Department for Companion Animals and Horses
University of Veterinary Medicine Vienna
University Clinic for Small Animals, Small Animal Surgery
(Ass. Prof. Dr. Britta Vidoni)

and

Department for Biomedical Sciences
University of Veterinary Medicine Vienna
Institute of Pharmacology and Toxicology
(Univ. Prof. Dr. Veronika Sexl)

Pharmacological inhibition of vascular endothelial growth factor

A novel therapeutic approach for corneal vascularization in veterinary ophthalmology

PhD thesis submitted for the fulfilment of the requirements for the degree of

DOCTOR OF PHILOSOPHY (PhD)

University of Veterinary Medicine Vienna

submitted by

Dr. Lisa-Marie Müllerleile

Vienna, April 2021

PhD committee

First supervisor

Ao. Univ. Prof. Dr. Barbara Nell, Dipl. ECVO
Department for Companion Animals and Horses, University Clinic for Small Animals, Small Animal Surgery

University of Veterinary Medicine Vienna

Veterinärplatz 1
1210 Vienna, Austria

Second supervisor

Ass. Prof. Priv.-Doz. Dr. Daniela Alexandra Fux
Department for Biomedical Sciences, Institute of Pharmacology and Toxicology

University of Veterinary Medicine Vienna

Veterinärplatz 1
1210 Vienna, Austria

This is for Miu.

Quand tu regarderas le ciel, la nuit,
puisque j'habiterai dans l'une d'elles, puisque je rirai dans l'une d'elles,
alors ce sera pour toi comme si riaient toutes les étoiles.
Tu auras, toi, des étoiles qui savent rire.

(Antoine de Saint-Exupéry)

Acknowledgements

First, I want to thank my supervisors, Prof. Barbara Nell and PD Dr. Daniela Andrea Fux. For their support, patience, and for their willingness to listen to my thoughts and troubles. I thank you for the thorough training and the commitment to find answers to my questions, to promote me without ceasing, and for the belief in me and my abilities.
Heartfelt thanks go to my vet colleagues, in particular Sarah Balmer, Verena Herb, Vera Prucha, and Ulrike Lessiak. As teammates and close friends, you have been incredibly supportive and I miss working with you. I thank you for your thought-provoking impulses and for catching me when turnarounds were inevitable.
I would also like to thank Bernhard Buxbaum, who was instrumental in laying the foundation for this work. My thanks go the VetCore, Stefan Kummer, Michael Bernkopf, Michael Wambacher, and Alexander Tichy for their highly professional advice and support.

I want to thank all dogs that were part of this work. You are the heart of everything and the reason for being a veterinarian. It was always a great pleasure to meet and work with you. This moments were always rewarded with joy and kindness. Many thanks are contributed to your human companions who gave me an incredible amount of trust. I hope this work that only came about with your help, can bring veterinary medicine a little further.

The last lines are for those who fill my heart and enable me to face each day with joy and happiness. In this way, you allow me to always carry out my work with satisfaction and dedication. My grandfather Karlfried, grandmother Elisabeth, and dad Thomas are calling me "Käpsele", and this means in Baden dialect to be a very ingenious person, thereby giving me the knowledge that I can make a difference. Thomas is not only dad to me, but also my best friend, great listener, critic, and motivational coach.
Words cannot express how great my gratitude is to Miu, Pepe, Maia, and Markus. You are my uphill momentum and the lifeline when something goes wrong. Miu, you had been the most cunning, warm-hearted, and strong-minded person I have ever known.
Thank you for being my family, my safe haven, and my home.

List of publications

- Muellerleile LM, Buxbaum B, Nell B; Fux DA. In-vitro binding analysis of anti-human vascular endothelial growth factor antibodies bevacizumab and aflibercept with canine, feline, and equine vascular endothelial growth factor. 2019. Research of Veterinary Science, 124:233-238.
  Impact factor: 1.892

- Muellerleile LM, Tichy A, Nell B. Serum vascular endothelial growth factor changes and safety after topical anti-human VEGF antibody bevacizumab in healthy dogs. 2019. Veterinary Ophthalmology, 22(5):600-606.
  Impact factor: 1.105

- Muellerleile LM, Bernkopf M, Wambacher M, Nell B. 2021. Topical bevacizumab for the treatment of corneal vascularization in dogs: A case series. Veterinary Ophthalmology. [Article submitted, March 2021]
  Impact factor: 1.105 (2019)

Contribution

- Muellerleile LM, Buxbaum B, Nell B; Fux DA. In-vitro binding analysis of anti-human vascular endothelial growth factor antibodies bevacizumab and aflibercept with canine, feline, and equine vascular endothelial growth factor. 2019. *Research of Veterinary Science,* 124:233-238.

  Muellerleile LM: experimental design, experiments, data analysis, writing of the paper
  Buxbaum B (shared first authorship): experimental design, writing of the paper
  Nell N: experimental design, contribution to materials, writing of the paper
  Fux DA: experimental design, contribution to materials, experiments, data analysis, writing of the paper

- Muellerleile LM, Tichy A, Nell B. Serum vascular endothelial growth factor changes and safety after topical anti-human VEGF antibody bevacizumab in healthy dogs. 2019. *Veterinary Ophthalmology,* 22(5):600-606.

  Muellerleile LM: experimental design, experiments, data analysis, writing of the paper
  Tichy A: data analysis, writing of the paper
  Nell B: experimental design, experiments, contribution to materials, writing of the paper

- Muellerleile LM, Bernkopf M, Wambacher M, Nell B. 2021. Topical bevacizumab for the treatment of corneal vascularization in dogs: A case series. *Veterinary Ophthalmology*. [Article submitted, March 2021]

  Muellerleile LM: experimental design, experiments, data analysis, writing of the paper
  Bernkopf M: experimental design, experiments, writing of the paper
  Wambacher M: contribution to materials, writing of the paper
  Nell B: experimental design, experiments, contribution to materials, writing of the paper

Declaration

I, Lisa-Marie Müllerleile, hereby declare that the rules of Good Scientific Practice have been followed in all aspects throughout this PhD study.

Content

1. Summary

Corneal vascularization is a major challenge in veterinary ophthalmology and is associated with a variety of adverse effects, including visual impairment due to corneal edema, deposition of pigments and lipids, persistent corneal inflammation, and loss of the corneal immune privilege (Dana and Streilein 1996, Andrew et al. 1998, Murphy et al. 2001, Andrew 2008, Dean and Meunier 2013, Lassaline et al. 2015, Plummer 2015, Hindley et al. 2016, Ledbetter et al. 2016, Krecny et al. 2018).

Normally, the mammalian cornea is free of blood and lymph vessels, which is one of the prerequisites for corneal transparency (Crispin and Barnett 1983, Werkmeister et al. 2017). Corneal avascularity is regulated by a balance of pro- and antiangiogenic mechanisms (Cursiefen et al. 2004b, Ambati et al. 2006, Cursiefen et al. 2006, Matsui et al. 2012, Zakaria et al. 2012). Stimuli such as hypoxia, inflammation, and infection can tip the scales to the angiogenic side that cause ingrowth of blood vessels into the cornea (Mastyugin et al. 2001, Gan et al. 2004, Goyal et al. 2010, Shi et al. 2010, Lutty et al. 2011, Chen et al. 2012, Park et al. 2018).

One of the key mediators of angiogenesis is the vascular endothelial growth factor (VEGF)-A, which is involved in both physiological and pathological vascularization (Shalaby et al. 1995, Amano et al. 1998, Gerhardt et al. 2003, Gan et al. 2004, Jia et al. 2004, Eming and Krieg 2006, Pieh et al. 2008). Vascular endothelial growth factor A is a glycoprotein that exerts its effect through two tyrosine kinase cell surface receptors, VEGF receptor (VEGFR)-1 and VEGFR-2. (Gerhardt et al. 2003, Jia et al. 2004). A causative function of VEGF-A and VEGFR-2 during corneal angiogenesis has been demonstrated in experimental animal models of corneal vascularization and in humans with naturally occurring corneal angiogenesis (Cursiefen et al. 2000, Mastyugin et al. 2001, Gan et al. 2004, Chen et al. 2012, Yun et al. 2020).

Nonetheless, the function of VEGF in ocular diseases in animal patients is largely unknown (Zarfoss et al. 2010, Binder et al. 2012, Sandberg et al. 2012, Balicki and Sobczyńska-Rak 2014). There are few studies indicating a possible role of VEGF in a number of canine ocular diseases, such as chronic superficial keratitis and pre-iridal fibrovascular membranes (Zarfoss et al. 2010, Sandberg et al. 2012, Balicki and Sobczyńska-Rak 2014). In experimental canine

models of retinopathy of prematurity, the usage of therapeutic VEGF inhibitors led to an efficient reduction of retinal vascularization (Lutty et al. 2011). In horses affected by equine recurrent uveitis, a causal role of VEGF has been postulated (Deeg et al. 2006, 2007, Curto et al. 2016).

Although studies in dogs, cats, and horses are sparse, it can be speculated that VEGF-driven mechanisms are causally involved in corneal vascularization in these species, too.

The discovery of drugs that inhibit VEGF-A and its effectiveness in neovascular disorders has revolutionized human ophthalmology, in particular in retinal neovascular disorders (Avery et al. 2006, Schmidt-Erfurth et al. 2014, Dugel et al. 2020, Sahni et al. 2020). Two of the most intensively studied VEGF inhibitors are bevacizumab and aflibercept (Presta et al. 1997, Holash et al. 2002). Bevacizumab is a humanized murine anti-VEGF monoclonal antibody that binds to human VEGF-A (Presta et al. 1997). Aflibercept is a fusion protein, consisting of regions of VEGFR-1, VEGFR-2 and human IgG1, that binds to human VEGF-A, VEGF-B and placenta growth factor (PIGF), and to VEGF-A and PIGF of mice (Holash et al. 2002, Papadopoulos et al. 2012). The use of bevacizumab and aflibercept is considered a promising approach to suppress corneal vascularization in humans (Dastjerdi et al. 2009, Aksoy 2019, Cholak et al. 2020) and a clinical applicability in dogs, cats, and horses is conceivable. This is of great interest in veterinary ophthalmology, as the incidence of corneal vascularization is high, persistent corneal blood vessels can result in vision-threatening complications, and existing standard therapies may be insufficient or carry a high risk of adverse events (Slatter et al. 1977, Bedford and Longstaff 1979, Kaswan and Salisbury 1990, Tolar et al. 2006, Bock et al. 2014, Plummer 2015, Dowling et al. 2016, Hindley et al. 2016, Rigas et al. 2020, Villar et al. 2020).

Bevacizumab and aflibercept were designed to bind specifically to human VEGF-A (Presta et al. 1997, Holash et al. 2002, Papadopoulos et al. 2012). The pharmacological binding properties to canine, feline, and equine VEGF are unknown to date. In addition, there is no knowledge about the in-vivo effects of topical usage in these species. Therefore, the aim of this PhD study was to evaluate the binding properties of bevacizumab and aflibercept to canine, feline, and equine VEGF and to further investigate the effects of topical bevacizumab in-vivo in dogs.

Amino acid sequence comparisons revealed a high homology and an identical sequence of the bevacizumab binding region of canine, feline, and equine VEGF compared to human VEGF. Using ELISA- and Western blot analyses, the binding ability of both bevacizumab and

aflibercept to canine VEGF was demonstrated. However, the results indicated that feline and equine VEGF may not be bound by aflibercept and bevacizumab or bind in a nonspecific manner only.

Subsequently, we set out to investigate bevacizumab eye drops in healthy dogs, derived from commercially available bevacizumab for intravenous use and diluted in sterile saline. The results showed that topical bevacizumab at a concentration of 2.5 mg/ml, administered twice a day over four weeks, was well tolerated. This finding allowed us to use topical bevacizumab in dogs with naturally occurring corneal vascularization refractory to standard therapy. A reduction in corneal vascularization, most notably as a significant decrease in vessel diameter, a reduction in distal vessel branching, an improvement in corneal edema and inflammatory cell infiltration, and a marked improvement in corneal clarity was observed. However, a French bulldog developed a corneal erosion and two Chihuahuas suffering from preexisting mitral valve insufficiency died during the treatment-free observation period. A causal relationship with the drug was considered unlikely. Nevertheless, dogs prone to corneal erosions and neurotrophic disease, as well as preexisting cardiovascular disorders, should be selected with caution (Kim et al. 2008, Yu et al. 2008a, Dong et al. 2017).

This PhD work demonstrated the capability of bevacizumab and aflibercept to bind canine VEGF and the potential of topical bevacizumab to reduce persistent corneal vascularization in dogs.

Overall, this PhD project provided an important step for future clinical use of therapeutic VEGF inhibitors in neovascular ocular disorders in animals.

2. Introduction

2.1. Corneal transparency and the impact of corneal blindness

The ability to see is considered the most important sensory perception (Scott et al. 2016). Visual impairment and blindness have a major impact on the quality of life and mental health (Alma et al. 2011, Renaud and Bédard 2013). Blindness due to a corneal disorder is one of the leading causes of vision loss (Whitcher et al. 2001, Bourne et al. 2018). Normally, the cornea is clear and acts as the major refractive interface of the eye, which is essential for transmission of light and the visual perception (Sun et al. 2005, 2011, Werkmeister et al. 2017).

The transparency of the cornea is based on its unique biological and structural properties, including a highly ordered hierarchical arrangement of the collagen-rich extracellular matrix (ECM) of the stroma (Crispin and Barnett 1983). The major stromal ECM components are dense, regularly packed, small diameter collagen fibrils (Crispin and Barnett 1983). A subtly balanced interaction between collagen fibrils, keratocytes, and ECM components like type V collagen and proteoglycans are essential for maintaining the regular collagen fibril growth, stromal extracellular matrix assembly, and water content within the cornea (Sun et al. 2011, Chen et al. 2014, 2015). Any alteration of this intricate system can result in deficiencies in corneal clarity and refraction (Chen et al. 2007, Sun et al. 2011).

In addition, corneal transparency is maintained by the nonkeratinized corneal epithelium moisturized by the preocular tear film, the deturgescence accomplished by the corneal epithelium and endothelium, and the absence of blood and lymphatic vessels within the cornea (Crispin and Barnett 1983, Bonanno 2012, Werkmeister et al. 2017).

In almost all mammalian species, the normal adult cornea is avascular, except for a capillary arched network at the limbus (Crispin and Barnett 1983, Werkmeister et al. 2017). Corneal angiogenesis can lead to a severe corneal opacities due to corneal edema, pigmentation, lipid deposition, and may result in persistent corneal inflammation and loss of the corneal immune privilege (Fig. 1) (Dana and Streilein 1996, Cursiefen et al. 2004a, Chung et al. 2009, Dubielzig et al. 2010, Gelatt et al. 2013a, Werkmeister et al. 2017).

Figure 1. Corneal vascularization can lead to severe complications and visual impairment in animal patients. (a) Pacman frog with keratoconus, corneal ulceration, and vascularization (b) Egyptian sand gecko with chronic keratitis, severe corneal scarring and pigmentation, and anterior synechia (c) Chihuahua with superficial immune-mediated keratitis associated with corneal vascularization and edema (d) Persian cat with corneal sequestrum, surrounded by corneal blood vessel infiltration (e) Domestic shorthaired cat with conjunctival flap (f) Leopard gecko with corneal pigmentation and lipid deposition (g) Tiger python with exophthalmos, defective spectaculum, chronic keratitis, and corneal vascularization (h) Holsteiner horse with immune-mediated keratitis associated with vascular sprouting and severe corneal edema.

2.2. Molecular pathways of angiogenesis

Angiogenesis describes the highly complex process of blood vessel growth from pre-existing vascular networks (Gerhardt et al. 2003, Chung and Ferrara 2011). Normally, the mammalian cornea is free of blood vessels (Crispin and Barnett 1983, Werkmeister et al. 2017). This avascular state of the cornea is actively maintained by a balance of anti- and proangiogenic factors and mechanisms (Cursiefen et al. 2004b, 2006, Ambati et al. 2006, Matsui et al. 2012, Zakaria et al. 2012).

2.2.1. Angiogenic factors

Some of the most important angiogenic and antiangiogenic factors will be addressed below, focusing on the role of the vascular endothelial growth factor (VEGF) and its interlinked agonists and antagonists.

Vascular endothelial growth factors represent a group of signal molecules and compromises five members, VEGF-A, VEGF-C, VEGF-D, and the placenta growth factors (PIGF) (Ferrara 2009). Additionally, viral VEGF-E and reptilian VEGF-F have been identified (Ogawa et al. 1998, Suto et al. 2005, Yamazaki et al. 2005).

The members of the VEGF family bind to three different tyrosine kinase cell surface receptors, the vascular endothelial growth factor receptors (VEGFR)-1, VEGFR-2, and VEGFR-3 (Park et al. 1994, Olofsson et al. 1998, Gerhardt et al. 2003, Gan et al. 2004, Jia et al. 2004). Vascular endothelial growth factor A binds to VEGFR-1 and VEGFR-2 (Gerhardt et al. 2003, Jia et al. 2004), PIGF and VEGF-B to VEGFR-1 (Park et al. 1994, Olofsson et al. 1998), and VEGF-C and VEGF-D to VEGFR-3 (Jia et al. 2004, Cursiefen et al. 2006, Chung et al. 2009).

Vascular endothelial growth factor receptor 3 is a major regulator of lymphangiogenesis (Cursiefen et al. 2004a, Goyal et al. 2010, Han et al. 2014, Du and Liu 2016). However, VEGFR-3 signaling via VEGF-C and -D is involved in angiogenesis, too (Chung et al. 2009). For example, corneal VEGF-C and -D implants are capable to induce corneal vascularization via VEGFR-3 and activate the recruitment of macrophages, which in turn secrete VEGF-A (Chung et al. 2009).

The function of VEGFR-1 is not yet fully understood and it is controversially debated whether VEGFR-1 has a more pro- or antiangiogenetic function (Park et al. 1994, Cursiefen et al. 2004a, Ambati et al. 2006, Li et al. 2011). Studies demonstrated that VEGFR-1 signaling amplifies inflammatory hem- and lymphangiogenesis, induced mainly by VEGF-A and to a presumably lesser extent by PIGF (Park et al. 1994, Cursiefen et al. 2004a). In a study with mice lacking VEGFR-1, significantly decreased VEGF values were detected, resulting in an inhibition of tumor angiogenesis and growth (Li et al. 2011). This conflicts with the hypothesis that VEGFR-1 has an inhibitory or trap function and does negatively regulate VEGFR-2 signaling (Ambati et al. 2006). This is supported by the observation that VEGFR-1 is not detectable in corneas of Florida and Antillean manatees, the only mammalian species known to have physiological corneal vascularization (Ambati et al. 2006). In contrast, dugongs, African, and Asian elephants, the closest extant terrestrial relatives of manatees, do exhibit corneal VEGFR-1 and do not have vascularized corneas (Ambati et al. 2006).

Vascular endothelial growth factor receptor 2 exerts its effects via VEGF-A (Gerhardt et al. 2003, Jia et al. 2004). Vascular endothelial growth factor A is a glycosylated, disulfide-linked homodimer and VEGF-A-driven VEGFR-2 signaling is postulated to be the dominant inducer of both pathological and physiological angiogenesis (Shalaby et al. 1995, Amano et al. 1998, Gerhardt et al. 2003, Gan et al. 2004, Jia et al. 2004, Eming and Krieg 2006, Pieh et al. 2008). This is supported by the finding that mice embryos deficient in VEGFR-2 have significant deficiencies in vasculogenesis (Shalaby et al. 1995). The VEGF-A-driven VEGFR-2 activation regulates the development of the vascular system by promoting vascular endothelial cell migration, mitosis, and survival (Jia et al. 2004) and by guiding angiogenic sprouting (Gerhardt et al. 2003). Additionally, VEGF-A is essentially required for the normal wound healing and corneal nerve regeneration (Amano et al. 1998, Eming and Krieg 2006, Yu et al. 2008a), induces blood vessel dilation, and increases vessel tortuosity and permeability (Edelman et al. 2005). In ocular disorders, VEGF-A is thought to be one of the key originators of pathological corneal, intraocular, and retinal angiogenesis (Aiello et al. 1994, Mastyugin et al. 2001, Gan et al. 2004, Haines et al. 2006, Pieh et al. 2008, Wuest and Carr 2010, Yun et al. 2020).

Human VEGF-A is encoded by the VEGF-A gene and due to alternative splicing, at least nine human VEGF-A isoforms exist ($VEGF_{121}$, $VEGF_{145}$, $VEGF_{148}$, $VEGF_{162}$, $VEGF_{165}$, $VEGF_{165b}$, $VEGF_{183}$, $VEGF_{189}$, $VEGF_{206}$) (Tischer et al. 1991). In the canine species, five VEGF isoforms

were identified ($VEGF_{120,}$ $VEGF_{144}$, $VEGF_{164}$, $VEGF_{182}$, $VEGF_{188}$) (Scheidegger et al. 1999, Jingjing et al. 2000). The most frequently expressed isoforms are $VEGF_{120}$, $VEGF_{164}$, and $VEGF_{188}$ in dogs and $VEGF_{121}$ and $VEGF_{165}$ in humans (Ballaun et al. 1995, Scheidegger et al. 1999, Jingjing et al. 2000).

The reason for the complexity of VEGF-A isoforms is not yet fully understood. Isoforms differ in their biological properties, their way to interact with VEGFR-2 and neuropilins (NP), and the binding affinity to heparin (Houck et al. 1992, Park et al. 1993, Soker et al. 1998, Lee et al. 2005). The isoforms $VEGF_{189}$ and $VEGF_{206}$ have two heparin-binding domains and are bound to the extracellular matrix (ECM) or cell surfaces (Houck et al. 1992, Park et al. 1993). The isoform $VEGF_{121}$ has a low affinity to bind heparin and is soluble (Houck et al. 1992). As $VEGF_{165}$ has only one heparin-binding domain, it is both soluble and ECM bound (Park et al. 1993). Processing of the C-terminus of VEGF isoforms by matrix metalloproteinases convert the bound to a soluble form, thereby affecting the bioavailability of VEGF (Lee et al. 2005). Some isoforms of VEGF-A can bind to the co-receptors NP-1 and NP-2, thereby potentiating their effect (Soker et al. 1998). Mice expressing only $VEGF_{120}$ show insufficiently developed vascular branching (Carmeliet et al. 1999, Ruhrberg et al. 2002), whereas mice exhibiting only $VEGF_{188}$ suffer from excessive branching and thin, disorganized blood vessels (Maes et al. 2004). Thus, the balance of these VEGF isoforms is essential for the normal development of the vasculature system and changes in VEGF-A isomer composition can lead to devastating implications for the physiological vasculogenesis and organogenesis (Carmeliet et al. 1999, Ruhrberg et al. 2002, Maes et al. 2004).

Besides VEGF, a plenty of pro- and antiangiogenetic factors and mechanisms are involved in the highly complex process of angiogenesis (Chung and Ferrara 2011). Within the scope of this work, only the interacting, enhancing, or influencing mechanisms of VEGF-A with those factors will be addressed.

Matrix metalloproteinases (MMPs) are a large group of zinc-dependent metalloproteinases (Sivak et al. 2004, Lee et al. 2005, Mimura et al. 2009, Ebrahem et al. 2010, Rajashekhar et al. 2014, Han et al. 2015, Du and Liu 2016, Park et al. 2018). A variety of functions are attributed to this group of molecules, including pro- and antiangiogenic properties (Lee et al. 2005,

Mimura et al. 2009, Ebrahem et al. 2010, Han et al. 2015, Du and Liu 2016). Angiogenesis describes the growth of new blood vessels from preexisting vasculature and sprouting into the cornea (Gerhardt et al. 2003, Chung and Ferrara 2011). However, the invasion of endothelial cells requires degradation of ECM structures which is induced by MMPs (Mimura et al. 2009). Additionally, MMPs affect corneal angiogenesis through an interplay with various cytokines, signaling molecules, growth- and transcription factors, including VEGF functions in multiple antagonistic and agonistic ways (Sivak et al. 2004, Lee et al. 2005, Ebrahem et al. 2010, Rajashekhar et al. 2014, Han et al. 2015). In ocular surface disorders, MMP-9 is known to be overexpressed and MMP-2 levels are increased in vascularized corneas (Shi et al. 2010, Park et al. 2018). Matrix metalloproteinases are able to increase VEGF values by releasing sequestered VEGF and cleavage of the C-Terminus of the heparin-binding domains of VEGF (Lee et al. 2005, Ebrahem et al. 2010). Experimental animal studies demonstrated that the angiogenic response of MMP-9-induced corneal neovascularization can be suppressed by the use of VEGF inhibitors (Ebrahem et al. 2010). Conversely, VEGF-induced corneal vascularization can be repressed by MMP inhibitors, indicating a mutually potentiating proangiogenic effect of VEGF and MMP-9 in the course of corneal vascularization (Ebrahem et al. 2010). The transmembrane MMP-14, whose effect on angiogenesis is still under debate, is capable to cleave VEGFR-1 into a 59.8 kDa fragment that binds $VEGF_{165}$, thereby having a VEGF-trapping mechanisms and an antiangiogenic effect (Han et al. 2015). In mice with suture-induced inflammatory corneal vascularization, MMP-14 was found to promote lymphangiogenesis via the VEGF-C/VEGFR-3 signaling pathway (Du and Liu 2016). In addition, it is known that via the VEGF/$\alpha v\beta 5$ pathway, MMP-14, MMP-2, and various types of integrins are expressed on newly formed corneal blood vessels during experimentally induced, corneal vascularization (Zhang et al. 2002).

Integrins are transmembrane proteins that have crucial functions in mediating between the ECM and cells (Senger et al. 2002, Chen et al. 2007, Muether et al. 2007). In murine models of corneal vascularization, integrin $\alpha_5\beta_1$ was increased and its inhibition resulted in a regression of corneal blood vessels (Muether et al. 2007). A significantly reduced vascular response was observed in mice lacking the $\alpha 1\beta 1$ integrin receptor follwing corneal allograft transplantation (Chen et al. 2007). Furthermore, integrins $\alpha_1\beta_1$ and $\alpha_2\beta_1$ are known to be involved in VEGF-driven

angiogenesis in the skin of mice (Senger et al. 2002). Zhang et al. showed that in rats with alkaline-burn-induced corneal angiogenesis, integrins $\alpha_v\beta_5$, $\alpha_1\beta_1$, and $\alpha_2\beta_1$ expression correlated with corneal vascularization and the author suggested that this was driven via the VEGF pathway (Zhang et al. 2002).

Fibroblast growth factors (FGF) are potent angiogenetic mediators (Gualandris et al. 1996, Zhang et al. 2001, Kano et al. 2005, Murakami et al. 2011, Gurung et al. 2018). Studies found that corneal inflammatory cells and injured corneal stroma itself secrete FGFs (Gualandris et al. 1996) and both FGF-2 and VEGF-A have been reported to promote and sustain corneal vascularization in herpetic keratitis (Gurung et al. 2018). Fibroblast growth factors possess a stimulatory effect on vascular endothelial cells via tyrosine kinase FGF receptors, similar to VEGF (Zhang et al. 2001). There is a collaborative interaction and interdependence of FGF and VEGF, as FGF-2 acts synergistically with VEGF-A on neoangiogenesis (Kano et al. 2005). Murakami et al. found that suppression of FGF leads to a reduced expression of VEGFR-2, resulting in loss of vascular integrity and an impaired formation of the vascular morphology in-vivo (Murakami et al. 2011).

Corneal inflammation and angiogenesis are interwoven, which is underlined by a close interplay of VEGF-A, macrophages, and interleukins (Il) (Cursiefen et al. 2004a, Ebrahem et al. 2006, Chung et al. 2009). Vascular endothelial growth factor A is capable to chemoattract immune cells (Cursiefen et al. 2004b, Chung et al. 2009). In turn, macrophages that are attracted to the injured or inflamed corneal tissue, potentiate the angiogenic effect by secretion of further VEGF-A (Cursiefen et al. 2004a, Chung et al. 2009). In addition, interleukins like Il-1β and Il-6 can promote corneal vascularization through the VEGF-A pathway (Ebrahem et al. 2006).

The proangiogenic factors are counterbalanced by a plethora of antiangiogenetic factors that are substantial in the regulation of corneal avascularity (Chung and Ferrara 2011). The key mechanisms, particularly in relation to VEGF-A, will be addressed below.

2.2.2. Antiangiogenic factors

There are various mechanisms that maintain corneal avascularity, such as the soluble VEGFR-1, which is postulated to act as an endogenous VEGF-A trap (Ambati et al. 2006). Additionally, there are negative feedback mechanisms that prevent profuse angiogenesis. For example, VEGF-A activates the delta-like ligand 4 (DLL4) in tip cells, cells at the tips of vascular sprouts that have an important role in the angiogenesis process, and DLL4 decreases the VEGFR-2 expression via the notch signaling pathway in stalk cells (Lobov et al. 2007). This negative feedback loop is in turn inversely regulated by other notch ligands, such as Jagged-1, that decrease the signaling in adjacent tip cells (Benedito et al. 2009).

Beyond these mechanisms, there are antiangiogenic factors that are essential for the maintenance of corneal vascularity, such as thrombospondin (TSP), pigment epithelium-derived factor (PEDF), endostatin, and angiostatin.

Thrombospondins are ECM-bound glycoproteins with multiple functions, including preserving the avascular ambit of the cornea and corneal wound healing (Cursiefen et al. 2011, Matsuba et al. 2011, Blanco-Mezquita et al. 2013). Studies demonstrated that corneas of mice deficient in TSP-1 have an impaired corneal wound healing, persistent opacity (Blanco-Mezquita et al. 2013), and develop profuse lymphangiogenesis (Cursiefen et al. 2011). It is thought that the lymphangiogenic effects are caused by TSP binding to CD36 on corneal macrophages, thereby decreasing the levels of lymphangiogenic VEGF-C and -D but not VEGF-A (Cursiefen et al. 2011). In addition, TSP-1 and TSP-2 inhibit several angiogenic factors, such as MMP-2 and MMP-9 (Bein and Simons 2000).

Pigment epithelium-derived factor is a glycoprotein that is localized in multiple ocular tissues and functions as an important inhibitor of corneal angiogenesis (Matsui et al. 2012, Eslani et al. 2017). In vascularized corneas of rats, the administration of synthetic PEDF yielded in a reduction of corneal vascularization and a decrease of VEGF values (Matsui et al. 2012).

Endostatin and angiostatin are proteins with antiangiogenic properties (Hanai et al. 2002, Kim et al. 2002, Sharma et al. 2004). Angiostatin and endostatin have multiple effects on vascular

endothelial cells, such as the induction of apoptosis and the inhibition of proliferation and migration (Hanai et al. 2002, Sharma et al. 2004). In vascularized rodent corneas, subconjunctivally applied endostatin leads to a suppression of corneal angiogenesis (Li et al. 2015) and has an indirect inhibitory effect on VEGF by interacting with VEGFR-2 (Kim et al. 2002)

The maintenance of corneal avascularity is based on the counterbalance of angiogenic and antiangiogenic factors (Cursiefen et al. 2000, 2004b, Mastyugin et al. 2001, Gan et al. 2004, Chen et al. 2012). However, this balance is considered to be redundantly regulated (Cursiefen et al. 2004b). This means that avascularity is not automatically lost in the absence of one of the antiangiogenic factors and that a threshold exists up to which angiogenesis does not occur even under mild angiogenic stimuli (Cursiefen et al. 2004b). However, events like injury, inflammation, or hypoxia may cause the threshold to be exceeded and tip the scales to the angiogenic side (Mastyugin et al. 2001, Gan et al. 2004, Goyal et al. 2010, Shi et al. 2010, Lutty et al. 2011, Chen et al. 2012, Park et al. 2018).

2.2.3. Sequelae of corneal angiogenesis

When the sprouting of blood vessels into the cornea occurs, undesirable effects may result, including decreased corneal clarity, edema, and deposition of pigments, crystals, and lipids (Figure 2) (Bedford and Longstaff 1979, Nasisse et al. 1998, Williams 2008, Dreyfus et al. 2011, Benayoun et al. 2012, Laguna et al. 2015, Narimatsu et al. 2019, Costa et al. 2021).

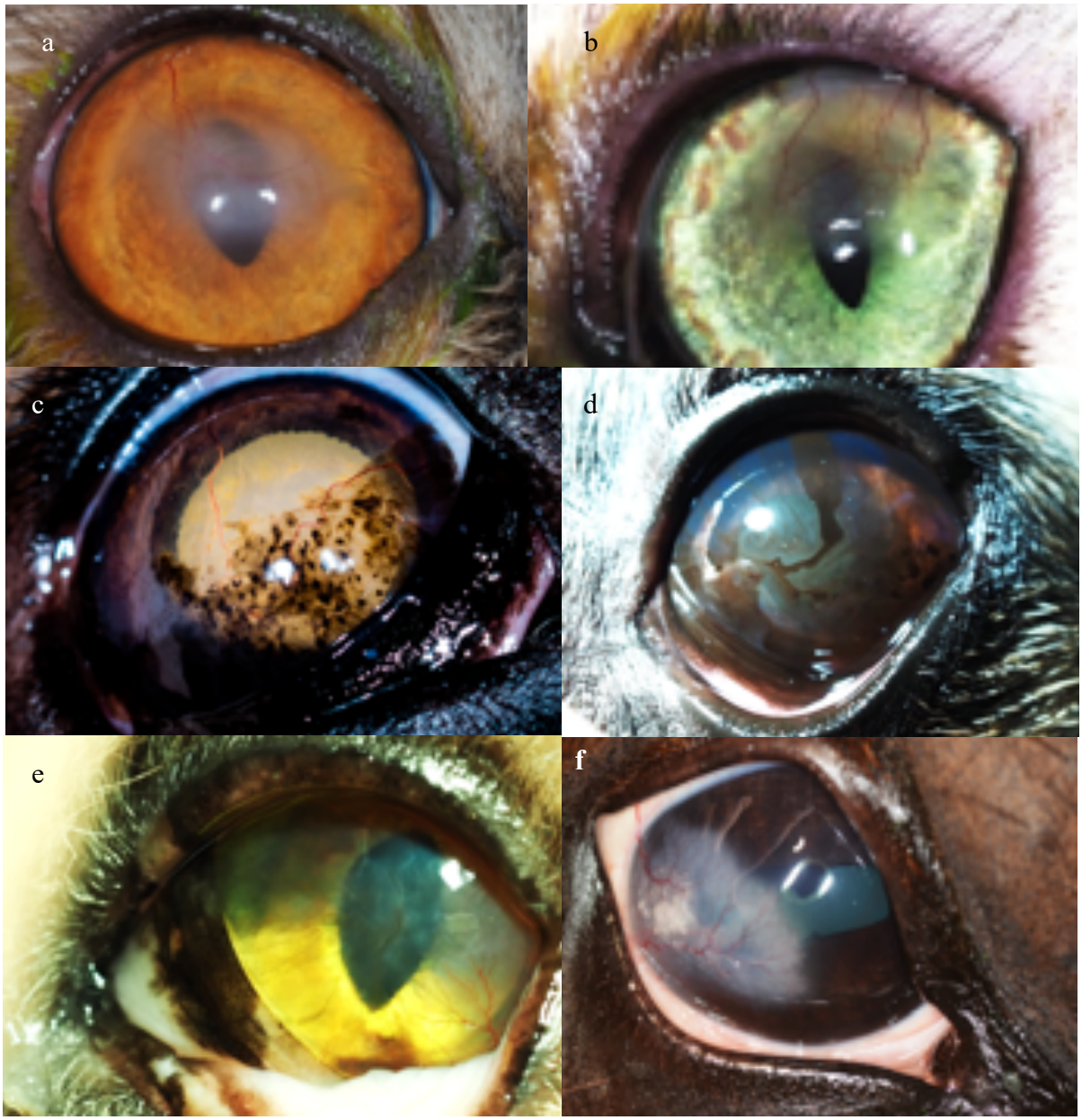

Figure 2. Undesired effects of corneal vascularization can lead to a decrease of corneal transparency and visual impairment. (a) Domestic shorthaired cat with corneal vascularization and edema (b) Persian cat following corneoconjunctival transposition due to a corneal sequestrum, showing corneal vascularization, edema, and pigmentation (c) German shepherd dog with chronic superficial keratitis associated with corneal pigmentation over more than half of the corneal surface (d) Pug with severe corneal pigmentation involving the entire corneal surface, resulting in significant visual impairment (e) Domestic shorthaired cat with corneal vascularization and deposition of pigment within the cornea (f) Quarter horse with invasive squamous cell carcinoma, showing corneal vascularization, corneal neoplastic infiltration, and deposition of lipids and calcium.

Besides these clinically obvious consequences of corneal vascularization, persistent blood vessels within the cornea constitute the risk of continuous inflammation and loss of the corneal immune privilege (Dana and Streilein 1996, Yamagami et al. 2002). Immune-privileged tissues are sites, where the insertion of foreign tissue can survive for a prolonged time, whereas similar transplants in conventional tissues are rejected (Streilein 2003). This becomes visible in clinical practice when tissue from bladder or pericardium is successfully used as corneal donor graft in animal patients (Featherstone et al. 2001, Balland et al. 2016). The underlying mechanisms of the ocular immune privilege are based on the anterior chamber-associated immune deviation, the immunosuppressive microenvironment of the eye, and anatomical, cellular, and molecular barriers (Dana and Streilein 1996, Streilein et al. 1997). The corneal immune privilege is essential to maintain the corneal functions and clarity and enables the cornea to self-regulate inflammatory responses (Dana and Streilein 1996). Corneal vascularization impairs the corneal immune privilege, which is of great clinical importance, as the risk for corneal graft rejections is significantly increased in vascularized corneas (Yamagami et al. 2002, Bachmann et al. 2010). There are no studies in veterinary medicine assessing a direct association between corneal graft rejection and corneal vascularization (McMullen et al. 2015, Lacerda et al. 2017). However, ophthalmologists recommend to treat corneal vascularization prior to surgery (Featherstone et al. 2001, Brooks et al. 2008, Lacerda et al. 2017, Hos et al. 2019).

2.3. Pathological angiogenesis in the eye

2.3.1. Molecular insights: The role VEGF in ocular pathological vascularization

The understanding of the relationship of VEGF and ocular neovascular disorders has been driven significantly by the investigations of ocular retinal disorders (Aiello et al. 1994, Pieh et al. 2008, Lutty et al. 2011). Retinopathy of prematurity (ROP) is a blinding condition in premature infants (Pieh et al. 2008) and was studied in a ROP-like model in newborn dogs (Lutty et al. 2011). In this experimental model, dogs were exposed to high levels of oxygen, which resulted in a stop of vasculogenesis and vasoobliteration. Subsequently, the dogs were returned to normal room air and as a response to the deficiently vascularized inner retina, a

pronounced vasoproliferation phase followed. Following intravitreal therapeutical VEGF inhibitors, a reduction of retinal vascularization was observed in these dogs (Lutty et al. 2011). Pieh et al. found that ROP infants had significantly increased levels of soluble VEGFR-2 in the plasma (Pieh et al. 2008). Interestingly, systemic VEGF-A concentrations were similar between ROP and healthy children, suggesting a local VEGF-A production in the retina in ROP (Pieh et al. 2008). Besides ROP, there is a variety of blinding retinal disorders in humans where VEGF appears to have a causal role and are treated routinely with therapeutic VEGF inhibitors, such as neovascular age-related macular degeneration and diabetic macular edema (Avery et al. 2006, Arevalo et al. 2007, Schmidt-Erfurth et al. 2014, Dugel et al. 2020).

The causal association of VEGF in corneal vascularization in humans, mice, and rabbits is well studied, too (Cursiefen et al. 2000, Mastyugin et al. 2001, Gan et al. 2004, Chen et al. 2012, Yun et al. 2020). In a rabbit model of hypoxic corneal vascularization induced by closed eye contact lens wear, a 12-fold expression of VEGF mRNA compared to baseline was described (Mastyugin et al. 2001). Corneal hypoxia can induce corneal angiogenesis via VEGF-A expression through the hypoxia-inducible factor-1 α (HIF-1α) (Chen et al. 2012). In humans, VEGF-A is immunohistochemically detectable in endothelial, stromal, intravascular inflammatory, and basal corneal epithelial cells in tissue samples of corneal vascularization, (Cursiefen et al. 2000) and is strongly expressed by corneal vascular endothelial cells in rabbits following corneal alkali burn (Gan et al. 2004). A recent study on herpetic keratitis indicated that herpes-infected cells themselves actively secrete VEGF-A, thereby promoting corneal angiogenesis (Yun et al. 2020).

In veterinary ophthalmology, knowledge about VEGF and its role during ocular diseases in dogs, cat, and horses are sparse.

