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Evidence of impaired mitochondrial cellular bioenergetics in ocular fibroblasts derived from glaucoma patients

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Highlights:

- Mitochondrial bioenergetics evaluated using Seahorse Bioscience Analyser were altered in Tenon's ocular fibroblast cells from glaucoma patients compared to non-glaucomatous control patients.
- Impaired mitochondrial cellular bioenergetics was detected in glaucomatous ocular fibroblasts including basal respiration, maximal respiration and spare capacity.
- Basal oxidative stress was elevated in glaucomatous Tenon's ocular fibroblasts and hydrogen peroxide (H_2O_2) induced reactive oxygen species (ROS) simulated the glaucomatous condition in normal Tenon's ocular fibroblasts.
- Mitochondrial dysfunction observed in glaucomatous patients within this study provides further evidence for the potential of neuroprotective bioenergetic based therapies for this irreversible cause of blindness.

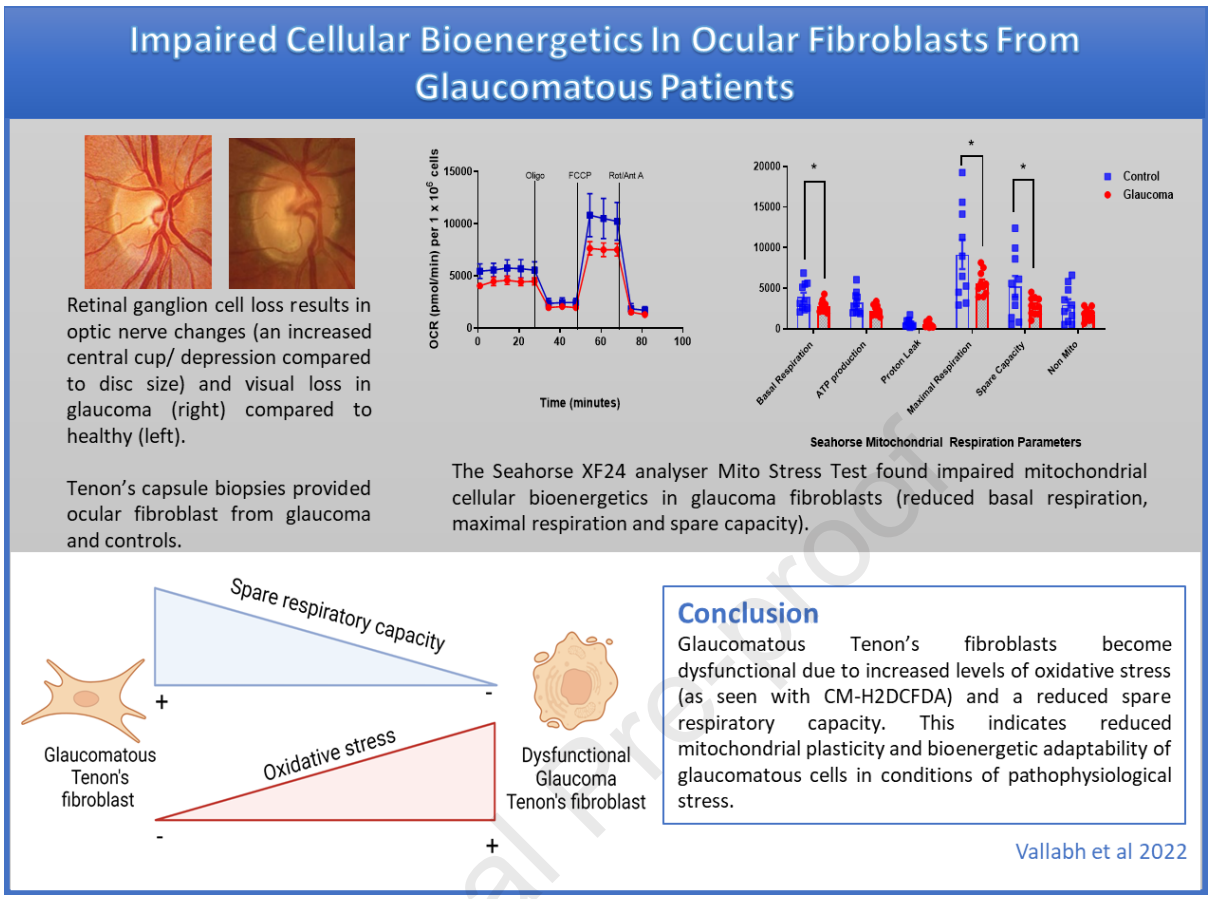
Abstract:

