Received: 22 March 2021 | Revised: 11 June 2021 | Accepted: 23 August 2021

DOI: 10.1111/vop.12931

WILEY

CASE REPORT

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

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Abstract

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

Animals 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 treatment response was variable. Some cases showed a marked reduction in vascularized area and edema, while 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 28 days. A thinning of vessels, consolidation of areal bleedings into fine vascular networks, decrease in distal vessel branching, and a change from blurry vascularized beds into demarcated thin vessels were observed. One dog developed a SCCED 6 months after the last bevacizumab administration. Two dogs died 4 and 4.5 months after the last bevacizumab administration, aged 16 and 12 years, respectively. In all events, a causal relationship is unlikely but cannot be ruled out with certainty.

Conclusions: Our findings suggest that topical 0.25% bevacizumab may be an effective treatment option for corneal vascularization in dogs. Further long-term placebo-controlled studies with larger patient cohorts are recommended to provide scientific evidence of efficacy and to investigate dosage, safety, possible use as a single treatment, and routes of administration.

KEYWORDS

bevacizumab, corneal vascularization, dog, keratitis, Vascular endothelial growth factors, VEGF

1 | 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 pigmentary keratitis 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).[8-10] Vascular endothelial growth factor A is a promotor of both physiological and pathological angiogenesis and is increased in inflamed and vascularized corneas, suggesting a causal role in corneal vascularization.[4,11] 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 have been used for the treatment of a variety of ocular disorders in humans.[12-14] One of these drugs, bevacizumab, is a humanized murine anti-VEGF monoclonal antibody that binds all isoforms of human VEGF-A.[15,16] It has also been shown to bind to canine VEGF in-vitro.[17] The anti-angiogenic effect of topical bevacizumab has already been demonstrated in-vivo in various animal models and in human patients with corneal vascularization.[9,10,18,19]

This case series describes the use of topical 0.25% bevacizumab administered twice a day for 28 days in dogs with corneal vascularization.

2 | MATERIALS AND METHODS

2.1 | 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 at 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.

2.2 | 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 on the same day of the study treatment initiation.[20] The pharmacist (MW) prepared single-dose containers for each dog, filled with 0.5 ml of 0.25% bevacizumab. The labels were inscribed based on GMP standards[21] and a detailed written instruction leaflet was enclosed.

2.3 | 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 lesser than 15 mm/min, treatment with VEGF-influencing drugs or agents comprising human proteins 28 days before the study start, known coagulation disorders, or clinical manifestations of systemic diseases.

2.4 | Treatment regime and study design

The dogs were treated and examined on an outpatient basis. The eye drops were administered by the owners after a detailed instruction by the first author (LM). Study treatment was initiated at the enrollment visit (visit 1). The study eye was treated with one drop of 0.25% bevacizumab twice a day for 28 days. Re-examinations were scheduled immediately after the 28-day treatment period (visit 2) and 6–7 months after treatment initiation (visit 3). 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 tolerability assessment, and photography of the cornea.

2.5 | 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 examination included slit-lamp biomicroscopy (Kowa SL-15; Kowa,

Tokyo, Japan), indirect ophthalmoscopy (Keeler Vantage; Keeler Instruments Inc, Broomall, USA), 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).

2.6 | Photography

Photographs were taken with a digital system camera (OM-D E-M10 Mark III, Olympus, Shinjuku, Tokyo, Japan) in combination with a magnification macro lens (M.ZUIKO DIGITAL ED 60mm F2.8 Macro /120mm, Olympus, Shinjuku, Tokyo, Japan) and a pincer-shaped macro flash light (STF-8 Macro Flash, Olympus, Shinjuku, Tokyo, Japan) in the same examination room by the same two investigators (LM and MB). The room was darkened by a black shutter and only one ceiling light was turned on. Light intensity was measured with a luxmeter to control consistent lighting conditions (Light Meter Model Nr. 4332004118, Urceri, USA). ISO was set to 200 and the camera exposure was controlled manually. The camera aperture was adjusted to eight or ten, the shutter speed to 1/200 or 1/160 s, and 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. 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., San Jose, USA). Files were stored LZW-compressed (lossless). 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 blood vessels in the target file; the parameters exposure, contrast, 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 blood vessel image.

2.7 | Tolerability and pain assessment

Any reported adverse event by the owners and any abnormal ocular or general finding observed during the study visits were recorded. Pain assessment was performed by the same investigator (LM) using a prespecified pain scale (Table S1).[22,23] Additionally, the owners were asked to score the sensation of pain according to a predefined pain scale (Table S2).

2.8 | Efficacy assessment and quantification of corneal vascularization

A modified scoring system was used to quantify macroscopic findings at each visit during slit-lamp biomicroscopy.[24] The following variables were scored for each eye: Corneal edema, vascularization, and pigmentation: $0 \leq 25\%$, $1 = 26\%–50\%$, $2 = 51\%–75\%$, $3 \geq 76\%$ (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 blood vessel incisions located on a given circle within the corneal surface were counted at visit 1, visit 2, and visit 3 (Figure 1). The entire corneal surface was divided into four concentric circles to count the number of blood vessels on each circle (Figure 1B). Only blood-filled vessels were included in the analysis. Each measurement was performed three times, and the mean value was used for analysis. Analyses were performed using an imaging software (Fiji; open-source software: https://imagej.net/Fiji) and Microsoft Excel (Microsoft Corporation, Mircosoft Excel 2016, Redmond, USA).

2.9 | Data analysis

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

3 | RESULTS

3.1 | Animals

Twenty eyes of fifteen dogs met the eligibility criteria and were included in the study. In two cases, visit 2 was delayed because of personal reasons of the owners. Two dogs died and one eye was enucleated before the final long-term examination, resulting in the loss of four eyes to follow-up. Signalment and ocular diagnosis is listed in Table 1. Detailed patient history and pretreatment information are outlined in (Tables S3 and S4).

