Cure Glaucoma Foundation Final Report

Development of an Enzyme Replacement Therapy for Primary Congenital Glaucoma

PI: Rachida Bouhenni, PhD

Akron Children's Hospital

Project Summary

Glaucoma is classified as a group of diseases that damage the eye's optic nerve, leading to vision loss and blindness. Elevation of the pressure within the eye due to improper drainage of the fluid (aqueous humor) in the front of the eye is the major risk factor for glaucoma. In the pediatric form of glaucoma, also known as primary congenital glaucoma (PCG), children are born with a defect in the angle (trabecular meshwork) of the eye that slows the normal drainage of aqueous humor from the eye. The disease is caused by a mutation in a gene called cytochrome p450 B1 (CYP1B1) which is an enzyme. The function of this gene is not known but it is thought that it is involved in vitamin A processing in the eye. Because PCG results from a defective enzyme, we hypothesize that replacing the defective enzyme could rescue the phenotype. The long-term goal of this proposal is to develop an enzyme replacement therapy for PCG. In this study, we sought to: 1) manufacture the enzyme (CYP1B1), 2) characterize the enzyme, 3) inject the enzyme into a mouse eye, 4) assess the delivery of the enzyme into the eye and, 5) study its effect on cellular stress and tissue growth at the molecular level.

Project Results

1. **CYP1B1 (candidate drug) protein manufacturing**

To manufacture the protein in house we used 2 different mammalian cells (HEK293T/17 and CHO-K1). Once introduced into the cells, the cells will start making the protein as they divide. The protein was introduced into the cells in the form of a gene cloned into a shuttle vector (CYP1B1/pME-HA) and a specific tag that we used to extract the protein from the cells. The protein was then extracted from cells using a lysis buffer and stored at -80 ^{o}C until used for injections. Using CHO-K1 cells, we were able to produce 13.518 mg of total CYP1B1 as determined by using a protein assay (Bicinchoninic Acid). To purify the protein from any contaminants, we dialyzed it against a buffer (50mM KPO4 pH 7.4). **Figure 1** is a western blot showing the overexpression of the enzyme in CHO cells (A) and the purified enzyme (B). The black bands represent the protein. The darker the band, the more protein it contains.

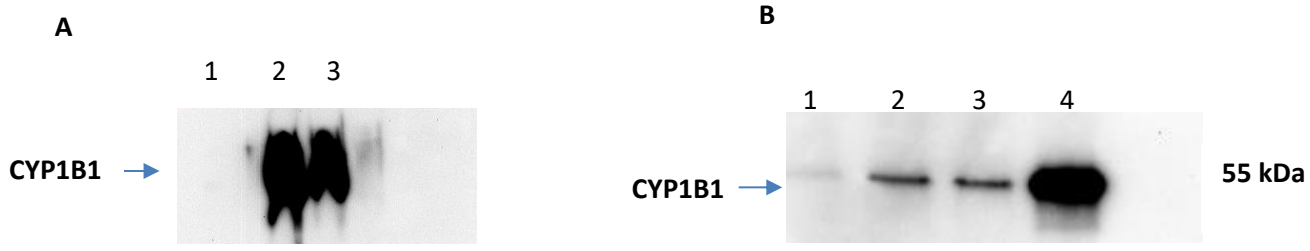

Figure 1 Western blotting showing **A:** overexpression of CYP1B1 in CHO cells (Lane 1: CYP1B1 in CHO cells before overexpression, Lanes 2 and 3: CYP1B1 in CHO cells after overexpression. **B:** purification of CYP1B1 from the cells. Lane 1: wash 1, lane 2: elution 1, lane 3: elution 3, lane 4: concentrated CYP1B1 (final product). Each lane represents a different step in the purification process.

1.1. Characterization of the enzyme (protein)

To be able to use the purified protein as a drug, it must be enzymatically active. The purification goes through several washing steps that may interfere with the protein activity and renders it inactive. To confirm our protein activity, we used three different assays: the ethoxyresorufin-O-deethylase (EROD) assay, CYP 450 P-Glo assay and cytochrome p450 reductase assay. We used samples from the liver as positive controls as the liver contains high levels of CYP1B1.