Glaucoma is a progressive optic neuropathy characterized by the neurodegeneration of the retinal ganglion cells (RGCs) resulting in irreversible visual impairment and eventual blindness. RGCs are extremely susceptible to mitochondrial compromise due to their marked bioenergetic requirements and morphology. There is increasing interest in therapies targeting mitochondrial health as a method of preventing visual loss in managing glaucoma. The bioenergetic profile of Tenon's ocular fibroblasts from glaucoma patients and controls was investigated using the Seahorse XF24 analyser. Impaired mitochondrial cellular bioenergetics was detected in glaucomatous ocular fibroblasts including basal respiration, maximal respiration and spare capacity. Spare respiratory capacity levels reflect mitochondrial bio-energetic adaptability in response to pathophysiological stress. Basal oxidative stress was elevated in glaucomatous Tenon's ocular fibroblasts and hydrogen peroxide (H_2O_2) induced reactive oxygen species (ROS) simulated the glaucomatous condition in normal Tenon's ocular fibroblasts. This work supports the role of therapeutic interventions to target oxidative stress or provide mitochondrial energetic support in glaucoma.

Keywords: Tenon's fibroblast, glaucoma, mitochondria, Seahorse XF analyser, oxidative stress, bioenergetics

Abbreviations: RGCs: Retinal ganglion cells, POAG: primary open angle glaucoma, IOP: intraocular pressure, LHON: Leber's Hereditary Optic Neuropathy, ADOA: Autosomal Dominant Optic Atrophy, H_2O_2: hydrogen peroxide, ROS: reactive oxygen species, GSS2: glaucoma staging system 2, TFs: Tenon's fibroblasts, GTFs: Glaucomatous Tenon's fibroblasts, NTFs: non-glaucomatous controls, DMEM: Dulbecco's Modified Eagle's Medium, PBS: phosphate buffered saline, SEM: standard error of the mean, OCR: oxygen consumption rate, DMSO: Dimethyl sulfoxide, mtDNA: mitochondrial DNA

Graphical abstract



Introduction

Glaucoma is a progressive optic neuropathy characterized by the neurodegeneration of the retinal ganglion cells (RGCs) resulting in irreversible visual impairment and eventual blindness [1]. In glaucoma, damage and degeneration of RGCs and their axons result in characteristic changes in the appearance of the optic nerve head and patterns of visual field loss [2]. Glaucoma is the leading cause of irreversible blindness worldwide and is estimated to affect over 60 million people globally of which approximately 10% are estimated to be blind from this disease [3]. Glaucoma is an umbrella term for a heterogenous group of optic neuropathies of which primary open angle glaucoma (POAG) is the most prevalent [4]. The pathogenesis of POAG is multifactorial and complex [5,6] but currently lowering intra-ocular pressure (IOP) medically or surgically is the only modifiable risk factor [7]. POAG can be clinically sub-divided into patients with normal IOP, termed normal-tension glaucoma, and those with raised IOP, termed high-tension glaucoma [2,4]. Given that POAG can develop with a normal IOP, and even when IOP is adequately treated and controlled POAG patients can still progress to blindness [8–10], supports the concept that other non-IOP mechanisms can drive glaucoma development and progression.