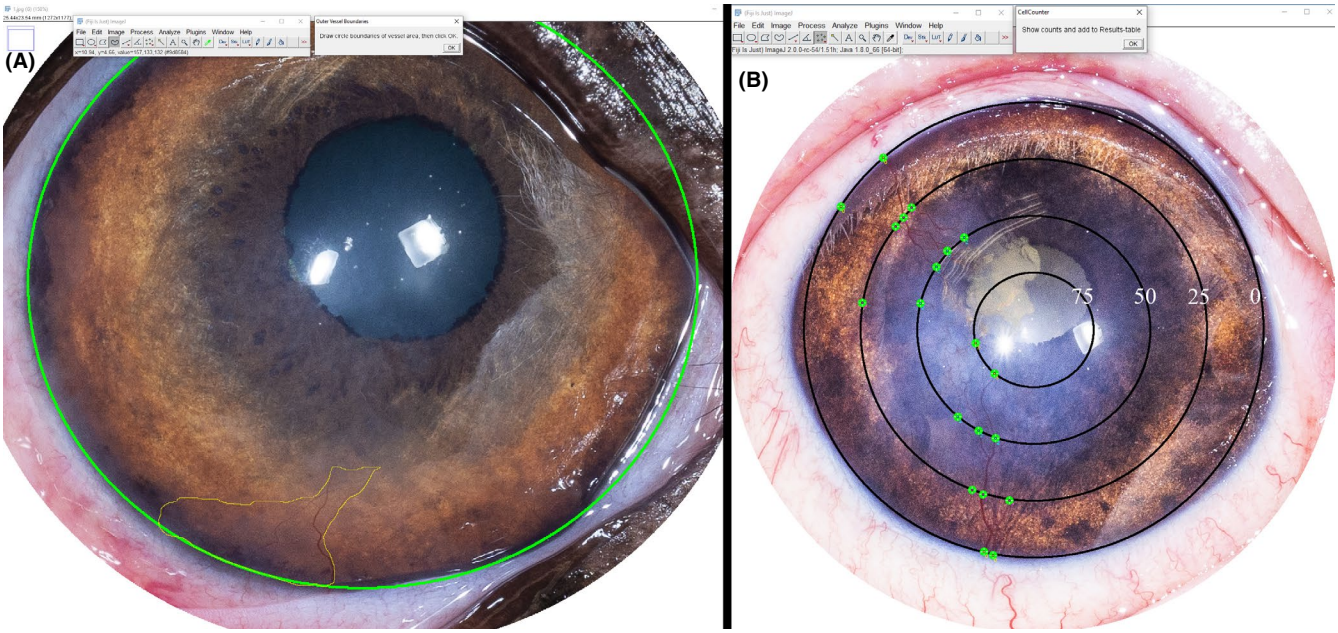

FIGURE 1 Measurement of the vascularized corneal area (A) and the number of blood vessel incisions (B). First, the entire corneal surface was surrounded by the green circle to calculate the total corneal area. The vascularized area was then delineated by the yellow line. By using the previously determined total corneal area, the imaging program calculated the vascularized corneal area (A). To count the blood vessel incisions, the corneal surface was divided into four concentric circles (black line), named as 0-circle, 25-circle, 50-circle, and 75-circle from outside to inside (B). The green dots on the black circles indicate the counted vessel incisions (B). Analyses were performed with an image processing program (Fiji; open-source software: https://imagej.net/Fiji)

3.2 | Tolerability and pain assessment

One dog (#20) showed increased photosensitivity and ocular pain in the study eye at an unscheduled visit 6 months after the last bevacizumab administration. According to the 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, USA) and systemic immunosuppressive therapy for severe allergic dermatitis. Ocular findings included corneal erosion with loose epithelium and a halo of fluorescein stain at the ulcer margins, consistent with the diagnosis of a superficial chronic corneal epithelial defect (SCCED) (Figure 2C). The SCCED was located in the ventronasal quadrant. The corneal vascularization, located in the ventrotemporal quadrant, was unchanged and the overlying epithelium was intact. A 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 (0.1 mg/kg SID p.o., Metacam®, Boehringer Ingelheim, Ingelheim am Rhein, Germany) were prescribed. A follow-up examination was scheduled after 2 weeks. However, the owner reported that ten days after the DBD, the erosion progressed into a deep ulcer and the eye was enucleated on an emergency basis at the referring veterinarian. No histological examination was performed.

Two dogs died during the treatment-free study period before visit 3. One dog died four and a half months after the last bevacizumab administration of unknown reasons at the age of 16 years. The patient was treated asynchronous on both eyes (#6 and #7) with topical bevacizumab. The dog suffered from mitral endocardiosis that was regularly examined by a cardiologist and 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 patient (#14) died 4 months after the last bevacizumab administration. According to the referring veterinarian, the dog died of an incurable pulmonary edema at the age of 12 years. The patient suffered from mitral valve insufficiency and was examined regularly by a cardiologist. Treatment included systemic furosemide and benazepril. The dog did not show any signs of cardiovascular decompensation, pulmonary dysfunction, or general incompatibility

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	OD	7	FC	23.0	Boxer	Superficial stromal keratitis
	9	OS					Superficial stromal keratitis
G *	10	OD	9	FC	30.0	German shepherd dog	CSK
	11	OS					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	Siberian husky	Superficial keratitis
L *	16	OD	7	MC	14.2	Mixed breed dog	Superficial keratitis
	17	OS					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		

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

*Bilaterally treated with topical bevacizumab.

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 bevacizumab eye drops. All clinical parameters remained within the physiological range with only clinically irrelevant minor fluctuations.

No change in behavior or touch response after administration of bevacizumab were reported by the owners. In the investigator's pain assessment, none of the dogs showed any sign of pain at study visit 2 and visit 3 (Figure 3).

3.3 | 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 (Table 2). While some cases showed a marked decrease in corneal vascularization (Figures 4, 5, 6, 7), other dogs had more subtle signs of clinical improvement, such as increased ocular comfort, reduction of ocular discharge, conjunctival hyperemia, and chemosis, and decrease in corneal edema and pigmentation (Figure 2).

