Corneal endothelium: normative data in primates and corneal endothelial dystrophy and endotheliitis in dogs

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ABSTRACT

The corneal endothelium (CE) is a single, highly metabolic layer of hexagonal cells that constitute the innermost layer of the cornea. The CE maintains corneal transparency by actively pumping ions into the anterior chamber thereby facilitating the exit of water to maintain deturgescence. The regenerative capacity of the CE is limited in humans, nonhuman primates and dogs such that loss of a critical number or function of endothelial cells can lead to CE decompensation, corneal edema, vision impairment, and in chronic cases, painful corneal ulcers.

Non-human primates (NHP), including rhesus macaques (*Macaca mulatta*), are highly valuable animal models to study ophthalmic diseases due to their similar ocular development, anatomy, and physiology to humans. Establishment of reference values for corneal thickness and corneal endothelial cell density, as well as their association with factors such as age or sex are critical to better understand the advantages and limitations of rhesus macaques as an animal model for the study of corneal endothelial diseases and therapeutics directed towards these important cells. In Chapter 2 of this dissertation, we determined normal ranges of corneal thickness and endothelial cell density as well as their association with age, sex, weight in 144 rhesus macaques from the California National Primate Research Center (CNPRC).

Corneal endothelial dystrophy (CED) is a bilateral, progressive disease in dogs characterized by premature degeneration and loss of corneal endothelial cells, which leads to corneal edema, bullous keratopathy and recurrent corneal ulceration. Secondary infectious keratitis and corneal perforation can occur in advanced cases, necessitating enucleation. In humans, an analogous condition exists, termed Fuchs' endothelial corneal dystrophy (FECD). Currently, corneal transplantation is the only definitive treatment for FECD. However, this surgical procedure is rarely performed in

veterinary medicine due to the lack of donor tissue, price, and risk of complications. In humans, topical rho-associated coiled-coil kinase (ROCK) inhibitors are in clinical trials to assess their efficacy in accelerating endothelial regeneration and preventing bullous keratopathy in FECD-affected patients. We recently completed a prospective, open label clinical trial demonstrating that the ROCK inhibitor, ripasudil, delayed progression of CED in dogs with early disease. However, adherence to this medication was difficult in some cases as ripasudil requires 4 times a day application. Netarsudil 0.02% is an FDA approved ROCK inhibitor for the treatment of glaucoma in humans that only requires twice daily administration. In Chapter 3 of this dissertation, we provide an interim analysis of for this clinical trial that tests the efficacy of topical netarsudil 0.02% as a treatment for CED in dogs.

The pathogenesis of FECD is complex, and both genetic and non-genetic factors are known to play critical roles. A retrospective study from our laboratory found that Boston Terriers are overrepresented among the breeds that have CED, suggesting the presence of an underlying genetic predisposition in this breed. The identification of a genetic mutation or variant associated with CED would facilitate the development of genetic testing to inform owners, veterinarians, and Boston terrier breeders on which dogs may be on risk of developing CED. In Chapter 4 of this dissertation is a genome-wide association study (GWAS) to identify risk associated loci for CED in the Boston Terrier.

Other causes of CE cell damage or degeneration include anterior uveitis, glaucoma, intraocular surgery, lens luxation, diabetes mellitus, canine adenovirus-1 infection (CAV-1), senility, and corneal endotheliitis. Corneal endotheliitis is the result of primary inflammatory damage to the CE that typically manifests with endothelial loss, corneal edema, keratic precipitates, and inflammatory debris on the endothelium.

Corneal endotheliitis as a primary entity is uncommon in canine patients, and some of the clinical signs and imaging features can be misinterpreted by the clinician. In Chapter 5, we describe the clinical findings, advanced imaging characteristics, and treatment of four canine endotheliitis cases. The purpose of this chapter was to inform the clinicians of those clinical and imaging features that are indicative of corneal endotheliitis, as well as the response we have observed in our patients to different treatments.

In summary, this dissertation analyzes different aspects of the physiology and disease of the corneal endothelium in comparative ophthalmology. Namely, we define standard corneal parameters in primates without ocular disease, explore the efficacy and safety of a new medical treatment for canine CED, investigate the genetics of CED in the Boston terrier, and offer a review on the clinical presentation and imaging characteristics of canine corneal endotheliitis.

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CHAPTER 1: INTRODUCTION

1. Cornea and corneal endothelium: anatomy and physiology

The cornea is a multilayered structure that protects the internal structures of the eye and facilitates light entry and refraction. Corneal function depends on the conservation of its morphology and physiology of each layer in order to maintain transparency and prevent light scatter. In human and non-human primates (NHP), the cornea is comprised of five layers: corneal epithelium, Bowman's layer, corneal stroma, Descemet's membrane (DM), and corneal endothelium (CE).[1] Domestic animal species, including dogs, lack Bowman's layer.[2]

Corneal epithelium is a stratified, non-keratinized epithelium composed by a single layer of columnar basal cells that produce a thin basement membrane with an overlying, few layers of polygonal wing cells, and several layers of the most superficial squamous cells.[1] The Bowman's layer is an acellular layer underlying the epithelial basement membrane that in humans gets thinner with age.[3] The corneal stroma is the thickest layer of the cornea, and it is composed primarily of extracellular matrix including collagens, proteoglycans, and glycoproteins.[4] The most common cell of the stroma is the keratocytes, which produces and remodels the extracellular matrix to maintaining its orderly structure.[4] In contrast to many other tissues, the cornea lacks pigment, myelinated nerves and blood vessels in order to maintain transparency. The corneal stroma and epithelium are nourished by the capillary loops at the limbus, the tear film, and the aqueous humor.[1]

The DM secreted by the CE and it composed primarily of collagens, including collagen IV and VIII.[4] Missense mutations in the gene encoding the subunit α-2 of

collagen VIII (*COL8A2*) have been associated with a form of early onset of Fuchs' endothelial corneal dystrophy (FECD) in humans.[5]

The CE is comprised of a single layer of flat, hexagonal cells that serves as barrier with the anterior chamber and regulates corneal hydration and nutrition. The endothelial cells join via *zonula occludens*, *macula adherens* and lateral gap junctions, forming a permeable barrier that facilitates the diffusion of small molecules, such as glucose, from the aqueous humor to the corneal stroma.[6–8] Because of the composition of the corneal stroma, there is a constant swelling pressure that drives water from the anterior chamber into it. The endothelium counteracts this process by active secretion of sodium and chloride ions, as well as bicarbonate, into the anterior chamber that generates an osmotic gradient to drives water from the stroma into the aqueous humor.[8] The endothelial cells have a high density of mitochondria due to this intense metabolic activity.[8] With aging, the number of corneal endothelial cells decline and the remaining cells increase in size, producing a variation in size (polymegathism) and lose their hexagonal shape and thus display pleomorphism.[9]

2. Corneal endothelial dystrophies in human and dogs.

The regenerative capacity of the corneal endothelium varies between species. Rabbits and rodents have a faster and higher regenerative capacity through mitosis of corneal endothelium when comparing with dogs, NHPs and humans, for whom the regenerative capacity is considered very limited.[10] Most mammals are born with enough CECs to maintain corneal transparency throughout life, despite a slow rate of CEC decline with age.[11,12] However, loss in function or a critical number of CECs can to leads to decompensation with resultant corneal edema. In humans, functional decompensation occurs when central endothelial cell density is ≤500 cells/mm^2.[12]

In 2015, the International Classification of Corneal Dystrophies recognized four different types of corneal endothelial dystrophies in humans: FECD, Posterior Polymorphous Corneal Dystrophy (PPCD), Congenital Hereditary Endothelial dystrophy (CHED), and X-linked Endothelial Corneal Dystrophy (XECD). Among them, FECD is the most common type.[13,14]

FECD is a bilateral, progressive disease characterized by the development of excrescences of DM, called guttata, and progressive endothelial cell loss that eventually can lead to corneal edema, bullous keratopathy, corneal ulceration, and impaired vision.[15] This disease most commonly affects patients in the fourth decade of life or older, although forms of early onset affecting children or teenagers in their first decade of life are also described. Women are overrepresented in cases of FECD.[13] Genetic and non-genetic factors are recognized to play a role in FECD. The genetics of FECD is complex, and both familial or sporadic presentations are described.[13] Specifically, intronic repeat expansion of the *TCF4*, and mutations in *COL8A2* or *SLC4A11*, amongst others, have been associated with this disease (summarized in **Table 1**).[16]

In dogs, CED was first described in 1876,[17] and is clinically defined as a bilateral, late onset, progressive disease, in which the endothelial cells prematurely degenerate (**Fig. 1 and 2**).[17,18] Although bilateral, it can be asymmetric with the disease, manifesting initially in one eye.[19] Similar to FECD in humans, some studies have reported a greater frequency of females versus males affected by canine CED.[19,20] There is also a strong breed predilection.[18] In one study, Boston Terriers were overrepresented in CED-patients in comparison to the hospital population (~10% of the dogs diagnosed with CED, observed vs expected ratio = 11.8, $P = 2.5 \times 10^{-23}$),[21] suggesting the presence of an underlying genetic predisposition in this breed.

3. GWAS and use of GWAS in corneal conditions both in humans and dogs

Genomic analysis has become a critical research tool for the identification and study of the genetic factors involved in multiple human diseases. Specifically, genome-wide association studies (GWAS) are a common approach employed to identify genetic variants associated with non-mendelian, complex diseases.[22] GWAS uses single chip-based microarrays that can test thousands of single nucleotide polymorphisms (SNPs). The purpose of a GWAS is identifying which allele/s are associated with the trait studied.[22] In humans, GWAS have been powerful tools to study genetic factors involved in common ocular diseases including as age-related macular degeneration,[23] glaucoma[23–26] and myopia,[22,23] as well as corneal diseases such as keratoconus[27] or FECD.[28,29]

Dogs and humans share many of the same diseases and occupy similar environments, thus making the dog a superb translational model for complex diseases such as diabetes, epilepsy or cancer.[30] Furthermore, the process of domestication, selective breeding and population bottlenecks have produced higher rates of certain diseases or conditions due to the homogenization the genome within different breeds as well as long linkage disequilibrium and long haplotype blocks.[31] These characteristics make the ideal scenario for the study of risk associated loci using GWAS. Since the overwhelming majority of canine breeds are highly inbred,[32] a small number of dogs can be utilized to investigate diseases with Mendelian inheritance patterns or identify risk associated loci in complex diseases.[33–36] With the improvement of the dog genome annotation and the development of more accessible, cutting-edge technology for genetic testing, GWAS are one of the most common and powerful

approaches for the study of the genetic component of complex diseases in veterinary medicine.

In dogs, genome-wide association mapping approaches have been successful in identifying loci associated with different ocular diseases, including cataract,[37] glaucoma,[37–39] and inherited retinal diseases,[37,40] among others. Genotyping studies has been also been useful on the study the of corneal disease in dogs: examples include the identification of a 30 bp deletion at a splice site in the *NOG* gene associated with spontaneous superficial chronic corneal epithelial defects (SCCED) in Boxers, or the association of a missense mutation in *CHST6* with macular corneal dystrophy in Labrador retrievers.[41,42]

4. Characteristics of corneal endothelial dystrophies in advanced imaging.

On ophthalmic examination, FECD patients typically present with guttae, thickening and lamination of the DM and stromal edema that can progress to bullous keratopathy and, in some cases, affect the corneal epithelium causing epithelial bullae.[13] As the disease progresses, corneal endothelial cell density decreases and polymegathism and pleomorphism increases as visualized with confocal or specular microscopy.[13] Differential diagnosis for canine CED include primary causes of corneal degeneration such as anterior uveitis, glaucoma, intraocular surgery, lens luxation, diabetes mellitus, senility, canine adenovirus-1 infection and endotheliitis. [43–46] While suspicion for CED is evident on a thorough ophthalmic examination, imaging of the CE with confocal or specular microscopy is necessary for definitive diagnosis.

Similar to humans, canine CED patients demonstrate thickening of DM and corneal stromal edema. This increase is evident using slit lamp examination or optical coherence tomography (**Fig. 2**). As in humans, it is possible to observe excrescences

on the DM (guttae-like lesions)[20,47] in some CED patients, as well as enlarged corneal endothelial cells, decreased corneal endothelial cell density and increased pleomorphism and polymegathism using confocal microscopy (**Fig. 2**).

The CE in most mammals possesses a limited regenerative capacity. The exception is rabbits, which exhibit a high proliferative capacity after endothelial injury while, in other mammalian species, mitosis of the endothelium is rarely observed.[10,48,49] Instead, the adjacent endothelial cells migrate and spread to cover the area.[48] Multinucleated corneal endothelial cells have been observed in non-human primates (NHPs) following endothelial injury in a transcorneal freezing model,[50] as well as in humans and in dogs with CED (**Fig. 3**), and are associated with CE healing.[10,51] It has been speculated that the origin of multinucleated cells could be either be through amitotic cell division or cell coalescence.[48,52]

Histologically, the CE consists of a single layer of cuboidal cells with clear, finely vacuolated cytoplasm and a thick, homogeneous, periodic Schiff positive basement membrane, termed DM.[43] In CED affected dogs, the corneal endothelial cells are attenuated, the DM is thicker and there is a lack of artifactual clefting in the stroma from edema. Some cases also demonstrate excrescences of disorganized, fibrillar extracellular matrix on the inner aspect (guttae-like lesions) or duplication of DM (**Fig. 4**).[43,47,53,54] A limitation of regular, sagittal and histological sections is the small number of endothelial cells that can be examined using this approach. Alternatively, whole-mounts preparations of CE allow to assess a larger number of endothelial cells, calculate endothelial cell density, and evaluate their shape and surface. Furthermore, CE whole-mounts are ideal for vital stains and IHC, key to study the characteristics of these cells as well as the relationships between them and with their environment.[10,55]

5. Medical treatments for FECD and canine CED, and potential applications of ROCK inhibitors as therapeutic for FECD and canine CED

Currently, FECD is the leading cause of corneal transplantation in the United States with penetrating keratoplasty (PK), Descemet's stripping automated endothelial keratoplasty (DSAEK), and DM endothelial keratoplasty (DMEK) all being utilized to treat FECD.[15,56,57] In mild cases, topical application of hyperosmotic saline solutions or ointments can help to relieve the clinical symptoms in patients with FECD or CED.[15,58,59]

While corneal transplantation has been described in dogs,[60–62] it is currently not a routinely performed due to the lack of donor tissue, cost, and risks of complications. Canine patients with corneal edema or bullous keratopathy can benefit from receiving sodium chloride 5% ophthalmic solution or palliative surgeries such as superficial keratectomy with conjunctival advancement hood flap (SKCAHF), that reduce corneal thickness and corneal edema and improves quality of vision.[58,63,64] However, surgical treatments are costly, invasive, and the effect is temporal. Thus, a medical therapy to delay the progression of FECD and canine CED is warranted.

The Rho family is a group of small GTP-binding proteins that are involved in multiple cellular functions including cell motility, adhesion, cell shape, apoptosis, and cell proliferation.[65] The ROCK family is a family of effector serine/threonine kinases and includes ROCK1 and ROCK2 downstream of Rho. Structurally, both isoforms are composed by an N-terminal kinase domain, a central coiled-coil region with a Rho-binding domain, and a C-terminal with a pleckstrin homology (PH) domain that contains a cysteine-rich region. The C-terminal portion acts as auto-inhibitory region, whereas interaction between the Rho-binding domain and the Rho-GTPs domain of active Rho GTPases promote ROCK activity by phosphorylation of a wide range of

substrates.[66] ROCK1 and ROCK2 are widely distributed in the human body and have multiple functions, including regulation of the cytoskeleton and microtubule network, participating of the cellular contraction and cellular motility, regulation of the cellular morphology and polarity, apoptosis, and cell division.[65–68] Dysregulation of the Rho/ROCK signaling pathway has been associated with multiple human diseases, including cardiovascular diseases, neurodegenerative disorders, cancer metastasis, and metabolic diseases, among others. Two ROCK inhibitors, ripasudil (Glanatec© 0.4% ripasudil ophthalmic solution) and netarsudil (Rhopressa© 0.02% netarsudil ophthalmic solution), have been approved for the treatment for glaucoma and ocular hypertension in humans in Japan and United States, respectively. Both inhibit ROCK1 and ROCK2 and decrease outflow resistance, thereby lowering the intraocular pressure.[69] In addition, netarsudil is also a norepinephrine transporter inhibitor. However, the therapeutic application of ROCK inhibitors has been expanded since *in vitro* studies have shown that activation of the Rho/Rho kinase pathway is involved in corneal endothelial cell apoptosis.[66,68,70]

Specifically, Rho kinase inhibitors promote corneal endothelial cell survival and cell adhesion *in vitro* [70,71] and accelerate corneal endothelial regeneration *in vivo* in rabbit, canine and NHP injury models.[72,73] Preliminary results from pilot studies in patients with corneal endothelial decompensation indicate that topical application of different ROCK inhibitors, including topical netarudil 0.02% ophthalmic solution, reduced corneal edema and improved vision acuity.[72,74,75]

Furthermore, ROCK inhibitors have also shown to enhance the efficacy cell-based therapies,[76] and to accelerate recovery of corneal transparency in FECD patients when applied after Descemet stripping surgery.[77] The proliferative stimuli of releasing of the cell-to-cell contact inhibition after removing damaged corneal

endothelium is thought to be enhanced by ROCK inhibitors;[78] a hypothesis that opens a new, promising avenue on the use of ROCK inhibitors as medical therapy in combination with corneal surgical procedures.

ROCK inhibitors can cause vasodilation as a result of the relaxation of vascular smooth muscle.[69] Indeed, the main adverse effect of topical ROCK inhibitors in ophthalmology is conjunctival hyperemia.[79] Other adverse events reported are much rarely reported and include transient reticular epithelial edema, eye pruritus, conjunctival hemorrhage, or headache.[80,81]

Preliminary results of a clinical trial that employed the ROCK inhibitor ripasudil (Glanatec© 0.4% ripasudil ophthalmic solution), applied 4 times a day as a treatment for canine CED, showed stabilization or improvement of corneal disease in more than 50% of the eyes after 1 year of treatment. Patients in early stage of disease showed a more favorable response.[82] The most common adverse reaction in this study, similar to human, were conjunctival hyperemia and reticulated intraepithelial bullae.[82]

6. Concluding remarks

In summary, the corneal endothelium is key to maintaining corneal transparency and has a variable regenerative capacity depending on the species, with both humans and non-human primates presenting a very limited regenerative capacity. In humans, genetics play a role in corneal endothelial dystrophies and in veterinary medicine a genetic predisposition is suspected in some breeds, such as the Boston terrier. ROCK inhibitors can promote endothelial cell survival *in vitro* and have shown promising results in pilot studies *in vivo*, thus are excellent candidates as a medical treatment for corneal endothelial degeneration in humans and dogs.

