The significance of pre-iridal monocellular membranes in dogs and those with anterior segment dysgenesis associated glaucoma, secondary glaucoma, and primary glaucoma

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Abstract

Purpose: The etiology and pathogenesis of pre-iridal monocellular membranes in dogs with glaucoma is poorly understood. The objectives of this study were to define the association of pre-iridal monocellular and fibrovascular membranes with canine primary glaucoma with light microscopy and compare this to congenital glaucoma associated with anterior segment dysgenesis (ASD) and secondary glaucoma; and evaluate the immunohistochemical characteristics of the cells present in these membranes and characterize their ultrastructure by scanning electron microscopy.

Methods: 108 Enucleated eyes from dogs categorized clinically and histologically with primary/goniodysgenesis-associated glaucoma, congenital glaucoma associated with anterior segment dysgenesis (ASD) and secondary glaucoma were included in the study. The type of pre-iridal membrane and intraocular histologic findings were reviewed and compared statistically for each group. From this population, 10 globes from each group were selected to complete immunohistochemical labelling with CD18, Smooth Muscle Actin (SMA) and CD117. All of these globes had either a pre-iridal monocellular or fibrovascular membrane. Additionally, 10 normal globes were included as a control. SEM was completed on 10 globes selected from these 40 globes.

Results: Pre-iridal monocellular membranes appear to be more frequent in globes with primary glaucoma, whereas fibrovascular membrane are more common in secondary glaucoma. There was no statistical significance with type of membrane with breed, gender and age between glaucoma groups. Monocellular membranes were seen in 10/10 normal globes. CD18 was positive in 9/10 normal globes, and in 15 globes with monocellular membranes and 7 with fibrovascular membranes in glaucomatous eyes. SMA and CD117 were not detected in monocellular membranes of normal globes. SMA was positive in 10 monocellular and 7 fibrovascular membranes of glaucomatous globes. CD117 was present in 7 monocellular and 5 fibrovascular membranes glaucomatous globes. Monocellular membranes examined with SEM were a continuous sheet of spindle cells with scattered round cells that extended over the anterior iris face in normal and all glaucomatous globes examined.

Conclusions: Pre-iridal monocellular membranes are common in all types of canine glaucoma and also in normal eyes. These membranes seem to be a normal component of the anterior iris

surface and their immunoreactivity to CD18 suggests that some cells on this membrane are of hematopoietic stem-cell origin. In contrast the immunoreactivity to SMA and CD117 labelling of monocellular membranes in glaucomatous, but not normal globes, suggest metaplastic cellular change secondary to glaucoma.

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List of Abbreviations

AH: Aqueous humor

AS: Anterior synechia located elsewhere than the base of the iris

ASD: Anterior segment dysgenesis

CC: Ciliary cleft

CP: Ciliary process cyst

Factor VIII: Von Willebrand's factor

FBN1: Fibrilin-1

GD: Goniodysgenesis

H&E: Hematoxylin & Eosin stain

ICA: Iridocorneal angle

IOP: Intraocular pressure

MmHg: Millimetre of mercury

MOMC: monocyte-derived multipotential cells

NSE: Neuro specific enolase

OCT: Optical coherence tomography

PAS: Peripheral anterior synechia

PCG: Pigmentary cystic glaucoma

PIFM: Pre-iridal fibrovascular membrane

PL: Pectinate ligament

PLD: Pectinate ligament dysplasia

POAG: Primary open angle glaucoma

PPMS: Persistent pupillary membranes

PS: Posterior synechia

RPE: Retinal pigment epithelium

SEM: Scanning electron microscopy

SMA: Smooth muscle actin

THGP: Tamm-Horsfall glycoprotein

TM: Trabecular meshwork

UBM: Ultrasound Biomicroscopy

1.Literature Review

1.1 Glaucoma

Glaucoma has been diagnosed since antiquity and it was Hippocrates, who described "glaykoseis" as blindness occurring in elderly people.[1] In veterinary medicine, glaucoma in dogs was first studied by Magrane approximately 70 years ago. [2,3,4,5,6]

Glaucoma is characterized clinically by an elevated intraocular pressure (IOP). Elevated IOP develops when aqueous humor (AH) outflow is obstructed or reduced below the level of production.[7] The elevated IOP affects all ocular tissues. The retinal ganglion cells die by apoptosis and necrosis due to multiple factors [8, 9, 10] The loss of their axons results in optic nerve atrophy and blindness.[8, 11]

1.2 Pathogenesis of glaucoma

AH is a transparent fluid that is produced by the ciliary epithelium and flows through the posterior chamber and pupil into the anterior chamber. A small amount also enters the vitreous. AH exits the anterior chamber via the filtration angle. The rate of aqueous humour production in the dog is approximately 5.22 ± 1.87 microliters per minute. [12] AH provides nutrition to the avascular ocular tissues (lens and cornea) with small amounts of proteins, immunoglobulins, enzymes, lipids, and inorganic compounds, including carbohydrates (80%), urea (80%), and amino acids (0.08 to 3.14). [13] AH is also responsible for removing metabolic waste products, and it provides a fluid refraction that is part of the ocular refractive index (1.335).[14] The production of AH is relatively constant in the non-diseased state, therefore, the rate of exit of the AH from the globe determines the IOP. AH production by the non-pigmented ciliary epithelial cells is via the processes of active secretion, ultrafiltration and simple diffusion.[15] Active secretion is the most important mechanism accounting 80-90% of the production. [16] The non-pigmented ciliary epithelial cells contain carbonic anhydrase (an isoenzyme) which is responsible for the formation and transport of bicarbonate into the AH. [15] Sodium (Na+) ions are actively transported from the blood into the AH by a sodium/potassium adenosine triphosphate (ATPase) pump.[15] Ultrafiltration differs in that soluble components cross the ciliary capillaries into the ciliary body stroma due to differences in pressure between the ciliary body capillaries and the IOP.[17]

After the AH enters the anterior chamber it flows in a dorsal to ventral circulation along the endothelium of the cornea as it cools.[18] Eventually the AH enters and passes through the filtration angle. The major structures of the uveal outflow system are the iridocorneal angle (ICA), pectinate ligament (PL) and ciliary cleft (CC) which contains the uveal and scleral trabecular meshwork (TM).[19] The AH exits the eye through two pathways after entering the TM: the conventional and unconventional pathway.[15] In the conventional pathway of most domestic animals the AH drains from the TM and enters the aqueous collecting veins, where it mixes with blood in the episcleral veins. In primate species, AH flows from the TM, through the inner wall of Schlemm's canal, then into its lumen, and into draining collector channels, aqueous veins and episcleral veins. [19]

There are four processes that allow the aqueous humor to traverse the angular aqueous plexus through transcellular pores, large vacuoles, pinocytosis or by phagocytosis by macrophages and cells from the TM.[20] When the Schwalbe's line located in the ICA decrease in number, the amount of aqueous humor passing through the corneoscleral trabecular meshwork will decrease increasing the outflow resistance.[21] Also, the extracellular matrix in the TM plays an important role in regulation the IOP in normal and glaucomatous eyes.[22] Changes in the amount and quality of the TM extracellular matrix due to intraocular inflammation, cytokines, growth factors and medications etc., will affect the outflow resistance.[22]

The conventional pathway constitutes 85-90% of the volume of aqueous outflow depending on the species.[23] The unconventional uveoscleral outflow pathway accounts for the rest of the outflow and it is relatively independent of the IOP. [19] When the ciliary muscle contracts, the movements result in spreading of the TM which decreases outflow resistance and AH gains access to the choroid and suprachoroidal space, bypassing the aqueous venous plexus.[19] This route accounts 10-15% of the total AH drainage in dogs .[23,24]

Increased resistance to outflow of the AH can occur due to many reasons. Many underlying ocular diseases cause anatomic changes within the globe that obstruct AH outflow within either or both the conventional and non-conventional pathways. These include intumescent cataract, lens luxation, hyphema, intraocular neoplasia, and these are commonly implicated in the pathogenesis of *secondary glaucoma*.[25] Congenital anomalies of anterior segment development may induce congenital and neonatal glaucoma.[25,26,27] Finally, inherited abnormalities of ocular

development such as PL dysplasia (PLD) /goniodysgenesis are associated with, or at least serve as a marker for, the development of some cases of *primary glaucoma*. [25,28,29,30]

Many additional factors have been associated with the development of primary glaucoma in dogs and these include age, gender and breed.[30,31,32] Age-related changes of the anterior segment include exfoliation of the pigmented cells of the iris, pupillary atrophy and fibrosis, pigment dispersion with phagocytosis by macrophages, and accumulation of collagen in the ciliary processes.[31,32,33] Another risk factor for development of primary glaucoma includes gender, with females having approximately 2:1 frequency comparted to males.[30] The angle opening distance has been shown to be smaller in females than in males and a shorter axial globe length has also been documented.[31,32,34] Genetic risk factors have been reported in primary glaucoma in dogs with different breeds having been classified to have higher risk to develop glaucoma.[30,34,35,36]

1.3 Types of glaucoma

Canine glaucoma may be classified as congenital/neonatal, secondary, or primary (idiopathic). Congenital and neonatal glaucoma is associated with anterior segment dysgenesis (ASD) and manifest clinically at birth or within few weeks of life.[27,37] Secondary glaucoma occurs secondary to an obvious antecedent ocular disease that is impairing aqueous drainage.[7] Primary glaucoma is an elevated IOP without other antecedent intraocular disease and is often assumed to have genetic etiologies based on breed associations.[30,34,38,39]. Primary glaucoma in the beagle has been investigated extensively.[11,30,35] The mutation responsible for this disorder is known and it is within the ADAMTS10 gene.[35] However, primary glaucoma associated with goniodysgenesis remains idiopathic and the pathogenesis of this late onset, bilateral, breed specific disease remains unknown.[25]

1.3.1 Congenital/neonatal glaucoma

In contrast to the plethora of literature on primary and secondary glaucoma in dogs, sparse references exist for congenital glaucoma.[26,27,37,40] Congenital/neonatal glaucoma implies a diagnosis of glaucoma or buphthalmos at birth. Animals with anterior segment dysgenesis (ASD) develop glaucoma within the first few months to years of life .[25,27,37] This form of congenital glaucoma is associated with ASD in some dogs. The anterior segment anomalies of ASD affect the cornea, lens and anterior uvea and include small lenses (microphakia), cataracts, hypoplastic

uvea and cornea.[41,42,43] Persistent pupillary membranes (PPMs), and defects in corneal stroma and endothelium may also be present.[26]

1.3.2 Secondary glaucoma

Secondary glaucoma develops when there is concurrent ocular pathology that is most likely reducing or stopping the circulation and drainage of the AH, therefore leading to an increase in IOP.[44] and it is the most common type in dogs.[45,46,47] It may manifest as a unilateral, or bilateral condition. Secondary glaucoma develops secondary to several acquired or inherited ocular conditions such as Cairn terrier melanocytosis, and Golden retriever pigmentary uveitis. [48,49] The AH flow may be impeded at the level of the pupil, ICA or TM.[7] Obstruction at the pupil may occur due to blockage by the lens, vitreous, inflammatory material, tumor or synechiae.[7] Obstruction in the ICA or TM may be secondary to the presence of blood, inflammatory material, remains of uveal cysts, neoplasia, peripheral anterior synechia, or pre-iridal fibrovascular membranes (PIFM).[7,50]

Uveitis is one of the most common causes and accounts for 45% of secondary glaucoma in dogs.[51] The pathogenesis of elevated IOP caused by uveitis may include inflammatory debris occluding the TM,CC, pupil or ICA; complete posterior synechiae causing pupillary block and iris bombe; and peripheral anterior synechia blocking the ICA.[7] PIFM may develop with chronic uveitis which also cover the ICA.[7] Primary lens luxation secondary to zonular degeneration will lead to development of glaucoma resulting from vitreous being displaced into the anterior chamber with obstruction of the pupil and iridocorneal angle.[52] Intumescent cataracts that develop secondary to diabetes mellitus may induce uveitis, may compress the ciliary cleft and there is potential risk of lens capsule rupture and the formation of posterior synechiae and peripheral anterior synechia and complete CC collapse.[7,53] Dogs that have had phacoemulsification have some risk to develop glaucoma, secondary to synechia, and the presence of fibrin and inflammatory cells obstructing the pupil or ICA.[54,55,56] Chronic retinal detachments stimulate the formation of a PIFM which may occlude the angle or pupil.[50,57] Intraocular masses or neoplastic cells can cover or infiltrate the IC, and they may also promote formation of a PIFM.[50,58] Pigmentary uveitis of the Golden Retriever commonly leads to secondary glaucoma, although the exact mechanism is not fully understood. Pigmentary uveitis is associated with the formation of multiple translucent uveal cysts as well as pigment dispersion

within the globe. Globes removed due to development of glaucoma typically have amorphous fibrin-like accumulations, ruptured and intact thin walled uveal cysts, multiple synechia, dispersed uveal pigment and PIFM. All of these may disrupt AH outflow.[59,60,61] Ocular melanocytosis of the Cairn terrier and other dog breeds is an inherited condition in which the iris becomes thickened by pigment-laden cells which are released into the AH resulting in pigment deposition in the iridocorneal angle, and sclera. This infiltration of the drainage angle with pigment-laden cells leads to obstruction of AH outflow and secondary glaucoma.[62]

1.3.3 Primary glaucoma

Primary glaucoma is an idiopathic, assumed to be genetically based, condition that begins in one eye and will affect the contralateral eye months to years later usually late in life (average 9 years old).[29,30,34,63] The most common form of primary glaucoma is associated with PLD where there is a lack of fenestration of the ICA, also known as goniodysgenesis.[64] Goniodysgenesis is a bilateral congenital abnormality of the PL.[11,64] The etiology and the pathogenesis are largely unknown, although yet to be identified mutations and coincidental inflammation, pigment dispersion, vascular and aging changes in the eye all may predispose to the development of primary glaucoma.[7,30,33] It is important to note that only a small percentage of dogs with goniodysgenesis will develop bilateral glaucoma in their lifetime.[65] Thus, there are other factors at play in the pathogenesis of primary glaucoma associated with goniodysgenesis as mentioned above.

