular Endothelial Cell

RBD = Receptor-Binding Domain

CNV = Choroidal Neovascularization

VISION = VEGF Inhibition Study in Ocular Neovascularization

GA = Geographic Atrophy

PED = Pigment Epithelium Detachment

BFV = blood flow velocities (s) in the

CRA = Central Retinal Artery

TPCA = Temporal Posterior Ciliary Artery

OA = Ophthalmic Artery

ADR = Adverse Drug Reaction

PDGFR = PDGF Receptor

siRNA = small interfering RNA

DDIT4 = DNA-Damage-Inducible Transcript 4

KSP = Kinesin Spindle Protein

Contents

1. Introduction

Age-related macular degeneration (AMD) and diabetic retinopathy (DR) are the most common ocular diseases dramatically affecting the quality of life of patients and causing an enormous burden to the healthcare system in Europe and USA [1; 2]. AMD, whose pathogenesis is multifactorial and not yet fully elucidated, is classified in early and late stages, atrophic dry (85%) and exudative wet neovascular (15%) degeneration categories [3]. Quite similarly, DR, a sight-threating complication of diabetes developed by more than one-third of diabetic individuals, is classified in two stages: non proliferative and proliferative [4; 5]. In some cases, both non proliferative AMD and DR may progress and convert to proliferative neovascular forms, in which the formation of new vessels from the existing ones, a process named "ocular angiogenesis", represents a major cause of visual loss. Neovascular AMD and DR are the leading causes of blindness in elderly and working age people, respectively [6; 7]. These pathologies are associated with neovascularization in the posterior segment of the eye; in particular, the hallmark of wet AMD is the formation of new blood vessels arising from the choroidal microvascular bed and invading the sub-retinal space, while DR presents alterations preferentially at retinal level [8; 9]. Although with some differences, both neovascular AMD and DR are characterized by endothelial cell (EC) proliferation and migration, increase in vascular permeability and inflammation, all processes in which Vascular Endothelial Growth Factor-A (VEGF-A) plays a key role. In mammals, VEGF-A belongs to a family that also includes VEGF-B, -C, –D, and placental growth factor (PLGF). Among these closely-related growth factors, VEGF-A is the most potent mediator of both retinal and choroidal angiogenesis, and its inhibition via intraocular anti-VEGF treatments currently represents the cornerstone of therapies for both AMD and DR [10; 11]. The outcomes of anti-VEGF treatments are to counteract pathological neovascularization and disease progression, to arrest visual impairment and, in the best case, to gain the recovery of vision. Some molecules targeting VEGF-A pathway and acting at multiple levels are currently used in ophthalmology, and much more are under investigation in clinical trials for either AMD, DR, or other eye diseases characterized by neovascularization. These anti-VEGF-A

drugs can be divided in three main pharmacological classes: 1) molecules targeting VEGF isoforms, 2) molecules inhibiting VEGF receptors, and 3) molecules inhibiting VEGF downstream signaling [12]. This review will focus on current therapies and novel substances under development belonging to the first pharmacological class, with a special attention to oligonucleotide-based interventions targeting VEGF at ocular level.

2. Pharmacodynamic classification and clinical application of drugs acting upon VEGF

Several agents have been developed to interfere with the VEGF system and various molecules are already used especially in cancer therapy. A few of them are approved as ophthalmic therapies, such as ranibizumab, pegaptanib and aflibercept, others are under investigation and/or currently used off-label like bevacizumab. They mainly act on VEGF itself or on its gene expression and comprise some promising molecules, under preclinical or clinical investigation, that will be briefly illustrated later on in this review.

A fundamental class of therapeutics acting as angiogenesis inhibitors is represented by monoclonal antibodies characterized by a high specificity for a given target, which they bind and neutralize. In this category, ranibizumab is an engineered recombinant humanized Fab fragment of 48 kDa designed from the full-length monoclonal antibody bevacizumab to optimize retinal penetration. Ranibizumab binds with high affinity a site present in all VEGF-A isoforms (see below) and their bioactive proteolytic fragments [13]. On the market since 2006, ranibizumab is approved by FDA for the treatment of all lesion types in neovascular AMD, diabetic macular edema (DME), and macular edema secondary to retinal vein occlusion (RVO) [14]. Its intraocular administration is recommended at a dosage of 0.5 mg/month, and treatment protocols usually advice an initial loading dose of three monthly injections followed by administration *pro re nata* (PRN) based on the disease activity. However, as for the other anti-VEGF treatments, the optimal regimen has yet to be established, although recently the ophthalmic community has made available guidelines on the

160 treatment of patients with advanced AMD also to help clinicians to prevent over- and/or under-
161 treatment with anti-VEGF therapy [11; 15].
162 Bevacizumab is a full-length humanized monoclonal IgG antibody of 149 kDa targeting the same
163 site of ranibizumab, and thus inhibiting all VEGF-A isoforms. Approved in 2004 by FDA for
164 systemic use in the treatment of certain metastatic cancers, it is widely used off-label as intravitreal
165 therapy in proliferative eye diseases, especially neovascular AMD and DME, although with various
166 limitations of medical, financial and ethical nature [16; 17; 18]. The recommended dose for
167 intraocular treatment is 1.25 mg/month.
168 Aflibercept, or VEGF Trap-Eye, is a fully human recombinant protein of 115 kDa consisting of key
169 binding domains from VEGF receptor (VEGFR) -1 and VEGFR-2 fused to an IgG Fc fragment
170 [19]. It acts as a soluble decoy receptor recognizing and neutralizing all VEGF-A isoforms, with the
171 establishment of a tighter binding than the native receptors. Unlike the anti-VEGF monoclonal
172 antibodies currently in use, aflibercept is purposely designed to inhibit also VEGF-B and PLGF-1
173 and -2 [20]. It has been approved by FDA for the treatment of neovascular AMD at the end of 2011,
174 and for RVO in 2012 [21]. The same molecular structure has been approved in 2012 by FDA also
175 for systemic use within the combination therapy for patients with metastatic colorectal cancer with
176 the name of ziv-aflibercept. The two products present substantial differences in both the preparation
177 of the purified aflibercept and the drug formulation, indeed aflibercept/VEGF Trap-Eye undergoes
178 more purification steps during manufacturing than ziv-aflibercept, and it is formulated with proper
179 buffers to minimize the risk of ocular irritation. The recommended regimen is an intravitreal dose of
180 2 mg/month for three consecutive treatments, followed by one injection every two months; a
181 possible variant is represented by bimonthly injections from the beginning of the therapy [22].
182 Besides the just mentioned drugs, which are all proteins targeting pan-VEGF-A isoforms, there is
183 another drug somehow unique in its own way: pegaptanib, a 28-nucleotide RNA aptamer of ~50
184 kDa with a high selectivity for the VEGF-A_{165} isoform. Pegaptanib sodium was the first drug
185 officially registered for the treatment of neovascular AMD in 2004, and also the first aptamer to

enter in therapy. Aptamers can be envisioned as "chemical antibodies" since they offer the advantages of antibodies - high specificity and affinity - in a relatively small, chemically synthesized molecule without cell-culture-derived contaminants. Moreover, aptamers are highly versatile and their commercial synthesis by large-scale manufacturing is fairly uncomplicated and cost-effective [23]. Pegaptanib is used off-label also for DME and branch RVO (BRVO). The intravitreal injection is at a dose of 0.3 mg once every six weeks.

3. The main target of anti-VEGF agents: $VEGF_{165}$ among pro-angiogenic and anti-angiogenic function - from one main actor to two opponents

The human *VEGF-A* gene is organized in 8 exons separated by 7 introns [24; 25] resulting in the generation of at least 7 isoforms of VEGF-A_{xxx}, where $_{xxx}$ is the number of amino acids encoded. The various isoforms contain between 121 and 206 amino acids, where VEGF-A_{165} is the predominant member, followed by VEGF-A_{121} [26].

In recent years, great interest raised the discovery by Bates et al. of the VEGF-A_{165}b isoform, formed by an alternative splicing at exon 8 of *VEGF-A* gene [27]. Now we know that the VEGF-A family is composed by two subfamilies of splice variants which differ for an alternative C-terminal sequence: the "conventional" VEGF-A_{xxx} (or VEGF-A_{xxx}a), formed by proximal splice-site selection, and the "novel" VEGF-A_{xxx}b, generated by a splicing 66 bases downstream. The alternative splicing of *VEGF-A* (since now referred as *VEGF*) thus results in proteins of the same length but with different amino acids at the C-terminus: Cys-Asp-Lys-Pro-Arg-Arg in $VEGF_{xxx}$a isoforms, and Ser-Leu-Thr-Arg-Lys-Asp in $VEGF_{xxx}$b subfamily, respectively [27]. More importantly, the alternative splicing of exon 8 is the key determinant of isoform switching from "pro-angiogenic" $VEGF_{xxx}$a to "anti-angiogenic" $VEGF_{xxx}$b.