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11. Tables

Table 1. Genes identified as associated with Fuchs' endothelial corneal dystrophy (FECD) by the International Committee for Classification of Corneal Dystrophies (IC3D, 6th column) or involved in CE metabolism. The type of dystrophy associated with the gene, or the function of the gene are described in the 3rd column. PPCD: Posterior polymorphous corneal dystrophy. CHED: Congenital hereditary endothelial dystrophy CHED

Gene	Protein encoded	Associated corneal disease or function of the gene	Type of mutation	Reference	IC3D
COL8A2	Subunit α-2 of collagen VIII	Early onset FECD, PPCD 1 and 2	Two non-synonymous mutations, L450W and Q455K. Same mutations were reproduced in murine transgenic model	[5,83–85]	Yes
TCF4	E-protein family	Late onset FECD	>50 intronic CTG repeats (normally 10-37 repeats)	[28,86,87]	Yes
SLC4A11	Sodium Bicarbonate Transporter-Like protein 11	Late onset FECD, CHED2	Missense and deletion mutations	[88,89]	Yes
ZEB1	Transcription factor Zinc finger E-Box binding homeodomain 1	Late onset FECD, PPCD	Missense and non-sense mutations	[87,90]	Yes
LOXHD1	Lipoxygenase Homology Domain-containing protein 1	Late onset FECD	Autosomal dominant inheritance. Missense mutation.	[91]	Yes
AGBL1	Cytosolic Carboxypeptidase 4	Late onset FECD	Nonsense and missense mutations	[92]	Yes
KANK4	KN motif and Ankyrin repeat domain-containing protein 4	Late onset FECD	Loci associated	[28]	Yes
LAMC1	Laminin subunit gamma-1	Late onset FECD	Loci associated	[28]	Yes

ATP1B1	Sodium/potassium-transporting ATPase subunit beta-1	Late onset FECD	Loci associated	[28]	Yes
YAP1	Yes-Associated Protein	Role in mechanotransduction, involved on the development of the eye	Single copy deletion in mice, experimental model	[93]	No
WWTR1	Transcriptional coactivator with PDZ-binding motif	Involved in corneal endothelial cell development, DM biomechanics	Single and double copy deletion in mice, experimental model	[94]	No
CTNNB1	Catenin Beta-1	Regulates endothelial cell proliferation, nuclear translocation activates mitosis		[95]	No
TGF-B	Transforming Growth Factor Beta-1	Blocks β-catenin nuclear translocation		[95]	No
AQP1	Aquaporin-1	Involved in fluid transport and restoration of corneal water content and transparency following corneal edema		[96]	No
CDH2	Cadherin 2	Cell adhesion protein found in cell membrane in corneal endothelial cells		[97]	No
TJP1	Tight junction protein ZO-1	Component of endothelial cells tight junctions. Associated with central corneal thickness and central topography		[98]	No

12. Figures

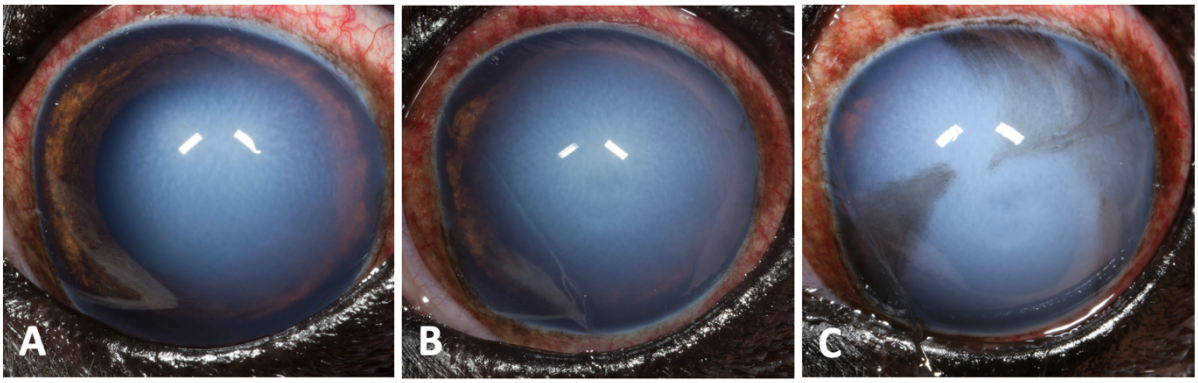

Figure 1: Clinical progression of corneal edema in a 10-year old, female spayed Boston terrier diagnosed with CED. (A) On initial exam, there is a moderate corneal edema in the central as well as temporal cornea, affecting 60% of the corneal surface. **(B)** Four months later, the edema covers 83% of the cornea with only the medial perilimbal cornea spared **(C)** Ten months after the initial examination, the edema affects nearly 100% of the cornea, and there are wisps of corneal melanosis extending from the dorsolateral and ventromedial limbus towards the central cornea.

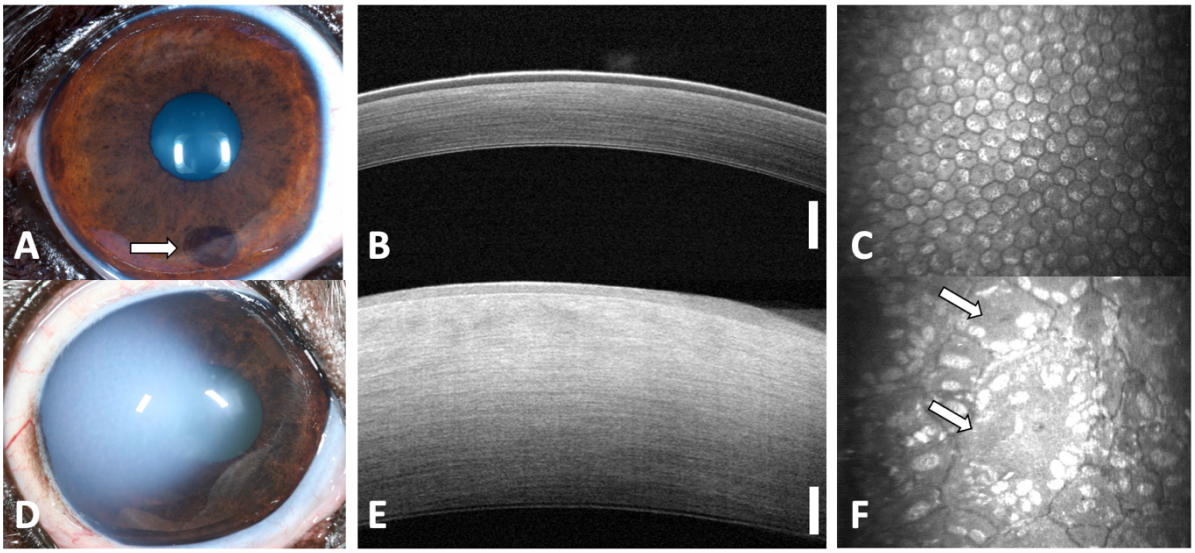

Figure 2: Corneal edema with increased corneal thickness and decreased corneal endothelial cell density with altered morphology are hallmarks of CED. (**A, B**) An 8-year old neutered unaffected male Boston terrier with a clear cornea. Normal corneal central corneal thickness (CCT) of 569 µm by FD-OCT, and one mobile, pigmented, iris cyst on the anterior chamber (arrow). (**C**) Corneal endothelial cell density of 2187 cells/mm^2, and normal cell morphology by IVCM. (**D**) A nine-year old spayed female affected Boston terrier with moderate corneal edema involving the central, temporal paraxial, and perilimbal cornea. (**E**) With FD-OCT, this patient has thickening of the central corneal stroma (CCT=1556 µm) and loss of the lamellar arrangement of the collagen fibrils in the anterior stroma. (**F**) By IVCM, there is decreased corneal endothelial density as well as marked polymegathism, pleomorphism, and presence of multinucleated cells (arrows).

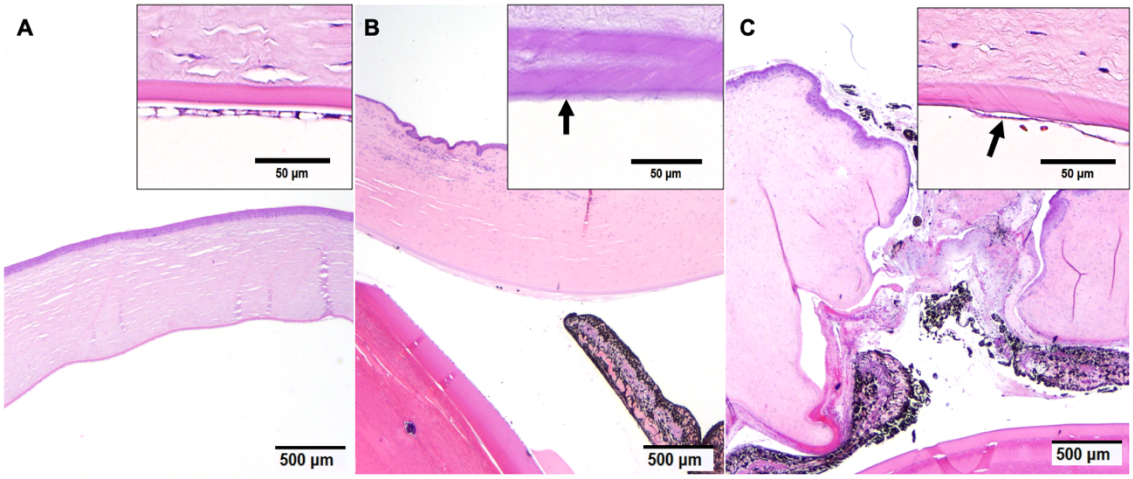

Figure 3: Characteristic attenuation and loss of CE was observed in CED-diagnosed Boston terrier. (**A**) Non-affected, male neutered 10 years old Boston terrier. HE. Inset: The CE is composed by a single layer of cuboidal cells. HE. (**B**) Fifteen years old, female spay Boston terrier diagnosed with CED with corneal edema and suppurative keratitis. HE. Inset: Duplication of DM (arrowhead) and loss of corneal endothelial cells. PAS. (**C**) Corneal perforation with iris prolapse in an 8 years old male Boston terrier diagnosed with CED. HE. Inset: Attenuation of corneal endothelial cells (arrow). The inner surface of the DM is irregular (remodeling)

CHAPTER 2: Normal corneal thickness and endothelial cell density in rhesus macaques (*Macaca mulatta*)

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1. Abstract

Purpose: To define the normal range of central corneal thickness (CCT) and corneal endothelial cell density (ECD) in rhesus macaques *(Macaca mulatta*) and the effects of age, body weight, sex and intraocular pressure (IOP) on these parameters.

Methods: Ophthalmic examinations were performed on 144 rhesus macaques without anterior segment pathology. The CCT was measured via ultrasound pachymetry (USP) and specular microscopy, and the ECD was semi-automatically and manually counted using specular microscopy. Rebound tonometry was used to measure IOP. Linear regression and mixed-effects linear regression models were used to evaluate the effects of age, body weight, sex, and IOP on CCT and ECD.

Results: 98 females and 46 males were included, with an age range of 0.2-29.4 years. Mean ± SD CCT by USP and specular microscopy statistically differed at 483 ± 39 and 463 ± 33 μm, respectively ($P < 0.001$). The ECDs were 2717 ± 423 and 2747 ± 438 cells/mm^2 by semi-automated and manual analysis, respectively. Corneal endothelial degeneration was identified in one aged rhesus macaque.

Conclusions: Mean USP and specular microscopy CCT values differed significantly, while semiautomatic and manual ECD were not. CCT was associated to IOP and sex, while ECD was associated with body weight and age ECD ($P < 0.05$). As in humans, corneal disease in rhesus macaques is uncommon.

Translational relevance: Establishing reference values is fundamental in order to utilize rhesus macaques as a model for corneal disease or to identify toxicity in studies of ocular drugs or devices.

2. Introduction

In humans, corneal diseases are a common cause of blindness.[1] Non-human primates (NHP) are valuable animal models to study ocular diseases due to their similar ocular development, anatomy and physiology with humans.[2–5] In particular, rhesus macaques (*Macaca mulatta*) have been useful in studying the development of therapies for diseases that affect the corneal stroma and endothelium.[2,6–10] The corneal endothelium is the innermost layer of the cornea, and is composed of a single layer of highly metabolic cells responsible for maintaining corneal deturgescence, transparency, and refractive power.[11] Disturbance of the stromal architecture or corneal endothelial cell dysfunction can lead to changes in corneal thickness with concomitant decreases in visual acuity.[11]

Normative data are a valuable reference for research and can aid in understanding the limitations and translatability of animal models to human biology. Normative data on central corneal thickness (CCT) and endothelial cell density (ECD) are useful to understand corneal physiology. A previous study reporting ECD in rhesus macaques[12] did not include cell area data or provide correlations with other ocular and biometric parameters. Thus, the purpose of this work was to provide normative data for CCT and ECD from 144 rhesus macaques and determine their relationship to body weight, age, sex, intraocular pressure (IOP). Axial length (AXL) of the eye and refractive error were also measured.

Finally, we also compared agreement between manual and semiautomated analysis for determining ECD as well as use of ultrasound pachymetry (USP) versus specular microscopy for measuring CCT.

3. Material and methods

Animal care

All rhesus macaques in the present study were cared for and examined at the California National Primate Research Center (CNPRC), an accredited Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International institution. All procedures were performed following the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals, the guidelines of the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research, and the protocol approved by the Institutional Animal Care and Use Committee at UC Davis.

Ophthalmic examination

A total of 144 rhesus macaques underwent a single, complete ophthalmic examination. All data were derived from normal animals either prior to use in a study, or from a screening program to identify animals with age-related or potentially inherited ocular abnormalities. Primates with anterior chamber abnormalities were excluded from this study. Ketamine (5–30 mg/kg intramuscularly [IM]), dexmedetomidine (0.015– 0.075 mg/kg IM), and midazolam (0.10 mg/kg IM) were administered prior to the examination and imaging. In a minority of cases, an additional smaller dose of these medications was given to

extend time under anesthesia when necessary. Anesthetics were administered by CNPRC staff under the direction of a veterinarian. Animals were always monitored by a trained technician and veterinarian. Ocular exams were performed with the primate in supine position. The IOP measurements took place with the animal held upright. Ocular imaging tests were done prone with the chin on a chin rest. The eyelids were kept open using a speculum, and regular corneal hydration (GenTeal tears, Alcon, Geneva, Switzerland) was applied regularly during the exam. Studies included external color photography (Rebel T3 EOS, Canon, NY, USA) and rebound tonometry (TonoVet, Icare Oy, Vantaa, Finland) for both eyes. Non-contact specular microscopy (Topcon SP-2000P; Topcon Corp., Tokyo, Japan) was performed to evaluate the corneal endothelium in one eye per animal with laterality chosen at random. After hand-held slit lamp examination (SL-17, Kowa Optics, CA USA), streak retinoscopy (Welch-Allyn, Inc., Skaneateles, NY) was performed following cycloplegia with tropicamide 1% (Akorn Inc, Lake Forest, IL), phenylephrine 2.5% (Paragon BioTeck, Inc., Portland, OR), and cyclopentolate 2% (Alcon Laboratories Inc, Fort Worth, TX) to estimate refractive error in both eyes. Corneal thickness was measured using USP (Pachette 4, DGH Technology Inc., PA, USA) in both eyes. Finally, A-scan ocular biometry (Sonomed Pacscan Plus, Escalon, Wayne, PA) was performed to determine the axial length of both globes. To reverse the anesthesia, atipamezole at a comparable dose to dexmedetomidine was employed after examinations were completed.

Data for CCT was collected using specular microscopy and USP. The ECD was semiautomatically calculated with the same specular microscope with the Endothelial Cell Analysis Module in the IMAGEnet 2000 software package

(Topcon Corp., Tokyo, Japan). The ECD measurements from the central cornea were used for this study as the central ECD is considered to be representative of the full cornea.[12] Simplified cell analysis method was employed in the IMAGEnet 2000 endothelial cell analysis software to determine ECD and cell area using the center method, in which the analyst manually selects the center of the endothelial cell.[13,14] For this study, at least 30 contiguous corneal endothelial cells were selected. To ensure accuracy of the ECD values, one of the authors (M.I.C) estimated the corneal endothelial cell density by planimetry, which involved selecting images of good quality (114) and manually calculating the area of five representative corneal endothelial cells within an area of 0.036 mm^2 using the standardized grid displayed by the Endothelial Cell Analysis Module as a reference.

Statistical analysis

To calculate agreement between USP and specular microscope for CCT values and between manual and semiautomated ECD counts, a concordance correlation coefficient (CCC) and coefficient interval were calculated using values obtained from the same eye. For the CCC, the results were interpreted as previously described, with values > 0.75 indicating good agreement, values between 0.40 and 0.75 indicating moderate agreement, and values < 0.4 indicating poor agreement.[15–17] Normality was determined by the Anderson-Darling test for normality, and *t*-test with Welsh correction was employed to compare statistical differences between USP and specular microscope for CCT values and between manual and semiautomated ECD counts.

A mixed-effects linear regression model was used to evaluate the correlation of an individual's body weight, age, and sex to CCT and ECD. Each NHP was treated as a random effect and all other variables were considered fixed effects. Reference ranges were calculated as a range of ± 2 SD from the mean.[18] The statistical analysis was carried out in R using R packages epiR, *lme4*, and lmerTest and GraphPad Prism version 9.3.1.[19]

4. Results

A total of 144 rhesus macaques were examined in this study, of which 98 were female and 46 were male, with ages ranging from 0.2-29.4 years (**Fig. 1**). Out of the 144 rhesus macaques examined, one presented an abnormal corneal endothelial cell density and cell morphology and was excluded from further analysis.

Mean IOP for the remaining 143 primates was 16 ± 4 mmHg, with a range of 7 to 29 mmHg (**Table 1**). Mean CCT was 483 ± 39 µm using USP and 463 ± 33 µm using specular microscopy significantly differed between the two techniques ($P < 0.001$, **Table 1**). The CCC was 0.47 (CI 0.36-0.57), indicating moderate consistency between CCT generated by USP and specular microscopy (**Fig. 2A1, 2A2**). For the 114 NHPs that had ECD estimated by semiautomatic and manual analyses, mean ECD was 2719 ± 439 and 2747 ± 438 cells/mm^2, respectively (**Table 1**), which was not significantly different ($P = 0.24$). The CCC was 0.88 (CI 0.83-0.91), indicating strong consistency between the ECD calculated by the two analysis techniques (**Fig. 2B1, 2B2**). The mean ECD and corneal endothelial cell area for the 143 primates receiving specular microscopy were 2717 ± 423 cells/mm^2 and 377 ± 59 µm^2, respectively (**Table 1**). Regarding

refractive error and AXL, one highly myopic NHP was found (-17 diopters OU), with a markedly high AXL (26.24 and 25.20 mm for OD and OS, respectively). This primate was deemed to be an outlier and thus excluded from statistical analysis for these parameters. The median refractive error for the remaining 142 primates was 0.75 D (interquartile range 0.25 and 1.25 D) and ranged from -3.75 to +11.5 D (**Table 1**). The median AXL calculated for 142 primates was 19.95 mm (interquartile range 19.51 to 20.49 mm), and values ranged from 17.48 to 22.36 mm (**Table 1**). A linear model analysis found a direct relationship between AXL and age, with an increase of 1 mm in 24 years ($P < 0.0001$).

A mixed-effects linear regression model including sex, age, weight, and differences between left and right to study CCT by USP revealed that females had significantly thicker corneas than males, at 487 ± 41 and 477 ± 35 µm respectively, ($P = 0.024$). A significant correlation was found between IOP and CCT values, with an increase of 1.26 mmHg for each 100 µm increase in CCT ($P = 0.015$, **Fig. 3**). No significant correlations were observed between CCT and age ($P = 0.833$), CCT and weight ($P = 0.123$), CCT and refractive error ($P = 0.574$), or CCT and AXL ($P = 0.470$).

Using a mixed-effects linear regression model for semi-automated ECD measurements using the same parameters, body weight and age were significantly negatively correlated with ECD ($P = 0.006$ and $P < 0.0001$, respectively). The ECD decreased 29 cells/mm^2 for every 1 kg increase in weight and decreased 23 cells/mm^2 for each 1 year of increase in age (**Fig. 4A, 4B and 5**). As expected, ECD and corneal endothelial cell area were strongly and significantly correlated ($R^2 = 0.9985$, $P < 0.0001$). A significant correlation was also observed between semiautomatic ECD and AXL ($P = 0.032$), with an

increase of 80 cells/mm^2 per each mm of increase in AXL, however, this correlation was not significant when using the manual ECD data (P = 0.860). No significant differences were observed between ECD and eye laterality (P = 0.380) or ECD and sex (P = 0.453). No significant correlations were observed between ECD and refractive error (P = 0.166), semiautomatic ECD and USP CCT (P = 0.894) or semiautomatic ECD and specular CCT (P = 0.891).