Goniodysgenesis is present at birth and is a dysplastic lesion diagnosed in the dog with gonioscopy.[30] In the last two decades several papers have documented narrowing or perhaps recession of the angle in dogs with goniodysgenesis and these studies have proposed that goniodysgenesis is a progressive disorder associated with angle closure.[66,67,68,69,70] We believe that it is unlikely, as congenital PLD is not progressive, and the reported changes are more likely explained by age-related changes including enlarging lens size, age related pigment dispersion and uveal atrophy, alterations in collagen, and recession of the filtration angle.[11,33]

The term “closed angle” is most common term used today to describe goniodysgenesis. However, a closed anatomical angle implies apposition of the base of the iris to Descemet’s membrane. This is in conflict with the true anatomically closed angle seen when peripheral

anterior synechiae develops causing secondary glaucoma.[43] Therefore, goniodysgenesis is not a closed angle, as the anatomic position of the base of the iris and Descemet's membrane are not in apposition. Rather, there is a lack of fenestration of the PL tissue. Bauer *et. al.* published a double blinded study on the histologic evaluation of filtration angles from dogs with known secondary and confirmed primary glaucoma in dogs with goniodysgenesis. They reported that chronic glaucoma alters the filtration angle substantially and experienced pathologists and ophthalmologists were unable to differentiate the angles as affected with PLD or normal .[71] Thus, chronicity of elevated IOP causing stretching of the globe changes the anatomy of the ICA. This results in the angle associated with goniodysgenesis affected by chronic glaucoma, and those filtration angles that were clinically normal but affected by chronic secondary glaucoma are indistinguishable by light microscopic examination.

Glaucoma associated with goniodysgenesis is most frequently seen in the American Cocker Spaniel, English Cocker Spaniel, Basset Hound, Bouvier des Flandres, Welsh Springer Spaniel, Chow Chow, Samoyed, and Norwegian Elkhound. [30,38, 65, 67, 71,72,73] Genetic mutations may play a role in canine glaucoma associated with goniodysgenesis including COL1A2, RAB22A, and NEB genes in Basset Hounds, olfactomedin like 3 (*OLFML3*) in Border Collies and also SRBD1 in Shiba inus and Shih Tzus.[74,75,76]

There are forms of primary glaucoma that are not associated with goniodysgenesis that have been termed primary open angle glaucoma (POAG). This condition has been extensively studied in the beagle.[35] POAG was identified as an inherited autosomal recessive trait on canine chromosome 20.[35,76] The ICA in dogs with POAG does not have typical gonioscopic features of goniodysgenesis. Rather the PLs are present and appear similar to those of normal dogs, and the ICA remains open until the end stages of the disease when the CC collapses.[34,35] A genetic mutation responsible for this form of POAG was identified as ADAMTS10.[35] Specific mechanisms of glaucoma are unknown, however ADAMTS10 is involved in the production of matrix metalloproteinase involved in the function and structure of microfibrils.[77] The variant Gly661Arg in ADAMTS10 disrupts the microfibrils structure increasing the resistance of the AH outflow.[77]

Other breeds can rarely be affected by forms of non-goniodysgenesis associated primary glaucoma. This includes the Norwegian Elkhound, where affected dogs develop a slowly progressive accumulation of FBN1 plaques located in the TM that may increases the AH outflow resistance similar to the glaucoma seen in Beagles.[34,38] IOP increases around 8-16 months of age due to an alteration of the extracellular matrix and defects in the microfibril structure that will decrease the AH outflow.[31,38]

The study of primary glaucoma is confounded by the variety of potential genetic, aging and inflammatory predisposing triggers, the lack of a canine model that will consistently develop primary or idiopathic glaucoma associated with goniodysgenesis, and the inability to reliably confirm goniodysgenesis histologically in buphthalmic globes. Therefore, the most consistent diagnostic criteria for idiopathic glaucoma is a combination of the clinical confirmation of goniodysgenesis, and elevated IOPs that eventually affects both globes in the absence of other intraocular pathology known to induce secondary glaucoma.[71]

1.4 Diagnosis of Glaucoma

IOP can be measured directly by manometry or it can be estimated by tonometry.[78,79] The direct measurement of the IOP is done via paracentesis by cannulating the anterior chamber of the eye.[78] Although this method is very accurate, it is invasive and involves general anesthesia and therefore not a practical procedure in a clinical setting.[78] Therefore IOP is usually estimated in clinical practice by a large variety of tonometers: rebound tonometer such as the ICARE Tonovet ®, applanation tonometer or TonoPen XL® , Perkins®, MacKay-Marg, pneumatic tonometers such as the Pulsair® or Reichert Non-Contact® tonometer, and indentation tonometers called Schiotz ®.[78,80,81] In human medicine the Goldman applanation tonometer is most commonly used and is considered the gold standard instrument.[82] However, this is not a practical instrument to use in animals.[83] Although the Schiotz ® and applanation tonometers are diagnostically useful, the rebound tonometers are subjectively easier to use, produce consistent results by a variety of relatively unskilled examiners, and they are considered to be the most useful tonometer in multiple species.[78,83]

Tonography is a technique that estimates AH outflow. This procedure requires the animal to be sedated or anesthetized as the probe needs to rest on the cornea for a prolonged period of time. This procedure is not used commonly in the clinical settings.[81,84]

IOP in dogs varies considerably and a range of 15-25 mm Hg is considered normal by many ophthalmologists.[79,85] The clinical diagnosis of glaucoma is confirmed by tonometry and an IOP greater than 30 mmHg is considered consistent with a diagnosis of glaucoma.[31,85] Once diagnosed, glaucoma is categorized as congenital/neonatal, secondary, or primary by taking into consideration the signalment and history, biomicroscopic, ophthalmoscopic, and gonioscopic findings.[7,71,72,78,86]

Gonioscopy allows the examiner to visualize the ICA including the PLs and anterior CC in the normal eye.[68,72] Gonioscopy utilizes a biomicroscope and a contact lens such as the Koeppe, Barkan or multiple other hand-held and mirrored lenses.[68]

Ultrasound Biomicroscopy (UBM) has been studied to evaluate the structure of the ICA in dogs.[86,87,88] UBM provides high-resolution, in vivo image of the anterior segment architecture providing objective and quantitative information of the entire ICA and CC. [86] UBM, provides an image of the cross-sectional structures of the entire ICA and ciliary cleft that may not be seen with gonioscopic examination.[86]

Optical coherence tomography (OCT) is a non-contact imaging modality that offers high-resolution cross-sectional images of the cornea and retina similar to light microscopy.[89,90,91] Normal ICA of 6 animal species have been evaluated using Visante ® AS-OCT (Carl Zeiss Meditec, Inc., Dublin,CA) to measure the ICA.[92] The ability to image the ICA utilizing OCT depends on the machine and can be limited in dogs due to the size of the canine globe and location of the ICA. However, OCT images of the ICA and retina is useful to evaluate the amount of retinal and optic nerve damage due to glaucoma and therefore assists the clinician during the management of the disease.[89,90,91]

1.4.1 Clinical signs

The presenting signs of glaucoma vary, depending on type of glaucoma, stage (acute versus chronic), and age of the affected animal.[31,64] The acute manifestations of primary glaucoma include episcleral injection, corneal edema, and often a dilated pupil. Episcleral injection occurs due to episcleral vasculature congestion secondary to the ocular inflammation due to the impaired AH drainage through the scleral venous plexus and non-convectional outflow pathways.[64] Corneal edema occurs due to a decompensation in the corneal epithelium pump.[64]A dilated pupil may be due to impaired iris muscle and intraocular neurological dysfunction, and has the potential to promote the development of peripheral anterior synechia that will further reduce AH outflow.[64]

With chronicity, ocular manifestations include: buphthalmos, corneal striae, corneal vascularization and pigmentation, mydriasis, lens luxation or subluxation, vitreous degeneration and retinal and optic nerve degeneration.[64,93] Enlargement of the globe (buphthalmia, hydrophthalmos, megaglobus or macrophthalmia) occurs due to stretching of the ocular tissues secondary to high IOP.[64] Breaks in Descemet's membrane (corneal striae) are caused by the stretching of the less elastic inner cornea.[41,64] Vascularization and pigmentary keratitis occur secondary to exposure.[64,79] Enlargement of the globe leads to stretching and breakdown of the zonular fibers supporting the lens causing luxation or subluxation.[64]

Dogs with secondary glaucoma commonly present with signs of severe uveitis, i.e. aqueous flare, aqueous fibrin, hypopyon, and/or hyphema. Pupil size and shape may be variable as uveitis commonly results in miosis, however, as the IOP increases the pupil may become more dilated. Also, posterior synechia may develop causing irregularity in pupil shape and even iris bombe. Many clinical signs of secondary glaucoma are similar to the signs of primary glaucoma as they also include episcleral vascular congestions and corneal edema. Similar to chronic primary glaucoma, secondary glaucoma induces with buphthalmos, corneal striae, corneal vascularization and pigmentation, lens luxation and subluxation, vitreous degeneration and retinal and optic nerve degeneration.[64]

The ICA can be classified as closed (peripheral anterior synechiae): manifested by a shallow

anterior chamber when examined with a biomicroscope, and a closed angle when viewed with gonioscopy; the angle may be obstructed by cellular infiltrates (neoplasia, inflammatory cells and fibrin, hemorrhage and cellular debris); or the angle may be open and appear normal and the anterior chamber is deep. It is important to realize that secondary glaucoma may develop in globes with goniodysgenesis due to all the aetiologies mentioned above.

1.5 Microscopic examination of canine globes with glaucoma (light, scanning and transmitting electron microscopy)

All intraocular and ocular tissues are affected by glaucoma and most globes examined contain a plethora of non-specific histologic changes.[11]Canine globes are rarely examined in the acute stages of any category of glaucoma and the histologic signs of all of these include varying amounts of uveitis, pigment dispersion, mild neutrophilic infiltrate in the lamina cribosa, ganglion cell death, optic nerve axon, ganglion cell, inner plexiform and inner nuclear loss and optic nerve atrophy and gliosis.[8,11,43,64]

Histologic examination of canine glaucoma is most often completed in the chronic stages and changes are again very similar in all three categories of glaucoma. In addition to the histologic features noted in acute glaucoma. The features of chronic glaucoma include degeneration of the outer plexiform layer of the retina, thinning and stretching of the sclera and breaks in Descemet's membrane.[11,64]

Unique histologic features of congenital/neonatal glaucoma include small lenses (microphakia), hypoplastic anterior uvea, filtration angle and ciliary cleft.[27,41,43,94,] Unique histologic features of secondary glaucoma relate to the ocular lesions leading to alteration in the AH dynamics in the eye. These include features of uveitis, lens luxation, intraocular neoplasia, retinal detachments, pre-iridal vascular membranes and cellular proliferation.[7,43] In addition, pre-iridal monocellular membranes have also been noted to be common in dogs with primary glaucoma.[95]

Fixation, processing, sampling, orientation artefacts and folding of the globe when placed on the slides for light microscopy can lead to erroneous interpretation[11] Electron microscopy preserves the ultrastructure of tissues giving a three-dimensional image and precise specific cell structures,

from the molecular anatomy to the whole cell.[96] Ultrastructural changes associated with glaucoma examined by transmitting (TEM) and scanning electron microscopy (SEM) are reported sparingly in the veterinary literature.[97,98,99] SEM has been used in veterinary ophthalmology in canine eyes to examine the vessels of aqueous drainage and the PL structure of the dog.[97,99] ICAs showed different types and degrees of abnormalities on SEM.[98] One study examining five abnormal canine ICAs assessed by gonioscopy showed holes or imperfections in the sheet that covers the ICA in four of the five cases on SEM.[98]

1.6 Pre-iridal membranes

Histologically, the normal appearance of the iris surface has been described as a discontinuous layer composed of fibroblasts, melanocytes and connective tissue.[100] Peiffer *et al* was the first to report the presence of PIFM in 83 diseased canine eyes.[57] The presence of three types of membranes were described (cellular, vascular or fibrous). It was postulated that these membranes likely formed in response to angiogenic and fibroblastic stimuli from leucocytes, neoplasia or ischemic retina. These membranes were most commonly seen in chronic endophthalmitis, glaucoma, and intraocular neoplasia. Cellular membranes were described as a monolayer of plump cells and, occasionally spindle cells that extended over the anterior iris surface. Vascular membranes were formed from budding iridal capillaries and fibrous membranes from fibrous and vascular component.[57] PIFMs are most commonly associated with lens induced uveitis, hyphema, retinal detachment, endophthalmitis and ocular trauma and intraocular neoplasms.[57,58,95,101] Moore *et al* reported PIFMs in 86% of canine globes enucleated after cataract surgery and intractable glaucoma and uveitis.[101]

Recently it was noted on a light microscopic study that cellular membranes are commonly present in goniodysgenesis-associated primary glaucoma.[95] The monocellular membranes were noted to cover the ICA and merge seamlessly with the corneal endothelium in some (5/16) globes, however in a few (2/12) globes with a PIFM, the membrane was just covering the iris and ICA and did not extend onto the corneal endothelium.[95] Although similarities exist with both, the true relationship between cellular membranes and the corneal endothelium, as well as PIFMs remain unknown.