A major contribution to enhance the understanding of VEGF in horses was made by Deeg and colleagues, who studied equine recurrent uveitis (ERU), a leading cause for equine blindness. To date, the etiopathogenesis and treatment of ERU is still controversially discussed (Gerding and Gilger 2016). In an equine model of uveitis, Deeg et al. showed that retinal vessels and Mueller glial overexpress VEGF (Deeg et al. 2006). Immunohistochemical findings revealed that VEGF was detectable in the retina of ERU eyes and was associated with an increase of

PEDF (Deeg et al. 2007). Additionally, VEGF was 19-fold higher in the aqueous humor of horses with ERU compared to healthy and non-ERU uveitis eyes (Curto et al. 2016).

In dogs, besides the already discussed canine animal models in ROP, there are only single studies which assessed the causative role of VEGF during ocular diseases. Abrams et al. found that VEGF is higher in the aqueous humor compared to plasma in diabetic dogs (Abrams et al. 2011). Anyway, there was no detectable difference between VEGF values in the aqueous humor between diabetic and non-diabetic dogs. Interestingly, another study demonstrated a significant increase of VEGF in a variety of intraocular canine disorders, except for diabetic cataracts (Sandberg et al. 2012). Thus, it is hypothesized that the lack of VEGF increase in diabetic dogs represents a protective mechanism against diabetic retinopathy, which is a widespread blinding condition in diabetic humans (Abrams et al. 2011).

Pre-iridal fibrovascular membranes (PIFMSs) are a feared complication of ocular diseases in animal patients due to their association with synechiae, glaucoma, and hyphema (Pfeiffer et al. 1990). In dogs that underwent cataract surgery, PIFMs are one of the leading causes of enucleation (Moore et al. 2003). An immunohistochemical analysis demonstrated a pronounced presence of VEGF in canine PIFMs associated with intraocular inflammatory and neoplastic disorders (Zarfoss et al. 2010). These findings are consistent with the observation of significant increased VEGF values in the aqueous humor of dogs with PIFMs compared to normal eyes (Sandberg et al. 2012). Thus, a causative relationship between PIFMs and VEGF is postulated (Zarfoss et al. 2010, Sandberg et al. 2012) and VEGF inhibition may be an interesting treatment option.

Data on VEGF and its association during corneal diseases in animal patients are sparse. Binder et al. found that VEGF receptors are immunohistochemically detectable in both normal and vascularized canine corneas, highlighting that VEGF has both physiological and pathological functions in the canine cornea (Binder et al. 2012). In dogs with chronic superficial keratitis (CSK), plasma VEGF values are significantly increased and a causal relationship is suggested (Balicki and Sobczyńska-Rak 2014).

2.3.2. Clinical insights: Corneal vascularization in animal patients

There are no overall epidemiological data on the prevalence of corneal blindness or corneal vascularization in dogs. Nevertheless, persistent corneal blood vessels are a common finding in veterinary ophthalmology (Andrew et al. 1998, Murphy et al. 2001, Andrew 2008, Dean and Meunier 2013, Lassaline et al. 2015, Plummer 2015, Hindley et al. 2016, Ledbetter et al. 2016, Krecny et al. 2018). In the following, some of the diseases accompanied by corneal vascularization will be discussed, with emphasis on the species dog and on disorders that are the subject of this PhD work.

2.3.2.1. Keratoconjunctivitis sicca

One of the main causes of canine corneal vascularization is a disturbance of the precorneal tear film, which is termed keratoconjunctivitis sicca (KCS) in veterinary medicine and dry eye disease (DED) in human beings (Kaswan and Salisbury 1990, Glaze 2005, Hendrix 2005, Lemp et al. 2007, Sanchez et al. 2007, Labetoulle et al. 2018).

Keratoconjunctivitis sicca describes a chronic inflammatory disease of the ocular surface and is an umbrella term for a variety of different causes, manifestations, and disease characteristics (Kaswan and Salisbury 1990, Herrera et al. 2007, Sanchez et al. 2007, Williams 2008). In cats and horses, KCS is rare (Glaze 2005, Hendrix 2005). In contrast, a prevalence of up to 27 % has been reported in dogs, with a large number of undiagnosed cases likely leading to an even higher percentage (Gemensky-Metzler et al. 2015).

In canine KCS, a distinction is made between hereditary, primary, immune-mediated and secondary or acquired KCS (Kaswan and Salisbury 1990), with immune-mediated KCS occurring most frequently (Sanchez et al. 2007). Usually, canine KCS occurs bilaterally and both the quality and quantity of tears can be disturbed (Kaswan and Salisbury 1990, Krecny et al. 2018). The disease typically manifests as purulent ocular discharge, discomfort, conjunctival hyperemia, and chemosis (Fig. 3a) (Kaswan and Salisbury 1990, Herrera et al. 2007, Sanchez et al. 2007). Corneal edema, tissue damage, vascularization, and pigmentation are frequent complications and ulceration up to corneal perforation may occur (Kaswan and Salisbury 1990, Herrera et al. 2007, Sanchez et al. 2007). In addition, there is a positive interrelation between

chronic keratitis and corneal squamous carcinoma, with strongest correlation to keratoconjunctivitis sicca in brachycephalic dogs (Dreyfus et al. 2011).

On the molecular level, dry eyes have a disturbed ocular surface osmolarity that impairs the immunohaemostasis and activates a complex cascade of inflammatory events (Luo et al. 2005). These include an increased release of autoreactive T-cells, the activation of innate immune mechanisms like toll-like receptor and natural killer cells, and an upregulation of proinflammatory cytokines and chemokines (De Paiva et al. 2007, Goyal et al. 2010, 2012). The resulting corneal inflammation is driven by infiltration of cells of the CD4+ T-cell compartment (De Paiva et al. 2007). As discussed earlier, corneal inflammation and angiogenesis can enhance and sustain each other (Cursiefen et al. 2004a, Ebrahem et al. 2006, Chung et al. 2009). In DED, VEGF-A, VEGF-C, VEGF-D, and VEGFR-3 are increased (Goyal et al. 2010), and VEGF-A is capable to chemoattract immune cells that in turn secrete further VEGF-A (Cursiefen et al. 2004a, Chung et al. 2009). Thus, vascularization and inflammation persist, which results in corneal epithelial damage, loss of goblet cells, pathological alteration of the mucin layer, tear film instability, and hyperosmolarity of the ocular surface (Cursiefen et al. 2004a, Chung et al. 2009, Goyal et al. 2010, 2012, Alam et al. 2020).

Due to the high incidence and disease burden, novel treatment approaches are the topic of current eye research and the therapeutic inhibition of VEGF-A, VEGF-C, and VEGF-D is described as an effective treatment option in human DED (Goyal et al. 2012, Jiang et al. 2018, Cursiefen et al. 2019, Kasetsuwan et al. 2020). In dogs, current treatment options include lifelong topical and/or systemic immunosuppressants, lubricants, and lubricant stimulants (Kaswan and Salisbury 1990, Morgan and Abrams 1991, Ofri et al. 2009, Hendrix et al. 2011). The proven standard treatment is topical cyclosporine and tacrolimus, pimecrolimus, or glucocorticoids may be indicated as a second-line therapy (Nell et al. 2005, Ofri et al. 2009, Hendrix et al. 2011, Labetoulle et al. 2018, Kallab et al. 2020). Topical cyclosporine in dogs with KCS results in a significant decrease of inflammatory cells of the ocular surface (Izci et al. 2015) and increases the tear film quantity (Morgan and Abrams 1991, Nell et al. 2005, Ofri et al. 2009, Hendrix et al. 2011). However, in the presence of advanced lacrimal gland fibrosis (Kaswan and Salisbury 1990) or neurogenic KCS (Matheis et al. 2012), topical cyclosporine may be ineffective and the ability to repress blood vessels is still under debate (Bock et al. 2014,

Villar et al. 2020). Overall, a reported number of 25 - 50 % of dogs with immune-mediated KCS do not response to cyclosporine therapy (Morgan and Abrams 1991, Ofri et al. 2009). Therefore, novel therapeutic approaches targeting both angiogenesis and inflammation are needed, especially as the prevalence of KCS in dogs is increasing and untreated KCS can lead to corneal blindness (Kaswan and Salisbury 1990).

2.3.2.2. Immune-mediated Keratitis

Chronic superficial keratitis (*CSK)* is a progressive, incurable, bilateral disorder of the cornea in dogs (Bedford and Longstaff 1979). German shepherd dogs and German shepherd dog mixes are overrepresented (Bedford and Longstaff 1979). An immune-mediated cause combined with a genetic component and ultraviolet light is suspected (Campbell et al. 1975, Slatter et al. 1977). The disease is characterized by corneal blood vessel infiltration, raised vascularized granulation tissue, deposition of pigment, crystals and lipids, and corneal inflammatory infiltrates (Bedford and Longstaff 1979, Nell et al. 2005). Typically, the signs begin at the temporal limbus. From there, the corneal changes may involve the entire corneal surface (Fig. 3b) (Bedford and Longstaff 1979, Nell et al. 2005). The disease is incurable and can lead to bilateral, irreversible blindness (Slatter et al. 1977, Bedford and Longstaff 1979, Nell et al. 2005).

Studies found that CSK occurs more often in dogs exposed to intense solar radiation and a stronger cellular hypersensitivity to corneal and iris antigens has been shown in CSK affected dogs (Campbell et al. 1975, Slatter et al. 1977). The cornea possesses tissue-specific antigens that may be modified by external factors like ultraviolet light. Ultraviolet light and hypoxia induces an increase of HIF-1α and VEGF expression through the phosphatidylinositol 3-kinase (PI3K)-Akt signaling pathway (Li et al. 2006). The PI3K-Akt pathway is a major regulator of both normal and cancer angiogenesis (Karar and Maity 2011) and the occurrence of corneal squamous cell carcinomas following CSK is reported (Dreyfus et al. 2011). Balicki and Sobczyńska-Rak demonstrated that dogs with CSK have increased plasma VEGF values and this observation was significant in dogs during early disease (Balicki and Sobczyńska-Rak 2014).

Current treatment options for CSK include topical dexamethasone, cyclosporine, tacrolimus, and radiotherapy (Nell et al. 2005, Allgoewer and Hoecht 2010, Balicki 2012, Gelatt et al.

2013b). The use of UV light-blocking contact lenses used to slow down disease progression has been described but was associated with ocular side effects (Denk et al. 2011). Unfortunately, current treatment options are often ineffective and corneal changes are progressive despite therapy (Bedford and Longstaff 1979, Nell et al. 2005, Gelatt et al. 2013b). Additionally, the younger the dog is at onset, the more severe is the disease progression (Gelatt et al. 2013b). In milder courses, the inflammatory component might improve under topical long-term immunosuppressive therapy, but corneal blood vessels often persist (Bedford and Longstaff 1979, Nell et al. 2005). Thus, therapeutical approaches that directly target corneal angiogenesis and the underlying causal mechanisms are needed. Vascular endothelial growth factor might be an interesting target in CSK, as a causal relationship is speculated and an association to VEGF and UV light-induced angiogenesis exists (Li et al. 2006, Balicki and Sobczyńska-Rak 2014).

Superficial punctate keratitis (SPK*)* describes a bilateral corneal inflammation frequently observed in Shetland sheep dogs and longhaired dachshunds (Nasisse 1995, Andrew 2008). The disease is often associated with allergies or autoimmune diseases and an immune-mediated genesis is suspected (Andrew 2008). In the acute phase, SPK manifests as bilateral, superficial keratitis with multiple, punctate corneal erosions (Nasisse 1995, Andrew 2008). The lesions can develop into recurrent, subepithelial corneal ulcers that may extend deep into the corneal stroma (Andrew 2008). The disease occurs in episodes and with each new episode, there is an increased risk for corneal vascularization, tissue scarring, and corneal deposition of crystals and pigmentation (Andrew 2008). In advanced stages, complete loss of vision may occur (Gelatt et al. 2013c). Therapy consists of local administration of cyclosporine and/or corticosteroids (Nasisse 1995, Clerc and Jegou 1996, Andrew 2008). In most cases, there is a rapid clinical improvement, but after treatment discontinuation, recurrencies may occur (Gelatt et al. 2013c). Therefore, lifelong therapy and frequent follow-up examinations may be required to find the optimal, safe, low-dose steroid maintenance therapy (Gelatt et al. 2013c).

2.3.2.3. Infectious keratitis

Infectious keratitis is a vision-threatening ocular disease in animal patients (Tolar et al. 2006, De Linde Henriksen et al. 2014, Hindley et al. 2016, Suter et al. 2018, Auten et al. 2020).
In dogs, any previous damage to the cornea can result in secondary bacterial infection, which can cause serious deteriorations in corneal health and severe complications like melting ulcers are common (Fig. 3c) (Tolar et al. 2006, Hindley et al. 2016, Suter et al. 2018, Auten et al. 2020). In more than half of the dogs with ulcerative keratitis, bacteria are detectable in the samples, with Gram-positive predominating over Gram-negative bacteria (Tolar et al. 2006, Hindley et al. 2016, Suter et al. 2018, Auten et al. 2020). Brachycephaly, previous surgical eye procedures, disorders of the ocular surface, and prior use of topical steroids increase the risk of bacterial keratitis (Tolar et al. 2006, Hindley et al. 2016, Suter et al. 2018, Auten et al. 2020). Topical antibiotics are the standard of care treatment of bacterial-induced keratitis (Tolar et al. 2006, Hindley et al. 2016). Anyway, persistent corneal vascularization is common and may cause impaired corneal clarity (Tolar et al. 2006, Hindley et al. 2016). A recent study in a murine model of Pseudomonas aeruginosa-induced keratitis found that corneal angiogenesis and lymphangiogenesis correlated positively with an early VEGF-A and a late VEGF-C and VEGFR-3 upregulation (Narimatsu et al. 2019). There are isolated case reports of the use of VEGF inhibitors for persistent corneal vascularization in bacterial keratitis describing a beneficial effect in suppressing corneal vascularization (Benayoun et al. 2012, Hos et al. 2019).

In dogs, fungal keratitis is a rare disease compared to other animal species (Ledbetter et al. 2016). Although rare, canine fungal keratitis may be associated with corneal vascularization and can lead to serious corneal morbidity and loss of vision (Ledbetter et al. 2016).
Fungal ulcerative keratitis is frequently observed in horses and is characterized by a severe course of disease (Andrew et al. 1998, De Linde Henriksen et al. 2014). Interestingly, a negative association between VEGF-A and the diagnosis of fungal keratitis in horses has been observed (De Linde Henriksen et al. 2014). Equine fungal ulcers tend to perforate rapidly and are characterized by a delayed wound healing with a lack of corneal vascular sprouting (Andrew et al. 1998, De Linde Henriksen et al. 2014). It is assumed that certain fungi are capable to produce antiangiogenic metabolites (Welch et al. 2000) and that a reduced VEGF-A expression is the reason for the delayed healing (De Linde Henriksen et al. 2014).

Similar to humans, herpetic keratitis is a common disorder in animal patients, in particular in cats and horses (Nasisse et al. 1998, Kershaw et al. 2001, Dean and Meunier 2013). In humans, HSV-1-induced keratitis has been intensively studied (Wuest and Carr 2010, Wuest et al. 2011, Yun et al. 2020). Findings demonstrated that HSV-1 infected cells, like CD4+ and myeloid cells, produce pathogenic VEGF-A levels in the cornea and can directly activate VEGF-A transcription. In cats, eosinophilic keratitis (EK) represents a common corneal disorder associated with various degrees of corneal vascularization (Fig. 3d) (Nasisse et al. 1998, Dean and Meunier 2013). Feline herpes virus (FHV)-1 is postulated to have a causal function during EK (Nasisse et al. 1998, Dean and Meunier 2013). The disease requires lifelong topical immunosuppressive therapy, frequent re-examinations, and is often associated with persistent corneal vascularization refractory to standard therapy (Spiess et al. 2009, Dean and Meunier 2013). In addition, FHV-1 is thought to be involved in the etiopathogenesis of feline corneal sequestrum, a common disease in cats that can lead to severe corneal health impairment and even corneal perforation (Fig. 3e) (Nasisse et al. 1998, Laguna et al. 2015).

In mature dogs, keratitis caused by canine herpesvirus-1 (CHV-1) and corneal vascularization is rare (Ledbetter 2013). However, it is discussed whether CHV-1 is an underestimated condition.

There are no studies that investigated the relationship of VEGF and herpetic keratitis in animal patients. In human herpetic keratitis, clinical studies indicated that VEGF inhibitors might represent a promising treatment approach (Koenig et al. 2009, Krizova et al. 2014, Hos et al. 2019). Since herpetic keratitis is a common disorder in animals that is often refractory to standard therapy (Dean and Meunier 2013), and even if not yet proven, a causal relationship between VEGF and herpetic keratitis is conceivable, inhibition of VEGF would be an interesting treatment approach in this indication.

2.3.2.4. Ulcerative keratitis and superficial chronic corneal defects

The sprouting of blood vessels into the cornea may develop in the course of any kind of corneal ulcers and is initially part of the normal healing process (Fig. 3f) (Amano et al. 1998, Murphy et al. 2001, Eming and Krieg 2006, Hindley et al. 2016, Eaton et al. 2017, Wu et al. 2018).

A frequently observed ulcer type that is not secondary to another underlying primary ocular disease are superficial chronic corneal defects (SCCED), with a breed disposition for boxer dogs (Murphy et al. 2001, Wu et al. 2018, Hung et al. 2020). Superficial chronic corneal defects are characterized by loose epithelial tissue at the defect margins, frequently do not heal on conventional conservative therapy, and can be accompanied by corneal vascularization (Murphy et al. 2001, Chandler et al. 2010, Eaton et al. 2017, Wu et al. 2018, Hung et al. 2020). The underlying pathomechanism is not yet fully understood. Murphy et al. found that affected dogs show abnormal hyperinnervation of the subepithelial stroma and epithelium at the defect margins (Murphy et al. 2001). Is it assumed that degenerated, dying, or metabolically impaired epithelial cells secrete neurotrophic factors (Murphy et al. 2001). In this way, the body may attempt to provide increased amounts of neurotransmitters and neuropeptides through hyperinnervation to promote tissue repair (Murphy et al. 2001), as corneal sensory nerves and neurotransmitters have a crucial corneal trophic function (Müller et al. 2003, Wang et al. 2012). The current proven method of therapy is a diamond burr debridement (DBD) and, in the absence of tear deficiencies, the insertion of a bandage lens (Wu et al. 2018, Hung et al. 2020). In about 70 % of cases, the erosions heal after one DBD procedure and in refractory or recurrent cases, further DBD sessions or keratotomy may be required (Wu et al. 2018, Hung et al. 2020). In addition to a DBD, topical and/or systemic tetracycline and doxycycline treatment is recommended, and the use of protease inhibitors and substance P is conceivable (Murphy et al. 2001, Chandler et al. 2010, Eaton et al. 2017, Wu et al. 2018). However, a DBD may be associated with severe adverse events, such as infectious keratitis and keratomalacia (Wu et al. 2018, Hung et al. 2020). Some dogs develop severe corneal scarring, fibrosis, pigmentation, and persistent vascularization (Murphy et al. 2001, Eaton et al. 2017, Wu et al. 2018).

2.3.2.5. Corneal disorders caused by anatomical malformations

Breeding measures that fulfill the desire for a very prominent brachycephaly, steep forehead, and prominent skin folds, are associated with significant injury to the ocular surface, including chronic ulcerations, inflammation, and in severe cases blindness or loss of the globe (Fig. 3e and Fig. 3g)(Dreyfus et al. 2011, Labelle et al. 2013, Plummer 2015, Hindley et al. 2016, Lacerda et al. 2017, Costa et al. 2021). Additionally, pugs are predisposed to a meibomian gland

dysfunction and tear film abnormalities, which in turn disrupts corneal health further and increases the risk of bacterial keratitis, corneal vascularization, and ulceration (Krecny et al. 2018). An impairment of corneal nerve fiber density is postulated in brachycephalic dogs and cats, which predisposes them for impaired corneal wound healing (Kafarnik et al. 2008).
The major therapeutic approach consists of the surgical correction of the eyelid malformations and to improve the tear film status (Nell et al. 2005, Plummer 2015). However, blood vessels may remain and complications, such as persistent inflammation and progressive pigmentation of the cornea, are common (Plummer 2015, Krecny et al. 2018). For these reasons, safe and targeted therapies against corneal inflammation and angiogenesis are needed.

2.3.2.6. Corneal neoplasms

Tumor growth and the development of metastases are dependent on the formation of blood vessels (Liang et al. 2017). Vascular endothelial growth factor has been identified as one of the dominant inducers of tumor angiogenesis in mammalian species (Waldner et al. 2010, Peterson et al. 2016, Liang et al. 2017). Vascular endothelial growth factor is expressed by a variety of cells in dogs suffering from canine lymphoma (Wolfesberger et al. 2007). Plasma levels of VEGF was found to be significantly higher in high-grade compared to low-grade T-cell lymphomas, making VEGF a conceivable future biomarker and possible treatment target for canine lymphoma (Aresu et al. 2014). In addition to lymphoma, an association with VEGF has been reported in a variety of other canine tumors, including breast and prostate carcinomas, soft tissue sarcomas, and mastocytomas (Rebuzzi et al. 2007, Queiroga et al. 2011, Adelfinger et al. 2015).
However, there are only few studies investigating the association of VEGF in canine ocular neoplasms (Binder et al. 2012, Sandberg et al. 2012, Edelmann et al. 2013). In humans, VEGF, VEGFR-1, and VEGFR-2 expression is increased in uveal melanomas, retinoblastomas, uveal adenomas, and choroidal melanomas (Stitt et al. 1998, Sahin et al. 2007). In dogs, VEGF was detected in the aqueous humor of few cases of intraocular benign and malignant melanomas, adenomas, and lymphomas (Sandberg et al. 2012). In an immunohistochemical study, the expression of VEGF receptors of canine intraocular disorders was assessed, revealing that

VEGFR-1 was expressed in all sampled intraocular tumors and VEGFR-2 was detected in only malignant and metastatic intraocular neoplasms (Binder et al. 2012).

There are single case reports of subconjunctival or topical applied VEGF inhibitors in humans with corneal squamous cell carcinomas (Faramarzi and Feizi 2013, Asena and Altınörs 2015). In dogs and cats, primary corneal neoplasms are rare (Haeussler et al. 2011, Leis et al. 2017). However, malignant neoplasms of the eyelids in cats and limbal squamous cell carcinomas in horses are frequently observed and novel treatment strategies are required (Fig. 3h) (Newkirk and Rohrbach 2009, Lassaline et al. 2015, Bellone et al. 2017) There is only one case report in veterinary medicine that described the successful intralesional anti-VEGF bevacizumab therapy in a palpebral sebaceous carcinoma relapse of an Amur tiger (Edelmann et al. 2013). Thus, drugs that inhibit VEGF may be a future treatment option for ocular neoplasms in animal patients.

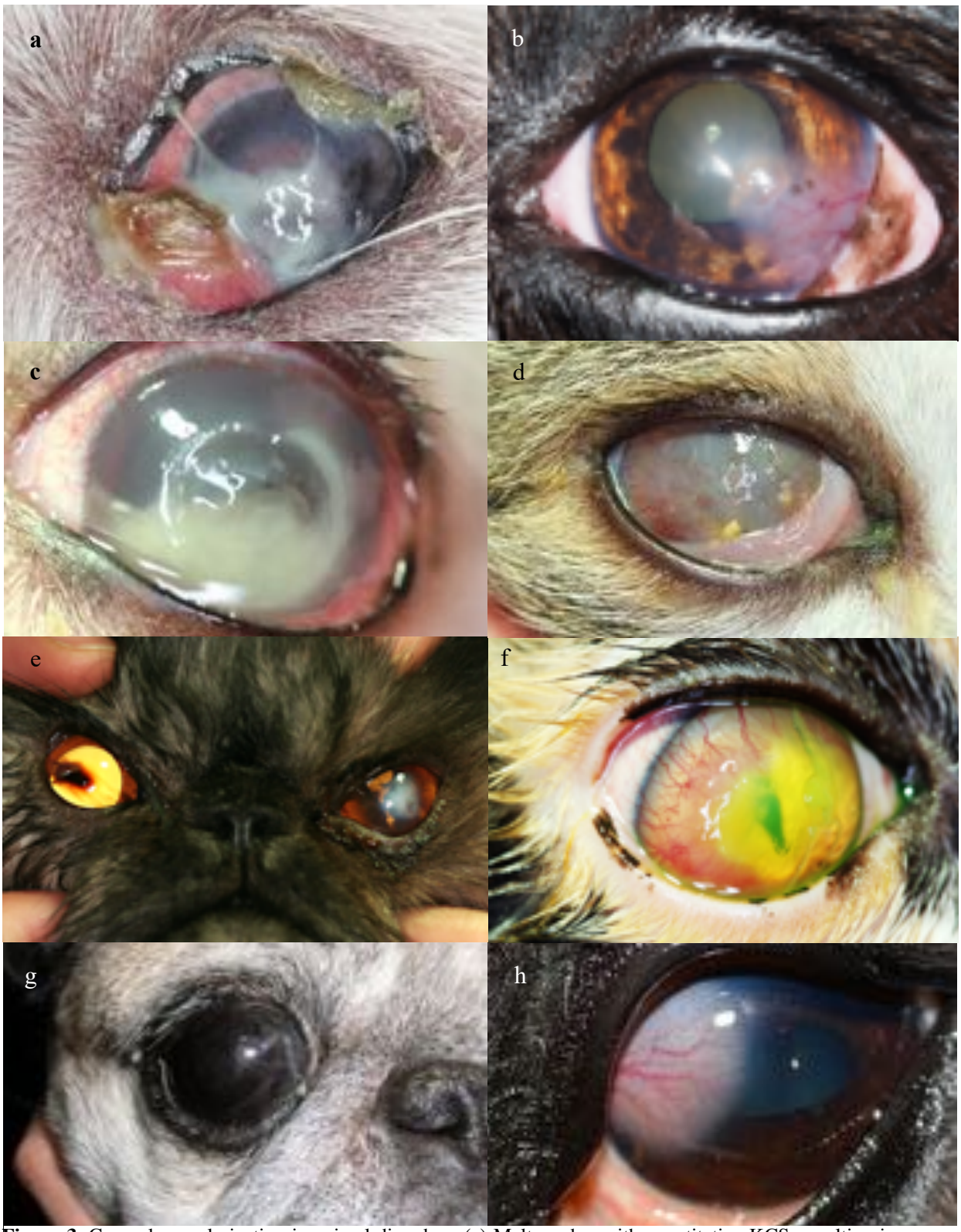

Figure 3. Corneal vascularization in animal disorders. (a) Maltese dog with quantitative KCS, resulting in severe injury to the cornea and conjunctiva (b) German pointer with CSK associated with blood vessel infiltration, corneal edema, and pigmentation (c) Chihuahua with melting ulcer (d) Domestic shorthaired cat with eosinophilic keratitis associated with corneal blood vessel infiltration (e) Persian cat with brachycephalic ocular syndrome, corneal sequestrum OD, and perforation and anterior synechia after corneal sequestrum rejection OS (f) Persian cat with corneal ulcer with severe corneal vascularization (g) Pug with entropion of the lower eyelid, macroblepharon, KCS, and corneal blindness due to severe corneal pigmentation (h) Appaloosa mare with corneal stromal invasive squamous cell carcinoma.

2.4. The dilemma: Treatment options for corneal vascularization in dogs

There are various options to treat corneal vascularization, including medical therapies, radiation, and surgical procedures.

Cyclosporine is routinely used for the treatment of canine corneal vascularization, particularly during tear film disorders or when topical steroids pose a major risk (Kaswan and Salisbury 1990, Morgan and Abrams 1991, Herrera et al. 2007, Hendrix et al. 2011, Villar et al. 2020). The usage of topical cyclosporine is debated, as the drug is a potent T-cell inhibitor and potentially increases the risk of opportunistic herpetic or fungal infection (Kaswan and Salisbury 1990, Dowling et al. 2016) and was found to delay corneal wound healing (Villar et al. 2020). Furthermore, it is discussed whether cyclosporine has no or only insufficient inhibitory effects on corneal vascularization (Bucak et al. 2013, Bock et al. 2014, Villar et al. 2020).

Topical glucocorticoids may be indicated in some cases of corneal vascularization (Bedford and Longstaff 1979, Andrew 2008, Kallab et al. 2020). The exact antiangiogenic mode of action is complex. It results from a variety of mechanisms, such as the inhibition of proliferation of lymphatic endothelial cells and proinflammatory cytokines, by interfering with VEGF-driven angiogenesis via the mifepristone-sensitive steroid receptor, and the reduction of VEGF-induced vascular leakage (Robin et al. 1985, McNatt et al. 1999, Edelman et al. 2005, Ebrahem et al. 2006, Hos et al. 2011). However, steroids are considered to suppress corneal blood vessels only incompletely and are known to carry a variety of risks (Tolar et al. 2006, Dowling et al. 2016, Hindley et al. 2016, Murtagh et al. 2018, Sebbag et al. 2020). Possible complications are the secondary colonization with opportunistic pathogens, an impaired wound healing, and the development of corneal perforations or melting ulcers (Tolar et al. 2006, Dowling et al. 2016, Hindley et al. 2016, Murtagh et al. 2018). Moreover, a recent study showed that topical prednisolone in dogs reaches the systemic blood circulation and systemic side effects are conceivable (Sebbag et al. 2020). Thus, clinicians have to calculate between treatment benefits and potential risks when using glucocorticoids (Tolar et al. 2006, Dowling et al. 2016, Hindley et al. 2016, Murtagh et al. 2018, Sebbag et al. 2020).

Besides glucocorticoids, non-steroidal anti-inflammatory drugs (NSAID) are used to treat corneal vascularization, in particular when the lower risk of side effects must be considered (Rigas et al. 2020). However, NSAIDs can be associated with corneal melting and the response to therapy may be insufficient (Pakneshan et al. 2008, Murtagh et al. 2018, Rigas et al. 2020). In human medicine, various efficient alternatives to medical treatment are described, such as argon laser (Gerten 2008), Nd:YAG laser (Kumar et al. 2016), photodynamic therapy (Hou et al. 2017), and fine needle diathermy (Pillai et al. 2000, Spiteri et al. 2015). However, complications of laser therapy are reported including corneal hemorrhage, damage of iridal tissue, corneal crystalline deposits, and inadvertent retinal photocoagulation (Pillai et al. 2000, Spiteri et al. 2015, Kumar et al. 2016). In healthy dogs, usage of Nd-YAG laser caused ulcerative keratitis and nerve fiber damage (Weigt et al. 2002). The use of radiotherapy in dogs suffering from severe CSK has been described as a treatment option to delay disease progression (Allgoewer and Hoecht 2010). Anyway, in animal patients general anesthetic or a deep sedation would be necessary to perform these procedures and may be unfeasible for clinical use in veterinary practice.

2.4.1. Novel therapies for corneal vascularization: VEGF inhibitors

The European Vision Institute defined corneal vascularization as a highly relevant ocular topic that needs to be addressed in near-term future research (Cursiefen et al. 2019). The discovery of drugs that inhibit VEGF has revolutionized human ophthalmology and numerous studies have proven their potential to reduce pathological vascularization in the eye (Arevalo et al. 2007, Kim et al. 2008, Dastjerdi et al. 2009, Koenig et al. 2009, Lutty et al. 2011, Nork et al. 2011, Krizova et al. 2014, Dugel et al. 2020, Sahni et al. 2020). Therapeutic VEGF inhibitors vary in molecular size, structure and charge, as well as binding capability to VEGF family members and binding affinity to VEGF-A (Presta et al. 1997, Holash et al. 2002, Avery et al. 2006, Genentech Inc. 2011, Papadopoulos et al. 2012, Tietz et al. 2015). Bevacizumab and aflibercept, two of the best studied VEGF inhibitors, are widely used for various neovascular eye disorders in humans (Avery et al. 2006, Arevalo et al. 2007, Korobelnik et al. 2014, Schmidt-Erfurth et al. 2014, Olmos et al. 2016).

Bevacizumab is a humanized murine anti-VEGF monoclonal antibody with a molecular weight of 149 kDa and an isoelectric point (pI) of 8.3 that binds to all isoforms of human VEGF-A (Presta et al. 1997, Vlčková et al. 2008, Genentech Inc. 2011). Bevacizumab was engineered by site-directed mutagenesis of a human antibody framework and murine anti-human VEGF monoclonal antibody A.4.6.1 and consists of amino acid sequences of 93 % to human IgG1 and of 7 % to murine antibody (Presta et al. 1997, Rodrigues et al. 2009). The use of monoclonal antibodies has become a fast growing class of drugs with a wide field of application in humans (Ferrara et al. 2004, Rodrigues et al. 2009). Therapeutic monoclonal antibodies exert their therapeutic effects by inhibiting receptors through interaction with their ligands or by binding to specific soluble ligands, thereby blocking their cellular signaling pathways (Presta et al. 1997, Muller et al. 1998, Ferrara et al. 2004, Rodrigues et al. 2009).

Bevacizumab is approved for various human cancers (Genentech Inc. 2011) and is used off-label for the treatment of ocular conditions associated with pathological vascularization, such as neovascular age-related macular edema, diabetic edema, and neovascular glaucoma (Avery et al. 2006, Dastjerdi et al. 2010b, Olmos et al. 2016). In ophthalmology, bevacizumab is administered primarily via the intravitreal administration route for neovascular disorders of the posterior eye segment (Avery et al. 2006, Arevalo et al. 2007, Olmos et al. 2016). The topical administration of bevacizumab is debated, as the intact corneal epithelium is not permeable for large molecules (Mun et al. 2014). Indeed, in-vivo studies found that topical bevacizumab is not able to permeate the intact corneal epithelium, whereas subconjunctivally injected bevacizumab gets into the cornea (Dastjerdi et al. 2011, Moisseiev et al. 2014). However, in vascularized corneas, studies demonstrated that topical bevacizumab and aflibercept are capable to penetrate into the corneal stroma (Yoeruek et al. 2008, Dastjerdi et al. 2011, Sella et al. 2016).

Bock et al. were among the first to investigate the effect of topical and subconjunctival bevacizumab in mice with suture-induced corneal vascularization (Bock et al. 2007). They found that the use of 5 mg/ml QID (quarter in die, four times a day) bevacizumab for five days resulted in a significant suppression of hem- and lymphangiogenesis. The angioregressive effect observed by Bock et al. was confirmed in subsequent studies in rats and rabbits, using various drug concentrations, administration routes, and treatment durations (Dastjerdi et al. 2010a, Ozdemir et al. 2014, Sella et al. 2016).

Based on the promising results obtained in these experimental animal studies, topical bevacizumab was explored in human beings affected by corneal vascularization. For this purpose, bevacizumab eye drops were prepared by diluting commercial available intravenous bevacizumab in 0.9 % saline (Kim et al. 2008, Koenig et al. 2009) or 0.01 % benzalkonium chloride (Dastjerdi et al. 2009, Cheng et al. 2012) and were applied in different treatment regimes, including 2.5 mg/ml BID (bis in die, two times a day) for two weeks (Krizova et al. 2014), 5 mg/ml BID for up to twelve weeks (Koenig et al. 2009), 10 mg/ml BID/QID for three weeks (Dastjerdi et al. 2009, Cheng et al. 2012), and 12.5 mg/ml BID for three months (Kim et al. 2008).

Overall, the results indicated that topical bevacizumab has the potential to prevent further progression of outgrowing blood vessels into the cornea, reduce corneal blood vessel diameter, and decrease the extent of corneal vascularization (Kim et al. 2008, Dastjerdi et al. 2009, Koenig et al. 2009, Cheng et al. 2012, Krizova et al. 2014).

Aflibercept is a humanized recombinant dimeric glycoprotein with a molecular weight of 115 kDA and a pI of 8.82 (Holash et al. 2002). It is engineered Holash et al. by fusing the second binding domain auf VEGFR-1 and the third binding domain of VEGFR-2 to the Fc portion of human IgG1. Thus, aflibercept acts as a soluble decoy receptor and is called „VEGF-Trap“. Aflibercept binds with high affinity to all human VEGF-A isoforms, VEGF-B, and PIGF (Papadopoulos et al. 2012). Additionally, Aflibercept is able to bind murine VEGF-A and PIGF (Papadopoulos et al. 2012).

The topical use of 0.01-0.1 % aflibercept in corneal vascularization in experimental animals is proven to be effective in suppressing hem- and lymphangiogenesis (Sella et al. 2016, Devarajan et al. 2019). Results on the efficacious use of aflibercept for corneal vascularization have recently been published and are conflicting (Aksoy 2019, Cholak et al. 2020, Sella et al. 2021). In one case report, a child with persistent corneal vascularization unresponsive to glucocorticoids was treated with aflibercept eye drops (Aksoy 2019). The treatment regime included topical aflibercept (50 μl per application, derived from of a 40mg/ml solution) three times a day for seven days and yielded in complete regression of corneal blood vessels without any side effects. In another case report, an adult woman with severe corneal vascularization due to an ocular surface burn received a single subconjunctival injection of 4 mg aflibercept,

resulting in a decrease of corneal vascularization without any adverse events (Cholak et al. 2020). However, most recently Sella et al. reported that a single subconjunctival injection of 2 mg aflibercept in patients with formed corneal vascularization did not lead to a reduction of corneal vascularization (Sella et al. 2021).

There are no reports of clinical applicability and compatibility in dogs, cats, or horses for either bevacizumab or aflibercept. Due to the high incidence of corneal vascularization as a blinding condition and the partly insufficient treatment options, therapeutic VEGF inhibition may represent a novel approach for the treatment of corneal vascularization in animals.

2.5. Hypotheses

Persistent corneal vascularization unresponsive to standard therapies is a great challenge in veterinary ophthalmology (Dean and Meunier 2013, Lassaline et al. 2015, Plummer 2015). The persistence of corneal blood vessels results in continuous corneal inflammation and an impairment of corneal health and clarity, thereby representing a leading cause for corneal blindness. Current treatment options may be insufficient and can bear the risk of side effects (Tolar et al. 2006, Bock et al. 2014, Dowling et al. 2016, Hindley et al. 2016, Villar et al. 2020). In human ophthalmology, therapies that target VEGF are promising (Dastjerdi et al. 2009, Koenig et al. 2009). Due to the high need for targeted treatment options in canine corneal vascularization, therapeutical VEGF inhibitors are of great interest.

The purpose of this PhD thesis was to investigate the binding ability of VEGF inhibitors bevacizumab and aflibercept with canine, feline, and equine VEGF. Furthermore, the PhD project aimed to assess the tolerability of topical bevacizumab in healthy dogs and the safety and efficacy in dogs affected by persistent corneal vascularization.

The following hypotheses of this PhD study were defined:

Project 1

a) Bevacizumab and Aflibercept are capable to bind canine, feline, and equine VEGF.

Project 2

b) Topical bevacizumab (2.5 mg/ml, BID, over four weeks) is topically and systemically safe in healthy dogs.

c) Topical bevacizumab (2.5 mg/ml, BID, over four weeks) does not increase arterial blood pressure, does not alter coagulation and blood parameters, and does not decrease serum VEGF values in healthy dogs.

Project 3

d) Topical bevacizumab (2.5 mg/ml, BID, over four weeks) is topically and systemically safe in dogs with persistent corneal vascularization.

e) Topical bevacizumab (2.5 mg/ml, BID, over four weeks) leads to a decrease of vascularized area in dogs with persistent corneal vascularization.

3. Manuscripts

- Muellerleile LM, Buxbaum B, Nell B; Fux DA. In-vitro binding analysis of anti-human vascular endothelial growth factor antibodies bevacizumab and aflibercept with canine, feline, and equine vascular endothelial growth factor. 2019. Research of Veterinary Science, 124:233-238.

- Muellerleile LM, Tichy A, Nell B. Serum vascular endothelial growth factor changes and safety after topical anti-human VEGF antibody bevacizumab in healthy dogs. 2019. Veterinary Ophthalmology, 22(5):600-606.

- Muellerleile LM, Bernkopf M, Wambacher M, Nell B. 2021. Topical bevacizumab for the treatment of corneal vascularization in dogs: A case series. Veterinary Ophthalmology.

  [Article submitted, March 2021; submitted journal version for peer review]

Research in Veterinary Science 124 (2019) 233–238

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

In-vitro binding analysis of anti-human vascular endothelial growth factor antibodies bevacizumab and aflibercept with canine, feline, and equine vascular endothelial growth factor

Lisa-Marie Muellerleile[a,*,1], Bernhard Buxbaum[a,1], Barbara Nell[a], Daniela A. Fux[b]

[a] Department for Companion Animals and Horses, University of Veterinary Medicine Vienna, Austria
[b] Department for Biomedical Sciences, Institute of Pharmacology and Toxicology, University of Veterinary Medicine Vienna, Austria

ARTICLE INFO

Keywords:
Bevacizumab
Aflibercept
VEGF-A
Anti-VEGF
Species cross-reactivity

ABSTRACT

Purpose: Promising results have been described for antibodies binding vascular endothelial growth factor (VEGF) in patients with corneal neovascularization. Whether veterinary patients would also benefit from this therapeutic approach has not been investigated yet. We examined binding properties of anti-human VEGF antibodies bevacizumab (Avastin®) and aflibercept (Zaltrap®) for canine, feline, and equine VEGF.
Methods: Human, equine, feline, and canine VEGF were analyzed for sequence similarity using the "Basic Local Alignment Search Tool" (BLAST). Western-blot analysis and ELISA were used to assess binding properties.
Results: BLAST analysis revealed a sequence homology of canine, feline, and equine VEGF to human VEGF-A of 93%, 92%, and 89%, respectively. Western-blot analysis showed immunoreactivity of bevacizumab with human, canine, and feline VEGF, but not with equine VEGF. Aflibercept recognized VEGF of all tested species. ELISA data indicated that bevacizumab and aflibercept bind canine VEGF in a dose-dependent manner. Feline VEGF was bound by bevacizumab and aflibercept in a dose-independent manner. ELISA study further confirmed the lack of bevacizumab binding to equine VEGF, and yielded also a dose-independent binding by aflibercept.
Conclusions: Bevacizumab and aflibercept turned out to bind VEGF with species-specific differences. Further studies are required to investigate their efficacy and safety under clinical conditions.

