Evaluation of Therapeutic Interventions for the Treatment of Eye Disorders

By

JOSEPH D. BOGAARD
B.S.E., University of Iowa, 2009

THESIS

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Defense Committee:

Iris S. Kassem, Advisor, Ohthalmology and Visual Sciences, Neuroscience
John Larson, Chair, Neuroscience
Mark I. Rosenblatt, Ophthalmology and Visual Sciences
Nancy Freitag, Microbiology and Immunology
David Pepperberg, Ophthalmology and Visual Sciences, Bioengineering

This thesis is dedicated to my family, friends, and mentors, without whom it would never have been accomplished.

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Contribution of Authors

Chapter 1 is a literature review that places my dissertation question in the context of the larger field and highlights the significance of my research question. Chapter 2 represents a manuscript being submitted later this year for which I was the primary author and major driver of the research. My research mentor, Dr. Iris Kassem contributed to the writing of the manuscript. Chapter 3 represents a manuscript being submitted later this year for publication for which I was the primary author and a major driver of the research. My research mentor, Dr. Iris Kassem, and Erica Oltra gathered the data from the electronic medical records at University of Illinois at Chicago. I was responsible for synthesizing the data, running the statistical analysis, creating the figures, and writing the manuscript. Dr. Iris Kassem contributed to the writing of the manuscript. Chapter 4 represents a series of unpublished experiments directed at drug development and discovery for retinitis pigmentosa. I was the major driver of the cell based experiments, with the liver microsome studies (Figures x and x) done by Bhargava Karumundi and assistance with the animal studies from Dr. Zhiqian Dong and Dr. Krzysztof Palczewski. Chapter 5 represents my synthesis of the research presented in this thesis/dissertation and my overarching conclusions. The future directions of this field and this research question are discussed.

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LIST OF ABBREVIATIONS

AMD	Age Related Macular Degeneration
BCVA	Best Corrected Visual Acuity
BSS	Balanced Salt Solution
CYP	Cytochrome P450
ERG	Electroretinogram
IATS	Infant Aphakia Treatment Study
IOL	Intraocular Lens
IOP	Intraocular Pressure
MCW	Medical College of Wisconsin
OCT	Optical Coherence Tomography
OTS	Ocular Trauma Score
POTS	Pediatric Ocular Trauma Score
RP	Retinitis Pigmentosa
SD OCT	Spectral Domain Optical Coherence Tomography
tPA	Tissue Plasminogen Activator
UIC EEI	University of Illinois Ear and Eye Infirmary
VA	Visual Acuity
WHO	World Health Organization

SUMMARY

Biochemical, cellular, animal, and retrospective human studies were performed to evaluate the effectiveness of different drugs and treatment strategies. Rabbits were used as a model for clear cornea lensectomy procedures to evaluate enoxaparin, triamcinolone, and tissue plasminogen activator. A retrospective examination of all the pediatric cases of ocular trauma that were seen in UIC's EEI for over 13 years were used to evaluate if the timing of amblyopia therapy affects visual outcomes. Biochemical studies of CYP inhibition, cell death studies with 661W and a mouse model of retinal degeneration were used to evaluate a candidate therapy and its analogs.

Rabbit studies demonstrated a significant treatment benefit to both enoxaparin, a low molecular weight heparin, for the prevention fibrosis. The effect was augmented by enoxaparin in combination with a low dose intraocular steroid, preservative-free triamcinolone, with a reduction of multiple measures of inflammation and fibrosis. Tissue plasminogen activator was found to be an effective treatment to solubilize fibrin formed in the anterior chamber after lensectomy. In children under the age of 8 with a history of ocular trauma, visual acuity outcomes were improved if amblyopia therapy was initiated within the first three months after the initial injury. Despite promising results in the cell-based studies, our top sulfaphenazole analog, KB-2-001, showed no protection in animal studies.

I. INTRODUCTION

A. Anatomy and physiology of the eye

The eyes are responsible for our most used sense, vision. More than 75% of the information that humans gather about their surroundings is through vision and a significant portion of the brain is involved in visual processing. The human eye is a spheroid-shaped globe usually just under an inch in diameter. It is a complex system that developed from four different layers of embryologic tissues: neural tube, neural crest, surface ectoderm, and mesoderm (1). It resides in the orbit of the skull along with all of the extraocular muscles, nerves, and blood vessels necessary for the eye to function (2, 3). Figure 1 shows a labeled drawing of the eye and a schematic of the retina.

Externally, the front of the eye consists of the cornea, limbus, and sclera. The cornea and limbus comprise one sixth of the eye surface. The cornea is a clear 11-12mm structure with about an 8mm radius of curvature, giving the anterior of the eye a steeper appearance compared to the 12mm radius of curvature of the sclera. Because the cornea is normally transparent, the colored part of the eye known as the iris, and dark appearance of the center known as the pupil are seen instead. The iris is responsible for controlling the amount of light that gets into the eye, and the crystalline lens which helps focus the light onto the back of the eye. The cornea is joined to the sclera by a ring-like structure called the limbus. The sclera constitutes about five sixths of the external visible part of the eye and continues posteriorly into the orbit covering the entire eye. The extraocular muscles attach to the sclera and give the eye the ability to move within the orbit.

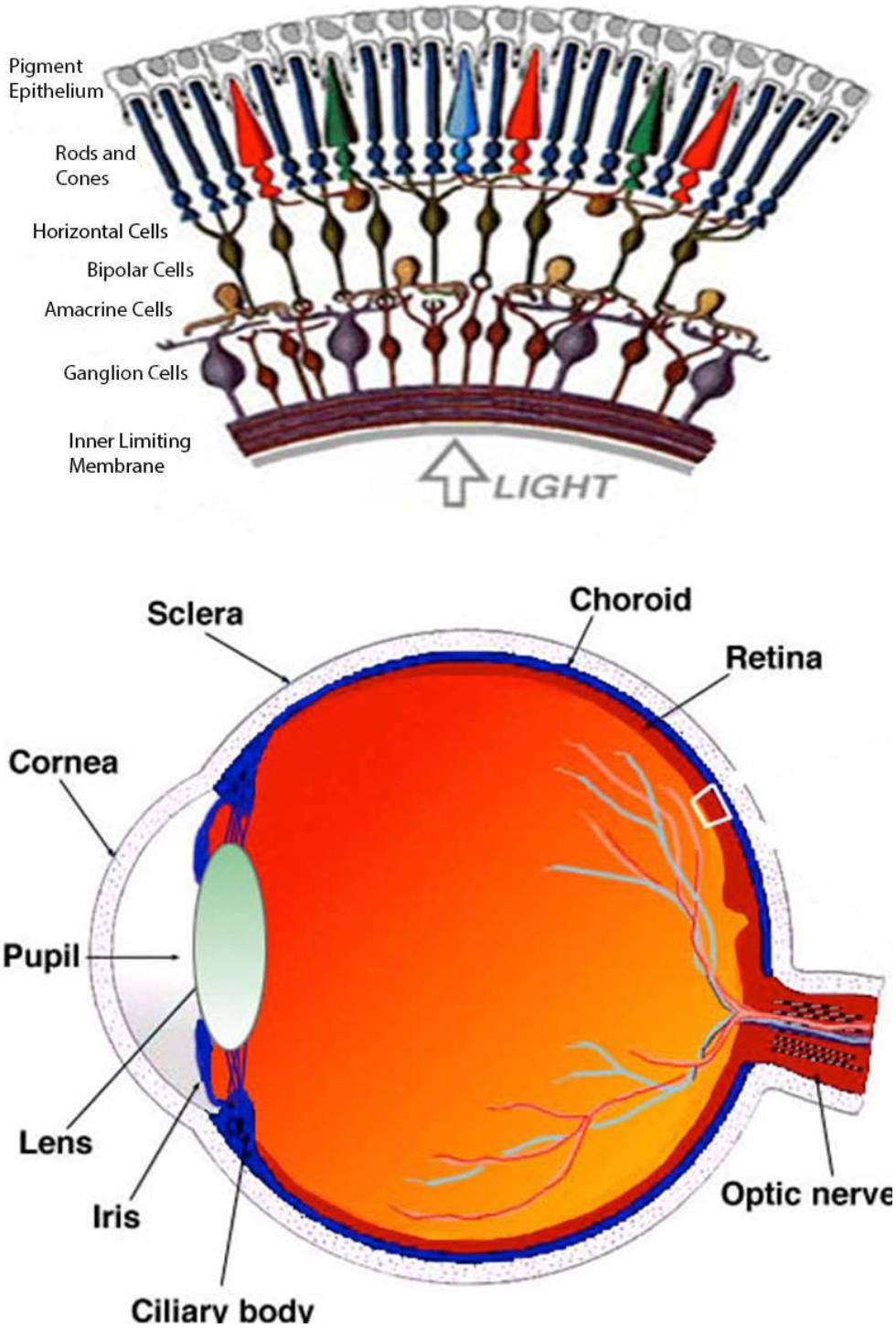

Figure 1 - Graphical representation of the human eye and retina. Many of the important structures are labeled. Adapted from: http://webvision.med.utah.edu/

The eye is divided into 3 segments, the anterior chamber, posterior chamber, and vitreous cavity. The anterior segment is between the cornea and iris and contains a clear fluid, the aqueous humor. The aqueous humor has many functions, including maintaining the structure and intraocular pressure globe, providing nutrients to the avascular tissues of anterior of the eye, and maintaining the immune response. Aqueous humor is also found in the posterior chamber, the space behind the posterior iris and anterior to the lens and vitreous. This aqueous is constantly being made by the ciliary body in the posterior chamber and drains out of the eye through the trabecular meshwork before eventually exiting through one of the veins of the orbit.

The lens provides about one third of the focusing power of the eye, the rest is provided by the corneal air-tear interface. The crystalline lens is a mostly acellular biconvex structure made up of several components that, along with the cornea, serves as a major source of refractive correction of the eye to focus light onto the retina (Figure 2). The lens is held inside a capsule that is an elastic, transparent basement membrane of type IV collagen fibers. The thickness of the lens capsule is asymmetric; it is the thickest anteriorly and at the posterior preequitorial poles and is as thin as two micrometers in humans at the central posterior pole. The capsule serves as an attachment point for the zonular fibers that are responsible for suspending the lens inside the eye at the proper position. When the zonular fibers relax tension during ciliary muscle contraction, they allow for a range of focusing power of the lens, called accommodation. Beneath the capsule is a single layer of special epithelial cells. These cells are metabolically active and mitotic, with most of the cell division occurring in a ring near the center of the anterior capsule.

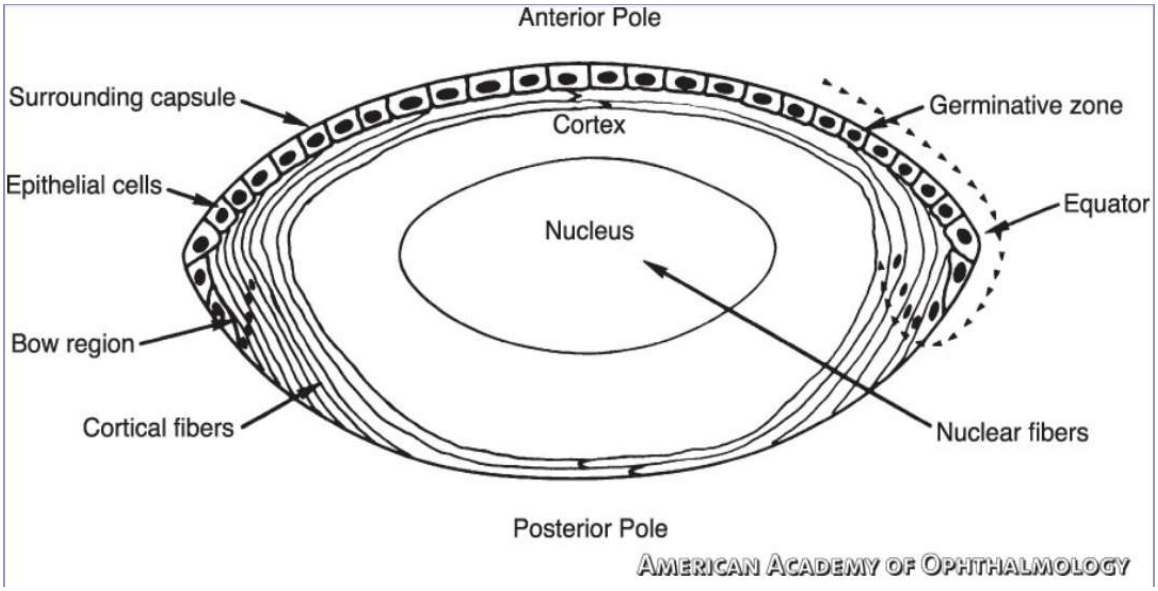

Figure 2 - Schematic drawing of the lens and capsule.
Adapted from: American Academy of Ophthalmology BCBS 2011-2012.

New epithelial cells slowly migrate towards the edges of lens before they begin to terminally differentiate into transparent lens fibers.

Behind the lens is the vitreous cavity, which contains a transparent jelly-like substance, which comprises about two thirds of the volume of the eye and also gives the eye its form and shape. Unlike the aqueous fluid that is constantly being replenished, the vitreous is present at birth and changes slowly over the course of our lifetime.

External to the vitreous is the retina, the tissue in the eye that is responsible for detecting light and encoding it as signal that is sent back to the brain through the axons of the optic nerve. Between the retina and the sclera is a layer called the choroid, which is a connective tissue that contains the blood vessels responsible for providing nourishment and oxygen to the outer retina. The vertebrate retina is a complex tissue with 10 distinct layers. These layers from internal to external are: the internal limiting membrane, optic or nerve fiber layer, ganglion cell layer, inner plexiform layer, inner nuclear layer, outer plexiform layer, outer nuclear layer, external or outer limiting membrane, photoreceptor layer with rods and cones, and the retinal pigment epithelium (Figure 3). All of these layers are visible in vivo through the use of a well-established imaging technique known as optical coherence tomography (OCT) (Figure 4). OCT is projects infrared light into the eye and captures the light that is reflected from the various features to generate a cross-sectional image of ocular structures. OCT is able to generate much higher resolution images than the ultrasonography techniques because OCT uses light instead of sound waves (4).

While the eye is responsible for focusing light so that the retina can detect the information, it must be processed and interpreted by the visual system. Shapes, color, depth, edges, contrast, and movement, are a result of how the brain interprets the information sent from the retina.

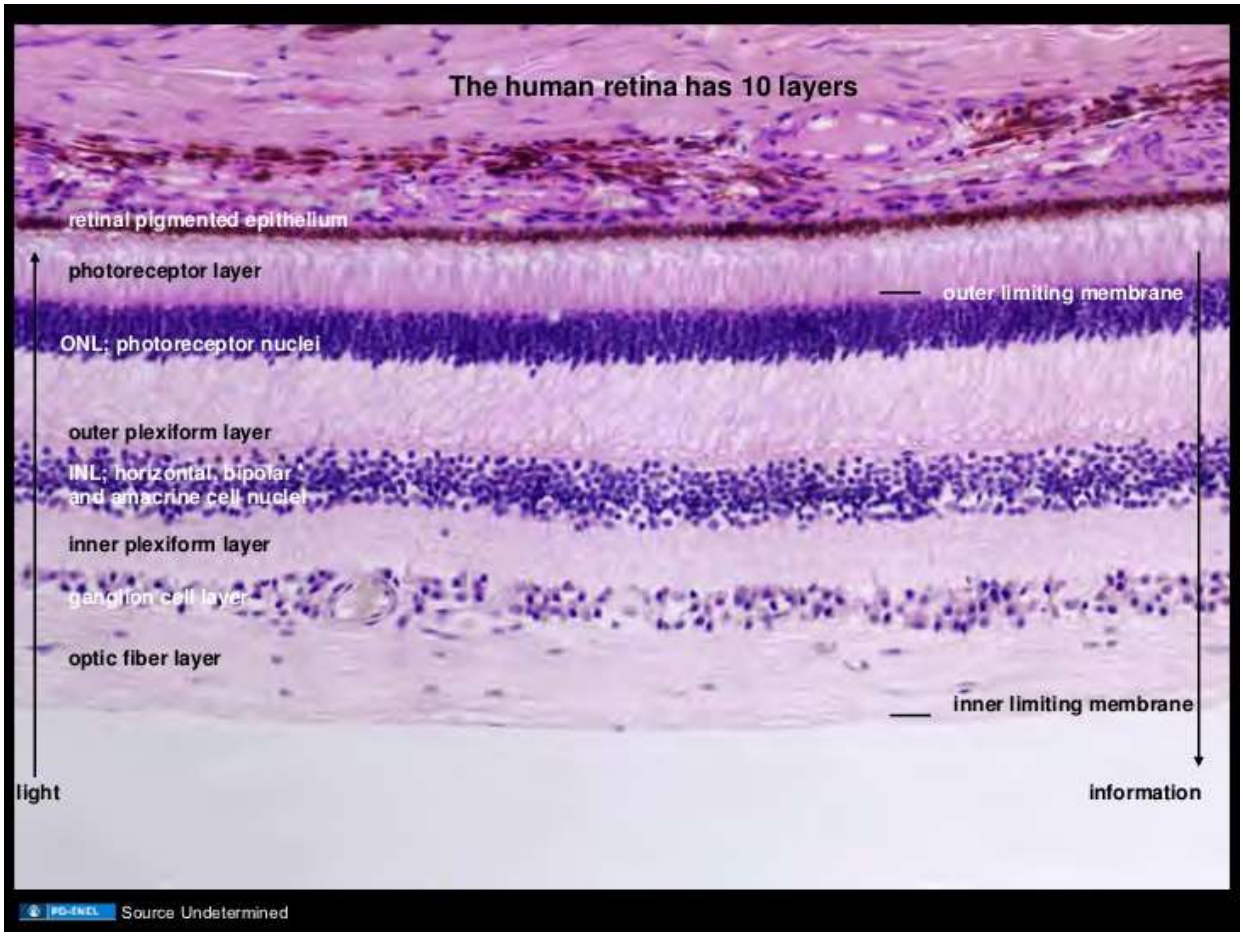

Figure 3 - Hematoxylin and eosin stain of a section of the human retina with the 10 layers of the retina labeled.
Adapted from: http://www.slideshare.net/openmichigan/032009p-hitchcockretina-andvisualsystemlecture

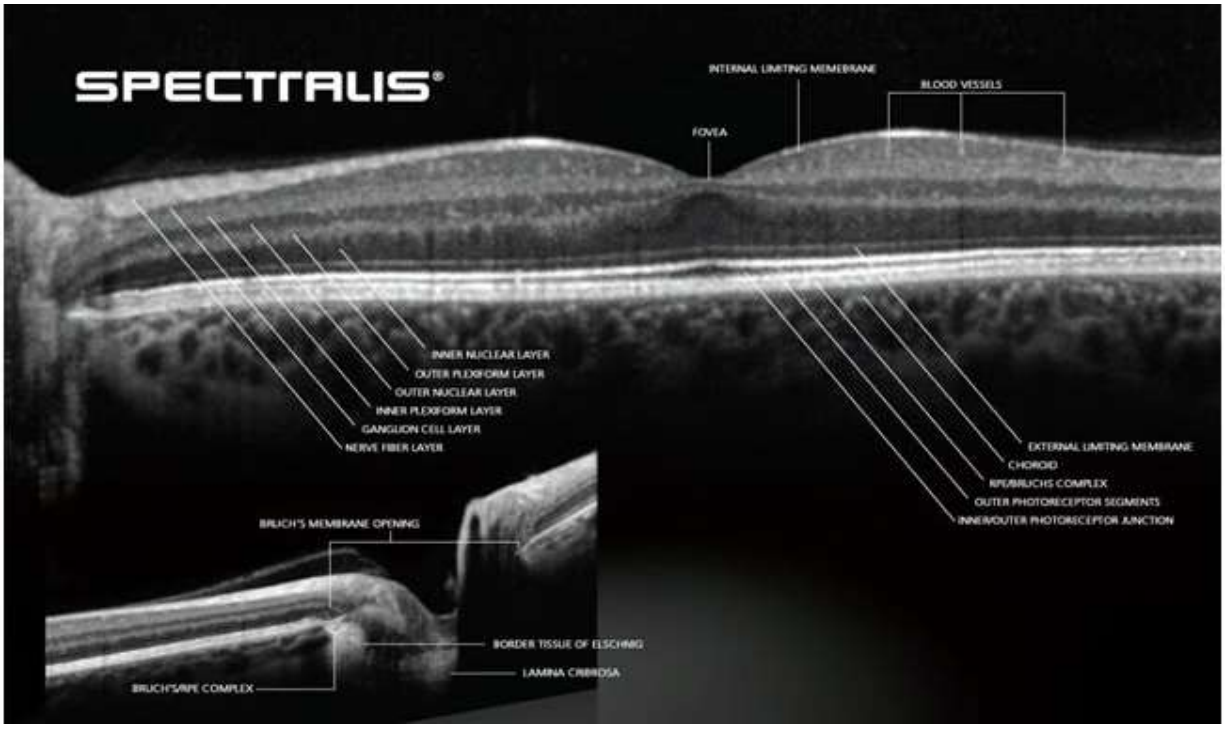

Figure 4 - Spectral Domain Optical Coherence Tomography (SD-OCT) image of a normal human retina, labeled with the different layers the retina. Upper image is the fovea of the retina and the bottom left image is of the optic nerve. Adapted from: http://www.eyeoptics.dk/429/spectralis-spectralisoct-spectralishra

Our understanding of how this complex, multi-level, multi-region process occurs was first explored in a landmark series of experiments performed by David Hubel and Torsten Wiesel in 1959 titled 'Receptive Fields of Single Neurons in the Cat's Striate Cortex (5). They continued to study visual processing for over 25 years and published over 30 manuscripts together. In 1981, they were awarded the Nobel Prize in Medicine "for their discoveries concerning the visual system," shared with Roger W. Sperry. Of their many discoveries, Hubel and Wiesel described how visual information is processed from 2 eyes into one cohesive picture of the world, and how the lack of normal vision during critical periods of visual development affected the brain.

After photoreceptor cells detect photons, retinal ganglion cells transmit the information to the thalamus, a structure in the brain responsible for aggregating and relaying sensory and motor information from the body to cortical tissue. From there, thalamic cells innervate of the layers of the striate cortex. Hubel and Wiesel showed that cells in the striate cortex show a fixed specificity in their responses to different retinal stimulations (5, 6). These cells are responsible for detecting 'edges' or straight-lined boarders that separate areas of different brightness's in a fixed orientation. Additionally, cells in the striate cortex are divided into columns that extend from the external surface deep to the white matter of the brain. All cells in a column respond to the same edge orientation (6, 7). While each individual cell in a column responds to the same edge orientation, each cell has its own preference for which eye it detects from. Each cell could be driven by one eye nearly exclusively, by both eyes equally, or anywhere in-between (6). While eighty percent of cells within the edge orientation column can be influenced by both eyes, the relative influence of the two eyes on a specific cell varies in healthy adult cats from cell to cell (8). Cells that are dominated by one eye are grouped together in columns that are perpendicular to the edge orientation columns, referred to as ocular dominance columns (8).

In addition to describing the processing of visual information, Hubel and Wiesel demonstrated that there is a critical period for visual development in primate and non-primate species (9-12). Ocular dominance columns are formed early in life during the critical period. If the striate cortex doesn't receive normal input from both eyes these columns will not form properly (9, 12-14). In cats, this period starts around the fourth week of life and lasts for several weeks. Monocular deprivation for even short periods of a few days during this critical period resulted in drastic changes. Not only was there a shift in the number of cells that were driven by the normal eye, but there was also a significant decline in the number of binocularly driven cells.

