Award Number: W81XWH-19-1-0824

TITLE: Targeting Metabolic Reprogramming for the Prevention and Treatment of Proliferative Vitreoretinopathy

PRINCIPAL INVESTIGATOR: Dr. Leo Kim

CONTRACTING ORGANIZATION: Schepens Eye Research Institute of Mass. Eye and Ear, 20 Staniford St, Boston, MA, 02114

REPORT DATE: October 2022

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2022	Annual	15 SEPT 2021 – 14 SEPT 2022

4. TITLE AND SUBTITLE

Targeting Metabolic Reprogramming for the Prevention and Treatment of Proliferative Vitreoretinopathy

5a. CONTRACT NUMBER
W81XWH-19-1-0824

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

6. AUTHOR(S)
Leo Kim, Daisy Shu

E-Mail: leo_kim@meei.harvard.edu

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Schepens Eye Research Institute of Mass. Eye and Ear,
20 Staniford St, Boston, MA, 02114

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT This is our third year on the project, and we have continued to publish our new findings identifying the key metabolic reprogramming traits associated with proliferative vitreoretinopathy (PVR) using our in-vitro model of epithelial-mesenchymal transition (EMT) and inflammation of human retinal pigment epithelial cells (RPE). Our research findings have yielded the identification of several promising metabolic drugs which effectively block retinal EMT in vitro to be tested in our in vivo rabbit ocular surgery model of PVR. We have now collected a total of 43 human vitreous samples. We have sent through primary human RPE cells for metabolomics processing and will compare these results with our ARPE-19 cell line data. We have identified another cytokine that induces EMT of RPE, namely, tumor necrosis factor-alpha, and found that it exerts opposite effects to metabolic reprogramming compared to transforming growth factor-beta and found a novel metabolic drug, dimethyl fumarate (DMFu) that blocks to activity of tumor necrosis factor-alpha.

15. SUBJECT TERMS
Proliferative vitreoretinopathy, retinal pigment epithelial cells, epithelial-mesenchymal transition, metabolism, mitochondria, transforming growth factor-beta

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRDC
U	U	U	UU	23	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

1. INTRODUCTION**:**

Proliferative vitreoretinopathy (PVR) is a common and serious complication of retinal detachment surgery and retinal trauma. PVR is characterized by the uncontrolled proliferation and migration of retinal cells, predominantly retinal pigment epithelial (RPE) cells, resulting in the formation of a fibrotic membrane at the retinal surface. A key established mechanism in PVR is conversion of RPE cells into mesenchymal cells through epithelial-mesenchymal transition (EMT). The purpose of our project is to investigate the role of metabolic rewiring and mitochondrial dysfunction during EMT of RPE. In doing so, we aim to find novel drug targets to combat PVR. The scope of our research involves 1) using an in vitro human RPE model to understand the metabolic changes and identify drug candidates to inhibit PVR formation and 2) validation of the top candidate drugs using an in vivo rabbit model of PVR.

2. KEYWORDS**:**

- Proliferative vitreoretinopathy
- Epithelial-mesenchymal transition
- Wound healing
- Transforming growth factor-beta
- Retina pigment epithelial cells
- Metabolism
- Mitochondria

3. ACCOMPLISHMENTS**:**

- **What were the major goals of the project?**

Specific Aim 1	**Timeline (months)**	**Completion (%)**
Major Task 1: To characterise the metabolic reprogramming involved in EMT of RPE and PVR		
Subtask 1: characterization of the metabolic traits characteristic of RPE mesenchymal transition by metabolic and gene expression profiling	1-12	100
Subtask 2: Untargeted metabolic analysis of extracellular and intracellular compartments of RPE cells following EMT	6-12	95
Milestone: identification of the precise metabolic changes associated with human RPE mesenchymal conversion	8-12	95
Major Task 2: To determine the key metabolic changes associated with PVR by metabolic profiling of human vitreous samples		
Subtask 3: Collection of vitreous samples from PVR and control patients	1-18	50
Milestone: HRPO Approval	6	100
Subtask 4: Metabolomics profiling by Mass Spectrometry of human PVR samples and control patients	18-24	0
Milestone: Identification of potential metabolic biomarkers of PVR progression and severity	20-24	0
Specific Aim 2	**Timeline (months)**	**Completion (%)**
Major Task 3: To evaluate the efficiency of pharmacological metabolic regulation in preventing and treating PVR		
Subtask 5: Submission of Animal Protocol to IACUC	1-3	100
Milestone: Local IACUC approval	6	100
Milestone: ACURO approval	9	100
Subtask 6: Selection of metabolic drugs able to prevent/revert RPE mesenchymal transition in vitro	12-24	100
Subtask 7: Testing the top 3 pre-screened metabolic drugs in the rabbit model of PVR	24-36	30

- **What was accomplished under these goals?**

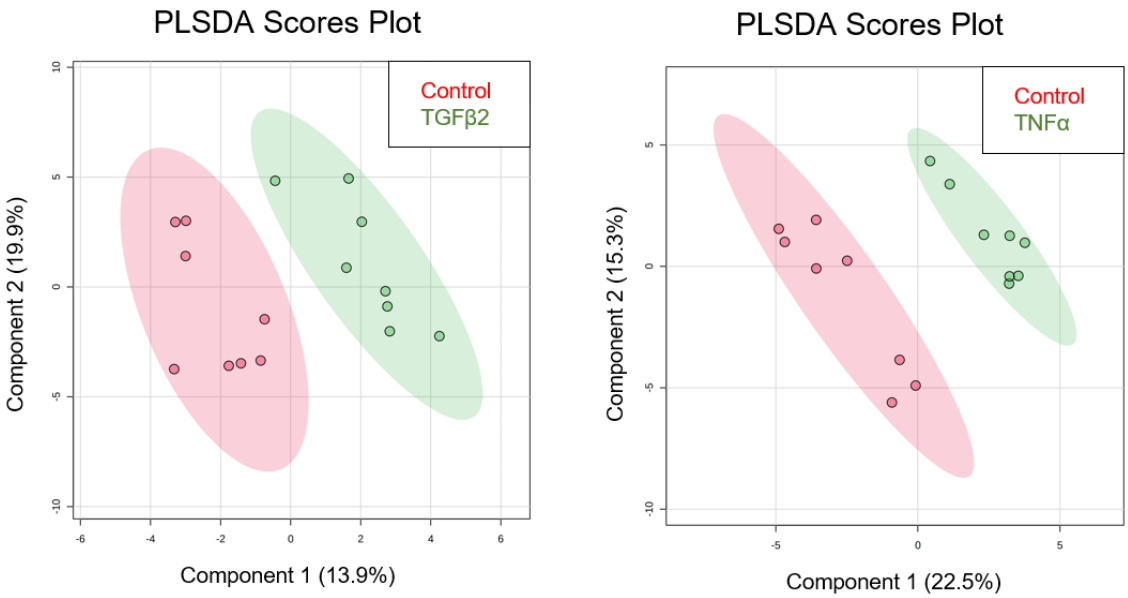
Major Activities

1. Published our characterization of the metabolic changes associated with TGFβ2- and TNFα-induced EMT in RPE and the novel ability of ZLN005 to block this EMT response
    a. Shu DY, Butcher ER, Saint-Geniez M. Suppression of PGC-1α Drives Metabolic Dysfunction in TGFβ2-Induced EMT of Retinal Pigment Epithelial Cells. International Journal of Molecular Sciences. 2021; 22(9):4701.
    b. Shu DY, Frank SI, Fitch TC, Karg MM, Butcher ER, Nnuji-John E, Kim LA and Saint-Geniez M (2022) Dimethyl Fumarate Blocks Tumor Necrosis Factor-Alpha-Driven Inflammation and Metabolic Rewiring in the Retinal Pigment Epithelium. Front. Mol. Neurosci. 15:896786. doi: 10.3389/fnmol.2022.896786
2. We attended and presented our poster at the recent Military Health System Research Symposium entitled “Metabolic reprogramming of the retinal pigment epithelium drives TGFβ2-induced epithelial-mesenchymal transition”.
3. IACUC and ACURO approval for in vivo rabbit PVR model has been obtained and we are now in the process of ordering all the reagents/consumables/animals required for our animal experiments.
4. IRB and HRPO approval for human sample collection for metabolomics study has been obtained and we have now collected a total of 43 human vitreous samples.
5. Due to validation issues with our first round of metabolomics data, we sent through another set of metabolomics samples for processing and have obtained the data and identified various metabolic pathways impacted during TGFβ2-driven EMT of RPE. Since we are now working with human primary RPE cells (H-RPE), we have also sent through another set of metabolomics samples on H-RPE – this set has been processed and we are currently analyzing the data ready for publication.
6. We will be running our in vivo rabbit PVR experiments in November 2022.