Before purification from cells, the protein activity was high as determined by all 3 assays, however, when purified the protein lost most of its activity during purification or storage. Experiments are underway to improve the protein purification. A commercially available CYP1B1 was purchased from Creative Biomart (catalog # CYP1B1-01H) and was used for the enzyme delivery into the in vivo (animal) experiments to establish the enzyme delivery (therapy) proof of concept.

2. Animal (in vivo) experiments

The animals we used in this study were: **1)** normal mice that were used as controls and were designated as **wild type** throughout this report and, **2)** mice that had a mutation in CYP1B1 (CYP1B1-/-) which we referred to as **knockouts** throughout this report. These knockouts do not make the CYP1B1 protein therefore their eyes are undeveloped and show a phenotype that is similar to Primary Congenital Glaucoma in humans (**Figure 2**).

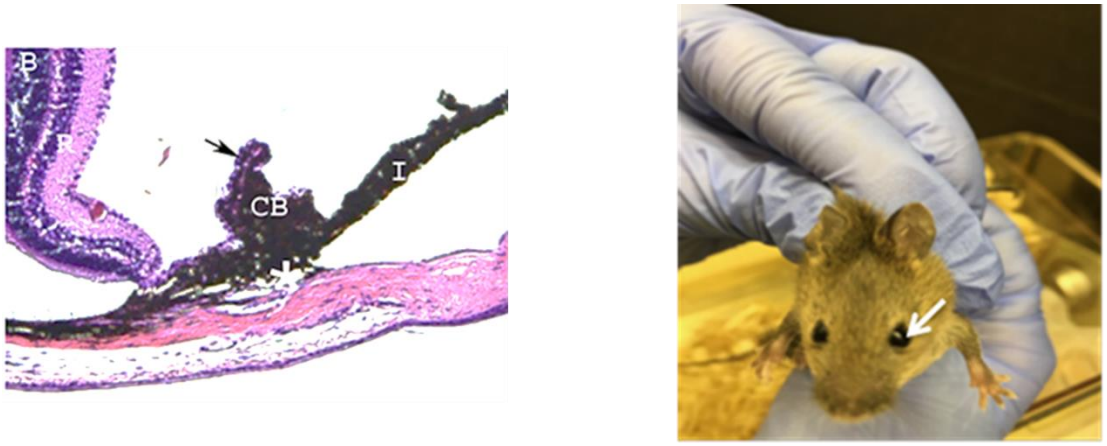

Figure 2. Characterization of the CYP1B1 knockout mouse. **Left**: A cross section of the anterior chamber (anterior part of the eye) showing the different ocular structures (R: retina, CB: ciliary body, I: iris, * : trabecular meshwork) and **Right**: a photograph showing the bulging of the eye in the CYP1B1 knockout mouse due to glaucoma

2.1. CYP1B1 treatment design

Animals were treated in 4 groups (young with and without a steroid, old with and without a steroid), with either 1 µl, 1.5 µl or 2 µl of saline or CYP1B1 protein (enzyme). Control groups consisted of those animals (wild type or knockouts) that did not receive an injection in either if their eyes (**Table 1**). All injections were performed in the right eye. The left eye for each animal was used an internal control.