1.6.1 Immunohistochemistry of pre-iridal membranes

Immunohistochemistry detects cellular markers in tissue sections by immunological labelling target cells.[102] This technique is very sensitive and specific and can detect a wide variety of antigens in animals.[102] Immunohistochemistry has been used to study PIFMs and monocellular membranes. Differences in immunohistochemical profiles between PIFMs and monocellular membranes suggest different origins of these membranes. PIFMs and monocellular membranes are similar in that they both label with vimentin and are negative for cytokeratin AE1/AE3.[95] However, PIFMs are positive to Factor VIII-related antigen, while monocellular membranes are negative.[95] This suggests that both PIFMs and monocellular membranes have a mesenchymal origin.[95]

Gornik *et al* evaluated 17 enucleated globes with pre-iridal fibrovascular membranes categorized as early (cellular) or chronic (fibrovascular) in canine eyes with various ocular diseases and found that all were positive to CD34 (a hematopoietic and stem cell marker) and 88% were positive for CD 18 (a monocyte marker of leukocyte lineage).[103] This suggests that pre-iridal membranes may have a hematopoietic progenitor stem cell and monocyte origin.[103]
Recent immunophenotyping of normal canine corneal endothelium and pre-iridal cellular membranes revealed these cellular membranes and the corneal endothelium in normal canine eyes are positive for S100, vimentin, neuro specific enolase (NSE), weakly positive for Tamm-Horsfall glycoprotein (THGP) and negative for pancytokeratin.[104] The authors suggested that normal canine endothelium is immunophenotypically similar to human corneal endothelium, and pre-iridal monocellular membranes are likely to originate from corneal endothelium.[104]
PIFMs are commonly observed with light microscopy in canine globes with secondary glaucomas occurring due to retinal detachment, uveitis and intraocular neoplasia.[57,58,95] PIFMs can obstruct AH outflow at the level of the pupil or the ICA to cause elevated IOP.[57,58] Pre-iridal monocellular membranes are also commonly seen in canine globes with primary glaucoma histologically at the Prairie Diagnostic Services at the Western College of Veterinary Medicine (WCVM). The origin, pathogenesis, and significance of these membranes in relation to PIFMs and glaucoma is of interest.

1.7 Hypothesis

The origin and significance of pre-iridal monocellular membranes are unknown; they may be a consequence of disease, they may contribute to development of primary glaucoma, pre-iridal fibrovascular membranes, or simply be an insignificant finding in canine globes. There are two possibly related cell populations reported in humans which may be associated with the origin of monocellular membranes. These are termed monocyte-derived multipotential cells (MOMC) and fibrocytes.[105,106] MOMC may originate from circulating monocytes and differentiate at the site of tissue injury/inflammation.[105,107] Fibrocytes may enter sites of tissue injury and contribute to scar tissue formation.[106]

Cellular membranes in canine eyes appear to have characteristics of MOMC and fibrocytes and these could be involved in the pathogenesis of primary glaucoma. MOMC are a population of cells with a fibroblast-like morphology. They have a unique phenotype positive for CD14 and CD45 (hematopoietic and monocyte lineage markers), CD34 (stem cell marker), CD117 (stem cell marker) and type 1 collagen.[105] MOMC appear to originate from circulating monocytes. Differentiation from monocyte to MOMC requires binding of fibronectin and soluble factor(s) from CD14-blood cells encountered at the site of tissue injury and inflammation.[105] Subsequent differentiation into tissue-specific cells is thought to occur in response to organ-specific cues provided by surrounding cells.[105] Additionally, a potentially related population of circulating cells with fibroblast properties has been described.[106] These cells are believed to enter sites of tissue injury and are termed fibrocytes. They are characterized by distinctive phenotype positive for CD 45, CD 34, and type 1 collagen, and are negative for CD14. Fibrocytes contribute to scar formation and may play an important role in normal wound repair and pathological fibrotic responses.[106] Fibrocytes may be related to MOMC. In vitro, MOMC may differentiate into a variety of mesenchymal cell types in permissive culture conditions developed for mesenchymal stem cells. Upon full differentiation they lose expression of CD45 and CD14.[105]

As noted above, previous studies have investigated pre-iridal membranes in canine eyes with various ocular diseases and found that they were positive to CD18 and the stem-cell marker

CD34 suggesting there may be some similarities to MOMC and fibrocytes.[103] We wished to investigate these similarities further. Therefore, the research questions we had were:

1. What is the association of the pre-iridal monocellular membrane with canine primary, secondary and congenital forms of glaucoma?
2. What is the origin of pre-iridal monocellular membranes?
3. What is the relationship of the pre-iridal monocellular membrane to the pre-iridal fibrovascular membrane?

1.8 Purpose and Objectives of the Research

The primary objective of this study is to define the prevalence of pre-iridal monocellular and fibrovascular membranes with globes affected with congenital glaucoma associated with anterior segment dysgenesis (ASD), primary glaucoma associated with goniodysgenesis (GD), and secondary glaucoma. We evaluated enucleated globes under light microscopy and histologic features were recorded. Statistical analysis was performed for each of the histological lesions compared to globes with monocellular versus fibrovascular membranes.

The second objective was to see if there are associations of these membranes with breed, gender, age and histopathologic ocular changes. For this we calculated the prevalence of membranes within age and gender categories and compared the age of dogs with congenital/ASD-associated glaucoma with primary or secondary glaucoma, and between dogs with primary and secondary glaucoma. The median age of dogs in each type of glaucoma were also calculated. The associations between monocellular and fibrovascular membranes with light microscopic findings and type of glaucoma were calculated statistically.

The third objective was to evaluate the origin of pre-iridal monocellular membranes by further investigating the immunohistochemical characteristics of pre-iridal membranes. For this we completed immunohistochemical staining of globes with pre-iridal monocellular and fibrovascular membranes in primary, secondary, and congenital glaucoma as well as in normal eyes with CD 18, CD 117, smooth muscle actin (SMA).

In the fourth objective, we characterized the pre-iridal monocellular membrane ultrastructure with scanning electron microscopy (SEM). We performed SEM on sections of 5 globes with monocellular membranes: 3 normal globes, 1 globe with primary glaucoma, 1 globe with congenital glaucoma; and 3 with secondary glaucoma all with fibrovascular membranes. Additionally, we selected two globes from our archives with no visible membrane on light microscopy, both of which had been diagnosed with secondary glaucoma.

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2. Prevalence and significance of pre-iridal membranes in 108 glaucomatous canine eyes.

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2.1Abstract

Purpose: (i) To evaluate the prevalence of pre-iridal monocellular and fibrovascular membranes in canine globes affected with congenital glaucoma associated with anterior segment dysgenesis (ASD), primary glaucoma associated with goniodysgenesis (GD), and secondary glaucoma, (ii) examine the associations of these membranes by breed, gender, age and histopathologic ocular changes.

Methods: The hospital and histopathologic records of dogs who had eyes enucleated due to blindness and uncontrolled glaucoma were reviewed. Glaucoma was categorized clinically and histologically into three groups: congenital/ASD associated, primary associated with goniodysgenesis (confirmed with Gonioscopy), and secondary associated with significant contributing intraocular disease. The presence or absence and type of pre-iridal membrane (monocellular or fibrovascular) and other intraocular histologic findings were reviewed and compared statistically for each group.

Results: 108 canine globes (101 dogs) were included in the study. The number of globes where pre-iridal monocellular membranes were detectable in congenital/ASD, primary, and secondary glaucoma were 10/19, 29/40, 23/49 respectively. In contrast 3/19, 9/40, and 24/49 respectively had fibrovascular membrane. There was no statistical significance with type of membrane with breed, gender and age between glaucoma groups.

Conclusions: Pre iridal monocellular membranes are common in all types of canine glaucoma. However, they appear to be frequent findings in globes with primary glaucoma, whereas fibrovascular membrane are more common in secondary glaucoma. The origin and significance of the pre-iridal monocellular membranes and their relationship to pathogenesis of glaucoma remains unknown. Further studies are needed that evaluate control globes and glaucomatous eyes with immunohistochemistry and electron microscopy.

Key words: dogs, eye, glaucoma, pre-iridal monocellular and fibrovascular membranes, goniodysgenesis, congenital/ASD associated glaucoma, secondary glaucoma, primary glaucoma associated with goniodysgenesis.

2.2 Introduction

Glaucoma is a common condition in veterinary ophthalmology characterized by elevated intraocular pressure (IOP) that induces significant ocular dysfunction and degeneration of all ocular tissues with buphthalmos and blindness as common sequelae. Although the pathogenesis of glaucoma is not fully understood, elevated IOP develops due to inadequate aqueous humor filtration which leads to progressive retinal and optic nerve degeneration related to axonal damage, apoptosis and necrosis of retinal ganglion cells.[1,2] In the dog, aqueous humor outflow is primarily through the iridocorneal angle (conventional outflow) but a small percentage leaves by diffusion through the iris, ciliary body, and choroid via venous drainage (unconventional outflow).[3] Decreased aqueous humor outflow occurs due to physiologic or structural modifications of the outflow pathways from the posterior chamber, pupil, filtration angle, trabecular meshwork (TM), vasculature of the scleral venous plexus, and the choroidal outflow pathways.[4,5,6]

Canine glaucoma may be classified into three basic categories. Congenital glaucoma is associated with anterior segment dysgenesis (ASD) and often manifests at birth or in young dogs. Primary glaucoma may be associated with goniodysgenesis (GD) or a normal appearing filtration angle in older dogs (7-10 years of age). Secondary glaucoma is when antecedent intraocular disease has been identified and is assumed to directly interfere with aqueous humor exit.

Congenital or early onset ASD-associated glaucoma manifests in neonatal animals or early in life, usually less than 3 years of age.[7,8,9,10,11] This form of glaucoma is associated with ASD which manifests with ocular anomalies including hypoplastic filtration angle and ciliary cleft, lenticular and anterior uveal hypoplasia.[7, 8,12] The pathogenesis is unknown, and some eyes affected with ASD fail to develop glaucoma. The percentage of canine globes affected with ASD that develop glaucoma is unknown.

Primary glaucoma is most commonly associated with GD and occurs in several breeds of dogs.[13] GD is a synonym for pectinate ligament dysplasia (PLD), where there is limited fenestration of the neural crest tissues within the iridocorneal angle during ocular development.[13] This form of glaucoma manifests in mid to late life and initially presents in one eye with the contralateral eye developing glaucoma months to years later.[15] Therefore, glaucoma associated with GD

eventually manifests bilaterally in affected dogs. Most assume a genetic predisposition for primary glaucoma associated with GD.[13, 14, 16] GD is considered to be a marker for primary glaucoma, as only a small percentage (<10%) of dogs with GD will develop glaucoma at mid to late life.[13]

Secondary glaucoma is an acquired condition and develops when there is antecedent ocular pathology resulting in reduced aqueous humor drainage. Secondary glaucoma may be unilateral or bilateral depending on the etiology.[4]

A common histologic finding in all types of canine glaucoma is the presence of a pre-iridal membranes that may span the filtration angle. Peiffer *et al* described these initially in 83 diseased canine eyes.[17] They classified these membranes as monocellular, vascular or fibrous. It was proposed that these membranes were likely formed in response to angiogenic and fibroblastic cytokines that provide stimuli to vascular iridal tissues. These cytokines are released from intraocular tissues related to chronic intraocular inflammation, neoplasia, and ischemic retina.[17] Pre-iridal membranes were most commonly seen in globes with chronic retinal detachments, endophthalmitis, chronic glaucoma and neoplasia (ciliary body adenoma, uveal melanoma, and metastatic tumors).[17] In a study of the light microscopic appearance of the ICA in chronic glaucoma, it was noted that pre-iridal monocellular membranes were more common in eyes diagnosed with primary glaucoma associated with GD, whereas fibrovascular membranes were more common in secondary glaucoma.[18]

It has been suggested that pre-iridal monocellular membranes seen in diseased canine eyes may represent an early form of the PIFM, or that they may originate from metaplastic corneal endothelium that traverses the filtration angle.[17,19]

The objectives of this study therefore were: (i) evaluate the prevalence of monocellular and fibrovascular pre-iridal membranes in dogs diagnosed with congenital/ASD-associated glaucoma, dogs with primary glaucoma associated with GD, and those with secondary glaucoma; and (ii) determine whether there is any association between the presence of monocellular pre-iridal membranes with breed, gender, age, type of glaucoma, and light microscopic examination findings.

2.3 Material and methods

2.3.1 Data collection

Medical records of dogs referred to the ophthalmology service and where an enucleated globe(s) submitted to an Ocular pathology service with the diagnosis of glaucoma between 2000 and 2018 were reviewed. Patient information collected included: clinical diagnosis and ophthalmic examination findings, breed, gender, neuter status, and age at presentation.

Clinically, only eyes examined by a board-certified veterinary ophthalmologist were included in the study. Histologically, Periodic acid-Schiff and/or Hematoxylin and Eosin (H&E)-stained slides were reviewed. The slides of each enucleated globe were examined by a board-certified veterinary ophthalmologist (DACVO, B.G) for the purposes of this study.
The inclusion criteria for congenital/ASD-associated glaucoma included age of ≤ 3 years with buphthalmos and ophthalmic and histologic findings of glaucoma and ASD (such as ciliary body and iris and filtration angle hypoplasia, microphakia, cataract, and elongated hypoplastic ciliary processes) (Figure 2-1a-c).

Inclusion criteria for primary glaucoma were gonioscopic findings confirming pectinate ligament dysplasia in the non-glaucomatous eye and, whenever possible, confirmation of eventual bilateral glaucoma that resulted in enucleation (n= 5 dogs) and light microscopic examination of both globes that excluded antecedent ocular disease (n=8 globes). (Figure 2-2).

Inclusion criteria for secondary glaucoma included globes with antecedent ocular disease (intraocular neoplasia, pigmentary uveitis, melanocytosis (melanosis) of Cairn Terriers, lens luxation, endophthalmitis, glaucoma secondary to phacoemulsification, lens induced uveitis) confirmed on clinical and light microscopic examinations, and a normal gonioscopic (n=5) examination of the non-glaucomatous eye provided that this clinical test was performed (Figure 2-3a-c) .

2.3.2 Light microscopic evaluation

Archived enucleated globes which had been formalin- or Davidson's–fixed and paraffin-embedded, were routinely sectioned, stained samples with Hematoxylin and Eosin or Periodic acid-Schiff were examined by light microscopy. Histologic features were recorded for each globe: type of neoplasia in secondary glaucoma, corneal striae, corneal ulcer, corneal edema, closed ciliary cleft, peripheral anterior synechia (PAS), type of uveal inflammation, uveal cysts (classified as thin walled cyst and pigmentary uveitis in the Golden Retriever, posterior iris epithelium atrophy, uveal atrophy, anterior or posterior synechia, cataract, tapetal sparing, vitreous degeneration, retinal detachment classified as exudative or serous when there was accumulation of fluid within the subretinal space (serous effusion, fresh blood or inflammatory exudates), rhegmatogenous retinal detachment when a full-thickness hole or tear in the neurosensory retina with vitreous in the subretinal space. Focal detachments included focal associated areas of RPE (retinal pigment epithelium) hypertrophy with loss of outer nuclear layer. When there was degeneration that reduced the retina to a glial scar with RPE hypertrophy we classified the retina (inner and outer) simply as degenerate.