As for the excluded primate, when examining the ECD data for outliers (**Fig 6A)**, a geriatric male presented low ECD both in the right (OD) and the left eye (OS). The OD was examined when he was 19.6 years old, and the ECD by semiautomatic specular microscopy was 1507 cells/mm^2, (average cell area 663 ± 121 μm^2, **Fig 6C**). Three years later, the OS was examined and was also confirmed to have low ECD values, at 1086 cells/mm^{2,} (cell area 920 ± 244 μm^2, **Fig 6D**). On ophthalmic examination of this animal, nuclear sclerosis and anterior and posterior incipient cataracts were noted in both eyes, as well as vitreous degeneration. The IOPs were normal (19 mm Hg OD, 20 mm Hg OS), and no signs of active or chronic anterior uveitis were present. Although both corneas were transparent, the CCT was slightly thicker than the average CCT of the cohort examined at 541 and 538 μm for OD and OS, respectively. Thus, this NHP's corneas were considered abnormal, and his data were excluded from analysis.

5. Discussion

The use of appropriate *in vivo* models that mimic the structure of the human cornea is key in the study of the pathophysiology of corneal diseases, as well as in the development of therapeutic strategies. While other laboratory

animal models have their own advantages, NHPs have similar corneal thickness, diameter, ECD, and corneal endothelial cell regenerative capacity, all of which are properties that make them an excellent model for the study of corneal disease.[20,21] In this regard, the establishment of reference ranges for healthy, NHP corneas are critical to better understand the advantages and limitations of this species for the study of corneal and corneal endothelial diseases. Herein, we have established reference values for CCT and ECD and elucidated factors that influence these values in rhesus macaques.

Preservation of physiologic IOP values is essential for the maintenance of correct homeostasis and corneal characteristics. In this study, the mean IOP was similar to previous studies in captive[5] and free-ranging rhesus macaques,[22] and humans.[23] As in humans and other studies in rhesus macaques, this study found that corneal thickness has a direct relationship with IOP.[5,24,25] Thus, CCT values should be taken into consideration when interpreting IOP measurements.

Determination of CCT is essential in the diagnosis and monitoring of a wide range of corneal diseases and prior to ocular surgical procedures.[26,27] While there are several techniques available to measure corneal thickness, USP remains the standard technique in humans.[26] Our study compared USP and specular microscopy for CCT measurements in rhesus macaques. Similar to human reports,[28,29] we reported mean CCT by USP to be significantly higher than specular microscopy with moderate concordance between the two types of measurements, suggesting these two devices should not be used interchangeably. The USP CCT in this study was similar to previous studies in rhesus macaques employing the same instrument (486 ± 38 μm)[5], and slightly thinner than human CCT measured by USP (535 ± 34 μm; 547 ± 35 μm).[30,31] In

our study population, females had slightly thicker corneas than males by a mean of 10 μm and that difference was considered statistically significant using a mixed-effect linear regression analysis. While most studies in humans do not find statistically significant differences between male and female CCT,[30,32] some studies have reported variation of CCT in females associated with the menstrual cycle.[33,34] Further studies would be required to deduce if hormonal variation plays a role in corneal thickness in female rhesus macaques. In concordance with some previous studies done in humans, there was no significant age-dependent CCT differences in our study.[32,35] However, there is an interesting debate regarding age-related changes in corneal thickness in humans, with some studies reporting increased CCT with age,[36,37] while others finding the opposite relationship.[27,38] Reasoning for these differences may include inadequate sample size, genetic differences among sample populations, or sampling bias due to the inclusion of patients with ocular, non-corneal alternations.[27]

Manual and semiautomatic ECD values were comparable in this study, supporting the reliability of semiautomatic determination of ECD with specular microscopy in rhesus macaques with healthy eyes. The ECD values obtained in this study are similar to the ones reported in humans (2800 cells/mm^2 and 2737 cells/mm^2 in 30 year-old adults).[39,40] Similar to humans, we have observed an age-related decline in ECD.[41,42] Our mixed-effects linear regression found a positive correlation between semiautomatic ECD and AXL, that was not significant when the analysis was performed with manual ECD values. Although a correlation between ECD and AXL has been previously described in humans,[43] the inconsistency between the results obtained using both datasets suggests that

the effect of AXL in ECD is questionable. In accordance with Lin *et al.,* we also found a correlation between AXL and age.[5]

Fuchs endothelial corneal dystrophy (FECD) is the most common endothelial dystrophy in humans and is characterized by guttae formation on Descemet's membrane and premature degeneration and progressive loss of corneal endothelial cells that leads to corneal edema, bullous keratopathy and vision loss.[11,44] With specular microscopy, patients diagnosed with FECD typically present with low ECD, enlarged endothelial cells with loss of hexagonality, and small multifocal hyporreflective round excrescences of extracellular matrix known as guttae.[11,45] Of the 144 primates examined, one 22.6-year-old male had low corneal ECD with mild pleomorphism and polymegathism, which are characteristics described in humans with corneal endothelial disease.[46] Both eyes had ECD below our lower limit of our reference range (1871 to 3563 cells/ mm^2), but were greater than what has been reported for corneal decompensation in humans (≤500 cells/mm^2).[47,48] Both corneas were clear with a thickness approaching the upper limit of our reference range (406 to 562 μm). In aggregate, the findings in this primate are suggestive of a bilateral endothelial degeneration in a compensated stage. A primary cause for endothelial degeneration was not evident. While guttae-like lesions were not observed in this NHP, it is possible that this rhesus macaque has a heritable corneal endothelial degeneration, similar to FECD in humans. In humans, peripheral corneal endothelial cell density has been shown to correlate with disease severity for FECD.[49]

Limitations of our study are the unbalanced number of females versus males included as well as the lack of peripheral corneal endothelial cell analysis.

Surveys of larger cohorts with specular microscopy that include the peripheral corneal endothelium may lead to the identification of additional NHPs with corneal endothelial disease and a better characterization of this condition.

In summary, in this study we report normative data and reference values of corneal endothelial cell density and corneal thickness and their relationships with age, body weight, sex, axial length, and IOP. As in humans, rhesus macaques with low ECD are rare but can be found within the population. The measurements obtained expectedly align with human parameters, highlighting the similarity in anatomy as well as the value of NHP research models for the study of corneal diseases.

6. Acknowledgments

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Tables

Table 1. Age, weight, and ocular biometric findings, and determined reference ranges from 143 rhesus macaques with healthy corneas included in the study**.**

	Mean ± SD	Range	Reference range
Age (years)	13.8 ± 7.1	0.2-29.4	
Weight (Kg)	10.1 ± 3.8	1.0 to 23.9	
CCT USP (µm)	484 ± 39	367 to 593	406 to 562
CCT Specular (µm)	463 ± 33	388 to 568	403 to 529
ECD (cells/mm^2)	2717 ± 423	1853 to 3864	1871 to 3563
EC area (µm^2)	377 ± 59	258 to 539	259-495
IOP (mm Hg)	16 ± 4	7 to 29	8 to 24

Figures

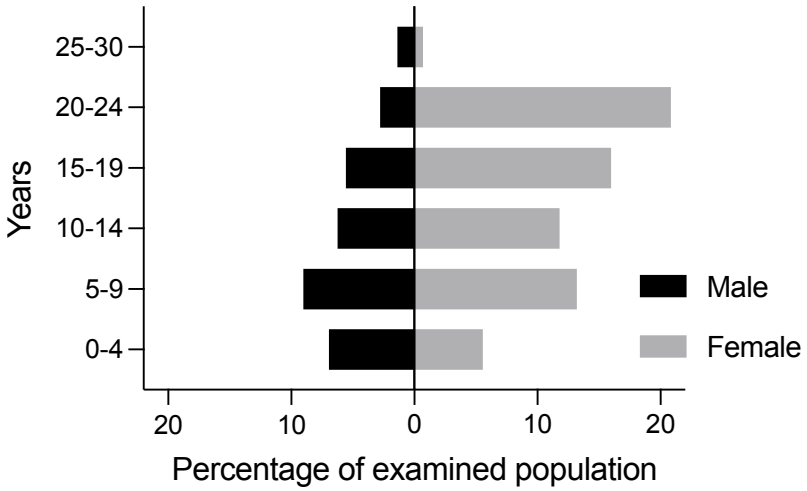

Figure 1. Age and sex of the 144 rhesus macaques examined in this study. The NHPs were divided into 6 age groups: 0 to 4 years (8 females, 10 males), 5 to 9 years (19 females, 13 males), 10 to 14 years (17 females, 9 males), 15 to 20 years, (23 females, 8 males), 20 to 24 years (30 females, 4 males) and 25 to 30 years (1 female, 2 males). Females were overrepresented (n=98, 68%).

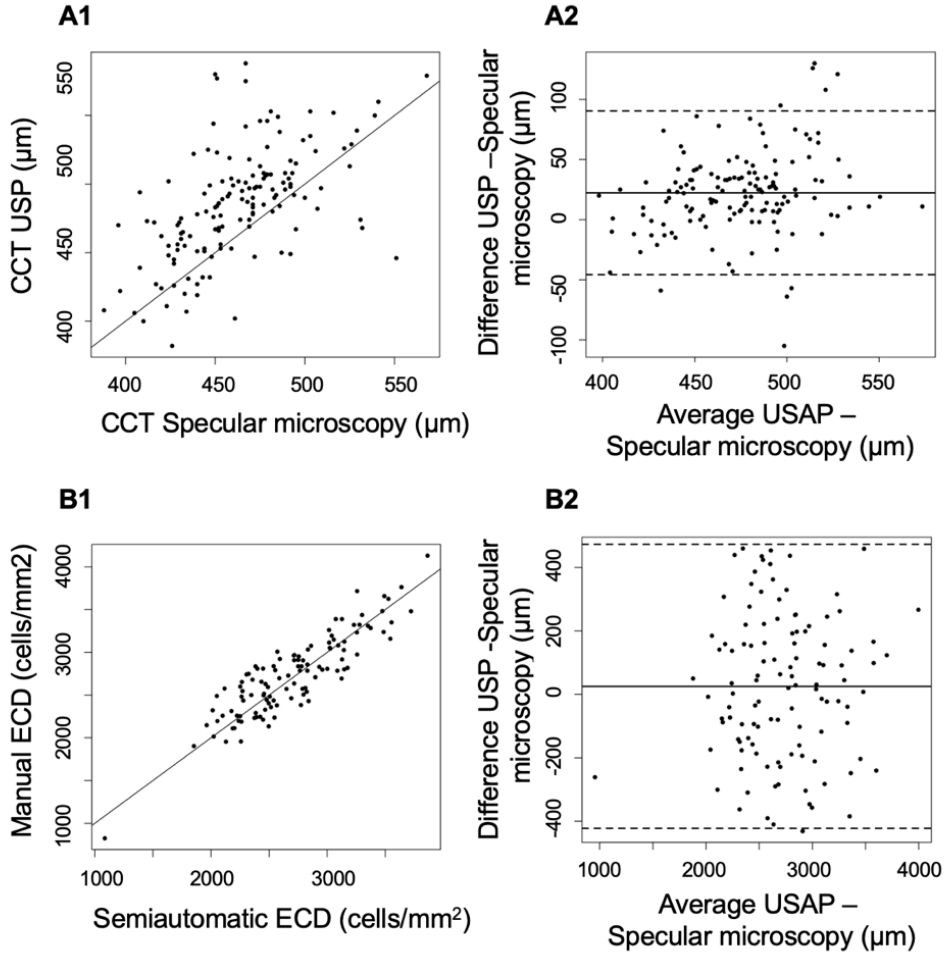

Figure 2. Scatterplots and Bland-Altman plots of USP and specular measurements for CCT (A1, A2), and ECD semiautomatic and manual measurements (B1, B2) in 143 primates without corneal disease. For the Bland-Altman plots (A2, B2), the vertical axis shows the difference among the two types of measurements and the horizontal axis is the mean value of the two types of measurements. The dashed lines represent the 95% limits of agreement and the black line the mean of the differences between the two types of measurements. The CCC was 0.47 (0.36-0.57) for CCT measurements, and 0.88 (0.83-0.91) for ECD, indicating moderate and strong agreement between techniques, respectively.

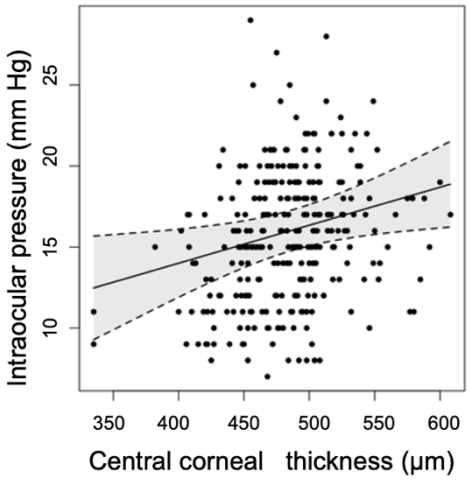

Figure 3. CCT measured with USP and IOP measured by rebound tonometry were directly related in 286 eyes of 143 primates with healthy corneas. The area in grey corresponds with the 95% CI. For every 1.26 mm Hg increase in IOP the CCT increases by 100 µm (P = 0.015, R^2 = 0.07).

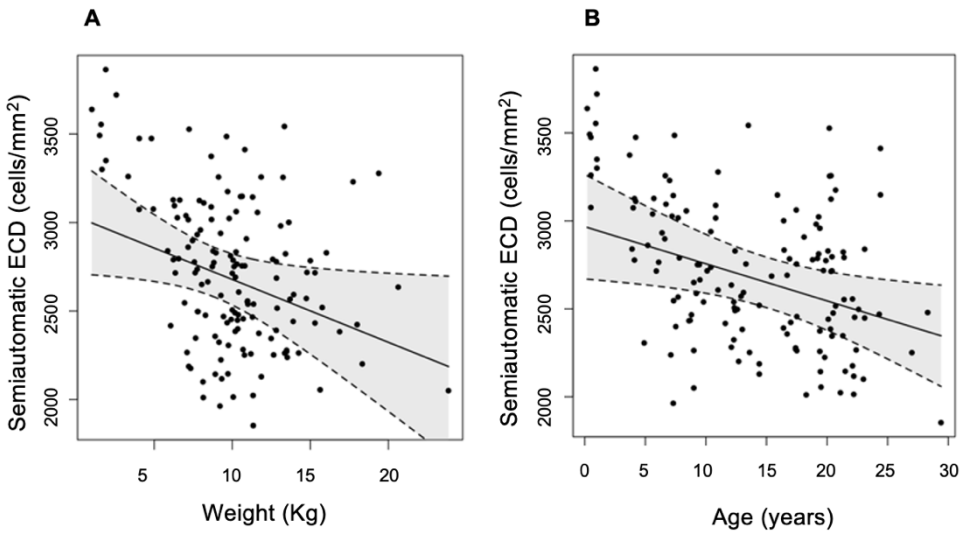

Figure 4. An indirect relationship was identified between (A) ECD and body weight, and (B) ECD and age in 143 eyes of 143 primates with healthy corneas. For each 1 kg increase in body weight, ECD decreases by 29 cells/mm^2 (P = 0.006, R^2 = 0.17). For each additional year of age, ECD decreases by 23 cells/mm^2 (P < 0.0001, R^2 = 0.22). The area in grey corresponds with the 95% CI.

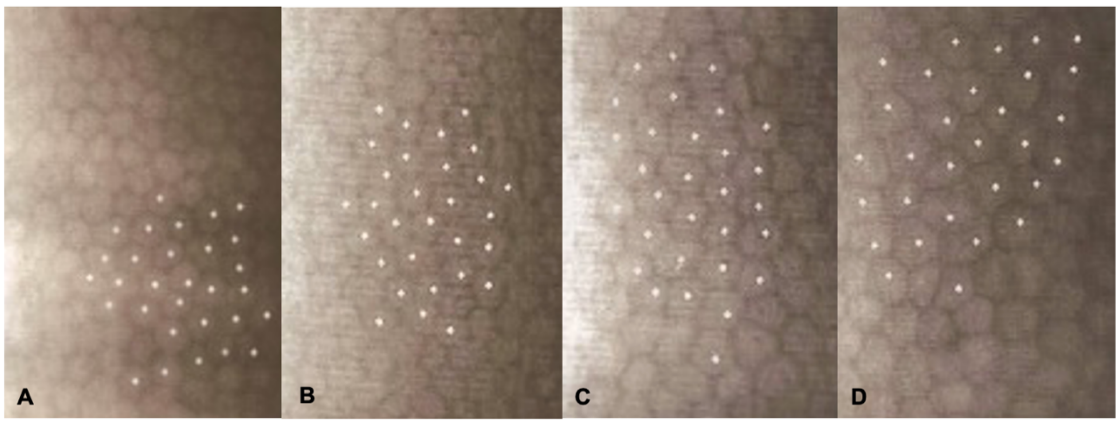

Figure 5. Corneal endothelial cell appearance using specular microscope in healthy rhesus macaques at different ages. While the cell morphology remains regular and mostly hexagonal, lower ECD and increased cell area were observed in older individuals. Rhesus macaques of 0.4 years (A, 3061 cells/mm^2, mean ±SD cell area 366 ± 69 μm^2), 10.8 years (B, 3088 cells/mm^2, mean cell area 323 ± 51), 22.6 years (C, 2497 cells/mm^2, mean cell area 400 ± 24 μm^2) and 29.4 years (D, 1853 cells/mm^2, mean cell area 539 ± 17) are shown. White dots in the center of some cells were placed manually for analytical processing.

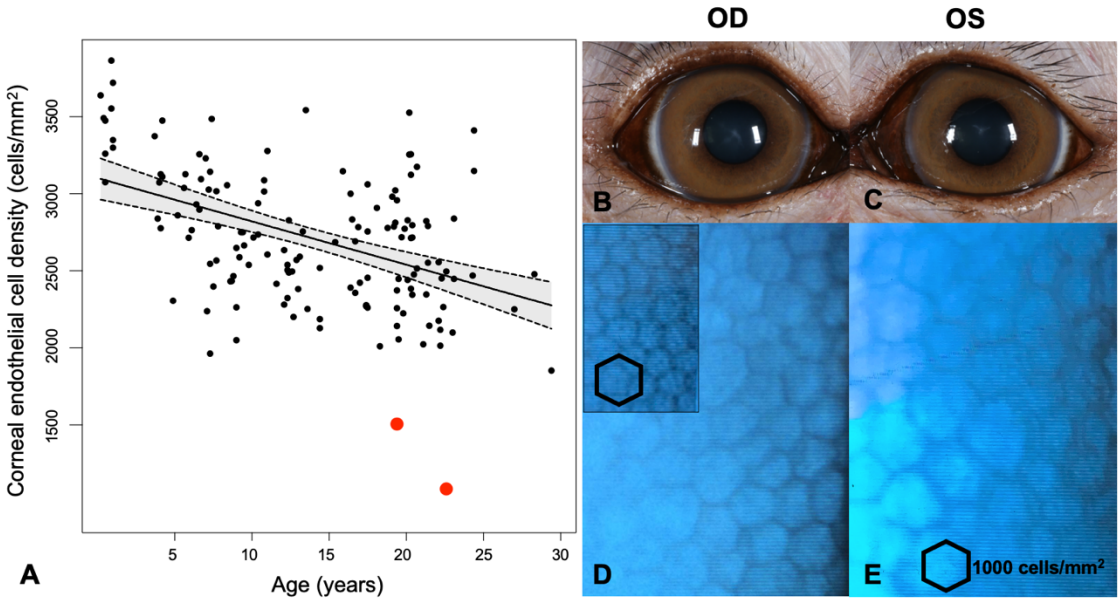

Figure 6: A geriatric male rhesus macaque with low corneal ECD, mild pleomorphism, and polymegathism in both eyes. In a scatterplot for analyzing outliers (A), the value for OD and OS of the rhesus macaque is outside of the cluster. The area in grey corresponds with the 95% CI. Anterior segment appearance was normal OD (B) and OS (C). Specular microscopy revealed low ECD at 19.3 years old (D, 1507 cells/mm^2, OD) and at 22.6 years old (E, 1086 cells/mm^2, OS). Both eyes also had larger cells when compared with other primates (663 ± 121 μm^2 and 920 ± 244 μm^2 for OD and OS, respectively). Subjective loss of hexagonality of the endothelial cells was also apparent. The inset in D includes detail of the specular microscopy at same magnifications of a 21.4-year-old female with normal corneal endothelial morphology (ECD 2457 cells/mm^2).