All VEGF-A isoforms present the VEGFR-binding site and activate both Flt-1/VEGFR-1 and Kdr/VEGFR-2, although with different affinity and potency. VEGFR-1 mediates VEGF-induced chemotaxis and inflammation, while VEGFR-2 is the main mediator of the mitogenic, angiogenic

212 and permeability-enhancing effects of VEGF [28; 29]. In endothelial cells VEGFR-2 can be co-
213 expressed with the neuropilin-1 (NRP1) co-receptor, which enhances VEGF binding to VEGFR-2
214 and its signal transduction up to 6-fold [30] (see also Figure 1). NRP1 is an isoform-specific
215 receptor for $VEGF_{165}a$ [31]; $VEGF_{165}a$ binding to VEGFR-2 and NRP1 results in receptor
216 dimerization, rotation of its intracellular domain, and its autophosphorylation [32; 33]. Conversely,
217 this full rotation likely does not occur when $VEGF_{165}b$ binds to VEGFR-2, resulting in an
218 inefficient autophosphorylation of the receptor [31; 32]. In respect to its angiogenic counterpart,
219 $VEGF_{165}b$ isoform is indeed a weaker agonist, since it binds and poorly activates VEGFR-2,
220 resulting in differential activation of intracellular pathways and, most relevantly, not inducing
221 vasculogenesis [32]. This feature seems to be due to the difference in the C-terminal end of
222 $VEGF_{165}b$ isoform. In fact, $VEGF_{165}b$ does not bind to NRP1 [30; 32], probably because its
223 adjacent C-terminal sequence lacks Cys-160, which instead is present in $VEGF_{165}a$, affecting its
224 secondary structure and folding, and likely VEGFR-2 signaling [27]. It is possible that also the
225 neutral charge conferred by the two terminal residues (Lys-Asp) of $VEGF_{165}b$ contributes to the
226 lack of interaction with NRP1 and the differential VEGFR-2 downstream signaling [31].

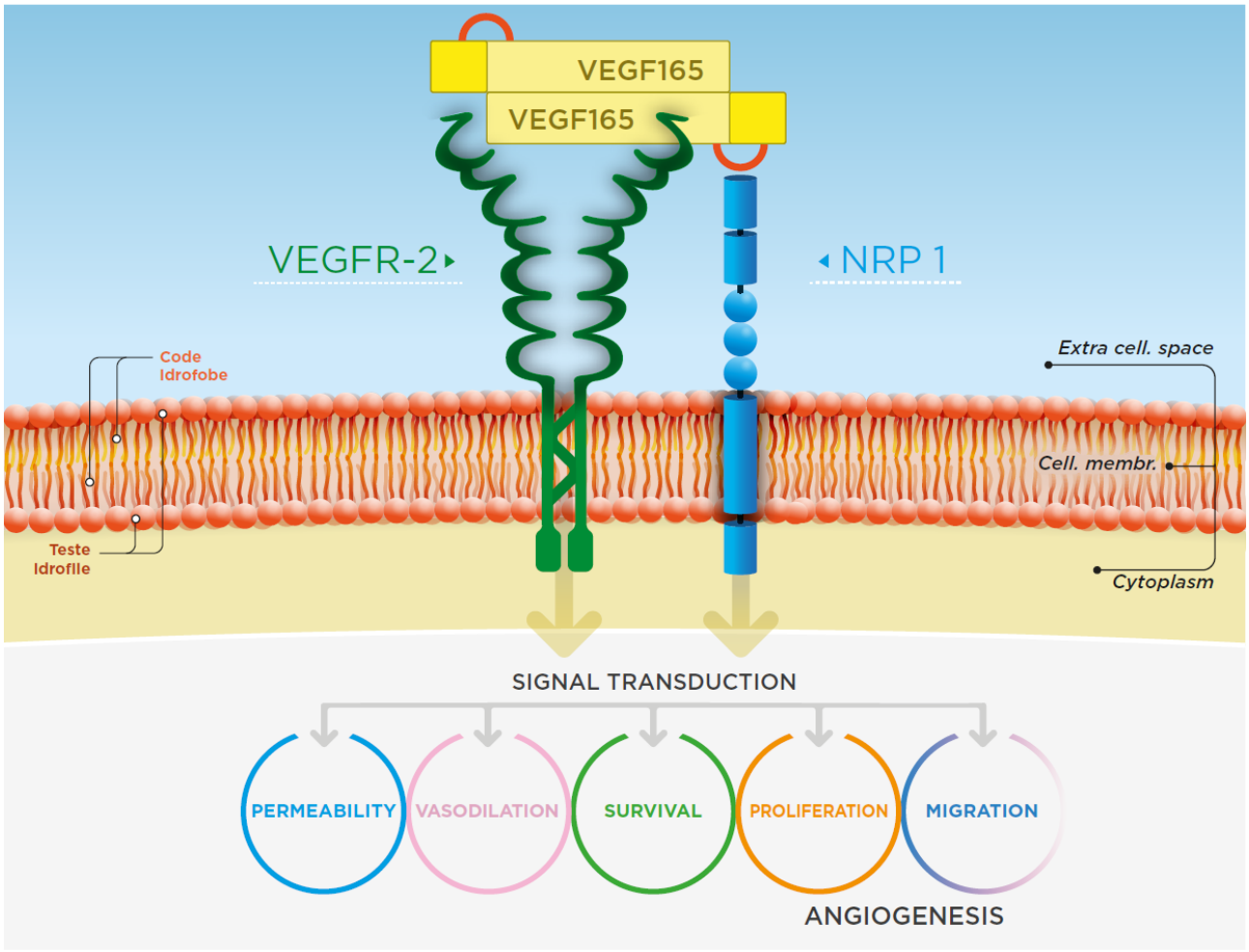


Figure 1. $VEGF_{165}$ interacts with VEGFR-2 and NRP1 in endothelial cells. $VEGF_{165}$ functions as a dimer and its binding to VEGFR-2 promotes the receptor dimerization with subsequent activation of tyrosine kinase domains and downstream signaling pathways responsible of the effects reported. In $VEGF_{165}$ is present a disulfide bond between Cysteines 146 and 160 (in orange color), necessary for VEGF activity and its interaction with NRP1 (for more details see text, paragraph 3).

4. Heterogeneous activities of the different VEGF isoforms: a hot topic to understand the effects of selective and non-selective VEGF inhibition

Since the beginning the predominant concept was that blocking all the VEGF-A isoforms, instead of only $VEGF_{165}$, was the winning strategy to face neovascularization, therefore the most commonly used anti-VEGF molecules injected for neovascular ocular diseases have been ranibizumab and bevacizumab. More recent studies on the role of the various VEGF isoforms and

their biological effects, as well as relevant clinical evidence raised in pharmacovigilance, impose to reconsider previous findings on the molecular mechanisms of anti-VEGF drugs in light of the advanced knowledge.

VEGF is secreted by several cell types in the retina, such as vascular ECs, retinal pigment epithelium (RPE), pericytes, retinal neurons, and astrocytes, indicating that VEGF plays important functions in ocular homeostasis [34]. In particular, VEGF is a critical factor for the homeostasis and plasticity of both blood vessels and neurons.

4.1. The "conventional" VEGF isoforms and the vasculature

VEGF is essential for EC survival in normal conditions, and adult blood vessels require autocrine VEGF for maintenance of homeostasis [35]. *In vivo* treatment with a soluble receptor VEGF-Trap in adult mice leads to early regression of normal blood capillaries, in a sequence of events comprising cessation of blood flow, EC apoptosis, pericytes migration from regressing vessels to surviving ones, and formation of acellular capillaries, also called basement membrane ghosts [36]. The features of the various VEGF isoforms and proteolytic products, their tissue expression and roles during development and in adult, have been recently reviewed [37]. The specific functions of VEGF isoforms in vascular patterning have been elucidated by transgenic mouse models. Mice selectively expressing only $VEGF_{164}$ present normal retinal vascular development, while mice expressing only $VEGF_{120}$ have severely impaired outgrowth and patterning of developing retinal vessels [38; 39], indicating that $VEGF_{164}$ plays the main role in vasculogenesis. However, $VEGF_{164}$-deficient mice expressing both $VEGF_{120}$ and $VEGF_{188}$ showed no difference in physiological neovascularization when compared with wild-type control animals, indicating that other VEGF isoforms, in combination, may compensate $VEGF_{164}$ lack and be sufficient to promote normal physiological neovascularization [40]. From the development through all the life, the VEGF isoforms act in concert to assure the optimal formation and maintenance of an adequately branched vessel network, guiding specific ECs in growing and shaping the vascular tree [41-44]. Importantly,

of the total amount of VEGF secreted by RPE cells, 75% is represented by $VEGF_{165}$ and 24% by $VEGF_{121}$ [45]. The various isoforms differ not only, as previously mentioned, for alternative splicing but also for their distribution: for instance, $VEGF_{121}$ is freely diffusible; $VEGF_{188}$, which contains two heparin sulfate binding sites, is sequestered by cell membrane or extracellular matrix; $VEGF_{165}$ is in both soluble and bound status. Soluble isoforms are essential for maintenance of RPE and choriocapillaries in the adult, indeed the absence of both $VEGF_{120}$ and $VEGF_{164}$ in mice leads to an age-dependent degenerative phenotype characterized by RPE dysfunction, loss of barrier properties, insoluble drusen-like deposits resembling atrophic AMD [46].

$VEGF_{165}$ appears to be a critical isoform for retinal angiogenesis not only under development but also in pathological conditions; among the VEGF isoforms, $VEGF_{165}$a is indeed the principal mediator of inflammation and cellular immunity occurring in pathological retinal neovascularization, acting as a proinflammatory cytokine targeting monocytes, macrophages and leukocytes, in a positive feedback loop involving primarily ECs and sustaining the neovascularization process [40; 47]. The expression pattern of VEGF isoforms, which is strictly regulated in normal conditions, is disrupted in diseases; for example, *in vivo* it has been shown that in pathological retinal neovascularization of rodents, the $VEGF_{164}/VEGF_{120}$ ratio undergoes a ten-fold increase (~25.5 versus ~2.2 in the physiologically developing retina), and this likely contributes to an angiogenic switch and to the appearance of inflammation-associated vessel invasion within the vitreous [40]. $VEGF_{121}$ has less affinity for VEGFRs than $VEGF_{165}$, explaining the lower mitogenic, proinflammatory potency of $VEGF_{121}$ relative to $VEGF_{165}$ [48; 49].