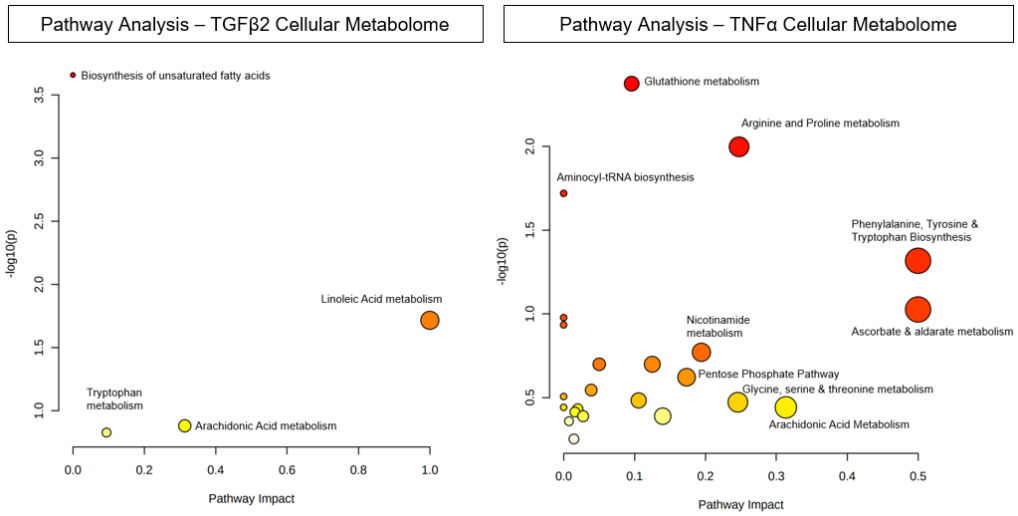
Major Task 1: Metabolomics on in vitro RPE

We have previously reported on metabolomics data on the immortalized human RPE cell line (ARPE-19) treated with TGFβ2 for 24 hours (both secreted and cellular metabolites were analyzed). We now report metabolomics data on primary human RPE cells (H-RPE, Lonza) treated with either TGFβ2 or TNFα for five days (both secreted and cellular metabolites were analyzed). Metabolomics data was analyzed using MetaboAnalyst, firstly by performing partial least-squares discriminant analysis (PLSDA) to assess separation of the clusters of samples between control and treated groups (either TGFβ2 or TNFα). Metabolites which showed a significant ($p \leq 0.05$) fold change were used to generate the enrichment pathway analysis plots.

Metabolomics profiling showed distinct metabolomics signatures associated with TGFβ2- and TNFα-treated primary RPE cells. PLSDA scores plots showed separation of control and treated groups.

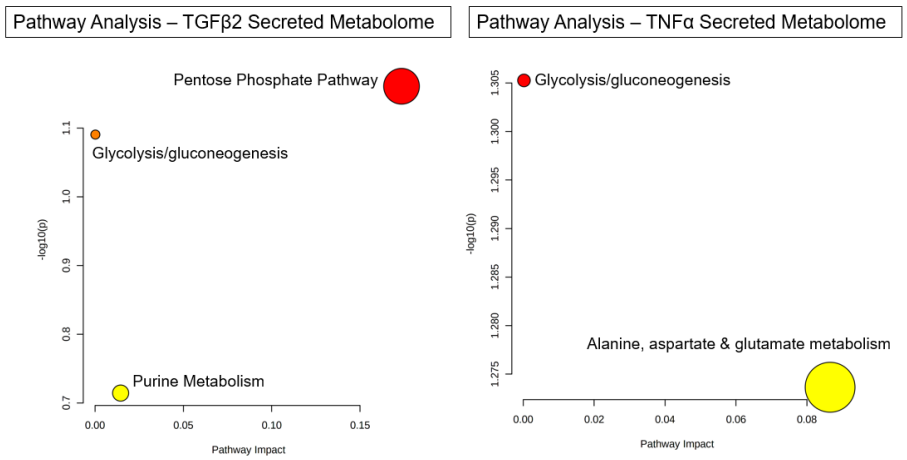


Of the 188 metabolites tested, 7 metabolites were significantly changed in TGFβ2-treated cells and 26 in TNFα-treated cells. Enrichment pathway analysis depicted altered glutathione metabolism, proline metabolism, aminoacyl-tRNA synthesis, nicotinamide metabolism, pentose phosphate pathway and arachidonic acid metabolism in TNFα-treated cells whereas TGFβ2-treated cells showed perturbations in the biosynthesis of fatty acids, tryptophan metabolism, linoleic acid metabolism and arachidonic acid metabolism.



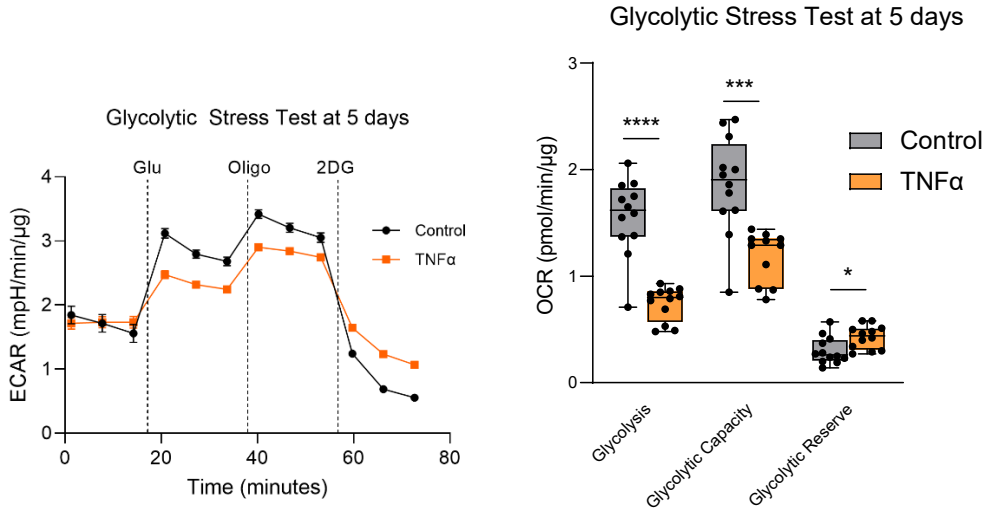
In terms of secreted metabolites, PLSDA scores plots also showed separation of control and treated groups.

Of the 188 metabolites tested, 7 metabolites were significantly changed in media from TGFβ2-treated cells and 4 in TNFα-treated cells. Enrichment pathway analysis depicted alterations in the glycolysis/gluconeogenesis pathways for media from both TGFβ2- and TNFα-treated cells compared to control. Additionally, the pentose phosphate pathway and purine metabolism were altered for media from TGFβ2-treated cells. For TNFα-treated cells, secreted metabolites in the alanine, aspartate and glutamate metabolism were altered compared to control.

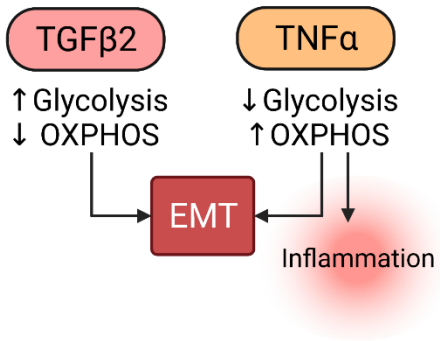


Further to our previous report showing that TNFα enhanced mitochondrial oxidative phosphorylation (OXPHOS) in both ARPE-19 and H-RPE, we now report that TNFα reduces glycolysis, glycolytic capacity and glycolytic reserve in H-RPE on the Seahorse XFe96 Glycolytic Stress Test.

Excitingly, we are now collaborating with Dr. Deeba Hussain (ophthalmologist and researcher) who is studying plasma metabolite changes in patients with retinal diseases to identify common pathways



While TGFβ2 and TNFα both have the capacity to induce EMT in RPE, the mechanistic routes to this common endpoint differ metabolically. TGFβ2 suppresses OXPHOS with compensatory enhancement of glycolysis whereas TNFα enhances OXPHOS and suppresses glycolysis. The divergent bioenergetic rewiring governing TGFβ2 and TNFα may be linked to their differential effect on inflammation. We have summarized our current understanding of how these two growth factors differ in their impact on metabolism in the schematic below.

