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REVIEW ARTICLE

Anti-VEGF Treatment in Corneal Diseases

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Abstract: ***Background*:** Corneal neovascularization (CN) is a clue feature of different ocular pathological conditions and can lead to corneal edema and opacification with subsequent vision loss. Vascular endothelial growth factor (VEGF), which plays a key role in new vessels formation, proliferation and migration, was found to be up-regulated in these conditions. Nowadays, it is possible to down-regulate the angiogenic process by using anti-VEGF agents administered by different routes.

***Objective*:** To evaluate the efficacy, safety and possible future directions of anti-VEGF agents used for the treatment of CNV owing to different aetiologies.

***Methods*:** A computerized search of articles dealing with the topic of anti-VEGF therapy in CN was conducted in PubMed, Scopus and Medline electronic databases. The following key phrases were used: anti-VEGF agents, corneal neovascularization, bevacizumab, ranibizumab, vascular endothelial growth factor, angiogenesis.

***Results*:** The use of anti-VEGF therapy in the treatment of CN reduced pathological vessel density without causing significant side effects. Various administration routes such as topical, subconjunctival and intrastromal ones are available, and the choice depends on patient and disease characteristics. Much more effectiveness is achieved in case of early administration before mature and well-established vessels take place. A combined approach between various drugs including anti-VEGF agents should be adopted in those cases at higher risk of neovascularization recurrence such as chronic long-standing diseases where ischemic and inflammatory stimuli are not definitively reversed.

***Conclusion*:** The efficacy and safety of anti-VEGF agents support their adoption into the daily clinical practice for the management of CN.

Keywords: Anti-VEGF, neovessels, cornea, bevacizumab, avastin, ranibizumab, corneal neovascularization, vascular endothelial growth factor.

1. INTRODUCTION

The human cornea is a transparent and avascular tissue located at the outermost part of the eye, serving as the main refractive diopter of the whole system. Avascularity is required to maintain its optical clarity that is essential in order to provide an optimal vision. Under normal conditions, ocular structures maintain a balance between the levels of angiogenic and anti-angiogenic factors to prevent the occurrence of pathological neovascularization (NV), which refers to a condition in which new blood vessels arise from pre-existing ones [1, 2]. Several paraphysiological and pathological conditions affecting the ocular surface such as contact lens wearing, infection, inflammation, dry eye and injury, including limbal stem cell deficiency (LSCD), can result in corneal neovascularization (CN) [3, 4]. This phenomenon may impair visual acuity due to the increased vascular permeability, with lipid exudation and corneal edema that can determine the opacification of the stroma and irregularity of the corneal surface [5-8]. Apart from cases secondary to LSCD in which only limbal stem cell transplantation can restore corneal transparency with neovessels regression, treatment strategies for CN include mainly topical corticosteroids, fine needle diathermy, photodynamic therapy, amniotic membrane transplantation and laser coagulation [9-12].

The key role of vascular endothelial growth factor (VEGF) in the pathogenesis of CN was demonstrated for the first time in a rat model through the induction of corneal injury and subsequent NV [13]. As a result, the use of anti-VEGF agents in the management CN has aroused increasing interest and several further studies that investigated their efficacy have provided promising results [14-17].

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In the present review, we will discuss the clinical efficacy of anti-VEGF agents in the treatment of CN owing to different aetiologies. Furthermore, we will evaluate also the safety profile, limitations of current data and possible future directions of anti-VEGF research lines.

1.1. Method of Literature Search

Relevant articles published till 1st October 2019 were searched using PubMed, Scopus and Medline databases, as well as through the reference lists of identified publications. Search terms included the following key phrases: anti-VEGF agents, corneal neovascularization, bevacizumab, ranibizumab, vascular endothelial growth factor, angiogenesis.

2. MECHANISMS OF CN

Angiogenesis is a complex multistep process regulated by the action of both stimulatory (angiogenic factors) and inhibitory (angiogenic inhibitors) molecules. Although a certain amount of CN can be beneficial in some cases to support infection resolution and prevent stromal melting, it is generally considered to be a detrimental process, as it is associated with impaired vision and increased risk of complications [18].

The main causing factors of CN are represented by inflammation and hypoxia. In cases mediated by inflammation (*e.g.* trauma, infections, chemical burns, degenerative and immune-mediated conditions, LSCD), corneal epithelial and endothelial cells, macrophages, and other inflammatory mediators produce angiogenic factors such as VEGF and fibroblast growth factors. VEGF regulates the production of proteolytic enzymes such as matrix metalloproteinases (MMPs), which promote limbal vascular endothelial cell proliferation and migration [13, 19-21]. Inflammation can also induce Langerhans cell migration into the cornea, leading to additional production of angiogenic cytokines such as interleukin (IL)-1, IL-6 and IL-8 and further recruitment of immune cells [22-25]. The angiogenic cascade, amplified by inflammation, tips the balance between pro- and anti-angiogenic factors in favour of angiogenesis. In particular, IL-1 acts as a key cytokine in inflammatory neovascularization. It is mainly secreted from injured corneal epithelial cells and directly stimulates proliferation as well as migration of endothelial cells while enhancing the production of strong pro-angiogenic agents such as VEGF and basic fibroblast growth factor (bFGF). IL-6 and IL-17A also promote CN by increasing VEGF production in corneal cells in the setting of herpes simplex virus (HSV) keratitis. As Biswas and co-authors showed, the secretion of IL-6 from virus-infected cells may stimulate noninfected resident cells and other inflammatory mediators to secrete VEGF in a paracrine manner, thus the down-regulation of the neovascularization cascade may result from IL-6 neutralization [23].

As it is well known, LSCD goes together with CN: besides the loss of the physical barrier effect, inflammation resulting from damaged limbal stem cells plays a crucial role in the pathogenesis of CN as it promotes the up-regulation of angiogenic factors and causes the destruction of the remaining limbal stem cells (LSCs), thus leading to the amplification of such pathological process [6].

On the other hand, the majority of cases of CN mediated by hypoxia result from contact lens wearing. Under hypoxic conditions, VEGF is up-regulated by corneal epithelial and endothelial cells in the attempt at enhancing the supply of oxygen to the cornea [26]. However, contact lens wearing was found to be associated with subclinical inflammation and focal LSCD which occur as a consequence of hypoxic, mechanical and toxic insults and then result in corneal edema, neutrophil chemotaxis and NV [27, 28]. Not least, impaired corneal innervation is strongly associated with CN. Indeed, the role of corneal nerves in maintaining a trophic cornea is confirmed by the evidence of CN in the setting of ocular diseases characterized by injured innervation, such as neurotrophic keratitis and aniridia. Ferrari and co-authors evaluated the regulatory cross-talk of corneal vascular and neural networks and interestingly showed that both superficial and deep stromal corneal nerves completely disappeared in the corneal sectors involved in neovessels ingrowth, while persisted in non-vascularized areas [29]. They hypothesized that this phenomenon is mediated by the reduction of angiostatic molecules such as epithelial VEGFR3 and platelet-derived growth factors (PDGF) following corneal denervation. In particular, the former acts as a sink for VEGF ligands, hence contributing to preserving corneal avascularity whereas the latter presents both neurotrophic and angiostatic properties and it is constitutively expressed by the corneal epithelium [29].

3. MOLECULAR PATHWAYS OF CN

3.1. VEGF

Numerous factors play a role in the regulation of angiogenesis. Examples include VEGF, MMPs, bFGF, PDGFs, IL-1, IL-8, angiogenin, and transforming growth factor alpha (TGF-α) and beta (TGF-β). VEGF is among the main angiogenic chemical mediators and consists of a 48 kilodalton (kD) secreted grown-factor peptide that promotes several steps of angiogenesis, including proteolytic activities (dissolution of the membrane of the original vessel), endothelial cell proliferation, migration, and capillary tube formation [18, 29].

The so-called VEGF family is composed of 5 members in mammals: VEGF-A, VEGF-B, VEGF-C, VEGF-D and PGF, the former being the most significant stimulating factor secreted by several heterogeneous cells such as macrophages, T-cells, fibroblasts, pericytes, astrocytes, retinal pigment epithelial cells, and corneal cells [30-32]. The interaction between VEGF-A and its tyrosine-kinase receptors, principally expressed on endothelial cells (VEGFR-1, VEGFR-2), results in the dimerization and the subsequent activation of the kinase domains located intracellularly followed by autophosphorylation of receptors, which finally initiates the angiogenesis process, increases vascular permeability and leads to vasodilatation [33-36].

3.2. PDGF

The PDGF family includes 5 dimeric ligands (PDGF-AA, -AB, -BB, -CC and –DD) secreted by platelet granules and other cells such as monocytes, vascular endothelial cells, smooth muscle cells, corneal fibroblasts, epithelial and endothelial cells. The interaction with PDGF receptor leads to the

recruitment, proliferation and maturation of vessels by attracting pericytes that also promote vascular stability, hence increasing VEGF efficacy. As a matter of fact, the inhibition of PDGF signaling pathway not only reduces the number of corneal vessels, but also increases neovessels susceptibility to anti-VEGF therapy by means of pericytes detachment and decreased endothelial pericyte coverage with subsequent loss of vascular stability [37, 38].

