IDENTIFICATION AND LOCALIZATION OF MATRIX METALLOPROTEINASES (MMPs) PRESENT WITHIN THE AQUEOUS HUMOR AND IRIDOCORNEAL DRAINGE ANGLE TISSUE OF NORMAL AND GLAUCOMATOUS CANINE EYES

By

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ABSTRACT

Matrix metalloproteinases (MMPs) have been found to have a diverse role in the development, normal physiology and pathology within numerous ocular tissues. To the author's knowledge, this is the first study to quantify intraocular MMP activity levels related to the aqueous humor outflow pathway within normal verses glaucomatous canine eyes. Using zymography, we identified the dominant MMP activity within the aqueous humor and iridocorneal drainage angle tissue for both normal and glaucomatous dogs. Immunohistochemical localization of MMPs within the entire globe was also performed. MMP-2 and MMP-9 were found to be the most active MMP involved with the aqueous humor outflow pathway in dogs. A significant correlation between increasing MMP-2 and MMP-9 activity levels and the presence of glaucoma was found. Further, localization of MMP-2 and MMP-9 within the iridocorneal angle tissue, iris and cornea were found to be altered in normal versus glaucomatous canine eyes.

INDEX WORDS: Dog, Glaucoma, Extracellular Matrix, Iridocorneal angle, Matrix Metalloproteinases, MMPs, Trabecular Meshwork, Zymography, Immunohistochemistry

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DEDICATION

This work is dedicated to my beautiful wife, Melissa, whom I owe greatly for her support throughout the long hours spent in the laboratory and during the writing of this project. And to my parents, Michael and Mimi Weinstein, for their continuous support as I worked towards this achievement.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Background

Matrix metalloproteinases (MMPs) have been found to have a diverse role in ocular development, normal physiology and pathology.[6,21,22] These zinc-dependent proteolytic enzymes have been shown to be active within the lens, retina, cornea, sclera, and trabecular meshwork. [6, 21, 22] These proteases contribute to the delicate balance of tissue synthesis and degradation as well as regulate wound healing.[6, 21] Recent human research has shown that MMPs become altered in ocular diseased states such as cataracts, diabetic retinopathy, corneal ulceration, and glaucoma.[21,22]

In veterinary medicine, the role of MMPs has been investigated in cardiac disease, septic arthritis, and laminitis.[27, 36, 38] In the eye, the role of MMPs within the cornea and pre-corneal tear film in dogs has been investigated.[24, 25] MMP-2 and MMP-9 have been found to be important in terms of remodeling of the cornea stromal collagen. [24, 25 , 29] MMP-2 is synthesized by the corneal stromal keratocytes and performs a surveillance function in normal cornea, becoming active to degrade collagen molecules when the cornea is damaged. [24,25] MMP-9 is produced by corneal epithelial cells, stromal fibroblasts and neutrophils following corneal wounding. [24,25] MMP-9 functions to further degrade wounded collagen and elastic fibers. [24,25] This parallels human medicine findings where the total MMP-2 and MMP-9 activity was increased within the cornea and pre-corneal tear films in patients with recurrent ulcers.[44] MMP-9 has been shown to be elevated in the tears of dogs with keratoconjunctivitis when compared to normal dogs .[40] In

horses, higher amounts of MMP-2, MMP-9 and the serine protease, neutrophil elastase (NE) were found in the tear film of ulcerated eyes. [40] In another study, altered expression of MMP-2 and MMP-9 was found in acute and chronic experimentally wounded canine corneas and keratectomy samples from canine patients with spontaneous chronic corneal epithelial defects (SCCEDs) .[29] As a corneal ulcer heals, the levels of these proteases diminish within the cornea and tears. [40] However, in "melting" ulcers these proteinases remain elevated. [40] Excess MMP-2 and MMP-9 can cause a cornea to soften as they degrade the collagen and elastic fibers of the stroma. [40] Successful treatment of melting corneal ulcers needs to lead to a rapid reduction in the protease activity within the cornea and tears.

Studies in human medicine have determined that matrix metalloproteinases (MMPs) play an important role in reducing resistance to aqueous humor outflow within the eye[1,3,4,6,8,14,16] and become altered with the disease state of glaucoma.[1,3,4,7,13,14,16,17,19,22] Recent studies investigating intraocular MMPs in humans, bovine and other laboratory animals have confirmed the presence of intraocular MMPs-1,2,3 and 9 along the aqueous humor outflow pathway (aqueous humor and iridocorneal angle tissue).[1,8,13,16] These enzymes play a role in the remodeling the collagen and elastic connective tissues which form the support of the meshwork. It has been suggested that MMPs may be involved in development and migration of the cranial neural crest cells that contribute to the formation of the trabecular meshwork tissue, although this has not been proven yet. [22]

These relevant MMPs have been found to play a role in the maintenance of the ECM (extracellular matrix) and trabecular outflow within the human eye.[1,3,16] The idea of a regulatory feedback loop which restores normal intraocular pressure (IOP) in response to increases in IOP has been investigated.[3, 16] In particular, the trabecular meshwork cells that line the corneoscleral

and inner uveal meshwork tissue can detect changes intraocular pressure and respond by increasing levels of MMPs.[1,3,4,6,14,22 ,23] Intraocular MMPs increase the extracellular matrix (ECM) turnover rate, reduce the resistance to aqueous outflow through the meshwork and restore normal IOP levels.[1,3,4,8,13,14,16,21,23]

In normal eyes, there appears to be a balance between MMPs and their inhibitors called TIMPs (tissue inhibitors of metalloproteinases).[15,19] This MMP/ TIMP balance becomes disrupted in glaucomatous eyes.[15, 19, 22, 31, 37, 49] In humans, changes in MMPs-2 , MMP- 9 and MMP-3 have been implicated as being involved with the disease process of glaucoma.[15,19,31,41] These studies have found that an increase in MMP-2, MMP-9 and MMP-3 activity in glaucomatous eyes when compared to normal eyes. [31,41] This change in MMP activity has been found within the aqueous humor, iridocorneal angle tissue and Tenon's capsule in patients with primary open angle glaucoma (POAG), primary angle closure glaucoma (PACG) and exfoliation glaucoma (ExG).[15, 19, 34, 39, 42] Alterations in the gene expressions of MMPs have been also found within glaucomatous human eyes and eyes with increased intraocular pressure.[37, 48] Transcripts of MMP-2 were found to become upregulated in human trabecular meshwork during pressure-induced homeostatic responses.[47] Patients with exfoliation glaucoma (ExG) had TIMP-2 up-regulated over MMPs, confirming an imbalance between MMPs and their inhibitors.[49] Genotyping of MMP-9 revealed an altered gene in humans with PACG, supporting that an abnormal MMP-9 gene may be down-regulating MMP-9 activity in human acute angle closure glaucoma.[48] Despite all of this recent human research, the endogenous MMP activity in normal and glaucomatous canine eyes is currently unknown.

Canine glaucoma, especially breed related primary canine glaucomas have increased in frequency over the past decade within the United States. [11] Currently, the treatment options

include medical management involving drugs which increase outflow through uveoscleral pathway and decrease production of aqueous humor. Surgery involves cyclodestructive procedures and placement of shunts which bypass the normal aqueous outflow. Currently, there are no therapies which target increasing the outflow by changing the resistance through the trabecular meshwork. In humans, novel research focuses on MMPs and altering their activity and control over conventional trabecular outflow resistance.[5,7,9,12,20] The relationship between MMPs within the aqueous outflow pathway and its relationship with glaucoma is currently unknown in dogs. This seems to be an important but overlooked topic, since the trabecular outflow or the conventional outflow pathway is the predominant pathway in the dog.[10]

It is known that certain breeds of dogs are particularly susceptible to primary glaucoma.[11] Among these are the Beagle, Basset Hound, Cocker Spaniel, Chow Chow, Shar-Pei and the Norwegian Elkhound.[11] Primary glaucoma is likely initiated by goniodysgenesis (congenital abnormalities in the pectinate ligaments and or ciliary cleft tissue). In breeds with primary glaucoma, collapse of the ciliary cleft and trabecular meshwork (TM) contributes to the pathology of this disease. In open angle glaucoma there is an accumulation extracellular matrix tissue that may be associated with obstruction of aqueous outflow .[11] We speculate that altered MMP activity may play a role in both these types of canine primary glaucoma. The role of MMPs, particularly in development of goniodysgenesis needs to be investigated. For example, the presence of a mesodermal band across the iridocorneal (ICA) angle causing pectinate ligament dyplasia may involve abnormal MMP regulation and activity. Failure of rarefaction or normal turnover of this mesodermal band may involve lower than normal MMP activity or an overexpression of TIMPs over MMPs during development of the iridocorneal angle. The accumulation of ECM within the ciliary cleft in primary open angle glaucoma (POAG) may also

be related to inadequate MMP activity. In humans they have found that TIMPs become overexpressed over MMPs in POAG favoring ECM accumulation.[15, 19, 22, 31, 37, 39, 49] It is speculated that this same de-regulation of MMP activity may contribute to the disease process of glaucoma in the dog. In addition to directly contributing to the pathologic changes early in disease, changes in MMPs activity may be occurring in dogs with glaucoma as an adaptive response to pressure changes that occur later in the disease process. For example, MMP-2 has been found to become elevated later in disease in humans with POAG. [19] Increased MMP-2 expression may go on to induce activation of MMP-9. [48] Increased MMP expression can cause a cascade of activation of other proteases, increased degradation of the cell adhesion molecules, turn on other cell signaling molecules and cytokines and cause release of extracellular matrix (ECM) bound factors, all having effects on neighboring trabecular cell functioning. [21] Manipulations of MMPs in the dog as potential therapy or prevention of glaucoma may be possible in the future. New therapies will target MMP regulation by making use of exogenous /endogenous substances, alteration of MMP receptors and gene therapies that alter the activity of MMPs and enhance aqueous humor outflow facility.[4,5,6,7,12,20] Prior to this research there was no current knowledge about MMP activity in normal dogs, let alone for dogs with glaucoma.

In our study of canine eyes we examined MMPs within the aqueous humor and aqueous outflow pathways (iridocorneal angle tissue) in normal and glaucomatous canine eye. Particularly, we were interested in investigating MMP activity within the trabecular meshwork tissue. In the first part of this study we determined which MMPs were most active. The preferred method utilized to detect these MMPs and their activity is through a technique called substrate gelatin zymography.[17] This technique allows for the identification and quantification of activity of the MMP by enzymatic degradation of a specific matrix.[17] All of the previously determined

relevant ocular human MMPs-1, 2, 3 and 9 will degrade a gelatin matrix and therefore can be identified using substrate gelatin zymography.[2,8,12-14,16,17] Zymography was used as a screening profile to identify the most relevant MMPs along the aqueous outflow pathway within normal dogs and dogs with glaucoma. Zymography utilizes MMP degradation of a preferential gelatin substrate and sorts the MMP proteins by their molecular weight.[17] Zymography is a two step process involving protein separation by SDS-PAGE followed by detection of proteolytic activity.[17] In the first step, SDS electrophoresis reversibly inhibits MMP activity and separates the MMP-TIMP complexes.[1,3,7,8,13,14] Then the MMPs are activated again by a buffer and observed degrading their respective substrate. [1,3,7,8,13,14] Zymography identifies MMPs based on activity and can differentiate latent from active forms.

Once the most active MMPs were identified by zymography, the second part of this study utilized immunohistochemistry to localize this MMP activity to a cellular level with the iridocorneal angle tissue. We also compared localization of MMP activity within the iridocorneal angle tissue to neighboring tissues within the eye. Immunohistochemistry involves the use of antibodies produced against MMPs coupled indirectly to an enzymatic detection system that effectively tags the MMPs within the tissues. Light microscopy was used to directly visualize this MMP staining. Western blots were performed to confirm specificity of our anti-human MMP antibodies towards canine MMPs.

Purpose of the study

This project will be the first step in determining the spectrum of MMP activity in normal dogs versus dogs with glaucoma. We hope to gain more knowledge about the pathologic process of glaucoma to further advance treatment for glaucoma in dogs. We hypothesize that MMP meshwork remodeling and control over aqueous outflow resistance will serve as an important

target for novel glaucoma therapies. To our knowledge this is the first study that will elicit the MMP activity within the aqueous humor outflow pathway within dogs. Human research has shed new light on the role of MMPs in regulation of the trabecular meshwork outflow. We intend to extrapolate these research applications for the dog. The specific aim of this study is to quantify the normal spectrum of MMP activity within the aqueous outflow pathway (aqueous humor and iridocorneal angle tissue) and to determine whether MMP activity and tissue localization becomes altered with the disease state of glaucoma. The goal of this study is to quantitatively determine if there is a statistically significant relationship between matrix metalloproteinase activity levels and the presence of glaucoma. We plan to 1) determine MMP activity within the aqueous humor and 2) determine MMP activity within the iridocorneal angle drainage tissue. We also plan to determine if there is a relationship between the cellular localization of MMPs (within iridocorneal angle tissue) and the presence of glaucoma. The knowledge obtained from these experiments may allow for the development of potential new therapies for glaucoma.

Expected Results

We will utilize the scientific techniques that are available to identify and quantify the normal level of activity of matrix metalloproteinases (MMPs) involved in the aqueous outflow pathway within normal canine eyes. Alterations in MMP activity effects the remodeling of the surrounding tissues, and potentially contributes to the diseased stated of glaucoma. Human research has shown that MMPs are involved with controlling the resistance to aqueous humor outflow through the trabecular meshwork and that experimental manipulation of MMPs influence outflow and intraocular pressure.[3,4,9-11] Recent studies have implicated several MMPs to play a major role in this process in both bovine and human eyes.[1,3-8,11] Similarly, we expect that in the canine eye, MMPs present within the aqueous humor and iridocorneal angles tissue

play a role in regulation of the aqueous humor outflow and become altered with the disease state of glaucoma. We expect to find a basal endogenous activity of MMPs present within normal aqueous humor outflow tissue. Zymography was used to differentiate MMPs present in an inactive or active form. In our diseased eyes we expect to find an alteration of MMPs compared to the normal tissue for both primary and secondary glaucomas. We will determine the MMP activity within the aqueous humor and expect to find that these MMPs to become altered with the diseased state of glaucoma. We localized the MMPs within the TM cells, ECM of the iridocorneal angle tissue and expected differences between distribution in normal and glaucomatous eyes. We also expected to find differences in distribution of MMPs in other ocular tissues.

CHAPTER 2

IDENTIFICATION OF OCULAR MATRIX METALLOPROTEINASES (MMPs) PRESENT WITHIN THE AQUEOUS HUMOR AND IRIDOCORNEAL DRAINAGE ANGLE TISSUE OF NORMAL AND GLAUCOMATOUS CANINE EYES[1]

[1]William L. Weinstein, Ursula M. Dietrich, John S. Sapienza, K. Paige Carmichael, Phillip A. Moore, and Thomas M. Krunkosky. 2007. *Veterinary Ophthalmology.* 10, Supplement 1, 108-116.

Abstract

Purpose The purpose of this study was to identify the most active MMPs within the aqueous humor and iridocorneal angle tissue involved in the normal canine eye and to compare these results to the MMP activity in dogs with glaucoma.

Animal Studied Aqueous humor samples from 32 normal eyes and 26 glaucomatous eyes were obtained through aqueous centesis and analyzed for MMP activity. Iridocorneal angle tissue from 16 enucleated normal canine eyes and 5 enucleated glaucomatous eyes were dissected and homogenized into solution.

Procedure Bradford total protein assays were determined for aqueous humor samples and iridocorneal angle tissue samples. Substrate gelatin zymography was performed using 0.2µl volumes of an MMP-2/ MMP-9 control, 2 µl volumes of aqueous humor samples and 10 µg weights of total protein from iridocorneal angle tissue. The presence of MMP gel bands were identified visually and measured quantitatively by densitometry technique. A statistical analysis was performed on the data using a student t-test, multiple logistic Wald's chi-squared regression, Pearson correlations and a repeated measures analysis.

Results Within the aqueous humor of canine eyes, MMP-2 latent form was found to be the most relevant MMP. The quantity of latent MMP-2 within the aqueous humor of the glaucoma samples was significantly increased compared to the normal aqueous samples ($P<0.0001$). Glaucoma occurrence is associated with elevated aqueous humor latent MMP-2 ($P=0.0002$). Within the canine iridocorneal angle tissue, MMP-9 latent form and MMP-2 active form were found to be the most relevant MMPs. MMP-2 active form was found to be significantly increased in the glaucoma tissue samples when compared to the normal tissue samples ($P=0.0044$). MMP-9 latent form was also found to be significantly increased in glaucomatous

tissue when compared to the normal eyes (P=0.0002). Tissue MMP-9 latent form was found to be associated with glaucoma status (P=0.042).

Conclusion Glaucoma aqueous humor samples expressed a statistically increased latent MMP-2 when compared to normal eyes. Iridocorneal angle tissue from glaucomatous eyes expressed a statistically significant increase in active MMP-2 and latent MMP-9 when compared to normal eyes. This data demonstrates that there is an association between elevated levels of intraocular MMP-2 and MMP-9 with the presence of glaucoma.

Introduction

Studies in human medicine have determined that matrix metalloproteinases (MMPs) play an important role in reducing resistance to aqueous humor outflow within the eye[1,3,4,6,8,14,16] and become altered with the disease state of glaucoma.[1,3,4,7,13,14,16,17,19,22] In particular, the trabecular meshwork cells can detect changes in intraocular pressure (IOP) and respond by increasing levels of MMPs.[1,3,4,6,14,22] Intraocular MMPs increase the extracellular matrix (ECM) turnover rate, reduce the resistance to aqueous outflow through the meshwork, and restore normal IOP levels.[1,3,4,8,13,14,16,21] The endogenous MMP activity in normal and glaucomatous canine eyes is currently unknown. In humans, it has been hypothesized that MMPs play a role in glaucoma.[3,4,8,12,14,15,19] To the best of our knowledge no studies have been performed that are aimed at identification of aqueous outflow related MMPs and that describe their role in ECM regulation in the canine eye.

New models of the trabecular meshwork (TM) reveal a dynamic matrix that is constantly remodeling and controlled by the activity of MMPs.[1,4,6,13,21] Recent research has recognized the importance of identifying the ocular MMPs involved in the aqueous outflow pathway of humans.[1,6,8] In these studies, MMPs and TIMPs (tissue inhibitory metalloproteinases) relevant to the maintenance of the ECM (extracellular matrix) and trabecular outflow in the human eye have been identified.[1,3,16] This knowledge allows for the development of potential new therapies for glaucoma. New therapies will target MMP regulation by making use of exogenous /endogenous substances and gene therapies that alter the activity of MMPs and enhance trabecular aqueous humor outflow facility.[4,5,6,7,12,20] These therapies will supplement current medical therapies that enhance outflow through the non-conventional (uveosleral) pathways.[9,18]

Trabecular meshwork (TM) cells are responsible for secreting the extracellular matrix (ECM) and this matrix is maintained by enzymes that are secreted by the trabecular cells.[8,12,14,22] These enzymes are known as matrix metalloproteinases (MMPs) and are characterized by the matrix substrate they degrade; collagenases, gelatinases or stromelysins.[2,22] All of these MMPs share common characteristics: amino acid similarities, a zinc ion at the active site, secretion in a latent form that needs activation for proteolytic activity, inhibition by TIMPs and they all degrade at least one component of the ECM.[2] In their inactive form, MMPs form a complex with a tissue inhibitory metalloproteinase (TIMP).[2] This inhibitor is bound to and blocks the active domain site of the MMP protein.[2] When an MMP is activated it becomes separated from its TIMP. A regulatory feedback loop exists between MMPs and their inhibitors or tissue inhibitory metalloproteinases (TIMPs). A homeostatic reflex mechanism occurs, in which the TM cells sense increases in IOP by stretch receptors within the ECM.[7,8,13] Increases in IOP cause the TM cells to respond by increasing MMP secretion and decreasing TIMP levels within the ECM.[3,4,14]Again, the end result is to increase the ECM turnover rate, to reduce trabecular aqueous humor outflow resistance and to restore normal IOP levels.[3,4,7,13,14] Likewise, elevations in TIMP levels increase ECM deposition and the resistance to outflow and tend to drive up intraocular pressures.[3,4,7,13,14]

It is known that certain breeds of dogs are particularly susceptible to development of primary glaucoma.[11] Among these are the Beagle, Basset Hound, Cocker Spaniel, Chow Chow, Shar-Pei and the Norwegian Elkhound.[11] Current therapy for canine glaucoma includes both medical and surgical approaches that decrease aqueous production and increase aqueous outflow.[18] In humans, novel research focuses on MMPs and altering their activity and control over conventional trabecular outflow resistance.[5,7,9,12,20] In dogs, this avenue of research is

equally important in pursuing, since trabecular outflow is a predominant aqueous outflow pathway in dogs.[11]

Recent studies investigating intraocular MMPs in humans have confirmed the presence of MMPs-1,2,3 and 9.[1,8,13,16] In humans, particularly changes in MMPs-2 and 9 have been implicated as being involved with the disease process of glaucoma.[15,19] Early decreases in MMPs-2, 9 and an increase in tissue inhibitory metalloproteinase (TIMP-2) have been shown to be associated with glaucoma in humans.[15,19] One of the methods utilized to detect these MMPs and their activity is through a technique called substrate gelatin zymography.[17] This technique allows for the identification and quantification of activity of the MMP by enzymatic degradation of a specific matrix.[17] Zymography utilizes MMP degradation of a preferential gelatin substrate and sorts the MMP proteins by their molecular weight.[17] Activity bands on a gel are visualized as clear bands representing digestion of the gelatin substrate. Zymography is a two step process involving protein separation by SDS-PAGE followed by detection of proteolytic activity.[17] In the first step, SDS electrophoresis reversibly inhibits MMP activity and separates the MMP-TIMP complexes.[1,3,7,8,13,14] Then the MMPs are activated again by a buffer and observed degrading their respective substrate.[1,3,7,8,13,14] Therefore, zymography demonstrates bands for both active and inactive MMPs.

All of the previously determined relevant ocular human MMPs-1, 2, 3 and 9 will degrade a gelatin matrix and therefore can be identified using substrate gelatin zymography.[2,8,12-14,16,17] This also includes other MMPs which exhibit gelatinase activity such as MMP-1, MMP-2, MMP-3, MMP-7, MMP-8, MMP-9, MMP-13, MMP-14, MMP-15, MMP-16, MMP-19, MMP-23 , MMP-24 and MMP-26.[2,17]

Inherent MMP gelatinase enzyme activity was utilized in this study to identify the presence and quantify MMP activity in both the aqueous humor and iridocorneal angle drainage tissues of normal dogs and dogs with glaucoma. The focus of this project was to identify the spectrum of MMP activity in normal versus glaucomatous canine eyes, in order to gain more knowledge about MMP involvement in the glaucoma pathologic processes. The long term clinical goal of this research is to further advance the knowledge and treatment of glaucoma in dogs.

Materials and Methods

Clinical ophthalmic exams on patients

Complete ophthalmic examination including applanation tonometry using a Tono-pen® vet (Medtronic, Jacksonville, FL, USA), slit lamp examination and indirect ophthalmoscopy were performed on all patients to confirm the diagnosis of normal and glaucomatous eyes. Gonioscopy using a 17mm Koeppe pediatric lens (Ocular Instruments, Bellevue, Washington, USA) was used to aid in the distinction between primary and secondary glaucoma cases. Clinical data including breed, age, gender, intraocular pressure, duration of glaucoma, types and frequency of medications used and prostaglandin F2α use was recorded for all patients.

Sample population for aqueous humor

Aqueous humor samples from 32 freshly enucleated normal canine eyes were collected by intracameral aqueous centesis at the University of Georgia College of Veterinary Medicine during the years 2005 and 2006. These normal eyes and aqueous humor samples were obtained from dogs that were euthanized for various non-ocular related terminal surgeries, as part of the junior surgery program. All animals were cared for according to the animal care and use committee and University of Georgia guidelines. The eyes were enucleated by a

transconjunctival technique [10] and immediately afterwards the aqueous humor was obtained. Aqueous humor samples from 26 glaucomatous eyes were obtained from clinical cases seen at The University of Georgia College of Veterinary Medicine and Long Island Veterinary Specialists (LIVS) during the years of 2005 and 2006. Inclusion criteria for glaucomatous cases included dogs diagnosed with glaucoma based on elevations of intraocular pressures (IOP) (IOP>25mmHg) and also the presence of changes within the fundus indicative of glaucoma (changes in optic nerve, retinal vasculature and reflectivity). The aqueous humor from the canine glaucoma clinical cases was collected by aqueous centesis under general anesthesia prior to transscleral cyclophotocoagulation and other ocular surgeries including enucleations, placement of intrascleral prosthesis and placement of gonioimplants. The aqueous humor was centrifuged for 10 minutes, supernatant poured off, aliquoted in Eppendorf tubes, frozen at –70 degrees Celsius and stored for substrate gelatin zymography. Bradford total protein assays were determined for each sample.

Sample population for iridocorneal angle tissue

Iridocorneal angle tissue was obtained from 16 enucleated normal canine eyes and 5 enucleated glaucomatous eyes seen at The University of Georgia College of Veterinary Medicine during the years 2005 and 2006. Normal tissue samples were obtained from the same 16 dogs that were utilized for the aqueous humor analysis. However, only one eye from each dog was utilized to obtain the normal tissue samples. Glaucomatous eyes were obtained by enucleation in dogs where medical therapies failed and owners elected removal of the eye. Again, inclusion criteria for dogs with glaucoma was based on elevations of intraocular pressures (IOP) (IOP>25mmHg) and the presence of changes within the fundus indicative of glaucoma (changes in optic nerve, retinal vasculature and reflectivity). No prior surgeries were performed

on these eyes. All globes were obtained through a transconjunctival enucleation technique.[10] Immediately after enucleation, the eyes were dissected microscopically as previously described.[1,4] Globes were transected 5 mm posterior to the limbus, and the iris and lens teased away from the angle with a fine tip forceps, leaving primarily ciliary body tissue and an irido-corneal-scleral region containing trabecular meshwork.[1,4] The corneal tissue was then removed from these anterior segment discs.[1,4] Tissues were frozen and stored at -70 degrees Celsius for tissue preparation.

Iridocorneal angle tissue preparation

During the preparation, tissues were kept frozen by placing them on liquid nitrogen. Small 100 µg pie slices of the anterior segment discs were obtained using a sharp razor blade. The tissue was weighed and added to 0.5ml of a western lysis buffer.

The tissue was manually ground up, homogenized with a pellet pestle, ultra-sonicated twice for 10 seconds, centrifuged at 14,000 rpm for 5 min at 4 degrees Celsius and incubated in buffer on ice for 30 min.[8,13,14] The supernatant was removed and total protein concentrations were determined for each of the samples. Sample liquid aliquots were frozen at -70 degrees Celsius for substrate gelatin zymography.

Bradford total protein assays

Bradford total protein assays were prepared using multi-well plates for unknowns and BSA standards (Bio-rad, Hercules, CA, USA). Active color changes of a dye reagent were measured by spectrophotometry at a 595 nm wavelength and analyzed by a computer program (Softmax Pro, Bio-rad, Hercules, CA, USA). Sample total protein concentrations were determined as µg/µl concentrations.

Substrate gelatin zymography

An 8% SDS-PAGE containing 1mg/ml gelatin was polymerized and covered with a 4% stacking gel to produce a row of 10 sample wells. 2µl volumes of sample aqueous humor, 10 µg weights of total protein from iridocorneal angle tissue and 0.2 µl volumes of MMP-2/ MMP-9 controls (MMP-2/9 control, Bio-rad, Hercules, CA, USA) were combined with a western lysis buffer , 4x sample buffer (under non-reducing conditions) and were loaded onto a gel that was electrophoresed at 100 volts for 120 minutes. Using an MMP detection method previously described [17] and following electrophoresis the gels were removed and washed for 1 hour in 2.5% Triton-X100 buffer to remove SDS. The gels were incubated for 16 hours in incubation buffer (100mM Tris/HCL 5mM $CaCl_2$,0.005% Brij-35 and 0.001%NaN_3) at 37 degrees Celsius then stained in 0.25% Coomassie dye for one hour and de-stained in 5% methanol/ 7.5% acetic acid for one hour. Inhibitory controls were run with 10mM of EDTA which was added to the incubation buffer to confirm that protease activity was effectively blocked.

Measuring MMP activity

The MMPs were qualitatively identified and detected as clear bands against a blue background of undigested substrate as previously shown.[13] Computer densitometry was used to quantitatively determine the MMP activity levels. Gels were visualized using a Bio-Rad Fluor-S Max2 Multi-Imager (Bio-rad, Hercules, CA, USA) and analyzed for relative density using Quantity One Quantization software (Bio-rad, Hercules, CA, USA). MMP activity bands were measured as an optical density units (ODu*mm2). Intensity was measured for each band and normalized to a positive control from each gel to compensate for any gel differences.

Statistical analysis

Multiple logistic regression was performed to determine a Wald's chi-squared test statistic of independence between MMP activity & total protein concentration for the binary trait of glaucoma (SAS® program, SAS Institute Inc.,Cary, NC, USA). A student t test was performed between the normal and glaucoma MMPs and total proteins (Microsoft Excel®, Microsoft Corp., Redmond, WA, USA). Pearson correlations were determined.(SAS® program, SAS Institute Inc.,Cary, NC, USA). A repeated measures analysis was performed to take into account normal dogs where both eyes were utilized from the same patient (SAS® program, SAS Institute Inc., Cary, NC, USA).