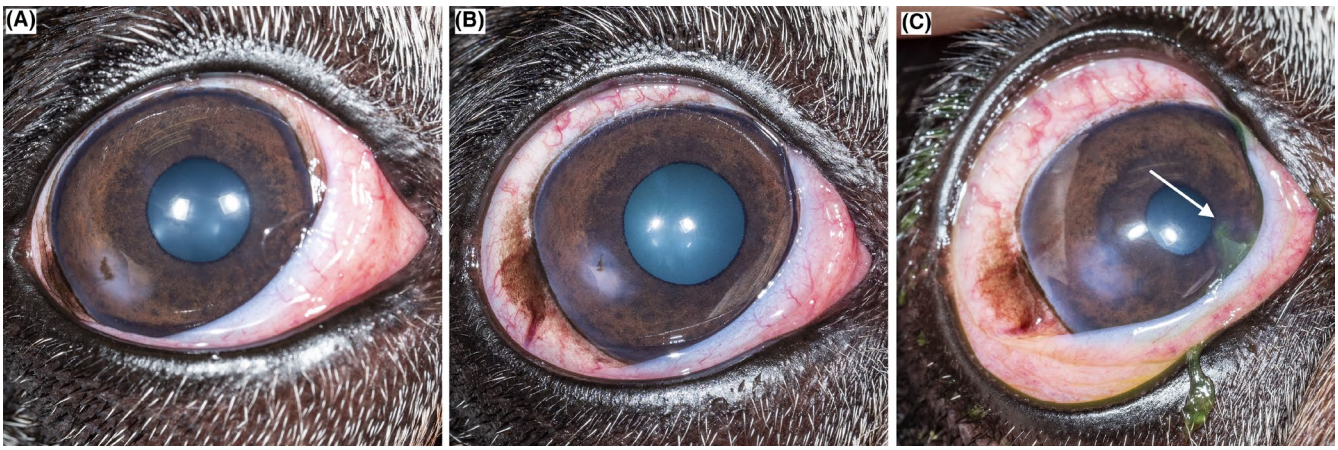

FIGURE 2 French bulldog (#20) with superficial corneal vascularization at baseline visit 1 (A), immediately after topical bevacizumab for 28 days at visit 2 (B), and 6 months after discontinuation of bevacizumab at an unscheduled visit (C). After topical bevacizumab, corneal blood vessels arising from the temporoventral limbus thinned or became bloodless. Corneal pigmentation and edema decreased (B). Six months after discontinuation of bevacizumab, a SCCED (white arrow) was detected in the study eye (C). Ocular findings included a corneal erosion with loose epithelium and a halo of fluorescein stain at the ulcer margin. The SCCED was located in the ventronasal quadrant. Corneal blood vessels were located in the ventrotemporal quadrant, were unchanged compared to visit 2, and were covered by intact corneal epithelium

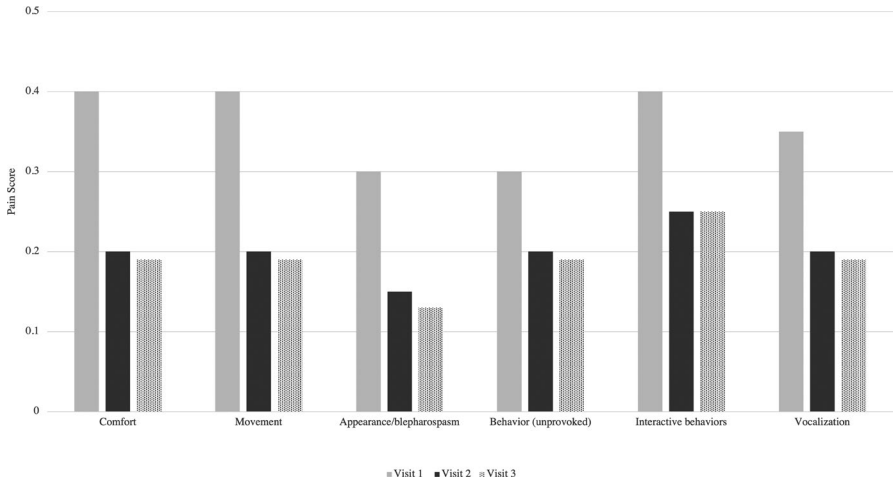

FIGURE 3 Results of the pain assessment performed by the investigator using a predefined pain scale. Displayed are the mean pain scores for each pain score category at baseline visit 1 (baseline), immediately after topical bevacizumab for 28 days at visit 2, and after discontinuation of bevacizumab at long-term follow-up visit 3. Pain score categories included comfort (0–3), movement (0–3), appearance/blepharospasm (0–4), behavior (unprovoked) (0–3), interactive behaviors (0–3), and vocalization (0–3) (Table S1)

The mean vascularized area was reduced by 48.8% (range 4.9%–100%) after 28 days of bevacizumab treatment ($n = 10$) (Table 2). In cases of diffuse corneal vascularization or scattered blood vessels distributed over the entire corneal surface, measurement of vascularized area was not performed.

The mean score of corneal edema and vascularization decreased from 1.6 to 1.0 and from 1.5 to 1.1, respectively ($n = 20$) (Figure 8). The count of blood vessel incisions showed a mean reduction of 28.0%, 31.1%, 4.6%, and 16.5% for the 0-, 25-, 50-, and 75- circle, respectively ($n = 17$) (Table 2). Counting of blood vessel incisions was not possible for areal corneal bleedings. The number of blood vessel incisions of the two innermost circles (50- and 75-circle) increased after bevacizumab treatment in four and two eyes, respectively. In these eyes, blood vessels became visible because corneal edema decreased or diffuse hemorrhages developed into well-defined fine blood vessels (Figures 4 and 6).

Subjectively, a reduction of the vascular caliber and a decrease in blood vessel branching was observed (Figures 2, 4, 6, 9, and 10). Some blood vessels contained less or no blood at all (Figure 7B) and diffuse or blurred corneal hemorrhages consolidated to well-defined thin blood vessels (Figures 4, 5, and 6).