3.3. bFGF

bFGF is a peptide involved in wound healing and angiogenesis and encompasses 23 structurally related heparin-binding angiogenic molecules. While FGF-2 is expressed in corneal epithelium and endothelium under normal conditions, FGF-1 has been shown to be upregulated in injured corneas [39]. The interaction between FGFs and their receptors leads to endothelial cell migration, proliferation and tubule formation of the endothelial cells which then supports vascular maturation. Furthermore, it promotes the degradation of extracellular matrix (ECM) by up-regulating urokinase-type plasminogen activator and MMP production in endothelial cells, thus allowing localized proteolytic digestion at the vascular migration front [40].

3.4. Nitric Oxide (NO)

Nitric oxide functions include angiogenesis regulation, pro- and anti-inflammatory activities, as well as modulation of mitochondrial oxygen consumption. In particular, NO plays both a pro- and anti-angiogenetic role, depending on its concentration and on the different available isoforms. Main corneal sources of NO are represented by fibroblasts, endothelium and inflammatory cells whereas major conditions associated with NO production are hypoxia and inflammation. NO acts as pro-angiogenic factor by mediating VEGF-induced proliferation of endothelial cells. Moreover, both short- and long- term exposures of human endothelial cells to VEGF were shown to stimulate the release of biologically active NO, thus supporting the evidence of an ambivalent interplay between these two agents [41].

3.5. RHO/RHO-Associated Kinase (ROCK) Pathway

Several studies have suggested an association between CN and cytoskeletal regulators including ROCK. The RHO/ROCK pathway features pleiotropic functions including stress fiber assembly and focal adhesion formation with subsequent regulation of endothelial cell polarity, cell adhesion, motility and apoptosis, all key processes of CN pathogenesis. As a consequence, the use of ROCK inhibitor, which goes under the name of Y27632, may lead to a substantial reduction of VEGF-mediated migration and tube formation [42].

3.6. WNT Pathway

The WNT signaling pathway also plays an established role in the angiogenic process by regulating various physiological activities including proliferation, migration, differentiation and apoptosis. The binding of WNT ligand to its receptor results in cytoplasmic β-catenin stabilization, which in turn translocates into the nucleus and leads to the transcription of WNT- target genes, including VEGF among the others [43].

3.7. Nucleotide-binding Oligomerization Domain 1 (NOD1) Pathway

Nod1 receptor is a member of Nodlike receptors (NLRs) that can be expressed by human corneal epithelial cells under inflammatory conditions. Indeed, its activation stimulates nuclear factor-kappa B (NF-κB) *via* a kinase called RICK and subsequently promotes the secretion of inflammatory cytokines with an eventual resultant NV. Not by chance, stimulation of Nod1 signaling in alkali-induced CN models resulted in increased vascularization, while Nod1 blocking reversed the condition [44].

4. TREATMENT STRATEGIES FOR CN

Several treatments, either medical and surgical, have been proposed in order to reduce neovessels density and to restore the angiogenic privilege of the cornea. Since CN is often associated and triggered by inflammation, the use of immunosuppressant agents such as topical corticosteroids reduces inflammatory stimuli and concomitantly decreases pathological vessels ingrowth. Unfortunately, the efficacy goes together with unpleasant side effects such as superinfection, glaucoma and cataract formation [45]. Other therapeutic agents exerting an immunomodulation activity include cyclosporine A, tacrolimus, sirolimus and nonsteroidal anti-inflammatory drugs. Interestingly, IL-1 receptor antagonist has a potential role in CN treatment since the neutralization of the related signaling pathway results in a double inhibition: the down-regulation of both VEGF and inducible NO synthase [46, 47].

Laser has been successfully applied to treat CN in the setting of graft rejection and lipid keratopathy. The maximum effect of the procedure is exerted on corneal efferent vessels that are characterized by a relatively slower blood flow. On the other hand, limited efficacy and frequent reopening are documented on thinner and deeper afferent vessels characterized by a faster blood flow. Complications of laser therapy include inadvertent damage to the corneal endothelium, hemorrhage, thinning, crystalline deposits on iris and pupil peaking [48]. A good alternative to laser treatment is represented by fine needle diathermy that obliterates both afferent and efferent vessels at different corneal depths. However, repeated treatment sessions may be needed, and attention should be paid to prevent serious adverse events such as corneal microperforation, intracorneal hemorrhages and transient opacification [49].

Currently, the most promising pharmacologic treatment is based on anti-VEGF drugs, which have the advantage to selectively act on the principle promotor of the angiogenic cascade, thus down-regulating all pathogenetic processes underlying CN, such as endothelial cell proliferation, inflammatory response, proteolytic activities and increased vascular permeability. Furthermore, both animal and human models showed that anti-VEGF therapies are safe and well-tolerated for corneal use [50, 51].

4.1. Types of Anti-VEGF Agents

The interaction between VEGF and its receptor can be modulated by the so-called anti-VEGF agents which represent the mainstay of treatment for rheumatological disorders,

tumors and metastasis [52]. Among the different agents, anti-VEGF antibodies have been proven to be very effective in the treatment of ocular diseases, such as age-related macular degeneration (AMD), macular edema, proliferative retinopathies and iris neovascularization [38]. They are mainly represented by bevacizumab (Avastin, Genentech, San Francisco, CA, USA), ranibizumab (Lucentis, Genentech, San Francisco, CA, USA/Novartis Ophthalmics, Basel, Switzerland), pegaptanib (Macugen, Phizer, New York) and aflibercept (Eylea, Regeneron, Tarrytown, NY, USA).

Pegatinib was the first agent to receive Food and Drug Administration (FDA) approval for the Ophthalmic use. It is a pegylated 28-base ribonucleic acid (RNA) oligonucleotide ligand which specifically binds and blocks the activity of extracellular VEGF-A165 isoform [53, 54]. This limited targeting explains its lower efficacy and long history of reduced side effects compared to other anti-VEGF antibodies [55].

Bevacizumab is a full-length recombinant humanized murine monoclonal antibody acting against the VEGF molecule that binds to and inhibits the activity of all human VEGF-A isoforms [54-56]. It has been approved by the FDA for the treatment of various solid tumors, whereas it is still an off-label drug in the Ophthalmic field [57-59]. It has been shown that Bevacizumab not only inhibits vessel growth and neovascularization, but also induces regression of pathological microvessels, stabilizes normal vessels, and prevents leakage and concomitant inflammatory response [60]. Ranibizumab acts against all VEGF-A isoforms but, rather than being a full antibody, it is a fragment of a humanized monoclonal anti-VEGF-A antibody. It was specifically designed for the intraocular use and was FDA-approved in 2006 for the treatment of neovascular AMD, macular edema (ME) after retinal vein occlusions (RVO), diabetic macular edema (DME) and diabetic retinopathy (DR) with DME [53, 54, 61]. A previous paper in the setting of HSV keratitis showed the positive outcomes of CN treated with Ranibizumab after a failed therapy with Bevacizumab [62]. However, although the average decrease of either area of NV and vessel caliber was greater in the Ranibizumab group, the difference was not significant. The lower molecular weight of Ranibizumab is potentially associated with greater corneal penetration and faster achievement of therapeutic concentrations compared to Bevacizumab, thus explaining the superiority of Ranibizumab in terms of onset of action and degree of efficacy [63]. Moreover, the binding affinity of ranibizumab is higher compared to bevacizumab, though the latter has a longer half-life.

The selective action of Pegaptanib on VEGF-A165 isoform may represent an advantage in terms of adverse events when compared to Bevacizumab and Ranibizumab since VEGF-A has been found to promote corneal nerve regeneration and epithelium wound healing, and its blockade may determine a major risk for superficial epitheliopathy and corneal thinning [64].

Aflibercept, also known as VEGF trap, is a decoy receptor fusion protein that contains a portion of the binding domain of VEGFR-1 and VEGFR-2 and binds all of the VEGF-A isoforms as well as placental growth factor with high affinity. It was approved by the FDA as a therapy for neovascular AMD, post-RVO macular edema, and diabetic macular edema [53, 54]. Aflibercept was found to have a greater binding affinity for VEGF compared to either Bevacizumab and Ranibizumab, thus suggesting its potential application as a second line therapy in those eyes not responding to the other anti-VEGF agents [65].