4.2. The “conventional” VEGF isoforms and the neurons

VEGF plays a fundamental role also in neurogenesis and neuroprotection, and VEGF abnormal expression has been linked to several neurodegenerative disorders [50-52]. *In vitro* studies demonstrated that VEGF, in particular the 165 isoform, increases the survival of various types of neurons under different stresses via VEGFR-2 through multiple molecular mechanisms including

292 phospholipase C, PI3K, p38/MAPK and MEK1/2 activation, caspase-3 inhibition, and modulation
293 of ion channel currents [53-57]. Protective effects are also exerted on supporting cells, such as
294 astrocytes and microglia, through VEGFR-1 [58]. *In vivo*, intraocular injection of either $VEGF_{120}$ or
295 $VEGF_{164}$ prevents retinal neuron apoptosis resulting from ischemic/reperfusion (I/R) injury in the
296 rat, acting via VEGFR-2 signaling; however, the retina injected with $VEGF_{164}$ develops
297 hemorrhages and edema that were not detected after $VEGF_{121}$ injection [59], suggesting that
298 $VEGF_{121}$ is more implicated in the homeostasis of neurons and vessels than in pathologic contexts.
299 It is well known that downstream the VEGF signaling is induction of Nitric Oxide Synthase
300 (iNOS), leading to a potent vasodilation [60], and that increasing volumetric blood flow enhances
301 neuroprotection in ischemic tissues [61]; interestingly, the authors showed that the $VEGF_{121}$-
302 mediated neuroprotection in the I/R retina is only partially dependent by an increase of iNOS and
303 blood flow, and that $VEGF_{121}$ exerts a pro-survival action also on retinal ganglion cells (RGCs) *in*
304 *vitro*, strongly suggesting a direct neuroprotective effect of $VEGF_{121}$ [59]. In the same *in vivo*
305 model, a brief ischemic preconditioning increases both $VEGF_{120}$ and $VEGF_{164}$ expression as a
306 neuroprotective response, and this benefic pro-survival effect on RGCs is suppressed by either
307 VEGFR-1/Fc fusion protein or an anti-VEGF antibody; conversely and of great interest, pegaptanib
308 does not impair RGCs viability, further suggesting that not fully abrogating VEGF responses, and
309 especially sparing $VEGF_{120/121}$, represent a key strategy to preserve retinal neurons [59]. More
310 recently the same group confirmed these findings in other *in vitro* and *in vivo* models on RGCs
311 death, showing that the pro-survival effects of $VEGF_{121}$ are mediated by VEGFR-2 and PI3K/Akt
312 signalings, and that total VEGF blockade significantly exacerbates neuronal cell death [62].
313
314 **4.3. The emerging role of $VEGF_{165}$b in blood vessels and neurons**
315 As mentioned, it has been discovered that VEGF family is actually constituted of two subfamilies,
316 whose differential functions still need to be fully investigated. It has been reported that in normal
317 conditions the "anti-angiogenic" $VEGF_{xxx}$b isoforms represent the predominant proportion of the

total VEGF in human eye tissues and fluids; in particular, $VEGF_{165}b$ is highly expressed in the normal eye (retina, lens, sclera, iris, vitreous) and it is down-regulated in the vitreous fluid of DR patients, where a switch in VEGF splicing from anti- to pro-angiogenic isoforms likely occurs [63]. This alteration in VEGF balance between the two subfamilies seems to be also present in other angiogenic-associated conditions, as shown in oxygen-induced retinopathy (OIR) mouse model and in laser-induced choroidal neovascularization of AMD mouse model [64; 65]. In humans, beside in DR, significantly lower levels of $VEGF_{165}b$ have been also revealed in the vitreous fluid from patients with RVO [66] but not with AMD, although a trend towards a reduction (-20% $VEGF_{165}b$ median vs control subjects) was detected [67]. Interestingly, in animal models of OIR and AMD, intraocular injections of recombinant human $VEGF_{165}b$ inhibit retinal neovascularization [64; 68; 69]. To this regard, *in vitro* and *in vivo* studies have demonstrated that $VEGF_{165}b$ inhibits several $VEGF_{165}a$-induced processes, such as EC migration and proliferation, vasodilatation [27] and pathological angiogenesis in many tumor types [70; 71]. And as, $VEGF_{165}b$ seems to be more than simply an anti-angiogenic factor, since in a dose dependent manner it is able not only to inhibit the pathologic pre-retinal proliferation of new vessels, but also to reduce the ischemic area in OIR animal model [68; 69]. Specifically, these studies show that $VEGF_{165}b$ favors physiological revascularization *in vivo* and acts as a survival factor for both retinal endothelial and epithelial cells *in vitro*, these latter effects being observed also with its sister isoform $VEGF_{165}a$ [69]. $VEGF_{165}b$ inhibits $VEGF_{165}a$-induced EC migration and proliferation but does not interfere with regrowth of blood vessels within previously vascularized areas, a process named “revascularization” [69; 72], suggesting that $VEGF_{165}b$ contrasts the invasive phenotype and promotes physiological angiogenesis, in agreement with its weak agonist activity. Consistently, in a rat model of proliferative retinopathy, $VEGF_{164}$ blockade by EYE001/pegaptanib inhibits pathological neovascularization but not physiological revascularization, resembling the $VEGF_{165}b$-mediated effects; in contrast, a VEGFR-1/Fc chimera blocking all VEGF isoforms suppresses both pathological and physiological retinal neovascularization [40].

In vitro and *in vivo* evidence also demonstrate that $VEGF_{165}b$ is neurotrophic and exerts neuroprotective effects in response to multiple insults in various types of neurons; in particular, *in vivo* $VEGF_{165}b$ pretreatment protects RGCs and the inner nuclear layer cells in rat retinal I/R injury model [73]. As for $VEGF_{165}a$, $VEGF_{165}b$-mediated neuroprotection is through VEGFR-2, p44/42 MAPK activation, and caspase-3 inhibition, but, in contrast to $VEGF_{165}a$, it does not involve either PI3K or p38 MAPK [73].

On the whole, these findings implicate that blocking all VEGF isoforms leads rapidly to an effective inhibition of ocular neovascularization, but in the long term this may result in detrimental effects at both vascular and neuronal retinal level, mainly due to the loss of the physiologic effects mediated by $VEGF_{121}$ and $VEGF_{165}b$. Local and systemic effects secondary to intraocular treatment with pan-VEGF drugs may be also reinterpreted in light of these evidence, as reported below.

5. Developing the aptamer strategy to interfere with VEGF

The story of pegaptanib discovery and development started in the '90s, when precursors of pegaptanib were selected among huge libraries of oligonucleotides, about 10^{14} RNA molecules containing 30 randomized positions [74]. By using the SELEX (Systematic Evolution of Ligands by Exponential Enrichment) technology developed by Larry Gold's group at the Colorado University in the early 1990s [75], specific inhibitors of VEGF-A_{165} (from now referred as $VEGF_{165}$) were searched. The pathogenic role of $VEGF_{165}$ in tumor vascularization and growth was evidenced few years earlier [76-78]. The identification of $VEGF_{165}$ as the main actor of pathological angiogenesis on one side and, on the other, the concurrent research on aptamers as novel therapeutics, converged to the development of some promising anti-VEGF RNA ligands. For the initial selection of the anti-VEGF aptamers the investigators used as target the recombinant human $VEGF_{165}$ [74].

The first work on anti-VEGF aptamers, published in 1994, reported an initial set of high affinity RNA ligands selected for their ability to inhibit the binding of [^{125}I]VEGF to its receptors in a concentration-dependent manner in human umbilical vein endothelial cells (HUVECs) [74].

Competition experiments on candidate anti-VEGF aptamers revealed that they all bound to a similar site within the heparin-binding domain (HBD) of $VEGF_{165}$, and indeed they were displaced by heparin [74]. To address the question of specificity of these high-affinity ligands towards $VEGF_{165}$, the scientists performed binding experiments with various heparin-binding proteins, such as PDGF (Platelet-Derived Growth Factor) and FGF (Fibroblast Growth Factor). In those years, specific inhibitors of VEGF, basically pan-VEGFs, were limited to monoclonal antibodies [79] and soluble VEGF receptor [80], thus the isolation of RNA molecules with unexpected binding selectivity to $VEGF_{165}$ aroused a great interest. The lead compounds were further modified and optimized [81; 82], t44-OMe having high binding affinity for $VEGF_{165}$ and the best activity in the Miles assay, an *in vivo* test to evaluate the capacity of a given substance to inhibit vascular leakage following VEGF intradermal injection in guinea pigs [82]. Interestingly, the addition of a 40 kDa polyethylene glycol (PEG) to the 5'-end of the 27 nucleotides long t44-OMe slightly decreased (about 4 fold) the binding affinity to $VEGF_{165}$, but markedly improved the inhibitory activity in the Miles assay (83% with PEG-conjugated t44-OMe; 48% with t44-OMe) [82]. The more efficient inhibition of VEGF-induced permeability displayed by PEG-t44-OMe might be the consequence of a prolonged tissue permanence of the aptamer due to its conjugation with PEG [83]. Starting from PEG-t44-OMe, and few intermediates such as NX1838 and EYE001, pegaptanib was finally generated [84; 85]. Pegaptanib sodium is a covalent conjugate of 28 nucleotides in length that terminates in a pentylamino linker, to which two 20 kDa monomethoxy PEG units are attached via the two amino groups on a lysine residue. This drug represented the first pharmacologic approach joining the laser photocoagulation and verteporfin photodynamic therapy, considered at that time the sole therapeutic interventions for neovascular ocular diseases.