Increased chronological age is an independent risk factor for glaucoma [11–14] and mitochondrial dysfunction is associated with age-related neurodegenerations [15]. RGCs are particularly susceptible to mitochondria dysfunction due to their high energy demands and unique morphology [16–18]. In the human glaucomatous retina, the RGC dendrites show early degeneration with remodelling and redistribution of the mitochondria, and a reduction in mitochondrial volume [19]. This mirrors glaucomatous degeneration in animal models in which RGCs are under metabolic stress [20,21]. Inherited optic neuropathies like Leber's Hereditary Optic Neuropathy (LHON) and Autosomal Dominant Optic Atrophy (ADOA) result from mitochondrial mutations or nuclear gene mutations encoding mitochondrial proteins [22]. Vision is lost in both LHON and DOA due to RGC death secondary to mitochondrial

dysfunction [22,23]. Due to the phenotypic similarities of these inherited optic neuropathies with glaucomatous optic neuropathy there has been increasing investigation of mitochondrial involvement in the pathogenesis of glaucoma [16,24–28]. Our group and others have reported mitochondrial DNA mutations in peripheral blood leucocytes from POAG patients [29–31]. Furthermore, defects in complex I oxidative phosphorylation and subsequent decreased mitochondrial respiration and ATP production have been detected in blood lymphocytes from POAG patients [23,32].

Cells derived from ocular tissues better represent the glaucomatous disease context and can be derived during ocular surgery [33,34] or from post-mortem studies [19,25]. Post-mortem studies are limited, expensive and challenging to obtain clinical data but have identified mitochondrial defects in the glaucomatous retina [19] and lamina cribrosa cells [25]. Mitochondrial dysfunction and autophagy have also been studied in glaucoma using Tenon's ocular fibroblasts [35]. Cataract surgery is an ocular procedure commonly performed in patients with and without glaucoma and allows the relatively simple harvesting of Tenon's ocular fibroblasts [36].

Herein, we report impaired mitochondrial cellular bioenergetics in Tenon's ocular fibroblasts derived from glaucoma (POAG) patients. Basal oxidative stress was elevated in glaucomatous Tenon's ocular fibroblasts and hydrogen peroxide (H_2O_2) induced reactive oxygen species (ROS) simulated the glaucomatous condition in normal Tenon's ocular fibroblasts. This work supports the role of therapeutic interventions to target oxidative stress or provide mitochondrial energetic support in glaucoma.

Methods

Subjects and Clinical Assessment

Participants with primary open angle glaucoma (POAG) and disease negative non-glaucomatous controls were recruited at the Royal Liverpool University Hospital, Liverpool, U.K. This study adhered to the tenets of Declaration of Helsinki and were approved by the relevant institutions, with all participants giving informed written consent. Ethical approval for the study was acquired from the NHS Research Ethics Committee (REC Ref 14/LO/1088). Clinical phenotyping included a detailed ocular and medical history, drug history, intra-ocular pressure (IOP) measurement by Goldmann tonometry, slit-lamp bio-microscopy with stereoscopic disc examination and gonioscopy, and visual field testing (Humprey Visual Field Analyzer, Zeiss; Swedish interactive algorithm standard 24-2 program). The diagnosis of POAG was based on open anterior chamber angles on gonioscopy, glaucomatous optic nerve damage on fundoscopy and a glaucomatous visual field defect. Glaucoma severity was graded by analysis of the visual field using the Glaucoma Staging System (GSS2) staging system [37]: mild (stage 0-1), moderate (stage 2-3), advanced (stage 4-5). Patients were excluded if below 18 years of age, if they had previous intraocular surgery or any findings on examination suggesting ocular hypertension or a secondary cause of glaucoma. Ethnically matched and age matched controls without glaucomatous optic neuropathy and a pressure less than 21mmHg, were also recruited to the study.

Isolation of human primary Tenon's ocular fibroblasts

Human primary Tenon's ocular fibroblasts (TFs) were cultured from subjects with POAG (GTFs) or non-glaucomatous controls (NTFs) undergoing glaucoma or cataract surgery using the explant method as previously described [38]. A limbal incision was created as a part of glaucoma surgery (or at the site of sub-Tenon's injection of local anaesthetic after administration of topical anaesthetic for cataract surgery) and a 5mm x 5mm square of Tenon's