FD006 is a novel anti-VEGF-A monoclonal antibody, structurally similar to Bevacizumab, obtained by using antibody phage display technology. The administration of this agent in an experimental model of alkali burn cornea induced the regression of corneal neovessels [66]. In particular, FD006 seemed to have a major efficacy during the early stage after chemical injury if compared to Bevacizumab. The reason for this finding could be related to the higher binding affinity and the slower dissociation rate of FD006. However, in the late stages of the disease, the two agents had an equal therapeutic effect on inhibiting CN [66]. Besides antibodies against VEGF for therapeutic use, soluble VEGF receptors can block the effect of VEGF ligand by VEGF trapping, thus preventing its interaction with the related receptor, in a similar way as Aflibercept. Several soluble receptors including VEGFR-1, -2, -3, which serve as decoy receptors for pro-angiogenic VEGF molecules, are expressed in the cornea to maintain its avascularity under normal homeostatic conditions. Therefore, the direct use of VEGF soluble receptors or recombinant fusion proteins of the soluble receptors could be used to inhibit CN. Singh and co-authors demonstrated that over-expression of sVEGFR-3 determined a 58% decrease in the lymphatic area and a 31% decrease in blood vessel area in a model of injured cornea [67]. Iriyama and co-authors used nanotechnology to create polyplex micelles containing sVEGFR-1 (sFlt-1) plasmid whereas Cho and co-authors tested the administration of VEGFR-1 morpholino. Both study groups successfully achieved a significant reduction of CN [68, 69]. Flt23k (coupling domains 2e3 of Flt with KDEL) and Flt24k (coupling domains 2e4 of Flt with KDEL) are two available types of VEGFR intraceptor, which represent an alternative way for blocking VEGF by means of a quadripeptide retention signal (KDEL) that binds to endoplasmic reticulum receptors and acts by preventing the secretion of proteins coupled to it. Thus, the recombinant construct intracellularly binds VEGF and subsequently blocks its functions [70].

Not least, silencing RNA sequences (siRNA), such as bevasirinib and SIRNA-027, are able to induce inhibition of post-transcriptional RNA processing, thus blocking VEGF synthesis and expression, whereas multi-kinase inhibitors such as sorafenib, sunitinib and pazopanib are able to reduce VEGF effects by blocking the kinase-mediated signaling pathway [55, 71]. A comparison of the main types of anti-VEGF agents is summarized in Table **1**.

5. ANTI VEGF AGENTS IN CORNEAL DISEASES: CLINICAL RESULTS

CN occurs as a nonspecific response to several corneal and ocular surface diseases such as infection, inflammation, degeneration, damage to the limbal stem cell barrier and trauma. Representative images of 4 eyes affected by CN owing to different underlying diseases are provided in Fig. (**1**).

Table 1. Comparison of the main anti-vascular endothelial growth factor agents.

Anti-VEGF Agents	Structure	Target	Efficacy	FDA Approval
Pegaptanib	Pegylated RNA oligonucleotide	Extracellular VEGF-A165 isoform	Lower efficacy	FDA approval for ophthalmic use
Bevacizumab	Full-length recombinant humanized monoclonal antibody	All VEGF-A isoforms	High efficacy; longer plasma half-life	FDA approval for solid tumors; off-label administration for ophthalmic use
Ranibizumab	Fragment of humanized monoclonal antibody	All VEGF-A isoforms	High efficacy; increased binding affinity; greater corneal penetration and faster achievement of therapeutic concentrations due to lower molecular weight	FDA approval for ophthalmic use
Aflibercept	Decoy receptor fusion protein	All VEGF-A isoforms	Greater long-term binding affinity; further inhibition of PDGF	FDA approval for ophthalmic use

VEGF, vascular endothelial growth factor; PDGF, platelet derived growth factor; FDA, food and drug administration.

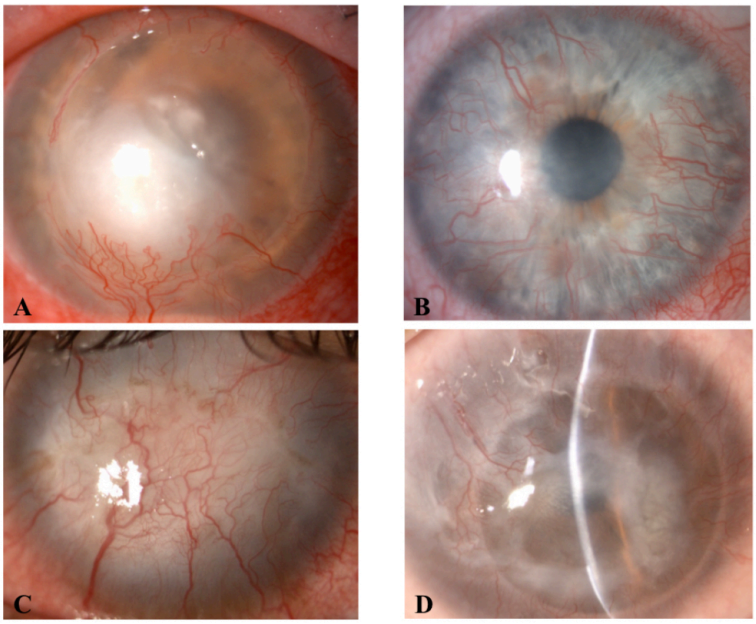

Fig. (1). Different clinical pictures characterised by corneal neovascularization. Part **A**: Herpes Simplex Virus Keratitis. Part **B**: Severe dry eye disease owing to graft *versus*-host disease. Part **C**: Total limbal stem cell deficiency; Part **D**: Immunological graft rejection after penetrating keratoplasty. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

The main conditions associated with pathological vessel growth are discussed below along with the clinical results of the related works.

5.1. LSCD

LSCD is characterized by a loss or deficiency of stem cells which are essential for the re-population of the corneal epithelium. Indeed, the stratified corneal epithelium constantly renews in order to ensure the transparency of the optical system, and any trauma or loss of epithelial cells must be repaired quickly [72]. Moreover, the limbus acts as a potent inhibitor of corneal vascularization owing to the conjunctivalization process [73]. Among the underlying diseases, there are a variety of ocular pathologies either congenital, such as aniridia, and acquired. The latter conditions include inflammatory and infective diseases, tumor, chemical burn and inappropriate use of contact lens [74]. Chemical injury of the cornea represents an ocular emergency requiring immediate treatment [75]. Chemical agents such as alkali and acids can penetrate and damage either the cornea and the anterior chamber, leading to a partial or total loss of limbal stem cells. Subsequent conjunctivalization of the cornea with massive neovascularization may develop, causing reductions in corneal clarity and visual acuity. Current standard treatment is based on topical steroids that can be associated with

significant short- and long-term complications such as glaucoma, cataract, increased risk of infections and impaired corneal epithelial healing. Several authors have investigated the efficacy of anti-VEGF agents in experimental models of alkali burn [30, 76-79]. The first studies date back to 2007 and showed the efficacy of topical and subconjunctival application of bevacizumab. Manzano and co-authors evaluated the effect of 4 mg/mL of bevacizumab drops administered twice a day in a rat model of chemical injury, reporting a 40% decrease in CN [76]. Barros and co-workers studied the effects of subconjunctival bevacizumab injections on angiogenesis in cauterized rat corneas. Animals were divided into 4 groups that received respectively saline solution on the day of the injury, bevacizumab just after the injury, bevacizumab on day 3 and bevacizumab on day 5. The data showed inhibition of angiogenesis in all treated groups compared to controls [77]. These results are in agreement with the ones from Hosseini and co-authors who evaluated the effects of subconjunctival bevacizumab on a chemical-induced CN in rabbits, showing a significant decrease in the total vascularization area and in the longest neovascular pedicle length [30].

On the other hand, Oh and co-workers aimed at evaluating the role of subconjunctival bevacizumab in ocular inflammation by using a model of chemical burn characterized by LSCD with the recruitment of various inflammatory mediators [78]. Bevacizumab was shown to significantly reduce the infiltration of inflammatory cells and the expression of inflammatory cytokines such as IL-2, IL-6 and Interferon-gamma (IFN-gamma), as well as CN and damage, thus confirming both the anti-inflammatory and anti-angiogenetic effect of anti-VEGF agents. Similar anti-angiogenetic and anti-inflammatory effects of anti-VEGF antibodies were demonstrated by Dogonay and co-authors who performed bevacizumab injections in experimental models of alkali-injured corneas. Neovascularization areas and inflammatory index scores including ciliary hyperemia, central corneal edema and peripheral corneal edema appeared to be significantly lower when compared to the untreated group [79].

Although there is still poor literature on anti-VEGF therapy in chemically-injured human corneas, the reported results are encouraging. DeStafeno and Kim tested with success for the first time in human patients the application of topical bevacizumab in the reduction of CN owing to a chemical burn. The use of 1% (10 mg/mL) bevacizumab eye drops at the posology of 4 times a day was able to significantly reduce either the superficial and deep stromal CN [80].