Results

Clinical findings for aqueous humor analysis-Normal cases

Aqueous humor was collected from 32 normal eyes (16 OS and 16 OD) (Table 2.1). Both eyes were samples from each dog. The study included 16 dog breeds consisting of 5 Pit Bull mixed breed dogs, 2 Chow Chow mixes, 2 Hound crosses, 2 Labrador Retriever mixes, 2 German Shepherd mixes and 2 mixed breed Husky dogs. Gender distribution for the group included 9 intact females, 1 spayed female, 1 intact male and 5 neutered males. Ophthalmologic exams revealed no clinical abnormal findings for this group of normal dogs. The average age for this sample population was 1.93 years. The average intraocular pressure for this group at the time of aqueous humor sampling was 13.73±3.36mmHg.

Clinical Findings for aqueous humor analysis-Glaucoma cases

Aqueous humor was collected from 26 glaucomatous eyes. 21 of these cases were primary glaucomas and 5 of these cases were determined to be secondary glaucomas (Table 2.1). Causes for these secondary glaucomas cases included: 2 uveitis, 1 trauma, 1 intra-ocular neoplasm and

one lens luxation. The group of dogs with primary glaucoma included 7 Cocker Spaniels, 3 Chow Chows, 1 Shar-Pei, 1 Basset hound, 1 Shibu-Inu, 2 Cock-a-poos, 1 Greyhound, 1 Maltese and 4 mixed breed dogs. The group of dogs with secondary glaucoma included 1 Pit Bull, 1 Golden Retriever, 1 Labrador Retriever, 1 Bulldog and 1 mixed breed dog. Gender distribution for the group consisted of 12 spayed females, 12 neutered males and 2 intact males. The average age for the glaucoma cases was 8.5 years with an average intraocular pressure at the time of sampling of 37.04±15.00mmHg. Duration of glaucoma was classified for the cases as follows: 21 chronic (7+ days duration), 3 sub-acute, (3-7days duration) 2 acute (1-2 days duration). The average number of glaucoma medications these dogs were maintained on at the time of sampling was determined to be 3 medications (including any combination of a topical carbonic anhydrase inhibitor, beta-blocker, oral carbonic anhydrase inhibitor, neuroprotectant, and a prostaglandin analogue). Average use of prostaglandin F2α use was 1-2 times (average=1.16) a day.

Clinical findings for iridocorneal angle tissue analysis-Normals

Iridocorneal angle tissue was collected from 16 normal eyes (8 OS and 8 OD) (Table 2.1). The group of 16 dogs included 5 Pit Bull mixed breed dogs, 2 Chow Chow mixes, 2 Hound crosses, 2 Labrador Retriever mixes, 2 German Shepherd mixes and 2 mixed breed Husky dogs. Gender distribution included 9 intact females, 1 spayed female, 1 intact male and 5 neutered males. These were the same dogs utilized for the aqueous humor analysis but only one eye was utilized for tissue collection. The average age for this sample population was 1.93years. The average intraocular pressure for this group at aqueous humor sampling was 14.063±3.32 mmHg.

Clinical Findings for iridocorneal angle tissue analysis-Glaucoma cases

The 5 glaucoma eyes (2 OD and 3 OS) were obtained from 1 West Highland White Terrier, 1 mixed breed dog, 1 Great Dane, and 1 Chow Chow and animals in this group had an average age

of 7.55years (Table 2.1). In one case both eyes were affected and utilized to obtain iridocorneal angle tissue. The group gender consisted of 3 spayed females and one intact male. Three eyes were classified as primary and 2 eyes classified as secondary glaucomas. The cause of these two secondary glaucoma cases includes a lens luxation and uveitis. Average IOP for this group was 27.78±13.57 mmHg. Four of these cases were classified as chronic glaucomas and one as an acute glaucoma using the same classification scheme as used for the aqueous humor group. On average, dogs in the group received 3 medications (including a combination of topical carbonic anhydrase inhibitors, beta-blockers, oral carbonic anhydrase inhibitors, neuroprotectant, and a prostaglandin analogue). Prostaglandin F2α average daily use was 1-2 times (average= 1.5 times) a day.

Experimental results for aqueous humor analysis-MMP results

Qualitative interpretation of the aqueous humor gels revealed that the normal aqueous humor samples consistently showed a small latent MMP-2 band in all the samples (Figure 2.1). The intensity of the latent MMP-2 band was increased for all the primary and secondary glaucoma samples. Some bands were so enlarged that it was difficult to determine if this was in fact only a latent band or whether we were seeing an increase in active MMP-2 and/or blending of latent MMP-2 into the active band region of the gel (Figure 2.1). In contrast, a latent MMP-9 band occurred less frequently and in only 4 of the glaucoma samples. Quantitative interpretation using densitometry showed that the average optical density of the measured latent MMP-2 band increased 6 fold for the glaucoma cases from 3.11 ± 2.10 ODu*mm^2 to 18.40±8.90 ODu*mm^2 (P=0.0001) (Table 2.2). No significant differences were found between aqueous humor MMP-2 latent levels between primary and secondary glaucomas. Both groups exhibited a statistically increased optical density measurements (P<0.0001). Average MMP-2 values of 17.278

ODu*mm^2 for primary glaucomas and 21.08 ODu*mm^2 for secondary glaucomas were obtained compared to the normal value of 3.11 ODu*mm^2. A repeated measures analysis which accounts for the correlation between eyes in normal patients where both eyes were used on the same patient was performed. Again this confirmed significant differences between normal and glaucoma samples for latent aqueous MMP-2 ($P<0.0001$). No significant correlation was found between duration of glaucoma and latent MMP-2 levels in aqueous humor samples from eyes with glaucoma. In addition no significant correlations were found between age of patient and latent MMP-2 levels within the aqueous humor.

Experimental results for aqueous humor analysis-Total protein results

Total protein also increased in the aqueous humor of the glaucoma cases when compared to the total protein concentration in normal eyes. The normal samples had an average total protein of 0.919±0.60 µg/µl and this was increased to 10.37±8.44µg/µl for the glaucoma samples ($P<0.0001$) (Table 2.2). This is a significant 11 fold increase in total protein concentration ($P<0.0001$)(Table 2.2).

Experimental results for aqueous humor analysis-Total protein and MMP results

A multiple logistic regression analysis suggests that latent MMP-2 levels and total protein concentration are dependent on glaucoma status. The incidence of glaucoma is higher with increasing latent MMP-2 levels ($P=0.0002$) and the incidence of glaucoma also increases with increasing total protein concentration ($P=0.0010$). The odds of glaucoma occurrence increased by 2.051 (95% confidence interval: 1.397, 3.011) for every increase in optical density measurement of the latent MMP-2 band. The odds of glaucoma occurrence increased by 4.014 (95% confidence interval: 1.758, 9.165) for every µg/µl increase in total protein. A Pearsons

correlation confirmed a strong positive correlation, r=0.5892, between latent MMP-2 levels and total protein concentration within aqueous humor (P<0.0001) (Table 2.3).

Experimental results for iridocorneal angle tissue analysis-MMP results

Qualitative interpretation of the iridocorneal angle tissue gels revealed the presence of both MMP-2 and MMP-9 activity within the iridocorneal angle in both normal eyes and canine eyes with glaucoma. A latent MMP-2 band was present in both the normal samples and glaucoma samples (Figure 2.3). The intensity of this band was not statistically different for the two groups (P>0.05). In contrast, the intensity of the MMP-2 active bands was increased when comparing the glaucoma samples to the normal samples (Figure 2.2). The iridocorneal angle tissue also contained a latent MMP-9 band and the intensity of this latent MMP-9 band was greater in the glaucoma samples (Figure 2.2). Quantitative interpretation using densitometry showed that MMP-2 active form was significantly increased in the glaucoma samples when compared to the normal tissue samples (P=0.023). The MMP-2 active form was increased from 0.122±0.011 ODu*mm^2 to 3.82±8.32ODu*mm^2 for the glaucoma samples (P=0.023)(Table 2). MMP- 9 latent form was also found to be significantly increased from 7.12±77.53 ODu*mm^2 to 17.86±10.96 ODu*mm^2 in glaucomatous tissue when compared to the normal eyes (P=0.000375) (Table 2.2). Strong positive correlations with r=0.78037 were found between the presence of glaucoma and increasing MMP-2 active form (P=0.0246). In addition, strong correlations with r=0.527 for the presence of glaucoma with increasing MMP-9 latent form were found (P=0.0164). Using multiple logistic regression analysis MMP-9 latent form was found to be associated with glaucoma status (P=0.042). For every point increase in measured latent MMP-9 the odds of glaucoma occurrence increases by 1.168 (95% confidence interval: 1.006, 1.355). A repeated measures analysis which accounts for correlation between eyes in which two eyes were

used from the same normal patients was run. In this analysis, active MMP-2 was found to be increased for combined primary and secondary glaucomas, compared to the normal eyes ($P<0.0044$). Taken independently, primary glaucomas had a significantly increased active MMP-2 levels when compared to normal eyes ($P=0.0204$). However, secondary glaucomas had an elevated but not statistically significant increase in MMP-2 active form. Tissue latent MMP-9 levels were shown to be increased for combined primary and secondary glaucomas ($P=0.0002$), for primary glaucomas alone ($P=0.0020$), and for secondary glaucomas alone ($P=0.0276$). For the tissue samples, there were no significant differences in MMP-2 latent levels between normal and glaucomatous eyes. No significant correlations were found between age and latent MMP-2 levels in tissue samples from eyes with glaucoma.

Inhibition of protease activity with EDTA

Gelatinolytic activity of MMP-2 and MMP-9 was completely inhibited when 10mM of EDTA was added to the incubation buffer. Figure 3 shows complete inhibition of normal and glaucoma aqueous humor samples when 10mM EDTA was added to the development buffer.

Experimental results for iridocorneal angle tissue analysis-Total protein results

Total protein was found to be increased in the glaucoma samples to $3.83\pm1.59\mu g/\mu l$ compared to the normal samples at $2.81\pm0.397\ \mu g/\mu l$ ($P=0.0114$) (Table 2.2).

Discussion

Gelatin zymography was used in this study to identify the most relevant MMPs along the aqueous outflow pathway within normal dogs and dogs with glaucoma. This technique was utilized to identify MMP-2 and MMP-9 as the most important MMPs within normal and glaucomatous canine eyes. We failed to see bands for all other MMPs that exhibit gelatinase activity and that should have displayed activity if present. There were no activity bands detected

for MMP-1, MMP-3, MMP-7, MMP-8, MMP-13, MMP-14, MMP-15, MMP-16, MMP-19, MMP-23, MMP-24 and MMP-26 which exhibit gelatinase activity. This rules out the presence of the above MMPs in normal and glaucomatous aqueous humor and iridocorneal angle tissue samples. Bands were detected for MMP-2 and MMP-9 in normal and glaucoma samples. Further, we identified changes in the levels of MMP-2 and MMP-9 activity which correlated with the presence of glaucoma.

Our study determined latent MMP-2 to be the most relevant MMP within the aqueous humor in canine eyes. Within the aqueous humor of normal canine eyes, there exists an endogenous level of latent MMP-2. This form of MMP-2 is inactive and therefore likely coupled to an inhibitory metalloproteinase or TIMP. For glaucoma, levels of the latent aqueous MMP-2 appear to be increased as shown by our zymography results. The latent MMP-2 aqueous reservoir appears to have increased 6 fold in size for the glaucoma samples. Analysis indicates that the incidence of glaucoma is associated with increasing latent MMP-2 levels. We speculate that latent MMP-2 circulates through the iridocorneal angle tissue where this latent form might be converted to its active form. Within the iridocorneal angle tissue, latent MMP-2 levels appear to be present in both normal and glaucomatous eyes at similar levels. However, active MMP-2 levels appear to be significantly increased with glaucoma. This indicates conversion of the latent form of MMP to an active enzyme. There also exists an endogenous reservoir of latent MMP-9 which becomes increased in eyes with glaucoma. At the same time, total protein also appears to increase within the aqueous humor and iridocorneal angle of glaucomatous eyes. This may reflect increased levels of MMPs as well as other increased protein expression not currently identified. Gelatinolytic activity of MMP-2 and MMP-9 was completely inhibited when 10mM of EDTA was added to the incubation buffer. This confirms metallic dependent protease activity.

Protease activity is dependent on metallic cat-ions (particularly zinc) and enzymatic activity was inhibited by effectively binding up available metallic cat-ions with a chelating agent (EDTA).

Human studies have shown that endogenous MMPs play a role with remodeling of drainage angle tissue within the eye.[1,3,4,6,13,14,21,22] It is speculation that deregulation in MMP/TIMP levels and their effect on aqueous outflow may contribute to the disease process of glaucoma. We suggest that our measured increase in MMP-2 and MMP-9 may represent physiologic adaptive changes that occur in response to elevated intraocular pressures that occurs with glaucoma. Likewise, increased MMP-2 and MMP-9 within canine glaucomatous globes may assist in partially restoring normal IOP levels. This is purely speculative and needs to be explored further with studies of prospective experimental research designs.

It is important to mention that MMP-2 and MMP-9 are not entirely specific for glaucoma and have been shown to be elevated and associated with intraocular inflammatory disease occurring with cataracts and uveitis.[15] It has been reported that MMPs become overexpressed with ocular inflammatory disease [15] and have a multitude of physiologic effects throughout the body which include activation of signaling molecules and receptors, degradation of extracellular molecules responsible for cell adhesion and also activation of other proteases. [6,21,21] For the purpose of this study we grouped primary and secondary glaucomas to look at the disease as a whole compared to normal eyes. We also separated out our data for the two groups and analyzed them separately. In comparing the MMP elevation there was no significant difference between the elevations in MMPs between primary and secondary glaucomas. Our analysis which groups primary and secondary glaucomas together shows that overall for the disease of glaucoma there is an association with elevated MMP-2 and MMP-9 levels and glaucoma. We also separated out primary and secondary glaucomas to compare MMP levels to normal dogs. A separate analysis

of the data has shown that MMP-2 and MMP-9 become elevated for both primary and secondary glaucoma individually. Latent MMP-2 becomes elevated within the aqueous humor for both primary and secondary glaucoma taken individually. Active MMP-2 becomes significantly elevated in the iridocorneal angle for primary glaucoma. It becomes elevated for secondary glaucomas but is not statistically significant when compared to normal tissue. Again, this is probably due to our smaller sample tissue size. We did not find any association between duration of the glaucoma and the levels of MMPs in this study.

We wish to point out some potential factors contributing to variability in this study. First, we acknowledge difference between average age for our normal and glaucomatous populations. However, even though the mean age was higher for the glaucoma population it still included a broad range of ages. A correlation was performed between varying age and MMP levels for the glaucoma samples and was found not to be significant for our data. Age is a variable to consider but was unlikely to account for the difference between MMP levels between our normal and glaucoma samples for this study. Second, we recognize that a source of variability may be the time from enucleation of eyes to aqueous humor collection for the normal samples. The time from enucleation to aqueous collection was kept constant and was for all purposes immediate. However, glaucomatous aqueous humor was collected before enucleation. Also in some cases aqueous was collected before placement of an intrascleral prosthesis and or laser cyclophotocoagulation, where no enucleation was performed. Since the enucleation/ aqueous collection interval time was so short the authors do not feel that this introduced a significant source of variability to the data. After enucleation the eyes and collected aqueous humor samples were placed immediately on ice until homogenized. We do not believe that this altered the MMP activity. Third, we recognize that our small sample size for the glaucoma tissue samples may be

a limiting factor. However, despite the small sample size overall there was a measurable increase in MMP-2 and MMP-9 levels for glaucoma verses normal samples.

Future studies which look specifically at MMP levels in goniodysgenic breeds need to be determined to elicit the activity of these enzymes. Measuring these enzymes in goiniodysgenic breeds may reveal MMP changes early in the disease process. In addition, prospective studies need to be performed which determine the relationship between elevation of intraocular pressure and these MMP levels in the dog. There is much promise for future research in the dog to follow. Manipulation of the trabecular outflow using exogenous substances has been shown to increase MMP activity altering outflow rates in humans.[7] Identification of responsible MMP genes and gene insertion has been performed in culture.[5,12] Genetic engineering has been used to increase endogenous MMP activity by insertion of MMP genes into trabecular meshwork cells in culture and *in vivo* for laboratory animals. [5,12] In the future, there may be the potential for MMP gene therapy for glaucoma in people and in dogs.

To the best of our knowledge this is the first study that quantifies the normal baseline endogenous activity and intraocular levels of MMPs-2 and 9 within the aqueous outflow pathway in the dog. In addition, we illustrate that MMP -2 and MMP-9 levels become altered with the disease state of canine glaucoma. Our study lays the groundwork for future studies investigating alteration of physiologic MMP-2 and MMP-9 activity in the dog in order to pursue effective therapies and preventative treatment in breeds that have a predilection for primary glaucoma.

CHAPTER 3

IMMUNOHISTOCHEMICAL LOCALIZATION OF MMP-2 AND MMP-9 WITHIN THE IRIDOCORNEAL DRAINAGE ANGLE TISSUE OF NORMAL AND GLAUCOMATOUS CANINE EYES[1]

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Introduction

Previous studies in human medicine have determined that matrix metalloproteinases (MMPs) play an important role in reducing resistance to aqueous humor outflow within the eye [1,3,4,6,8,14,16] and become altered with the disease state of glaucoma. [1,3,4,7,13,14,16,17,19,22] MMPs play an important role in the remodeling of the trabecular meshwork tissue and regulate the resistance through the iridocorneal angle tissue. [13,16] Within the dynamic extracellular matrix (ECM), the trabecular meshwork cells are responsible for secreting the MMPs which degrade collagenases, gelatinases and stromelysins.[1,3,4,6,14,22] Recent human studies have confirmed the presence of MMP-1, MMP-2, MMP-3, and MMP-9 within the aqueous humor outflow pathway.[1, 8, 13,16] It is hypothesized that early decreases in MMP-2 and MMP-9 and an increase in expression of tissue inhibitors of metalloproteinases (TIMPs) over these MMPs may to be contributing to glaucoma in humans.[25] Later in the disease process, increases in MMP-2 and MMP-9 may reflect a physiologic adaptive response to restore outflow in the diseased eye.[3]

In our previous study, using substrate gelatin zymography, we identified the aqueous outflow-related MMPs in the canine eye. MMP-2 and MMP-9 were identified as the most important MMPs within the normal and glaucomatous canine eyes. In normal eyes, latent MMP-2 was found within the aqueous humor in canine eyes. In glaucomatous eyes the latent MMP-2 aqueous reservoir appears to have increased six fold compared to normal eyes for glaucoma (for both primary and secondary glaucomas). Within the iridocorneal angle tissue we identified latent MMP-2 and latent MMP-9 levels. For the glaucoma samples, latent MMP-2, latent MMP-9 and active MMP-2 forms increased significantly with glaucoma (for both primary and secondary glaucomas). A significant correlation between increasing MMP-2 and MMP-9 with glaucoma supports an association between these altered MMPs and the disease state of canine glaucoma.

We speculate that our measured increase in MMP-2 and MMP-9 may represent physiologic adaptive changes that occur in response to elevated intraocular pressures with glaucoma. We also speculate that an overexpression of TIMPs over MMPs early in the disease process may contribute to the pathology within the pectinate ligaments and or ciliary cleft tissue early in the disease process of goniodysgenesis.

In this study, immunohistochemical analysis is further used to confirm the presence of MMPs within the fixed ocular tissue. The long term clinical goal of this research is to further advance the knowledge and treatment of glaucoma in dogs. To the author's knowledge, this is the first study which utilizes immunohistochemistry to compare MMP staining within the aqueous outflow pathway in normal and glaucomatous canine eyes.

Materials and Methods

Clinical ophthalmic exams

Complete ophthalmic examination including applanation tonometry using a Tono-pen® vet (Medtronic, Jacksonville, FL, USA), slit lamp examination and indirect ophthalmoscopy were performed on all patients to confirm the diagnosis of normal and glaucomatous eyes. Gonioscopy using a 17mm Koeppe pediatric lens (Ocular Instruments, Bellevue, Washington, USA) was used to aid in the distinction between primary and secondary glaucoma cases. Clinical data including breed, age, gender, intraocular pressure, duration and type of glaucoma was recorded for all patients.

Sample population of iridocorneal angle tissue:

Control eyes from necropsy cases that had died from natural causes or were euthanized for other non-ocular related problems were obtained by subconjunctival enulceation. These eyes were obtained within less than 1-2 hours time. After death, the eyes were removed and fixed in

Davidson for 24-48hrs. Glaucomatous eyes were obtained from dogs previously diagnosed with clinical glaucoma based on abnormal fundus examination and elevated intraocular pressures. These eyes were enucleated under general anesthesia and immediately placed into Davidson fixative. All animals used were cared for according to the animal care and use committee and the University of Georgia guidelines. Enucleations for clinical cases were performed within the hospital setting and met the standard for the University of Georgia Veterinary Teaching Hospital surgical protocols.

Immunohistological identification of ocular MMPs

Indirect immunohistochemistry (IHC) for MMP-2 and MMP-9 were performed on all tissue samples. Longitudinal cross sectional tissue preparations were prepared as follows. Control globes and glaucomatous globes were fixed in Davidson for 12-48hrs. The tissues were sectioned at 3 μm, mounted on probed slides, and parrafin-embedded. Slides were heated for 30 min at 70 degrees Celsius. Slides were deparaffinized in Hemo De solution and re-hydrated in ethanol and water. Epitope retrieval was performed by incubating in a commercial vector antigen unmasking solution (Vector Laboratories, Inc., Burlingame, CA). Rinsing was performed with a PST-T solution (PBS solution containing 0.1 % Tween-20, ph=7.4). Next, blocking with serum for nonspecific binding (serum of species which the secondary antibody was produced: sheep serum or rabbit serum for this experiment). Incubation was performed with polyclonal antibodies at 4 degrees Celsius overnight with primary antibody consisting of anti-human MMP-2 (Goat) and anti-human MMP-9 (Mouse). The secondary antibody was applied 12 hours later using anti-goat & anti-mouse IgG (Sheep & Rabbit). The secondary antibody was purchased and conjugated with substrate alkaline-phosphatase enzyme (Chemicon) . 100μl of Levamisole (Biogenex) was combined with 5ml of the chromogen fast red stain to block for endogenous

alkaline phosphastase within the tissue. Fast red chromogen stain was applied for 45 minutes. The slides were counterstained with hemotoxylin for 2 minutes, washed in water and allowed to dry overnight. Slides were than coverslipped using a Permount solution.

Antibody Specificity/ Western blots

Western blot analysis for MMP-2 and MMP-9 was performed to confirm specificity in the dog for the polyconal anti-human MMP-2 and MMP-9 antibodies used in this experiment. Protein concentrations from canine iridocorneal angle tissue and aqueous humor samples for were calculated using a Bio-Rad Bradford Protein assay, and a 96 well plate reader. Proteins were loaded into a gradient gel (BioRad Ready Gel 10-20%) at an amount of 30 µg/µl per well. The protein samples were resolved by SDS-PAGE and then transferred onto nitrocellulose membranes. The membranes were blocked in a 5% nonfat dried milk in 20 mMTris, 500mM NaCl, 0.1% Tween-20, pH 7.5 blocking solution, and then immunoblotted with the following antibodies (R&D systems) at the concentration of 1:1,000: Mouse Anti- human MMP-9 and Goat anti- human MMP-2. The primary antibodies were applied in blocking buffer at 4'C overnight. After incubation, the blots were treated with an anti-goat IgG HRP linked antibody with a concentration of 1:1,000 (R&D systems). The blots were then imaged with a Flour-S Max (Bio-Rad) multimager system after being treated with SuperSignal West Dura extended duration chemiluminescence substrate solution (Pierce).

Qualitative Hemotoxalin and Eosin staining interpretation

H &E stained sections were observed for all control and glaucomatous cases. If histological lesions were found in any of the control eyes, the sample was removed from the study.

Qualitative MMP staining interpretation

Immunohistochemistry MMP staining was qualitatively evaluated for presence and distribution within specific cells, matrix or tissue stroma of the iridocorneal angle tissue. MMP staining was observed in the iridocorneal angle of control and glaucomatous eyes. This included specifically examining tissue of the iridocorneal angle including part iris, corneal scleral meshwork and uveal trabecular meshwork. In addition, staining was observed in neighboring ocular tissue including iris, cornea, sclera, ciliary body, choroid, retina, optic nerve, lens and anterior, posterior and vitreous ocular chambers.

Quantitative MMP staining interpretation

Quantification of IHC cell counts was performed by counting the number of stained cells within 5 40X fields within each tissue of interest. The final average of 5 counts was determined. Histogram densitometry was performed on the corneoscleral and uveal trabecular meshwork tissue to quantitatively assess the intensity of extracellular matrix staining, using a photoshop based image analysis for the staining of MMPs. Histogram densities for the spectrum of colors staining blue, green and red was observed within the trabecular meshwork.

Statistical analysis

Student t test with 2 tail distribution for two samples with unequal variances was performed between the control and glaucoma MMPs counts and for histogram densitometry data using (Microsoft Excel®, Microsoft Corp., Redmond, WA, USA).

Results

Clinical findings for sample population-control cases

Nine eyes were obtained from necropsy cases that had died from natural causes or from other non-ocular related problems (Table 3.1). Breeds represented include the Dachshund, Labrador

Retriever, Wolf Hybrid, King Charles Cavalier Spaniel (KCCS), Neopolitan Mastiff, Bulldog and Mix breed dogs. The average age was 6.07 years at time of enucleation (Table 3.1).

Clinical findings for sample population-glaucoma cases

Seven eyes were obtained from clinical cases treated at University of Georgia Veterinary Teaching Hospital. Three eyes included were primary glaucomas and four were secondary glaucomas (Table 3.1). Breeds represented included 2 Chow Chows and a Sharpei (primary glaucomas) & a Bloodhound, Labrador, Shetland Sheepdog, Beagle (secondary glaucomas). All cases were considered chronic with the duration of the glaucoma at grater than 1 week. Average age at time of enucleation was 8.1 years (Table 3.1). Average intraocular pressure at the time of enucleation for these cases was 41.67 ±11.07mmHg.

The primary glaucomas and secondary glaucomas consisted of case #1-7 (Table 3.2).

Qualitative results for Hematoxilin and Eosin staining for glaucomatous cases

Primary glaucomas.

Case 1 (339-2): Examination of H+E staining of a section of complete globe revealed a closed iridocorneal angle with pigmented tissue extending from the base of the iris to connect to an arborized Descemet's membrane. There was a collapsed ciliary cleft (Figure 3.1). The retina was artifactually detached and was severely degenerated throughout all layers (Figure 3.2). The sclera appeared thinned. Morphologic diagnosis of goniodysgenesis and primary narrowed angle closure glaucoma was made.

Case 2 (485-2): Examination of H+E section revealed a narrowed iridocorneal angle (ICA), with the iris reflected against the back of the cornea. Pigmented tissue extended from the base of the iris to the cornea. The ciliary cleft appeared collapsed. The cornea contained anterior to mid stromal corneal neovascularization with corneal edema. A small to moderate number of

lymphocytes were present within the collapsed ciliary cleft. The retina showed decreased numbers of retinal ganglion cells and moderate gliosis of the optic nerve head (ONH). The morphologic diagnosis of goniodysgensis with primary narrowed angle glaucoma was confirmed.

Case #3 (485-1). H+E section revealed a closed iridocorneal angle, collapsed ciliary cleft with a shallow anterior chamber and iris bombe. A pigmented band of tissue extended across the iridocorneal angle (ICA) (Figure 3.1) and there was pigment present on the lens capsule. The cornea contained anterior to mid stromal neovascularization and corneal edema with infiltration of lymphocytes and neutrophils into the stroma. The retina was severely degenerated and the optic nerve exhibited moderate gliosis and cupping. The morphologic diagnosis of goniosysgenesis, primary angle closure glaucoma, and mild lymphopurulent keratitis was made.

Secondary Glaucomas.

Case #4 (5071) H+E staining of an aphakic globe revealed a closed iridocorneal angle and collapsed ciliary cleft. The anterior uveal tract contained an infiltration of lymphocytes, plasma cells, and lesser numbers of macrophages and neutrophils. Atrophy of retinal ganglion cells and moderate optic nerve head gliosis and the presence of gitter cells with necrosis of optic nerve head was seen. The morphologic diagnosis of moderate lymphoplasmocitic uveitis with secondary glaucoma was made.

Case # 5: (254): In the H +E section of complete globe the iris was adhered to the anterior surface of the lens and was infiltrated by many lymphocytes, neutrophils and macrophages. This inflammatory infiltration extended throughout the iridocorneal angle between the choroid and the retina. There was a sub-retinal exudate present and the retina was detached (Figure 3.3). Within this infiltration there were numerous lymphocytes and tissue macrophages that contained a large

amount of melanin pigment. The choroid was expanded with these inflammatory cells. There was infiltration of the cornea at the limbus with lymphocytes and tissue macrophages. This infiltration of inflammatory cells extended along the deep stroma and Descemet's into the sclera. Within the anterior chamber there was fibrinous material containing lymphocytes, macrophages and neutrophils. There were scattered neutropils adhered to the corneal endothelial layer. There was a complete retinal detachment as well as a large amount of hemorrhage within the vitreal chamber. The retinal pigmented epithelium was hypertrophied. Periodic acid-Schiff (PAS), Grocott-Gomori methenamine silver staining (GMS) and acid fast staining failed to reveal infectious organisms. The morphologic diagnosis of severe granulomatous endophthalmitis with posterior synichia was made. This type of inflammatory response was consistent with an immune mediated response, the cause of which could not be determined.

