CHANGES IN CHARACTERISTICS OF THE CANINE MYOCILIN GENE AND MYOCILIN PROTEIN IN GLAUCOMATOUS AND NORMAL DOGS

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL
OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

2007

This work is dedicated to
Dr. Kirk Gelatt for teaching me to love research
and
Audrey, Liam and my mother for making me finish it.

ACKNOWLEDGMENTS

My special thanks to all who helped me to get to this point, especially everyone who helped with the lab work.

I've been here too long. I have met so many amazing people, each who've helped me not only move my projects along, but helped me grow as a person.

I'd like to thank

Dr. Kirk Gelatt for all of his support, guidance, and kicks-in-the-pants over these last 12 years. Kirk is one of the most amazing people I've ever met, and the main reason I was never in a hurry to move on. He has done so much for his specialty, and I can't imagine anyone ever doing more. He helped bring the science of Veterinary Ophthalmology to the modern day. He has more papers, and books, and presentations than even God, I think.

Drs William Dawson and Don Samuelson. They proved to me that there is still a need for pure science. It sounds pretentious, but I don't believe that every project researched needs to be for a major pharmaceutical company or a big business R&D. They proved that while grants are great, choosing what to research is ultimately up to the researcher.

Dr Brooks has always been running around in the background while I've been here. So busy with the UF veterinary clinic, that I'm lucky to ever see him. It was he, however, that got me sucked into primate research and pattern ERG's and the business side of research.

And Dr Peggy Wallace for teaching me genomics, and how research works in a medical school. And I thought I was busy…

On the technician side, I must thank Pat Lewis. She has taught me more about not only histochemistry, but about how to survive in a academic laboratory environment. Even on her worst days, she always had at least one nice thing to say to me. Not to mention, she's probably the best instructor in histo I've ever known.

To Tommy Rinkoski, for putting me in a good mood, writing really creatively, and putting up with all my absences as I put this all together.

Linda Lee-Ambrose and Marc Salute for teaching me Western Blotting, and cursing. Beth Fisher, for holding my hand as I learned PCR reactions. Thanks to everyone in Dr. Peggy Wallace's lab for teaching me genomic and good bench top techniques. To Butch Sapp and Delena McTeer for teaching me how to talk back to doctors.

I've got to thank the million students that I've gotten to help, and been helped by. Each one of them taught me something, not always good…but something.

A special thanks to Hillary Hart, a very successful student. She did more with the protein localizations in this project than anyone. She was the moving force behind a lot of the project. She's also a great poster designer.

I'd also like to take a moment and thank the other graduate students I've worked with, Maria Kallberg, Andras Komaromy, Franck Ollivier. You guys gave me help and hope all the time. It was fun to work with you.

I'd like to thank my wife, Audrey, for all of her time and patience as I've finished this project. I know you've been buried under housework and baby-sitting, I love you.

And finally, to my mother, who never let me doubt myself. Who always knew I could be great. Thanks!

And to all the rest I've neglected. Thanks for everything!!!

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CHANGES IN CHARACTERISTICS OF THE CANINE MYOCILIN GENE AND MYOCILIN PROTEIN IN GLAUCOMATOUS AND NORMAL DOGS

By

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May 2007

Chair: Kirk N. Gelatt
Major Department: Veterinary Medicine

The glaucomas are a group of diseases causing blindness in millions of people every year. The causes of glaucoma are numerous, but in recent years, more attention has been paid to genetic causes of the inherited forms of this disease. Myocilin has been attributed to be involved in over 6% of inherited types of human glaucoma, the highest correlation to date. We have attempted to characterize this gene and protein in the anterior eye tissues, aqueous humor (AH), and irido-corneal angle of a colony of beagles with hereditary glaucoma; and aqueous humor samples from both normal and glaucomatous dogs of other breeds using protein characterization, histochemical localization and micro-array RNA analysis. Genomic *myoc* (coding regions and untranslated regions) differences were not seen in the DNA analysis of normal (n=1) and glaucomatous (n=1) beagles. Comparisons of AH myocilin levels between differing glaucoma severity beagle dogs showed relative differences with the early, moderate, and advanced forms (1.95, 7.66, 13.31 units respectively). Clinical samples showed differences between normal and glaucomatous dogs as well. Normal (cataractous) dogs had the lowest level of myocilin in the aqueous humor at 4140.27 ± 674.12 μg/ml. Primary glaucoma dogs were found to have an

aqueous humor myocilin protein level of 29404.18 ± 7449.11 µg/ml. Secondary glaucomas had the highest level of myocilin in the aqueous humor with 44797.03 ± 11659.83 µg/ml. Myocilin localization in the eye tissues showed an increase in the amount of myocilin in different tissue. The microarray chip study showed very little change in levels of mRNA for myocilin in glaucomatous dogs versus normal dogs. This study shows a strong correlation between amounts of myocilin protein and the presence and severity of glaucoma, but a mutation in the myocilin gene may not be the only myocilin mediated cause of glaucoma in diseased dogs. A mutation in the myocilin promoter or other genes involved with processing myocilin may also be at fault. Further, the increase in production or decrease in turnover of myocilin in all ocular tissues may be the result of the ocular hypertension rather than a cause.

CHAPTER 1
INTRODUCTION

Since the 1950s, glaucoma has been used to describe a number of ocular diseases involving optic nerve damage, a major risk factor of elevated intraocular pressure, and an eventual loss of vision. Glaucoma affects 66.8 million people around the world, with 6.8 million of those suffering bilateral blindness (Quigley, 1993; Quigley, 1996). In the United States, 12,000 are blinded yearly by glaucoma and up to 2% of the human population over 40 is affected by the disease (Kahn et al., 1980; Tielsch et al., 1991). African Americans are affected five times more by POAG than Caucasians, leading to the belief that there is a significant genetic component to the disease (Tielsch et al., 1991).

Individuals are eight times more likely to develop glaucoma than the general population if they have a first degree relative with the disease (Johnson et al., 1996; Armaly et al., 1968; Demenais, 1983; Lowe, 1972). Other signs indicating that POAG could have inheritable factors include the fact that as the disease appears to run in families, and some families show direct Mendelian inheritance (usually autosomal dominant) (Broughton et al., 1983; Budde, 2000; Francois, 1980; Francois, 1981; Francois, 1985; Merin et al., 1972).

The first eye disease linked to a chromosomal location was the X-linked color blindness gene (Deeb et al., 2003). Currently there are over 90 genes (or gene locations) that cause known inherited eye disorders (Cohen et al, 2004). There are several forms of glaucoma linked with specific genes and susceptibility loci (Table 1-1) (Cohen et al., 2004; Duggal et al., 2005).

Many have inferred from the lack of simple Mendelian inheritance that POAG is a more complex disease with a convoluted etiology (Fingert et al., 2002; Avramopoulos et al., 1996;

Francois, 1980; Francois, 1981; Francois, 1985). In humans, chromosome 1 was the first autosome implicated in the heredity of glaucoma. (Cotton, 1993). Further analysis was able to link it specifically to the Q arm of the chromosome (Johnson et al., 1996). In humans, six different genes and chromosomal points have been implicated in glaucoma to some extent. These were initially mapped to GLC1A, GLC1B, GLC1C, GLC1D, GLC1E, and GLC1F (Sheffield et al., 1993; Stoilova et al., 1996; Wirtz et al., 1997; Wirtz et al., 1998; Trifan et al., 1998; Sarfarazi et al., 1998; Lichter, 2001; Alward et al., 2003; Alward, 2000; Alward, 2003; Andersen et al., 1997; Andersen et al., 1996; Andersen et al., 2002; Aung et al., 2002; Baird et al., 2005; Belmouden et al., 1997; Booth et al., 1997; Borges et al., 2002; Brinkman et al., 2005; Brooks et al., 2004; Broughton et al., 1983; Clepet et al., 1996; Datson et al., 1996; Ikezoe et al., 2003; Lichter et al., 1996; Lichter et al., 1997; Mardin et al., 1999; Meyer et al., 1996; Morissette et al., 1995; Rozsa et al., 1998; Stoilova et al., 1996; Sunden et al., 1996; Wirtz et al., 1999).

Myocilin is the only gene and protein that can be directly linked to an inheritable glaucoma in humans (Stone et al., 1997). Analyzing the same gene family in other species may help determine the effects of mutations and on this disorder. Myocilin has been reported in some form in humans, mice, rats, bovine, rabbits, pigs and non-human primates. Recently, research has been reported in cats as well (Fautsch et al., 2006). In this study we concentrated on the dog as our animal of choice. The dog has long been a stable, accurate animal model for ophthalmologic studies (Gelatt et al., 1981; Gelatt et al., 1998; Gelatt et al., 1998; Weinreb et al., 2005; Fingert et al., 2001; Knepper et al., 1997; Obazawa et al., 2004). Any results gathered from this analysis would not only improve our knowledge of glaucomas in humans, but perhaps lead to tests for glaucoma in the canine as well.

Anatomy and Physiology of Aqueous Humor and Outflow

The eye in the canine breeds is usually a nearly spherical globe approximately 21 mm in diameter and very similar in size compared to man. As one of the most complex of sensory organs, it has many different regions with specific functions.

In the dog, the eye is composed of three basic layers. The outer layer is fibrous tunic, which is further divided into the cornea and sclera. The fibrous tunic gives the eye a constant shape and form, which are imperative for a functional visual system. In addition, the most anterior portion of the fibrous tunic, the cornea, is transparent, thus enabling light to pass through, and is shaped in a manner that makes it a powerful lens that refracts light rays centrally, toward the visual axis of the eye. The second and middle layer is the uvea (meaning "grape"). The uvea, which is further divided into the choroid, the ciliary body, and the iris, is heavily pigmented and vascularized. It functions to modify both external and internal light, including reflection and scatter, as well as to provide nourishment and remove wastes for most of the eye's components. The choroid and ciliary body are both attached to the internal surface of the sclera (Figure 1-1). Parts of the eye involved with aqueous outflow and glaucoma include the iris, ciliary body, cornea, and the iridocorneal angle.

The iris originates from the anterior portion of the ciliary body, and it extends centrally to form a diaphragm in front of the lens. The iris of the eye is a round, flat diaphragm with its base in the ciliary body. It completely covers the anterior lens, except for a small hole (pupil) in the center (Figure 1-2). It is used to control the amount of light striking the retina as well as the focal depth of the projected image. It divides the eye into the anterior and posterior segments (chambers). The only communication of aqueous humor fluids in the eye occur through the iris's pupil. In the dog the pupil is nearly perfectly circular. Other species may have horizontally

oriented pupils (horses, cows, goats and other ungulates), slit type pupils (cats), or even double pupils as seen in some fish (Anableps) (Figure 1-3).

The posterior surface of the iris faces the posterior chamber and has numerous surface projections. The posterior surface of the iris contains radial folds that extend to the base of the ciliary processes.

The ciliary body is an anterior continuation of the choroid, and it joins with the iris. The ciliary body removes wastes and provides nourishment for the cornea and lens. Nutrients for the refractive structures are primarily supplied by the aqueous humor of the eye. Aqueous humor is an optically clear fluid originating from vascular sinuses within the folds and processes of the ciliary body and later drains into the iridocorneal (or anterior chamber angle), which forms the anterior boundary of the ciliary body. In the continuous process of aqueous humor formation and drainage, intraocular pressure (IOP) is created, which is responsible for providing the eye most of its rigidity.

Topographically, the ciliary body is divided into an anterior pars plicata and a posterior pars plana. The pars plicata consists of a ring of 70 to 100 ciliary processes, depending on the species, with their intervening valleys (Figure 1-4) (Prince et al., 1960).

There are usually 74-76 processes in carnivores and primates, which greatly increases the surface area for the production of aqueous humor. In addition to aqueous production, ciliary processes play variable roles in lenticular accommodation, because these structures are intimately associated with the crystalline lens. In anurans, birds, and some reptiles, the ciliary processes are attached to the lens and participate directly in accommodation. By comparison, the processes in mammals primarily serve as a region for attachment of the lenticular zonules, which

connect the lens with the ciliary body and its musculature, which in turn is responsible for accommodation.

The appearance of individual ciliary processes are thin and blade-like, with rounded tips that are invested with zonular fibers in canines. Between the major ciliary folds, wide valleys with smaller, secondary folds are present. Many of the smaller secondary folds originating near the pars plana merge with the major processes at their base. The surface has numerous convolutions, but most of it is obscured by the zonular fibers (Figure 1-5) (Troncoso, 1942).

The zonular fibers pass down into the valleys, and many fibers pass posteriorly to their origin on the pars plana.

The pars plana is the first flat, posterior portion extending from the posterior termination of the processes to the peripheral termination of the retina (Figure 1-4). The width of the pars plana varies, because the retina extends more anteriorly in the inferior and medial quadrant in most species, enhancing peripheral vision. Therefore, the pars plana is wider superiorly and laterally. In the dog, the ora ciliaris retinae, the seam between pars plana and the retina, is 8 mm behind the limbus dorsally and laterally but only 4 mm ventrally and medially (Donovan et al., 1974). The main mass of the ciliary body consists of the smooth muscles. Contraction of these muscles draws the ciliary processes and body both forward and inward, thus relaxing the lenticular zonules (suspensory ligament of the lens) and changing the shape and refraction of the lens. This muscle is not as well developed as that of most non-primate species, and offers variably weakened accommodative ability. Both regions of the ciliary body are heavily pigmented.

The iridocorneal angle (ICA, or filtration angle or anterior chamber angle) is the anterior-most component of the ciliary body in nonprimates. The ICA is formed by the junction of the corneoscleral tunic at the limbus, base of the iris, and an anterior recession of the ciliary body,

which is known as the ciliioscleral sinus or cleft (Figure 1-1). The pectinate ligament spans the opening of the ciliioscleral sinus from the pigmented corneoscleral junction to the root of the iris (Figure 1-6) (Baulmann et al., 2002; Bhattacharya et al., 2005).

Beneath the pectinate ligament and within the ciliioscleral sinus is a matrix of loose tissue strands, the trabecular meshwork, which is divided into two regions: the uveal trabecular meshwork (UTM) and the uveoscleral trabecular meshwork (CSTM). The trabecular meshwork consists of a mesh of collagen cords that are covered by cells (Booth et al., 1999; Samuelson et al., 1983; Samuelson et al., 1984). In the ciliioscleral sinus, the inner CSTM appears to be anterior tendinous extensions of ciliary body musculature (Gum et al., 1992). At the expense of the ciliary body musculature, a proportionally larger sinus is found in most domestic animals than in humans. Adjacent to the meshwork are aqueous collecting channels, which in turn empty into the intrascleral venous plexus and then the vortex veins.

Aqueous Humor

Aqueous humor flows from the posterior chamber, in which it is produced by ciliary body epithelial cells and vasculature, and through the pupil, into the anterior chamber, and to the filtration angle. Aqueous flows between the pillars of the pectinate ligament and into the trabecular meshwork. Aqueous humor then leaves the eye either through the corneoscleral trabecular meshwork and associated outflow channels or through the ciliary body and anterior uvea (i.e., uveoscleral outflow). Most forms of increased IOP may be associated with increased resistance to aqueous outflow in both of these areas. The balance between production of aqueous humor and its exit through the iridocorneal angle are essential for maintaining the shape of the eye, its rigidity, and the close adherence of the retina to the choroid.

Production of the aqueous humor occurs in the ciliary processes. Each ciliary process is covered by a double layer of epithelium; an inner, nonpigmented, cuboidal epithelium, which

forms a complete, internal monocellular lining of the ciliary body; and an outer, pigmented, cuboidal epithelium, which also is one cell layer thick.

The sides of the nonpigmented epithelium have numerous villous processes along the bottom two-thirds. The intercellular spaces in this region and in the pars plana are filled with intercellular material that have the staining characteristics of glycosaminoglycans (GAGs) (Samuelson, 2007). The base of the cells also react positively for the same material. The ciliary body nonpigmented epithelium may produce the GAGs of the vitreous humor. These cells secrete the GAGs, which mostly consist of hyaluronans, laterally into the cystic intercellular spaces, which then communicate both with the vitreous base and basally (Fine et al., 1979; Huang et al., 2000).

These chemical factories not only nourish the lens, iris and cornea, but also help regulate intraocular pressure (Coca-Prados et al., 1999).

Cellular junctions between the two layers of epithelium (nonpigmented and pigmented) of the ciliary process are very important (Abe et al., 1999). The lateral intercellular junctions of the nonpigmented epithelium consist of desmosomes, except at the apical end (Figure 1-7). The apical ends possess gap junctions, zonula adherens, and zonula occludens, which probably represent the anatomic blood–aqueous humor barrier (Shabo et al., 1976; Smith, 1971; Smith et al., 1973; Smith et al., 1983; Streeten, 1988).

The protein composition of aqueous humor was first analysed in 1948 by von Sallmann and Moore, who attempted to separate aqueous humor proteins with basic free electrophoresis (von Sallmann et al., 1948). The method they used required a relatively large amount of aqueous humor (2 ml) and was performed by pooling samples from 80 rabbits. The advent of paper electrophoresis allowed not only smaller samples to be used, but better overall separation of the

aqueous humor proteins and in 1951 Witmer published the first analyses using this technique on pathological human samples (Witmer, 1951). The first true protein identification occurred in the early 1950's with Wunderly and Cagianut (1952) and Esser (1954) identifying albumin, alfaglobulin, betaglobulin, gammaglobulin, and pre-globulin in the aqueous humor. They were able to show minor changes between normal and pathologic samples (Wunderly et al., 1952; Esser et al., 1954). In 1967, Hemmingsen and Øther were able to show no significant changes between intraocular protein and total systemic protein when total protein was increased (Hemmingsen et al., 1967). More significant analyses were done in the 1970's (Table 1-2). Major components of aqueous humor were shown to be: potassium, anions (chloride and bicarbonate), ascorbic acid, amino acids (free and protein), and sugars (glucose) (Cole, 1974).

Description of Myocilin

The causes of glaucoma are numerous, but in recent years, more attention has been paid to genetic causes of the inherited forms of this disease. Most notably a mutation in the myocilin gene has been linked to juvenile onset open angle glaucoma (JOAG) as well as other forms of adult early onset primary open angle glaucoma (POAG) (Rozsa et al., 1998; Fingert et al., 2002; Francois, 1980; Kaur et al., 2005; Johnson, 2000).

Myocilin was discovered initially in a course of studies performed by Stone and 14 colleagues from seven other laboratories that analyzed proteins inducible by dexamethasone in a long-term treatment of cultured human trabecular meshwork cells (Stone et al., 1997; Polansky et al.,1997; Johnson, 2000; Ishibashi et al., 2002). Using this model of a steroid induced glaucoma, research demonstrated an increase in the production of myocilin (originally named Trabecular Meshwork Inducible Glucocorticoid Response protein [TIGR]) similar to that of in vivo dexamethasone glaucoma studies (Polansky et al., 1997; Huang et al., 2000). A cDNA sequence for TIGR was then found by using mRNA from cultured human trabecular meshwork

cells treated with dexamethasone for 10 days (Stone et al., 1997; Nguyen et al., 1998; Ishibashi et al., 2002). In a completely unrelated set of experiments, another laboratory cloned cDNA with the same sequence from mRNA from a human retina library and studied its effects on human and pig retinas. Kubota named this novel protein Myocilin (MYOC) which was the final name issued by the Human Genome Organization Genome Database Nomenclature Committee in 1998 (Kubota et al., 1997). The gene symbol is *MYOC*.

Kubota initially reported the open reading frame for *MYOC* coded for a protein of 490 amino acids, but later reported a correct 504 amino acids (Kubota 1998). The approximate molecular weight of *MYOC* is between 55 and 57 kilodaltons (kDa). By western blot multiple bands appear, most likely due to translational and post-translational processing since only a single gene copy is found in Southern and Northern blot analysis (Nguyen et al., 1998; Kubota et al., 1998; Ortego et al., 1997; Adam et al., 1997; Tamm et al., 1999).

In humans, there are two possible start (ATG) sites reported in the open reading frame of *MYOC* separated by 42 nucleotides (Figure 1-8) (Kubota et al., 1997). After the second start signal is a hydrophobic leucine-rich signal sequence with a cleavage site at amino acids 32 and 33 (Ala-Arg) (Nguyen et al., 1998; Kubota et al., 1997; Ortego et al., 1997). The first start signal site and cleavage site of the signaling sequences were later confirmed by amino terminal sequencing of immunoprecipitated *MYOC* (Nguyen et al., 1998). *MYOC* Genomic organization consists of three exons and a 5-kilobase promoter region (Figure 1-9). All exon-intron boundaries conform to the GT/AG consensus for intronic donor and acceptor splice signals (Nguyen et al., 1998; Kubota et al., 1998; Adam et al., 1997). The promoter region contains 13 predicted hormone response elements, including several glucocorticoid regulatory elements (Nguyen et al.,1998). mRNA size as determined by Northern Blot in humans is 2.37 to 2.5

kilobases (Nguyen et al., 1998; Kubota et al., 1997; Ortego et al., 1997; Tamm et al., 1999; Fingert et al., 1998). Two major domains have been determined by sequence alignment and BLAST analysis. The first is a myosin-like domain near the N-terminal, similar to the non-muscle myosin of *Dictyostelium discoideum,* a soil-living amoeba. The myosin homology at the N-terminal is relatively low and has been reported to show a 25% to 29% amino acid identity with the heavy chain of myosin of different species, now including canines (Kubota et al., 1997; Ortego et al., 1997). The second domain is similar to bullfrog olfactomedin (Kubota et al., 1997). This region is almost completely encoded by the third exon and is highly conserved across species.

Olfactomedin was originally identified as the major component of the mucus layer that surrounds the chemosensory dendrites of olfactory neurons in frogs (Snyder et al., 1991; Yokoe et al.,1993). Soon after, a homologous olfactomedin-related glycoprotein was identified in the neurons of the rat, mouse, and human brain (Danielsonet al., 1994; Nagano et al., 1998; Karavanich et al., 1998; Karavanich et al., 1998). Frog olfactomedin had 31% to 40% amino acid residues in common with *MYOC*, whereas the rat and human olfactomedin-related glycoprotein shared 45% to 50% amino acid residues with *MYOC/TIGR* (Kubota et al., 1997; Ortego et al., 1997; Adam et al., 1997).

Human myocilin may have evolved from the fusion of two different earlier genes (Mukhopadhyay et al., 2002; Challoner et al., 1985). These include the *Noelin* family of genes, consisting of *Noelin* 1, *Noelin* 2, and *Noelin* 3. *Noelin* 1 and 2 have been found expressed in ocular tissues, although all three can be found in brain/nerve tissues. It is hypothesized that myocilin may have evolved from a gene duplication/fusion event involving *Noelin* 2 (Mukhopadhyay et al., 2004; Chapman et al., 1996).

Trabecular meshwork cells in monolayer cultures and perfused anterior segment organ cultures secrete MYOC into the culture medium on treatment with dexamethasone. Under these conditions, a modified MYOC form with a molecular weight of 66 kDa is observed in addition to the 52- to 56- kDa forms (Nguyen et al., 1998). To date, myocilin has been located in the cornea, trabecular meshwork, lamina cribrosa, optic nerve, retina, iris, ciliary body, vitreous humor, corneal epithelium, corneal endothelium, the corneal stroma, sclera, uveal and corneoscleral meshwork, ciliary epithelium, ciliary muscle, lens epithelium, stromal and smooth muscles of the iris, throughout the vitreous body as fine filamentous fibers, surface of rods and cones, neurons of the inner and outer nuclear layer, and optic nerve ganglion cells (Karali et al., 2000; Aung et al., 2003). The 66 kDa form is most likely caused by N-glycosylation at amino acids 57 to 59 (Asn-Glu-Ser) since treatment with tunicamycin reduces significantly its formation in human trabecular meshwork cells (Nguyen et al., 1998; Raymond, 2000). Use of this site has been recently confirmed by Raymond,who reported at the Glaucoma Research Foundation meeting that COS-7 cells transfected with human MYOC/TIGR cDNA secrete two isoforms that migrate at approximately 57 and 63 kDa's (Raymond, 2000). Treatment with tunicamycin and PNGaseF (but not O-glycosidase) cleaved the 63-kDa form, leaving the 57-kDa form. Furthermore, cells transfected with cDNA mutated at Asn-57 did not secrete the 63-kDa form. Structural analyses of the *MYOC* cDNA sequence also predicted several potential O-glycosylation sites and phosphorylation sites, a hyaluronan-binding site, putative glycosaminoglycan initiation sites and a tripeptide C-terminal targeting signal for microbodies, a group of small, single-membraned organelles such as peroxisomes, glyoxysomes, and glycosomes (Polansky et al., 1997; Nguyen et al., 1998; Adam et al., 1997; Fingert et al., 1998). No functional role for these sites has been found as yet.

Myocilin has a leucine zipper motif defined by eight leucine residues evenly spaced among seven residues between amino acids 117 and 166 (Figure 1-10). This is consistent with many protein-protein interactions as well as characteristic for many DNA binding transcription factors. Many documented mutations that cause JOAG/POAG seem to have their roots in disrupting the secondary structure of the olfactomedin region (Nagy et al., 2003).

Myocilin in the mouse was first described by Abderrahim, et al. in 1998. The basic genomic structure of the murine myocilin is very similar to the human form. It has three distinct exons with highly conserved intron-exon boundaries. The first intron is approximately 7kb while the second is about 1.5kb, very similar to the human second intron (Abderrahim et al., 1998). In developing murine eyes, myocilin cannot be immunostained before embryonic day 17.5. The nerve fiber layer of the retina was observed to contain myocilin. By postnatal day 12, the cells of the trabecular meshwork and iris stroma began to immunolabel for myocilin, as well as the epithelial layers of the ciliary body and iris (Knaupp et al., 2003).

Coding DNA sequence comparisons between the mouse and human show a 82% sequence identity. There are no gaps in the alignment, although comparisons from the stop codon into the 3' untranslated region show similarity doesn't extend beyond the first 100 base pairs (Abderrahim et al., 1998). Predicted translation of the murine sequence shows a 490 amino acid protein that is 81% identical (90% similar) to human. The third exon is highly conserved between mouse and human and is still comparable to nematode (Adam et al., 1997). A potential glycosaminoglycan extension site is not conserved between species, and may suggest that this putative signal has no relevance to myocilin function (Abderrahim et al., 1998; Baird et al., 2001).

Functions of Myocilin

Myocilin was first described after exposing trabecular meshwork (TM) cells to dexamethasone (Stone et al., 1997). Later research showed this to be a trabecular meshwork specific response, and further exposure to dexamethasone only induced myocilin production in the TM and not in any other ocular tissue (Lo et al., 2003; Fautsch et al., 2000). Myocilin expression was investigated in the rat eye after three different stresses were applied. In one experiment, four male Brown Norway rats had their intraocular pressure increased by injection of 50µl of a 1.75M hypertonic saline solution through the episcleral vein. After six weeks of daily pressure readings, the rats were killed and graded on the degree of optic nerve damage (a scale of 1 [normal] to 5 [total degeneration]) by several observers. Subsequent quantitative analysis of myocilin mRNA levels in the rat eye showed a decrease in mRNA production in the rat retinas (as much as 33 fold) (Ahmed et al., 2001). In a second experiment, 62 adult female albino Wistar rats had their left (OS) eye IOP raised by cautery of 2 or 3 episcleral veins. Myocilin mRNA levels dropped significantly (2-2.5 times) in the irido-corneal angle and ciliary body tissues for the first 3 weeks, but then returned to normal. In the last experiment performed by Ahmed, et al., four rats had the optic nerve of their left eye surgically transected. After performing a sham operation in the right eye as a control, mRNA myocilin levels were again measured within two days. In the retina, myocilin levels increased (nearly doubled). Messenger RNA levels did not change significantly in the angle tissues of sham and transected eyes (Ahmedet al., 2001). In total, 74 different mRNAs in the retina increased in production after an increase in IOP. Seven mRNA levels decreased. (Ahmed et al., 2004) Interestingly enough, in a later paper by Ahmed et al., very little myocilin was found in the eye angle tissues of rats at all, although several analogues were seen (Ahmed et al., 2004; Alvarado et al., 2005).