Bahar and co-authors evaluated the effect of perilimbal subconjunctival injection of 2.5 mg/0,1 mL bevacizumab in chemically injured human cornea. The eye received at least 2 bevacizumab injections at monthly intervals unless the stabilization was achieved. No intra-operative or post-operative side effects were noted. Short-term results showed partial regression of CN, likely explained by the use of low dose and frequency of drug and the presence of cytokines other than VEGF causing CN. Moreover, bevacizumab was shown to have a cumulative effect as described in the setting of retinal disorders treated with the intravitreal anti-VEGF application [80-84]. Bock and co-authors obtained the same beneficial effects with the topical application of 5 mg/mL bevacizumab eye drop instilled 5 times a day up to 6 months [85]. As it is well known, bevacizumab better inhibits new, fresh blood vessels rather than old, well-established ones [64]. Surprisingly, Avastin eye drops were found to reduce the perfusion of also old and not-regressing vessels [85]. This finding may be related to different mechanisms: neutralization of corneal VEGF may tip the fine-tuned local balance of vasodilatory and vasoconstrictive factors towards vasoconstrictive factors such as endothelins [86]; in addition, the anti-inflammatory effect of anti-VEGF therapy may also cause reduced vessel dilatation merely by reducing the amount of pro-inflammatory cytokines present in the cornea. Therefore, anti-VEGF therapy may be efficiently used in the management of chemical burns as it avoids early tissue remodeling, scarring and subsequent opacification. However, in order to ensure its beneficial effect, anti-VEGF therapy should be started as soon as possible after the injury.

5.2. Infections

Infections can determine the onset of VEGF-mediated CN. The main sources of angiogenic factors are virus-infected epithelial cells, macrophages and inflammatory cells. In the stromal keratitis caused by herpes simplex virus (HSV), the formation of new vessels is an essential step for the pathogenesis of keratopathy [74]. The permanent presence of HSV-DNA and HSV-immune complexes contributes to inflammation and angiogenesis through increased levels of MMP-9 and VEGF [87, 88]. New vessels in-growth together with the higher recruitment of inflammatory agents lead to haze, scarring and vision impairment. Based on the high VEGF expression in infected eyes, several authors have proposed the use of anti-VEGF agents as a valuable therapeutic approach in the control of HSV keratitis [89]. Benayoun and co-workers evaluated the effect of a single subconjunctival injection of 2.5 mg (0.1 mL) of bevacizumab in patients suffering from viral keratitis, obtaining a reduction in CN ranging from 20% to 30% at 3 months [90]. Furthermore, greater efficacy was noted in the case of earlier administration and higher activity of the primary disease. This effect is probably the result of initial higher levels of VEGF and easier penetration of the drug into the inflamed tissue. Indeed, the acute phase of the inflammatory response is characterized by the great recruitment of inflammatory agents into the stroma that represents either a cause and a consequence of VEGF up-regulation. This also explains the reduced effect of bevacizumab on old vessels, being these less dependent on inflammatory mediators. Petsoglou and co-authors adopted a protocol consisting of 3 subconjunctival injections of 2.5 mg of bevacizumab, monthly administered. This therapy regimen had only a few and not clinically significant side effects and caused a greater regression of recent-onset CN compared to topical corticosteroid alone [91]. A similar beneficial effect was reported by Carrasco and co-workers who described the case of a patient with a history of herpetic stromal keratitis who experienced a dramatic regression of corneal vessels 1 week after subconjunctival injection of 0.05 mL (1.25 mg) of bevacizumab. After a 3-month follow-up, there was no recurrence of CN [92]. You and co-authors evaluated the dose-related effects of subconjunctival bevacizumab: of 29 eyes included, 7 received 1.25 mg/0.05 mL, 15 2.5 mg/0.1 mL and 7 5.0 mg/0.2 mL of

bevacizumab. The treatment was well-tolerated, and the efficacy correlated with the dose as the group treated with 5.0 mg bevacizumab experienced the greatest reduction in CN [93]. Other proposed administration modalities were the topical and the intrastromal ones. The former, as previously reported by Koenig and co-authors, led to a mean reduction of 61% in the vascularized area and of 24% in vessel diameter, thus providing the same efficacy profile of subconjunctival injections [94]. The latter was documented by Yeung and co-workers who performed a combined subconjunctival and intrastromal bevacizumab injections in patients with corneal scarring and NV owing to various aetiologies, such as HSV and zoster keratitis. The intrastromal injection had the advantage of reaching higher local concentrations in the deep layers of the cornea, thus being able to affect the proliferating vessels in the central area [96]. Lastly, anti-VEGF agents may also be effective in other infectious keratitis associated with CN, such as bacterial and interstitial keratopathy [81, 90].

5.3. Corneal Graft Rejection

Allograft rejection is a leading cause of failure of corneal transplantation and CN is among the major contributors to this event. Indeed, an insult to the cornea, such as keratoplasty, may enhance the production of angiogenic factors thus leading to a pathological NV, which starts from the recipient's bed and extends to the graft-host interface and to the graft itself. Close follow-up of patients at risk is necessary to detect and treat this complication as soon as possible in order to achieve better outcomes [96-100]. Corticosteroids remain the treatment of choice to control CN and reduce the risk of immunological graft rejection. However, they exhibit several limitations in their ability to reduce pathological vessels and are often accompanied by adverse events. Alongside the well-detectable blood vessels, tiny and almost-invisible lymphatic vessels seem to play an important role in the pathogenesis of graft rejection. In this contest, VEGF agents mediate not only angiogenesis but also lymphangiogenesis, as documented by previous studies [99, 101-103]. Starting from this evidence, anti-VEGF agents have been used to improve corneal graft survival in experimental models and humans. Harooni and co-authors reported a case of graft rejection with stromal vascularization following penetrating keratoplasty (PK) that was managed by a single subconjunctival injection of bevacizumab (1.25 mg/0.05 mL) [104]. Subsequently, other studies confirmed the beneficial effect of subconjunctival bevacizumab: all patients experienced a decrease in the number and caliber of vessels after the procedure; however, a single injection was shown to achieve only short-term effects, suggesting the need for eventually repeating the treatment over time [81, 105, 106]. It is well known that corneal transplantation performed in a recipient eye without signs of inflammation and NV is the ideal scenario with a success rate higher than 90% [107]. On the other hand, corneal grafts performed in inflamed and vascularized beds are at much risk of failure. Examples of high-risk transplantation are eyes with HSV keratitis, chemical burn, ulcer and Stevens-Johnson syndrome (SJS). In order to increase the graft survival rate in these cases, some studies have proposed the administration of anti-VEGF agents prior to surgery [108]. Fasciani and co-workers investigated whether pre-operative subconjunctival and/or intrastromal bevacizumab could help to prevent graft rejection in high-risk keratoplasty, reporting no graft failure during a mean follow-up period longer than 2 years. Since the first injection, all eyes presented less congestion, perfusion and activity of vessels with a concomitant decrease of conjunctival hyperemia and edema [108]. Corneal confocal microscopy showed the reduction of immunological mediators, such as Langerhans cells into the graft, whose presence has been associated with the rejection event [109]. Jarrin and co-workers reported a case of vascularized host-graft interface completely reversed by a single injection of bevacizumab, whereas Hashemian and co-authors performed a deep intrastromal injection to treat a CN spot not responding to topical or subconjunctival regimens [110, 111]. Another study reported the long-term follow-up of topical bevacizumab therapy in patients with graft rejection following PK [112]. Topical bevacizumab was administered twice daily for 15 days and allowed the regression of either major and minor vessels, with no relapse during a 9-month follow-up.

5.4. SJS

SJS is an acute inflammatory polymorphic disease affecting the skin and mucous membranes. Ocular involvement occurs in half of the patients and is an important cause of corneal blindness characterized by chronic ocular surface inflammation, dry eye, LSCD, stromal opacification, keratinization and NV [113]. Current treatments lack efficacy or require complex surgical procedures such as transplantation of conjunctival limbal stem cells, amniotic membrane or autologous oral mucosal epithelial cells, photodynamic therapy (PDT) and keratoprosthesis [114-119]. Being CN a major feature of this sight-threatening condition, anti-VEGF agents have been proposed as a therapeutic strategy with promising results. Uy and co-authors evaluated the efficacy and safety of topical bevacizumab on CN among patients with SJS: patients received bevacizumab eye drops (25 mg/mL) 4 times daily for a period of 3 months. At the end of the study, all subjects experienced a significant improvement in ocular comfort and visual acuity. Moreover, all eyes presented decreased corneal opacification, CN and conjunctival injection [120]. Kesarwani and Yoon analyzed the effect of subconjunctival bevacizumab on the ocular surface and showed the regression of CN and the subsequent improvement in patient's symptoms such as pain, tearing and photophobia [121, 122]. In particular, Kesarwani and co-authors reported the presence of a concomitant decrease of CN in the contralateral eye following bevacizumab injection, thus supporting the recent findings of a bilateral effect of bevacizumab after intravitreal and subconjunctival injections in animal studies. [121, 123, 124]. Yoon and co-authors, on the other hand, investigated the effectiveness of a combined approach including photodynamic therapy with verteporfin and intrastromal injection of bevacizumab which was shown to inhibit with success CN [122].