CHAPTER 3: TOPICAL NETARSUDIL FOR THE TREATMENT OF PRIMARY CORNEAL ENDOTHELIAL DEGENERATION IN DOGS

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1. Abstract

Purpose: To evaluate the tolerability and efficacy of the topical rho-kinase inhibitor netarsudil applied twice daily for the treatment of canine primary corneal endothelial degeneration (PCED).

Methods: Twenty-five eyes of 20 client-owned dogs with PCED were enrolled in a prospective, randomized, placebo-controlled clinical trial. Ophthalmic examination, intraocular pressure measurement, Schirmer tear test I, ultrasonic pachymetry, Fourier-domain optical coherence tomography, and *in vivo* confocal microscopy were performed prior to and at one, two, four, six and eight months

of enrollment. The patients received topical netarsudil 0.02% (Rhopressa®) or vehicle control twice daily for the initial four months and all patients received netarsudil for the final four months.

A preliminary analysis was completed using the data from the first 14 dogs enrolled (18 eyes). The effect of treatment on central corneal thickness (CCT), corneal stromal thickness, percentage of cornea affected by edema, and endothelial cell density (ECD) was evaluated by repeated measures ANOVA. Additionally, Kaplan-Meier curves and log-rank test were used to compare the corneal edema progression of eyes in the treatment group versus eyes in placebo group. Eyes were classified as improved, progressed, or stable at four and eight months using clinical response criteria.

Results: All dogs developed conjunctival hyperemia in at least one eye during the study period. Unilateral transient reticulated intraepithelial bullae and a stromal hemorrhage were observed in 2 dogs in the treatment group. Two dogs showed persistent lower tear production while receiving netarsudil, requiring topical immunomodulatory treatment. There were no significant differences in CCT, corneal stromal thickness or ECD between treated and non-treated eyes.

Conclusions: Netarsudil is generally well-tolerated in dogs with PCED. With the data analyzed so far, Netarsudil treatment did not result in significant clinical improvement in dogs with PCED. Inclusion of the data from all 20 dogs enrolled in this study is warranted to determine if topical netarsudil slows progression of PCED in dogs.

2. Introduction

The corneal endothelium is responsible for the maintenance of stromal dehydration by active transport of ions using Na^+/K^+ ATPase pumps.[1] The normal adult canine corneal endothelium is thought to have very limited to no regenerative capacity, similar to the human corneal endothelium,[2] thus, a dramatic loss of function or of the number of corneal endothelial cells can lead to decompensation and corneal edema.[1] Age-related endothelial degeneration and corneal endothelial dystrophy are the two recognized causes of primary corneal endothelial degeneration (PCED).[3,4] In humans, the most common form of corneal endothelial dystrophy is Fuchs' endothelial corneal dystrophy (FECD), which is one of the leading causes of penetrating and endothelial keratoplasty in humans.[5,6] Lamellar and endothelial keratoplasty have also proven to improve corneal thickness and corneal edema in canine patients with corneal endothelial dystrophy.[7,8] However, the lack of donor tissue is a major limitation to the implementation of these surgical procedures in veterinary medicine, and while corneal transplantation is centerpiece on treatment of FECD in humans,[9] it is rarely performed in canine patients. Instead, palliative therapy to relieve the clinical symptoms are available.[10] Topical application of sodium chloride hypertonic ophthalmic ointment (5%) has been employed to reduce or delay corneal edema progression,[11] despite limited efficacy at reducing corneal thickness in normal dogs.[12] Palliative surgical procedures such as superficial keratectomy and conjunctival advancement hood flap (SKCAHF) may be advised in some dogs in advanced stage of disease.[13,14] However, these surgical treatments are invasive, entail surgical and anesthetic risks, and the effect is temporary, which are limitations that must be taken into consideration.

The Rho-associated coiled-coil kinases (ROCK) 1 and 2 are widely distributed in the human body and have multiple functions, including regulation of the cytoskeleton, cellular contraction and cellular motility, and regulation of the cellular morphology, polarity, apoptosis, and cell division.[15] *In vitro* studies have shown that activation of the Rho/Rho kinase pathway is involved in corneal endothelial cell apoptosis,[16] and that ROCK inhibitors promote corneal endothelial cell survival and adhesion.[17] *In vivo* studies in rabbit,[18] dogs,[19] and non-human primates (NHP)[20] employing cryoinjury models have shown that topical ROCK inhibitors accelerate corneal endothelial regeneration after corneal endothelial wounding.[18,20] A pilot study in humans reported improvement in corneal clarity and vision associated with topical netarsudil 0.02% ophthalmic solution administration in patients with FECD.[21] Furthermore, ROCK inhibitors have also shown to enhance the efficacy of cell-based therapies when combination with injected corneal endothelial cells in rabbit and NHP models.[22] These inhibitors facilitated a faster recovery in vision in human patients with endothelial disease undergoing Descemet stripping surgery.[23] Based on these promising data, we previously completed a prospective, open-label clinical trial to determine the safety and efficacy of the ROCK inhibitor ripasudil (Glanatec®, 0.4% ripasudil) as a treatment for canine PCED. This study demonstrated stabilization or improvement of clinical disease in more than half of the eyes included in the study after one year of treatment.[24] A more favorable response was found in canine patients in early stage of disease.[24] However, ripasudil is not commercially available at the US and requires four times a day (QID) application.

Rhopressa® (netarsudil 0.02% ophthalmic solution) is an FDA-approved ROCK inhibitor and norepinephrine transporter inhibitor for the treatment of

glaucoma in humans that is typically administered once or twice a day (BID). The purpose of our study was to evaluate the tolerability and efficacy of topical ROCK inhibitor netarsudil administered twice daily for the treatment of canine PCED using a prospective, double-masked, placebo-controlled clinical trial.

3. Material and methods

Animals

Dogs included in this study were exclusively client-owned. This study was approved by the Institutional Animal Care and Use Committee of the University of California-Davis (#21327) and was in concordance with the Association for Research in Vision and Ophthalmology resolution on the use of animals in research. Dogs with a presumptive diagnosis of PCED assigned by board-certified veterinary ophthalmologists from the University of California-Davis Veterinary Medical Teaching Hospital and other specialty hospitals in California and Nevada between March 2020 and October 2021 were included in this study. Dogs diagnosed with causes of secondary corneal endothelial cell injury such as glaucoma, intraocular surgery, lens instability, diabetes, and endotheliitis were excluded,[25–28] and only eyes with <40% of the cornea affected by edema were included on the study. Prior to enrollment, informed consent was obtained for all dogs.

Treatment:

During the first four months, study patients were randomly assigned into treatment or placebo group, and either received one drop of netarsudil 0.02% ophthalmic solution (Rhopressa®, Aerie Pharmaceuticals, NY, USA) or placebo

(Rhopressa® vehicle) BID. Owners and clinicians were masked to the treatment during the first four months to objectively evaluate potential adverse reactions and benefits of receiving topical netarsudil. To evaluate long-term effects of the medication and as an incentive for owners, all dogs then received topical netarsudil for four additional months.

Owners recorded in a drug log form the time of application of the medication during the study. Additional medications, such as hypertonic saline ophthalmic ointment for the treatment of corneal edema and/or bullous keratopathy or any other ophthalmic medications, were prescribed when needed and noted for all dogs.

Ophthalmic examination and imaging

Prior to enrollment, all patients received a complete, baseline ophthalmic examination to confirm the diagnosis of PCED (**Fig. 1**). The exam included a Schirmer tear-test 1 (STT1; Intervet, Inc., Summit, NJ, USA), intraocular pressure (IOP) measurement by rebound tonometry (TonoVet; Icare® Finland), handheld and digital slit-lamp biomicroscopy (SL-15; Kowa American Corporation, Torrance, CA, USA and Imaging Module IM 900; Haag Streit, Koeniz, Switzerland), and binocular indirect ophthalmoscopy (Keeler Instruments Inc., Broomall, PA, USA) using a 28 diopter (D) indirect lens (Volk Optical, Inc., Mentor, OH, USA). Color photography (Canon OD 5S) with ImageJ software[29] were employed to evaluate the area of the cornea affected by edema.

The central corneal thickness (CCT) was measured using ultrasound pachymetry (USP Pachette 3; DGH Technology, Inc., Exton, PA, USA). Intravenous sedation with acepromazine (0.005-0.02 mg/kg) and butorphanol

(0.1-0.3 mg/kg) or dexmedetomidine (1-3 mcg/kg) was administered as needed for imaging. The FD-OCT (RTVue 100, software version 6.1, 26000 A scan/sec, 5-µm axial resolution, 840-nm superluminescent diode, Optovue, Inc., Fremont, CA, USA) was employed to identify the different layers of the cornea and manually measure their thickness, as previously described.[25,30] *In vivo* confocal microscopy (IVCM) (ConfoScan 4 Nidek Technologies, Gamagori, Japan and ConfoScan 4 NAVIS imaging software) of the central cornea was performed to evaluate corneal endothelial cell morphology as described previously (**Fig. 1**).[25,30,31] The corneal endothelial cell density (ECD) was calculated from IVCM scans following a system previously reported.[24,32] For ECD calculations, the individual analyzing the images was masked to both the patient identity and timepoint at which the images were obtained. Lastly, corneas were stained with fluorescein sodium (Ful-Glo strips USP 1mg; Akorn Inc) to assess for corneal ulceration. At the completion of the exam, intramuscular atipamezole (1-3 mcg/kg) was administered as reversal for dogs receiving dexmedetomidine. Afterwards, ophthalmic examinations with the same advanced imaging as in baseline were performed one, two, four, six, and eight months after starting the medication. Additionally, patients received a basic ophthalmic exam including IOP measurement 1 week after starting the treatment to identify any potential adverse reactions.

Clinical response criteria

Eyes were classified as progressed, stable or improved using the following response criteria combined when compared with baseline: (1) eyes were considered improved if they demonstrated a >10% increase in ECD, a >10%

decrease in percentage of cornea affected by edema, and/or a >20% decrease in CCT; (2) eyes were considered progressed if they demonstrated a >10% decrease in ECD, a >10% increase in percentage of cornea affected by edema, and/or a >20% increase in CCT; (3) eyes were classified as stable if they did not meet criteria for improved nor progressed disease.

The percentage of change for CCT, corneal stromal thickness, ECD, and corneal edema for eyes in the treatment group was calculated comparing the 8-month and the baseline exam. A significant decrease on CCT was deemed to be below 20% from the previous value taking into consideration that some patients would receive sodium chloride hypertonic ophthalmic ointment that might influence CCT values.

The percentage of change for eyes in the placebo group was calculated in two steps: difference comparing the 4-month and baseline exam (period receiving placebo) and difference comparing the 8-month and the 4-month exam (period receiving topical netarsudil).

Statistical analysis:

A power analysis was performed and demonstrated that 20 dogs would allow us to detect a 29% difference in central corneal thickness between netarsudil (n = 10) and vehicle treated (n = 10) groups with a power of 0.8 and an alpha of 0.05; a previously reported difference in mean ± SD of 483 ± 357 µm between baseline and 2 months post- SKCAHF was used.[13] Data from 7 dogs with CED and a CCT of >1000 mm at baseline were used in the analysis (baseline = 1271 ± 247 µm and 2 months post-SKCAHF surgery = 788 ± 226 µm).[13] The differences in CCT, percentage of the cornea affected by edema, and ECD

across timepoints were evaluated by repeated measured analysis of variance (ANOVA) or a Friedman test for normally and non-normally distributed data, respectively. Normality was determined for each data set by the Shapiro Wilk test for normality. Post-hoc tests were performed using Wilcoxon signed ranks. Corneal edema progression was represented using Kaplan-Meier curves for the treatment and placebo group, in which more than 10% of increase of corneal edema between timepoints was considered progression. Log-rank test was employed to evaluate differences between curves. For all statistical analysis, the data from each eye included in each group were considered individually. The statistical analysis was carried out in GraphPad Prism version 9.3.1.

4. Results

Study population:

Eighteen eyes of 14 PCED-affected dogs were included in this preliminary analysis. Seven dogs were included in the placebo and seven dogs were included in the treatment group. The study population consisted of 4 Boston terriers, 3 Chihuahua mixed breed dogs, 3 Jack Russell terriers, 1 Labrador retriever, 1 basset hound, 1 standard poodle, 1 shih tzu mixed breed (**Table 1**). Mean age at enrollment in the treatment group was 11.2 ± 0.7 years and 10.7 ± 2.9 years in the placebo group. Out of the 14 dogs, 10 (4 Boston terrier, 3 Jack Russell terriers and 3 Chihuahuas) were diagnosed with corneal endothelial dystrophy, while the remaining 4 dogs were diagnosed with age-related endothelial degeneration. In the placebo group, five dogs were treated unilaterally, and two dogs were treated bilaterally during the first four months and then received netarsudil in the aforementioned eyes for the last four months. The remaining seven dogs

received topical netarsudil during the entire study period (five received unilateral treatment and two received bilateral treatment). One patient was humanely euthanized for reasons unrelated to ocular disease five months after enrolling the study.

Tolerability

The most common adverse reaction observed with drug treatment was conjunctival hyperemia, which was recorded at least once during ophthalmic examination in all dogs (**Table 1**). Reticulated intraepithelial bullae were observed in one eye receiving topical netarsudil of one dog at a single timepoint that resolved without discontinuing the medication. One patient that had previously diagnosed with corneal pigmentation and vascularization and was receiving cyclosporine 0.2% ophthalmic ointment (Optimmune®, USP, Merk & Co, Kenilworth, NJ) developed a corneal stromal hemorrhage in one eye at the end of the trial. After discontinuation of topical netarsudil for one month, the hemorrhage resolved within 1 month but had corneal melanosis was observed at four months after the diagnosis of the stromal hemorrhage (**Fig. 2**).

No differences in STT or IOP were observed between treatment and placebo groups at any timepoint (**Fig. 3**). Of the fourteen dogs included in this study, three were presented with decreased tear production while receiving topical netarsudil (bilaterally in two patients, and unilaterally in one). Two of the three patients had low tear production 2 months after receiving Rhopressa® that required cyclosporine 0.2% ophthalmic ointment (Optimmune®) in one (n=1) or both (n=1) eyes with a frequency that ranged from every other day to BID. One patient had a IOP decrease from 17 to 10 mm Hg one week after initiating topical

netarsudil. No signs of ocular inflammation were observed. After receiving ketorolac tromethamine 0.5% ophthalmic solution once daily for a week, the IOP returned to a normal value (14 mm Hg) and remained at that pressure over the rest of the study; the ketorolac was discontinued at that time. One patient developed ocular hypertension in one eye (28 mm Hg) 6 months after starting the topical netarsudil, that was managed with dorzolamide HCl 2% ophthalmic solution applied 3 times a day during the next 2 months. The IOP was then stable (21) and the frequency of topical dorzolamide was reduced to BID for the remainder of the study.

Efficacy

No significant differences were observed in CCT measured at baseline with USP between the placebo-treated (mean 746 ± 115 μm) and netarsudil-treated (mean 646 ± 128 μm) groups (P = 0.136). No significant differences in CCT or corneal stromal thickness were observed between netarsudil- and placebo-treated groups at four and eight months (P = 0.99 and P = 0.97 respectively, **Fig. 4a**).

Corneal stromal thickness measured with FD-OCT at baseline did not significantly differ between the placebo (mean 625 ± 122 μm) and the treatment group (mean 569 ± 193 μm, P = 0.474). No significant differences in stromal thickness were observed between the groups at four (P = 0.484) and eight months (P = 0.588 **Fig 4b**), nor were there differences between baseline and 4 months (P =0.245 and P = 0.885 for the placebo and treatment group, respectively) and baseline and 8 months (P = 0.460 and P = 0.717).

At baseline, mean ECD did not significantly differ between the groups (1035 $\pm$ 572 cells/mm^2 in the placebo group and 1423 $\pm$ 607 cells/mm^2 in the treatment group; P = 0.181). No significant differences were observed in ECD between the placebo group and the treatment group at four and eight months (P = 0.998 and P = 0.878 respectively, **Fig. 4c**), nor between baseline and 4 months (P =0.269 and P = 0.753 for the placebo and treatment group, respectively) and baseline and 8 months (P = 0.362 and P = 0.964).

Of the eighteen eyes included on this study, two eyes from two dogs in the placebo group and two eyes from two dogs in the treatment group had corneal edema at baseline, and fourteen had clear corneas. At the end of the study, four eyes from four dogs (two in the treatment group, two in the placebo group) developed corneal edema. The percentage of the cornea affected by edema did not differ between baseline and four months (P = 0.999) and eight months (P = 0.909) of treatment (**Fig. 4d**).

When evaluating the nine eyes in the treatment group using the clinical response criteria, seven eyes progressed, one improved, and one remained stable at the end of the trial. In the placebo group, during the first four months, four eyes progressed, four were stable and one improved (**Table 2**). Eight eyes remained in the trial after the placebo phase and received netarsudil for four months. Of those, two eyes improved, four eyes remained stable, and two eyes progressed (**Table 2**).

The response for each eye using the clinical response criteria in dogs that received medication in both eyes was disparate. Two dogs received netarsudil in both eyes for eight months, with one dog experienced a bilateral decrease in ECD. By contrast, the other dog showed progression of corneal edema, CCT and

reduced ECD in one eye, and marked improvement in the second eye with a >50% increase in ECD values at the end of the trial (**Fig. 5**).

Hypertonic saline ointment as additional medication for corneal edema

Five out of eighteen eyes (three in treatment group and two in the placebo group) started the study receiving QID topical hypertonic saline ointment. Two eyes in the treatment group and two eyes in the placebo group started hypertonic saline ointment QID during the study (**Table 1**).

6. Discussion

The purpose of this study was to evaluate the safety and efficacy of topical netarsudil twice a day as a non-surgical alternative therapy to stabilize or slow disease progression in patients diagnosed with PCED that had <40% of the cornea affected by edema. Our interim analysis indicates that netarsudil is well-tolerated in dogs, however, with the data analyzed to date no efficacy was detected in dogs with PCED.

In this clinical trial we confirmed that similar to previous studies,[24,33] twice a day topical netarsudil is well tolerated in dogs, with conjunctival hyperemia being the most common adverse reaction. In our study, one patient experienced transient reticulated intraepithelial edema, which is an adverse reaction described in physician-based and veterinary-based ophthalmology following ROCK inhibitor treatments. [24,34] Another patient developed a corneal stromal hemorrhage by the end of the study, which is an adverse reaction that was previously reported in canine patients receiving a different ROCK inhibitor.[24] Other adverse reactions described in human patients receiving topical netarsudil such as corneal verticillata were not observed in this study.[9] Two patients were diagnosed with

dry eye and required topical immunomodulatory treatment. A review of the literature did not identify any known association between ROCK inhibitors and alterations on tear production. One possibility may be that the patients in this study developed age-related dry eye, which is common in older dogs, but a potential association between topical netarsudil and dry eye cannot be excluded. As such, we recommend monitoring tear production in patients receiving topical netarsudil.