Table 1. Number of animals used in the study

	Young wild type (2-4 weeks old)				**Old wild type (8-12 weeks old)**				**Young CYP1B1 knockout (2-4 weeks old)**				**Old CYP1B1 knockout (8-12 weeks old)**			
# Injections/animal	**1**		**4**		**1**		**4**		**1**		**4**		**1**		**4**	
Steroid added	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+
Control	0	3	3	0	0	2	3	0	0	2	3	0	0	3	3	0
1 µl saline	0	3	0	0	0	3	0	0	0	3	0	0	0	2	0	0
2 µl saline	0	2	0	0	0	2	0	0	0	3	0	0	0	6	0	0
1 µl enzyme	0	4	3	0	0	4	3	0	0	4	3	0	0	4	4	0
1.5 µl enzyme	0	0	3	0	0	0	3	0	0	0	4	0	0	0	4	0
2 µl enzyme	0	2	3	0	0	2	3	0	0	3	0	0	0	4	17	0

Injections were performed intracamerally (in the front part of the eye through the cornea) as shown in **Figure 3**. Each batch included wild type young (2-4 weeks) and old (8-12 weeks), CYP1B1 knockout young (2-4 weeks) and old (8-12 weeks). Animals received either one injection and sacrificed 1-week post-treatment or 4 injections, one week apart and were sacrificed 24 hours following the last injection. Animals that developed cataract or inflammation after the injection were excluded from the analysis.

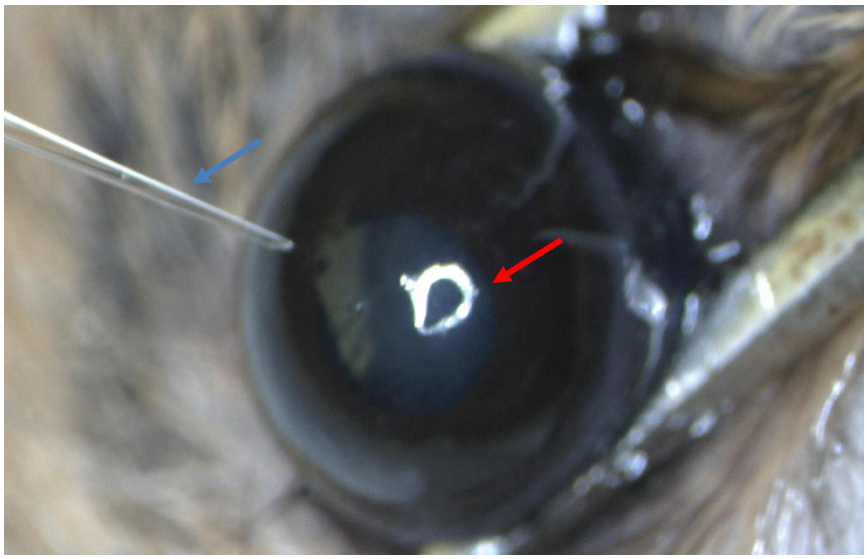

Figure. 3. A photograph demonstrating the injection process. Blue arrow: a glass pipette pulled used as a needle, Red arrow: the cornea.

After performing the first round of injection, we noticed that animals developed an inflammatory reaction, so we added a steroid that was applied immediately after

the injection in the animals that received only one injection (**Table 1**). The steroid seemed effective, and no inflammation was noticed in the eyes, or the sections of the injected eyes compared to non-injected eyes. A total of 123 animals were used in this experiment.

Sample collection: Following euthanasia, whole eyes were enucleated and fixed (preserved) in formalin for histological and histo-immunological analyses or frozen immediately for western blot analysis.

2.2. Delivery of the enzyme (protein) into the mouse eye

We successfully delivered the enzyme into the eye of the mouse using the method described above. **Figure 4** shows the amount of the enzyme detected in the anterior part of the eye of a wild type of mouse after injection of the enzyme (shown in the red fluorescence in the right panel, white arrow) compared to before injection (left panel). Note wild type still expresses CYP1B1.