tissue was excised from beneath the conjunctiva after separation by blunt dissection. Petri dishes were scored with blade with a middle 'X' and the Tenon's tissue explant was mechanically applied into this central 'X'. TFs were cultured in complete medium (Dulbecco's Modified Eagle's Medium/ Nutrient Ham F12 (1:1) medium: DMEM/F12) supplemented with L-glutamine, 10% fetal calf serum, penicillin/ streptomycin mix (1:1) and amphotericin (all from Sigma-Aldrich, UK). 5ml of complete medium was applied and incubated at 37°C with 5% CO_2 and 95% humidity in an incubator (Sanyo CO_2 Incubator MCO-17A, Sanyo, Japan) and cells were passaged until they reached passage 4. The cells were tested for mycoplasma using previously described techniques [39] and then used for further experiments or conserved at -80°C using 10% dimethyl sulfoxide (DMSO) (Sigma-Aldrich, UK) until further use. Vimentin (V9) mouse monoclonal antibody (MA5 11883) (Thermofisher Scientific, USA) immunocytochemical staining (2µg/ml in 1% BSA for 1 hour at 37°C) was performed to confirm that the cells were fibroblasts.

Measurement of mitochondrial content

Citrate synthase activity was used as a quantitative marker of mitochondrial content in TFs. Intact mitochondria were isolated from TFs using a commercial Mitochondrial Isolation Kit (Thermoscientific, USA) from a Citrate Synthase Activity Assay (Sigma-Aldrich, USA). Citrate synthase activity is reported as nmole/min/mL = milliunit/mL. One unit of citrate synthase is the amount of enzyme that generates 1.0 mmole of CoA per minute at 25 °C and pH 7.2. Tenon's ocular fibroblasts from subjects with POAG (GTFs; n=5) or non-glaucomatous controls (NTFs; n=5) were tested in duplicate and citrate synthase activity assay analysed. Statistical significance was determined using unpaired t testing.

Seahorse XF24 Analyzer measurement of cellular bioenergetics

Cellular bioenergetics of human primary Tenon's ocular fibroblasts (TFs) was determined using the extracellular flux analyser (Seahorse XF24 Analyzer; Seahorse Bioscience, Agilent Technologies, UK). TFs (2×10^4) were seeded in a 24 well Seahorse XF plate and incubated at 37°C with 5% CO_2 and 95% humidity for 24 hours. Prior to the experiment the medium was removed from the cells and incubated for one hour with serum free medium with or without hydrogen peroxide (Sigma-Aldrich, UK) to a final concentration of 100µM and 200µM in 450µl of serum free medium. Hydrogen peroxide (H_2O_2) was used to induce oxidative stress [40] and the concentration and duration of H_2O_2 treatments were determined through optimisation experiments and mirror previous studies in Tenon's ocular fibroblasts [41]; a 1-hour exposure of 50µM H_2O_2 is considered physiological and 100-200µM H_2O_2 is deemed pathological[42,43]. Medium was then removed and the cells were washed twice with Seahorse medium (DMEM supplemented with 10mM D-glucose (Sigma-Aldrich, UK), 2mM L-glutamine (Sigma-Aldrich, UK) and 2mM pyruvate (Sigma-Aldric, UK), pH 7.4) prior to applying 450µl of seahorse medium as previously described [44]. The plates were then incubated at 37°C with no CO_2 for a further hour. XF Cell Mito Stress Test assays (Seahorse Bioscience, Agilent Technologies, UK) were performed to assess mitochondrial respiration through real-time, non-invasive measurement of oxygen consumption rate (OCR). The sequence of the Seahorse XF24 Mito Stress Test involves five measurements of the OCR at 7-minute intervals, three measurements after addition of 1.26µM oligomycin (Sigma-Aldrich, UK), three measurements after addition of 1.0µM of FCCP (Sigma-Aldrich, UK) and two final measurements after the addition of a combination of 1µM antimycin A (Sigma-Aldrich, UK) and 1µM rotenone (Sigma-Aldrich, UK). Within the assay empty wells were used as blanks and two wells had cells without the Mito Stress test reagents as a control. Six replicates were performed of each test condition and enabled the mitochondrial respiration parameters to be calculated, including basal respiration, ATP-linked respiration, proton leak respiration and spare capacity (see **Supplement Figure S1** and **Table S1**). The data was normalised by cell number using the CyQUANT Cell Proliferation Assay (Thermo Scientific, USA). For standardisation purposes, as the individual values per cell are small, these were then

multiplied by 1 x 10^6 and all the results from the Seahorse XF24 assay were presented in this manner [45].