1. Introduction

Corneal diseases in dogs, cats, and horses like keratoconjunctivitis sicca, superficial pigmentary keratitis, and chronic superficial keratitis are frequently accompanied by corneal neovascularization (CNV) (Labelle et al., 2013; Sanchez et al., 2007; Slatter et al., 1977). Although neovascularization is essentially required for corneal wound repair and prevents stromal melting (Conn et al., 1980), CNV also leads to undesired events like persistent inflammation, loss of the immune privilege, and visual impairment through corneal edema, tissue scarring, and lipid deposition (Dana and Streilein, 1996; Epstein et al., 1987; Maddula et al., 2011). To combat CNV several therapeutic options are considered. Beside different surgical approaches, topical application of corticosteroids, cyclosporine, and non-steroidal anti-inflammatory drugs are often used to suppress CNV (Dean and Meunier, 2013; Gilger et al., 2005; Nell et al., 2005; Spiess et al., 2009; Williams et al., 1995). However, these drugs show variable efficacy and various adverse side effects (Gaarder et al., 1998; Kaswan and Salisbury, 1990; Nasisse et al., 1989; Petroutsos et al., 1982). As none of these drugs selectively addresses the process of corneal neovascularization, more effective and targeted treatment approaches are still required for veterinary patients.

A promising therapeutic approach for CNV in human patients is the topical or subconjunctival application of monoclonal anti-vascular endothelial growth factor (VEGF) antibodies such as bevacizumab (Avastin®) and aflibercept (Zaltrap®). Bevacizumab is a humanized murine monoclonal antibody (Presta et al., 1997) and is reported to be effective in treating ocular neoangiogenesis during wet age-related macular degeneration, diabetic macular edema, and macular edema secondary to retinal vein occlusion (Arevalo et al., 2007; Avery et al., 2006; Martin et al., 2012). Moreover, bevacizumab is reported to be effective in patients with naturally occurring corneal neovascularization (Dastjerdi et al., 2009; Kim et al., 2008; Krizova et al., 2014). The

* Corresponding author at: Lisa-Marie Muellerleile, Department of Companion Animals and Horses, University of Veterinary Medicine Vienna, Veterinaerplatz 1, A-1210 Vienna, Austria.
E-mail addresses: lisa-marie.muellerleile@vetmeduni.ac.at (L.-M. Muellerleile), barbara.nell@vetmeduni.ac.at (B. Nell), daniela.fux@vetmeduni.ac.at (D.A. Fux).
[1] The first and second authors contributed equally to this work.

https://doi.org/10.1016/j.rvsc.2019.03.018
Received 28 August 2018; Received in revised form 20 February 2019; Accepted 21 March 2019

VEGF-trap aflibercept is a recombinant fusion protein of components of the VEGF receptors VEGFR-1 and VEGFR-2, and the constant region (Fc) of human IgG1 (Holash et al., 2002). Compared to other anti-VEGF drugs aflibercept shows the highest affinity to human VEGF (Papadopoulos et al., 2012). Aflibercept appears to be more efficacious than bevacizumab in improving visual acuity in patients with diabetic macular edema (Wells et al., 2016) and age-related macular degeneration (The Diabetic Retinopathy Clinical Research Network, 2015). Intravitreal injection of aflibercept (Eylea®) is approved for the treatment of wet age-related macular degeneration, macular edema following retinal vein occlusion, diabetic macular edema, but is also reported to be efficient in vascular glaucoma and myopic choroidal neovascularization (Brown et al., 2015; Heier et al., 2014; Ikuno et al., 2015; SooHoo et al., 2015).

As central driver of vessel formation, VEGF is also involved in CNV seen in veterinary patients (Binder et al., 2012; Henriksen et al., 2014). However, data concerning the use and efficacy of bevacizumab and aflibercept in animals are limited. Single experiments showed that topical or subconjunctival application decreases or prevent corneal neovascularization in rats (Ozdemir et al., 2014; Sella et al., 2016), which implies that VEGF from non-human species may be also scavenged by these antibodies. Cross-reactivity of aflibercept is also postulated for mouse and rabbit VEGF, whereas bevacizumab interaction was only reported for non-human primate VEGF (Papadopoulos et al., 2012). However, whether dogs, cats, and horses – most common patients in veterinary ophthalmology - would also benefit from bevacizumab and aflibercept is unclear. The current in-vitro study was designed to assess the binding reactivity of these anti-VEGF antibodies with canine, feline, and equine VEGF.

2. Materials and methods

2.1. Reagents

Recombinant human $VEGF_{165}$ (CAT 293-VE), canine (CAT # 1603-CV/CF) and feline VEGF (CAT # 5844-CV) were obtained from R&D Systems, Minneapolis, USA; equine VEGF (CAT # RP0252D-025) was from Kingfisher, St Paul, USA. Bevacizumab (25 mg/ml; Avastin®) was purchased from Roche (Switzerland, Basel) and aflibercept (25 mg/ml; Zaltrap®) from Sanofi (France, Paris). Mouse anti-human IgG1 Fc Secondary Antibody (CAT # MH1015) was from ThermoFischer, (Waltham, USA), and the horseradish-peroxidase conjugated anti-mouse IgG (Cat#7076S) from Cell Signaling Technology® (Cambridge, United Kingdom). The Canine (Cat # CAVE00) and Human VEGF Quantikine ELISA Kits (Cat # DVE00) were purchased from R&D Systems (Minneapolis, USA); the Equine VEGF-A VetSet ELISA Development Kit (CAT # VS0409E-002) was obtained from Kingfisher (St Paul, USA); the Feline Nori VEGF ELISA Kit (Cat # GR188042) from Genorise Scientific (Pennsylania, USA).

2.2. Protein sequence analysis

Protein sequences of human (NCBI NP_003367.4), equine (XP_023479773.1), feline (XP_023109318.1) and canine VEGF-A (AAD29684.1) were analyzed by using the Basic Local Alignment Search Tool (BLAST) algorithm (BLASTP2.8.0 +) (Altschul et al., 1997). The multiple sequence alignment was performed by the usage of the T-COFFEE Multiple Sequence Alignment Server (Notredame et al., 2000).

2.3. Immunoblotting

Recombinant human, canine, feline, and equine VEGF (1 µg) was solved in non-reducing sample buffer (62.5 mM Tris–HCl, pH 6.8, 10% glycerol, 2% SDS, 0.1% bromophenol blue), subjected to 15% SDS-PAGE and transferred to polyvinylidene fluorid (PVDF) membrane (Thermo Fisher Scientific, Illinois, USA, CAT #88585). Membranes were incubated in StartingBlock™ PBS Blocking Buffer (Thermo Fisher Scientific, Illinois, USA, CAT # 37538) for 1 h at room temperature, washed three times with Tris-buffered saline containing 0.1% Tween-20 (TBS/T) and incubated with bevacizumab (1:10000 in TBS/T) or aflibercept (1:10000 in TBS/T) for 24 h at 4 °C. Subsequently, membranes were washed with TBS/T and incubated with anti-human immunoglobulin G (IgG) FC secondary antibody (1:1000) for 1 h at room temperature. Afterwards membranes were washed again with TBS/T and incubated with horseradish-peroxidase conjugated anti-mouse IgG (1:3000) for 1 h at room temperature. After washing with TBS/T, membranes were incubated with Clarity™ Western ECL Substrate (Bio-Rad Laboratories, Inc., California, USA) and immunoreactivity was detected by using ChemiDoc™ XRS + and ImageLab™ Software (Bio-Rad Laboratories, Inc., California, USA).

2.4. VEGF binding assay

Quantitative binding of bevacizumab and aflibercept to VEGF were investigated by using commercially available, species-specific ELISA kits. Canine, human, feline, and equine VEGF with an end concentration of 2.5 nM were incubated without and with different concentrations of bevacizumab (ranging from 0.54–33.6 µM; corresponding to 0.08–5 mg/ml) or aflibercept (ranging from 0.70–43.4 µM; corresponding to 0.08–5 mg/ml) for 1 h at 37 °C.

After incubation, the reaction mixtures were transferred to the ELISA microplates and analysis for free VEGF was performed in concordance to the manual instructions. ELISA plates were finally recorded by using an EnSpire Multimodal Plate Reader (PerkinElmer, Waltham, USA). Each measurement was performed in duplicates.

3. Results

3.1. Canine, feline, and equine VEGF show high sequence homology to human VEGF

To find out whether feline, equine, and canine VEGF represent potential binding partners for bevacizumab and aflibercept, protein sequences were compared with the sequence of human VEGF. Analysis revealed 93% homology of canine and human VEGF. Feline VEGF showed 92%, and equine VEGF 89% homologous regions with the human VEGF. Sequence differences are located between glutamine acid 40 and prolin 166, whereas the N-terminal region from position 1 to 39, and the C-terminal region from position 166 to 215 are identical (Fig. 1). Compared to human VEGF, canine, feline, and equine VEGF protein is shorter by one, two or seven amino acids, respectively.

3.2. Binding of bevacizumab and aflibercept to canine, feline and equine VEGF

To get insights whether bevacizumab and aflibercept may bind canine, feline, and equine VEGF, an immunoblot was performed. Canine, feline, and equine VEGF were blotted on a PVDF membrane and exposed to 2.5 µg/ml bevacizumab or 2.5 µg/ml aflibercept. Binding to human VEGF served as internal control. As shown in Fig. 2A, membranes incubated with bevacizumab showed immunoreactive bands for human, canine, and feline VEGF. No immunosignal was observed for equine VEGF. Also, higher concentrations of bevacizumab (2.5 µg/ml–0.25 mg/ml) or increasing VEGF amounts (1–100 µg) did not yield an immunoreactive band for equine VEGF (data not shown). In contrast to bevacizumab, membrane incubation with aflibercept showed immunoreactive bands for human, canine, feline, and equine VEGF proteins (Fig. 2B). The findings indicate that bevacizumab may interact with canine and feline, but not with equine VEGF, whereas aflibercept binds to VEGF from all tested species.

Fig. 1. Sequence alignment of VEGF. A multiple amino acid alignment of VEGF protein from human, dogs, cats and horses was performed. Amino acids homologous to human sequence are indicated as dots, variants with respective amino acid code, and missing acids as dash (–).

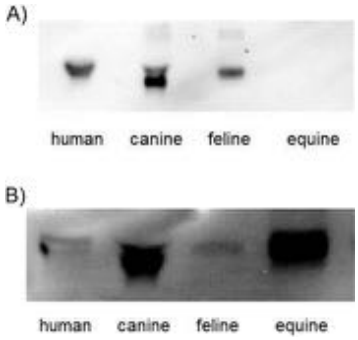

Fig. 2. Immunoblotting of VEGF with bevacizumab (A) and aflibercept (B). Equal amounts of human, canine, feline and equine VEGF (1 µg) were blotted on PVDF membrane and incubated with bevacizumab (1:10000; A) or aflibercept (1:10000; B). Immunoreactive signals were detected by chemiluminescence.

3.3. Determination of bevacizumab and aflibercept binding affinities

To further characterize the binding properties, canine, feline, and equine VEGF were incubated with 0.08–5 mg/ml bevacizumab or aflibercept, corresponding to clinically relevant and safe doses (Koenig et al., 2009; Krizova et al., 2014). Subsequently, the amount of unbound VEGF was quantified by ELISA. In parallel experiments, binding properties for human VEGF were determined. As shown in Fig. 3A, bevacizumab and aflibercept bound human VEGF in a dose-dependent manner with K_d values of 40 nM and 190 nM, respectively. In similar, a dose-dependent binding of bevacizumab and aflibercept was observed for canine VEGF with calculated K_d values of 130 nM and 250 nM, respectively (Fig. 3B). A reduction of free feline VEGF by 59.9% and 83.8% was observed in presence of 0.54 µM bevacizumab (corresponds to 0.08 mg/ml) and 0.70 µM aflibercept (corresponds to 0.08 mg/ml), respectively (Fig. 3C). However, incubation of feline VEGF with higher antibody concentrations did not further reduce the amount of free VEGF (Fig. 3C). Binding analysis of equine VEGF showed that different concentrations of bevacizumab did not affect the amount of free VEGF (Fig. 3D). In contrast, 0.70 µM aflibercept (corresponds to 0.08 mg/ml) reduced free equine VEGF by 55.5%, whereas increasing concentrations had no further effect on VEGF amount (Fig. 3D).

4. Discussion

Binding of VEGF by therapeutic antibodies is an innovative strategy to interfere with corneal neovascularization in humans. To assess whether these anti-VEGF molecules would also represent a therapeutic option for dogs, cat and horses suffering from CNV, the present study investigated the binding properties of bevacizumab and aflibercept to canine, feline, and equine VEGF.

Bevacizumab and aflibercept were designed to specifically bind human VEGF-A and its different splicing variants with $VEGF_{165}$ being the most abundant isoform in humans (Holash et al., 2002; Papadopoulos et al., 2012; Presta et al., 1997; Tischer et al., 1991). Bevacizumab binds VEGF between Arg 82 and Gly 91, which reflects the VEGF receptor binding region (Muller et al., 1997). The VEGF binding region of aflibercept has not been postulated so far. However, aflibercept consists of VEGFR-1 and VEGFR-2 components (Holash et al., 2002) suggesting that VEGF binding may also occur via the receptor binding domain. VEGF from dogs, cats, and horses differ in eight to ten amino acids and in total length compared to the human $VEGF_{165}$. Nevertheless, the regions between Arg 82 and Gly 91 were identical, which renders binding of bevacizumab and aflibercept to VEGF from dogs, cats, and horses very likely.

A common approach to test antibody binding to a target protein is immunoblotting. The approach confirmed binding of aflibercept to canine, feline, and equine VEGF, whereas bevacizumab only bound canine and feline, but not equine VEGF. The finding was surprising as equine and human VEGF showed identical protein sequence within the bevacizumab binding region. Beside epitope recognition, efficient antibody binding also requires epitope accessibility. It is well known that different protein folding and three-dimensional protein structures may hide the binding region, which may lead to lack of antibody interaction despite the presence of a respective epitope. As equine VEGF exhibits most variations in the protein sequence, structural differences to human VEGF are thus likely and might explain the lack of bevacizumab binding.

The next step was to investigate the binding strength by measuring the equilibrium constant (KD). Bevacizumab and aflibercept are described to bind human VEGF with high affinity. Binding experiments based on surface resonance technology yielded K_d values of 58 pM to 4.5 nM (Papadopoulos et al., 2012; Yang et al., 2014) for bevacizumab and 0.49 pM to 9.3 nM (Papadopoulos et al., 2012; Yang et al., 2014) for aflibercept. By using an ELISA, we calculated a K_d value of 40 nM for bevacizumab binding to human VEGF, which was 10-fold higher as reported by Yang et al. (Yang et al., 2014). Surprisingly, aflibercept with 190 nM displayed a much higher K_d value for human VEGF in our hands than reported by others (Holash et al., 2002; Yang et al., 2014). It is well known that peptide – antibody interactions may be affected by several factors. Beside temperature and pH values, different chemical additives and buffer compositions modulate binding kinetics (Andersson et al., 2001). In contrast to others, our binding reaction was performed at 37 °C and without further buffer supplements like urea or

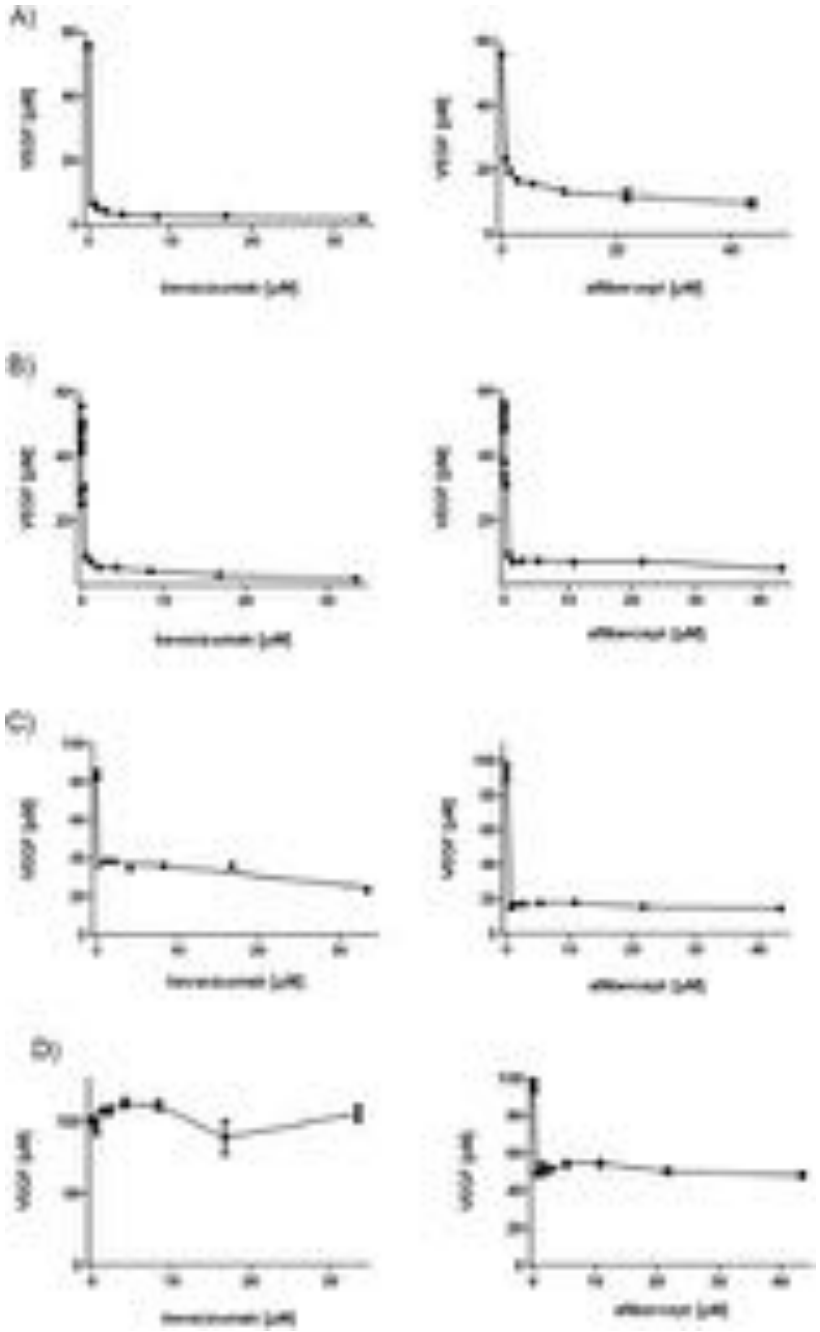

Fig. 3. Binding studies of bevacizumab and aflibercept. Equal amounts of human (A), canine (B), feline (C), and equine VEGF (D) were incubated with varying concentrations of bevacizumab (left graph) or aflibercept (right graph) for 60 min at 37 °C. Subsequently, unbound VEGF (ordinate) was quantified by ELISA and data obtained were used for Kd calculation by GraphPad Prism. Data shown represent mean ± S.D. of three independent binding experiments done in duplicates.

NaCl (Andersson et al., 2001). It is thus very likely that differences in K_d values observed for human VEGF in our study result from distinct experimental conditions.

By using our protocol, bevacizumab was found to bind canine VEGF with a 4-fold higher K_d value than calculated for human VEGF. Whereas bevacizumab was thus less affine, aflibercept showed similar affinity for canine and human VEGF. As human and canine VEGF protein displayed the highest homology, comparable affinities for the therapeutic antibodies are likely. Surprisingly, no K_d values could be calculated from the binding data obtained for feline and equine VEGF. In contrast to dose-dependent binding of canine VEGF, binding of feline VEGF to bevacizumab and aflibercept was linear. Independent of the antibody concentration used, equal amounts of feline VEGF were scavenged by the therapeutic antibodies. Such a linear or dose-independent protein – protein interaction may be indicative for non-specific binding, which was reported for peptides at the Fc fragment of human IgG (Medina et al., 1999). As bevacizumab and aflibercept both contain the Fc fragment of human IgG (Holash et al., 2002; Presta et al., 1997), feline VEGF binding data might be thus considered to result from non-specific interaction with bevacizumab and aflibercept. Whether small variants in the peptide sequence of feline VEGF accounts for the non-specific binding properties remains to be investigated.

The ELISA approach also found a dose-independent binding of aflibercept to equine VEGF, whereas no VEGF scavenging was detected for bevacizumab. This observation is in line with the lack of bevacizumab binding seen for immobilized VEGF using the immunoblotting approach. Both findings suggest that bevacizumab may interact with equine VEGF neither in specific nor nonspecific manner. Compared to canine and feline VEGF, equine VEGF is the peptide with the most variants in the protein sequence compared to human VEGF. Hence, it is close to speculate that these interspecies differences may account for the absence of equine VEGF binding to bevacizumab.

Although the results of the current study are promising and provide a basis for future strategies to use anti-VEGF treatments in veterinary ophthalmology, we are aware that the data were obtained from isolated in-vitro experiments and does not consider pathophysiological, immunological and pharmacokinetic parameters, which may affect drug efficacy and tolerability in veterinary patients. Although VEGF-A plays a major role in pathological angiogenesis, vessel formation is a highly complex process which is regulated by an interplay of various other pro- and anti-angiogenic factors (Chung and Ferrara, 2011). Our study, however, only tested the interaction of aflibercept and bevacizumab with VEGF. The clinical benefit of an exclusive VEGF neutralization in animal patients remains to be further confirmed. Aflibercept is considered more anti-angiogenic in human patients than bevacizumab as it binds the proangiogenic factors VEGF-B, PIGF-1, and PIGF-2 as well (Papadopoulos et al., 2012). Whether the extended binding profile applies for animals, still needs to be tested. Moreover, aflibercept and bevacizumab are humanized proteins with a molecular weight of 115 and 149 kDa proteins, which may act as antigens and evoke an immune response in animals. The different molecular sizes of bevacizumab and aflibercept may further affect the ocular half-live, bioavailability, size-dependent tissue penetration, and diffusion rates. Future studies are indispensable to determine the clinical efficacy and safety of the therapeutic antibodies in diseased veterinary patients.

Together our data indicate that bevacizumab and aflibercept interact with canine VEGF in a similar manner to human VEGF, but not to feline and equine VEGF. A therapeutic application in dogs with CNV is conceivable. However further studies are needed to provide evidence for a therapeutic benefit and clinical efficacy in diseased canine patients with naturally occurring CNV.

Acknowledgements

The authors want to thank Alexandra Schoos for her technical assistance. The research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflict of interest

The authors declare no conflicts of interest.

Authors' contribution

All authors have read and approved the final manuscript.

LMM contributed to the design of the study; performed analysis; interpreted data; wrote the manuscript; and agreed to be responsible for all aspects of work ensuring its integrity and validity. BB is contributed equally to this work; he contributed to the design of the study; performed analysis; helped to interpret data; wrote the manuscript; and agreed to be responsible for all aspects of this work ensuring integrity and validity. BN contributed to the study design; helped to interpret data; critically revised the manuscript; and agreed to be responsible for all aspects of work ensuring its integrity and validity. DAF contributed to the study design; performed analysis; helped to interpret data; helped to write the manuscript and critically revised the manuscript; and agreed to be responsible for all aspects of work ensuring its integrity and validity.

References

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST : a new generation of protein database search programs. Nucleic Acids Res. 25 (17), 3389–3402.

Andersson, K., Choulier, L., Hämäläinen, M.D., van Regenmortel, M.H.V., Altschuh, D., Malmqvist, M., 2001. Predicting the kinetics of peptide-antibody interactions using a multivariate experimental design of sequence and chemical space. J. Mol. Recognit. 14 (1), 62–71.

Arevalo, J.F., Fromow-Guerra, J., Quiroz-Mercado, H., Sanchez, J.G., Wu, L., Maia, M., Berrocal, M.J., Solis-Vivanco, A., Farah, M.E., 2007. Primary intravitreal bevacizumab (Avastin) for diabetic macular edema. Results from the pan-American collaborative retina study group at 6-month follow-up. Ophthalmology 114 (4), 743–750. https://doi.org/10.1016/j.ophtha.2006.12.028.

Avery, R.L., Pieramici, D.J., Rabena, M.D., Castellarin, A.A., Nasir, M.A., Giust, M.J., 2006. Intravitreal bevacizumab (Avastin) for neovascular age-related macular degeneration. Ophthalmology 113 (3), 363–372. https://doi.org/10.1016/j.ophtha.2005.11.019.

Binder, D.R., Herring, I.P., Zimmerman, K.L., Phillip Pickett, J., Huckle, W.R., 2012. Expression of vascular endothelial growth factor receptor-1 and -2 in normal and diseased canine eyes. Vet. Ophthalmol. 15 (4), 223–230. https://doi.org/10.1111/j.1463-5224.2011.00973.x.

Brown, D.M., Schmidt-Erfurth, U., Do, D.V., Holz, F.G., Boyer, D.S., Midena, E., Heier, J.S., Terasaki, H., Kaiser, P.K., Marcus, D.M., Nguyen, Q.D., Jaffe, G.J., Slakter, J.S., Simader, C., Soo, Y., Schmelter, T., Yancopoulos, G.D., Stahl, N., Vitti, R., Berliner, A.J., Zeitz, O., Metzig, C., Korobelnik, J.F., 2015. Intravitreal aflibercept for diabetic macular edema: 100-week results from the VISTA and VIVID studies. Ophthalmology 122 (10), 2044–2052. https://doi.org/10.1016/j.ophtha.2015.06.017.

Chung, A.S., Ferrara, N., 2011. Developmental and pathological angiogenesis. Annu. Rev. Cell Dev. Biol. 27, 563–584. https://doi.org/10.1146/annurev-cellbio-092910-154002.

Conn, H., Berman, M., Kenyon, K., Langer, R., Gage, J., 1980. Stromal vascularization prevents corneal ulceration. Investig. Ophthalmol. Vis. Sci. 19 (4), 362–370.

Dana, M.R., Streilein, W., 1996. Loss and restoration of immune privilege in eyes with corneal neovascularization. Investig. Ophthalmol. Vis. Sci. 37 (12), 2485–2494.

Dastjerdi, M.H., Al-Arfaj, K.M., Nallasamy, N., Hamrah, P., Jurkunas, U.V., Pineda, R., Pavan-Langston, D., Dana, R., 2009. Topical bevacizumab in the treatment of corneal neovascularization: results of a prospective, open-label, non-comparative study. Arch. Ophthalmol. 127 (4), 381–389. https://doi.org/10.1001/archophthalmol.2009.18.

Dean, E., Meunier, V., 2013. Feline eosinophilic keratoconjunctivitis: a retrospective study of 45 cases (56 eyes). J. Feline Med. Surg. 15 (8), 661–666. https://doi.org/10.1177/1098612X12472181.

Epstein, R.J., Stulting, R.D., Hendricks, R.L., Harris, D.M., 1987. Corneal neovascularization. Pathogenesis and inhibition. Cornea 6 (4), 250–257.

Gaarder, J.E., Rebhun, W.C., Ball, M.A., Patten, V., Shin, S., Erb, H., 1998. Clinical appearances, healing patterns, risk factors, outcomes of horses with fungal keratitis: 53 cases (1978-1996). J. Am. Vet. Med. Assoc. 213 (1), 105–112.

Gilger, B.C., Michau, T.M., Salmon, J.H., 2005. Immune-mediated keratitis in horses: 19 cases (1998-2004). Vet. Ophthalmol. 8 (4), 233–239. https://doi.org/10.1111/j.1463-5224.2005.00393.x.

Heier, J.S., Clark, W.L., Boyer, D.S., Brown, D.M., Vitti, R., Berliner, A.J., Kazmi, H., Ma, Y., Stemper, B., Zeitz, O., Sandbrik, R., Haller, J.A., 2014. Intravitreal aflibercept injection for macular edema due to central retinal vein occlusion: two-year results from the COPERNICUS study. Ophthalmology 121 (7), 1414–1420. https://doi.org/

10.1016/j.ophtha.2014.01.027.
Henriksen, M. de L., Andersen, P.H., Mietelka, K., Farina, L., Thomsen, P.D., Plummer, C.E., Mangan, B.G., Heegaard, S., Coleman, J.K., Toft, N., Brooks, D.E., 2014. Equine deep stromal abscesses (51 cases - 2004-2009) - part 2: the histopathology and immunohistochemical aspect with attention to the histopathologic diagnosis, vascular response, and infectious agents. Vet. Ophthalmol. 17 (Suppl. 1), 14–22. https://doi.org/10.1111/vop.12102.
Holash, J., Davis, S., Papadopoulos, N., Croll, S.D., Ho, L., Russell, M., Boland, P., Leidich, R., Hylton, D., Burova, E., Ioffe, E., Huang, T., Radziejewski, C., Bailey, K., Fandl, J.P., Daly, T., Wiegand, S.J., Yancopoulos, G.D., Rudge, J.S., 2002. VEGF-trap: a VEGF blocker with potent antitumor effects. Proc. Natl. Acad. Sci. 99 (17), 11393–11398. https://doi.org/10.1073/pnas.172398299.
Ikuno, Y., Ohno-Matsui, K., Wong, T.Y., Korobelnik, J.F., Vitti, R., Li, T., Stemper, B., Asmus, F., Zeitz, O., Ishibashi, T., 2015. Intravitreal aflibercept injection in patients with myopic choroidal neovascularization: the MYRROR study. Ophthalmology 122 (6), 1220–1227. https://doi.org/10.1016/j.ophtha.2015.01.025.
Kaswan, R.L., Salisbury, M.A., 1990. A new perspective on canine keratoconjunctivitis sicca: treatment with ophthalmic cyclosporine. Vet. Clin. N. Am. Small Anim. Pract. 20 (3), 583–613. https://doi.org/10.1016/S0195-5616(90)50052-2.
Kim, S.W., Ha, B.J., Kim, E.K., Tchah, H., Kim, T., 2008. The effect of topical bevacizumab on corneal neovascularization. Ophthalmology 115 (6), 33–38. https://doi.org/10.1016/j.ophtha.2008.02.013.
Koenig, Y., Bock, F., Horn, F., Kruse, F.E., Straub, K., Cursiefen, C., 2009. Short- and long-term safety profile and efficacy of topical bevacizumab (Avastin®) eye drops against corneal neovascularization. Graefes Arch. Clin. Exp. Ophthalmol. 247, 1375–1382. https://doi.org/10.1007/s00417-009-1099-1.
Krizova, D., Vokrojova, M., Lieheneova, K., Studeny, P., 2014. Treatment of corneal neovascularization using anti-VEGF bevacizumab. J. Ophthalmol. 2014, 1–8.
Labelle, A.L., Dresser, C.B., Hamor, R.E., Allender, M.C., Disney, J.L., 2013. Characteristics of, prevalence of, and risk factors for corneal pigmentation (pigmentary keratopathy) in pugs. J. Am. Vet. Med. Assoc. 243 (5), 667–674.
Maddula, S., Davis, D.K., Maddula, S., Burrow, M.K., Ambati, B.K., 2011. Horizons in therapy for corneal angiogenesis. Ophthalmology 118 (3), 591–599.
Martin, D.F., Maguire, M.G., Fine, S.L., Ying, G.S., Jaffe, G.J., Grunwald, J.E., Toth, C., Redford, M., Ferris, F.L., 2012. Ranibizumab and bevacizumab for treatment of neovascular age-related macular degeneration: two-year results. Ophthalmology 119 (7), 1388–1398. https://doi.org/10.1016/j.ophtha.2012.03.053.
Medina, E., Molinari, G., Rohde, M., Haase, B., Chhatwal, G.S., Guzmán, C.A., 1999. Fc-mediated nonspecific binding between fibronectin-binding protein I of Streptococcus pyogenes and human immunoglobulins. J. Immunol. 163 (6), 3396–3402. https://doi.org/ji_v163n6p3396.
Muller, Y.A., Christinger, H.W., Keyt, B.A., De Vos, A.M., 1997. The crystal structure of vascular endothelial growth factor (VEGF) refined to 1.93 Å resolution : multiple copy flexibility and receptor binding. Structure 5 (10), 1325–1338. https://doi.org/10.1016/S0969-2126(97)00284-0.
Nasisse, M.P., Guy, J.S., Davidson, M.G., Sussman, W.A., Fairley, N.M., 1989. Experimental ocular herpesvirus infection in the cat. Investig. Ophthalmol. Vis. Sci. 30 (8), 1758–1768.
Nell, B., Walde, I., Billich, A., Vit, P., Meingassner, J.G., 2005. The effect of topical pimecrolimus on keratoconjunctivitis sicca and chronic superficial keratitis in dogs: results from an exploratory study. Vet. Ophthalmol. 8 (1), 39–46. https://doi.org/10.1111/j.1463-5224.2005.04062.x.
Notredame, C., Higgins, D.G., Heringa, J., 2000. T-Coffee : a novel method for fast and accurate multiple sequence alignment. J. Mol. Biol. 302 (1), 205–217. https://doi.org/10.1006/jmbi.2000.4042.
Ozdemir, O., Altintas, O, Altintas, L., Ozkan, B., Akdag, C., Yüksel, N., 2014. Comparison of the effects of subconjunctival and topical anti-VEGF therapy (bevacizumab) on experimental corneal neovascularization. Arq. Bras. Oftalmol. 77 (4), 209–213.
Papadopoulos, N., Martin, J., Ruan, Q., Rafique, A., Rosconi, M.P., Shi, E., Pyles, E.A., Yancopoulos, G.D., Stahl, N., Wiegand, S.J., 2012. Binding and neutralization of vascular endothelial growth factor (VEGF) and related ligands by VEGF trap ranibizumab and bevacizumab. Angiogenesis 15, 171–185. https://doi.org/10.1007/s10456-011-9249-6.
Petroutsos, G., Guimaraes, R., Giraud, J.P., Pouliquen, Y., 1982. Corticosteroids and corneal epithelial wound healing. Br. J. Ophthalmol. 66, 705–708.
Presta, L.G., Chen, H., O'Connor, S.J., Chisholm, V., Meng, Y.G., Krummen, L., Winkler, M., Ferrara, N., 1997. Humanization of an anti-vascular endothelial growth factor monoclonal antibody for the therapy of solid tumors and other disorders. Cancer Res. 57, 4593–4599.
Sanchez, R.F., Innocent, G., Mould, J., Billson, F., 2007. Canine keratoconjunctivitis sicca : disease trends in a review of 229 cases. J. Small Anim. Pract. 48, 211–217.
Sella, R., Gal-Or, O., Livny, E., Dachbash, M., Nisgav, Y., Weinberger, D., Livnat, T., Bahar, I., 2016. Efficacy of topical aflibercept versus topical bevacizumab for the prevention of corneal neovascularization in a rat model. Exp. Eye Res. 146, 224–232. https://doi.org/10.1016/j.exer.2016.03.021.
Slatter, D.H., Lavach, J.D., Severin, G.A., Youg, S., 1977. Ueberreiter's syndrome (chronic superficial keratitis) in dogs in the Rocky Mountain area - a study of 463 cases. J. Small Anim. Pract. 18 (12), 757–772.
SooHoo, J.R., Seibold, L.K., Pantcheva, M.B., Kahook, M.Y., 2015. Aflibercept for the treatment of neovascular glaucoma. Clin. Exp. Ophthalmol. 43 (9), 803–807.
Spiess, A.K., Sapienza, J.S., Mayordomo, A., 2009. Treatment of proliferative feline eosinophilic keratitis with topical 1.5% cyclosporine: 35 cases. Vet. Ophthalmol. 12 (2), 132–137. https://doi.org/10.1111/j.1463-5224.2008.00679.x.
The Diabetic Retinopathy Clinical Research Network, 2015. Aflibercept, Bevacizumab, or Ranibizumab for Diabetic Macular Edema. N. Engl. J. Med. 372 (13), 1193–1203.
Tischer, E., Mitchell, R., Hartman, T., Silva, M., Gospodarowicz, D., Fiddes, J.C., Abraham, J.A., 1991. The human gene for vascular endothelial growth factor. J. Biol. Chem. 266 (18), 11947–11954.
Wells, J.A., Glassman, A.R., Ayala, A.R., Jampol, L.M., Bressler, N.M., Bressler, S.B., Brucker, A.J., Ferris, F.L., Hampton, G.R., Jhaveri, C., Melia, M., Beck, R.W., 2016. Aflibercept, Bevacizumab, or Ranibizumab for Diabetic Macular Edema: Two-Year Results from a Comparative Effectiveness Randomized Clinical Trial. Ophthalmology 123 (6), 1351–1359. https://doi.org/10.1016/j.ophtha.2016.02.022.
Williams, D.L., Hoey, A.J., Smitherman, P., 1995. Comparison of topical cyclosporin and dexamethasone for the treatment of chronic superficial keratitis in dogs. Vet.Rec. 137 (25), 635–639. https://doi.org/10.1136/vr.137.25.635.
Yang, J., Wang, X., Fuh, G., Yu, L., Wakshull, E., Khosraviani, M., Day, E.S., Demeule, B., Liu, J., Shire, S.J., Ferrara, N., Yadav, S., 2014. Comparison of binding characteristics and in vitro activities of three inhibitors of vascular endothelial growth factor a. Mol. Pharm. 11 (10), 3421–3430. https://doi.org/10.1021/mp500160v.

Received: 17 July 2018 | Revised: 3 September 2018 | Accepted: 23 October 2018

DOI: 10.1111/vop.12628

ORIGINAL ARTICLE

WILEY

Serum vascular endothelial growth factor changes and safety after topical anti-human VEGF antibody bevacizumab in healthy dogs

Lisa-Marie Muellerleile[1] | **Alexander Tichy**[2] | **Barbara Nell**[1]

[1]Department of Companion Animals and Horses, University of Veterinary Medicine Vienna, Vienna, Austria

[2]Department of Biomedical Sciences, University of Veterinary Medicine Vienna, Vienna, Austria

Correspondence
Lisa-Marie Muellerleile, Dr.med.vet., University of Veterinary Medicine Vienna, Department of Companion Animals and Horses, Vienna, Austria.
Email: lisa-marie.muellerleile@vetmeduni.ac.at

Abstract

Objective: To evaluate ocular and general safety of topical anti-human VEGF bevacizumab and the effect on serum vascular endothelial growth factor (VEGF) values in healthy dogs.

Procedures: Nine university-owned beagles received 0.05 mL of 0.25% bevacizumab eyedrops (Avastin®, Roche) in one eye and 0.05 mL of 0.9% saline solution in the other eye as a control, administered at 12 hours intervals over a period of 28 days. Continuous monitoring for vital parameters and ocular examinations were conducted. Complete blood counts including hematology and coagulation parameters were performed before trial start as well as 24 hours, 7 days, and 28 days after trial start. Measurements of serum VEGF values were obtained using an ELISA-based approach at days 0, 7, and 28. The experiment was designed as a masked placebo-controlled study.

Results: No clinical signs of ocular toxicity or systemic incompatibility were noted in any dog at any time point of the study. No signs of pain were present in any dog at any time point. All blood count values remained in normal clinical ranges without relevant variation. There was no significant change in mean serum VEGF values between day 0 and day 7 and between day 0 and day 28.

Conclusions: The results indicate that topical bevacizumab treatment is safe in healthy dogs. However, further studies are needed to assess safety and efficacy in diseased dogs with naturally occurring corneal neovascularization.