It has also been shown that an increase in myocilin production in transgenic *Drosophila* eyes also leads to changes in the expression in other protein products (Borras et al., 2003). High-density oligonucleotide microarrays have identified changes in the expression of at least 50 transcripts. Among these are the *Drosophila* homologs of aquaporin-4 and cytochrome-P450, previously linked to some forms of glaucoma (Borras et al., 2003).

Myocilin is secreted into the aqueous humor as both a single protein and as two separate parts. Intracellularly, myocilin is found in vesicles and processed by the endoplasmic reticulum. It is often secreted into the aqueous humor of various species as a doublet of 55-57 kDa. This doublet is caused by glycosylated and nonglycosylated versions of the protein being present (Aroca-Aguilar et al., 2005; Stamer et al., 1998; Zimmerman et al., 1999; Caballero et al., 2000; Rao et al., 2000; Jacobson et al., 2001; Nguyen et al., 1998). A segment of the myocilin protein containing only the olfactomedin segment perfused directly into human cadaver or porcine eyes has shown no effect on outflow facility (Goldwich et al., 2003). It has also been shown that misfolding of the mutant myocilin in the endoplasmic reticulum can lead to ER stress and cytotoxicity (Zimmerman et al., 1999; Joe et al., 2003). It has recently been proposed that a mutation in myocilin (such as mutation P370L) could cause the endoproteolytic processing within the endoplasmic reticulum to malfunction causing a regulation problem for the normal activity for myocilin (Aroca-Aguilar et al., 2005).

Myocilin has also been linked to other tissues with transendothelial fluid flow (Goldwich et al., 2005). Myocilin has been found in the podocytes of the kidney of the rat and induced in the mesangial cells during glomerulonephritis. This knowledge helps support the possibility that myocilin functions in cell-cell adhesion and/or signaling processes (Goldwich et al., 2005). Myocilin has also been found in gliotic tissue of injured cerebral cortex, leading to the concept of

myocilin as an inhibitor to neuronal regeneration (Jurynec et al., 2003). It is also present normally in the paranodal terminal loops of the nodes of Ranvier, and outer mesaxons and basal/abaxonal regions of the myelin sheath of the peripheral nerves (Ohlmann et al., 2003). It has been found in the sciatic nerve of rats as early as the 15th postnatal day (Ohlmann et al., 2003).

Recently, et al, transgenic mice were created that would either express myocilin in the eye at up to 15 times the normal level, or have it be completely absent (Gould et al., 2004). These mice were able to demonstrate almost no clinically identifiable changes in the eye. Pathogenic signs of glaucoma were absent (Gould et al., 2004). Similar results have been found in human patients with the Arg46Stop mutation, which almost completely removes the entire myocilin coding sequence, with no overt disease symptoms present (Gong et al., 2004).

It is also proposed that complexes may form between myocilin proteins in the aqueous humor. It has been shown that hetero-oligomers formed by wild type myocilin and some mutant types of myocilin can actually form larger complexes than wildtype alone, on the range of 150kDa (Gobeil et al., 2004). Myocilin has also been implicated in blocking the functions mediated by the Heparin II domain of fibronectin. This activity also limits the number and type of focal adhesions that can form in a human fibroblast plate (Peters et al., 2005). Reciprocally, it has also been demonstrated that a mutation in the myocilin gene could be a gain of function, actually causing the over production on myocilin or mis-regulating other functions (Kim et al., 2001).

Causes and Risks of Myocilin Related Glaucoma

Glaucoma has been defined in many ways. Its simplest definition had been merely an increase in intraocular pressure. In recent years the best definition has been as a group of

diseases of the optic nerve involving loss of retinal ganglion cells in a characteristic pattern of optic neuropathy, often due to an increase in intraocular pressure.

Juvenile open angle glaucoma and adult onset open angle glaucoma have been linked to mutations in the 3rd exon of the human myocilin gene (Adam et al., 1997; de Vasconcellos et al., 2003). Most of the mutations are missense (Table 1-3 and 1-4) (Adam et al., 1997; Bunce et al., 2003; Mackey et al., 2003).

In 2000, Angius et al. found a Gln368stop *MYOC* mutation and analyzed it in all living members of a 5 generation family. The Gln368stop defect was found in 19 patients with primary open angle glaucoma, 5 with ocular hypertension, and 22 healthy carriers. They found that the presence of the mutation did not signify POAG at a later age and there must be other risk factors involved (Angius et al., 2000; Chen, 2004).

Two Italian families were found to carry a mutation in myocilin, p.K423E or p.C25R, out of 26 families tested (an 8% incidence). The evidence shows that a molecular genetic exam should be included in the management of glaucoma cases (Bruttini et al., 2003; Angius et al., 1998). French patients were shown to have significant association between mutations in MYOC and raised IOP (Melki et al., 2003; Brezin et al., 1997; Brezin et al., 1998). In Chinese patients, however, no link in myocilin mutations could be found in a phenotypically similar form of glaucoma called chronic primary angle-closure glaucoma (PACG). Although there were a number of mutations found in the 106 chinese patients tested, many of the mutations were also found in the normal control group with no adverse effects (Aung et al., 2005). However, it is possible that the normal controls could later develop glaucoma. Similarly, in studies of 91 and 492 Chinese patients, no link could be found between POAG and myocilin mutations (Lam et al., 2000; Pang et al., 2002). In fact, a negative association was seen in the case of a Gly12Arg in

that it may have protective effects against POAG (Pang et al., 2002). In the United Kingdom, a lower than average number of linked mutations were found in the myocilin of the people in that region. Of 426 people tested, only six had any mutations in their myocilin (1.4%), much lower than the average in other countries (Aldred et al., 2004). Challa et al., showed there was an overall 4.4% prevalence of myocilin mutations in the POAG population in Ghana, West Africa (Challa et al., 2002). Finnish glaucoma families were found not to have any major mutations, just a few polymorphisms in the Myoc gene and OPTN gene (Forsman et al., 2003). Indian populations with POAG were found to have a 2% prevalence of myocilin mutations including novel myocilin mutations (Kanagavalli et al., 2003; Mukhopadhay et al., 2002; Acharya et al., 2002; Chakrabarti et al., 2005).

Some research has led to the conclusion that although a mutation in the myocilin gene is not necessary for POAG or even JOAG, those individuals that develop glaucoma and have a mutation in the myocilin gene generally have a earlier onset or higher peak intraocular pressure in the disease (Craig et al., 2001; Abecia et al., 1996). But in other research, when cases of glaucoma containing the myocilin Gln368Stop mutation were compared to cases without the mutation, no significant differences were seen. Both IOP peak and age of onset were similar (Graul et al., 2002).

The myocilin promoter MYOC.mt1 was screened for mutations as a cause of glaucoma by using a series of 779 unrelated human patients, 652 with open-angle glaucoma and 127 glaucoma suspects. When analyzed, plausible disease causing mutations were found in 3% of the entire group. No link was made between polymorphisms in the MYOC.mt1 region and disease state, except by Polansky, et al. who found it to be a strong marker for the progression of glaucoma (Alward et al., 2002; Fan et al., 2004; Fan et al., 2004; Polansky et al., 2003; Ozgul et al., 2005;

Kirstein et al., 2000; Klein et al., 2004). It has also been demonstrated that a polymorphism in the gene promoter may affect the severity or age of onset of glaucoma (Colomb et al., 2001).

One of the newest proposals for myocilin causative glaucoma is the mis-processing of myocilin in the endoplasmic reticulum. The ER endoproteolytically processes the myocilin into a 35 kDa portion containing the C-terminal olfactomedin-like domain, and a 20 kDa portion containing the N-terminal leucine zipper-like domain (Aroca-Aguilar et al., 2005; Ahmed et al., 2004; Caballero et al., 2001; Carelli et al., 2004; Kong, 2001; O'Brien et al., 2000). It has been shown the mis-processing of the myocilin may lead to an insoluble aggregate in the cells of the ciliary body and accumulation in the irido-corneal angle of the eye, leading to a cascade of cellular toxicity and mechanical blockage of the angle (Caballero et al., 2000; Caballero et al., 2001; Jacobson et al., 2001).

I hypothesized the myocilin gene would be mutated in a hereditary glaucomatous dog colony versus a group of normal healthy animals; myocilin protein content in the aqueous humor would be higher in advanced glaucomatous animals than in mildly affected and normal animals; and myocilin protein content would be greater in cells of the ciliary body, iris and trabecular meshwork in glaucomatous animals than in normal animals. The goals of this study were to demonstrate the possibilities of these three hypotheses, using an inherited glaucoma beagle primary open angle glaucoma model, as well as other normal and spontaneous glaucomatous dogs of different breeds.

Table 1-1: Loci and Genes Associated with Glaucoma

Gene	Loci	Chromosome	Protein	Phenotype	Inheritance
MYOC	GLC1A	1q23-24	Myocilin	JOAG, POAG	Autosomal Dominant
	GLC1B	2cen-q13		POAG	
	GLC1C	3q21-24		POAG	
	GLC1D	8q23		POAG	
OPTN	GLC1E	10p14-15	Optineurin	POAG	
	GLC1F	7q35-36		POAG	
CYP1B1	GLC3A	2p21	Cytochrome P1B1	Congenital glaucoma	Autosomal Recessive
	GLC3B	1p36		Congenital glaucoma	
PITX2	RIEG1, IRID2	4q25-27	Homeobox transcription factor	Rieger syndrome	
FOXC1		6p25	Forkhead transcription factor	Congenital glaucoma; Rieger/Axenfeld anomaly	
LMX1B	ABO adenylate kinase	9q34	Lim homeodomain	Nail patella syndrome	Autosomal Dominant

Table 1-2: Composition of aqueous humor

Property or constituent	Value	Remarks	Reference
Volume	350 µl		Mestrezat and Magitot, 1921
Ascorbate	1.06 ± 0.31 µM/g H_2O	Plasma = 0.042 ± 0.023 µM/g H_2O	de Bernardinis et al., 1965
B12	29.9 pg/ml	Plasma = 271.9 pg/ml	Phillips et al., 1968
Bicarbonate	19.64 ± 1.4 µM/g H_2O	Plasma = 26.47 ± 2.64 µM/g H_2O	de Bernardinis et al., 1965
Carbon dioxide	Pco2 = 38.6 mm Hg	16 cataract patients	Thiel, 1967
Chloride	134 ± 22.4 µM/g H_2O	Plasma = 109 ± 18.4 µM/g H_2O	de Bernardinis et al., 1965
Glucose	3.00 ± 2.04 µM/g H_2O	Plasma = 6.33 ± 2.45 µM/g H_2O	de Bernardinis et al., 1965
	3.70 – 4.78 µM/g H_2O	Plasma = 4.72 – 6.55 µM /ml	Pohjola, 1966
Glycoprotein	63-75 µg/ml		Cardia and Coriglione, 1962
Hexosamine	14.9-18.3 µg/g		Meyer et al., 1938
Lactate	4.28 ± 1.30 µM/g H_2O	Plasma = 1.78 ± 0.80 µM/g H_2O	de Bernardinis et al., 1965
Oxygen	PO2 = 59.7 mm Hg	Cataract patients, 64-90 yrs	Thiel, 1967
pH	7.21		Becker. 1957
	7.38 (7.31 - 7.42)	Cataract patients, 60-94 yrs	Thiel, 1967
Protein	Total protein: 31-1000 mg/100 g Fractions as % of total : Prealbumin 1 = 4.1 Prealbumin 2 = 8.2 Albumin = 31.0 α_1-globulin = 10.6 α_2-globulin = 11.2 β-globulin = 20.1 γ-globulin = 12.7 τ-fraction = 2.1	Results on one case with secondary Cataract Total protein = 55 mg/100 mg	Kronfield et al., 1941 Praus, 1961
Sodium	162.9 ± 4.3 µM/g H_2O	Serum = 176.4 µM/g H_2O	Cagainut, 1957

Table 1-3. Human myocilin mutations detected in population

Mutation	POAG	Controls	p-value	Protein Solubility	References	Year
17 bp DUP 56-72bp	1/1703 (0.058%)	0/793	N/A	N/A	Fingert et al.	1999
ARG82CYS	2/1703 (0.12%)	0/793	N/A	N/A	Fingert et al.	1999
ARG91STOP	1/91 (1.1%)	0/113	N/A	N/A	Lam et al.	2000
GLY252ARG	1/74 (1.4%)	0/43	N/A	N/A	Shimizu et al.	2000
GLU261LYS	3/79 (3.8%)	0/90	N/A	N/A	Vázquez et al.	2000
ARG272GLY	1/74 (1.4%)	0/60	N/A	N/A	Shimizu et al.	2000
TRP286ARG	1/1703 (0.058%)	0/793	N/A	N/A	Fingert et al.	1999
THR293LYS	2/1703 (0.12%)	0/793	N/A	N/A	Fingert et al.	1999
GLY323LYS	1/74 (1.4%)	0/43	N/A	N/A	Shimizu et al.	2000
GLN337GLU	1/79 (1.3%)	0/90	N/A	N/A	Vázquez et al.	2000
GLU352LYS	5/1703 (0.29%)	1/793 0.13%)	N/A	Insoluble	Fingert et al.	1999
PRO361SER	1/1703 (0.058%)	0/793	N/A	N/A	Fingert et al.	1999
GLY364VAL	2/1703 (0.12%)	0/793	N/A	Insoluble	Fingert et al.	1999
GLY367ARG	1/50 (2.0%)	0/5	N/A	N/A	Suzuki et al.	1997
GLN368STOP	27/1703 (1.6%)	1/793 0.13%)	P=0.0025	Insoluble	Fingert et al.	1999
	3/152 (2.0%)	0/104	N/A		Wiggs et al.	1998
	2/74 (2.7%)	0/60	N/A		Shimizu et al.	2000
	1/79 (1.3%)	0/90	N/A		Vázquez et al.	2000
PRO370LEU	1/50 (2.0%)	0/5	N/A	Insoluble	Suzuki et al.	1997
	1/74 (1.4%)	0/43	N/A		Shimizu et al.	2000
	1/152 (0.66%)	0/104	N/A		Wiggs et al.	1998
	1/25 (4%)	0/130	N/A		Vasconcellos et al.	2000
THR377MET	2/1703 (0.12%)	0/793	N/A	Insoluble	Fingert et al.	1999
	1/74 (1.4%)	0/60	N/A		Shimizu et al.	2000
	1/152 (0.66%)	0/104	N/A		Wiggs et al.	1998
SER393ARG	1/1703 (0.058%)	0/793	N/A	N/A	Fingert et al.	1999
VAL426PHE	2/74 (2.7%)	0/60	N/A	N/A	Shimizu et al.	2000
CYS433ARG	7/25 (28%)	0/130	N/A	N/A	Vasconcellos et al.	2000

Table 1-3. Continued

Mutation	POAG	Controls	p-value	Protein Solubility	References	Year
TYR437HIS	4/1703 (0.23%)	0/793	N/A	Insoluble	Fingert et al.	1999
	1/152 (0.66%)	0/104	N/A		Wiggs et al.	1998
ALA445VAL	1/1703 (0.058%)	0/793	N/A	N/A	Fingert et al.	1999
lbp DEL codon 453	1/1703 (0.058%)	0/793	N/A	N/A	Fingert et al.	1999
ILE465MET	1/1703 (0.058%)	0/793	N/A	N/A	Fingert et al.	1999
ARG470CYS	1/1703 (0.058%)	0/793	N/A	N/A	Fingert et al.	1999
ILE477ASN	1/1703 (0.058%)	0/793	N/A	Insoluble	Fingert et al.	1999
	1/74 (1.4%)	0/43	N/A		Shimizu et al.	2000
PRO481THR	1/1703 (0.058%)	0/793	N/A	N/A	Fingert et al.	1999
PRO481LEU	1/1703 (0.058%)	0/793	N/A	N/A	Fingert et al.	1999
GLU483STOP	1/1703 (0.058%)	0/793	N/A	N/A	Fingert et al.	1999
1544ins489STOP	1/79 (1.3%)	0/90	N/A	N/A	Vázques et al.	2000
ILE499SER	1/74 (1.4%)	0/60	N/A	N/A	Shimizu et al.	2000

Table 1-4: Human myocilin mutations detected in glaucoma pedigrees

Mutation	Pedigrees	Affected Members with the Mutation	Z max	Protein Solubility	References	Year
GLY246ARG	1	7	N/A	N/A	Adam et al.	1997
GLY252ARG	1	5	N/A	N/A	Booth et al.	2000
ARG272GLY	1	4	N/A	N/A	Shimizu et al.	2000
GLU323LYS	1	11	N/A	Insoluble	Shimizu et al.	2000
PRO334SER	2	3	N/A	N/A	Kee et al.	1997
GLN337ARG	1	5	N/A	N/A	Stoilova et al.	1997
GLY364VAL	2	20	3.5	Insoluble	Alward et al.	1998
GLY367ARG	1	5	N/A	N/A	Mansergh et al.	1998
	1	2	N/A		Michels-Rautenstrauss	1998
1177GACA->T	4	20	6.6	Insoluble	Angius et al.	1998
GLN368STOP	15	25	N/A	Insoluble	Stone et al.	1997
	3	8	N/A		Allingham et al.	1998
	2	8	N/A		Shimizu et al.	2000
	1	19	N/A		Angius et al.	2000
PRO370LEU	2	14	N/A	Insoluble	Adam et al.	1997
	1	15	N/A		Shimizu	2000
	1	2	N/A		Suzuki et al.	1997
	1	4	N/A		Stoilova et al.	1998
	1	7	N/A		Michels-Rautenstrauss	1998
THR377MET	2	15	1.3	Insoluble	Alward et al.	1998
	1	3	N/A		Shimizu et al.	2000
ASP380ALA	1	14	N/A	Insoluble	Kennan et al.	1998
	1	3	N/A		Stoilova et al.	1998
396INS397	1	6	N/A	N/A	Alward et al.	1998
LYS423GLU	1	79	N/A	Insoluble	Morissette et al.	1998
VAL426PHE	1	10	N/A	Insoluble	Mansergh et al.	1998
	1	12	N/A		Shimizu et al.	2000
TYR437HIS	2	34	13.8	Insoluble	Alward et al.	1998
THR448PRO	1	2	N/A	N/A	Yokoyama et al.	1999
ILE477ASN	1	19	11.6	Insoluble	Alward et al.	1998
	1	15	N/A	Insoluble	Richards et al.	1998
	1	16	N/A		Shimizu et al.	2000
ILE477SER	1	20	N/A	Insoluble	Adam et al.	1997
ASN480LYS	3	52	N/A	Insoluble	Adam et al.	1997
ILE499PHE	1	7	N/A	Insoluble	Adam et al.	1997
ILE499SER	1	2	N/A	N/A	Shimizu et al.	2000
SER502PRO	1	7	N/A	N/A	Stoilova et al.	1998

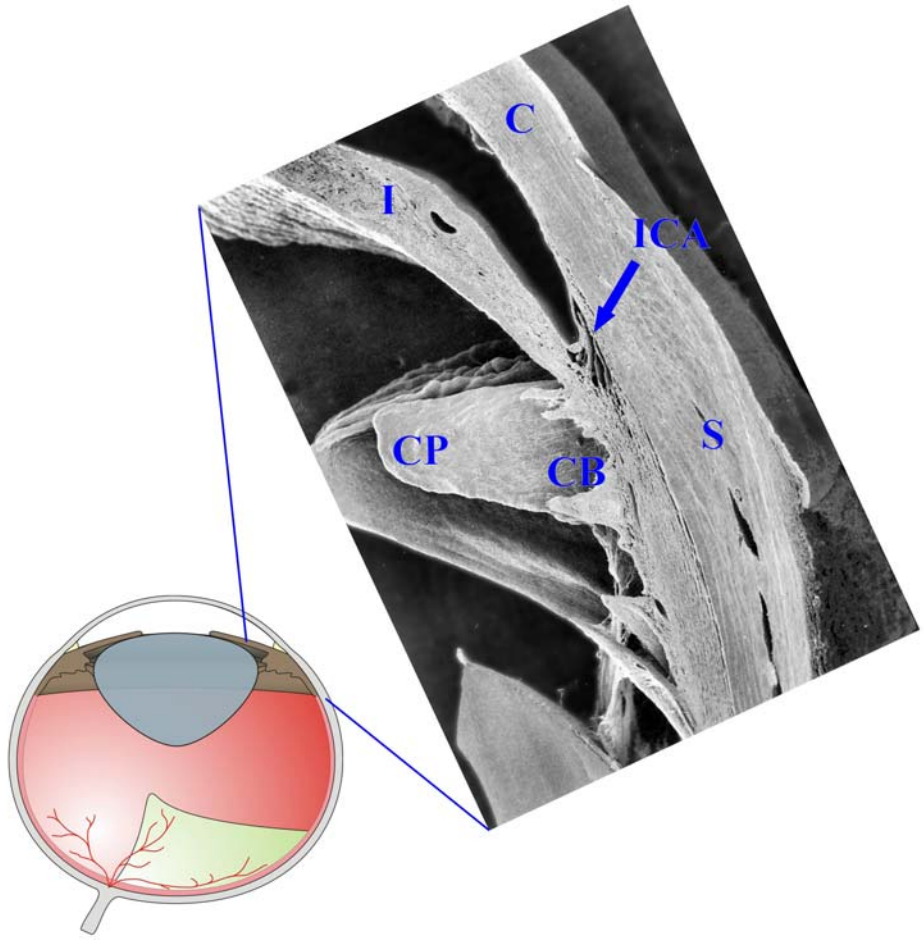

Figure 1-1: Basic structures of the eye.

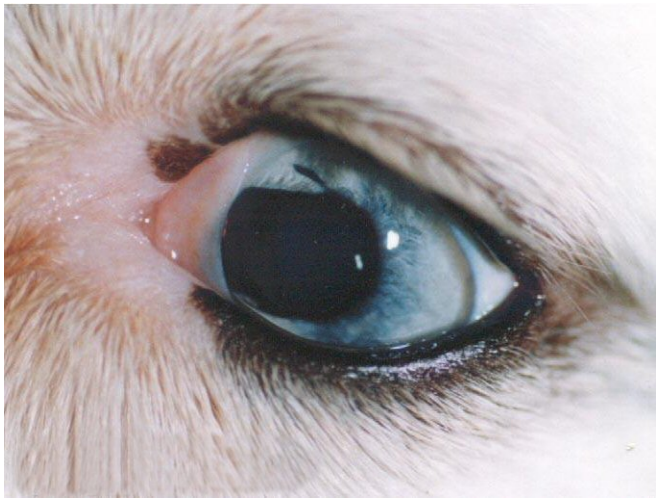

Figure 1-2: The Canine iris controls the amount of light entering the eye.

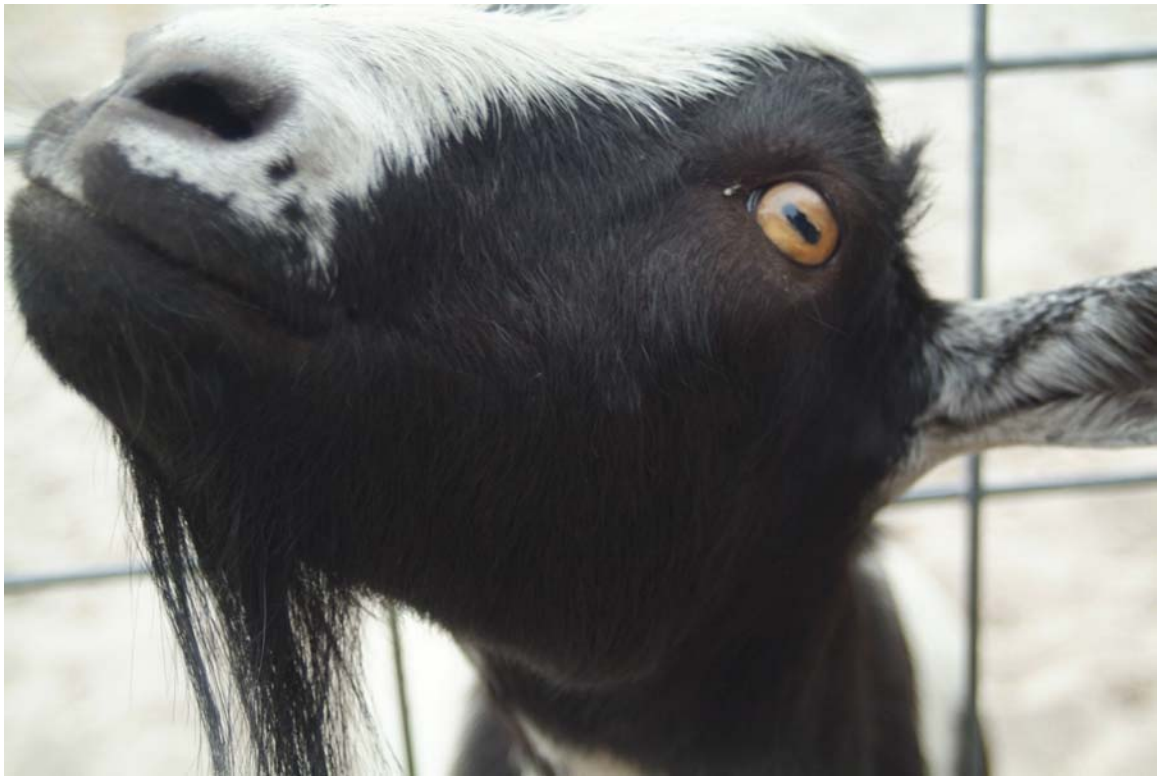

Figure 1-3: Example of a horizontal pupil.

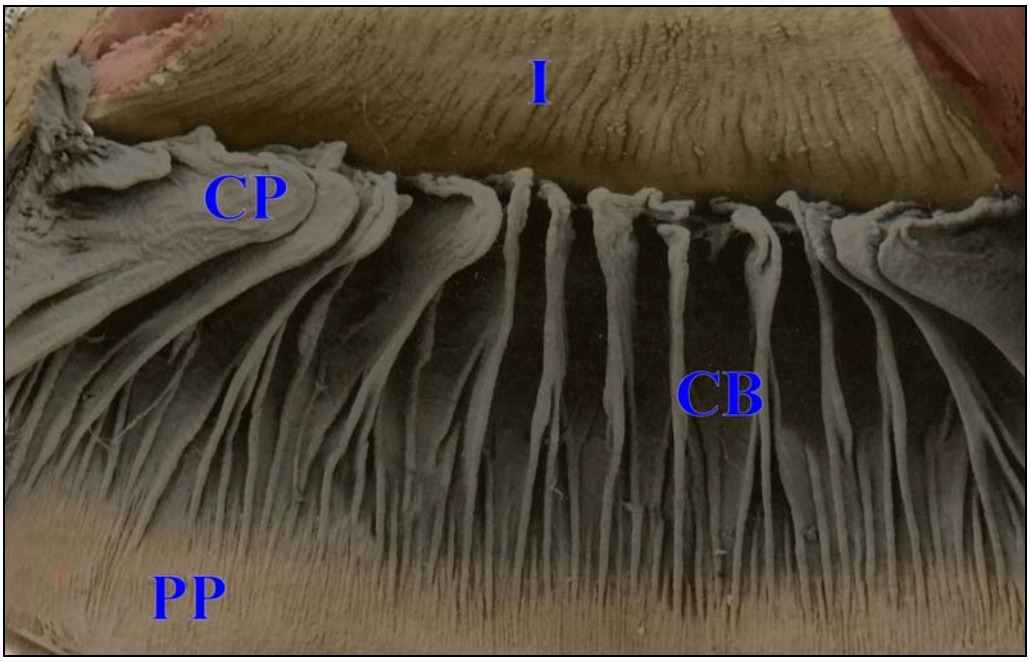

Figure 1-4: Rear of the Iris (I), Ciliary Body (CB), Ciliary Processes (CP), and Pars Plicata (PP).