Case # 6 (110): H+E staining of a section of complete globe revealed that the anterior chamber was shallow. There was iris bombe and iridal vessels were distended (rubeosis iridis). The iris was infiltrated by moderate numbers of lymphocytes and plasma cells. There was peripheral corneal neovascularization with anterior to mid stromal neovascularization and corneal edema. There was a complete retinal detachment and degeneration of all layers. There was hypertrophy of the retinal pigmented epithelial cells. Subretinal hemorrhage was present containing neutrophils and macrophages filled with hemosiderin. Hemorrhage was present in the anterior chamber and there were also infiltration of neutrophils and macrophages. Neutropils were seen adhered to Descemet's membrane. The diagnosis of moderate to severe lymphoplasmocytic uveitis with secondary glaucoma was made.

Case #7 (339-1): H+E section revealed a uveal tract that was severely infiltrated by lymphocytes, plasma cells and neutrophils with a few macrophages. The vessels of the iris

appeared distended (rubeosis iridis) (Figure 3.5). The iridocorneal angle was infiltrated by these same inflammatory cells (Figure 3.4). The cornea was edematous with peripheral neovascularization and deep to mid stromal neovascularization. Infiltration by lymphocytes, neutrophils and plasma cells within the anterior chamber was evident. The retina was severely degenerated in all layers, especially within the retinal ganglion cell layer. Morphologic diagnosis of panuveitis with secondary glaucoma was made.

Qualitative results for in situ indentification of MMP-2 & MMP-9 staining

Cornea. Staining for MMP-2 and MMP-9 and was observed at a low to moderate level within normal corneal epithelium (Table 3.3). In normal eyes, MMP staining was seen within the cytoplasm of squamous, polygonal, and basal corneal epithelial cells. A normal baseline MMP staining was seen within the corneal epithelial cells and corneal stroma (Figures 3.6 and 3.7) In primary glaucoma cases, there was also staining throughout the corneal epithelium but there appeared to be increased staining intensity of MMPs to moderate levels throughout the corneal epithelial layers. (Figure 3.8) Staining of MMP-2 and MMP-9 was evident within the corneal stroma in normal and glaucomatous eyes from low to moderate levels and moderate to high levels respectively. Within normal eyes MMP-2 and MMP-9 staining was visible within the cytoplasm of corneal fibroblasts (keratocytes). In glaucomatous eyes, staining appeared within cytoplasm of stromal fibroblasts, lymphocytes and neutrophils (for primary glaucoma and secondary glaucomas) (Figure 3.9). The corneal endothelium had a positive staining for MMP-2 and MMP-9 in both normal and glaucomatous cases with low to moderate intensity. Subjectively, there did not appear to be a difference between endothelial staining in normal and glaucomatous cases. In secondary glaucomatous cases, neutrophils adhered to the corneal endothelium also stained positive for MMP-2 and MMP-9 (Figure 3.3). This appeared as more

intense staining of the endothelial layer in secondary glaucomas vs. primary glaucomas and normals.

Iris. Within normal iris tissue there appeared to be a low level staining of MMP-2 and MMP-9 within the cytoplasm of pigmented cells, non-pigmented cells, fibroblasts and within the endothelium of blood vessels (Figure 3.11). For the glaucoma cases, there was increased staining in these same cells (Figure 3.12) with the addition of staining within the cytoplasm of a number of inflammatory cells seen infiltrating in these secondary glaucoma cases (neutrophils, lymphocytes, plasma cells, and macrophages) (Figure 3.13). Staining increased to moderate numbers for these glaucoma cases (Table 3.3).

Ciliary body. There was a low to moderate level of staining intensity for MMP-2 and MMP-9 for both normal (Figure 3.20 and 3.21) and glaucomatous eyes within the cytoplasm of non-pigmented and pigmented ciliary body epithelium (Table 3.3). For secondary glaucoma cases, staining appeared in the cytoplasm of inflammatory cells associated with cyclitic membranes in some cases (lymphocytes, plasma cells, neutrophils and macrophages) (Figure 3.22). Primary glaucomas had low to moderate levels of staining within the cytoplasm lymphocytes seen occasionally infiltrating adjacent to ciliary body epithelium.

Iridocorneal angle tissue. For the iridocorneal angle tissue, there was a low level of MMP-2 and MMP-9 staining of trabecular cells of the corneal scleral and inner uveal meshwork in the controls (Table 3.3). There was also a less specific "dusting" of MMP staining dispersed between the ECM tissue (Figure 3.14). This was seen in very low levels for the controls but become more prominent in the glaucomatous eye. Primary glaucomas showed increased staining to low to moderate levels observed within the TM and in some primary glaucoma cases within the cytoplasm of inflammatory cells such as lymphocytes (Figures 3.15, 3.16 and 3.17)). For the

secondary glaucoma cases increased intensity of cytoplasmic staining was observed within the infiltration of inflammatory cells into the meshwork, such as lymphocytes, plasma cells, neutrophils and macrophages. These cells accumulated within the ciliary cleft as well as adhered to pectinate ligaments and at the entrance to the ICA (Figures 3.18 and 3.19).

Sclera. The sclera in normal eyes had a low to moderate level of staining for MMP-2 (Table 3.3). Staining in normal eyes occurred within the cytoplasm of neutrophils within blood vessels, endothelial cells of blood vessels and within fibroblasts within the scleral connective tissue (Figures 3.25). Staining seemed to be increased to moderate levels within these cells for glaucomatous cases (Table 3.3). In secondary glaucomatous cases, there was also staining within the cytoplasm of inflammatory cells including neutrophils and fibroblasts.

Choroid. MMP-2 and MMP-9 staining within the choroid in normal eyes occurred in low intensity within the cytoplasm of endothelial cells of blood vessels, non-pigmented cells and pigmented cells (Figures 3.25 and 3.26). Staining within these cells increased to moderate to high numbers for glaucoma cases. Cytoplasmic MMP staining of neutrophils invading the choroid was seen in several secondary glaucoma cases (Figures 3.27 and 3.28).

Retina. Staining in the retina for MMP-2 and MMP-9 occurred in low levels within the nerve fiber layer, ganglion cells, inner nuclear layer (INL) and outer nuclear layer (ONL) in normal eyes (Figure 3.29). Staining increased to moderate levels within these cells in glaucomatous eyes (Table 3.3). MMP staining was evident within neutrophils and macrophages infiltrating the retina in some secondary glaucoma cases (Figure 3.30).

Optic nerve. Staining was observed within glial cells in low intensity in normal eyes (Figure 3.1). For glaucomatous cases the cytoplasmic staining for MMP-2 and MMP-9 increased within glial cells to moderate numbers (Figure 3.32). Staining was also seen in the neuropil tissue

surrounding the axons. In a few glaucomatous cases, gitter cells stained positive for MMP-2 and MMP-9.

Lens. Staining within the lens occurred at low intensity in normal globes within lens epithelium and a diffuse homogenous fast red staining occurred in the nucleous and cortex at low to moderate levels (Figures 3.23). This homogenous background staining was more prominent within glaucomatous eyes. Staining increased to moderate levels within these regions in glaucomatous globes (Figure 3.24).

Ocular chambers. Staining was not observed in the anterior chamber, posterior chamber and vitreous chambers in normal globes (Table 3.3). For secondary glaucomas MMP staining was observed within the cytoplasm inflammatory cells consisting of neutrophils and macrophages within the anterior chamber and or vitreous in several cases. (Figure 3.33 and 3.34).

Quantitative results for immunohistochemical identification of MMP-2

Immunohistochemical MMP-2 staining within the cytoplasm of cells was expressed as cell counts for all tissues, representing the average number of cells counted in five 40X fields.

Overall globe. Overall the average MMP-2 count for the entire eye was 5.739 cells/40X for control eyes and was 10.51 cells/40X for glaucomatous eyes (Table 3.4). This overall count was significantly increased for glaucomatous eyes (P=0.037). Further classification of the glaucomas as primary and secondary revealed that overall count of control MMP-2 (5.739cells/40X) was significantly less than overall count for secondary glaucomas (15.54 cells/40X) (P=0.0058.) However, overall average control MMP-2 count was not significantly less than that for the primary glaucomas (3.8cells/40X).

Iridocorneal angle tissue. Within the iridocorneal angle tissue (pectinate ligaments, ciliary cleft tissue, containing the trabecular meshwork) overall MMP-2 staining for glaucomas

(18.57cells/40X) was elevated above the controls (13.4cells/ 40X), however this elevation was not significant. Further, when the glaucomas were broken down among primary and secondary it was found that normal MMP-2 staining (13.4cells/40X) was decreased compared to secondary glaucomas (28.69cells)/40X but this increase was not significant. Also, MMP-2 staining was not found to be elevated for the primary glaucomas compared to control eyes.

Iris. For the complete iris: MMP-2 staining was elevated in the glaucoma samples (9.68cells/40X) vs. control (4.20cells/40X) but this elevation was not significant.

Cornea. For the complete cornea: MMP-2 staining in glaucomatous eyes (12.4cells/40X) was raised but not significantly compared to the controls (7.3cells/40X).

Other ocular tissue. MMP-2 counts were elevated (but not significantly) for glaucomatous eyes vs. control eyes within the sclera, choroid, ciliary body, retina, optic nerve, lens, anterior chamber and vitreous. For secondary glaucomas only there was staining for MMP-2 within anterior chamber and or vitreal chambers within inflammatory cells. (Table 2).

Quantitative results for immunhistochemical identification of MMP-9

Immunohistochemistry staining within the cytoplasm of cells was expressed as cell counts for all tissues, representing the average number of cells counted in 5 40X fields.

Overall globe. Overall the average MMP-9 count for the entire eye was 2.921cells/40X for control eyes and was 14.95 cells/40X for glaucomatous eyes (Table 3.4). This average overall count for all the tissues was significantly increased for glaucomatous eyes (P=0.00000407). Further classification of the glaucomas as primary and secondary revealed that the overall ocular count of control MMP-9 (2.921 cells/40X) was significantly less than overall count for secondary glaucomas (21.963 cells/40X) (P=0.00000449) However, overall control MMP-9

count (2.921 cells/40X) was not significantly less than that for the primary glaucomas (5.59cells/40X).

Iridocorneal angle. Within the iridocorneal angle tissue (pectinate ligaments, ciliary cleft tissue) overall MMP-9 staining for glaucomas (25.94 cells/40X) was elevated significantly above the controls (2.06 cells/40X) (Table 3.4). Further, when the glaucomas were broken down among primary and secondary it was found that control MMP-9 staining (13.4 cells/ 40X) was decreased significantly compared to secondary glaucomas (42.783/ 40X) (P=0.00102). MMP-9 staining was not elevated for the primary glaucomas compared to control eyes.

Iris. For the complete iris: MMP-9 staining was significantly elevated in the glaucoma samples (0.477 cells/ 40X) vs. controls (12.029 cells/40X) (P=0.04) (Table 3.4).

Cornea. For the complete cornea: MMP-9 staining in glaucomas (16.58 cells/ 40X) was raised significantly compared to the controls (4.44 cells/40X) (P=0.007).

Other ocular tissue. MMP-9 counts were elevated (but not significantly) overall for glaucomatous eyes vs. control eyes within the sclera, choroid, ciliary body, retina, optic nerve, lens , anterior chamber and vitreous. For secondary glaucomas only there was staining for MMP-9 within anterior chamber and or vitreal chambers within inflammatory cells. (Table 3.5).

Quantitative results for immunohistochemical comparison of MMP-2 and MMP-9

Entire globe. A comparison for overall staining in the entire eye was performed to compare MMP-2 and MMP-9 staining in control eyes. Overall average MMP-2 staining (5.739cells/40X) was increased compared to MMP-9 staining (2.921cells/40X) within control eyes (P=0.0174) (Table 3.4). A comparison for overall staining in the entire eye was performed to compare MMP-2 and MMP-9 staining in glaucomatous eyes. Overall average MMP-2 staining

(10.51cells/40X) was decreased compared to MMP-9 staining (14.95cells/40X) in glaucomatous eyes, but not significantly.

Histogram densitometry of Trabecular Meshwork (TM) in control vs. glaucomatous canine eyes

Histogram densitometry was performed over the region of the corneoscleral meshwork and uveal trabecular meshwork to access the more intense staining qualitatively observed within the extracellular matrix (ECM) of glaucomatous trabecular meshwork (TM) vs. control TM tissue (Table 3.6). This ECM MMP staining was dispersed and not necessarily confined within any cells and appeared as increased background staining within the ECM. Staining was quantified in the blue, green and red color range for both MMP-2 and MMP-9 staining. A 40X field was centered over the corneal scleral and uveal meshwork using the termination of Descement's membrane and operculum as a landmark that was consistently placed in the top portion of the circular field and the field was centered over the corneal corneoscleral meshwork. The average densitometry measurements were increased in glaucomatous vs. control eyes. However, we found no significant statistical difference between measured color intensity for blue, green and red color spectrum between control and glaucomatous eyes for both MMP-2 and MMP-9.

Antibody specificity/Western blot analysis

Western blot analysis using goat anti-human MMP-2 and MMP-9 confirmed positive MMP bands that lined up against the human MMP-2 and MMP-9 controls (Figures 3.35 and 3.36). Positive MMP-2 and MMP-9 bands were found to be present within iridocorneal angle tissue and aqueous humor samples. This supports specificity of anti-human MMP-2 and MMP-9 antibodies towards MMP-2 and MMP-9 within canine ocular tissue.

Discussion

MMPs have been identified along the aqueous outflow pathway in humans. In this study, immunohistochemistry revealed a significant increase in staining intensity for MMP-2 and MMP-9 throughout all major tissues in the eye for all glaucomatous cases (primary and secondary glaucoma), over control eyes. In previous human studies, immunohistochemistry has been utilized to identify MMPs within trabecular meshwork tissue. MMP-1 was localized within the normal human ICA outflow pathway through peroxidase immunohistochemistry to reveal immunoreactivity within the ciliary body, iris, trabecular meshwork and cornea.[8] The most intense immunohistochemical staining was localized within the iris and ciliary body (smooth muscle cells, pigmented & non-pigmented cells), moderate staining was located within the corneal (epithelial cells and endothelium) and light to moderate staining was observed in the trabecular meshwork. [8] In another study, expression and distribution of MMPs were localized within the human iris and ciliary body via immunohistochemistry.[13] In the ciliary body, staining for MMP-1, 2, 3, 9 was observed to be present in the following order of intensity: non-pigmented epithelium > ciliary muscle > pigmented epithelium> stroma cells. [13] Within the iris order of staining intensity was observed as follows: anterior border> anterior epithelium> stromal cells> posterior epithelium.[13]

Dysregulation of MMPs may be associated for the development of glaucoma. Human research supports this theory as it has been shown that abnormalities in MMP activity are associated with the development of primary open angle glaucoma (POAG) and exfoliation glaucoma (ExG).[15, 19, 34, 37, 39] MMP-2, MMP-3 and MMP-9 have been localized within the human normal TM and in POAG and ExG eyes.[42] Increased staining intensity was seen in

glaucomatous eyes vs. normal human eyes. [42] Staining was found to be intracellular (within TM), extracellular (within ECM) and in homogenous (non-specific).[42]

At the same time, alterations in MMP activity may be contributing to similar pathology in secondary glaucomas. MMPs also become elevated in uveitis-related secondary glaucomas reflecting that increased MMP activity can be seen also with inflammatory disease and may be associated with development and complications of secondary glaucoma.[15, 43 16] In humans, MMP-2 and MMP-9 activity and distribution has been shown to differ for normal eyes vs. eyes with POAG, ExG and secondary glaucoma.[15, 19, 22, 31, 31, 37, 39] These MMPs are believed to influence the aqueous outflow dynamics through their effects on the trabecular meshwork.[1,3,4,8,13,14,16,21] Our recent findings show that MMPs are also present within the eyes of dogs. We determined the MMP activity within the canine ICA for normal vs. glaucomatous canine eyes using substrate gelatin zymography. In this current study, localization along the aqueous humor pathway has been performed using immunohistochemistry to further determine the distribution of MMPs within the tissues for control vs. glaucomatous canine eyes.

Zymography data determined that MMP-2 and MMP-9 are active within normal canine iridocorneal angle tissue. Within the iridocorneal angle, latent MMP-2, latent MMP-9 and active MMP-2 were found to be increased within glaucomatous eyes. Using immunhohistochemical techniques, we further identified both MMP-2 and MMP-9 within the iridocorneal drainage angle tissue, as well as within neighboring ocular tissues for both control and glaucomatous canine eyes The focus of this study was to further compare the distribution of previously identified canine MMP-2 and MMP-9 within the ICA tissue between normal and glaucomatous canine eyes to gain more knowledge about MMP involvement in the glaucomatous pathologic process. In this study, indirect immunohistochemistry (alkaline phosphatase-fast red detection

system) in conjunction with light microscopy was utilized to further localize the MMPs within the iridocorneal angle tissue at the cellular level. We also compared the distribution of MMPs within the aqueous outflow tissue to other neighboring ocular tissues. Quantitative light microscopy counts of stained cells were performed in conjunction with histogram densitometry of the trabecular cornealscleral and uveal trabecular outflow tissue.

Secondary glaucoma overall tissue staining for MMP-2 was significantly increased but this overall staining was not significantly increased in primary glaucomas. This increase was largely due the increased staining within inflammatory cells (mainly neutrophils). Within the iridocorneal angle tissue MMP-2 positive cell counts were numerically increased for glaucoma over normal, but this count was not statistically significant. Although numerical counts for the staining of the TM cells was increased in the secondary glaucomas, this elevation was not statistically significant. In all remaining ocular tissues MMP-2 counts appeared to be increased, although this increase was not statistically significant. Average MMP-9 staining within all tissues was increased in glaucomatous eyes when compared to control tissue. MMP-9 cell counts were increased significantly in glaucomatous secondary glaucoma vs. control tissues. Increased staining within trabecular meshwork tissue was also significantly increased for MMP-9 secondary glaucomas. MMP-9 was significantly elevated within the iris and cornea for all glaucoma cases. MMP-2 was found to be increased within the iris and cornea for glaucomatous cases but not statistically significant. MMP-9 counts became elevated for sclera, ciliary body, chorid, retina, ONH and lens. MMP-2 levels became elevated in sclera, choroid, retina, ONH and lens. Anterior chamber and vitreous MMP-2 and MMP-9 staining occurred within the cytoplasm of inflammatory cells for secondary glaucomas cases. Qualitative interpretation of tissue staining clearly showed increased MMP staining intensity within ocular tissues of

glaucomatous eyes. Although increased the numerical counts were not always found to be statistically significant. This could very much reflect sample size.

In a comparison of the IHC staining intensity to the zymography results, we found a parallel increase in both MMP-2 and MMP-9. Zymography confirmed increased latent MMP-9 and active and latent MMP-2 within glaucomatous tissues. This parallels the increased staining of MMP-9 and MMP-2 found within our iridocorneal angle tissue. Zymography is very sensitive and can often pick up picagram levels of enzyme activity present. This may explain why the MMP-2 activity was found to be significantly elevated by our zymography but the numerical counts although elevated, were not always statistically significant in many of our tissues using immunohistochemistry technique. IHC counts reflect all MMPs, regardless of whether active or inactive. Our IHC counts reveal that total MMP-9 staining amounts do increase significantly in glaucoma over controls for overall globe, ICA tissue, cornea and iris. Total MMP-2 staining increased significantly for average overall staining of globes in glaucoma vs. controls. It is possible that in glaucoma in dogs, the increase in MMP amounts may be less relevant than the increase in MMP activation. Within the ICA, MMP up-regulation occurs by increased synthesis and increased activation of MMPs by the TM cells. It has been found that cell cultures which are stressed and strained (to mimic increased IOPs) show a sustained increased levels protease activity over a few days. [3] Activation of MMPs can result in a cascade of activation of MMPs each one further activating each other. MMP-2 will activate MMP-1 to activate MMP-9 or MMP-2 can activate MMP-9 directly.

When we separate the primary glaucomas from the secondary we found more pronounced staining of the secondary glaucomas. IHC staining was useful in confirming the location of MMPs within tissues of the TM cells, within inflammatory cells and within the extracellular

matrix of the trabecular meshwork. The background staining of the ECM was found to be increased within the glaucomatous eyes over the normal. Subjectively the ECM of the corneal scleral meshwork and uveal meshwork appeared to have a denser patchy fast red staining. However, although this appeared to be qualitatively increased upon light microscopic examination, our densitometry analysis of the cornealscleral TM revealed that this subjective increased staining of the ECM was not necessarily statistically significant. Although not significant, densitometry measurements were elevated so we might expect that with a larger sample sizes that these p- values could become significant.

Increased staining levels of MMP-2 and MMP-9 found for these chronic cases could represent a physiologic response to chronically increased pressures. MMP-2 and MMP-9 cause degredation and turnover of the meshwork and may be increasing trabecular meshwork outflow. However, this response is not able to fully compensate when the disease process is so far advanced. We still need to investigate the activity of these enzymes early in the disease process, for example in goniodysgenic dogs. Looking at the MMP activity levels throughout stages of the disease process would help to complete the overall picture. However, it is often difficult to obtain eyes earlier than the chronic cases as owners often desire to treat medically or surgically until the cases become chronic and non- responsive. We can only speculate that a deregulation of MMPs early on could predispose for glaucoma. It is important to mention that MMP-2 and MMP-9 are not entirely specific for glaucoma and have been shown to become elevated in uveitis cases in people. MMPs become expressed with other types of inflammatory disease. To support this we found elevations of these proteases within our secondary inflammatory glaucomas. This was evident as we saw increased staining within inflammatory cells within the

iris, cornea, and iridocorneal angle. With uveitis cases we might also expect to find MMP staining within inflammatory cells.

One of the limitations of this study is that we only were able to determine an association between MMP levels, localization and activity, in disease versus normal eyes. We were not able to determine if a causal relationship exists between elevated MMP staining and glaucoma or vise versa. It is difficult to lo at intraocular pressure (IOP) retrospectively and determine its effects on MMP levels, since we only have limited IOP measurement at sampling. Future prospective studies need to investigate IOPs and MMPs activity throughout the disease process. Looking at the progression of MMP activity in relation to IOPs would further shed light on the role that these proteases are playing and link them to trabecular resistance changes.

Specific polyclonal antibodies against canine MMP-2 and MMP-9 were not available at the time of this study and so we had to use anti-human MMPs. Since we did not know the specificity of anti- human MMPs antibodies toward the canine MMPs, Western blots were run to confirm this specificity. We are confident of the specificity of our antibodies to binding canine MMPs. MMPs are fairly consistent across species so we would expect that canine MMPs should bind to polyclonal antibodies made in other species. This assumption was confirmed by our Western blots. One major limitation of this study is that our sample numbers were limited by the numbers of obtainable enucleated eyes from clinical cases. A large majority of enucleations are performed at the regular veterinarians and not referral centers. This limited the number of clinically enucleated cases obtained. Also cases were usually of chronic duration as most owners elect to enucleate after prolonged unsuccessful medical treatment

We have confirmed the presence of matrix metalloproteinases MMP-2 and MMP-9 within the iridocorneal angle tissue using immunohisotochemistry and have further localized

MMPs within trabecular meshwork cells, the ECM, and inflammatory cells seen within the iridocorneal angle. In addition, MMP staining was evident within adjacent ocular tissues within the eye. MMP-2 and MMP-9 are present in normal and glaucomatous ocular tissues. The spectrum of MMP staining was found to differ between normal eyes and the diseased state for ICA tissue as well as neighboring tissues. Basal levels of MMPs-2 and MMP-9 staining were found highest in the ciliary body, iris, cornea and sclera in normal eyes. Lighter MMP staining was found in the iridocorneal angle, choroid, retina, ONH and lens. This basal level staining appeared to increase within glaucomatous tissue to highest levels within the cornea, ICA, ciliary body, iris, choroid and retina. Increased MMP-9 staining was most significantly increased for glaucomatous eyes within the cornea, iris and iridocorneal angle. MMP staining became most evident within the cytoplasm of inflammatory cells for secondary glaucoma cases. This imparted increased staining to the secondary glaucoma cases. These inflammatory cells appeared throughout tissues of the cornea, sclera, iris, ciliary body, choroids, TM, retina, optic nerve, lens and anterior and vitreal chambers in cases. Interestingly, our zymography data showed that there was no difference in the activity elevations above normals between primary and secondary glaucomas. MMPs became elevated equally for both primary and secondary glaucomas. Although there appears to be quantitatively more staining of MMPs in secondary glaucomas it is the activity of the MMPs which is most relevant. The MMP activity not the total quantity determines the role of these enzymes within the tissue. This IHC study supports our previous research which utilized zymography to determine the activity within the aqueous humor and iridocorneal angle tissue for glaucoma and for both primary and secondary glaucoma cases. Future prospective investigations need to determine if the increased MMP activity and increased

MMP accumulation within tissues associated with glaucoma is the result of the disease process or a direct trigger of the disease.

CHAPTER 4

CONCLUSIONS

Human research has shown that a regulatory feedback loops exists between the MMPs and their inhibitors, the tissue inhibitory metalloproteinases or TIMPs. [3] When TM cells detect distortion they up-regulate MMP-2 and MMP-9 activity and help restore aqueous outflow.[24, 31] After outflow is restored the TIMPs become up-regulated and bind up the MMPs sites to again curb the MMP activity.[24] This regulation is responsible for the daily trabecular meshwork resistance maintenance.

We have shown that in the canine eye there is a basal activity of MMP-2 and MMP-9 present within normal tissue of the trabecular meshwork (TM). Specifically, within our iridocorneal angle tissue we found latent MMP-2 and latent MMP-9 present. These MMPs are present but in an inactive form and are ready to be activated if needed. We speculate that their role is one of degrading extracellular matrix (ECM) material and regulating the normal daily turnover of the meshwork, similar to what was found in human studies. MMPs are also likely to play an adaptive role in restoring some aqueous outflow within the disease state of glaucoma.

Activation and regulation of MMPs within the human eye has been determined to be a tightly regulated event. With regards to activation of MMPs, certain MMP activator proteins have been found such as MMP activator protein 1 (AP-1).[16,31] Increased MMP production was found dependent on this activator protein. Further, exogenous substances that activate AP-1 have been found to activate MMPs.[16,31] Growth factors, interleukins and prostaglandins in the aqueous humor may also influence MMP activity in the TM. [46] Interleukin -1 and TNF have been shown

to increase MMP expression in the trabecular meshwork over TIMPs.[30, 32, 35, 45] TNF alpha has been shown to effect activities of MMPs and TIMPs in the TM, via the ERK-MAP kinase pathway and protein kinase C [24,25] and TNF alpha and IL-1 via the c-Jun kinase transduction pathway. [32] This supports a series of intracellular trabecular cellular signaling events upstream before MMPs are secreted and activated. Prostaglandins have been shown to increase the activity of MMPs 1, 2, and 3 in the monkey uveoscleral outflow pathway[9] and to also increase trabecular meshwork outflow facility in cultured human anterior segments.[28] With regards to inhibition of MMPs, it has been shown that TGF-beta 2 and plasminogen activator inhibitor -1 inhibit activity of MMP-2 within human TM cells.[22] Inhibition of MMP-2 activity by TGF-beta 2 inhibitor may lead to accumulation of ECM in the TM of human glaucomatous eyes. These MMP activation and regulatory pathways within the dog still need to be elicited. We speculate that abnormalities in any of these pathways could lead to altered MMP activity within glaucomatous globes.

Within our glaucoma tissue samples, we found that MMP-2 latent form was increased and that MMP-9 became active. For primary and secondary glaucomas both MMP-2 and MMP-9 became elevated. The aqueous humor pool of MMP-2 increased in the diseased state of glaucoma. This could represent a spillover from the diseased tissue or a direct source of MMP-2 circulating to the diseased tissue. Increased levels of MMPs in the aqueous humor paralleled increase activity of MMPs within the iridocornal angle tissue. Immunohistochemistry further indicated that there is a normal basal level of MMP staining throughout most ocular tissues of all three tunics of the eye. This normal basal immunostaining increases with glaucoma. Staining for MMP-2 and MMP-9 becomes most evident when there is infiltration by inflammatory cells seen within secondary glaucomas. This supports the notion that when MMPs become abnormally active they could be involved in tissue damaged associated with in the disease process of

glaucoma. Also, that inflammation can serve as a source of increased MMP activity within eyes with glaucoma.