The statistical analysis of the data was performed using GraphPad Prism 6.0 software (La Jolla, CA, USA). Additionally, linear mixed effect model was run in R [46]. This method of analysis was preferred as the analysis was performed on all data (120 data points from 20 subjects), while adjusting for correlation on measurements from same subjects, which was achieved by introducing a random intercept parameter for data that came from the same subject. This method of analysis pulls information from all variables into one model and hence has higher effective sample size to estimate the variability due to subject differences [47].

Measurement of oxidative stress

Tenons fibroblasts were tested for mitochondrial function changes using two probes: Mitosox Red (Thermo Fisher Scientific, USA) and CM-H2DCFDA (Thermo Fisher Scientific, USA). In order to perform these test the cells were prepared in a similar manner. 8 x 10^4 cells Tenon's ocular fibroblasts were incubated with serum free medium at 37°C with 5% CO^2 and 95% humidity in an incubator for 24 hours prior to testing.

MitoSOX™ Red (Thermo Fisher Scientific, USA) was used for detection of mitochondrial superoxide production. On the day of testing the medium was removed and washed twice with Hanks' Balanced Salt Solution (HBSS) (Gibco, Thermo Fisher Scientific, UK). Thereafter 5µM MitoSOX™ in HBSS was applied and incubated in the dark for thirty minutes. The cells were washed twice with HBSS, trypsinised and centrifuged at 1500RPM for 5 minutes. The pellet was washed with HBSS and centrifuged at 1500RPM for 5 minutes and resuspended in 500µl of HBSS and flow cytometry was performed.

CM-H2DCFDA (Thermo Fisher Scientific, USA) was used to measure intracellular reactive oxygen species (ROS). 5µM CM-H2DCFDA in phenol free and serum free DMEM/F12, (HEPES no phenol red) (Gibco, Thermo Fisher Scientific, UK) was applied and incubated in the dark for thirty minutes. The cells were washed twice with 1x phosphate-buffered-saline

(PBS)(Gibco, Thermo Fisher Scientific, UK), trypsinised and centrifuged at 1500RPM for 5 minutes. The pellet was washed with PBS and centrifuged at 1500RPM for 5 minutes and resuspended in 500µl of phenol free and serum free DMEM/F12, (HEPES no phenol red) and flow cytometry was performed.

Flow cytometry data was collected on the BD Accuri™ C6 Flow cytometer (BD Biosciences, USA) by collecting 5000 events and by setting an FSC-H threshold of 1,000,000. All experiments were performed in triplicate and statistical analysis of the data was performed using GraphPad Prism 6.0 software (La Jolla, CA, USA). Mann-Whitney U test was performed for each parameter.

Results

Primary Tenon's ocular fibroblasts (TFs) were cultured from POAG patients (GTFs; n=10) and disease negative non-glaucomatous controls (NTFs; n =10). All subjects were Caucasian and the POAG group were 70.03 (SD ±11.90) years of age (mean ages (± standard deviation/SD) (n=5 female) and the control group were 78.30 (SD ± 7.59) years of age (n=6 female). The phenotypic data for each individual donor is given in **Supplement Table S2**. (n=6 had advanced glaucoma and n=4 had moderate glaucoma). There were no significant differences in the mitochondrial content of Tenon's ocular fibroblasts from disease negative non-glaucomatous and glaucomatous subjects as measured by citrate synthase activity (**Fig.1**). This confirmed that the differences in subsequent experiments was not observed due to variations in mitochondrial content.

Impaired mitochondrial cellular bioenergetics in glaucomatous Tenon's ocular fibroblasts