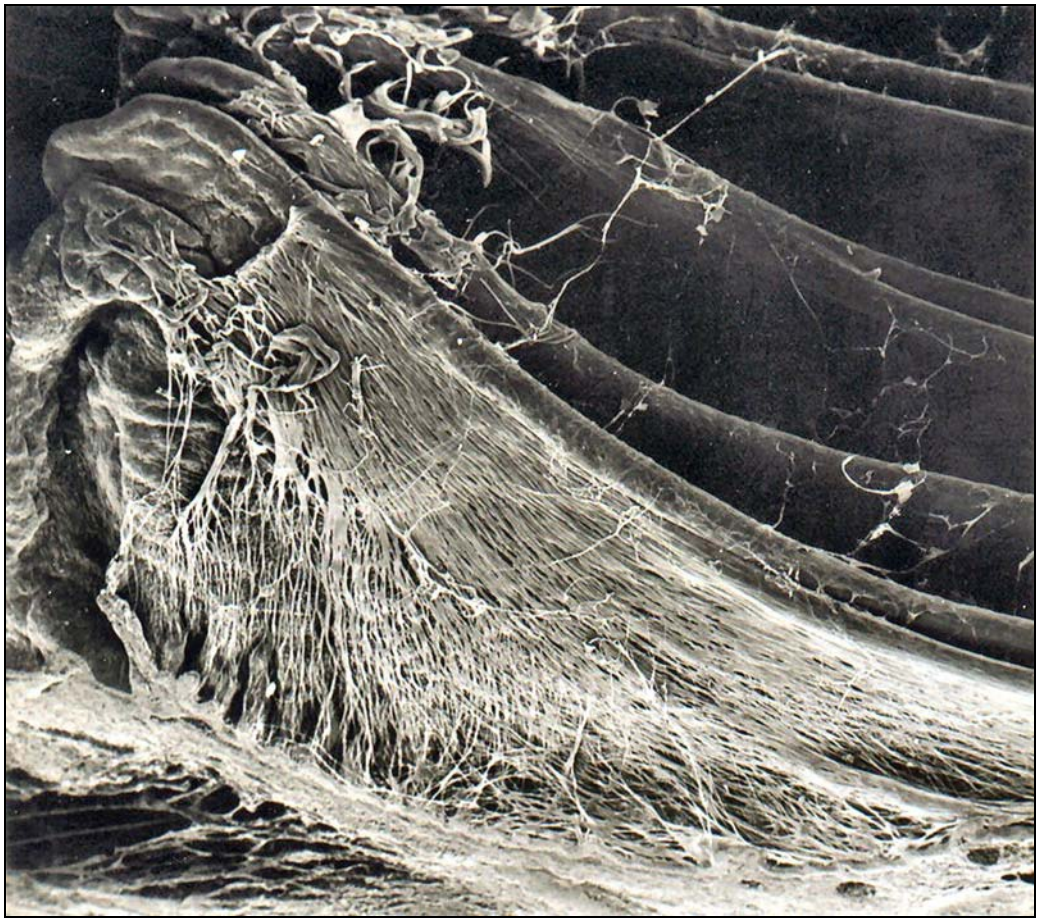

Figure 1-5: Zonules over the ciliary processes.

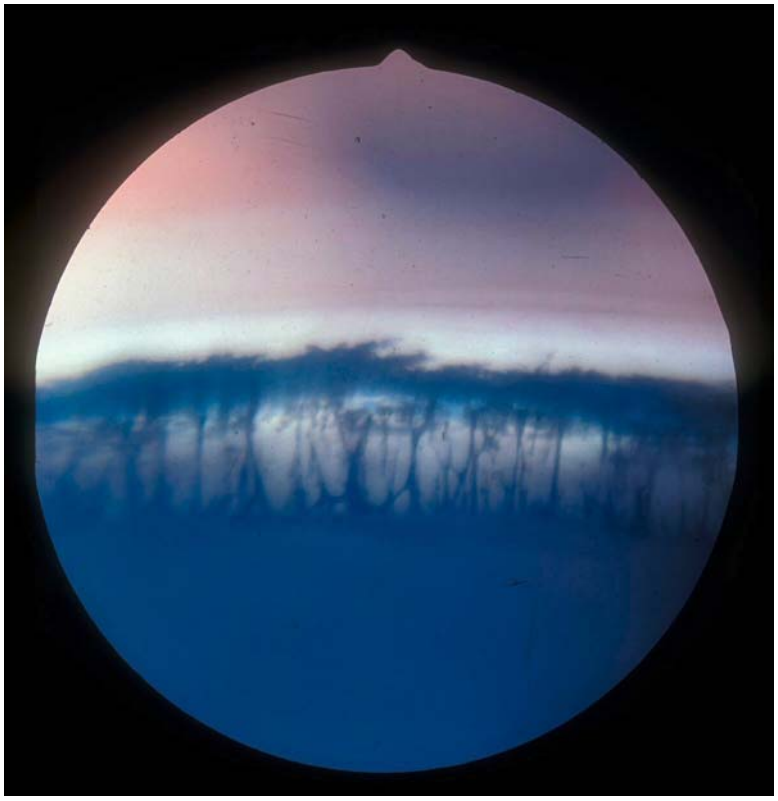

Figure 1-6: Pillars of the pectinate ligament from the base of the iris to the inner cornea.

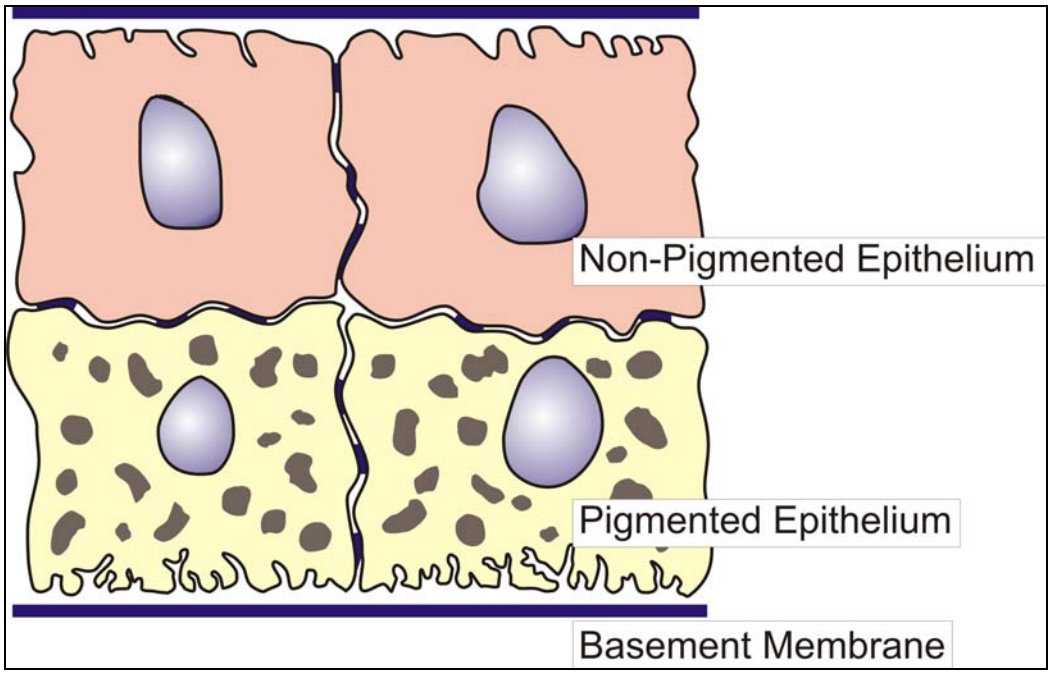

Figure 1-7: Pigmented and non-pigmented epithelium of the ciliary body. Cellular junctions between the two layers of epithelium of the ciliary process are very important. The lateral intercellular junctions of the nonpigmented epithelium consist of desmosomes, except at the apical end.

Figure 1-8: Human myocilin mRNA overlaid on human complete DNA for the region, showing exons and areas removed by post processing. ATG's show two possible start points. Blue underlined = Exon 1. Green underlined = Exon 2. Red underlined = Exon 3.

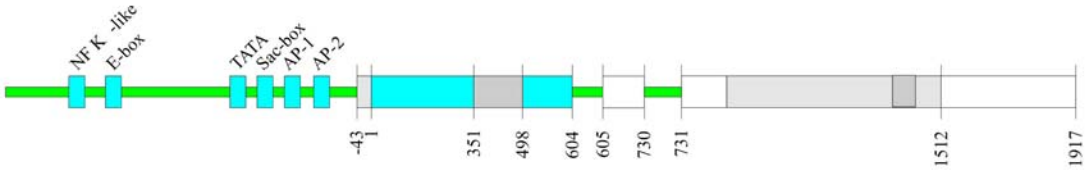

Figure 1-9: Myocilin consists of 3 exons and a 5-kilobase promoter region.

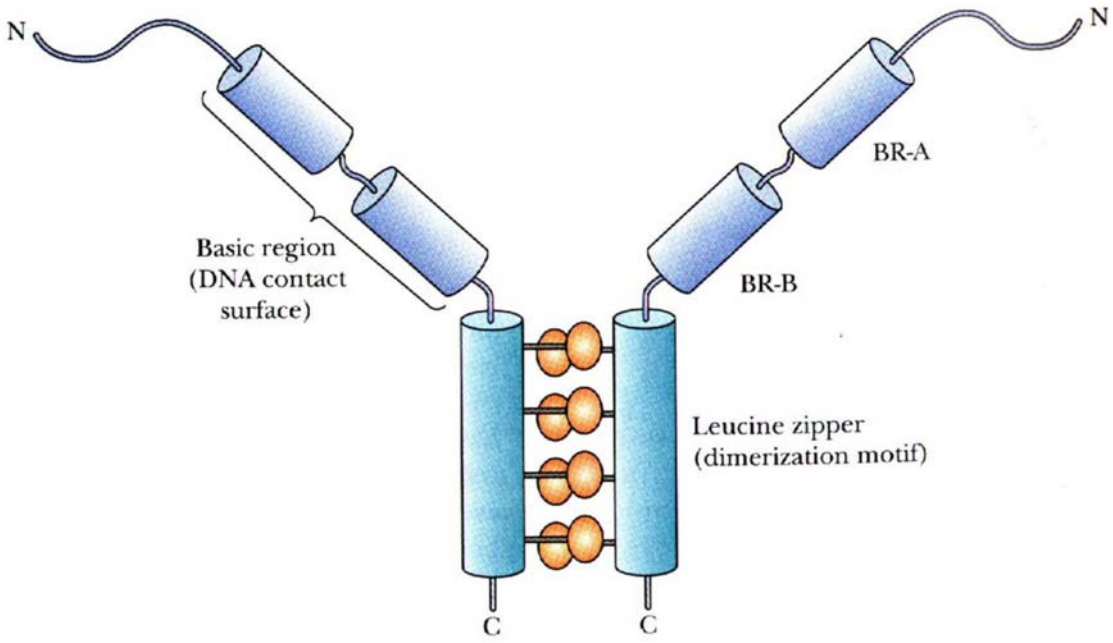

Figure 1-10: Basic leucine zipper motif.

CHAPTER 2
MATERIALS AND METHODS

To analyze the myocilin gene, blood samples and/or buccal swabs were taken from both normal and glaucomatous dogs. Dr. Gelatt's glaucoma beagle colony at the University of Florida was used as a known, inherited glaucoma model, to compare later with primary, secondary glaucomatous samples as well as non-glaucoma aqueous humor samples collected from around the nation (Gelatt et al., 1998; Gelatt et al., 1998; Demenais et al., 1979; Gelatt et al., 1981; Gelatt et al., 2004). The glaucomatous dogs were further divided into mild, moderate and severe glaucoma (See protein analysis).

Gene Analysis

The confirmation of the structure of the myocilin gene in the canine was the first phase of this study. To obtain DNA samples, two milliliters of blood were collected from one primary open angle glaucomatous beagle and one normal dog in EDTA treated vacuum tubes. The blood sample was then mixed with enough red blood cell lysis solution (Madisen et al., 1987) to bring the total volume to 45 ml. This solution was heated in a water bath at 37° for 15 minutes. The solution was then placed in a centrifuge at 2000 rpm for 10 minutes to pellet the white blood cells containing the DNA. The pellet was then broken up by tapping and 200µl of Gentra cell lysis solution (Gentra PureGene Systems, Minneapolis, MN) was then added. The solution was then transferred to a sterile 2 ml eppendorf tube using a sterile pipette. It was then vortexed for 10-40 seconds. The solution was quite thick. Two hundred and fifty microliters of Gentra Protein Precipitation solution was then added and vortex mixed as little as possible (to avoid mechanical shearing of the DNA). It was then centrifuged at 6000 g for 10 minutes to precipitate the

proteins. The pellet that formed was fairly tight and tannish brown. No strings of protein were still visible. If the pellet didn't form well after the first centrifuging, the sample was placed on ice for 5 minutes, and then re-centrifuged. The supernatant, containing the DNA, was carefully removed with a wide bore pipette tip into a new 2 ml eppendorf tube. We were careful to avoid disturbing the protein pellet. 200 µl of chloroform was then added to the solution in the new tube and mixed vigorously by inversion several times. The solution was then centrifuged at 10,000 rpm for 5 minutes. The top layer of the resulting solution was then transferred to a new sterile eppendorf tube, carefully avoiding the interphase. The final DNA sample needs to be completely protein-free or the DNA will degrade with time. The tube was then inverted gently with 1 ml isopropanol (2-propanol) until the DNA completely precipitated (Schleren lines disappeared). This final solution was then centrifuged at 2000 g for 2 minutes to cause the DNA to pellet. The supernatant was then poured off, and the DNA pellet was washed with 300 µl of genomic quality 70% ethanol (ETOH). It was then centrifuged for 2 minutes at 2000 g to re-pellet the DNA. The ethanol was then carefully poured off and the DNA left to air dry for a few minutes (careful not to let it completely dry). The pellet was then resuspended in 200-500 µl of sterile 1X TE (40 mM TRIS, 1 mM EDTA, pH 8.0) and inverted for a few days to allow the DNA to dissolve.

When our study first began, the canine genome had not yet been released. We extrapolated a possible "best guess" set of primers by aligning known myocilin sequences from other species. These included: human, mouse, rat, cow, rhesus, pig, and rabbit. Once an alignment of these seven species was made, the most conserved areas of the gene were used to make primers for PCR replication and amplification (Table 2-1).

Later (summer 2004), new primers were made based on the newly released canine genome from a team led by Kerstin Lindblad-Toh, Ph.D., of the Broad Institute of MIT and Harvard,

(Cambridge, MA), and Agencourt Bioscience Corp., (Beverly, MA) based on a 6-fold shotgun sequence of a common canine (*canis familiaris)* Boxer dog (Figure 2-1). The known sequences for human, mouse, rat, cow, rhesus, pig, and rabbit were aligned against the new canine genome, and the sequence for myocilin was extrapolated in the dog. Primers were based on this sequence in the dog. Eleven primers were designed following common primer design guidelines from Dr. Margaret Wallace at the University of Florida:

- Design primers only from areas with unambiguous sequences.
- Choose a priming site that is 30-50 bases from where new sequence is required.
- Primers with one or more G or C residues at the 3'-end will increase binding efficiency.
- Primers with long runs of a single base (i.e., more than three or four, especially G or C) should be avoided.
- Use of primers longer than 18 bases minimizes the chances of encountering problems with non-specific hybridization.
- For cycle sequencing, primers with melting temperatures (Tm) above 45°C generally produce better results than primers with lower melting temperatures. Tm's in the range of 55°C to 65° work well. Avoid using primers with Tm's above 65°C-70°C.
- For primers with a GC content of less than 50% (GC content of 55% is ideal), it may be necessary to extend the primer sequence beyond 18 bases to keep the melting temperature above the recommended lower limit of 45°C.
- Avoid primers which could form secondary structures (i.e., inverted repeats).
- Avoid primers that can hybridize to each other to form dimers (complementary regions ≤ 4 bp).
- And finally, do not confuse "picomoles" with "picomolar" when calculating primer quantities.

These primers are listed in table 2-2. Primer pairs were then used to sequence the entire open reading frame and untranslated regions of the gene.

To sequence a complete copy of the myocilin gene, we first ran PCR on multiple matched pairs of our final primers. One quarter microliter of purified DNA sample was combined with 2.5

µl 10x PCR buffer solution, 2.0 µl dNTP solution, 0.5 µl of each 5' and 3' primers, 0.25 µl Taq polymerase, and water to bring the volume to 25 total microliters. This recipe would shift slightly depending on the concentration of the purified DNA, and the strength of the Taq.

The solution would then be placed in a PCR reactor for 25-35 cycles. The cycle would include a 45 second denaturing phase at 94° C, a 45 second annealing phase at 45-66° C (depending on the primers used), and an extension phase of 45 seconds at 72° C. A final extension phase at the end of the cycles would lat at least 20 minutes at 72° C.

The PCR products were then direct sequenced. Direct sequencing involves sequencing double stranded PCR products. Both alleles are present in the sequencing reaction resulting in one double peak in the case of a base pair change (for a heterozygote) and a more complicated pattern of double peaks in cases of insertions, deletions, alternate splicing, etc. While it is important to align the sequence against the known normal or published sequence, careful perusal of the sequence chromatogram is essential to detect mutations when using Direct Sequencing.

The first step in direct sequencing is PCR product filtering to remove all foreign / non-DNA material. Millipore microcon-PCR centrifugal filtration devices were used for this step by inserting a filter into one of the eppendorf tubes provided in the kit with the purple side up. We then added as much of our PCR product as possible (usually 20-22 µl) and added sterile water to bring the total volume up to 400 µl. We then closed the cap and centrifuged in the adjustable-speed microfuge at 1300 x g for 15 minutes.

Next, we removed the filter device and placed purple side up into a fresh Millipore eppendorf tube, and added 20 µl of sterile distilled water, being careful not to touch the membrane. Finally, we inverted the filtration device so that the white part was sticking out of

the tube. The cap did not close in this position. After spinning at 1300 x g for 2 minutes in the adjustable-speed microfuge, our filtered product was recovered in the bottom of the tube.

To check our purified results, and confirm that we still had present DNA, we ran a basic check gel on agarose. We then ran a Big Dye 3 Sequencing Reaction (ABI – Applied Biosystems, Foster City, CA). Starting with 3 µl purified PCR product, we added 2 µl Big Dye 3 Reaction Mixture, 2 µl 5x sequencing buffer, 0.2 µl primer, and 2.8 µl sterile distilled water. One drop of oil was placed on top of reaction to minimize evaporation during the PCR reaction. A PCR reaction programmed at 96° C for 30 seconds, 50° C for 15 seconds, and 60° C for 4 minutes for 25 cycles was used. An Edge Biosystem column was prepared by spinning in the adjustable-speed centrifuge for 3 minutes at 850 g. The column was transferred to a clean 1.5 ml eppendorf tube. Using a pipette set for just under 10 µl, the PCR reaction was removed from its tube and transferred to the center of the now dry column in the clean eppendorf tube. We carefully avoided transferring any of the oil used in the PCR reaction. The cap was closed and centrifuged for 3 minutes at 850 g. After the sequencing sample had been run through the column, we dried the volume collected down in the speed-vac (caps open) until sample was completely dry. Approximately 30 minutes with full vacuum and medium temperature to dry. We finally stored the dry sample in the freezer, after it colled to room temperature until we were ready to sequence.

To the thawed dry samples from before, we added 17ul of TSR (ABI Prism Template Suppression Reagent) and vortexed the sample for several seconds. The tubes were then centrifuged for a few moments to recollect the samples in the tubes. The tubes were then heated to 94°C for 2-3 minutes and then placed on ice for 5 minutes. This final solution was then vortexed for 15-20 seconds and then centrifuged to re-collect in the tube. Approximately 16µl of

the samples was now removed from the tube, careful to avoid air bubbles, and placed in a Perkin Elmer 0.5 ml sequencing tube and capped with Perkin Elmer Septa gray rubber stoppers. The samples were finally run on the Applied Biosystems (ABI 310) Sequencer. All results were analysed using SeqEd v.1.0.3.

Protein Analysis

Myocilin protein levels were analyzed in the aqueous humor of glaucomatous and normal dogs. Dr. Gelatt's glaucomatous beagle colony was used initially as a source of inherited glaucoma dogs. Sixteen dogs were used and classified as either having mild, moderate or advanced glaucoma as diagnosed by Dr. Gelatt. Mild glaucoma was defined as little or no visible changes to the eye, with only low intraocular pressure increase. Moderate glaucoma was classified as a spike in intraocular pressure and still little or no externally visible problems. Advanced glaucoma was defined as a large spike in IOP, and visual eye problems (ie, enlarged globe, corneal edema, lens luxation and ocular irritation). Clinical samples were also collected from around the nation.

To compare to the beagle POAG model, aqueous humor samples were collected from a number of veterinary ophthalmology specialty clinics of several different breeds affected with spontaneous glaucoma. Those with suspected inherited glaucoma included both POAG as well as PCAG types. A total of 353 samples (141 Male, 194 Female, 18 unreported) with average ages of 107 months for the males, 104 months for the females and 105 months for the unreported genders were analyzed. These were classified by the attending veterinary ophthalmologist as either primary glaucoma (1°), secondary glaucoma (2°), cataractous, diabetic cataractous or other. Primary glaucoma has no other signs of ocular disease except a spontaneous ocular hypertension and occurs in several purebred dog breeds. Secondary glaucoma is described as secondary to a primary disease (such as a uveitis) or trauma. Cataractous aqueous humor samples

were used as a non-glaucoma control, as the samples are relatively easy to collect during a cataract surgery without any additional trauma to the dog. Diabetic cataracts were separated from the cataractous group, to check for any diabetic changes that may affect this study.

A 0.1 ml sample of aqueous humor was drawn from the anterior chamber each dog in the study. This was done by anesthetizing the dog with 3-5 ml propofol IV, and maintaining with 2-3% isoflurane gas anesthetic. The eye was sterilized with a small amount of iodine wash and rinsed with sterile water. The eye was held with a small single toothed forcep while a 1cc tuberculin syringe with a 0.75 inch 30 gauge needle was carefully inserted just center of the limbus. Careful not to make contact with the iris, the 0.1 µl sample was drawn. A sterile gauze applicator stick was placed at the point of entry as the needle was withdrawn to stop aqueous outflow. The sample was then either kept cold on ice, frozen on dry ice, or placed in a vial with 100 µl protease inhibitor (Mini-Complete, Roche Scientific), and then transferred to a -80° ultra-freezer for storage. A Western blot or Comassie stain was run on each sample to compare these myocilin levels. A polyclonal anti-body was used from either Alcon Pharmaceuticals (from human trabecular cell culture) or Santa Cruz Biotechnologies (from mouse anti-human myocilin).

Comassie Stain

To analyze the total proteins in the aqueous humor we used a modified *SimplyBlue SafeStain Microwave Protocol* from Invitrogen. An SDS reducing buffer (Cell Signaling Technology) was prepared by combining 1 part reducing agent, 10 parts buffer, and 20 parts sterile deionized water. Two µl of each sample was then combined with 38 µl of the SDS reducing buffer. The samples were then heated at 100°C for 5 minutes in a boiling water bath. They were then moved to an ice bath for a further 5 minutes. The samples were then placed in a centrifuge for 5 minutes at 14,000 rpm.

12% Bis-Tris gels were loaded into our electrophoresis apparatus with 1x running buffer (Invitrogen). Each gel was then loaded with sample and controls. The first lane of the gel was loaded with 8 µl of a control ladder (SeeBlue Plus 2, Invitrogen®). Lane two contained 8 µl of a human myocilin control (Alcon Labs), prepared similarly to the samples. Lanes 3 and higher were inoculated with 4 µl of each sample/buffer solution. The gel was then placed in an electric current for 3 hours at 60V.

At the end of 3 hours, the gel was removed from its cassette and trimmed down. The gel was placed in 100 ml of sterile deionized water and heated in a 1000w microwave oven for one minute and 25 seconds. It was then placed on a clinical rotator for 1 minute. This was repeated 2 more times. After the final wash, 30 ml of Simply Blue Safe Stain (Invitrogen®) was added and the gel heated for 35 seconds and then placed on the rotator for 10 minutes. The stain was decanted from the gel dishes and a single water wash was performed, followed by a 20 ml 20% sodium chloride wash for 10 minutes. The gels were then taken to a Biorad ChemiDoc XRS imager for scanning and densitometry.

Western Blot

Western blotting with a human anti-myocilin antibody was begun exactly the same way as our Comassie protocol. After the samples were run for 3 hours at 60V, the gels were removed and the cassettes discarded. Two pieces of filter paper and 4 sponges were liberally soaked with 1x Transfer buffer (Invitrogen). A sandwich was made with two sponges, 1 filter paper, the gel, one piece of 0.2µm nitrocellulose membrane, another filter paper, and the last two sponges careful to minimize air bubbles. The sandwich was placed in a western blotting press with the gel towards the cathode and the membrane towards the anode. The western blot transfer was run at 30V for 1.5 hours.

When the transfer to the nitrocellulose membrane was complete, the membrane was removed to a dish and a small amount of 1% Ponceau S was added. Gently shaking for 1 minute reveals any protein bands in the membrane, and shows that the transfer was successful. The Ponceau S was gently rinsed from the dish with deionized water. The ladder was marked with a permanent marker for later measurements. A Block solution was made with 1x TBA, 0.5% Tween 20, and 1% BSA. The membrane was then covered with the block solution for 30 minutes at room temperature and gently shaken. While the blocking was being performed, the primary antibody was prepared, in this case rabbit anti-human myocilin (Santa Cruz Biotechnologies). An antibody dilution buffer was prepared by using a 1:10 dilution of the block solution. Using 2.5ml of the antibody dilution buffer, a 1:500 antibody dilution is made by adding 5 µl of primary antibody. The blocked membrane was then placed in a small bag with the diluted primary antibody and gently rocked overnight at 4°C.

The next morning, the nitrocellulose membrane was then washed in a solution containing 0.05% Tween 20 in 1% TBS (USB Corporation) four times for 5 minutes each. During final wash, a new bag was prepared with a1:500 dilution of donkey serum (5 µl) in 2.5 ml antibody dilution buffer. The sealed bag with the membrane and the serum dilution was then placed on a nutator for 30 minutes. A secondary anti-body 1:2000 dilution was then prepared using 5ml of the antibody dilution buffer and 2.5 µl donkey anti-rabbit antibody (Santa Cruz Biotechnology). A new bag was prepared and the membrane moved into it with 2.5 ml of the secondary antibody dilution and gently agitated for 90 minutes at room temperature. Eighty minutes into the agitation, our Avidin/Biotin conjugate (ABC) solution was prepared (Pierce Biotechnology). The membrane was then washed 4 times for 5 minutes each, as before. At the end of the final wash, the membrane was placed in a new bag with 3 ml of the ABC solution and rocked on the nutator

for 30 minutes at room temperature. The membrane was then removed and washed four times as before. During the final wash we prepared 6 ml of Supersignal West Pico Chemiluminescent Substrate (Pierce Biotechnology). The membrane was covered by the chemiluminescent substrate in a small dish for 1 minute and then sealed in plastic wrap and taken for imaging on a Biorad ChemiDoc XRS. The membrane was exposed to the digital imager for 10 minutes and the image used for analysis.

Myocilin Protein Localization

Specimens from the anterior uveas of 10 beagles, five with inherited glaucoma (3-mos- to 13-yrs-of age) and five age-matched normals, 1 normal walker hound, 1 normal schnauzer, and 2 cocker spaniels with spontaneous glaucoma were used in this study. The presence and localization of myocilin in the normal and glaucomatous canine anterior eye were studied by the use of immunohistochemical and immunocytochemical techniques.