The literature on precursors of pegaptanib provides information on their binding to VEGF, and it is thus useful to better understand the molecular mechanism of inhibition of this aptamer and to clarify its biological activity. Since the beginning it was evident that the candidate aptamers under development were displaced from $VEGF_{165}$ by heparin, suggesting that the HBD of VEGF was

involved in the ligand-protein binding [74]. Accordingly, it was then shown that the amino acid sequence of VEGF that remained photo-cross-linked to the aptamer after digestion corresponded to a specific site within the HBD of $VEGF_{165}$; more precisely, an uridine residue within the minimal RNA sequence capable of high affinity binding to VEGF formed a cross-link with the residue cysteine-137 within the exon 7-encoded domain of $VEGF_{165}$, mediating much of the heparin binding activity of VEGF [82]. These findings *in vivo* were confirmed, demonstrating that the uridine-14 of the therapeutic aptamer forms a cross-link with cysteine-137 and that the HBD is the primary determinant for the affinity and specificity in the complex formed by the aptamer and $VEGF_{165}$ [86]. Notably, the HBD is completely lacking in the $VEGF_{121}$ isoform.

6. Sparing $VEGF_{165}$b while targeting VEGF

The previously described differences in the C-terminal structures of VEGF may also explain the fact that pegaptanib selectively binds the angiogenic $VEGF_{165}$a, likely sparing the anti-angiogenic counterpart, as better elucidated below. In other words, the C-terminus of $VEGF_{165}$a may be the key determinant for both the interaction between HBD and NRP1, and the binding between pegaptanib and HBD. In agreement, NMR data and three dimensional solution structures of aptamer-ligand complexes reveal that specificity and affinity for a given binding site are profoundly influenced by the near residues, which affect the adaptive recognition by the aptamer [87].

Interestingly, as later reported by the same group which first identified [27] and characterized $VEGF_{165}$b in terms of mechanism of action and expression [72], pegaptanib does not bind to $VEGF_{165}$b [69]. Specifically, to evaluate whether a direct interaction between pegaptanib and $VEGF_{165}$b exists, the authors incubated the RNA aptamer with $VEGF_{165}$a or $VEGF_{165}$b protein, separated the samples on acrylamide gel under non-denaturating conditions, and probed the membranes with either an anti-$VEGF_{165}$ antibody detecting both VEGF isoforms, or a specific anti-$VEGF_{165}$b antibody directed to the nine C-terminal amino acids of $VEGF_{165}$b [69], which is currently being used to detect this specific isoform in human tissues [88]. The blot showed a band

shift of $VEGF_{165}a$, but not $VEGF_{165}b$, toward higher molecular weight when the aptamer was added, suggesting that pegaptanib does not physically interact with $VEGF_{165}b$ [69]. The same authors reported that pegaptanib and $VEGF_{165}b$ given separately to human microvascular endothelial cells (HMVECs) inhibit the $VEGF_{165}$-induced cell migration; however, the concomitant treatment with the two inhibitors removes the benefit of each agent in the same assay, suggesting that, although there is not a direct interaction between pegaptanib and $VEGF_{165}b$, the combination of the two molecules does not add benefit [69]. This may be explained considering that VEGF functions as a dimer and, although till now it has been solely proven the existence of $VEGF_{165/110}$ heterodimers [48], an alternative is that $VEGF_{165}b$ may pair with $VEGF_{165}a$ reducing the angiogenic potential of the latter through a differential VEGFR signaling, and that pegaptanib may interfere with the formation of this heterodimer. However, further studies are needed to clarify this point.

In contrast to pegaptanib, it has been shown that bevacizumab binds to both $VEGF_{165}a$ and $VEGF_{165}b$ with equal affinity [89], and likely it is the same for ranibizumab, since it was designed on the parent molecule bevacizumab. *In vivo* studies in a cancer model report that $VEGF_{165}b$ strongly impairs the efficacy of bevacizumab and this fact implicates that patients expressing high levels of $VEGF_{165}b$ may be no-responders to bevacizumab and other pan-VEGF drugs [89]. These findings may also explain some of the undesired side effects of pan-VEGF agents.

7. Gaining insight into pegaptanib mechanism of action and its biological target

The differential biological effects, and related therapeutic profile, exerted by pegaptanib in comparison with pan-VEGF drugs mainly reside in the aptamer inhibitory activity selectively targeted toward $VEGF_{165}$ – or we may even say the pro-angiogenic $VEGF_{165}a$ isoform.

It has been suggested that pegaptanib, by binding to a site within HBD, contrasts HBD interaction with NRP1 co-receptor and thus only exerts an inhibitory effect on the NRP1-mediated amplification of VEGFR signaling, instead of an efficient inhibition of the VEGFR signaling itself [90]. In support of this hypothesis there is the observations that $VEGF_{121}$, which contains the

448 receptor-binding domain (RBD) but lacks the HBD and does not interact with NRP1, activates both
449 VEGFR-1 and VEGFR-2, although with much lower potency than $VEGF_{165}$ [48]. However, from
450 the beginning, experimental evidence showed that the pegaptanib precursor t44-OMe efficiently
451 blocked the binding of $VEGF_{165}$ to both Flt-1/VEGFR-1 and Kdr/VEGFR-2 [82]. Accordingly, it
452 was then clearly shown that also the pegaptanib precursor NX1838 bound Kdr/VEGFR-2; in
453 contrast, unambiguous data on VEGFR-1 were not produced because of the lack of specific anti-Flt-
454 1/VEGFR-1 antibodies at that time [84]. Moreover, internal data Bausch & Lomb [referred by S.
455 Giuffrida] support the assertion that pegaptanib can effectively inhibit $VEGF_{165}$ binding to its
456 receptors, VEGFR-1 (IC_{50}=0.47 nM), VEGFR-2 (IC_{50}=1.10 nM), and NRP1 (IC_{50}=0.23 nM). This
457 report is based on cell-free receptor plate binding studies and shows that the maximal inhibition
458 exerted by pegaptanib on $VEGF_{165}$ binding to the different VEGF receptors is 75-90% (the lowest
459 for VEGFR-1; the highest for VEGFR-2 and NRP1), suggesting subtle differences in the binding of
460 $VEGF_{165}$ to its receptors. To this regard, an hypothesis is that the aptamer inhibits VEGFR signaling
461 by providing a steric interference between RBD and the cell-surface receptors, as previously
462 evidenced for some anti-angiogenic HBD-binding proteins [86].
463 *In vitro* studies on HUVECs assessed that NX1838 inhibited $VEGF_{165}$ receptor binding and
464 downstream signaling events, including phosphorylation of VEGFR-2, phospholipase Cγ activation,
465 calcium mobilization, and cellular proliferation [84]. In this report, the inhibition of $VEGF_{165}$-
466 mediated cellular events exerted by NX1838 was comparable to that observed with an anti-VEGF
467 monoclonal antibody; in contrast, this aptamer was ineffective as an inhibitor of $VEGF_{121}$-induced
468 HUVECs proliferation [84]. NX1838 indeed did not bind $VEGF_{121}$ isoform lacking HBD, site for
469 pegaptanib binding. Internal data from Bausch & Lomb [referred by S. Giuffrida] report that
470 pegaptanib binds $VEGF_{189}$ with a lower but significant affinity than $VEGF_{165}$; we cannot exclude
471 that pegaptanib also binds exon 7-containing $VEGF_{183}$ and $VEGF_{206}$, which are expressed at very
472 low level and play a marginal role in angiogenesis.

Ranibizumab and bevacizumab bind to the RBD sequence [13; 91] which is common to all the VEGF isoforms, thus blocking the binding of all of them to VEGFRs and the related angiogenic signaling; this justifies the more potent effect of these drugs in inhibiting EC migration, proliferation and vascular permeability in comparison to pegaptanib. On the other side, this strength of pan-VEGF drugs may also represent their weakness, since such molecules counteract also the physiologic effects of $VEGF_{121}$ and $VEGF_{165}$b.

8. Pegaptanib from biological target definition to current therapeutic use

Preclinical *in vitro* and *in vivo* studies demonstrated that pegaptanib precursors inhibit two main $VEGF_{165}$-mediated functions, the enhancement of EC proliferation and vascular permeability [82; 84], providing the rationale for the therapeutic use of this aptamer for pathologies characterized by angiogenesis and vascular leakage. EYE001 (later on designated pegaptanib) was tested in human tumor xenograft mouse model and in various animal models of ocular neovascularization, such as the Miles assay, the rat corneal neoangiogenesis and the mouse retinopathy of prematurity models, showing significant attenuation of $VEGF_{165}$-mediated effects in eye diseases [85]. The open-label phase IA safety study on 15 patients with subfoveal choroidal neovascularization (CNV) secondary to AMD revealed no significant safety issues at 0.25-3 mg doses of EYE001/pegaptanib, and that 80% of subjects showed stable or improved vision 3 months after a single intravitreous injection [85], opening the way to larger clinical trials on patients with exudative AMD. Pegaptanib was then evaluated in two concurrent, multi-center, prospective, randomized, double-blinded, sham-controlled, dose-ranging trials on patients with all types of wet AMD: the VEGF Inhibition Study in Ocular Neovascularization (VISION) trials. These studies showed that pegaptanib treatment every 6 weeks reduced vision loss by about 50% in the first year and maintained this benefit stabilizing vision acuity at the second year [92; 93]. In particular, the pooled analysis of these phase III trials showed that 70% of patients treated with pegaptanib 0.3 mg versus 55% of patients receiving the sham injection lost fewer than 3 lines of visual acuity on an ETDRS vision chart. Within the context