These changes to the organization and sensitivity of striate cortex cells from monocular deprivation are the physiologic basis for amblyopia, or "lazy eye." Amblyopia is a manifestation of a visual disruption during the critical period of visual development. Clinically, amblyopia is the reduction of the best-corrected visual acuity of an eye that cannot be attributed to any structural or functional abnormality of the eye or optic nerve. In humans, amblyopia can develop before the age of 8. There are several different types of amblyopia. Refractive amblyopia is caused by a high refractive error in one or two eyes and is the most common type of amblyopia. When there is a significant difference in the refractive error between two eyes causing decreased vision in one eye, it is referred to as anisometropic refractive amblyopia. Strabismic amblyopia is caused by a misalignment of the eyes, resulting in both eyes not receiving congruous visual input to the fovea. This results in suppression of one eye in order to reduce visual confusion and double vision. Deprivational amblyopia, the third type of amblyopia, results from an obstruction to central vision, or when the visual input is not transmitted to the brain.

The neuroplasticity of the early visual system also means there is a window of opportunity to treat amblyopia and improve visual acuity. To accomplish this, the treatment of amblyopia

involves first correcting the cause of the amblyopia such as correcting refractive error or eliminating the obstruction. Amblyopia therapy also includes penalization of the sound eye with occlusion or pharmacological methods. Limiting the use of the better eye can be accomplished by either patching the better eye or by using cycloplegic drops such as atropine, limiting accommodation and blurring the vision at near.

With all of these different structures of the eye and visual system that make it possible for sight, it should not be surprising that even minor disruption to any of these tissues is often enough to cause dramatic changes in vision. An opacity or disruption of the corneal contour, anterior chamber inflammation or bleeding, lens clouding, opacities of the vitreous from inflammation or bleeding, acquired or inherited retinal problems, high refractive errors, or problems with the brain's interpretation of the visual input are all potential causes of vision loss.

In this thesis, I sought to investigate therapeutic strategies to several causes of vision loss through cellular, pre-clinical, and clinical evaluations. I specifically investigated the potential treatment of two problems that affect children, congenital cataracts and amblyopia from open globe ocular trauma. I also focused on a potential therapy for an inherited retinal degeneration, retinitis pigmentosa.

B. Cataracts

For the lens to function properly, it must maintain its own clarity. A reduction in the optical clarity of the lens is referred to as a cataract. According to the World Health Organization (WHO), cataracts are the leading cause of blindness and visual impairment worldwide. In 2002, the WHO estimated that cataracts were responsible for over 17 million cases of reversible blindness of the 37 million people in the world suffering from blindness. This number is expected to rise to over 40

million by 2020 because of the aging of the general population (15). Because there is currently no treatment other than surgery for a visually significant cataract, they are significant socioeconomic burden both around the world. Here in the United States, the government spends over 3.4 billion dollars each year through Medicare and over 8000 cataract surgeries are performed per million of population. Furthermore, patients with vision loss have significantly higher medical costs, more than 90% of which are not directly related to their vision loss such as depression, injury, and long term care admission (16). Due to limitations of cost and availability, fewer than 50 cataract surgeries are performed per million people in developing countries, less than one percent of the United States. The socioeconomic burden for vision impairment is worse in developing nations because they remove two people from the workforce, the affected individual and another person to care for them.

While cataracts are predominant in the aging population, they can also be present at birth, develop in childhood, or be secondary to trauma or systemic conditions. Congenital cataracts are estimated to have a prevalence between 3 to 40 per 10,000 newborns and are estimated to be responsible for as much as 10% of all childhood blindness worldwide (17, 18). Pediatric cataracts can be unilateral or bilateral as an isolated occurrence or as part of a syndrome. Cataracts can be stable or progressive. Regardless of the etiology, if a cataract is visually significant in a child, it requires prompt treatment because of the risk of decreased vision from amblyopia. Earlier removal of visually significant cataracts is beneficial for better visual outcomes and most sources recommend removal of a unilateral cataract by 6 to 8 weeks of age (19). Removal is recommended earlier with unilateral cataracts than with bilateral cataracts because if one eye has normal vision, it is difficult to obtain good vision secondary to amblyopia of the affected eye. Bilateral cataracts can be removed slightly later because both eyes have decreased vision. However, since the first

several months of life are most critical for visual development, there is a risk of nystagmus in children with bilateral cataracts. Once nystagmus is seen in a child, the potential for a good visual outcome is significantly decreased.

C. Cataract Surgery

Once a cataract becomes visually significant and there is no metabolic issue that can be corrected to reverse the lens opacity, the only option is to remove it surgically. The most common current treatment is extracapsular cataract surgery. In adults, this involves tearing a hole in the anterior lens capsule and removing the lens nucleus and cortex while leaving the posterior lens capsule intact. By leaving the posterior lens capsule intact in the eye, the anterior and posterior chambers are able to maintain their separation from the vitreous. The lens nucleus of an adult can be removed by expressing the nucleus in large fragments or in its entirety or by breaking it up into small fragments with phacoemulsification. Phacoemulsification uses ultrasonic energy to break up the hard nucleus of the lens into smaller pieces that can be removed with vacuum suction, allowing lens removal through a small incision. In children and young adults, the nucleus is usually soft enough that the entire lens can be aspirated. Before the widespread use of phacoemulsification, extracapsular cataract surgery involved removal of large fragments and utilized a tool known as a Simcoe, which is a co-axial double lumen cannula. One lumen irrigates a fluid into the eye replacing aqueous humor, while the other aspirates the lens material from the chamber. Simcoe allows for manual control of suction using a syringe and can be used for removal of soft lenses such as those in pediatric cataracts or for the cortical material of older patients.

Pediatric lensectomy is different than adults not just by the softness of the lens and amblyopia, but also by the postoperative course. Children have a rapid opacification of the lens

capsule that is so robust the center of the posterior capsule is often removed along with a portion of the anterior vitreous in order to prevent opacification of the visual axis. In addition, the inflammatory response to lensectomy in children is more robust. In young children, a lens is often not implanted as a primary surgical procedure because of the high incidence of complications resulting in the need for re-operation (20, 21). If a lens is not implanted in the eye, the eye is said to be aphakic. If an eye is left aphakic, there is usually a residual high refractive error that must be treated with a contact lens. Contact lenses for aphakia have their own challenges and risks that will be discussed in detail in the next chapter. In chapter 2, I investigated the efficacy of available therapies to reduce this response and allow insertion of an intraocular lens using a rabbit model of lensectomy.

D. Treatment of Children with Open Globe Trauma

Information gained from studies of novel therapeutics in rabbits can potentially help improve visual outcomes in children who need cataract surgery, including those who have had an ocular injury. Children who have suffered an injury resulting in an open eye need immediate surgery to close the eye. Patients often have an unpredictable postoperative course with widely variable visual outcomes (22-25). This variability in outcomes may be in part be because of the risk of amblyopia, and explains why younger age is an independent risk factor for poorer visual outcomes (25-27). I hypothesized that by treating amblyopia early, before decreased visual acuity is established and possibly before the onset of physiological and anatomical changes described by Hubel and Wiesel, there may be a measured effect on visual outcome in children with eye trauma. In chapter 3 of this thesis I determined the effect of early amblyopia therapy on visual outcomes of children with open globe ocular trauma with a retrospective case series.

E. Potential Therapeutics for Retinitis Pigmentosa

Retinitis Pigmentosa (RP) is a group of inherited eye diseases that share common clinical phenotypic presentation, namely, the degeneration of the retina. The term was first used in 1857 to describe the spicules of pigment seen throughout a patient's degenerated retina that was believed to be caused by an infection. With over 100,000 people affected in the United States and over 1.5 million worldwide, RP is the most common cause of inherited visual impairment. Annual total healthcare costs for RP patients were found to be over $7000 a year higher than unaffected individuals even before considering additional expenses such as caregivers, rehabilitation, home assistance, and institutional care (28). Additionally, there are substantial emotional costs for patients and their families. Anxiety, disorientation, depression, loss of independence, and difficulty with work or activities of daily living are some of the problems faced by patients and loved ones.

The vision loss in patients with RP is progressive and can be severe. RP is a highly variable disorder, with some patients developing symptoms in the first decade of life and others as late as the sixth decade. Symptoms include night blindness and dark adaptation difficulties early, followed by a slow insidious loss of their peripheral vision. This constricts their visual field making it difficult to drive. As the disease continues to advance, some eventually lose their central vision (29). Visualization of the retina invariably shows narrowing of the retinal vasculature. Optic disc waxy pallor, bone-spicule like pigmentation, and a thinning appearance to the retina are also common findings in RP patients, which become more prevalent as the disease progresses (30).

Research efforts over the past thirty years have led to an exponential growth of knowledge regarding inherited eye diseases such as RP. Since the first retinitis pigmentosa gene was discovered in 1989, over 200 genes that cause any inherited eye disease have been discovered (31). Retinitis pigmentosa is a genetically complex disease that has 61 non-syndromic known

causal genes that show a broad range of expressivity (31-34). Specifically, genes involved in each of the following groups have been identified: phototransduction cascade, vitamin A metabolism, structural/cytoskeletal, cell signaling, cell-cell interaction, synaptic transmission, RNA intron splicing, protein trafficking, maintenance of cilia, pH regulation, and phagocytosis. Unfortunately, this explosion of knowledge has not yet lead to an approved treatment for RP. The genetic causes of RP are as varied as the disease's presentation. It can be inherited as an autosomal dominant (30%-40%), autosomal recessive (50%-60%), or X-linked recessive condition (5-15%) (32-34). Additionally, affected family members with the same mutation(s) can present with different phenotypes (35, 36). Many early treatment attempts failed to consider this heterogeneity. More recent gene-directed treatment approaches have focused on treating a subset of patients with a specific gene mutation.

While individual family members can be affected by the disease differently, examining these mutations on a population scale shows that there is a good amount of predictability in a patient's disease course from their genotype (37). There is some deal of predictability of a patient's genotype from their disease presentation and course (35). This allows physicians specializing in inherited retinal diseases to predict the most likely causal genes in a patient based on their presentation and disease course, making for easier and faster identification of a patient's genotype.

In order to identify novel therapeutics and pathways for the treatment of retinitis pigmentosa, we created a high-throughput cellular light-induced toxicity assay in an SV40T transformed murine cone photoreceptor cell line known as 661W to allow for the screening of thousands of compounds simultaneously. 661W is a well-established cell line that has been used as a model for light-induced retinal degeneration (38-47). Bright white light-induced retinal

degeneration has been used as a model for over 40 years to study the mechanisms of photoreceptor cell death in retinal degenerative disease (48). Our lab has previously used this model to successfully identify pathways involved in photoreceptor death after establishing that this assay mimics the cell death of photoreceptors seen in retinal degeneration (49). We conducted a high-throughput screen using the Prestwick library, which contains 1,200 FDA approved small molecule drugs using this model. Bioinformatics analysis implicated several pathways with previously understood roles in retinal degenerative diseases.

Our top screening candidate was sulfaphenazole (SPZ), a selective cytochrome P450 2C9 inhibitor that is a novel target for neuroprotection in retinal degenerative disease. Secondary validation experiments have confirmed sulfaphenazole and CYP450 2C as a potential target in this model (50). In Chapter 4 I will discuss how molecular modeling of CYP2C9 and sulfaphenazole was used to perform intelligent drug design. Candidate inhibitors were created to develop a compound with improved potency and efficacy in both cell-based phenotypic and liver microsome screens. Several lead compounds were generated based on this approach and are presented in Chapter 4. One of the top compounds was also tested using animal models of retinal degeneration.

II. JUVENILE RABBIT ANIMAL MODEL OF LENSECTOMY TO EVALUATE THERAPEUTIC INTERVENTIONS FOR POSTOPERATIVE INFLAMMATION AND FIBROSIS

A. Introduction

Cataracts are one of the most important causes of treatable childhood blindness while lensectomy is one of the most common intraocular surgeries in children. Studies in the UK and Sweden put the incidence of cataracts at approximately 3 per 10,000 children (51, 52). Even with such a low incidence, pediatric cataracts are responsible for approximately 10% of blindness in children in India (53). This highlight how a condition such as cataracts with a relatively low incidence can have a profound effect on the population if there is no access to treatment. In contrast to adults, children present a unique challenge compared to adults because of the risk of amblyopia. Because the visual system is still has plasticity during the first 8 years of life, any decrease in vision, such as those from a visual axis opacity from a cataract can cause lifelong visual impairment if the amblyopia is not properly treated. In children, the earlier a visually significant cataract is removed, the better the visual potential (19). However, the younger the child the more vigorous the immune response is to intraocular surgery, resulting in more inflammation and fibrosis. This often manifests as anterior chamber inflammation, fibrin membrane formation, corectopia or pupil displacement due to iris synechiae, a fibrotic posterior capsule opacity, or other complications that can impair vision (20, 21). Because of the risk of amblyopia, any complications that compromise the clarity of the visual axis require prompt intervention.

Because of the difference in the response to cataract surgery, lensectomy is often performed differently in a child than an adult. Like adults, a circular opening, or capsulorhexis, is made in the anterior lens capsule. A child's lens is soft compared to the hardened lens of an older

adult, so it is aspirated from of the lens capsule instead of using phacoemulsification. In younger children, there a circular opening is often made in the posterior capsule, or posterior capsulotomy, with removal of the anterior vitreous. This is done in younger children because of the risk of rapid and fibrotic opacification of the posterior capsule and anterior vitreous interface. If the child needs a posterior capsulotomy and anterior vitrectomy, some surgeons will approach the lens from an area called the pars plana just posterior the limbus and anterior the retina. In adults, posterior capsule opacification is much less frequent and slower in onset, so the posterior capsule is left intact and a lens is inserted into the capsular bag.

A lens may or not be inserted into the capsular bag and is at the discretion of the surgeon. The visual outcomes and complications with implantation of an intraocular lens versus leaving the child aphakic is being studied with an ongoing prospective multi-center randomized clinical trial, the Infant Aphakia Treatment Study (IATS). The Infant Aphakia Treatment Study randomized children between 4 weeks and 7 months of age at the time of surgery with a unilateral cataract to placement of an intraocular lens at the time of lensectomy or left aphakic with a contact lens for optical correction. The study showed that subjects with unilateral cataract had similar visual outcomes with or without IOL placement. Subjects treated with an IOL needed more surgery in the first year secondary to complications, many of which were related to scarring and membrane formation (54). The median visual acuity of the treated eyes in each group was 20/159 with their fellow eyes having a median visual acuity of 20/25. At 4.5 years of age, approximately 50% of patients in each group had poor best-corrected visual acuity worse than 20/200. However, subjects with intraocular lens implantation at the time of surgery had significantly more postoperative adverse events (81% IOL, 56% CL), and additional intraocular surgeries (72% IOL, 16% CL), often due to visual axis opacification than those treated with contact lenses (20, 21). In

the first year, the majority of the adverse events were seen in the IOL group. After the first year, there was a trend towards higher adverse events in the aphakic subjects treated with a contact lens (25% IOL, 42% CL), but this was not statistically significant with a p value of 0.073.

Therefore, physicians may be disinclined to insert and IOL during the primary surgery and consequently treat with a contact lens. However, significant issues arise from either form of treatment. Contact lenses place a significant burden on caretakers who must clean, maintain, and place the contact lens in an often uncooperative child, who may not tolerate the lens. The contact lens needs to appropriately match the refractive error of the child, leading to multiple appointments each year. In addition, contact lenses are also expensive and not readily available in developing countries, making it difficult to acquire new contact lenses when one is lost or when a new lens is needed. There are also risks of contact lens use. In the IATS, 18% of the subjects left aphakic experienced a contact lens-related adverse event including corneal abrasion, ulcer, or keratitis. Failure to correctly monitor the status of a contact lens or treat a corneal infection can leave the child with permanently reduced visual potential due to amblyopia.

Given the complications with primary IOL implantation and the limitations with contact lenses, our goal was to investigate treatments with the potential to reduce the postoperative adverse events and additional surgeries associated with the use of an intraocular lens in pediatric cataracts. This would make primary insertion of an IOL more appropriate, potentially improving visual outcomes and significantly reducing the burden of the caretaker.

1. Therapeutic Targets

The goal of the therapeutic agents used was to prevent or treat complications after lensectomy without needing to rely on topical administration of drops by the caretaker. I was

most interested in preventing pupillary membranes and corectopia since they accounted for 17 and 11 of the 62 postoperative complications in the IATS (20). These complications are believed to be the result of scarring and fibrin membrane formation. It is likely that inflammation is not the only mechanism involved in these adverse events. Inhibition of fibrin formation to prevent these complications should also be evaluated. In addition, I investigated the potential treatment of intraocular fibrin using a plasminogen activator to break up fibrin into fibrin degradation products as an alternative to surgical removal or repair.

2. Intraocular Preventative Therapies

While topical ophthalmic steroid use is the standard of care following many ophthalmic surgeries, an intraocular injection is often used to treat ocular inflammatory disease. Triamcinolone is a corticosteroid that is 5 times more potent than the natural compound cortisol, making it an extremely potent anti-inflammatory agent (57). Intraocular injections of preservative-free triamcinolone are already FDA approved and used to visualize certain ocular structures during surgery and to treat uveitis where its anti-inflammatory effects may last several months (58). Given the limited access, cost, and compliance to topical medications in developing parts of the world, effective preventative treatment with intraocular steroid at the time of surgery would be a great benefit. Triamcinolone has been studied to prevent postoperative complications after pediatric lensectomy (59). In this study, 4 to 8 mg of intraocular triamcinolone in addition to topical and oral steroids were shown to decrease postoperative complications compared to without intraocular steroid. However, long-term use of steroids, including triamcinolone, is not without it's risks. Steroid-response elevations in intraocular pressure can result in glaucoma and the reduced immune response from triamcinolone can make the eye more prone to infection (60).

Even when intraocular steroids are used during pediatric cataract surgery, pupillary membranes and corectopia may still occur. Because of these potential complications, I also investigated an alternative non-steroidal therapy for the prevention of fibrin formation after intraocular surgery.

Fibrin is formed from the conversion of fibrinogen to fibrin by thrombin (Figure 5). Prothrombin is converted to thrombin by factor Xa, which is activated either by the classical clotting cascade, or the alternate clotting cascade. Heparin is an anti-coagulant that decreases fibrin clot formation by inactivating thrombin through binding to anti-thrombin III and by preventing the conversion of prothrombin to thrombin. Heparin has also been noted to have some anti-inflammatory properties as well (61). Enoxaparin is a low molecular weight heparin with a longer half-life in the blood than heparin that is often used as a subcutaneous injection as an anticoagulant. The half-life for unfractionated heparin in the blood is about ninety minutes, but enoxaparin has a four and a half hour half-life. Figure 5 shows the final common pathway of clotting and how enoxaparin interacts with that pathway to prevent the formation of fibrin. In this chapter, I investigated the effect of intraocular injection of fibrin into the anterior chamber at the end of lensectomy in a rabbit animal model of lensectomy. I also evaluated if there is a synergistic effect of the combination of enoxaparin with low-dose triamcinolone (0.5mg) on the prevention of inflammation and fibrosis after lensectomy.

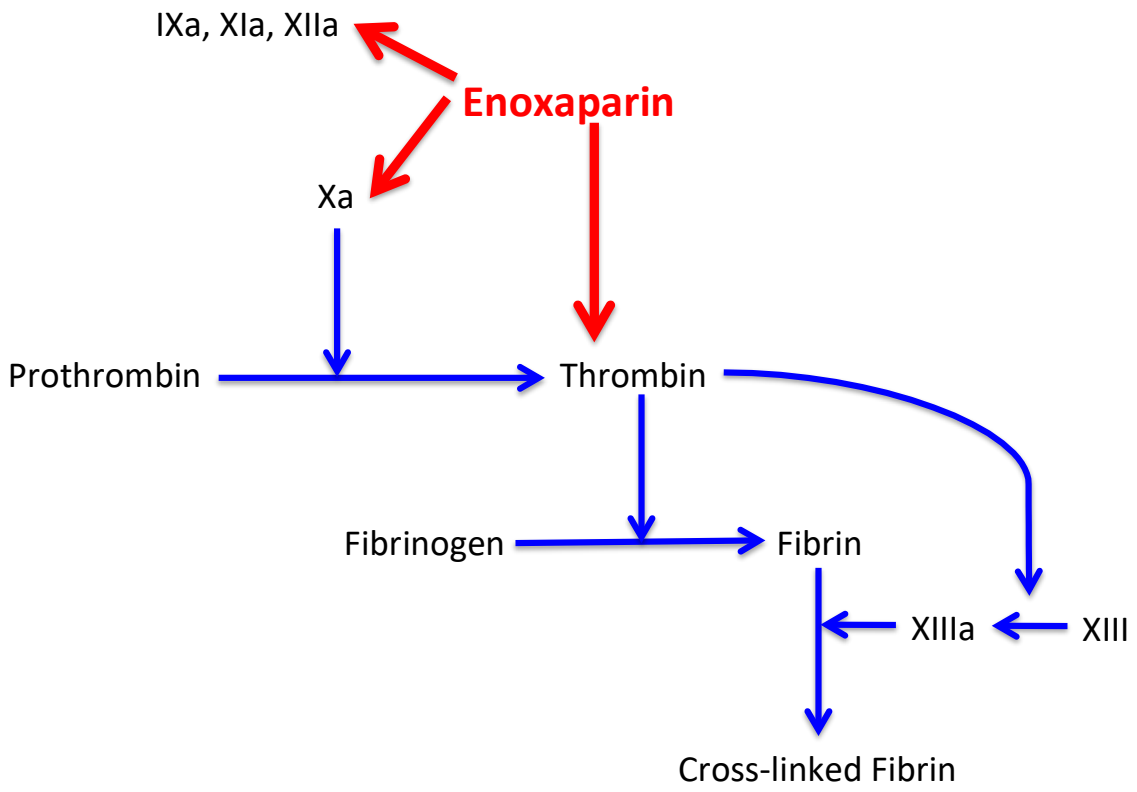

Figure 5 - Schematic diagram of the formation of cross linked fibrin and the role of enoxaparin to block its formation.

3. Treatment of Fibrin after Lensectomy

Once fibrin has formed in the eye, surgical removal or revision is often the only available treatment. I investigated the potential of pharmacologic dissolution of fibrin as an alternative to surgery. Plasmin is the major enzyme responsible for fibrin clot breakdown into degradation products (Figure 6). Tissue Plasminogen Activator (tPA) is a serine protease that enhances the conversion of plasminogen to plasmin. tPA has been used extensively as a thrombolytic agent in clinical medicine over the past two decades to treat acute myocardial infarctions, acute ischemic stroke, or pulmonary embolisms. The short timing window for the use of tPA is because after that time, the lack of blood flow has caused irreversible damage to the tissue, not because tPA would be ineffective at breaking up the clot. Given this efficacy in breaking up clots, I investigated intraocular tPA as an potential alternative to surgery in patients with fibrin in the anterior chamber.