For immunohistochemistry, the samples were incubated sequentially, first in peroxidase block, followed by goat serum, and then with the rabbit polyclonal primary antibody for human myocilin. The samples were then incubated with an anti-rabbit donkey antibody with a biotinylated link followed by peroxidase-labeled streptavidin and then by substrate-chromogen AEC. Normal, mild, moderate, and severe glaucoma samples were compared, and examined for similarities and differences using light microscopy.

With immunocytochemistry, samples were embedded in L.R. white resin. Sections were cut at 90 nm and mounted on nickel grids. These grids were incubated with bovine serum albumin followed by goat serum. The samples were then incubated with primary antibody and then the secondary antibody, 18 nm colloidal gold labeled goat anti-rabbit IgG. Grids were then examined using transmission electron microscopy.

Microarray Gene Chips

Microarrays, often called DNA chips, usually consist of a piece of glass or nylon on which hundreds of pieces of DNA strands, known as probes, are arranged in a regular pattern. Analysis of those strands provides a picture of gene expression that takes place in a cell during a given moment. New microarray gene chips, developed with our help, were used to screen for RNA transcription in the eye. Tissue samples from both glaucomatous and normal dogs were collected (Table 2-3). These included ciliary body, iris and trabecular meshwork / irido-corneal angles. The samples were fast frozen, or stored in RNA-later (Qiagen). The samples were then ground down and the cells lysed. The mRNA was extracted using University of Florida ICBR protocols. The mRNA was then reverse transcribed into cDNA and hybridized with a biotin transcript. The samples were heated in the presence of Mg^{2+} and fragmented into biotinylated cRNA segments. These segments were then hybridized on the DNA chip for 16 hours and scanned in the ICBR core laboratories. The results were analyzed with proprietary software and normalized using canine GAPDH as a housekeeping gene.

Table 2-1: Original canine myocilin primers

Primers	Sequence 5' to 3'
CMYOCA01F	CAGGGAGGGCTCTCCAGTAT
CMYOCA01R	GCTGGCCACACTGAAAATATAC
CMYOCA02F	CTGCTACTTCTGGCCTGTCTCT
CMYOCA02R	CTTGGGTTTCCAGCTGTTCT
CMYOCA03F	GAGACCTGGAGTCCACCAAA
CMYOCA03R	CTCTTCTCAGCCTTGCTACCTC
CMYOCA04F	CAAGTCAGCTCTGGAAGAAGAA
CMYOCA04R	CTTGGCCCTCCTTAATTCATCT
CMYOCB01F	GCCTATTAAATACCATCCTCAGCA
CMYOCB01R	CTCTTGCCAGGCTGACCTGT
CMYOC01F	CCTTCATATCTTTCTTGATCTTAGGG
CMYOC01R	TTTTTCAATTTACTCTCAGAAGCTG
CMYOC02F	TTTTCAGAAACTGTGACAATCTG
CMYOC02R	AAAAATGAAACAAAAATCAAGTCA
CMYOC03F	CCGCTAAATCTAAGTTTTCAATCA
CMYOC03R	TTGTTTATTCATGAGAGACACACAGAG
CMYOC04F	GTGGCTCAGTGGTTGCTTAAC
CMYOC04R	ACCCAGGTGGTTCAGTCAGTTA
CMYOC05F	TTAGAGAGAGAGAGCATGAGCA
CMYOC05R	TGATCGATGTGTCATATGAATTG
CMYOC06F	AAAATGAAGTAAATCAGATATGGAAGT
CMYOC06R	AGACACAGAGCGTCCAGGTTTA
CMYOC07F	GGCATTTACCCTCTACCCAGTA
CMYOC07R	CTCATCCACACCCCGTACTT
CMYOC08F	TGTCCTTAATTCTCCAGGATGC
CMYOC08R	GTCTCAGCGGTCAGCTCGTAG
CMYOC09F	AGACCACGTGGAGAATCGAC
CMYOC09R	ACTGCTTCCGGATGTTAGTCTC
CMYOC10F	GCTACACGGACATCGACCTG
CMYOC10R	CTTCTCCAGGGGGTTGTAGTC
CMYOC11F	CCACCTGTACACCATCAGCAG
CMYOC11R	CTTAAAAAGCCGAGAAAAGCTG
CMYOC12F	GACAACTTCAACATGGTCACCT
CMYOC12R	GAAGAGACTACTATGCATGACAGCTT
CMYOC13F	GTCTGACCGTGTGGAAACAG
CMYOC13R	CCCCTCCTCTAATTATGGCTTG
CMYOC14F	AAAAGGCCCTTACTGGCTAAAG
CMYOC14R	CAACCCCAAGAGATGACCTG
CMYOC15F	TAGTGGGCTTCTAAGGCTTCAC
CMYOC15R	AGAGAGGGGAAGTTGAGGAAAG
CMYOC16F	GGAGCATTACTGGTGTGTGTGT
CMYOC16R	GCAGGTTCAGTTACCAAAAAGTC

Table 2-2: Final canine myocilin primers

Name	Sequence 5' to 3'
DMYOC1	GARGA ARCCT CACCM AGCCT C
DMYOC2	TTCTG GCCKG CYTGG TGTGG G
DMYOC3	TCCCT GGAGA GYCTC CTCCA C
DMYOC4	ACCTC CTSGC TGCTG CTYTC
DMYOC5	TGGAA TTTGG ACACK TTGGC C
DMYOC6	CTCBG ACTTC ARYTC CTGGA AG
DMYOC7	GCAAG TATGG HGTGT GGATG
DMYOC8	GTGGG CTTGG GGTCT CKCAT
DMYOC9	GTGAA GGCHG AGAAG GAAAT YC
DMYOC10	GTCAA TGTCY GTGTA GCCAC
DMYOC11	TTGAT RTCAT ARGTG ACCAT GTT
Key:	R=A or G, Y=C or T, M=A or C, K=G or T, S=C or G, W=A or T, B=C or G or T, D=A or G or T, H=A or C or T, V=A or C or G, N=A or C or G or T

Table 2-3 : Dogs used for microarray analysis with short history.

Dog	Age	Gender	Glaucoma	Previous treatments:
Samson	14 yr	Male	Advanced	1992 Dexamethasone, 1995 Timolol, Pilocarpine, 1996 Trusopt, Betagan, Methazolamide, Humersol, 2001 Travaprost, Latanoprost, Bimatoprost, Rescula, Brimonidine
Victoria	11 yr	Female	Advanced	1996 Trusopt, Betagan, Methazolamide, Humersol, 2001 Travaprost, Latanoprost, Bimatoprost, Rescula, Brimonidine
Hunter	6 m	Male	Normal	None.
XPX2	1 yr	Male	Normal	None.
AZG2	1 yr	Female	Normal	None.
XKX2	1 yr	Male	Normal	None.
1DG2	1 yr	Female	Normal	None.

Figure 2-1: Boxer made famous for providing the DNA for the first shot-gun DNA sequence for canines.

CHAPTER 3
RESULTS

Genomic Findings

The newly designed primers were successful in amplifying sequences from the canine genome on the canine chromosome 7. Fragments were amplified from normal and glaucomatous beagle DNAs and sequenced. These were then aligned using SeqEd (ABI). Comparisons between our normal control dog and the reported canine genome were extremely similar although there were a few insignificant differences. Comparisons between the glaucoma dogs and the reported canine genome DNA were near identical. The glaucoma dog genomic sequence (all 3 exons, untranslated regions, and flanking intronic sequences) was identical to normal beagle sequence. No structural or mutational differences were seen between the glaucoma dog and the published normal canine genome. The 1451 bp dog *MYOC* cDNA sequence is shown in figure 3-1.

Expected differences with other known species sequences of the myocilin gene were present, which were not expected to be pathogenic. None of the published known mutations of myocilin were present in any of the samples tested. RT-PCR analysis of trace amounts of RNA from the trabecular meshwork failed to amplify any fragments, so we could not rule out a deep intronic mutation that could affect splicing.

Protein Analysis

Using Western blot with human polyclonal antibodies made by Santa Cruz Biotechnologies, we were able to demonstrate the presence of myocilin in the aqueous humor of the dog. Its banding appearance was very similar to reported findings at approximately 55kDa,

with two other expected bands nearby (Figure 3-2). Immunohistochemistry also showed this (see sections below).

Inherited Model Glaucomatous Dogs

The myocilin protein was shown to be present in the Gelatt glaucoma beagle colony using Comassie staining and Western blot techniques (Table: 3-1, Figures 3-2, 3-3, 3-4, 3-5). A human trabecular meshwork derived myocilin protein control (Alcon) was used on every gel for relative comparisons, and given a rank of 1 unit on each gel. In the younger, nearly normal (mild glaucoma) beagle eyes (n= 4 dogs) a relatively low level of myocilin was detected. It was characterized as a single band approximately 53-57 kDa. In the youngest dogs (1.5 yrs of age), two faintly visible bands above and below the main band can be seen. The level of myocilin for the mild glaucoma dogs was approximately 1 to 2 versus the human myocilin protein control. The intensity of the myocilin bands increased as the glaucoma severity classification increased. The moderately affected dogs showed a marked increase in the presence of myocilin protein (five to six-fold versus the human control). The highest levels of myocilin were found in the most severe cases of inherited glaucoma, with levels up to 15 times the human control amount.

Clinical Samples

A total of 353 samples (141 Male, 194 Female, 18 unreported gender) with average ages of 107 months for the males, 104 months for the females and 105 months for the unknown gender dogs were analyzed. Breeds seen included: Airedale (1), Akita (1), Alaskan Malamute (2), Australian Shepherd (5), Australian Cattle Dog (1), Basset Hound (16), Bearded Collie (1), Bichon Frise (10), Border Collie (2), Boston Terrier (5), Boykin Spaniel (2), Cairn Terrier (2), Cavalier King Charles Spaniel (1), Chihuahua (3), Chow Chow (8), American Cocker Spaniel (62), Dachshund (3), Miniature Dachshund (1), Dalmatian (2), English Cocker Spaniel (1), Golden Retriever (3), Gordon Setter (1), Great Dane (1), Great Pyrenees (1), Italian Greyhound

(4), Jack Russell Terrier (4), Japanese Chin (1), Keeshond (2), Labrador Retriever (10), Lhasa Apso (9), Maltese (6), Manchester Terrier (1), Mixed Breeds (68), Newfoundland (1), Papillon (1), Pembroke Welsh Corgi (2), Miniature Pinscher (3), Pomeranian (4), Miniature Poodle (19), Standard Poodle (6), Pug (5), Rhodesian Ridgeback (1), Saint Bernard (1), Samoyed (2), Schipperke (1), Miniature Schnauzer (12), Standard Schnauzer (2), Scottish Terrier (2), Shar Pei (2), Shiba Inu (4), Shih Tzu (21), Siberian Husky (5), Welsh Terrier (1), West Highland White Terrier (4), Wheaten Terrier (2), and Yorkshire Terrier (12). Cataractous dog aqueous humor, used as non-glaucomatous controls, was shown to have little or no myocilin present at the dilutions used to visualize the glaucoma animals. With few exceptions, myocilin protein levels were found to be increased in animals with primary spontaneous glaucoma and secondary glaucomas (Figures 3-6, 3-7, 3-8). The level of the myocilin present could be correlated to the severity of the disease with the most advanced cases having the greatest apparent amounts of myocilin. Comparisons between differing glaucoma groups showed significant differences. Normal (Cataractous) dogs had the lowest level of myocilin at 4140.27 ± 674.12 μg/ml (average ± std dev). Diabetic cataractous dogs were found to have similar levels at 3339.37 ± 774.71 μg/ml. Primary spontaneous glaucoma dogs were found to have an aqueous humor myocilin protein level of 29404.18 ± 7449.11 μg/ml. Secondary glaucomas had the highest level of myocilin in the aqueous humor with 44797.03 ± 11659.83 μg/ml. Severe cases of glaucoma also had extra banding and a more globular appearance on the Western blot and Comassie gels (Figure 3-6). No obvious correlation could be made between myocilin levels and age or sex. Different breeds showed some but no statistical significant differences in severity, only in number of samples received (Table 3-2). This may show a predominance of spontaneous

glaucomatous cases in certain breeds or may be bias of ascertainment based on frequency of those breeds in the United States.

Immunohistochemistry

Specimens from the anterior uveas of 10 beagles, five with inherited glaucoma (3-mos- to 13-yrs-of age) and five age-matched normals, 1 normal walker hound, 1 normal schnauzer, and 2 cocker spaniels with spontaneous glaucoma were used in this study.

Within normal, mild and moderately glaucomatous canine specimens from age groups three months to thirteen years of age, identical immunolabeling of myocilin was observed by light microscopy. Immunolabeling in these specimens showed that myocilin was homogeneously distributed in ocular tissues (Figure 3-9). Immunolabeling of specimens from dogs with advanced glaucoma, however, exhibited an increased aggregation in areas surrounding the ICA, nonpigmented epithelium of the ciliary processes, and anterior cornea (Figure 3-10).

Within the cornea, intense staining for myocilin was seen throughout the cytoplasm of cells within the corneal epithelium (Figure 3-11 and 3-12) and endothelium (Figure 3-13). Within the corneal stroma, mild labeling was evident as thin extracellular lines running parallel to collagen bundles. There was no staining of Descemet's membrane.

Within the iris, cell membranes of smooth muscle cells of the sphincter and dilator muscles stained positive, as well as most resident cells of the iris stroma (Figures 3-14 and 3-15). Positive staining of vascular endothelial cells within the iris vessels was observed.

Within the ciliary body, the cell membrane of ciliary smooth muscle cells stained intensely. Vascular smooth muscle cells surrounding the ciliary body arteries and arterioles stained intensely. The cytoplasm of cells within the nonpigmented layer of the ciliary epithelium of the ciliary body and processes stained intensely (Figure 3-16 and 3-17). Labeling in the ciliary

epithelial cells of the pars plana was weaker than in the pars plicata. There was localized labeling in stroma of the ciliary processes.

Trabecular meshwork cells were homogenously labeled (Figure 3-18). The sclera adjacent to the angular aqueous plexus as well as other parts of the sclera stained positive. Scleral staining was observed extracellularly between collagen bundles (Figure 3-19). Vascular smooth muscle cells of arterioles and arteries within the sclera labeled intensely (Figure 3-20).

In samples with advanced glaucoma, greater intensity of staining was observed within the sclera adjacent to the ICA; trabecular meshwork cells of the ICA; and the nonpigmented epithelium of the ciliary processes in tissues surrounding the collapsed irideocorneal angle (Figure 3-10). In addition, vitreal membrane-like material labeled intensely within the posterior chamber (Figure 3-21 and 3-22) and corneal epithelium (Figure 3-12).

Immunocytochemistry

Myocilin was identified in the trabecular meshwork cells of older glaucomatous dogs as well as normal dogs (Figure 3-23, 3-24, and 3-25). It was also found extracellularly in the extracellular matrix around the trabecular meshwork (Figure 3-26). Lastly, it was also observed in the non-pigmented epithelium of the ciliary body and vitreal-like material within the posterior chamber of a 10 year old glaucomatous beagle (Figure 3-27 and 3-28).

Microarray

In comparisons of 6 dogs (4 normal and 2 glaucomatous), there were some differences noted in the mRNA levels of certain genes. The analysis of the final microarray run showed a signaling ratio of 1:0.4 in normal beagle dogs versus glaucomatous beagle dogs when hybridized with the human myocilin cDNA probe and 1:1.9 increase in *MYOC* mRNA signaling in normal beagle dogs versus glaucomatous beagle dogs when hybridized with the bovine myocilin cDNA probe. Two of the normal dogs had higher values, more similar to the glaucomatous dogs, based

on hybridization to the bovine myocilin cDNA probe. The overall difference between normal and glaucoma dogs was barely significant with the bovine myocilin probe ($p=0.05$). All the dogs, glaucomatous included, had very similar low results on the human myocilin pattern ($p=0.31$).

There are many other genes showing greater signaling differences. A signaling ratio greater than 1:2 is generally significant. A selection of significant genes can be seen in table 3-3.

Table 3-1: Relative myocilin protein levels in inherited glaucomatous dogs.

			Relative to control myocilin level on:				
Beagle	Initial Glaucoma State	Eye	05/06/04	09/03/04	11/03/04	05/09/05	11/21/05
Vanessa	Advanced	OD	10.56	15.65			
Vanessa	Advanced	OS	11.14	18.08			
Vanna	Advanced	OD	7.43	11.48			
Vanna	Advanced	OS	9.65	10.99			
Vick	Advanced	OD	10.46	13.30			
Vick	Advanced	OS	11.79	14.90			
Victoria	Advanced	OD	7.60		12.44	13.05	13.86
Victoria	Advanced	OS	11.07		15.12	15.85	14.77
Whoopie	Moderate	OD	8.46		7.15	8.33	7.40
Whoopie	Moderate	OS	8.84		9.30	10.20	9.97
Woody	Moderate	OD	11.37		11.94	10.84	11.28
Woody	Moderate	OS	12.49		11.89	12.32	10.71
Wrigley	Moderate	OD	7.86		9.19	9.75	9.91
Wrigley	Moderate	OS	7.52		10.09	9.57	9.37
Xmas	Moderate	OD			8.57	10.31	10.16
Xmas	Moderate	OS			6.98	7.55	8.79
Yoyo	Moderate	OD	7.91		3.70	9.12	9.65
Yoyo	Moderate	OS	6.63		4.57	8.05	8.04
Zag	Moderate	OD	8.90		4.50	3.70	4.13
Zag	Moderate	OS	7.93		3.63	4.70	4.96
Zig	Moderate	OD	5.79		4.16	5.46	5.95
Zig	Moderate	OS	7.71		2.66	5.49	8.80
America	Moderate	OD	7.10		2.34	3.33	5.16
America	Moderate	OS	7.32		6.02	4.52	3.57
Bridgette	Mild	OD			2.14	2.86	3.09
Bridgette	Mild	OS			2.51	1.64	2.28
Brooke	Mild	OD			2.44	2.39	1.25
Brooke	Mild	OS			2.25	1.89	0.93
Candy	Mild	OD			1.61	2.05	2.36
Candy	Mild	OS			0.89	1.20	2.09
Daisy	Mild	OD			2.41	1.53	1.18
Daisy	Mild	OS			1.75	2.29	1.76

Table 3-2: Aqueous humor myocilin levels from major breeds of dogs

Breeds	Number of M/F/U	Average Age M/F/U	1° Glaucoma	2° Glaucoma	Cataract	Diabetic Cataract	Unknown
Airedale	0/0/1	0/0/122		17689.14 ± 0.00			
Akita	0/1/0	0/146/0	6143.82 ± 0.00				
Alaskan Malamute	0/2/0	0/95/0	17958.50 ± 4134.91				
Australian Shepherd	0/5/0	0/120/0		10096.35 ± 0.00	2318.06 ± 902.70		
Australian Cattle Dog	0/1/0	0/195/0		78348.52 ± 0.00			
Basset Houndv	7/9/0	104/93/0	20069.07 ± 8034.04			2072.23 ± 498.38	18647.65 ± 0.00
Bearded Collie	0/0/1	0/0/72					
Bichon Frise*	6/4/0	114/53/0	30689.94 ± 0.00	16742.35 ± 0.00	3565.69 ± 1188.51	2318.80 ± 0.00	1560.73 ± 0.00
Border Collie	2/0/0	37/0/0		10004.99 ± 1903.02			
Boston Terrier	1/4/0	120/114/0		13104.03 ± 1733.02	2125.77 ± 478.34		
Boykin Spaniel	0/2/0	0/168/0			3168.39 ± 1337.77		
Cairn Terrier	1/1/0	140/148/0		21456.58 ± 2035.90			
Cavalier King Charles Spaniel	0/1/0	0/60/0		1303.11 ± 0.00			
Chihuahua	0/3/0	0/98/0	48243.20 ± 0.00	28969.56 ± 6919.48			
Chow Chow*	2/6/0	120/121/0	30487.78 ± 9050.08				
Cocker Spaniel, American*	19/33/10	110/111/130	36590.07 ± 10246.25	34478.16 ± 8303.43	4073.40 ± 872.38		6458.55 ± 2088.85
Dachshund	2/1/0	102/141/0	5183.85 ± 0.00	6031.20 ± 288.59			
Dachshund, Miniature	0/1/0	0/22/0		94535.50 ± 0.00			
Dalmatian	1/1/0	100/U/0	187287.31 ± 0.00	9396.99 ± 0.00			
English Cocker Spaniel	0/1/0	0/108/0			2249.88 ± 0.00		
Golden Retriever	0/3/0	0/109/0		51884.36 ± 30387.85			
Gordon Setter	1/0/0	132/0/0			3526.84 ± 0.00		
Great Dane	0/1/0	0/132/0			4492.88 ± 0.00		
Great Pyrenees	1/0/0	88/0/0	2972.81 ± 0.00				
Italian Greyhound	0/4/0	0/101/0	6596.57 ± 4428.47	4732.02 ± 797.41			
Jack Russell Terrier*	2/2/0	98/93/0		17993.97 ± 9138.39			
Japanese Chin	0/1/0	0/120/0	7692.16 ± 0.00				
Keeshond	2/0/0	96/0/0			1062.52 ± 552.89		
Labrador Retriever*	2/8/0	111/72/0	54973.09 ± 42329.15	23476.86 ± 6217.79	458.13 ± 458.13	2354.47 ± 1177.23	
Lhasa Apso*	3/6/0	164/98/0	34781.93 ± 0.00	80265.99 ± 0.00	2043.81 ± 2043.81	8139.65 ± 3887.96	
Maltese*	5/1/0	118/163/0	66452.89 ± 53788.70		2696.47 ± 1569.93	2313.07 ± 205.07	
Manchester Terrier	0/1/0	0/108/0		21909.08 ± 0.00			
Mixed Breed*	34/30/4	106/112/139	47030.84 ± 35453.88	25499.47 ± 8187.94	5401.32 ± 2112.58	5681.94 ± 1904.40	2513.38 ± 0.00
Newfoundland	0/1/0	0/44/0	15857.43 ± 0.00				
Papillon	0/1/0	0/48/0	7830.83 ± 0.00				

Table 3-2: Continued

Breeds	Number of M/F/U	Average Age M/F/U	1° Glaucoma	2° Glaucoma	Cataract	Diabetic Cataract	Unknown
Pembroke Welsh Corgi	1/0/1	120/0/120			1463.97 ± 0.00		2990.30 ± 0.00
Pinscher, Miniature	3/0/0	112/0/0			4401.68 ± 0.00	629.53 ± 629.53	
Pomeranian	4/0/0	72/0/0			18684.91 ± 10844.96		
Poodle, Miniature*	7/12/0	114/117/0		3254.03 ± 3254.03	5790.49 ± 4060.35	10314.82 ± 6637.16	
Poodle, Standard*	2/4/0	156/116/0			4984.69 ± 1399.11	547.26 ± 0.00	
Pug	2/3/0	69/96/0		284436.83 ± 277425.09		1635.71 ± 195.54	
Rhodesian Ridgeback	0/1/0	0/67/0		77590.46 ± 0.00			
Saint Bernard	1/0/0	94/0/0	33208.09 ± 0.00				
Samoyed	2/0/0	108/0/0			3157.13 ± 588.61		
Schipperke	0/1/0	0/120/0			2189.07 ± 0.00		
Schnauzer, Miniature*	3/9/0	68/88/0		38042.73 ± 0.00	8688.65 ± 5472.76	549.76 ± 549.76	
Schnauzer, Standard	0/2/0	0/120/0			4595.14 ± 188.38		
Scottish Terrier	2/0/0	84/0/0			3264.79 ± 79.62		
Shar Pei	0/2/0	0/91/0	2497.16 ± 1426.95				
Shiba Inu	0/4/0	0/68/0	19802.13 ± 7674.53				
Shih Tzu*	11/9/1	123/123/51	10115.54 ± 2122.09	139317.16 ± 119087.88	8419.50 ± 5915.79	4124.78 ± 1449.25	
Siberian Husky*	3/2/0	70/14/0	16918.81 ± 9366.35		3236.53 ± 0.00		13562.18 ± 0.00
Welsh Terrier	1/0/0	84/0/0					3326.34 ± 0.00
West Highland White Terrier	4/0/0	156/0/0			1424.43 ± 338.64	2423.12 ± 0.00	
Wheaten Terrier	1/1/0	73/149/0	12586.31 ± 0.00	54163.23 ± 0.00			
Yorkshire Terrier *	3/9/0	168/118/0	13134.43 ± 0.00		4303.14 ± 970.56	3646.07 ± 1512.08	
Totals/Averages	141/194/18	107/104/105	29404.18 ± 7449.11	44797.03 ± 11659.83	4140.27 ± 674.12	3339.37 ± 774.71	7008.45 ± 2479.97

* Generally thought to have inherited glaucoma (Gelatt et. al, 2004)

Table 3-3 : Selected DNA chip microarray results. Ratio and probability show major differences between glaucoma and normal mRNA results. Items that may be of interest in bold.