of the 2-year trial, an exploratory analysis at week 54 of vision outcomes of a subgroup of naïve patients with early CNV secondary to AMD, suggested that pegaptanib treatment is associated with enhanced vision benefits, likely due to increased preservation of photoreceptors and/or RPE [94]. Analogously, the efficacy of pegaptanib as primary therapy for patients with early CNV-AMD was evaluated in a retrospective study with a mean follow-up of about 9 months (range 6-14 months), which showed a 90% rate of improvement or stabilization of vision outcomes for pegaptanib, benefits that exceeded those reported in the VISION trial [95]. A retrospective study of patients with exudative AMD with small lesion size and followed up over 1 year compared the effect of pegaptanib versus ranibizumab, concluding that the visual outcomes of the two drugs were equivalent [96]. However, after the approval of pan-VEGF inhibitors, these latter have been preferred, pegaptanib monotherapy was reconsidered, and this drug was evaluated as a maintenance therapy following non-selective anti-VEGF agents in wet AMD [97]. To this regard, in a small number of patients with all types of wet AMD, induction therapy with bevacizumab followed by pegaptanib maintenance produced visual acuity and anatomical improvements at 54-week [97]. The efficacy and safety of pegaptanib as a maintenance therapy was then assessed on a larger scale with a phase IV, open-label, uncontrolled exploratory study including patients with subfoveal wet AMD [LEVEL study; 98]. The results showed that pegaptanib was safe and well tolerated, and that the visual acuity and anatomical improvements gained during the induction phase were well preserved at 54-week, with only 50% of patients requiring a booster treatment given after a mean of 5 months post-baseline. Similar results were reported at 54-week for Japanese patients with neovascular AMD enrolled in the multi-center, prospective LEVEL-J study [99], and were further confirmed on a small subgroup of patients after a 3-year follow up [100]. According to these findings, pegaptanib is presently used mainly as a maintenance therapy following pan-VEGF agents in long term treatment of wet AMD. Besides this indication, pegaptanib is used off-label for proliferative DR, DME, BRVO, and myopic choroidal neovascularization [101-107].

9. Selective versus non-selective anti-VEGF drugs side effects: an ongoing debate from the bench to the eye of patients

Notably, non-selective anti-VEGF drugs, especially monoclonal antibodies, obtained very good results in numerous clinical trials (such as ANCHOR, CATT, IVAN, MARINA, HORIZON studies) to the extent that they are considered the most effective therapies for neovascular eye diseases, but there is some concern about their potential local and systemic side effects, especially in the long-term period [as reviewed in 34]. Ideally, an effective and safe anti-VEGF therapy should reduce neovascularization without damaging the normal vessels, and also preserving the physiologic functions of the retinal neurons and other cells. As mentioned, pan-VEGF agents exert a potent anti-angiogenic action that exceeds the blockade of neoangiogenesis; for example, *in vivo* studies showed that non-selective VEGF inhibition causes capillary regression, robust and early changes in ECs, pericytes, and basement membrane in the adult normal vasculature [108; 109], all effects likely due to the block of the physiological functions of VEGF isoforms, besides the inhibition of the pathological effects, as described in the previous paragraphs.

Local side effects are possible for theoretically every drug used in ophthalmology, and include toxicity related to the substance itself or due to the route of administration, such as in this case to single or repeated intraocular injections. Among others, the most common local adverse effects of anti-VEGF treatments comprise endophthalmitis, retinal detachment, intraocular pressure increase, eye inflammation, hyperaemia and hemorrhage, which can occur with major or minor incidence following treatment with any of the anti-VEGF agents [for comprehensive reviews see: 110-112]. Instead, the main occurrence of some adverse effects in non-selective anti-VEGF agents might be explained with their indiscriminate inhibition of all VEGF isoforms, including $VEGF_{121}$ and $VEGF_{165}b$, which indeed seem to play a role mainly in physiological processes at vascular and neuronal level.

9.1. Anti-VEGF drugs and atrophy of the retina

In both MARINA and ANCHOR trials on ranibizumab at 2 years, the increase in RPE abnormalities was one of the most significant characteristic lesions associated to visual acuity loss [as reviewed in 113]. Analogously, the number of ranibizumab injections was significantly associated with the progression of RPE atrophy in wet AMD patients followed for a median of 16 months (range 3–36 months) [114]. It has been proposed that pan-VEGF blockade is responsible of increasing geographic atrophy (GA) in AMD patients, a gradual complication characterized, among others, by choriocapillaries and RPE atrophy, photoreceptors death, and leading to a progressive visual loss [115; 116]. Monthly or PRN injection regimen with ranibizumab or bevacizumab led to development of GA by 2 years in 18.3% of wet AMD subjects included in the CATT trials [117]; an update of this study indicates that GA growth rate does not differ between eyes treated monthly or PRN, but it may be accelerated by ranibizumab [118]. Moreover, the multicenter cohort SEVEN UP study assessing long-term outcomes 7 to 8 years after initiation of intensive ranibizumab therapy in patients previously enrolled in the MARINA, ANCHOR and HORIZON trials, evidenced that, although ranibizumab is efficacious in wet AMD, one third of subjects demonstrated visual benefits and another third had poor outcomes; more alarming, macular atrophy was detected by fundus autofluorescence in 98% of all studied eyes, with the area of atrophy mainly localized in the fovea and significantly correlated with a poor visual outcome [119]. Interestingly, to our knowledge in literature there is only one report of rapid development of foveal GA possibly related to a single injection of pegaptanib in one patient presenting an already imbalanced foveal choroidal circulation due to AMD complicated by chronic serous drusenoid pigment epithelium detachment (PED) [120], suggesting that selectively inhibiting $VEGF_{165}$ and preserving other isoforms may avoid GA occurrence.

9.2. Anti-VEGF drugs, RPE tears and other lesions

Other complications possibly occurring in wet AMD patients are fibrosis and formation of scars; in addition, since the approval of intravitreal pharmacotherapy, there has been a huge number of reports of RPE tears developing after anti-VEGF injections, and thus a debate raised whether in these cases anti-VEGF therapy is beneficial, or not, on the anatomical and visual outcomes. To cite only a few of these evidence, RPE tears were diagnosed about 2 months after the first injection with an anti-VEGF agent, and observed in 12–15% of all eyes treated for PED in wet AMD [121]. The SEVEN UP study on ranibizumab reported the absence of fibrotic lesions in almost 40% of the examined eyes, although central fibrotic scars were demonstrated in approximately one third of the retina, with significant repercussions on visual acuity [119]. Approximately 45% of eyes treated with either ranibizumab or bevacizumab and enrolled in the CATT study developed scar by 2 years [122]. However, the majority of these RPE tear cases identified after ranibizumab, bevacizumab or pegaptanib therapy were associated with a pre-existing complication, a baseline vascularized PED, which in most cases evolves into RPE tears [123; 124]. A recent study with an average follow-up of 42 months in patients with a diagnosis of RPE tear developed spontaneously or after anti-VEGF therapy, stated that the formation of atrophic or fibrotic disciform scars occurred equally in both ranibizumab-treated and discontinued groups, and finally suggested that, in general, continuing anti-VEGF therapy is beneficial, reduces adverse outcomes and improves prognosis [125]. In support of this conclusion, *in vitro* and *in vivo* studies showed that fibroblast proliferation is stimulated by VEGF and inhibited by administration of bevacizumab, which contrasts collagen deposition and improves the outcomes after glaucoma surgery [126]. It has been suggested that VEGF isoforms play distinct roles in scar formation, with $VEGF_{189}$ being mainly involved in fibrosis; for this reason, pan-VEGF blockade may have a better anti-scarring potency than the selective $VEGF_{165}$ inhibitor pegaptanib, which *in vitro* inhibits fibroblast growth only at the highest doses tested, and whose benefits in post-operatory seem to be mediated mainly by inhibition of angiogenesis, but not reduction of inflammation or collagen deposition [127].

9.3. Anti-VEGF agents and the haemodynamics of eye vessels

Studies on the effects of anti-VEGF drugs on retrobulbar and retinal haemodynamics in wet AMD patients indicate that non-selective molecules may induce hypoperfusion. Specifically, 4 weeks after a single injection of bevacizumab, the flow of all retrobulbar arteries - in particular the blood flow velocities (BFVs) in the central retinal (CRA), temporal posterior ciliary (TPCA) and ophthalmic arteries (OA) - has been shown to be reduced [128]. A significant vasoconstriction of the retinal arterioles lasting thirty days after each injection of ranibizumab was observed in wet AMD patients [129]. A more recent study showed that ranibizumab leads to an early impairment of the native choroidal and retinal vascular networks, but most of these effects are reversible after its discontinuation; however, the study evidenced a significant correlation between the number of injections and percentage of changes in BFVs of CRA at month 6 [130]. The sole study on pegaptanib, specifically on DME and BRVO patients, only shows a significant decrease of blood flow velocity in the CRA after the third injection, possibly due to a cumulative effect for repeated treatments; no effects on retinal capillary blood flow, velocity or resistance index in the OA or TPCA were evidenced in the small number of subjects examined [131]. However, further and larger studies are needed to confirm the pegaptanib's better profile than other anti-VEGF agents on the haemodynamics of retrobulbar and retinal vessels.