The ciliary cleft was described as open or collapsed. The ICA was described as being open if there was no apposition of the iris base to the peripheral cornea and closed if there was peripheral anterior synechia attaching the iris to the corneal endothelium. Angle recession was noted if the ICA was stretched so that the iris base was far from the termination of Descemet's membrane and pectinate ligaments or dysplastic filtration angle were not observed and assumed to be flattened against the inner sclera near the aqueous collection veins.

The presence and type of pre-iridal membrane (monocellular or fibrovascular) were also recorded. Criteria for a monocellular membrane were a single layer of multiple contiguous non-pigmented plump and flattened spindle cells, closely apposed or slightly lifted from the anterior surface of the iris (Figure 2-4). Criteria for a pre-iridal fibrovascular membrane (PIFM) were a vascular membrane that covered the surface of the iris and were formed from small blood vessels that arose from the underlying iridal vessels and fibrous tissue at different stages of maturity (Figure 2-5).

2.3.3 Statistical Analysis

Contingency tables were created, and Fisher's exact test was used to compare the prevalence of the presence of membranes across congenital glaucoma/ASD, primary glaucoma, and secondary glaucoma as well as the presence of each membrane type (Table 2-1).

Contingency tables were created for each of the histological lesions compared to globes with monocellular vs fibrovascular membranes. Odds ratio were calculated as a measure of clinical effect for those relationships that have a p-value less than 0.05 based on a Chi-square test (Table 2-3).

Non-parametric t-test was calculated to know the prevalence of membranes within age and gender categories and to compare the age of dogs with congenital/ASD-associated glaucoma with primary or secondary glaucoma, and between dogs with primary and secondary glaucoma. The median age of dogs with congenital/ASD-associated glaucoma, primary and secondary glaucoma were also calculated.

Statistical significance for all tests was set at $P \leq 0.05$ and Stata14 software (StataCorp) was used for data analyses. If dogs had more than one globe included within the study, one eye was excluded from the statistical dataset to ensure that all observations were independent within the statistical analysis. Also, only globes with the same type of membrane on each side were included to avoid bias (n=105)

2.4 Results

2.4.1 Association of mono-cellular and fibrovascular pre-iridal membranes with type of glaucoma

A total of 108 canine globes (101 dogs) were included in the study. From those eyes, 19 were congenital/ ASD-associated glaucoma, 40 GD-associated glaucoma, and 49 secondary glaucoma. There was no significant difference between the presence of monocellular and fibrovascular membranes in the congenital/ASD group (P=0.092). Monocellular membranes were significantly more common than fibrovascular membranes in the primary glaucoma group (P<0.001). There

was no significant difference in presence of monocellular and fibrovascular membranes in the secondary glaucoma group (P=1.0).

There was significant difference with the presence of any type of membrane across the three glaucoma types (P=0.033). There was a significant difference with the presence of a monocellular membrane across the three glaucoma categories likely driven by primary glaucoma (P=0.037) There was a significant difference with the presence of a fibrovascular membrane across the three glaucoma categories likely driven by secondary glaucoma (P=0.010). These results are summarized in Table 2-1.

2.4.2 Associations of demographics with type of glaucoma and pre-iridal membranes

The most representative breed in the congenital/ASD-associated glaucoma group (n=18) were the Siberian Husky (n=3) and German Shepherd (n=2) however these numbers were too small to be meaningful.

In primary glaucoma associated with GD (n=37), the most common breeds were Cocker Spaniel (n=6), Shih Tzu (n=5), Labrador Retriever (n=5), and Shiba Inu (n=5).

The most common breeds included in secondary glaucoma (n=46) were Cocker Spaniel (n=8), Golden Retriever (n=5), Shih Tzu (n=4), and Boston Terrier (n=4). We could not assess statistical associations of breed and type of glaucoma or presence of a pre-iridal membrane due to low statistical power.

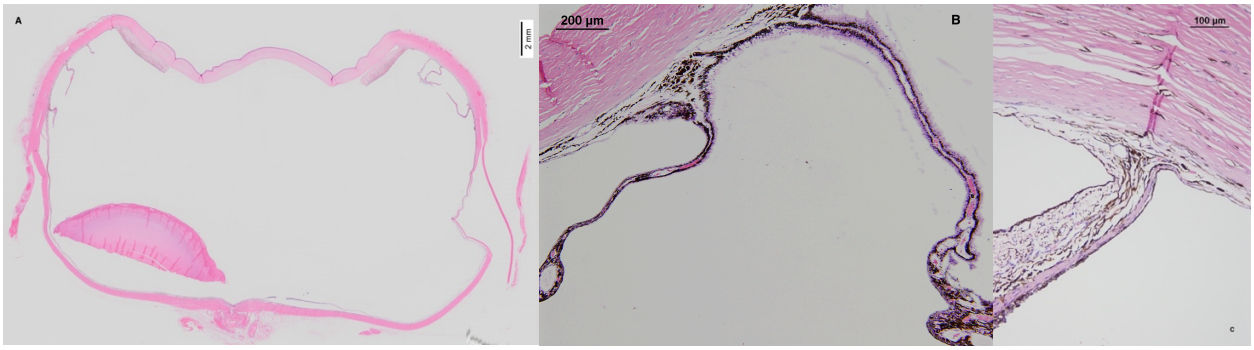
The median age of dogs with congenital/ASD-associated glaucoma was 1.3 years (range 0.3-3), primary glaucoma associated with GD was 8.3 (1-16), secondary glaucoma was 8.9 (1-16). The median age of dogs with congenital/ASD-associated glaucoma was significantly younger when compared to dogs with primary or secondary glaucoma (P<0.0001). Age was not significantly different between dogs with primary and secondary glaucoma (P=0.3847). There was no association with age and presence of a pre-iridal membrane, either monocellular (P=0.76) or fibrovascular (P=0.90).

In the congenital/ASD-associated glaucoma group, 9 were female, 5 were male dogs and 4 were unknown gender. In GD glaucoma group there were 17 females, 19 males and 1 unknown gender. In secondary glaucoma there were 24 females, 21 males and 1 of unknown gender. There

was no statistical difference with sex and presence of a pre-iridal monocellular (*P*=0.98) or fibrovascular membrane (*P*=0.5).

2.4.3 Association of pre-iridal membranes with light microscopic findings.

Associations between monocellular and fibrovascular membranes with light microscopic findings and type of glaucoma are summarized in Tables 2-2 & 2-3. The most common light microscopic findings overall were a collapsed ciliary cleft which was present in 72% of globes and generalized retinal degeneration present in 69% of globes (both inner and outer retinal degeneration).There was a significant statistical difference with the proportion of PAS (*P*= 0.0001) across monocellular and fibrovascular membranes. The odds of PAS were 0.094 (CI= 0.02-0.39) times less likely for globes with monocellular membranes than in globes with fibrovascular membranes. There was also a significant statistical difference with the proportion of globes with uveal atrophy (*P*= 0.02) across monocellular and fibrovascular membranes. The odds of uveal atrophy were 3.26 (CI=1.1-10.9) times more likely in globes with monocellular membranes than fibrovascular membranes.



Figures 2-1 A-C. (A) This is a subgross histologic section of a canine globe with ASD-associated glaucoma. Note the elongated and hypoplastic ciliary processes, and hypoplastic ciliary body (Hematoxylin and Eosin stain). (B) This is a higher power histologic section that reveals a hypoplastic and elongated ciliary processes in a young dog with ASD associated glaucoma (Hematoxylin and Eosin stain). (C) Hypoplastic filtration angle in a young dog with ASD associated glaucoma (Hematoxylin and Eosin stain).

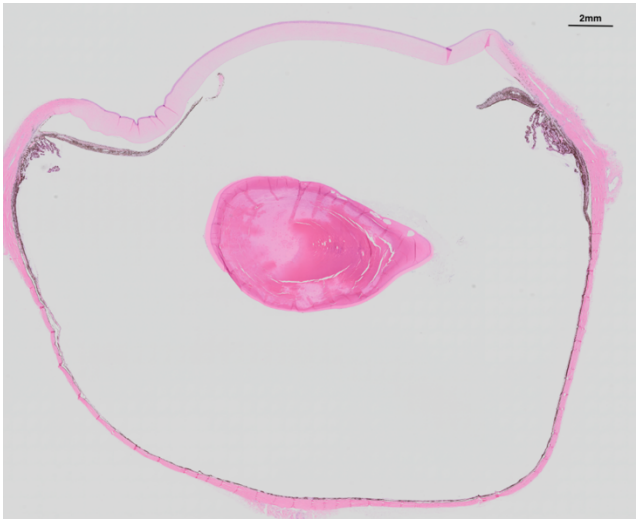


Figure 2-2. Low power picture of a buphthalmic eye in a dog with primary glaucoma (Hematoxylin & Eosin stain).

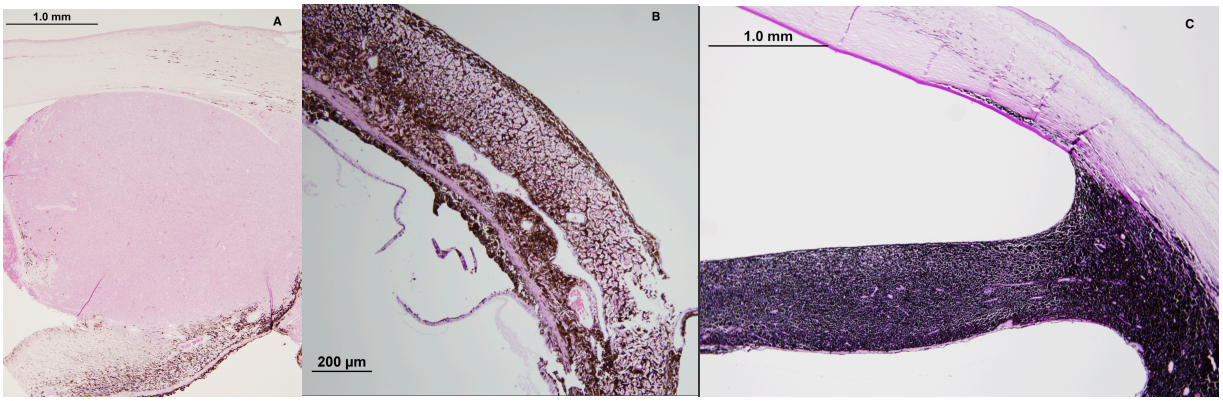


Figure 2-3 A-C. (A) This is a low power histologic section of the iris of a dog with ciliary epithelial adenocarcinoma (Hematoxylin & Eosin stain). (B) Microscopic photograph of a thin walled cyst on the posterior aspect of the iris in a Golden Retriever (Hematoxylin and Eosin stain). (C) Microscopic photograph of an iris with melanocytosis in a Cairn Terrier (Periodic acid-Schiff stain). All of these were designated as secondary glaucoma.

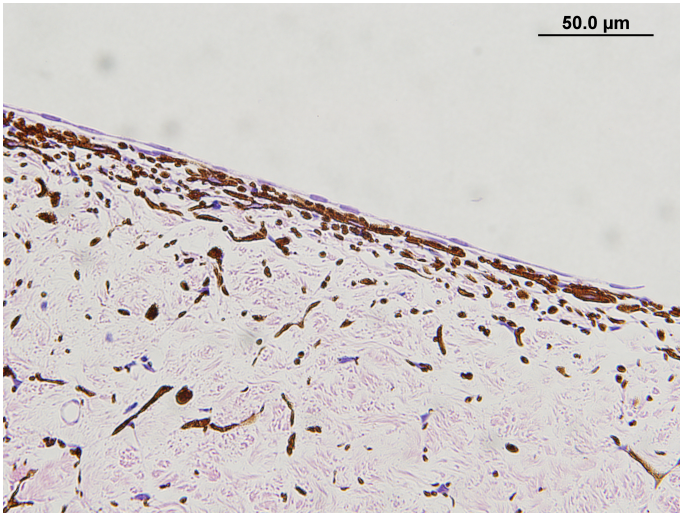


Figure 2-4. A low power histologic section of a pre-iridal monocellular membrane in a dog with primary glaucoma. (Hematoxylin & Eosin stain).

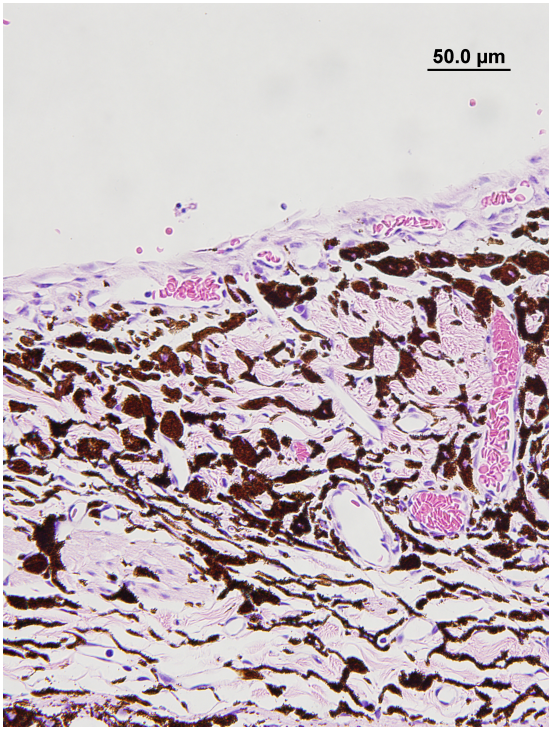


Figure 2-5. Low power histologic section of a pre-iridal fibrovascular membrane in a dog with secondary glaucoma. (Hematoxylin & Eosin stain).

Table 2-1: Prevalence of membranes in each type of glaucoma.