KEYWORDS
bevacizumab, dog, safety, VEGF

1 | INTRODUCTION

Corneal neovascularization (CNV) and corneal opacification arise concomitantly in several corneal diseases in dogs, for instance keratoconjunctivitis sicca, superficial pigmentary keratitis, and chronic superficial keratitis.[1-3] Although corneal neovascularization is initially essential for corneal wound healing and hindering stromal melting, corneal neovascularization can cause blindness, tissue scarring, lipid deposition, edema, and potentially sustains inflammation.[4,5] Furthermore, avascularity is one of the prerequisites of the corneal immune privilege.[6] Hence, inhibition of corneal

neovascularization represents a therapeutic strategy in animals with chronic keratitis and CNV. Promising therapeutic results in animal models and human patients with CNV have been reported for anti-human vascular endothelial growth factor (VEGF) therapies.[7-10]

VEGF-A is a glycosylated endothelial mitogen that is involved in pathological angiogenesis and is known to be increased in inflamed and vascularized corneas.[11-13] Hence, pharmacological inhibition of VEGF-A is a promising strategy to treat diseases driven by pathological neovascularization. There is an arsenal of distinct VEGF inhibitors differing in their antiangiogenetic activity, binding affinity for VEGF-A, and their binding spectrum to different VEGF isoforms and other members of the VEGF family.[14] One of the most potent VEGF inhibitors is bevacizumab. In human ophthalmology, an off-label intravitreal use of bevacizumab is already taking place and is reported to be effective for the treatment of wet age-related macular degeneration, diabetic macular edema, macular edema secondary to retinal vein occlusion, vascular glaucoma, and CNV.[7,8,15-17]

So far no investigations have been carried out to determine the safety and medical compatibility of topical bevacizumab in dogs. There are several animal models which have proven the safety and efficacy of subconjunctivally and topically administered bevacizumab in mice, rabbits, and chinchilla bastard rabbits.[10,18,19] It has been shown that topical bevacizumab treatment does not have a significant influence on corneal integrity, corneal wound repair, and corneal nerve fiber density in mice with experimentally induced corneal epithelial abrasions.[10,18-21]

Systemic side effects have been reported after intravitreal injections of bevacizumab in humans, such as systemic hypertension, cerebrovascular accidents, and facial skin redness.[22,23] A decrease of plasma VEGF values after intravitreal bevacizumab injections has been described.[24] However, there is no knowledge about effects on serum VEGF values and general safety after topically applied bevacizumab in dogs.

The aim of the current work was to investigate the safety and medical compatibility of topical administered bevacizumab in healthy dogs. Additionally, we studied the effect of topical bevacizumab treatment on serum VEGF values in healthy dogs. We hypothesized that topical administered bevacizumab is both systemically and topically safe and has no effect on serum VEGF values.

2 | MATERIAL AND METHODS

2.1 | Animals

The study was approved by the institutional ethics and animal welfare committee and the national authority according to §§ 26ff. of Animal Experiments Act, Tierversuchsgesetz 2012 —TVG 2012 (GZ 68.205/0134-WF/V/3b/2017). The trial was conducted on 10 university-owned beagle dogs aged at least 16 months and bred for experimental purpose. The experiment was designed as a masked placebo-controlled study.

Each dog underwent a full physical and ocular examination to ensure eligibility requirements prior to initiation of the study. The arterial blood pressure was measured and a complete blood count including hematology, baseline serum VEGF values, activated partial thromboplastin time, partial thromboplastin time, and thrombin time was conducted. A complete ophthalmic examination was performed in all dogs by the first author (LM) under supervision of a board-certified ophthalmologist (BN). The examination included slit-lamp biomicroscopy (Kowa SL-15; Kowa, Tokyo, Japan), indirect ophthalmoscopy (Keeler Vantage; Keeler Instruments Inc, Broomall, PA), Schirmer tear test-1 (Teststreifen, MSD, Unterschleissheim, Germany), fluorescein staining (Fluorotouch Ophthalmic Strips, Eickemeyer, Tuttlingen, Germany), and intraocular pressure measurements using rebound tonometry (TonoVet, Icare, Vantaa, Finland). Posterior segment examination was conducted after pharmacological mydriasis (Mydriaticum, Agepha, Senec, Slovakia). Dogs had to be free of any systemic and ocular disease and did not receive ocular drugs, any kind of systemic medication, VEGF affecting drugs or agents consisting of human proteins 14 days before the start of the trial.

2.2 | Drug preparation

A 0.25% solution of bevacizumab eyedrops was aseptically prepared from a commercial available intravenous bevacizumab solution (Avastin®, Roche, Basel, Switzerland) by the institute's pharmacy in compliance with good manufacturing practice. Sterile 0.9% saline solution was used as the solvent, and the study drug was stored at 2-8°C and protected from light with a durability of 28 days.[25] Sterile 0.9% saline solution served as the control.

The pharmacist prepared single-dose containers for each dog with a single dose of either the study medication (0.05 mL of 0.25% bevacizumab solution) or the placebo (0.05 mL of 0.9% saline solution). The containers were labeled with the information "left eye" or "right eye" and only the pharmacist had knowledge about the content. After the trial, the pharmacist revealed which eye received bevacizumab or placebo.

2.3 | Study design

All examinations were performed by the same experienced observer (LM). Beagle dogs received 0.05 mL of 0.25% bevacizumab eyedrops in one eye and 0.05 mL of 0.9% saline solution in the other eye as a control, administered at 12 hours intervals over a period of 28 days.

Continuous monitoring was scheduled as follows: Over a period of 3 hours after the first drug administration

monitoring of vital parameters (including respiratory and heart rate, mucous membrane color, capillary refill time, and arterial blood pressure) was performed hourly. Thereafter, the parameters were taken every 3 hours for the duration of 24 hours after trial start. At day 2-7, dogs underwent a full physical examination and arterial blood pressure measurements once a day. The final examination was performed at day 28 after trial start.

Complete blood counts including hematology and coagulation parameters (activated partial thromboplastin time, partial thromboplastin time, and thrombin time) were performed 24 hours, 7 days, and 28 days after trial start. Venous blood sample was obtained via the cephalic vein.

Indirect blood pressure measurements were obtained by using an oscillometric device (PETMAP™ graph II, Ramsey Medical, USA, Tampa). Dogs were allowed to settle for several minutes before the blood pressure readings began. An appropriate cuff (Critter Cuff™, Ramsey Medical, USA, Tampa) with a width of about 40% of the forelimb circumference was applied and linked to the oscillometric pressure unit. Readings were taken from the median artery by putting the cuff around the right midradial region overlying the median artery. Dogs were restrained gently on the examination table in sternal recumbency, placing the cuff at the level of the right heart. For each dog, three readings (each systolic, diastolic, and mean arterial pressure) were taken successively.

Ophthalmic examinations were performed directly after drug administration, as well as 3 hours, 24 hours, once a day at day 2-7, and at day 28. They were performed without pharmacological mydriasis except the final examination at day 28. A modified Hackett-McDonald scoring system[26] and a pain score system[27] were used to determine ocular and systemic toxicity (see below).

2.4 | Ocular irritation and assessment of ocular toxicity potential

Ocular examination findings were scored using a modified Hackett-McDonald scoring system.[26] Examination scores were recorded for conjunctival congestion/hyperemia (0-3+), conjunctival chemosis/swelling (0-3+), conjunctival/ocular discharge (0-3+), corneal edema (0-3+), and corneal vascularization (0-3+). Moreover, all dogs were assessed using a subjective pain scoring system[27]: Pain scoring categories included comfort, movement, degree of blepharospasm, unprovoked behavior, interactive behaviors, and vocalization (Table 1).

2.5 | Serum VEGF values: Enzyme-linked Immunosorbent Assay

Serum samples were centrifuged for 20 minutes at 1000× g within 30 minutes of collection. Afterward, samples were stored in sterile polypropylene tubes at ≤−80°C. Measurement of serum VEGF values was obtained using an ELISA-based approach. For that a commercially available canine enzyme-linked immunosorbent assay was used (Canine VEGF Quantikine ELISA Kit, R&D Systems, Minneapolis, USA, Cat # CAVE00). VEGF values were measured before, 7 days, and 28 days after trial start. The ELISA was conducted in accordance with the assay instructions. All standards and samples were analyzed in duplicate.

2.6 | Statistical analysis

The current work was designed as a pilot study. Currently, safety and medical compatibility of topical bevacizumab in dogs are uncharted, and effect sizes and statistical variation are unknown. Sample size planning based upon comparable study schedules of former studies in rodent models.[20,28]

Statistical analysis was performed using the software program SPSS (IBM SPSS Statistics 24).

The clinical endpoint was the occurrence of ocular or systemic adverse events. The frequency and type of systemic and ocular recorded side effects were analyzed in a descriptive manner.

Quantitative data were summarized as mean ± standard deviation. The confidence interval was computed from the observed data, using confidence values of 95%. Furthermore, a paired-sample *t* test was used to investigate differences of the arterial blood pressure, heart rate, respiratory rate, and coagulation parameters between day 0, day 1, day 7, and day 28. Changes in VEGF serum values were compared between day 0, day 7, and day 28 using a paired-sample *t* test. The assumption of normal distribution was tested using Kolmogorov-Smirnov test. A *P*-value <0.05 was considered as statistically significant.

3 | RESULTS

3.1 | Animals

Nine of 10 clinic-owned beagle dogs were included in the study. One of the beagles was not concordant with the eligibility requirements as he showed a slight thrombocytopenia and thus was excluded from the study. All study dogs were male with a mean weight of 14.7 ± 2.5 kg and the median age was 30 (range 23-39) months.

3.2 | Ocular toxicity potential

No clinical signs of ocular toxicity or ocular adverse events such as conjunctival hyperemia or chemosis, ocular discharge, corneal edema, corneal vascularization, or corneal defects were noted in either eye of any dog at any time point of the study. Intraocular pressure measurements and values of Schirmer

TABLE 1 Pain score system modified from Clark et al[27] to determine ocular and systemic toxicity in healthy dogs after topical bevacizumab application

Pain scoring category	Manifestation
Comfort	0 = dog is calm and interested in surroundings
	1 = dog shows mild agitation or is depressed, not interested in surroundings
	2 = dog shows moderate agitation and is restless
	3 = dog is extremely agitated
Movement	0 = dog is quiet
	1 = about 1-2 position changes per minute
	2 = about 2-6 position changes per minute
	3 = dog shows continuous changes
Appearance/ blepharospasm	0 = eyelids are completely open and in physiological position
	1 = eyelids are partially closed (ca. 25%)
	2 = eyelids are partially closed (ca. 50%); dog shows mild tearing
	3 = eyelids are partially closed (ca. 75%); dog shows moderate tearing
	4 = eyelids are completely closed; dog shows marked tearing
Behavior (unprovoked)	0 = normal
	1 = minor changes
	2 = moderately abnormal (less mobile or alert than normal, not interested in surroundings, fretful)
	3 = noticeably abnormal (fretful, vocalizing, self-mutilation, groaning)
Interactive behaviors	0 = normal
	1 = pulls head away when eyes getting touched
	2 = vocalizes when eyes getting touched
	3 = violent reaction to touching of eye (biting, snapping, groaning)
Vocalization	0 = quiet
	1 = dog cries but responds to be quiet
	2 = dog intermittently cries without response to quiet voice
	3 = dog constantly cries without response to quiet voice

tear test-1 values remained within normal limits, with minimal variations without clinical relevance in any dog at any time point. Thus, only the confidence interval and standard deviation was computed and are illustrated in Table 2.

No signs suggestive of pain using a subjective pain scoring system were present in any dog at any time point.

3.3 | Systemic toxicity potential

No clinical signs of systemic incompatibility or adverse events were noted in any dog at any time point. All values remained in normal clinical ranges without relevant variation. Thus, only the confidence interval and standard deviation were computed.

Values of the differential blood count and coagulation parameters remained within the normal range.

There was no significant change in mean serum VEGF values between day 0 and day 7 (50.8 ± 18.6 pg/mL vs 55.8 ± 11.2 pg/mL, respectively; $P = 0.72$) and between day 0 and day 28 (50.8 ± 18.6 pg/mL vs 52.9 ± 17.0 pg/mL, respectively; $P = 0.47$).

All recorded data are shown in Table 3.

4 | DISCUSSION

In human medicine, there is a widely off-label use of bevacizumab for the treatment of various eye diseases accompanied by pathological angiogenesis.[7,8,15-17] Most of them are retinal, choroidal, and corneal diseases such as neovascular age-related macular degeneration (AMD), diabetic macular edema and macular edema secondary to retinal vein occlusion (RVO), and superficial corneal diseases associated with corneal neovascularization. Chronic keratitis and corneal neovascularization are also common in veterinary ophthalmology[1-3] and a high need for target-directed treatments exists. However, data concerning medical compatibility, safety, and efficacy of bevacizumab in animals are rare.

Abrams et al[29] found that VEGF is higher in aqueous humor than in plasma of diabetic and nondiabetic cataractous dogs suggesting a local production of VEGF within the eye. This assumption is further supported by the observation, that there is a constitutive expression of VEGF receptor-1 by endothelial cells and nonvascular cells of the cornea, uvea, lens, and retina of dogs.[30] It is known that dogs with glaucoma, uveitis, and intraocular neoplasia show a higher VEGF receptor-2 expression than healthy dogs, suggesting a role of VEGF in pathologic angiogenesis in canine eyes.[30] This leads us to the assumption that VEGF is also contributing to pathological vascularization in the cornea of dogs and a therapeutic application of anti-VEGF substances in canine patients with keratitis and CNV is conceivable.

There is an arsenal of various anti-VEGF substances.[31] We chose bevacizumab as it is commercially available and fits a cost-effectiveness ratio suitable for veterinary use.

For the future development of anti-VEGF treatments in veterinary medicine, it is important to keep in mind that

TABLE 2 Mean ± SD of Schirmer tear test (mm/min) and intraocular pressure (mm Hg) in healthy dogs after topical bevacizumab application at baseline and on days 1, 7, and 28 of the study

	Day 0	Day 1	Day 7	Day 28
STT (mm/min)	20.0 ± 2.2	20.6 ± 1.8	21.2 ± 2.3	20.6 ± 1.8
IOP (mm Hg)	18.2 ± 1.0	17.1 ± 1.1	17.4 ± 1.4	17.2 ± 1.2

IOP, intraocular pressure; SD, standard deviation; STT, Schirmer tear test.

bevacizumab is a humanized murine antibody that is designed to bind human VEGF-A. Hence, pharmacological suitability of bevacizumab in veterinary patients might be critically questioned. Recent in vitro experiments demonstrated that bevacizumab binds canine VEGF dose-dependently (Muellerleile et al, submitted to journal 2018). Interestingly, feline and equine VEGF showed linear, dose-independent binding characteristics, suggesting only a non-specific interaction in cats and horses (Muellerleile et al, submitted).

Aside from pharmacologically suitability, the safety profile of bevacizumab for possible future clinical applications is important. Medical compatibility and the occurrence of side effects depend on the application type and the drug dose.[14]

In the present study, no ocular and systemic side effect occurred after topical bevacizumab treatment (2.5 mg/mL BID). These results correlate with those previously reported in people and rodent models.[8,20,21] However, Kim et al described the occurrence of corneal erosions and corneal thinning in people with CNV after long-term bevacizumab treatment (12.5 mg BID).[18] It is noteworthy that the frequency and dose of bevacizumab were much higher in this study and diseased people were examined. However, we decided to use clinically relevant and viable doses with respect to possible future applications of bevacizumab.

Systemic side effects of anti-VEGF therapies have been reported after intravenous and intravitreal bevacizumab injections in human patients. These application types are associated with serious and sometimes fatal side effects, for example, gastrointestinal perforations, hemorrhage, hypertension, cardiac ischemia, cerebrovascular ischemia, and arterial thromboembolic events.[22,23]

It is known that bevacizumab is detectable in serum after intravitreal injections.[24] These observations lead to the concern that topical bevacizumab has the potential to enter the systemic circulation and thus causing systemic effects. We considered it unlikely as there were no documented side effects after topical bevacizumab treatment. Nevertheless, as a precaution, we investigated systemic effects to estimate a potential systemic reaction after topical treatment.

Furthermore, we tested the effect of topical bevacizumab on serum VEGF values. It was so far unknown if topical bevacizumab treatment leads to serum VEGF changes. This topic has been intensively studied for intravitreal injections.[24] In those studies, bevacizumab concentrations reached a maximum serum concentration 7 or 8 days after intravitreal injection.[24] In our study, there was no significant effect on serum VEGF values after topical bevacizumab treatment (2.5 mg/mL BID) over a treatment period of 28 days.

There were some limitations in this study. The sample size was small. Although our findings were favorable, a larger sample size would have gained confidence in the statistics. All analyzed beagles were healthy, male, and relative young. Discrepancies between healthy corneas, corneas of older dogs, and corneas suffering from chronic inflammation are possible due to different corneal VEGF concentrations and structural integrity alterations.

Another shortcoming of our study is that no untreated dogs were included. As safety after topical bevacizumab in healthy dogs was the major question of our study, we decided

TABLE 3 Mean ± SD of heart rate, respiratory rate, systolic and diastolic blood pressure, and serum VEGF levels in healthy dogs after topical bevacizumab application at baseline and on days 1-7 and day 28 of the study

	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 28
Heart rate (heartbeat/minute)	98.7 ± 11	95.6 ± 8.1	94.2 ± 6.0	92.9 ± 4.8	97.3 ± 8.7	96.4 ± 7.6	94.2 ± 7.0	95.6 ± 8.1	94.2 ± 4.5
Respiratory rate (breaths/minute)	23.6 ± 4.2	24.0 ± 2.8	23.1 ± 3.3	23.6 ± 2.4	23.6 ± 3.1	23.6 ± 2.4	22.2 ± 2.9	23.1 ± 3.3	24.0 ± 2.8
BP (systolic) (mm Hg)	147.1 ± 5.5	143.4 ± 3.4	142.7 ± 4.4	141.6 ± 3.2	141.8 ± 3.9	144.4 ± 3.9	142.8 ± 3.5	145.6 ± 4.7	148.6 ± 6.6
BP (diastolic) (mm Hg)	73.6 ± 9.6	73.7 ± 7.3	73.6 ± 7.6	72.3 ± 10.2	74.6 ± 8.3	74.0 ± 8.3	74.2 ± 7.8	73.6 ± 8.6	73.1 ± 10.1
Serum VEGF (pg/mL)	50.8 ± 18.6							55.8 ± 11.2	52.9 ± 17.0

BP, blood pressure; SD, standard deviation; VEGF, vascular endothelial growth factor.

to use the contralateral eye as control. Baseline blood values and baseline vital parameters before treatment were compared to those after treatment start to detect systemic effects. No systemic side effects are reported in human medicine[8] and no side effects occurred in our study so one might argue the value of a separated control group. Anyway, we considered that there was no real control group and we confessed that a separation into a control group and a treatment group would have obtained a better reasonable assurance particularly for systemic effects.

Chosen by the institutional pharmacist the left eye was the treated one. One might argue that laterality plays a role and the left eyes could have reacted differently. There is no publication supporting this theory. Bilateral administration might lead to a higher serum drug concentration, however probably not high enough to cause side effects. In human medicine, no systemic side effects have been reported after topical bevacizumab even in higher concentrations and after bilateral use.[8] However, further studies are required to study larger animal groups with uni- and bilaterally affected and treated dogs.

Another limitation of our study is the treatment duration and drug concentration. Currently, there is no knowledge about safety of topical bevacizumab in dogs. In accordance to treatment protocols of former clinical human studies, an approved concentration of 2.5 mg/mL[7] was tested over a period of 28 days to find a basic idea. Anyway, it would have been interesting to investigate the safety, changes of serum VEGF values, and particularly the effectiveness of bevacizumab in higher drug concentrations. We are aware that patients with chronic keratitis require long-term therapy and future studies will be necessary to evaluate the effect and safety over a longer period than 28 days.

5 | CONCLUSION

In summary, topical bevacizumab (2.5 mg/mL BID) seems to be topically and systemically safe in healthy dogs. Additionally, no changes in serum VEGF values after topically administered bevacizumab were observed. Our results provide a basis for the future development of anti-VEGF treatments for veterinary use. However, further studies are needed to assess differences between uni- and bilateral use, laterality, and a longer treatment period. It will be an issue of future studies to investigate the safety and efficacy in diseased dogs with naturally occurring corneal neovascularization.

REFERENCES

1. Labelle A, Dresser C, Hamor R, Allender M, Disney J. Characteristics of, prevalence of, and risk factors for corneal pigmentation (pigmentary keratopathy) in Pugs. *J Am Vet Med Assoc*. 2013;243(5):667-674.
2. Sanchez RF, Innocent G, Mould J, Billson F. Canine keratoconjunctivitis sicca: disease trends in a review of 229 cases. *J Small Anim Pract*. 2007;48:211-217.
3. Slatter DH, Lavach JD, Severin GA, Youg S. Überreiter's Syndrome (chronic superficial keratitis) in dogs in the Rocky Mountain area - A study of 463 cases. *J Small Anim Pract*. 1977;18(12):757-772.
4. Maddula S, Davis DK, Maddula S, Burrow MK, Ambati BK. Horizons in Therapy for Corneal Angiogenesis. *Ophthalmology*. 2011;118(3):591-599.
5. Epstein R, Stulting R, Hendricks R, Harris D. Corneal neovascularization. Pathogenesis and inhibition. *Cornea*. 1987;6(4):250-257.
6. Dana MR, Streilein W. Loss and restoration of immune privilege in eyes with corneal neovascularization. *Invest Ophthalmol Vis Sci*. 1996;37(12):2485-2494.
7. Krizova D, Vokrojova M, Liehneova K, Studeny P. Treatment of corneal neovascularization using anti-VEGF bevacizumab. *Journal of Ophthalmology*. 2014;2014:1-8.
8. Dastjerdi MH, Al-Arfaj KM, Nallasamy N, et al. Topical bevacizumab in the treatment of corneal neovascularization: results of a prospective, open-label, non-comparative study. *Arch Ophthalmol*. 2010;127:381-389.
9. Sella R, Gal-or O, Livny E, et al. Effifacy of topical aflibercept versus topical bevacizumab for the prevention of corneal neovascularization in a rat model. *Exp Eye Res*. 2016;146:224-232.
10. Ozdemir O, Altintas L, Altintas O, Ozkan B, Akdag C, Yüksel N. Comparison of the effects of subconjunctival and topical anti-VEGF therapy (bevacizumab) on experimental corneal neovascularization. *Arq Bras Oftalmol*. 2014;77(4):209-213.
11. Binder DR, Herring IP, Zimmerman KL, Phillip Pickett J, Huckle WR. Expression of vascular endothelial growth factor receptor-1 and -2 in normal and diseased canine eyes. *Vet Ophthalmol*. 2012;15(4):223-230.
12. Henriksen ML, Andersen PH, Mietelka K, et al. Equine deep stromal abscesses (51 cases - 2004–2009) - Part 2: The histopathology and immunohistochemical aspect with attention to the histopathologic diagnosis, vascular response, and infectious agents. *Vet Ophthalmol*. 2014;17(SUPPL.1):14-22.
13. Philipp W, Speicher L, Humpel C. Expression of vascular endothelial growth factor and its receptors in finlamed and vascularized human corneas. *Invest Ophthalmol Vis Sci*. 2016;41(9):2514-2522.
14. Ferrara N, Hillan KJ, Gerber H, Novotny W. Discovery and development of bevacizumab, an anti-VEGF antibody for treating cancer. *Nat Rev Drug Discovery*. 2004;3:391-400.
15. Avery RL, Pieramici DJ, Rabena MD, Castellarin AA, Nasir MA, Giust MJ. Intravitreal bevacizumab (Avastin) for neovascular age-related macular degeneration. *Ophthalmology*. 2006;113(3):363-372.
16. Martin DF, Maguire MG, Fine SL, et al. Ranibizumab and bevacizumab for treatment of neovascular age-related macular degeneration: Two-year results. *Ophthalmology*. 2012;119(7):1388-1398.
17. Arevalo JF, Fromow-Guerra J, Quiroz-Mercado H, et al. Primary intravitreal bevacizumab (Avastin) for diabetic macular edema. Results from the Pan-American collaborative retina study group at 6-month follow-up. *Ophthalmology*. 2007;114(4):743-750.
18. Kim SW, Ha BJ, Kim EK, Tchah H, Kim T. The effect of topical bevacizumab on corneal neovascularization. *Ophthalmology*. 2008;115(6):33-38.

19. Papadopoulos N, Martin J, Ruan Q, Rafique A, Rosconi M, Shi E, et al. Binding and neutralization of vascular endothelial growth factor (VEGF) and related ligands by VEGF Trap, ranibizumab and bevacizumab. *Angiogenesis*. 2012;15:171-185.
20. Yoeruek E, Ziemssen F, Henke-Fahle S, Tatar O, Tura A, Grisanti S, et al. Safety, penetration and efficacy of topically applied bevacizumab : evaluation of eyedrops in corneal neovascularization after chemical burn. *Acta Ophthalmol*. 2008;86:322-328.
21. Bock F, Onderka J, Dietrich T, et al. Bevacizumab as a potent inhibitor of inflammatory corneal angiogenesis and lymphangiogenesis. *Invest Ophthalmol Vis Sci*. 2007;48(6):2545-2552.
22. Shima C, Sakaguchi H, Gomi F, Kamei M, Ikuno Y, Oshima Y, et al. Complications in patients after intravitreal injection of bevacizumab. *Acta Ophthalmol*. 2008;86:372-376.
23. Wu L, Martínez-Castellanos M, Quiroz-Mercado H, et al. Twelve-month safety of intravitreal injection of bevacizumab (Avastin): results of the Pan-America Collaborative Study Group (PACORES). *Graefe's Arch Clin Exper Ophthalmol*. 2008;246(1):81-87.
24. Zehetner C, Kirchmair R, Huber S, Kralinger MT, Kieselbach GF. Plasma levels of vascular endothelial growth factor before and after intravitreal injection of bevacizumab, ranibizumab and pegaptanib in patients with age-related macular degeneration, and in patients with diabetic macular oedema. *Br J Ophthalmol*. 2013;97:454-459.
25. Krämer I, Thiesen J. In: Kraemer I, Thiesen J, eds. *Stabil-Liste: Physikalisch-chemische Stabilität, Kompatibilität und Inkompatbillität parenteral applizierbarer Zytostatika, Virustatika und Supportivtherapeutika*. 6th ed. München: ADKA, Bundesverband Dt. Krankenhausapotheker; 2013.
26. Munger RJ. Veterinary ophthalmology in laboratory animal studies. *Vet Ophthalmol*. 2002;5:167-175.
27. Clark JS, Bentley E, Smith LJ. Evaluation of topical nalbuphine or oral tramadol as analgesics for corneal pain in dogs: A pilot study. *Vet Ophthalmol*. 2011;14(6):358-364.
28. Papathanassiou M, Theodossiadis PG, Liarakos VS, Rouvas A, Giamarellos-Bourboulis EJ, Vergados IA. Inhibition of corneal neovascularization by subconjunctival bevacizumab in an animal model. *Am J Ophthalmol*. 2008;145(3):424-431.
29. Abrams KL, Stabila PF, Kauper K, Elliott S. Vascular endothelial growth factor in diabetic and nondiabetic canine cataract patients. *Vet Ophthalmol*. 2011;14(2):93-99.
30. Binder DR, Herring IP. Evaluation of nasolacrimal fluorescein transit time in Ophthalmically Normal Dogs and nonbrachycephalic cats. *Am J Vet Res*. 2010;71(5):570-574.
31. Chang J-H, Garg NK, Lunde E, Han K-Y, Jain S, Aszar DT. Corneal neovascularization: an anti-VEGF therapy review. *Surv Ophthalmol*. 2012;57(5):415-429.

How to cite this article: Muellerleile L-M, Tichy A, Nell B. Serum vascular endothelial growth factor changes and safety after topical anti-human VEGF antibody bevacizumab in healthy dogs. *Vet Ophthalmol*. 2019;22:600–606. https://doi.org/10.1111/vop.12628

Veterinary Ophthalmology

Topical bevacizumab for the treatment of corneal vascularization in dogs: A case series

Journal:	*Veterinary Ophthalmology*
Manuscript ID	VOP-21-03-2625
Wiley - Manuscript type:	Case Report
Keywords:	bevacizumab, VEGF, Vascular endothelial growth factor, corneal vascularization, keratitis, dog

Topical bevacizumab for the treatment of corneal vascularization in dogs: A case series

Lisa-Marie Muellerleile, *[1] Michael Bernkopf, ‡[2] Michael Wambacher, §[3] Barbara Nell, *[4]

**Department of Companion Animals and Horses, University of Veterinary Medicine Vienna, Veterinärplatz 1, Vienna, A-1210, Austria; ‡E-Learning and New Media, University of Veterinary Medicine Vienna, Veterinärplatz 1, Vienna, A-1210, Austria, and §Hospital Pharmacy, University of Veterinary Medicine Vienna, Veterinärplatz 1, Vienna, A-1210, Austria*

Running title: Bevacizumab in canine corneal vascularization

Key words: bevacizumab, VEGF, Vascular endothelial growth factors, corneal vascularization, keratitis, dog

Corresponding author

Lisa-Marie Muellerleile, Dr.med.vet.

University of Veterinary Medicine Vienna

Department of Companion Animals and Horses

Veterinaerplatz 1,

A-1210 Vienna, Austria

Tel.: +43 1 25077 5332

Fax.: +43 1 25077 5391

e-mail: l-m.muellerleile@gmx.de

Michael Bernkopf, Dr.med.vet.

Michael Wambacher, Mag.

Barbara Nell, Prof. Dr., Dipl. ECVO

ABSTRACT

Objective To evaluate the effect and safety of topical anti-human vascular endothelial growth factor bevacizumab in dogs with persistent corneal vascularization.

Animal studied Prospective case series of 15 adult dogs (20 eyes).

Procedures Dogs received 0.25% bevacizumab eye drops BID for 28 days. Follow-ups were scheduled 28 days and 6-7 months after treatment start. Macroscopic findings were scored for conjunctival hyperemia, chemosis, ocular discharge, corneal edema, vascularization, and pigmentation. Vascularized area was assessed by analyzing photographs using an imaging software.

Results The extent of treatment response was variable. Some cases showed a marked reduction in vascularized area and edema, other eyes had subtle signs of improvement. Vascularization score decreased from 1.5 to 1.1 and vascularized area was reduced by 48.8% after 4 weeks. A thinning rather than a shortening of vessels, a consolidation of areal bleedings into fine vascular networks, decrease of distal vessel branching, and a change from blurry vascularized beds into demarcated thin vessels were observed. One dog developed a corneal erosion 6 months after last bevacizumab administration. Two dogs died 4.5 and 4 months after the last bevacizumab administration at the age of 16 and 12 years, respectively. In all events, a causal relationship is unlikely but cannot be ruled out with complete certainity.

Conclusions Topical 0.25% bevacizumab may be an effective treatment option for corneal vascularization in dogs. Further long-term and placebo-controlled studies with larger patient cohorts are needed to investigate dosage, safety, possible use as a single treatment, and routes of administration.

INTRODUCTION

The healthy canine cornea is clear and avascular and any angiogenic activity within the cornea, whether caused by hypoxia, a corneal insult or an inflammatory process, is pathological. [1,2] Corneal vascularization can lead to visual impairment through corneal edema, tissue scarring, and lipid- and pigment deposition, and is associated with persistent corneal inflammation and loss of the corneal immune privilege.[1–4] Several diseases in dogs are accompanied by corneal vascularization, for instance keratoconjunctivitis sicca, immune mediated keratitis, chronic superficial keratitis, and the pigmentary keratitis syndrome in brachycephalic breeds.[5–7]

A promising new therapy for corneal vascularization has been investigated in human ophthalmology and targets the Vascular Endothelial Growth Factor A (VEGF-A).[8–11] VEGF-A is a promotor of the physiological and pathological development of blood vessels and is increased in inflamed and vascularized corneas, suggesting a causative role of corneal vascularization [4,12] Hence, the therapeutic inhibition of VEGF-A could be a novel treatment option for canine patients with corneal vascularization.

Several types of therapeutic VEGF inhibitors that are used for the treatment of a variety of ocular disorders in humans.[13–15] One of these drugs is bevacizumab, a humanized murine VEGF antibody that binds all isoforms of human VEGF-A and its pharmacological suitability to canine VEGF has been shown, too. [16–18] The antiangiogenic effect of topical bevacizumab has already been demonstrated in vivo in various animal models and in human patients with corneal vascularization. [8–11,19,20]

This case series describes the effect, the safety profile, and the long-term outcome of topical 0.25% bevacizumab administered twice a day over 28 days in dogs with corneal vascularization.

MATERIAL & METHODS

Animals

The protocol involving client-owned dogs in this study was approved by the institutional ethics and animal welfare committee and the national authority according to §§ 26ff. of Animal Experiments Act, Tierversuchsgesetz 2012 —TVG 2012 (GZ 68.205/0046-V/3b/2018 and 68.205/0042-V/3b/2019). Client-owned dogs were presented within the ophthalmological service of the University of Veterinary Medicine, Vienna. Signalment information was recorded for each dog during the enrollment visit as indicated by the owner. The owners signed an informed consent form as part of the enrollment procedure.

Drug preparation and labelling

A 0.25% solution of bevacizumab eye drops was aseptically prepared and filled from a commercially available bevacizumab solution (Avastin®, Roche, Grenzach-Wyhlen, Germany) by the hospital pharmacy according to good manufacturing practice (GMP). Sterile 0.9% saline (B. Braun Melsungen AG, Melsungen, Germany) served as the solvent. With regard to the shelf life of bevacizumab, the eye drops were prepared at the same day of the study treatment initiation.[21] The pharmacist (MW) prepared single dose containers for each dog and each administration with a single dose of 0.5 mL of 0.25% bevacizumab. The labels were inscribed based on GMP standards [22] and a detailed written instruction leaflet was enclosed.

Recruitment criteria

Dogs had to be adult (≥16 months) and were included if they had persistent corneal vascularization for at least 28 days. Exclusion criteria were corneal surface defects, Schirmer tear test-1 measurements smaller than 15 mm/min, treatment with VEGF-influencing drugs or agents

made up of human proteins 28 days before the study start, known coagulation disorders, or clinical manifest general diseases.

Treatment regime and study design

The dogs were treated and examined on an outpatient basis. The eye drops were administered by the dog owners after a detailed instruction by the first author. (LM). On day 0 study treatment was initiated. The study eye was treated with one drop of 0.25% bevacizumab twice a day over 28 days. At least 4 weeks and about 6-7 months (long-term follow-up) after treatment initiation, a reexamination was scheduled. In some cases, depending on the indication and disease progression, additional follow-up examinations were planned at the discretion of the study investigators. Each study visit included a full physical and ocular examination, a safety assessment, and photographs of the cornea.

Ocular examination

A complete ocular examination was carried out in all dogs by the same investigator (LM) under supervision of a board-certified ophthalmologist (BN). Each exam included slit-lamp biomicroscopy (Kowa SL-15; Kowa, Tokyo, Japan), indirect ophthalmoscopy (Keeler Vantage; Keeler Instruments Inc, Broomall, PA), Schirmer tear test-1 (Teststreifen, MSD, Unterschleissheim, Germany), fluorescein staining (Fluorotouch Ophthalmic Strips, Eickemeyer, Tuttlingen, Germany), and measurement of intraocular pressure using rebound tonometry (TonoVet, Icare, Vantaa, Finland). The examination of the posterior eye segment was conducted after pharmacological mydriasis (Mydriaticum, Agepha, Senec, Slovakia).

Photography

Photographs were taken with a digital system camera (OM-D E-M10 Mark III, Olympus) in combination with a magnification macro lens (M.ZUIKO DIGITAL ED 60mm F2.8 Macro

/120mm, Olympus) and a pincer-shaped macro flash light (STF-8 Macro Flash, Olympus) in the same examination room by the same 2 investigators (LM and MB). The room was darkened by a black shutter and only one ceiling light was turned on. The light intensity was measured with a luxmeter to control consistent lighting conditions (Light Meter Model Nr. 4332004118, Urceri, USA). The ISO was set to 200 and the camera exposure was controlled manually. The camera aperture was set to 8 or 10 and shutter speed to 1/1200 or 1/160 sec. The autofocus was set on a central point in the center of the image. The macro flash was used in automatic exposure (TTL) mode, and in some cases overruled by manual exposure compensation. The image files were saved in RAW format and were processed to TIF images under standardized conditions (MB) using Adobe Photoshop and Adobe Camera Raw (Adobe Inc., USA). Files were stored LZW-compressed (lossless). The images were cropped to fill the entire format in an aspect ratio of 1:1. During image processing, attention was paid to the best possible presentation of the corneal vessels in the target file; the parameters exposure, contrasts, highlights, blacks, clarity, vibrance, sharpness, and saturation were adjusted accordingly. The settings chosen when a patient's eye was first captured in Adobe Camera Raw, were saved as an xmp metafile and applied to subsequent images of that patient. Starting from this basic setting, the image was then fine-tuned, if necessary, by adjusting the above-mentioned parameters in order to achieve a comparable vessel image (Fig. 1).

Tolerability assessment

Any reported adverse event by the dog owners and any abnormal ocular or general finding observed during the study visits was recorded. Pain sensation was assessed by the same investigator (LM) using a subjective pain scoring system [23,24] (Supplemental Table 1).

Additionally, the owners were asked to rate the sensation of pain according to a predefined pain scorings (Supplemental Table 2).

Efficacy assessment and quantification of corneal vascularization

A modified scoring system was used to quantify macroscopic findings at each visit during slit-lamp biomicroscopy.[25] The following variables were scored for each eye: Corneal edema, vascularization, and pigmentation: 0 = <25%, 1 = 26% to 50%, 2 = 51% to 75%, 3 = >76% (area relative to total corneal area). Conjunctival hyperemia, conjunctival chemosis, and ocular discharge: 0 = none, 1 = mild, 2 = moderate, 3 = severe.

Additionally, the vascularized corneal area was assessed and the vessels incisions located on a given circle within the corneal surface (4 circles in total, each 25% smaller: 0-25-50-75-circle) were counted at baseline visit, 4 weeks after treatment start and at long-term follow-up visit (Fig.2). Only blood-filled vessels were included in the analysis. Each measurement was performed three times and the mean value was used for analysis. Analysis were performed using an imaging software (Fiji; open-source software: https://imagej.net/Fiji) and Microsoft Excel (Microsoft Corporation, Mircosoft Excel 2016).

Data analysis

Data collection and analysis were performed using Microsoft Excel (Microsoft Corporation, Mircosoft Excel 2016). Quantitative data were listed as mean ± standard deviation. The rate and type of side effects were evaluated in a descriptive manner.

RESULTS

Animals

Twenty eyes of 15 dogs met the eligibility criteria and were included in the study. In two cases, the first follow-up after 28 days was delayed because of personal reasons of the dog owners. Two dogs died shortly before the final long-term examination and were therefore lost to follow-up, which is discussed below. Signalment and ocular diagnosis is listed in Table 1. Detailed patient history and pretreatment information are outlined in Supplemental Table 3 and Table 4.

Tolerability assessment

One dog (O #20) showed increased photosensitivity and ocular pain of the studied eye at the final examination, 6 months after the last bevacizumab administration. According to the dog owner, the symptoms had been present for 2 weeks, the dog was rubbing the eye due to a generalized pruritus, and all medications were discontinued because meningitis was suspected 6 weeks earlier. The discontinued medication included topical cyclosporine (Optimmune® BID OU, MSD Animal Health, Kenilworth) and systemic immunosuppressive therapy for severe allergic dermatitis. Ocular findings included corneal erosion with a loose epithelium and a halo of less intensive fluorescein-staining at the ulcer margins OD, consistent with the diagnosis of superficial chronic corneal epithelial defect (SCCED). The SCCED was located in the ventronasal quadrant. The corneal vascularization, located in the ventrotemporal quadrant, was unchanged and the overlying epithelium was intact. Diamond burr debridement (DBD) was performed under topical anesthesia (Novain 0.4% eye drops, Agepha Pharma, Bratislava, Slovakia). Topical ofloxacin (Ofloxa-Vision® sine, Omnivision, Puchheim, Germany) and systemic meloxicam (0.1mg/kg SID p.o., Metacam®, Boehringer Ingelheim, Ingelheim am Rhein, Germany) were prescribed. A follow-up was scheduled after 2 weeks, however the dog

owner reported that 10 days after the DBD the erosion progressed into a deep ulcer and the eye was therefore enucleated on an emergency basis at the referring veterinarian. No histological examination was performed.

Two dogs died after the bevacizumab treatment just before the final examination. One dog (E #6 and #7) died 4.5 months after the last bevacizumab administration of unknown reasons at the age of 16 years. It was treated asynchronous on both eyes with topical bevacizumab. The dog suffered from mitral endocardiosis which was regularly examined by a cardiologist. It was treated with systemic angiotensin converting enzyme (ACE) inhibitors (substance unknown). Blood pressure and vital parameters were normal during cardiac examinations before and during this study. There were no signs of cardiovascular or pulmonary dysfunction at any time point prior and during the study. A histopathological examination was not performed. Another dog (J #14) died 4 months after the last bevacizumab administration. According to the referring veterinarian, he died of a incurable pulmonary edema at the age of 12 years. The patient suffered from mitral valve insufficiency and was examined regularly by a cardiologist. The dog was treated with systemic furosemide and benazepril and did not show any signs of cardiovascular decompensation, pulmonary dysfunction, or general incompatibility neither during the study visits nor at the cardiological examinations at any timepoint. A histopathological examination was not carried out.