4. Rabbit Animal Model for Lensectomy

We performed intraocular lens extraction and IOL insertion in juvenile New Zealand White rabbits. Rabbits are one of the most frequently used animal models for ophthalmic studies with the first laboratory experiments using a rabbit eye model dating back to 1827 (62-70). The rabbit eye model has been used to evaluate many new surgical techniques and tools such as phacoemulsification systems, IOLs, IOL insertion systems, irrigating solutions, and other novel technologies (71). The similarities in the anterior chamber and lens diameters make the rabbit an excellent animal model for evaluating the biocompatibility of IOLs in pre-clinical trials (72, 73). Rabbits are often used in paired and unpaired studies where both eyes are used in studies, reducing the number of animals necessary (71). Like humans, younger rabbits have a more robust

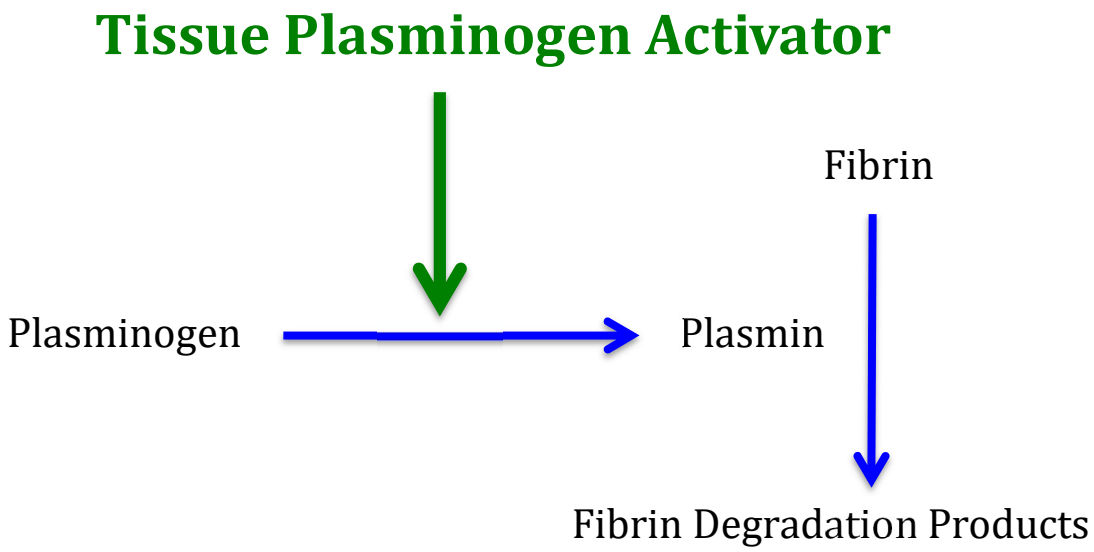

Figure 6 - Schematic diagram of the degradation of cross linked fibrin and the role of tissue plasminogen activator (Alteplase) to speed the breakdown of fibrin.

response to lensectomy than adults, making it an ideal model to study the effect of intraocular lens implantation in a juvenile model (71). Using this model, we investigated the intraoperative use of pharmacological agents triamcinolone and enoxaparin (low molecular weight heparin) on postoperative inflammation and fibrosis.

5. Optical Coherence Tomography

OCT is a well-established imaging technique developed over 20 years ago that uses light to capture micrometer resolution images from optical scattering media light biological tissues (4, 74). OCT is similar to ultrasound, except by using light instead of sound, higher resolution images are acquired. Keane and colleagues recently demonstrated that OCT signal strength is useful as an objective measurement for vitreous inflammation in humans (75). Figure 7 shows a high definition scan of the rabbit retina adapted from Muraoka et. al. (2012) with the layers of the rabbit retina labeled (76). These layers are similar those seen in the human retina (Figure 4), but the OCT does not have the central foveal contour because there is no macula in the rabbit. In addition to clinical examinations in rabbits with the therapies described above, I also used OCT signal strength to quantify the clarity of the entire visual axis. Together, these multiple measures allowed me to determine multiple aspects of the efficacy of the therapeutic interventions on the postoperative course in response to juvenile lensectomy in a rabbit animal model.

B. Methods

6. Animal Preparation & Lensectomy

All experiments were approved and in compliance with the Institutional Animal Care and Use Committee at the University of Illinois at Chicago (UIC) and the Medical College of Wisconsin (MCW). New Zealand White Rabbits (Harlan or Covance) were anesthetized with intravenous

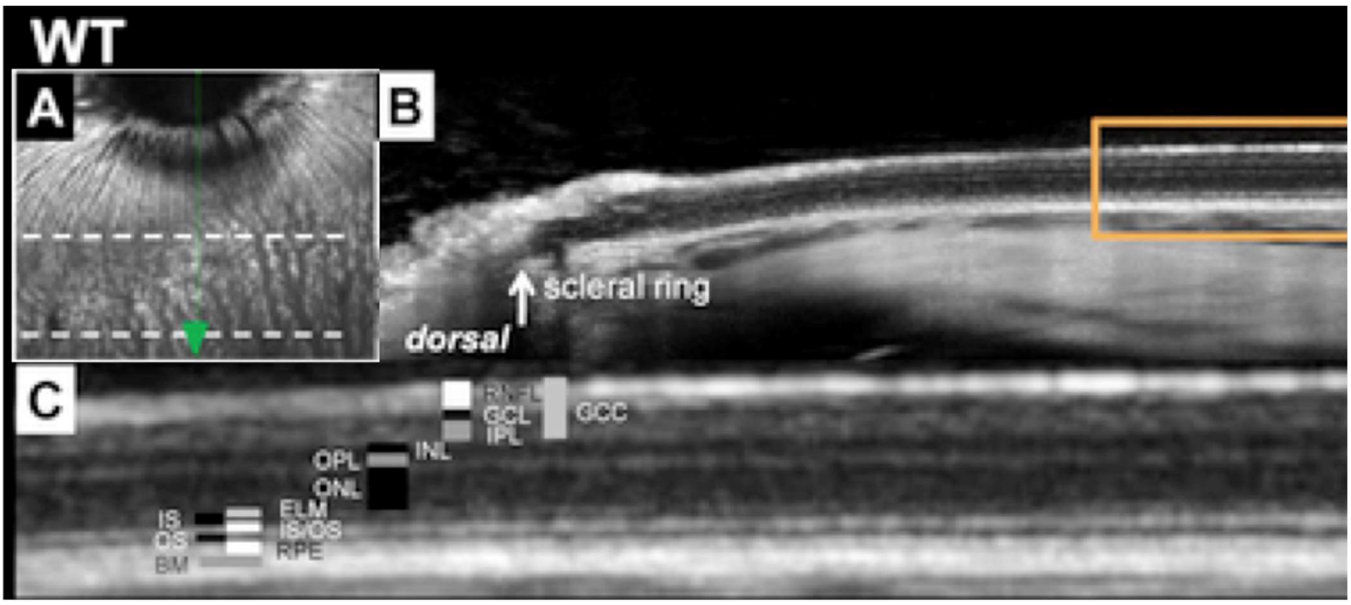

Figure 7 - A high definition Spectral Domain Optical Coherence Tomography image of a rabbit retina.
Adapted from Muraoka et. al. (2012). The different layers and sections of the retina are labeled. (A) red free image showing the section of retina being imaged by OCT (B) OCT of a normal rabbit (C) zoomed in section of the retina from B, with the layers labeled.

ketamine and xylazine 50-60 and 5mg/kg at UIC and isoflurane at MCW. Bilateral clear-cornea lensectomy surgery was performed as follows. The eye was prepped with 5% betadine solution into the eye and surrounding area was prepped with 10% betadine and draped. A clear-corneal incision was made just anterior to the limbus using a 20 gauge MVR blade. The anterior chamber was then reformed with viscoelastic. A cystotome needle was used to puncture the anterior capsule followed by micro-utrata forceps to make a continuous curvilinear capsulorhexis. The lens was then removed by irrigation and aspiration via a Simcoe double lumen irrigation/aspiration cannula. The anterior chamber and capsular bag were then filled with viscoelastic and the wound was expanded with a 2.4mm keratome blade. A 10-0 nylon mattress suture was pre-placed into the corneal incision. An acrylic foldable intraocular lens (Alcon SN60WF 30D; Fort Worth, TX), was inserted into the capsular bag. The viscoelastic was then removed with Simcoe irrigation and aspiration. The preplaced suture was tied to close the wound and the knot was buried. Once the wound was deemed to be watertight, either 8mg enoxaparin, 0.5mg preservative-free triamcinolone, a combination of 8mg enoxaparin and 0.5mg triamcinolone, or balanced salt solution was injected into the anterior chamber. Rabbits received the same treatment in both eyes with 6 eyes for each treatment group. The rabbit was then awakened from anesthesia. Rabbits received topical erythromycin twice daily for 4 days and analgesia with subcutaneous buprenorphine (0.01-0.05mg/kg) perioperatively, then twice a day for 3 days as needed. The rabbits were not given heparin flush or NSAIDs at any time pre or postoperatively.

7. Postoperative Examinations

Postoperatively, rabbits were examined under sedation with ketamine and dexmeditomidine 1-5/0.5mg/kg subcutaneously on days 3 through 7 and on day 14. Examinations

consisted of Spectral-domain Optical Coherence Tomograph (OCT, Spectralis OCT, Heidelberg Engineering), intraocular pressure and corneal pachymetry measurements, and slit lamp biomicroscopy examinations with photography. We used SD-OCT to quantify the clarity of the visual axis by measuring one half disc diameter just below the optic nerve. The thickness of the cornea was measured using either corneal OCT or a pachymeter **(**PachPen, Accutome**)** and the intraocular pressure in each eye was also measured using an ICare rebound tonometer. Slit lamp biomicroscopy was used to evaluate each eye in the following categories: iris synechiae, iris dilation in millimeters, hemorrhage in the anterior chamber, the amount of fibrin in the anterior chamber, the cell grade, the flare grade, and the amount of posterior capsule opacity that was present. Pupil synechiae were documented as the clock hours where the iris is adherent to either the anterior capsule or the lens itself. The amount of fibrin in the anterior chamber was measured as a percentage of the 6mm optic that was covered. Figure 8 shows examples of eyes that were estimated at 0% to 100% in 20% increments along with their corresponding OCT signal strengths. Cell and flare grade were measured using the SUN classification system criteria, a well-established and accepted clinical grading scale for uveitis (77).

A subset of rabbits had unilateral lensectomy and received no treatment at the time of surgery. Examinations occurred as above on postoperative day 3. Then under sterile conditions prepped with betadine, 100 to 200 microliters of fluid were removed from the eye with a sterile 30-gauge needle on a 1cc syringe. Then, an injection of either 25 micrograms of sterile-filtered rabbit tissue plasminogen activator in 50uL of BSS (tPA, Molecular Innovations) or in 50uL of BSS (control) was injected into the anterior chamber of the eye. Postoperative examinations were performed on days 4-7 after lensectomy (1-4 after treatment) and day 14 (day 11 after treatment) as described above.

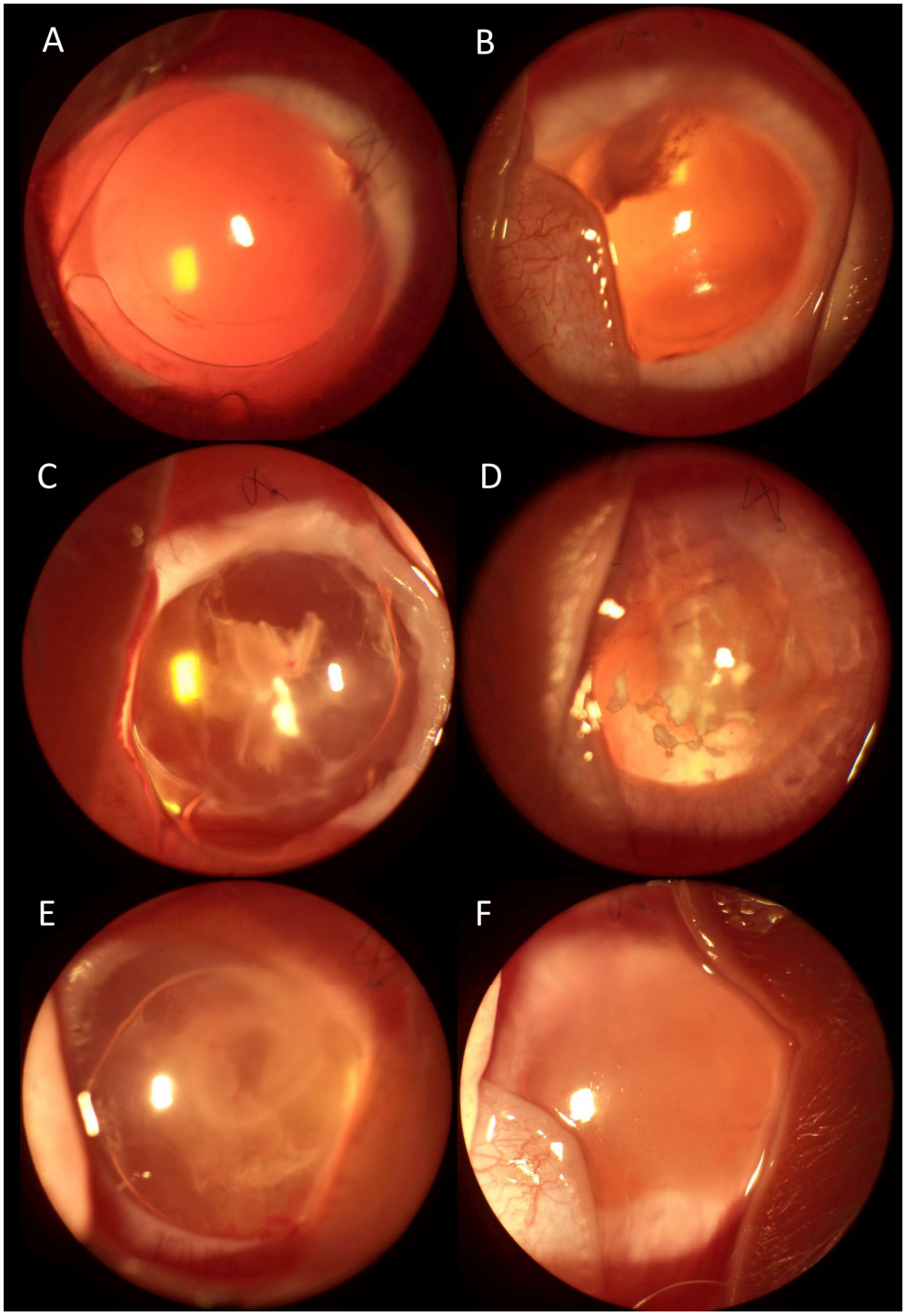

Figure 8 – Eyes with anterior chamber fibrin
Representative examples of eyes with anterior chamber fibrin graded from 0% to 100% in 20% steps. (A) 0% fibrin; postop day 3 OD; OCT signal strength: 28.33 (B) 20% fibrin; postop day 5 OS; OCT signal strength: 18.89 (C) 40% fibrin; postop day 5 OS; OCT signal strength: 16.06 (D) 60% fibrin; postop day 4 OS; OCT signal strength: 15.11 (E) 80% fibrin; postop day 4 OS; OCT signal strength: 0.2 (F) 100% fibrin; postop day 3 OS; OCT signal strength: 0.2

8. Statistics

The data curves were compared using a generalized linear mixed model with interactions (R version 3.1.2). Categorical data such as cell and flare was compared using Fisher's exact tests in R version 3.1.2. A p-value < 0.01667 was considered to be statistically significant to correct for 3 comparisons with a p-value of <0.05.

C. Results

All of the rabbits had bilateral surgery with three rabbits per group for a total n of 6 eyes in the following groups: untreated or control (balanced salt solution), 8mg enoxaparin, 0.5mg preservative-free triamcinolone, or a combination of 8mg enoxaparin and 0.5mg triamcinolone. Untreated eyes had a large amount of fibrin in the anterior chamber after lensectomy with intraocular lens implantation in the juvenile rabbit that decreased over time. This corresponded to the decrease in OCT signal strength observed with increasing amounts of fibrin in the anterior chamber (Figure 9). There was a significant decrease of fibrin in the eye when treated with enoxaparin or a combination of enoxaparin and triamcinolone. (Figure 10 c, d and f, Figure 11), which significantly increased the OCT signal strength (Figure 12, $p < 0.005$).

Examining the average amount of fibrin in the anterior chamber over time in all eyes in each group over time, (postoperative day 3 – 7 and day 14), both enoxaparin and the combination of enoxaparin and triamcinolone significantly reduced the amount of fibrin present in the anterior chamber compared to control (BSS) (Figure 11, $p<0.005$). Compared to control, 0.5mg of triamcinolone did not have a significant decrease in anterior chamber fibrin ($p = 0.3853$).

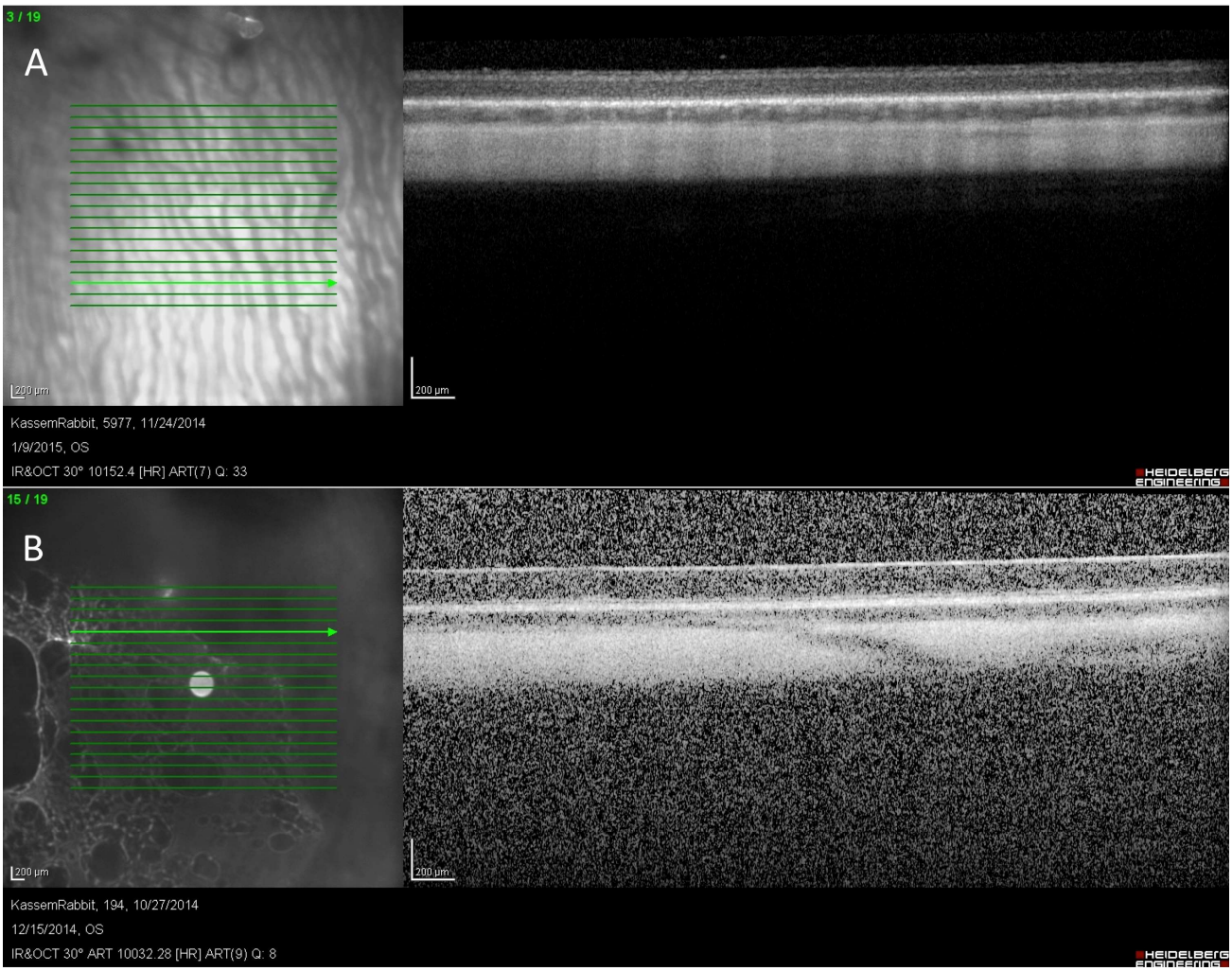

Figure 9 - Representative OCT images with and without treatment
(A) an eye treated with enoxaparin and triamcinolone with high signal strength quality (32dB) on postop day 3 (B) an untreated eye on postop day 3 with poor signal strength quality (8dB)

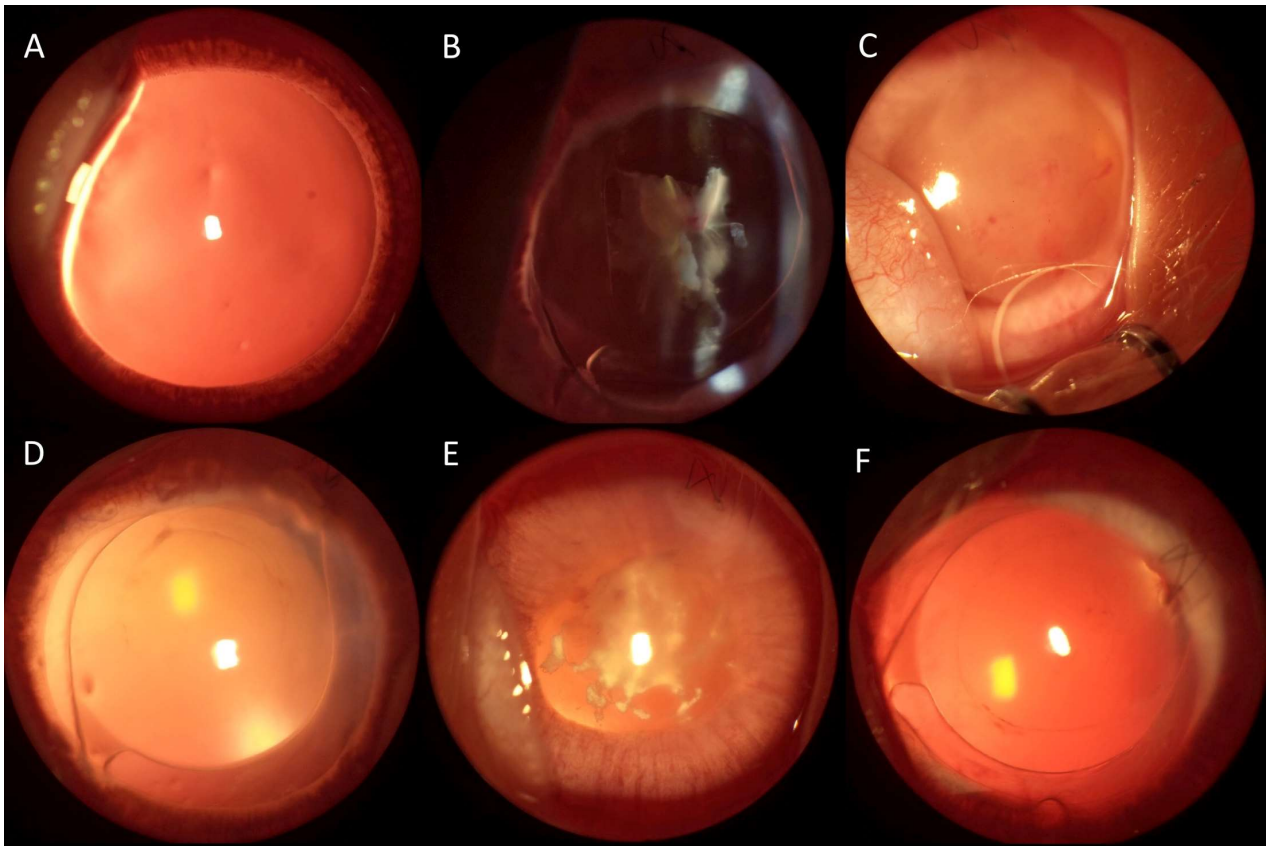

Figure 10 - Representative slit lamp photos of rabbit eyes (A) prior to lensectomy (B) post lensectomy with cell/flare. Images C-F are post lensectomy with intraocular lens implantation postoperative day 3 (C) post lensectomy treated with balanced salt solution (untreated) (D) treated with 8mg of enoxaparin (E) treated with 0.5mg of triamcinolone (F) treated with both 8mg of enoxaparin and 0.5mg of triamcinolone