Due to their IOP-lowering effects, ROCK inhibitors are used to treat glaucoma in physician-based ophthalmology.[9] Consistent with a previous study,[35] IOP did not significantly differ from baseline or between groups. However, one eye of one dog was presented with a decrease in IOP where treatment with an anti-inflammatory medication was initiated for one week. Thus, monitoring of IOP in canine patients receiving topical netarsudil is recommended.

Our study, using 14 dogs and our clinical criteria, did not identify a significant improvement associated with the netarsudil treatment. Additionally, in the treatment group, only 1 eye was stable and 1 eye improved whereas, in the other 7 eyes, there was progression in at least one of the clinical parameters studied at the end of the study. Our previous study with another ROCK inhibitor, ripasudil, demonstrated a more favorable response in canine PCED patients in early stage of disease, with 7 out of 11 eyes classified as clinically stable or improved 1 year after starting topical ripasudil.[24] However, the current analysis is incomplete and underpowered, as we still need to include the data from 6 additional dogs to confirm whether netarsudil is effective for PCED in patients in early stage of disease.

Cell-to-cell contact inhibition is an antiproliferative regulatory mechanism, present in corneal endothelial cells.[36] In humans, removal of a portion of damaged corneal endothelium can stimulate proliferation and migration of the adjacent healthy endothelial cells, and the effect is thought to be boosted with the addition of ROCK inhibitors treatment.[37] Indeed, ROCK inhibitors have been shown to facilitate a faster recovery of corneal transparency in FECD patients undergoing surgical procedures or transcorneal freezing.[20,23] Similarly, our previous study employing a canine model with healthy endothelium showed significant reduction in CCT and increase in ECD after cryoinjury in eyes treated with the ROCK inhibitor Y27632 when comparing with vehicle control;[19] however, the potential benefit of a combined therapy in canine patients with PCED is still unknown. Future studies to test the synergic effectivity of combining ROCK inhibitors and corneal surgical interventions in canine PCED-patients are warranted.

When conducting a double-masked, prospective clinical trial there are certain limitations that need to be acknowledged. In our study, we detected a significant difference in corneal stromal thickness at baseline between the treatment and placebo group. The incorporation of sodium chloride hypertonic ophthalmic ointment as regular treatment for some patients during the trial might have also impacted corneal edema and corneal thickness values, as it is well known that this medication helps to reduce corneal edema and reduce corneal thickness.[10,12] In addition, the inclusion of data from the remaining 6 patients included in this clinical trial will improve the power and reliability of the results in future analyses.

7. Conclusion

Topical netarsudil is well tolerated in PCED-affected canine patients. Conjunctival hyperemia that did not require medical treatment was the most common adverse reaction. An association between the treatment and thinner cornea, higher ECD values, or slower corneal edema progression was not observed in this study. Inclusion of data from more patients is required to strengthen the results of this interim analysis.

8. Acknowledgments

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9. Conflict of interest

Netarsudil (Rhopressa®) and its vehicle were donated by Aerie Pharmaceuticals. Aerie Pharmaceuticals had no input into the study design, analysis or interpretation of the data presented in the manuscript.

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1. Tables

Table 1. Summary of patient demographics, hypertonic saline ophthalmic ointment prescription, and adverse reactions of animals included in our clinical trial

	Group	Eyes in study	Breed	Age	Sex	Diagnosis	Start hypertonic ointment	Adverse reactions
1	Treatment	2	Boston terrier	11.3	M	CED	During trial	Conjunctival hyperemia
2	Treatment	2	Jack Russell terrier	10.2	F	CED	No	Conjunctival hyperemia, unilateral reticulated intraepithelial bullae
3	Treatment	1	Chihuahua mix	11.1	M	CED	Before trial	Conjunctival hyperemia, decreased IOP
4	Treatment	1	Boston terrier	9.8	F	CED	Before trial	Conjunctival hyperemia
5	Treatment	1	Chihuahua mix	11	F	CED	Before trial	Conjunctival hyperemia
6	Treatment	1	Chihuahua mix	10.7	F	CED	Before trial	Conjunctival hyperemia
7	Treatment	1	Basset hound	12.26	F	Age-related endothelial degeneration.	No	Conjunctival hyperemia
8	Placebo	2	Jack Russell terrier	6.8	M	CED	No	Conjunctival hyperemia, bilateral dry eye
9	Placebo	2	Jack Russell terrier	7.2	F	CED	No	Conjunctival hyperemia
10	Placebo	1	Boston terrier	9.6	F	CED	During trial	Conjunctival hyperemia, unilateral dry eye
11	Placebo	1	Labrador retriever	11.7	M	Age-related endothelial degeneration.	During trial	Conjunctival hyperemia
12	Placebo	1	Boston terrier	14.1	F	CED	Before trial	Conjunctival hyperemia
13	Placebo	1	Standard poodle	11.1	M	Age-related endothelial degeneration.	No	Conjunctival hyperemia
14	Placebo	1	Shih tzu mix	13.2	F	Age-related endothelial degeneration.	Before trial	Conjunctival hyperemia

Table 2. Summary of patient progression on the trial

	Group	Baseline to 4 months	4 to 8 months	Baseline to 8 months
Dog 1 eye 1	Treatment	Progressed	Progressed	Progressed
Dog 2 eye 1	Treatment	Improved	Progressed	Progressed
Dog 3 eye 1	Treatment	Stable	Progressed	Progressed
Dog 4 eye 1	Treatment	Progressed	Progressed	Progressed
Dog 4 eye 2	Treatment	Improved	Improved	Improved
Dog 5 eye 1	Treatment	Progressed	Stable	Progressed
Dog 5 eye 2	Treatment	Progressed	Stable	Progressed
Dog 6 eye 1	Treatment	Progressed	Improved	Stable
Dog 7 eye 1	Treatment	Progressed	Improved	Progressed
Dog 8 eye 1	Placebo	Progressed	Improved	Improved
Dog 9 eye 1	Placebo	Stable	Improved	Improved
Dog 10 eye 1	Placebo	Stable	Stable	Stable
Dog 10 eye 2	Placebo	Stable	Improved	Improved
Dog 11 eye 1	Placebo	Progressed	Stable	N/A
Dog 12 eye 1	Placebo	Improved	Progressed	Improved
Dog 12 eye 2	Placebo	Progressed	Improved	Improved
Dog 13 eye 1	Placebo	Progressed	Progressed	Progressed
Dog 14 eye 1	Placebo	Stable	Stable	Progressed

The overall clinical response criteria progression observed on the patients participating in the trial. Eyes were considered improved if they demonstrated a >10% increase in ECD, a >10% decrease in percentage of cornea affected by edema, and/or a >20% decrease in CCT. Eyes were considered progressed if they demonstrated a >10% decrease in ECD, a >10% increase in percentage of cornea affected by edema, and/or a >20% increase in CCT. Eyes were classified as stable if they did not meet criteria for improved nor progressed disease.

11. Figures

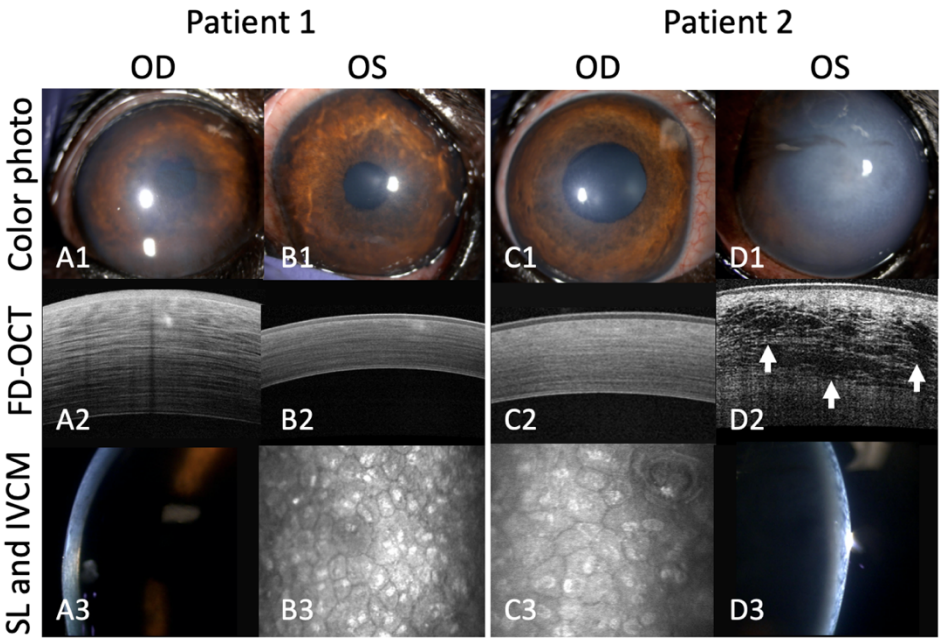

Figure 1: Corneal appearance, structure, and cell morphology for two PCED patients enrolled in the clinical trial: Both patient 1 and 2 were spayed female Boston Terriers, aged 9.8- and 9.6-years old, respectively. At baseline exam, mild focal and moderate diffuse corneal edema in >40% of the corneal surface is evident on the right (A1) and left eye (D1) of each patient, which excluded those eyes from participating in the trial. The contralateral eyes of both patients were in a compensated stage at baseline (B1, C1). Characteristic disorganization of stromal collagen fibrils (A2, D2) and anterior stromal water clefts (D2, arrows) were observed with SD-OCT. Imaging of the contralateral eyes in compensated stage (no corneal edema) is displayed for reference (B2, C2). Moderate pleomorphism and polymegathism of the corneal endothelium as well as multinucleated cells were observed with IVCM in both patients (B3, C3). With slit-lamp, increase in corneal thickness (A3, D3) and anterior stromal bullae (bullous keratopathy, D3) were also identified.

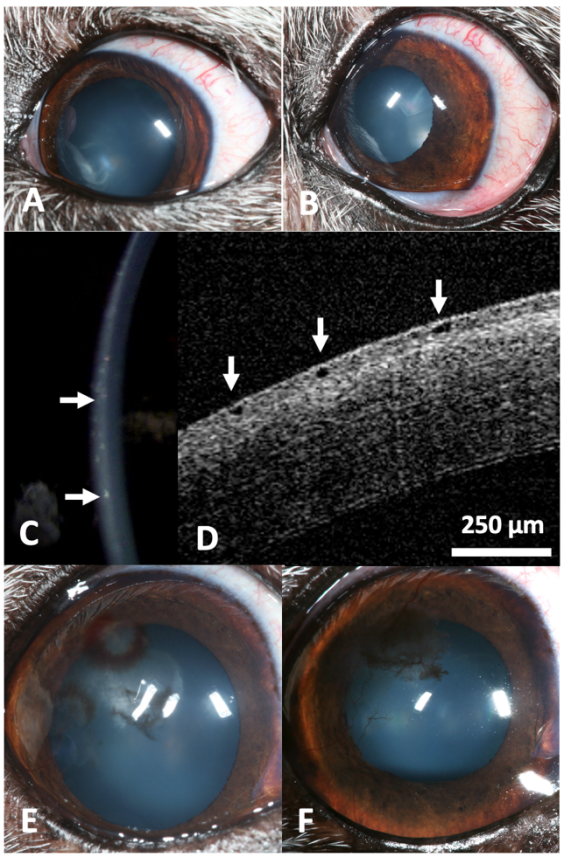

Figure 2. Adverse reactions observed in a 10-year-old female Jack Russell terrier (A, B, C) and a 12-year-old Chihuahua mix (D) receiving topical netarsudil twice daily. Mild conjunctival hyperemia developed after 1 week of topical netarsudil application (A2) in comparison to before the use of topical netarsudil (A1). With slit lamp (B) and FD-OCT (C), intraepithelial bullae were observed 1 month after starting topical netarsudil (arrows). Focal corneal stromal hemorrhage associated with corneal vascularization was identified at eight months. This dog had been receiving topical netarsudil twice daily for the entire study period (D1). The hemorrhage resolved 1 month after discontinuation of topical netarsudil and four months later, subepithelial melanin was observed in the superior-temporal paraxial cornea (D2).

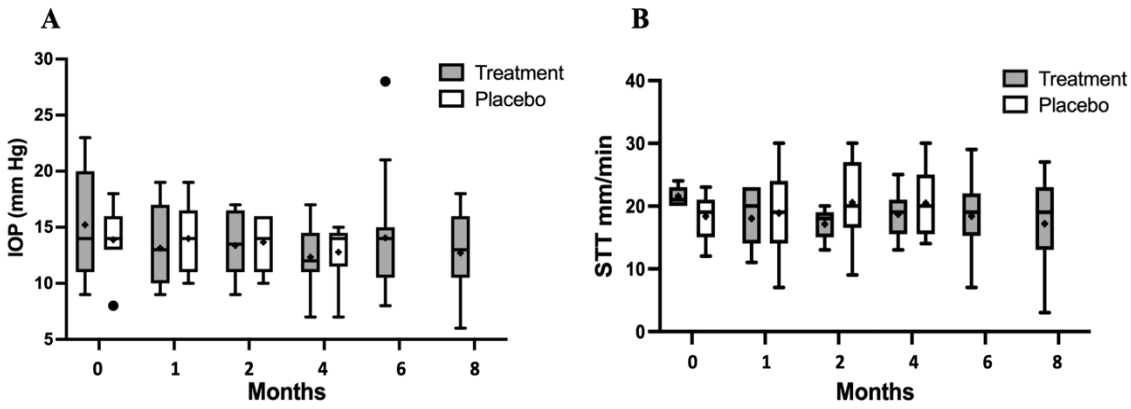

Figure 3: No significant differences were observed in IOP (A) or STT values (B) between the netarsudil-treated (7 dogs, nine eyes) and placebo-treated (7 dogs, nine eyes) groups. Box plots depict median, mean (**+**), and 25th and 75th percentiles. Whisker plots show maximum and minimum values. Circles indicate outliers. Two-way repeated measures ANOVA. IOP: intraocular pressures measured by rebound tonometry. STT: Schirmer tear test.

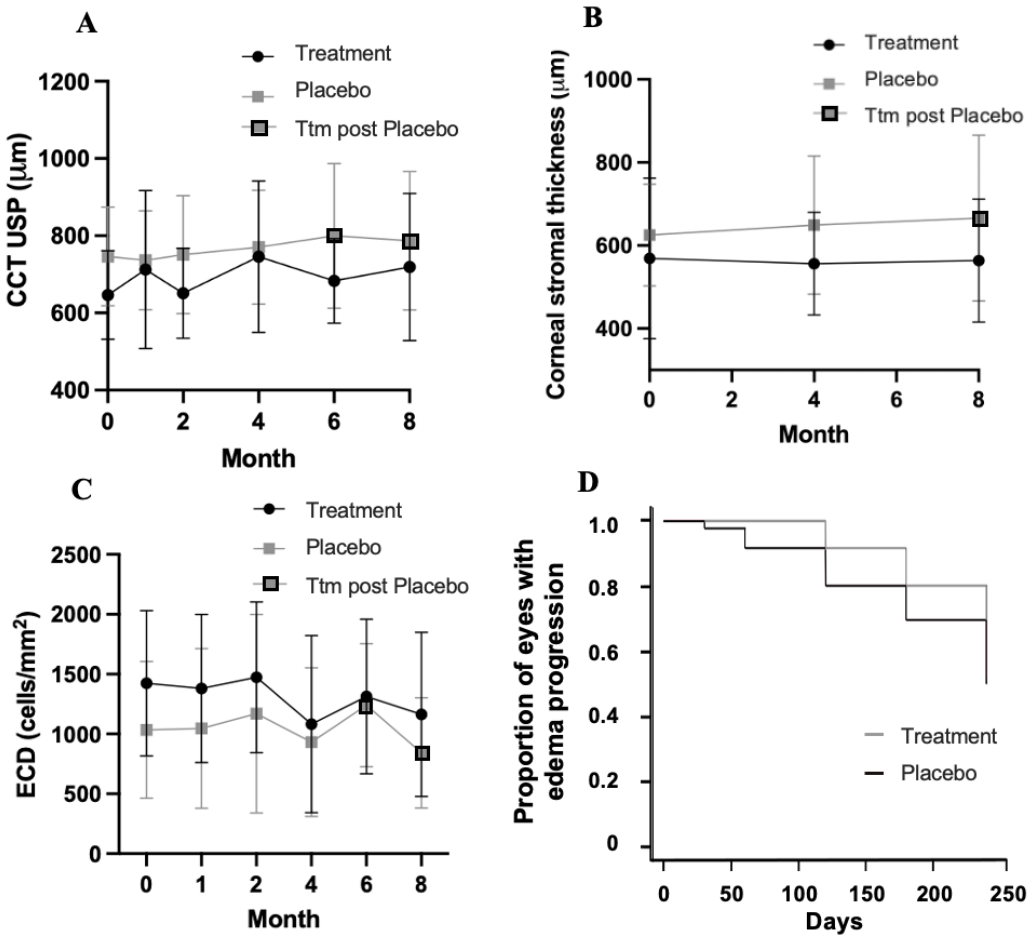

Figure 4. Plots (A, B, C) and Kaplan Meier curves (D) comparing CCT, corneal stromal thickness, ECD, and corneal edema progression in patients treated with netarsudil during eight months versus patients receiving placebo during the first four months. No significant differences were observed between patients treated with netarsudil and patients treated with placebo in corneal thickness (A) nor corneal stroma thickness (B). Similarly, no significant differences were observed in ECD nor edema progression between dogs receiving netarsudil and placebo (C, P = 0.95 and D, P = 0.88, respectively). Ttm: dogs receiving treatment (netarsudil) post placebo. For the Kaplan Meier curves, progression was established when the patient had an increase of >10% in area of the cornea affected by edema. Ttm: Netarsudil treatment post placebo.

CHAPTER 4: CORNEAL ENDOTHELIAL DYSTROPHY (CED) IN THE BOSTON TERRIER: A GENOME-WIDE ASSOCIATION STUDY

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1. Abstract

Purpose: Corneal endothelial dystrophy (CED) is a progressive disease in dogs characterized by early degeneration and loss of corneal endothelial cells, which clinically manifest as progressive corneal edema, bullous keratopathy and recurrent corneal ulcers. In humans, an analogous condition called Fuchs' endothelial corneal dystrophy (FECD) has genetic factors that contribute to the pathogenesis of disease. Boston terriers are overrepresented among dogs diagnosed with CED, suggesting there is an underlying genetic predisposition. The purpose of this study was to identify risk-associated genetic loci for CED in the Boston terrier.

Methods: DNA was collected from venous blood or formalin-fixed paraffin embedded tissue samples from 85 CED-affected Boston terriers and 114 breed-matched controls. Clustering analysis and quality control measures were applied to each sample. A case-control genome-wide association study (GWAS) was employed with 80 cases and 107 controls. Bonferroni correction was applied to *P* values. A quantitative GWAS was employed with endothelial cell density values of 29 cases and 46 controls.

Results: After quality control measures were applied, a case-control GWAS (λ=1.17) and a quantitative GWAS (λ=1.12) were performed. Both GWAS failed to identify any SNP significantly associated with CED in Boston terriers.

Conclusions: Using the current genotype data available, we did not identify any region of genetic association with CED in Boston terriers. The results of this genetic study, in combination with the knowledge about CED in Boston terriers, suggests that CED is a complex disease. Thus, our study is likely underpowered. Future GWAS including a larger number of samples might identify potential risk associated loci for CED in Boston terriers.