10. Risk of inhibiting VEGF beyond the eye

Since their appearance in clinics, many observational studies, reviews and meta-analyses have been published on the systemic tolerability of ophthalmic anti-VEGF drugs and related adverse drug reactions (ADRs). Indeed, all the intraocular injected anti-VEGF agents can pass through the blood-retinal barrier and enter the systemic circulation, causing a decrease in VEGF plasma levels at various degrees, with several consequences. For instance, it was documented that intravitreally administered bevacizumab rapidly penetrates the rabbit retina before leaking into the blood circulation [132], and that in patients the intraocular injection of bevacizumab strongly decreases

629 the VEGF serum concentration, to the extent that after 1 month after the antibody treatment blood
630 VEGF is still 23% of baseline [133]. The circulating VEGF protects the vascular patency and
631 integrity, and up-regulates NOS, thus a prolonged anti-VEGF treatment potentially increases the
632 risk of hypertension and thromboembolic events [134]. Relevantly, in comparison with healthy age-
633 and sex-matched populations, neovascular AMD patients are elderly people presenting an increased
634 prevalence of hypertension, myocardial infarction, stroke, diabetes, and thus they may be more
635 susceptible to cardiovascular and cerebrovascular toxicities and prone to manifest ADRs [135-139].
636 In particular, the most frequently documented comorbidities with wet AMD are hypertension and
637 other cardiovascular diseases, accounting 57.5% of cases [140]. After a 10-year period, people with
638 early-stage AMD have almost a 2-fold cumulative incidence of stroke than controls (4.08% vs.
639 2.14%) [141]. As well, DR subjects are more likely to have an increased risk for vascular events
640 [139].
641 A recent review of some relevant clinical trials shows that the rates of serious thromboembolic
642 events, such as stroke, heart attack and death, are similar for AMD patients treated with different
643 anti-VEGF agents. In particular, the authors state that in these subjects the arterial thrombotic risk
644 appears sufficiently low, when compared with the natural incidence of thromboembolic events in
645 this category of elderly people, to be considered acceptable and counterbalanced by the advantage
646 of a visual improvement [142]; in few words, the risk of thrombotic events is seen as the worthy
647 price for ocular benefits. Similarly, a recent meta-analysis in DME patients evidences no significant
648 difference between anti-VEGF treated subjects and controls for arterial thromboembolic events;
649 however, the authors judge the quality of the evidence on these ADRs as moderate due to an
650 incomplete report of safety data, and the exclusion of high-risk participants (people with previous
651 cardiovascular events) in some studies [106]. Another systematic review of pre- and post-marketing
652 safety data on ranibizumab, pegaptanib and aflibercept, including 7,720 spontaneous reports from
653 the European database EudraVigilance, highlights an increased number of thromboembolic events
654 (0.8%–5%) and mortality (2.8%–4%) with anti-VEGF agents evidenced by post-marketing studies,

and suggests the need to properly evaluate the risk for such serious and long-term ADRs with further studies [112]. Again, data from real life evidence relevant safety issues for some non-selective anti-VEGF agents; a comparative analysis of ADRs in the WHO database shows an elevated disproportionality for cardiovascular events in patients treated with ranibizumab, in particular myocardial infarction, cardiac failure congestive, and cerebrovascular accidents [111]. This analysis was performed on 3,180 reports of worldwide pharmacovigilance from 2002 to 2012, corresponding to 7,753 drug-reaction pairs concerning ranibizumab (5,130, 66%), bevacizumab (2,069, 27%), and pegaptanib (554, 7%). Interestingly, although the number of reports on pegaptanib were more limited in comparison with other agents, no relevant safety issues were identified for this drug. In agreement, safety data from year 2 and 4 of the VISION trial previously suggested no evidence of an increased risk of systemic adverse events associated with long-term treatment with pegaptanib [143]. As AMD patients, DR population may require long-term anti-VEGF therapy, thus it is important to consider potential systemic effects in subgroups prone to vascular events when deciding between non-selective and selective agents [144]. In light of this consideration, and being pegaptanib potentially safer than non-selective anti-VEGF agents, some authors have suggested to use pegaptanib as an initial therapy for DME, substituted with a pan-VEGF blocker in case the desired result is not achieved [145].

VEGF regulates vascular permeability in various districts and it exerts neurotrophic and neuroprotective effects on blood-retinal and blood-brain barriers [146], thus it is conceivable that pan-VEGF suppression induced by intravitreal treatment may be deleterious also at cerebral level. According to the above mentioned spontaneous reports from the WHO database, a potential increased risk of cerebrovascular events associated with ranibizumab, especially with a more intensive treatment, was evidenced also by meta-analyses on five clinical trials with this drug (FOCUS, MARINA, ANCHOR, PIER, and SAILOR) [147; 148].

Clinical and experimental findings report that the use of anti-VEGF agents can result in neuronal damage, often leading to pain [149]. Recent evidence have shown that both $VEGF_{165}a$ and

681 $VEGF_{165}b$ are neuroprotective, but they have pro- and anti-nociceptive effects, respectively [150];
682 the authors thus suggest that pain associated with anti-VEGF agents, and especially with molecules
683 non-discriminating among the two 165 isoforms, is not fully attributable to a loss of a
684 neuroprotective effect, but possibly also involves the modulation of nociception by VEGF-A
685 isoforms.
686 Another organ dependent on VEGF and potentially exposed to injury from systemic absorption of
687 ophthalmic anti-VEGF drugs is the kidney. Hypertension and proteinuria were described soon as
688 adverse effects of systemic treatment with bevacizumab [151]. In general, anti-VEGF agents have
689 common adverse vascular effects attributable directly or indirectly to VEGF blockade, including
690 hypertension and renal vascular injury, usually manifested by proteinuria and thrombotic
691 microangiopathy [152]. The renal toxicity is likely due to the loss of VEGF functions in the
692 developed kidney, and to the close relationships existing among VEGF, NO, endothelin-1 and
693 angiotensin-II expression [153]. In particular, $VEGF_{121}$ is fundamental for renal function [154] and
694 it has been shown to protect rats from kidney infarction in thrombotic microangiopathy through
695 maintaining NO production and/or preventing EC death [155]. Down-regulation of NO by anti-
696 VEGF has been also implicated among the mechanisms underlying hypertension, besides
697 rarefaction of the microvasculature induced by anti-VEGF agents [156].
698 Further studies are needed to better identify the main thromboembolic events related to the use of
699 anti-VEGF agents, in particular non-selective inhibitors, and their occurrence. However, the risk for
700 some systemic ADRs may be increased in patients treated with anti-VEGF agents due to their
701 intrinsic characteristics; moreover, since most of the patients are aged 65 or older, age-related
702 physiologic changes, such as impairment of hepatic and renal function, may increase the odds for
703 ADRs. To this criticism we should add potential comorbidities and polypharmacy, which are
704 common in the elderly and contribute to increase risk factors for cardio- and cerebro-vascular
705 events. In light of these evidence, pegaptanib maintenance strategy after a loading phase with pan-
706 VEGF drugs may represent a safer therapeutic option in AMD patients with various comorbidities,

offering clinically meaningful benefits with a minimal systemic exposure to non-selective VEGF inhibition (and related potential side effects), reduced number of intravitreal injections required for treatment, and thus an improved cost/effectiveness profile [157].

11. Tachyphylaxis: a relevant issue in anti-VEGF therapy

Anti-VEGF therapy is efficacious in the majority of patients; however, in the long-term, repeated intravitreal injections of these molecules seem to be associated with a reduced bioefficacy. To this regard, still up to one-fourth of all treated patients, defined as no-responders, does not benefit from intravitreal injections and visual acuity deteriorates even under treatment [158]. In general, long-term efficacy of a drug can be affected by tachyphylaxis, a phenomenon which is often confused with tolerance, both determining a reduced drug efficacy. When drugs are administered repeatedly over a short period, tachyphylaxis can develop quite quickly and no response is observed following a dosage increase, although the efficacy can be restored if the compound is suspended for a short while [159].

Keane and collaborators [160] first speculated the possible implication of the tachyphylaxis phenomenon following administration of ranibizumab for the treatment of neovascular AMD, and a diminished therapeutic response due to tachyphylaxis was also indicated for intravitreal bevacizumab [161; 162].