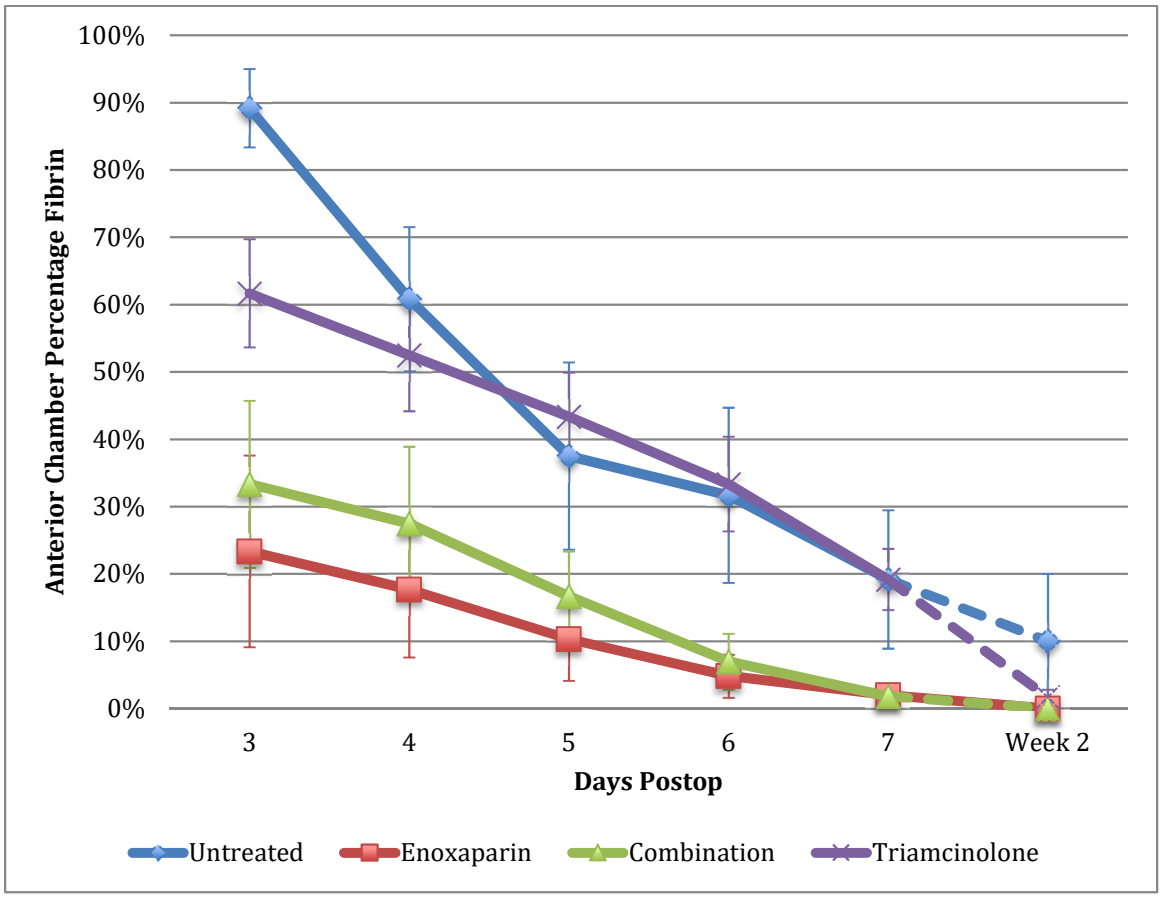

Figure 11 – Anterior chamber fibrin by treatment and day
The average percentage of the anterior chamber with fibrin quantified by slit lamp examination.

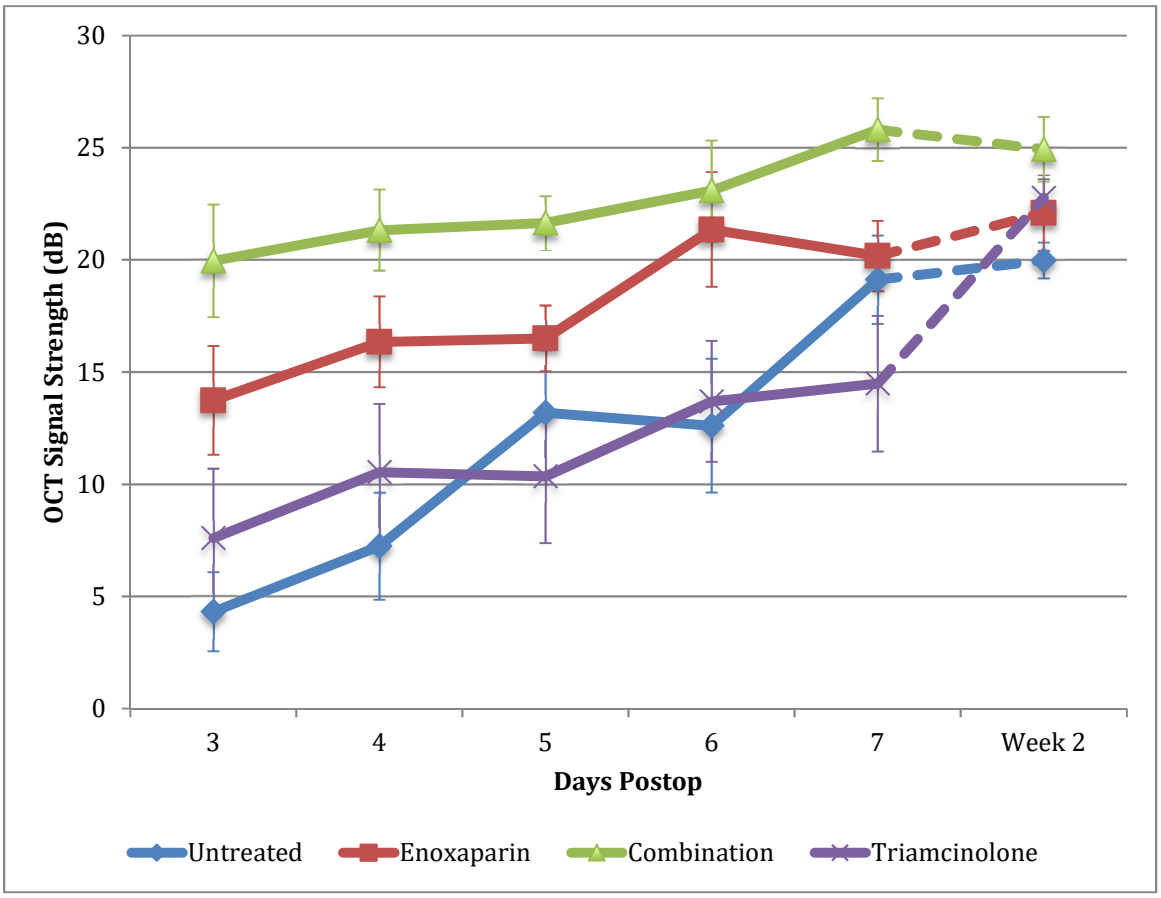

Figure 12 – OCT signal strength by treatment and day
The average OCT signal strength measured using in decibels (log scale). This represents an objective measure of visual axis clarity.

The anterior chamber fibrin results correlated with the pupil synechiae. There were pupil synechiae in the control and triamcinolone groups that were not present in the eyes treated with enoxaparin or the combination of enoxaparin and triamcinolone (Figure 10 c-f). Consistent with less synechiae, eyes treated enoxaparin or enoxaparin and triamcinolone combination dilated more, with the entire optic and parts of the haptic being visible compared to either the untreated or the triamcinolone eyes (Figure 10 d and f) with the average day 3 untreated dilation of 5.95mm ± 0.373mm vs. 7.65mm ± 0.273mm in the combination ($p=0.00426$).

Next, we examined the anterior chamber for cell and flare (Figures 13, 14). Enoxaparin alone showed no beneficial effect on cell or flare when compared to controls (Fisher's Exact test, $p>0.1$). Triamcinolone, at the low dose of 0.5mg, did show a trend towards reducing the amount of cells in the anterior chamber, but it was not statistically significant (p-value day 3 = 0.0601). The combination of triamcinolone and enoxaparin significantly decreased amount of eyes with flare (p-value = 0.01515 days 3, 6, and 7; p-value = 0.002165 day 5).

Finally, intraocular pressure of the eyes was significantly decreased compared to eyes that did not have lensectomy (Figure 15). IOP on day 3 was an average of 10.5mmHg in untreated eyes compared to 13.8mmHg in eyes treated with both enoxaparin and triamcinolone. After the first 5 days, eyes treated with enoxaparin and triamcinolone had an increased intraocular pressure compared with lensectomy control, approaching that of the unoperated eyes.

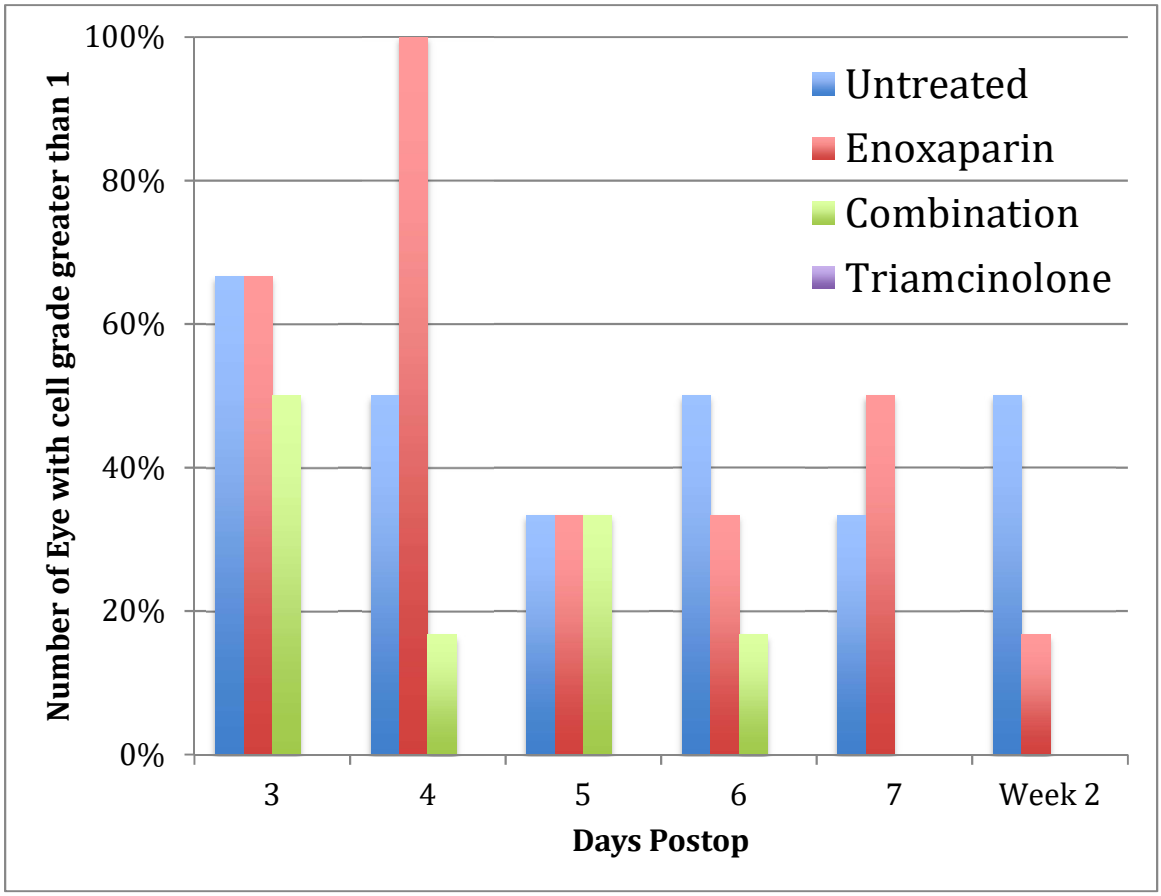

Figure 13 – Cell grade by treatment and day
The percentage of eyes with a cell grade > 1 (more than 15 cells per 1 x 1mm high powered field) measured on slit lamp beam examination.

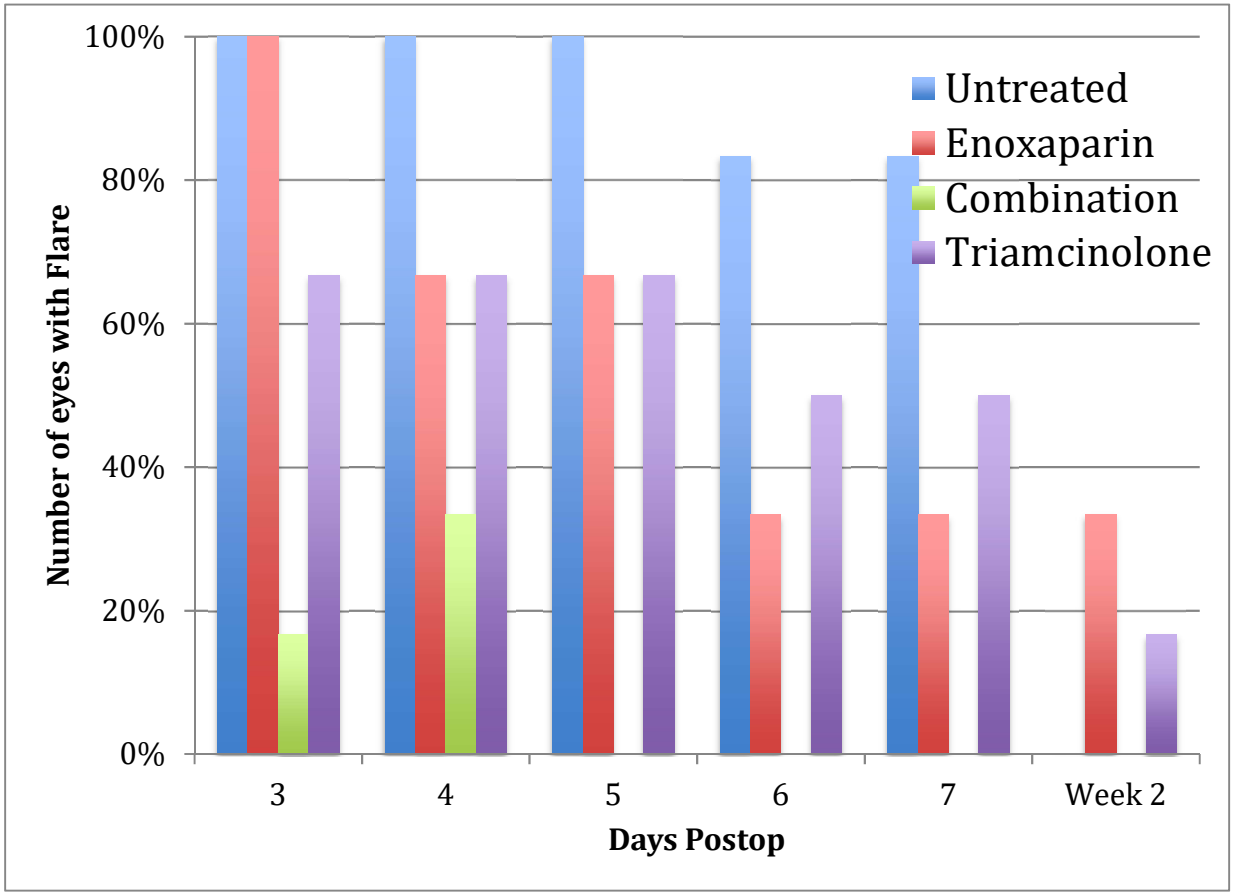

Figure 14 – Presence of flare by treatment and day
The percentage of eyes with a flare grade > 0 measured on slit lamp examination.

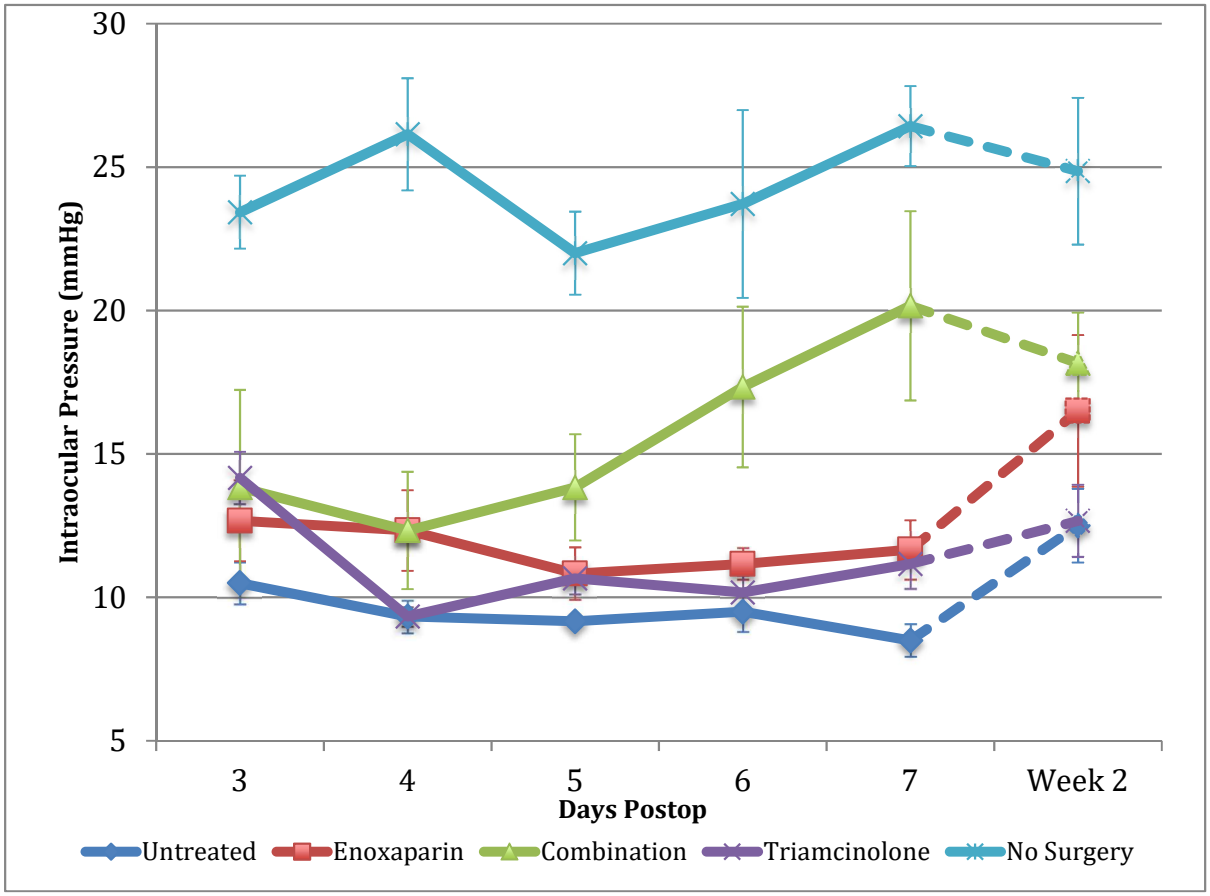

Figure 15 – Intraocular pressure in mmHg by treatment and day, measured with tonometry.

9. Intraocular Postoperative Treatment of Fibrin

Next, we evaluated eyes without any intraocular therapy at the end of lensectomy surgery for treatment of postoperative fibrin. After examinations on postoperative day 3, eyes were injected with either 25 micrograms of rabbit tPA or BSS (control). The baseline examinations on postoperative day 3 were not significantly different between the two groups prior to therapy (Figure 16, 17, Figure 18). The results starting on postop day 4, or 1 day after anterior chamber injection with either tPA or BSS show a dramatic divergence of the two groups in both the amount of fibrin in the anterior chamber. Fibrin was significantly reduced in the group treated with 25 micrograms of tPA versus control (Figure 16; $p=7.907x10^{-08}$) with increased anterior chamber cell. OCT signal strength was also significantly better after injection with 25 micrograms of tPA versus control (Figure 17; p=0.0004112). Representative photos showing treated versus control eyes demonstrate remarkable similarity on day 3 but a dramatic change on day 4 (Figure 18).

D. Discussion

The surgical and postoperative management of pediatric cataracts still represent a major challenge in ophthalmology. Reducing the incidence of adverse events after lensectomy is a priority so that children may have improved visual outcomes. Given the limited access, cost, and compliance to topical medications in developing parts of the world, effective preventative treatment with intraocular drugs at the time of surgery would provide a significant benefit. It may also allow for the insertion of an intraocular lens at the time of surgery. This would decrease the need for contact lenses and the potential risk of refractive amblyopia.

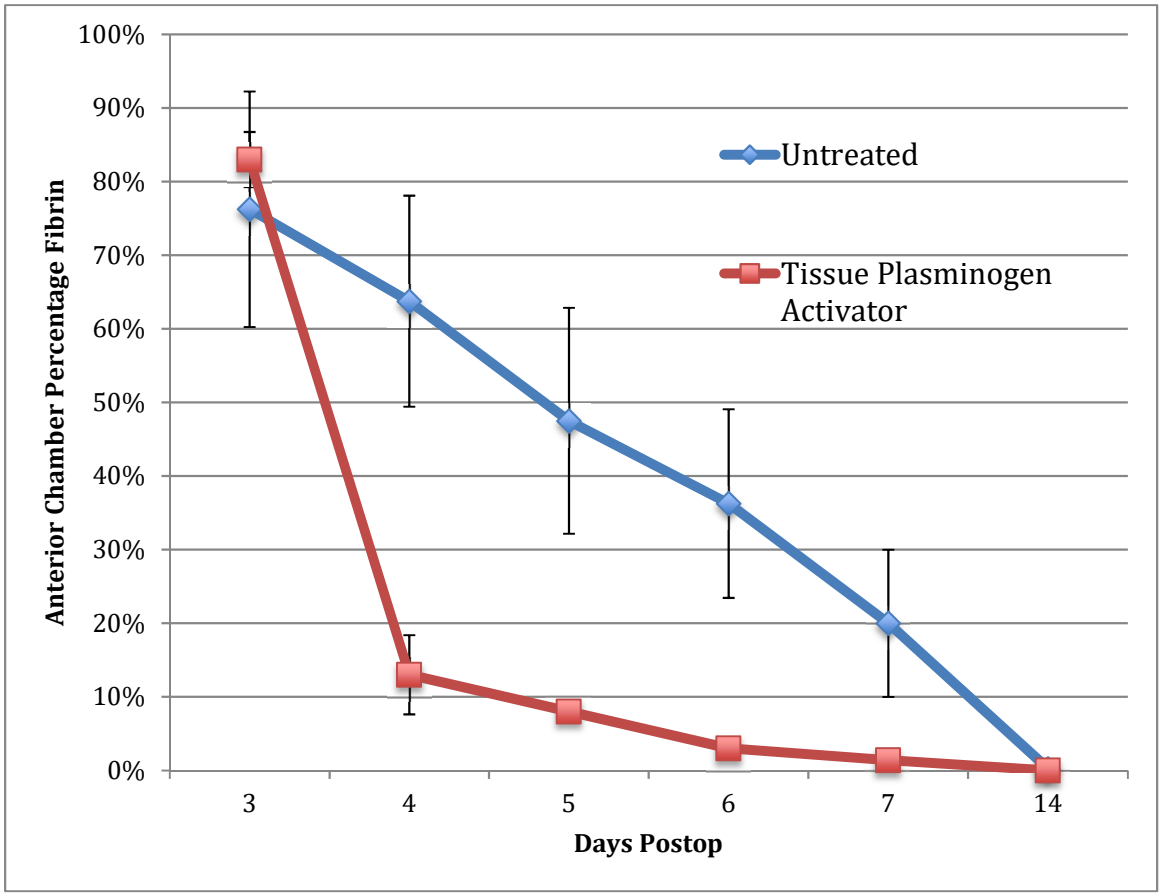

Figure 16 – Anterior chamber fibrin by day, tPA
The average percentage of the anterior chamber with fibrin quantified by slit lamp examination.