2. Introduction

Canine corneal endothelial dystrophy (CED) is a late-onset, bilateral disease characterized by premature degeneration and loss of corneal endothelial cells. Corneal endothelial cells are responsible for the maintenance of corneal deturgescence and transparency.[1] Canine patients diagnosed with CED typically present progressive corneal edema, bullous keratopathy and recurrent corneal ulcers that in severe cases may lead to enucleation of the globe.[2,3] Fuchs endothelial corneal dystrophy (FECD) is a similar condition found in humans that, in early stages,

manifests with low corneal endothelial cell density (ECD), thickening of the Descemet's membrane and presence of small deposits of extracellular matrix (corneal guttae).[4,5] Advanced cases of FECD demonstrate corneal decompensation characterized by corneal edema, bullous keratopathy, and impaired vision.[5,6] The incidence of FECD in US is estimated to be up to 4% in patients older than 40 years of age, with women at higher risk (relative risk of 2.5 to 3).[4,5] The genetics of FECD is complex, and mutations in *COL8A2* or *SLC4A11*, amongst others, have been associated with FECD. Both familial or sporadic presentations are described. [4,5]

Boston terriers with CED demonstrate similar clinical presentation and *in vivo* imaging features to human patients with FECD, with decrease in ECD and increase in corneal and Descemet's membrane-endothelium complex thickness.[7] Similar to humans, a female predisposition for CED has been described in the Boston terrier as well.[7] Furthermore, previous studies have identified Boston terriers as overrepresented among the dogs diagnosed with CED (observed vs expected ratio = 11.8, $P = 2.5 \times 10^{-23}$), with up to 10% of the dogs diagnosed with CED being Boston Terriers,[2,8] suggesting a possible underlying genetic predisposition to CED in this breed. While certain mutations have been demonstrated to be associated with the diagnosis of FECD in humans,[5,9,10] the role of genetic factors in canine CED is yet to be determined.

Genotypic studies are currently a powerful research tool for the identification of genetic risk factors involved in human diseases, including ophthalmological conditions.[11] With the improvement of the dog genome annotation and the development of more accessible genetic testing,[12] there has been an increase in the use of genome-wide association studies (GWAS) for mapping canine diseases.

For this prospective study, we aim to identify potential risk-associated loci for CED in Boston terriers using a GWAS approach.

3. Materials and methods

Animals

The DNA collected for this study was obtained from venous blood or formalin-fixed paraffin embedded tissue samples of client-owned pets. This study was approved by the Institutional Animal Care and Use Committee of the University of California-Davis and in concordance with the Association for Research in Vision and Ophthalmology resolution on the use of animals in research. Dogs that were diagnosed with CED by board-certified veterinary ophthalmologists from the University of California-Davis Veterinary Medical Teaching Hospital (UCD-VMTH) and other specialty hospitals across the USA between December 2013 and November 2021 were included in this study.

Phenotype analysis

Only client-owned Boston terriers were included in this study. Boston terriers diagnosed by a veterinarian or a board-certified veterinary ophthalmologist with any stage of disease severity were included as cases on the study. For both cases and controls, dogs with bilateral anterior uveitis, glaucoma, or lens luxation that may cause secondary endothelial injury were excluded from the study. To avoid potential cases of age-related endothelial degeneration, dogs that displayed the first clinical symptoms and/or received the diagnosis of CED at >13 years of age were also excluded. For controls, only purebred Boston terriers ≥ 10 years old without clinical signs of CED or Boston terriers ≥ 8 years with *in vivo* confocal microscopy (IVCM) data confirming normal endothelium were included.

Dogs examined at UCD-VMTH underwent an ophthalmic examination that included handheld and slit-lamp biomicroscopy (SL-15; Kowa American Corporation, Torrance, CA, USA) and intraocular pressure (IOP) measurement by rebound tonometry (TonoVet; Icare® Finland). When *in vivo* confocal microscopy (IVCM) was performed, the ECD was calculated and the endothelial cell morphology analyzed as previously described (ConfoScan 4 Nidek Technologies, Gamagori, Japan, and ConfoScan 4 NAVIS imaging software).[7] Previously published ECD values in normal Boston terriers (2297 ± 372 cells/mm^2) were used to evaluate the ECD of the candidate controls.[7] Information about the pedigree and disease status of relatives was also recorded, when available. The statistical analysis was carried out in GraphPad Prism version 9.3.1.

DNA extraction

The DNA was extracted from either venous blood or FFPE tissue from 85 purebred Boston terriers diagnosed with CED and 114 breed matched controls. The DNA was extracted from blood for 79 cases and 92 controls following the standard protocol for DNA extraction from blood (Gentra Puregene, Qiagen, Germantown, MD) and from FFPE tissue for 6 case and 22 controls (Quick-DNA FFPE Miniprep Kit, Zymo Research, Irvine, CA). The Zymo Research protocol was modified by doubling the amount of deparaffinization solution to ensure paraffin was completely removed from the sample.

Genome-wide association studies (GWAS)

Samples that had a minimum of 20 ng/µl of DNA were genotyped using the Illumina CanineHD 220K BeadChip (Illumina, San Diego, CA) array. The version of the canine

genome assembly CanFam2.1 was used as reference. A case-control genome-wide association study (GWAS) was performed based on disease status using PLINK 1.9.[13] Dog samples with missing genotype call (mind) of >10%, variants with minor allele frequencies (maf) of > 5%, and variants with missing genotype calls (geno) of >10% were excluded from the association study. A Bonferroni correction was applied to correct for multiple comparisons using PLINK. Population stratification was evaluated using the Q-Q plots and multi-dimensional scaling (MDS) plots. To test the stratification within the population of Boston terriers, a cluster analysis was performed. Available genotyping information from five other breeds from different studies from coauthors of this Chapter were incorporated on the analysis to identify non-purebred Boston terriers. The genotypes employed included 58 American cocker spaniels, 55 West Highland white terriers, 46 standard poodles and 33 German shorthaired and wirehaired pointers. Additionally, linear mixed model analysis (GEMMA)[14] was performed to correct for population stratification. The results were evaluated with Manhattan plot and quantile-quantile (Q-Q) plot carried out in R (qqman package).[15]

Subsequently, a quantitative GWAS was run using ECD values from 29 CED-diagnosed Boston Terriers and 46 controls examined at the UCD-VMTH following the same methodology.

4. Results

Phenotype analysis

Among the potential CED cases evaluated for this study, eleven dogs were excluded: four dogs presented concomitant glaucoma at the time of diagnosis, six dogs had endothelial damage suspected to be secondary to chronic corneal ulcers or uveitis, and one dog was a mixed breed dog rather than purebred.

Of the 44 patients diagnosed with CED at the UCD-VMTH, two owners were aware of their pets having another relative diagnosed with CED. None of the dogs included for GWAS in this study were known to be related.

Mean ± SD age for cases was 10.1 ± 2.0 (range 6.0-14.0 years). For dogs older than 13 years old, the owners reported ongoing corneal opacity for at least one year prior to diagnosis by a veterinary ophthalmologist. The number of females affected was higher (46 females versus 34 males). But, when comparing with the total population of Boston terriers presented to the UCD-VMTH between 1/1/2013 and 1/1/2022 (406 females, and 446 males), there were no differences between sex (Chi-square test $P = 0.09$). Mean age in the control group was 11.1 ± 1.5 (range 8.0-14.9) years; 56 were males and 51 were females.

A total of 45 controls and 29 CED-diagnosed cases were analyzed by IVCM at the UCD-VMTH and included in the quantitative GWAS. The difference on ECD values between CED-affected dogs and controls was statistically significant ($P < 0.001$) at 1181 ± 306 cells/mm^2 for CED-diagnosed dogs and 463 ± 33 µm, and 2421 ± 274 cells/mm^2 for controls (**Fig. 1. B**). In seven patients with advanced disease stage, the markedly thickened (1323 ± 390 microns) and edematous cornea prevented acquisition of endothelial images by IVCM. The ECD and the endothelial cell morphology observed in the cases and controls were similar to the ones described in a previous study of CED in Boston terriers (**Fig. 2**).[7]

GWAS

Out of the 171 DNA samples obtained from blood that were successfully genotyped, one did not pass the GWAS quality control filters. Out of the 28 DNA samples obtained from FFPE tissue, 10 did not pass the filters and one was excluded

after cluster analysis demonstrated that the dog was likely not a purebred Boston terrier (**Fig. 3**, arrow)

A case-control GWAS was performed, employing 80 cases and 107 controls. A total of 136,857 SNPs were included in this GWAS. The genomic inflation (λ) was 1.14 before mixed model analysis and 0.94 after correction. The corrected p-value for genome-wide association was determined to be $3.7x10^{-7}$ and the results were expressed in a Q-Q plot and in a Manhattan plot (**Fig. 4**). There were no SNPs that were significantly associated with CED in our cohort.

A second, quantitative GWAS was run using ECD values from 29 CED-diagnosed and 45 control Boston terriers. All samples from this dataset passed the quality control filters and cluster analysis. A total of 136,855 variants passed the filters, and the λ was 1.12. The corrected p-value for genome-wide association was determined to be $3.7x10^{-7}$. A single SNP in chromosome 7 (57861638) had a *P* value of $4.62x10^{-7}$. After running a mixed model analysis for correction of the stratification, the revised λ value was 1.06, and the *P* value for that SNP on chromosome 7 was corrected ($9.54x10^{-2}$). Similar to the case-control GWAS, there were no significant SNPs that were associated with CED using the dataset for this study (**Fig. 5**)

5. Discussion

The Boston terrier is one of the most common breeds frequently presented to ophthalmology services due to corneal complications.[16] A retrospective study conducted at the UCD-VMTH found 10 Boston terriers affected with CED between August 1991 and October 2014 (observed vs expected ratio = 11.8, $P = 2.5x10^{-23}$). In this study, a case-control GWAS using 80 CED-affected Boston terriers and 107 matched-breed controls and a quantitative GWAS using 29 CED-affected Boston

terriers and 46 controls, respectively, did not identify candidate regions for CED in Boston terriers. The clinical presentation and imaging characteristics of affected dogs included on this study were similar to those previously described for canine CED.[7] In our study, IVCM facilitates early diagnosis when clinical symptoms were not apparent (compensated stage) and provided reliable ECD for quantitative GWAS.

In humans, a diverse number of genetic alterations have been associated with FECD, such intronic repeat expansion in *TCF4*, or mutations in *COL8A2*, *SLC4A11* or *ZEB1*, amongst others,[5,10,17,18] highlighting the complexity of this disease in humans. Most cases of FECD are sporadic, with a smaller amount presenting on a familial basis.[5,10] FECD is classified as either early or late onset, with early onset usually manifesting clinical signs at the first decade of life, and late onset after the fourth decade of life.[5,10] Early onset FECD is typically more severe, observed in individuals with familial predisposition, and usually associated with genetic mutations in *COL8A2*.[10] Late onset FECD is usually milder, more common than early onset, and less frequently associated with familial predisposition.[10]

Due to its unique genetic architecture, with long linkage disequilibrium and resultant long haplotype blocks, the dog is an attractive model for the study of genetic traits through genome-wide association mapping-approach strategies.[12,19] After the completion of the Dog Genome Sequencing Project in 2005, the improvement of SNP array platforms for dogs, there has been an increase in the use of genetic studies to investigate canine diseases.[12,19] In particular, genome-association approaches have been remarkably useful in the identification of mutations and loci associated with canine diseases that follow a mendelian inheritance pattern.[20-23] With complex diseases,[24,25] these approaches improve the understanding of the pathogenesis of these disorders. Furthermore, identification of mutations causative of disease or

associated with specific conditions have allowed the development of genetic tests available to veterinarians, owners, and breeders to guide diagnosis, prevent disease, and make more informed breeding decisions. Some examples of successful GWAS on the study of ocular diseases in dogs include the identification of loci associated with cataract, glaucoma, or retinal diseases.[27-29] As for corneal diseases, a GWAS allowed the identification of a defect in the NOG gene associated with spontaneous superficial chronic corneal epithelial defects (SCCED) in the Boxer.[30]

In this study, we found two owners that were aware of their pets having another relative diagnosed with CED, however, we were unable to collect pedigree information for other affected dogs. Additionally, the late onset of the condition limited our ability to examine other family members, particularly parents that were no longer living, thus, making very challenging to study any potential pattern of inheritance for CED in Boston terriers.

When analyzing the quantitative GWAS, a single SNP presented a low *P* value close to statistical significance, however, the P value was corrected after mixed model analysis, indicating that the P value was likely due to population stratification.

Unfortunately, our case-control and quantitative GWAS did not lead to the identification of any candidate regions for CED in Boston Terriers. The low λ value on the case-control GWAS after mixed model analysis correction (0.94), and the Q-Q plots from both our binary and our quantitative GWAS suggest that our study is likely underpowered. Previous studies demonstrate that a GWAS for multigenic traits in dogs using 100 cases and 100 controls have a power of 97% when the risk multiplicative factor is 5 and of 50% when the risk multiplicative factor is 2.[19] Future studies including a larger number of samples might be able to detect regions of association.

6. Conclusions

In our study, a case-control GWAS did not identify candidate regions for CED in Boston terriers using 80 CED-affected Boston terriers and 107 matched-breed controls. This study is likely underpowered. Future studies employing a larger number of samples might be able to detect regions of association with CED in Boston terrier.

7. Acknowledgements

This research was supported by the Center for Companion Animal Health, School of Veterinary Medicine, University of California, Davis, the American Kennel Club Canine Health Foundation, the National Institutes of Health P30 EY12576, R01EY016134 (SMT), and the Jane-Lin Fong Ophthalmic Clinical Trial Support Fund. We also thank the collaboration of all veterinarians that sent samples and clinical information, and all the owners that participated in this study.

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6. Figures

1A **1B**

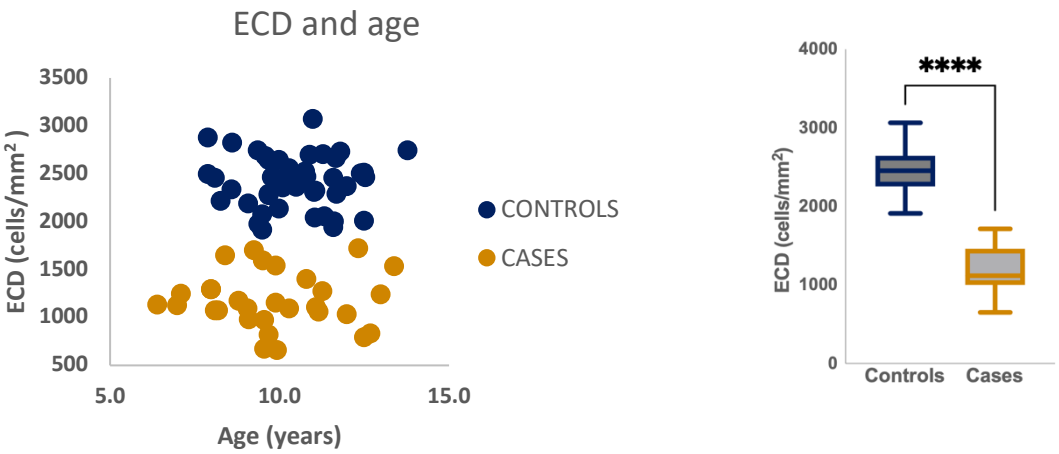

Figure 1. Scatterplot and whisker box plot showing the ECD and ages of 29 CED-diagnosed Boston terriers and 46 breed-matched controls included on the quantitative GWAS. CED-diagnosed Boston terriers have lower ECD when compared with controls (1A, scatterplot). The difference on ECD values between CED-affected dogs and controls was statistically significant ($P < 0.001$) at 1181 ± 306 cells/mm^2 for CED-diagnosed dogs and 463 ± 33 µm, and 2421 ± 274 cells/mm^2 for controls (A2, whisker box plot representing the median and the maximum and minimum range, *** $P < 0.001$)

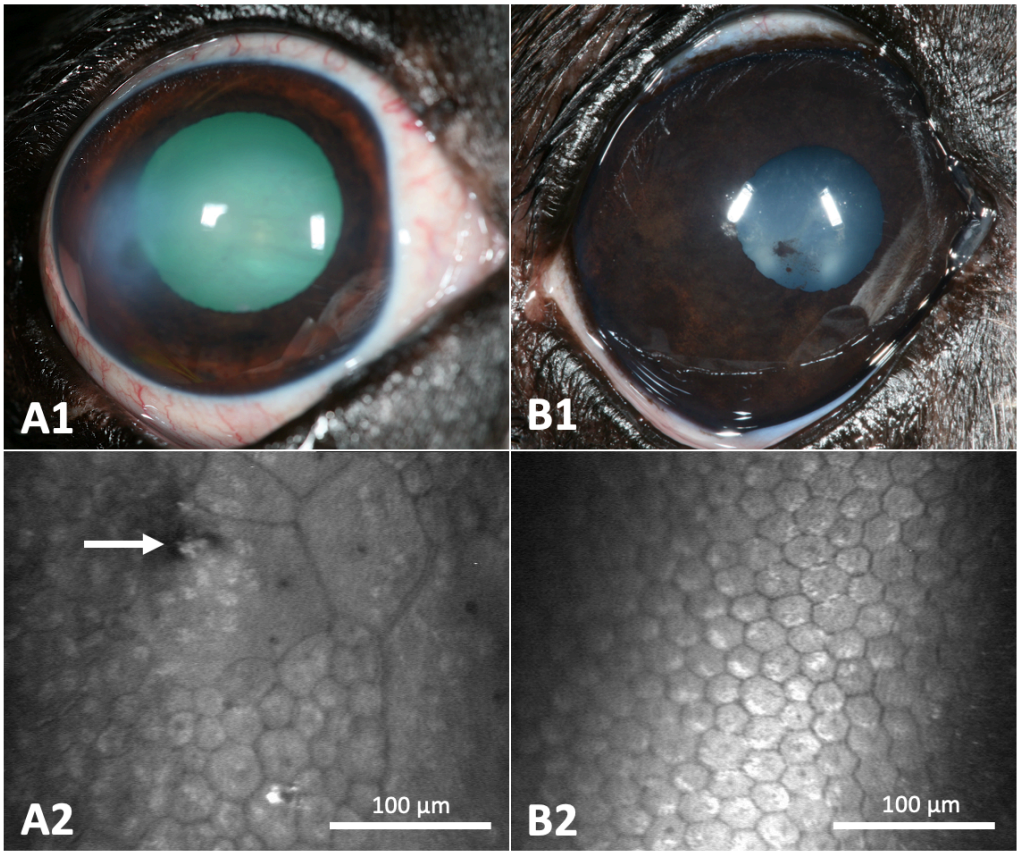

Figure 2. Corneal edema, lower ECD and abnormal corneal endothelial morphology are the hallmarks for canine CED. Focal corneal edema observed in the right eye in 11.5-year-old male, Boston terrier diagnosed with CED (A1). Loss of hexagonality (pleomorphism) and marked variation in corneal endothelial cell size (polymegathism) were observed with IVCM on that same eye (A2). The arrow indicates a multinucleated cell that overlaps with a guttae-like lesion (A2). Normal corneal appearance (B1) and the endothelial cells examined with IVCM (B2) of a 11-year-old male Boston terrier without corneal disease are displayed for reference.