5.5. Allergic and Immunological Disorders

There is growing evidence that VEGF plays a role in idiopathic and allergic keratoconjunctivitis: cytokines from inflammatory cells may induce VEGF production that stimulates CN and the formation of giant papillae [125]. Nguyen

and co-authors reported the efficacy of subconjunctival steroids and bevacizumab injections in children with blefaroker-atoconjunctivitis (BKC) causing progressive, bilateral CN and scarring [126]. Similar efficacy was reported by Elbaz and co-workers, who performed FND plus adjuvant intrastromal and subconjunctival bevacizumab in children with refractory BKC. In these patients, once bevacizumab was injected, there was no occurrence of CN even after long-term follow-up longer than 2 years [127].

Ocular pemphigoid is another immune-mediated disorder that can positively respond to anti-VEGF therapy. Patients diagnosed with this disease develop in more severe cases conjunctival scarring, progressive CN and opacification with subsequent vision loss [128]. Case reports documented the use of bevacizumab in ocular mucosal and cicatricial pemphigoid unresponsive to the standard steroid regimen. De-Stafeno and co-authors adopted the use of topical bevacizumab (1.0%; 5 mL) 4 times daily, obtaining the reduction of both superficial and deep vessels with no side effects [80]; another study reported a single case of ocular graft-*versus*-host disease (GVHD) that partially responded to subconjunctival bevacizumab [81]. Lim and co-workers used the Boston Ocular Surface Prosthesis as bevacizumab delivery system in a patient with complicated Sjogren syndrome, obtaining a significant objective improvement of the clinical picture [8]. Overall, anti-angiogenic drugs act as useful tools in the management of immune-mediated diseases; however, due to the chronic nature of these conditions, there is a need for repeated treatments over time.

5.6. Dry Eye

Dry eye is a multifactorial disease of the ocular surface characterized by a loss of homeostasis of the tear film, whose instability and hyperosmolarity together with ocular surface inflammation, damage and neurosensory abnormalities play a pathogenetic role [129]. The core mechanism of the dry eye starts with the reduced lacrimal flow or increased evaporation, which results in the onset of tear hyperosmolarity that causes damage to the surface epithelium [130, 131]. The subsequent tear instability further exacerbates tear hyperosmolarity and completes the vicious spiral of the dry eye mechanism. VEGF has been shown to be up-regulated in the tears of patients with ocular chronic inflammatory disorders, such as dry eye, thus confirming its role in the pathogenesis of this complex disease [125]. In fact, VEGF is the key factor promoting the crosstalk between angiogenesis and inflammation; it directly stimulates corneal and inflammatory cells which produce inflammatory and pro-angiogenic cytokines while controlling the proliferation and migration of new vessels [132, 133]. Erdurmus and Totan were the first to report a case of subconjunctival bevacizumab used to treat a patient with dry eye and filamentary keratitis, showing a good response after treatment [105]. These promising results were confirmed by Jiang and co-authors who performed bevacizumab subconjunctival injection (100 μL, 25 mg/mL) in 64 eyes of 32 dry eye patients. Significant improvements in symptoms, tear break-up time, goblet cells density and NV area were observed during a 3-month follow-up period compared to the baseline. No adverse side effects were noted [134]. The same research group proposed also a novel technique, consisting of the intra-meibomian gland (MG) injection of bevacizumab to treat meibomian gland dysfunction (MGD) which is the leading cause of evaporative dry eye. Patients included in the protocol received multiple intra-MG injections of bevacizumab (150 μL, 2.5 mg/0.1 mL) in the area of severe telangiectasia. The treatment was effective and safe in ameliorating MG function and discomfort symptoms by improving lid-margin vascularity, conjunctival injection, expressed secretion quality, MG expressibility, tear break-up time (TBUT) and corneal staining [135]. Thus, anti-VEGF agents confirm their capability to relieve symptoms and signs of the disease safely and effectively for a period of at least 3 months after therapy, which may open up a new potential treatment for dry eye disease.

5.7. Contact Lens Wearing

People wearing contact lenses are at a major risk to develop CN by means of both hypoxic and inflammatory mechanisms. Many of them must reduce the amount of time of wearing or totally discontinue their use in order to allow CN regression. Anti-VEGF therapy was adopted by some authors, who showed its beneficial effect in terms of reduced CN and discomfort symptoms relief [136]. Zaki and co-authors aimed to study and evaluate the effect of subconjunctival bevacizumab injections in patients with corneal ischemia caused by contact lens wearing. All eyes received a single subconjunctival injection of 2.5 mg (0.1 ml) bevacizumab. Significant regression of both major and minor vessels density was observed in all eyes 2 weeks after the injection. The level of CN continued to decrease for 3 months and then remained approximately stable for the remaining 6-month follow-up period [137]. Petsoglou and co-authors conducted a double-masked placebo-controlled trial to evaluate the use of subconjunctival bevacizumab: patients received 3 0.1 ml injections containing either 2.5 mg bevacizumab or 0.9% saline at monthly intervals. Eyes treated with bevacizumab experienced a significant reduction in the area of CN compared to controls [91].

5.8. Degenerative Conditions

Pterygium is a degenerative condition characterized by pathological in-growth of blood vessels. It is defined as a common ocular disorder in which conjunctival tissue proliferates as a fibrovascular pannus over the perilimbal conjunctiva and then extends onto the corneal surface, causing chronic conjunctival hyperemia, corneal astigmatism, irritation and impaired vision [138]. Standard treatment regimen is based on its surgical excision together with adjunctive measures to prevent recurrence after excision (*e.g.* use of mitomycin) [139, 140]. However, the efficacy of these modalities is limited because of potential ocular side effects, such as superficial punctuate keratitis, poor epithelial healing, scleral ulceration, bacterial infection, and increased intraocular pressure (IOP) [74]. Anti-VEGF agents can be potentially included in this therapeutic algorithm since it has been previously demonstrated that VEGF is over-expressed in this condition [141-143]. Bahar and co-authors reported 5 cases of recurrent pterygium, in which corneal NV did not regress with success after 2 injections of subconjunctival bevacizumab (2.5 mg/0.1 mL, 1 month apart) [144]. There might be several reasons for this result. First of all, it is possible that the dose or the frequency of the administration

were insufficient to block VEGF functions; furthermore, cytokines other than VEGF contribute to pterygium formation and are not antagonized by bevacizumab. Lastly, since the pathogenesis of pterygium is multifactorial, all therapies that have an isolated target may be inadequate to antagonize the growth of the lesion [144]. Recently, Nava-Castaneda and co-workers evaluated the efficacy of 3 subconjunctival bevacizumab injections in patients with early pterygium recurrence [145]. In disagreement with the results of Bahar, the vascularized area and the corneal opacification were significantly reduced in all recurrent pterygia, suggesting the potential role of anti-VEGF agents in the management of pterygium recurrence. Moreover, a similar beneficial effect was reported by Wu and co-workers who performed a 3-weeks topical bevacizumab administration in patients with impeding recurrent pterygium [146]. On the other hand, Hurmeric investigated the effect of a single *versus* multiple subconjunctival ranibizumab injections in patients with an early pterygium recurrence and stated that some patients had a decreased vessel area over time, with a higher effect recorded in the group of patients undergone multiple injections [147].

6. ROUTES OF ADMINISTRATION

6.1. Topical

A wide range of topical bevacizumab concentrations have been shown to be safe and effective for treating CN. In particular, the concentration, the frequency, and duration of application should be adjusted to the type and severity of the underlying disorder. In rat models, topical bevacizumab (4 mg/ml) applied twice daily for 1 week was shown to attenuate chemically-induced CN [76]. The efficacy of topical anti-VEGF agents has been confirmed also in humans with various concentrations (5, 10, and 25 mg/mL) and frequencies (up to 5 times daily). Despite the majority of studies have used bevacizumab as anti-VEGF agents, the anti-angiogenic efficacy of topical ranibizumab has been recently documented. However, its application may be limited due to the expensive costs [147]. The topical route of administration is reported to be generally well-tolerated in long-term therapy as confirmed by Koenig and co-authors [94]. Conversely, Kim observed the development of corneal epitheliopathy in 6 of 10 eyes and corneal thinning in a case with CN during the second month of treatment with 1.25% topical bevacizumab twice daily [148]. In fact, it is known that VEGF inhibitors may impair the wound healing process in a dose-dependent manner. However, no side effects were detected using a slightly lower dose (1.0%) for 3 weeks [148]. Starting from this assumption, drug dose should be tapered after the first month of treatment in order to prevent similar complications. Furthermore, in order to minimize systemic absorption, patients should be instructed to close their lids for 1 minute after the application or to apply digital pressure on the puncta. Silicone punctal plugs placed in the lower eyelids represent other tools useful to further minimize systemic absorption [149, 150]. Clinical studies that investigated the topical administration of anti-VEGF are summarized in Table **2**.