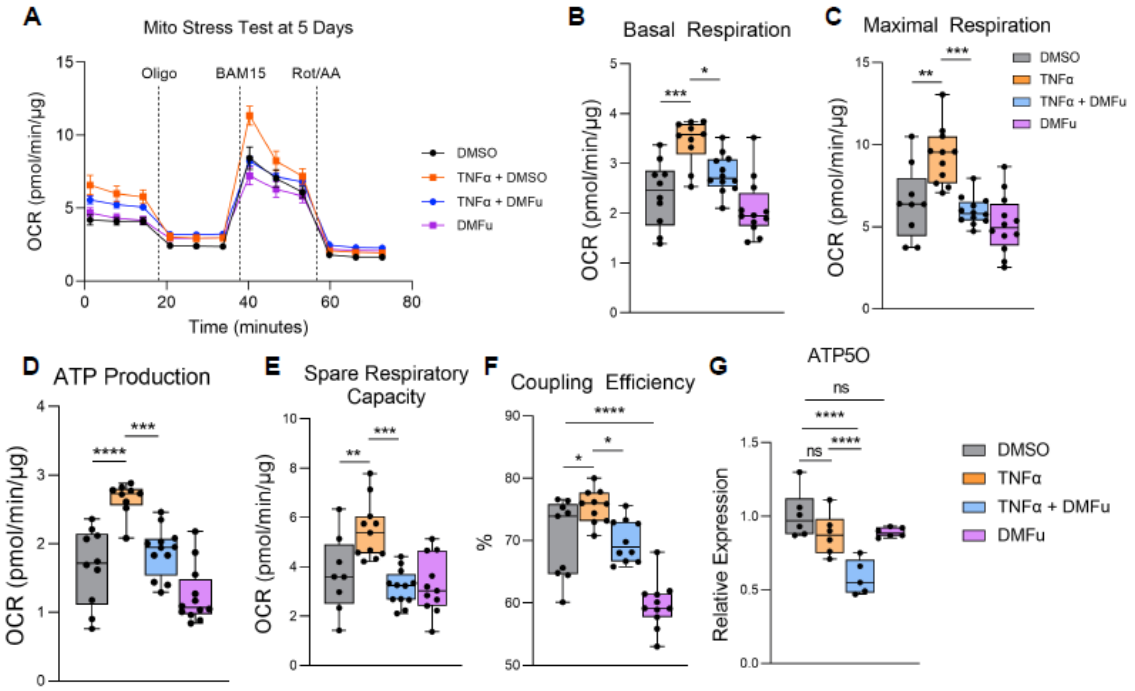


Further research is required to determine whether the enhanced OXPHOS induced by TNFα is required for exerting its inflammatory effects on RPE. Elucidating the metabolic nodes that underpin the metabolic rewiring processes driven by TGFβ2 and TNFα will enable the development of more effective drug targets for combating retinal fibrosis.

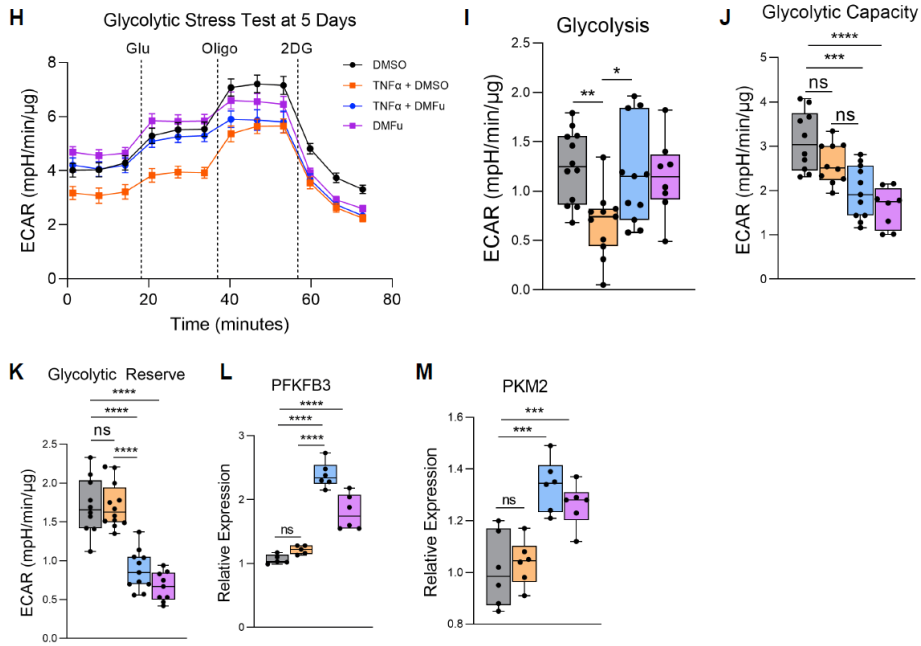
Major Task 3: Evaluation of the efficiency of pharmacological metabolic regulation in preventing and treating PVR

We have further explored the capacity of the metabolic drug, dimethyl fumarate (DMFu), in blocking TNFα-driven inflammation in primary human RPE (H-RPE). Building on from our previous report on the ability of DMFu 80 µM to block TNFα-induced upregulation of IL-6 secretion and gene expression, we found that DMFu is able to normalize the metabolic changes induced by TNFα. Specifically, we showed that DMFu blocked TNFα-induced upregulation of mitochondrial oxidative phosphorylation (OXPHOS), maintaining basal levels of oxygen consumption rate (OCR) using the Seahorse XFe96.

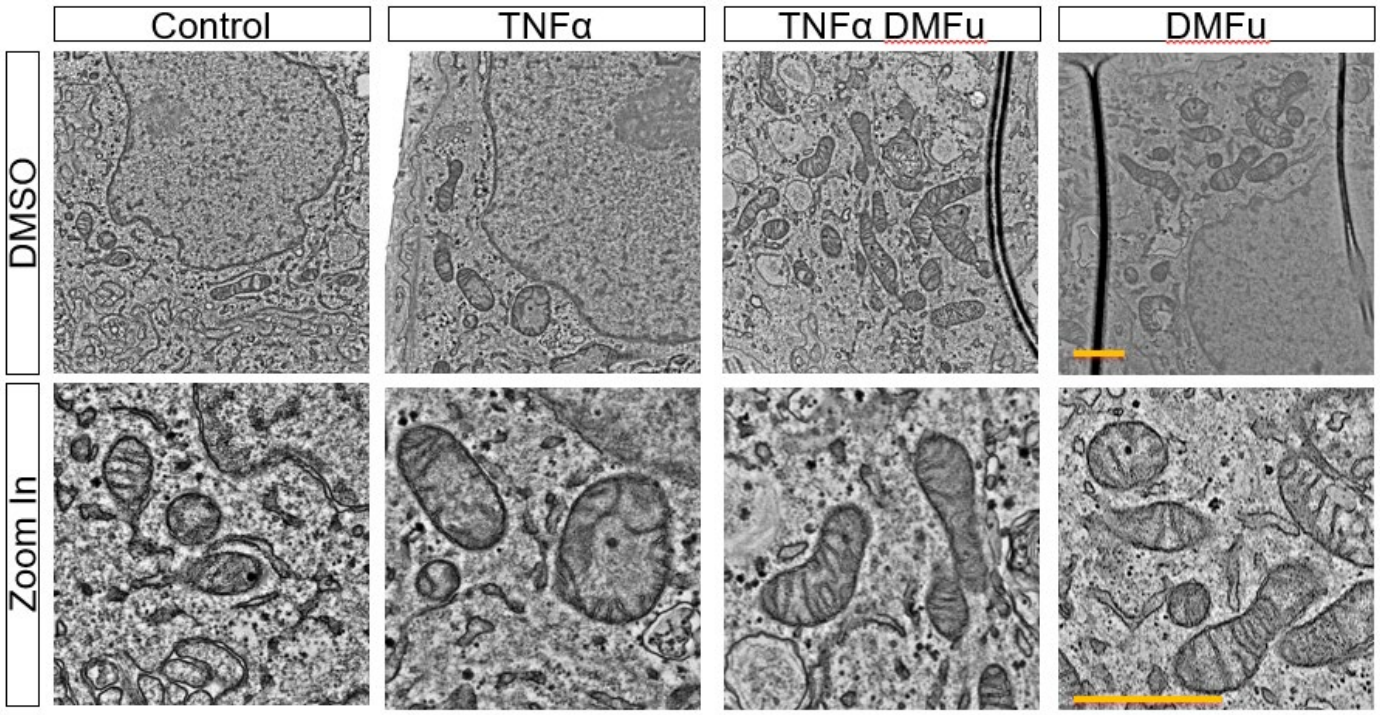
On the Seahorse Mito Stress Test, DMFu blocked the elevated mitochondrial respiration induced by TNFα (Figure 5A). Specifically, DMFu blocked TNFα-induced elevation of basal respiration (Figure 5B), maximal respiration (Figure 5C), ATP production (Figure 5D), spare respiratory capacity (Figure 5E) and coupling efficiency (Figure 5F). This blockade was accompanied by a reduction in gene expression of ATP5O, a key component of the ATP synthase in the electron transport chain (Figure 5G).