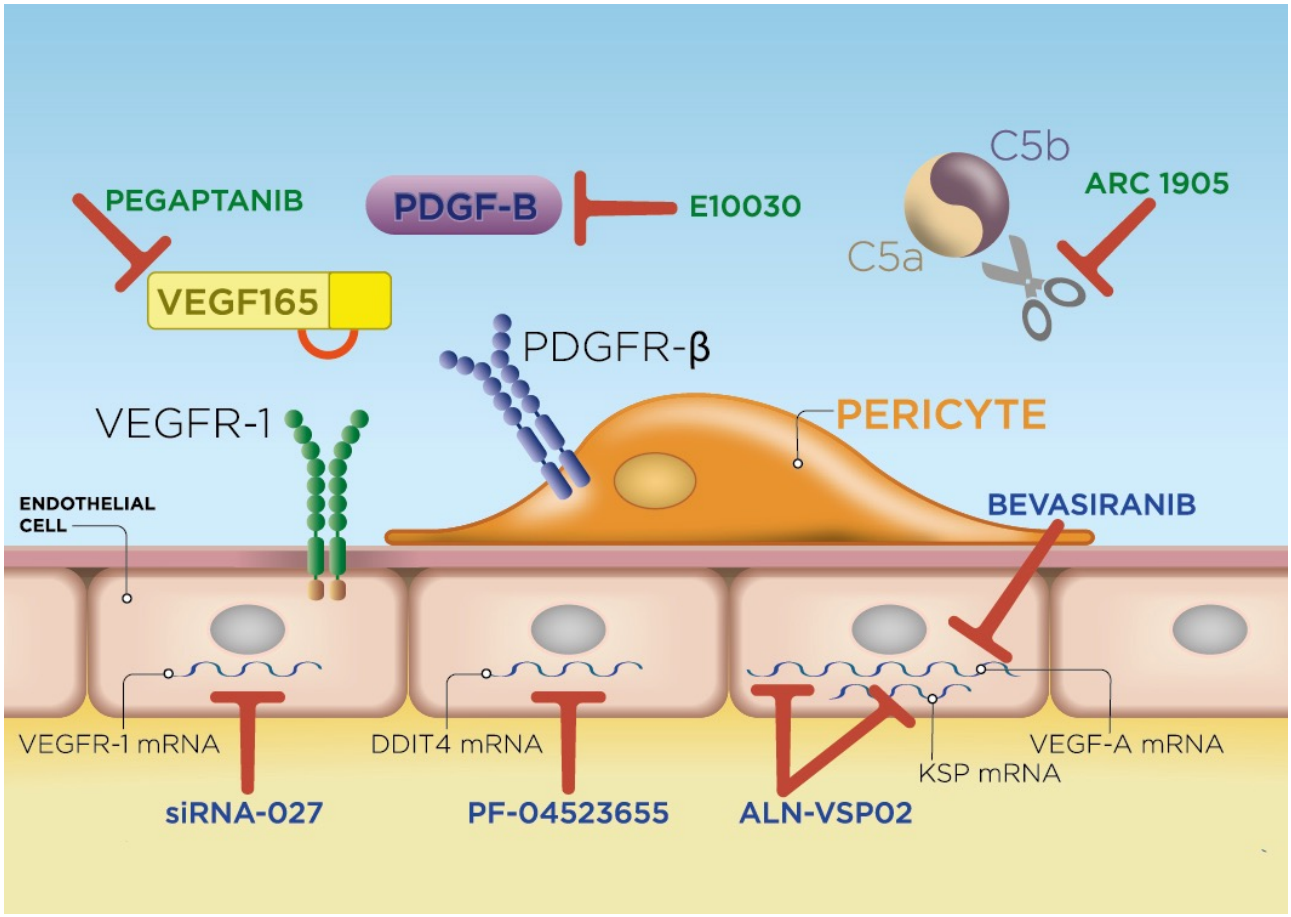
Although some cases of presumed tachyphylaxis may be ascribed to a poor or suboptimal response to treatment [11], several mechanisms have been proposed to contribute to the diminished drug response, such as the alteration of the neovascular membrane including increased fibrosis, chronic changes in the vessel wall and in relevant neighboring structures as photoreceptors or RPE [159]. The attenuated response after repeated administration may be also explained in terms of a raise in other angiogenic signaling pathways which are aimed to compensate the blocked activity of VEGF; for example, macrophages located within the choroidal neovascular tissue may respond to VEGF inhibition by upregulating the production of VEGF itself [164]. As already reported for other

733 therapeutic humanized monoclonal antibodies, the formation of circulating neutralizing antibodies
734 may also take part to tachyphylaxis; indeed, neutralizing antibodies have been already documented
735 against ranibizumab [163] and bevacizumab [164]. Clearly, this last aspect is in favor of pegaptanib
736 since aptamers are nonimmunogenic and are less likely to cause tolerability issues [86]. Genetic
737 variants of the *VEGF* gene seem also to alter the response to anti-VEGF treatment [165], therefore,
738 it has been suggested that even minor differences in the binding properties might explain a
739 differential response to the various anti-VEGF therapeutics, offering the possibility of a response
740 even in patients who developed a tolerance to one drug [158]. Within this general context, clinical
741 evidence demonstrated that no-responders to either bevacizumab or ranibizumab benefit from a
742 switch to the other drug [158; 166; 167]. Moreover, a significant improvement in visual and
743 anatomical outcomes was also described after switching therapy to aflibercept in 34 eyes with
744 persistent subfoveal fluid formerly treated with ranibizumab [168]. More recently, switching to
745 pegaptanib monotherapy has been also documented to be strongly effective in those AMD patients
746 who did not respond to ranibizumab or ranibizumab combined with photodynamic therapy [169].
747 The authors ascribe the efficacy of pegaptanib mainly to its selectivity towards the $VEGF_{165}$
748 isoform and to the fact that it is immunologically lenient. Moreover, since according to Pfizer's
749 internal data pegaptanib has a weak binding ability towards PDGF, they also assert that this feature
750 can additionally contribute to pegaptanib activity [169]. To this regard, several lines of evidence
751 suggest that the response of blood vessels to anti-VEGF therapy is influenced by vessel maturation
752 which involves pericytes [see 170]. The recruitment of pericytes on endothelial cells is mediated by
753 PDGF-B signaling via PDGF receptor-type β (PDGFR-β). Indeed, transgenic mice lacking PDGF-B
754 and PDGFR-β are characterized by abnormal vessel stabilization and maturation [171], and
755 inhibition of PDGF cascade increases EC sensitivity to anti-VEGF agents [172]. Therefore, a
756 combined therapy targeting PDGF-B and VEGF-A may represent a more effective pharmacological
757 approach to face neovascular AMD and possibly to avoid tachyphylaxis challenge. Within this
758 context, pegaptanib itself, although provided with a weak binding activity towards PDGF, may

759 further benefit of this combination therapy strategy not only in inhibiting new vessel growth but
760 also vessel regression, as documented by Jo and collaborators in mice [170].
761
762 **12. Future perspectives in eye neovascularization: oligonucleotide-based interventions to**
763 **modulate VEGF pathway**
764 Anti-VEGF therapy has certainly represented a breakthrough intervention to counteract pathological
765 angiogenesis, although it should be taken into account that these agents usually help to delay further
766 vision loss rather than improve it. This latter aspect underscores the need to identify novel
767 approaches, also considering that novel VEGF-dependent [173] as well VEGF-independent
768 pathways [174] may be involved, the latter may contribute to better explain the resistance, observed
769 in some patients, to the anti-VEGF treatment itself, as previously mentioned.
770 Within this general context, as recently reviewed [175], efforts have also been directed to develop
771 advanced drug-delivery devices to reduce treatment burden, such as using the encapsulated cell
772 technology (designed to deliver active compounds directly into the vitreous following trans-scleral
773 implantation) or utilizing colloidal drug carriers (consisting of suspensions of
774 microparticles/nanoparticles or liposomes), and also taking into account the need to develop eye
775 drop or oral formulations to improve patient compliance. Moreover, considering that, as recently
776 reviewed [175-177], single nucleotide polymorphisms can play a predictive role in AMD
777 progression or, in general, in the response to treatment, pharmacogenomics studies may help in the
778 choice of a more appropriate therapy.
779 The present paragraph specifically deals with novel oligonucleotide-based interventions in the eye
780 mainly targeting, directly or indirectly, VEGF mRNA/protein/receptors or to be used in
781 combination with the currently used anti-VEGF agents (Table 1; Figure 2), leaving the reader to
782 other recent publications for differential pharmacological approaches or biological targets [i.e. 22;
783 172; 178; 179].
784

NAME	TYPE OF MOLECULE	BIOLOGICAL TARGET	CLINICAL STAGE
RANIBIZUMAB	Recombinant humanized monoclonal antibody fragment	ALL VEGF-A ISOFORMS	**CURRENT DRUGS**
BEVACIZUMAB	Recombinant humanized monoclonal antibody		
AFLIBERCEPT	Fusion protein	ALL VEGF-A & VEGF-B ISOFORMS + PLGF	
PEGAPTANIB	RNA aptamer	VEGF-A_{165} ONLY	
BEVASIRANIB	siRNA	VEGF-A mRNA	**Phase III**
SIRNA-027	siRNA	VEGFR-1 mRNA	**Phase I**
PF-04523655	siRNA	DDIT4 mRNA	**Phase II**
ALN-VSP02	Dual -siRNA	VEGF-A and KSP mRNAs	**Phase I**
E10030	DNA aptamer	PDGF-BB	**Phase III**
ARC1905	RNA aptamer	C5 complement	**Phase I**

Table 1. Current drugs and novel oligonucleotide-based molecules to face ocular neovascularization. The drugs currently used (also off-label) in therapy or the oligonucleotide-based molecules under clinical trials and potentially useful to counteract ocular neovascularization in different eye diseases are reported. When not specifically indicated, the biological target is referred to the protein (for more details see text, paragraph 12). PLGF: Placental Growth Factor.



790

791 **Figure 2. Oligonucleotide-based interventions targeting VEGF and other molecules involved**
792 **in neovascularization.** Aptamers (in green color), siRNAs (in blue color) and their targets,
793 expressed in endothelial cells and pericytes, are depicted. These under-trials molecules may be used
794 in combination with the currently used anti-VEGF agents (for more details see text, paragraph 12).

795

796 **12.1. Small interfering RNAs (siRNAs)**

797 Sometimes also named short interfering RNAs or silencing RNAs, siRNAs are double-stranded
798 RNA molecules, 20-25 base pairs in length, capable of operating gene silencing at
799 posttranscriptional level. Indeed, their catalytic nature allows for one siRNA to guide the cleavage
800 of thousands of mRNAs, resulting in effective gene silencing with no translation of the related
801 proteins. These molecules hold a great potential since can be easily designed and are characterized
802 by high efficacy and specificity [180].

Bevasiranib is a double-stranded RNA of 21 nucleotides in length directed to VEGF-A mRNA. Preclinical studies documented bevasiranib efficacy in inhibiting neovascularization in both mice and nonhuman primate models [181; 182]. Moreover, in rabbits, an extensive uptake into the retina was observed following intravitreal injections of a single dose of either 0.5 mg/eye or 2.0 mg/eye of ^{3}H-bevasiranib [183]. Promising results for the treatment of AMD and DME originated from Phase I and II clinical trials, also showing that bevasiranib effects were not manifest until 6 weeks after treatment. However, in March 2009 OPKO Health Pharmaceuticals decided to terminate its Phase III clinical study for the treatment of wet AMD.