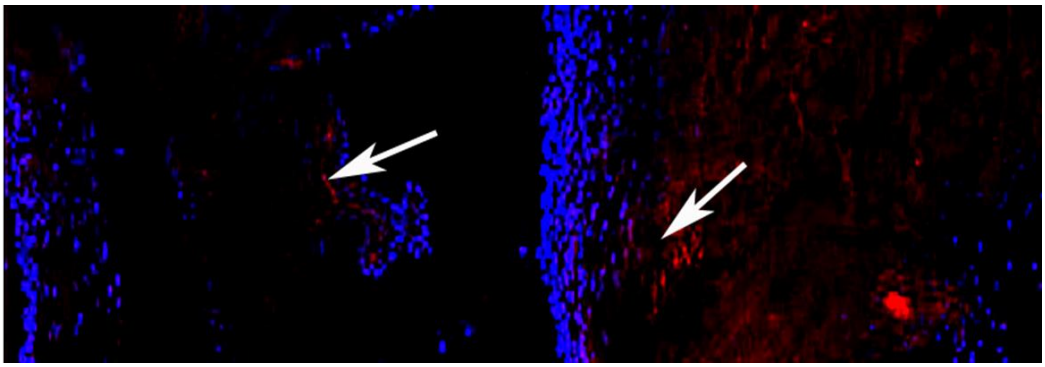

Figure 4. A microscope photograph showing the iridocorneal angle (the angle between the cornea, retina and iris, the site for the ocular fluid drainage from the eye) for an injected eye (left) and non-injected eye (right). 20 x original magnification. Blue color represents the nuclei of the cells.

2.3. Microscopic analysis of the injected mouse eyes

Injection of the enzyme into the mouse eye did not result in noticeable structural changes in any of the tissues (**Figure 5**) using light microscopy.

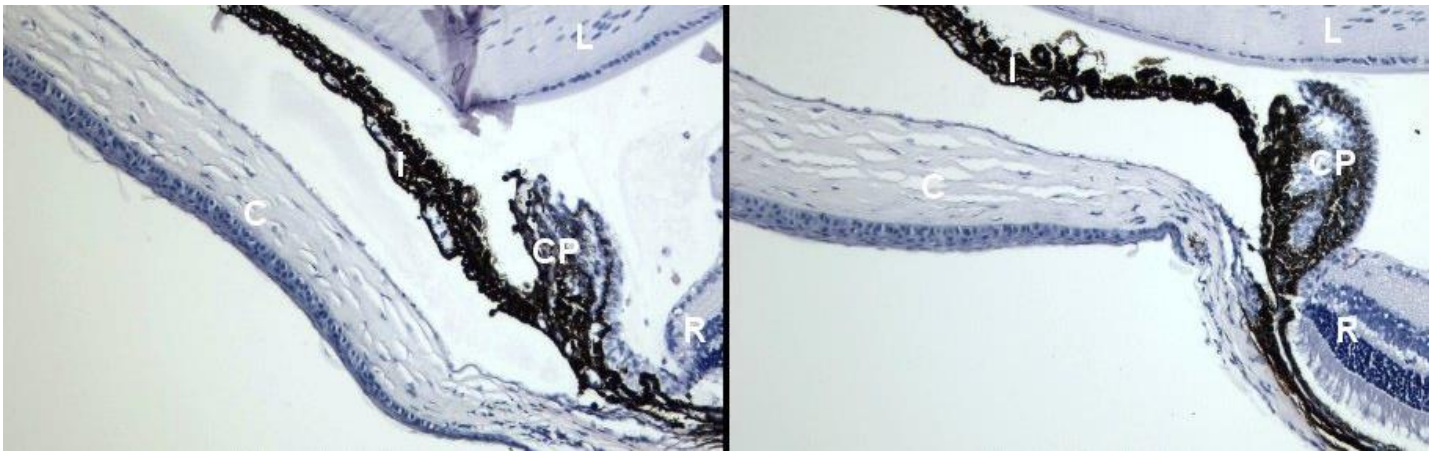

Figure 5. A representative photograph of a mouse eye after the injection of the enzyme (left) compared to before the injection of the enzyme (right). C: cornea, I: iris, CP: ciliary processes, R: retina, L: lens. Original magnification 10x. No structural changes were noticed.