The Seahorse Glycolytic Stress Test showed that DMFu restored the suppression of glycolysis induced by TNFα (Figure 5H) with a statistically significant increase in glycolysis (Figure 5I) but no changes in glycolytic capacity (Figure 5J) and a reduction in glycolytic reserve (Figure 5K). The enhanced glycolysis with co-treatment of DMFu and TNFα is supported by the enhanced gene expression of glycolytic enzymes, namely, PFKFB3 (Figure 5L) and PKM2 (Figure 5M).



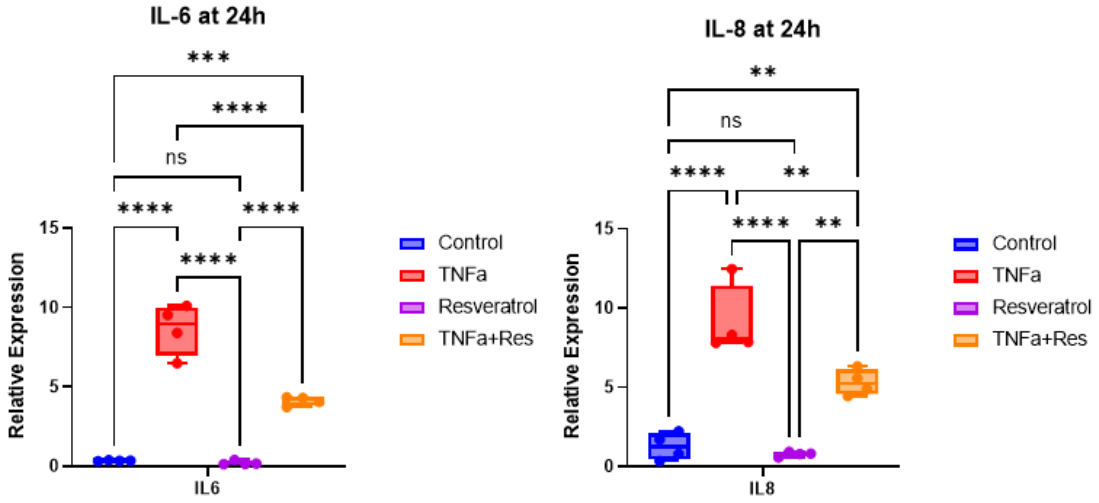
On an ultrastructural level, TEM showed that mitochondria parameters including the integrity of cristae architecture was maintained with DMFu. Scale bar is 2500 µm.



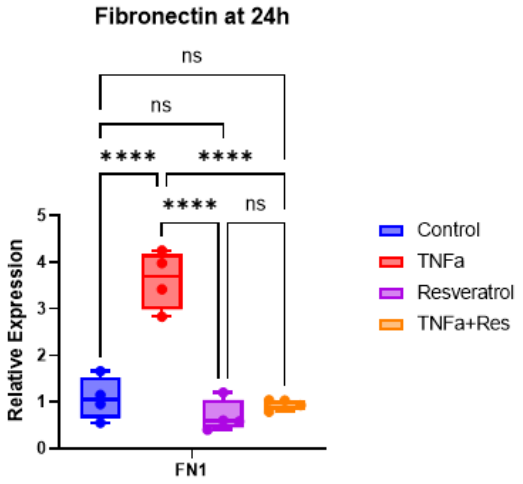
Our recent work shows that resveratrol is effective in blocked TNFα-induced EMT and inflammation in human primary retinal pigment epithelial cells (H-RPE). Resveratrol is a natural polyphenol found in red wine that has anti-aging and anti-inflammatory properties against cellular stress. Resveratrol is a sirtuin1 (SIRT1) activator and can activate mitochondrial function through SIRT1 activation. SIRT1 is critical in regulating glucose homeostasis, lipid metabolism and mitochondrial and energy homeostasis.

Matured H-RPE were serum starved for 3 days in 24-well plates and treated with TNFα (10 ng/ml) and/or resveratrol (50 μM) or the vehicle control (DMSO) for 24 hours before harvesting for qPCR analysis.

TNFα significantly increased inflammatory gene expression of IL-6 and IL-8 at 24h which was significantly blocked by concurrent resveratrol administration:



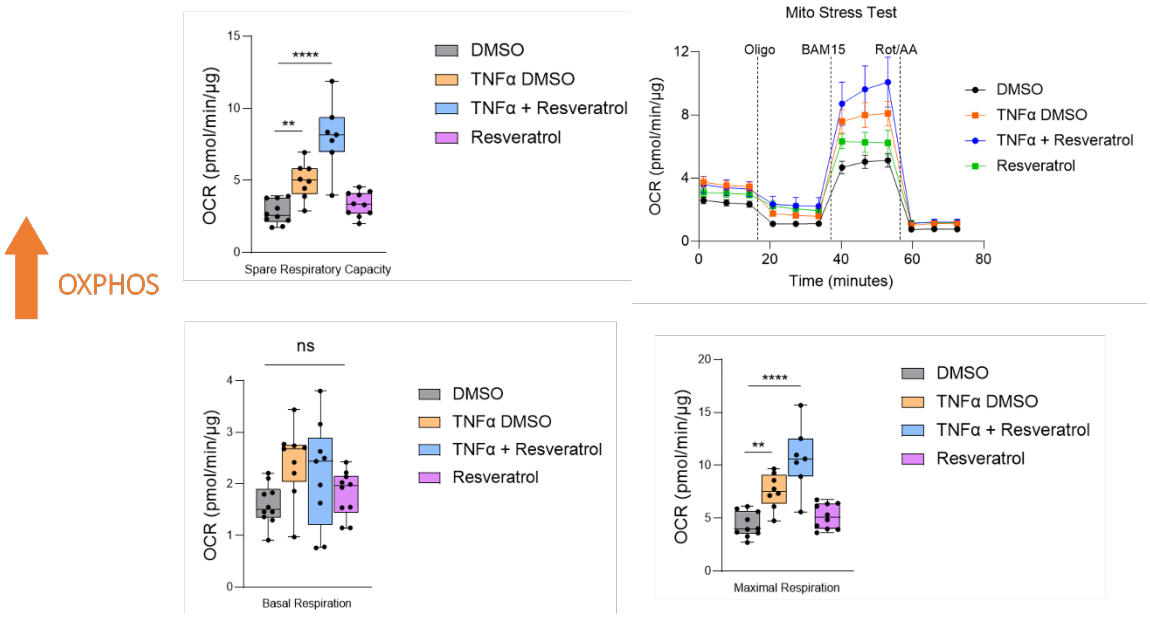
Furthermore, resveratrol significantly blocked TNFα-induced upregulation of the EMT marker, fibronectin at 24h.



Major Task 3: Evaluation of the efficiency of pharmacological metabolic regulation in preventing and treating PVR

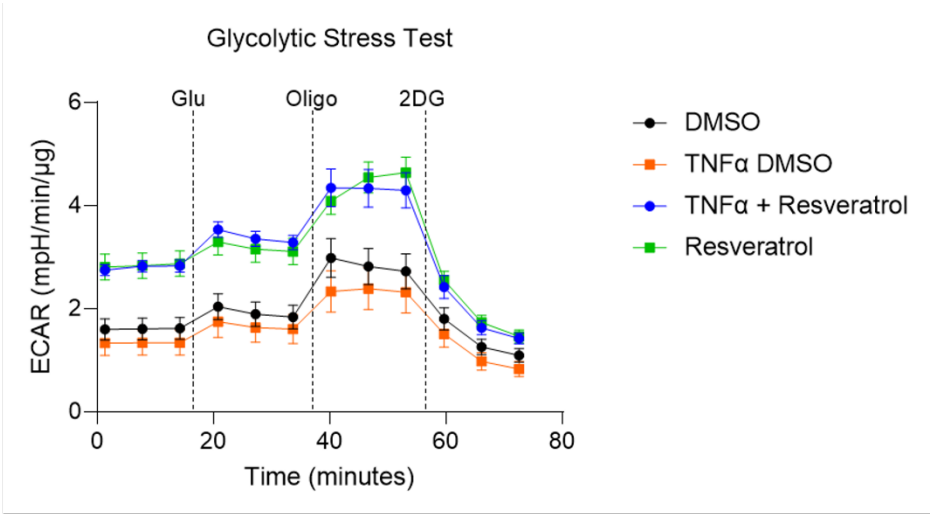
Our recent work shows that resveratrol is effective in blocked TNFα-induced EMT and inflammation in human primary retinal pigment epithelial cells (H-RPE). Resveratrol is a natural polyphenol found in red wine that has anti-aging and anti-inflammatory properties against cellular stress. Resveratrol is a sirtuin1 (SIRT1) activator and can activate mitochondrial function through SIRT1 activation. SIRT1 is critical in regulating glucose homeostasis, lipid metabolism and mitochondrial and energy homeostasis.