6.2. Subconjunctival

Several studies have reported the efficacy of subconjunctival bevacizumab without significant ocular or systemic adverse reactions. Once injected, it diffuses into the corneal stroma and persists for a few days after the injection [151]. The optimal dose and timing of injection for subconjunctival bevacizumab have not yet been defined; many authors used doses of 5.0 mg/mL, 2.5 mg/0.1 and 1.25 mg/0.05 mL [81, 105, 152, 153]. As previously reported, subconjunctival bevacizumab is characterized by a cumulative effect, so injection should be repeated to maximize the therapeutic effect [81, 144]. Furthermore, better results are achieved with increasing doses as suggested by You and co-authors [93]. A limitation of this administration route is the difficulty for the injected drug to reach the central cornea as it exerts its maximum effect on the adjacent region and this issue can justify a combined approach including both subconjunctival and intrastromal injections. However, there are still conflicting results in the literature about the best route of administration between topical and subconjunctival. Dastjerdi and co-authors investigated corneal graft survival rates in a model of vascularized corneal transplantation following their treatment with either topical and subconjunctival bevacizumab, and showed that the regression of CN was more intense when treatment was applied subconjunctivally [154]. Furthermore, Ahmed and co-workers compared topical (12.5 mg⁄ml, 3 times daily) and subconjunctival (5 mg and 10 mg) bevacizumab treatments in an experimental rabbit model of CN, and found a significant decrease in the amount of neovascularization, particularly in the subconjunctival group [155]. On the other hand, Ozdemir and co-authors did not find any statistically significant difference between the two routes because both inhibited with success CN and reduced inflammation [156]. Overall, it can be postulated that the therapeutic efficacy of subconjunctival administration seems to be clinically more efficient than the topical one, even though the difference is not statistically relevant. This difference of effect may be related to the limited penetration of topical bevacizumab through the corneal epithelium and/or to its rapid clearance by tear flow [155, 156]. Similar profiles of efficacy have been described using other anti-VEGF agents, such as ranibizumab and aflibercept; the latter antagonize not only VEGF but also PDGF. However, bevacizumab continues to be the most widely used option as a result of lower costs and comparable efficacy. Clinical studies that investigated the subconjunctival administration of anti-VEGF are summarized in Table **3**.

6.3. Intrastromal

Compared to the aforementioned routes of administration, intrastromal injections may possibly allow greater exposure of the corneal vessels to the drug, as well as local delivery of a known concentration. As previously described in an experimental model, animals treated with intrastromal injection achieved the same response of the subconjunctival group using a dose 463 times lower [157]. Furthermore, intrastromal injections allow the drug to reach vessels in the central cornea, in contrast to subconjunctival administration that exerts its effect mainly in the area adjacent to the injection [157, 158]. In the study from Vieira, the injection of bevacizumab into the corneal stroma was performed without complications during the entire follow-up period, which ranged from 2 to 17 months [158]. Clinical studies that investigated the intrastromal administration of anti-VEGF are summarized in Table **3**.

Table 2. Clinical results for topical anti-vascular endothelial growth factor agents in the treatment of corneal diseases.

First Author (Year)	Study Design	Number of Patients (Eyes)	Diagnosis	Dosage	Duration of Treatment	Adverse Reactions	Efficacy on CN	References
DeStafeno (2007)	Prospective case series	2 (2)	Ocular trauma and pemphigoid, steroid treatment failed	10 mg/ml (1%), 4 drops per day	Not specified	None	Regression	[80]
Uy (2008)	Retrospective case series	2 (3)	Stevens–Johnson syndrome	25 mg/ml (2.5%), 4 drops per day	3 months	None	Regression	[120]
Bock (2008)	Prospective case series	5	Following limbar cell stem transplant and/or keratoplasty	12.5 mg/ml (1.25%) 2 drops per day	3		Regression	[85]
Kim (2008)	Prospective case series	7 (10)	Various etiologies	12.5 mg/ml (1.25%) 2 drops per day	3	Epitheliopathy and corneal thinning	Regression	[148]
Koenig (2009)	Prospective case series	27 (30)	Various etiologies	5 mg/ml (0.5%), 5 drops per day	0.5–12 months	None	Vessel diameter reduction	[94]
Dastjerdi (2009)	Prospective case series	10 (10)	Various etiologies	10 mg/ml (1%), 2*vs*4drops per day	3 weeks	None	Regression	[150]
Lim (2009)	Retrospective case series	5	HSV, SJS, LSCD, neovascular pannus and scar	One drop of 1% bevacizumab to be instilled each morning in the fluid reservoir of the BOSP.	3 months	None	Regression	[8]
Saxena (2009)	Case report	1 (1)	Graft rejection	Bevacizumab (4 mg/4 mL) one drop twice a day	15 days	None	Regression	[112]
Petsoglou (2013)	Prospective controlled double-masked	30	Allergic eye disease, HSV/HZV, non-viral keratitis, corneal surgery	2.5 mg/o.1 mL at monthly intervals 0.1% Dexamethasone drops were used 4 times daily for the first month, then the dose was modified if clinically indicated	3 injections	None	Regression	[91]

CN, corneal neovascularization; HSV, herpes simplex virus; SJS, Stevens-Johnson syndrome; LSCD, limbal stem cell disease; HZV, herpes zoster virus.

Table 3. Clinical results of subconjunctival and intrastromal anti-vascular endothelial growth factor agents in the treatment of corneal neovascularization.

First Author (Year)	Study Design	Number of Patients (Eyes)	Diagnosis	Dosage	Duration of Treatment	Adverse Reactions	Effects on CN	References
Erdurmus (2007)	Retrospective case series	2 (2)	Dry eye, graft failure	2.5 mg/0.1 ml	Single application	None	Regression	[105]
Doctor (2008)	Retrospective case series	7 (8)	Various etiologies	2.5 mg/0.1 ml, monthly injections	Up to 3 months	None	Regression	[153]
Bahar (2008)	Retrospective case series	10 (10)	Various etiologies	2.5 mg/0.1 ml,	-	None	Regression	[81]
Carrasco (2008)	Case report	1 (1)	HSV	Bevacizumab 0.05 mL (1.25 mg)	Single injection	None	Regression	[92]
Oh (2009)	Prospective case series	3 (3)	Lipid keratopathy, corneal vascularisation of unknown origin	1.25 mg/0.05 ml subconjunctivally, 1.25 mg/0.05 ml intracorneally, 2–3 injections	Monthly intervals	Intrastromal haemmorhage (resolved spontaneously)	Regression	[78]
You (2009)	Prospective case series	29 (29)	Keratoplasty, corneal scar, lipid keratopathy, herpetic keratitis	1.25 mg/0.05 ml - 2.5 mg/0.1 ml - and 5.0 mg/0.2 ml	Up to 1 month	Pain at the injection site, subconjunctival haemor- rhage and ocular irritation	Partial regression	[51]
Symes (2010)	Case report	1 (1)	High risk keratoplasty	Bevacizumab 2.5 mg in 0.1 mL, one injection repeated after surgery	Single inejction	None	Regression	[162]
Zaki (2010)	Prospective case series	10 (10)	Chronic inflammation, healed ulcers	2.5 mg/0.1 ml	Single application	None	Regression	[137]
Jarrin (2012)	Case report	1 (1)	Graft rejection	Bevacizumab 1.25 mg	Single injection	None	Regression	[110]
Hashemian (2011)	Case report	1 (1)	Graft rejection	Intrastromal Bevacizumab (2.5 mg/1 mL	Single injection	None	Regression	[88]
Yeung (2011)	Retrospective case series	12 (12)	Progressive vascularisation, steroid treatment failed, various aetiologies	1.25 mg/0.05 ml subconjunctivally, 1.25 mg/0.05 ml intracorneally, 1 to 3 injections	Up to 8 months	None	Regression	[72]

(Table 3) contd….