In all other dogs, there was no evidence of ocular or systemic intolerance or pain associated with brolucizumab eye drops. All clinical parameters remained within the physiological range with only clinically irrelevant minor fluctuations.

No dog owner reported any change in behavior, signs of pain, or touch response after administration of bevacizumab. In the investigator's pain assessment, none of the dogs showed a

change in pain perception at any time point, and pain scores remained unchanged (data not shown).

Efficacy assessment and quantification of corneal vascularization

The extent of clinical outcomes was highly variable, as reflected by the high variation of the minimum and maximum values recorded for the vascularized area and the count of vessel incision (Table 2). While some cases showed a marked decrease of corneal vascularization (Fig.3 and 4), other dogs had more subtle signs of clinical improvement, such as increased ocular comfort, less conjunctival hyperemia and chemosis, decrease of corneal edema and pigmentation, or decrease of corneal pigmentation and less ocular discharge (Fig.5)

The mean vascularized area was reduced by 48.8% (range 4.9%-100%) after 4 weeks of bevacizumab treatment (n=10) (Table 2). In cases of diffuse corneal vascularization or scattered vessels distributed over the entire corneal surface, measurement of vascularized area was not performed.

The mean score of macroscopic assessed corneal vascularization and edema decreased from 1.6 to 1.0 and from 1.5 to 1.1, respectively (n=20) (Fig.6).

The count of vessel incisions showed a mean reduction of 28.0%, 31.1%, 4.6%, 16.5% for the 0-, 25-, 50-, and 75- circle, respectively (n=17). Counting the vessel incisions was not possible for areal corneal bleedings or in cases of generalized corneal vascularization. In some cases, the number of vessel incisions of the two innermost circles (50- and 75-circle) increased after bevacizumab treatment (Table 2). In these cases, blood vessels became visible because of a decrease of corneal edema or diffuse and blurred hemorrhages at the end of each blood vessel developed into well-defined fine blood vessels (Fig.7). Subjectively, we rather observed a reduction of the vascular caliber and a decrease of vessel branching, than a shortening of blood

vessels (Fig.7-9). Some blood vessels contained less or no blood at all and diffuse or blurred corneal hemorrhages consolidated to well defined thin vessels.

Long-term follow up

The clinical improvement observed in week 4 was maintained for all but one patient (K#15) in the long-term follow up visit (Fig.9-13). The owner had discontinued the prescribed topical cyclosporine treatment. Clinical findings included a relapse of superficial corneal vessels OS (studied eye), new corneal vessels OD, and a worsening of the quantitative tear film deficiency OU.

DISCUSSION

The present study indicates that topical 0.25% bevacizumab may be an effective treatment option for persistent corneal vascularization in dogs. The range of response to therapy was high, which was also observed in human medicine.[8] The permeability of a soluble substance is affected by the porosity, conductivity, and the sinuousness of the medium to be permeated.[26,27] Because of the great heterogeneity in etiology, severity, disease duration, and varying tissue scarring of the studied dogs, the corneas may exhibit varying degrees of structural alteration, drug permeability and bioavailability.

The involvement of other factors in neoangiogenesis, such as fibroblast growth factors, are not inhibited by bevacizumab and this might be another reason for partial treatment response. [28] Bevacizumab can inhibit the proliferation in growing blood vessel, but in mature blood vessels already covered with pericytes, which most likely do not require VEGF-A for proliferation.[29,30] Therefore VEGF inhibitors might lead to an insufficient therapy response.[29,30]

We observed that it was not so much a reduction in vessel length, but a thinning of blood vessels. These observations are consistent with reports in human patients.[8,10,31] VEGF is as an effective vasodilator and increases the vascular permeability.[32,33] Thus, VEGF inhibition lowers the blood flow rate and narrows the blood vessel diameter, which can explain our findings.[8,32,33]

All but one patient showed stationary corneal vascularization until at long-term follow-up visit. This also correlates with clinical studies in human patients. [8,9] Anyway, patients may have individual needs and there are many factors that can influence the response to treatment, as discussed earlier. We assume that in some cases, a longer treatment duration, a second therapy session, and a higher dose may be necessary.

In general, topical application is the preferred route for the treatment of corneal diseases in animal patients.[34] The efficacy of topical drug administration depends on the ability to penetrate the corneal epithelium to reach the target tissue in a sufficiently high therapeutic dose. Bevacizumab has a molecular weight of 149kD and is too large to permeate the intact corneal epithelium. [18,35,36] However, in corneas with insufficient barrier function, bevacizumab is known to penetrate into the corneal stroma.[19,36]

Besides bevacizumab, there are other VEGF inhibitors with a smaller molecular size, different molecular charge, binding affinity, and binding spectrum. [15,16,37] These drugs should be considered in future studies. Besides the topical application just discussed, another route of administration is conceivable. Studies have shown that subconjunctival bevacizumab is able to reduce corneal vascularization effectively.[20,36]

VEGF-A is not only involved in pathological vascularization, but also has vital functions such as corneal nerve regeneration and wound healing. [38,39] Topical bevacizumab has been reported to increase the risk of corneal erosions, especially with prolonged use or at a higher dose.[8,10,40] In our study one eye developed a corneal erosion 6 months after the last bevacizumab administration. Unfortunately, the erosion developed into a deep corneal ulcer. The dog suffered from an immune mediated keratitis and severe allergic dermatitis. We consider it unlikely that a drug-related side effect was the cause, since the last bevacizumab dose was 6 months ago, all immunosuppressive systemic and topical medications had been discontinued shortly before the adverse event occurred, and anamnestically the dog scratched the eye. Nevertheless, we cannot exclude a causal relationship with complete certainty. Unfortunately, no histopathologic examination was performed. Concerning patient selection, it has to be considered, that

276 therapeutic VEGF inhibition can contribute to an increased risk of corneal tissue damage,
277 particularly in patients who are prone to spontaneous epithelial defects.[40]
278 In rare cases the administration of intravitreal bevacizumab in human patients is associated with
279 severe systemic adverse events.[41,42] In our study two dogs died 4 and 4.5 months after the last
280 bevacizumab administration at the age of 12 and 16 years. Both patients suffered from mitral
281 valve insufficiency and were examined regularly by a cardiologist. Both patients had no signs of
282 cardiovascular or pulmonary dysfunction prior or during the study exams. A causal relationship
283 cannot be ruled out completely. Since the dogs died a long time after the last bevacizumab
284 administration, the patients showed no abnormalities in the physical examinations during
285 bevacizumab treatment, and the dose was very low a causal relation is unlikely. Topical 0.25%
286 bevacizumab for 28 days is known not to alter systemic VEGF levels in healthy dogs.[43] Although
287 the uptake of bevacizumab into the bloodstream may be different in an inflamed cornea and
288 conjunctiva, we do not believe that bevacizumab given for a short time and at a low dose of
289 0.25% would reach a therapeutic concentration high enough to cause systemic side effects.[35]
290 Anyway, prolonged neutralization of VEGF may have unintended local or systemic
291 consequences and systemic VEGF concentrations in diseased dogs would help answer that
292 question.
293 Sandberg et al. found that dogs with intraocular disorders such as glaucoma, lens-induced
294 uveitis, retinal detachment, intraocular tumors, and grade-3 preiridal-fibrovascular membranes
295 (PIFM) had significantly higher VEGF concentrations in the aqueous humor compared with
296 normal eyes.[44] Zarfoss et al. demonstrated that blood vessels and nonvascular spindle cells of
297 canine PIFMs were immunohistochemically positive for VEGF.[45] VEGFR2 is highly expressed
298 in the vascular endothelium of diseased eyes of dogs.[46] These findings suggest that VEGF is an

essential promoter in numerous canine intraocular disorders and treatments that inhibit VEGF are of great interest. However, topical bevacizumab cannot penetrate the normal cornea. In human medicine, intracameral and intravitreal bevacizumab injections are used routinely to treat various ocular disorders (off-label).[35,42,47] Intraocular administration routes are also conceivable in dogs, but the safety profile is unexplored.

Bevacizumab was developed and approved for the treatment of several cancer types.[18] In veterinary medicine, there is one report on an Amur tiger with a palpebral sebaceous gland carcinoma that was treated successfully with intralesional bevacizumab as an adjuvant to surgery. [48] At the University of Veterinary Medicine Vienna, a female Appaloosa suffering from a relapsed corneal stromal invasive squamous cell carcinoma has been treated with intrastromal bevacizumab as an adjuvant to surgical excision, topical mitomycin C, and systemic firocoxib. The horse has been relapse-free for at least 9 months (K.-O. Blohm, personal correspondence).

The study has several limitations. In some cases it was difficult to detect the exact margins of the vessels, because of corneal pigmentation or edema. It must be assumed that corneal vessels also run indiscernibly under the pigmentation or edema. In some eyes, it was not useful to quantify the vascularized area because the vessels were distributed over the entire surface. In contrast to focal changes, which can certainly be assessed with this approach, generalized or diffuse corneal vascularization should be examined with another method. Krizova et al. divided the corneal surface into triangular segments of identical size and evaluated the number of segments affected by corneal vascularization.[9] Dastjerdi et al. assessed the surface area of the corneal blood vessels themselves by tracing the vessels and erasing the nonvascular area. The remaining vascularized area was then pixelated and measured.[8] Another limitation is the heterogeneity of our patient cohort. In all but two eyes, topical bevacizumab was given parallel to topical cyclosporine. This

was decided from an ethical point of view, as we did not want to run the risk of worsening the disease status. It cannot be said with certainty that the effect was achieved by bevacizumab alone. Nevertheless, the studied dogs received topical cyclosporine as a long-term therapy over months or years without further improvement of corneal vascularization. Additionally, recent research found that topical cyclosporine 1% TID did not inhibit corneal angiogenesis in rats.[49] Based on these arguments, it can be assumed that the observed anti-angiogenic effect is due to bevacizumab. Another limitation is the study design. The study was planned as a prospective open-label case series with no control group. Future studies with larger patient cohorts and a controlled and masked study setting are needed to answer this pending questions. In this study, due to the lack of knowledge of bevacizumab's safety profile in dogs, we opted for a low dose over a short period of time. However, it is of great interest and the subject of future studies to discover the clinical impact of higher bevacizumab concentrations, longer treatment durations, and another route of administration, such as subconjunctival or intrastromal application.

Conclusions

In conclusion, the clinical improvement and reduction of the corneal vascularization indicates that topical bevacizumab can be an effective therapy to reduce corneal vascularization in dogs. Attention has to be paid on dogs with recurrent superficial epithelial defects. Further research with larger patients cohorts and placebo-controlled long-term studies are warranted to gain further insights into different dosing regimens, safety profile, clinical impact as a monotherapy, routes of administration, and the use for other indications.

342 ACKNOWLEDGEMENTS

343 This research was supported using resources of the VetCore Facility (VetImaging | VetBiobank)

344 of the University of Veterinary Medicine Vienna.

345

346 **Conflict of Interest**

347 This study was funded by the incomes of the ophthalmology unit of the Department of Companion

348 Animals and Horses, University of Veterinary Medicine Vienna.

349 Lisa-Marie Muellerleile wants to disclose that she is working full-time for Novartis Pharma AG.

350 At no time she was paid for any part of this work nor was she otherwise influenced by her

351 employment in the creation of this research project.

REFERENCES

1. Dubielzig RR, Ketring KL, McLellan GJ, Albert DM. *Veterinary Ocular Pathology: A Comparative Review*. Philadelphia: Saunders Ltd.; 2010.202-203 p.
2. Gelatt K, Gilger BC, Kern TJ. *Veterinary Ophthalmology*. 5th ed. Hoboken: John Wiley & Sons, Inc.; 2013.62, 977 p.
3. Dana MR, Streilein JW. Loss and restoration of immune privilege in eyes with corneal neovascularization. *Investig Ophthalmol Vis Sci*. 1996;37(12):2485-2494.
4. Philipp W, Speicher L, Humpel C. Expression of Vascular Endothelial Growth Factor and Its Receptors in Inflamed and Vascularized Human Corneas. *Investig Ophthalmol Vis Sci*. 2000;41(9):2514-2522.
5. Labelle AL, Dresser CB, Hamor RE, Allender MC, Disney JL. Characteristics of, prevalence of, and risk factors for corneal pigmentation (pigmentary keratopathy) in Pugs. *J Am Vet Med Assoc*. 2013;243(5):667-674. doi:10.2460/javma.243.5.667.
6. Sanchez RF, Innocent G, Mould J, Billson FM. Canine keratoconjunctivitis sicca: Disease trends in a review of 229 cases. *J Small Anim Pract*. 2007;48(4):211-217. doi:10.1111/j.1748-5827.2006.00185.x
7. Allgoewer I, Hoecht S. Radiotherapy for canine chronic superficial keratitis using soft X-rays (15 kV). *Vet Ophthalmol*. 2010;13(1):20-25. doi:10.1111/j.1463-5224.2009.00750.x
8. Dastjerdi MH, Al-Arfaj KM, Nallasamy N, et al. Topical bevacizumab in the treatment of corneal neovascularization; results of a prospective, open-label, noncomparative study. *Arch Ophthalmol*. 2009;127(4):381-389. doi:10.1001/archophthalmol.2009.18
9. Krizova D, Vokrojova M, Liehneova K, Studeny P. Treatment of corneal neovascularization using anti-VEGF bevacizumab. *J Ophthalmol*. 2014;2014:178132.

doi:10.1155/2014/178132

10. Koenig Y, Bock F, Horn F, Kruse F, Straub K, Cursiefen C. Short- and long-term safety profile and efficacy of topical bevacizumab (Avastin®) eye drops against corneal neovascularization. *Graefe's Arch Clin Exp Ophthalmol*. 2009;247:1375-1382. doi:10.1007/s00417-009-1099-1

11. Cheng S-F, Dastjerdi MH, Ferrari G, et al. Short-term topical bevacizumab in the treatment of stable corneal neovascularization. *Am J Ophthalmol*. 2012;154(6):940-948.e1. doi:10.1016/j.ajo.2012.06.007

12. Cursiefen C, Rummelt C, Küchle M. Immunohistochemical localization of vascular endothelial growth factor, transforming growth factor α, and transforming growth factor β1 in human corneas with neovascularization. *Cornea*. 2000;19(4):526-533. doi:10.1097/00003226-200007000-00025

13. Schmidt-Erfurth U, Kaiser PK, Korobelnik JF, et al. Intravitreal aflibercept injection for neovascular age-related macular degeneration: ninety-six-week results of the VIEW studies. *Ophthalmology*. 2014;121(1):193-201. doi:10.1016/j.ophtha.2013.08.011

14. Korobelnik JF, Do D V., Schmidt-Erfurth U, et al. Intravitreal aflibercept for diabetic macular edema. *Ophthalmology*. 2014;121(11):2247-2254. doi:10.1016/j.ophtha.2014.05.006

15. Tietz J, Spohn G, Schmid G, et al. Affinity and Potency of RTH258 (ESBA1008), a Novel Inhibitor of Vascular Endothelial Growth Factor A for the Treatment of Retinal Disorders. *Investig Ophthalmol Vis Sci*. 2015;56(7):1501.

16. Papadopoulos N, Martin J, Ruan Q, et al. Binding and neutralization of vascular endothelial growth factor (VEGF) and related ligands by VEGF Trap, ranibizumab and

bevacizumab. *Angiogenesis*. 2012;15(2):171-185. doi:10.1007/s10456-011-9249-6

17. Muellerleile LM, Buxbaum B, Nell B, Fux DA. In-vitro binding analysis of anti-human vascular endothelial growth factor antibodies bevacizumab and aflibercept with canine, feline, and equine vascular endothelial growth factor. *Res Vet Sci*. 2019;124:233-238. doi:10.1016/j.rvsc.2019.03.018

18. US Food and Drug Administration (Center for Drug Evaluation). Prescribing Information. U.S. BL 125085 Supplement: Bevacizumab–Genentech, Inc. Published 2011. Accessed March 2nd, 2021. https://www.accessdata.fda.gov/drugsatfda_docs/label/2011/125085s225lbl.pdf

19. Sella R, Gal-Or O, Livny E, et al. Efficacy of topical aflibercept versus topical bevacizumab for the prevention of corneal neovascularization in a rat model. *Exp Eye Res*. 2016;146:224-232. doi:10.1016/j.exer.2016.03.021

20. Kim JY, Kim DY, Kim ES, Kim MJ, Tchah H. Topically administered bevacizumab had longer standing anti-angiogenic effect than subconjunctivally injected bevacizumab in rat corneal neovacularization. *Int J Ophthalmol*. 2013;6(5):588-591. doi:10.3980/j.issn.2222-3959.2013.05.06

21. Kraemer I, Thiesen J. *Stabil-Liste: Physikalisch-Chemische Stabilitaet, Kompatibilitaet Und Inkompatibilitaet Pareneteral Applizierbarer Zytostatika, Virostatika, Und Supportivtherapeutika. 6. Edition. Muenchen: ADKA, Bundesverband Dt. Krankenhausapotheker*.; 2013.

22. European Commission. Good Manufacturing Practice (GMP) guidelines. In: *Medicinal Products for Human and Veterinary Use: Annex 13 Investigational Medicinal Products Document*. Vol 4. European Commission - Enterprise and industry directorate-general;

2010.

23. Clark JS, Bentley E, Smith LJ. Evaluation of topical nalbuphine or oral tramadol as analgesics for corneal pain in dogs: A pilot study. *Vet Ophthalmol*. 2011;14(6):358-364. doi:10.1111/j.1463-5224.2011.00883.x

24. Hackett R, McDonald T. *Ophthalmic Toxicology and Assessing Ocular Irritation*. 5th edn. (Marzulli FN, Maibach HI, eds.). Boca Raton: CRC Press Inc; 1996.

25. Ledbetter E, Mun J, Kowbel D, Fleiszig S. Pathogenic phenotype and genotype of Pseudomonas aeruginosa isolates from spontaneous canine ocular infections. *Investig Ophthalmol Vis Sci*. 2009;50(2):729-36.

26. Stewart JM, Schultz DS, Lee O-T, Trinidad ML. Collagen cross-links reduce corneal permeability. *Investig Ophthalmol Vis Sci*. 2009;50(4):1606-1612. doi:10.1167/iovs.08-2727

27. Bear J, Bachmat Y. A Generalized Theory On Hydrodynamic Dispersion In Porous Media. *Int Union Geod Geophys Publ*. 1967;72:7-16.

28. Gurung H, Bryant K, Chucair-Elliott A, Carr D. Fibroblast growth factor-2 drives and maintains progressive corneal neovascularization following HSV-1 infection. *Mucosal Immunol*. 2018;11(1):172-185. doi:10.1038/mi.2017.26

29. Cursiefen C, Hofmann-Rummelt C, Kuechle M, Schloetzer-Schrehardt U. Pericyte recruitment in human corneal angiogenesis: An ultrastructural study with clinicopathological correlation. *Br J Ophthalmol*. 2003;87(1):101-106. doi:10.1136/bjo.87.1.101

30. Benjamin LE, Golijanin D, Itin A, Pode D, Keshet E. Selective ablation of immature blood vessels in established human tumors follows vascular endothelial growth factor

withdrawal. *J Clin Invest*. 1999;103(2):159-165. doi:10.1172/JCI5028

31. Bock F, Koenig Y, Kruse F, Baier M, Cursiefen C. Bevacizumab (Avastin) eye drops inhibit corneal neovascularization. *Graefe's Arch Clin Exp Ophthalmol*. 2008;246(2):281-284. doi:10.1007/s00417-007-0684-4

32. Laham RJ, Li J, Tofukuji M, Post M, Simons M, Sellke FW. Spatial heterogeneity in VEGF-induced vasodilation: VEGF dilates microvessels but not epicardial and systemic arteries and veins. *Ann Vasc Surg*. 2003;17(3):245-252. doi:10.1007/s10016-001-0299-x

33. Ourradi K, Blythe T, Jarrett C, Barratt SL, Welsh GI, Millar AB. VEGF isoforms have differential effects on permeability of human pulmonary microvascular endothelial cells. *Respir Res*. 2017;18(1):116. doi:10.1186/s12931-017-0602-1

34. Gelatt KN, Gilger BC, Kern TJ. *Veterinary Ophthalmology*. 5th ed. Hoboken: John Wiley & Sons, Inc.; 2013. 353 p.

35. Moisseiev E, Waisbourd M, Ben-Artsi E, et al. Pharmacokinetics of bevacizumab after topical and intravitreal administration in human eyes. *Graefe's Arch Clin Exp Ophthalmol*. 2014;252(2):331-337. doi:10.1007/s00417-013-2495-0

36. Dastjerdi MH, Sadrai Z, Saban DR, Zhang Q, Dana R. Corneal penetration of topical and subconjunctival bevacizumab. *Investig Ophthalmol Vis Sci*. 2011;52(12):8718-8723. doi:10.1167/iovs.11-7871

37. Holash J, Davis S, Papadopoulos N, et al. VEGF-Trap: A VEGF blocker with potent antitumor effects. *Proc Natl Acad Sci U S A*. 2002;99(17):11393-11398. doi:10.1073/pnas.172398299

38. Li Z, Burns AR, Han L, Rumbaut RE, Smith CW. IL-17 and VEGF are necessary for efficient corneal nerve regeneration. *Am J Pathol*. 2011;178(3):1106-1116.

doi:10.1016/j.ajpath.2010.12.001

39. Gan L, Fagerholm P, Palmblad J. Vascular endothelial growth factor (VEGF) and its receptor VEGFR-2 in the regulation of corneal neovascularization and wound healing. *Acta Ophthalmol Scand*. 2004;82(5):557-563. doi:10.1111/j.1600-0420.2004.00312.x

40. Kim SW, Ha BJ, Kim EK, Tchah H, Kim T. The Effect of Topical Bevacizumab on Corneal Neovascularization. *Ophthalmology*. 2008;115(6):33-38. doi:10.1016/j.ophtha.2008.02.013

41. Shima C, Sakaguchi H, Gomi F, et al. Complications in patients after intravitreal injection of bevacizumab. *Acta Ophthalmol*. 2008;86(4):372-376. doi:10.1111/j.1600-0420.2007.01067.x

42. Wu L, Martínez-Castellanos MA, Quiroz-Mercado H, et al. Twelve-month safety of intravitreal injections of bevacizumab (Avastin®): Results of the pan-american collaborative retina study group (PACORES). *Graefe's Arch Clin Exp Ophthalmol*. 2008;246(1):81-87. doi:10.1007/s00417-007-0660-z

43. Muellerleile LM, Tichy A, Nell B. Serum vascular endothelial growth factor changes and safety after topical anti-human VEGF antibody bevacizumab in healthy dogs. *Vet Ophthalmol*. 2019;22(5):600-606. doi:10.1111/vop.12628

44. Sandberg CA, Herring IP, Huckle WR, LeRoith T, Pickett JP, Rossmeisl JH. Aqueous humor vascular endothelial growth factor in dogs: association with intraocular disease and the development of pre-iridal fibrovascular membrane. *Vet Ophthalmol*. 2012;15:21-30. doi:10.1111/j.1463-5224.2011.00931.x

45. Zarfoss MK, Breaux CB, Whiteley HE, et al. Canine pre-iridal fibrovascular membranes: morphologic and immunohistochemical investigations. *Vet Ophthalmol*. 2010;13(1):4-13.

doi:10.1111/j.1463-5224.2009.00739.x

46. Binder DR, Herring IP, Zimmerman KL, Pickett PJ, Huckle WR. Expression of vascular endothelial growth factor receptor-1 and -2 in normal and diseased canine eyes. *Vet Ophthalmol*. 2012;15(4):223-230. doi:10.1111/j.1463-5224.2011.00973.x

47. Martin DF, Maguire MG, Fine SL, et al. Ranibizumab and Bevacizumab for Treatment of Neovascular Age-Related Macular Degeneration: 2-Year Results: Comparison of Age-related Macular Degeneration Treatments Trials (CATT) Research Group. *Ophthalmology*. 2012;119(7):1388-1398. doi:10.1016/j.ophtha.2012.03.053.

48. Edelmann ML, Utter ML, Klein L V., Wotman KL. Combined excision and intralesional bevacizumab for sebaceous carcinoma of the eyelid in an Amur tiger (Panthera tigris altaica). *Vet Ophthalmol*. 2013;16(3):219-224. doi:10.1111/j.1463-5224.2012.01051.x

49. Villar T, Capistrano E, Galera G, Fleming K, Roady P, Martins B. Evaluation and Comparison of Inhibitory Effects of Topical Tacrolimus, Cyclosporine, and Dexamethasone on Corneal Neovascularization AND Wound Healing in an Experimental Rat Study. In: *Conference Proceedings of the 51st Annual Scientific Meeting of the American College of Veterinary Ophthalmologists, Virtual Conference, September 23-27, 2020. Https://Onlinelibrary.Wiley.Com/Doi/Abs/10.1111/Vop.12844. Accessed March 2nd, 2021*. https://onlinelibrary.wiley.com/doi/abs/10.1111/vop.12844

509 FIGURE LEGENDS

510 **Figure 1.** Example of image processing and standardization of technical shooting conditions to
511 achieve best possible comparability.

512 **Figure 2**. Measurement of the (a) vascularized corneal area and (b) the count of vessel incisions,
513 including 4 circles, each 25% smaller (0-25-50-75-circle). The green spots on the circles show
514 the counted blood vessel incisions (c). Analysis were performed with an image processing
515 program (Fiji; open-source software: https://imagej.net/Fiji).

516 **Figure 3.** Siberian Husky (#15) with immune mediated keratitis (a) before and (b) after
517 bevacizumab treatment for 28 days. Superficial blood vessels (black arrow) arising from the
518 dorsal limbus (a) completely disappeared after bevacizumab treatment (b).

519 **Figure 4.** Chihuahua (#7) with immune-mediated keratitis (a) before and (b) 1 month after last
520 bevacizumab. In (a), ocular findings included a planar stromal hemorrhage adjacent to the
521 ventrotemporal limbus. In (b) it consolidated into a fine vascular network.

522 **Figure 5.** French bulldog (#20) with superficial corneal vascularization (a) before and (b) after
523 bevacizumab. After topical bevacizumab, corneal blood vessels arising from the temporoventral
524 limbus thinned or were bloodless. Corneal pigmentation and edema decreased.

525 **Figure 6.** Scoring results of macroscopic ocular findings during slit-lamp biomicroscopy.
526 Displayed are the mean scores at baseline, 4 weeks after bevacizumab treatment and 5 months
527 after the last bevacizumab administration. Conjunctival hyperemia/chemosis and ocular
528 discharge: 0 = none, 1 = mild, 2 = moderate, 3 = severe, corneal edema, vascularization and
529 pigmentation (area relative to total corneal area): 0 = <25%, 1 = 26% to 50%, 2 = 51% to 75%, 3
530 = >76%. bva = bevacizumab

Figure 7. Maltese dog (#3) with immune mediated keratitis (a) before and (b) 4 weeks after treatment start with bevaciczumanb. In (b), distal vessels were thinner, fewer in number, less branched, and more sharply demarcated.

Figure 8. Prague Rattler (#18) with superficial keratitis (a) before and (b) after bevacizumab. After 4 weeks of topical bevacizumab, vessel diameter decreased, ocular discharge, eye comfort, and conjunctival hyperemia and chemosis improved.

Figure 9. Great Pyrenees dog (#13) with severe unilateral stromal keratitis (a) before and (b) 2 months after bevacizumab treatment start. In (b), blood vessels became thinner and fewer, and corneal edema improved, in particular in the ventral third of the corneal surface. The blood vessels in the central corneal area were less branched and fewer in number.

Figure 10. German Shepherd Dog (#10 and #11) with bilateral CSK. (a) OD and (b) OS before bevacizumab treatment. Clinical improvement was observed for both eyes after 4 weeks of bevacizumab treatment. At long-term follow-up, (c) OD and (d) OS, a further decrease of corneal edema and thinning of corneal blood vessels was observed.

Figure 11. Boxer with persistent corneal vascularization after delayed healing of a stromal ulcer (#9) before (a) and 7 months after (b) bevacizumab. In (b), blood vessel thinning, a decrease of corneal inflammatory cell infiltration and edema is observed. The dog showed persistent corneal vascularization prior to bevacizumab start with only systemic NSAIDs; the dog did not receive any topical medication before and after bevacizumab. Interestingly, further clinical improvement in the months after the last bevacizumab administration was noted.

Figure 12. Chihuahua (#6) with an immune mediated keratitis (a) before and (b) 4 months after

552 treatment start with bevacizumab (patient was lost for long-term follow-up). In (b), the
553 vascularized area and corneal vessel diameter decreased, and the vessels became more sharply
554 demarcated. Corneal cell infiltration and corneal edema improved.
555

556 TABLES

557 ***Table 1.*** *Signalment and ocular diagnosis of the studied dogs.*

Patient	**Eye #**		**Age** (years)	**Gender**	**Weight** (kg)	**Breed**	**Diagnosis**
A	1	OD	10	MC	11.8	French Bulldog	Superficial stromal keratitis, pigmentary keratitis
B	2	OD	7	FC	2.7	Chihuahua	Superficial stromal keratitis, pigmentary keratitis
C *	3	OD	2	F	5.3	Maltese dog	Superficial stromal keratitis, pigmentary keratitis
	4	OS					Superficial stromal keratitis, pigmentary keratitis
D	5	OD	14	FC	12.0	French Bulldog	Superficial keratitis, pigmentary keratitis
E *	6	OD	16	MC	3.2	Chihuahua	Superficial stromal keratitis
	7	OS					Superficial stromal keratitis

F *	8 9	OD OS	7	FC	23.0	Boxer	Superficial stromal keratitis Superficial stromal keratitis
G *	10 11	OD OS	9	FC	30	German Shepherd dog	CSK CSK
H	12	OD	3	M	13.6	French bulldog	Superficial keratitis
I	13	OS	7	M	12.0	Great Pyrenees dog	Stromal keratitis
J	14	OS	12	M	2.5	Chihuahua	Superficial stromal keratitis, pigmentary keratitis
K	15	OS	12	MC	34.2	Husky	Superficial keratitis
L *	16 17	OD OS	7	MC	14.2	Mixed breed dog	Superficial keratitis Superficial keratitis
M	18	OS	11	MC	4.0	Prague Rattler	Superficial keratitis, pigmentary keratitis
N	19	OD	4	MC	7.0	Shih Tzu	Superficial stromal keratitis, pigmentary keratitis

O	20	OD	8	FC	9.8	French Bulldog	Superficial keratitis
Mean ± SD			**8.5 ± 4**		**12.4 ± 9.8**		

F = Female; FC = Female castrated; M = Male; MC = Male castrated; OD = Oculus dexter; OS = Oculus sinister; CSK = Chronic superficial keratitis; SD = standard deviation

* = bilaterally treated with topical bevacizumab

Table 2. *Efficacy assessment: Decrease in the vascularized area and number of vessel incisions between baseline and 4 weeks after bevacizumab treatment. Data were analyzed using the imaging software Fiji.*

Category	***Difference between baseline and 4 weeks after topical bevacizumab***
Vascularized area (%)	
n=(10)	
Mean ± SD	*48.8 ± 33.5*
Min	*4.9*
Max	*100*

Vessel incisions on a certain circle (%)	
(n=17)	
0-Circle	28.0 ± *36.0*
25-Circle	31.1 ± *39.4*
50-Circle	4.6 ± *51.5*
75-Circle	16.5 ± *35.3*

567

568 SD = standard deviation

569 Min = minimum (lowest observation)

570 Max = maximum (highest observation)

SUPPLEMENTAL TABLES

Supplemental Table 1*: Modified pain score system assessed by the investigator to investigate ocular and systemic toxicity in dogs with corneal vascularization before and after topical bevacizumab application.*[23,24]

Pain scoring category	**Manifestation**
Comfort	0 = dog is calm and interested in surroundings
	1 = dog shows mild agitation or is depressed, not interested in surroundings
	2 = dog shows moderate agitation and is restless
	3 = dog is extremely agitated
Movement	0 = dog is quiet
	1 = about 1-2 position changes per minute
	2 = about 2-6 position changes per minute
	3 = dog shows continuous changes
Appearance/ blepharospasm	0 = eyelids are completely open and in physiological position
	1 = eyelids are partially closed (25%)
	2 = eyelids are partially closed (50%); dog shows mild tearing
	3 = eyelids are partially closed (75%); dog shows moderate tearing
	4 = eyelids are completely closed; dog shows marked tearing
Behavior (unprovoked)	0 = normal
	1 = minor changes

	2 = moderately abnormal (less mobile or alert than normal, not interested in surroundings, fretful)
	3 = noticeably abnormal (fretful, vocalizing, self-mutilation, groaning)
Interactive behaviors	0 = normal
	1 = pulls head away when eyes getting touched
	2 = vocalizes when eyes getting touched
	3 = violent reaction to touching of eye (biting, snapping, groaning)
Vocalization	0 = quiet
	1 = dog cries but responds to be quiet
	2 = dog intermittently cries without response to quiet voice
	3 = dog constantly cries without response to quiet voice

575

Supplemental Table 2. *Modified pain score system assessed by the owner to investigate ocular and systemic toxicity directly after, 6 hours and 3 days after the first topical bevacizumab application.*[23,24]

Pain Scoring category	Behavior	Touch response
0	General: calm, interested in surroundings, normal behavior Eye: eyelids are completely open, no rubbing/scratching	Normal
1	General: mild agitation, more restless than usually Eye: eyelids are open, but might show slightly tearing or a slight higher frequency of blinking	Seems to be uncomfortable when getting touched, slightly pulls head away
2	General: mild to moderate agitation, restless, whining/moaning Eye: eyelids are partially closed, mild tearing, redness	Pulls head away when eyes getting touched
3	General: moderate agitation, less mobile or alert than usually, not interested in surroundings, fretful, whining/moaning Eye: moderate tearing, moderate redness, partially closed eyelids	Vocalization when eyes getting touched

4	General: extremely agitated, noticeably abnormal (fretful, vocalizing, self-mutilation, groaning), constantly crying without response to quiet voice, vomiting, salivation Eye: eyelids are completely closed, marked tearing	Violent reaction (biting, snapping, groaning), vocalization

Supplemental Table 3. *Systemic disease details of the studied dogs.*

Dog	**Eye #**	**Systemic disease**	**Systemic treatment**
A	1	Congenital vertebral malformations without neurological deficits	None
B	2	None	None
C *	3 4	Dental problems	None
D	5	None	None
E *	6 7	Mitral endocardiosis without pulmonary arterial hypertension, blood pressure normal	ACE inhibitors
F *	8 9	Hypothyroidism, spondylosis	Meloxicam
G *	10 11	Omarthrosis, biceps tendinopathy	None
H	12	Gastroenteritis	None

I	13	None	None
J	14	Mitral valve insufficiency, no deficits (accidental finding during routine examination)	Benazepril, furosemide
K	15	Pancreatitis and gastroenteritis (since 2014 asymptomatic), abscess (leg), food allergy	None
L *	16 17	Hip fracture, otitis media	None
M	18	Mitral endocardiosis (no deficits), allergies	Imidapril, ceterizine
N	19	None	None
O	20	Congenital vertebral malformation, patellar luxation, environmental allergies, allergenic dermatitis, canine malassezia dermatitis, brachycephalic obstructive airway syndrome	Gabapentin, cyclosporine

581

582 * = bilaterally treated with bevacizumab eyedrops

583 ACE inhibitors = Angiotensin-converting-enzyme inhibitors

584

585

586

587 ***Supplemental Table 4.*** *Ocular disease details of the studied dogs.*

588

Eye #	**Morphologic diagnosis**	**Ocular diseases details**	**CYA** (DOT)	**DEX** (DOT/time distance to last DEX)
1	Superficial stromal keratitis, pigmentary keratitis	St.p. 360 degree conjunctival flap due to stromal collagenolytic ulcer; macroblepharon, nasal entropion, qualitative tear deficiency	2a [a]	N/A
2	Superficial stromal keratitis, pigmentary keratitis	St.p. 360 degree conjunctival flap due to perforated corneal ulcer; macroblepharon, shallow orbit	5a 8m [a]	N/A
3	Superficial stromal keratitis, pigmentary keratitis	Suspected immune mediated keratitis, blepharitis, trichiasis of caruncle and nasal fold; iris atrophy, immature cataract, nuclear sclerosis	2m [a]	N/A
4	Superficial stromal keratitis, pigmentary keratitis	Suspected immune mediated keratitis, blepharitis, trichiasis of caruncle and nasal fold; iris atrophy, immature cataract, nuclear sclerosis	2m [a]	N/A

5	Superficial keratitis, pigmentary keratitis	St.p. DBD and pTR due to a SCCED; marcoblepharon, nasal entropion, nuclear sclerosis	11m [a]	N/A
6	Superficial stromal keratitis	Suspected immune mediated keratitis, shallow orbit	4m [a]	7w/3w [c]
7	Superficial stromal keratitis	Suspected immune mediated keratitis, shallow orbit	4m [a]	7w/3w [c]
8	Superficial stromal keratitis	St.p. collagenolytic ulcer post DBD; distichiasis, macroblepharon, ectropion lower eyelid	N/A	N/A
9	Superficial stromal keratitis	St.p. DBD; severe corneal edema and infiltration of inflammatory cells, conjunctivitis, distichiasis, macroblepharon, ectropion lower eyelid, iris coloboma, nuclear sclerosis	N/A	N/A
10	CSK	Accompanied by plasmacytic conjunctivitis	1a 2m [a]	6w/1w [c]
11	CSK	Accompanied by plasmacytic conjunctivitis	1a 2m [a]	6w/1w [c]
12	Superficial keratitis	St.p. excision of a palpebral dermoid in the lower eyelid and refractory corneal erosion (immune mediated cause suspected) accompanied by spastic	1a 4m [a]	4w/1a 4m [c]

		entropion of lower eyelid; St.p. nasal canthoplasty due to macroblepharon/nasal entropion		
13	Stromal keratitis	Unilateral keratitis suspected association with corneal cat scratch, generalized stromal edema and fibrosis, recurrent blepharitis	2a 4m [a]	N/A
14	Superficial stromal keratitis, pigmentary keratitis	qualitative tear deficiency, distichiasis, shallow orbit, macroblepharon	9m [a]	N/A
15	Superficial keratitis	suspected immune mediated keratitis and/or qualitative and quantitative tear deficiency (tear deficiency responded well to topical DEX/CYA), corneal vascularization persisted	3m [a]	4w/8w [c]
16	Superficial keratitis	qualitative and quantitative tear deficiency (tear deficiency responded well to topical DEX/CYA), corneal vascularization persisted	1a 10m[a]	2w/1a 10m [c]
17	Superficial keratitis	qualitative and quantitative tear deficiency (tear deficiency responded well to topical DEX/CYA), corneal vascularization persisted	1a 10m[a]	2w/1a 10m [c]

18	Superficial keratitis, pigmentary keratitis	qualitative tear deficiency, conjunctivitis, persistent corneal vascularization despite topical CYA	2a 11m[a]	N/A
19	Superficial stromal keratitis, pigmentary keratitis	St.p. nasal canthoplasty due to nasal fold trichiasis, entropion, macroblepharon St.p. superficial stromal corneal ulcer, unresponsive to conventional ulcer treatment, but responded well to topical DEX (suspected immune mediated cause), quantitative tear deficiency	1a 8m[a,b]	5w/1w [c]
20	Superficial keratitis	St.p. deep stromal ulcerative keratitis, ulcer healed after 5w but with persistent corneal vascularization despite topical CYA/DEX; macroblepharon	2m[a]	8w/3w [d]

589

590 CSK = Chronic superficial keratitis; St.p. = Status post; DBD = diamond burr debridement; pTR
591 = partial Tarsorrhaphy; SCCED = superficial chronic corneal epithelial defect; CYA =
592 cyclosporine A; DOT = duration of therapy; DEX = dexamethasone; a = year(s); m = month(s);
593 w = week(s); [a] = 0.2% cyclosporine (Optimmune®, MSD Animal Health, Kenilworth); [b] = 2.0%
594 cyclosporine (prepared by hospital pharmacy of University of Veterinary Medicine, Vienna); [c] =
595 dexamethasone + gentamicin sulfate (DexaGenta-Pos® eye ointment, Ursapharm,
596 Saarbruecken); [d] = dexamethason dihydrogen posphate (Monodex® 1mg/ml, Théa PHARMA
597 SA, Clermont-Ferrand, France)

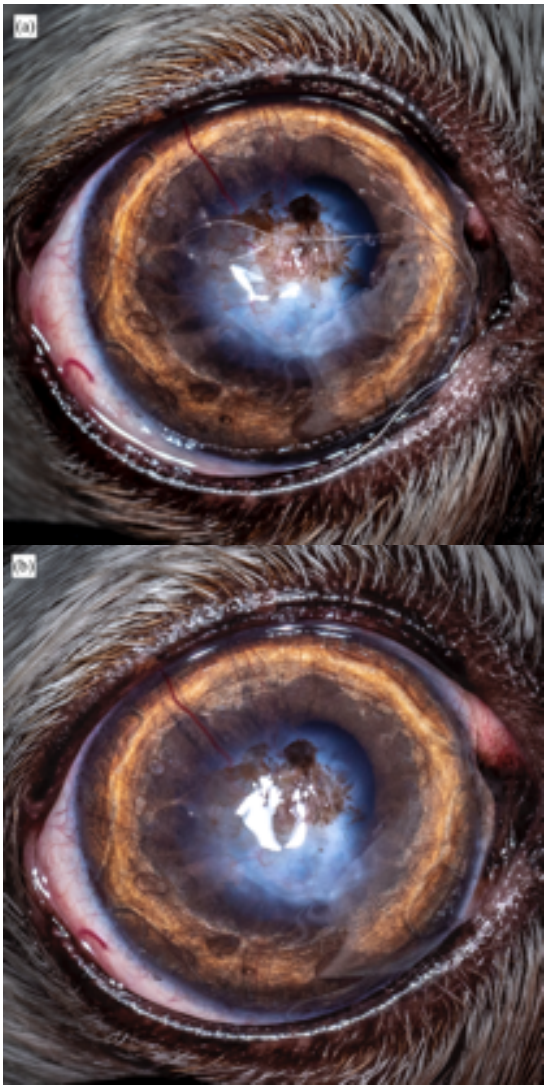

Figure 1. Example of image processing and standardization of technical shooting conditions to achieve best possible comparability.