Glaucoma	Congenital/ASD	Primary	Secondary	*P* value
Total	19	40 (39)*	49 (47)*	108 (105)*
Presence of membrane	13/19 (68%)	36/39 (92%)	43/47 (91%)	0.033
Monocellular	10/19 (53%)	29/39 (74%)	22/47 (47%)	0.037
Fibrovascular	3/19 (16%)	8/39 (20%)	22/47 (47%)	0.010

*For the P value only globes with the same type of membrane on each side were included to avoid bias (n=105).

Table 2-2: Histological findings associated with each type of glaucoma.

	Congenital			Primary			Secondary			Total
# eyes cases	19			40			49			108
Corneal striae	3 (17%)			1 (3%)			1 (2%)			5 (4.6%)
Corneal ulcer	1 (5.5%)			3 (7.5%)			4 (9%)			8 (7.4%)
Corneal edema	5 (28%)			21 (58%)			24 (49%)			50 (46%)
CC: collapsed	11 (58%)			35 (87.5%)			32 (65%)			78 (72%)
PAS	0 (0%)			3 (7.5%)			13 (26.5%)			16 (15%)
Uveal cyst	1 (5.5%)			5 (13.8%)			9 (19.5%)			15 (14%)
	TW	CP	A	TW	CP	A	TW	CP	A	
	0	0	1(100%)	0	2 (40%)	3(60%)	5(55%)	1 (11%)	3(33%)	
	4 (22.2%)			15 (41.6%)			22 (47.8%)			
Uveal inflammation	L	LP	LPN	L	LP	LPN	L	LP	LPN	
	0	4(100%)	0	0	14(93%)	1(7%)	2(9%)	13(59%)	7(32%)	41 (38%)
Uveal atrophy	18 (100%)			13 (36%)			6 (13%)			
AS	5 (27%)			6 (17%)			15 (33%)			26 (24%)
PS	2 (11%)			8 (20%)			7 (15%)			17 (16%)
Cataract	5 (27%)			9 (25%)			27 (55%)			41 (38%)
Retinal detachment	5 (33%)			6 (11%)			17 (39%)			28 (26%)
	S/E	F	R	S/E	F	R	S/E	F	R	
	1(20%)	0	4(80%)	2(33%)	4(66%)	0	7(41%)	8(47%)	2(12%)	

Retinal degeneration (inner and outer)	15 (79%)	26 (65%)	33 (67%)	74 (69%)
Dorsal/tapetal sparing	1 (5.5%)	18 (45%)	7 (15%)	26 (24%)
Angle recession	1 (5.5%)	6 (17%)	8 (17%)	15 (14%)

CC, ciliary cleft; PAS, peripheral anterior synechia; AS, anterior synechia located elsewhere than the base of the iris (PAS); PS, posterior synechia; L, lymphocytic uveitis; LP, lymphoplasmacytic uveitis; LPN, lymphoplasmacytic neutrophilic uveitis; TW, thin walled cyst; CP ciliary process cyst; A, posterior iris epithelium atrophy; S, serous; E, exudative; F, focal; R, rhegmatogenous; NA, no applicable.

Table 2-3: Association between histologic findings and type of membrane.

Histologic findings	Monocellular membrane (62 eyes)	Fibrovascular membrane (36 eyes)	*P* value	Odds Ratio (95% confidence interval)
Corneal striae	2(3%)	2(5%)	0.59	0.58(0.04-8.42)
Corneal ulcer	5(8%)	3(8%)	1	1(0.18-6.8)
Corneal edema	31(50%)	15(40%)	0.36	1.4(0.6-3.6)
CC: close	42(68%)	28(76%)	0.40	0.67(0.23-1.8)
PAS	3(5%)	13(35%)	0.0001	0.094(0.02-0.39)
Uveal cyst	9(15%)	5(13%)	0.89	1(0.3-4.5)
Uveal inflammation	25(41%)	13(38%)	0.61	1.2(0.5-3.2)
Uveal atrophy	24(38%)	6(16%)	0.02	3.26 (1.1-10.9)
AS	12(19%)	9(24%)	0.56	0.75(0.25-2.3)
PS	9(15%)	6(16%)	0.82	0.87(0.25-3.3)
Cataract	21(34%)	19(51%)	0.08	0.48(0.2-1.2)
Retinal detachment	14(22%)	12(32%)	0.28	0.6(0.22-1.7)
Retinal degeneration (inner and outer)	39(63%)	26(70%)	0.45	0.7(0.26-1.85)

Dorsal/tapetal sparing	19(31%)	6(16%)	0.11	2.3(0.75-7.7)
Vitreous degeneration	2(3%)	2(5%)	0.60	0.58(0.40-8.4)
Angle recession	9(14%)	3(8%)	0.34	1.9(0.43-11.75)

CC, ciliary cleft; FA, filtration angle; AS, anterior synechia located elsewhere than the base of the iris (PAS); PS, posterior synechia.

2.5 Discussion

Light microscopic examination of glaucomatous eyes revealed pre-iridal membranes present in the majority 98/108 (91%) of the diseased canine globes. Of these, most 62/98 (63%) were monocellular while 36/98 (37%) were fibrovascular membranes. Fibrovascular membranes were significantly more common in secondary glaucoma compared to primary glaucoma and monocellular membranes were significantly more common in primary glaucoma compared to secondary glaucoma, which is consistent with the study done by Bauer *et al*. [18] However, monocellular membranes were not exclusive to the primary glaucoma group and, in fact, they were a common histologic finding in all forms of glaucoma.

Studies of pre-iridal membranes have theorized that monocellular membranes may be associated with development of primary glaucoma, that they may be an early form of a fibrovascular membrane, or that they may originate from metaplastic corneal endothelium.

[17,18,19]

With small numbers and multiple breeds represented, it was not possible to analyze the effect of breeds statistically. Primary glaucoma associated with GD has been reported to be more common in American Cocker Spaniel, English Cocker Spaniel, Basset Hound, Bouvier des Flandres, Welsh Springer Spaniel, Chow Chow, Samoyed, and Norwegian Elkhound. [13, 14, 20] Of these breeds we had just 1 American Cocker Spaniel, 6 English Cocker Spaniel, 2 Basset Hound, 2 Chow Chow, and 1 Norwegian Elkhound. Secondary glaucoma can develop in any breed subsequent to other inherited conditions such as melanocytosis, and pigmentary cystic glaucoma (PCG). [21, 22] In our secondary glaucoma group, 1 Cairn Terrier with melanocytosis and 5 golden retrievers with PCG were included. We did not document congenital/ASD-associated glaucoma to be overrepresented in any canine breed.

The most common light microscopic findings in globes with glaucoma was a collapsed ciliary cleft and outer and inner retinal degeneration. These findings are commonly reported in the veterinary literature[2,5, 23]. The ciliary cleft is closed or collapse in most dogs presented with chronic glaucoma.[12] In dogs with primary GD associated glaucoma this may be related to either increase in the IOP or by a slow progressive change in the CC itself, however the mechanism is unknown.[24]

When light microscopic findings were evaluated for associations with membrane type, only PAS and uveal atrophy showed any association. PAS was 10.7 times (CI=2.5-61.8) more likely to be present in globes with fibrovascular membranes. PAS has already been reported to be a common finding in globes with fibrovascular membranes and likely occurs secondary to the fibrovascular membrane extending across the filtration angle to the corneal endothelium effectively closing the iridocorneal angle.[26] Uveal atrophy is common with all categories of chronic glaucoma and may be a direct result of tissue hypoxia and pressure induced atrophy secondary to a loss of pigment, smooth muscle and vasculature.[15] The significance of age in dogs with congenital/ASD glaucoma ($P<0.0001$) is attributed to the fact that these dogs are all young while the primary and secondary glaucoma manifests much later in life.

Limitations of this study are those common to all retrospective studies including incomplete clinical and histopathological information which reduced the number of animals that met our inclusion criteria. We were unable to truly assess severity or duration of glaucoma prior to enucleation due to difficulties confirming historical information. Some dogs with primary glaucoma were lost to follow up and, thus we were unable to confirm if they developed glaucoma in the contralateral eye. Gonioscopy was not performed in all secondary glaucoma cases, nor was it possible when glaucoma presented bilaterally simultaneously in dogs with congenital/ASD associated, primary GD associated glaucoma, or secondary glaucoma.

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3.Immunohistochemistry and SEM evaluation of pre-iridal monocellular and fibrovascular membranes of dogs with primary goniodysgenesis associated glaucoma, secondary glaucoma, and anterior segment dysgenesis glaucoma, with comparison to normal canine globes.

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3.1 Abstract

Purpose: (i) To evaluate immunohistochemical labelling of pre-iridal monocellular and fibrovascular membranes and (ii) describe the light and scanning electron microscopic (SEM) characteristics of these membranes in glaucomatous and normal canine globes.

Methods: Immunohistochemical labelling for CD18, smooth muscle actin (SMA) and CD117 was completed on 40 canine globes including those with congenital/anterior segment dysgenesis-associated glaucoma (n=10), primary/goniodysgenesis-associated glaucoma (n=10), secondary glaucoma (n=10), and normal globes (n=10). SEM was completed on 10 globes including: 5 with monocellular membranes, 3 with fibrovascular membranes and 2 without a histologically detectable membrane. **Results:** Monocellular membranes were noted in all normal globes on light microscopy and appeared to be morphologically very similar to those in diseased globes. CD18 was detected in 9/10 monocellular membranes in normal globes, 15/23 monocellular and 7/8 fibrovascular membranes in globes with glaucoma. SMA and CD117 were not detected in monocellular membranes of normal globes. SMA was positive in 10/23 monocellular and 7/8 fibrovascular membranes of glaucomatous globes. CD117 was present in 7/23 monocellular and 5/8 fibrovascular membranes glaucomatous globes. SEM of monocellular membranes revealed a continuous sheet of mostly spindle cells and few individual round cells that extended over the anterior iris face in normal and all glaucomatous globes examined. **Conclusion***:* Pre-iridal monocellular membranes are a normal component of the anterior iris surface and CD18 positivity suggests some cells within these are of histiocytic origin. SMA and CD117 labelling of monocellular membranes in glaucomatous, but not normal globes, suggest metaplastic cellular change secondary to intraocular pathology related to glaucoma.

3.2 Introduction

Pre-iridal membranes are a common finding in diseased canine globes. The presence of pre-iridal membranes in dogs was first described in 1990 by Peiffer *et al.*[1] Pre-iridal membranes were classified into 3 types: monocellular, vascular and fibrous. Pre-iridal fibrovascular membranes (PIFMs) were most commonly seen in globes with chronic endophthalmitis, glaucoma, and intraocular neoplasia from different animals (dogs, cats, horses). Monocellular membranes were described as a single cell layer of plump to spindle shaped cells on the anterior iridal surface. Fibrovascular membranes consisted of multiple layers of small vessels with supporting fibroblasts, arising from the anterior stroma of the iris, with subsequent deposition of collagen that occasional covered both iridal surfaces.[1] Fibrovascular membranes develop in response to angiogenic factors released within the globe secondary to conditions such as retinal detachment, intraocular neoplasia, and chronic uveitis.[1, 2] The origin of the pre-iridal monocellular membrane, however, remains unknown. Peiffer *et al* suggested that monocellular membranes were an early stage of fibrovascular membranes. [1]

Recently, Bauer *et al* reported monocellular iridal membranes to be more common in canine globes with primary glaucoma, while PIFMs were more common in canine globes with secondary glaucoma.[3] These two types of membranes were evaluated with immunohistochemical labels in glaucomatous canine globes to determine if endothelial cell metaplasia or iridal vascular budding participates in the development of the monocellular membrane.[3] Canine globes were labeled with vimentin, cytokeratin AE1/AE3 and Von Willebrand's factor (Factor VIII). Both fibrovascular and monocellular membranes were positive for vimentin, and negative for cytokeratin AE1/AE3. Fibrovascular membranes additionally expressed positivity to Factor VIII-related antigen whereas the monocellular membranes were negative for this antigen. Based on these findings, Bauer *et al* concluded that monocellular membranes are most likely of mesenchymal origin and are unlikely to be derived from vascular or corneal endothelial cell metaplasia.[3] This is in contrast to a recent study of normal canine corneal endothelium and monocellular membranes, which revealed monocellular membranes and the corneal endothelium in normal canine globes have a similar immunophenotype.[4] These authors noted that canine corneal endothelium and the monocellular membranes were positive for S100, Vimentin, Neuro-

specific enolase (NSE), weakly positive for Tamm-Horsfall glycoprotein (THGP), and negative for pancytokeratin. These results suggested that normal canine endothelium is immunophenotypically similar to human corneal endothelium, and that monocellular membranes were likely to originate from corneal endothelium.[4]

Gornik *et al* evaluated canine globes with varied ocular disease using immunohistochemistry and categorized types of pre-iridal membranes into different stages of membrane development.[5] Monocellular membranes were categorized as an early developmental stage, while fibrovascular membranes were intermediate or chronic, similar to Peiffer's original paper.[1] Early and intermediate pre-iridal membranes were found to be positive to CD18 and CD34, which label leucocytes and stem cells, respectively, which led them to suggest that monocellular membranes arose from a hematopoietic stem cell origin. [5,6,7]

Bone marrow-derived mesenchymal precursors present in the circulation are attracted to sites of injury where they differentiate into spindle-shaped fibroblast like cells called fibrocytes, which are different from fibroblasts. [6,8, 9, 10,11,12, 13, 14] Fibrocytes are believed to differentiate from CD14+ peripheral blood monocytes. [12, 13, 15, 16] Fibrocytes are a subpopulation of leucocytes characterized by positive immunophenotype for CD45, CD34, CD18 and type 1 collagen.[6,11] It has been suggested that these cells have pluripotential features of both leucocyte and connective tissue, and are able to differentiate along fibroblast, smooth muscle, or osteogenic lines depending on the environment and wound site.[6] They may act as antigen-presenting cells, provide contractile forces of wound closure via α-smooth muscle actin (SMA) induction, promote angiogenesis, produce cytokines, chemokines, and growth factors that induce fibroblast hyperplasia, and secrete components of extracellular matrix.[11] Fibrocytes have been implicated in a wide range of fibrotic processes involving the eye.[11] Based on their spindle shape, their immunophenotype, and their behaviour, we speculated that these cells may be involved in the formation of pre-iridal membranes of diseased globes in dogs, and possibly contribute to development of glaucoma.