The Mito Stress Test from the Seahorse XF24 Analyzer was used to investigate mitochondrial cellular bioenergetics in glaucomatous Tenon's ocular fibroblasts (GTFs) compared to non-glaucomatous Tenon's ocular fibroblasts (NTFs). An oxygen consumption rate (OCR) curve was generated from NTFs and GTFs obtained from POAG (n =10) and disease negative

controls (n=10) and run in six replicates per subject (**Fig. 2A**) from which mitochondrial respiration parameters were calculated (**Fig. 2B**). Basal respiration is a measure of ATP synthase (ATP production) and proton leak. There was a significant reduction in basal respiration (basal OCR) between NTFs (3933+/-536 pmol/min/10^6cells) compared to GTFs (2803 ± 231 pmol/min/10^6cells). Maximal respiration which is a measure of the maximum rate of respiration that the cell can achieve and was significantly reduced in GTFs (5617±463 pmol/min/10^6cells) compared to NTFs (9163±1798 pmol/min/10^6cells). The spare capacity was also significantly reduced in GTFs (2813±354 pmol/min/10^6cells) compared to NTFs (5230±1288 pmol/min/10^6cells). The spare capacity describes the amount of additional ATP than can be generated by oxidative phosphorylation in the event of a sudden increase in energy demand or cell stress. The extracellular acidification rate was measured throughout the Mito Stress Test to calculate the baseline ECAR and ECAR oligomycin which reflects the glycolytic capacity. There was no significant difference in the ECAR in control fibroblasts compared to glaucoma. Overall, the reductions in basal and maximal respiration coupled with spare capacity defects highlights significant defects in mitochondrial bioenergetics in GTFs.

Oxidative stress and mitochondrial cellular bioenergetics in Tenon's ocular fibroblasts

Oxidative stress was induced by pre-treatment of H_2O_2 at two concentrations (100µM and 200µM) for 1 hour prior to the Mito Stress Test using the Seahorse XF24 Analyzer in NTFs and GTFs (**Fig. 3** and **Table 1**). In both GTF and NTF, the basal respiration increased with exposure to 100 µM H_20_2 but not 200µM H_20_2. Mitochondrial basal respiration responded to lower levels of oxidative stress but at higher levels of oxidative stress resulted in mitochondrial bioenergetic compromise. Proton leak increased at both 100µM and 200µM H_20_2 indicating mitochondrial damage due to increased uncoupling protein activity, damage to the inner mitochondrial membrane and/or electron transport chain complexes. In NTFs increasing concentrations of H_20_2 reduced the OCR curve with significant reductions in maximal respiration and spare capacity mirroring the findings in the GTFs without H_2O_2 treatment (**Fig.3**

and **Table 1**). These findings demonstrate increasing mitochondrial dysfunction in response to oxidative stress in NTFs with reduced cellular bioenergetics. This reflected the pre-treatment state of GTFs and the induction of further oxidative stress only impacted spare capacity in GTFs. Pre-treatment with 200µm H_2O_2 significantly reduced spare capacity (1262±446 pmoles/min/10^6 cells vs. from 2813±354 pmoles/min/10^6 cells) in GTFs (**Fig.3G.**). H_2O_2 induced oxidative stress further compromises spare capacity in GTFs hindering the cells already compromised ability to respond to cell stress. Given that mitochondrial cellular bioenergetics were already compromised in GTFs and the induction of oxidative stress resulted in similar OCR profiles in the NTFs while further impacting spare capacity in the GTFs we sought to determine the basal oxidative stress in both GTFs and NTFs. The level of intracellular ROS was measured using a CM-H2DCFDA assay and a MitoSOX Red assay and in GTFs compared to NTFs there was a significant increase in general ROS in the cell, but no changes were observed in mitochondrial derived superoxide (**Fig.4**). GTFs are already under oxidative stress prior to H_2O_2 treatment which exacerbates already compromised mitochondrial bioenergetics.

Discussion

The role of mitochondria in glaucoma pathogenesis has gained increasing interest as they are considered potential targets for therapeutic intervention [48–51]. In this study we have demonstrated impaired mitochondrial cellular bioenergetics in Tenon's ocular fibroblasts derived from glaucoma (POAG) patients. Furthermore, we have shown elevated basal oxidative stress in GTFs compared to NTFs. H_2O_2 induced ROS simulated the glaucomatous condition in NTFs and further compromised mitochondrial function in GTFs.

Using the Seahorse XF Mito Stress Test our study demonstrated that the mitochondrial respiration profile was globally impaired in GTFs compared to NTFs. Specifically, there were significant reductions in basal respiration, maximal respiration and the spare capacity in GTFs.