2.4. Determining the effect of delivering the enzyme into the mouse eye:

Since the CYP1B1 knockout mouse does not develop high intraocular pressure (AMIRMOKHTARI; FORESI; DEWAN; BOUHENNI *et al.*, 2021), one way to assess the success of our enzyme therapy is to measure the levels of p53 and GADD45 proteins in the injected eyes. In our preliminary work, we showed that CYP1B1 binds to all *trans* retinal, an intermediate in the processing of vitamin A (retinol) in the eye. We therefore hypothesized that when CYP1B1 is absent as in primary congenital glaucoma, vitamin A is not processed completely. This leads to accumulation of all *trans* retinal, an intermediate in vitamin A processing pathway (**Figure 6**). All *trans* retinal is toxic and its accumulation leads to cellular stress that we can measure by p53, a cellular stress marker. Elevation of the latter leads to the arrest of the growth of the ocular tissues that could be measured by GADD45 (BOUHENNI, ROWE, ARVO 2021).

Figure 6. Vitamin A (Retinol) processing in the eye. Retinol is the form vitamin A is obtained from the diet. Retinoic acid is the active biological form of vitamin A that regulates cell growth during embryonic development. Retinol has to be converted to retinoic acid before it can be used by cells. CYP1B1 could be involved in the second oxidation step, which is the conversion of all *trans* retinal to all *trans* retinoic acid.

2.5. Reduction of p53 and GADD45 in the CYP1B1 injected eyes

Figures 7, 8 and 9 are immunohistochemistry photographs showing the effect of CYP1B1on the levels of the cellular stress marker p53 and the growth arrest marker GADD45 proteins.

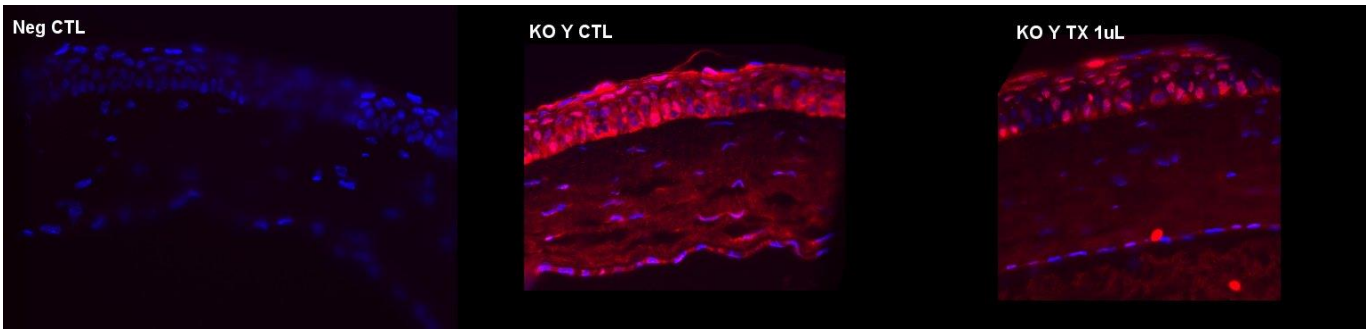

Figure 7. Cornea sections showing levels of p53 protein (cellular stress marker) levels after CYP1B1 injection. **Left**: neg control, **Middle**: CYP1B1 knockout non-injected and **Right**: CYP1B1 knockout injected. Levels of p53 are shown in red). Original magnification 10x. Note the reduced levels of p53 in the injected eye (right). Blue color represents the nuclei of the cells.