TABLE 2 Results of the efficacy assessment

Category	Difference between baseline and 28 days after topical bevacizumab
Vascularized area (%) $n = (10)$	
Mean ± SD	48.8 ± 33.5
Min	4.9
Max	100
Blood 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

Note: Displayed are the differences in the vascularized area and the number of blood vessel incisions between visit 1 (baseline) and visit 2 after 28-day treatment with bevacizumab expressed as a percentage. Data were analyzed using the imaging software Fiji.

Abbreviations: SD, standard deviation; Min, minimum (lowest observation); Max, maximum (highest observation).

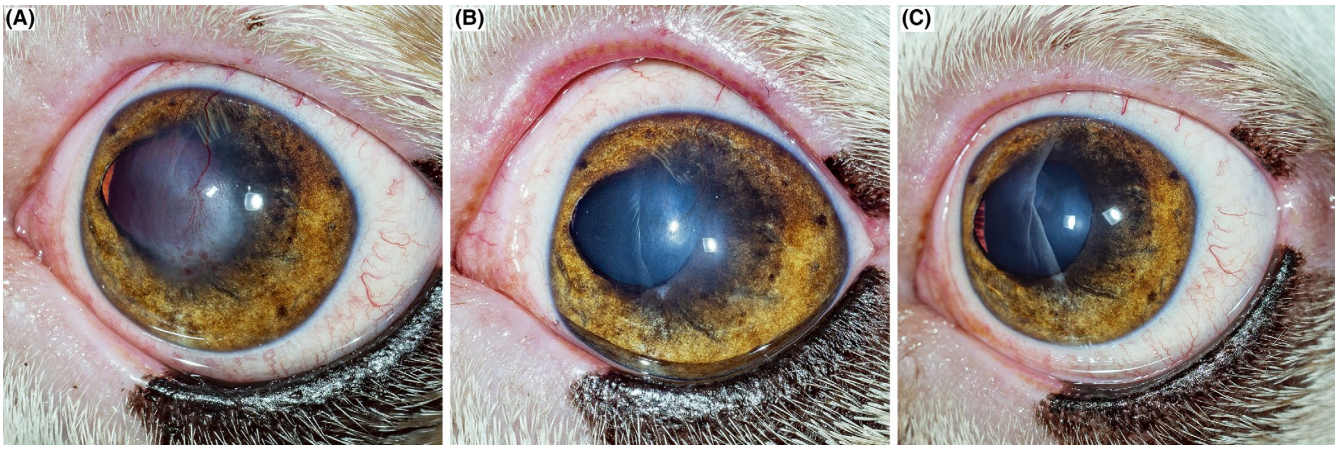

FIGURE 4 Boxer (#9) with persistent corneal vascularization after delayed healing of a stromal ulcer at baseline visit 1 (A), immediately after topical bevacizumab for 28 days at visit 2 (B), and after discontinuation of bevacizumab at long-term follow-up visit 3 (C). Prior to the initiation of bevacizumab treatment, the dog was medicated with systemic NSAIDs and the eye had persistent corneal vascularization. The patient did not receive any topical medication before and after bevacizumab. A thinning of blood vessels and a decrease in corneal inflammatory cell infiltration and edema were observed at visit 2 (B). Further clinical improvement after discontinuation of bevacizumab was noted at visit 3 (C)

3.4 | Long-term follow-up

The clinical improvement observed immediately after 28 days of bevacizumab treatment (visit 2) was maintained until long-term follow-up (visit 3) in all but one patient. In the latter, the owner had discontinued the prescribed topical cyclosporine treatment. Interestingly, the dog also showed corneal blood vessel sprouting in the previously unaffected right eye. Clinical findings included a relapse of superficial corneal blood vessels OS (study eye), new corneal blood vessels OD, and a worsening of quantitative tear film deficiency OU (Figure 7C).

4 | DISCUSSION

In the present case series, client-owned dogs with naturally occurring corneal vascularization were treated with 0.25% bevacizumab eye drops twice a day for the duration of 28 days. The results suggest that topical bevacizumab may have the potential to reduce corneal vascularization in dogs.

However, the range of response to therapy was high, which was also observed in human medicine.[10] There are several possible explanations for this observation. Firstly, we included an inhomogeneous group of client-owned

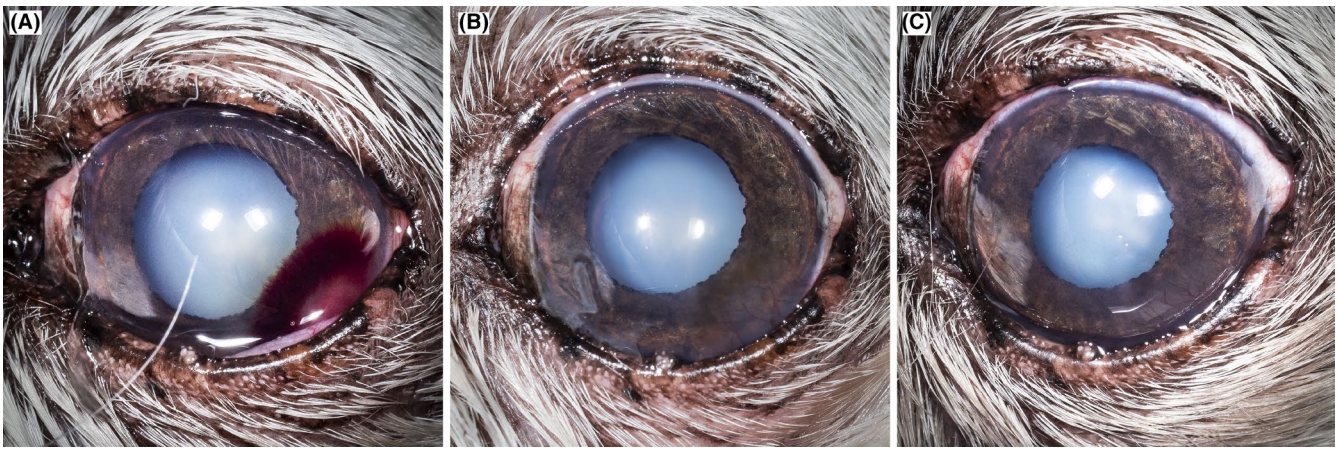

FIGURE 5 Chihuahua (#7) with immune-mediated keratitis at baseline visit 1 (A), immediately after topical bevacizumab for 28 days at visit 2 (B), and after discontinuation of bevacizumab at long-term follow-up visit 3 (C). In (A), ocular findings included a planar stromal hemorrhage adjacent to the ventrotemporal limbus. In (B), it consolidated into a fine vascular network. Clinical improvement was maintained for at least 6 months after drug discontinuation (C)