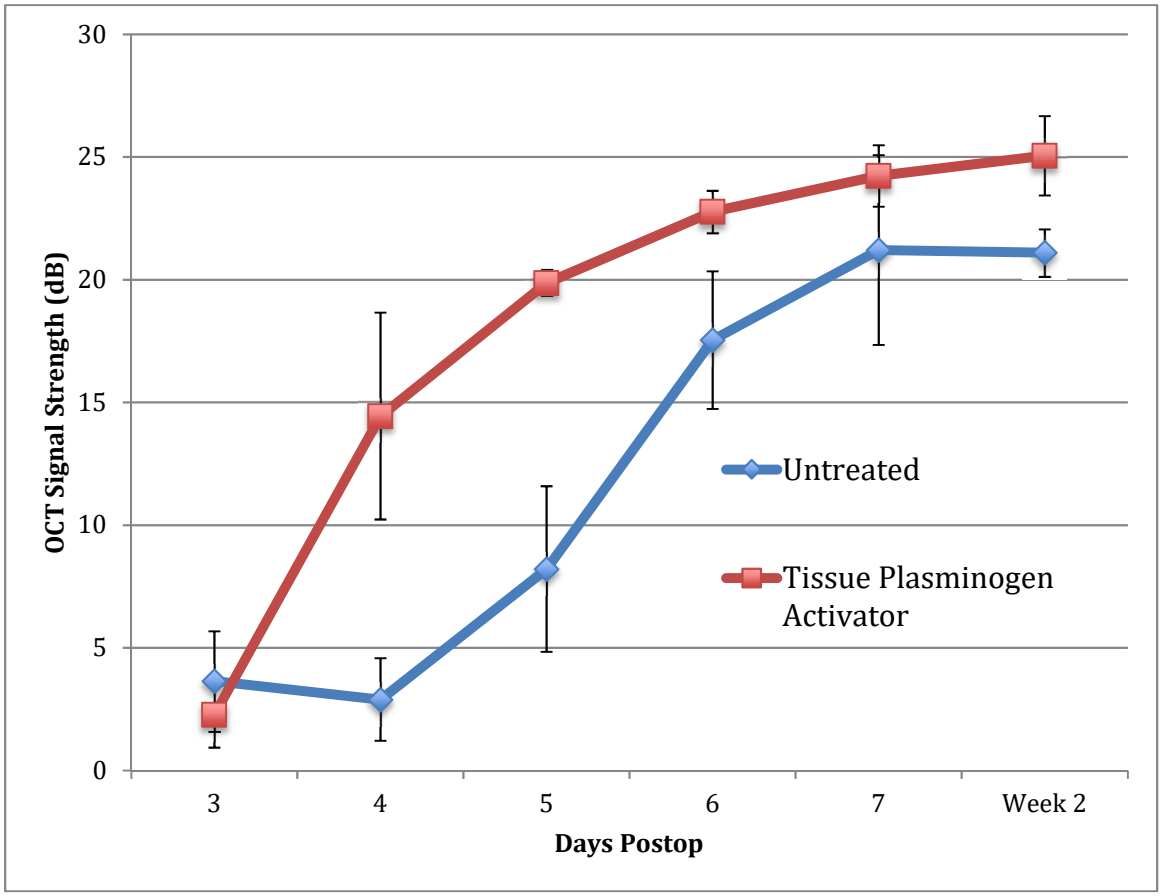

Figure 17 – OCT signal strength by day, tPA
The average OCT signal strength in decibels (log scale). OCT signal strength was used as a measure of visual axis clarity.

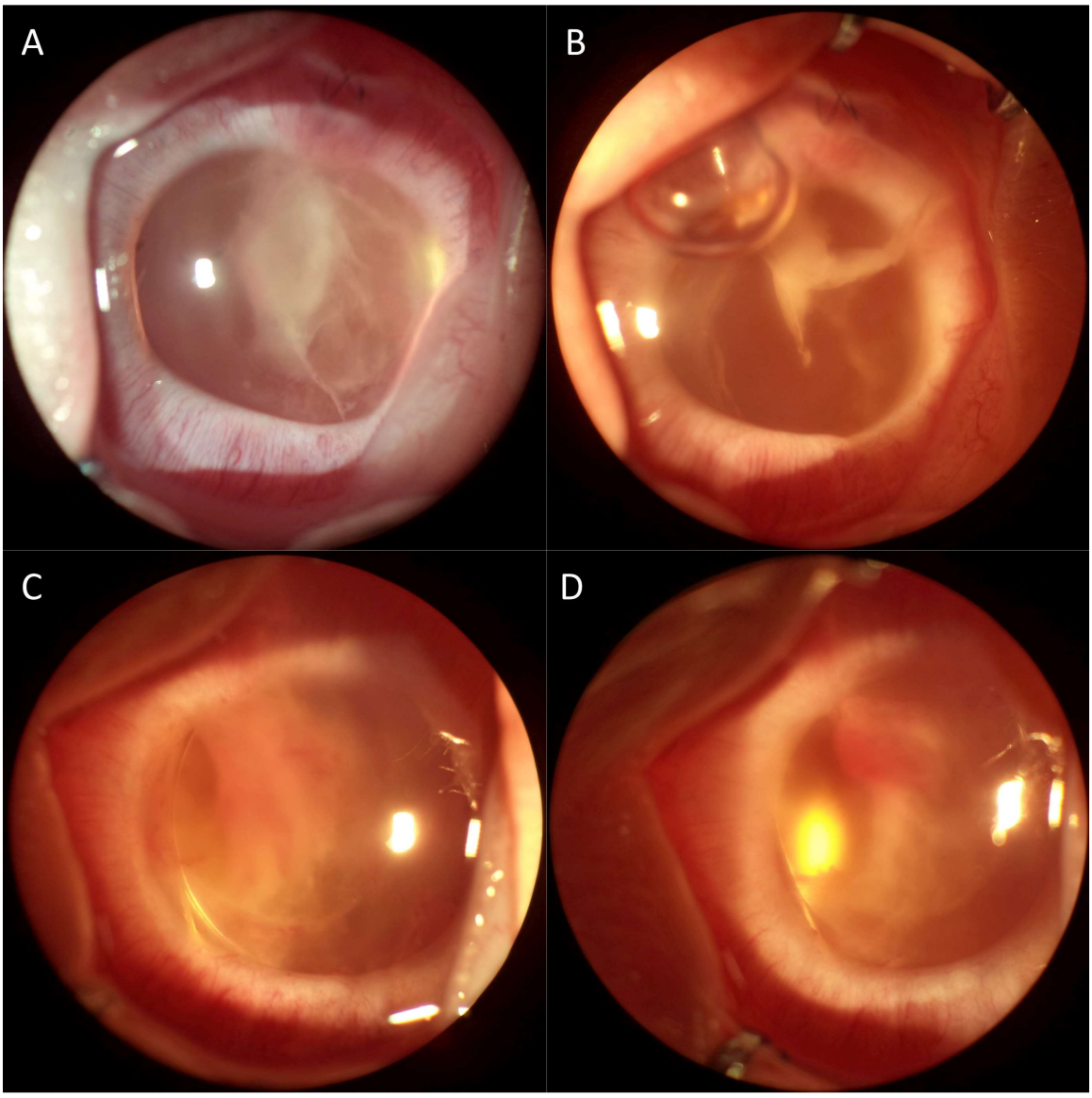

Figure 18 – Slit lamp photos before and after tPA treatment
Representative slit lamp photos before and after tissue plasminogen activator treatment. (A) 1714 OD on postop day 3 (85% fibrin). (B) 1714 on postop day 4 after receiving 25 micrograms of tPA in 50 microliters of BSS (30% fibrin). (C) 3149 OD on postop day 3 (95% fibrin). (D) 3149 on postop day 4 after receiving 50 microliters of BSS (85% fibrin).

These results show that a significant reduction of postoperative scarring with enoxaparin alone and in combination with a low dose of triamcinolone when examined on slit-lamp photography (Figure 11). This results in an improvement in the clarity of the visual axis as measured by increased OCT signal strength (Figure 12). Triamcinolone alone did not significantly improve fibrin in the anterior chamber, anterior chamber cell or flare, or OCT signal strength. This correlates with other studies where a lower dose of only 1.2mg did not affect outcomes in subjects (78). In comparison, a higher dose of 4-8mg did improve outcomes (59). In both studies, oral steroid was also used, but no effect was seen with the lower dose. However, enoxaparin combined with an even lower dose of triamcinolone in this study augmented the effect of enoxaparin by reducing flare, likely resulting the increased the OCT signal strength (Figures 2.7, 2.9).

One potential confounding variable for this study was the variation in surgical technique and complications. Surgical complications such as posterior capsule tears, intraocular lenses that were displaced, or corneal thickening in response to surgery could increase postoperative inflammation and fibrosis or decrease the OCT signal strength. Surgical technique was standardized between surgery days and the same surgeon performed all of the procedures for each experiment. While there were at least one complication in each of the treatment groups, there were no complications in the untreated group. Given that these studies show a treatment effect for enoxaparin with or without triamcinolone, this effect would likely be even more apparent in the absence of surgical complications in the enoxaparin groups.

Using heparin or its derivatives for cataract surgery is not an entirely novel idea. Several studies evaluated using enoxaparin or heparin in the irrigating solution for human congenital cataract surgery (79-82). Previously, 40mg was diluted into 500ml (0.08mg/ml), as opposed to 10mg diluted into approximately 0.2ml total fluid (40mg/ml) (82). This dose of enoxaparin in this

study is 500-fold more concentrated than that used in the irrigation fluid for the randomized study of children. There are advantages to having enoxaparin dissolved in the irrigating solution. The main benefit of treatment in the irrigating solution is that it can have its effect throughout the entire procedure instead of having to wait until the end. However, this does have the potential of increasing the risk of bleeding because of the instability of the anterior chamber. By injecting enoxaparin when all wounds are closed with a stable anterior chamber, it is possible that there is a decreased the risk of bleeding. By injecting enoxaparin when all wounds are closed with a stable anterior chamber, it is possible that there is a decreased the risk of bleeding.

Other groups have investigated coating intraocular lenses in heparin as a way to both decrease intraocular inflammation and prevent posterior capsule opacity in children (83, 84). Results were inconsistent, with one study finding a benefit and the second smaller study finding no benefit. However, all of these studies done in human were in addition to the standard of care, treatment with topical steroids. While the advantages of being able to treat postoperative inflammation and fibrosis with a single therapeutic dose at the time of surgery, future studies need to compare new treatment strategies to long-term treatment with topical steroids.

The studies in this chapter did not evaluate the postoperative course beyond 2 weeks, so the effect of therapies on posterior capsule opacity formation cannot be determined from this study. The Infant Aphakia Treatment Study indicates that the critical period for follow up where most complications occur is 1 year. Without an understanding how long a human year correlates in rabbits, we cannot definitively say that these intraocular therapies would reduce all complications due to fibrin for the entire first year. However, in children, time is of the essence with better outcomes with earlier surgery. The same can be said for clarity of the visual axis due to complications involving opacification of the visual axis. Perhaps by following the rabbits past 2

weeks, we would get a clearer picture of how posterior capsule opacification is affected by these interventions.

Once a fibrin membrane is formed, the preventative strategies investigated above would have little effect. In this chapter, I also investigated the effect of an intraocular therapy for treating a fibrin once it forms, namely with tissue plasminogen activator. This gives a potential alternative to surgical intervention. Intravenous administration of tPA has been FDA approved for thrombolysis in coronary arteries since 1988. Since then, this drug has been used for many off-label indications, including some in the eye (85, 86). Studies have found that there is as much as 30 times more tPA present in the aqueous humor than there is in plasma in normal human eyes (87). One small study in adults showed complete fibrinolysis within four hours in 18 of the 19 eyes treated with 25 micrograms of tPA (88). Another small study (34 eyes, 26 patients) over a decade ago examined giving pediatric patients (age 3-14) 20 micrograms of tPA at the end of lensectomy (89). The group found that the incidence of intraocular fibrin membranes were significantly lower on days 1, 3, 7, and 14 after injection, (p values = 0.02 or 0.01) but no significant difference on days 30 and 90. The insignificant difference may be due to the low incidence, with none in the treated group and only 1 subject in the control group having a fibrin membrane at 30 days. Similarly, there were no subjects in the tPA group and 3 in the control group with pupil synechiae, but this was not statistically significant. Rather than try to treat prophylactically, because of the short half-life of tPA, subjects should have been treated when membranes were seen, and continued to treat, potentially repeatedly, with the primary outcome as visual acuity and the number of additional surgeries needed in treated patients versus controls. While studies have shown that there were no adverse effects from a single tPA injection, repeated injections might have added toxicity, so additional safety studies would be necessary.

The results from these studies suggest that enoxaparin and tissue plasminogen activator warrant further investigation for their potential as therapeutic modalities for children who need cataract surgery. Enoxaparin may be a useful preventative therapy and may be augmented in the presence of a lower dose of intraocular steroid. When complications related to fibrin do occur, tissue plasminogen activator may be useful as a non-surgical option. These modalities may allow for placement of an intraocular lens in young children with reduced complications. This may help with treatment of amblyopia, potentially improving visual outcomes versus aphakia with contact lens correction of refractive error.

Future studies will examine the effect of lower doses of enoxaparin on postoperative fibrosis. In addition, determining the duration of half-life and clearance in the anterior chamber will help us further titrate the most effective intraocular dose of enoxaparin in the context of lensectomy. Before these treatments are ready for clinical trials, dose response curves will be necessary, as is the proteomic analysis of the anterior chamber fluid samples gathered with this project. Understanding the effect of enoxaparin, triamcinolone, or the combination on the inflammatory cascade of the eye may give us clues on the mechanism of action and synergy as well as other potential therapeutic targets.

III. EARLY AMBLYOPIA THERAPY IMPROVES VISUAL OUTCOMES IN PEDIATRIC OPEN GLOBE TRAUMA

A. Introduction

Open globe trauma is a vision-threatening event with an often-unpredictable clinical course. Ocular trauma is one of the leading causes of monocular blindness in children worldwide (90, 91). In 2002, Kuhn and colleagues reviewed the visual outcomes of over 2500 patients to determine characteristics associated with visual outcomes, giving rise to the Ocular Trauma Score (OTS) (92). The OTS is difficult to extrapolate to children because they often are unable to provide a preoperative quantifiable vision and because younger children are at risk for amblyopia (27). Acar and colleagues recognized the unique characteristics of the pediatric population with development of the Pediatric Ocular Trauma Score (POTS) in 2011 (26).

Risk factors for poor visual outcomes at any age include endophthalmitis, retinal detachment, vitreous hemorrhage, surgical delay, hyphema, and a more posterior wound location (22-25, 93-97). Younger age is an independent risk factor for poor visual outcome (22, 98). Since young children are at risk for amblyopia, it is possible that the development of amblyopia may be a factor contributing to poorer visual outcomes in younger children. Management of pediatric ocular trauma may involve waiting until the postoperative issues have been resolved, sometimes many months after the injury, before considering amblyopia as part of the management plan. In this situation, there is a normal visual system with an external factor causing amblyopia, which is then treated. Hubel and Weisel demonstrated that there are physiological and anatomical changes in ocular dominance columns in response monocular deprivation (12). By treating patients with amblyopia therapy in the perioperative period, which is likely before there are any changes to the

nervous system, we may be able to prevent of the onset of changes associated with amblyopia. This would maintain the normal anatomy and physiology of the visual system, potentially improving final visual outcomes. To investigate this, we retrospectively reviewed pediatric open globe trauma cases seen at the University of Illinois at Chicago Eye and Ear Infirmary (UIC EEI) to determine if amblyopia therapy and its time of initiation are related to the final visual outcomes.

B. Methods

Approval was obtained from the Institutional Review Board at the University of Illinois at Chicago. The study and data collection protocols conformed to all local laws, complied with the rules and regulations concerning the privacy and security of Patient Health Information (PHI) under the Health Insurance Portability and Accountability Act of 1996 (HIPAA), and complied with the principles of the Declaration of Helsinki. The records of children less than 18 years old who presented with open globe eye injuries between August 2001 and July 2014 were retrospectively reviewed. Subjects that had previous intraocular surgery, a history of subnormal vision, had less than 3 months of follow up, or final vision of hand motion, light perception, or no light perception were excluded from the analysis. Subjects with a final vision of hand motion or worse were excluded because of the likelihood of poor visual outcomes regardless of intervention. Subject charts were examined to gather age, gender, mechanism of injury, time of injury, time of surgery, initial visual acuity (VA), final best corrected VA (BCVA) and concomitant eye pathology. Ocular trauma scores were calculated for each injury using the POTS as per Acar et al (26). Amblyopia therapy was recorded as initiated if the subject if patching or atropine therapy of the sound eye was started or if spectacle correction was given for anisometropic refractive error. The subjects

were divided into five groups (higher points is presumed to be better prognosis) based on the POTS:

Group 1: ≤ 45 points.

Group 2: 46–64 points.

Group 3: 65–79 points.

Group 4: 80–89 points.

Group 5: 90–100 points.

Statistical analysis was performed using a two-sided Student's t-test for continuous variables or a two-sided Fisher's exact test for categorical variables with R statistical software (64 bit, version 3.1.2). A p-value ≤ 0.05 was considered statistically significant.

C. Results

99 children (77 male, 22 female; 78% male) with a mean age of 6.78 ± 4.18 years (range: 7 weeks – 17 years old) presented to UIC EEI with open globe eye injuries without previous ocular pathology. Wood (22.2%), metal (19.2%), and glass (9.1%) accounted for the majority of the observed injuries seen among the 99 subjects (Table I). 44 subjects had less than 3 months of follow-up or had a final best corrected visual acuity of hand motion or worse and were excluded from the remaining analysis. Of the 55 children remaining, 74.5% were male and 25.5% were female with a mean age of 7.05 ± 4.03 years. Subject demographics for this cohort can be seen in Table II. Complications at presentation, initial vision, POTS category, and final visions for the entire cohort can be seen in Table II. Of the remaining subjects, 38 (69.1%) were below the age of 9 years old at the time of injury. Twenty-one subjects (38.2%) received amblyopia therapy with 9 subjects initiated within 3 months after the initial injury. All subjects where amblyopia therapy was initiated were below the age of 9 years old. For subjects less than 9 years old, the final BCVA was better in those with early amblyopia therapy started within 3 months after the injury than those without amblyopia therapy with a LogMAR BCVA of 0.30 and 0.96, respectively (Figure 19 and Table II, p=0.011). Subjects under 9 years old treated within 3 months after the initial injury had better final VA than those treated later with a LogMAR BCVA of 0.30 versus 0.96 (p = 0.004). There was no statistical difference between subjects with early amblyopia treatment and those 9 years of age or older with a BCVA of 0.30 and 0.57 (p>.05, Figure 19). The distribution of the POTS categories and concomitant pathologies between subject groups was not significantly different (Figure 20, p>0.05). Finally, we compared the visual outcomes of subjects at UIC's EEI based on their POTS in Table III.

Table I - THE CAUSES OF PENETRATING INJURIES AT UIC EEI

Cause of Injury	Percent of cases[a]
Wood	22.2
Metal	19.2
Glass	9.1
Pen/Pencil	8.1
Knife	7.1
BB Pellet	6.1
Plastic	4.0
Animal Scratch/Bite	3.0
Stone	3.0
Scissors	2.0
Wire	2.0
Human Scratch	1.0
Other	7.1
Unknown	6.1

[a] n=99

Table II - SUBJECT DEMOGRAPHICS, COMPLICATIONS, POTS CATEGORIES, PRESENTING VISIONS AND FINAL VISIONS BY TREATMENT GROUP

	All Subjects	<9 years old			≥9 years old
		Untreated	Treated Early	Treated Late	
Number of subjects	55	17	9	12	17
Average years of age (STDEV)	7.05 (4.03)	5.56 (2.18)	3.50 (1.97)	4.58 (2.07)	12.18 (2.18)
Number of Males (% male)	41 (74.55)	11 (64.71)	6 (66.67)	10 (83.33)	14 (82.35)
Average POTS score (STDEV)	40.64 (23.85)	43.53 (22.62)	50.56 (19.76)	32.50 (22.88)	38.24 (22.62)
Complications					
Endopthalmitis	0	0	0	0	0
Retinal Detachment	12	2	0	4	6
Vitreous Hemorrhage	18	3	2	4	9
Cataract	30	8	2	7	13
Delay of Surgery >48h	6	2	0	2	2
Organic/Unclean Injury	18	5	4	5	4
Hyphema	10	3	0	2	5
Iris Prolapse	39	15	7	9	8
Central Visual Axis Wound	28	11	4	7	6
POTS Categories					
Group 1 (≤45)	31	9	2	7	13
Group 2 (46-64)	15	5	6	4	0
Group 3 (65-79)	8	3	1	1	3
Group 4 (80-89)	0	0	0	0	0
Group 5 (90-100)	1	0	0	0	1
Presenting Vision					
NLP	1	0	0	0	1
LP/HM	14	5	1	3	5
1/200-19/200	13	2	3	1	7
20/200 – ≤20/40	2	2	0	0	0
>20/40	1	0	0	0	1
NOT QUANTIFIED	24	8	5	8	3
Average LogMAR acuity (STDEV)	1.76 (0.98)	1.75 (0.90)	1.85 (0.93)	1.85 (NA)	1.75 (0.90)

Table II - SUBJECT DEMOGRAPHICS, COMPLICATIONS, POTS CATEGORIES, PRESENTING VISIONS AND FINAL VISIONS BY TREATMENT GROUP

	All Subjects	<9 years old			≥9 years old
		Untreated	Treated Early	Treated Late	
Final Vision					
NLP	0	0	0	0	0
LP/HM	0	0	0	0	0
1/200-19/200	18	9	0	6	3
20/200 – 20/50	18	4	4	4	6
≥20/40	19	4	5	2	8
Average LogMAR acuity (STDEV)	0.76 (0.70)	0.96 (0.69)	0.30 (0.23)	1.11 (0.70)	0.57 (0.66)

Table III - COMPARISON OF VISUAL OUTCOMES BY POTS SCORE

POTS category	Number of eyes	NLP	LP/HM	1/200-19/200	20/200-20/50	≥20/40
1	**48**	**22.9**	**12.5**	**18.8**	**22.9**	**22.9**
2	**16**	**0**	**6.3**	**43.8**	**31.3**	**18.8**
3	**9**	**0**	**11.1**	**22.2**	**22.2**	**44.4**
4	**0**	N/A	NA	NA	NA	NA
5	**1**	0	**0**	**0**	**0**	**100**