The role of MMPs in the development of glaucoma in relation to the TM abnormalities within goniodysgenic dog breeds still needs to be determined. MMP activity needs to be tightly regulated as disease may be associated with abnormally increased or decreased MMP activity. Excessively elevated levels of MMPs in chronic glaucoma could lead to angle closure and clilary cleft collapse. At the same time expression of TIMPs over MMPs could lead to ECM accumulation and increased trabecular resistance. An early deregulation of TIMPs expressed over tissue MMPs could be contributing ECM accumulation in primary open angle glaucomas (POAG) and PL dysplasia or mesodermal failure of rarifaction seen with goniodysgenesis. At the same time we also hypothesize that an overexpression of MMPs could be leading to trabecular meshwork or clilary cleft collapse in primary angle closure glaucoma (PACG) in the dog. Chronic up-regulation of these proteases in tissue would lead to tissue damage, decreasing the viability of the TM cells. Excess MMP activity could cause tissue destruction of the ECM and support for the TM cells. In theory, this could lead to the ECM disintegrating and cause the TM collapse.

There are no specific therapies that target changing the trabecular meshwork resistance via MMP modification. MMP induced meshwork remodeling and trabecular resistance can serve to be future targets for therapy. Manipulation of trabecular meshwork and MMP activity has been shown to increase outflow rates in humans through exogenous substances, for example Selenium induced secretion of endogenous MMP-2 by trabecular meshwork cells. [7] Alteration of the adenosine A_1 receptor in TM cells can stimulate secretion of MMP-2 from the trabecular meshwork, indicating the possibility of drugs that target the MMP secretion pathway. [20] Genetic

engineering has been performed using recombinant adenovirus vectors to insert genes encoading for MMPs into trabecular meshwork cell cultures and the trabecular meshwork of laboratory animals.[12,33] Gene therapy could prove to be beneficial in preventing impediment of trabecular outflow. In the future, there may be the potential for MMP gene therapy for glaucoma in both animals and people. Also, the potential for therapy that targets susceptible eyes early on in the disease process before pressure increases or aqueous humor resistance occurs. Prior to this the complete spectrum of MMP activity need to be determined for goniodysgenic and early glaucomatous globes.

These studies are the first step in identifying the normal and glaucomatous MMP level differences in the canine eye to further gain more knowledge about the pathologic process in this species.

Table 2.1: Clinical findings for sample populations

Samples	**Breeds**	**Av. Age (years)**	**Gender**	**# Eyes**	**Av. IOP (mmHg)**	**Duration of glaucoma**	**Average # medications used**	**Prostaglandin F2α use**
32 normal (aqueous humor)	5 Pit Bull 2 Chow X 2 Hound X 2 Labrador X 2 Shepherd X 2 Husky X	1.93 years	9 FI 1 FS 1 MI 5 MN	16 OS 16 OD	13.73 mmHg	NA	NA	NA
16 normal (iridocorneal angle)	5 Pit Bull 2 Chow X 2 Hound X 2 Labrador X 2 Shepherd X 2 Husky X	1.93 years	9 FI 1 FS 1 MI 5 MN	8 OS 8 OD	14.063 mmHg	NA	NA	NA
26 glaucoma (aqueous humor)	<u>21 Primary</u> 7 Cocker 3 Chow Chow 1 Shar-Pei 1 Basset 1 Shibu-Inu 2 Cock-a-poo 1 Grey Hound 1 Maltese 4 Mix breed <u>5 Secondary</u> 1 Pit Bull 1 Golden Ret. 1 Labrador Ret. 1 Bulldog 1 mix breed	8.5 years	12 FS 12 MN 2 MI	14 OS 12 OD	37.04 mmHg	21 (Chronic) 3 (Subacute) 2 (Acute)	3 different medications	1-2 times a day (average=1.2)

Samples	**Breeds**	**Av. Age (years)**	**Gender**	**# Eyes**	**Av. IOP mmHg**	**Duration of glaucoma (days)**	**Average # medications used**	**Prostaglandin F2α use**
5 glaucoma (iridocorneal angle tissue)	<u>3 Primary</u> 1 WHWT 1 Mixed breed 1 Chow Chow <u>2 Secondary</u> 1 WHWT 1 Great Dane	7.55 years	3 FS 1 MI	2 OS 2 OD	27.78 mmHg	4 (Chronic) 1 (Acute)	3 different medications	1-2 times a day (average=1.5)

Table 2.2: Results of aqueous humor MMPs and total protein levels

●Aqueous humor latent MMP-2 and total protein (TP) levels

Cases	Av. latent MMP-2 (ODu*mm2)	Av. TP (µg/µl)	Increase in MMP	Increase in TP
32 Normal	3.11 ± 2.10	0.919 ± 0.60	NA	NA
26 Glaucoma	18.40 ± 8.90	10.37± 8.44	6X	11X
			(P<0.0001)	(P<0.0001)

●Iridocorneal angle tissue active MMP-2 and total protein (TP) levels

Cases	Av. active MMP-2 (ODu*mm2)	Av. TP (µg/µl)	Increase in MMP	Increase in TP
16 Normal	0.122±0.011	2.81±0.397	NA	NA
5 Glaucoma	3.82±8.32	3.83±1.59	31X	1.4X
			(P=0.023)	(P=0.0114)

●Iridocorneal angle tissue latent MMP-9 levels

Cases	Av. latent MMP-9 (ODu*mm2)	Increase in MMP
16 Normal	7.12±77.53	NA
5 Glaucoma	17.86±10.96	2.5X
	(P=0.000375)	

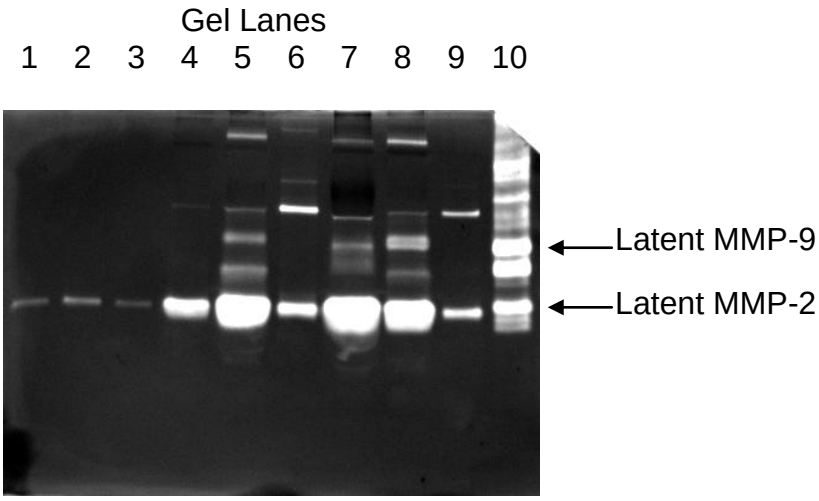

Figure 2.1: Gelatin zymography of aqueous humor

Legend:

Lanes 1-3 represent normal aqueous humor samples

Lanes 4-9 represent glaucoma aqueous humor samples

Lane 10 represents purified MMP-2 and MMP-9 positive controls

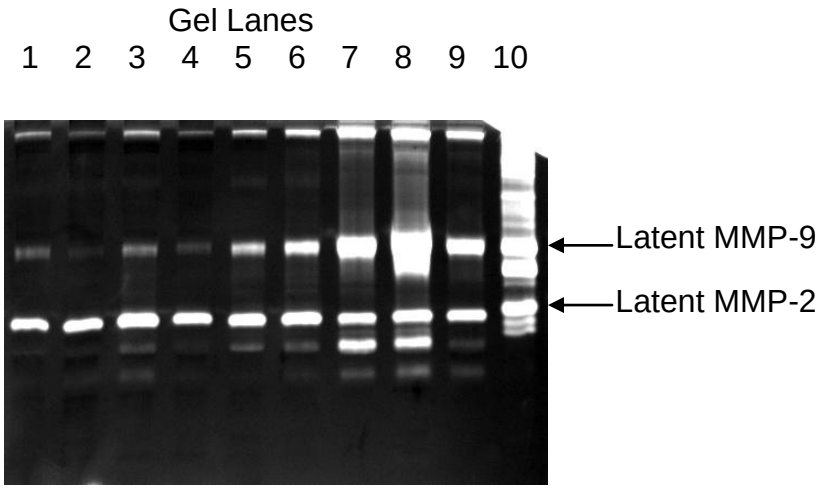

Figure 2.2: Gelatin zymography of iridocorneal angle tissue

Legend:

Lanes 1-4 represent normal iridocorneal angle tissue samples

Lanes 5-9 represent glaucoma iridocorneal angle tissue samples

Lane 10 represents purified MMP-2/ MMP-9 positive controls

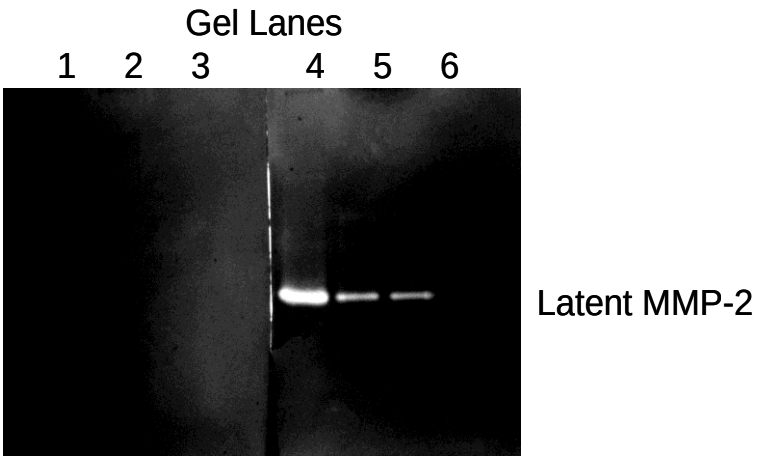

Figure 2.3: Gelatin zymography inhibition with EDTA

Legend:

Lanes 1-3: represent sample inhibition with 10mM EDTA

Lane 4: represents glaucoma aqueous sample

Lane 5-6: represent normal aqueous samples

Table 3.1: Clinical findings for sample population for immunohistochemistry

Control Samples	Breeds	Av Age	Sex		
9 Dogs/ 9 Eyes	Dachshund Labrador Retriever Wolf Hybrid KCCS Neopolitan Mastiff 2 Bulldogs 2 mix breed	6.07 years	2 F/S 4 F 3 M		
Glaucoma Samples (Chronic)	Breeds	Av Age	Sex	Type	Average IOP at enucleation
7 Dogs/ 7 Eyes	Sharpei Chow Chow Chow Chow _____ Bloodhound Labrador Shetland Sheepdog Beagle	8.1 years	6 F/S 1 M/N	3 Primary _____ 4 Secondary	41.67 ±11.07mmHg

Table 3.2: Clinical findings for glaucoma population for immunohistochemistry

Case #	*Glaucoma type*	*Breed*	*Sex*	*Age*	*Diagnosis*
#1	Primary	Sharpei	F/S	5yr	Goniodysgenisis PACG
#2	Primary	Chow Chow	F/S	9yr	Goniodysgensis PACG
#3	Primary	Chow Chow	F/S	9yr	Goniodysgenesis PACG
#4	Secondary	Bloodhound	F/S	1.5yr	Lens induced uveitis, post phacoemulsification (aphakic)
#5	Secondary	Labrador	M/N	6 yr	Immune mediated uveitis
#6	Secondary	Shetland Sheepdog	F/S	10 yr	Uveitis-toxoplasmosis
#7	Secondary	Beagle	F/S	16yr	Phacoclastic uveitis, cataract

Table 3.3: Qualitative MMP-2 and MMP-9 staining intensities

	Sclera	Cornea	Iris	Ciliary Body	Iridocorneal angle	Choroid	Retina
MMP-2 Control	++	++	+	++	+	+	+
MMP-9 Control	++	++	+	++	+	+	+
MMP-2 Glaucoma	+++	++++	+++	+++	++	++++	+++
MMP-9 Glaucoma	+++	++++	+++	+++	++	++++	+++

	ONH	Lens	AC	Vitreous
MMP-2 Control	+	+	None	None
MMP-9 Control	+	+	None	None
MMP-2 Glaucoma	+++	++	++ (secondary)	++ (secondary)
MMP-9 Glaucoma	+++	++	++ (secondary)	++ (secondary)

+=low staining, ++moderate staining, +++moderately high staining, ++++=high staining

Table 3.4: MMP staining overall globe, ICA, complete iris, & cornea

IHC staining	Globe average	ICA tissue	Complete iris	Complete cornea
MMP-2 control	5.739 (±6.7)	13.4 (±10.9)	4.20 (±4.5)	7.3 (±7.1)
MMP-2 glaucoma Primary Secondary	10.51 (±12.1) 3.8 (±4.5) 15.54 (±16.6)	18.57 (±18.9) 3.49 (±1.91) 28.69 (±25.8)	9.68 (±12.5) NE NE	12.4 (±12.5) NE NE
P value *Primary* *Secondary*	*0.037* *>0.05* *0.0058*	>0.05 >0.05 >0.05	>0.05 NE NE	>0.05 NE NE
MMP-9 control	2.921 (±4.3)	2.06 (±2.6)	0.477 (±0.73)	4.44 (±5.7)
MMP-9 glaucoma Primary Secondary	14.95 (±16.8) 5.59 (±7.38) 21.96 (±21.4)	25.94 (±31.2) 5.2 (±2.98) 42.783 (±39.1)	12.029 (±16.4) NE NE	16.58 (±16.4) NE NE
P value *Primary* *Secondary*	*0.00000449* *>0.05* *0.00000449*	*0.016* *>0.05* *0.00102*	0.04 NE NE	0.04 NE NE

Cell counts given as cells counted per 40X power fields (average of N=5 counts)

Table 3.5: Quantitative MMP staining for remaining ocular tissues

IHC Staining	Sclera	Ciliary Body	Choroid	Retina	Optic Nerve	Lens	Anterior Chamber	Vitreous
MMP-2 norm.	4.24 (±2.3)	16.36 (±15.5)	2.36 (±13.69)	5.62 (±6.34)	0.088 (±0.15)	0.844 (±1.1)	0 (±0)	0 (±0)
MMP-2 glau.	11.66 (±13.2)	19.22 (±20.3)	15.19 (±20.5)	22.31 (±27.5)	5.74 (±7.29)	5.029 (±5.17)	2.69 (±3.1)	3.46 (±4.3)
MMP-9 norm.	4.22 (±4.8)	10.2 (±14.7)	1.77 (±2.20)	5.13 (±6.7)	5.09 (±8.9)	0.78 (±1.0)	0 (±0)	0 (±0)
MMP-9 glau.	15.74 (±13.2)	20.11 (±23.8)	23.11 (±26.6)	39.23 (±33.5)	5.63 (±6.4)	5.46 (±5.2)	2.94 (±3.4)	4.71 (±5.4)

Cell counts given as cells counted per 40X power fields (average of N=5 counts)

Table 3.6: Densitometry histogram for corneoscleral uveal trabecular meshwork tissue

MMP-2	Blue	Green	Red	MMP-9	Blue	Green	Red
Control	0.17784	0.22887	0.22515	Normal	0.13857	0.17075	0.14935
Glaucoma	0.20279	0.24959	0.24750	Glaucoma	0.20149	0.19279	0.15702

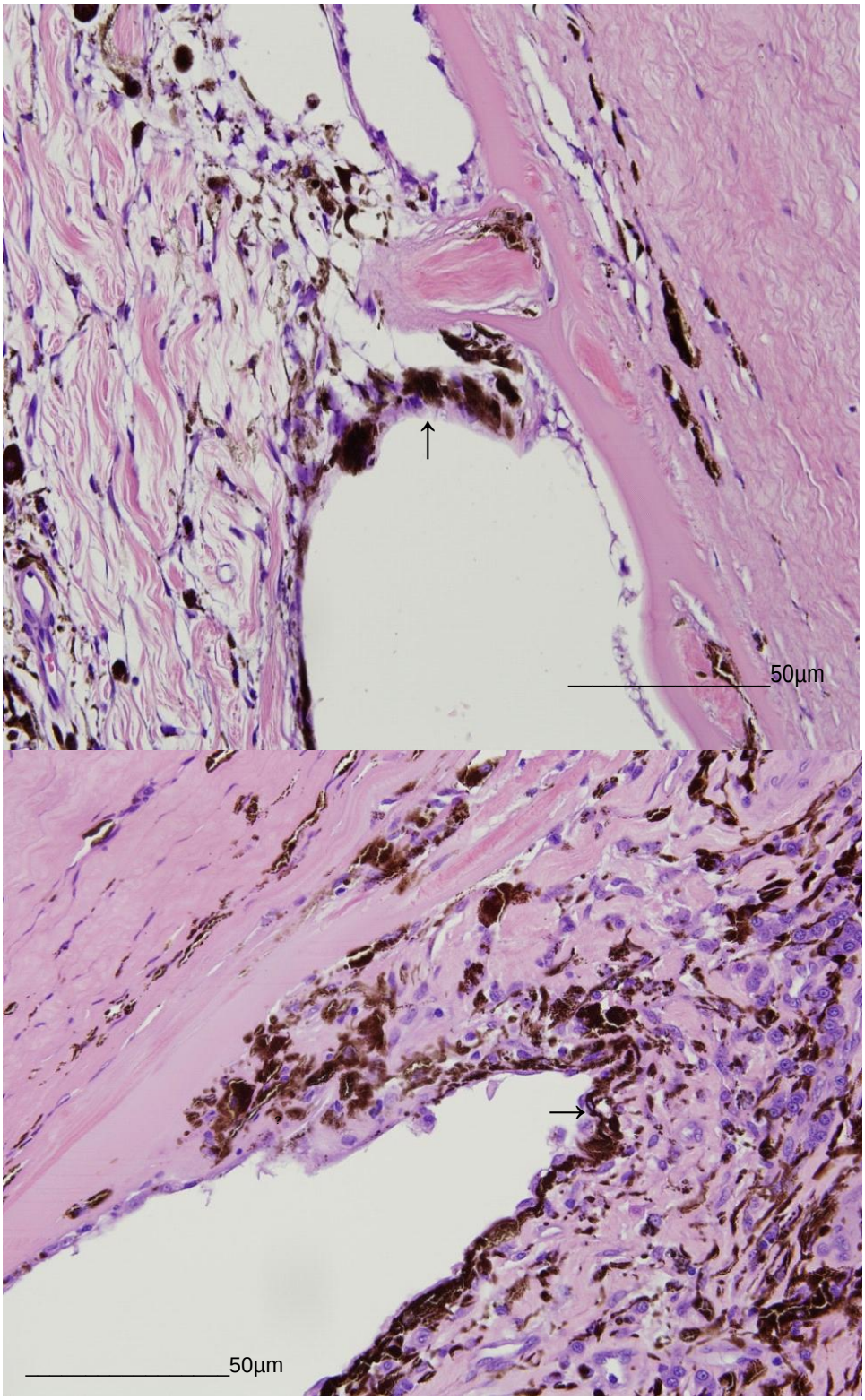

Figure 3.1: Primary Glaucoma, Iridocorneal angle
Legend: Goniodysgenic pigmented band extending across iridocorneal angle (arrows). H+E staining, 40X magnification-top & bottom.

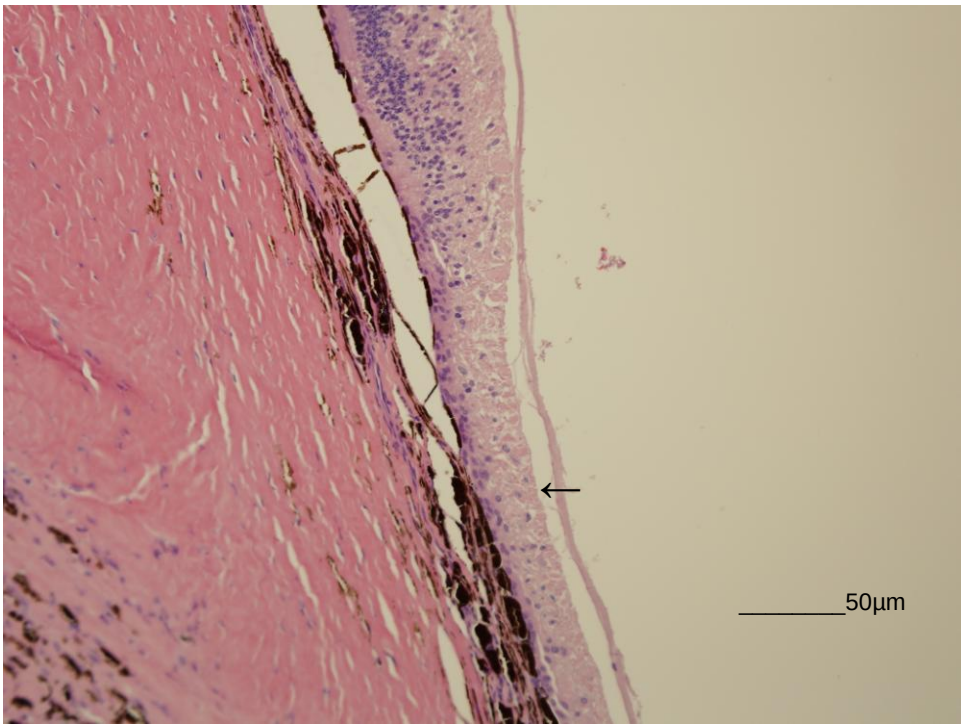

Figure 3.2: Primary Glaucoma, Retina
Legend: Retinal degeneration secondary to chronic glaucoma (arrows). H+E staining, 20X magnification.

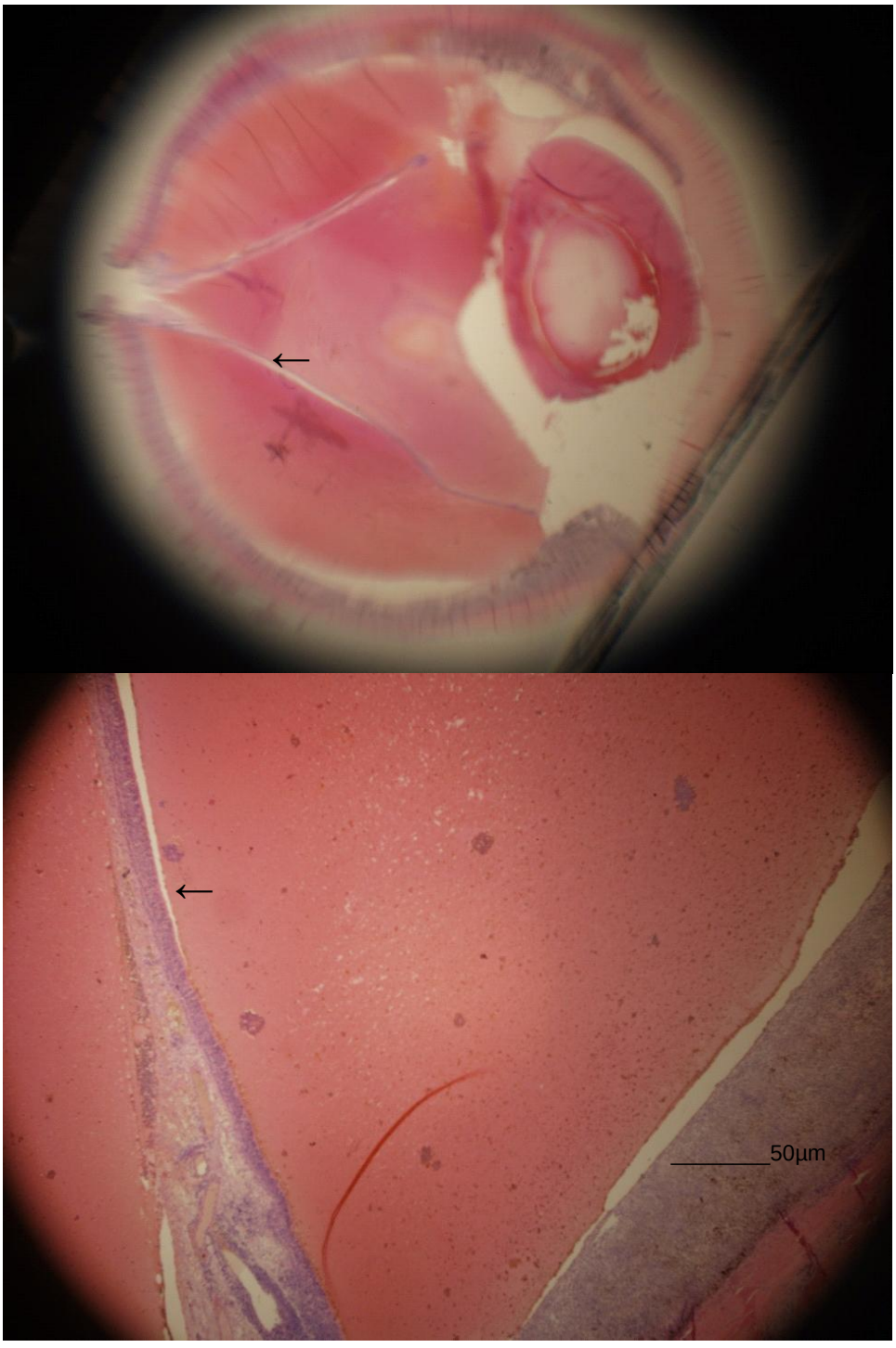

Figure 3.3: Secondary Glaucoma, Globe & Posterior segment
Legend: Globe with severe endophthalmitis with exudate filling vitreous, Arrows show retinal detachment. H+E staining, Subgross (top) & 40X magnification (bottom).

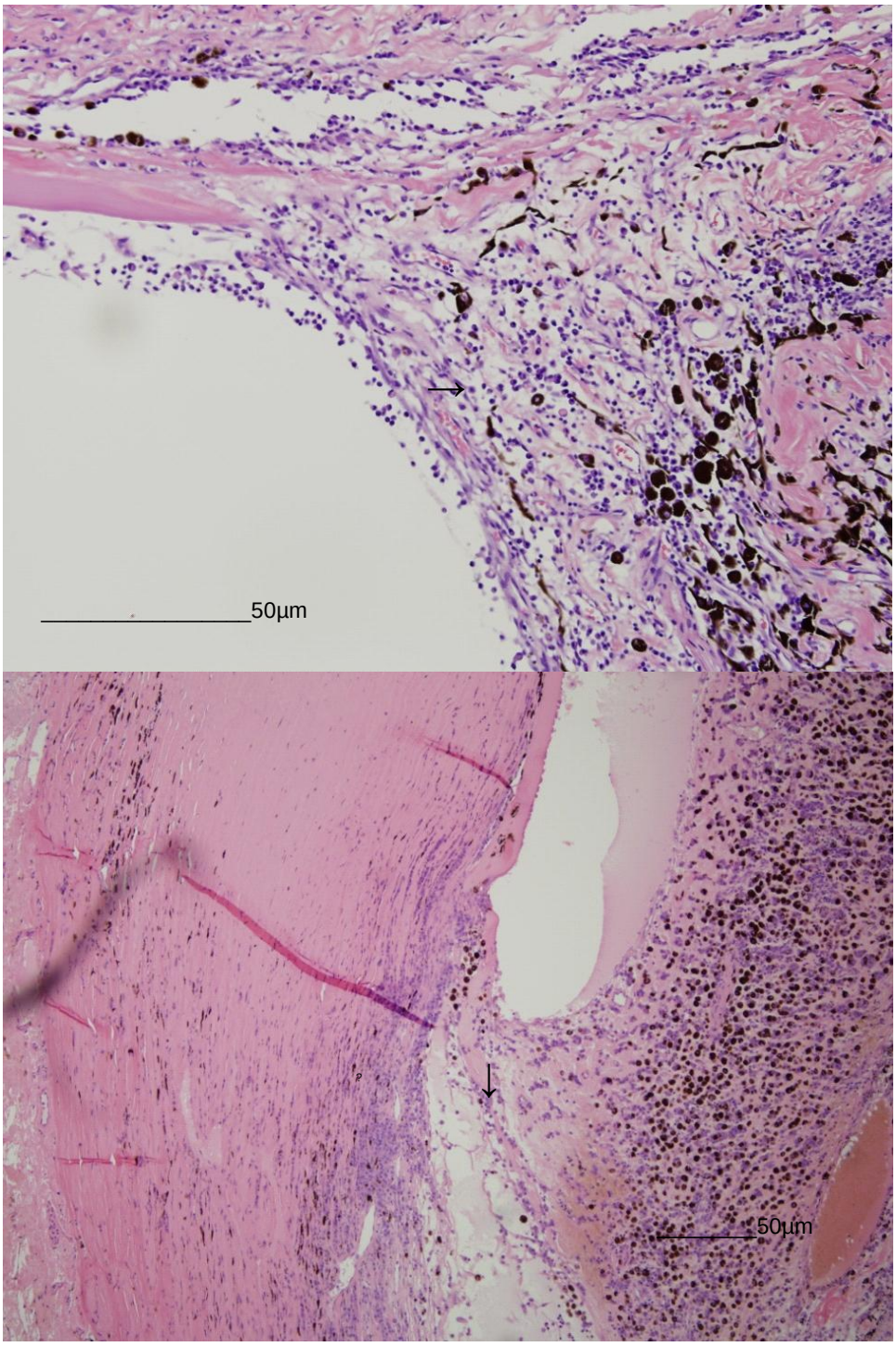

Figure 3.4: Secondary Glaucoma, Iridocorneal angle
Legend: Inflammatory cells invading the iridocorneal angle in this case of secondary glaucoma (arrows). H+E staining, 20X magnification (top), 40X magnification (bottom).