First Author (Year)	Study Design	Number of Patients (Eyes)	Diagnosis	Dosage	Duration of Treatment	Adverse Reactions	Effects on CN	References
Benayoun (2012)	Prospective case series	12(11)	HSV, HZV, chemical burn, bacterial keratitis, pemphigoid, keratoplasty	bevacizumab 2.5 mg (0.1 mL)	Single injection	None	Regression	[90]
Kesarwani (2012)	Case report	1(1)	SJS	Bevacizumab 0.05 mL (1.25 mg)	Single inejction	-	Regression	[121]
Kim (2014)	Prospective case series	16(16)	HSV, graft rejection, chemical burn, pemphigoid, recurrent ulcer	Sunconjunctival/intratsromal injections of 2.5 mg bevacizumab or 1 mg of ranibizumab	-	Epitheliopathy	Regression	[157]
Nava-Castaneda (2015)	Prospective case series	38	Pteryigum	Bevacizuamb 2.5 mg/0.1 mL	3 injections	-	Regression	[145]
Elbaz (2015)	Retrospective case series	9(9)	HSV, complete cornea anesthesia, BKC, suture tract infection	FND and subcoonjucntival/intrastromal injections of bevacizumab 25 mg/mL	Single injection	Intracorneal hemorrhage	Regression	[127]
Fasciani (2015)	Prospective case-control series	27(27)	High risk keratoplasty	Subconjunctival/intrastromal injections of bevacizumab 5 mg/0.2 ml	3 injections	None	Regression	[108]
Jiang (2015)	Prospective case series	32(64)	Dry Eye	Bevacizumab 100 iL 25 mg/mL	Single injection	None	Regression	[134]
Yoon (2019)	Prospective case series	8(8)	SJS	Intrastromal bevacizumab 2.5 mg/0.1 ml and verteporfin +6 mg/m^2	-	-	Regression	[122]
Jiang (2018)	Prospective case series	13(26)	Dry Eye, MGD	Intrameibomian injection of bevacizumab 150 µL, 2.5 mg/0.1 mL	Single injection	None	Regression	[135]
Nguyen (2018)	Retrospective case series	2(2)	BKC	Triancinolone 4mg/0.1mL × 0.55mL and subconjunctival bevacizumab 2.5mg/0.1mL × 0.05mL 2/3 times per week	-	None	Partial regression	[126]

CN, corneal neovascularization; HSV, herpes simplex virus; HZV, herpes zoster virus; BKC, blepharokeratoconjunctivitis; SJS, Stevens-Johnson syndrome; MGD, meibomian gland dysfunction.

6.4. Intravitreal

Intravitreal injection is the standard administration route to treat retinal disorders. As previously documented, high doses of bevacizumab can penetrate ocular tissues after intravitreal inoculation [156]. Assuming that some drug can slowly diffuse into the anterior segment, this procedure may have also a positive effect on CN; however other systems that deliver anti-VEGF agents in the anterior tissues are preferable to avoid some of the complications associated

with the intravitreal route such as uveitis and endophthalmitis [159, 160].

7. SAFETY

The high amount of studies evaluating anti-VEGF use in corneal diseases support the safety of this treatment [78, 81, 92, 105, 153, 161, 162]. However, given the trophic role of VEGF and its receptor expression on corneal endothelial and epithelial cells, several side effects following VEGF neutralization at the ocular surface could occur. In fact, VEGF might have a role in maintaining normal corneal function and/or epithelial healing [163]. *In vitro* blockage of VEGF signaling with anti-VEGF antibody reduced the growth of cultured trigeminal neurons by 17% and the regeneration of sub-basal neurons by 23%, so anti-VEGF therapy may have a detrimental effect on corneal sensation, especially in conditions such as HSV keratitis and diabetes [164]. This finding was confirmed by Goldhardt and co-authors who found the impaired function of corneal nerves in eyes with a history of anti-VEGF injections compared to eyes without [165]. Nishijima and co-workers demonstrated that VEGF-A is a survival factor for retinal neurons and a critical neuroprotectant during the adaptive response to ischemic injury [166]. Thus, high concentrations of anti-VEGF factors might have an adverse effect on the proliferation of these cells [166]. Nonetheless, the dose administered intravitreally in the daily practice has been shown to be safe and no toxicity for any investigated concentration was noted on human optic nerve head astrocytes and human trabecular meshwork cells [167]. Corneal epithelial defects have been reported as possible complications of anti-VEGF treatment. Kim *et al.* documented epitheliopathy in 6 of 10 treated eyes following prolonged treatment with topical bevacizumab (1.25% twice daily) [125]. However, these adverse events occurred only in the second month of treatment. Conversely, Lee and co-authors reported that subconjunctival injections of bevacizumab improved corneal wound healing after alkali burn injury in a mouse model [7]. The possible cytotoxic effect on endothelial cells has been ruled out by several authors. In particular, Lichtinger and co-workers did not report any decrease in endothelial cell count nor cell morphology 6 months after combined subconjunctival and intrastromal bevacizumab injections [168].

As already reported for intravitreal administration, possible side effects include also transient and sustained IOP elevation, conjunctival hemorrhage, pain at the injection site, uveitis and endophthalmitis [6]. Considering the multiple functions of VEGF in both physiological and pathological processes, some concerns have been raised regarding the potential systemic side effects associated with the ophthalmic use. In fact, the administration of intravenous bevacizumab in adjuvant chemotherapeutic regimens was found to be associated with hypertension, proteinuria, hemorrhages and thromboembolic events [169]. Although the confined site of injection and the lower dosage used in the setting of corneal diseases, even locally administered anti-VEGF agents are ultimately cleared through the systemic circulation. The evaluation of Pegaptanib pharmacodynamics in humans showed a mean plasma level of 80 ng/mL following a single intravitreal 3 mg injection with a plasma half-life of approximately 10 days, whereas plasma VEGF levels in the healthy human adult are typically less than 100 pg/mL [170]. Besides involvement in pathological neovascularization, VEGF plays a key role in the wound healing process, bone growth, cyclic endometrial development and, last but not least, in the formation of collateral vessels, which is of critical importance in ischemic accidents, such as myocardium infarction. In addition, patients with cardiovascular systemic diseases such as diabetes and ocular vascular conditions such as wet age-related macular degeneration are at higher risk for cardiac and peripheral vascular accidents, therefore attention should be paid regarding the systemic implications of long-term VEGF inhibition [169, 170]. A higher risk for systemic complications has been previously reported in patients with poor health conditions treated with anti-VEGF therapy for colon cancer. These patients experienced a double incidence of serious thromboembolic events [170]. However, the most severe adverse events recorded after the intravitreal administration of anti-VEGF agents have not been reported after topical, subconjunctival or intrastromal administrations [74].

8. CURRENT LIMITATIONS

Despite many studies showed the beneficial effects of anti-VEGF agents in treating corneal disorders, several questions still remain to be answered. First of all, it is difficult to identify which patients would best benefit from the topical or the subconjunctival administration of bevacizumab. Furthermore, the treatment effect could depend on the underlying disease responsible for CN that could influence vessel activity. In fact, CN occurring in the setting of an acute insult, such as a chemical burn or microbial keratitis, might differ in susceptibility to treatment compared to CN associated with chronic, ongoing inflammatory processes, such as the dry eye. Since no guidelines about posology, timing and duration of treatment are available for the use of anti-VEGF agents in the contest of corneal diseases, controlled prospective trials comparing different protocols are needed to establish the proper regimen able to provide long-term safety and efficacy.

9. FUTURE DIRECTIONS

VEGF is not the only molecule involved in the angiogenesis process. Other angiogenic factors in the corneal tissue include FGF-1 and FGF-2, MMPs, angiostatin, endostatin, pigment endothelium-derived factor, thrombospondin, insulin-like growth factor, placental growth factor (PIGF) and PDGF.

PDGF binds to receptors on pericytes and enhances the stability of new vessels, partly by reducing their requirement for VEGF. Therefore, the removal of PDGF should lead to the reduction of pericytes and the subsequent regression of vascularization, as previously shown in mice models. During the angiogenic process, in fact, new vessels undergo a period of "fine tuning" where vascular endothelial cells become apoptotic if sufficient supply of angiogenic factors is lacking. Morphologically, this period correlates with the absence of pericyte coverage of new vessels. Mature, pericyte covered vessels, in contrast, do not depend on elevated levels of angiogenic factors for survival.

Since pathological new vessels in human CN are rapidly covered by pericytes, anti-VEGF therapy may not be

Table 4. Clinical studies registered on ClinicalTrials.Gov about anti-vascular endothelial growth factor agents for the treatment of corneal neovascularization.

Site	Study Design	Number of Patients	Diagnosis	Status	Drug	Route	Dosage	Posology	Comparator Arm
Ramathibodi Hospital Bangkok, Thailand	Interventional comparative randomized single masked	80	Pterygium	Completed	Bevacizumab	Subconjunctival injection	1.25 mg/0.05mL 2.5 mg/0.1mL 3.75 mg/0.15mL bavacizumab	Single injection	Topical 0.1% fluorometholone eye drops
Instituto de Olhos de Goiania, Brazil	Interventional noncomparative single masked	8	CN	Completed	Bevacizumab	Subconjunctival injection	-	Single injection	-
Service d'Ophtalmologie Limoges, France	Interventional comparative randomized double-masked	38	CN	Completed	Bevacizumab	Subconjunctival injection	0.5 ml bevacizumab	Three subconjunctival injections of 0.5 ml of bevacizumab at inclusion, 1 month, 2 months.	Three subconjunctival injections of 0.5 ml of Nacl at inclusion, 1 month, 2 month
Massachusetts Eye and Ear Infirmary Boston, Massachusetts, United States	Interventional comparative randomized quadruple masked	90	Graft rejecton	Completed	Bevacizumab	Subconjunctival injection and topical eye drops	0.1 mL (2.5 mg) bevacizumab and bevacizumab (1% solution)	injection of 0.1 mL (2.5 mg) bevacizumab immediately upon the conclusion of the penetrating keratoplasty procedure Day 1 post-transplant surgery, treatment with topical bevacizumab (1% solution), 4 times daily for 4 weeks	0.9% NaCl & Refresh Liquigel injection of 0.1 mL (2.5 mg) immediately upon the conclusion of the penetrating keratoplasty procedure Day 1 post-transplant surgery, treatment with topical regimen 4 times daily for 4 weeks
Chang Gung Memorial Hospital, Taipei, Taiwan	Interventional noncomparative nonrandomized	10	CN	Active, not recruiting	Bevacizumab	Subconjunctival injection and topical eye drops	Topical 10 mg/cc or subconjunctival 2.5 mg/0.1cc	-	-

(Table 4) contd….