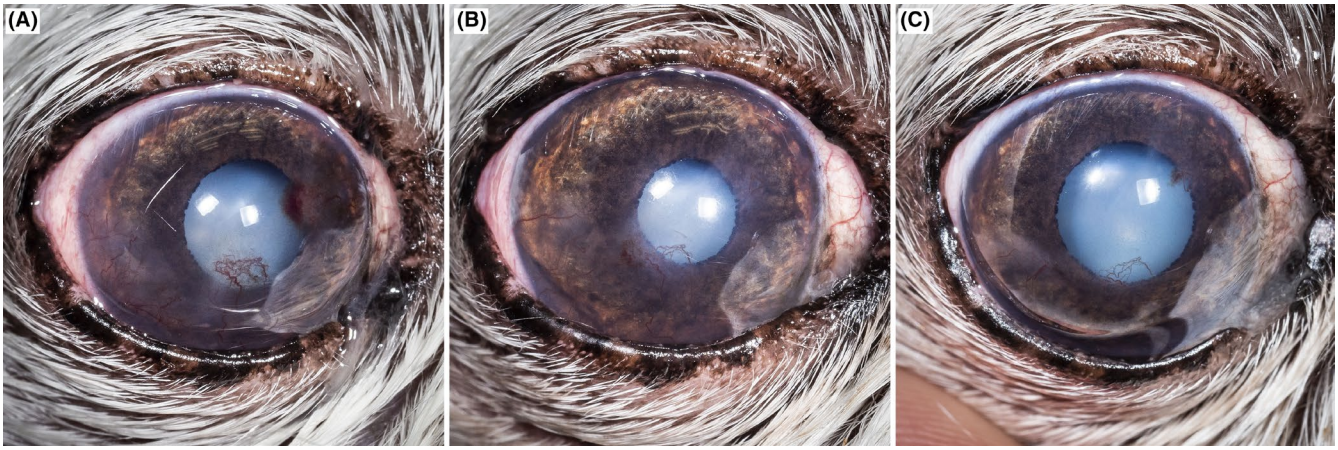

FIGURE 6 Chihuahua (#6) with immune-mediated keratitis at baseline visit 1 (A), immediately after topical bevacizumab for 28 days at visit 2 (B), and after discontinuation of bevacizumab at long-term follow-up visit 3 (C). In (B), the vascularized area and corneal vessel diameter decreased, and the vessels became more sharply demarcated. Corneal cell infiltration and corneal edema improved. Clinical improvement after drug discontinuation was maintained for at least 14 weeks (C). The patient died 4 months after study start and was therefore lost for follow-up visit 3

dogs with a large variety of etiologies for corneal vascularization, disease severity, and progression.[25] Secondly, the studied dogs exhibited a varying degree of tissue scarring. The permeability of a soluble substance is affected by the porosity, conductivity, and the sinuousness of the medium to be permeated.[26,27] Thus, the corneas exhibited varying degrees of structural alteration, potentially resulting in variable degrees of drug permeability and bioavailability. Thirdly, we included dogs with persistent, established corneal blood vessels. Studies demonstrated that bevacizumab can inhibit the proliferation in growing blood vessels but not in established blood vessels already covered with pericytes that most likely do not require VEGF-A for proliferation.[28,29] Koenig et al. found that topical bevacizumab was most effective in humans with corneal vascularization at early disease stages.[25] The authors hypothesized that in patients with established corneal blood vessels, inflammation is less pronounced and drug permeability is decreased, which may contribute to an inadequate response to treatment in advanced disease stages. Fourthly, there is a large number of proangiogenic factors other than VEGF-A that are involved in the complex process of corneal blood vessel formation and are not inhibited by bevacizumab.[15,30,31,32,33,34,35,36,37,38] Fifthly, therapeutic monoclonal antibodies are reported to be immunogenic and can induce the formation of anti-drug antibodies (ADA).[39] Anti-drug antibodies may be directed against components of the drug and can lead to hypersensitivity