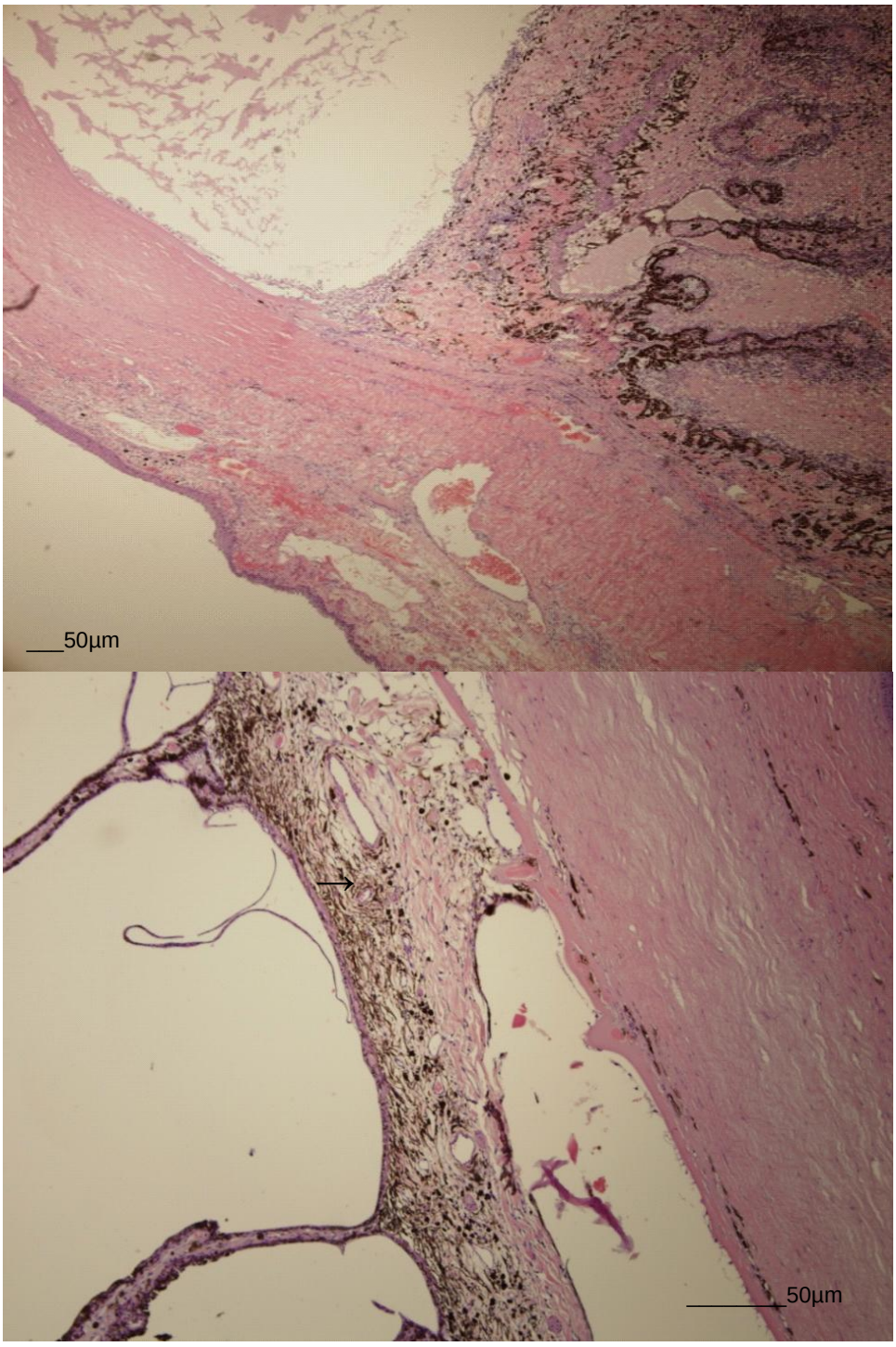

Figure 3.5: Secondary Glaucoma, Uvea
Legend: Inflammatory infiltration of the uveal tract (arrows). H+E staining, 10X magnification (top) and 20X magnification (bottom).

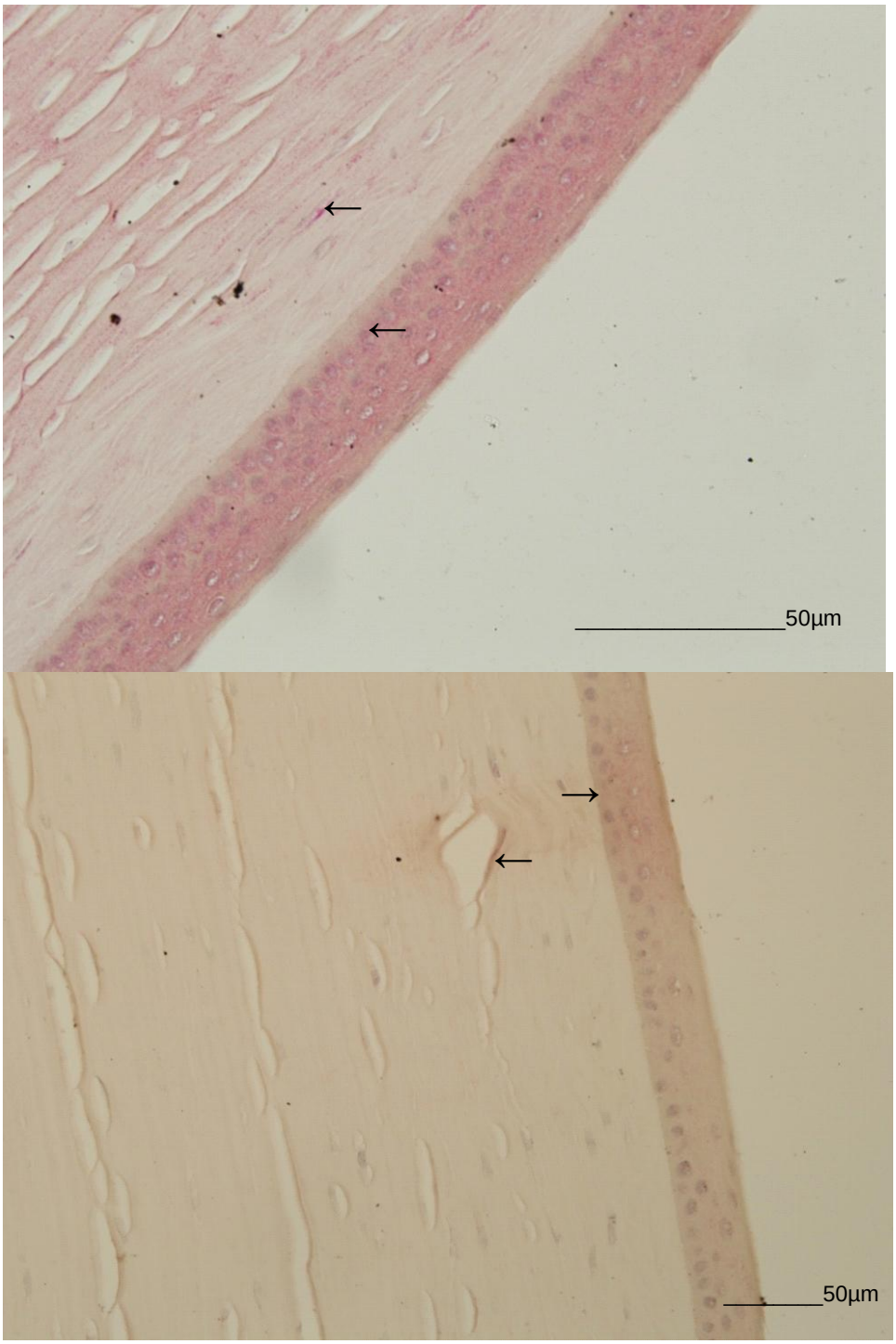

Figure 3.6: Control Cornea, IHC for MMP-2 and MMP-9
Legend: MMP staining within corneal epithelium and stroma (arrows).
IHC fast red staining for MMP-2, 40X magnification (top),
IHC fast red staining for MMP-9, 20X magnification (bottom).

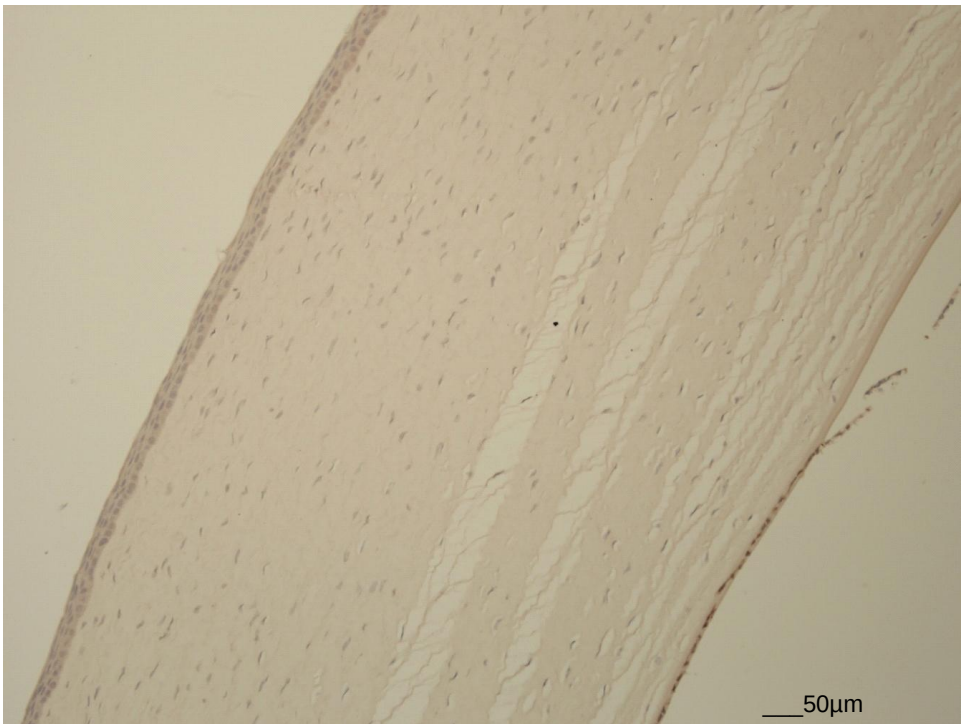

Figure 3.7: Control Cornea, IHC for MMP-9
Legend: Very low levels of staining of the cornea. IHC fast red staining for MMP-9, 10X magnification.

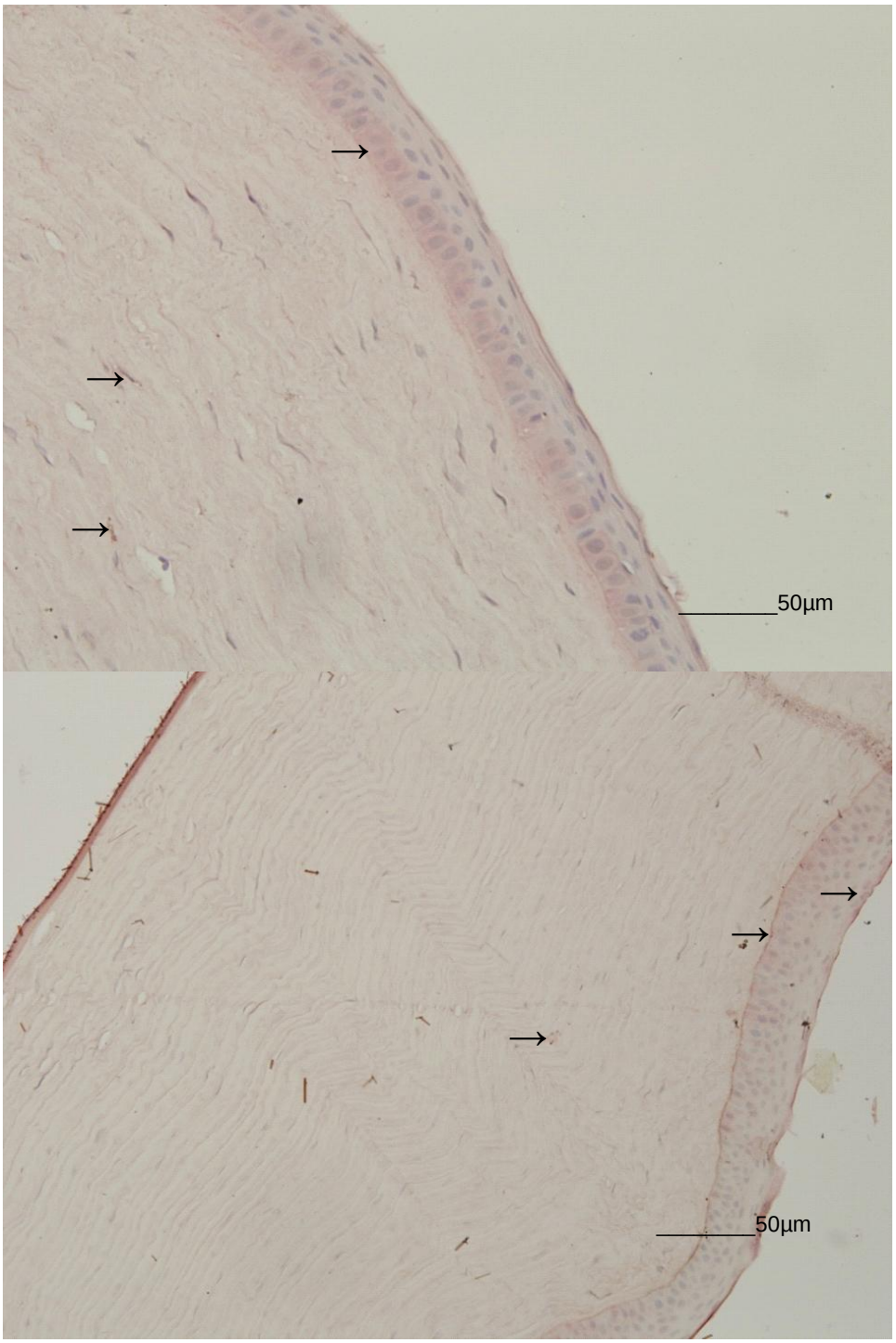

Figure 3.8: Primary Glaucoma, Cornea, IHC staining for MMP-2
Legend: Increased MMP-2 staining within basal, polygonal and squamous corneal epithelium, stromal keratocytes and corneal endothelium (arrows). IHC fast red staining for MMP-2, 20X magnification (top & bottom).

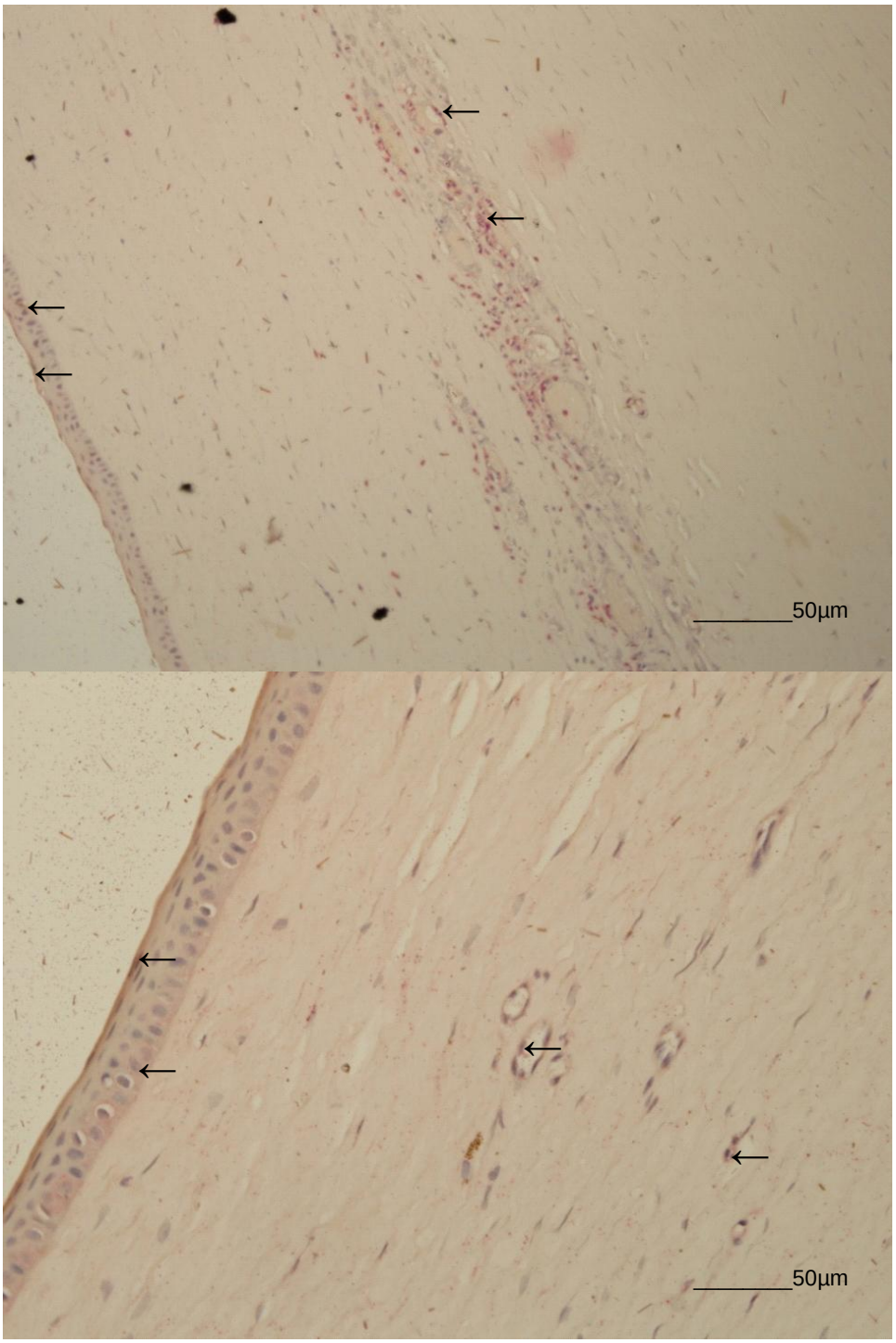

Figure 3.9: Secondary Glaucoma, Cornea, IHC for MMP-9
Legend: Increased MMP-9 staining within basal corneal epithelium, polygonal wing cells, squamous cells, keratocytes, endothelium of blood vessels and neutrophils within the corneal stroma (arrows). IHC fast red staining for MMP-9, 20X magnification (top & bottom).

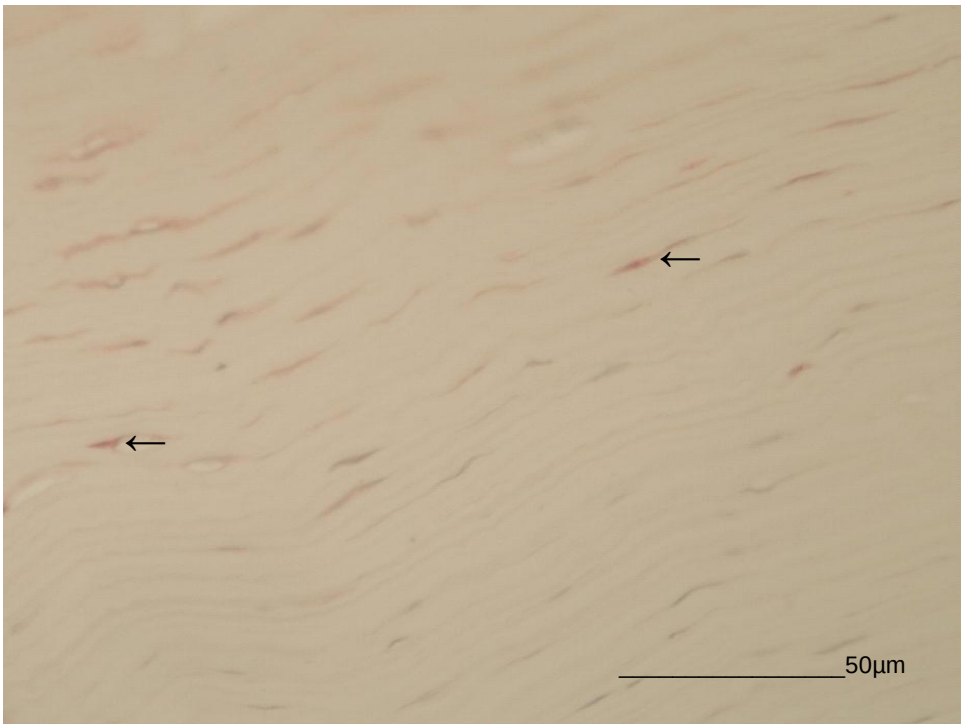

Figure 3.10: Secondary Glaucoma, Cornea, IHC for MMP-9
Legend: MMP-9 staining within the cytotoplasm of keratocytes (arrows). Fast red staining for MMP-9, 40X magnification.

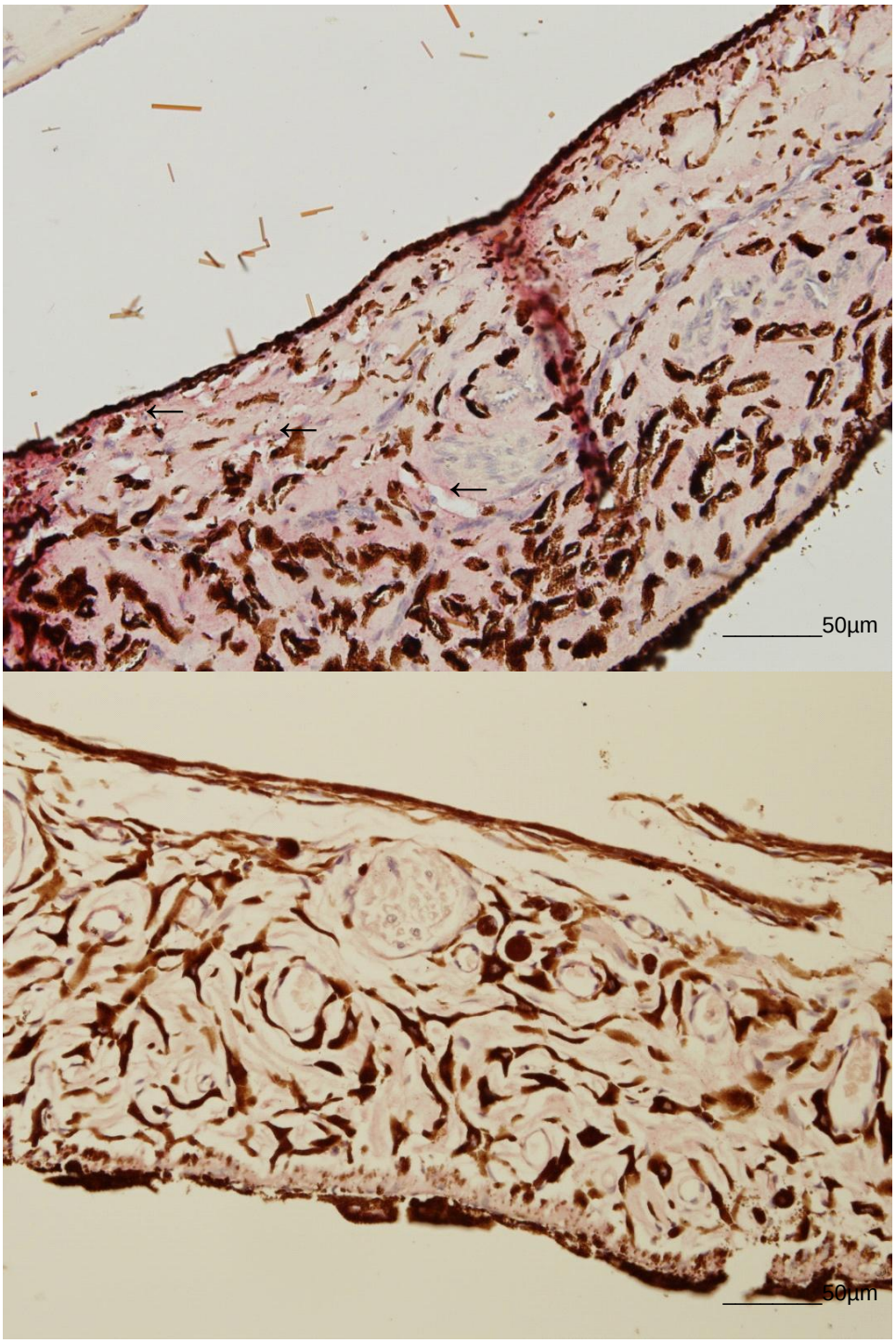

Figure 3.11: Control Iris, IHC for MMP-2 and MMP-9
Legend: MMP staining within the iris pigmented and non-pigmented cells, fibroblasts of the iris stroma and the endothelium of blood vessels. IHC fast red staining for MMP-2 (top) and MMP-9 (bottom), 20X magnification (top & bottom).

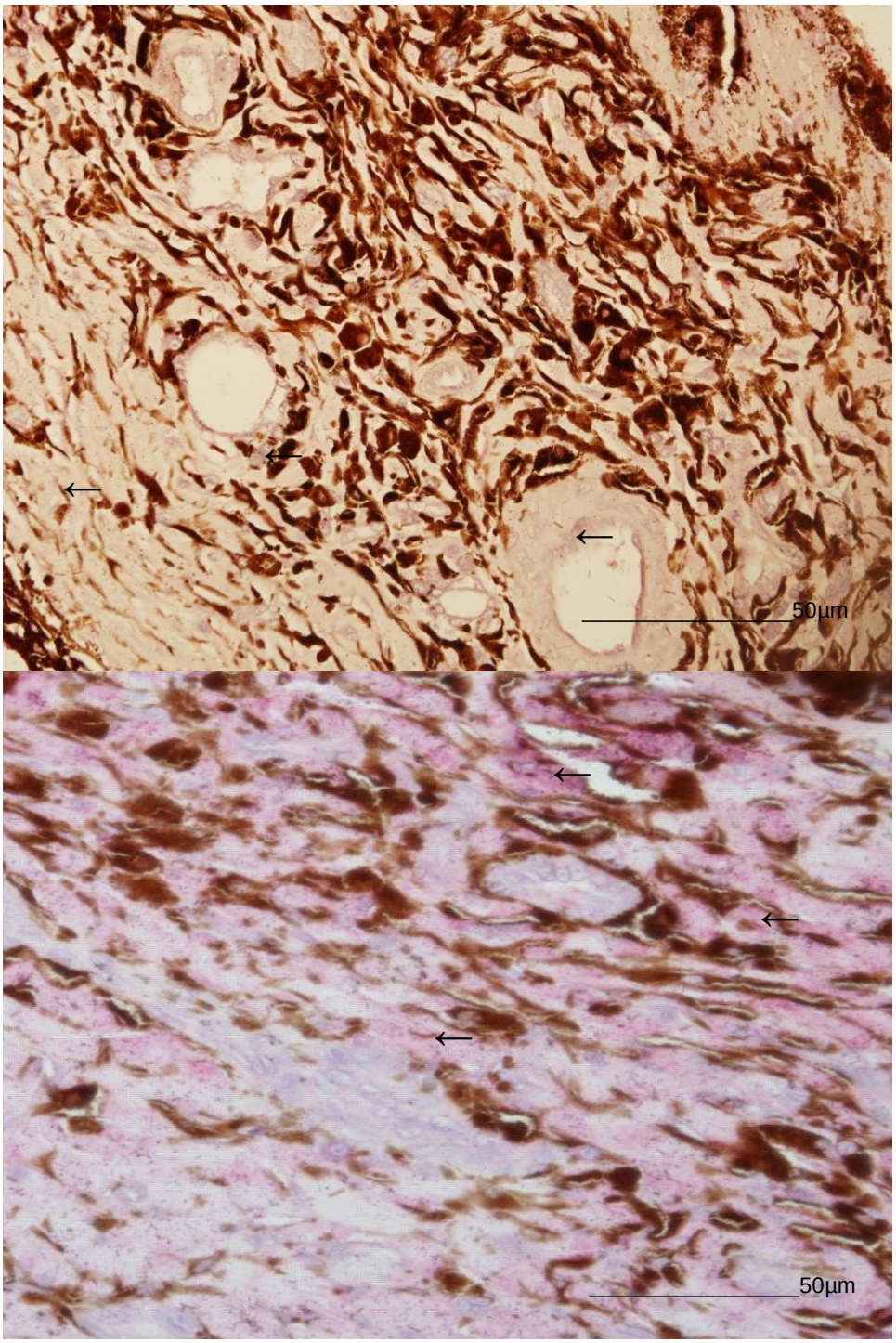

Figure 3.12: Primary and Secondary Glaucoma, Iris, IHC for MMP-2
Legend: Increased MMP staining within the iris pigmented and non-pigmented cells, fibroblasts of the stroma and endothelium of blood vessels (arrows). IHC fast red staining for MMP-2,40X magnification, primary glaucoma (top), IHC fast red staining for MMP-2, 40x magnification, secondary glaucoma (bottom).