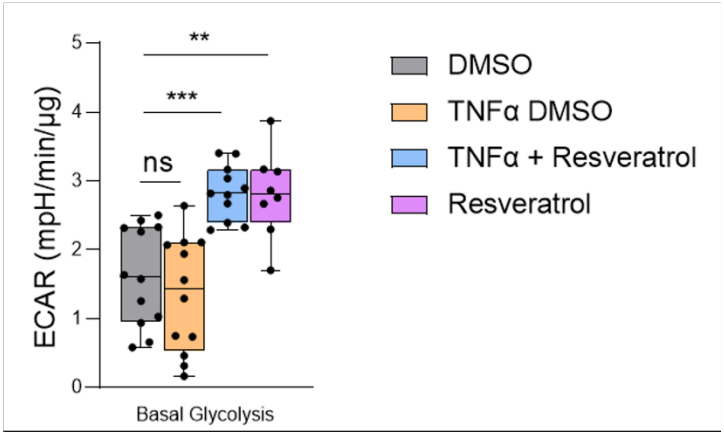
Matured H-RPE were serum starved for 3 days in 24-well plates and treated with TNFα (10 ng/ml) and/or resveratrol (50 μM) or the vehicle control (DMSO) for 24 hours before harvesting for Seahorse XFe96 analysis.



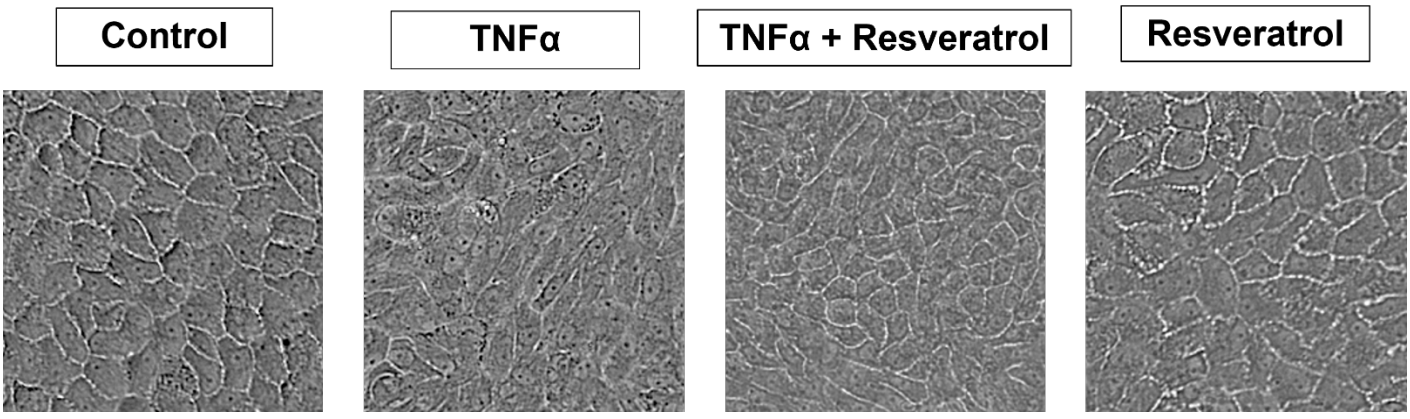
Maximal respiration and mitochondrial spare respiratory capacity (SRC) were significantly enhanced with resveratrol on the Seahorse Mito Stress Test. Co-treatment with resveratrol and TNFα further enhances oxygen consumption rate (OCR). There were no statistically significant

differences in basal respiration. Drug injections were Glucose (Glu), Oligomycin (Oligo), BAM15, Rotenone and Antimycin A (RotAA).

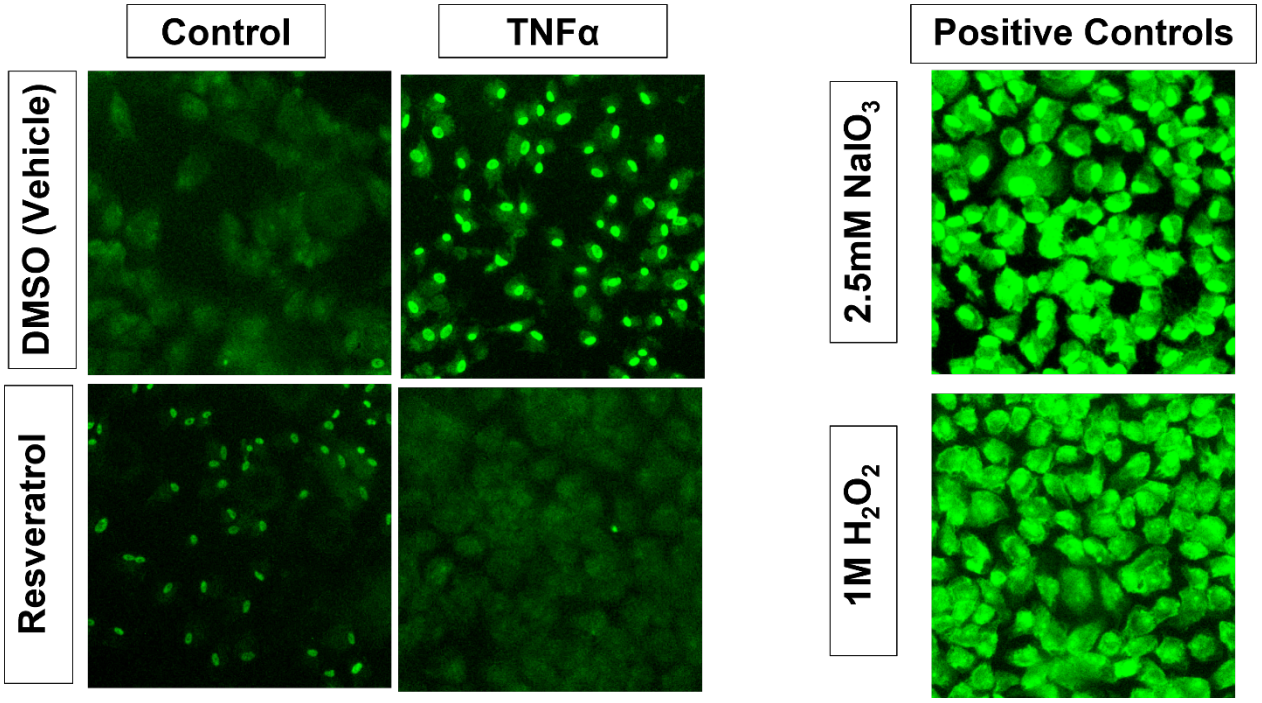




On the Seahorse Glycolytic stress test, basal glycolysis levels are significantly enhanced with resveratrol alone and cotreatment of resveratrol and TNFα. TNFα did not impact on basal glycolysis. Drug injections were Glucose (Glu), Oligomycin (Oligo), and 2-Deoxyglucose (2DG).

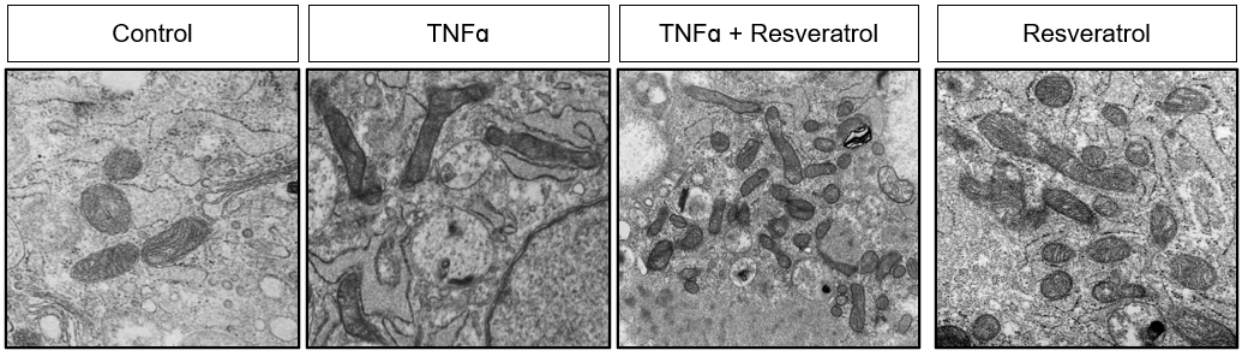


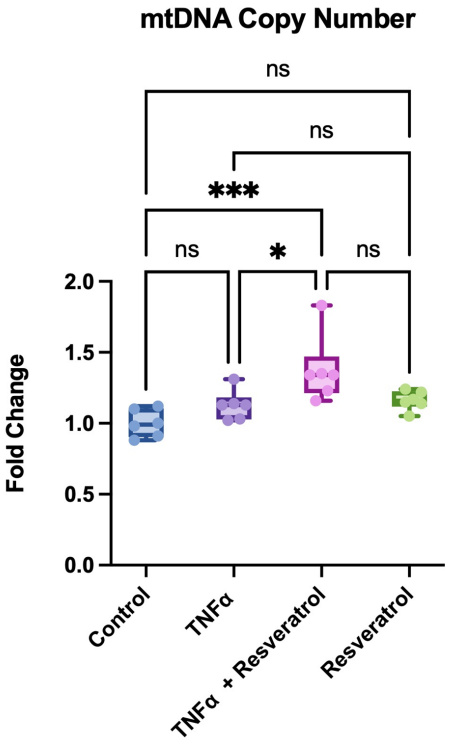
H-RPE cells treated with TNFα showed an elongated morphology with loss of the regular cuboidal cobblestone morphology. Concurrent **treatment with resveratrol rescued the epithelial morphology** with cells appearing similar to the control. Cells with resveratrol alone showed an enhanced epithelial border and maintained the regular cobblestone morphology.