Sirna-027 (also named AGN211745) is a chemical-modified siRNA that targets a conserved region of VEGFR-1 mRNA. In mice, it was shown to reduce pathological angiogenesis in a laser-induced CNV model [184], and it was proven to be safe and effective in nonclinical safety studies [185]. Based on its preclinical activity and tolerability, a Phase I study was subsequently conducted concluding that a single intravitreal dose of Sirna-027, up to 1600 μg/eye, is well tolerated in patients with CNV resulting from neovascular AMD [185]. However, no additional trials are currently running on this molecule (search up to July 16th 2015 at: ClinicalTrials.gov and EU Clinical Trials Register).

PF-04523655 is a 19-nucleotide, O-methyl stabilized, siRNA that specifically targets the DNA-damage-inducible transcript 4 (DDIT4) genes, also known as REDD1 or RTP-801, indirectly leading to a decrease in VEGF-A production [22]. RTP801 is a hypoxia-inducible factor 1–responsive gene, which displays strong hypoxia-dependent up-regulation both *in vitro* and *in vivo*. Indeed, in diabetic rats its expression usually increases by up to 70% in RPE/choroid and it is reduced by the administration of PF-04523655 [186]. Furthermore, RTP801 knockout mice show a significant reduction in retinal neovascularization in a model of retinopathy of prematurity [187]. A Phase I multicentre study has been completed on AMD in 2010, showing that a single intravitreal injection of 50 to 3000 μg of PF-045237655 is generally safe and well tolerated over 24 months [188]. A Phase II interventional clinical trial (the DEGAS study) was subsequently conducted to

829 evaluate the safety and efficacy of three doses of PF-04523655 (0.4mg, 1mg and 3mg) for the
830 treatment of DME in comparison with focal/grid laser. In general, PF-04523655 was proven to be
831 safe and well-tolerated, with few adverse events judged treatment-dependent. All the three dose
832 levels of the siRNA continued to improve visual acuity from baseline through month 12 in patients
833 with DME. Moreover, at month 12, a trend for a greater improvement in visual acuity from baseline
834 was observed in the 3mg PF-04523655 group with respect to the laser photocoagulation one.
835 Unfortunately, the study was terminated early at month 12 based on the high patient discontinuation
836 rate, mainly due to lack of efficacy [189]. Two Phase II studies have been additionally run to
837 investigate the benefits of a combined therapy of this siRNA with ranibizumab in wet AMD and in
838 DME, respectively. The first clinical trial, the MONET study, which assessed the efficacy of
839 different dosing paradigms of PF-04523655 versus ranibizumab (0.5mg) showed that, in subjects
840 with neovascular AMD, the combined therapy leads to an average gain in visual acuity that is more
841 elevated than with ranibizumab monotherapy, with no safety concerns identified [190]. In relation
842 to the second clinical trial, the dose escalation study and evaluation of PF-04523655 with/without
843 ranibizumab (MATISSE study) carried in DME patients, although already completed, no results
844 have been posted thus far (search up to July 16th 2015 at: ClinicalTrials.gov and EU Clinical Trials
845 Register).
846 Although no specific studies have been yet performed on ocular diseases, it is worth to mention
847 ALN-VSP02 since it is a lipid nanoparticle-formulated dual-targets drug candidate. It contains 2
848 different siRNAs, chemically modified (with 2′-O-methyl groups to minimize immunostimulation)
849 in a 1:1 molar ratio, directed to two different pathways: VEGF-A and kinesin spindle protein (KSP).
850 KSP is a member of the kinesin superfamily of microtubule-based motor proteins whose inhibition
851 determines cycle arrest at mitosis, finally leading to cell death [191]. To assess the activity and
852 safety of intravenous ALN-VSP02 in humans, a Phase I trial was initiated in patients with advanced
853 solid tumors with liver involvement. On the whole, ALN-VSP02 was well tolerated, with an
854 adverse event profile favorable in comparison with chemotherapy and with other orally or

intravenously targeted therapies administered in oncology. At molecular level, ALN-VSP02 counteracts the translation of both VEGF-A and KSP proteins, which results in growth inhibition of tumor cells and complete regression of liver metastases in endometrial cancer [192].

No Phase II clinical trials are currently running (search up to July 16th 2015 at: ClinicalTrials.gov and EU Clinical Trials Register).

12.2. Aptamers

Aptamers represent a step forward with respect to siRNAs; indeed, although still oligonucleotide-based molecules, they do not necessarily require transfection and their stability/bioavailability can be largely improved since the *in vitro* production allows to manipulate their kinetic properties. In particular, 2'F or 2'OMe modifications as well as the presence of conformationally restricted nucleotides confer resistance to nucleases, while PEG-conjugation limits renal filtration [178].

As previously said, pegaptanib has been the first aptamer entered in therapy. Besides pegaptanib, later on other aptamers have been synthesized to target VEGF. For example Nonaka and collaborators identified a DNA aptamer (named Vap7) able to bind both $VEGF_{121}$ and $VEGF_{165}$ isoforms through the RBD region. However, they subsequently optimized this aptamer for a diagnostic potential application as biosensor for VEGF detection [193; 194].

As cited earlier, current theories suggest that blocking simultaneously VEGF-A and PDGF results in a more effective inhibition of neovascularization [170; 195]. PDGF is a family of proteins comprising four different polypeptides (PDGF A-D) which can combine either as homodimers or heterodimers. The homodimer PDGF-BB has been involved in pericyte recruitment, maturation and survival through the binding on its specific receptor, namely the PDGFR-β, on pericytes [196]. Indeed, PDGF inhibition causes a loss of pericytes, leaving ECs vulnerable to anti-VEGF therapy, an effect that can also help avoid tachyphylaxis.

On these premises it has been designed E10030, a DNA aptamer specifically targeting PDGF-BB. This drug candidate is a PEGylated, 2'F- and 2'OMe-modified aptamer of 29 nucleotides. E10030

881 has been successfully assessed in association with anti-VEGF molecules in inducing neovascular
882 regression [197]. A Phase I trial was performed to determine the combined effect of E10030 and
883 ranibizumab on subjects with subfoveal CNV, showing a significant vascular regression and a
884 superior efficacy in comparison to ranibizumab monotherapy after 12 weeks of treatment [198].
885 Similar results were obtained in a Phase II trial where a greater efficacy was observed, following 6
886 monthly injections, especially with the higher dose of E10030 in combination with ranimizumab
887 with respect to ranibizumab alone [179]. Phase III studies are currently running/recruiting to
888 evaluate the safety and efficacy of E10030 in combination with ranibizumab or bevacizumab or
889 aflibercept in comparison to the respective anti-VEGF alone (search up to July 16th 2015 at:
890 ClinicalTrials.gov and EU Clinical Trials Register).
891 Still remaining in the context of combined therapies, another promising aptamer to be associated to
892 anti-VEGF agents is ARC1905 which targets C5 complement. C5 is a serum glycoprotein that is
893 cleaved in two fragments, C5a (active) and C5b, during complement activation. C5a is chemotactic
894 and plays a key role in stimulating neutrophil-endothelial adhesion [199]. The aptamer antagonizes
895 C5 cleavage thus preventing complement activation. A Phase I trial has been completed on the
896 safety, tolerability, and pharmacokinetic profile of multiple doses of ARC1905 in combination with
897 ranibizumab in subjects with subfoveal CNV secondary to AMD, however up to now no results
898 have been posted (search up to July 12th 2015 at: ClinicalTrials.gov and EU Clinical Trials
899 Register).
900

901 **13. Conclusions**

902 The study of anti-VEGF strategies in the treatment of ocular diseases linked to abnormal
903 vascularization raises several questions relevant for both the understanding of the biology of the
904 VEGF system and the rational design of the interventions directed to counteract VEGF. The
905 observations on the role of VEGF in AMD and DR also underscore the need to increase the
906 knowledge of the molecular bases of ocular diseases due to an impaired angiogenesis control.

907 The discovery of several isoforms of VEGF having different biological activity has revealed a
908 previously unforeseen biological complexity which needs to be addressed when studying the
909 clinical activity of currently available anti-VEGF drugs and while exploring new molecules active
910 on this target. In particular, an answer has to be provided to the question whether for a full
911 antiangiogenic activity is better to act against all the existing VEGF isoforms or to selectively block
912 few or one of them. The fact that some of the VEGF isoforms have antiangiogenic and
913 neuroprotective action suggests that the VEGF system is physiologically balanced and that in
914 presence of an angiogenic process it would be preferable in the long run to hit the angiogenic
915 isoforms leaving unaffected those isoforms having a different biological activity, the neutralization
916 of which may be responsible for a derangement of vasculature control as well as of tissue reparative
917 processes. When matching the molecular profiles and comparing the clinical activities of the
918 available drugs, in particular of the pan-VEGF antibodies and of the aptamers, such as pegaptanib,
919 displaying a preferential affinity toward the $VEGF_{165}$a isoform, one is tempted to speculate that a
920 selective action is sufficient to sustain in the time the antiangiogenic effect, while to quickly
921 develop a full block of the angiogenic process an action directed toward all the isoforms is more
922 effective. The preclinical studies with some of the newer and more selective siRNAs will help to
923 clarify this point. In the meantime, the new molecular advances may be used to better tailor the
924 existing therapies and to explain some of the cases of therapy resistant patients and to understand
925 the possible mechanisms underlying their side effects.
926 The discovery that, in addition to VEGF, other factors, such as PDGF, may participate to the control
927 of the angiogenic process, also raises the question whether it is necessary to simultaneously act also
928 on these other molecules, or VEGF inhibition is sufficient for an effective therapy. On the other
929 hand it is possible that different patients, so far included in the same diagnostic category, indeed
930 have different molecular dysfunctions underlying their disease requiring a more refined profiling of
931 the VEGF isoforms and of the other factors regulating angiogenesis in that particular
932 patient/disease.

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