There is still much that is unknown about pre-iridal membranes. The origin and significance of monocellular membranes is undetermined; as is their relationship to fibrovascular membranes.

Additionally, they are common in canine primary glaucoma, however, it is not clear if monocellular membranes contribute to the pathogenesis of glaucoma.[3] It is possible that monocellular membranes may be a consequence of ocular disease or even a normal finding. The objectives of this study were (i) to evaluate the immunohistochemical characteristics of the cells of pre-iridal monocellular and fibrovascular membranes and (ii) to characterize their histologic and ultrastructure with scanning electron microscopy with comparison of normal canine globes to enucleated globes with varied types of chronic glaucoma.

3.3 Material and Methods

3.3.1 Globe selection for Immunohistochemistry

Forty archived formalin-fixed, paraffin embedded enucleated canine globes were selected. These included four groups (n=10 for each), which were normal globes, globes diagnosed with congenital and anterior segment dysgenesis (ASD)-associated glaucoma, globes from dogs with primary glaucoma associated with goniodysgenesis, and globes with common forms of secondary glaucoma.

Selection criteria for normal globes were those without clinical or histologic detectable disease. All glaucomatous globes were clinically and histologically confirmed to have glaucoma based on a board-certified ophthalmologist and pathologists' examinations. Selection criteria for congenital/ASD-associated glaucoma were young animals (≤ 3 years) with histologically confirmed ASD. Selection criteria for the primary glaucoma group included globes with gonioscopically confirmed goniodysgenesis, lacking potential causes of secondary glaucoma, and with both globes affected by glaucoma at the end of the follow up in older dogs (≥6 years old). For each of the above groups, 8 globes with monocellular membranes and 2 with fibrovascular membranes were chosen for evaluation with immunohistochemical labels. Selection criteria for secondary glaucoma included globes with glaucoma secondary to common intraocular aetiologies including ciliary body adenoma/adenocarcinoma (n=5), pigmentary/uveitis/glaucoma of the Golden Retriever (n=4) and Cairn Terrier ocular melanosis (n=1). In this group 7 globes with monocellular membranes and 4 globes had fibrovascular membranes were chosen for evaluation with immunohistochemical labels. One globe with

secondary glaucoma had a monocellular membrane on one iris leaflet and a fibrovascular membrane was present on the other leaflet.

Monocellular membranes were a histologically discernable single layer of contiguous non-pigmented plump and flattened spindle cells, closely apposed or slightly lifted from the anterior surface of the iris. The anterior layer of the iris was defined as the outmost layer of flattened pigmented cells. Care was taken to ensure that monocellular membranes were not mistaken as detached corneal endothelium. Globes were determined to have pre-iridal fibrovascular membranes when the surface of the iris was at least partially covered by small blood vessels sprouting (or arising) from the underlying iridal vessels and fibrous tissue at different stages of maturity.

3.3.2 Light microscopy and Immunohistochemistry

Six-micron sections were routinely prepared and stained with hematoxylin and eosin (H&E stain) and periodic acid Schiff (PAS stain). Immunohistochemistry was completed at the Ocular Pathology Service using an automated slide stainer (Autostainer Plus, Dako Canada Inc., Mississauga, ON). Heat-induced epitope retrieval was performed in a Tris/EDTA pH 9 buffer for 20 minutes.

We utilized CD18 which labels leucocytes, specially histiocytes. SMA as a marker of smooth muscle actin filament. CD117 as a marker for stem cell growth factor receptor (Table 3-1).[4,6,8,10] The primary antibodies for the detection of CD 18 (Monoclonal, Mouse anti-canine CD18 clone CA16.3C10, P. Moore, UC-Davis, Davis, CA) SMA (Monoclonal, Mouse anti-smooth muscle specific actin, Leica Microsystems Inc, Concord, ON) CD117 (Rabbit anti-CD117, Dako Canada Inc., Mississauga, ON) were applied for 30 minutes, and binding of the primary antibodies was detected using an HRP-labelled polymer detection reagent (EnVision+ System - HRP Labelled Polymer, Dako Canada Inc., Mississauga, ON). Positive controls included the intestine for SMA, gastrointestinal stromal tumor for CD117, and spleen for CD18.

Slides were reviewed by an ophthalmology resident, a board-certified veterinary pathologist and a board-certified ophthalmologist. Immunoreactivity was scored using these criteria: location of the label in the cell (membrane, cytoplasmic, nuclear), shape of cell labelled in the pre-iridal membrane (spindle cell, round cell), and percentage of immunopositive cells, round or spindle (< 25%, 25-50%, 50-75% or >75 %). The slides were randomized, and examiners were masked as to the type of glaucoma. Round cells were only counted when they were within or closely apposed on the membranes on the iridal surface.

3.3.3 Scanning electron microscopy (SEM)

Ten globes were selected for SEM, including 5 with monocellular membranes: 3 normal globes, 1 primary glaucoma, 1 congenital glaucoma; and 3 with a fibrovascular membrane (all secondary glaucoma). Additionally, we selected two globes from our archives with no visible membrane on light microscopy, both of which had been diagnosed with secondary glaucoma.

SEM was completed with a Hitachi SU8010 field emission scope with an accelerating voltage of 3kV. All eye samples were further fixed with 1% OsO4 in 0.2M sodium cacodylate. Samples were then sequentially dehydrated for 20 minutes at a time using 30, 50, 70, 80,95,100% ethanol (EtOH), repeated three times and then substitution proceeded with a 1:3 mixture of Amyl acetate: 100 EtOH for 20 minutes, followed by 1:1, 3:1 and lastly pure amyl acetate. Samples were critical point dried in a CO_2, then sputter coated with 10nm thick Au films using the Quorum Q150T ES sputtering unit and then they were imaged.

3.3.4 Statistics analysis

Fisher's exact test was used to compare the prevalence of each immunohistochemical label between monocellular and fibrovascular membranes, and the association between type of glaucoma and the immunohistochemical labels. McNemar's Chi Square was used to compare the three different immunohistochemical labels on monocellular and fibrovascular membranes, and to compare the association between immunohistochemical labels and normal globes versus glaucoma. Statistical significance for all tests was set at P value ≤ 0.05. Stata14 software (StataCorp) was used for data analyses.

3.4 Results

3.4.1 Histologic characteristics of pre-iridal monocellular and fibrovascular membranes

Monocellular membranes appeared as a histologically discernable layer on the surface of the iris primarily formed of flattened spindle cells, although most also contained some round cells (Figure 3-1a, Table 3-2). Monocellular membranes were noted in all control globes where they appeared like those present in diseased globes. In contrast, fibrovascular membranes were multicellular, layered, formed from blood vessels that originated from capillaries within the iris stroma. All fibrovascular membranes also contained both round and spindle cells assumed to be remnants of or complete portions of the monocellular membrane (Figure 3-1b, Table 3-2), often on the surface of the fibrovascular membranes.

3.4.2 CD18

Results of immunohistochemistry by type of glaucoma are summarized in Table 3-3. CD18 labelling was detected in 9/10 monocellular membranes of control globes and 15/23 monocellular membranes of globes with glaucoma (Figure 3-2). CD18 labelling was detected in monocellular remnants and some cells within 7/8 fibrovascular membranes of globes with glaucoma (Figure 3-3). CD18 labelling was present in the perinuclear cytoplasm of both round and spindle cells within membranes.

Round cells were fewer in number than spindle cells within monocellular membranes, however *in glaucomatous globes* labelling for CD18 was more common in round cells than spindle cells being positive in greater than or equal to 75% of the round cells present in 12/31 positive membranes, and less than 25% of the spindle cells in 22/31 positive membranes (Tables 3-4, 3-5 & 3-6).

3.4.3 SMA

SMA labelling of monocellular membranes was absent in all normal globes but was present in 10/23 monocellular membranes of glaucomatous globes (Figure 3-4a) and in 7/8 fibrovascular membranes of glaucomatous globes (Figure 3-4b). SMA labelling was present in the cytoplasm of primarily spindle cells within positive membranes. From all the membranes positive to SMA, labelling was present in round cells in only one monocellular membrane. SMA labelling was

detected in less than 25% of the spindle cells in 13/17 positive membranes (8/10 monocellular membranes and 5/7 fibrovascular membranes within glaucomatous globes) greater than 75% of spindle cells in 2/17 positive membranes (1/10 monocellular and 2/7 fibrovascular membranes).

3.4.4 CD117

Like SMA labelling, CD117 labelling was absent in all normal globes. CD117 was positive in 7/23 of the monocellular membranes (Figure 3-5a), and 5/8 of the fibrovascular membranes from globes with glaucoma (Figure 3-5b). One globe in the secondary glaucoma group with both a monocellular and a fibrovascular membrane was positive for CD117 on the monocellular membrane side only. CD117 labelling was positive in the cytoplasm in less than 25% of the round and spindle cells and was primarily present in the round cells in both monocellular and fibrovascular membranes, with only one monocellular membrane and one fibrovascular membrane with positive spindle cell labelling for CD117.

3.4.5 Association of membrane type with immunohistochemical labelling

There was no difference in presence of CD18 labelling between monocellular and fibrovascular membranes ($p = 0.65$). However, SMA ($p = 0.014$) and CD117 ($p = 0.005$) were significantly associated with the presence of fibrovascular membrane. Fibrovascular membranes were more likely to be positive for SMA ($p = 0.029$) and CD117 ($p = 0.028$) compared to CD18. Whereas, monocellular membranes were more likely to be positive for CD18 compared to SMA ($p = 0.004$) and CD117 ($p < 0.0001$).

The proportion of globes positive for CD18 did not differ between glaucoma cases and controls. ($p = 0.2744$). There was a significant difference in the proportion of globes that labeled positive for SMA between glaucoma cases and controls ($p = 0.001$) and CD117 status ($p = 0.0168$) as controls were negative for these immunohistochemical labels.

3.4.6 Scanning electron microscopy (SEM)

The high resolution achieved by SEM permitted detailed examination of the anterior surface and cross sections of the irides (Figures 3-6-10). From the 5 globes with monocellular membranes SEM revealed a remarkably similar appearance in all groups (normal, primary and congenital). It

consisted of a mostly continuous sheet of primarily spindle cells that extended over the anterior face of the iris. The round cells were dotted across this spindle cell membrane (Figure 3-6). There was no difference in appearance of monocellular membranes within normal globes and all glaucomatous eyes from each groups of glaucoma (Figure 3-7 & 3-8). In contrast, pre-iridal fibrovascular membranes consisted of sheets of branching vessels and extracellular matrix that provided a thick covering over the anterior surface of the iris (Figure 3-9a-9b).
Globes selected with no visible membrane on light microscopy (n=2) SEM revealed monocellular membranes of very similar appearance on the iris surface (Figure 3-10).

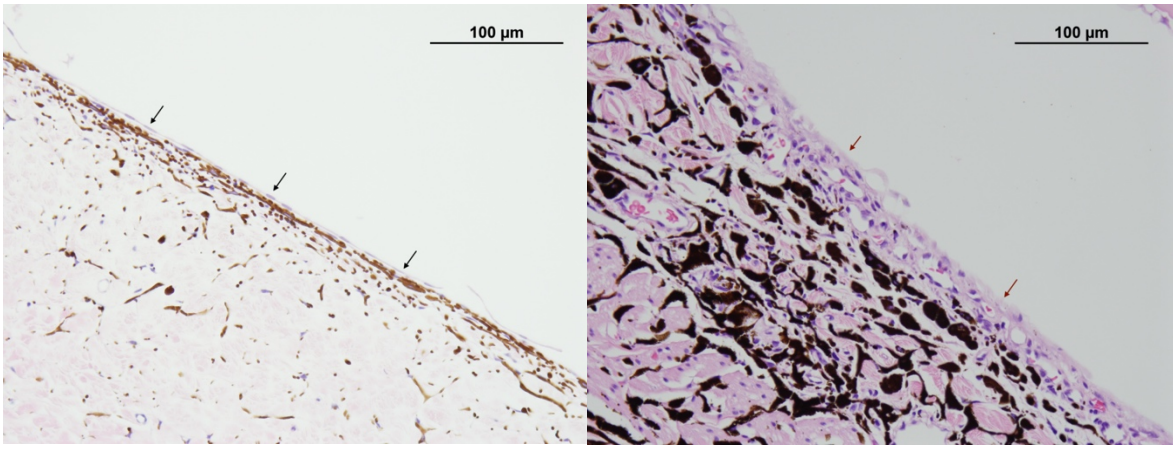

Figure 3-1 A-B. (A) This is a histologic section of an iridal leaflet in a canine globe with pre-iridal monocellular membrane. Note the subtle monolayer of cells over the anterior iris surface (black arrows) (Hematoxylin and eosin stain). (B) Histologic section of a canine globe with a pre-iridal fibrovascular membrane. Note the blood vessels originating from the iridal vessels expanding into the anterior surface of the iris (red arrows) (Hematoxylin & Eosin stain).