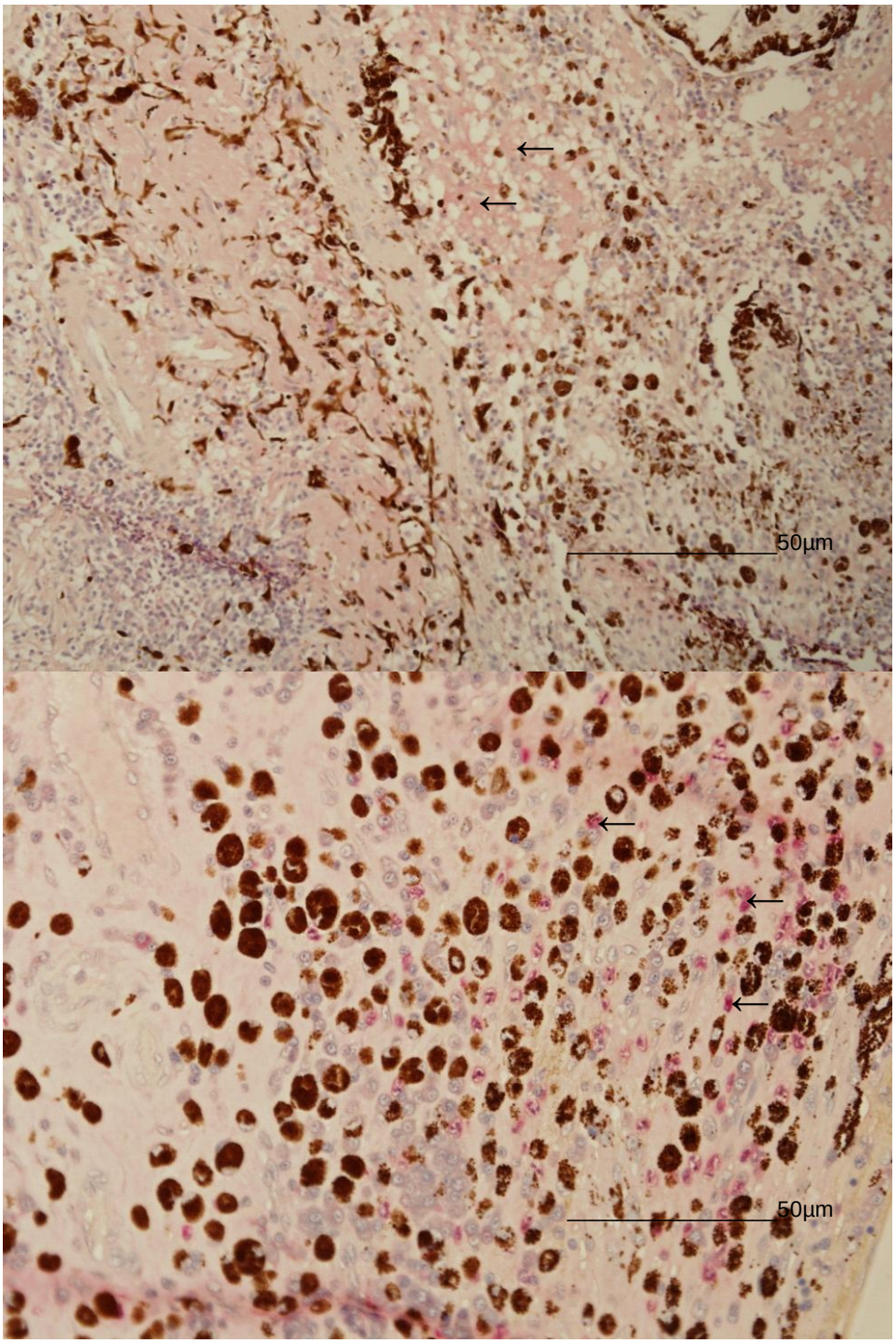

Figure 3.13: Secondary Glaucoma, Iris, IHC for MMP-9
Legend: Increased MMP-9 staining within iris inflammatory cells, pigmented and non-pigmented cells and fibroblasts within the iris stroma (arrows). IHC fast red staining for MMP-9, 40X magnification (top &bottom).

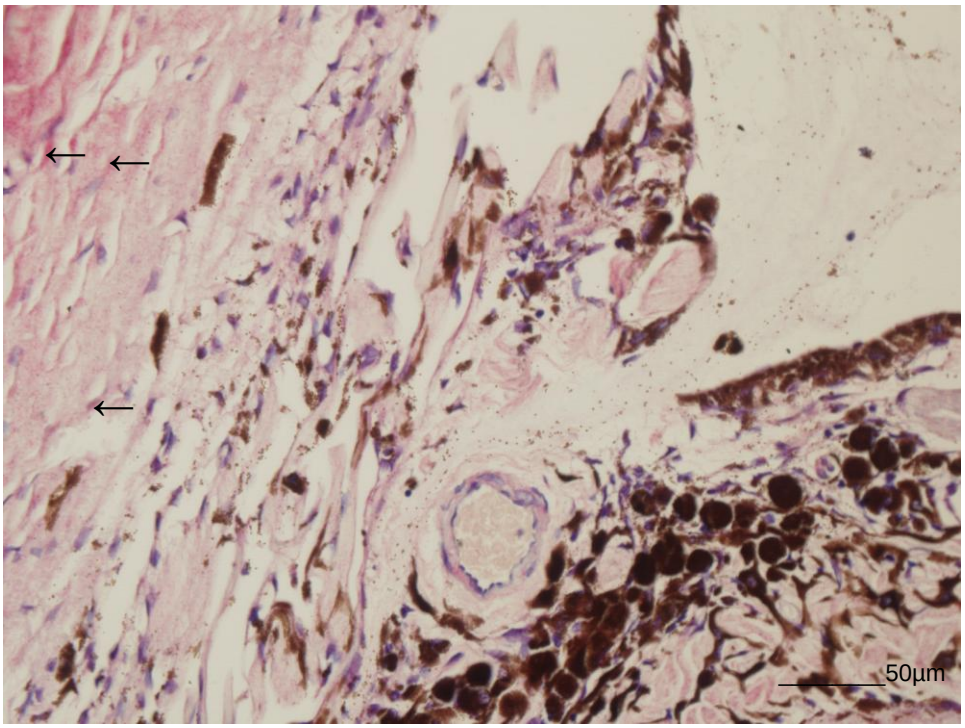

Figure 3.14: Control Iridocorneal angle, IHC staining for MMP-2
Legend: Staining of trabecular cells of the corneoscleral trabecular meshwork (arrows). IHC fast red staining for MMP-2, 20X Magnification.

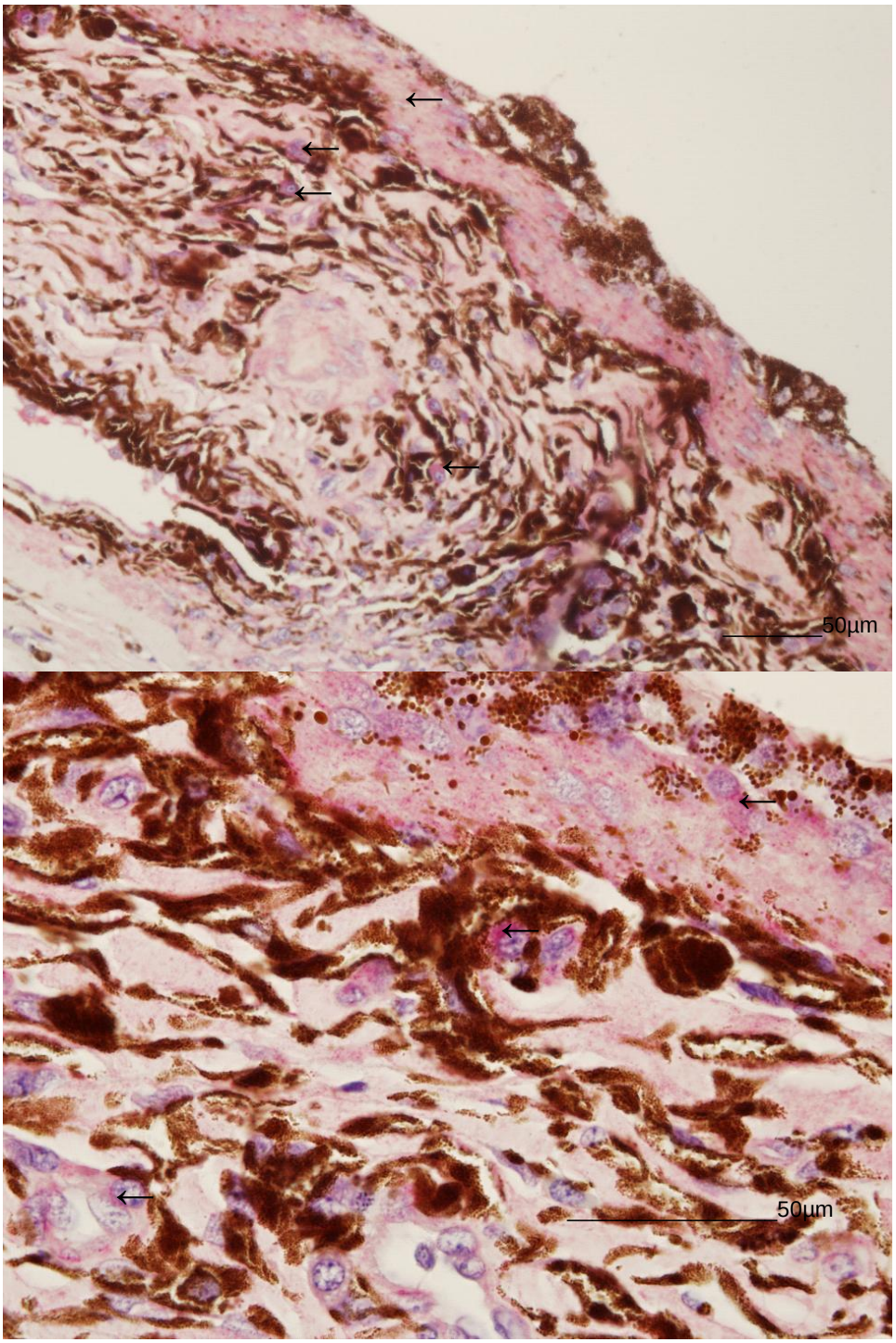

Figure 3.15: Primary glaucoma, Iridocorneal angle, IHC staining for MMP-2
Legend: Increased MMP-2 staining within iris anterior border, iris stroma, pigmented and non-pigmented cells and region of collapsed trabecular Meshwork (arrows). IHC fast red staining for MMP-2, 20X magnification (top), 40X magnification (bottom).

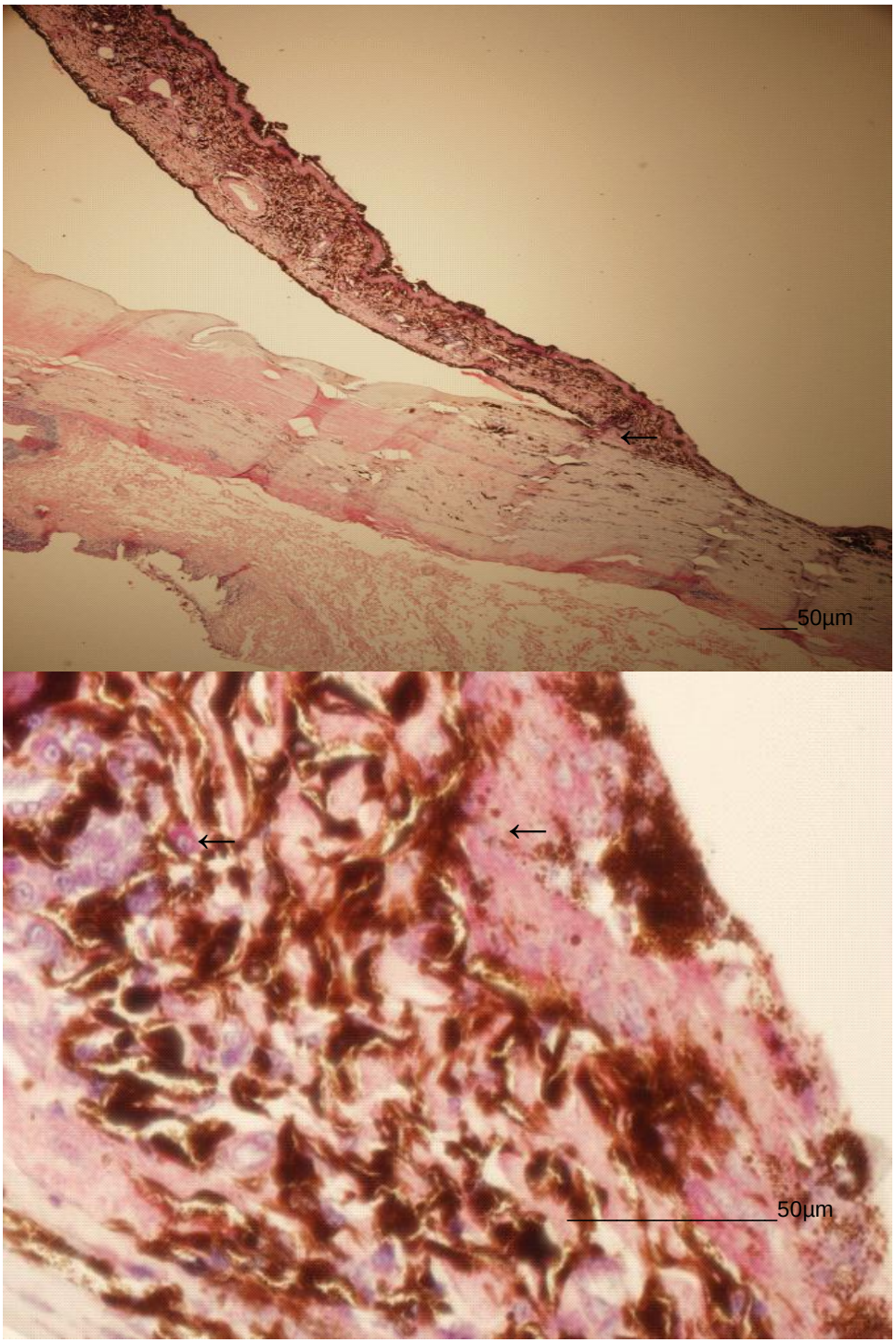

Figure 3.16: Primary Glaucoma, Iridocorneal angle, IHC staining for MMP-9
Legend: Collapsed ciliary cleft and increased staining within iridocorneal angle (arrows). IHC fast red staining for MMP-9,10X magnification (top), 40X magnification (bottom).

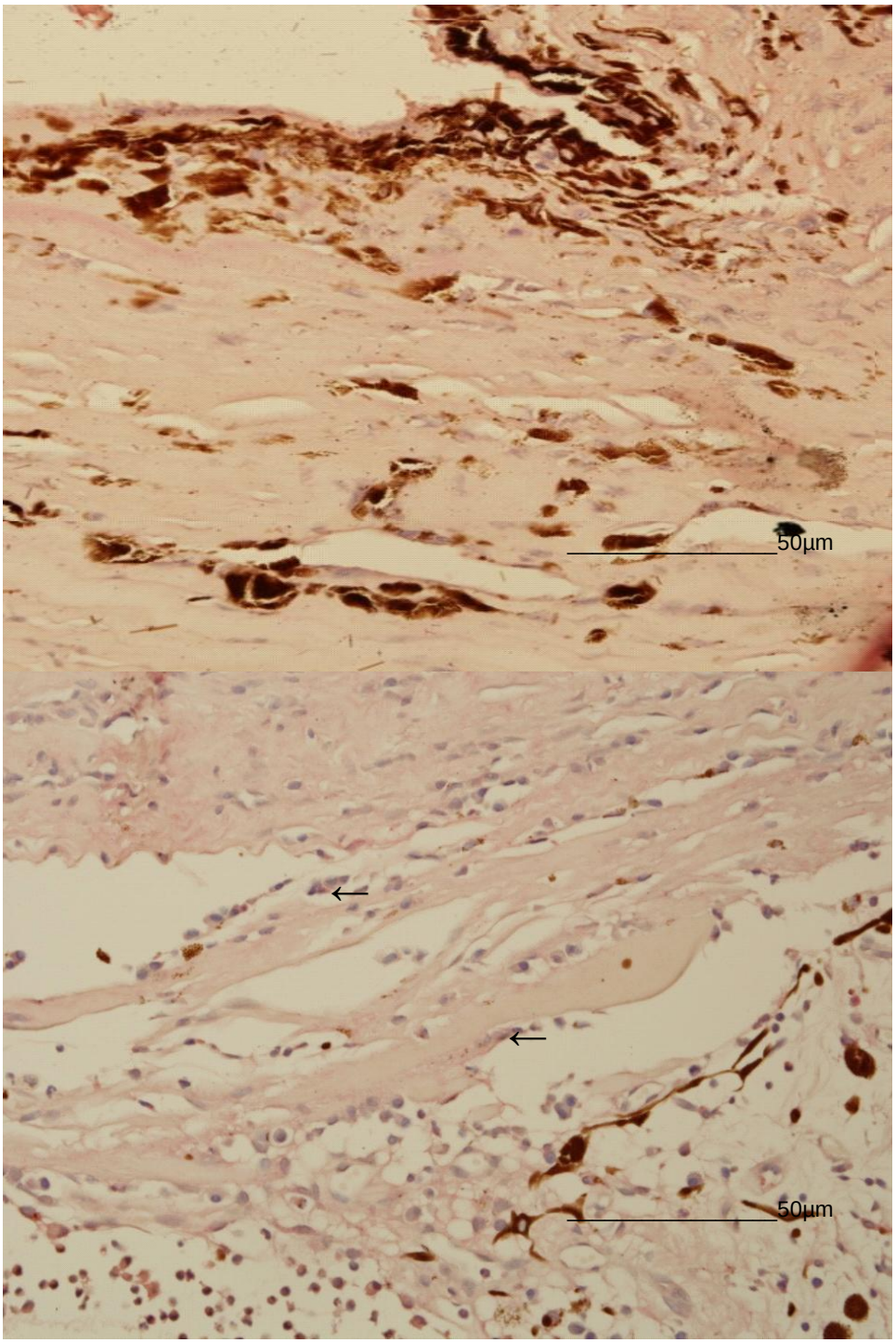

Figure 3.17: Primary and Secondary Glaucomas, Iridocorneal angle, IHC for MMP-2 and MMP-9

Legend: Increased MMP staining within the iridocorneal angle and trabecular meshwork (arrows). IHC fast red staining for MMP-2, primary glaucoma, 40X magnification (top), IHC fast red staining for MMP-9, secondary glaucoma, 40X magnification (bottom).

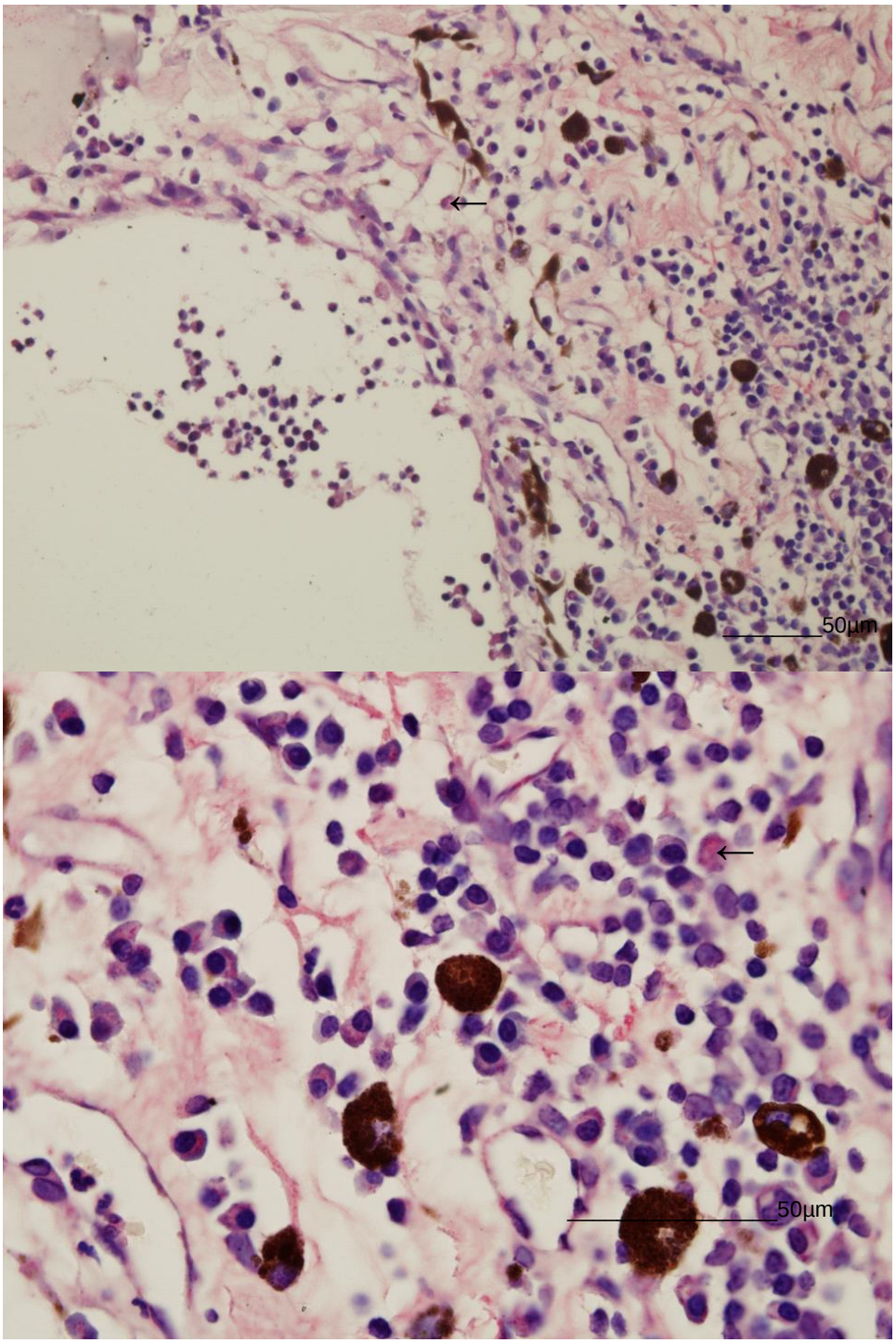

Figure 3.18: Secondary Glaucoma, Iridocorneal angle, IHC for MMP-2
Legend: Increased MMP-2 staining of inflammatory cells within the iridocorneal angle (arrows). IHC fast red staining for MMP-2, 20X magnification (top) and 40X magnification (bottom).

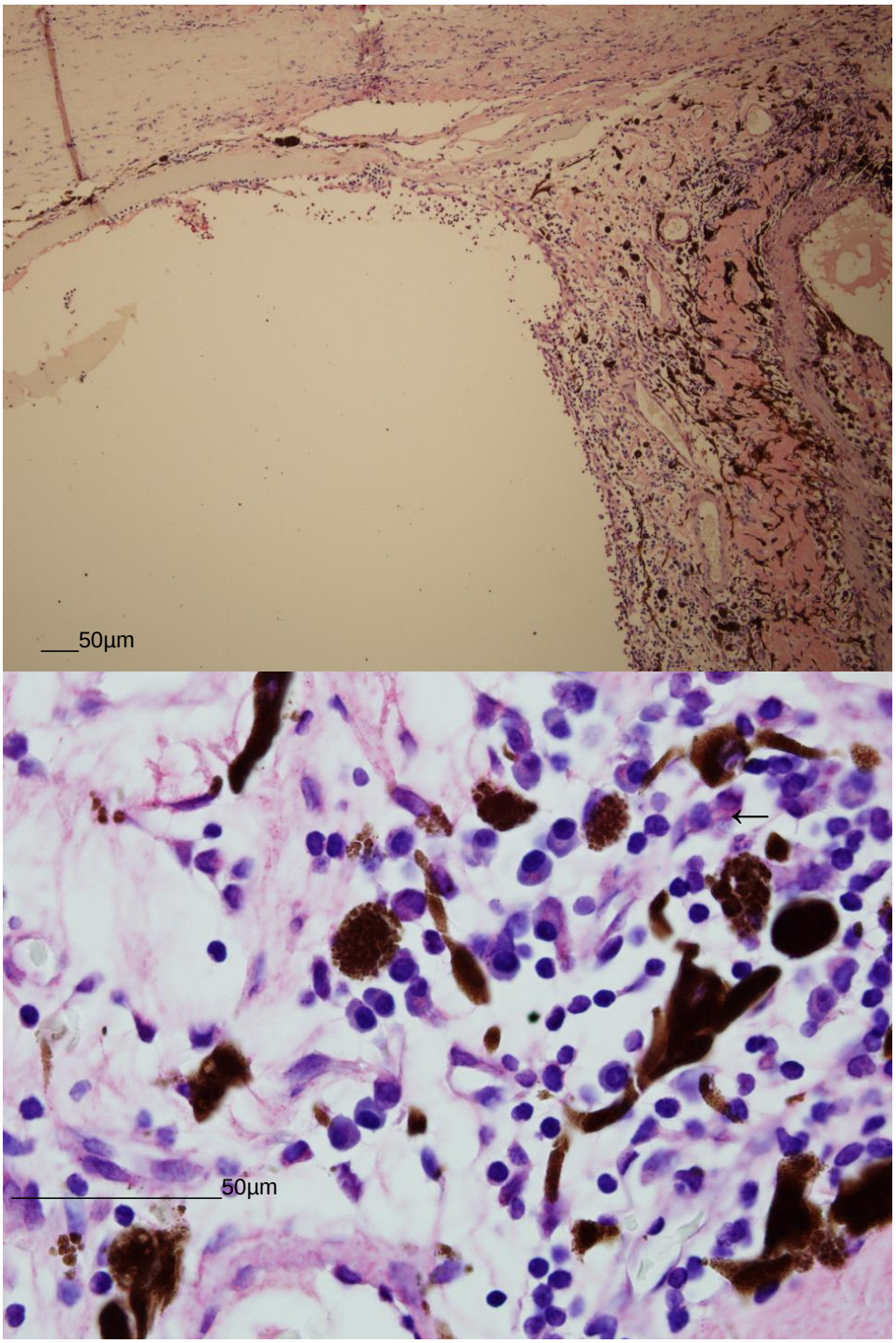

Figure 3.19: Secondary Glaucoma, Iridocorneal angle, IHC for MMP-9
Legend: Increased MMP-9 staining within inflammatory cells within the iridocorneal angle (arrows). IHC fast red staining for MMP-9, 20X magnification (top), 40X magnification (bottom).

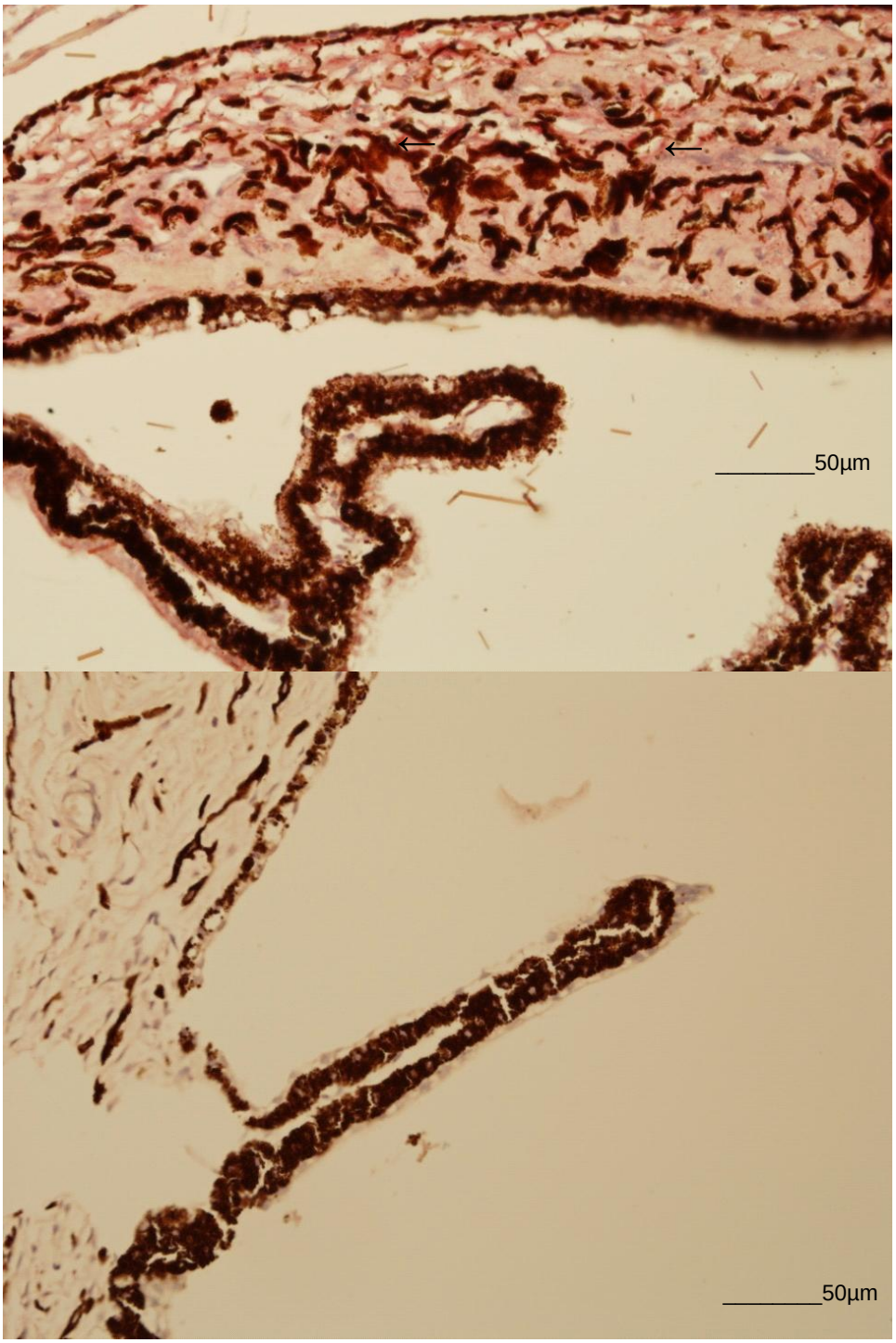

Figure 3.20: Control Ciliary Body, IHC for MMP-2
Legend: Low levels of MMP-2 staining within ciliary body pigmented and non-pigmented epithelium. IHC fast red staining for MMP-2, 20X magnification (top & bottom).