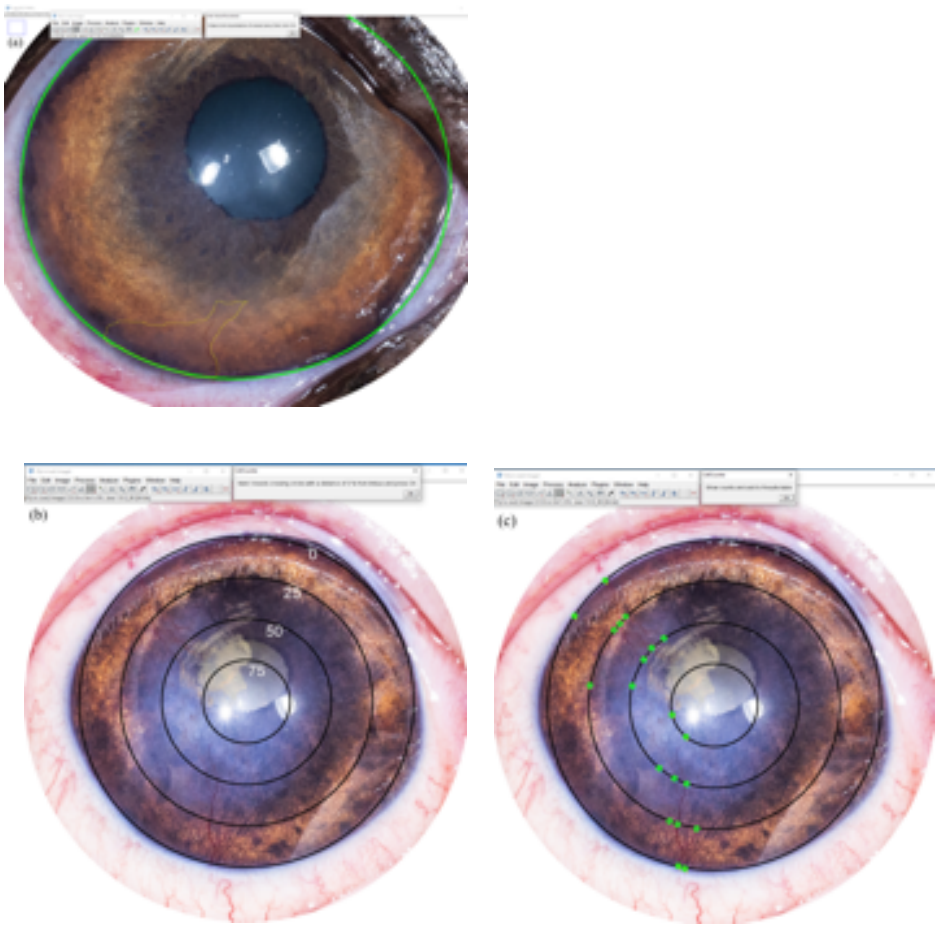

Figure 2. Measurement of the (a) vascularized corneal area and (b) the count of vessel incisions, including 4 circles, each 25% smaller (0-25-50-75-circle). The green spots on the circles show the counted blood vessel incisions (c). Analysis were performed with an image processing program (Fiji; open-source software: https://imagej.net/Fiji).

Figure 3. Siberian Husky (#15) with immune mediated keratitis (a) before and (b) after bevacizumab treatment for 28 days. Superficial blood vessels (black arrow) arising from the dorsal limbus (a) completely disappeared after bevacizumab treatment (b).

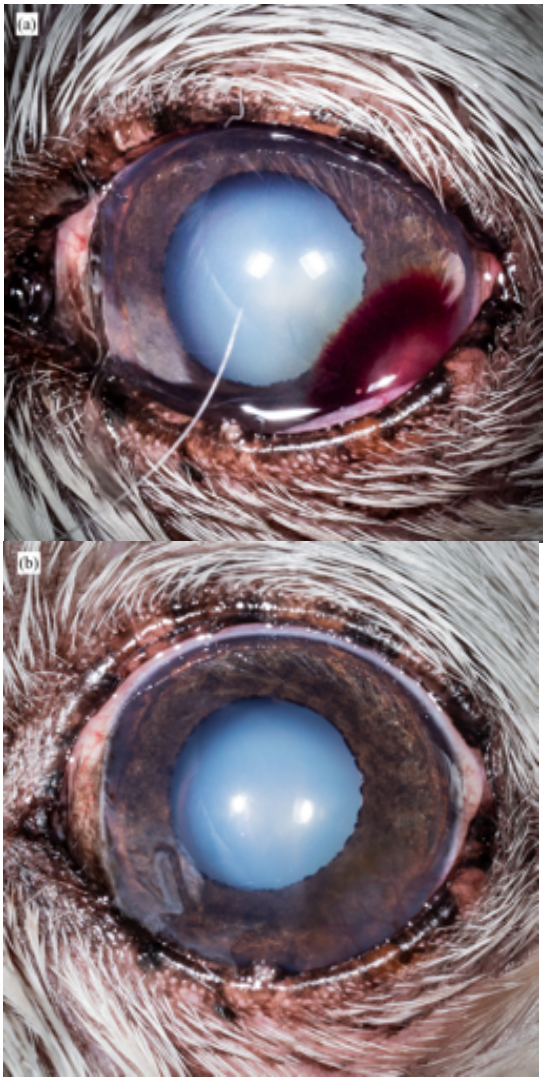

Figure 4. Chihuahua (#7) with immune-mediated keratitis (a) before and (b) 1 month after last bevacizumab. In (a), ocular findings included a planar stromal hemorrhage adjacent to the ventrotemporal limbus. In (b) it consolidated into a fine vascular network.

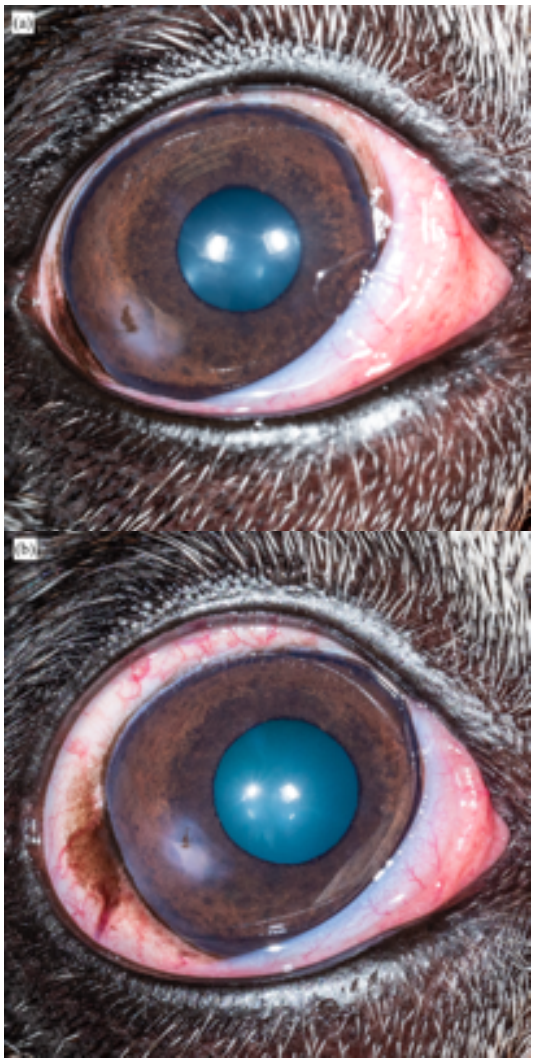

Figure 5. French bulldog (#20) with superficial corneal vascularization (a) before and (b) after bevacizumab. After topical bevacizumab, corneal blood vessels arising from the temporoventral limbus thinned or were bloodless. Corneal pigmentation and edema decreased.

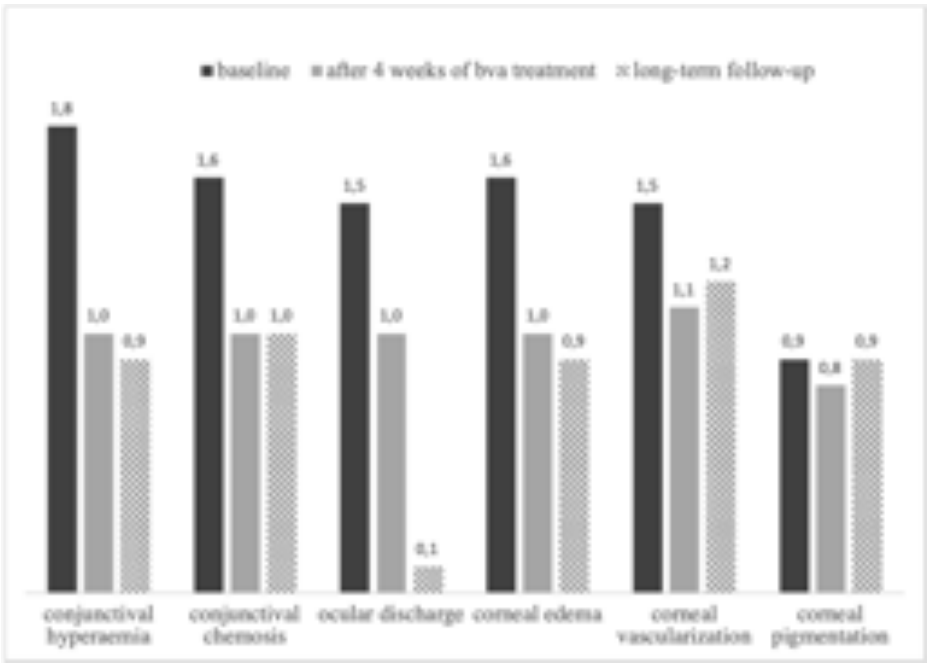

Figure 6. Scoring results of macroscopic ocular findings during slit-lamp biomicroscopy. Displayed are the mean scores at baseline, 4 weeks after bevacizumab treatment and 5 months after the last bevacizumab administration. Conjunctival hyperemia/chemosis and ocular discharge: 0 = none, 1 = mild, 2 = moderate, 3 = severe, corneal edema, vascularization and pigmentation (area relative to total corneal area): 0 = <25%, 1 = 26% to 50%, 2 = 51% to 75%, 3 = >76%. bva = bevacizumab

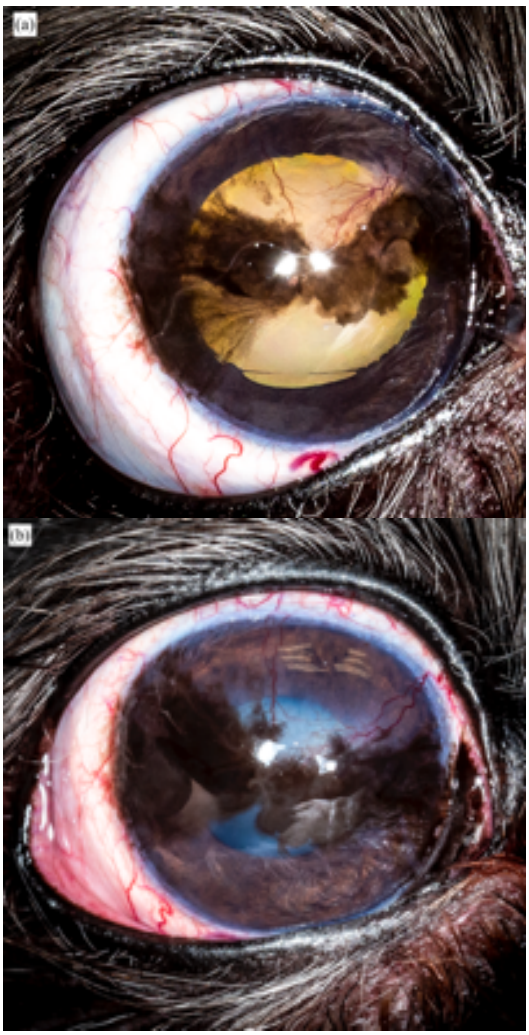

Figure 7. Maltese dog (#3) with immune mediated keratitis (a) before and (b) 4 weeks after treatment start with bevaciczumanb. In (b), distal vessels were thinner, fewer in number, less branched, and more sharply demarcated.

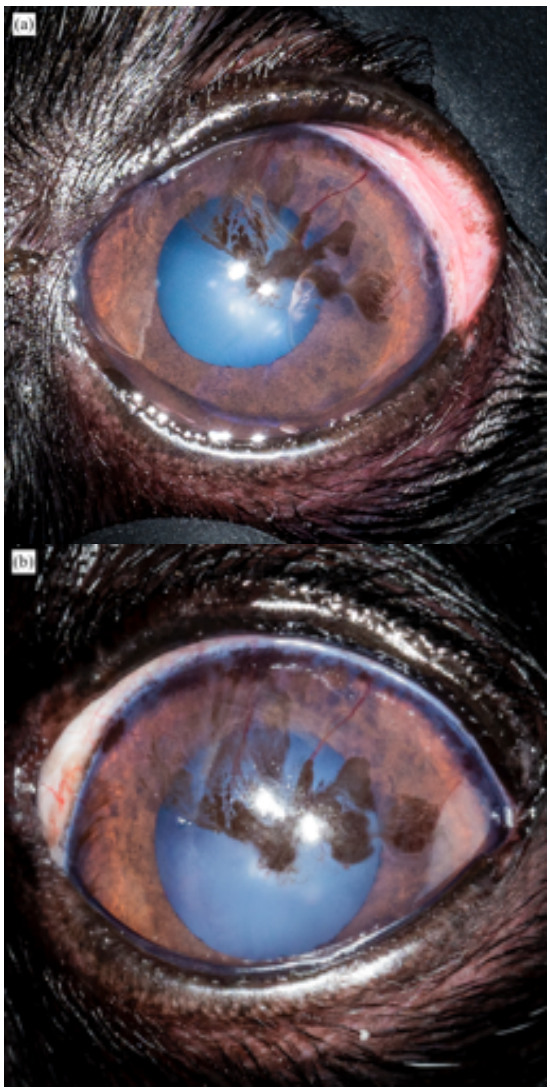

Figure 8. Prague Rattler (#18) with superficial keratitis (a) before and (b) after bevacizumab. After 4 weeks of topical bevacizumab, vessel diameter decreased, ocular discharge, eye comfort, and conjunctival hyperemia and chemosis improved.

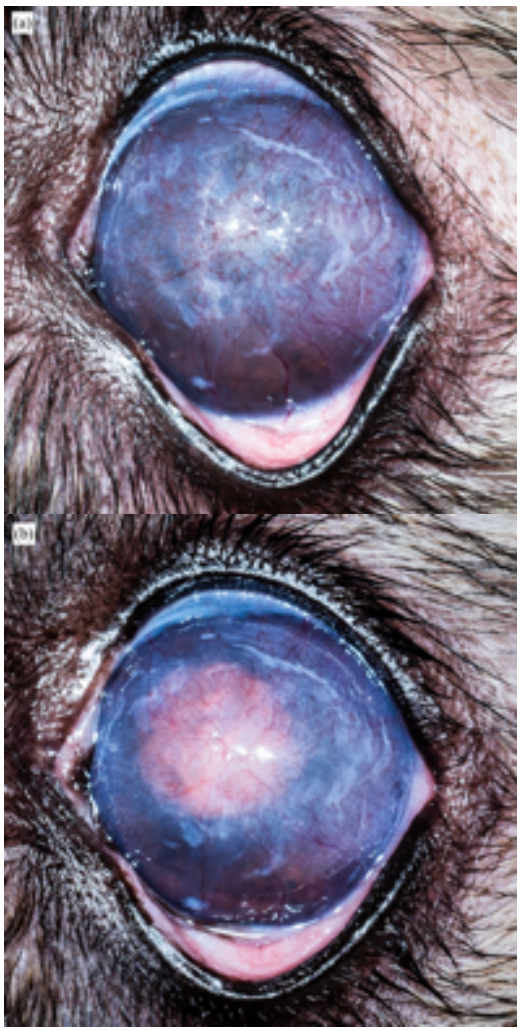

Figure 9. Great Pyrenees dog (#13) with severe unilateral stromal keratitis (a) before and (b) 2 months after bevacizumab treatment start. In (b), blood vessels became thinner and fewer, and corneal edema improved, in particular in the ventral third of the corneal surface. The blood vessels in the central corneal area were less branched and fewer in number.

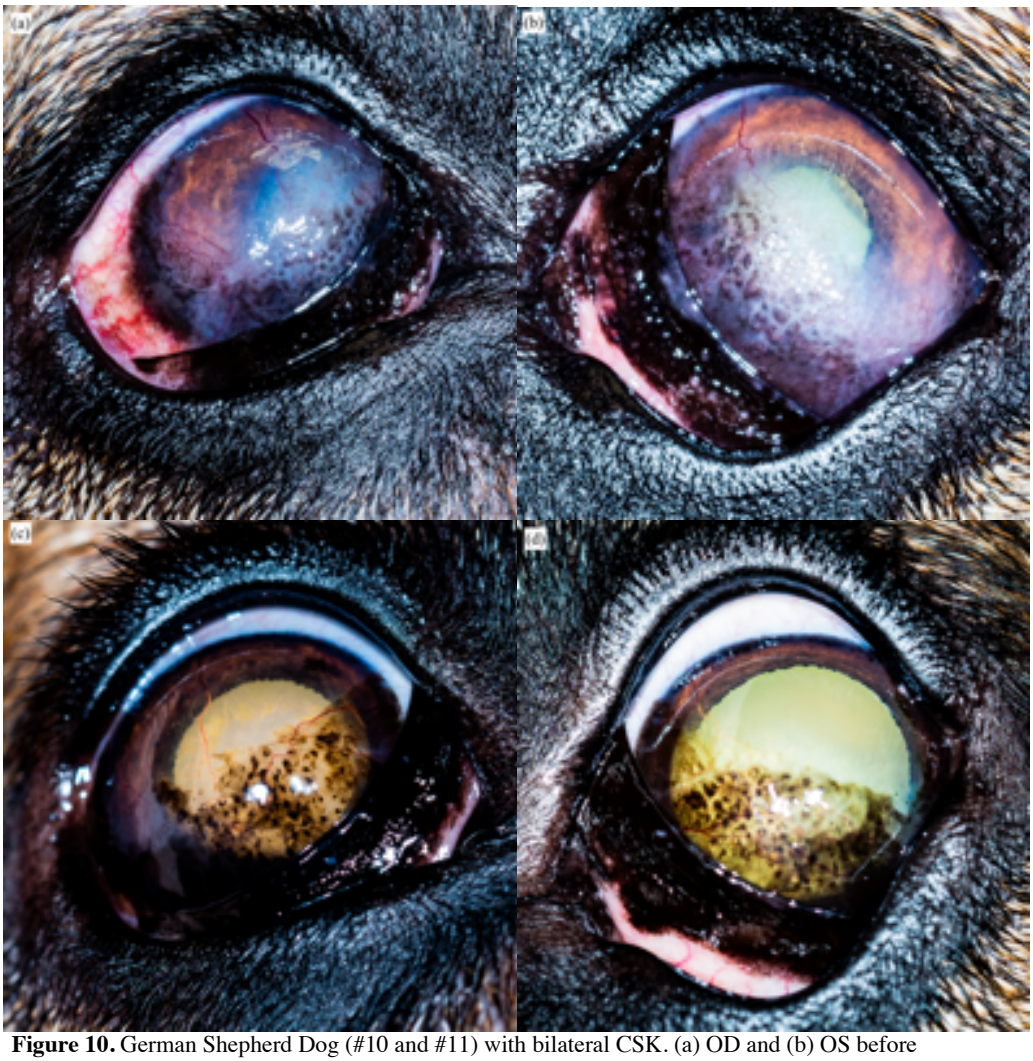

Figure 10. German Shepherd Dog (#10 and #11) with bilateral CSK. (a) OD and (b) OS before bevacizumab treatment. Clinical improvement was observed for both eyes after 4 weeks of bevacizumab treatment. At long-term follow-up, (c) OD and (d) OS, a further decrease of corneal edema and thinning of corneal blood vessels was observed.

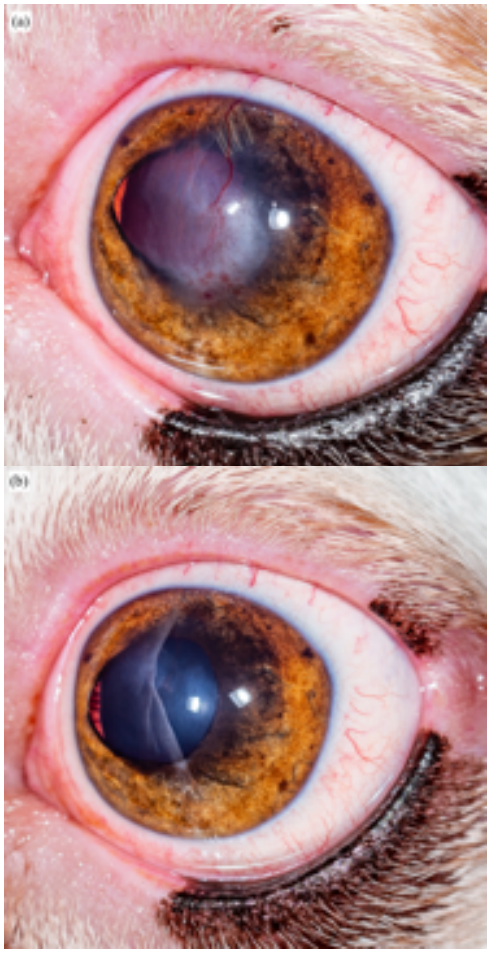

Figure 11. Boxer with persistent corneal vascularization after delayed healing of a stromal ulcer (#9) before (a) and 7 months after (b) bevacizumab. In (b), blood vessel thinning, a decrease of corneal inflammatory cell infiltration and edema is observed. The dog showed persistent corneal vascularization prior to bevacizumab start with only systemic NSAIDs; the dog did not receive any topical medication before and after bevacizumab. Interestingly, further clinical improvement in the months after the last bevacizumab administration was noted.

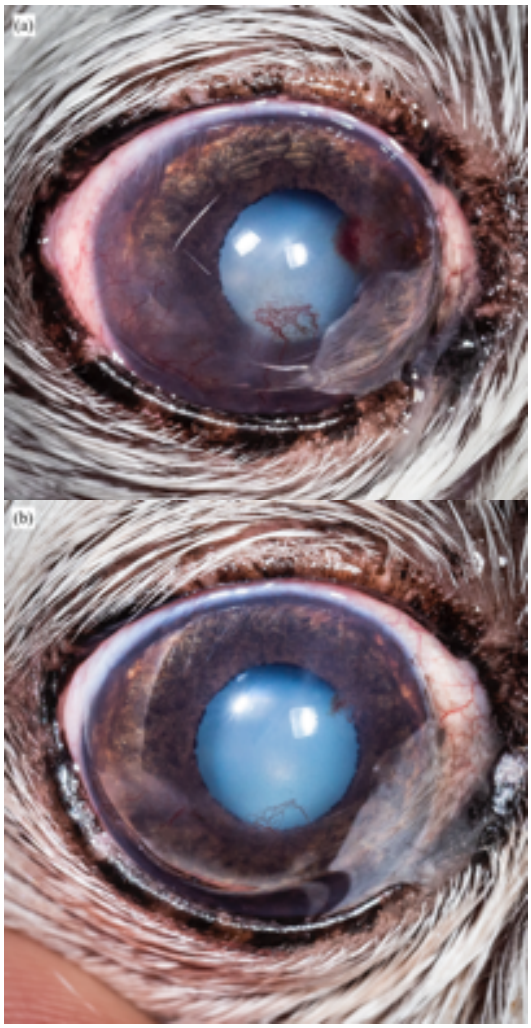

Figure 12. Chihuahua (#6) with an immune mediated keratitis (a) before and (b) 4 months after treatment start with bevacizumab (patient was lost for long-term follow-up). In (b), the vascularized area and corneal vessel diameter decreased, and the vessels became more sharply demarcated. Corneal cell infiltration and corneal edema improved.

4. Discussion

This PhD project aimed to investigate the potential of VEGF inhibitors bevacizumab and aflibercept in animal patients. Vascular endothelial growth factors have been intensively studied in humans and experimental rodent and rabbit models and its role in neovascular eye diseases is undisputed (Cursiefen et al. 2000, Gan et al. 2004, Pieh et al. 2008, Wuest et al. 2011, Chen et al. 2012, Yun et al. 2020).

Until now, there is a lack of knowledge about VEGF and VEGF inhibitory drugs in dogs, cat, and horses.

In our first study, we investigated the binding characteristics of anti-human VEGF bevacizumab and aflibercept to canine, feline, and equine VEGF in-vitro.

The purpose of the second and third study was to assess bevacizumab eye drops in-vivo in healthy dogs and in dogs with naturally occuring corneal vascularization. The first step was to establish a scientifically proven safe dose of bevacizumab in healthy individuals. After confirming a safe dose of 2.5 mg/ml BID, we explored the effects of bevacizumab in the previously studied regime in dogs with corneal vascularization.

The outcome of this PhD project is of great interest because persistent corneal vascularization is frequently observed in veterinary ophthalmology and can cause a variety of severe sequelae (Dana and Streilein 1996, Andrew et al. 1998, Murphy et al. 2001, Andrew 2008, Dean and Meunier 2013, Lassaline et al. 2015, Plummer 2015, Hindley et al. 2016, Ledbetter et al. 2016, Krecny et al. 2018). The dilemma of current treatment options is the lack of directly targeted inhibition of angiogenesis that may be associated with an insufficient response to therapy and the occurrence of side effects (Tolar et al. 2006, Bock et al. 2014, Dowling et al. 2016, Hindley et al. 2016, Murtagh et al. 2018, Rigas et al. 2020, Sebbag et al. 2020). The application of therapies that inhibit VEGF are not only of interest for corneal vascularization but also for other indications like ocular neoplasms and PIFMs with probable causal VEGF association (Zarfoss et al. 2010, Sandberg et al. 2012). This work has laid the foundation for the future clinical application of VEGF inhibitors in dogs, cats, and horses.

4.1. In-vitro analysis

Bevacizumab and aflibercept were designed to bind specifically human VEGF-A and are used for targeted therapies for pathological angiogenesis in cancer and eye disorders (Presta et al. 1997, Holash et al. 2002, Arevalo et al. 2007, Korobelnik et al. 2014, Schmidt-Erfurth et al. 2014, Olmos et al. 2016). Therapeutic VEGF inhibition has been extensively studied in experimental rodent and rabbit models (Bock et al. 2007, Dastjerdi et al. 2010a, Ozdemir et al. 2014, Sella et al. 2016, Devarajan et al. 2019). Thus, the studied VEGF inhibitors are supposed to be able to bind VEGF of mice and rabbits. However, there are contrary findings and the ability of bevacizumab to bind VEGF of mice and rabbits remains controversial (Bock et al. 2007, Yu et al. 2008b, Papadopoulos et al. 2012). In dogs, cats, and horses, there is no knowledge of the VEGF binding properties of bevacizumab and aflibercept.

Thus, our first approach was to compare amino acid sequences of human, equine, canine, and feline VEGF using the "Basic Local Alignment Search Tool"(BLAST). We found that there is high similarity of canine, feline, and equine VEGF to human VEGF-A of 93 %, 92 %, and 89 %, respectively. These findings correlate with those of other studies (Scheidegger et al. 1999, Jingjing et al. 2000). Our analysis revealed an identical amino acid sequence between Arg 82 and Gly 91 of all tested species, which is considered to be the essential sequence for binding to bevacizumab (Muller et al. 1998).

Following the comparison of amino acid sequences, we studied qualitative and quantitative binding properties of bevacizumab and aflibercept in equine, feline, and canine VEGF using a Western blot analysis and an enzyme-linked immunosorbent assay (ELISA).

In the Western blot, we found immunoreactive signals for aflibercept for canine, feline, and equine VEGF. However, immunoreactivity of bevacizumab was only detectable for canine and feline VEGF, but not for equine VEGF.

In order to explore quantitative binding characteristics, we conducted an ELISA. Different concentrations of bevacizumab or aflibercept were incubated with an ascending concentration series of VEGF and subsequently, unbound VEGF was measured. The analyses revealed that bevacizumab and aflibercept bind canine VEGF in a dose-dependent manner. In contrast, the binding of bevacizumab and aflibercept to feline VEGF was linear, suggesting an unspecific binding (Medina et al. 1999). ELISA analyses identified a dose-independent binding of aflibercept to equine VEGF, whereas no equine VEGF binding was detected for bevacizumab.

This finding was in line with the absence of bevacizumab binding to equine VEGF previously observed in the Western blot analysis.

This finding was unexpected because equine and human VEGF have an identic amino acid sequence of the bevacizumab binding region. Anyway, proteins do not function as linear polymers but as complex three-dimensional structures (Berg et al. 2017). In general, the structure of proteins can be described on four levels, the primary, secondary, tertiary, and quaternary structure (Berg et al. 2017). The primary structure refers to the amino acid sequence. The secondary structure corresponds to the conformation that local regions of the polypeptide chain form, such as α-helix, β-sheet, β-turn, Ω-loops (Berg et al. 2017). The secondary structure is built by the formation of hydrogen bonds of peptide N-H and -C=O groups of adjacent amino acids (Muller et al. 1998, Berg et al. 2017). The compact, asymmetric structure to which the polypeptides are arranged is called the tertiary structure (Suto et al. 2005, Berg et al. 2017). The function of a protein is directly related to this tertiary structure and the amino acid sequence of a protein determines its three-dimensional structure (Berg et al. 2017). Extensive research including deep learning systems has been conducted on how to predict the three-dimensional structure based on the amino acid sequence, which has proven to be very difficult (Roche et al. 2011). Furthermore, it is known that the tertiary structure is not only influenced by the amino acid sequences that are close to each other, but are caused by interactions over larger amino acid sequences, too (Berg et al. 2017).

In our BLAST analysis, equine VEGF had a high sequence similarity of 89 % and an identical sequence in the bevacizumab binding region compared to human VEGF. Nevertheless, we found that equine VEGF had the highest sequence variations and was shorter by seven amino acids compared to human VEGF. These differences might cause variations in the three-dimensional protein structure, thereby hiding the binding region and impairing antigen accessibility. These interspecies differences of equine VEGF may explain the lack of bevacizumab binding to equine VEGF.

The binding region of aflibercept is not yet published. However, aflibercept consists of parts of VEGFR-1 and -2, assuming that VEGF binding may occur via the receptor binding domain, too (Holash et al. 2002). Therefore, one would have to assume that just like bevacizumab, aflibercept is also unable to bind equine VEGF. Anyway, looking at how aflibercept and bevacizumab bind their targets may explain the lack of bevacizumab and the capability of

aflibercept binding to equine VEGF. MacDonald et al. found that binding of bevacizumab to VEGF-A_{165} can result in large, multimeric complexes mediated by surface-captured heparin and NP-1 (MacDonald et al. 2016). They also stated that, in contrast to bevacizumab, aflibercept binding to human VEGF-A_{165} formed monomeric 1:1 complexes, which did not bind epithelial or endothelial cells to a greater extent than unbound aflibercept and speculated that the formation of this complexes hide the heparin-binding region of VEGF. Thus, these findings underscored an exceptional trapping mechanism of aflibercept-VEGF binding that differs from classical antibody-ligand mechanisms (MacDonald et al. 2016). Therefore, we assumed that this different binding manner of VEGF-trap aflibercept may be the reason for our observation. In conclusion, the results of this in-vitro experiment indicated that bevacizumab and aflibercept bind VEGF with species-specific differences. The results demonstrated that bevacizumab and aflibercept are capable to bind canine VEGF and represent the cornerstone for the future clinical applicability of VEGF inhibitors in this species. This is of great interest, as novel targeted strategies like VEGF inhibition are needed for a variety of neovascular, potentially blinding ocular diseases in animals.

4.2. In-vivo analysis

The second part of this PhD project aimed to investigate topical bevacizumab in-vivo both in healthy dogs and dogs with corneal vascularization. Based on previous human studies, we selected a dose of 2.5 mg/ml BID over a four week period (Krizova et al. 2014). Bevacizumab eyedrops were aseptically prepared from commercial bevacizumab solution for intravenous use (Avastin®, Roche, Basel, Switzerland) by the institute's pharmacist according to good manufacturing practice. Sterile 0.9 % saline served as the solvent (Kim et al. 2008, Koenig et al. 2009).

4.2.1. Topical bevacizumab in healthy dogs

There is no knowledge about topical and systemic safety following bevacizumab eye drops in dogs. Our first in vivo study aimed to evaluate the tolerability of topical bevacizumab in healthy dogs, particularly in terms of corneal epithelial loss and ocular hypersensitivity reactions. Yet another question of interest was the effect of topical bevacizumab on systemic VEGF values and blood and coagulation parameters.

Ocular safety was assessed by biomicroscopic eye examination and by using a predefined pain scoring system. Additionally, conjunctival hyperemia and chemosis, ocular discharge, corneal edema, and corneal vascularization was determined by using predefined scorings. Any observed clinical abnormality was recorded. Full physical examinations, systemic blood pressure, and complete blood count, including hematology, serum coagulation parameters, and serum VEGF values, were performed.

There was no evidence of ocular and systemic intolerance. All clinical values stayed within normal physiologic limits with only minor, clinically irrelevant changes. Topical bevacizumab had no effect on blood counts, coagulation parameters, and serum VEGF values. At this point it should be mentioned that it would had been interesting to investigate an ascending concentration of bevacizumab. Anyway, due to ethical considerations and of special caution, we decided to use a scientifically proven safe dose of 2.5 mg/ml BID.

4.2.2. Topical bevacizumab in dogs with corneal vascularization

After verifying the safe applicability of topical bevacizumab in healthy dogs, we assessed bevacizumab with the same treatment regime in dogs with naturally occurring corneal vascularization refractory to standard therapy. The study was designed as a prospective planned case series involving adult, client-owned dogs, which were presented to the ophthalmology ambulatory service of the University of Veterinary Medicine of Vienna.

After confirming study eligibility, a dose of 2.5 mg/ml BID bevacizumab dissolved in 0.9 % sterile saline and filled in single dose containers, was administered over a period of four weeks by the dog owners. Re-examinations were scheduled four weeks after study start with a minimum follow-up of six months.

Full physical and ocular examinations were performed at each study visit. A predefined pain scoring was conducted by the same study investigator. Additionally, the dog owners were asked to estimate their dog's sensation of pain according to prespecified pain scores at certain points of time. The parameters hyperemia, ocular discharge, corneal edema, pigmentation, and vascularization were determined be using a prespecified grading system. Standardized corneal photographs were taken to determine the size of the vascularized corneal area and to count the blood vessel incisions on a specific circle on the corneal surface. Vessel morphology, including

vessel caliber and blood filling, distal vessel tortuosity and branching, was assessed subjectively during the clinical examination and recorded in a descriptive manner.

Twenty eyes of fifteen adult dogs were included, all of which were pretreated with systemic and/or topical anti-inflammatory agents with a lack of improvement of corneal vascularization. Brachycephalic breeds were overrepresented in our study. Overall, the patient population was very inhomogeneous, with a wide range of age and weight, a large variety of underlying diseases and pretreatments, and various degrees of disease severity and corneal scarring. Clinical appearance varied from subepithelial to deep stromal vessels, and fine superficial vascularized networks to planar stromal hemorrhages. The most common underlying diseases of corneal vascularization were immune-mediated keratitis, qualitative tear film deficiency, and the state after poorly healing corneal ulcerative keratitis, such as melting ulcers and complications after DBD.

4.2.2.1. Safety

In this study, a female French bulldog developed a SCCED at the study eye in the treatment-free interval, six months after the last bevacizumab administration. The SCCED progressed into a deep corneal ulcer, and the eye was enucleated by the referring veterinarian. Unfortunately, a histopathological examination was neglected.

The dog suffered from bilateral immune-mediated keratitis and allergic dermatitis that flared up repeatedly in severe episodes and was controlled under systemic immunosuppression. Shortly before the SCCED occurred, immunosuppressive therapy had to be stopped because of suspected meningitis. Thus, a causal relation to the drug discontinuation is conceivable.

In the literature, the occurrence of corneal epithelial defects and nerve fiber regeneration following topical bevacizumab is disputed (Bock et al. 2007, Yoeruek et al. 2008, Kim et al. 2009)(Yoeruek et al. 2008, Bock et al. 2009, Kim et al. 2009). In some animal studies with experimental-induced corneal erosions, topical bevacizumab did not delay wound healing and did not change nerve fiber density or epithelial cell morphology (Yoeruek et al. 2008, Bock et al. 2009). However, VEGF is essentially required for corneal wound and nerve repair (Amano et al. 1998, Eming and Krieg 2006, Yu et al. 2008a). Thus, conflicting findings indicated a delayed corneal wound closure in rabbits following topical and an impaired nerve fiber

regeneration after intrastromal or subconjunctival bevacizumab (Kim et al. 2009, Dong et al. 2017). In humans, topical bevacizumab has been reported to potentially cause corneal erosions, delay wound healing, and promote a corneal thinning (Kim et al. 2008, Koenig et al. 2009, Krizova et al. 2014). The occurrence of corneal epitheliopathies appears to correlate with the dose and duration of therapy (Kim et al. 2008). A single two to three week treatment duration at a dose of 2.5 mg-10 mg/ml two to four times a day is described to be topically safe and effective (Dastjerdi et al. 2009, Krizova et al. 2014). Thus, the use of bevacizumab as a single treatment rather than continuous therapy for months should be considered (Dastjerdi et al. 2009, Krizova et al. 2014).

Corneal sensory nerves and neurotransmitters have important corneal trophic functions and VEGF is known to mediate corneal nerve regeneration (Nakamura et al. 1997, Müller et al. 2003, Yu et al. 2008a). Abnormal corneal innervation is postulated to be causal in the development of SCCEDs (Murphy et al. 2001). Additionally, brachycephalic breeds are known to have an impaired nerve fiber density and morphology (Kafarnik et al. 2008). Unfortunately, brachycephalic breeds in particular have a very high prevalence of persistent corneal vascularization (Plummer 2015) and VEGF inhibitory therapies would be of great interest in these breeds. This is reflected by the fact that 73 % of our study population suffered from ocular brachycephalic syndrome. Unfortunately, we did not assess corneal sensitivity in our study, for example by using Cochet-Bonnet esthesiometry (Murphy et al. 2001). Therefore, we were not able to conclude with certainty that there was no causal relationship between the brachycephalic breed predisposition, altered and/or preexisting corneal sensitivity, the drug, and the development of the SCCED. We considered a causal drug relationship unlikely because we chose a low dose, the last bevacizumab dose was administered six months before the adverse event occurred, and there were no signs of epithelial thinning, ulceration, or pain during the study visits. Instead, the discontinuation of the immunosuppressive therapy that resulted in a relapse of immune-mediated keratitis, dermatitis, and self mutilation, may have contributed to the SCCED development and its progression into a deep ulcer.

However, bevacizumab should be carefully used in brachycephalic breeds and in individuals prone to recurrent corneal erosions or neurotrophic corneal disorders (Kim et al. 2008, Yu et al. 2008a, Dong et al. 2017).