A significant reduction in basal respiration has also been demonstrated in glaucoma lamina cribrosa cells [52]. The maximal respiration shows the maximum activity of electron transport chain and substrate oxidation that the cell can achieve. In GTFs the reduction in maximal respiration indicates a global defect in the electron transport chain and is a strong indicator of potential mitochondrial dysfunction [53]. Maximal respiration was reduced in blood lymphoblasts from POAG subjects [54] and complex I enzyme specific activity was significantly reduced by 18% in POAG lymphoblasts [55]. A significant reduction of maximal respiration has also been observed in other ocular age-related conditions (age related macular degeneration in RPE cells) using the Seahorse XF analyser [56].

There was a significant reduction in spare capacity in ocular Tenon's fibroblasts from glaucoma patients. The spare capacity is a measure of the mitochondrial capacity to meet additional cellular energy requirements in response to cellular stress to avoid an ATP crisis [57]. In effect, spare capacity indicates how close a cell is to operating at its bioenergetic limit [53]. In this respect spare capacity is a measure of mitochondrial fitness, and low spare capacity reflects mitochondrial dysfunction which might not be apparent under basal conditions and has been reported in cardiovascular and chronic neurological diseases [58]. A significant reduction in spare capacity has also been demonstrated in human glaucoma lamina cribrosa cells from the optic nerve head [52]. Ocular Tenon's fibroblasts therefore mirror mitochondrial dysfunction in the optic nerve head and are therefore also likely to reflect altered mitochondrial bioenergetics in the optic nerve and RGCs in glaucoma. Patient derived cells, and specifically ocular cells, provide an excellent platform to assess mitochondrial dysfunction in glaucoma [19,25,28,34,35]. Tenon's ocular fibroblasts provide an accessible cell type to provide enough material and case numbers for study and evaluate future metabolic and mitochondrial therapies in glaucoma.

Spare capacity depends on the functional integrity of the electron transport chain and the inner mitochondrial potential, the availability of energetic substrates for oxidation and the maintenance of mitochondrial homeostasis via biogenesis and mitophagy [57]. Oxidative stress has a significant impact on mitochondrial spare capacity [59,60]. Under conditions of oxidative stress, the spare capacity of cells is further depleted, and if the basal respiratory threshold is breached, cell death occurs [59–62]. Spare respiratory capacity levels correlate with the degree of mitochondrial plasticity, allowing bio-energetic adaptability in response to pathophysiological stress, and hence inadequate levels are associated with pathological conditions [57]. In GTFs there was elevated basal oxidative stress compared to NTFs which could represent one mechanism resulting in a reduced spare capacity. Oxidative stress and ageing can induce mitochondrial DNA (mtDNA) mutations impacting mitochondrial bioenergetics including spare capacity, in addition, to contributing to further ROS production [63]. Previous work by our group has demonstrated pathogenic variants in mtDNA extracted from peripheral blood leucocytes and Tenon's ocular fibroblasts from glaucoma patients [30,64]. The results demonstrate that the source of ROS in glaucomatous TFs is not mitochondrial in origin. In this paper we demonstrate elevated ROS and impaired mitochondrial bioenergetics in glaucoma, but the underlying mechanism of ROS induced mitochondrial dysfunction requires further investigation. The mechanistic basis is important to identify therapeutic strategies to reduce ROS, and mitigate impaired mitochondrial bioenergetics, to prevent or reduce RGC loss and protect vision in glaucoma. Several antioxidant-based therapies have been evaluated in experimental glaucoma models and clinical trials [65,66]. Coenzyme Q10 (ubiquinone) is a molecule that shuttles electrons from complex I and I to complex III which maintains the mitochondrial membrane potential, supporting ATP synthesis and inhibiting reactive oxygen species generation [67]. Improvements in retinal ganglion cell health following the topical administration of coenzyme Q10 have been demonstrated in rodent glaucoma models [67–70]