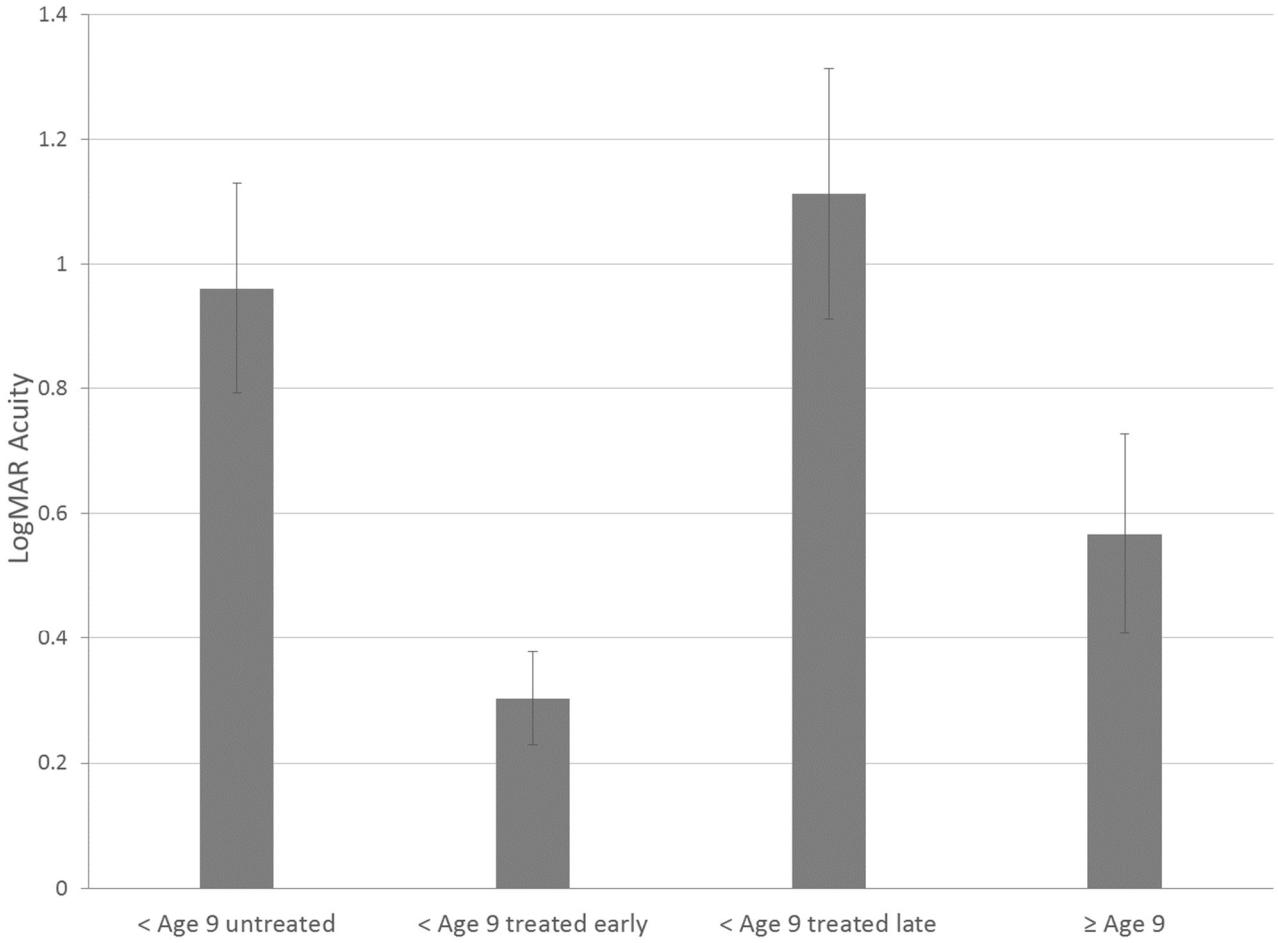

Figure 19 – Final visual acuity by treatment group
Subjects treated with amblyopia therapy within 3 months after the original injury had better final visual acuities compared to if amblyopia therapy was initiated later or not at all

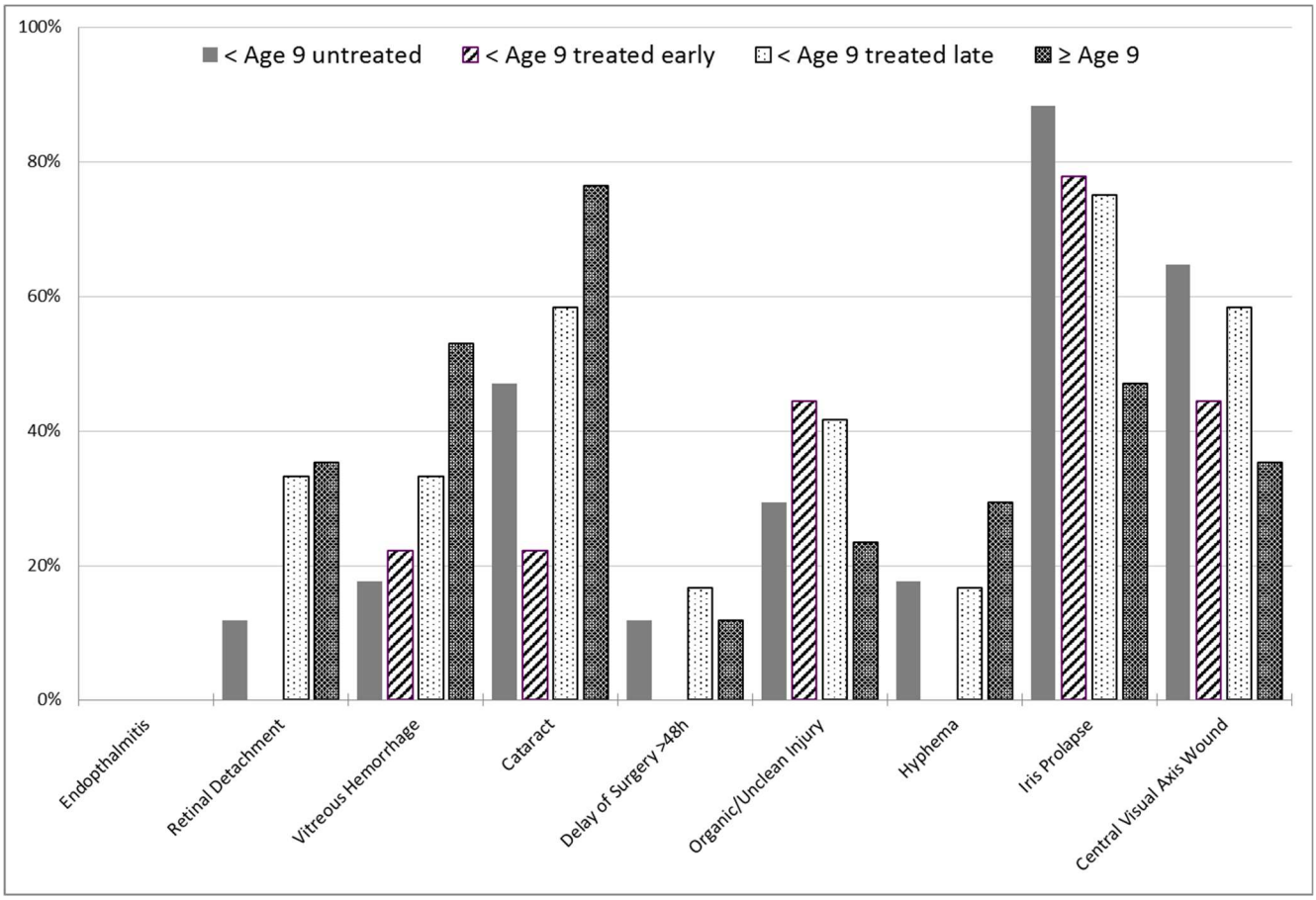

Figure 20 – Complications by treatment group
Percent of subjects with each pathology by treatment group. Multiple pathologies may have presented for the same subject. Concomitant pathologies were similar in subjects with or without amblyopia therapy for children with a final visual acuity of counts fingers (1/200-19/200) or better (n=55) ($p > 0.05$, Fisher exact test).

D. Discussion

Ocular trauma remains a common and preventable cause of decreased vision, especially in young children (22, 26, 91, 93). In this series, we presented over 13 years of pediatric open globe ocular trauma cases that presented to UIC's EEI. Of the 99 that presented, 55 had final measurable visions better than hand motions and the minimum 3 months of follow-up to be included for analysis. . We chose to exclude patients with final visual acuities of hand motion, light perception or no light perception to decrease selection bias, since the visual potential was likely poor due to their injury or subsequent complications that amblyopia therapy was often not initiated for these subjects. A large number of subjects were not followed for at least 3 months, which may have occurred for two reasons. First, our institution is a referral center from other physicians for complicated ocular problems, so subjects may have returned to their primary ophthalmologist before 3 months. Secondly, there may be a lower follow-up rate in our urban patient population that could not be controlled for because this is a retrospective study.

A prospective observational or randomized study is needed to validate the outcomes observed in our study. A randomized prospective trial would allow us to include all subjects, including injuries with poor visual potential. By evenly distributing all subjects including those with injuries indicative poor visual potential, selection bias would be decreased. The nature our institution being a tertiary care facility and care for patients with worse pathologies may also explain the low POTS scores of subjects in our study. However, the distribution of final visual outcomes of our series of subjects was significantly better when compared to prior studies, especially for the more severe injuries, with 11 of 48 subjects in the worst POTS category achieving a visual acuity of $\geq$ 20/40 while none were reported in previous studies. This may be in part due to differences in amblyopia management or to the experience with complicated ocular

pathologies. Being a subspecialty referral center gives our facility significant experience with severe traumatic injuries for patients of all ages. Multiple procedures are often performed within the same surgical time, even when unpredictable complications occur intraoperatively. A prospective multicenter observational study would be needed to determine the nature of the outcome differences in this study.

Despite the relatively small sample size of subjects treated for amblyopia, subjects below the age of 9 who received amblyopia treatment had significantly better visual outcomes. In addition, subjects with early amblyopia therapy had no statistical difference in visual outcomes to children who were 9 years old or over and unlikely to develop amblyopia (Figure 19). This was not likely due to the severity of the injury since both the POTS category and the pathologies were similar between the treated and untreated groups (Figure 20). These results suggest that the effects of ocular trauma and potential deprivational or refractive amblyopia may be counterbalanced with early patching, atropine penalization, or refractive correction, improving the visual outcomes in children at risk for amblyopia.

Amblyopia after open globe trauma is a unique situation in which amblyopia develops in the setting of previously normal eyes and visual system and may provide a perspective on prevention versus recovery of vision once amblyopia has developed. In our study, subjects who received amblyopia treatment early had significantly better visual outcomes than those who were treated later. Several of the subjects patched the sound eye in the immediate postoperative period before any evidence of amblyopia. Although the vision, when quantified, was often lower than the sound eye, it is unknown if decreased vision was due to postoperative pathologies causing decreased vision or amblyopia. This is because there were often anatomical abnormalities such as a corneal scar or vitreous hemorrhage that may have reduced the best-corrected visual acuity.

Although any form of amblyopia therapy including patching, atropine penalization, or anisometropic refractive error management may be employed, refractive error management is likely to be the least practical in this situation. Although wearing spectacles does provide protection of the eye from subsequent injury, the dynamic changes in refractive error require multiple prescription changes in short periods of time. Therefore, patching or atropine penalization of the sound eye is the most practical approach in subjects after open globe injury.

The improved outcomes with early amblyopia therapy may also be due in part to improved amblyopia therapy compliance. During the immediate postoperative period, many things have changed for the child and caretaker, so initiation of amblyopia therapy is simply another component of the postoperative care regimen. By establishing the routine early, it is possible that children are less likely to resist their caretaker. Patching may be preferable as first-line amblyopia therapy because you can also the caretaker to return earlier for a sudden change in behavior in a preverbal child or a subjective decrease in vision. These may signify a new problem, such as a rapidly developing cataract or retinal detachment. Children are not as aware of monocular changes in vision, so having a period where the injured eye is being used exclusively is advantageous and allows the patient to be aware of any acute vision changes.

E. Conclusions

The results of this study suggest that early amblyopia therapy can improve visual outcomes in children after open globe trauma. The improved final visions of subjects could not be attributed to differences in presenting pathologies or severity of injuries. However, because of the small sample size and lack of randomization, a larger prospective study is needed. A larger study may be able to identify children most at risk of vision loss without amblyopia therapy, allowing us to

better counsel patients and their caretakers. It may also help identify ocular pathologies that may benefit from preventative care or early intervention, potentially further improving visual outcomes.

IV. RETINITIS PIGMENTOSA

A. Introduction

Retinitis Pigmentosa (RP) is a group of inherited eye diseases that share common clinical phenotypic presentation, namely, the degeneration of the retina. The term was first used in 1857 to describe the spicules of pigment seen throughout a patient's degenerated retina that was believed to be caused by an infection. With over 100,000 people affected in the United States and over 1.5 million worldwide, RP is the most common cause of inherited visual impairment. Annual total healthcare costs for RP patients were found to be over $7000 a year higher than unaffected individuals even before considering additional expenses such as caregivers, rehabilitation, home assistance, and institutional care (28). Additionally, there are substantial emotional costs for patients and their families. Anxiety, disorientation, depression, loss of independence, and difficulty with work or activities of daily living are some of the problems faced by patients and loved ones.

Research efforts over the past thirty years have led to an exponential growth of knowledge regarding inherited eye diseases. Since the first retinitis pigmentosa gene was discovered in 1989, over 200 genes that cause any inherited eye disease have been discovered (31). Unfortunately, this explosion of knowledge has not yet lead to an approved treatment for RP.

One of the difficulties developing successful treatments for RP is due to the condition's complexity. RP is very genetically heterogeneous with over 50 causal genes currently known (31). It can be inherited as an autosomal dominant (30%-40%), autosomal recessive (50%-60%), or X-linked recessive condition (5-15%) (32-34). Additionally, affected family members with the same mutation(s) can present with different phenotypes (35, 36). Many early treatment attempts failed

to consider this heterogeneity. More recent gene directed treatment approaches have focused on treating a subset of the disease population, patients with a specific gene mutation.

The vision loss in patients with RP is progressive and severe. RP is a highly variable disorder, with some patients developing symptoms in the first decade of life and others as late as the sixth decade. Symptoms include night blindness and dark adaptation difficulties early in life, followed by a slow insidious loss of their peripheral vision, which constricts their visual field making it impossible to drive. As the disease continues to advance, many eventually lose their central vision (29). Visualization of the retina invariably shows narrowing of the retinal vasculature. Optic disc waxy pallor, bone-spicule like pigmentation, and a thinning appearance to the retina are also common findings in RP patients, which become more prevalent as the disease progresses (30). The genetic causes of RP are as varied as the disease's presentation. While individual family members can be affected by the disease differently, examining these mutations on a population scale shows that there is a good amount of predictability in a patient's disease course from their genotype (37). Additionally, there is some deal of predictability of a patient's genotype from their disease presentation and course (35). This allows physicians specializing in inherited retinal diseases to predict the most likely causal genes in a patient based on their presentation and disease course, making for easier and faster identification of a patient's genotype. With over 50 genes known to cause RP, it is not surprising that nearly any pathway can be compromised in RP. Specifically, genes involved in each of the following groups have been identified: phototransduction cascade, vitamin A metabolism, structural/cytoskeletal, cell signaling, cell-cell interaction, synaptic transmission, RNA intron splicing, protein trafficking, maintenance of cilia, pH regulation, and phagocytosis.

Despite the varied functions of these genes, it has been shown that a major cause of cell death in RP and other retinal degenerations is apoptosis (99-103). A hallmark of apoptotic cell death not seen other mechanisms of cell death is internucleosomal DNA fragmentation which is detectable on agarose gel electrophoresis via its appearance as a DNA ladder (104). The DNA fragments can also be detected by *in situ* labeling of apoptotic nuclei using TUNEL (terminal dUTP nick end labeling) (105). To demonstrate that apoptosis is a major cause of cell death in RP the histological sections with staining (apoptosis, rods, cones) from three mouse models of inherited retinal degeneration (*Pde6b*^{rd1} formerly r*d1* or *rd*, *Rds*^{Rd2} formerly *rds*, and transgenic rhodopsin Q334Ter) was examined using TUNEL in mice ranging in age from P0 to P60 (100). In control mice there was almost no apoptosis seen in either the photoreceptor or ganglion cell layer from days P0 to P30. There was a significant amount of apoptosis occurring before P14 in the inner nuclear layer, which contains amacrine cells, horizontal cells, and bipolar cells, a finding which had already been well documented (106). Comparatively, all three mouse models of RP showed significant photoreceptor apoptosis starting at P10. Additionally, there was an increase in the amount of apoptosis seen in the inner nuclear layer prior to P14, but similar to the wild type mice no apoptosis was seen in the inner nuclear layer after P15. Recently, evidence that necroptosis or a controlled necrosis plays a role in the cone cell death seen in RP has been demonstrated in cell and animal models (49, 107-109). While most necrosis is an uncontrolled process, necroptosis, is triggered by the interaction of receptor interacting protein 1 (RIP1) with receptor interacting protein 3 (RIP3) (110, 111). Normally when RIP1 is activated, it activates Fas-associated death domain and caspase-8 (112), but when a cell fails to undergo apoptosis, RIP1 binds to RIP3 and triggers necroptosis.

1. Bystander effect

The bystander effect or innocent bystander effect is the loss of cone photoreceptors seen in inherited eye diseases where the genetic cause of the disease is found only in the rods such as RP (101, 102). Typically, the majority or all of the rods are lost first before the cones start to die. Cones are responsible for color vision and our central visual acuity. Without cones present in the macula (center part of the eye) our visual acuity is 20/200 at best. Several theories for the mechanism of the bystander effect have been proposed. These theories include the release of a toxin during rod cell death (113), loss of rod produced trophic factors (114), activated microglia (115), oxidative stress (116), oxygen overload (117), and starvation (101). Given the time course typically seen both in patients and animal models, toxin release is unlikely to be the cause of the cone cell death. While the rest of these mechanism have been demonstrated to be a part of the bystander effect, both insulin to combat starvation and the exogenous addition of rod produced trophic factors have demonstrated a rescue effect in animal models and are active areas of research (101, 118).

2. Vitamin Supplementation

One of the first major (randomized, controlled, blinded) attempts at treating retinitis pigmentosa came in 1993 in the form of nutritional supplementation of vitamins A and E (119). The trial focused on electroretinogram (ERG) measurements with their primary outcome measure being cone ERG amplitude. Patients in the trial received either 15,000 IU/d of vitamin A, 400 IU/d of vitamin E, both, or neither for four to six years. They showed that while other, more important, measures of visual function such as visual field size or visual acuity were statistically unchanged among the groups, vitamin A treatment slowed the rate of ERG signal decline and vitamin E

treatment had an adverse effect on ERG signal decline. Enrolled patients had an average baseline ERG amplitude of approximately 0.23 microvolts at 30Hz and approximately 2.1 microvolts at 0.5Hz. While these findings were statistically significant, their clinical relevance remains in question, given that the lower bounds of normal for an ERG are 50 microvolts at 30Hz and 350 microvolts at 0.5Hz. The authors made no attempt to demonstrate why they were able to detect a difference in ERG between the groups. Two reasonable explanations are that patients who received vitamin A supplementation on average lost fewer photoreceptors or that the photoreceptors of patients who received vitamin A supplementation were more sensitive to light and therefore evoked a larger response. A recent randomized crossover trial with a washout period where patients received either 9-cis β-carotene or placebo for 90 days demonstrated that supplementation with vitamin A like moieties increased ERG measurements (120). Given that this effect was seen all patients, it is unlikely that the cause of the observed ERG changes were due cell loss, but rather there was a functional difference in the cells that were still viable in patients currently receiving 9-cis β-carotene.

Follow up work to the vitamin A and E study attempted to test if there was a beneficial effect of lutein in patients with retinitis pigmentosa already receiving 15,000 IU/d of vitamin A (121). Humphrey Field Analyzer 30-2 (30 degrees of vision tested using point location set "2" which tests points every 6 degrees starting 3 degrees off the meridians using 76 points) and Humphrey Field Analyzer 60-4 (from 30 degrees to 60 degrees of vision using 68 points) visual field measurements, more clinically relevant measurements were chosen as outcomes. After four years of follow up, patients treated with 12mg/d of lutein in addition to the 15,000 IU/d of vitamin A showed a statistically significant difference in the HFA 60-4 field measurements (p value = 0.03) but not in HFA 30-2 or the combined analysis. Given that they performed multiple hypotheses

testing, they failed to correct their α to give an overall α of 0.05, which would have resulted in no statistically significant findings. It is unclear if this study was underpowered or if the difference was simply too small to be clinically relevant. None of these studies included work to determine the underlying mechanism. Given the success of AREDS (Age Related Eye Disease Study) vitamins (a combination of Vitamins A, C, E, Zinc, Copper) for the treatment of AMD (122), it is not surprising that vitamin supplementation for RP is an active area of interest to researchers; but it may not be without its drawbacks. One group found that vitamin A supplementation in ABCA4 -/- mice (a gene in humans that is known to cause RP and other inherited retinal degenerations) increased toxic substance buildup in the retina which may be harmful to vision (123). Even in non-ABCA4 associated patients, high doses of vitamin A are known to be toxic. Women planning to conceive or who have severe osteoporosis should avoid vitamin A supplementation, and all patients receiving high doses of vitamin A should have their liver enzymes, serum retinol, and triglyceride levels checked regularly.

3. Gene Therapy

Another active area of research for the treatment of retinal degenerations is gene therapy. As of July 2013, over 1900 gene therapy clinical trials have been approved for study [http://www.abedia.com/wiley/index.html]. So far, only a single drug, Glybera, has been approved for use in the EU or US despite only having 27 patients in its clinical trial (124, 125). For inherited eye diseases, clinical trials of RPE65 associated Leber congenital amaurosis (LCA - an inherited eye disease with poor vision from birth), ABCA4 associated Stargardt's (a jouvenile inherited retinal degeneration that primarily affects the center of vision), and USH1B associated RP (USH1B mutations often cause hearing loss in these patients as well) are in various phases of

study (I-III) [clinicaltrials.gov], with new trials being planned. These trials have used adeno-associated virus (AAV) vectors injected intravitreally or subretinally deliver the gene of interest to the retina. Initial results from the RPE65-LCA trials have been promising, with an unblemished safety record and patients demonstrating a significant improvement in several aspects of vision (126-130). Treated patients showed decreased nystagmus in both eyes, improvement in the pupillary light response in the treated eye, increase in visual acuity in the treated eye, improvement from "unable to perform" in a mobility test to being able to avoid 12+/14 obstacles and spending approximately 1 minute in the maze. Additionally, the persistence of these findings after over a year demonstrates the stability and longevity of the treatment. The first patients in these trials treated were adults, but there is currently a phase III trial using the same viral vector and gene product to treat children younger than 3 years of age with RPE65-LCA. Based on these early results, gene therapies represent a powerful treatment option for similar diseases where patients lack a necessary protein for vision. However, many treatment strategies including gene therapy are only effective if the underlying architecture of the tissue is still intact. Attempts to treat patients with neuroprotective agents or gene therapy that have already lost photoreceptors or ganglion cells or retinal pigment epithelial cells in their retina will not have an effect. Similarly, autosomal dominant diseases are not caused by a lack of a functional protein, but a toxic allele or gain of function mutation, requiring different strategies for treatment with gene therapy. Additionally, while early gene therapy results have been promising with in restoring vision, gene therapy does not slow the progression of retinal degeneration in treated eyes compared to control eyes (131). These limitations stress the importance of a multi-factorial approach to treating these complex diseases.