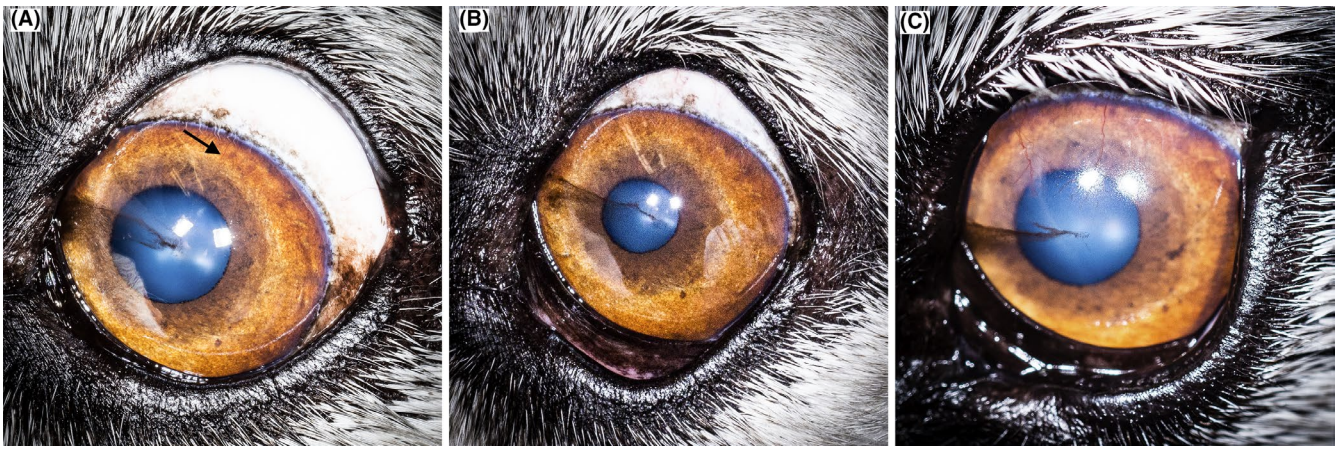

FIGURE 7 Siberian husky (#15) with immune-mediated keratitis at baseline visit 1 (A), immediately after topical bevacizumab for 28 days at visit 2 (B), and after discontinuation of bevacizumab at long-term follow-up visit 3 (C). Superficial blood vessels (black arrow) arising from the dorsal limbus (A) disappeared completely after bevacizumab treatment (B). After drug discontinuation, corneal vascularization relapsed (C)

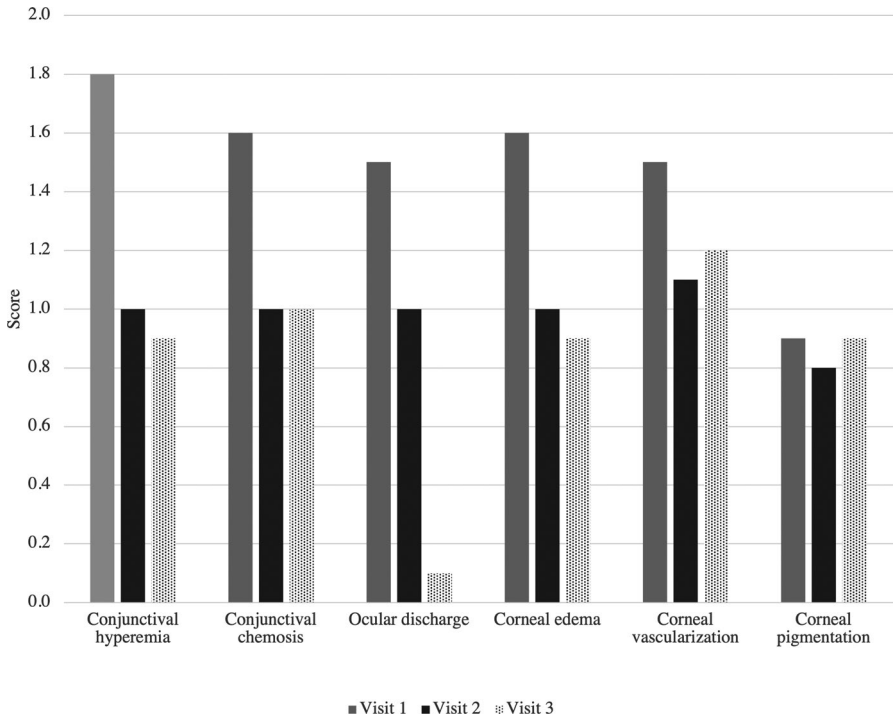

FIGURE 8 Results of the efficacy assessment. Macroscopic ocular findings were scored according to a predefined scale for the categories conjunctival hyperemia, conjunctival chemosis, ocular discharge, corneal edema, corneal vascularization, and corneal pigmentation. Displayed are the mean scores at baseline visit 1, immediately after topical bevacizumab for 28 days at visit 2, and after discontinuation of bevacizumab at long-term follow-up visit 3. 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%–50%, 2 = 51%–75%, 3 ≥ 76%. bva, bevacizumab

reactions, accelerate drug clearance, reduce drug efficacy, and increase interindividual variability of response to therapy.[40] Thus, the induction of ADAs may have contributed to the variable treatment response observed in our study. However, we did not analyze the status of ADAs in our study. To learn more about the development of ADAs after topical bevacizumab in dogs, future research could explore the change in ADA concentration during topical bevacizumab treatment.

We observed that there was not so much a reduction in blood vessel length, but a thinning of blood vessels. This observation is consistent with reports in human

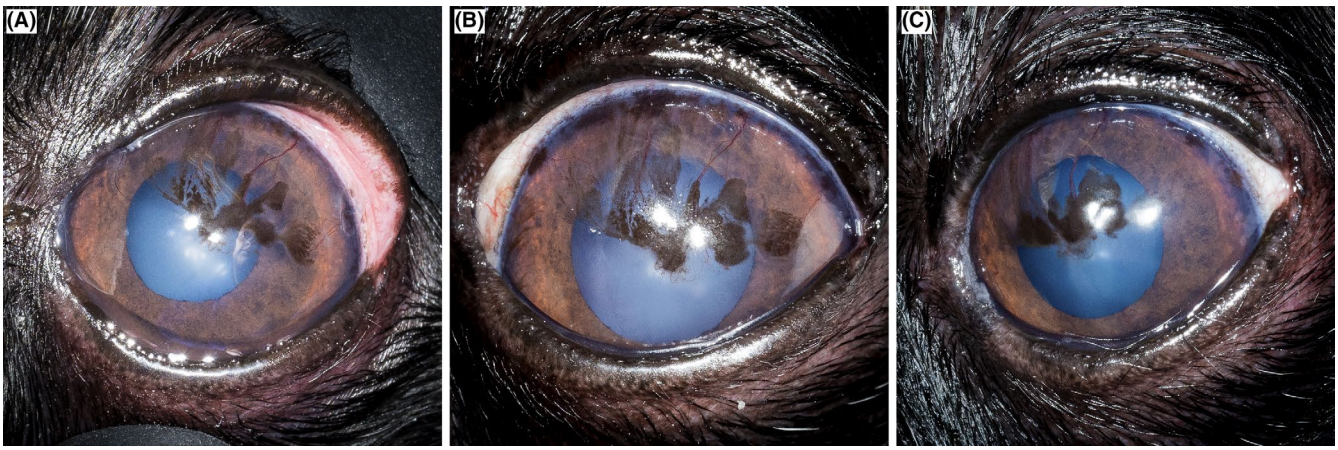

FIGURE 9 Prague rattler (#18) with superficial keratitis at baseline visit 1 (A), immediately after topical bevacizumab for 28 days at visit 2 (B), and after discontinuation of bevacizumab at long-term follow-up visit 3 (C). In (B), vessel diameter decreased, ocular discharge, eye comfort, and conjunctival hyperemia and chemosis improved. Clinical improvement after drug discontinuation was maintained for at least 6 months (C)