TNFα enhanced the cytoplasmic reactive oxygen species (ROS) levels and **resveratrol suppressed TNFα enhanced ROS down to basal levels** in the CellROX ROS stain.

We further showed that resveratrol was associated with increased number of mitochondria on TEM imaging which was accompanied by an increase in mitochondria DNA copy number. These data suggest that resveratrol may enhance mitochondrial biogenesis, further highlighting the key link between inflammation and metabolism.





Human vitreous samples collection

Currently, we have collected a total of 43 human vitreous samples as listed in the table below. We are still actively seeking PVR samples to perform quantitative metabolomics.

	Control (e.g. ERM)	RD	PVR
1	VPVR_006	VPVR_002	VPVR_001
2	VPVR_008	VPVR_003	VPVR_009
3	VPVR_011	VPVR_004	VPVR_013
4	VPVR_014	VPVR_005	VPVR_033
5	VPVR_021	VPVR_007	
6	VPVR_022	VPVR_010	
7	VPVR_024	VPVR_012	
8	VPVR_025	VPVR_015	
9	VPVR_027	VPVR_016	
10	VPVR_028	VPVR_017	
11	VPVR_030	VPVR_018	
12	VPVR_031	VPVR_019	
13	VPVR_032	VPVR_020	
14	VPVR_038	VPVR_023	
15	VPVR_039	VPVR_026	
16	VPVR_040	VPVR_029	
17		VPVR_034	
18		VPVR_035	
19		VPVR_036	
20		VPVR_037	
21		VPVR_041	
22		VPVR_042	
23		VPVR_043	

- **What opportunities for training and professional development has the project provided?**

Training activities for both Dr. Saint-Geniez and Dr. Shu include the hands-on rabbit handling training and rabbit ocular surgery training at the Schepens Eye Research Institute Animal Facility. Dr. Shu has also participated in the Schepens Eye Research Institute Postdoctoral Training Fellowship which include workshops such as the Molecular Basis of Eye Diseases Lecture Series, Responsible Conduct of Research (RCR) Lectures and Data Analysis Workshop.

Dr. Shu has been promoted from postdoctoral research fellow to a faculty position as Instructor in the Department of Ophthalmology of Harvard Medical School.

- **How were the results disseminated to communities of interest?**

Publications, posters, and talks are listed below.

- **What do you plan to do during the next reporting period to accomplish the goals?**

Over the next year, we plan to:

1. Finalize analysis of metabolomics data and publish the findings.
2. Collect human vitreous samples from normal, PVR and retinal detachment patients for metabolomics analysis.
3. Test metabolic drugs for their efficacy in blocking EMT of RPE and designate the top 3 candidates to be selected for the in vivo rabbit PVR model

4. IMPACT**:**

- **What was the impact on the development of the principal discipline(s) of the project?**

To date, our findings reveal that proliferative vitreoretinopathy (PVR) is associated with dramatic changes to the metabolic pathways and mitochondria of retinal pigment epithelial cells. This is an exciting finding as it presents a potential novel therapeutic window for treatment of PVR by targeting specific metabolic pathways and promoting mitochondrial health.

- **What was the impact on other disciplines?**

Nothing to Report

- **What was the impact on technology transfer?**

Nothing to Report

- **What was the impact on society beyond science and technology?**

Nothing to Report

5. CHANGES/PROBLEMS**:**

- **Changes in approach and reasons for change**

Nothing to Report

Actual or anticipated problems or delays and actions or plans to resolve them

The lab shutdown due to the COVID-19 pandemic delayed our progress in performing experiments and collecting human vitreous samples. There have been delays in obtaining all the human vitreous samples for metabolomics processing and rabbit experiments due to the pandemic. We have reached out to ophthalmologists Dr. Elizabeth Rossin and Dr. Dean Eliott for more vitreous samples.

- **Changes that had a significant impact on expenditures**

Nothing to Report

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

Nothing to Report

- **Significant changes in use or care of human subjects**

Nothing to Report

- **Significant changes in use or care of vertebrate animals.**

Nothing to Report

- **Significant changes in use of biohazards and/or select agents**

Nothing to Report

6. PRODUCTS**:**

- **Publications, conference papers, and presentations**

1st year

1. Departmental Presentation by postdoctoral fellow Daisy Shu presented on November 4th 2019 titled "TGFβ and the retinal pigment epithelium: integrating metabolic reprogramming with epithelial-mesenchymal transition (EMT)"
2. Conference abstract accepted for a talk at the ARVO annual meeting in Baltimore 2020 titled "Metabolic alterations during TGFβ2-induced EMT in retinal pigment epithelial (RPE) cells". Note that ARVO has been cancelled due to public health concerns regarding COVID-19 pandemic.
3. Review article accepted for publication: Shu DY, Butcher E, Saint-Geniez M. EMT and EndMT: Emerging Roles in Age-Related Macular Degeneration. International Journal of Molecular Sciences. 2020; 21(12), 4271.

2nd year

Peer Reviewed Publications:

1. Shu DY, Butcher ER, Saint-Geniez M. Suppression of PGC-1α Drives Metabolic Dysfunction in TGFβ2-Induced EMT of Retinal Pigment Epithelial Cells. International Journal of Molecular Sciences. 2021; 22(9):4701.

Talks (virtually presented)

1. Shu DY, Butcher E, Cai S, Senthilkumar I, Frank S, Gollapalli D, Saint-Geniez M. Suppression of PGC-1α drives metabolic dysfunction in TGFβ2-induced EMT of retinal pigment epithelial cells. [Abstract]. AOPT XV Meeting on March 6th, 2021, in the session on "Novel therapeutic targeting for age-related macular degeneration – overcoming the challenging path to success" moderated by Goldis Malek and Aprana Lakkarju, Virtually

presented and Daisy Shu was awarded the BrightFocus Foundation Honorable Mention Paper Award at the XV AOPT meeting online.

2. Dr. Saint-Geniez presented “Targeting metabolic dysfunction for the treatment of retinal degeneration and fibrosis” at the New England Eye Center Grand Rounds, Boston, MA. May 20th, 2021
3. Shu DY, Saint-Geniez M. Suppression of PGC-1α drives metabolic dysfunction in TGFβ2-induced EMT of retinal pigment epithelial cells. [Abstract for Departmental Seminar]. Schepens Eye Research Institute Trainees’ Work in Progress Seminar, April 2021, Virtual
4. Shu DY, Butcher E, Cai S, Senthilkumar I, Frank S, Gollapalli D, Saint-Geniez M. Differential effects of TNFα on mitochondrial function and metabolic activity in the retinal pigment epithelium. [Abstract] ARVO Annual Meeting, May 5th, 2021, Virtual
5. Shu DY, Saint-Geniez M. Integrating metabolic reprogramming and epithelial-mesenchymal transition (EMT): insights from the retina. [Abstract]. ASIP Young Investigator Keynote Seminar Series, June 16th, 2021, 12pm EST, Virtual
6. Shu DY, Saint-Geniez M. EMT, mitochondria and metabolic reprogramming: insights from the retinal pigment epithelium. [Abstract]. AOIP Young Investigator Seminar Series, September 10th, 2021, 3pm EST, Virtual. Followed by sharing career development tips in the AOIP Trainee Roundtable.
7. Shu DY, Butcher ER , Nnuji-John E, Frank S, Shah R, Cai S, Gollapalli D, Saint-Geniez M. Interplay Between TNFα-Induced Inflammation and Metabolic Dysfunction in Retinal Pigment Epithelial Cells [Abstract]. PISA 2021 (Pathobiology for Investigators, Students, and Academicians Young Investigators Virtual Meeting by the American Society for Investigative Pathology) on October 6th, 2021, 4:45-5pm, Virtual