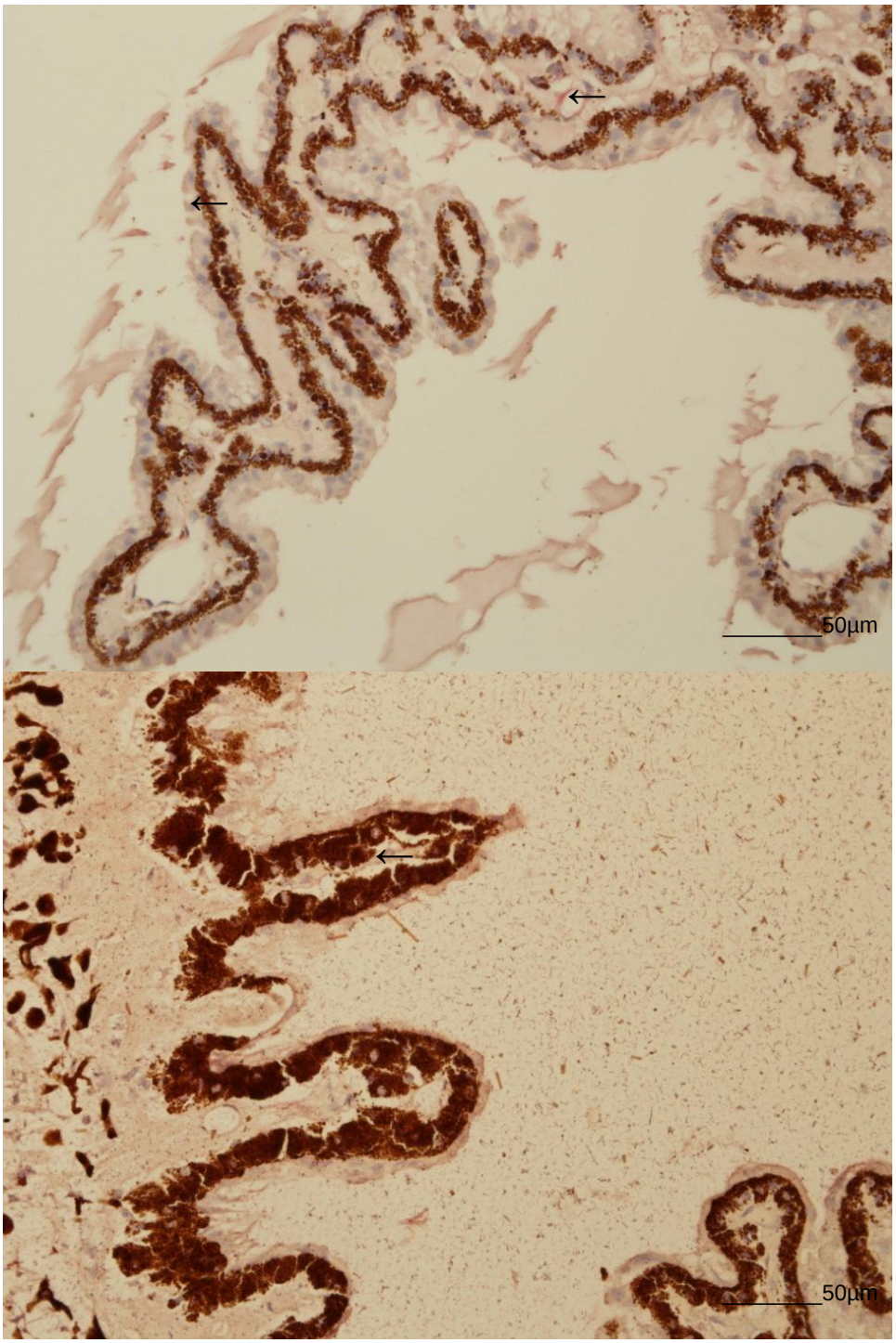

Figure 3.21: Control Ciliary Body, IHC for MMP-9
Legend: MMP-9 staining within ciliary body vessels, pigmented and non-pigmented epithelium (arrows). IHC fast red staining for MMP-9, 20X magnification (top & bottom).

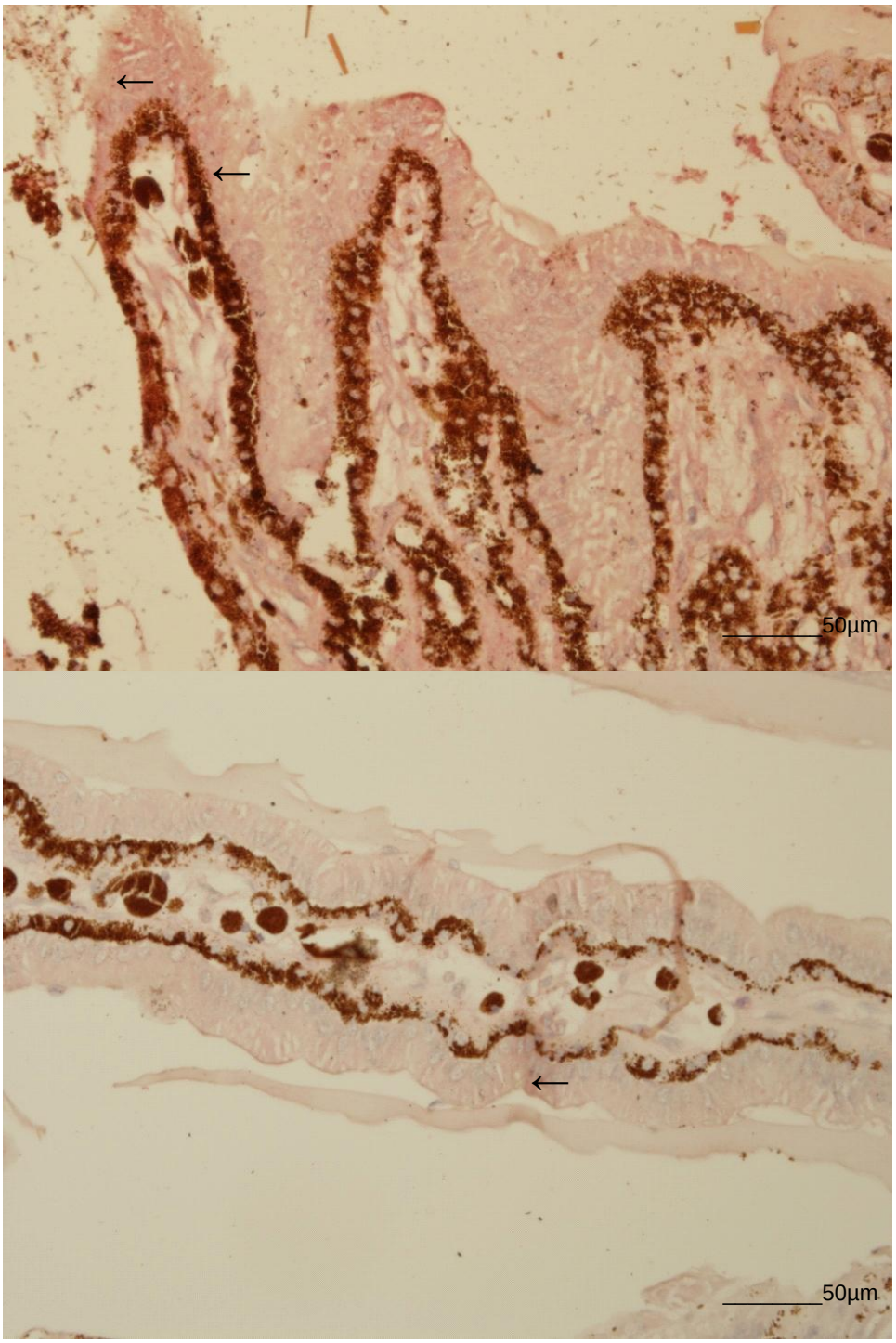

Figure 3.22: Secondary Glaucoma, Ciliary Body, IHC for MMP-2 and MMP-9
Legend: Increased MMP staining of ciliary body, pigmented and non-pigmented epithelium and within inflammatory cells (arrows). IHC fast red staining for MMP-2 (top) and MMP-9 (bottom), 20X magnification (top & bottom).

Figure 3.23: Control Lens, IHC for MMP-2 and MMP-9
Legend: MMP staining within lens capsule, lens epithelium and cortex and nucleus (arrows). IHC fast red staining for MMP-2 (top), 40X magnification, IHC fast red staining for MMP-9, 20X magnification (bottom).

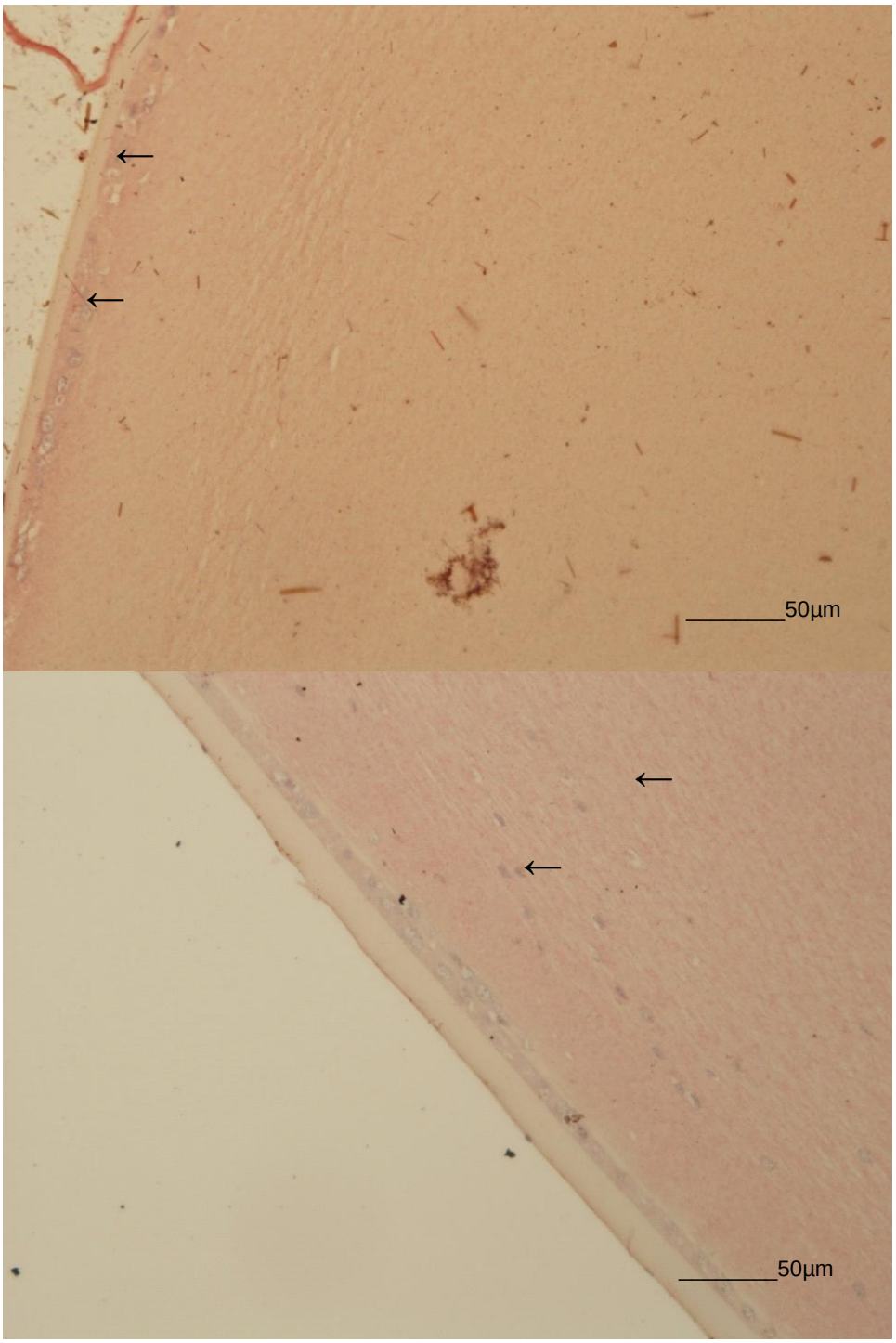

Figure 3.24: Secondary Glaucoma, Lens, IHC for MMP-2 and MMP-9
Legend: Increased MMP staining within lens epithelium, cortex and nucleus (arrows). IHC fast red staining for MMP-2 (top) and MMP-9 (bottom), 20X magnification (top & bottom).

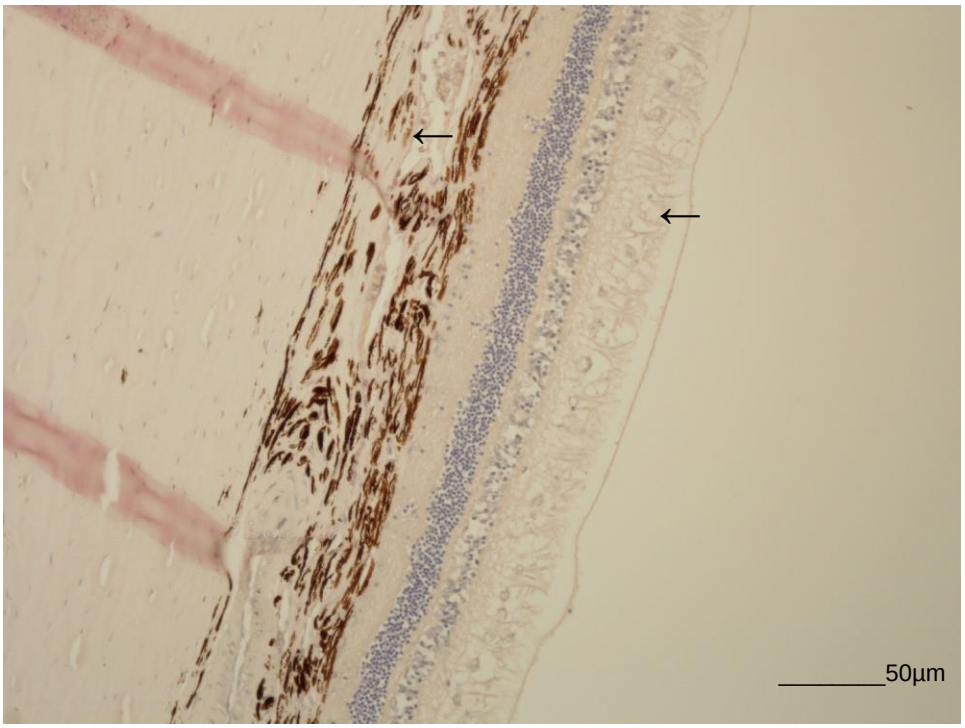

Figure 3.25: Control sclera, choroid and retina, IHC for MMP-9
Legend: Low levels of MMP-9 staining within sclera, choroid and retina (arrows). IHC fast red staining for MMP-9, 20X magnification.

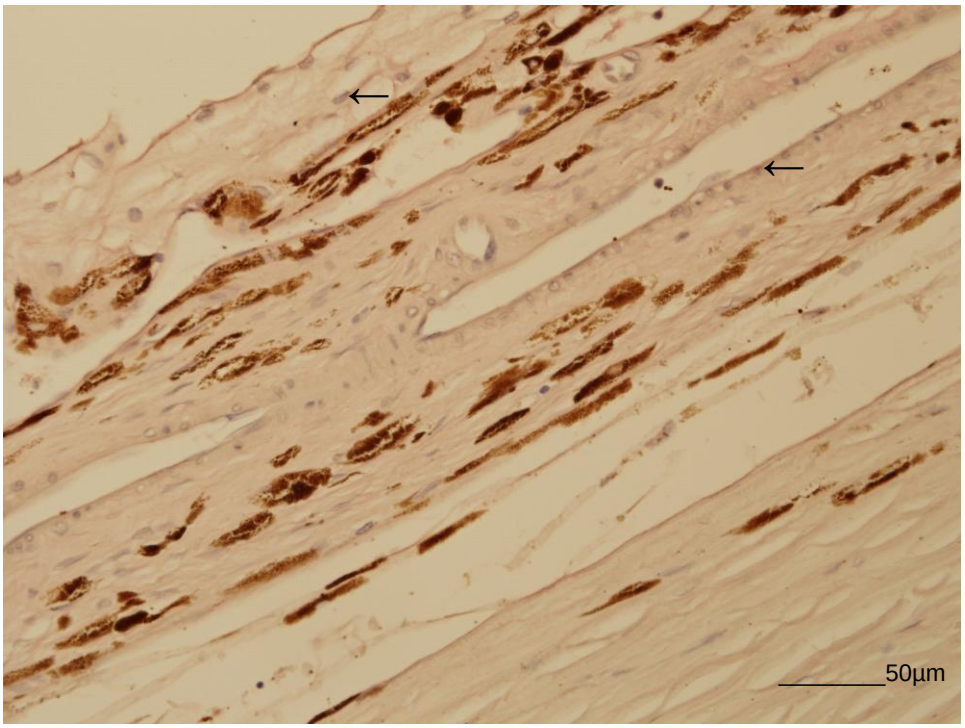

Figure 3.26: Control Choroid, IHC for MMP-2
Legend: MMP-2 staining within normal choroids within endothelium of blood vessels and pigmented and non-pigmented cells (arrows). IHC fast red staining for MMP-2, 20X magnification.

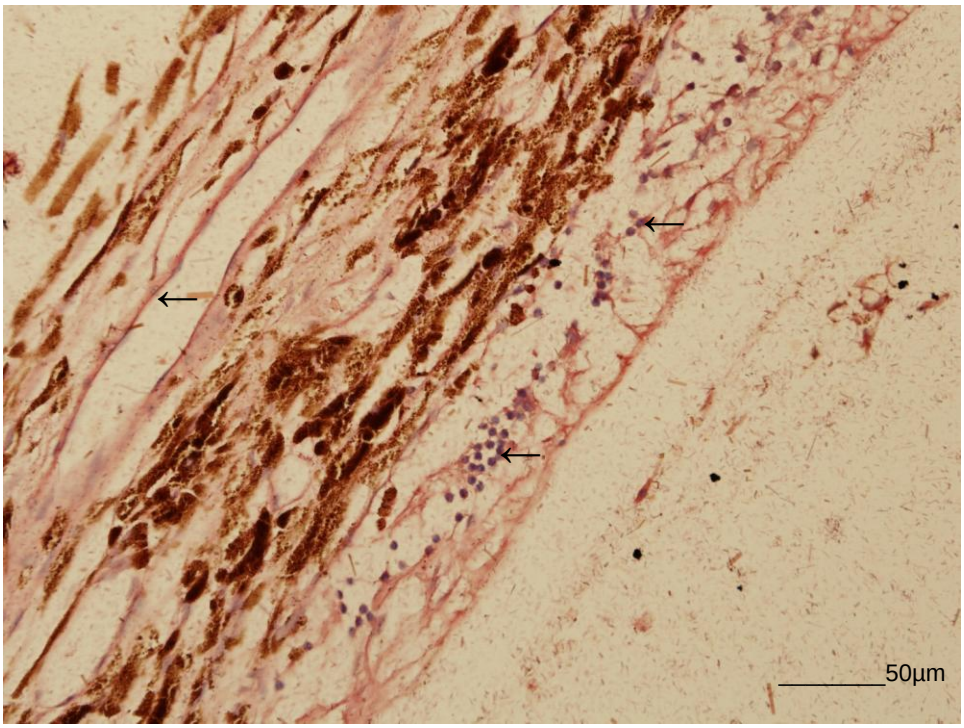

Figure 3.27: Secondary Glaucoma, Choroid, IHC for MMP-2
Legend: Increased MMP staining within the choroid in inflammatory cells, endothelium of blood vessels, pigmented and non-pigmented cells (arrows). IHC fast red staining for MMP-2, 40X magnification.

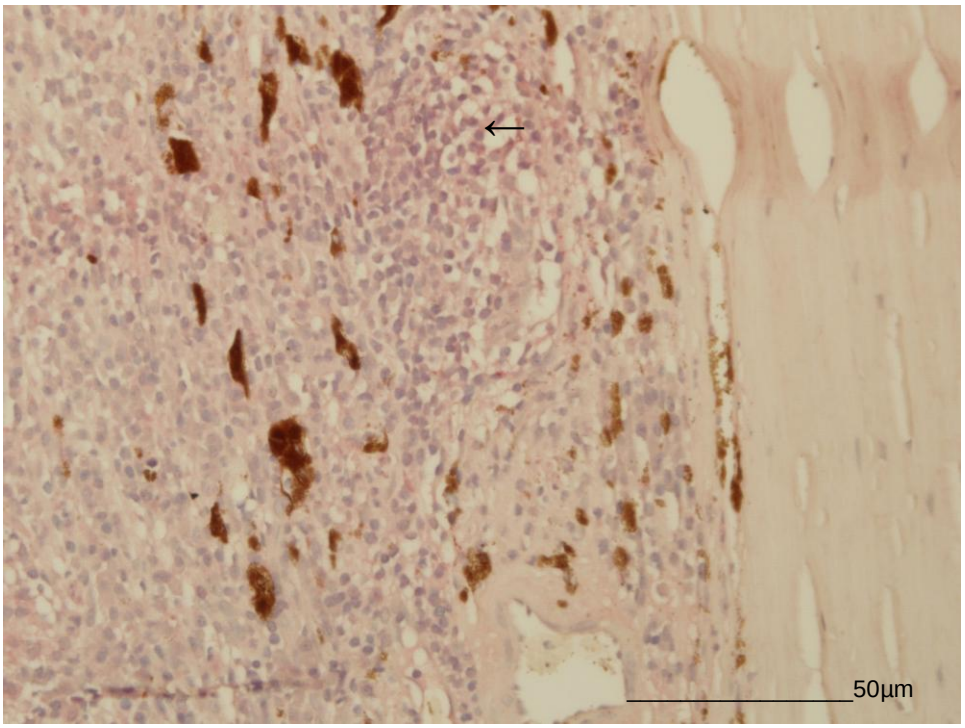

Figure 3.28: Secondary Glaucoma, Choroid and Sclera, IHC for MMP-9
Legend: Increased MMP-9 staining within inflammatory cells within the choroid and sclera (arrows). IHC fast red staining for MMP-9, 40X magnification.

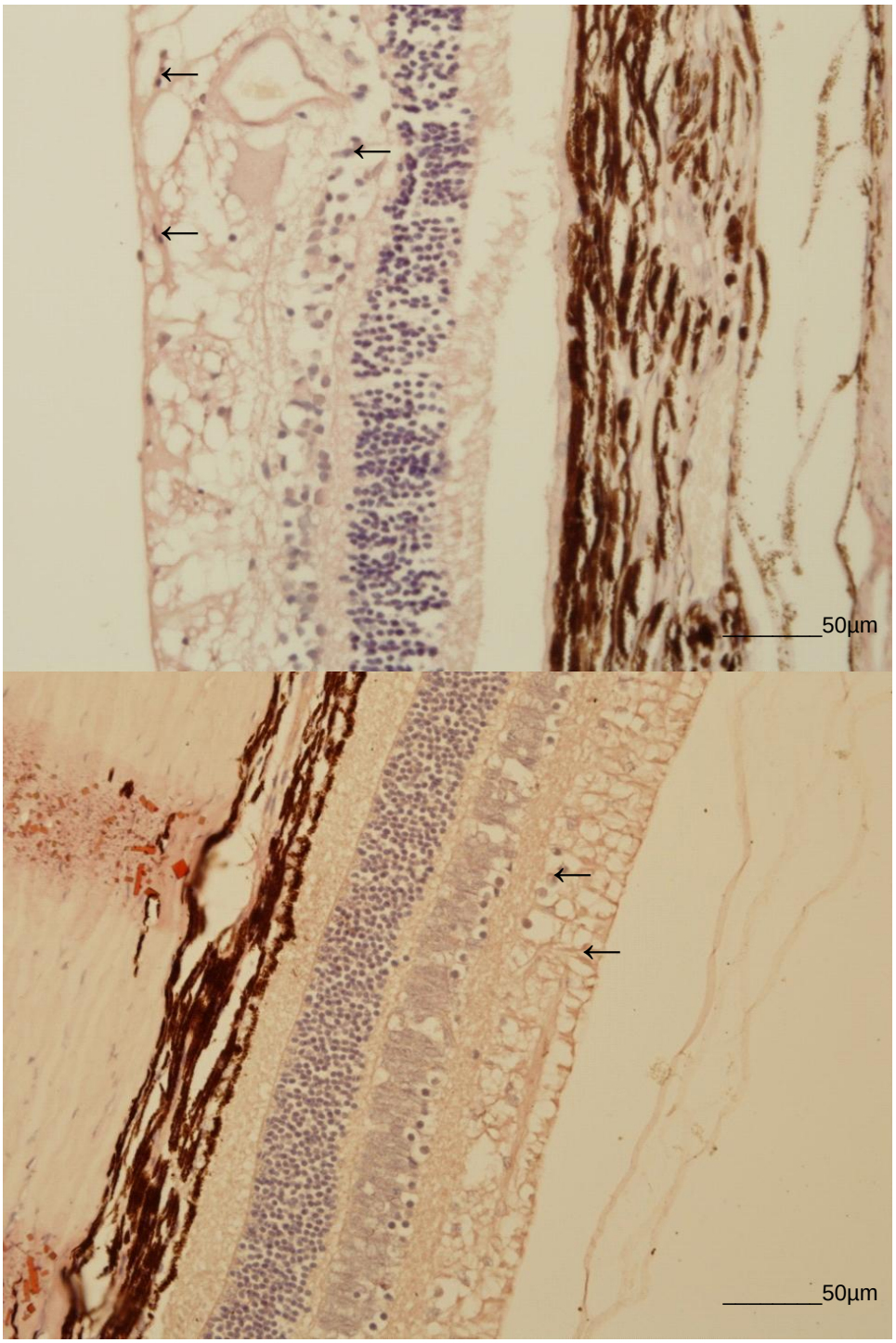

Figure 3.29: Control retina, IHC for MMP-2 and MMP-9
Legend: MMP staining within the retina (arrows). IHC fast red staining for MMP-2 (top) and MMP-9 (bottom), 20X magnification (top & bottom).

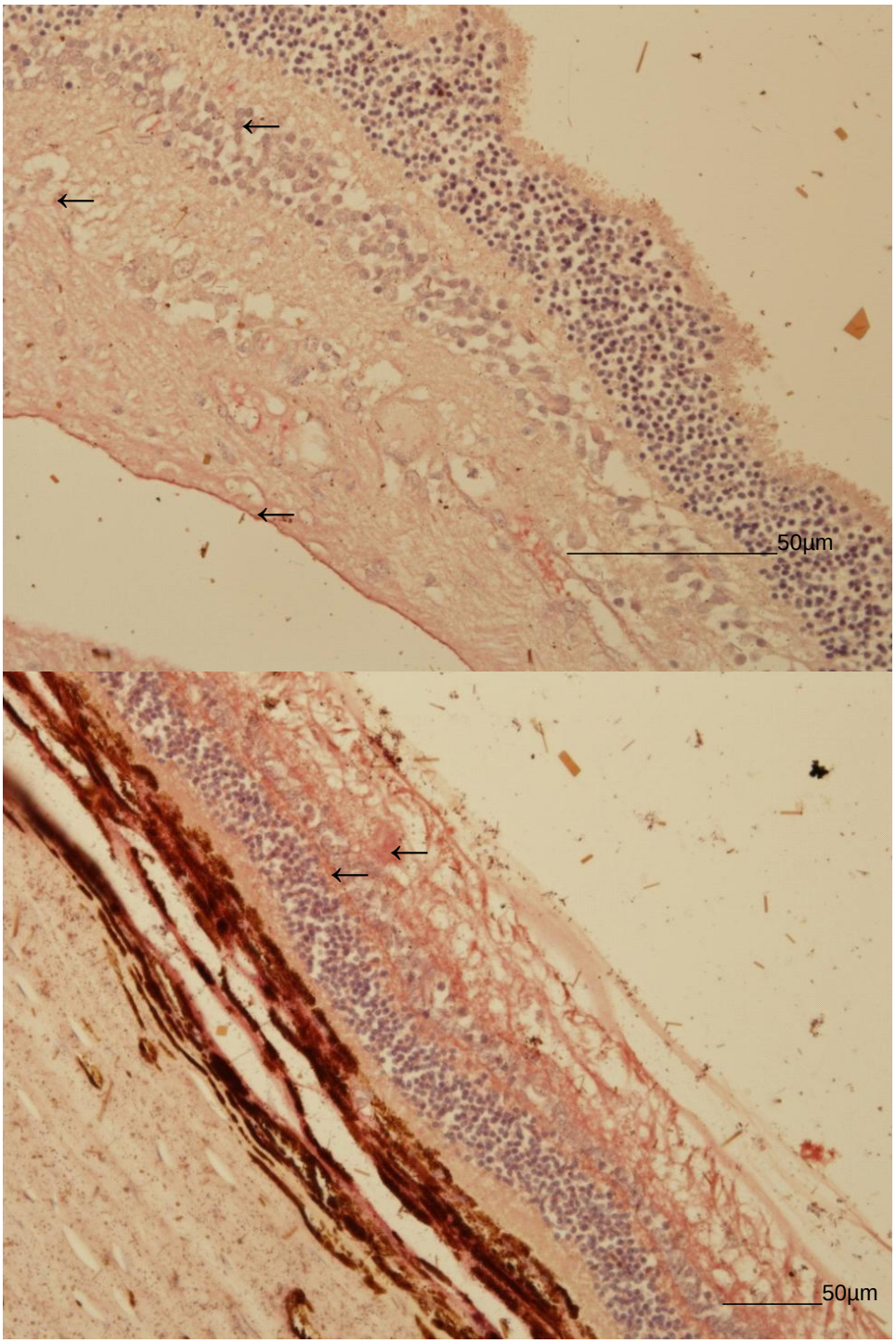

Figure 3.30:Secondary Glaucomas, IHC for MMP-2 and MMP-9
Legend: Increased MMP staining within inflammatory cells and retina (arrows). IHC fast red staining for MMP-2 (top) and MMP-9 (bottom), 40X magnification (top) and 20X magnification (bottom).