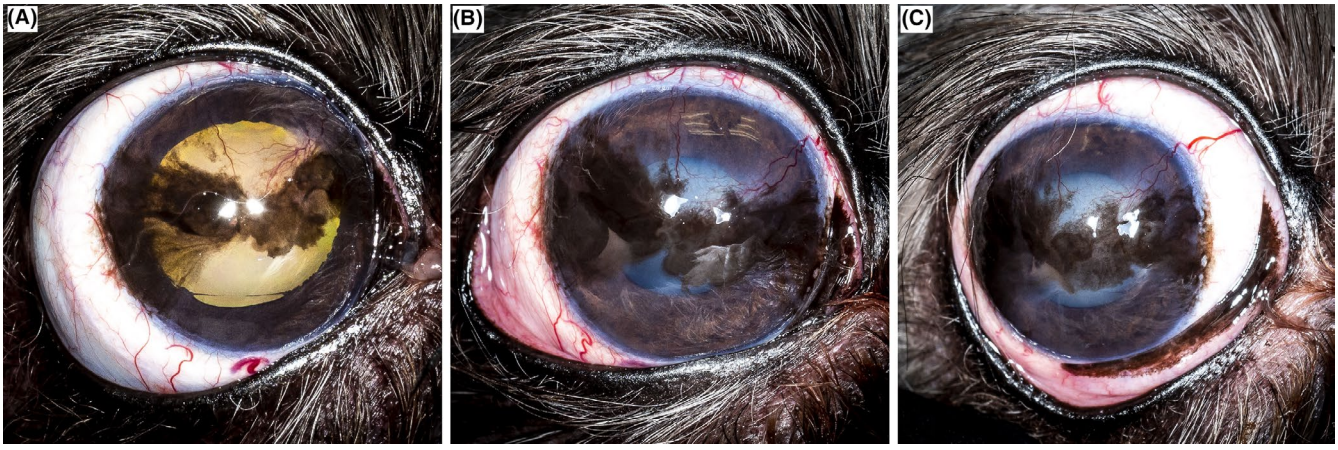

FIGURE 10 Maltese dog (#3) with immune-mediated keratitis at baseline visit 1 (A), immediately after topical bevacizumab for 28 days at visit 2 (B), and after discontinuation of bevacizumab at long-term follow-up visit 3 (C). In (B), distal vessels were thinner, fewer in number, less branched, and more sharply demarcated. Clinical improvement after drug discontinuation was maintained for at least 6 months (C)

patients.[10,25] VEGF is an effective vasodilator and increases the vascular permeability.[41] Thus, VEGF inhibition lowers the blood flow rate and narrows the blood vessel diameter, which can explain our findings.[10,42]

The clinical improvement observed directly after discontinuation of bevacizumab was maintained until long-term follow-up visit 3 in all but one dog. This is in accordance with past research in humans, where topical bevacizumab for 3 weeks had a sustained angioregressive effect for at least 6 months.[9,43]

In our study, we investigated the dosage of 2.5 mg/ml for the duration of 28 days. Due to the lack of knowledge of bevacizumab's safety profile in dogs, we opted for a low dose for a short period of time.[44] Various treatment regimes and drug doses of topical bevacizumab have been investigated.[9,10,25,45] Higher doses up to 25 mg/ml four times a day for 2 weeks,[45] 10 mg/ml either two or four times a day for 3 weeks,[9,10] or 5 mg/ml one to ten times a day for up to 12 months[25] are reported to be effective for the treatment of corneal vascularization in humans. You et al. found that the efficacy of subconjunctival bevacizumab correlated with drug dose, with higher doses being more effective than lower doses.[46] Based on these findings, a longer treatment duration or a higher dose of topical bevacizumab for the treatment of corneal vascularization in dogs could be considered in future studies.

In general, topical application is the preferred route for the treatment of corneal diseases in animal patients.[47] 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 149 kD and is too large to permeate the intact corneal epithelium.[16,48,49] However, in corneas with insufficient barrier function, bevacizumab is known to penetrate into the corneal stroma.[18,49] Besides topical application, an alternative route of administration could be considered in future research. For example, subconjunctival bevacizumab has been found to effectively reduce corneal vascularization.[19,49]

In addition to bevacizumab, there are various other VEGF inhibitors that differ in molecular size, molecular charge, binding affinity, and binding spectrum.[14,15,50] Some of these drugs, such as aflibercept and ranibizumab, have been shown to be effective in suppressing hem- and lymphangiogenesis in animal models of corneal vascularization.[18,51] These drugs could be investigated in future studies.

Vascular endothelial growth factor A is not only involved in pathological vascularization, but has also vital functions, such as corneal nerve regeneration and wound healing.[52,53] Topical bevacizumab has been reported to increase the risk of corneal erosions, especially with prolonged use or at a higher dose.[54] 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. However, it is unlikely that a drug-related side effect was the cause, since the last bevacizumab dose was 6 months ago, long-term immunosuppressive systemic and topical medications had been discontinued shortly before the adverse event occurred, and 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 should be considered that therapeutic VEGF inhibition can contribute to an increased risk of corneal tissue damage, particularly in patients who are prone to spontaneous epithelial defects.[54]

In rare cases, the administration of intravitreal bevacizumab in human patients is associated with severe systemic adverse events.[55,56] In our study, two dogs died four and four and a half months after the last bevacizumab administration at the age of 12 and 16 years, respectively. Both patients suffered from mitral valve insufficiency and were examined regularly by a cardiologist. Both dogs had no signs of cardiovascular or pulmonary dysfunction prior or during the study. In healthy dogs, administration of topical 0.25% bevacizumab for 28 days was shown to have no effect on systemic VEGF levels.[44] Although the uptake of bevacizumab into the bloodstream may be different in an inflamed cornea and conjunctiva, we do not believe that bevacizumab given for a short time and at a low dose of 0.25% would reach a therapeutic concentration high enough to cause systemic side effects.[48] Since the dogs died several months after the last bevacizumab administration, showed no abnormalities in the physical examinations during bevacizumab treatment, and the dose was very low, a causative relation is considered unlikely. However, a causal relationship cannot be excluded with certainty. In future studies, analyzing the systemic VEGF concentrations during bevacizumab treatment could be considered to rule out systemic side effects of topical bevacizumab in dogs with corneal vascularization.