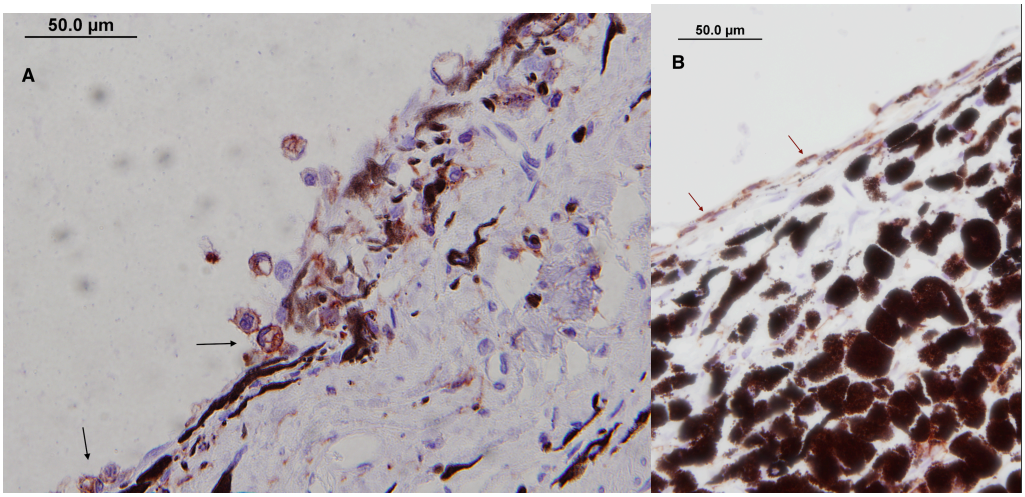

Figure 3-2 A-B. (A) Pre-iridal monocellular membrane in a dog with primary glaucoma. Black arrow is pointing round cell (CD18 stain). (B) Pre-iridal fibrovascular membrane in one dog with secondary glaucoma. The red arrow indicates the spindle cell (CD18 stain).

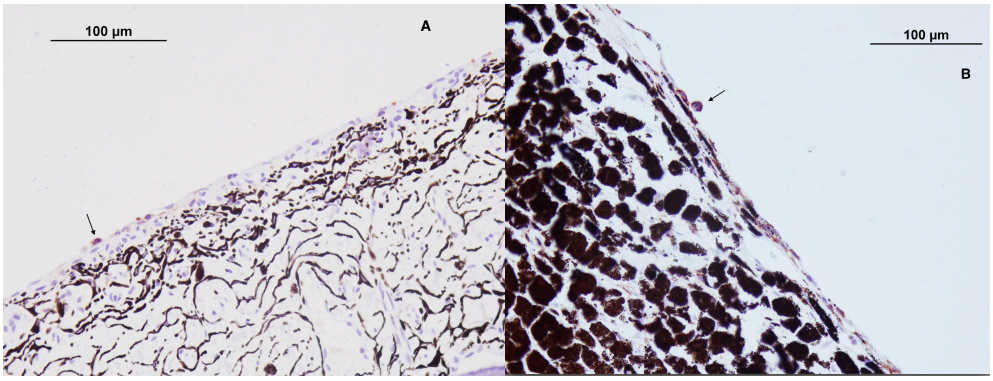

Figure 3-3 A-B. Two fibrovascular membranes in a dog with (a) congenital glaucoma and (b) secondary glaucoma. Black arrows are pointing the round cells (CD18 stain).

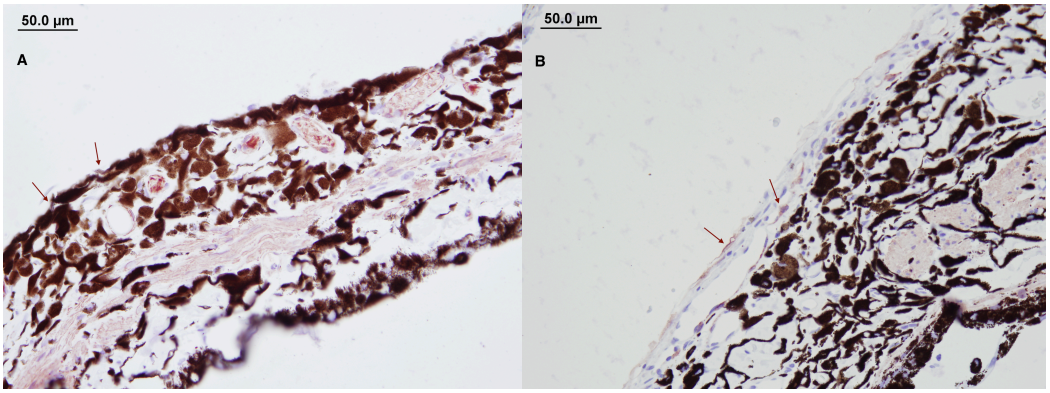

Figure 3-4 A-B. (A) Pre-iridal monocellular membrane in a dog with primary glaucoma. The spindle cells of the membrane are staining in red (red arrow). (SMA stain). (B) Pre-iridal fibrovascular membrane in a dog with secondary glaucoma. The spindle cells on and within the membrane are staining in red (red arrow). (SMA stain)

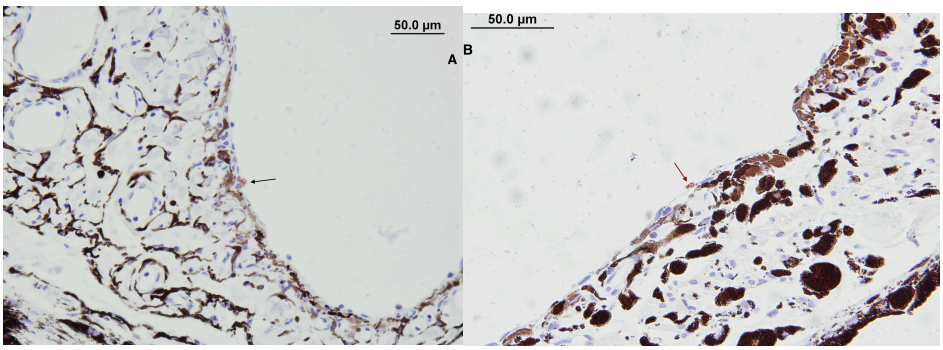

Figure 3-5 A-B. (A) Monocellular (B) and fibrovascular membrane in two dogs with primary glaucoma. The black arrow is showing the round cell on the monocellular membrane and red arrow the spindle cell on the fibrovascular membrane. (CD117 stain)

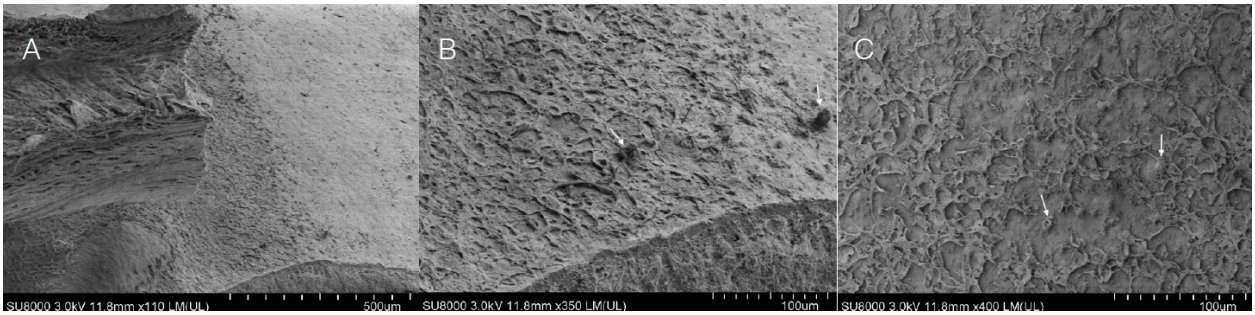

Figure 3-6 A-C. SEM at different powers on a pre-iridal monocellular membrane in a normal eye. White arrows are pointing the round cells. Spindle cells appear to cover the surface of the iris under the round cells.

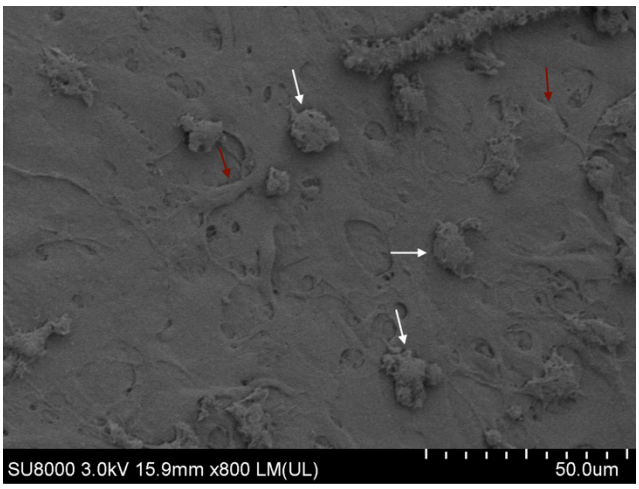

Figure 3-7. SEM of a monocellular membrane in a dog with congenital glaucoma. White arrow is pointing the round cell and red arrows the spindle cells.

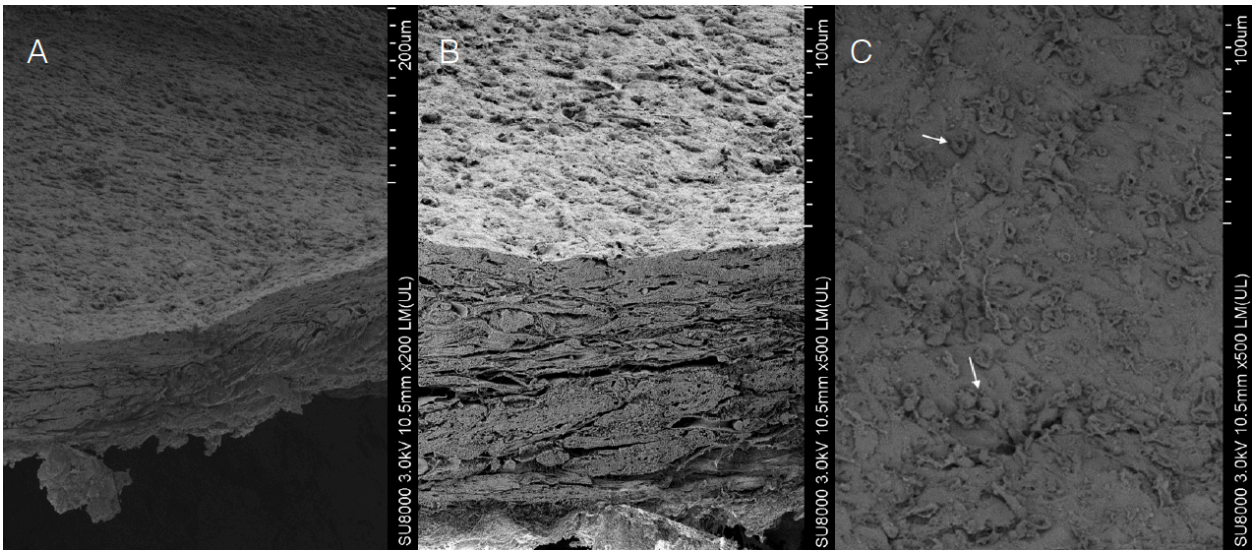

Figure 3-8 A-C. SEM at different powers on a pre-iridal monocellular membrane in a dog with primary glaucoma. White arrows are pointing the round cells.

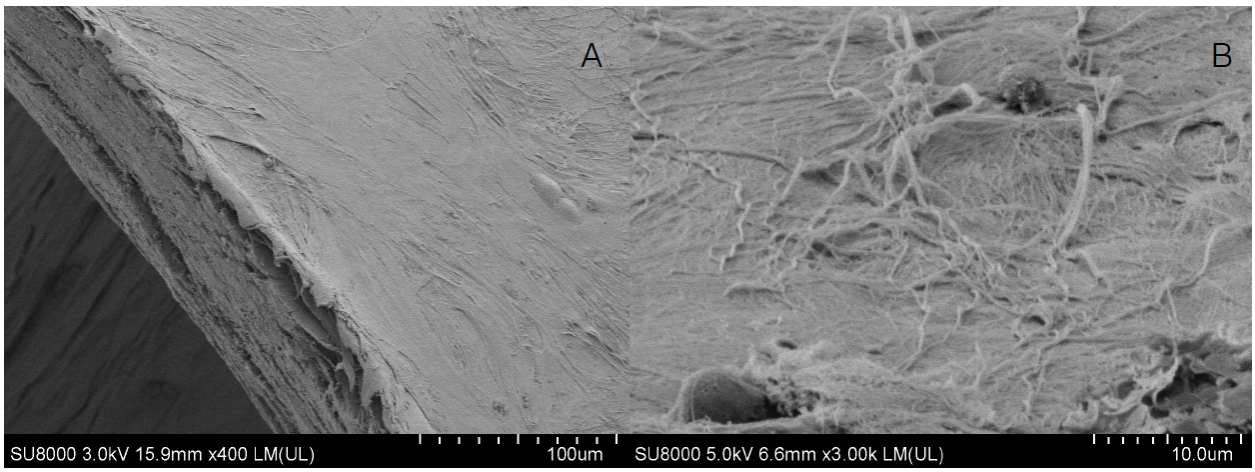

Figure 3-9 A-B. (A) SEM of a dog with secondary glaucoma and fibrovascular membrane. Note pre-iridal fibrovascular membrane bedding covering the anterior surface of the iris. (B) Higher magnification of the fibrovascular membrane where the vessels can be seen.

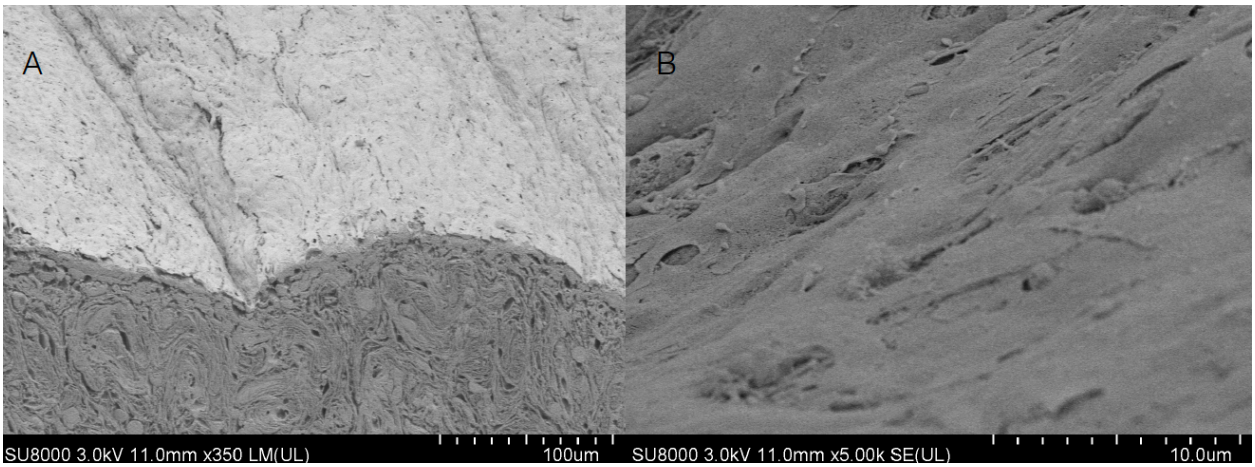

Figure 3-10 A-B. SEM on a normal eye with no identifiable pre-iridal membrane on light microscopy. However, SEM revealed monocellular membranes of very similar appearance on the iris surface.