In our study, two Chihuahuas died four and a half and four months after the last bevacizumab administration at the age of twelve and sixteen years, respectively. Both dogs had a history of well-controlled mitral valve insufficiency and were monitored regularly by a veterinarian cardiologist. In either case, there was no sign of cardiovascular or pulmonary dysfunction during the cardiological examinations and study visits. Vascular endothelial growth factor A is involved in physiological processes, such as the regulation of blood pressure, hemostasis, and vasculogenesis (Shalaby et al. 1995, Carmeliet et al. 1999, Ruhrberg et al. 2002, Gerhardt et al. 2003, Maes et al. 2004, Edelman et al. 2005). Thus, a prolonged systemic neutralization of VEGF may lead to unintended systemic complications (Shima et al. 2008, Wu et al. 2008). Following intravitreal administration of VEGF inhibitors in humans, bevacizumab and aflibercept cause a decrease of systemic VEGF values (Zehetner et al. 2013, 2015). Thus, systemic effects after intravitreal administration are plausible and thromboembolic events and systemic hypertension have been reported in rare cases (Shima et al. 2008, Wu et al. 2008). After topical administration, systematic bevacizumab concentration is significantly lower than after intravitreal or subconjunctival application and there are no reports of severe systemic side effects using the topical administration route (Koenig et al. 2009, Nomoto et al. 2009). This correlates well with our finding that bevacizumab did not alter systemic blood pressure and has no effect on differential blood count, coagulation parameters, and serum VEGF valuess in healthy dogs. However, it is well known that corneal permeability and drug bioavailability may be different in vascularized corneas compared to corneas with an intact barrier function (Yoeruek et al. 2008, Dastjerdi et al. 2011, Sella et al. 2016). Nevertheless, we administered a very low dose in our study, assuming that this dose is not high enough to cause systemic side effects. In addition, both dogs died months after the last bevacizumab administration during the treatment-free phase and showed no signs of cardiovascular or pulmonary dysfunction before or during the treatment period, making a causal drug relationship unlikely.

In future studies, measurement of systemic VEGF values, anti-drug antibodies, and drug levels in dogs with corneal vascularization may be considered.

Patients with preexisting cardiovascular disorders or any risk of embolic events should be carefully selected for VEGF inhibiting therapies.

4.2.2.2. Efficacy

To quantify the effect on corneal vascularization, photographs of the cornea were taken at each study visit. The major challenge was to take photos as standardized as possible, otherwise the image evaluation could be distorted. This included not only keeping the dogs still for a few minutes and getting the right angle of view and framing but also technical conditions, such as the identical light conditions measured with a luxmeter for consistency, optimal camera settings like white balance, ISO sensitivity, exposure time, and camera aperture, and working on a calibrated monitor. In advance, we thoroughly considered whether we would be able to shoot without flash, as this would cause reflections. Unfortunately, this was not possible, but we decided against the round flash and for a forceps shaped flash, because the reflected images appeared much smaller. The photographs were analyzed using an image processing software (Fiji; open-source software) by measuring the vascularized area and by counting of vessel incisions. For the latter, we divided the corneal surface into four circles, each 25 % smaller than the respective outer one, and counted the number of vessel incisions on each circle.

The results indicated that topical bevacizumab has the potential to reduce corneal vascularization. We found that it was not so much a complete disappearance or shortening of blood vessels but rather a reduction in vessel thickness and the formation of sharped demarcated blood vessels out of diffuse and areal hemorrhages. This observation is in line with those of other studies (Dastjerdi et al. 2009) and is plausible, considering that VEGF increases vascular permeability, vessel tortuosity, and vasodilatation (Edelman et al. 2005).

Clinically, we found that the tortuosity and branching of the vessel endings decreased notably, which is reported in retinal blood vessels following bevacizumab administration, too (Feng et al. 2014). This was particularly impressive in a male Great Pyrenees dog. The dog had unilateral stromal vascularization occupying the complete corneal surface. It was probably caused by an infectious keratitis following an untreated corneal cat scratch. The dog was treated for years with various glucocorticoids and cyclosporine without improving visual acuity or vascularization. Even though the vascularized area did not decrease after topical bevacizumab, distal vessel branching, tortuosity, vessel diameter, and corneal edema decreased markedly. The dog owner reported that the dog is now able to move more confidently on the ipsilateral side of the body through the obstacle course during agility training.

Additionally, we observed a marked reduction in inflammatory intracorneal infiltrates following topical bevacizumab which is plausible, as corneal inflammation and VEGF-driven angiogenesis are interlinked (Cursiefen et al. 2004a, Chung et al. 2009) and in inflamed corneas, VEGF-A is strongly expressed on various corneal cells, such as vascular endothelial cells, T-lymphocytes, and macrophages (Cursiefen et al. 2000).

Particularly in a German shepherd dog with CSK, we observed a marked decrease of corneal inflammatory infiltrations. The dog's vision was severely impaired, as the corneal opacity extended over more than two thirds of the corneal surface. Despite topical combined dexamethasone and cyclosporine therapy, there was no clinical improvement. In this dog, clinical improvement after bevacizumab treatment was mainly characterized by marked reduction of inflammatory cell infiltration and edema, resulting in a significant increase of corneal clarity. Stromal thick blood vessels remained. However, thin and superficial blood vessels became bloodless and were visible as ghost vessels. A recent study indicated that VEGF might be involved in the etiopathogenesis of CSK (Balicki and Sobczyńska-Rak 2014). For the first time, we showed that bevacizumab may have a beneficial effect on angiogenesis and inflammation in CSK.

In our study population, there was a high incidence of preexisting qualitative tear film deficiencies, which might have contributed causally to corneal vascularization in these dogs. Interestingly, we found that the tear-film-breakup time (TFBT), a measure that determines the tear film quality, increased during bevacizumab treatment. This finding correlates with the report of significant improvement of TBUT in humans with meibomian gland dysfunction following intra-meibomian gland injection of bevacizumab (Jiang et al. 2018). It remains the subject of future studies, to investigate therapeutic VEGF inhibition in qualitative KCS in dogs. Nevertheless, tear film disorders are accompanied by a deterioration of the ocular surface and corneal epithelium health and VEGF is required for normal wound repair (Amano et al. 1998, Eming and Krieg 2006). Thus, inhibition of VEGF should be used with caution in this indication.

We observed a high variety of treatment response to therapy. Overall, the mean vascularized corneal area was reduced by 48.8 %, with a high range of 4.9 - 100 %. This was reported in human studies, too (Dastjerdi et al. 2009, Koenig et al. 2009). There are various explanations for this finding. In our study, the dogs displayed a highly variable degree of corneal scarring

and depth of blood vessels within the cornea. The ability of a substance to penetrate a tissue depends on the porosity, conductivity, and tortuosity of the tissue (Bear and Bachmat 1967, Stewart et al. 2009). Reduced tissue permeability in porcine corneas with an increased corneal cross-link content following corneal crosslinking is reported (Stewart et al. 2009), suggesting that more heavily scarred corneas are less permeable. Some of the studied dogs showed massive corneal fibrosis and pigmentation prior to the study, whereas other ones had only thin subepithelial blood vessels without any sign of tissue scarring. In future research studies with larger patient cohorts, patients could be grouped according to their extent of corneal vascularization and scarring in order to assess correlations between degree of tissue scarring and the extent of drug efficacy. In addition, a variety of other pro- and antiangiogenic factors are involved in corneal angiogenesis, such as VEGF-C, VEGF-D, MMPs, FGF, and integrins (Gualandris et al. 1996, Zhang et al. 2001, Senger et al. 2002, Kano et al. 2005, Muether et al. 2007, Chung et al. 2009, Mimura et al. 2009, Murakami et al. 2011, Gurung et al. 2018). Bevacizumab solely binds VEGF-A and is not capable to inhibit other factors that may promote angiogenesis, too (Papadopoulos et al. 2012). Thus, the selective inhibition of only VEGF-A may lead to the impartial response to therapy. Furthermore, the duration and progression of a disease influences the temporal occurrence of angiogenic factors. For example, in DED animal models, VEGF expression increased earliest, whereas VEGF-C and VEGF-A expression occurred later in the course (Goyal et al. 2010). Furthermore, it is postulated that bevacizumab can only suppress proliferation of growing blood vessels and VEGF-A is probably not needed for proliferation of mature blood vessels (Benjamin et al. 1999, Cursiefen et al. 2003, Koenig et al. 2009). Besides this, humanized therapeutic monoclonal antibodies are reported to be immunogenic in animals and might induce immune responses that result in the production of anti-drug antibodies (ADA) (Van Meer et al. 2013). Anti-drug antibodies are directed against elements of the drug and can lead to hypersensitivity responses, a faster drug clearance, reduced effectiveness, and an elevated interindividual variability of treatment response (Chirmule et al. 2012). We studied a very low dose, and one might assume that this dose is not high enough to cause an ADA induction. However, studies indicated that lower rather than initially higher doses are associated with the occurrence of ADAs (Somerfield et al. 2010). Thus, ADAs potentially contributed to the variable interindividual treatment responses observed in our

study. Future research may consider determining ADA status before, during, and after bevacizumab treatment in dogs.

In our study, all but one dog maintained clinical improvement until long-term follow-up, which correlates well with studies in human patients (Dastjerdi et al. 2009, Koenig et al. 2009). Thus, a therapy episode of four weeks every six months with intermittent therapy-free periods could be conceivable. This would be of particular importance in veterinary medicine because multiple daily drug therapy is not always possible due to lack of patient compliance, long travel distances to the stables, or possible side effects under other long-term ophthalmic medications. Further studies are needed to investigate the optimal duration of therapy and therapy-free intervals, required timing of follow-up examinations, and the benefit of single subconjunctival injections. Moreover, the effect of bevacizumab in other indications, such as PIFMs, ERU, EK, and ocular tumors, remains unknown. In our study, we did not have any dog with ocular neoplasia, which is also due to the fact that primary corneal neoplasms are rare in this species (Haeussler et al. 2011, Leis et al. 2017). However, corneal neoplasms occur occasionally in horses, with squamous cell carcinomas (SSC) being the most frequent tumor type of the ocular surface (Lassaline et al. 2015). A heritable basis in Haflinger horses, a widespread breed in Austria, is postulated (Lassaline et al. 2015) and a causal relationship to ultraviolet light is suspected (Bellone et al. 2017). In cats, there is a high need for new targeted therapies for feline palpebral neoplasms (Newkirk and Rohrbach 2009). These neoplasms tend to be malignant and even with aggressive surgical excision and extensive palpebral lid reconstructions, can run lethally (Newkirk and Rohrbach 2009). There is only one published case report in animal medicine, dealing with an Amur tiger with a palpebral sebaceous carcinoma relapse that was successfully treated with intralesional bevacizumab as an adjuvant therapy to surgery (Edelmann et al. 2013). Taken together, therapeutic VEGF inhibition may represent a promising approach to combat ocular surface neoplasms in animals. However, our in-vitro results indicated that feline and equine VEGF may not be bound by aflibercept and bevacizumab or may interact only in a nonspecific manner. It would be of great interest to investigate other VEGF inhibitors, such as ranibizumab, brolucizumab, and the bispecific antibody faricimab that inhibits both VEGF-A and angiopoietin-2 (Stevenson et al. 2012, Dugel et al. 2020, Sahni et al. 2020).

Besides the usage of VEGF inhibitors in cancer diseases, novel treatment strategies for the treatment of PIFMs are needed (Pfeiffer et al. 1990). In our study, we solely assessed the effect

of bevacizumab on the ocular surface and the penetration through the intact corneal epithelium into the anterior chamber is unlikely (Dastjerdi et al. 2011, Moisseiev et al. 2014). To date, the safety and efficacy of intravitreal or subconjunctival bevacizumab administration in adult dogs is unknown. However, there are studies investigated intravitreal VEGF inhibitors in animal models of experimentally induced choroidal and retinal vascularization in monkeys and in newborn dogs (Lutty et al. 2011, Nork et al. 2011). These studies indicated that VEGF inhibition is effective in repression of pathological vascularization with no or only mild adverse events. It may be the topic of future research, to investigate the safety and efficacy of intraocular administered bevacizumab to treat intraocular or retinal disorders in dogs.

There are several limitations of this study. In our study, we included dogs with refractory corneal vascularization despite the use of topical/and or systemic medications, such as topical cyclosporine, dexamethasone, and systemic NSAIDs and cyclosporine. No dog was treated with topical or systemic glucocorticoids during the study period. However, almost all dogs were pretreated with topical cyclosporine and were maintained on the drug during the study. We decided against discontinuing cyclosporine for ethical reasons, because we did not want to take the risk for deterioration of the stable condition of the cornea. One may criticize that the angioregressive effect observed was not due to bevacizumab alone. However, cyclosporine was used in all dogs for months or years without further improvement of corneal vascularization. This is supported by the finding that topical cyclosporine did not inhibit corneal vascularization in rats (Bock et al. 2014, Villar et al. 2020). Future studies are needed, to investigate bevacizumab as a single treatment option.

Another limitation was the way we quantified the blood vessels, as discussed earlier. It has to be mentioned that even in 30-fold magnification with biomicroscopic slit lamp examination and high-quality photographs, not all corneal blood vessels are visible, in particular when corneal edema, pigmentation, and scarring are present. To assess each vessel as comprehensively as possible, further imaging techniques, such as ultrahigh-resolution OCT, indocyanine green angiography, or fluorescein angiography can be considered (Anijeet et al. 2012, Werkmeister et al. 2017).

Furthermore, the validity of this study is limited by its study design, as it was conceptualized as a prospectively planned case series. We did not have a control group, thereby we could solely

investigate the effect before and after treatment. Future studies with larger patient cohorts in a placebo-controlled, masked study setting are of interest.

5. Conclusion

In summary, this PhD project demonstrated that anti-human VEGF bevacizumab and aflibercept were able to bind canine VEGF in a dose-dependent manner in-vitro. However, our results indicated that feline VEGF is bound by aflibercept and bevacizumab solely in a nonspecific manner. In horses, there was a lack of VEGF binding to bevacizumab. Aflibercept bound equine VEGF in a linear manner, assuming a nonspecific binding.

Moreover, this study showed that bevacizumab eye drops are topically and systemically safe in healthy dogs and did not cause changes in vital signs, blood counts, blood pressure, and serum VEGF values in healthy dogs. Furthermore, our results indicated that topical bevacizumab may be beneficial for the treatment of corneal vascularization in dogs. However, a high range of response to treatment was noted and the angioregressive effect was characterized rather by a reduction of blood vessel caliber and branching, then by a complete disappearance of the vascularized area. In all cases affected by corneal edema or inflammatory cell infiltration, a clinical improvement and increase of corneal clarity was achieved.

Topical bevacizumab may be used with caution in dogs prone to corneal erosions and neurotrophic corneal disorders, and in the presence of pre-existing systemic cardiovascular disease, systemic hypertension, or risk for embolic events.

Finally, this PhD project is an important step to improve the understanding and knowledge of VEGF in dogs, cats, and horses. It might pave the ways for novel therapies for neovascular and cancer diseases in animals that may be addressed in future research.

6. References

Abrams K, Stabila P, Kauper K, Elliott S. 2011. Vascular endothelial growth factor in diabetic and nondiabetic canine cataract patients. Veterinary Ophthalmology, 14(2):93–99.

Adelfinger M, Bessler S, Frentzen A, Cecil A, Langbein-Laugwitz J, Gentschev I, Szalay A. 2015. Preclinical testing oncolytic vaccinia virus strain GLV-5b451 expressing an anti-VEGF single-chain antibody for canine cancer therapy. Viruses, 7(7):4075–4092.

Aiello L, Avery R, Arrigg P, Keyt B, Jampel H, Shah S, Pasquale L, Thieme H, Iwamoto M, Park J, et al. 1994. Vascular Endothelial Growth Factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders. The New English Journal of Medicine, 331(22):1480–1487.

Aksoy S. 2019. Treatment of corneal vascularization with topical aflibercept in a case of exposure keratopathy following cerebellar astrocytoma surgery. Indian Journal of Ophthalmology, 67(1):145–147.

Alam J, De Souza R, Yu Z, Stern M, De Paiva C, Pflugfelder S. 2020. Calcineurin inhibitor voclosporin preserves corneal barrier and conjunctival goblet cells in experimental dry eye. Journal of Ocular Pharmacology and Therapeutics, 36(9):679–685.

Allgoewer I, Hoecht S. 2010. Radiotherapy for canine chronic superficial keratitis using soft X-rays (15 kV). Veterinary Ophthalmology, 13(1):20–25.

Alma M, van der Mei S, Melis-Dankers BJ, van Tilburg T, Groothoff J, Suurmeijer T. 2011. Participation of the elderly after vision loss. Disability and Rehabilitation, 22(1):63–72.

Amano S, Rohan R, Kuroki M, Tolentino M, Adamis A. 1998. Requirement for vascular endothelial growth factor in wound- and inflammation-related corneal neovascularization. Investigative Ophthalmology and Visual Science, 39(1):18–22.

Ambati B, Nozaki M, Singh N, Takeda A, Jani P, Suthar T, Albuquerque R, Richter E, Sakurai E, Michael T. 2006. Corneal avascularity is due to soluble VEGF receptor-1. Nature, 443(7114):993–997.

Andrew S. 2008. Immune-Mediated Canine and Feline Keratitis. Veterinary Clinics of North America - Small Animal Practice, 38(2):269–290.

Andrew S, Brooks D, Smith P, Gelatt K, Chmielewski N, Whittaker C. 1998. Equine

ulcerative keratomycosis: Visual outcome and ocular survival in 39 cases (1987-1996). Equine Veterinary Journal, 30(2):109–116.

Anijeet D, Zheng Y, Tey A, Hodson M, Sueke H, Kaye S. 2012. Imaging and evaluation of corneal vascularization using fluorescein and indocyanine green angiography. Investigative Ophthalmology and Visual Science, 53(2):650–658.

Aresu L, Aricò A, Comazzi S, Gelain M, Riondato F, Mortarino M, Morello E, Stefanello D, Castagnaro M. 2014. VEGF and MMP-9: Biomarkers for canine lymphoma. Veterinary and Comparative Oncology, 12(1):29–36.

Arevalo J, Fromow-Guerra J, Quiroz-Mercado H, Sanchez J, Wu L, Maia M, Berrocal M, Solis-Vivanco A, Farah M. 2007. Primary Intravitreal Bevacizumab (Avastin) for Diabetic Macular Edema. Results from the Pan-American Collaborative Retina Study Group at 6-Month Follow-up. Ophthalmology, 114(4):743–750.

Asena L, Altınörs D. 2015. Topical bevacizumab for the treatment of ocular surface squamous neoplasia. Journal of Ocular Pharmacology and Therapeutics, 31(8):487–490.

Auten C, Urbanz J, Dees D. 2020. Comparison of bacterial culture results collected via direct corneal ulcer vs conjunctival fornix sampling in canine eyes with presumed bacterial ulcerative keratitis. Veterinary Ophthalmology, 1(23):135–140.

Avery R, Pieramici D, Rabena M, Castellarin A, Nasir M, Giust M. 2006. Intravitreal bevacizumab (Avastin) for neovascular age-related macular degeneration. Ophthalmology, 113(3):363–372.

Bachmann, Taylor R, Cursiefen C. 2010. Corneal neovascularization as a risk factor for graft failure and rejection after keratoplasty: An evidence-based meta-analysis. Ophthalmology, 117(7):1300-1305.e7.

Balicki I. 2012. Clinical study on the application of tacrolimus and DMSO in the treatment of chronic superficial keratitis in dogs. Polish Journal of Veterinary Sciences, 15(4):667–676.

Balicki I, Sobczyńska-Rak A. 2014. Serum vascular endothelial growth factor concentration in dogs diagnosed with chronic superficial keratitis. Acta Veterinaria Hungarica, 62(1):22–32.

Balland O, Poinsard A, Famose F, Goulle F, Isard P, Mathieson I, Dulaurent T. 2016. Use of a porcine urinary bladder acellular matrix for corneal reconstruction in dogs and cats.

Veterinary Ophthalmology, 19(6):454–463.

Ballaun C, Weninger W, Uthman A, Weich H, Tschachler E. 1995. Human keratinocytes express the three major splice forms of vascular endothelial growth factor. Journal of Investigative Dermatology, 104(1):7–10.

Bear J, Bachmat Y. 1967. A generalized theory on hydrodynamic dispersion In porous media. International Union of Geodesy and Geophysics, 72:7–16.

Bedford P, Longstaff J. 1979. Corneal pannus (chronic superficial keratitis) in the German Shepherd Dog. Journal of Small Animal Practice, 20:41–56.

Bein K, Simons M. 2000. Thrombospondin type 1 repeats interact with matrix metalloproteinase 2. Regulation of metalloproteinase activity. Journal of Biological Chemistry, 275(41):32167–32173.

Bellone R, Liu J, Petersen J, Mack M, Singer-Berk M, Drögemüller C, Malvick J, Wallner B, Brem G, Penedo M, et al. 2017. A missense mutation in damage-specific DNA binding protein 2 is a genetic risk factor for limbal squamous cell carcinoma in horses. International Journal of Cancer, 141(2):342–353.

Benayoun Y, Adenis J, Casse G, Forte R, Robert P. 2012. Effects of subconjunctival bevacizumab on corneal neovascularization: Results of a prospective study. Cornea, 31(8):937–944.

Benedito R, Roca C, Sörensen I, Adams S, Gossler A, Fruttiger M, Adams R. 2009. The Notch Ligands Dll4 and Jagged1 have Opposing Effects on Angiogenesis. Cell, 137(6):1124–1135.

Benjamin L, Golijanin D, Itin A, Pode D, Keshet E. 1999. Selective ablation of immature blood vessels in established human tumors follows vascular endothelial growth factor withdrawal. Journal of Clinical Investigation, 103(2):159–165.

Berg J, Tymoczko J, Gatto G, Stryer L. 2017. Stryer Biochemie. 8th. Edition. Berlin: Springer Spektrum, pp.48-73.

Binder D, Herring I, Zimmerman K, Phillip J, Huckle W. 2012. Expression of vascular endothelial growth factor receptor-1 and -2 in normal and diseased canine eyes. Veterinary Ophthalmology, 15(4):223–230.

Blanco-Mezquita J, Hutcheon A, Zieske J. 2013. Role of thrombospondin-1 in repair of penetrating corneal wounds. Investigative Ophthalmology and Visual Science,

54(9):6262–6268.

Bock F, Onderka J, Dietrich T, Bachmann B, Kruse FE, Paschke M, Zahn G, Cursiefen C. 2007. Bevacizumab as a potent inhibitor of inflammatory corneal angiogenesis and lymphangiogenesis. Investigative Ophthalmology and Visual Science, 48(6):2545–2552.

Bock F, Koenig Y, Kruse F, Baier M, Cursiefen C. 2008. Bevacizumab (Avastin) eye drops inhibit corneal neovascularization. Graefe's Archive for Clinical and Experimental Ophthalmology, 246(2):281–284.

Bock F, Onderka J, Rummelt C, Dietrich T, Bachmann B, Kruse F, Schlötzer-Schrehardt U, Cursiefen C. 2009. Safety profile of topical VEGF neutralization at the cornea. Investigative Ophthalmology and Visual Science, 50(5):2095–2102.

Bock F, Matthaei M, Reinhard T, Böhringer D, Christoph J, Ganslandt T, Cursiefen C. 2014. High-dose subconjunctival cyclosporine a implants do not affect corneal neovascularization after high-risk keratoplasty. Ophthalmology, 121(9):1677–1682.

Bonanno J. 2012. Molecular mechanisms underlying the corneal endothelial pump. Experimental Eye Research, 95(1):2–7.

Bourne R, Jonas J, Bron A, Cicinelli M, Das A, Flaxman S, Friedman D, Keeffe J, Kempen J, Leasher J, et al. 2018. Prevalence and causes of vision loss in high-income countries and in Eastern and Central Europe in 2015: Magnitude, temporal trends and projections. British Journal of Ophthalmology, 102(5):575–585.

Brooks D, Plummer C, Kallberg M, Barrie K, Ollivier F, Hendrix D, Baker A, Scotty N, Utter M, Blackwood S, et al. 2008. Corneal transplantation for inflammatory keratopathies in the horse: Visual outcome in 206 cases (1993-2007). Veterinary Ophthalmology, 11(2):123–133.

Bucak Y, Erdurmus M, Terzi E, Kükner A, Çelebi S. 2013. Inhibitory effects of topical cyclosporine A 0.05 % on immune-mediated corneal neovascularization in rabbits. Graefe's Archive for Clinical and Experimental Ophthalmology, 251(11):2555–2561.

Campbell L, Okuda H, Lipton D, Reed C. 1975. Chronic superficial keratitis in dogs: detection of cellular hypersensitivity. American Journal of Veterinary Research, 36(5):669–671.

Carmeliet P, Ng Y, Nuyens D, Theilmeier G, Brusselmans K, Cornelissen I, Ehler E, Kakkar V, Stalmans I, Mattot V, et al. 1999. Impaired myocardial angiogenesis and ischemic

cardiomyopathy in mice lacking the vascular endothelial growth factor isoforms VEGF164 and VEGF188. Nature Medicine, 5(5):495–502.

Chandler H, Gemensky-Metzler A, Bras I, Robbin-Webb T, Saville W, Colitz C. 2010. In vivo effects of adjunctive tetracycline treatment on refractory corneal ulcers in dogs. Journal of the American Veterinary Medical Association, 237(4):378–386.

Chen L, Huq S, Gardner H, De Fougerolles A, Barabino S, Dana M. 2007. Very late antigen 1 blockade markedly promotes survival of corneal allografts. Archives of Ophthalmology, 125(6):783–788.

Chen P, Yin H, Wang Y, Wang Y, Xie L. 2012. Inhibition of VEGF expression and corneal neovascularization by shRNA targeting HIF-1α in a mouse model of closed eye contact lens wear. Molecular Vision, 18:864–873.

Chen S, Young MF, Chakravarti S, Birk DE. 2014. Interclass small leucine-rich repeat proteoglycan interactions regulate collagen fibrillogenesis and corneal stromal assembly. Matrix Biology, 35:103–111.

Chen S, Mienaltowski M, Birk D. 2015. Regulation of corneal stroma extracellular matrix assembly. Experimental Eye Research, 133:69–80.

Cheng S, Dastjerdi M, Ferrari G, Okanobo A, Bower K, Ryan D, Amparo F, Stevenson W, Hamrah P, Nallasamy N, et al. 2012. Short-term topical bevacizumab in the treatment of stable corneal neovascularization. American Journal of Ophthalmology, 154(6):940–948.

Chirmule N, Jawa V, Meibohm B. 2012. Immunogenicity to therapeutic proteins: Impact on PK/PD and efficacy. American Association of Pharmaceutical Scientists Journal, 14(2):296–302.

Cholak A, Nasinnyk I, Kostenko P, Iakimenko S, Korol A. 2020. A case of vascularized corneal opacity treated with aflibercept. Journal of Ophthalmology (Ukraine), 1(492):54–56.

Chung A, Ferrara N. 2011. Developmental and pathological angiogenesis. Annual Review of Cell and Developmental Biology, 27:563–584.

Chung E, Chauhan S, Jin Y, Nakao S, Hafezi-Moghadam A, Van Rooijen N, Zhang Q, Chen L, Dana R. 2009. Contribution of macrophages to angiogenesis induced by vascular endothelial growth factor receptor-3-specific ligands. American Journal of Pathology, 175(5):1984–1992.

Clerc C, Jegou J. 1996. Superficial punctuate keratitis. Canine Practice, 21:6–11.

Costa J, Steinmetz A, Delgado E. 2021. Clinical signs of brachycephalic ocular syndrome in 93 dogs. Irish Veterinary Journal, 74:1–8.

Crispin SM, Barnett KC. 1983. Dystrophy, degeneration and infiltration of the canine cornea. Journal of Small Animal Practice, 24(2):63–83.

Cursiefen C, Rummelt C, Küchle M. 2000. Immunohistochemical localization of vascular endothelial growth factor, transforming growth factor α, and transforming growth factor β1 in human corneas with neovascularization. Cornea, 19(4):526–533.

Cursiefen C, Hofmann-Rummelt C, Kuechle M, Schloetzer-Schrehardt U. 2003. Pericyte recruitment in human corneal angiogenesis: An ultrastructural study with clinicopathological correlation. British Journal of Ophthalmology, 87(1):101–106.

Cursiefen C, Chen L, Borges L, Jackson D, Cao J, Radziejewski C, D'Amore P, Dana M, Wiegand S, Streilein J. 2004a. VEGF-A stimulates lymphangiogenesis and hemangiogenesis in inflammatory neovascularization via macrophage recruitment. The Journal of Clinical Investigation, 113(7):1040–1050.

Cursiefen C, Masli S, Ng TF, Dana MR, Bornstein P, Lawler J, Streilein JW. 2004b. Roles of thrombospondin-1 and -2 in regulating corneal and iris angiogenesis. Investigative Ophthalmology and Visual Science, 45(4):1117–1124.

Cursiefen C, Chen L, Saint-Geniez M, Hamrah P, Jin Y, Rashid S, Pytowski B, Persaud K, Wu Y, Streilein J, et al. 2006. Nonvascular VEGF receptor 3 expression by corneal epithelium maintains avascularity and vision. Proceedings of the National Academy of Sciences of the United States of America, 103(30):11405–11410.

Cursiefen C, Maruyama K, Bock F, Saban D, Sadrai Z, Lawler J, Dana R, Masli S. 2011. Thrombospondin 1 inhibits inflammatory lymphangiogenesis by CD36 ligation on monocytes. Journal of Experimental Medicine, 208(5):1083–1092.

Cursiefen C, Cordeiro F, Cunha-Vaz J, Wheeler-Schilling T, Scholl H. 2019. Unmet needs in ophthalmology: A European Vision Institute-Consensus roadmap 2019-2025. Ophthalmic Research, 62(3):123–133.

Curto E, Messenger K, Salmon J, Gilger B. 2016. Cytokine and chemokine profiles of aqueous humor and serum in horses with uveitis measured using multiplex bead immunoassay analysis. Veterinary Immunology and Immunopathology, 182:43–51.

Dana M, Streilein J. 1996. Loss and restoration of immune privilege in eyes with corneal neovascularization. Investigative Ophthalmology and Visual Science, 37(12):2485–2494.

Dastjerdi M, Al-Arfaj K, Nallasamy N, Hamrah P, Jurkunas U, Pineda R, Pavan-Langston D, Dana R. 2009. Topical bevacizumab in the treatment of corneal neovascularization; results of a prospective, open-label, noncomparative study. Archives of Ophthalmology, 127(4):381–389.

Dastjerdi M, Saban D, Okanobo A, Nallasamy N, Sadrai Z, Chauhan S, Hajrasouliha A, Dana R. 2010a. Effects of topical and subconjunctival bevacizumab in high-risk corneal transplant survival. Investigative Ophthalmology and Visual Science, 51(5):2411–2417.

Dastjerdi M, Sadrai Z, Saban D, Zhang Q, Dana R. 2011. Corneal penetration of topical and subconjunctival bevacizumab. Investigative Ophthalmology and Visual Science, 52(12):8718–8723.

Dean E, Meunier V. 2013. Feline eosinophilic keratoconjunctivitis: A retrospective study of 45 cases (56 eyes). Journal of Feline Medicine and Surgery, 15(8):661–666.

Deeg C, Pompetzki D, Raith A, Hauck S, Amann B, Suppmann S, Goebel T, Olazabal U, Gerhards H, Reese S, et al. 2006. Identification and functional validation of novel autoantigens in equine uveitis. Molecular and Cellular Proteomics, 5(8):1462–1470.

Deeg C, Altmann F, Hauck S, Schoeffmann S, Amann B, Stangassinger M, Ueffing M. 2007. Down-regulation of pigment epithelium-derived factor in uveitic lesion associates with focal vascular endothelial growth factor expression and breakdown of the blood-retinal barrier. Proteomics, 7(9):1540–1548.

Denk N, Fritsche J, Reese S. 2011. The effect of UV-blocking contact lenses as a therapy for canine chronic superficial keratitis. Veterinary Ophthalmology, 14(3):186–194.

Devarajan K, Ong H, Lwin N, Chua J, Schmetterer L, Mehta J, Ang M. 2019. Optical coherence tomography angiography imaging to monitor anti-VEGF treatment of corneal vascularization in a rabbit model. Scientific Reports, 9:17576.

Dong M, Di G, Zhang X, Zhou Q, Shi W. 2017. Subconjunctival Bevacizumab injection impairs corneal innervations and epithelial wound healing in mice. Investigative Ophthalmology and Visual Science, 58(3):1469–1477.

Dowling S, Webb J, Foster J, Ginn J, Foy D, Trepanier L. 2016. Opportunistic fungal

infections indogs treated with ciclosporin and glucocorticoids: Eight cases. Journal of Small Animal Practice, 57(2):105–109.

Dreyfus J, Schobert C, Dubielzig R. 2011. Superficial corneal squamous cell carcinoma occurring in dogs with chronic keratitis. Veterinary Ophthalmology, 14(3):161–168.

Du H, Liu P. 2016. Matrix metalloproteinase 14 participates in corneal lymphangiogenesis through the VEGF-C/VEGFR-3 signaling pathway. Experimental and Therapeutic Medicine, 12(4):2120–2128.

Dubielzig RR, Ketring KL, McLellan GJ, Albert DM. 2010. Veterinary ocular pathology: A comparative review. 1st. Edition. Philadelphia: Saunders Ltd., pp.202-203.

Dugel P, Singh R, Koh A, Ogura Y, Weissgerber G, Gedif K, Jaffe G, Tadayoni R, Schmidt-Erfurth U, Holz F. 2020. HAWK and HARRIER: Ninety-Six-Week Outcomes from the Phase 3 Trials of Brolucizumab for Neovascular Age-Related Macular Degeneration. Ophthalmology, 1–9.

Eaton J, Hollingsworth S, Holmberg B, Brown M, Smith P, Maggs D. 2017. Effects of topically applied heterologous serum on reepithelialization rate of superficial chronic corneal epithelial defects in dogs. Journal of the American Veterinary Medical Association, 250(9):1014–1022.

Ebrahem Q, Minamoto A, Hoppe G, Anand-apte B, Sears J. 2006. Triamcinolone Acetonide inhibits IL6- and VEGF-Induced Angiogenesis Downstream of the IL-6 and VEGF Receptors. Investigative Ophthalmology and Visual Science, 47(11):4935–4941.

Ebrahem Q, Chaurasia S, Vasanji A, Qi J, Klenotic P, Cutler A, Asosingh K, Erzurum S, Anand-Apte B. 2010. Cross-talk between vascular endothelial growth factor and matrix metalloproteinases in the induction of neovascularization in vivo. American Journal of Pathology, 176(1):496–503.

Edelman J, Lutz D, Castro M. 2005. Corticosteroids inhibit VEGF-induced vascular leakage in a rabbit model of blood-retinal and blood-aqueous barrier breakdown. Experimental Eye Research, 80(2):249–258.

Edelmann M, Utter M, Klein L, Wotman K. 2013. Combined excision and intralesional bevacizumab for sebaceous carcinoma of the eyelid in an Amur tiger (Panthera tigris altaica). Veterinary Ophthalmology, 16(3):219–224.

Eming S, Krieg T. 2006. Molecular mechanisms of VEGF-A action during tissue repair.

Journal of Investigative Dermatology Symposium Proceedings, 11(1):79–86.

Eslani M, Putra I, Shen X, Hamouie J, Afsharkhamseh N, Besharat S, Rosenblatt M, Dana R, Hematti P, Djalilian A. 2017. Corneal mesenchymal stromal cells are directly antiangiogenic via PEDF and sFLT-1. Investigative Ophthalmology and Visual Science, 58(12):5507–5517.

Faramarzi A, Feizi S. 2013. Subconjunctival bevacizumab injection for ocular surface squamous neoplasia. Cornea, 32(7):998–1001.

Featherstone H, Sansom J, Heinrich C. 2001. The use of porcine small intestinal submucosa in ten cases of feline corneal disease. Veterinary Ophthalmology, 4(2):147–153.

Feng F, Cheng Y, Liu Q. 2014. Bevacizumab treatment reduces retinal neovascularization in a mouse model of retinopathy of prematurity. International journal of Ophthalmology, 7(4):608–60813.

Ferrara N. 2009. Vascular endothelial growth factor. Arteriosclerosis, Thrombosis, and Vascular Biology, 29(6):789–791.

Ferrara N, Hillan K, Gerber H, Novotny W. 2004. Discovery and development of bevacizumab, an anti-VEGF antibody for treating cancer. Nature Reviews Drug Discovery, 3(5):391–400.

Gan L, Fagerholm P, Palmblad J. 2004. Vascular endothelial growth factor (VEGF) and its receptor VEGFR-2 in the regulation of corneal neovascularization and wound healing. Acta Ophthalmologica Scandinavica, 82(5):557–563.

Gelatt K, Gilger B, Kern T. 2013a. Veterinary Ophthalmology. 5th. Edition. Hoboken: John Wiley & Sons Inc., p.62 and p.977.

Gelatt K, Gilger B, Kern T. 2013b. Veterinary Ophthalmology. 5th. Edition. Hoboken: John Wiley & Sons Inc., p.1012.

Gelatt K, Gilger B, Kern T. 2013c. Veterinary Ophthalmology. 5th. Edition. Hoboken: John Wiley & Sons Inc., p.1015.

Gemensky-Metzler A, Sheahan J, Rajala-Schultz P, Wilkie D, Harrington J. 2015. Retrospective study of the prevalence of keratoconjunctivitis sicca in diabetic and nondiabetic dogs after phacoemulsification. Veterinary Ophthalmology, 18(6):472–480.

Genentech Inc. 2011. AVASTIN® (Bevacizumab)[Prescribing information] U.S. Food and Drug Administration website.

https://www.accessdata.fda.gov/drugsatfda_docs/label/2009/125085s0169lbl.pdf Revised May 2009. Accessed April 14, 2021.

Gerding J, Gilger B. 2016. Prognosis and impact of equine recurrent uveitis. Equine Veterinary Journal, 48(3):290–298.

Gerhardt H, Golding M, Fruttiger M, Ruhrberg C, Lundkvist A, Abramsson A, Jeltsch M, Mitchell C, Alitalo K, Shima D, et al. 2003. VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. Journal of Cell Biology, 161(6):1163–1177.

Gerten G. 2008. Bevacizumab (avastin) and argon laser to treat neovascularization in corneal transplant surgery. Cornea, 27(10):1195–1199.

Glaze M. 2005. Congenital and hereditary ocular abnormalities in cats. Clinical Techniques in Small Animal Practice, 20(2):74–82.

Goyal S, Chauhan S, El Annan J, Nallasamy N, Zhang Q, Dana R. 2010. Evidence of corneal lymphangiogenesis in dry eye disease: A potential link to adaptive immunity? Archives of Ophthalmology, 128(7):819–824.

Goyal S, Chauhan S, Dana R. 2012. Blockade of prolymphangiogenic vascular endothelial growth factor C in dry eye disease. Archives of Ophthalmology, 130(1):84–89.

Gualandris A, Rusnati M, Belleri M, Nelli E, Bastaki M, Molinari-Tosatti M, Bonardi F, Parolini S, Albini A, Morbidelli L, et al. 1996. Basic fibroblast growth factor overexpression in endothelial cells: An autocrine mechanism for angiogenesis and angioproliferative diseases. Cell Growth and Differentiation, 7(2):147–160.

Gurung H, Bryant K, Chucair-Elliott A, Carr D. 2018. Fibroblast growth factor-2 drives and maintains progressive corneal neovascularization following HSV-1 infection. Mucosal Immunology, 11(1):172–185.

Haeussler D, Rodríguez L, Wilkie D, Premanandan C. 2011. Primary central corneal hemangiosarcoma in a dog. Veterinary Ophthalmology, 14(2):133–136.

Haines J, Schnetz-Boutaud N, Schmidt S, Scott W, Agarwal A, Postel E, Olson L, Kenealy S, Hauser M, Gilbert J, et al. 2006. Functional candidate genes in age-related macular degeneration: Significant association with VEGF, VLDLR, and LRP6. Investigative Ophthalmology and Visual Science, 47(1):329–335.

Han K, Dugas-Ford J, Lee H, Chang J, Azar D. 2015. MMP14 cleavage of VEGFR1 in the cornea leads to a VEGF-trap antiangiogenic effect. Investigative Ophthalmology and

Visual Science, 56(9):5450–5456.

Han L, Su W, Huang J, Zhou J, Qiu S, Liang D. 2014. Doxycycline inhibits inflammation-induced lymphangiogenesis in mouse cornea by multiple mechanisms. Public Library of Science ONE, 9(9):1–9.

Hanai J, Dhanabal M, Karumanchi A, Albanese C, Waterman M, Chan B, Ramchandran R, Pestell R, Sukhatme V. 2002. Endostatin causes G 1 arrest of endothelial cells through inhibition of cyclin D1. Journal of Biological Chemistry, 277(19):16464–16469.

Hendrix D. 2005. Eye examination techniques in horses. Clinical Techniques in Equine Practice, 4(1):2–10.

Hendrix D, Adkins E, Ward D, Stuffle J, Skorobohach B. 2011. An investigation comparing the efficacy of topical ocular application of tacrolimus and cyclosporine in dogs. Veterinary Medicine International, 2011:487592.

Herrera H, Weichsler N, Gómez J, De Jalón J. 2007. Severe, unilateral, unresponsive keratoconjunctivitis sicca in 16 juvenile Yorkshire Terriers. Veterinary Ophthalmology, 10(5):285–288.