	Normal Beagle Dogs					Glaucoma Beagle Dogs						
UF Probe	AZG2	IDG2	Hunter	1-XX2	XKX2	Victoria	Sampson	Avg Glau	Avg Non	Ratio	Probability	Hit Definitions / Active Genes
UF_Cf_11404	-4.96	-4.63	-1.05	-0.53	1.31	-4.12	-3.42	-3.77	-1.97	1.91	0.05	myocilin [Bos taurus]
UF_Cf_15108	0.52	-0.75	-0.39	0.33	0.90	0.35	-0.45	-0.05	0.12	-0.41	0.31	myocilin; trabecular meshwork-induced glucocorticoid response protein [Homo sapiens]
UF_Cf_15247	0.49	0.03	-0.18	-0.46	-0.36	0.95	0.62	0.79	-0.10	-8.18	0.02	ABC transporter [Homo sapiens]
UF_Cf_17624	-2.07	-1.08	0.08	0.89	0.89	1.72	1.41	1.57	-0.26	-6.07	0.05	ABC transporter subunit [Naegleria gruberi
UF_Cf_13238	-0.29	0.28	0.00	0.13	0.15	0.52	0.63	0.58	0.05	10.65	0.02	ABC transporter, permease protein, putative
UF_Cf_16780	-0.48	-0.24	-0.29	-0.09	-0.11	0.52	0.72	0.62	-0.24	-2.56	0.02	asparaginyl-tRNA synthetase [Homo sapiens]
UF_Cf_15485	-0.25	1.54	1.40	-0.32	-0.15	1.51	0.88	1.20	0.44	2.69	0.05	aspartic protease family member (5T521) [Caenorhabditis elegans]
UF_Cf_12820	0.41	0.95	0.44	-0.46	-0.20	0.84	0.71	0.78	0.23	3.40	0.02	ASPN protein [Homo sapiens]
UF_Cf_11698	-0.37	0.08	-0.71	0.53	0.32	1.13	1.26	1.20	-0.03	-39.83	0.02	ATP synthase, H+ transporting mitochondrial F1 complex, beta subunit
UF_Cf_17024	-0.19	0.55	0.23	0.06	0.08	-0.61	-0.66	-0.64	0.15	-4.35	0.00	ATP/GTP-binding protein; homolog of yeast CFIA subunit Clp1p [Homo sapiens]]
UF_Cf_12948	0.36	0.13	0.19	-0.44	-0.37	-0.18	-0.17	-0.18	-0.03	6.73	0.02	atrium potassium channel IRK [Canis familiaris]
UF_Cf_18223	-0.64	0.28	2.27	0.11	0.55	-1.62	-1.52	-1.57	0.51	-3.05	0.01	B. burgdorferi predicted coding region BBG21 [Borrelia burgdorferi]
UF_Cf_19717	0.10	-2.59	0.70	1.17	1.13	0.79	0.81	0.80	0.10	7.84	0.00	B9 protein; likely ortholog of mouse endothelial precursor protein B9 [Homo sapiens]
UF_Cf_19320	0.92	-0.34	0.43	-1.02	-0.76	0.26	0.85	0.56	-0.15	-3.60	0.05	Bardet-Biedl syndrome 7 [Mus musculus
UF_Cf_15882	0.34	-0.62	0.06	0.09	0.24	0.56	0.68	0.62	0.02	28.18	0.04	basic fibroblast growth factor [Canis familiaris]
UF_Cf_12228	1.12	0.33	-0.70	-0.22	0.03	1.17	0.90	1.04	0.11	9.24	0.03	calcium-activated potassium channel beta 4 subunit [Meriones unguiculatus]
UF_Cf_19290	-0.49	0.34	-0.94	-0.83	-0.61	1.40	2.43	1.92	-0.51	-3.78	0.04	Calcyphosine (Thyroid protein P24) (TPP) (Protein 5 [Canis familiaris]
UF_Cf_15631	-0.42	-0.09	-0.04	0.07	0.11	0.47	0.60	0.54	-0.07	-7.23	0.02	calpain 1, large subunit; calpain, large polypeptide L1; calcium-activated neutral proteinase [Homo sapiens
UF_Cf_14128	0.50	0.22	0.90	0.27	-0.05	-0.74	-0.79	-0.77	0.37	-2.08	0.03	calponin like transmembrane domain protein [Homo sapiens]
UF_Cf_13871	-1.37	-1.05	-0.96	1.00	1.27	0.52	0.40	0.46	-0.22	-2.07	0.04	Canis familiaris vascular anastomotic upregulated protein mRNA, partial cds
UF_Cf_16999	-0.09	-0.24	-0.04	-0.01	-0.08	-0.54	-0.37	-0.46	-0.09	4.95	0.05	CD44 antigen precursor (Phagocytic glycoprotein I) (PGP-1) [Canis familiaris]
UF_Cf_15216	0.22	0.49	-0.23	0.03	0.13	-0.26	-0.38	-0.32	0.13	-2.50	0.04	cell adhesion molecule, neural [Bos taurus]
UF_Cf_18695	-1.21	-0.31	-0.59	-0.10	0.48	-2.31	-2.29	-2.30	-0.35	6.65	0.01	chondroitin sulfate proteoglycan 4 [Mus musculus]
UF_Cf_13051	-0.50	-0.15	1.10	0.60	-0.29	-2.50	-2.25	-2.38	0.15	-15.63	0.03	CocoaCrisp [Homo sapiens]
UF_Cf_10845	0.84	1.00	0.11	0.00	-0.20	1.22	1.10	1.16	0.35	3.31	0.01	COG0587: DNA polymerase III, alpha subunit [Burkholderia fungorum]

Table 3-3 : Continued

	Normal Beagle Dogs					Glaucoma Beagle Dogs						
UF Probe	AZG2	IDG2	Hunter	1-XX2	XKX2	Victoria	Sampson	Avg Glau	Avg Non	Ratio	Probability	Hit Definitions / Active Genes
UF_Cf_13181	-0.62	0.39	0.99	0.01	0.32	-1.21	-0.84	-1.03	0.22	-4.70	0.04	COG0845: Membrane-fusion protein [Nostoc punctiforme]
UF_Cf_12407	0.73	0.69	0.46	0.45	0.05	1.15	1.21	1.18	0.48	2.48	0.04	COG1033: Predicted exporters of the RND superfamily [Burkholderia fungorum]
UF_Cf_17066	-0.21	-0.16	1.22	0.39	0.31	-1.17	-1.00	-1.09	0.31	-3.50	0.00	COG1305: Transglutaminase-like enzymes, putative cysteine proteases [Microbulbifer degradans 2-40]
UF_Cf_14766	-0.18	-0.22	0.01	0.27	0.29	0.48	0.48	0.48	0.03	14.12	0.00	COG2219: Eukaryotic-type DNA primase, large subunit [Methanosarcina barkeri]
UF_Cf_10276	-0.62	-0.30	0.10	-0.01	-0.09	0.44	0.57	0.51	-0.18	-2.74	0.02	Component of oligomeric golgi complex 1 [Mus musculus]
UF_Cf_15801	0.05	-0.79	-0.20	0.41	0.40	-0.47	-0.53	-0.50	-0.03	19.23	0.00	DNA polymerase III, tau subunit, putative [Chlamydia muridarum]
UF_Cf_12504	-0.30	0.13	-0.16	-0.03	-0.14	0.35	0.37	0.36	-0.10	-3.60	0.02	DNA-DIRECTED RNA POLYMERASE BETA' CHAIN [Plasmodium falciparum]
UF_Cf_12161	0.00	-0.17	-0.46	-0.12	-0.18	-0.60	-0.48	-0.54	-0.19	2.90	0.03	DNA-directed RNA polymerase subunit A' [Methanosarcina mazei Goe1]
UF_Cf_10209	-0.09	0.03	0.09	-0.04	-0.08	0.47	0.37	0.42	-0.02	-23.33	0.01	envelope glycoprotein [Simian-Human immunodeficiency virus]
UF_Cf_14307	0.78	0.60	-0.39	0.02	-0.17	0.40	0.50	0.45	0.17	2.68	0.04	glycoprotein 150 [murid herpesvirus 4]
UF_Cf_13331	0.08	-0.34	-1.15	0.07	0.17	0.93	0.93	0.93	-0.23	-3.97	0.00	glycoprotein Ib [Canis familiaris]
UF_Cf_15935	-0.02	0.21	-1.22	-0.20	-0.21	0.54	0.64	0.59	-0.29	-2.05	0.00	glycoprotein precursor [Lassa virus]
UF_Cf_15817	-2.53	-0.76	0.41	-0.09	-0.53	1.74	1.13	1.44	-0.70	-2.05	0.04	golgi membrane protein GP73 [Homo sapiens
UF_Cf_11878	-0.58	-0.80	0.19	0.09	0.21	0.53	0.63	0.58	-0.18	-3.26	0.03	human immunodeficiency virus type I enhancer binding protein 2; human immunodeficiency virus type I enhancer-binding protein 2 [Homo sapiens]]
UF_Cf_10393	0.18	0.00	0.36	-0.26	-0.36	0.19	0.05	0.12	-0.02	-7.50	0.04	hyaluronoglucosaminidase 4; hyaluronidase 4 [Homo sapiens]
UF_Cf_15731	-0.55	-2.01	-0.69	1.17	1.11	0.47	0.33	0.40	-0.19	-2.06	0.01	lipoprotein lipase [Canis familiaris]
UF_Cf_10095	-0.54	0.39	0.37	-0.02	0.32	-0.67	-0.93	-0.80	0.10	-7.69	0.05	low density lipoprotein B; low density lipoprotein receptor defect B complementing; conserved oligomeric Golgi complex protein 1 [Homo sapiens]
UF_Cf_10185	0.38	0.46	0.37	-0.04	0.14	-0.66	-0.49	-0.58	0.26	-2.19	0.04	low molecular mass ubiquinone-binding protein (9.5kD) [Bos taurus]
UF_Cf_11745	-0.58	-0.63	-2.03	0.21	-0.56	-2.86	-2.45	-2.66	-0.72	3.70	0.03	L-type calcium channel alpha-1c subunit [Rattus norvegicus]
UF_Cf_11046	-0.44	-0.15	-0.06	0.01	0.03	0.27	0.26	0.27	-0.12	-2.17	0.00	mitochondrial carrier protein MGC4399 [Homo sapiens]
UF_Cf_11708	1.02	0.43	0.11	0.24	0.01	0.89	0.69	0.79	0.36	2.18	0.05	mitochondrial NADH:ubiquinone oxidoreductase B14.7 [Bos taurus]
UF_Cf_13315	-0.47	0.13	0.19	0.15	0.24	0.45	0.43	0.44	0.05	9.17	0.03	mitochondrial ribosomal protein S14 [Mus musculus]
UF_Cf_15730	0.06	-0.09	-0.02	-0.15	-0.11	0.21	0.14	0.18	-0.06	-2.82	0.02	mitochondrial ribosomal protein S18C; mitochondrial ribosomal protein S18-1 [Homo sapiens]

Table 3-3 : Continued

	Normal Beagle Dogs					Glaucoma Beagle Dogs						
UF Probe	AZG2	IDG2	Hunter	1-XX2	XKX2	Victoria	Sampson	Avg Glau	Avg Non	Ratio	Probability	Hit Definitions / Active Genes
UF_Cf_11954	-1.11	0.80	0.54	0.32	-0.03	1.38	1.23	1.31	0.10	12.55	0.03	mucin glycoprotein [Homo sapiens]
UF_Cf_12399	-0.08	0.13	-0.38	-0.08	-0.11	-0.33	-0.37	-0.35	-0.10	3.37	0.01	Myosin Ib (Myosin I alpha) (MMI-alpha) (MMIa) (MIH-L)
UF_Cf_16152	-1.36	-1.80	-0.56	0.60	0.48	1.29	1.27	1.28	-0.53	-2.42	0.01	myosin regulatory light chain 2 - human
UF_Cf_11710	-0.35	0.13	-0.24	0.00	-0.10	-0.43	-0.43	-0.43	-0.11	3.84	0.02	nuclear receptor co-repressor [Homo sapiens]
UF_Mm_19983	-0.25	0.05	0.02	0.13	-0.04	0.50	0.56	0.53	-0.02	-29.44	0.03	nuclear RNA export factor 1; tip associating protein; nuclear RNA export factor 1 (Mex67, yeast, homolog) [Homo sapiens]
UF_Cf_11546	-0.27	0.24	-0.80	0.04	0.02	-0.82	-0.89	-0.86	-0.15	5.55	0.00	nucleolar phosphoprotein Nopp34 [Homo sapiens]
UF_Cf_19677	0.04	0.33	0.34	0.24	0.25	0.66	0.73	0.70	0.24	2.90	0.01	orf 19 [Staphylococcus aureus prophage phiPV83]
UF_Cf_15414	-0.83	-2.31	0.54	-0.25	-0.11	1.47	0.91	1.19	-0.59	-2.01	0.04	orf; hypothetical protein [Salmonella typhi]
UF_Cf_15265	-0.66	0.76	-0.69	0.16	0.50	-0.46	-0.65	-0.56	0.01	-39.64	0.05	ORF2 [Canis familiaris]
UF_Cf_10109	-0.29	-0.28	-0.18	0.00	0.25	-0.52	-0.55	-0.54	-0.10	5.35	0.03	ORF24 [Alcelaphine herpesvirus 1]
UF_Cf_13593	0.40	0.43	0.26	0.20	0.05	-0.68	-0.69	-0.69	0.27	-2.56	0.01	orf274 [Euglena gracilis]
UF_Cf_17364	0.82	-2.31	-0.64	0.97	1.01	0.04	0.22	0.13	-0.03	-4.33	0.01	orf296 [Podospora anserina]
UF_Cf_18948	-1.16	-0.93	-0.81	-0.07	0.14	-1.50	-1.67	-1.59	-0.57	2.80	0.01	orf98 [Tetrahymena pyriformis]
UF_Cf_14836	-0.21	0.02	1.94	-0.03	0.39	-1.06	-1.43	-1.25	0.42	-2.95	0.04	prostaglandin E2 receptor EP3A subtype [Canis familiaris]
UF_Cf_13620	0.14	0.10	0.06	0.30	0.03	1.35	1.06	1.21	0.13	9.56	0.03	prostaglandin E2 receptor EP3B subtype [Canis familiaris]
UF_Cf_13325	-1.31	-0.57	-0.11	0.37	-0.04	-1.61	-1.27	-1.44	-0.33	4.34	0.03	retinaldehyde binding protein 1; retinaldehyde-binding protein 1 [Mus musculus]
UF_Cf_10691	-0.61	-0.57	0.37	0.02	0.21	-0.67	-0.55	-0.61	-0.12	5.26	0.02	stem-loop binding protein [Mus musculus]
UF_Cf_18898	-1.10	-0.62	0.48	-0.16	0.13	-1.33	-1.38	-1.36	-0.25	5.33	0.01	sterol regulatory element binding protein-1 [Canis familiaris]
UF_Cf_12694	-0.01	-0.10	-0.02	-0.01	0.03	0.51	0.56	0.54	-0.02	-24.32	0.00	stromal cell derived factor 4 [Rattus norvegicus]
UF_Cf_14149	-0.27	-0.81	-0.29	0.52	0.31	-0.40	-0.32	-0.36	-0.11	3.33	0.02	succinate dehydrogenase complex, subunit C, integral membrane protein, 15kDa [Bos taurus]
UF_Cf_10619	1.36	0.73	-0.93	-0.56	-0.44	0.96	0.80	0.88	0.03	27.50	0.01	T cell receptor beta chain hcvb4 [Canis familiaris]
UF_Cf_15898	-0.27	0.00	-0.04	0.18	0.11	0.44	0.43	0.44	-0.00	-108.75	0.01	T-cell leukemia translocation altered gene [Homo sapiens]
UF_Cf_14027	0.55	0.05	-0.03	0.03	0.03	-0.37	-0.50	-0.44	0.13	-3.45	0.02	uronyl-2-sulfotransferase; dermatan/chondroitin sulfate 2-sulfotransferase; uronyl 2-sulfotransferase [Homo sapiens]
UF_Cf_14046	-0.02	0.09	0.64	0.00	-0.06	-0.80	-0.81	-0.81	0.13	-6.19	0.00	vascular endothelial growth factor B [Homo sapiens]
UF_Cf_11361	-0.92	-0.32	-0.64	0.61	0.31	-0.55	-0.89	-0.72	-0.19	3.75	0.03	vir12 [Plasmodium vivax]
UF_Cf_17158	0.69	-0.95	-0.36	-0.02	0.19	-0.54	-0.68	-0.61	-0.09	6.78	0.03	WD repeat-containing protein 3 [Homo sapiens]
UF_Cf_11438	0.44	0.31	-0.18	0.12	0.08	0.51	0.38	0.45	0.15	2.89	0.04	Wim protein [Mus musculus]

Table 3-3 : Continued

	Normal Beagle Dogs					Glaucoma Beagle Dogs						
UF Probe	AZG2	IDG2	Hunter	1-XX2	XKX2	Victoria	Sampson	Avg Glau	Avg Non	Ratio	Probability	Hit Definitions / Active Genes
UF_Cf_13297	0.33	0.19	-0.24	0.14	0.14	1.49	1.51	1.50	0.11	13.39	0.00	Wolfram syndrome 1; Wolfram syndrome 1 (wolframin) [Rattus norvegicus]
UF_Cf_14834	0.48	-0.42	-0.06	-0.25	-0.21	0.50	0.33	0.42	-0.09	-4.51	0.02	X-ray repair cross complementing protein 2; X-ray repair, complementing defective, repair in Chinese hamster; DNA repair protein XRCC2 [Homo sapiens]
UF_Cf_18469	-1.02	0.89	0.68	0.66	0.86	1.62	1.54	1.58	0.41	3.82	0.02	zinc finger protein 140 (clone pHZ-39) [Homo sapiens]
UF_Mm_20096	0.59	0.80	0.00	-0.22	-0.20	-0.42	-0.45	-0.44	0.19	-2.24	0.01	Zinc finger protein 397 >gi|28901622|gb|AAM95991.2| zinc finger protein [Homo sapiens]
UF_Cf_15064	-0.45	-0.67	-0.51	0.04	0.14	-0.61	-0.66	-0.64	-0.29	2.19	0.01	zinc finger protein 445; zinc finger protein 168 [Homo sapiens]
UF_Cf_13933	0.03	0.75	1.01	0.04	0.00	1.01	1.43	1.22	0.37	3.33	0.03	zinc finger, DHHC domain containing 4 [Homo sapiens]
UF_Cf_15249	-0.78	0.88	0.58	-0.17	-0.03	-1.05	-1.01	-1.03	0.10	-10.73	0.01	ZONA PELLUCIDA SPERM-BINDING PROTEIN 3 PRECURSOR

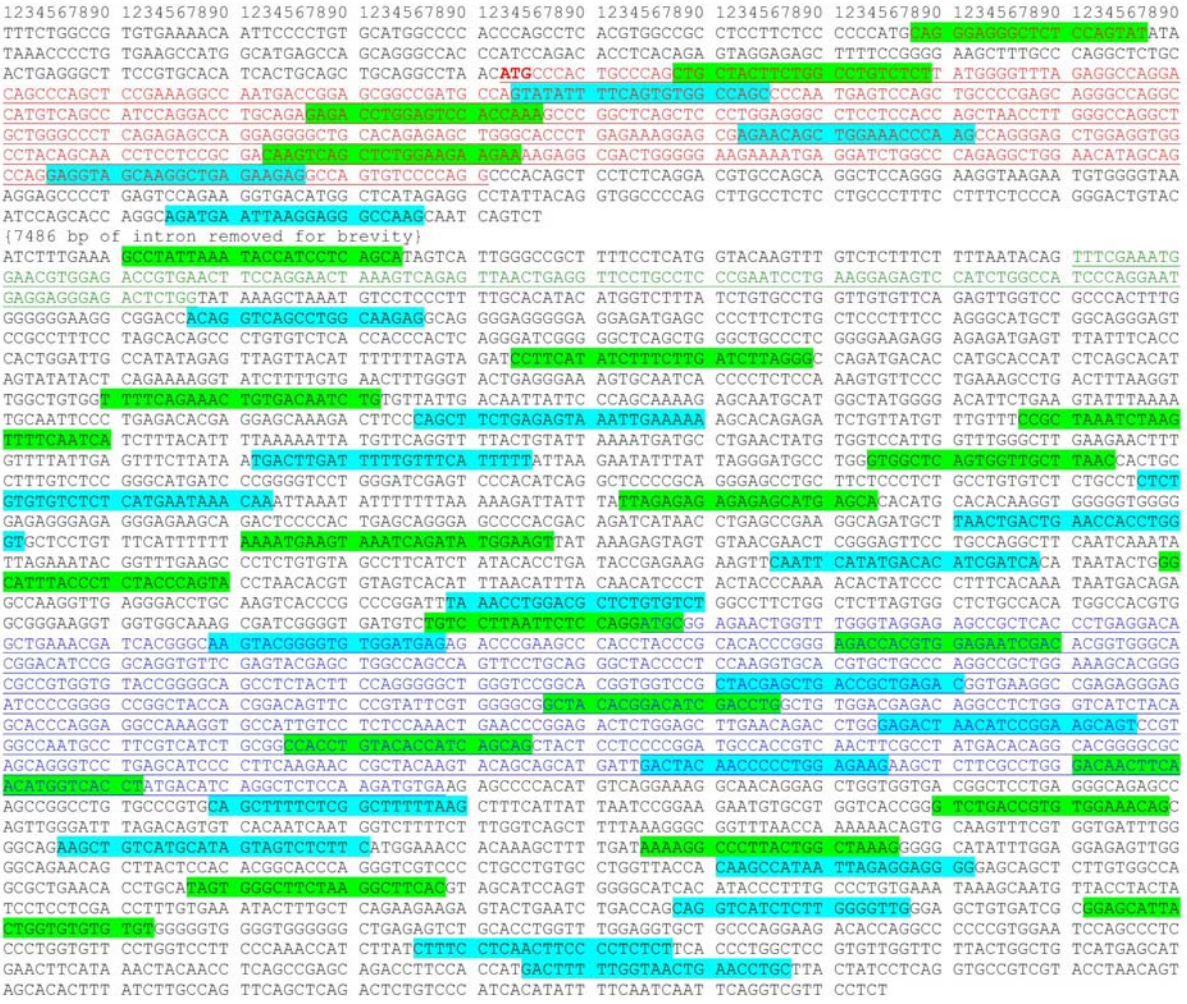

Figure 3-1: Region of Canine Chromosome 7 containing *MYOC*. The 1451 bp dog *MYOC* cDNA sequence is shown underlined. Red – Exon 1, Green – Exon 2, Blue – Exon3. Green highlighting represents "start" primers used to piece the gene together. Blue highlighting represents "end" primers.

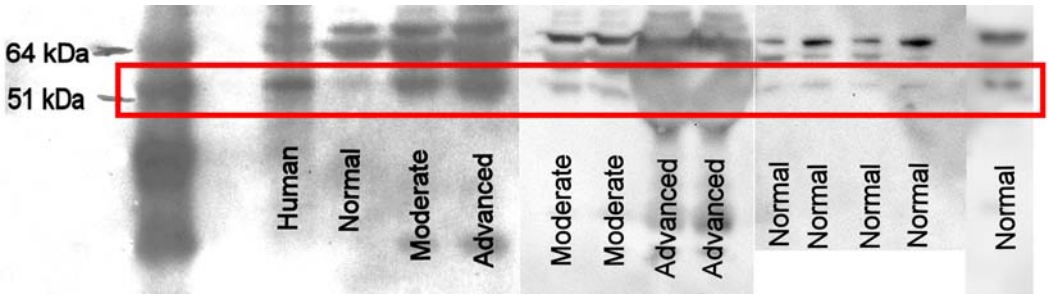

Figure 3-2: Western blot of aqueous humor samples from beagles with primary open angle glaucoma with human myocilin control. Box shows typical primary band.

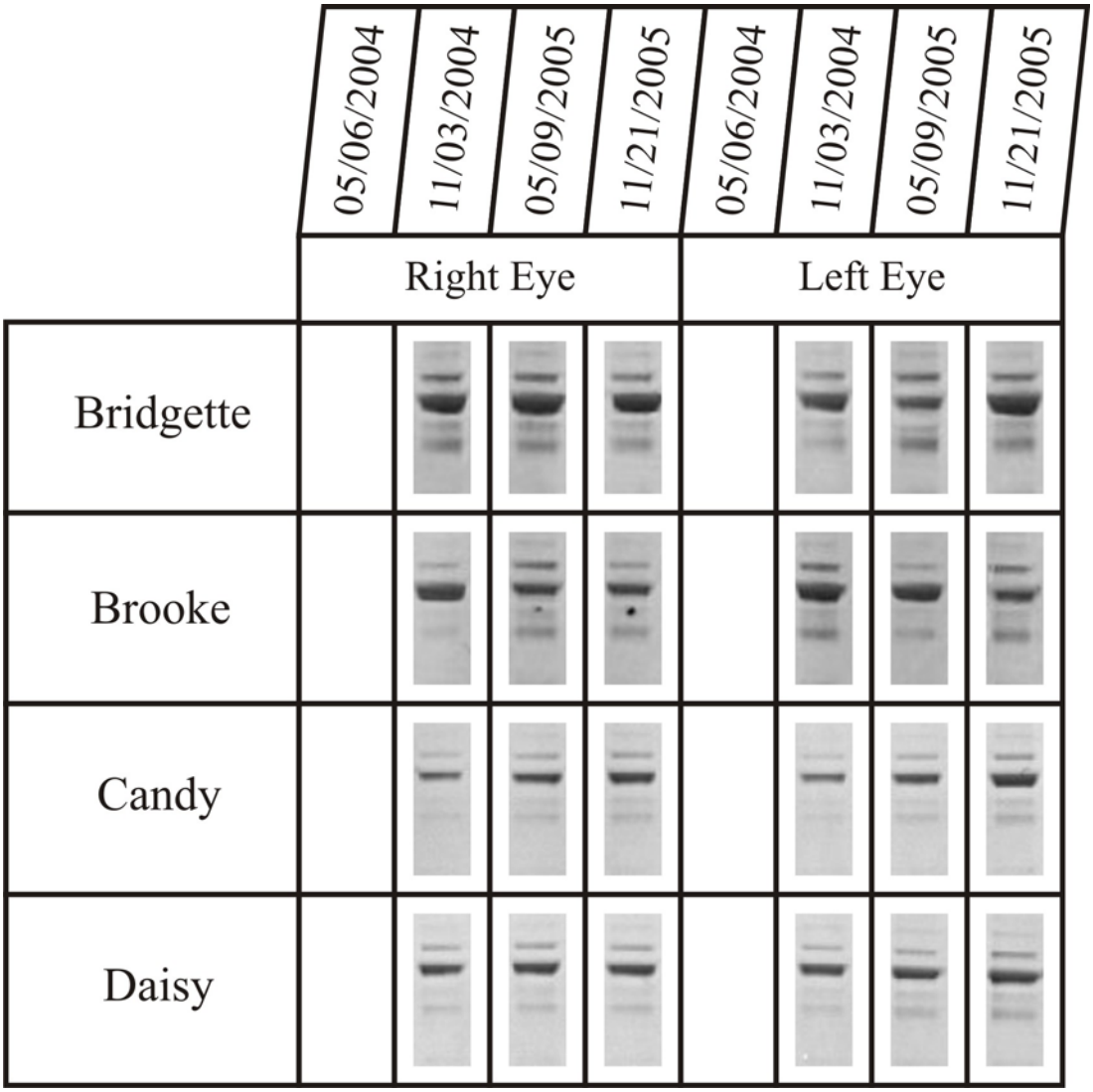

Figure 3-3: Comassie gel strips from aqueous humor of mildly affected glaucoma beagles over time. The darkest band is approximately 57 kDa.

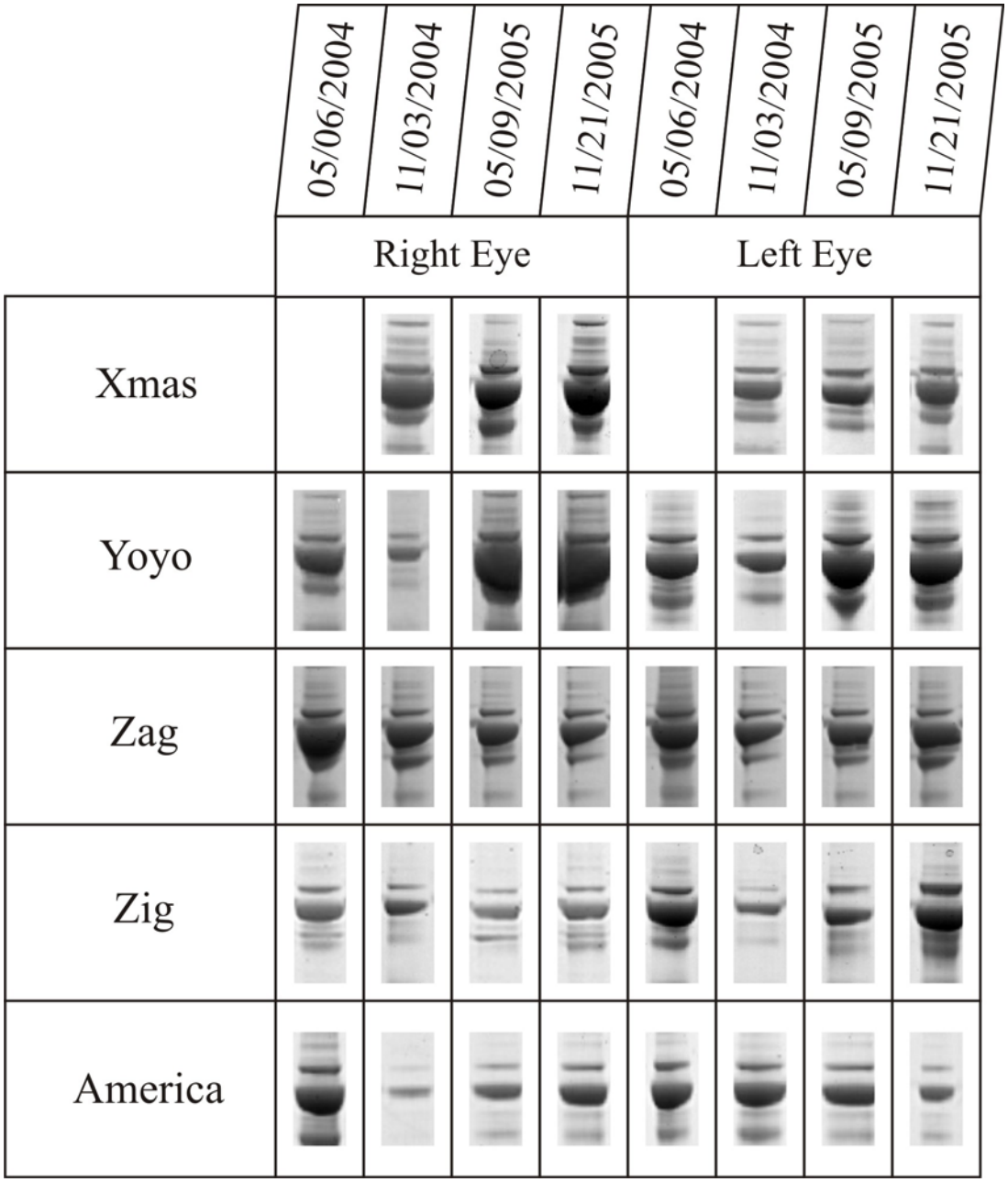

Figure 3-4: Comassie gel strips from aqueous humor of moderately affected glaucoma beagles over time. The darkest/ largest band centers at approximately 57 kDa.