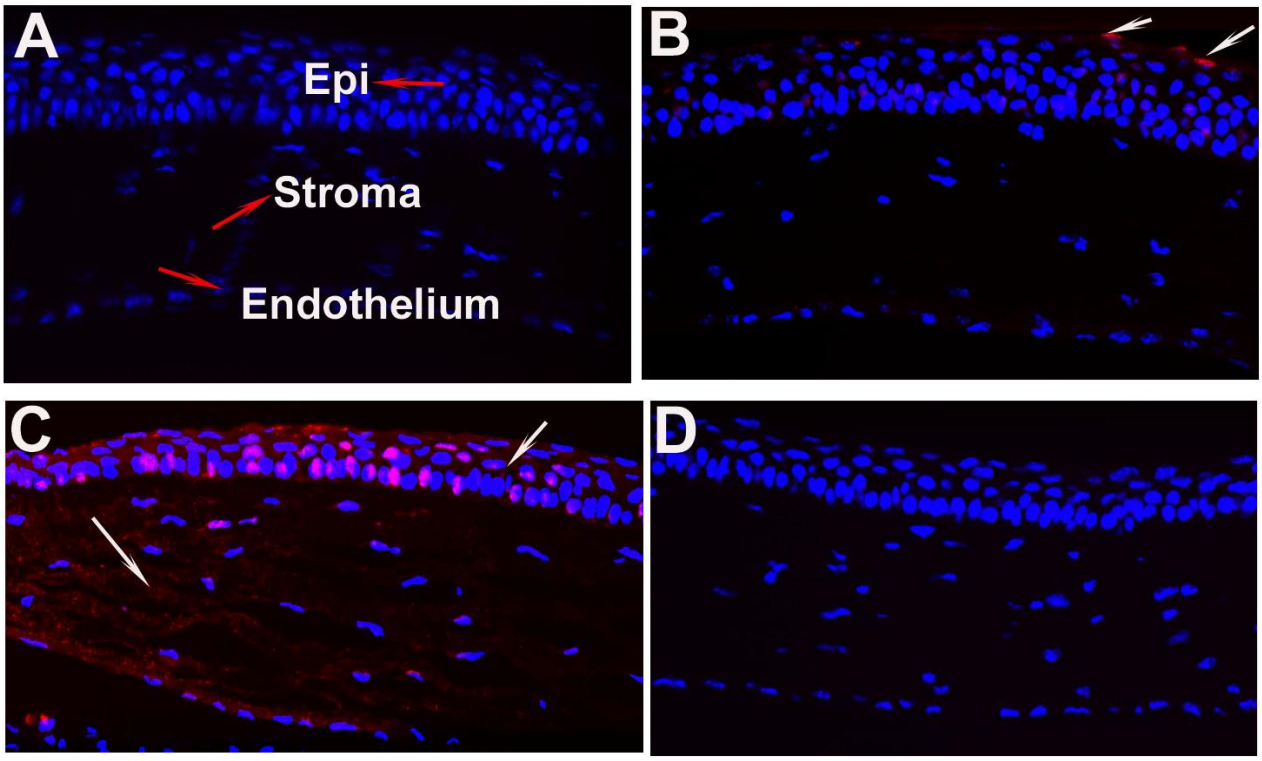

Figure 8. Cornea sections showing levels of GADD45 protein (the growth arrest marker) levels after CYP1B1 injection. **A**: neg control, **B**: wild type, **C**: CYP1B1 knockout non-injected and, **D**: CYP1B1 knockout injected. Levels of GADD45 are shown in red, white arrows). Original magnification 20x. Note the reduced levels of GADD45 in the injected eye (D) compared to the non-injected eye (C). Blue color represents the nuclei of the cells. Epi: epithelium, stroma: corneal stroma, endothelium: endothelial cells.