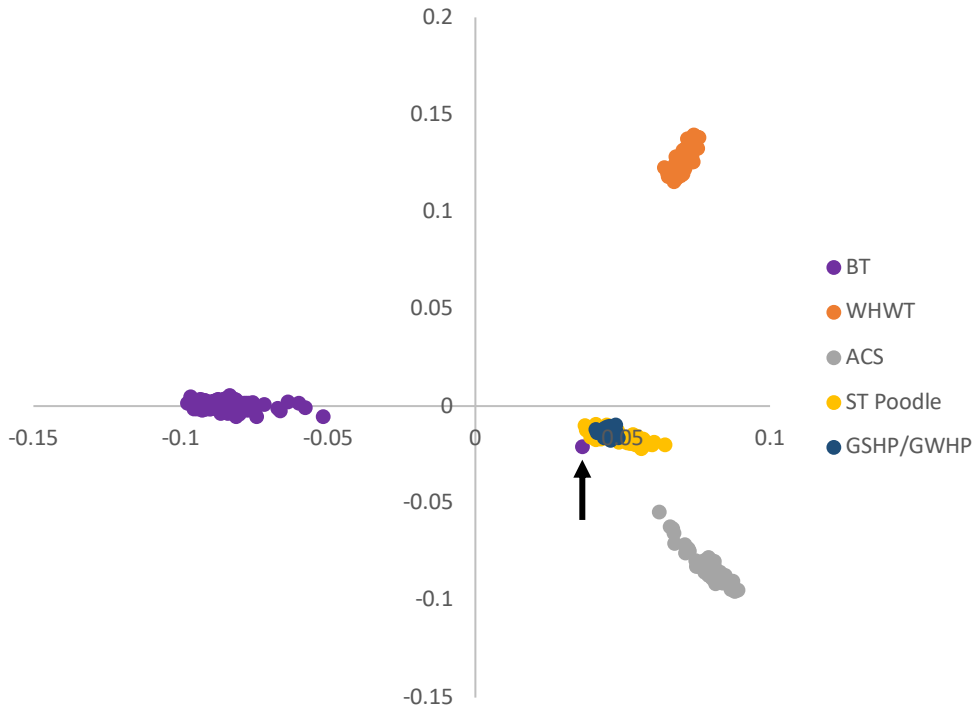

Figure 3. MDS plot showing the genotypes of Boston terriers included on the study after exclusion of outliers and when comparing with 5 other breeds. The Boston Terrier genotype dataset (BT, purple, 81 cases and 107 controls, purple) was combined with available genotypes from American cocker spaniels (ACS, grey, 58 dogs), West Highland white terriers (WHWT, orange, 55 dogs), standard poodles (yellow, 46 dogs) and German shorthaired and wirehaired pointers (GSHP/GWHP, blue, 33 dogs). One outlier was identified and excluded from the GWAS (arrow).

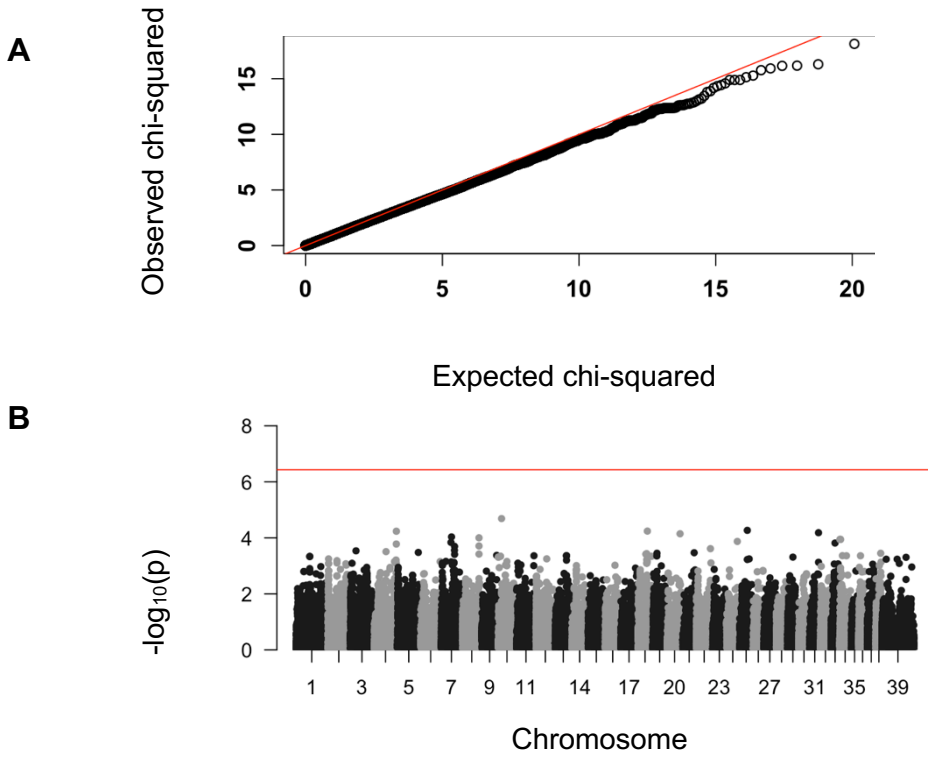

Figure 4. Results of the case-control genetic association test for CED in Boston terriers using 76 CED-affected Boston terriers and 99 non-affected breed-matched controls after mixed model analysis for correction of the stratification. Q-Q plot (A, $\lambda = 0.94$). and Manhattan (B). The Manhattan plot did not reveal any genome-wide significant association CED in Boston terriers using phenotyping data. The y axis of the Manhattan plot corresponds with -log 10 *P* values and the x axis indicates the chromosome location. The red line denotes genome wide significance ($P_{genomewide} = 3.6x10^{-7}$)

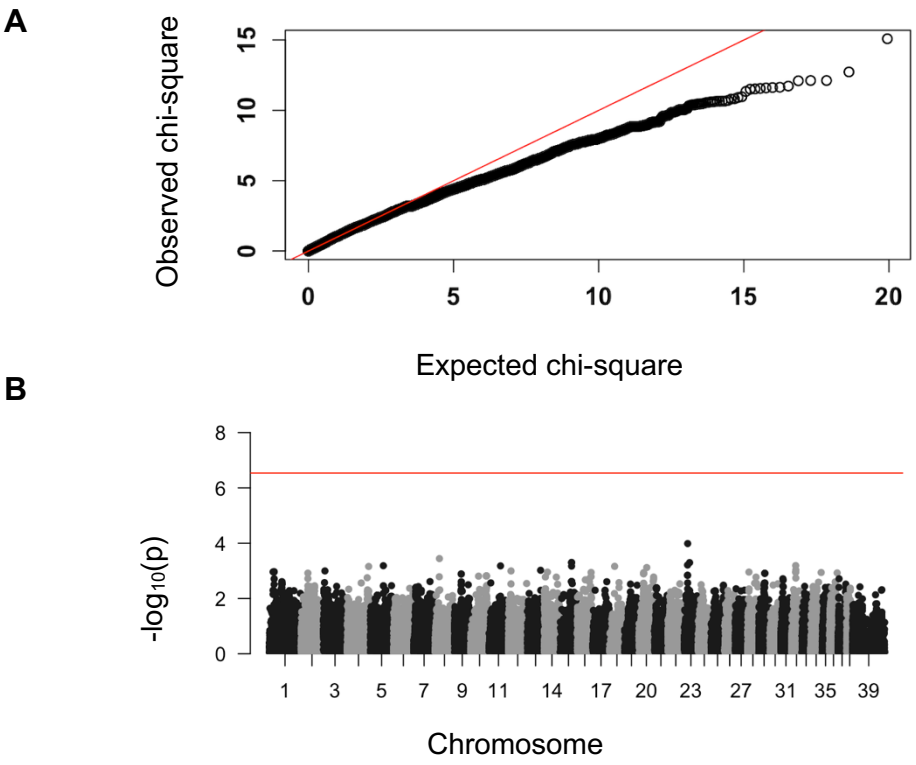

Figure 4. Results of the quantitative genetic association test for CED in Boston terriers using 29 CED-affected Boston terriers and 46 non-affected breed-matched controls after mixed model analysis for correction of the stratification. A Q-Q plot (A, λ = 1.06). After mixed model analysis for correction of the stratification, the quantitative Manhattan plot did not reveal any genome-wide significant association CED in Boston terriers using ECD data. The y axis of the Manhattan plot corresponds with -log 10 *P* values and the x axis indicates the chromosome location. The red line denotes genome wide significance ($P_{genomewide}$ = 3.7x10^{-7})

CHAPTER 5: CANINE ENDOTHELIITIS: CLINICAL CHARACTERISTICS, ADVANCED IMAGING FEATURES AND TREATMENT

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Running title: *Diagnostic features of canine endotheliitis*

1. Abstract

Objective: To describe the clinical findings, multimodal corneal imaging features and treatment in canine patients diagnosed with endotheliitis.

Animals studied: Four canine patients met inclusion criteria for bilateral corneal disease with endothelial inflammation and secondary corneal edema that responded to topical anti-inflammatory treatment.

Methods: The patients selected underwent a complete ophthalmic examination with emphasis on the cornea including ultrasound pachymetry (USP), Fourier-domain optical coherence tomography (FD-OCT), *in vivo* confocal microscopy (IVCM), and digital slit lamp photography.

Results: All patients in this study demonstrated thickened corneas due to edema with USP and FD-OCT. With IVCM, mild to severe polymegathism and pleomorphism of corneal endothelial cells, reduced endothelial cell density (ECD), hyperreflective keratic precipitates (KPs) and extracellular debris as well as hyporeflective pseudoguttata were observed. With FD-OCT, hyperreflective KPs were commonly observed on the inferior cornea. Clinical examination and advanced imaging results were consistent with a diagnosis of endotheliitis. All patients initially responded to topical anti-inflammatory treatment and required continued therapy. All patients received topical anti-inflammatory treatment and two patients received topical netarsudil, a rho-associated coiled-coil kinase (ROCK) inhibitor.

Conclusion: Endotheliitis should be considered for canine patients with bilateral edema that is most severe in the inferior cornea. Careful inspection of Descemet's membrane-endothelial complex should be performed for KPs or

inflammatory debris. Chronic administration of topical anti-inflammatories may be necessary to prevent flare-ups of endotheliitis.

Keywords: endotheliitis, ROCK inhibitor, *in vivo* confocal microscopy, optical coherence tomography, corneal edema

2. Introduction:

The corneal endothelium maintains corneal deturgescence and transparency. In dogs, causes of corneal endothelial degeneration include endothelial dystrophy, anterior uveitis, glaucoma, intraocular surgery, lens luxation, diabetes mellitus, canine adenovirus-1 infection (CAV-1), senility, and endotheliitis.[1–5] Corneal endotheliitis is the result of primary inflammatory damage to the corneal endothelium that typically manifests with corneal edema, keratic precipitates, and inflammatory changes in the anterior chamber, such as aqueous flare. With chronicity, endotheliitis can result in permanent endothelial degeneration.[6] In this case series, we describe the clinical findings, advanced imaging characteristics, and treatment of four canine endotheliitis cases.

3. Materials and methods:

Four canine patients were presented to the Comparative Ophthalmology Service at the University of California, Davis William R. Pritchard Veterinary Medical Teaching Hospital (UCD-VMTH). Two cases were suspected to have corneal endothelial dystrophy while the other two were suspected to have anterior uveitis with endotheliitis. All presented cases had bilateral corneal edema that responded to anti-inflammatory medication. Cases in which a primary cause of anterior uveitis were identified were excluded from this study. Patients underwent an ophthalmic examination with multimodal corneal imaging including anterior segment photography (Canon ROS 5D; Tokyo, Japan), intraocular pressure (IOP) measurement by rebound

tonometry (Tonovet, Icare, Vantaa, Finland), digital slit lamp imaging (Topcon SL-D7, Tokyo, Japan), ultrasound pachymetry (USP, Pachette 4; DGH Technology, Inc., Exton, PA), Fourier-domain optical coherence tomography (FD-OCT, RTVue 100; Optovue, Inc., Fremont, CA) and *in vivo* confocal microscopy (IVCM, ConfoScan 4; Nidek Technologies, Gamagori, Japan). Corneal endothelial cell density (ECD) was calculated as previously described.[1]

4. Case descriptions:

4.1 Case 1:

A 6-year-old male castrated Chihuahua mix was referred to the UCD-VMTH with blepharospasm, serous discharge, moderate conjunctival and episcleral hyperemia and mild chemosis in both eyes (oculus uterque, OU). Corneal edema OU was present and was most severe inferiorly (**Figure 1**), with pinpoint keratic precipitates (KPs). Mild and trace aqueous flare was present in the right (oculus dexter, OD) and left eye (oculus sinister, OS), respectively; IOPs were normal (OD 11; OS 14) (reference range 7-22 mm Hg).[7] Non-ulcerative keratoconjunctivitis and anterior uveitis OU were diagnosed, and prednisolone acetate 1% ophthalmic suspension (PA) was prescribed OU six times daily for one week and tapered and replaced by diclofenac 0.1% ophthalmic solution twice a day (BID). Four weeks after initial presentation, and one week later after discontinuing PA, the patient developed corneal edema; dexamethasone 1% ophthalmic solution three times daily (TID) was prescribed. At the 5-week recheck, no anterior uveitis was present, and the corneal edema was improved but still present OU. On FD-OCT and IVCM few, small, hyperreflective deposits consistent with KPs were identified (**Figure 1**). The dexamethasone was slowly tapered and replaced by diclofenac TID. Four months after

initial presentation, the patient was comfortable, and corneal edema was resolved OD. Subtle edema with mild increased inferior corneal thickness (729 µm, reference range 575-623 µm) was still present in OS.[8] On IVCM, hyporeflective pseudoguttata and hyperreflective KPs were identified OU (**Figure 1**); ECD was markedly lower than normal at 870 (OD) and 878 (OS) cells/mm^2; (reference range: 2300 to 2500 cells/mm^2).[5] Discontinuation of diclofenac resulted in a relapse of corneal edema one week later. Dexamethasone 1% ophthalmic suspension was resumed and one month later, the patient was receiving 1 drop OU BID and was comfortable with clear corneas. Eight months after the initial presentation, the patient was comfortable and with no grossly visible corneal edema. The ECD were low at 610 (OD) and 544 cells/mm^2 (OS). Topical netarsudil 0.02% ophthalmic solution (Rhopressa®) BID was prescribed in addition to topical dexamethasone. Eight months later, the ECD was slightly increased OS (791 cells/mm^2) and increased OD (1235 cells/mm^2), but there were marked regional differences in ECD and cell morphology OD (**Figure 1**). Few hyperreflective KPs were still visible on the corneal endothelium (**Figure 1**).

4.2 Case 2:

A 2-year-old male castrated Chihuahua mix was presented after receiving a diagnosis of anterior uveitis and corneal edema two months prior. The patient was receiving topical 5% sodium chloride ointment (NaCl) four times daily (QID) and neomycin-polymyxin B-dexamethasone (NPD) ophthalmic ointment BID OU. The corneas were clear (**Figure 2**) and corneal thicknesses were normal, including inferiorly (OD 571 µm, OS 558 µm); IOPs were 23 (OD) and 22 (OS) mm Hg. Mildly enlarged and irregularly shaped endothelial cells with enlarged, hyperreflective nuclei OU were observed with IVCM (**Figure 2**); ECD was low at 1371 (OD) and 1018 (OS) cells/mm^2. The patient was tapered off the NPD ointment. Two weeks later,

intermittent blepharospasm, severe conjunctival hyperemia, mild chemosis and diffuse corneal edema with the inferior cornea more severely affected were observed OU; superficial blood vessels were visible in the inferior and nasal cornea. The IOPs were 15 (OD) and 14 (OS) mmHg. Punctate KPs were most dense in the inferior cornea and visible with slit lamp biomicroscopy, FD-OCT and IVCM (**Figure 2**); the inferior cornea thickness had increased >50% from the previous visit (901 µm OD; 861 µm OS). Topical NPD ophthalmic suspension QID OU was prescribed then tapered to once a day over a 5-week period.

Seven months later, the corneas were clear and inferior corneal thickness was normal (OD: 529 µm; OS: 536 µm). Subtle punctate crystalline opacities in the temporal paraxial cornea were observed OU consistent with steroid keratopathy.[2] The NPD ophthalmic suspension was tapered over 4 weeks and replaced with topical diclofenac BID. Six weeks later, the patient was comfortable; however, trace flare OU, subtle corneal edema and increased inferior corneal thickness (OD 636 µm, OS 714 µm) were observed. With FD-OCT, small hyperreflective KPs and few cells in anterior chamber were observed and small hyporeflective pseudo-gutatta and hyperreflective deposits compatible with KPs were found with IVCM (**Figure 2**). Oral carprofen (2 mg/kg BID) was prescribed for five days, along with NPD ophthalmic suspension, diclofenac 0.1% ophthalmic solution and tacrolimus 0.03% ophthalmic suspension OU BID.

4.3 Cases 3 and 4:

Two 1.3-year-old littermate Australian cattle dogs were vaccinated at 6 weeks of age with a modified live vaccine (Canine Spectra 5, Durvet Inc., Blue Springs, MO) that protects against canine distemper virus, CAV-1 and -2, parainfluenza, and

parvovirus type 2b. The dogs were then adopted by different households. Case 3 was a spayed female while case 4 was an intact female.

Case 3 developed corneal opacity OU at 3 months of age. A veterinary ophthalmologist diagnosed corneal endothelial degeneration secondary to anterior uveitis OU and prescribed NPD ophthalmic suspension TID. After three months, the corneal edema had resolved, and the NPD was tapered to once a day. Ten months later, the patient was presented to the UCD-VMTH. Subtle stromal and endothelial corneal opacities were present OU (**Figure 3**); IOPs were mildly elevated (OD 29, OS 26 mm Hg) but the patient was excitable and easily stressed. With USP, corneal thicknesses were normal including inferiorly at 533 µm (OD) and 532 µm (OS). Descemet's membrane (DM)-endothelial complex was hyperreflective OU with FD-OCT and slit lamp biomicroscopy (**Figure 3**). With IVCM, KPs were observed (**Figure 3**). While most endothelial cells were hexagonal in shape (**Figure 3**), ECD was decreased at 1480 (OD) and 1893 (OS) cells/mm^2.

Case 4 presented to a veterinary ophthalmologist at 4 months of age and diagnosed with endotheliitis OU; IOPs were low at 3 (OD) and 8 (OS) mmHg. Topical PA and NaCl OU were prescribed TID. After improvement, the PA was slowly tapered over 5 months then discontinued. Six weeks after discontinuing topical PA, a relapse of clinical signs was observed with corneal edema and hypopyon OU, and PA was restarted QID. A month later, the hypopyon had resolved but corneal edema remained static, and the PA was tapered to BID.

One year after initial diagnosis the patient was referred to the UCD-VMTH. Severe inferior corneal edema was observed OD with multifocal to coalescing KPs present on the endothelium (**Figure 4**); IOPs were 19 (OD) and 14 (OS) mm Hg. Severe, diffuse corneal edema was observed OS with sparing of only the superior

perilimbal cornea (**Figure 4**); inferior corneal thickness was 1300 µm (OD) and 1703 µm (OS) as measured with FD-OCT. Thick hyperreflective deposits on the DM-endothelial complex were observed OU with FD-OCT consistent with KPs (**Figure 4**). With IVCM, the corneal endothelium was visible only OD and ECD was low (974 cells/mm^2, OD) with occasional elongated, hyperreflective nuclei present. Multifocal web-like hyperreflective deposits were present over the endothelial cells and were interpreted as KPs.[9] The patient was prescribed topical PA QID, NaCl QID, netarsudil BID, and tacrolimus 0.03% ophthalmic suspension BID OU. Four months later, the previously identified areas of corneal edema were markedly reduced OD but unchanged OS (**Figure 4**). The FD-OCT and IVCM findings were static from the previous visit other than a thin, hyperreflective band in the anterior stroma of the nasal cornea consistent with steroid keratopathy.[2] After ophthalmic exams, serum was submitted for ancillary testing and the patient had a positive titer against CAV-1.