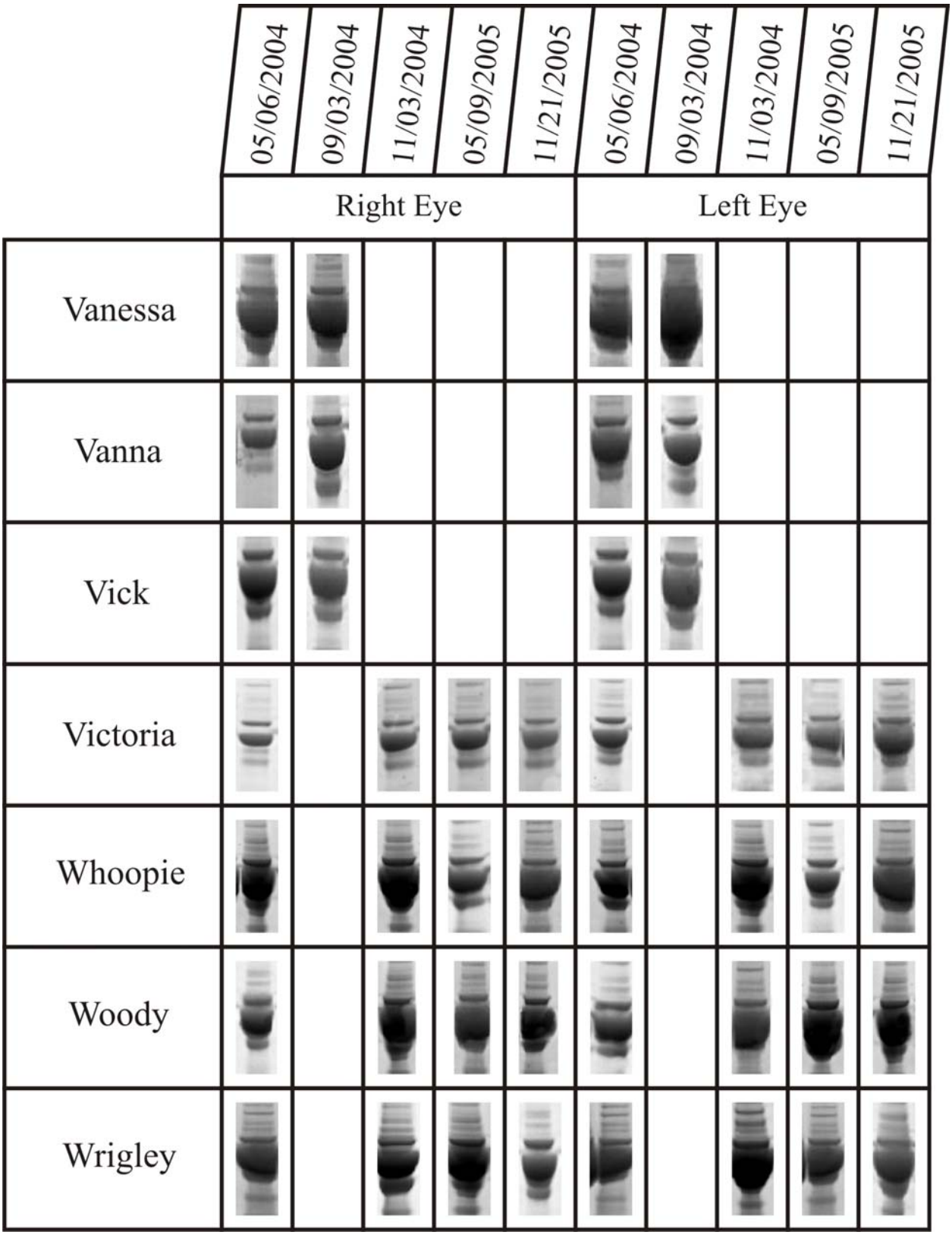

Figure 3-5: Comassie gel strips from aqueous humor of advanced glaucoma beagles over time. The darkest/ largest band centers at approximately 57 kDa.

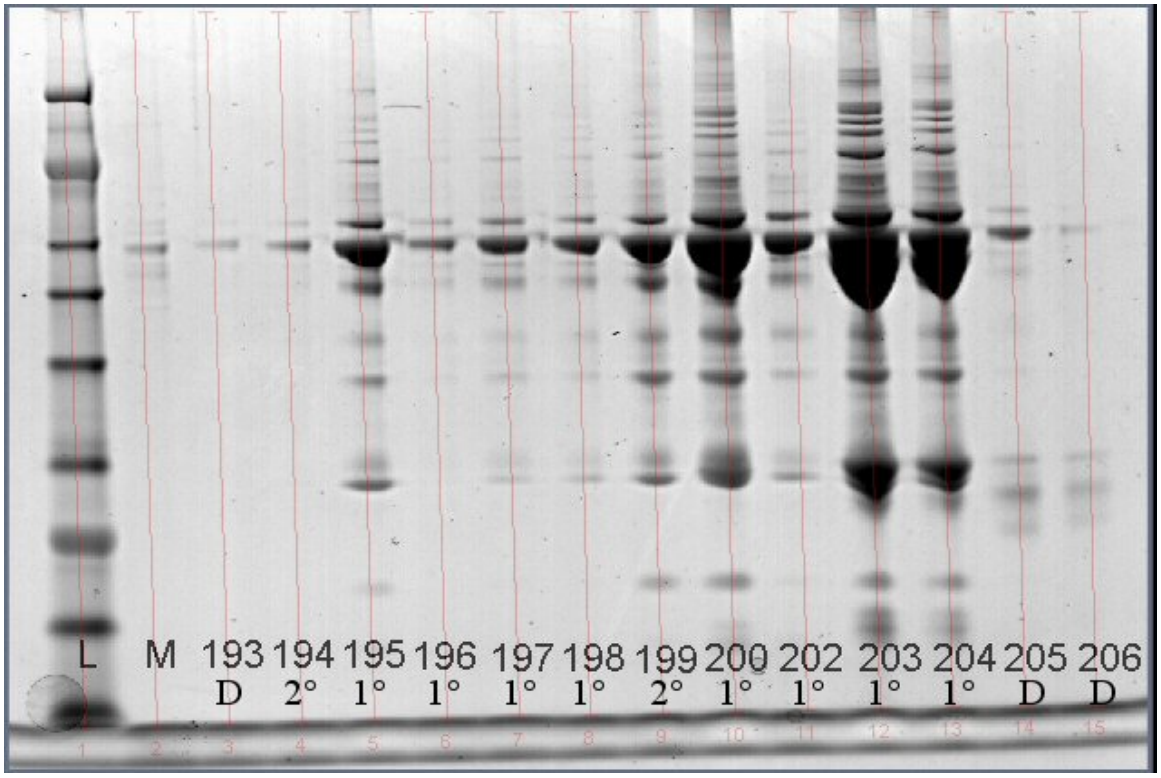

Figure 3-6 : Samples 193 through 206, examples of Comassie staining of aqueous humor. 1°=primary glaucoma, 2°=secondary glaucoma, C=cataract, D=diabetic cataract, L=ladder, M=myocilin control.

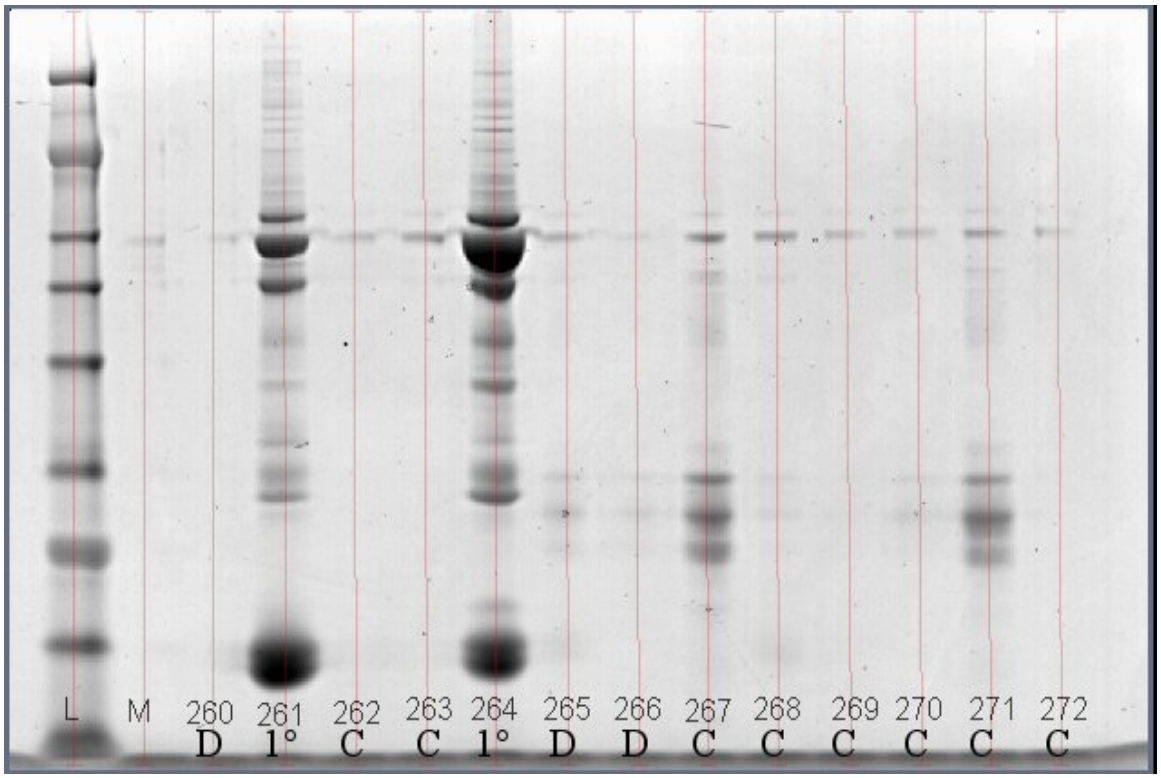

Figure 3-7 : Samples 260 through 272, examples of Comassie staining of aqueous humor. 1°=primary glaucoma, 2°=secondary glaucoma, C=cataract, D=diabetic cataract, L=ladder, M=myocilin control.

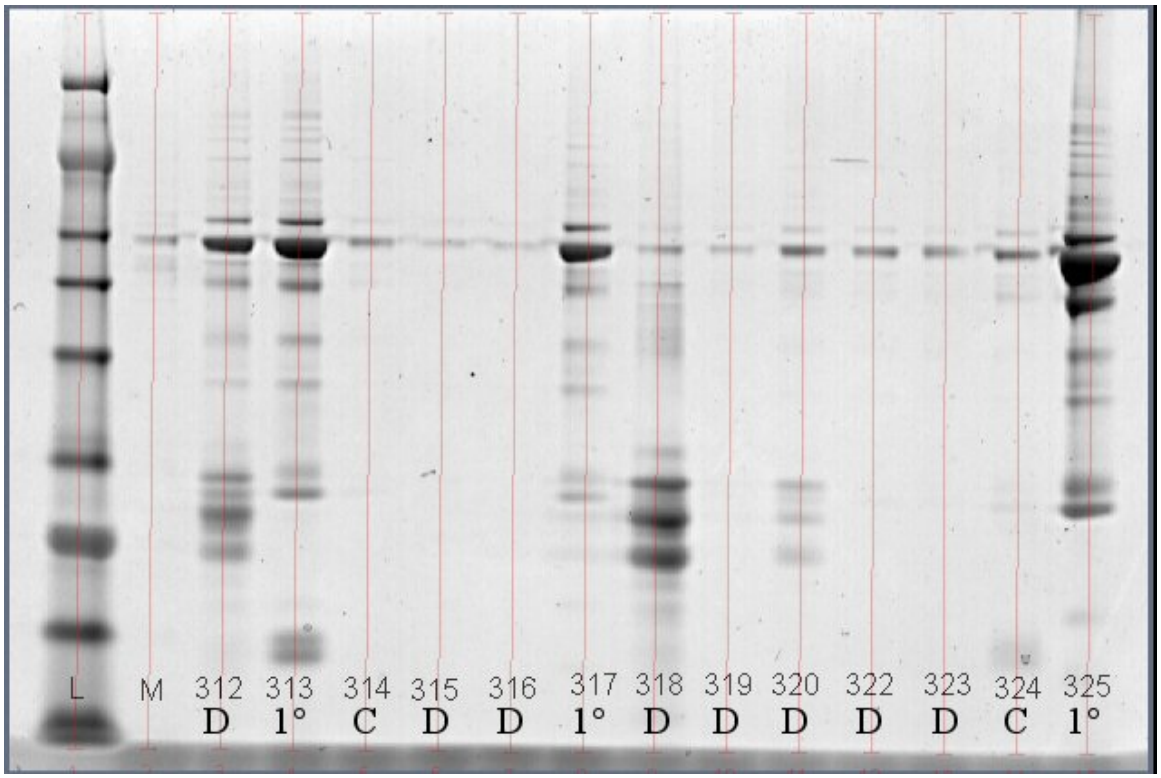

Figure 3-8 : Samples 312 through 325, examples of Comassie staining of aqueous humor. 1°=primary glaucoma, 2°=secondary glaucoma, C=cataract, D=diabetic cataract, L=ladder, M=myocilin control.

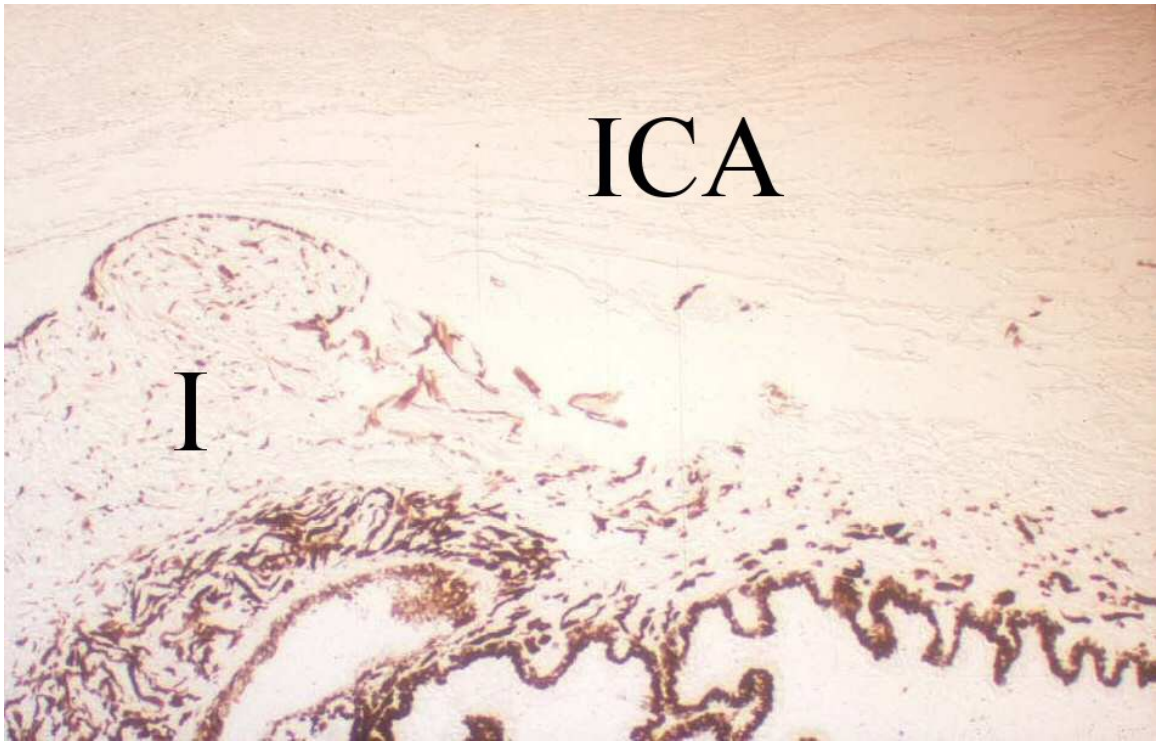

Figure 3-9: Overall view of iridocorneal angle (ICA) of a normal 1.5 yr. old (X100) beagle. Normal myocilin localization is observed in the Irido-Corneal Angle (ICA). Iris (I).

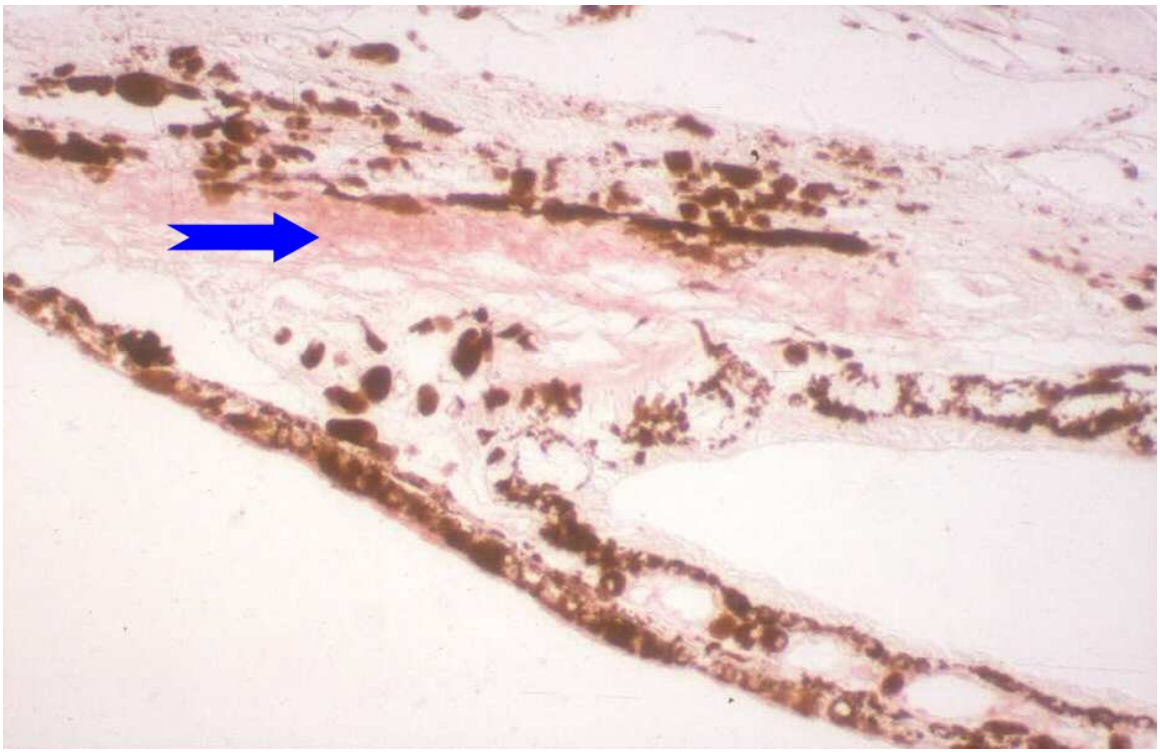

Figure 3-10: Overall view of irideocorneal angle (ICA) of a glaucomatous 6 yr. old beagle (X200). Increased myocilin localization is observed in the ICA (arrow) of the glaucomatous canine.

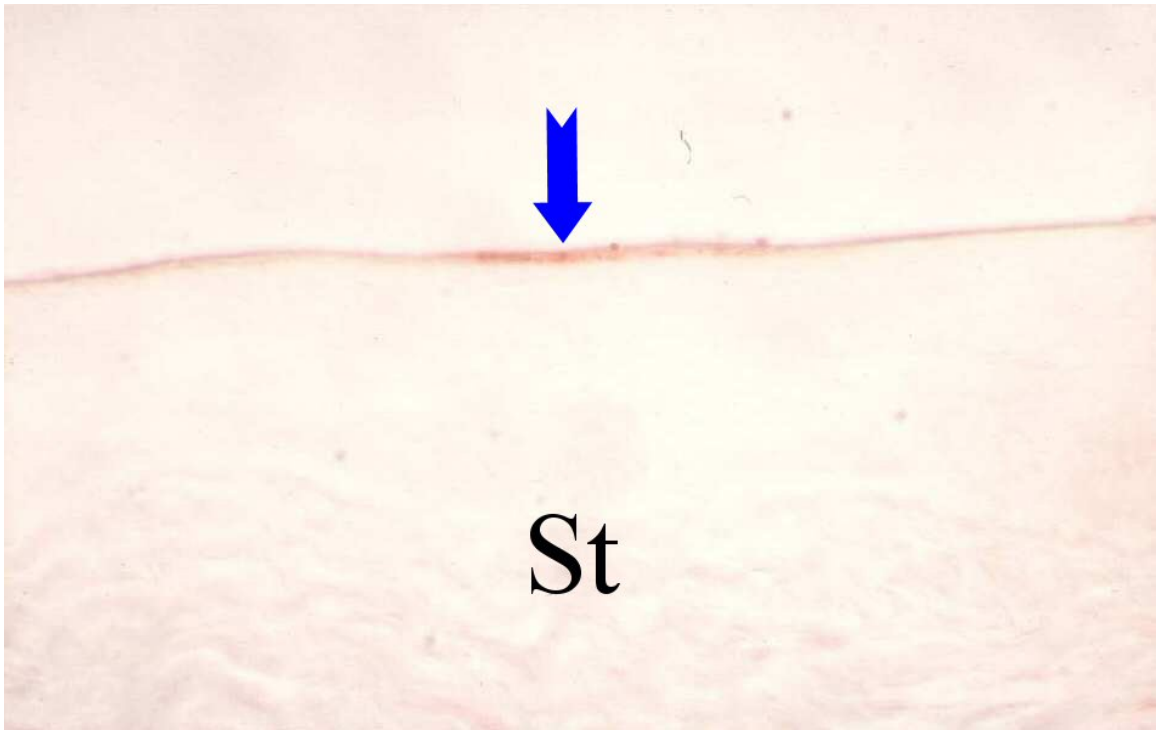

Figure 3-11: Positive staining in the corneal epithelium (solid arrows) and stroma of a normal beagle (X200).

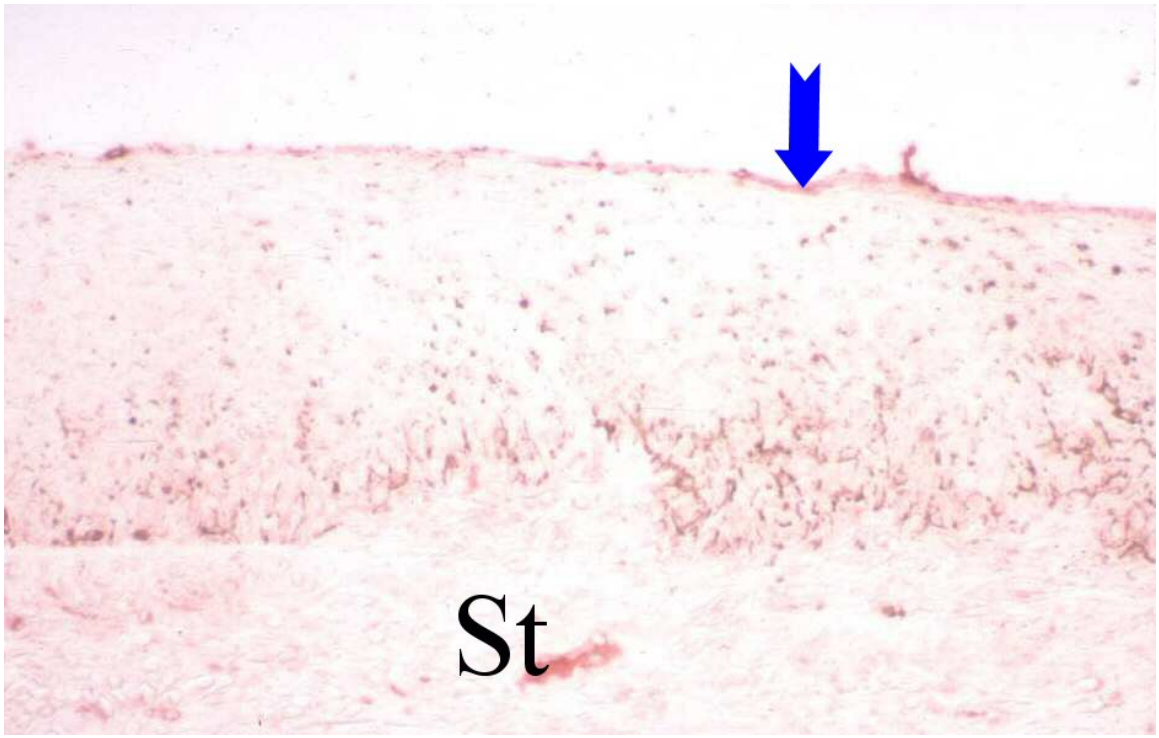

Figure 3-12: Positive staining in the corneal epithelium (arrow) and stroma of a glaucomatous cocker spaniel (X200).

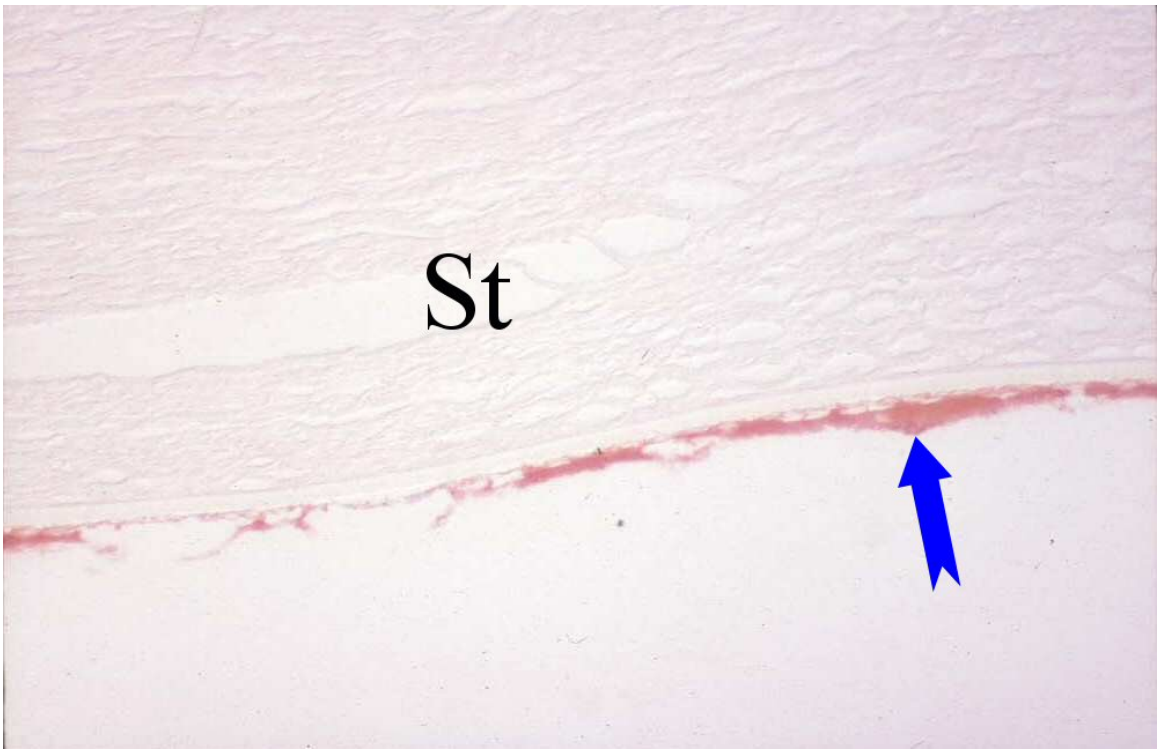

Figure 3-13: Positive myocilin localization in corneal endothelium (arrow) of a preglaucomatous 3 mo. old beagle (X200).