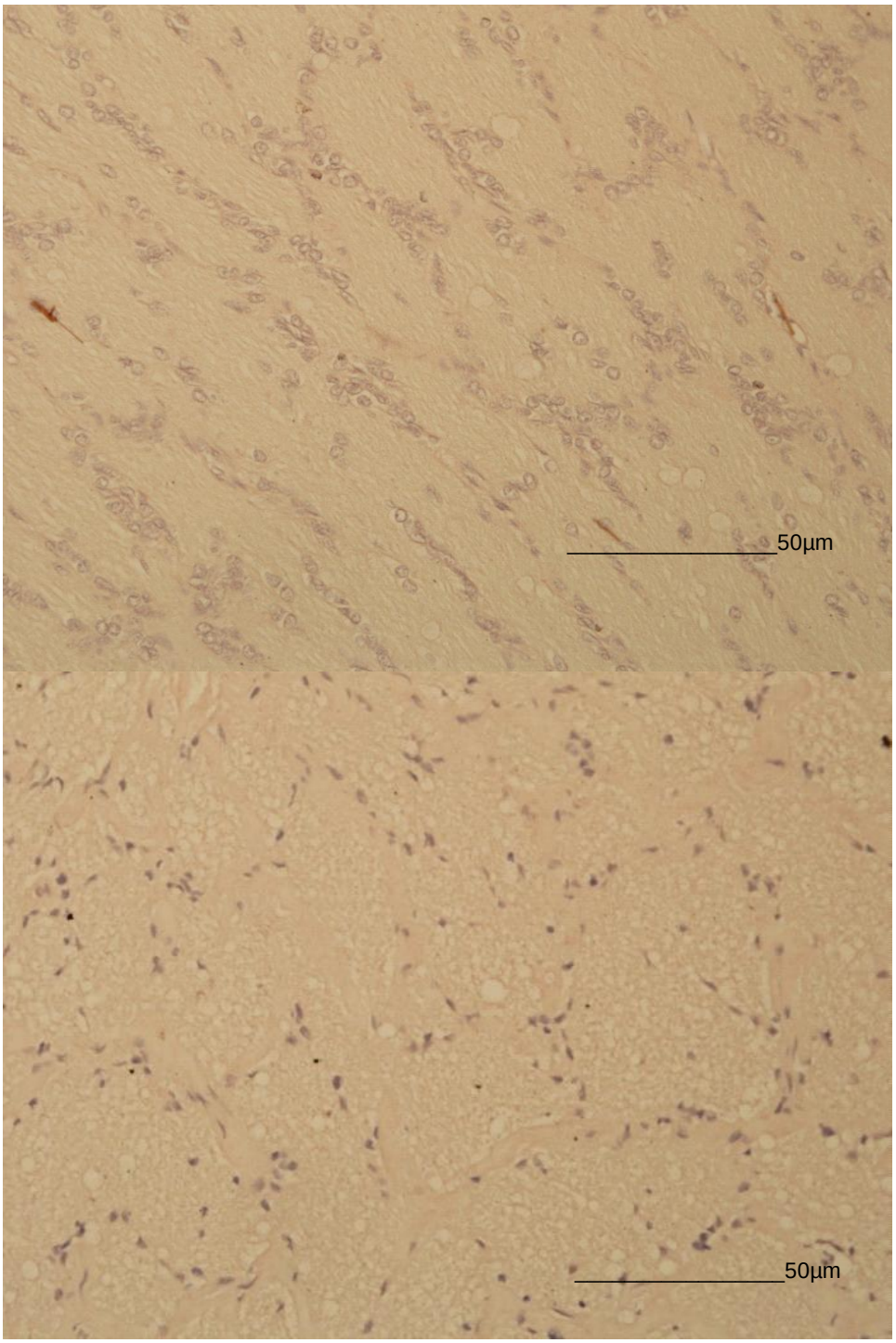

Figure 3.31: Control Optic Nerve, IHC for MMP-2 and MMP-9
Legend: Controls show minimal staining. IHC fast red staining for MMP-2 (top) and MMP-9(bottom), 40X magnification (top & bottom).

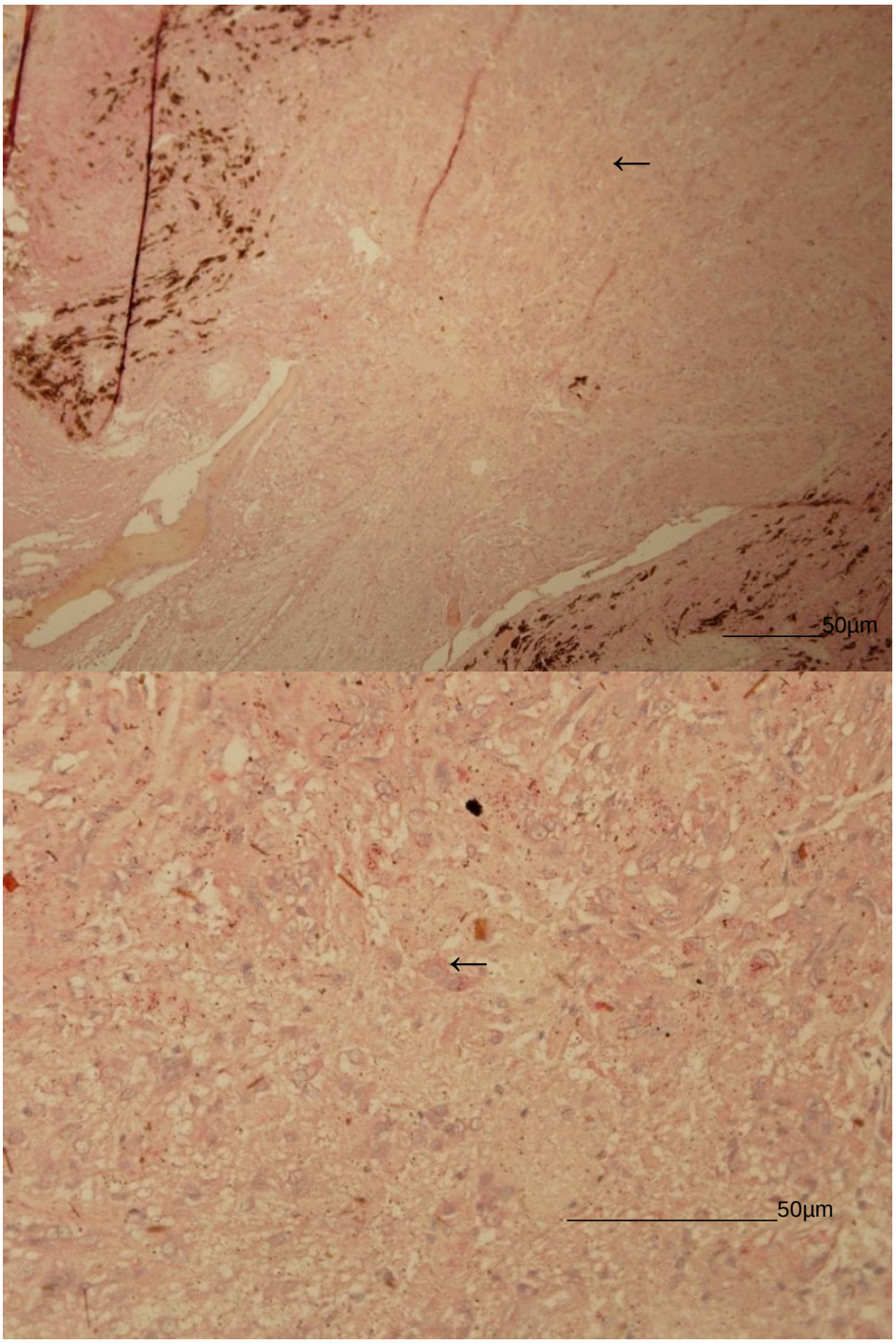

Figure 3.32: Primary and Secondary Glaucoma, Optic Nerve, IHC for MMP-2 and MMP-9
Legend: Increased staining within optic nerve (arrows). IHC fast red staining for MMP-2 (top) and MMP-9 (bottom), 20X magnification (top) and 40X magnification (bottom).

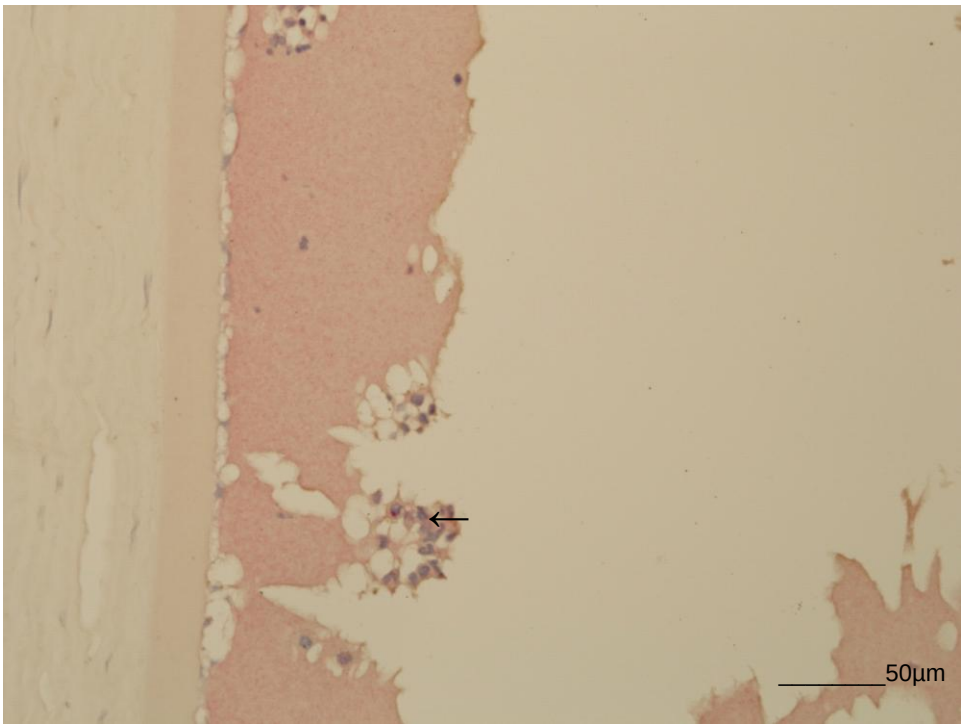

Figure 3.33: Secondary glaucoma, Anterior Chamber, IHC for MMP-9
Legend: Staining within inflammatory cells in the anterior chamber (arrows). IHC fast red staining for MMP-9, 20X magnification.

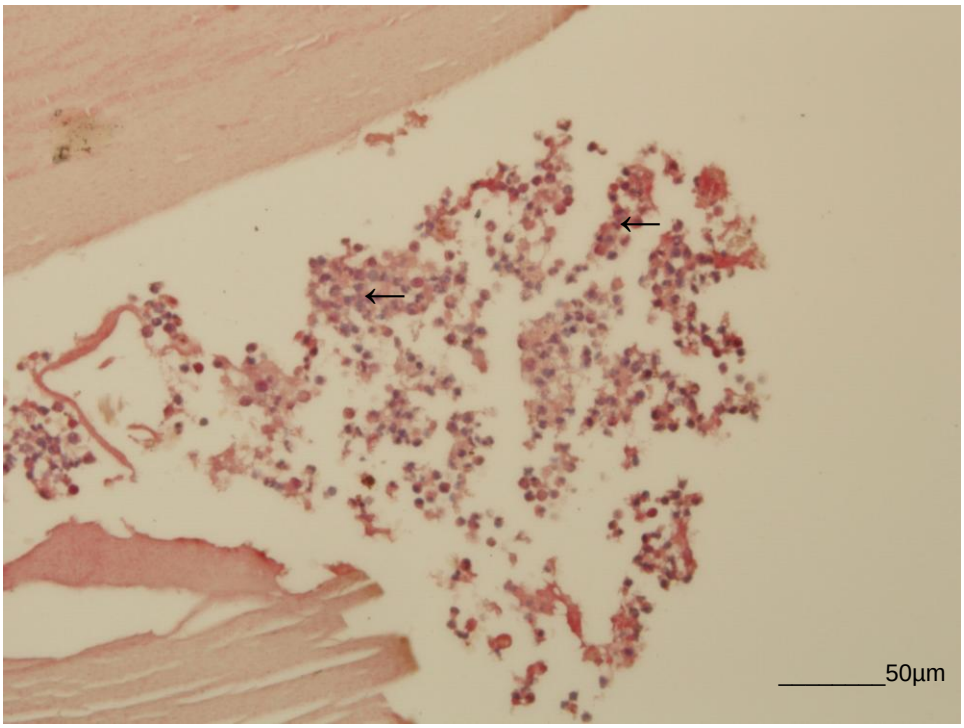

Figure 3.34: Secondary Glaucoma, Vitreous, IHC for MMP-9
Legend: Staining within inflammatory cells in the vitreous (arrows). IHC fast red staining for MMP-9, 20X magnification.

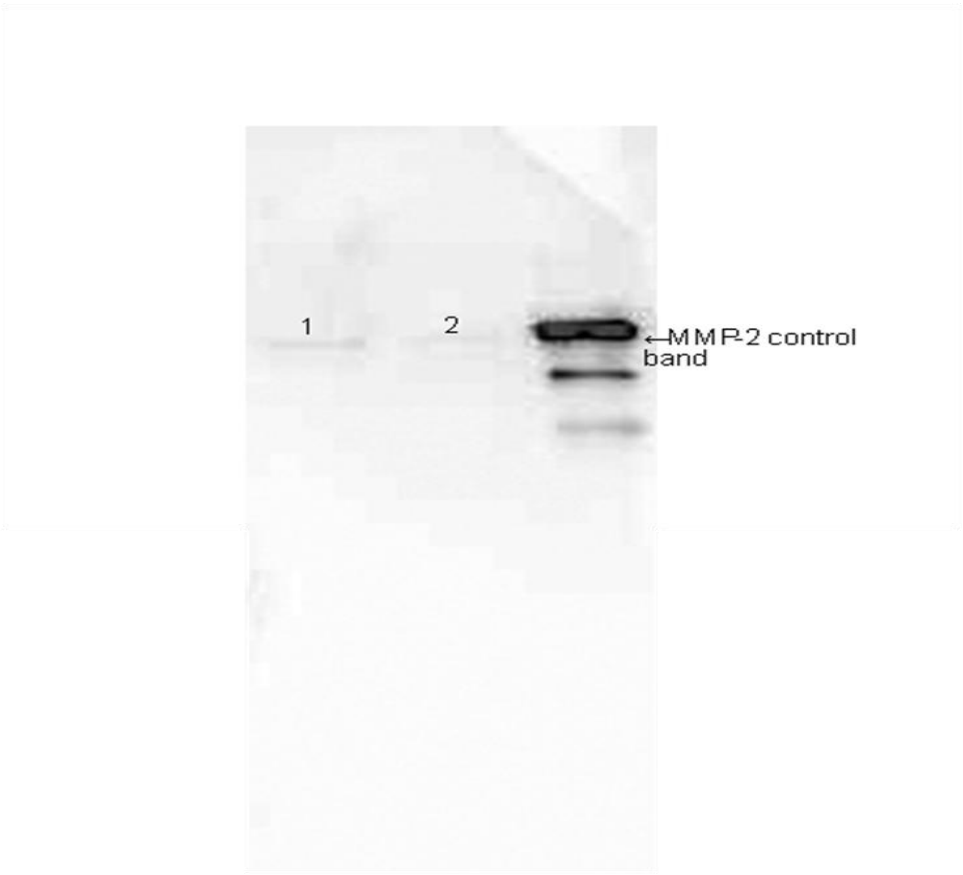

Figure 3.35: Western blot for MMP-2 confirming specificity of anti-human MMP-2 antibody in the canine eye
Legend: 1=glaucomatous iridocorneal angle tissue, 2=glaucomatous aqueous humor

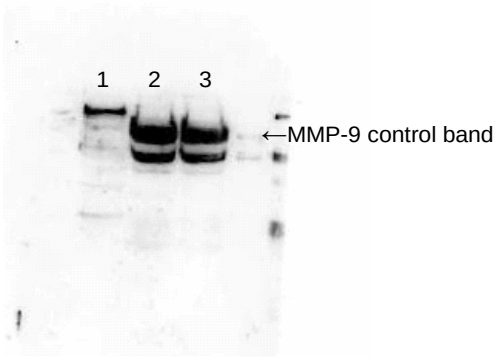

Figure 3.36: Western blot for MMP-9 confirming specificity of anti-human MMP-9 antibody in the canine eye
Legend: 1=normal canine white blood cells, 2=canine glaucomatous aqueous humor, 3=canine glaucomatous iridocorneal angle tissue

REFERENCES

1. Alexander JP, Samples JR, Van Buskirk EM, et al. Expression of matrix metalloproteinases and inhibitor by human trabecular meshwork. *Investigative Ophthalmology and Visual Science*. 1991; 32, 1: 172-190.

2. Baramova E and Foidard JM. Matrix Matalloproteinase Family. p. 239-242. In *Cell Biology International*, Academic Press, Ltd: 1995; 19, 3: 239-242.

3. Bradley JM, Kelley MJ, Zhu XH, et al. Effects of mechanical stretching on trabecular matrix metalloproteinases. *Investigative Ophthalmology and Visual Science.* 2001; 42, 7:1505-1513.

4. Bradley JM, Vranka J, Colvis CM, et al. Effect of matrix metalloproteinases activity on outflow in perfused human organ culture. *Investigative Ophthalmology and Visual Science*. 1998; 39,13:2649-2658.

5.Changwon K, Shon S, Hwang JM. Stromelysin gene transfer into cultured human trabecular cells and rat trabecular meshwork in vivo. *Investigative ophthalmology and visual science*. 2001;42,12: 2856-2860.

6. Clark AF. New Discoveries on the roles of matrix metalloproteinases in ocular cell biology and pathology. *Investigative Ophthalmology and Visual Science*. 1998; 39, 13:2514-2516.

7. Conley SM, Bruhn RL, Morgan PV, et al. Selenium's effects on MMP-2 and TIMP-1 secretion by trabecular meshwork cells. *Investigative Ophthalmology and Visual Science*. 2004; 45, 2:473-479.

8. Gaton DD, Sagara T, Lindsey JD, et al. Matrix metalloproteinase-1 localization in the normal human uveoscleral outflow pathway. *Investigative Ophthalmology and Visual Science*. 1999; 40,2:363-369.

9. Gaton DD, Sagara T., Lindsey JD. Increased matrix metalloproteinases 1,2, and 3 in the monkey uveoscleral outflow pathway after topical prostaglandin F2α-isopropyl ester treatment. *Arch Ophthalmology*. 2001;119:1165-1170.

10. Gelatt KN. Diseases of the canine orbit. In *Veterinary Ophthalmology*, Lippincot Williams and Wilkins: 1999; Third Edition, p.524-525.

11. Gelatt KN, MacKay EO, et al. Prevalence of the breed-related glaucomas in pure-bred dogs in North America. *Veterinary Ophthalmology*. 2004;7, 2,97-111.

12. Kee, C, Sohn S, Hwang, JM. Stromelysin gene transfer into cultured human trabecular cells and rat trabecular meshwork in vivo. *Investigative Ophthalmology and Visual Science*.2001;42,12:2856-2860.

13. Lan J, Kumar RK, Girolamo ND et al. Expression and distribution of matrix metalloproteinases and their inhibitors in the human iris and ciliary body. *Br J Ophthalmol* 2003;87:208-211.

14. Llobet A, Gasull X, Gual A. Understanding trabecular meshwork physiology: A key to the control of intraocular pressure. *News Physiol. Sci*. 2003;18:205-209.

15. Maatta M, Tervahartiala T, Vesti E et al. Levels and activation of matrix metalloproteinases in aqueous humor are elevated in uveitis-related secondary glaucoma. *J Glaucoma*. 2006;15(3):229-27.

16. Pang IH, Hellberg PE, Fleenor DL et al. Expression of matrix metalloproteinases and their inhibitors in human trabecular meshwork cells. *Investigative ophthalmology and Visual Science*. 2003; 44,8:3485-3493.

17. Patricia AM, Snoek-van B, von den Hoff JW. Zymographic techniques for the analysis of matrix metalloproteinases an their inhibitors. *BioTechniques*. 2005; 38,1:73-83.

18. Sapienza JS, Van der Woerdt A. Combined transscleral diode laser cyclophotocoagulation and Ahmed gonioimplantation in dogs with primary glaucoma:51 cases (1996-2004). *Veterinary. Ophthalmology*. 2005 Mar-Apr;8(2):121-7.

19. Schlotzer-Schrehardt U, Lommatzsch J, Kuchle A, et al. Matrix metalloproteinases and their inhibitors in aqueous humor of patents with pseudoexfoliation syndrome/glaucoma and primary open-angle glaucoma. *Invesitgative Ophthalmology and Visual Science.* 2003;44, 3:1117-1125.

20. Shearer TW, Crosson CE. Adenosine A_1 receptor modulation of MMP-2 secretion by trabecular meshwork cells. *Investigative Ophthalmology and Visual Science*. 2002;43, 9:3016-3020.

21. Sivak JM, Fini ME. MMPs in the eye: emerging roles for matrix metalloproteinases in ocular physiology. *Progress in Retinal and Eye Research*. 2002; 21:1-14.

22. Wride MA, Geatrell J, Guggenheim JA. Proteases in eye development and disease. *Birth Defects Research*. 2006;78:90-105.

23. WuDunn D. The effect of mechanical strain on matrix metalloproteinase production by bovine trabecular meshwork cells. *Current Eye Research*. 2001; 22,5:394-397.

24. Alexander JP and Acott TS. Involvement of the Erk-MAP kinase pathway in TNF alpha regulation of trabecular matrix metalloproteinases and TIMPs. *IOVS* 2003; 44 (1): 164-169.

25. Alexander JP and Acott TS. Involvement of protein kinase c in TNF alpha regulation of trabecular matrix metalloproteinases and TIMPs. *IOVS*. 2001; 42 (12): 2831-2838.

26. Arican M, Ceylan C. Metalloproteinases in canine experimental traumatic keratoconjunctivits. *Zentralbl Veterinarmed A*. 1999; 46, 527-32.

27. Aupperle H, Thielebein J, Kiefer B, et al. An immunohistochemistry study of the role of matrix metalloproteinases and their tissue inhibitors in chronic mitral valvular disease (valvular endocardiosis) in dogs. *Vet J*. 2008 (Epub ahead of print).

28. Bahler CK, Howell KG, Hann CR, et al. Prostaglandins increase trabecular meshwork outflow facility in cultured human anterior segments. *American Journal of Ophthalmology*. 2008; 145 (1):114-119.

29. Carter RT, Kambampati R, Murphy CJ, et al. Expression of matrix metalloproteinase 2 and 9 in experimentally wounded canine corneas and spontaneous chronic corneal epithelial defects. *Cornea*. 2007; 26 (10):1213-9.

30. Ehrich D, Tripathi B, Tripathi R., et. al. Effects of interleukin-1 beta and dexamethasone on the expression of matrix metalloprotease m RNA by trabecular cells exposed to elevated hydrostatic pressure. *Acta Ophthalmol Scand*. 2005; 83(1):104-8.

31. Fleenor DL, Pang IH, Clark AF. Involvement of AP-1 in interleukin-1 alpha-stimulated MMP-3 expression in human trabecular meshwork cells. *IOVS*. 2003; 44 (8):3494-3501.

32. Hosseini M, Rose AY, Sone K. IL-1 and TNF induction of MMP-3 by c-jun n-terminal kinase in trabecular meshwork. *IOVS*. 2006; 47 (4):1469-1476.

33. Hudde T, Apitz J, Bordes-Alonso R. Gene transfer to trabecular meshwork endothelium via direct injection into the schlemn canal and in vivo toxicity study. *Current Eye Research*. 2005; 30:1051-1059.

34. Kee C, Son S, Ahn BH. The relationship between gelatinase A activity in aqueous humor and glaucoma. *J Glaucoma*. 1999; 8 (1): 51-5.

35. Kelley MJ, Rose AY, Song K. Synergism of TNF and IL-1 in the induction of matrix metalloproteinase-3 in trabecular meshwork. *IOVS*. 2007; 48 (6): 2634-2643.

36. Kidd JA, Barr AR, Tarlton JF. Use of matrix metalloproteinases 2 and 9 and white blood cell counts in monitoring the treatment and predicting the survival of horses with septic arthritis. *Vet Rec*. 2007; 161 (10):329-24.

37. Liu CJ, Huang YL, Ju JP. Altered transcripts expression of matrix metalloproteinases and their tissue inhibitors in tenon capsule of patients with glaucoma. *J Glaucoma*. 2004; 13(6):486-91.

38. Loftus JP, Black SJ, Pettigrew A, et al. Early laminar events involving endothelial activation in horses with black walnut-induced laminitis. *AJVR*. 2007;68(11):1205-11.

39. Maatta M, Tervhartiala T, Harju M et al. Matrix metalloproteinases and their tissue inhibitors in aqueous humor of patients with primary open-angle glaucoma, exfoliation syndrome, and exfoliation glaucoma. *J Glaucoma*. 2005; 14(1): 64-9.

40. Ollivier et al. Proteinases of the cornea and precorneal tear film. *Veterinary Ophthalmology*. 2007; 10,4,199-206.

41. Pang IH, Fleenor DL, Hellberg PE, et al. Aqueous outflow-enhancing effect of tertbutylhydroquinone: involvement of AP-1 activation and MMP-3 expression. *Invest. Ophthalmology Vis Sci.* 2003; Aug 44 (8); 3502-10.

42. Ronkko S, Rekonen P, Kaarniranta K. Matrix metalloproteinases and their inhibitors in the chamber angle of normal eyes and patients with primary open-angle glaucoma and exfoliation glaucoma. *Graefe's Arch Clin Exp Ophthalmol*. 2007; 245: 697-704.

43. Sachdev NH, Girolamo ND, Nolan TM. Matrix metalloproteinases and their tissue inhibitors of matrix metalloproteinases in the human lens: implication for cortical cataract formation. *IOVS*. 2004, Vol. 45, No.11:4075-4082.

44. Sakimoto T, Shoji J, Yamada A., et al., Expression of matrix metalloproteinases in tear fluid of patients with recurrent corneal erosion*, Jpn J Ophthalmol*. 2007; 51(5):343-6.

45. Samples JR, Alexander JP, Acott TS. Regulation of the levels of human trabecular matrix metalloproteinases and inhibitor by interleukin-1 and dexamethasone. *IOVS*. 1993; 34 (12):3386-95.

46. Tripathi BJ, Tripathi RC, Gotsis S, et. al. Trabecular cell expression of fibronectin and MMP-3 is modulated by aqueous humor growth factors. *Exp Eye Res.* 2004; 78 (3):653-60.

47. Vittitow J, Borras T. Genes expressed in the human trabecular meshwork during pressure-induced homeostatic response. *J. Cell Physiol.* 2004; 201 (1):126-37.

48. Wang IJ, Chiang TH, Shih Y-F. The association of single nucleotide polymorphisms in the MMP-9 genes with susceptibility to acute primary angle closure glaucoma in Taiwanese patients. *Molecular vision*. 2006; 12:1223-32.

49. Zenkel M, Poschl E, Mark KV, et al. Differential gene expression in pseudoexfoliation syndrome. *IOVS*. 2005; 46 (10):3742-3752.