4. Cell Replacement Therapy

In patients with advanced disease whose retinal architecture is disrupted, one way to restore vision is with cell replacement therapy. With the combination of easy surgical access and a clear vitreous media to easily visualize the transplanted cells in follow up, the retina is an ideal organ for cell replacement therapies (132). Cell replacement of photoreceptors was originally shown to be viable using either human embryonic stem cells or embryonic retinal precursor cells, even in immunocompetent mice (133, 134). Since then, several groups have demonstrated the ability to (1) generate induced pluripotential stem cells (iPSCs) from mice or human adult or embryonic fibroblasts or keratinocytes, (2) use various transcription factors or small molecules to force iPSCs to differentiate into retinal photoreceptor precursors, and (3) transplant these retinal photoreceptor precursors into animals, demonstrating the safety and efficacy of their technique to repopulate photoreceptors and restore retinal function (135-139).

5. Retinal Prosthesis

An alternative to replacing using new cells to replace lost ones is the use electronic retinal implants. The most widely known example of using an implantable electronic device is the cochlear implant to treat sensorineural hearing loss (140). Like in the ear, the goal of the implant is to replace the function of the lost/nonfunctional cells, in this case the photoreceptors. A successful device would capture images, convert the images into an electrical signal, and transmit that signal to secondary retinal neurons capable of transmitting to visual cortex. The four main strategies for these devices are subretinal (141, 142), epiretinal (143-145), supra-chorodial (146), and direct cortical stimulation (147). Both epiretinal and subretinal implants are already in clinical trials, with over 40 patients total receiving devices. One implant in particular, the Argus II

retinal prosthesis system, was implanted in 27 patients. These patients who were no light perception prior to implantation demonstrated a significant improvement in their accuracy and repeatability in a spatial-motor task (seeing a bright white square on a 19" black touch screen 12" in front of them and touching the center of that square) with the Argus II on compared with the device off, demonstrating an improvement in visual acuity (143). These devices demonstrate a strong proof of concept for implantable devices as a long-term strategy for restoration of functional sight. As with cochlear implants, increasing the number of electrodes used will continue to improve the quality of the device and the quality of vision.

6. Neuroprotection

Given the genetic heterogeneity seen in RP and other inherited retinal degenerations, genetic strategies, which are dependent on the identification and targeting of gene mutations, have limited applications. Prolonging the viability of retinal cells through the use of growth factors, viability factors, or inhibiting apoptotic pathways provides a therapeutic strategy for the treatment of retinal degeneration that is not limited by disease etiology. NT-501, an intraocular encapsulated cell implant which releases ciliary neurotrophic factor (CNTF), has been implanted in over 180 patients, including a 51 patient study of geographic atrophy in AMD patients (148) and a 133 patient study for early and late state retinitis pigmentosa (149). In the geographic atrophy clinical trial, NT-501 was found to be effective in improving retinal thickness and best-corrected visual acuity in a dose dependent fashion over the 12-month study time. Unfortunately, the RP clinical trial with NT-501 was unable to detect a benefit after either 12 or 24 months with the device, which was surprising given the positive result demonstrated in RP dogs (150).

Additionally, patients receiving the high dose of CNTF in the RP study saw a toxic effect: a decrease in sensitivity to perimetery that was reversible after implant removal.

With apoptosis being a final common pathway in RP photoreceptor cell death (99-103), the blockade of apoptosis represents a widely applicable therapeutic strategy that is independent of genetic cause. D-cis diltiazem, a calcium channel blocker, was shown to inhibit photoreceptor apoptosis in both a light induced retinal degeneration model using BALB/c mice and a genetic retinal degeneration model with *Pde6b*^{rd1} mice (151, 152). Follow up experiments with calcium channel blockers using a P23H rhodopsin rat (153), a *Pde6b*^{rd1} canine (154), and the *Pde6b*^{rd1} mouse failed to replicate the original D-cis diltiazem findings. An alternative to using pharmaceuticals to block apoptosis is gene therapy to deliver an anti-apoptotic gene to photoreceptors. X-linked inhibitor of apoptosis (XIAP), which binds to and inhibits caspases 3, 7, and 9(155) was delivered to P23H and S334ter rhodopsin transgenic rats using an AAV vector carrying either XIAP or GFP (156). Eyes treated with XIAP-AAV had a significantly ($p<0.0001$) increased outer nuclear layer thickness (~35 micrometers in treated vs. ~15 micrometers in controls).

7. Genetic animal models for Retinitis Pigmentosa

There are many robust, well-studied models of retinal degeneration in rodents. There are at least 21 different genetic models of retinitis pigmentosa in mice, each with a well characterized phenotype and unique genetic cause (157). Additional genetic models have been created using knocking outs such as rhodopsin (Rho) (158) and PDE6G (159), or with transgenics like rhodopsin P23H (160), and rhodopsin S334ter (161). Several of the genetic models of RP have demonstrated that there is widespread apoptosis in these retinal degenerations (162). *Pde6b*^{rd1} mice appear to

be the most commonly used and published mouse model of RP, with the mutation already homozygous in over 20 backgrounds such as C3H; CBA/J; CBA/NJ; FVB/NJ; and NON/LtJ (157). Additionally, *Pde6b*^{rd1} mice are among the fastest to lose photoreceptors. Most of their outer nuclear layer is gone after first month of life with no significant disruption in the rest of the retinal architecture, making *Pde6b*^{rd1} mice a logistical and economical choice for many experiments (100, 157).

8. Light induced animal models for Retinitis Pigmentosa

Light induced retinal degeneration in animals has been well studied since its first use in rats in 1966 (163). Histologic changes and an ELISA-based cell death assay in BALB/c mice exposed to 13,000 lux (10,000-25,000 lux is full daylight but not direct sun) of light for two hours established that light-induced the retinal degeneration through photoreceptor apoptosis. Further tests demonstrated a dose-response relationship between the light exposure duration and the appearance of free nucleosomes (a marker for apoptosis) in the cytoplasm of retinal cells (48). Other studies showed that the light induced damage is highly replicable within a strain while further demonstrating the dose dependence of the light damage (164). Both Wistar rats and BALB mice have been used to evaluate a compounds efficacy in light induced models of retinal degeneration (151, 165). While BALB is the most common inbred albino mice strain used, albino strains show variable sensitivity to light. To demonstrate this, over 100 mice of 9 different albino strains and C57BL/6J mice were kept in approximately 1300 lux of light for three weeks before their outer nuclear layer thickness was measured (166). It demonstrated that at 1300 lux, C57 mice suffer no adverse effects, but the albino strains demonstrated a reduction in outer nuclear layer thickness, to varying degrees. Additionally, light appears to be a cofactor in many animal

models of inherited retinal degeneration. Many genetic models showed either protection by dark rearing, exacerbation of their degeneration in higher than normal levels of light, or both (167).

9. 661W photoreceptor cell line

The 661W cell line is a SV40T transformed murine photoreceptor adherent cell line. While two human retinoblastoma cell lines exist (WERI and Y79) both grow in suspension making them ill-suited for light exposure experiments. 661W has been shown to have cone photoreceptor-like qualities such as the expression of cone opsins, transducin and arrestin (168). Additionally, 661W can uptake retinoids (vitamin A derivatives), transform 9-cis retinal to all-trans retinal with light exposure, and are more sensitive to light following the introduction of retinoids (39). 661W has been used as a model for light induced retinal degeneration for over a decade, all with similar methodology (38-47). Briefly, cells were plated onto multi-well plates, given serum free or low serum media, exposed to fluorescent light ranging from 2,500 lux for 24 hours to 30,000 lux for 2 hours at room temperature or 37 degrees Celsius before cell death was measured. Light induced cell death occurs primarily through apoptosis. Apoptosis is modulated by NF-κB, decreasing cell viability when NF-κB is blocked (38, 40, 45, 47). This NF-κB activation following light exposure was also demonstrated in BALB/cJ mice after exposure to 3,000-3,500 lux (169). Chang *et al.* demonstrated that blockade of apoptosis was not necessarily sufficient, depending on the amount of light given. When the light exposure is very high and apoptosis inhibitors are given, necrosis increases in a compensatory fashion. Only when both apoptosis and necrosis are blocked is a large increase in the number of viable cells seen (49).

B. The Cytochrome P450 System

Cytochrome P450 (CYP) is a superfamily of heme containing monooxygenase enzymes that catalyze the oxidation of organic substrates. With CYPs found in all biological kingdoms, there are over 5000 enzymes known to belong to this family. CYPs are often classified based on their substrates (sterols, xenobiotics, fatty acids, eicosanoids, vitamins, and unknown) (170). CYP2C9 is an important human CYP that metabolizes both xenobiotic and endogenous compounds. It metabolizes over 100 known drugs including warfarin, phenytoin, tolbutamide, and many NSAIDs and endogenous compounds such as arachidonic acid (AA), 5-hydoxytryptamine, and linoleic acid (171). Together with cyclooxygenase and lipoxygenase CYP2C9 generates primarily 14,15-Epoxyeicosatrienoic acid (EET) and 11,12-EET from arachidonic acid (172). Additionally eicosapentaenoic acid (EPA) or docohexaenoic acid (DHA) can compete with AA to be converted by CYP2C and CYP2J enzymes into epoxy- and hydroxyl- metabolites (173). While some CYP enzymes like CYP2E1 are well conserved between humans and other animals, others have different substrates and specificity (174). For example, R-warfarin and S-warfarin are metabolized in humans CYP1A2/CYP3A4 and CYP2C9 respectively (175). In mice, R-warfarin is metabolized by CYP2C29; an enzyme with is considered to be the homolog to CYP2C9 (176). Sulfaphenazole, which is a potent inhibitor of human CYP2C9, has no effect on the metabolism of warfarin or tolbutamide in mouse liver microsomes (data unpublished). Determining an orthologous gene in mouse to human CYP2C9 is challenging because humans have 4 CYP2C genes and mice have 15 CYP2C genes. Additionally, many of the mouse CYP2C genes are not well characterized.

C. Methods

1. Cell Culture

The 661W photoreceptor cell line was generously provided by Dr. Muayyad Al-Ubaidi (Department of Cell Biology, University of Oklahoma Health Sciences Center, Oklahoma City, OK) through an approved material transfers agreement (177). The 661W cell line was maintained in Dulbecco's modified Eagle's medium with high glucose and GlutaMAX (Gibco, Life Technologies, Carlsbad, CA) containing 10% fetal bovine serum (Gibco, Life Technologies, Carlsbad, CA), and 1x Antibiotic-Antimycotic (Gibco, Life Technologies, Carlsbad, CA). Cells were grown at 37 degrees C in a humidified atmosphere of 5% CO2 and 95% air.

2. Phototoxicity

Phototoxicity experiments were performed using methods described previously (49, 50). Briefly, 661W cells (p36-p43) were seeded onto 96 well plates by adding 100µL to each well at density of $4x10^6$ cells/mL in DMEM+GlutaMAX + 10% FBS in the morning (8am-11am). The media was changed 6 hours later to DMEM+GlutaMAX + 10% FBS and 10µM 9-cis retinal to sensitize the cells to light overnight. Then the media was changed again the following morning and the cells were given serum free DMEM+GlutaMAX with a compound of interest. Then cells were exposed to 11,000 lux of light for 4 hours to inflict cell death. Cell viability was measured with CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Madison, WI) and normalized by using neighboring control wells to determine a percent protection. Controls included: media only wells; untreated wells on the plate; and untreated wells on an identical plate that was unexposed to light. Luminescence was measured in a plate reader luminometer (luminometer info).

3. Liver Microsomes

Human (20 individual pool, mixed gender) or mouse (C57/BL6 male) liver microsomes were incubated with a CYP2C9 substrate (warfarin or tolbutamide) and the inhibitor of interest. Compounds were tested at [0.5μM] in Human Liver Microsomes and [10μM] in Mouse Liver Microsomes. Warfarin and tolbutamide were selected because they are common substrates of human CYP2C9. Fresh NADPH (Sigma Aldrich, N7505 - 100MG) was added to a final concentration of 1.3mM and incubated for 15 minutes. Samples were then run on LC/MS to quantify the enzyme products.

4. FAS Ligand

661W cells (p17-20 and p37-40) were seeded onto Costar 96-well black flat clear-bottom plates (Corning Life Sciences) with 100μL to each well at a density of $2x10^3$ cells/mL in DMEM+GlutaMAX + 10% FBS 24 hours prior to treatment. The media was changed the following morning and the cells were given serum free DMEM+GlutaMAX, 5000ng/mL or 20,000ng/mL of Fas-agonistic Jo2 monoclonal antibody (BD Biosciences, Franklin Lakes, NJ), and a compound of interest. Cell viability was measured with CellTiter-Glo® Luminescent Cell Viability Assay at 48 h after treatment by incubating the cells with the pro-luminescent substrate in 96-well plates following manufacturer's instructions. Controls included untreated cells and wells with no cells. Luminescence was measured in a TEACAN plate reader.

5. Animals and treatments

$Abca4^{-/-}$ $Rdh8^{-/-}$ double knockout mice were generated as previously described (178). Mice were housed in the animal facility at the School of Medicine, Case Western Reserve University, where they were maintained under a 12-hour light (~10 lux) and 12-hour dark cycle.

Animals received an intraperitoneal injection of 70uL containing a 10µg/g dose of the treatment 30 minutes prior to the induction of light induced retinal degeneration. All animal procedures and experiments were approved by the Case Western Reserve University Animal Care Committees and conformed to recommendations of the American Veterinary Medical Association Panel on Euthanasia and the Association of Research for Vision and Ophthalmology.

6. Induction of light-induced retinal degeneration in Abca4$^{-/-}$ Rdh8$^{-/-}$

Abca4$^{-/-}$ Rdh8$^{-/-}$ mice were dilated with 1% tropicamide for 30 minutes and then exposed to fluorescent light (10,000 lx; 150-W spiral lamp, Commercial Electric) for 30 min in a white plastic bucket (Papersmith) with food and water and then kept in the dark. OCT and biochemical experiments were performed 48 hours after exposure.

7. Ultra-high resolution spectral-domain OCT

Ultra-high resolution spectral-domain OCT (SD-OCT; Bioptigen) was used for in vivo imaging of mouse retinas. Mice were anesthetized by intraperitoneal injection of a cocktail (20 µl g^{-1} body weight) containing ketamine (6 mg ml^{-1}) and xylazine (0.44 mg ml^{-1}) in 10 mM sodium phosphate, pH 7.2, and 100 mM NaCl. Pupils were dilated for 30 minutes with 1% tropicamide. Four pictures acquired in the B-scan mode were used to construct each final averaged SD-OCT image.

8. Quantification of KB-2-001 in mouse tissues

Product extraction from intravascular blood and whole eyes of mice treated with KB-2-001 was performed. Extracted compounds were separated by reverse-phase HPLC chromatography.

MS scans were recorded in the selected ion-monitoring (SIM) mode for each individual compound. Identities of detected adducts were confirmed on the basis of their MS/MS spectra.

D. Results

1. Phototoxicity

43 novel compounds were iteratively generated in three groups. Investigators were masked to the structure of the compounds (including sulfaphenazole) until after all initial phototoxicity data for a group of compounds was gathered and distributed to all members of the team. A variety of functional modifications at the presumed active site of sulfaphenazole as well as structural changes to the scaffold were made to generate a diverse group of compounds. Compounds were initially tested to identify the most successful modifications to drive the iterative creation of a second group of compounds (data not shown). Compounds were then tested together on a 96 well plate along with a number of additional control compounds: Minocycline, Ketoconazole, Sulforaphane, and Cyclosporin A (Figure 4.1A). Each compound was randomly placed on the plate twice and the experiment was repeated three times with the location of the compounds on the plate randomized between the plates. Modifications were then made to the most successful two compounds, KB-2-001 and KB-2-003, to generate 4 new compounds. KB-2-001, KB-2-003, Sulfaphenazole (KB-2-022), Tienilic acid (TNA) and the four new group 3 compounds were then tested together on a 96 well plate (Figure 4.1B). Each compound was randomly placed on the plate six times and the experiment was repeated three times with the compound's location on the plate randomized between the plates. Because none of the compounds in the final group were superior to KB-2-001 or KB-2-003, these two compounds (KB-2-001 and KB-2-003) were selected as the candidate drugs for animal trials.

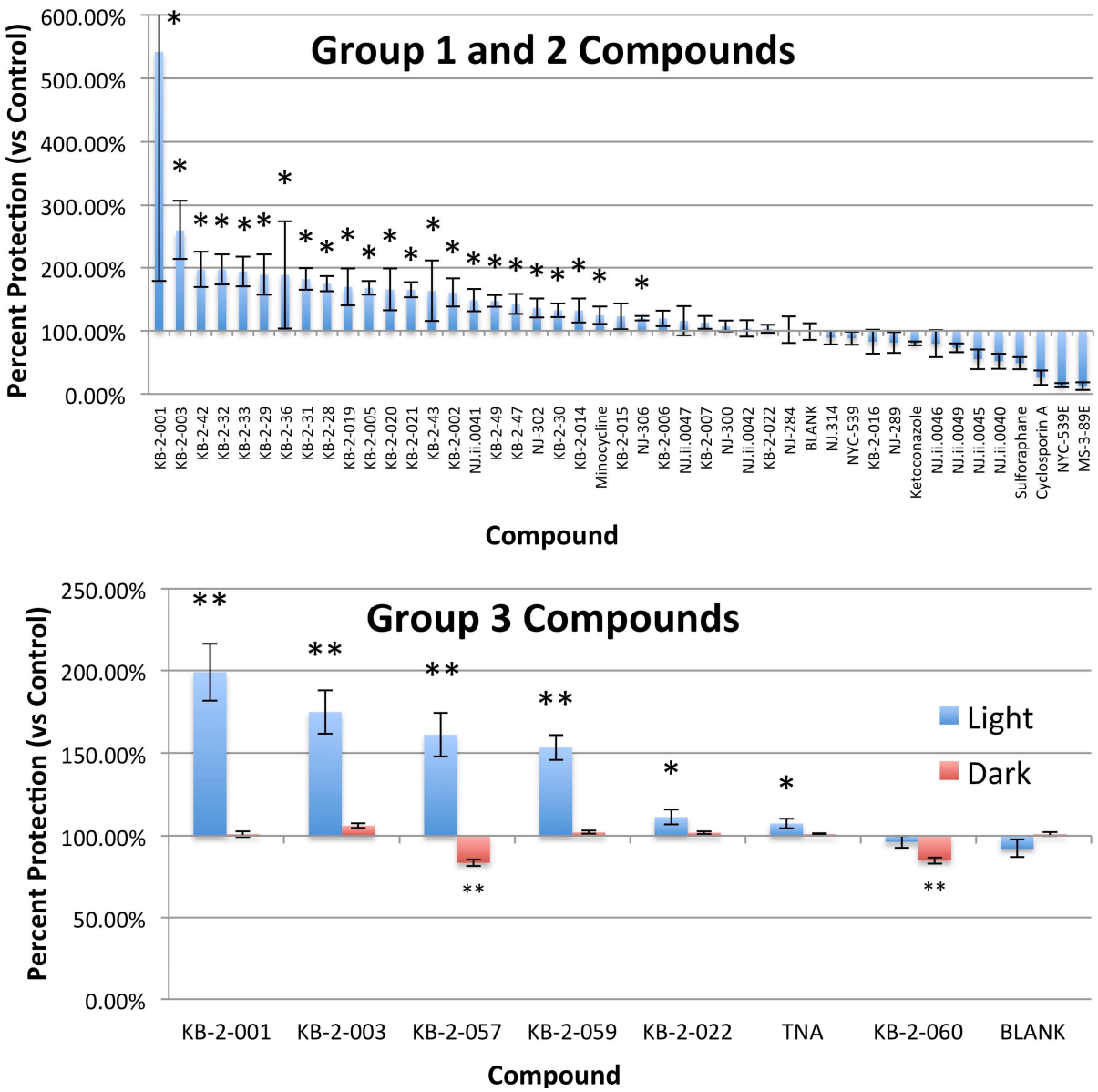

Figure 21 – Phototoxicity results by compound
(Top) Percent protection of the compounds from group 1, group 2, and additional previously published compounds of the light exposed wells with SEM bars. 100% protection indicates no change from control (BLANK). Compounds with a * indicate a statistically significant p-value (p-value < 0.05). Each compound was randomly placed on the plate twice and the experiment was repeated three times with the location of the compounds on the plate randomized between the plates. (Bottom) Percent protection of the compounds tested in group 3 with SEM bars for both the light exposed wells and the dark control wells. 100% protection indicates no change from control (BLANK). *, indicates a compound with a statistically significant p-value (* = p-value < 0.05, ** = p-value < 0.0001). Each compound was randomly placed on the plate six times and the experiment was repeated three times with the location of the compounds on the plate randomized between the plates.**

Interestingly, half of the group 3 compounds were toxic to cells in the absence of light, despite providing neuroprotective under light exposure (Figure 4.1B).

2. Liver Microsomes

Next, the inhibitory activity of sulfaphenazole and the analogs were examined using liver microsomes. The goal was to be able to find a correlation between the activities of the compounds seen in the biochemical based liver microsomes and that seen in the cell based phototoxicity. Figure 4.2 shows the remaining activity of the human liver microsomes and mouse liver microsomes to modify warfarin or tolbutamide, both of which are well-studied substrates of human CYP2C9. While there was no correlation seen between the liver microsome ranking and the photoxoticity ranking, compounds that performed well in the photoxicity screen showed some inhibition of the liver microsomes. This helped confirm that CYP2Cs were our likely target, but also confirmed that the behavior of the drug in 661W cells is more complex than solely inhibiting the CYP2C genes.

3. FAS Ligand

Initial studies with sulfaphenazole as well as liver microsome experiments demonstrate that sulfaphenazole and its derivatives have activity against CYP2C9 as well as mouse equivalents (50). The pathway through which CYP2C9 acts to induce retinal degeneration is not well characterized. Currently, there are no known pathways for cell death and retinal degeneration that these CYP proteins participate. Our prior work in 661W cells has demonstrated that FAS signaling participates in cell death caused by phototoxicity (49). To investigate the mechanism of action of our compounds involved in the FAS pathway, we stimulated the FAS receptor on 661W cells directly using Fas-agonistic Jo2 monoclonal antibodies (BD Biosciences, Franklin Lakes, NJ).