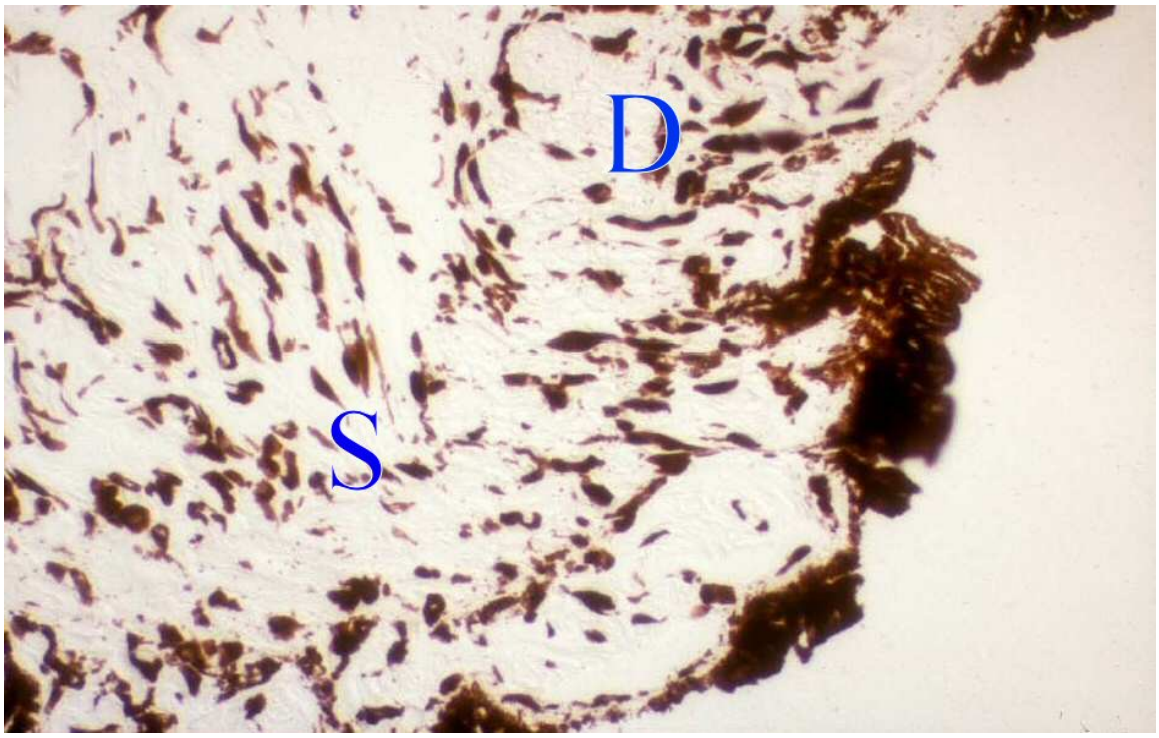

Figure 3-14: Localization in the sphincter (S) and dilator (D) muscles of the iris of a normal 1.5 yr. old beagle (X200).

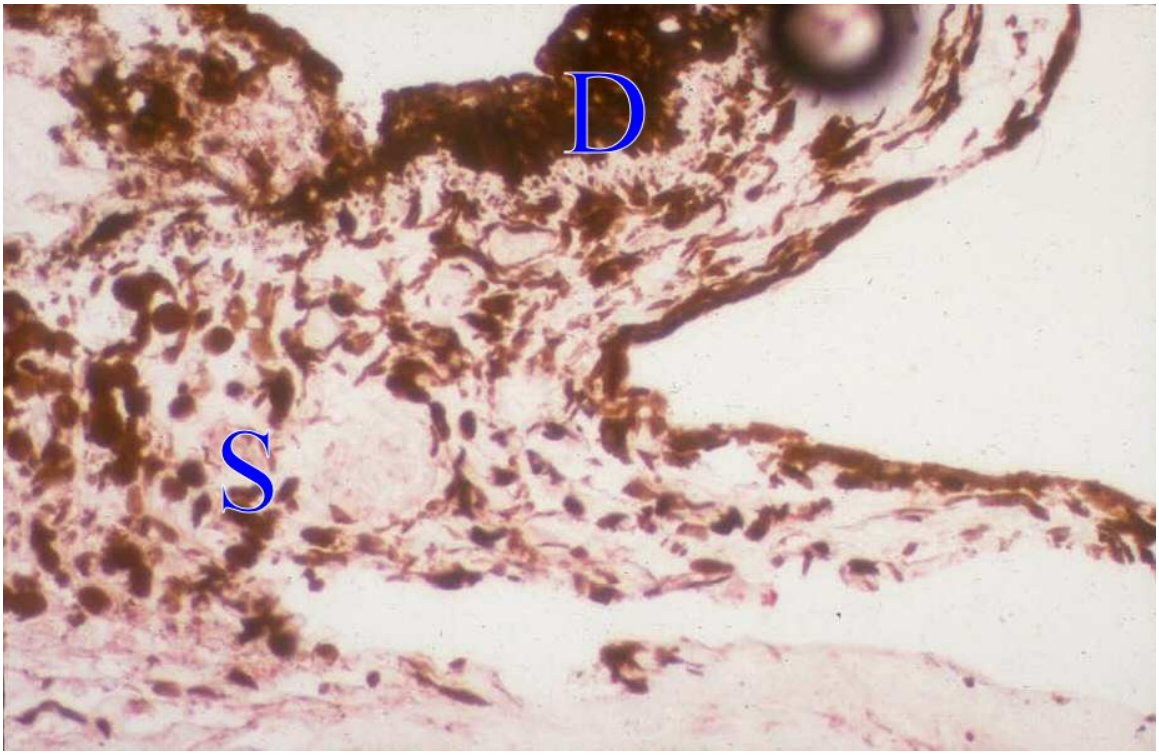

Figure 3-15: Localization in the sphincter (S) and dilator (D) muscles of the iris of a 10 year old glaucomatous beagle (X200).

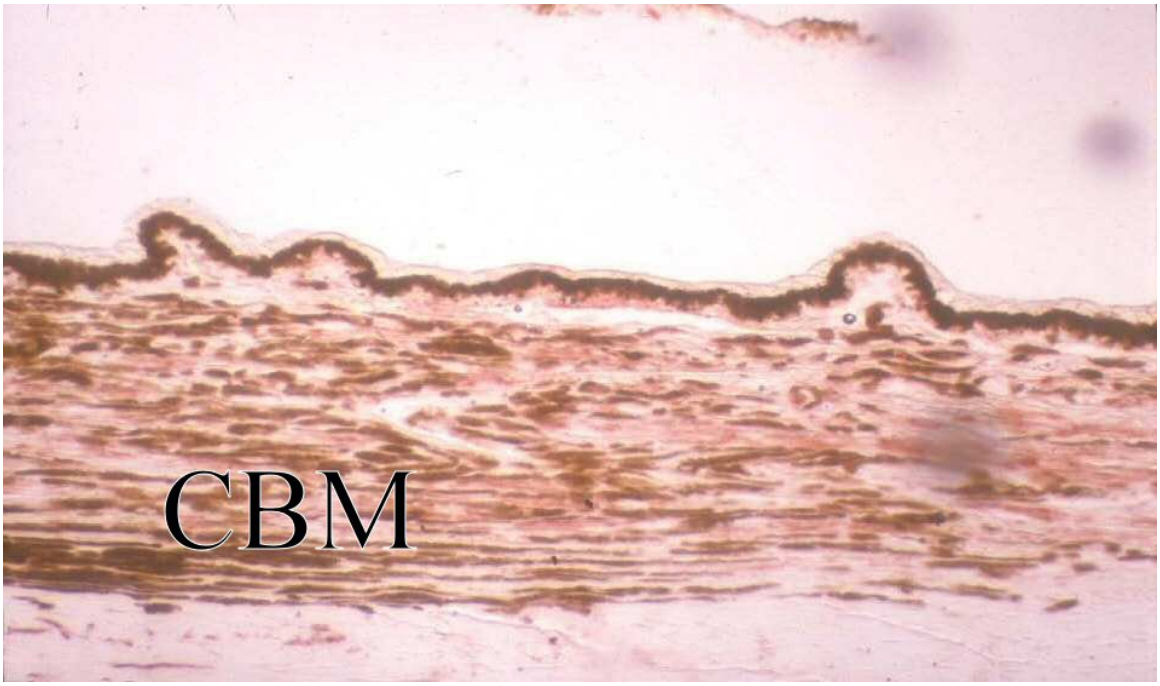

Figure 3-16: Myocilin localization along the cell membranes of the ciliary body musculature of a 3 mo. old normal beagle (X200).

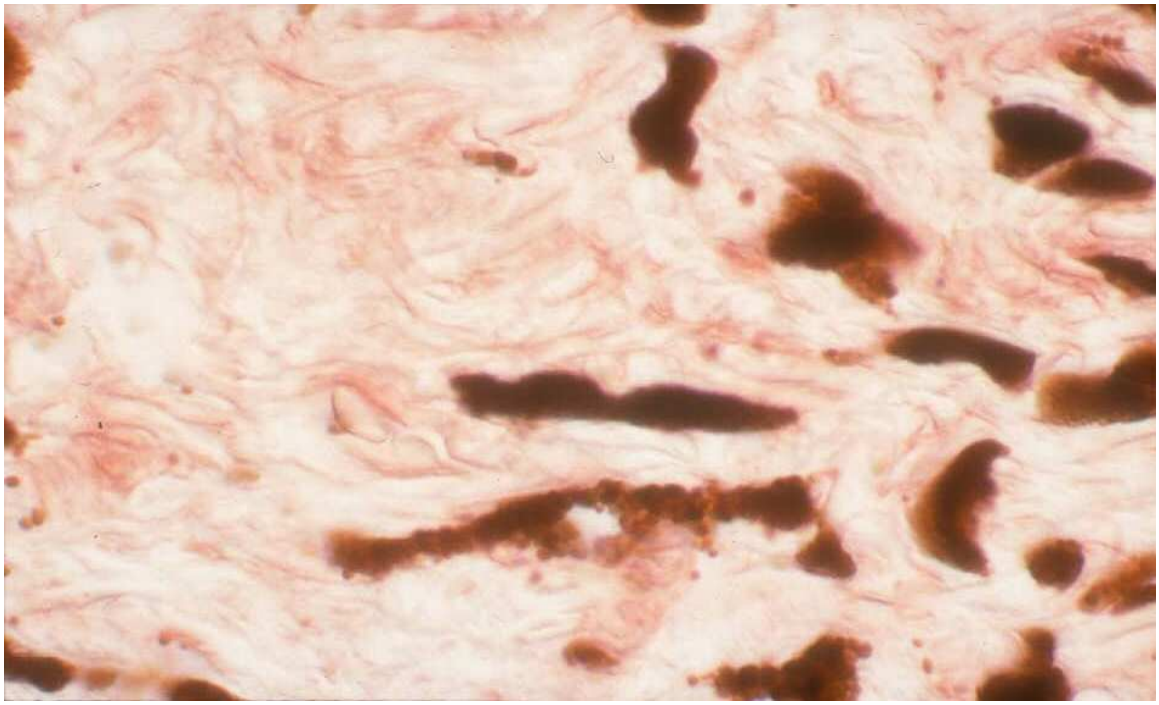

Figure 3-17: Myocilin localization along the cell membranes of the ciliary body musculature of a 1.5 yr. old normal beagle (X1000).

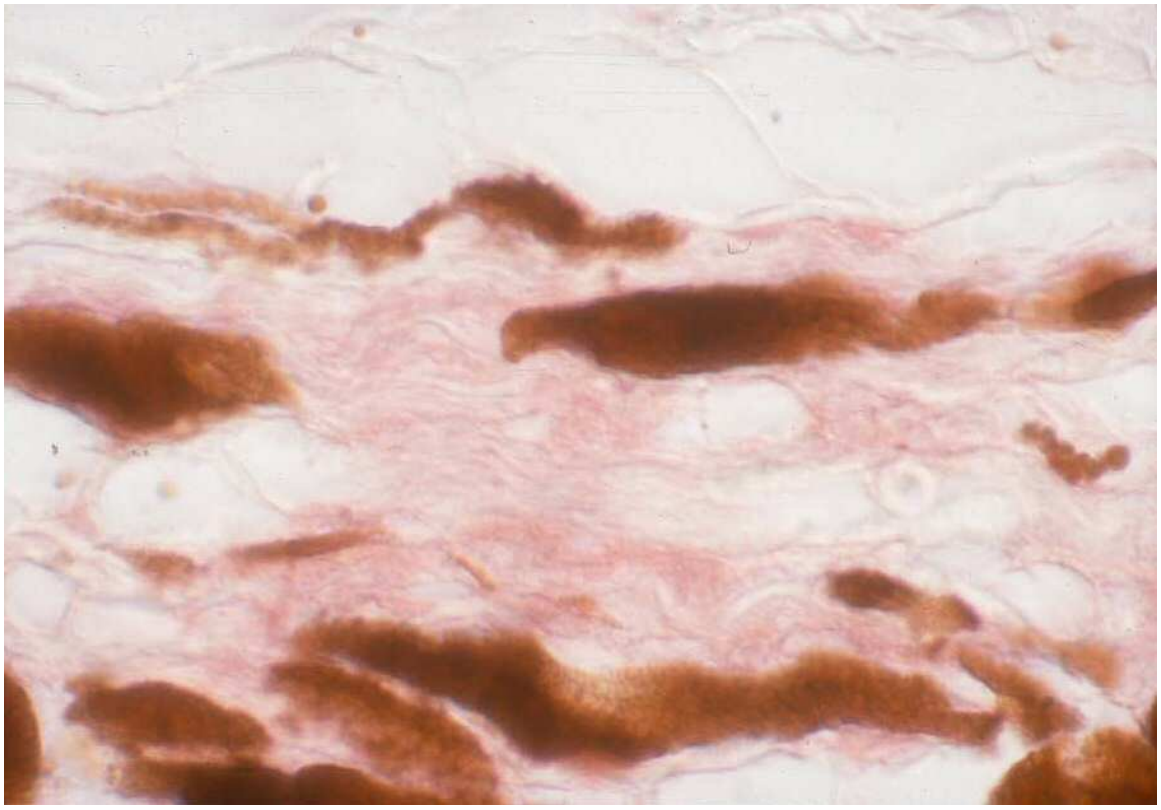

Figure 3-18: Positive myocilin localization in the trabecular meshwork cells of a 7 yr. old glaucomatous beagle (X1000).

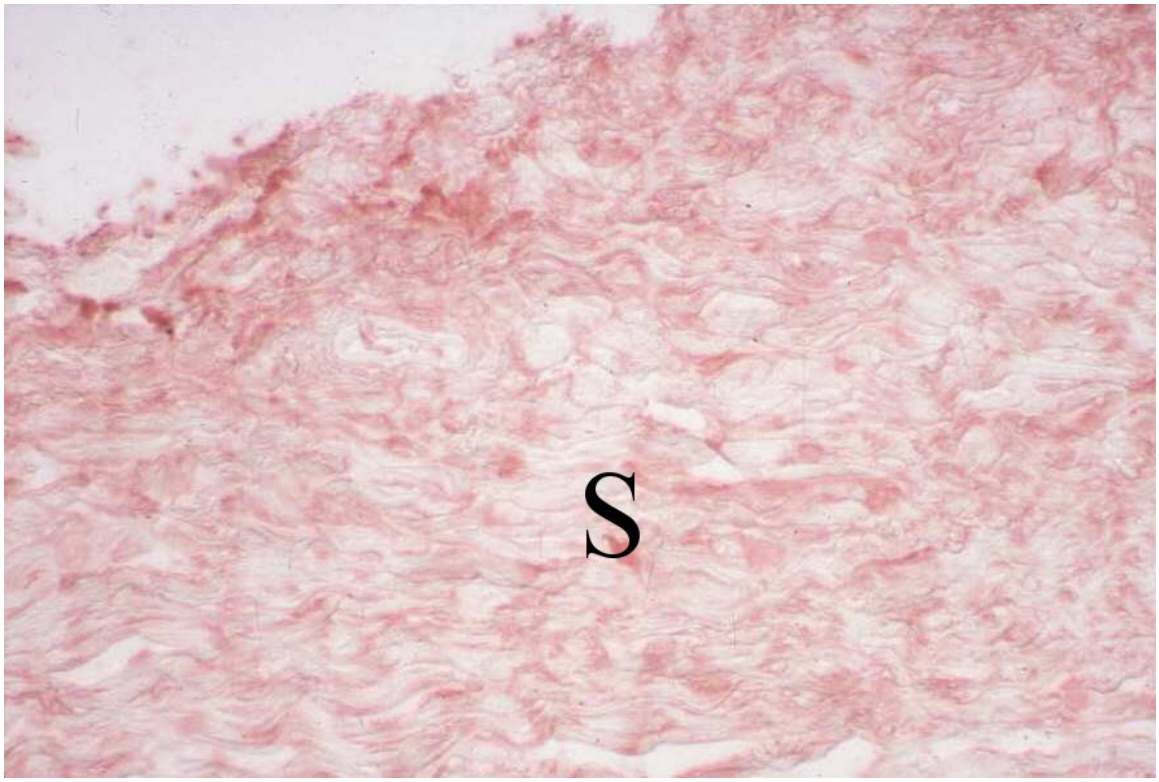

Figure 3-19: Positive myocilin localization in the outer sclera of a 13 yr. old glaucomatous cocker spaniel (X400).

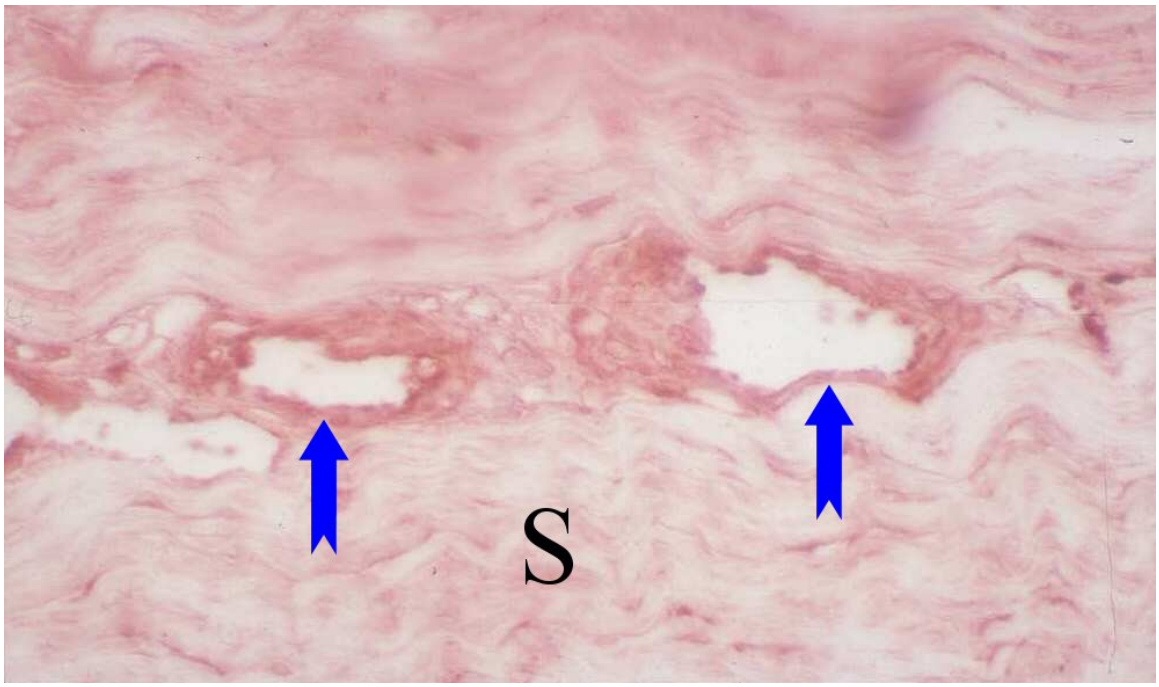

Figure 3-20: Positive myocilin localization in the vascular smooth muscle cells (arrows) within the sclera (S) of an 8 yr. old glaucomatous cocker spaniel (X400).

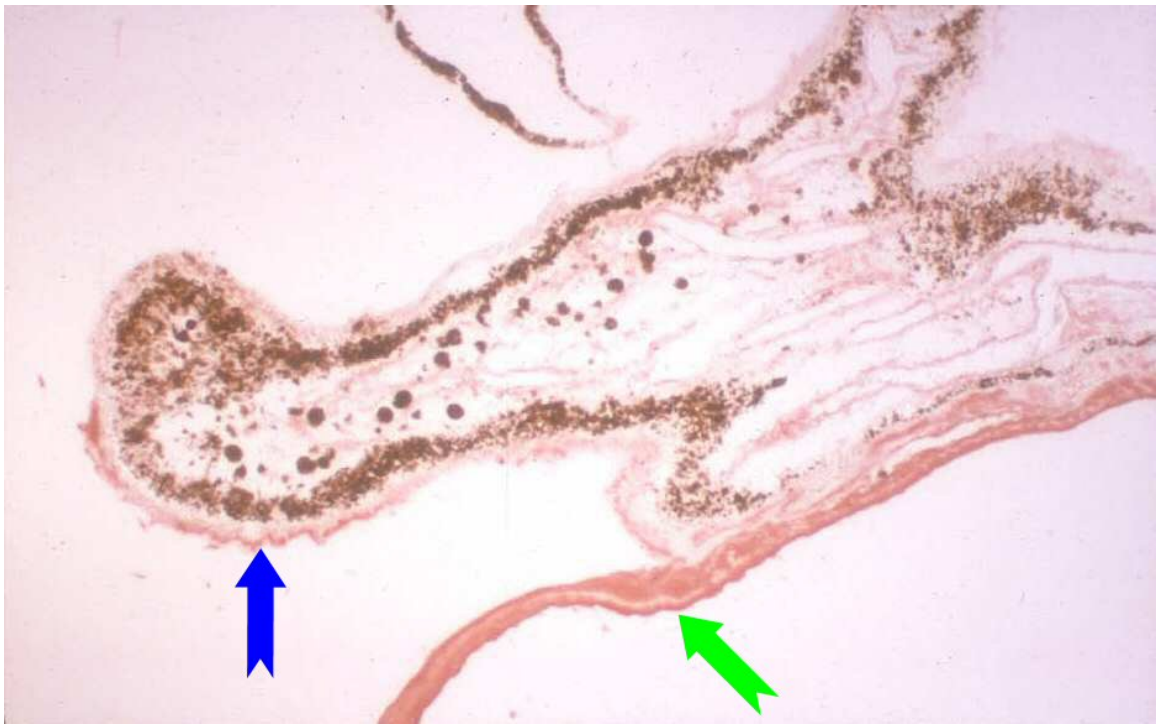

Figure 3-21: Intense myocilin labeling within the nonpigmented epithelium (Blue arrow) of the ciliary processes, and vitreal membrane-like material (Green arrow) of a 6 yr. old glaucomatous beagle (X200).

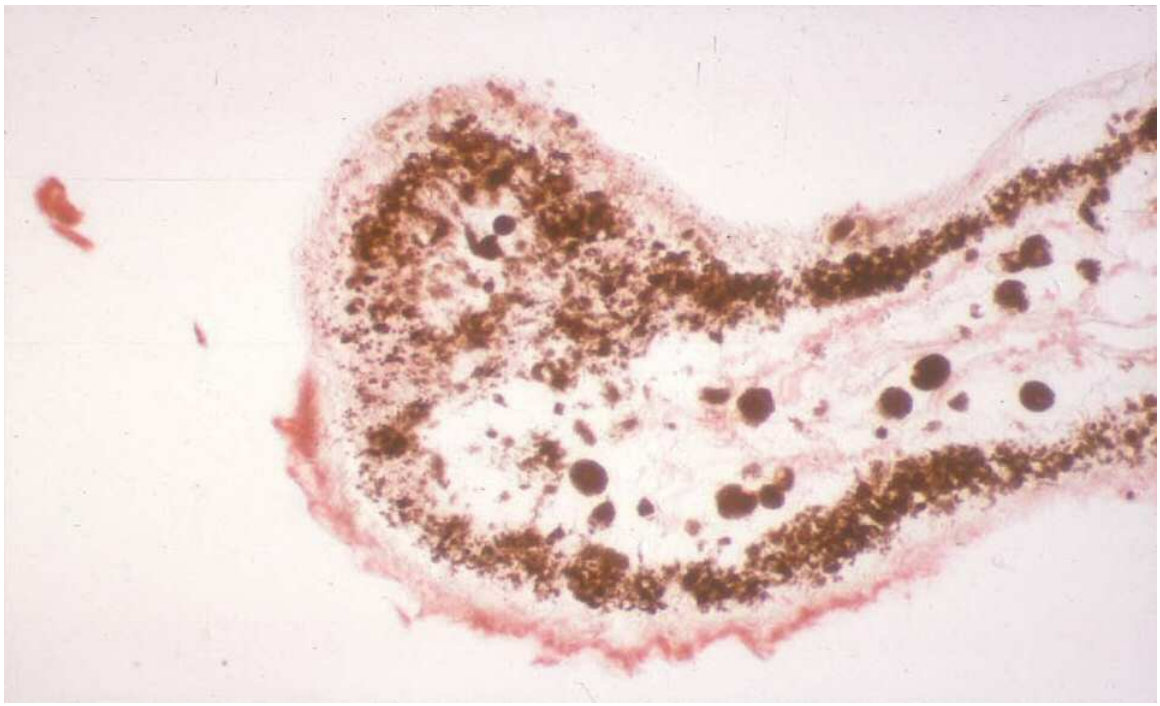

Figure 3-22: Detail of Figure 3-13. Intense myocilin labeling within the nonpigmented epithelium of the ciliary processes of a 6 yr. old glaucomatous beagle (X400).

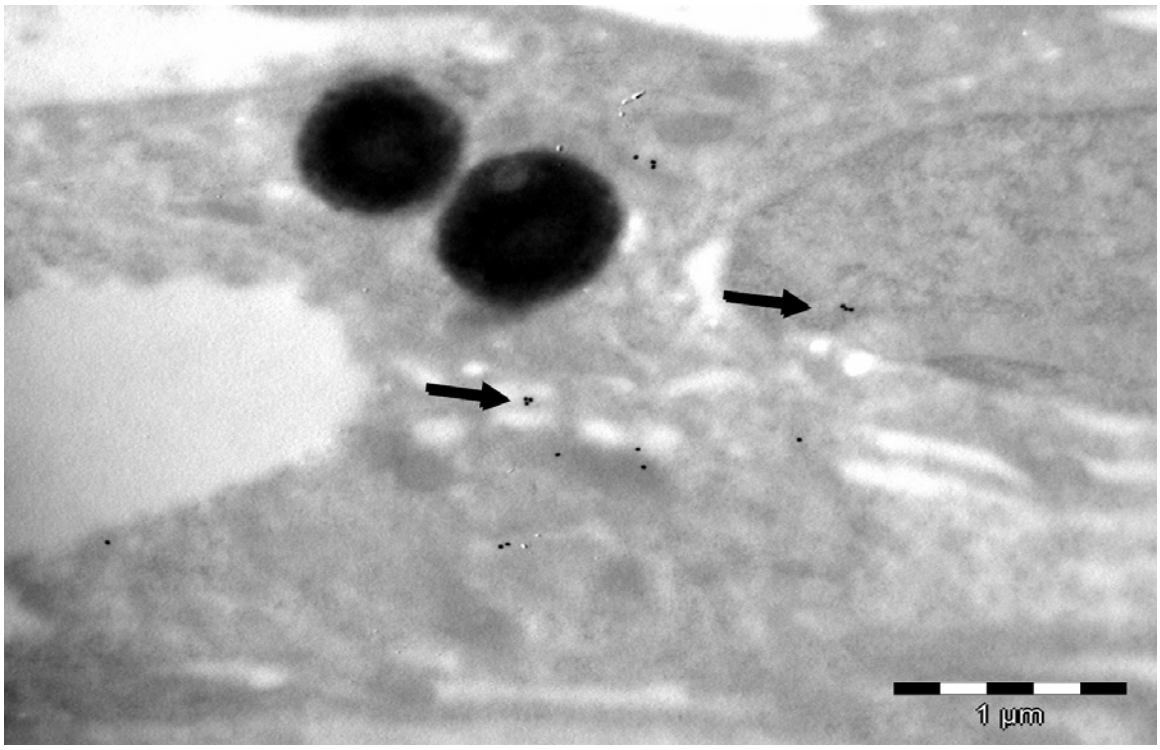

Figure 3-23: Trabecular meshwork cell of a 10 year old beagle with moderate glaucoma. TEM: Localization of myocilin with 18 nm colloidal gold particles (black arrows).