Sandberg et al. found that dogs with intraocular disorders such as glaucoma, lens-induced uveitis, retinal detachment, intraocular tumors, and grade-3 preiridal-fibrovascular membranes (PIFM) had significantly higher VEGF concentrations in the aqueous humor compared with normal eyes.[57] Zarfoss et al. demonstrated that blood vessels and nonvascular spindle cells of canine PIFMs were immunohistochemically positive for VEGF[58] and VEGFR-2 was found to be highly expressed in the vascular endothelium in canine malignant and metastatic intraocular tumors.[59] These findings suggest that VEGF is an essential promoter in numerous canine intraocular disorders and VEGF-inhibiting therapies are of great interest. However, topical bevacizumab cannot penetrate the normal cornea. In human medicine, intravitreal bevacizumab injections are used routinely to treat various ocular disorders.[48,60,61] Intraocular administration routes are also conceivable in dogs, but the safety profile in adult dogs remains unexplored to date.

Vascular endothelial growth factor has been described as one of the major inducers of tumor angiogenesis in mammalians.[62-64] Bevacizumab was developed and approved for the treatment of various neoplastic conditions.[16] In veterinary medicine, an Amur tiger with a palpebral sebaceous gland carcinoma was successfully treated with intralesional bevacizumab as an adjuvant to surgery.[65] At the University of Veterinary Medicine Vienna, an Appaloosa mare suffering from a corneal stromal invasive squamous cell carcinoma has been treated surgically and adjuvantly with topical mitomycin C, systemic firocoxib, and intrastromal bevacizumab (K.-O. Blohm, personal correspondence).

The study has several limitations. In some cases, it was difficult to detect the exact margins of the blood vessels due to corneal pigmentation or edema. It is assumed that corneal blood vessels also run indiscernibly under the pigmentation or edema. In some eyes, it was not useful to quantify the vascularized area because the blood vessels were distributed over the entire surface. In contrast to focal changes that can 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.[8] Dastjerdi et al. assessed the surface area of the corneal blood vessels themselves by tracing the vessels and erasing the nonvascular area.[10] The remaining vascularized area was then pixelated and measured.[10] Another limitation is the design of our study, which was planned as a prospective case series without a control group and with a highly inhomogeneous patient cohort. We included only client-owned dogs with a variety of comorbidities and disease etiologies, making the scientific validity in terms of efficacy difficult. For better comparability and identical baseline conditions, it would be necessary to study animals with experimentally induced, standardized corneal vascularization, as has been done in rodent animal models.[18,19,66]

In all but two eyes, topical bevacizumab was given concurrently with 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. There are contradictory findings regarding the angioregressive potential of cyclosporine.[67,68] Topical cyclosporine has been described to reduce corneal angiogenesis in rodent models induced by alkali burn, xenotransplantation, or cautery.[67] In contrast, recent research on rats with cautery induced corneal vascularization found topical cyclosporine 1% TID to be ineffective concerning the inhibition of corneal angiogenesis.[68] This study examined the effect of cyclosporine on newly formed corneal blood vessels. In our study, we included patients with chronic corneal vascularization and established blood vessels, which makes it impossible to draw direct conclusions. In human medicine, high-dose subconjunctival cyclosporine implants following high-risk corneal transplants, including patients with more than one quadrant of stromal vascularization or graft loss, did not significantly reduce corneal neovascularization.[69] The authors suggested that local cyclosporine has negligible angioregressive effects in the human cornea for this indication. Even though the studied dogs received topical cyclosporine as a long-term therapy for months or years without further improvement of corneal vascularization, we cannot prove that the antiangiogenic effect observed in our study was achieved by bevacizumab alone nor can evidence that cyclosporine did not contribute to the improvement of corneal vascularization.

Therefore, future studies with larger patient cohorts in a controlled and masked study setting are recommended to provide evidence for the efficacy of topical bevacizumab treatment to reduce corneal vascularization in dogs.

5 | CONCLUSIONS

Considering the limitations of this study and the design as a case series with various interindividual patient characteristics, the clinical improvement and reduction in corneal vascularization suggested that topical bevacizumab may be an effective therapeutic approach to reduce corneal vascularization in dogs. Caution should be exercised when using topical bevacizumab in dogs with recurrent superficial epithelial defects. Further research with larger patient cohorts and placebo-controlled, long-term studies are recommended to learn more about the efficacy of bevacizumab, gain further insights into different dosing regimens, safety profile, clinical impact as a monotherapy, routes of administration, and other indications for its use.

ACKNOWLEDGEMENT

This research was supported using resources of the VetCore Facility (VetImaging | VetBiobank) of the University of Veterinary Medicine Vienna.

CONFLICT OF INTEREST

This study was funded by the incomes of the ophthalmology unit of the Department of Companion Animals and Horses, University of Veterinary Medicine Vienna. Lisa-Marie Muellerleile wants to disclose that she is working full-time for Novartis Pharma AG. At no time she was paid for any part of this work nor was she otherwise influenced by her employment in the creation of this research project.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Muellerleile L-M, Bernkopf M, Wambacher M, Nell B. Topical bevacizumab for the treatment of corneal vascularization in dogs: A case series. *Vet Ophthalmol*. 2021;24:554–568. https://doi.org/10.1111/vop.12931