5. Discussion:

This case series demonstrates multimodal corneal imaging features of canine endotheliitis. Clinically, the patients presented bilateral diffuse corneal edema that improved after anti-inflammatory treatment. In contrast to acute anterior uveitis whereby edema is typically diffuse and mild to moderate in severity, these cases demonstrate more severe edema that persists beyond resolution of uveitis and is typically worse inferiorly. The IVCM findings included endothelial pleomorphism and polymegathism, pseudoguttata, and hyperreflective deposits consistent with what is observed in humans and horses with endotheliitis and endothelial immune-mediated keratitis, respectively.[10,11] Pseudoguttata are dark, acellular regions that result from endothelial cell edema and occur during bouts of inflammation.[12] The hyperreflective

deposits overlying the DM-endothelial complex in the inferior cornea were interpreted as KPs that can persist for months after anti-inflammatory treatment.[9] While the composition of this material is unknown and histopathological investigation is warranted, we presume these aggregates represent inflammatory debris. Our findings suggest that inspection of the DM-endothelial complex at high magnification with slit-lamp biomicroscopy should be performed in patients suspected to have endotheliitis, and that IVCM and FD-OCT are useful in cases where endotheliitis cannot be confirmed solely on ophthalmic examination. Exclusion of primary anterior uveitis through complete ophthalmic examination and additional imaging, if necessary, is also recommended in these cases.

In all four cases, ECD was markedly reduced suggesting that corneal edema not only results from endothelial dysfunction due to inflammation, but also from endothelial decompensation as a critical low number of cells is reached. In addition, two patients had elongated, hyperreflective nuclei which is associated with corneal endothelial trauma or disease in humans.[13] Dogs have a moderate endothelial regenerative capacity, particularly at a young age.[14] Since our patients were young to middle aged, it is possible that some endothelial regeneration could occur. We prescribed netarsudil to two patients and observed improvement in ECD in one patient and reduction in percentage of the cornea affected by edema in the less severe eye of a second patient. Topical ROCK inhibitors accelerate endothelial proliferation, migration and reduce apoptosis *in vitro* and *in vivo* in animal models as well as human patients.[15–17] While acknowledging regional variation in ECD and cell morphology in some of these patients, these data suggest that ROCK inhibitors may have a role in the treatment of endotheliitis in combination with anti-inflammatory treatment, and further studies are warranted.

In humans, cytomegalovirus and human herpesviruses cause endotheliitis.[9] A positive titer against CAV-1 was detected in patient 4. However, since both CAV-1 and CAV-2 are antigenically very close and cross-reaction is possible, it was not possible to determine whether the titer in this particular case was associated with natural infection or vaccination. It is possible that the endotheliitis in cases 3 and 4, who are littermates, could be attributable to natural infection with CAV-1 or vaccination with the attenuated CAV-2.

During natural infection, CAV-1 enters the eye during the viremic phase and replicates in corneal endothelial cells, causing endotheliitis. In a posterior phase or after vaccination, the production of neutralizing antibodies can lead to further damage through type III hypersensitivity reaction. During this process, immunocomplexes deposit in the anterior chamber, resulting in complement fixation and leukocyte recruitment which causes severe uveitis and endotheliitis with corneal edema; no antiviral treatment is currently available.[18,19] The potential role of viruses other than CAV-1 in canine endotheliitis has yet to be determined and necessitates further study.

In 3 patients, discontinuation of anti-inflammatory treatment led to relapse of clinical signs requiring re-institution of therapy. Furthermore, case 4 has had continuous anti-inflammatory treatment since the initial diagnosis and displayed the mildest changes in endothelial morphology and density of the presented cases. Thus, canine endotheliitis patients should be closely monitored if anti-inflammatories are discontinued; continuous treatment with a topical steroid at a low frequency may be preferable. Topical tacrolimus, a calcineurin inhibitor, may be beneficial in the long-term management of canine endotheliitis given that it can achieve adequate intraocular concentrations in human patients;[20] it was used in two patients in the present study in combination with other medications. This case series suggests that

some canine patients with endothelial disease may require lifelong topical anti-inflammatory therapy.

6. Conclusions

The presentation of canine endotheliitis can vary but should be considered in patients with corneal edema that is more severe inferiorly and persists beyond resolution of anterior uveitis. Careful inspection of DM-endothelial complex for KPs should be performed with slit-lamp biomicroscopy, FD-OCT and/or IVCM. Long-term administration of topical anti-inflammatories may be necessary to manage canine endotheliitis.

7. Acknowledgements

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8. Conflict of interest

While the netarsudil (Rhopressa®) was donated by Aerie Pharmaceuticals and could be viewed as a possible conflict of interest, we highlight that they had no input in the design, analysis or interpretation of the data presented in the manuscript or the manuscript itself.

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10. Figures

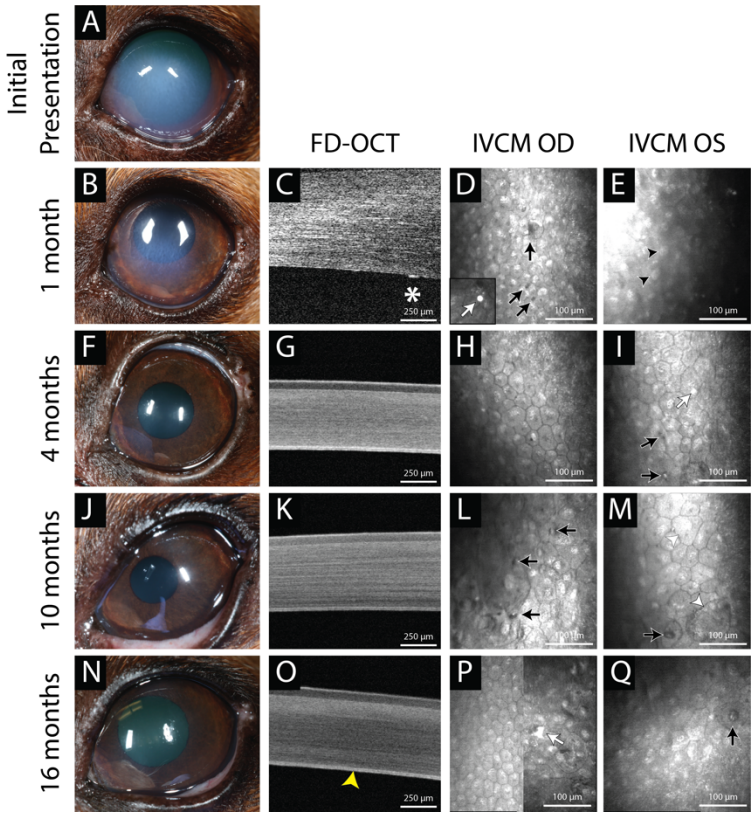

Figure 1. Clinical course of canine endotheliitis in a 6-year-old male castrated Chihuahua mix with 16 months of follow up. At initial presentation, the patient had bilateral corneal edema (A). One month after the patient was receiving dexamethasone 1% ophthalmic solution TID, the corneal edema was improved OS (B) and resolved OD (not shown) and was completely resolved OU four months later (F). At one month after initial presentation, FD-OCT demonstrated hyperreflective deposits on the endothelium consistent with KPs (C, asterisk). With IVCM, mild to moderate pleomorphism (D, E), endothelial cells with hyperreflective, elongated nuclei (E, black arrowhead), pseudogutatta (D, black arrows) and hyperreflective deposits consistent with pigmented KPs (D inset, white arrow) were observed 1 month after initial presentation. At four and ten months later, the corneas were clear (F, J). With FD-OCT, DM-endothelial complex was thickened

(G, K) and hyperreflective on FD-OCT. With IVCM, progressive endothelial pleomorphism and polymegathism (M, white arrowhead), hyperreflective KPs (I, white arrow) and pseudogutatta (I, L, M, black arrows) were observed. Sixteen months after initial presentation and after 8 months of topical netarsudil, the endothelial complex was still thickened with FD-OCT (O, yellow arrowhead). Endothelial morphology was improved OS but variable with IVCM (P). Hyperreflective KPs (P inset, white arrow) and pseudogutatta (Q, black arrow) persisted in this patient.

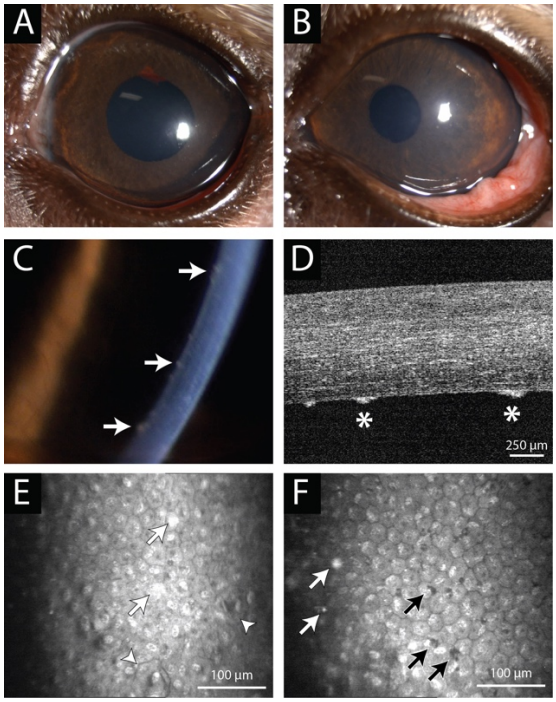

Figure 2. Active endotheliitis was present in a 2-year-old castrated male Chihuahua receiving topical diclofenac 1% ophthalmic solution twice daily. At initial presentation, the patient was receiving topical NPD ophthalmic ointment with no apparent clinical signs (A, OS). Two weeks after replacement of the NPD ophthalmic ointment by diclofenac twice daily, the patient was presented for ocular discomfort, mild corneal edema, and conjunctival hyperemia (B, OS). KPs were observed with slit lamp biomicroscopy (C, white arrows OS), FD-OCT (D, asterisk, OS) and IVCM (E, white arrows, OD). After replacing topical NPD with diclofenac BID, pseudogutatta (black arrows) and hyperreflective deposits compatible with KPs were observed with IVCM (F, white arrows, OD).

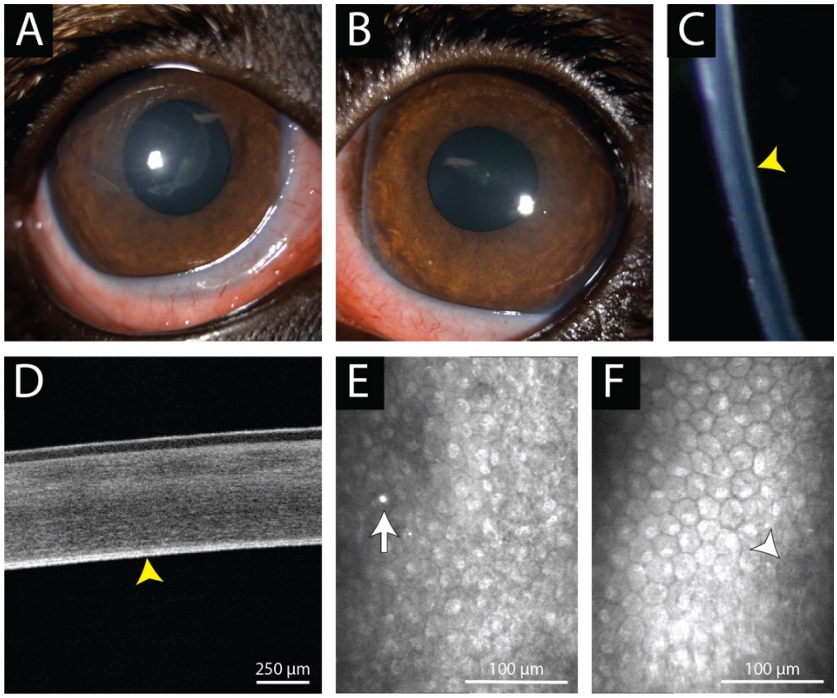

Figure 3. Changes to the endothelium and Descemet's membrane were visible in a 1-year-old female Australian cattle dog diagnosed with endotheliitis at 3 months of age. This patient was receiving NPD ophthalmic ointment once daily OU. Subtle opacity was present in the axial cornea OU (A, B) due to increased hyperreflectivity of the DM-endothelial complex observed with slit lamp biomicroscopy at 10X magnification and FD-OCT (C and D, yellow arrowheads). With IVCM, few, mildly pleomorphic endothelial cells (F, white arrowhead) and scant hyperreflective KPs were observed (E, white arrow).

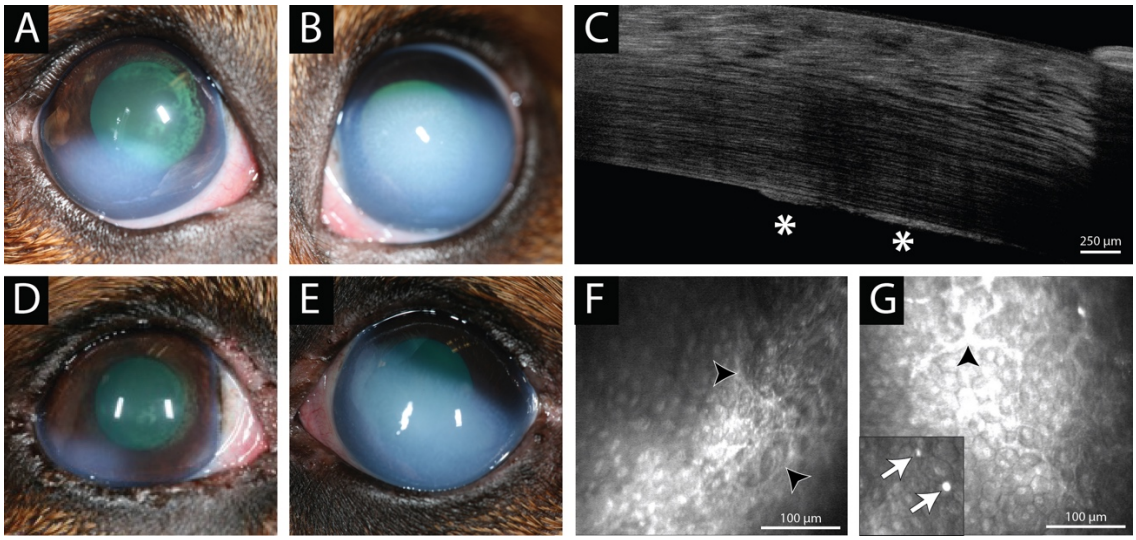

Figure 4. A 1-year-old female Australian cattle dog with repeated bouts of severe canine endotheliitis demonstrates marked changes to the corneal endothelium OU; corneal edema improved in the less affected OD four months later with topical netarsudil, anti-inflammatory and immunosuppressive therapy. One year after initial diagnosis of endotheliitis, marked inferior edema OD (A) and severe diffuse edema with only sparing of the superior perilimbal OS (B) was observed. With FD-OCT, hyperreflective deposits interpreted as KPs in the inferior cornea attached at the corneal endothelium were observed OD (C, asterisks) and OS (not shown). With IVCM, multifocal hyperreflective deposits partially cover a mildly pleomorphic endothelium (F, black arrowheads). After 4 months of topical netarsudil BID in combination with increased frequency of PA and NaCl to QID and the addition of tacrolimus BID, the corneal edema is improved OD (D) and static OS (E); With IVCM, persistence of hyperreflective deposits consistent with KPs (G, black arrows) were observed.

SUMMARY AND CONCLUSIONS

The corneal endothelium plays a key role in the maintenance of the corneal structure and transparency, and it is composed of a single layer of endothelial cells that have a limited regenerative capacity in some species including dogs, humans, and non-human primates (NHP). Corneal endothelial dystrophy (CED) in dog shares similar imaging features with Fuchs' endothelial corneal dystrophy (FECD) in humans, and it is characterized by premature degeneration and loss of corneal endothelial cells. During the completion of this work, one of the 144 NHPs examined for Chapter 2 demonstrated bilateral low endothelial cell densities and abnormal endothelial cell morphology reminiscent of FECD in humans or CED in dogs. This data suggests that, similar to other species with low regenerative capacity, NHPs also have corneal endothelial dystrophies. While it is exciting to ponder all the potential applications of a spontaneous NHP model for corneal endothelial dystrophy, the incidence of this condition within the colony appears very low (0.7%). Chapter 2 also provides relevant data for thickness and corneal endothelial cell density in a colony of rhesus macaques, and associated relationships with age, sex, and weight. Understanding the similarities and differences between humans and macaques, as well as establishing reference values, is key to recognize the value and limitations of rhesus macaques as animal models for studying corneal endothelial diseases and treatments.

Treatment options for canine CED include hypertonic saline ointment and palliative surgical treatments, that can help to reduce corneal thickness and improve vision. However, none of those treatments address the primary problem in CED patients, which is the progressive deterioration of the endothelial cells. The ROCK inhibitors have been shown promote endothelial cell survival *in vitro* and accelerate endothelial regeneration *in vivo* in dogs and rabbits. In dogs, a previous clinical trial

by our laboratory tested the efficacy of ripasudil 0.4% (Glanatec®) for CED. We demonstrated stabilization or improvement of clinical disease in more than half of the eyes included in the study after one year of treatment. However, one of the limitations of this medication is that it needs to be applied 4 times a day and adherence to the treatment is not always possible or constant. Furthermore, it is also not commercially available in the United States and thus owners must purchase it from Japanese online pharmacies. In humans, a pilot study in patients with FECD reported improvement in corneal clarity and vision associated with administration of topical netarsudil, a ROCK inhibitor that only requires twice daily application. With these promising results, we design a double-mask, placebo-controlled clinical trial to the efficacy and safety of netarsudil 0.02% ophthalmic solution (Rhopressa®) applied twice daily for canine CED or primary corneal endothelial degeneration (PCED). Preliminary results are detailed in Chapter 3. As expected, the most common adverse reaction observed was conjunctival hyperemia. Unfortunately, our interim analysis did not find statistically significant differences between the placebo and the treatment group. While there is ongoing data collection that needs to be included in the analysis, this preliminary study indicates that efficacy of netarsudil applied twice daily is inferior to ripasudil 4 times a day. While this study does not intend to explain the reason of the differences on efficacy observed between the two medications, it is possible that the effect of the medication is dependent on maintenance of the concentration of the medication in the cornea that requires more repeated applications per day.

In humans, genetic and non-genetic factors have been shown to play a role in FECD, and in dogs, a genetic predisposition is suspected in Boston terriers, given that this breed was overrepresented in a retrospective study of canine CED. In Chapter 4, we did not find candidate loci associated with CED in Boston terriers with GWAS-

approach using 80 cases and 107 controls. This data suggests that CED is inherited in a complex manner in Boston Terriers and additional dogs are needed to find loci associated with a risk of CED in this breed.

In Chapter 5, we discuss canine corneal edothelliitis. Although uncommon, canine corneal endotheliitis should be considered within the differential diagnosis for dogs with bilateral corneal edema. Canine corneal endotheliitis typically causes edema that is more severe in the inferior cornea, that persists beyond resolution of anterior uveitis, and responds to anti-inflammatory medication.

As a conclusion, the major findings of this work include:

1. The corneal thickness and endothelial cell density in rhesus macaques as well as the relationship of those parameters with age, sex and weight are similar to humans. This data highlights the value of rhesus macaques as animal models

2. Topical netarsudil has similar tolerability to other ROCK inhibitors when administered to CED or PCED patients. The current data does not show differences on efficacy between the placebo and the treatment group.

3. CED is likely inherited as a complex trait in Boston terriers.

4. Canine corneal endotheliitis should be considered a differential consideration in patients with bilateral corneal edema particularly when it is more severe inferiorly, associated with anterior uveitis, and responds to anti-inflammatory medication. Life-long topical corticosteroids may be necessary to prevent recurrence.