Posters (virtually presented)

1. Virtual Conference poster presentation at the American Society for Investigative Pathology PISA Meeting 9-13 November 2020 entitled “Metabolic Rewiring and Mitochondrial Dysfunction in Transforming Growth Factor-Beta 2-Induced Retinal Epithelial-Mesenchymal Transition”.
2. Shu DY, Butcher E, Cai S, Senthilkumar I, Frank S, Saint-Geniez M. [Abstract]. Metabolic Rewiring and Mitochondrial Dysfunction in Transforming Growth Factor-Beta 2-Induced Retinal Epithelial-Mesenchymal Transition. MGH Scientific Advisory Committee (SAC) 2021 Virtual Poster Session on April 7th, 2021
3. Shu DY, Butcher E, Cai S, Senthilkumar I, Frank S, Saint-Geniez M. [Abstract]. Metabolic Reprogramming of the Retinal Pigment Epithelium Drives TGFβ2-Induced Epithelial-Mesenchymal Transition. Experimental Biology 2021 Virtual Poster Session April 27-30th 2021
4. Shu DY, Butcher E, Cai S, Senthilkumar I, Frank S, Saint-Geniez M. [Abstract]. Metabolic Reprogramming of the Retinal Pigment Epithelium Drives TGFβ2-Induced Epithelial-Mesenchymal Transition. Experimental Biology 2021 Virtual Poster Session April 27-30th 2021
5. Shu D, Butcher E, Cai S, Frank S, Nnuji-John E, Gollapalli D, Saint-Geniez M. [Abstract]. Role of metabolic dysfunction in TNFαinduced inflammation in retinal pigment epithelial cells. XIX International Symposium on Retinal Degeneration, RD2021 Hybrid Format, Attended virtually. Sept. 27 – Oct. 2, 2021, Online and in person at the Sonesta Nashville Airport Hotel, Nashville, TN

3rd year

Peer reviewed publications

1. Shu DY, Frank SI, Fitch TC, Karg MM, Butcher ER, Nnuji-John E, Kim LA and Saint-Geniez M (2022) Dimethyl Fumarate Blocks Tumor Necrosis Factor-Alpha-Driven Inflammation and Metabolic Rewiring in the Retinal Pigment Epithelium. Front. Mol. Neurosci. 15:896786. doi: 10.3389/fnmol.2022.896786

Talks

1. **Shu DY**, Fitch TC, Frank SI, Butcher E, Karg MM, Cai S, Shah R, Gollapalli D, Saint-Geniez M. Dimethyl fumarate blocks TNFα-driven inflammation and metabolic rewiring in

retinal pigment epithelial cells [Abstract]. ARVO 2022 in Retinal Metabolism Session on May 1st, 2022, in Denver, Colorado.

2. Butcher ER, Frank SI , Saint-Geniez M, Kim LA , **Shu DY**. Mitochondrial Transplantation as a Novel Inducer of Retinal Pigment Epithelium Epithelial-Mesenchymal-Transition and Fibrosis, PISA2022, Wednesday, September 28, 2022, Neuropathology and Ocular Pathology Session.
3. Fitch TC, Frank SI , Saint-Geniez M, Kim LA , **Shu DY**. Protective Role of Resveratrol Against TNF-induced Inflammation and Mitochondrial Dysfunction in Retinal Pigment Epithelial Cells, PISA2022, Wednesday, September 28, 2022, Neuropathology and Ocular Pathology Session.

Posters

1. Shu DY, Frank SI, Karg MM, Butcher ER, Nnuji-John E, Shah R, Gollapalli D, Saint-Geniez M. [Abstract]. Contrasting Metabolic Profiles of TGFβ2 and TNFα in the Induction of Retinal Epithelial-Mesenchymal Transition (EMT) at Experimental Biology (Philadelphia, PA) on Saturday April 2nd ASIP Highlight Trailblazer Session (of 20 selected top posters from Trainees) and Sunday April 3rd, 2022, in the Ocular Pathobiology Session.
2. Fitch TC, Frank S, Kim LA, Shu DY. Resveratrol protects RPE against TNFα-induced inflammation in Age related Macular Degeneration. Harvard Ophthalmology Annual Meeting and Alumni Reunion, June 24, 2022, Boston, MA.
3. Shu DY, FitchTC , Frank SI, Karg MM , Butcher ER, Nnuji-John E, Kim LA , Saint-Geniez M. Metabolic Targeting of RPE Mesenchymal-transition for the Treatment of Proliferative Vitreoretinopathy. September 13, 2022, Military Health System Research Symposium (MHSRS), Gaylord Palms Resort & Convention Center, Kissimmee, Florida.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**:**
    - **What individuals have worked on the project?**

Name:	*Dr. Leo Kim*
Project Role:	*Collaborator*
Researcher Identifier (e.g. ORCID ID):	*0000-0001-9106-6416*
Nearest person month worked:	*4*
Contribution to Project:	*Dr. Kim is now the PI on this grant*
Funding Support:	*Salary is funded by this DoD grant*

Name:	*Dr. Daisy Shu*
Project Role:	*Postdoctoral Research Fellow*
Researcher Identifier (e.g. ORCID ID):	*https://orcid.org/0000-0002-5382-6450*
Nearest person month worked:	*12*
Contribution to Project:	*Dr. Shu has performed the experiments and data analysis and helped complete the forms for local IACUC and HRPO submission.*
Funding Support:	*Salary is funded by this DoD grant*

Name:	*Dr. Magali Saint-Geniez*
Project Role:	*Principal Investigator*
Researcher Identifier (e.g. ORCID ID):	*https://orcid.org/0000-0001-9897-138X*
Nearest person month worked:	*1*
Contribution to Project:	*Dr. Saint-Geniez led the conceptualization of the experiments, assisted with data curation, data analysis, project administration and helped complete and submit the forms for local IACUC and HRPO submission.* *She has now moved on to a new position in March 2022.*
Funding Support:	*NA*

Name:	*Dr. Deviprasad Gollapalli*
Project Role:	*Research technician*
Researcher Identifier (e.g. ORCID ID):	*NA*
Nearest person month worked:	*1*
Contribution to Project:	*Dr. Deviprasad Gollapalli provided technical support specific to this project by maintaining cell cultures, assisting the fellow with in vivo and in vitro experimentation, masked data collection and analysis, collecting and storing vitreous samples, coordinating reagent orders and storage, and performing primary human cells genotyping.* *He has now moved on to a new position in March 2022.*
Funding Support:	*NA*

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**

Dr. Leo Kim is now the PI on this grant, taking over the role from the previous PI, Dr. Magali Saint-Geniez, who has moved on to another position. Furthermore, Dr. Deviprasad Gollapalli (the former lab manager) has also moved to another position. With Dr. Leo Kim's strong expertise in PVR and retinal wound healing, on both clinical and research fronts, this has been a smooth transition. Dr. Daisy Shu, the postdoctoral research fellow, is still maintaining her role on the grant and will take over Dr. Deviprasad Gollapalli's lab managerial roles.

- **What other organizations were involved as partners?**

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS: N/A
9. APPENDICES:

MHSRS 2022

Title: Metabolic Targeting of RPE Mesenchymal-transition for the Treatment of Proliferative