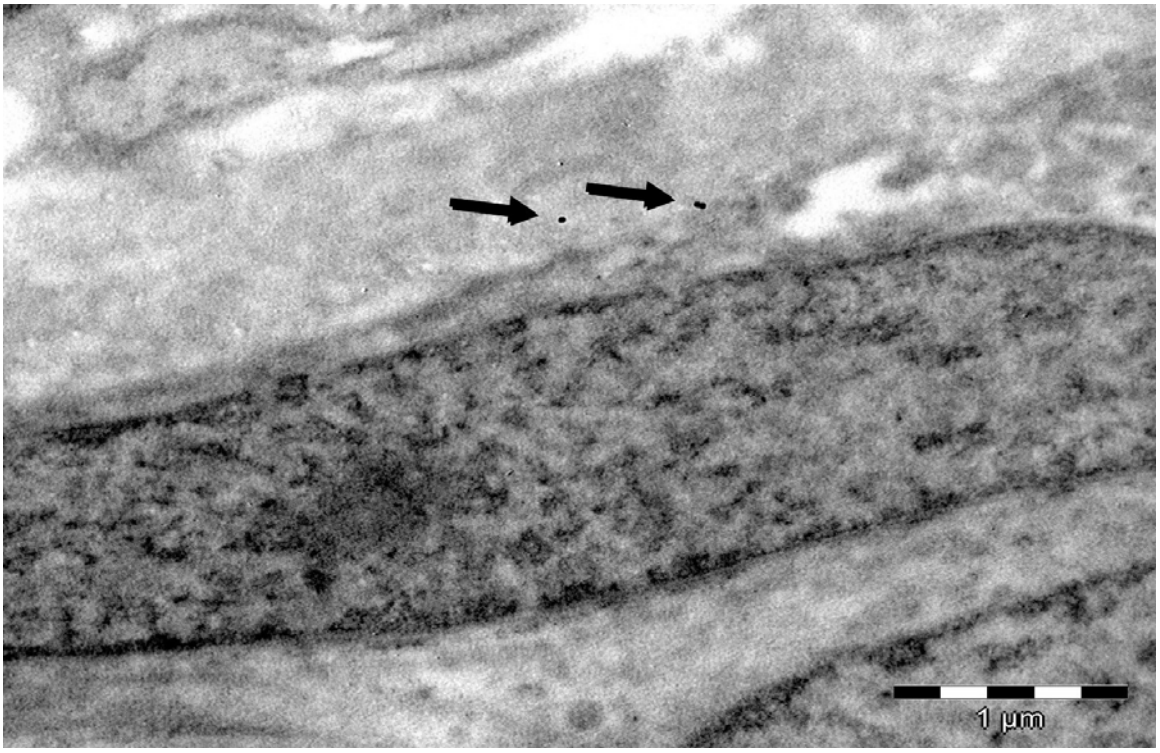

Figure 3-24: Trabecular meshwork cell of a 10 year old beagle with moderate glaucoma. TEM: Localization of myocilin with 18 nm colloidal gold particles (black arrows).

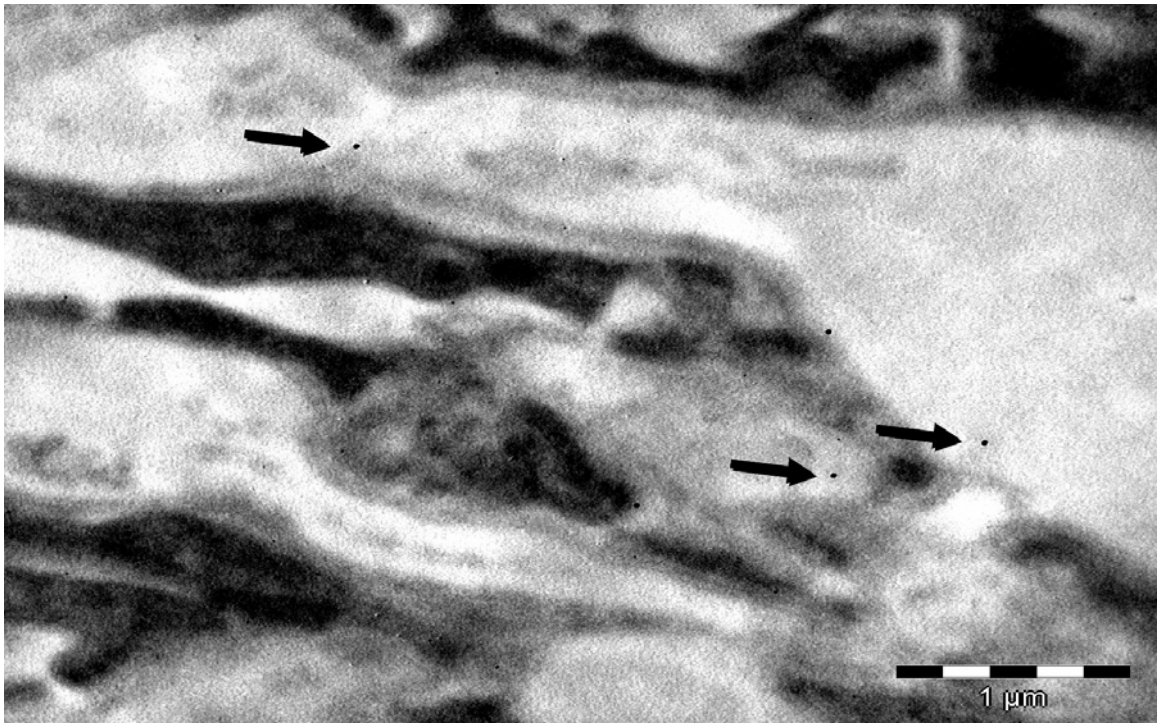

Figure 3-25: Trabecular meshwork cell of a 1.5 year old normal walker hound. TEM: Localization of myocilin with 18 nm colloidal gold particles (black arrows).

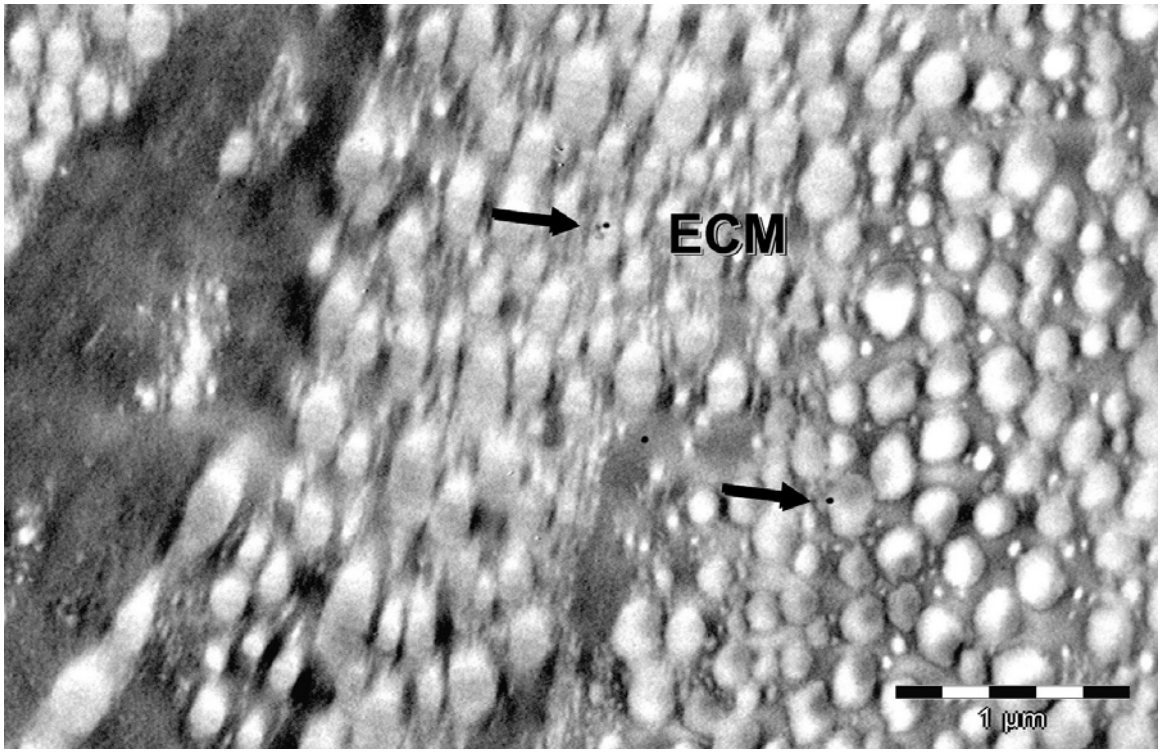

Figure 3-26: Extracellular matrix (ECM) or the trabecular meshwork of a 10 year old beagle with moderate glaucoma. TEM: Localization of myocilin with 18 nm colloidal gold particles (black arrows).

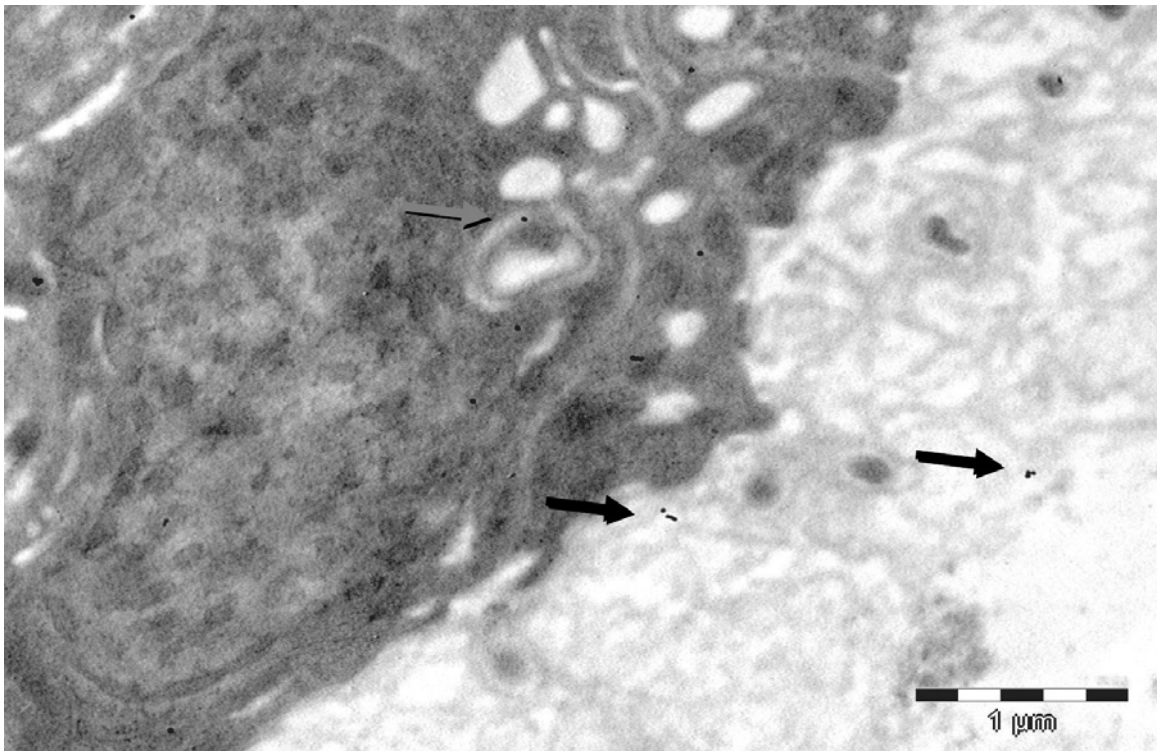

Figure 3-27: Nonpigmented epithelium of the ciliary body of a 10 year old beagle with moderate glaucoma. TEM: Localization of myocilin with 18 nm colloidal gold particles (black arrows).

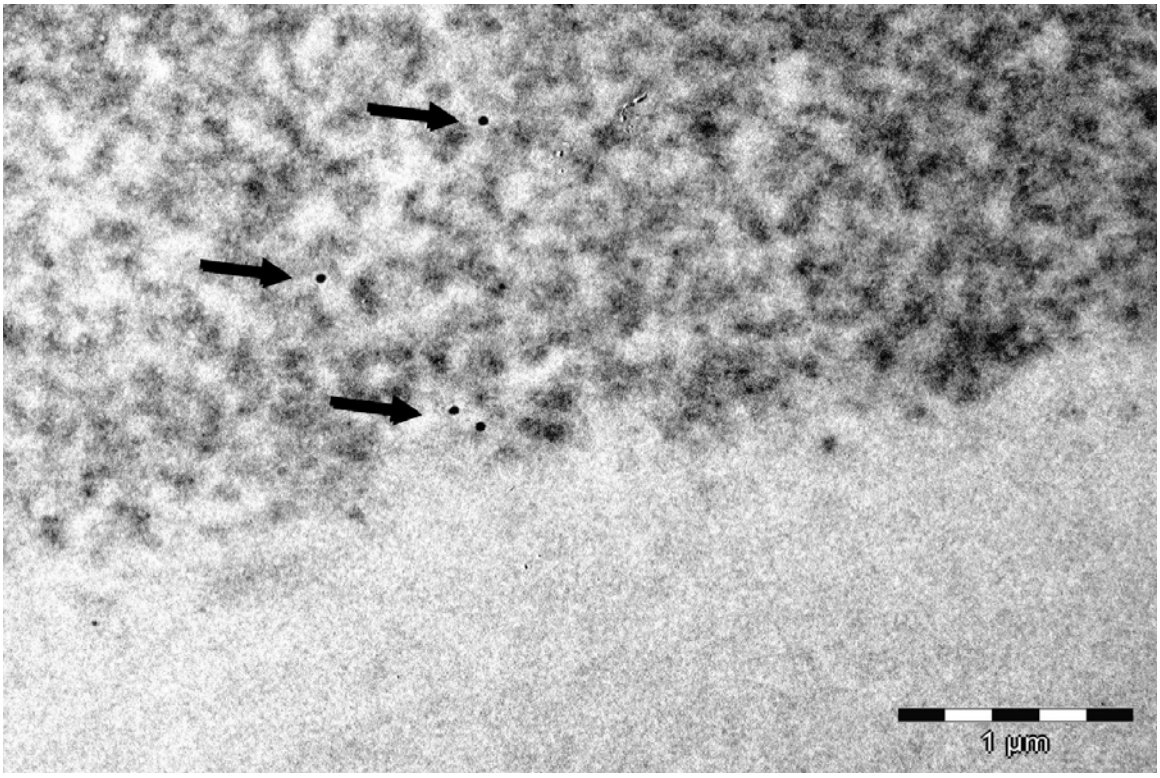

Figure 3-28: Vitreal membrane-like material within the posterior chamber of a 10 year old beagle with moderate glaucoma. TEM: Localization of myocilin with 18 nm colloidal gold particles (black arrows).

CHAPTER 4
DISCUSSION

The inherited glaucomas certainly have many causes. Myocilin is still one of the only gene/protein products clearly linked to glaucoma. The exact role of the myocilin protein is still not well defined and probably involves multiple functions (Gould et al., 2004). The association of the gene and its mutations to several different forms of early and late onset POAG in man continues to be strengthened (Tamm et al., 2001; Clark et al., 2001). New views of the trabecular meshwork encourage us to look increasingly at myocilin for regulatory problems in addition to typical mutations (Alvarado et al., 2005). Like many forms of POAG in man, POAG in the beagle is also demonstrated to have elevated myocilin within the aqueous humor and ocular tissues. A definite correlation can be made between the severity of glaucoma in both the human and canine eye and the amount of myocilin in the aqueous humor, and increase of myocilin found in the iridocorneal angle (Alward et al., 1998; Colomb et al., 2001; Fan et al., 2004). The presence of such a great amount of "free" myocilin in the aqueous humor could be indicative of an increase in the production of myocilin, from a change in mechanical or oxidative stress, or the lack of sufficient outflow of the myocilin (Aroca-Aguilar et al., 2005).

Myocilin exists both intra- and extra-cellularly, and has both glycosylated and nonoglycosylated forms (with molecular weights of approximately 66 kd and 55 kd respectively) (Caballero et al., 2001). In addition to its leucine zipper region, myocilin has multiple sites for glycosylation and phosphorylation, presumed binding sites for hyaluronic acid and heparin (which has been reported in the dog trabecular meshwork), and a signal sequence of 32 amino acids usually found in molecules secreted extracellularly. When extracellular, myocilin may bind

to other extracellular molecules or to the cell membrane of the trabecular cells and influence aqueous outflow resistance. As a sticky molecule, myocilin has binding sites for several components of the basement membrane.

The low amount of visible myocilin in the normal and mildly affected beagles demonstrates that myocilin is a normal aqueous humor protein in the dog. No differences between either age or gender were observed in these normal dogs. Obtaining a baseline, normal level of myocilin may be a step towards a future early test for glaucoma. The advanced glaucoma beagles showed a large increase in the amount of free myocilin. Although no mutation was found in the myocilin gene in the dogs from the colony tested, this higher level still links myocilin to glaucoma. The significant increase in myocilin may be related to errors in the post-transcriptional or post-translational processing of the myocilin in these dogs, or errors in the trabecular meshwork in the removal of aqueous humor and myocilin. These alterations could impair dynamics of the removal of myocilin, possibly having a binding effect to extracellular components such as hyaluronic acid or the basal laminae of trabecular meshwork cells. The inability of the myocilin-bound components to degrade properly would result in its accumulation the eye of individuals with POAG causing a rise in intraocular pressure and further development of the disease.

This study supports the hypothesis that changes in the level or activity of myocilin within the aqueous humor outflow pathway of beagles with POAG are associated with the rise of intraocular pressure and subsequent development of the disease. This is the first study in which the analysis of the myocilin gene, the myocilin protein and the localization of myocilin in the normal and glaucomatous canine eye was successful and observed by multiple techniques.

Myocilin has been localized in human normal and POAG trabecular meshwork (Lutjen-Drecoll et al., 1998; Tawara et al., 2000; Ueda et al., 2003; Clark et al., 2001). Myocilin appeared to be localized to the long-spacing collagens and the surrounding sheath of elastic-like fibers, interacting with microfibril-associated elements and codistributed with fibronectin, fibrillin-1, MAGP-1, decorin, and type VI collagen (Ueda et al., 2003). Myocilin, as a protein, occurs not only in the ocular tissues, but has also been reported in heart, skeletal muscle, bone marrow, lung, stomach, thyroid, prostate, pancreas, kidney, intestine, and lymph node (Tamm et al., 2001; Shepard et al., 2003).

Previous myocilin immunolocalization studies have been reported in humans (Karali et al., 2000, Lutgen-Drescoll et al., 1998; Tavara et al., 2000). In this study, myocilin localization in the normal, moderately glaucomatous, and advanced glaucomatous canine eye, was nearly identical to localization previously reported in the normal human eye. The localization of myocilin in the trabecular meshwork of the iridocorneal angle of the glaucomatous dog was much greater than that of a normal animal. In the nonpigmented epithelium of the ciliary processes localization of myocilin was unevenly distributed and stained with significantly greater intensity than that of normal ocular tissue. There was strong localization in the stroma of the ciliary processes in severe POAG, which is absent in normal and less severe POAG ocular tissue. This leads us to believe the non-pigmented epithelium of the ciliary body may be highly underrated in its importance in function for regulation of IOP. Generalized staining was observed in the stratified corneal epithelium of the normal eye, while in the severe glaucomatous eye, basal corneal cells stained most intensely. It was interesting to note the higher free myocilin levels in the aqueous humor coincided well to the changes noted in the intracellular myocilin in the

immunolocalization study. Further work on the post-transcriptional and post-translational processing of the myocilin may allow more insight into this relationship.

The data gathered from the DNA chip microarrays is potentially exciting. The relatively low amount of myocilin in both normal and advanced glaucomatous dogs (compared to protein increase) reported by the chip analysis make us wonder about the hybridization efficiency of the probes on the chip. Is there just enough evolutionary change between dog and the human and bovine probes on the chip that perhaps they don't hybridize robustly? A small increase in the production of mRNA for the myocilin protein was still observed based on the bovine myocilin probe. A nearly two-fold increase in mRNA would not be expected to cause the large increase in protein. However, there is increased protein stability or decreased protein turnover. The significance of this is unknown, but could support a theory that a promoter mutation could increase *MYOC* RNA and this increase in protein alone overwhelms the protein turnover system, or there are other factors involved. Certainly the protein data are more convincing than the RNA data, which could be wrong given lack of a canine probe. More important to future research is the large number of other genes and markers that were significantly different between the normal and glaucomatous beagles at least in the advanced stages of the disease (Table 3-3). These could be validated by real-time PCR, and legitimate results followed with protein studies or analysis of gene sequences.

When looking at differences in clinical samples and multiple breeds of dogs, myocilin again seems to be significant. Of the 353 dogs analyzed there was no significant difference between ages, genders or breeds. We chose to utilize cataractous dogs as our non-glaucomatous controls for multiple reasons: ease of collection during scheduled cataract surgeries, the large number of available samples, and the avoidance of procedures on otherwise healthy dogs. Later,

we decided to separate diabetic cataract cases from primary cataract cases in case of other diabetes-related changes. In advanced cases of primary (spontaneous) and secondary canine glaucomas, some dramatic differences were observed. Of greatest significance was a substantial increase in levels of secreted myocilin in the aqueous humor. Up to a ten-fold increase in myocilin is of clinical and research interest. In this study 118 primary glaucoma and 64 secondary glaucoma dogs were analyzed. Most of these dogs had higher than normal levels of free myocilin, similar to the POAG beagles. This may indicate aqueous humor myocilin levels are elevated in response rather than causing the initial ocular hypertension. Later analysis of other proteins in these dogs indicated that there was also an increased amount of CD-44 protein in these aqueous samples (Kallberg et al., 2006). The alignment of Western blot bands of myocilin and CD-44 indicated there may be a strong correlation for a complex between the two proteins. This could be tested using immunoprecipitation approaches. Levels in cataractous dogs were much lower for the myocilin and CD-44 (Kallberg et al., 2006).

This study also supports the original hypothesis that there is an increase of myocilin in both accumulation and localization in glaucomatous canine eyes. With further study, improved therapeutic strategies may be developed to treat glaucoma by altering the trabecular meshwork cells and decreasing aqueous outflow resistance (Borras et al., 2002; Borras 2003). The presentation of advanced POAG in clinical patients is very similar to advanced primary angle closure glaucoma (PACG) (Aung et al., 2005). This may prove to have some effect on changes in the myocilin levels between some of the primary glaucoma groups.

With the knowledge collected here, screening and genetic testing may soon be a reality for both dogs and man (Harasymowycz et al., 2005; Mackey et al., 2003). A simple way to check for the onset of glaucoma, before any damage or loss of vision takes place would be desirable. A

genetic pre-disposition would be interesting to test for with a blood sample or buccal swab. Or perhaps a slightly more invasive test to check for free myocilin in the aqueous humor or a fast test of corneal anterior epithelium may be of help some day.

Dexamethasone treatment, oxidative stress, stretching, and treatment with a transforming growth factor in culture human anterior segments and monolayer culture trabecular cells increase myocilin levels. Dexamethasone treatment on beagles can increase IOP in under a week (Gelatt et al., 1998). Perfusion studies have been performed in man recently, but further study into these areas with animal models and POAG beagles are needed (Goldwich et al., 2003; Fautsch et al., 2006; Sakai et al., 2006).

We reported myocilin in the aqueous humor of normal dogs and elevations of aqueous myocilin in the beagles with POAG in 2004 (MacKay et al., 2004). In that same report normal and glaucoma eye tissues were immunolabeled with the same rabbit anti-myocilin polyclonal antibody and the myocilin tissue levels paralleled the aqueous humor levels with the glaucomatous trabecular meshwork and ciliary body nonpigmented epithelium demonstrating the highest levels in the glaucoma eyes. Subsequent light, confocal and electron microscopy studies in 2004-05 of both normal and glaucomatous beagle eyes at different stages of the disease have confirmed these initial observations (Samuelson et al., 2005; Kallberg et al., 2006; MacKay et al., 2004). In the normal, mild, and moderate glaucomatous beagle eye, immunolabeling of myocilin occurred and the protein was homogeneously distributed within the ocular tissues. Immunolabelling of advanced glaucoma eyes revealed increased aggregation and staining in the iridocorneal angle, nonpigmented epithelium of the ciliary body processes, and anterior cornea. The confocal and electron microscopy results demonstrated the myocilin was in the apically

positioned vesicles in the nonpigmented epithelium, in melanocytes and adhering to the surface of individual melanosomes.

In summary, aqueous myocilin levels in POAG beagles were significantly increased in moderate and severe glaucomatous eyes with three discreet bands often seen. Mild and normal beagles demonstrated myocilin present at much lower levels. Myocilin was found intracellularly in greater amounts and in more tissues in glaucomatous beagles versus normal beagles, including the non-pigmented epithelium of the ciliary body. In other breeds, myocilin levels were shown to be higher in primary and secondary glaucomatous dogs versus non-glaucomatous (cataractous and diabetic cataractous) dogs. No significant differences were seen between genders or ages. The only breed differences seen were related to the number of samples received for each breed, which may be related to incidence in the population.

My hypothesis for the presence of a mutation in the myocilin region of the inherited glaucomatous beagle DNA has been proven false thus far. Typical mutations were ruled out. The idea that myocilin protein would be found in greater quantities in glaucomatous dogs has been proven true. Both the advanced glaucomatous beagles and the glaucomatous clinical aqueous humor samples were shown to have greater concentrations of myocilin than mild glaucomatous and normal animal samples. The hypothesis of a greater presence of myocilin intracellularly was also proven true. Additionally, other cell groups were shown to exhibit the presence of myocilin as well.

These studies suggest similar pathways may be involved in the canine primary or breed-related glaucomas, and additional studies need to focus on the promoter regions of the myocilin gene that may be 'upstream' from the protein encoding DNA sequence, perfusion studies to link cause and effect issues, and other proteins and their interactions in the aqueous humor.

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BIOGRAPHICAL SKETCH

Ed MacKay was born in 1972 in North Carolina to an Air Force family. He traveled extensively living in Okinawa, Japan; Las Vegas, Nevada; and Fort Walton Beach, Florida; before settling down in Gainesville to go to school.

Ed attended the University of Florida from 1989-1993, graduating with a Bachelor of Science from the College of Animal Sciences. He was soon after hired to help Dr. Kirk Gelatt as both a statistical analyst and research technician. Upon discovering his love of the field, Ed decided to pursue a PhD in the field of Veterinary Ophthalmology, and began slowly in 1997. He's now married and has one son, with a second baby on the way.

All else is prone to change without notice.