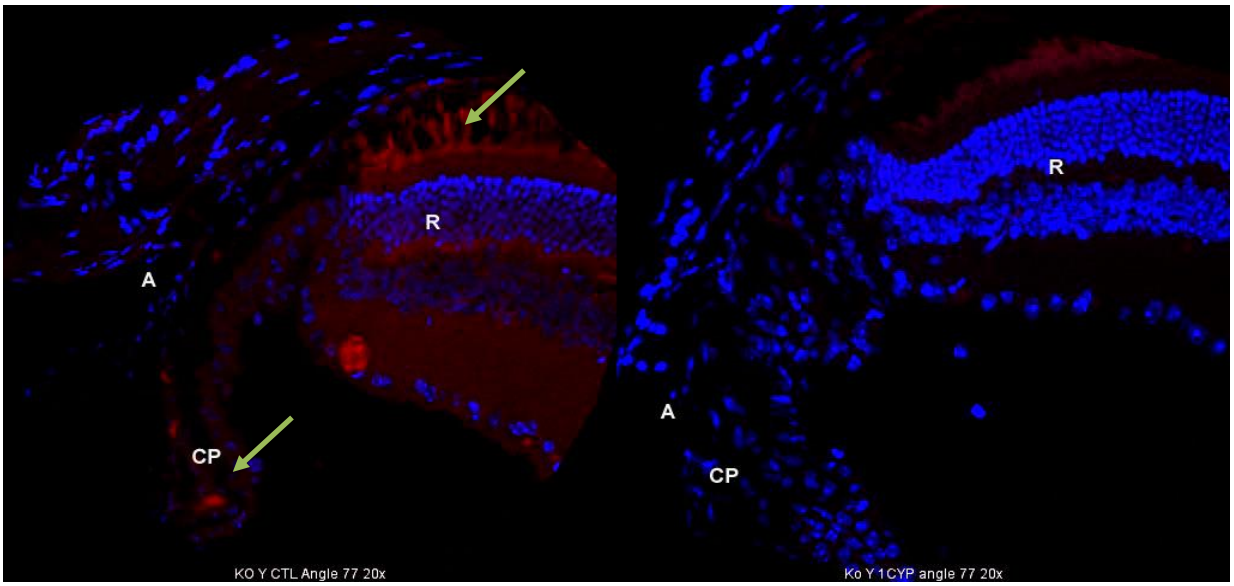

Figure 9. Angle sections showing levels of GADD45 protein levels after CYP1B1 injection. **Left**: CYP1B1 knockout control, no injection, **Right**: CYP1B1 knockout injected. Levels of GADD45 are shown in red, green

arrows). (R: retina, CP: ciliary processes, A: angle). Original magnification 20x. Note the reduced levels of GADD45 in the injected eye (D) compared to the non-injected eye (C). Blue color represents the nuclei of the cells.

Project conclusions: In this project we were able to determine the feasibility of delivering the CYP1B1 enzyme into the mouse eye. Studies are warranted to:

1. Improve the manufacturing of the enzyme.
2. Optimize the delivery of the enzyme into the eye.
3. Perform ultrastructural studies (using electron microscopy) to study the uptake of the enzyme by the cells in the eye and its effect on the ocular tissues.

References

AMIRMOKHTARI, N.; FORESI, B. D.; DEWAN, S. S.; BOUHENNI, R. A. *et al.* Absence of Cytochrome P450-1b1 Increases Susceptibility of Pressure-Induced Axonopathy in the Murine Retinal Projection. **Front Cell Dev Biol**, 9, p. 636321, 2021.

BOUHENNI RA, ROWE T, BENMERZOUGA I, SHIN WS, EDWARD DP. Cytochrome p450 1b1 binds to all trans retinal and affects p53 expression and growth arrest in human trabecular meshwork cells. ARVO, May 2021

PRESENTATIONS AND PUBLICATIONS

1. Rachida Bouhenni. Cytochrome p450 1b1 binds to all trans retinal and affects p53 expression and growth arrest in human trabecular meshwork cells. World Glaucoma Congress, July 3rd, 2021 (oral presentation).

2. Rachida Bouhenni, Theresa Rowe, Imaan Benmerzouga, Shik Shin, Deepak Paul Edward. Cytochrome p450 1b1 binds to all trans retinal and affects p53 expression and growth arrest in human trabecular meshwork cells. ARVO, May 2021

3. Naseem Amirmokhtari, Brian D. Foresi, Shiv S. Dewan, Rachida A. Bouhenni and Matthew A. Smith. Absence of Cytochrome P450-1b1 Increases Susceptibility of Pressure-Induced Axonopathy in the Murine Retinal Projection. Front. Cell Dev. Biol., 05 March 2021 | https://doi.org/10.3389/fcell.2021.636321

FINANCIAL REPORT (SEE ATTACHED).