Vitreoretinopathy

Proliferative vitreoretinopathy (PVR), a major cause of irreversible blindness, occurs in ≈50% of patients with penetrating ocular injuries or traumatic retinal detachment. PVR is caused by the epithelial-mesenchymal-transition (EMT) of retinal pigment epithelial (RPE) cells leading to the formation of a fibrotic membranes on the retina. Contraction of these membranes cause recurrent retinal detachment and tears resulting in dramatic vision loss. Emerging evidence implicates metabolic and mitochondrial dysfunction as key drivers of EMT. Both transforming growth factor-beta 2 (TGFβ2) and tumor necrosis factor-alpha (TNFα) induce EMT in RPE; however, little is known about their impact on RPE metabolism. We hypothesized that TGFβ2- and TNFα-induced EMT of RPE is driven by specific metabolic programs that can be targeted as a novel strategy for the treatment of PVR. Matured human primary RPE (H-RPE) were treated with TGFβ2 or TNFα (both at 10 ng/ml) for up to 5 days for Seahorse metabolic assays (Mito and Glycolytic Stress Tests), qPCR and untargeted metabolomics analysis (West Coast Metabolomics Center) analyzed using MetaboAnalyst. Both TGFβ2 and TNFα induced the upregulation of mesenchymal genes (fibronectin and matrix metalloproteinase-2) in H-RPE. While TNFα significantly upregulated inflammatory genes (IL-6, IL-8, IL-1β, MCP-1), no pro-inflammatory changes were evident in TGFβ2-treated cells. Metabolically, TGFβ2 induced the “Warburg effect” in H-RPE with enhanced glycolysis and a suppression of oxidative phosphorylation (OXPHOS) capacity. Intriguingly, TNFα triggers the opposite effect with enhanced oxidative phosphorylation (higher basal levels of oxygen consumption and maximal respiration) and reduced glycolysis and glycolytic capacity. Metabolomic profiling revealed distinct metabolomics signatures of TGFβ2- and TNFα-treated primary RPE cells. Of the 188 metabolites measured, 7 were significantly changed in TGFβ2-treated cells and 26 in TNFα-treated cells. Enrichment analysis depicted altered glutathione metabolism, proline metabolism, aminoacyl-tRNA synthesis, nicotinamide metabolism, pentose phosphate pathway and arachidonic acid metabolism in TNFα-treated cells whereas TGFβ2-treated cells showed perturbations in the biosynthesis of fatty acids, tryptophan metabolism, linoleic acid metabolism and arachidonic acid metabolism. The divergent bioenergetic rewiring governing TGFβ2 and TNFα in their induction of EMT may be linked to their differential effect on inflammation. Elucidating the contributions of TGFβ2 and TNFα and their mechanistic differences has enabled the discovery of effective drug targets for treating retinal fibrosis. For example, since TGFβ2 suppresses PGC-1α gene expression, a master regulator of mitochondrial biogenesis and respiration, we found that ZLN005, a small molecule activator of PGC-1α was effective in blocking TGFβ2-induced EMT in RPE through enhancement of mitochondrial OXPHOS. Furthermore, we found that the fumaric acid ester, dimethyl fumarate, effectively blocked TNFα-driven inflammation in RPE by suppressing TNFα-induced enhancement of OXPHOS and restoration of glycolysis. Our future research is dedicated to translating our findings in vivo by testing these novel metabolic drugs in the preclinical rabbit model of PVR.

American Society for Investigative Pathology (ASIP) PISA2022, Wednesday, September 28, 2022, Neuropathology and Ocular Pathology Session

Title: Protective role of resveratrol against TNFα-induced inflammation and mitochondrial dysfunction in retinal pigment epithelial cells

Introduction: Inflammation and oxidative stress are key drivers of retinal pigment epithelium (RPE) dysfunction in the pathogenesis of age-related macular degeneration (AMD), a leading cause of irreversible blindness globally. Tumor necrosis factor-alpha (TNFα), a pro-inflammatory cytokine involved in AMD, induces defects in mitochondrial health and function in the RPE. Sirtuins, a family of enzymes involved in maintaining metabolic homeostasis, mitochondrial function, and longevity, are downregulated in aged RPE cells and AMD retinal specimens. Here we investigated

the efficacy of resveratrol, a potent activator of sirtuin1 (SIRT1), in suppressing TNFα-induced inflammation, metabolic dysfunction and oxidative stress in RPE.
Methods: Matured primary human RPE (H-RPE) cells were treated with TNFα (10 ng/ml) and/or resveratrol (50 μM) or DMSO at equal volume as the vehicle control. Oxidative phosphorylation (OXPHOS) and glycolytic metabolic profiles were determined by the Seahorse Xfe96 Mito Stress Test and Glycolytic Stress Test, respectively. Gene expression of metabolic and inflammatory markers was assessed using qPCR. Interleukin-6 (IL-6) secretion was quantified by enzyme-linked immunosorbent assay (ELISA). Intracellular levels of reactive oxygen species (ROS) were measured using CellROX green reagent.
Results: TNFα induced elongation and loss of the regular cuboidal cobblestone morphology of H-RPE cells. Concurrent treatment with resveratrol blocked TNFα-induced H-RPE morphological changes. TNFα robustly upregulated IL-6 levels in H-RPE conditioned media with a 19-fold increase while concurrent treatment with resveratrol significantly suppressed TNFα-induced IL-6 secretion by almost 50% to a 10-fold decrease. Moreover, resveratrol suppressed TNFα-induced transcriptional upregulation of proinflammatory genes (IL-6, IL-8, TLR2, and MCP-1) and metabolic genes (ENO1, PFKFB3, HK2). Bioenergetic profiling using the Seahorse Xfe96 showed enhanced maximal mitochondrial respiration, spare respiratory capacity, and basal glycolysis levels in H-RPE treated with resveratrol. While co-treatment with resveratrol and TNFα further increased oxygen consumption rate, resveratrol suppressed TNFα-dependent accumulation induction of the ROS-producing NADPH oxidase NOX4. The protective effect of resveratrol on TNFα was further validated by evidenced reduction of TNFα-dependent accumulation of cytoplasmic ROS measured with the CELLROX assay.
Conclusions: RPE cells are profoundly affected by the pro-inflammatory cytokine TNFα. TNFα not only disrupts the structural epithelial morphology of H-RPE cells, but it also causes dysfunction of mitochondria and metabolism. Treatment with the natural organic compound, resveratrol, efficiently blocks TNFα-induced proinflammatory activation and bioenergetic reprogramming of RPE. These results reveal a critical interplay between inflammation and metabolic dysfunction in RPE, identifying resveratrol as a potential drug against AMD progression.

International Society for Eye Research (ISER2023) Meeting in Gold Coast, Australia for February 19-23, 2023

Title: Role of mitochondria and cellular metabolism during epithelial-mesenchymal transition of RPE

Epithelial-mesenchymal-transition (EMT) is a process whereby epithelial cells transdifferentiate into matrix-producing mesenchymal cells. EMT of retinal pigment epithelial cells (RPE) plays a crucial role in severe ocular pathologies including proliferative vitreoretinopathy and subretinal fibrosis in age-related macular degeneration. Emerging evidence implicates metabolic and mitochondrial dysfunction as key drivers of EMT. Both transforming growth factor-beta 2 (TGFβ2) and tumor necrosis factor-alpha (TNFα) have the capacity to induce EMT in RPE; however, little is known about their impact on mitochondrial metabolism. Matured human primary RPE (H-RPE) were treated with TGFβ2 or TNFα (both at 10 ng/ml) for up to 5 days for Seahorse metabolic assays (Mito and Glycolytic Stress Tests), qPCR and untargeted metabolomics analysis (West Coast Metabolomics Center) analyzed using MetaboAnalyst. Both TGFβ2 and TNFα induced the upregulation of mesenchymal genes (fibronectin and matrix metalloproteinase-2) in H-RPE. While TNFα significantly upregulated inflammatory genes (IL-6, IL-8, IL-1β, MCP-1), no changes were evident in TGFβ2-treated cells. Metabolically, TGFβ2 induced the “Warburg effect” in H-RPE with enhanced glycolysis and a suppression of oxidative phosphorylation capacity. Surprisingly, TNFα showed the opposite effect with enhanced oxidative phosphorylation (higher basal levels of oxygen consumption and maximal respiration) and reduced glycolysis and glycolytic capacity. Metabolomics profiling showed distinct metabolomics signatures associated with TGFβ2- and TNFα-treated primary RPE cells. Of the 188 metabolites tested, 7 metabolites were significantly changed in TGFβ2-treated cells and 26 in TNFα-treated cells. Enrichment analysis depicted altered glutathione metabolism, proline metabolism, aminoacyl-tRNA synthesis, nicotinamide metabolism, pentose phosphate pathway and arachidonic acid metabolism in TNFα-treated cells whereas TGFβ2-treated cells showed perturbations in the biosynthesis of fatty acids, tryptophan metabolism, linoleic acid metabolism and arachidonic acid metabolism. The divergent bioenergetic rewiring governing TGFβ2 and TNFα in their induction of EMT may be linked to their differential effect on inflammation. Elucidating the contributions of TGFβ2 and TNFα and their

mechanistic differences will enable the development of more effective drug targets for combating retinal fibrosis.

Review Article (attached at the end of the document)
Shu DY, Butcher E, Saint-Geniez M. EMT and EndMT: Emerging Roles in Age-Related Macular Degeneration. International Journal of Molecular Sciences. 2020; 21(12), 4271.

Original Research Papers (attached at the end of the document)
Shu DY, Butcher ER, Saint-Geniez M. Suppression of PGC-1α Drives Metabolic Dysfunction in TGFβ2-Induced EMT of Retinal Pigment Epithelial Cells. International Journal of Molecular Sciences. 2021; 22(9):4701.

Shu DY, Frank SI, Fitch TC, Karg MM, Butcher ER, Nnuji-John E, Kim LA and Saint-Geniez M (2022) Dimethyl Fumarate Blocks Tumor Necrosis Factor-Alpha-Driven Inflammation and Metabolic Rewiring in the Retinal Pigment Epithelium. Front. Mol. Neurosci. 15:896786. doi: 10.3389/fnmol.2022.896786