Table 3-1: IHC markers and positive cell expression.

Marker	Type of cells labelled	Expected staining	References
CD18	β-2 integrin subunits. All leucocytes, but upregulated in histiocytes	Membranous and/or cytoplasmic	Affolter 2002
SMA	Smooth muscle actin filament in smooth muscle cells, myofibroblasts, myoepithelial cells	Cytoplasmic	Mori 2005, Keeley 2010 Jooske 2006
CD117	Tyrosine kinase receptor (stem cell growth factor) Melanocytes, mast cells (normal and neoplastic) Cells of Cajal (gastrointestinal tumors) In Humans: multiple normal and neoplastic tissues, hematopoietic progenitor stem cells (myeloid and lymphoid lineage), stem cell in iris	Membranous, cytoplasmic, paranuclear	Morine 2004 Vrapciu 2014, Leong 2016

Table 3-2: Type of cell present in the monocellular and fibrovascular membranes of normal and glaucomatous canine globes.

Group	Monocellular Membrane	Cell type		Fibrovascular Membrane	Cell type	
	Total	Round and spindle cells	Spindle cells only	Total	Round and spindle cells	Spindle cells only
Control	10/10	5/10	5/10	NA	NA	NA
Congenital	8/10	8/8	0/8	2	2/2	0/2
Primary	8/10	7/8	1/8	2	2/2	0/2
Secondary	7/10	5/7	2/7	4	4/4	0/4

For congenital and primary glaucoma 8 globes were chosen with a monocellular membrane and 2 with fibrovascular membranes. For secondary glaucoma, 7 globes with a monocellular

membrane and 4 with a fibrovascular membrane were chosen. One globe with secondary glaucoma was chosen that had a monocellular membrane on one iris leaflet and a fibrovascular membrane on the other. NA; not applicable

Table 3-3: Immunohistochemistry marker by membrane type and type of glaucoma.

	Total	**CD18**	**SMA**	**CD117**
	41	31/41	17/41	12/41
Globes with monocellular membrane	**33/41**	**24/33**	**10/33**	**7/33**
Control	10/10	9/10	0/10	0/10
Primary	8/10	5/8	4/8	2/8
Secondary	7/10	4/7	3/7	2/7*
Congenital	8/10	6/8	3/8	3/8
Globes with pre-iridal fibrovascular membrane	**8/41**	**7/8**	**7/8**	**5/8**
Control	0/10	NA	NA	NA
Primary	2/10	2/2	2/2	1/2
Secondary	4/10	3/4	3/4	2/4
Congenital	2/10	2/2	2/2	2/2
***Both membranes**	**1/1**	**0**	**0**	**1/1 (monocellular side)**

*One globe eye with secondary glaucoma had fibrovascular membrane on one side and cellular on the other that we included in each group (fibrovascular and cellular). NA; not applicable

Table 3-4: Immunohistochemical labelling of cells within monocellular membranes of control globes.

Marker	**CD18**		**SMA**		**CD117**	
Total	9/10		0/10		0/10	
Cell type	Round	Spindle	Round	Spindle	Round	Spindle
<25%	1/9	9/9	0	0	0	0
25-50%	1/9	0	0	0	0	0
50-75%	0	0	0	0	0	0
>75%	2/9	0	0	0	0	0

Table 3-5: Immunohistochemical labelling of cells within monocellular membranes of glaucomatous globes.

Marker	**CD18**		**SMA**		**CD117**	
Positive	24/33		10/33		7/33	
Cell type	Round	Spindle	Round	Spindle	Round	Spindle
<25%	5/24	18/24	1/10	8/10	7/7	1/7
25-50%	2/24	1/24	0	1/10	0	0
50-75%	1/24	0	0	0	0	0
>75%	10/24	1/24	0	1/10	0	0

Table 3-6: Immunohistochemical labelling of cells within fibrovascular membranes of glaucomatous globes.

Marker	**CD18**		**SMA**		**CD117**	
Total	7/8		7/8		5/8	
Cell type	Round	Spindle	Round	Spindle	Round	Spindle
<25%	1/7	4/7	0	5/7	3/5	1/5
25-50%	0	1/7	0	0	0	0
50-75%	1/4	0	0	0	0	0
>75%	2/7	0	0	2/7	0	0

3.5 Discussion

The data in our study reveals that monocellular membranes are a normal finding in canine globes and may, in part be derived from circulating monocytes. Monocellular membranes of normal globes are similar in light microscopic and ultrastructural appearance to those of glaucomatous globes. However, monocellular membranes of normal globes have different immunohistochemical characteristics than pre-iridal membranes (both monocellular and fibrovascular) in glaucomatous globes suggesting that disease results in metaplastic transformation of some cells within them.

To our knowledge, SEM of pre-iridal monocellular and fibrovascular membranes has not been reported in dogs. Pre-iridal monocellular membranes were formed by a sheet of interconnected spindle and sparse round cells only present on the iridal surface. Whereas pre-iridal fibrovascular membranes consisted of sheets of branching vessels, spindle cells, and extracellular matrix extending out of the iris and covering the anterior surface of the iris and monocellular membranes can be visualized on the surface in some places.

Therefore, the normal iris appears to be composed of four, not three, different layers: a monocellular membrane composed of spindle and round cells, an anterior melanocytic layer, the stroma and sphincter and dilator muscles, and the bilayer posterior neuro-epithelial layers. [21] The anterior border layer has been previously described as a discontinuous cell layer formed of fibroblasts, melanocytes and connective tissue. [22] Additional reports of transmission electron microscopic images describe the anterior iris surface cells to appear fibrocytic in nature, lacking a basement membrane, and forming an almost continuous layer with their cellular processes. [23,24,25]

Transmission electron microscopy has demonstrated that the anterior surface of the normal canine iris is lined by a *discontinuous* layer of flat cells.[22] Our study revealed that the anterior surface of the iris in normal globes was mostly covered by a *continuous* sheet of spindle-like cells with a few round cells. SEM revealed this membrane to be present on the iris surface of the two globes where a cellular membrane was not visible on light microscopy. We believe that

monocellular membranes are likely present on all normal canine irides. SEM characteristics of monocellular membranes in normal globes and those affected with all forms of glaucoma are very similar.

The majority (9/10) of monocellular membranes in normal globes had positive immunolabelling for CD18 in both spindle and round cells. CD18 labelling was also positive within the majority of monocellular and fibrovascular membranes in globes diagnosed with various forms of glaucoma. This is similar to the findings by Gornik *et al* who evaluated PIFMs in canine globes with various ocular diseases and found that they were positive to CD18 and CD34 with immunohistochemestry.[5] CD18 labels leucocytes which are critical in regulating hematopoietic stem cells in the circulating blood and bone marrow. [6,7] In both normal and diseased globes the CD18 positive round cells within pre-iridal monocellular membranes likely represent these leucocytes. Therefore, it is possible that the cells staining positive to CD18 on the fibrovascular membranes are either the monocellular membrane within the fibrovascular membrane or the leucocytes itself.

Round cells were only counted as part of the membranes when they appeared to be within or closely apposed to the membrane itself. However, it is not clear if these cells are an intrinsic component of the membrane or leucocyte derived cells travelling through diseased globes, as would be expected when the blood ocular barriers are disrupted. In the normal globe however, the blood-aqueous barrier should prevent leukocytes from exiting the vascular system. Therefore, it is somewhat surprising to discover that monocellular membranes in normal dogs arise from leucocyte origins. We speculate that these cells represent an immune surveillance system within the irides.

Some spindle cells of pre-iridal monocellular membranes in both normal and glaucomatous globes were also positive for CD18. These spindle cells may be the fibrocytes described by Bucala *et.al*. which are described to be fibroblast-like shaped cells believed to differentiate from bone marrow-derived mesenchymal precursors in the peripheral blood circulation.[6] These precursors are thought to be attracted to sites of injury where they differentiate into spindle-shaped fibroblast-like cells. [2,4,5,8,10, 13, 17, 18, 21] Fibrocytes are believed to act as antigen-presenting

cells, and promoters of angiogenesis via production of cytokines, chemokines, and growth factors that also induce fibroblast hyperplasia, and secretion of components of extracellular matrix.[11] The exact role played by these precursor cells on the surface of the normal iris is unknown, but it is possible that through these mechanisms they contribute to the transformation and development of pre-iridal fibrovascular membranes in the face of injury or disease.

The major differences in immunohistochemical labelling characteristics of pre-iridal membranes between normal and glaucomatous globes was the presence of SMA and CD117 positivity in glaucomatous globes, while this was lacking in the normal controls. CD117 labels mesenchymal stem cells, which have a fibroblastoid phenotype.[27] In this study, the spindle cells were suspected to be either reservoir stem cells within the iris or recruited from circulation fibrocytes and were involved in the maintenance and repair of the iris.[27] SMA labels smooth muscle actin and is present in smooth muscle cells such as those in smooth muscle of blood vessels and myofibroblasts.[11, 18,19,] SMA and CD117 labelling were more common in fibrovascular membranes compared to monocellular membranes of glaucomatous globes. This may be due to cytokines recruiting vascular endothelium and fibroblasts from the iris tissue as part of the pathogenesis of fibrovascular membrane formation.[28,29] SMA labelling of monocellular membranes in glaucomatous eyes without vascular components indicates differentiation of cells within these monocellular membranes into myofibroblasts.[18] Complementary studies have demonstrated that fibrocytes undergo phenotypic differentiation into SMA expressing myofibroblasts.[19, 20]

Previous authors have suggested that monocellular membranes are an early developmental stage of a fibrovascular membrane.[1,2] In contrast we suggest that monocellular membranes are present normally, but that there are cells within them, that when faced with injury or disease, undergo metaplastic transformation and are associated with fibrovascular membranes when those are present. They may even promote the development of fibrovascular membranes. In our secondary glaucoma group, we included one globe with a monocellular membrane on one iris leaflet and a fibrovascular membrane on the other. The presence of both types of membranes in one globe, as well as expression of CD117 within the monocellular portion supports this theory.

Limitations of the current study include small numbers of globes in each group, inconsistent historical data, and the difficulty in determining chronicity of disease in each globe within the glaucoma groups. These cases represent a small proportion of the canine enucleated globes with pre-iridal membranes in our database. Further studies examining a larger number of glaucomatous globes with additional immunohistochemical markers such as CD14 (monocyte lineage markers), CD45 (nucleated cells of hematopoietic origin) and CD34 (bone marrow stem cell marker) as well as type collagen are needed to confirm the origin of the cells within pre-iridal membranes.

3.6 Chapter 3 References

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4. Conclusions

We have demonstrated that monocellular membranes are present in eyes with all types of glaucoma (primary glaucoma associated goniodysgenesis, secondary glaucoma and congenital/ASD glaucoma) and also in normal eyes.

When we compared the prevalence of monocellular an fibrovascular membranes amongst the different types of glaucoma, monocellular membranes were seen more commonly in globes with primary glaucoma whereas fibrovascular membranes were seen more commonly in globes with secondary glaucoma.

After performing immunohistochemical labelling of these membranes, monocellular membranes in normal globes labelled positive for CD18 in 90% of the cases. This positive immunoreaction to the membrane was not exclusive to monocellular membranes in normal globes but also present in the majority of monocellular and fibrovascular membranes in globes diagnosed with congenital/anterior segment dysgenesis-associated glaucoma, primary/goniodysgenesis-associated glaucoma and secondary glaucoma.

In comparison, PIFMS have already been reported to be positive to CD18, and also CD34. We therefore hypothesized that round cells present in fibrovascular and monocellular membranes derive from leucocyte origin.

The presence of round cells and immunohistochemical characteristics in normal globes lead us to think that these cells could represent a type of immune surveillance system present within irides. Whereas spindle cells could represent fibroblast-like shaped cells differentiated from bone marrow-derived hematopoietic precursors in the peripheral blood circulation that contribute to the development of pre-iridal fibrovascular membranes in the presence of injury or disease.

We therefore suggest that monocellular membranes represent a normal component of canine irides and when the cells present within these membranes are exposed to any type of injury they may undergo metaplastic transformation. In the presence of a more severe disease (i.e. intraocular tumor) these monocellular membranes may induce fibrovascular membrane development over the monocellular membrane.

When comparing monocellular membranes vs fibrovascular membranes utilizing SEM we were able to differentiate between monocellular and fibrovascular membranes. Interestingly, monocellular membranes were also noted on SEM in globes that had no visible membrane under light microscopy, and we believe these may be missed on light microscopy due to the thin nature of the monocellular membrane. Based on the SEM findings we believe that the normal iris is composed of four layers: a monocellular membrane (with spindle and round cells), an anterior melanocytic layer, the stroma and sphincter and dilator muscles, and the bilayer posterior epithelial layers.

In order to further evaluate the origin and significance of pre-iridal monocellular membranes, and to see if they could be related to MOMC, studies including immunohistochemical stains with CD14 and CD45 (hematopoietic and monocyte lineage markers), CD34 (stem cell marker), CD117 (stem cell marker) and type 1 collagen are necessary. However, this has major limitations due to the unavailability of certain antibodies for most veterinary species in paraffin embedded tissues, which impacts the ability to accurately perform IHC on retrospective studies and interpret results. Therefore more studies are necessary to increase the applicability and specificity of antibodies in animals. Additionally, the lack of standardized guidelines between different veterinary laboratories is a problem when performing and evaluating IHC.