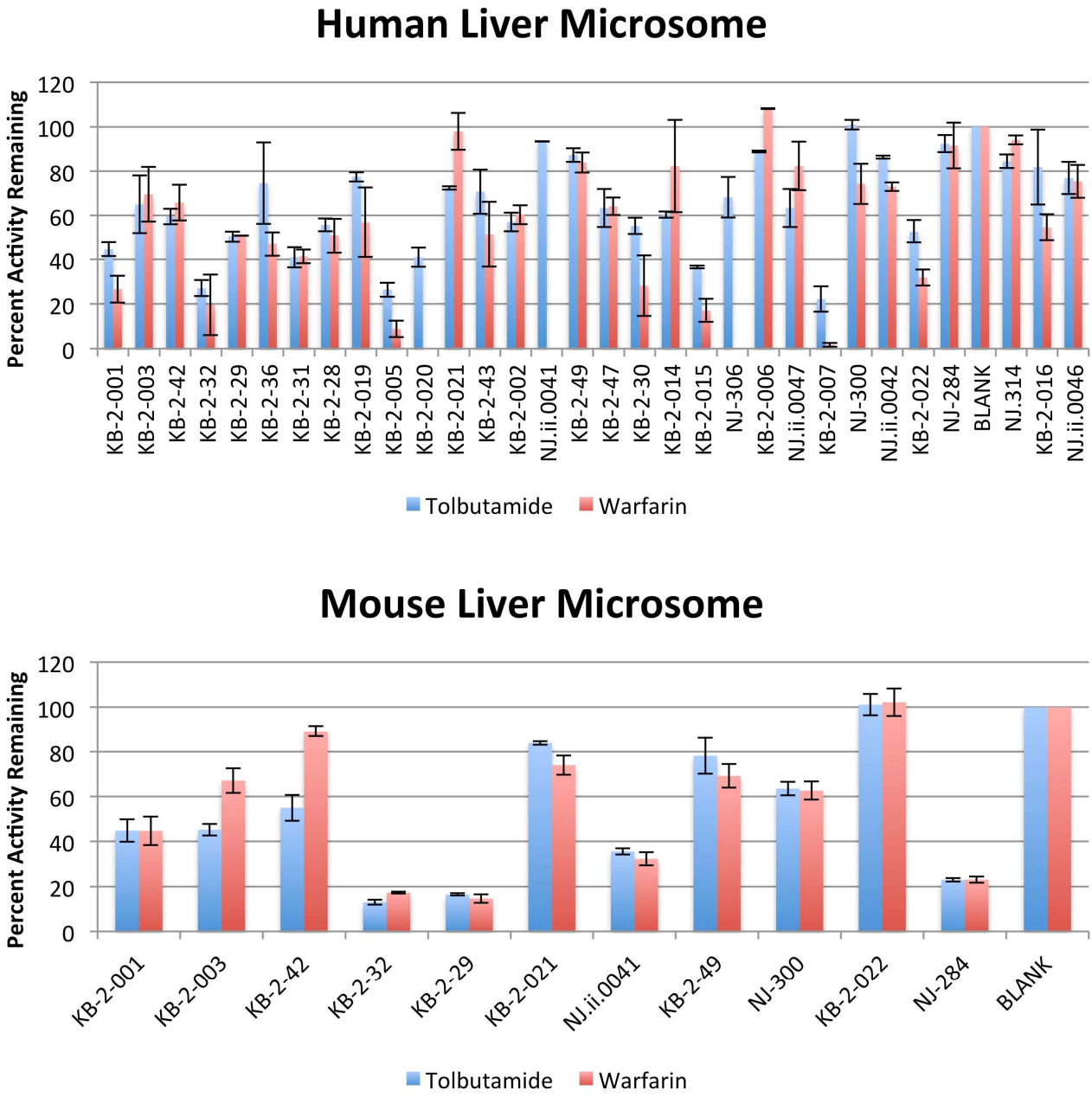

Figure 22 - Compound activity in liver microsomes
Percent activity remaining of the liver microsome following treatment with each compound. 100 percent activity represents no inhibitory action for this assay. Compounds were tested at [0.5µM] in Human Liver Microsomes (A) and [10µM] in Mouse Liver Microsomes (B). Warfarin and tolbutamide were selected because they are common substrates of Human CYP2C9. No data was collected for warfarin for compounds KB-2-020, NJ.ii.0041, NJ.306 with human liver microsomes. (Error bars are ± SD, N = 4)

To investigate if the mechanism of action of our compounds involved the FAS pathway, we stimulated the FAS receptor on 661W cells directly using the Fas-agonistic Jo2 monoclonal antibodies (BD Biosciences, FranklinLakes, NJ). To determine if these compounds achieve neuroprotection through modulation of this pathway, we treated half the wells with one of the test compounds or DMSO and 20,000ng of the Jo2 antibody for 48 hours to induce cell death. The other half of the wells was treated with one of the test compounds or DMSO but no Jo2 antibody. Wells with test compounds that did not receive the Jo2 antibody were compared to DMSO wells to test for toxicity. To determine if a compound was protective from the Jo2 antibody, Jo2 treated and untreated wells for each compound were compared to determine a percent survival. Figure 4.3 shows the percentage of surviving cells in Jo2 treated and untreated wells based on the compound of interest. KB-2-057 and KB-2-060 were toxic compared to DMSO. 10% FBS showed a significant increase in the number of viable cells, however this did not translate into any improvement in the percent survival after treatment with the Jo2 antibody (Figure 4.3A). In fact, none of these compounds offered any protection compared to DMSO. Next, we tested other compounds that have been shown to have neuroprotective effects in our phototoxicity assay: minocycline, cyclosporine, D-L sulfurophane, ketoconazole, a caspase inhibitor, and caspsin. D-L sulfurophane was the only compound that showed any protection from the Jo2 antibody (Figure 4.3B), but sulfurophane was toxic to cells in the absence of the Jo2 antibody (Figure 4.3B).

4. KB-2-001 in Abca4$^{-/-}$ Rdh8$^{-/-}$ mice

We tested KB-2-001 to determine its effect on acute, light-induced retinal damage in 4-week-old Abca4$^{-/-}$ Rdh8$^{-/-}$ mice, an animal model of human retinal degeneration using retinylamine as a positive control (178). Mice were given a single dose of test compound

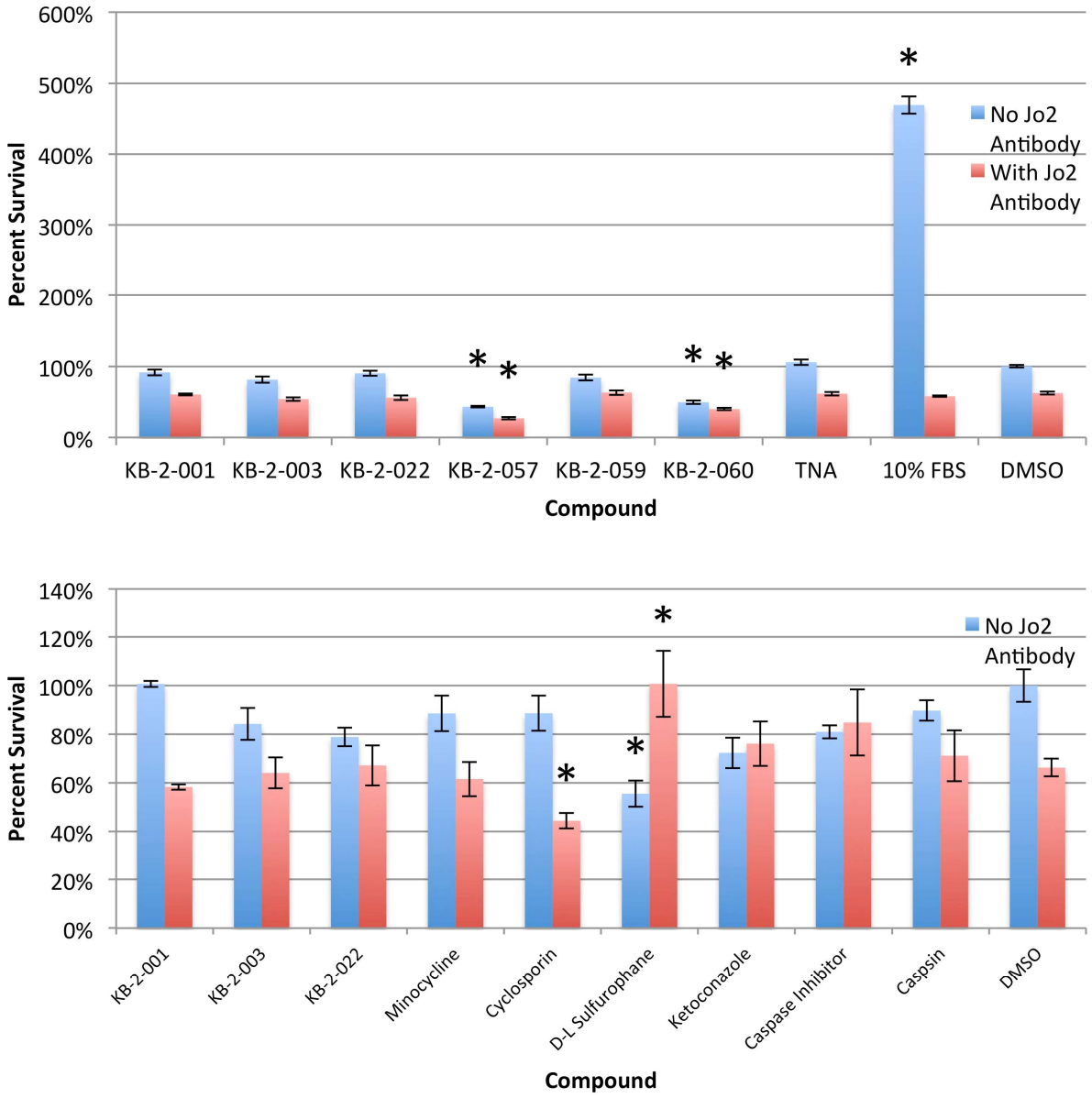

Figure 23 – Cell survival by compound against Jo2 antibody
Shows the survival percentage of cells treated with compounds when compared to controls. (A) KB-2-057 and KB-2-060 showed a statistically significant decrease compared to controls both with and without the FAS stimulating Jo2 antibody ($p < 1 \times 10^{-5}$). 10% FBS treated cells showed over a 400% increase in the number of cells compared to controls when not treated with the Jo2 antibody ($p < 1 \times 10^{-5}$), but the same fraction of cells survived as DMSO when treated with the Jo2 antibody. (n = 8 or more) (B) Additional compounds were tested demonstrated that only D-L sulfurophane was protective against the Jo2 antibody ($p < 0.01$) but it was also toxic in the absence of the Jo2 antibody, killing nearly 50% the cells ($p < 0.01$). Cyclosporin was toxic in the presence of the Jo2 antibody ($p < 0.01$)

(or DMSO) by intraperitoneal injection 30 minutes before light exposure at an intensity of 10,000 lux for 30 minutes. Mice were then kept in the dark (0 lux) for 3 days until their final evaluation via *in vivo* retinal imaging by optical coherence tomography (OCT). Mice treated with retinylamine showed significant protection in the form of a thicker outer nuclear layer (Figure 4.4A) compared to mice that were treated with only DMSO (Figure 4.4B) Figure 4.4C shows the average retinal thickness of the outer nuclear layer for each treatment group (n = 8 animals in each group). Retinylamine showed a statistically significant improvement over both the DMSO control group and our KB-2-001 test group (p-value < 0.05) and KB-2-001 did not show any improvement compared to control. We also used LC/MS to check if KB-2-001 was present in both eye tissue and blood 30 minutes after the intraperitoneal injection (Figure 4.4D).

E. Discussion

In this chapter, we took our top candidate from a high throughput cellular screen and performed lead optimization through structure activity relationship. We then validated the optimized compounds through our primary and an orthogonal assay before pre-clinical testing in a genetic model of retinal degeneration. We also attempted to test these compounds using light induced models of retinal degeneration in mice and rats, but we were unable to elicit outer nuclear layer cell loss in control animals (data not shown). Analyzing the results of the different modifications to sulfaphenazole in the cellular study using structure-activity relationships showed that compounds with more electronegative groups than $-NH_3$ in the critical position were more protective. However, when we compare the ranking from the cellular study with the ranking in the biochemical exams, no

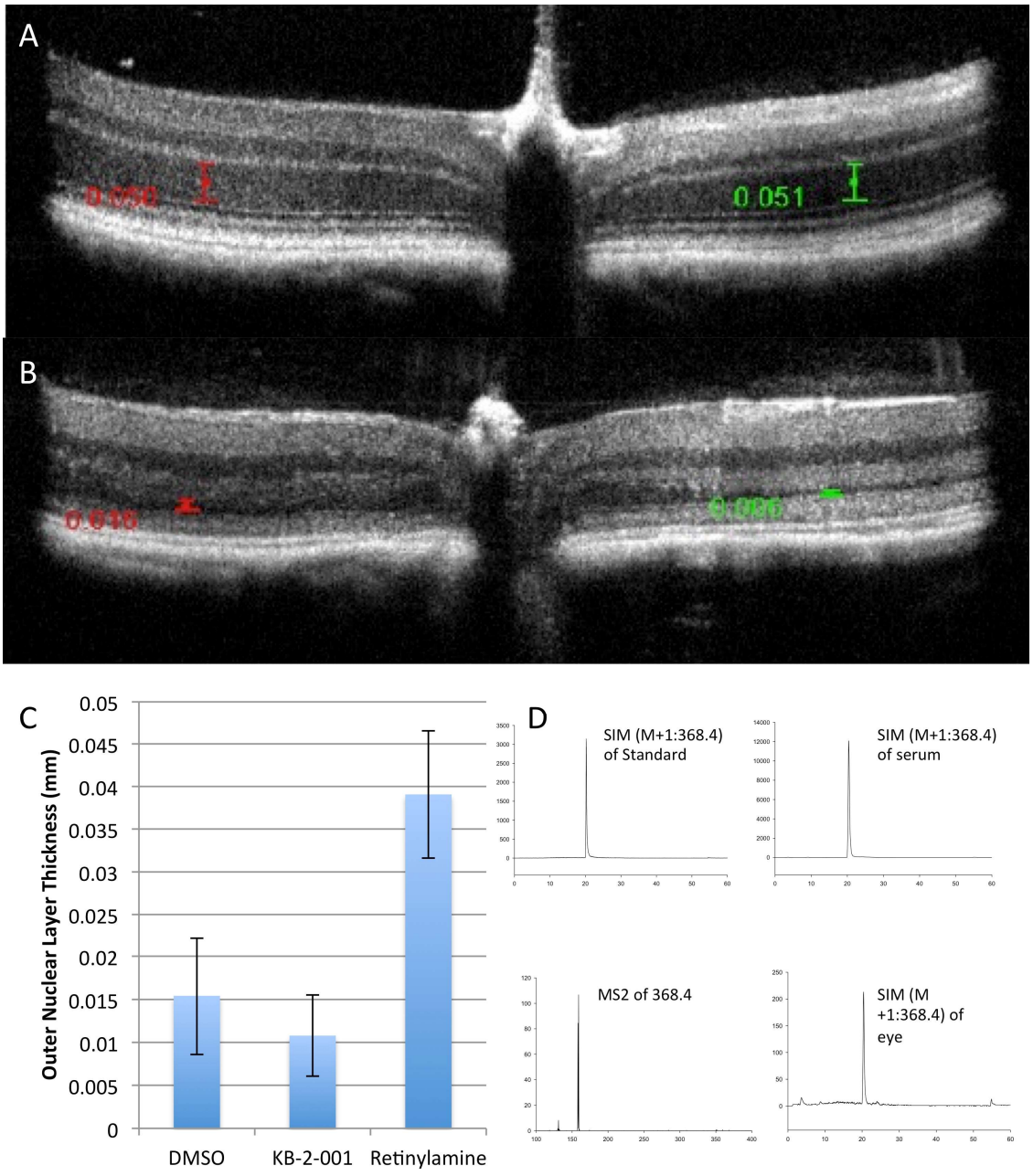

Figure 24 – KB-2-001 mouse study results
(A) OCT 3 days after light exposure in an Abca4$^{-/-}$ Rdh8$^{-/-}$ mouse treated with retinylamine. (B) OCT 3 days after light exposure in an Abca4$^{-/-}$ Rdh8$^{-/-}$ mouse treated with DMSO. (C) Outer nuclear layer thickness for each group. Retinylamine showed protection compared with either DMSO or KB-2-001 (p-value < 0.05). (D) Mass Spectrometry traces demonstrating that KB-2-001 (the Standard) was present in both the serum and eye tissue of a mouse 30 minutes after intraperitoneal injection.

clear correlation was present. There are two likely possibilities to explain this. First, our drug is having an off-target effect in 661W and the enzymes that are generating the protection are not the same as we are using in our biochemical assay. Another possibility is that the enzyme that is generating the protective effect is the CYP2C family, but because these enzymes are so promiscuous its possible using a different substrate for the biochemical exam would have yielded different results that line up more with the results from the 661W studies.

Next, we looked at an orthogonal assay, FAS signaling with the Jo2 antibody. Our previous work had shown that a major component of cell death in our 661W light assay was caused by FAS ligand. We hypothesized that since our drug was effective at preventing cell death in the light pathway it may be acting through the FAS pathway. Results from the FAS ligand assay showed that our drug has no effect on FAS ligand activated cell death. While this result was disappointing, it is still a useful result if future studies are performed. Having an orthogonal assay for a high throughput screen helps remove false positives that are acting through more general neuroprotective pathways rather than compounds that share our target of interest.

Finally, we attempted a small pilot animal study to determine if this drug and pathway merited further investigation, both with more cell and animal experiments. Our results showed that our drug had no effect in the double knockout mouse despite the drug being present in the retina. We also attempted to test the drug in two different animal models but technical difficulties prevented us from replicating the outer nuclear layer reported in the literature from light exposure in mice and rats, and based on the negative results from the double knockout, the decision was made to discontinue trials in these animal models.

Future studies should seek to determine why sulfaphenazole and its analogs were having a therapeutic effect. Once the true target of sulfaphenazole is in 661W is determined we can start to

look at pathway analysis and understand why our initial animal model work was unsuccessful. Results may show that the protection in 661W is an artifact of the cell transformation. If not, armed with the proper pathway analysis, we can repeat animal studies using the appropriate models as we try and move the project towards human trials.

V. DISCUSSION

A. General Discussion and Future Work

One of my goals in this thesis was to try to identify potential therapeutic modalities to improve visual outcomes for pediatric cataracts, which I investigated in chapter 2 of this dissertation. The major advantage of the method used in my studies is that by treating intraoperatively, these treatments that could be used in places in the world where care is scarce or with in situations where compliance may be poor. I hypothesized that reducing the incidence of adverse events will improve visual outcomes, especially in patients with socioeconomic disparities that have demonstrated poorer visual outcomes after lensectomy (19).

Our results showed that in untreated rabbits, the postoperative fibrin and inflammation occurs rapidly after surgery and slowly fades over two weeks. We found a significant reduction in the formation of postoperative fibrin with enoxaparin alone as well as in combination with a low dose of triamcinolone, which resulted in a significant improvement in the clarity of the visual axis as measured by OCT signal strength. Enoxaparin combined with low dose of triamcinolone in this study augmented the effect of enoxaparin by reducing flare, which was likely the cause of the increased OCT signal strength seen in this group.

While there are many potential confounders for my work in this study, one of the most important was the variation in surgical technique and complications. While the surgical technique was standardized for all surgery days and the same surgeon performed all of the procedures for each experiment, there were a variety of different complications in

each of the treatment groups while none of the control eyes had known surgical complications. One change we could have made was to exclude any eye with complications, but that may have biased the results while significantly increasing the number of procedures performed and animals utilized without affecting our conclusions.

Another limitation of this study is the time course. We chose to only follow the rabbits for two weeks postoperatively, but the Infant Aphakia Treatment Study indicates that the critical period for follow up in humans is 1 year. However, our observations showed that most of inflammation and fibrosis clinically self-resolved over two weeks in the rabbits. The spontaneous formation of new fibrin membranes more than two weeks after surgery seems unlikely, but was not tested. Instead, it may be that the first few weeks in rabbits are important for the formation of fibrin membranes and resolution of inflammation. It is difficult to know the actual time course of untreated humans because it is standard of care to prophylactically treat with topical medication and any significant opacity that forms is often treated with little delay. In future studies, following the rabbits past 2 weeks would give a clearer picture of how posterior capsule opacification is affected by these interventions and if further complications develop as the rabbits grow.

While enoxaparin and triamcinolone were studied for preventing postoperative complications, once a fibrin membrane is present these treatments likely have little benefit. This led to investigating an alternative to surgery for treating a fibrin membrane once it forms. tPA has been used in the eye in several small human trials before, but the risk benefit analysis has not yet led to it being a standard of care treatment (85, 86, 88, 89). One of the groups attempted to use tPA prophylactically at the time of surgery and found that the incidence of intraocular fibrin membranes were significantly lower on up to day 14 but

no significant difference on days 30 and 90. Rather than try to treat prophylactically, future studies should treat the membranes when they are seen, and continue to treat them, potentially repeatedly, for as deemed efficacious and safe for the patient. This would require additional safety studies because no one has published on the effects of repeated tPA injections intraocularly. Future animal studies should investigate the pharmacokinetics of these intraocular therapies as well as systemic absorption to determine safety, dose response, and time course of action. In addition, changes in the inflammatory pathway should also be investigated to better understand how these treatments affect the response to surgery and potentially identify new therapeutic targets.

With the potential use of enoxaparin or tPA in these subjects that often have traumatic cataracts, fibrin membranes, or corectopia, it is possible that we can further improve visual outcomes in patients after open globe ocular trauma. In chapter 3, I examined how the timing of amblyopia therapy for ocular trauma patients affects visual outcomes. Ocular trauma remains a common and preventable cause of decreased vision, especially in young children (22, 26, 91, 93). This project was a retrospective review of the treatment by multiple different attending physicians in the pediatric ophthalmology division after ocular trauma. To determine if there was a difference in visual acuity outcomes with or without amblyopia therapy, we looked retrospectively at all of the pediatric ocular trauma patients from August 2001 until July 2014 and analyzed the 74 patients that qualified for analysis.

Despite the relatively small sample size of subjects treated for amblyopia, we found that subjects below the age of 9 who received amblyopia treatment had significantly better visual outcomes. With the POTS categories and the pathologies being similar between the

treated and untreated groups, the results indicate that the treating deprivational or refractive amblyopia within the first three months after injury, even before amblyopia is diagnosed may improve visual outcomes. A large, multi-center prospective study is needed to further identify the most ideal treatment regimen and which patients are most likely to benefit from early amblyopia therapy. A prospective study would also identify other risk factors for poor visual outcomes, giving us future avenues for investigation for children with ocular trauma.

Finally, in chapter 4 I investigated the utility of a top candidate in a high throughput cellular screen and attempted to develop it into a novel therapeutic suitable for additional animal trials or early phase one clinical trial. I performed lead optimization through structure activity relationship before validating the optimized compounds through primary and orthogonal assays and moving onto animal testing with a genetic model of retinal degeneration. While the results were disappointing, these experiments gave me the tools and understanding necessary to repeat this process independently in my own lab in the future. If someone else were to continue these studies, it would be important to go back to the cell line and determine why sulfaphenazole and its analogs were having a therapeutic effect. Once the true target of sulfaphenazole in 661W is determined, the next step would be pathway analysis to understand why the initial animal model failed. I suspect that this work will show that the protection in 661W is an artifact of the cells transformation or of some other defect in the cell line. If not, armed with the proper pathway analysis, animal studies can be repeated using the appropriate models to try and move the project forward.

The studies in this thesis used preclinical and clinical studies to identify novel ways to improve outcomes for a variety of disorders. There are several promising future avenues

of study that may have clinical applicability in humans. Understanding the science behind some of the multiple causes of vision loss allows progress to be made in the methods of prevention and treatment of inherited, congenital, and acquired eye disorders.

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VITA

NAME:	Joseph D. Bogaard
EDUCATION:	B.S.E., Biomedical Engineering, University of Iowa, Iowa City, Iowa, 2009
	M.D., Medicine, University of Illinois at Chicago, Chicago, Illinois, 2017
	Ph.D, Neuroscience, University of Illinois at Chicago, Chicago, Illinois, 2017
TEACHING:	College of Engineering, University of Iowa, Iowa City, Iowa: Teaching Assistant Engineering Problem Solving II, 2007
	College of Engineering, University of Iowa, Iowa City, Iowa: Teaching Assistant Engineering Problem Solving II, 2008
PROFESSIONAL MEMBERSHIP:	American Medical Student Association American Medical Association Association for Research in Vision and Ophthalmology
ABSTRACTS AND PRESENTATIONS:	Joseph D. Bogaard, Jonathon Young, Iris S. Kassem. Tissue Plasminogen Activator for the Treatment of Fibrin After Lensectomy with Intraocular Lens Insertion in a Juvenile Rabbit Model. ARVO. May 1st 2016. Poster Presentation.

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