Hindley K, Groth A, King M, Graham K, Billson F. 2016. Bacterial isolates, antimicrobial susceptibility, and clinical characteristics of bacterial keratitis in dogs presenting to referral practice in Australia. Veterinary Ophthalmology, 19(5):418–426.

Holash J, Davis S, Papadopoulos N, Croll SD, Ho L, Russell M, Boland P, Leidich R, Hylton D, Burova E, et al. 2002. VEGF-Trap: A VEGF blocker with potent antitumor effects. Proceedings of the National Academy of Sciences of the United States of America, 99(17):11393–11398.

Hos D, Saban D, Bock F, Regenfuss B, Onderka J, Masli S, Cursiefen C. 2011. Suppression of inflammatory corneal lymphangiogenesis by application of topical corticosteroids. Archives of Ophthalmology, 129(4):445–452.

Hos D, Le V, Hellmich M, Siebelmann S, Roters S, Bachmann B, Cursiefen C. 2019. Risk of corneal graft rejection after high-risk keratoplasty following fine-needle vessel coagulation of corneal neovascularization combined with bevacizumab: A pilot study. Transplantation Direct, 5(5):1–5.

Hou Y, Le V, Clahsen T, Schneider A, Bock F, Cursiefen C. 2017. Photodynamic therapy leads to time-dependent regression of pathologic corneal (lymph) angiogenesis and

promotes high-risk corneal allograft survival. Investigative Ophthalmology and Visual Science, 58(13):5862–5869.

Houck K, Leung D, Rowland A, Winer J, Ferrara N. 1992. Dual regulation of vascular endothelial growth factor bioavailability by genetic and proteolytic mechanisms. Journal of Biological Chemistry, 267(36):26031–7.

Hung J, Leidreiter K, White J, Bernays M. 2020. Clinical characteristics and treatment of spontaneous chronic corneal epithelial defects (SCCEDs) with diamond burr debridement. Veterinary Ophthalmology, 23(4):764–769.

Izci C, Celik I, Alkan F, Erol M, Sur E. 2015. Clinical and light microscopic studies of the conjunctival tissues of dogs with bilateral keratoconjunctivitis sicca before and after treatment with topical 2% cyclosporine. Biotechnic and Histochemistry, 90(3):223–230.

Jia H, Bagherzadeh A, Bicknell R, Duchen M, Liu D, Zachary I. 2004. Vascular endothelial growth factor (VEGF)-D and VEGF-A differentially regulate KDR-mediated signaling and biological function in vascular endothelial cells. Journal of Biological Chemistry, 279(34):36148–36157.

Jiang X, Wang Y, Lv H, Liu Y, Zhang M, Li X. 2018. Efficacy of intra-meibomian gland injection of the anti-VEGF agent bevacizumab for the treatment of meibomian gland dysfunction with lid-margin vascularity. Drug Design, Development and Therapy, 12:1269–1279.

Jingjing L, Srinivasan B, Bian X, Downey H, Roque R. 2000. Vascular endothelial growth factor is increased following coronary artery occlusion in the dog heart. Molecular and Cellular Biochemistry, 214(1):23–30.

Kafarnik C, Fritsche J, Reese S. 2008. Corneal innervation in mesocephalic and brachycephalic dogs and cats: Assessment using in vivo confocal microscopy. Veterinary Ophthalmology, 11(6):363–367.

Kallab M, Szegedi S, Hommer N, Stegmann H, Kaya S, Werkmeister R, Schmidl D, Schmetterer L, Garhöfer G. 2020. Topical low dose preservative-free hydrocortisone reduces signs and symptoms in patients with chronic dry eye: A randomized clinical trial. Advances in Therapy, 37(1):329–341.

Kano M, Morishita Y, Iwata C, Iwasaka S, Watabe T, Ouchi Y, Miyazono K, Miyazawa K. 2005. VEGF-A and FGF-2 synergistically promote neoangiogenesis through

enhancement of endogenous PDGF-B-PDGFRβ signaling. Journal of Cell Science, 118(16):3759–3768.

Karar J, Maity A. 2011. PI3K/AKT/mTOR Pathway in Angiogenesis. Frontiers in Molecular Neuroscience, 4:51.

Kasetsuwan N, Chantaralawan K, Reinprayoon U, Uthaithammarat L. 2020. Efficacy of topical bevacizumab 0.05% eye drops in dry eye disease: A double-masked, randomized trial. Public Library of Science ONE, 15(6):1–14.

Kaswan R, Salisbury M. 1990. A new perspective on canine keratoconjunctivitis sicca: Treatment with ophthalmic cyclosporine. Veterinary Clinics of North America - Small Animal Practice, 20(3):583–613.

Kershaw O, Von Oppen T, Glitz F, Deegen E, Ludwig H, Borchers K. 2001. Detection of equine herpesvirus type 2 (EHV-2) in horses with keratoconjunctivitis. Virus Research, 80(1–2):93–99.

Kim S, Ha B, Kim E, Tchah H, Kim T. 2008. The Effect of Topical Bevacizumab on Corneal Neovascularization. Ophthalmology, 115(6):33–38.

Kim T, Chung J, Hong J, Min K, Seo K, Kim E. 2009. Bevacizumab application delays epithelial healing in rabbit cornea. Investigative Ophthalmology and Visual Science, 50(10):4653–4659.

Kim Y, Hawng S, Kim Y, Pyun B, Kim T, Lee S, Gho Y, Kwon Y. 2002. Endostatin blocks VEGF-mediated signaling via direct interaction with KDR/Flk-1. Journal of Biological Chemistry, 277(31):27872–9.

Koenig Y, Bock F, Horn F, Kruse F, Straub K, Cursiefen C. 2009. Short- and long-term safety profile and efficacy of topical bevacizumab (Avastin®) eye drops against corneal neovascularization. Graefe's Archive for Clinical and Experimental Ophthalmology, 247:1375–1382.

Korobelnik J, Do D, Schmidt-Erfurth U, Boyer D, Holz F, Heier J, Midena E, Kaiser P, Terasaki H, Marcus D, et al. 2014. Intravitreal aflibercept for diabetic macular edema. Ophthalmology, 121(11):2247–2254.

Krecny M, Witter K, Tichy A, Nell B. 2018. Tear film examinations and eyelid histology of pugs. Tierärztliche Praxis Ausgabe K: Kleintiere - Heimtiere, 46(6):380–385.

Krizova D, Vokrojova M, Liehneova K, Studeny P. 2014. Treatment of corneal

neovascularization using anti-VEGF bevacizumab. Journal of Ophthalmology, 2014:178132.

Kumar J, Gehra A, Sirohi N. 2016. Role of frequency doubled Nd: Yag laser in treatment of corneal neovascularisation. Journal of Clinical and Diagnostic Research, 10(4):NC01–NC04.

Labelle A, Dresser C, Hamor R, Allender M, Disney J. 2013. Characteristics of, prevalence of, and risk factors for corneal pigmentation (pigmentary keratopathy) in Pugs. Journal of the American Veterinary Medical Association, 243(5):667–74.

Labetoulle M, Leonardi A, Amrane M, Ismail D, Garrigue J, Garhöfer G, de la Maza M, Baudouin C. 2018. Persistence of Efficacy of 0.1% Cyclosporin A Cationic Emulsion in Subjects with Severe Keratitis Due to Dry Eye Disease: A Nonrandomized, Open-label Extension of the SANSIKA Study. Clinical Therapeutics, 40(11):1894–1906.

Lacerda R, Peña Gimenez M, Laguna F, Costa D, Ríos J, Leiva M. 2017. Corneal grafting for the treatment of full-thickness corneal defects in dogs: a review of 50 cases. Veterinary Ophthalmology, 20(3):222–231.

Laguna F, Leiva M, Costa D, Lacerda R, Peña Gimenez T. 2015. Corneal grafting for the treatment of feline corneal sequestrum: a retrospective study of 18 eyes (13 cats). Veterinary Ophthalmology, 18(4):291–296.

Lassaline M, Cranford T, Latimer C, Bellone R. 2015. Limbal squamous cell carcinoma in Haflinger horses. Veterinary Ophthalmology, 18(5):404–408.

Ledbetter E. 2013. Canine herpesvirus-1 ocular diseases of mature dogs. New Zealand Veterinary Journal, 61(4):193–201.

Ledbetter E, Norman M, Starr J. 2016. In vivo confocal microscopy for the detection of canine fungal keratitis and monitoring of therapeutic response. Veterinary Ophthalmology, 19(3):220–229.

Lee S, Jilan S, Nikolova G, Carpizo D, Iruela-Arispe L. 2005. Processing of VEGF-A by matrix metalloproteinases regulates bioavailability and vascular patterning in tumors. Journal of Cell Biology, 169(4):681–691.

Leis M, Salpeter M, Bauer B, Godson D, Grahn B. 2017. Primary bilateral corneal nerve sheath neoplasm in a dog. Veterinary Ophthalmology, 20(4):365–371.

Lemp M, Baudouin C, Baum J, Dogru M, Foulks G, Kinoshita S, Laibson P, McCulley J,

Murube J, Pflugfelder S, et al. 2007. The definition and classification of dry eye disease: Report of the definition and classification subcommittee of the international Dry Eye WorkShop (2007). The Ocular Surface, 5(2):75–92.

Li C, Liu B, Dai Z, Tao Y. 2011. Knockdown of VEGF receptor-1 (VEGFR-1) impairs macrophage infiltration, angiogenesis and growth of clear cell renal cell carcinoma (CRCC). Cancer Biology and Therapy, 12(10):872–880.

Li Y, Bi Z, Yan B, Wan Y. 2006. UVB radiation induces expression of HIF-1α and VEGF through the EGFR/PI3K/DEC1 pathway. International Journal of Molecular Medicine, 18(4):713–719.

Li Z, Yuan Z, Mu G, Hu M, Cao LJ, Zhang Y, Liu L, Ge M. 2015. Inhibitory effect of polysulfated heparin endostatin on alkali burn induced corneal neovascularization in rabbits. International Journal of Ophthalmology, 8(2):234–238.

Liang L, Yue Z, Du W, Li Y, Tao H, Wang D, Wang R, Huang Z, He N, Xie X, et al. 2017. Molecular imaging of inducible VEGF expression and tumor progression in a breast cancer model. Cellular Physiology and Biochemistry, 42(1):407–415.

De Linde Henriksen M, Haubro Andersen P, Mietelka K, Farina L, Thomsen P, Plummer C, Mangan B, Heegaard S, Coleman J, Toft N, et al. 2014. Equine deep stromal abscesses (51 cases - 2004-2009) - Part 2: The histopathology and immunohistochemical aspect with attention to the histopathologic diagnosis, vascular response, and infectious agents. Veterinary Ophthalmology, 17 Suppl 1:14–22.

Lobov I, Renard R, Papadopoulos N, Gale N, Thurston G, Yancopoulos G, Wiegand S. 2007. Delta-like ligand 4 (DII4) is induced by VEGF as a negative regulator of angiogenic sprouting. Proceedings of the National Academy of Sciences of the United States of America, 104(9):3219–3224.

Luo L, Li D, Corrales R, Pflugfelder S. 2005. Hyperosmolar saline is a proinflammatory stress on the mouse ocular surface. Eye and Contact Lens, 31(5):186–193.

Lutty G, McLeod D, Bhutto I, Wiegand S. 2011. Effect of VEGF trap on normal retinal vascular development and oxygen-induced retinopathy in the dog. Investigative Ophthalmology and Visual Science, 52(7):4039–4047.

MacDonald D, Martin J, Muthusamy K, Luo J, Pyles E, Rafique A, Huang T, Potocky T, Liu Y, Cao J, et al. 2016. Aflibercept exhibits VEGF binding stoichiometry distinct from

bevacizumab and does not support formation of immune-like complexes. Angiogenesis, 19(3):389–406.

Maes C, Stockmans I, Moermans K, Van Looveren R, Smets N, Carmeliet P, Bouillon R, Carmeliet G. 2004. Soluble VEGF isoforms are essential for establishing epiphyseal vascularization and regulating chondrocyte development and survival. Journal of Clinical Investigation, 113(2):188–99.

Mastyugin V, Mosaed S, Bonazzi A, Dunn M, Schwartzman M. 2001. Corneal epithelial VEGF and cytochrome P450 4B1 expression in a rabbit model of closed eye contact lens wear. Current Eye Research, 23(1):1–10.

Matheis F, Walser-Reinhardt L, Spiess B. 2012. Canine neurogenic Keratoconjunctivitis sicca: 11 cases (2006-2010). Veterinary Ophthalmology, 15(4):288–290.

Matsuba M, Hutcheon A, Zieske J. 2011. Localization of thrombospondin-1 and myofibroblasts during corneal wound repair. Experimental Eye Research, 93(4):534–540.

Matsui T, Nishino Y, Maeda S, Yamagishi S. 2012. PEDF-derived peptide inhibits corneal angiogenesis by suppressing VEGF expression. Microvascular Research, 84(1):105–108.

McMullen R, Gilger B, Michau T. 2015. Modified lamellar keratoplasties for the treatment of deep stromal abscesses in horses. Veterinary Ophthalmology, 18(5):393–403.

McNatt L, Weimer L, Yanni J, Clark A. 1999. Angiostatic activity of steroids in the chick embryo CAM and rabbit cornea models of neovascularization. Journal of Ocular Pharmacology and Therapeutics, 15(5):413–423.

Medina E, Molinari G, Rohde M, Haase B, Chhatwal G, Guzmán C. 1999. Fc-mediated nonspecific binding between fibronectin-binding protein I of Streptococcus pyogenes and human immunoglobulins. Journal of Immunology, 163(6):3396–3402.

Mimura T, Han K, Onguchi T, Chang J, Kim T, Kojima T, Zhou Z, Azar D. 2009. MT1-MMP-Mediated cleavage of decorin in corneal angiogenesis. Journal of Vascular Research, 46(6):541–550.

Moisseiev E, Waisbourd M, Ben-Artsi E, Levinger E, Barak A, Daniels T, Csaky K, Loewenstein A, Barequet I. 2014. Pharmacokinetics of bevacizumab after topical and intravitreal administration in human eyes. Graefe's Archive for Clinical and Experimental Ophthalmology, 252(2):331–337.

Moore D, McLellan G, Dubielzig R. 2003. A study of the morphology of canine eyes enucleated or eviscerated due to complications following phacoemulsification. Veterinary Ophthalmology, 6(3):219–226.

Morgan R, Abrams K. 1991. Topical administration of cyclosporine for treatment of keratoconjunctvitis sicca in dogs. Journal of American Veterinary Medical Association, 199(8):1043–6.

Muether P, Dell S, Kociok N, Zahn G, Stragies R, Vossmeyer D, Joussen A. 2007. The role of integrin α5β1 in the regulation of corneal neovascularization. Experimental Eye Research, 85(3):356–365.

Müller L, Marfurt C, Kruse F, Tervo T. 2003. Corneal nerves: Structure, contents and function. Experimental Eye Research, 76(5):521–542.

Muller Y, Chen Y, Christinger H, Li B, Cunningham B, Lowman H, De Vos A. 1998. VEGF and the Fab fragment of a humanized neutralizing antibody: Crystal structure of the complex at 2.4 Å resolution and mutational analysis of the interface. Structure, 6(9):1153–1167.

Mun E, Morrison P, Williams A, Khutoryanskiy V. 2014. On the barrier properties of the cornea: A microscopy study of the penetration of fluorescently labeled nanoparticles, polymers, and sodium fluorescein. Molecular Pharmaceutics, 11(10):3556–3564.

Murakami M, Nguyen L, Hatanaka K, Schachterle W, Chen P, Zhuang Z, Black B, Simons M. 2011. FGF-dependent regulation of VEGF receptor 2 expression in mice. The Journal of Clinical Investigation, 121(7):2668–2678.

Murphy C, Marfurt C, McDermott A, Bentley E, Abrams G, Reid T, Campbell S. 2001. Spontaneous chronic corneal epithelial defects (SCCED) in dogs: Clinical features, innervation, and effect of topical SP, with or without IGF-1. Investigative Ophthalmology and Visual Science, 42(10):2252–2261.

Murtagh P, Comer R, Fahy G. 2018. Corneal perforation in undiagnosed Sjögren's syndrome following topical NSAID and steroid drops post routine cataract extraction. British Medical Journal Case Reports, 2018:bcr2018225428.

Nakamura M, Nishida T, Ofuji K, Reid T, Mannis M, Murphy C. 1997. Synergistic effect of substance P with epidermal growth factor on epithelial migration in rabbit cornea. Experimental Eye Research, 65(3):321–329.

Narimatsu A, Hattori T, Koike N, Tajima K, Nakagawa H, Yamakawa N, Usui Y, Kumakura S, Matsumoto T, Goto H. 2019. Corneal lymphangiogenesis ameliorates corneal inflammation and edema in late stage of bacterial keratitis. Scientific Reports, 9:2984.

Nasisse M. 1995. Diagnosis and treatment of conjunctival and corneal diseases. Veterinary Quarterly, 17 Suppl 1:42–43.

Nasisse M, Glover T, Moore C, Weigler B. 1998. Detection of feline herpesvirus 1 DNA in corneas of cats with eosinophilic keratitis or corneal sequestration. American Journal of Veterinary Research, 59(7):856–858.

Nell B, Walde I, Billich A, Vit P, Meingassner J. 2005. The effect of topical pimecrolimus on keratoconjunctivitis sicca and chronic superficial keratitis in dogs: Results from an exploratory study. Veterinary Ophthalmology, 8(1):39–46.

Newkirk KM, Rohrbach BW. 2009. A retrospective study of eyelid tumors from 43 cats. Veterinary Pathology, 46(5):916–927.

Nomoto H, Shiraga F, Kuno N, Kimura E, Fujii S, Shinomiya K, Nugent AK, Hirooka K, Baba T. 2009. Pharmacokinetics of bevacizumab after topical, subconjunctival, and intravitreal administration in rabbits. Investigative Ophthalmology and Visual Science, 50(10):4807–4813.

Nork T, Dubielzig R, Christian B, Miller P, Miller J, Cao J, Zimmer E, Wiegand S. 2011. Prevention of experimental choroidal neovascularization and resolution of active lesions by VEGF trap in nonhuman primates. Archives of Ophthalmology, 129(8):1042–1052.

Ofri R, Lambrou G, Allgoewer I, Graenitz U, Pena T, Spiess B, Latour E. 2009. Clinical evaluation of pimecrolimus eye drops for treatment of canine keratoconjunctivitis sicca: A comparison with cyclosporine A. The Veterinary Journal, 179(1):70–77.

Ogawa S, Oku A, Sawano A, Yamaguchi S, Yazaki Y, Shibuya M. 1998. A novel type of Vascular Endothelial Growth Factor, VEGF-E (NZ-7 VEGF), preferentially utilizes KDR/Flk-1 receptor and carries a potent mitotic activity without heparin-binding domain. Journal of Biological Chemistry, 273(47):31273–31282.

Olmos L, Sayed M., Moraczewski A, Gedde S, Rosenfeld P, Shi W, Feuer W, Lee R. 2016. Long-term outcomes of neovascular glaucoma treated with and without intravitreal bevacizumab. Eye, 30(3):463–472.

Olofsson B, Korpelainen E, Pepper M, Mandriota S, Aase K, Kumar V, Gunji Y, Jeltsch M,

Shibuya M, Alitalo K, et al. 1998. Vascular endothelial growth factor B (VEGF-B) binds to VEGF receptor-1 and regulates plasminogen activator activity in endothelial cells. Proceedings of the National Academy of Sciences of the United States of America, 95(20):11709–11714.

Ozdemir O, Altintas O, Altintas L, Ozkan B, Akdag C, Yüksel N. 2014. Comparison of the effects of subconjunctival and topical anti-VEGF therapy (bevacizumab) on experimental corneal neovascularization. Arquivos Brasileiros de Oftalmologia, 77(4):209–213.

De Paiva C, Villarreal A, Corrales R, Rahman H, Chang V, Farley W, Stern M, Niederkorn J, Li D, Pflugfelder S. 2007. Dry eye-induced conjunctival epithelial squamous metaplasia is modulated by interferon-γ. Investigative Ophthalmology and Visual Science, 48(6):2553–2560.

Pakneshan P, Birsner A, Adini I, Becker C, D'Amato R. 2008. Differential suppression of vascular permeability and corneal angiogenesis by nonsteroidal anti-inflammatory drugs. Investigative Ophthalmology and Visual Science, 49(9):3909–3913.

Papadopoulos N, Martin J, Ruan Q, Rafique A, Rosconi M, Shi E, Pyles E, Yancopoulos G, Stahl N, Wiegand S. 2012. Binding and neutralization of vascular endothelial growth factor (VEGF) and related ligands by VEGF Trap, ranibizumab and bevacizumab. Angiogenesis, 15(2):171–185.

Park J, Keller G, Ferrara N. 1993. The vascular endothelial growth factor (VEGF) isoforms: Differential deposition into the subepithelial extracellular matrix and bioactivity of extracellular matrix-bound VEGF. Molecular Biology of the Cell, 4(12):1317–1326.

Park J, Chen H, Winer J, Houck K, Ferrara N. 1994. Placenta growth factor. Potentiation of vascular endothelial growth factor bioactivity, in vitro and in vivo, and high affinity binding to Flt-1 but not to Flk-1/KDR. Journal of Biological Chemistry, 269(41):25646–25654.

Park J, Kim B, Kim J, Hwang J. 2018. Matrix metalloproteinase 9 point-of-care immunoassay result predicts response to topical cyclosporine treatment in dry eye disease. Translational Vision Science and Technology, 7(5):31.

Peterson T, Kirkpatrick N, Huang Y, Farrar C, Marijt K, Kloepper J, Datta M, Amoozgar Z, Seano G, Jung K, et al. 2016. Dual inhibition of Ang-2 and VEGF receptors normalizes

tumor vasculature and prolongs survival in glioblastoma by altering macrophages. Proceedings of the National Academy of Sciences of the United States of America, 113(16):4470–4475.

Pfeiffer R, Wilcock B, Yin H. 1990. The pathogenesis and significance of pre-iridal fibrovascular membrane in domestic animals. Veterinary Pathology, 27:41–45.

Pieh C, Agostini H, Buschbeck C, Krueger M, Schulte-Moenting J, Zirrgiebel U, Drevs J, Lagrèze W. 2008. VEGF-A, VEGFR-1, VEGFR-2, and Tie2 levels in plasma of premature infants. Relationship to retinopathy of prematurity. British Journal of Ophthalmology, 92(5):689–93.

Pillai C, Dua H, Hossain P. 2000. Fine needle diathermy occlusion of corneal vessels. Investigative Ophthalmology and Visual Science, 41(8):2148–2153.

Plummer C. 2015. Addressing Brachycephalic Ocular Syndrome in the Dog. Today's Veterinary Practice, March/April:20–25.

Presta L, Chen H, O'Connor S, Chisholm V, Meng Y, Krummen L, Winkler M, Ferrara N. 1997. Humanization of an anti-vascular endothelial growth factor monoclonal antibody for the therapy of solid tumors and other disorders. Cancer Research, 57(20):4593–4599.

Queiroga F, Pires I, Parente M, Gregório H, Lopes C. 2011. COX-2 over-expression correlates with VEGF and tumour angiogenesis in canine mammary cancer. The Veterinary Journal, 189(1):77–82.

Rajashekhar G, Shivanna M, Kompella U, Wang Y, Srinivas S. 2014. Role of MMP-9 in the breakdown of barrier integrity of the corneal endothelium in response to TNF-α. Experimental Eye Research, 122:77–85.

Rebuzzi L, Willmann M, Sonneck K, Gleixner K, Florian S, Kondo R, Mayerhofer M, Vales A, Gruze A, Pickl W, et al. 2007. Detection of vascular endothelial growth factor (VEGF) and VEGF receptors Flt-1 and KDR in canine mastocytoma cells. Veterinary Immunology and Immunopathology, 115(3–4):320–333.

Renaud J, Bédard E. 2013. Depression in the elderly with visual impairment and its association with quality of life. Clinical Interventions in Aging, 8:931–943.

Rigas B, Huang W, Honkanen R. 2020. NSAID-induced corneal melt: Clinical importance, pathogenesis, and risk mitigation. Survey of Ophthalmology, 65(1):1–11.

Robin J, Regis-Pacheco L, Kash R, Schanzlin D. 1985. The Histopathology of Corneal

Neovascularization: Inhibitor Effects. Archives of Ophthalmology, 103(2):284–287.

Roche D, Buenavista M, Tetchner S, McGuffin L. 2011. The IntFOLD server: An integrated web resource for protein fold recognition, 3D model quality assessment, intrinsic disorder prediction, domain prediction and ligand binding site prediction. Nucleic Acids Research, 39 Suppl 2:171–176.

Rodrigues E, Farah M, Maia M, Penha F, Regatieri C, Melo G, Pinheiro M, Zanetti C. 2009. Therapeutic monoclonal antibodies in ophthalmology. Progress in Retinal and Eye Research, 28(2):117–144.

Ruhrberg C, Gerhardt H, Golding M, Watson R, Ioannidou S, Fujisawa H, Betsholtz C, Shima D. 2002. Spatially restricted patterning cues provided by heparin-binding VEGF-A control blood vessel branching morphogenesis. Genes and Development, 16(20):2684–2698.

Sahin A, Kiratli H, Tezel G, Soylemezoglu F, Bilgic S. 2007. Expression of vascular endothelial growth factor A, matrix metalloproteinase 9 and extravascular matrix patterns in iris and ciliary body melanomas. Ophthalmic Research, 39(1):40–44.

Sahni J, Dugel P, Patel S, Chittum M, Berger B, Del Valle Rubido M, Sadikhov S, Szczesny P, Schwab D, Nogoceke E, et al. 2020. Safety and efficacy of different doses and regimens of faricimab vs ranibizumab in neovascular age-related macular degeneration: The AVENUE phase 2 randomized clinical trial. Journal of the American Medical Association Ophthalmology, 138(9):955–963.

Sanchez R, Innocent G, Mould J, Billson F. 2007. Canine keratoconjunctivitis sicca: Disease trends in a review of 229 cases. Journal of Small Animal Practice, 48(4):211–217.

Sandberg C, Herring I, Huckle W, LeRoith T, Pickett J, Rossmeisl J. 2012. Aqueous humor vascular endothelial growth factor in dogs: association with intraocular disease and the development of pre-iridal fibrovascular membrane. Veterinary Ophthalmology, 15 Suppl 1:21–30.

Scheidegger P, Weiglhofer W, Suarez S, Kaser-Hotz B, Steiner R, Ballmer-Hofer K, Jaussi R. 1999. Vascular endothelial growth factor (VEGF) and its receptors in tumor-bearing dogs. Biological Chemistry, 380(12):1449–1454.

Schmidt-Erfurth U, Kaiser P, Korobelnik J, Brown D, Chong V, Nguyen Q, Ho A, Ogura Y, Simader C, Jaffe G, et al. 2014. Intravitreal aflibercept injection for neovascular age-

related macular degeneration: ninety-six-week results of the VIEW studies. Ophthalmology, 121(1):193–201.

Scott A, Bressler N, Ffolkes S, Wittenborn J, Jorkasky J. 2016. Public attitudes about eye and vision health. Journal of the Americam Medical Association Ophthalmology, 134(10):1111–1118.

Sebbag L, Kirner N, Wulf L, Mochel J. 2020. Tear film pharmacokinetics and systemic absorption following topical administration of 1% prednisolone acetate ophthalmic suspension in dogs. Frontiers in Veterinary Science, 7:571350.

Sella R, Gal-Or O, Livny E, Dachbash M, Nisgav Y, Weinberger D, Livnat T, Bahar I. 2016. Efficacy of topical aflibercept versus topical bevacizumab for the prevention of corneal neovascularization in a rat model. Experimental Eye Research, 146:224–232.

Sella R, Ben-Ishai M, Livny E, Nahum Y, Bahar I. 2021. Subconjunctival aflibercept for the treatment of formed corneal neovascularization. Eye & Contact Lens, 47(4):180–184.

Senger D, Perruzzi C, Streit M, Koteliansky V, De Fougerolles A, Detmar M. 2002. The α1β1 and α2β1 integrins provide critical support for vascular endothelial growth factor signaling, endothelial cell migration, and tumor angiogenesis. American Journal of Pathology, 160(1):195–204.

Shalaby F, Janet R, Yamaguchi T, Gertsenstein M, Wu X, Breitman M, Schuh A. 1995. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. Nature, 376(6535):62–66.

Sharma MR, Tuszynski GP, Sharma MC. 2004. Angiostatin-induced inhibition of endothelial cell proliferation/apoptosis is associated with the down-regulation of cell cycle regulatory protein cdk5. Journal of Cellular Biochemistry, 91(2):398–409.

Shi W, Liu J, Li M, Gao H, Wang T. 2010. Expression of MMP, HPSE, and FAP in stroma promoted corneal neovascularization induced by different etiological factors. Current Eye Research, 35(11):967–977.

Shima C, Sakaguchi H, Gomi F, Kamei M, Ikuno Y, Oshima Y, Sawa M, Tsujikawa M, Kusaka S, Tano Y. 2008. Complications in patients after intravitreal injection of bevacizumab. Acta Ophthalmologica, 86(4):372–376.

Sivak J, West-Mays J, Yee A, Williams T, Fini M. 2004. Transcription factors Pax6 and AP-2α interact to coordinate corneal epithelial repair by controlling expression of matrix

metalloproteinase gelatinase B. Molecular and Cellular Biology, 24(1):245–257.

Slatter D, Lavach J, Severin G, Young S. 1977. Überreiter's Syndrome (chronic superficial keratitis) in dogs in the Rocky Mountain area—A study of 463 cases. Journal of Small Animal Practice, 18(12):757–772.

Soker S, Takashima S, Miao H, Neufeld G, Klagsbrun M. 1998. Neuropilin-1 is expressed by endothelial and tumor cells as an isoform- specific receptor for vascular endothelial growth factor. Cell, 92(6):735–745.

Somerfield J, Hill-Cawthorne G, Lin A, Zandi M, McCarthy C, Jones J, Willcox M, Shaw D, Thompson S, Compston A, et al. 2010. A novel strategy to reduce the immunogenicity of biological therapies. The Journal of Immunology, 185(1):763–768.

Spiess A, Sapienza J, Mayordomo A. 2009. Treatment of proliferative feline eosinophilic keratitis with topical 1.5% cyclosporine: 35 cases. Veterinary Ophthalmology, 12(2):132–137.

Spiteri N, Romano V, Zheng Y, Yadav S, Dwivedi R, Chen J, Ahmad S, Willoughby C, Kaye S. 2015. Corneal angiography for guiding and evaluating fine-needle diathermy treatment of corneal neovascularization. Ophthalmology, 122(6):1079–1084.

Stevenson W, Cheng S, Dastjerdi M, Ferrari G, Dana R. 2012. Corneal neovascularization and the utility of topical VEGF inhibition: Ranibizumab (Lucentis) Vs bevacizumab (Avastin). The Ocular Surface, 10(2):67–83.

Stewart J, Schultz D, Lee O, Trinidad M. 2009. Collagen cross-links reduce corneal permeability. Investigative Ophthalmology and Visual Science, 50(4):1606–1612.

Stitt A, Simpson D, Boocock C, Gardiner T, Murphy G, Archer D. 1998. Expression of vascular endothelial growth factor (VEGF) and its receptors is regulated in eyes with intra-ocular tumours. Journal of Pathology, 186(3):306–312.

Streilein J. 2003. Ocular immune privilege: Therapeutic opportunities from an experiment of nature. Nature Reviews Immunology, 3(11):879–889.

Streilein J, Ksander B, Taylor A. 1997. Immune deviation in relation to ocular immune privilege. The Journal of Immunology, 158(8):3557–60.

Sun C, Cheng C, Chien C, Pang J, Ku W, Chen P, Yang C. 2005. Role of matrix metalloproteinase-9 in ex vivo expansion of human limbal epithelial cells cultured on human amniotic membrane. Investigative Ophthalmology and Visual Science,

46(3):808–815.

Sun M, Chen S, Adams S, Florer J, Liu H, Kao W, Wenstrup R, Birk D. 2011. Collagen V is a dominant regulator of collagen fibrillogenesis: Dysfunctional regulation of structure and function in a corneal-stroma-specific Col5a1-null mouse model. Journal of Cell Science, 124(23):4096–4105.

Suter A, Voelter K, Hartnack S, Spiess B, Pot S. 2018. Septic keratitis in dogs, cats, and horses in Switzerland: associated bacteria and antibiotic susceptibility. Veterinary Ophthalmology, 21(1):66–75.

Suto K, Yamazaki Y, Morita T, Mizuno H. 2005. Crystal structures of novel Vascular Endothelial Growth Factors (VEGF) from snake venoms: Insight into selective VEGF binding to kinase insert domain-containing receptor but not to fms-like tyrosine kinase-1. Journal of Biological Chemistry, 280(3):2126–2131.

Tietz J, Spohn G, Schmid G, Konrad J, Jampen S, Maurer P, Schmidt A, Escher D. 2015. Affinity and potency of RTH258 (ESBA1008), a novel inhibitor of Vascular Endothelial Growth Factor A for the treatment of retinal disorders [ARVO Annual Meeting Abstract]. Investigative Ophthalmology and Visual Science, 56(7):1501.

Tischer E, Mitchell R, Hartman T, Silva M, Gospodarowicz D, Fiddes J, Abraham J. 1991. The human gene for vascular endothelial growth factor: Multiple protein forms are encoded through alternative exon splicing. Journal of Biological Chemistry, 266(18):11947–11954.

Tolar E, Hendrix D, Rohrbach B, Plummer C, Brooks D, Gelatt K. 2006. Evaluation of clinical characteristics and bacterial isolates in dogs with bacterial keratitis: 97 Cases (1993-2003). Journal of the American Veterinary Medical Association, 228(1):80–85.

Van Meer P, Kooijman M, Brinks V, Gispen-De Wied C, Silva-Lima B, Moors E, Schellekens H. 2013. Immunogenicity of mAbs in non-human primates during nonclinical safety assessment. Monoclonal Antibodies, 5(5):810–816.

Villar T, Capistrano E, Galera G, Fleming K, Roady P, Martins B. 2020. Evaluation and comparison of inhibitory effects of topical tacrolimus, cyclosporine and dexamethasone on corneal neovascularization and wound healing in an experimental rat study.[ACVO Virtual Meeting Abstract, 2020].

Vlčková M, Kalman F, Schwarz M. 2008. Pharmaceutical applications of isoelectric focusing

on microchip with imaged UV detection. Journal of Chromatography A, 1181(1–2):145–152.

Waldner M, Wirtz S, Jefremow A, Warntjen M, Neufert C, Atreya R, Becker C, Weigmann B, Vieth M, Rose-John S, et al. 2010. VEGF receptor signaling links inflammation and tumorigenesis in colitis-associated cancer. The Journal of Experimental Medicine, 207(13):2855–2868.

Wang F, Gao N, Yin J, Yu F. 2012. Reduced innervation and delayed re-innervation after epithelial wounding in type 2 diabetic Goto-Kakizaki rats. American Journal of Pathology, 181(6):2058–2066.

Weigt A, Herring I, Marfurt C, Pickett J, Duncan R, Ward D. 2002. Effects of cyclophotocoagulation with a neodymium:yttrium-aluminum-garnet laser on corneal sensitivity, intraocular pressure, aqueous tear production, and corneal nerve morphology in eyes of dogs. American Journal of Veterinary Research, 63(6):906–915.

Welch P, Gabal M, Betts D, Whelan N, Studer M. 2000. In vitro analysis of antiangiogenic activity of fungi isolated from clinical cases of equine keratomycosis. Veterinary Ophthalmology, 3(2–3):145–151.

Werkmeister R, Sapeta S, Schmidl D, Garhöfer G, Schmidinger G, Aranha dos Santos V, Aschinger G, Baumgartner I, Pircher N, Schwarzhans F, et al. 2017. Ultrahigh-resolution OCT imaging of the human cornea. Biomedical Optics Express, 8(2):1221.

Whitcher J, Srinivasan M, Upadhyay M. 2001. Corneal blindness: A global perspective. Bulletin of the World Health Organization, 79(3):214–221.

Williams D. 2008. Immunopathogenesis of keratoconjunctivitis sicca in the dog. Veterinary Clinics of North America - Small Animal Practice, 38(2):251–268.

Wolfesberger B, Guija de Arespacohaga A, Willmann M, Gerner W, Miller I, Schwendenwein I, Kleiter M, Egerbacher M, Thalhammer J, Muellauer L, et al. 2007. Expression of Vascular Endothelial Growth Factor and its receptors in canine lymphoma. Journal of Comparative Pathology, 137(1):30–40.

Wu D, Smith S, Stine J, Michau T, Miller T, Pederson S, Freeman K. 2018. Treatment of spontaneous chronic corneal epithelial defects (SCCEDs) with diamond burr debridement vs combination diamond burr debridement and superficial grid keratotomy. Veterinary Ophthalmology, 21(6):622–631.

Wu L, Martínez-Castellanos M, Quiroz-Mercado H, Arevalo J, Berrocal M, Farah M, Maia M, Roca J, Rodriguez F. 2008. Twelve-month safety of intravitreal injections of bevacizumab (Avastin®): Results of the pan-american collaborative retina study group (PACORES). Graefe's Archive for Clinical and Experimental Ophthalmology, 246(1):81–87.

Wuest T, Carr D. 2010. VEGF-A expression by HSV-1-infected cells drives corneal lymphangiogenesis. Journal of Experimental Medicine, 207(1):101–115.

Wuest T, Zheng M, Efstathiou S, Halford W, Carr D. 2011. The herpes simplex virus-1 transactivator infected cell protein-4 drives VEGF-A dependent neovascularization. Public Library of Science Pathogens, 7(10):e1002278.

Yamagami S, Dana M, Tsuru T. 2002. Draining lymph nodes play an essential role in alloimmunity generated in response to high-risk corneal transplantation. Cornea, 21(4):405–409.

Yamazaki Y, Tokunaga Y, Takani K, Morita T. 2005. Identification of the heparin-binding region of snake venom vascular endothelial growth factor (VEGF-F) and its blocking of VEGF-A 165. Biochemistry, 44(24):8858–8864.

Yoeruek E, Ziemssen F, Henke-Fahle S, Tatar O, Grisanti S, Bartz-Schmidt K, Szurman P. 2008. Safety, penetration and efficacy of topically applied bevacizumab: Evaluation of eyedrops in corneal neovascularization after chemical burn. Acta Ophthalmologica, 86:322–328.

Yu C, Zhang M, Matis K, Kim C, Rosenblatt M. 2008a. Vascular Endothelial Growth Factor mediates corneal nerve repair. Investigative Ophthalmology and Visual Science, 49(9):3870–3878.

Yu L, Wu X, Cheng Z, Lee C, LeCouter J, Campa C, Fuh G, Lowman H, Ferrara N. 2008b. Interaction between bevacizumab and murine VEGF-A: A reassessment. Investigative Ophthalmology and Visual Science, 49(2):522–527.

Yun H, Yee M, Lathrop K, Kinchington P, Hendricks R, St Leger A. 2020. Production of the cytokine VEGF-A by CD4+ T and myeloid cells disrupts the corneal nerve landscape and promotes herpes stromal keratitis. Immunity, 53(5):1050–1062.

Zakaria N, Van Grasdorff S, Wouters K, Rozema J, Koppen C, Lion E, Cools N, Berneman Z, Tassignon M. 2012. Human tears reveal insights into corneal neovascularization. Public

Library of Science ONE, 7(5):3–10.

Zarfoss M, Breaux C, Whiteley H, Hamor R, Flaws J, Labelle P, Dubielzig R. 2010. Canine pre-iridal fibrovascular membranes: morphologic and immunohistochemical investigations. Veterinary Ophthalmology, 13(1):4–13.

Zehetner C, Kirchmair R, Huber S, Kralinger M, Kieselbach G. 2013. Plasma levels of vascular endothelial growth factor before and after intravitreal injection of bevacizumab , ranibizumab and pegaptanib in patients with age-related macular degeneration , and in patients with diabetic macular oedema. British Journal of Ophthalmology, 97:454–459.

Zehetner C, Kralinger M, Modi Y, Waltl I, Ulmer H, Kirchmair R, Bechrakis N, Kieselbach G. 2015. Systemic levels of vascular endothelial growth factor before and after intravitreal injection of aflibercept or ranibizumab in patients with age-related macular degeneration: A randomised, prospective trial. Acta Ophthalmologica, 93(2):e154–e159.

Zhang H, Li C, Baciu P. 2002. Expression of integrins and MMPs during alkaline-burn - Induced corneal angiogenesis. Investigative Ophthalmology and Visual Science, 43(4):955–962.

Zhang Z, Coomans C, David G. 2001. Membrane heparan sulfate proteoglycan-supported FGF2-FGFR1 signaling: Evidence in support of the „cooperative end structures“ model. Journal of Biological Chemistry, 276(45):41921–41929.