Site	Study Design	Number of Patients	Diagnosis	Status	Drug	Route	Dosage	Posology	Comparator Arm
Asociación Para Evitar la Ceguera en México, IAP, Hospital "Dr. Luis Sánchez Bulnes" **Mexico City, Mexico**	Interventional comparative randomized single masked	18	CN	Completed	Bevacizumab	Subonjunctival injection	0.1cc of subconjunctival Bevacizumab	Single injection	Patients with corneal neovascularization of infectious etiology, steroid reactors, and know glaucoma or glaucoma suspects *Versus* Patients with corneal neovascularization of any cause except for infectious 24disease (bevacizumab + 0.1cc of triamcinolone acetonide).
Walter Reed Army Medical Center **Washington, District of Columbia, United States**	Interventional noncomparative nonrandomized	24	CN	Completed	Bevacizumab	Topical	Bevacizumab 10mg/mL	1 drop daily for 3 weeks	-
Massachusetts Eye and Ear Infirmary **Boston, Massachusetts, United States**	Interventional noncomparative nonrandomized	24	CN	Completed	Bevacizumab	Topical	Bevacizumab 1.0%	-	-
Massachusetts Eye and Ear Infirmary **Boston, Massachusetts, United States**	Interventional comparative randomized triple masked	30	Graft rejecton	Completed	Bevacizumab	Subconjunctival injection and topical eye drops	0.1 mL (2.5 mg) bevacizumab and bevacizumab (1% solution)	injection of 0.1 mL (2.5 mg) bevacizumab immediately upon the conclusion of the penetrating keratoplasty procedure Day 1 post-transplant surgery, treatment with topical bevacizumab (1% solution), 4 times daily for 4 weeks	0.9% NaCl & Refresh Liquigel injection of 0.1 mL (2.5 mg) immediately upon the conclusion of the penetrating keratoplasty procedure Day 1 post-transplant surgery, treatment with topical regimen 4 times daily for 4 weeks

CN, corneal neovascularization.

effective in the elimination of mature and well-established vessels. Thus, early intervention and co-blockade of the VEGF and PDGF pathways can be performed to affect both the new and old vessels [55]. Jo and co-authors used an antibody against PDGFR-B along with Pegatinib and demonstrated that blocking both the pathways was more effective in reducing CN than blocking each pathway alone [171]. Perez-Santonja and co-workers used bevacizumab and sunitinib, which blocks VEGFR-1, VEGFR-2 and PDGF-B signaling cascades, and documented inhibition of CN almost 3 times greater compared to that one obtained with bevacizumab alone [172]. Moreover, a new combined approach including bevacizumab and suramin, a non-specific purinergic receptor antagonist, was proposed by Lopez and co-author, who showed the greater and longer inhibition of CN when adopting such therapy regimen [173].

Platelet-activating factor (PAF) is also a potent inducer of corneal angiogenesis, and PAF antagonists, such as LAU-0901, may determine therapeutic action against CN [174].

Therefore, the combination of different anti-angiogenic agents could achieve a synergistic effect and should be considered as an alternative treatment strategy, especially in those patients with long-standing chronic inflammatory disease that typically show less responsiveness to the anti-VEGF therapy alone. Further data to support future evolution are expected to come from ongoing clinical studies which are summarized in Table **4**.

10. AUTHORS INSIGHT ON THE TOPIC

CN is caused by a wide variety of events, including infection, inflammation, ischemia, degeneration, trauma, and loss of the limbal stem cell barrier. It impairs visual acuity, reduces the immune privilege of the cornea, and in the case of keratoplasty, affects also the graft survival by introducing circulating immune cells. Since a wide variety of events lead to the common pathway of corneal angiogenesis mediated by VEGF, its inhibition represents the main therapeutic strategy, regardless of the underlying cause of CN.

The only meta-analysis available in the literature provides evidence that anti-VEGF agents reduce significantly CN, regardless of the route of administration [175]. This finding is of special interest since topical instillation of full-length immunoglobulins is typically considered ineffective because such molecules are too large to penetrate the intact corneal epithelium. However, epithelium over the neovascularized area can be defective and result in incompetent barrier function thus facilitating the penetration of the drug. Furthermore, anti-VEGF agents are more effective in reducing active CN compared to a stable one.

The increasing popularity of anti-VEGF agents raises concerns about safety with long-term use of these agents. Although a meta-analysis on the side effects of subconjunctival or topical administration of anti-VEGF drugs has not yet been performed, the majority of the studies have reported no major local or systemic side effects. Though delivered in a small dose on the surface of the eye, anti-VEGF agents could also pass into the systemic circulation causing hypertension, proteinuria, and various cardiovascular events, and this issue should be taken into account, particularly in patients with poor health conditions.

Further controlled prospective randomized trials could help to elucidate the efficacy, safety and tolerability of anti-VEGF agents in CN. They should also identify the optimum regimen, in terms of dosing and duration of treatment, able to achieve a sustainable effect in the regression of CN while minimizing local and systemic side effects.

CONCLUSION

Corneal blindness is the third most common cause of visual impairment worldwide and CN is present in many cases [7]. In fact, CN is a relatively common complication occurring in the setting of ocular surface and corneal disorders that can lead to significant visual impairment and poor outcomes, if not addressed promptly. Moreover, since it acts as one of the major risk factors for graft rejection, its early detection and treatment are mandatory prior to proceeding with corneal transplantation. Several approaches including amniotic membrane transplantation, topical nonsteroidal anti-inflammatory and corticosteroids, photocoagulation, photodynamic therapy and fine needle diathermy have been used in the attempt of inhibiting the proliferation of new corneal vessels. However, these treatments have often partial or short-term efficacy, and may lead to unpleasant side effects.

Recent elucidations on the molecular pathways involved in the pathogenesis of CN highlighted the key role of VEGF, and subsequently opened up the perspective of the potential use of anti-VEGF agents for its management. Overall, studies assessing the efficacy of these agents in the treatment of CN showed good outcomes for both efficacy and safety, despite the response is based on multiple variables such as the chronicity and extent of CN as well as the underlying disease. Better results are achieved when the therapy is administered early in the course of the pathological process. Furthermore, CN can occur again after treatment if the inflammatory cascade is not reversed. In cases of long-standing and ongoing diseases, there might be a need for repeating anti-VEGF treatment at certain time intervals. Better outcomes can be obtained by adopting a combined approach rather than using anti-VEGF agents alone.

LIST OF ABBREVIATIONS

CN	=	Corneal neovascularization
VEGF	=	Vascular endothelial growth factor
NV	=	Neovascularization
LSCD	=	Limbal stem cell deficiency
MMPs	=	Matrix metalloproteinases
bFGF	=	Basic fibroblast growth factor
LSCs	=	Limbal stem cells
VEGFR	=	Vascular endothelial growth factor receptor
PDGF	=	Platelet derived growth factor
TGF-α	=	Transforming growth factor alpha
TGF-β	=	Transforming growth factor beta
ECM	=	Extracellular matrix
NO	=	Nitric oxide
ROCK	=	Rho-associated kinase
NOD1	=	Nucleotide-binding oligomerization domain 1
FND	=	Fine needle diathermy
AMD	=	Age-related macular degeneration
FDA	=	Food and Drug Administration

RNA	=	Ribonucleic acid
ME	=	Macular edema
RVO	=	Retinal vein occlusions
DME	=	Diabetic macular edema
DR	=	Diabetic retinopathy
HSV	=	Herpes simplex virus
PK	=	Penetrating keratoplasty
PDT	=	Photodynamic therapy
BKC	=	Blepharokeratoconjunctivitis
GVHD	=	Graft-*versus*-host disease
MG	=	Meibomian gland
MGD	=	Meibomian gland dysfunction
TBUT	=	Tear break-up time
IOP	=	Intraocular pressure
PIGF	=	Placental growth factor
PDGFR	=	Platelet derived growth factor receptor
PAF	=	Platelet-activating factor

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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