Neural tissue has significant energy demands and neurons can utilise 80% of their spare capacity to maintain ionic gradients and thus neuronal excitability [71,72]. This places neuronal function and survival vulnerable to mitochondrial dysfunction [73]. RGCs are extremely susceptible to mitochondrial compromise due to their marked bioenergetic requirements and morphology [16,17,51]. In the DBA/2J mouse model of glaucoma mitochondrial dysfunction is an early feature in the RGCs [74] and nicotinamide adenine dinucleotide (NAD) shows an age-dependent decline contributing to mitochondrial dysfunction and vulnerability to glaucoma in this model [51,74,75]. The prevention of NAD decline by dietary supplementation with nicotinamide (NAM; the amide form of vitamin B3) protected against mitochondrial and metabolic dysfunction and so RGC neurodegeneration in the DBA/2J glaucoma mouse model [51,76]. Recent human studies with oral nicotinamide supplementation with or without pyruvate have shown short term beneficial effects [77,78].

Conclusions

Bioenergetic based therapies in glaucoma face several challenges including the chronic nature of glaucoma, the clinical variability in disease progression and determining robust primary endpoints [51,79]. We have used Tenon's ocular fibroblasts derived from glaucoma (POAG) patients to detect elevated basal ROS levels and altered mitochondrial bioenergetics. This approach provides important insight into the pathogenesis of glaucoma but also could be employed as a strategy to risk profile patients for future bioenergetic based neuroprotection trials in glaucoma.

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Fig 1. The mitochondrial content measured by citrate synthase activity of glaucomatous Tenon's ocular fibroblasts (GTFs) and disease negative non-glaucomatous Tenon's ocular fibroblasts (NTFs). Citrate synthase is an exclusive marker of the mitochondrial matrix. There was no statistically significant difference (p=0.3845) in the citrate synthase activity assay between the GTFs and NTFs (n =5).

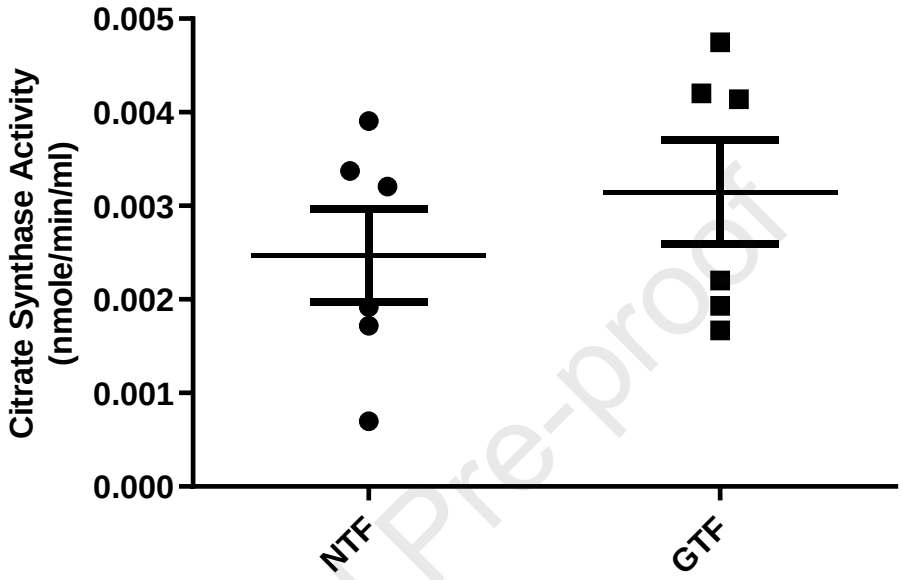


Fig 2. The Seahorse XF Analyzer Mito Stress Test detected altered mitochondrial cellular bioenergetics in glaucomatous Tenon's ocular fibroblasts. (A) The oxygen consumption rate (OCR) curve of the Mito Stress Test in disease negative non-glaucomatous Tenon's ocular fibroblasts (NTFs) (n=10) and glaucomatous Tenon's ocular fibroblasts (GTFs)) (n=10) after sequential addition of Oligo (oligomycin), FCCP and Rot/Ant A (rotenone/ antimycin A) (mean of six replicates); (B) Calculation of the mitochondrial respiration parameters demonstrated a significant reduction of the basal respiration (p=0.0449), maximal respiration (p=0.0113) and spare capacity (p=0.0481). Data on the graph represents the mean ± SEM (*=p<0.05, **= p< 0.01).

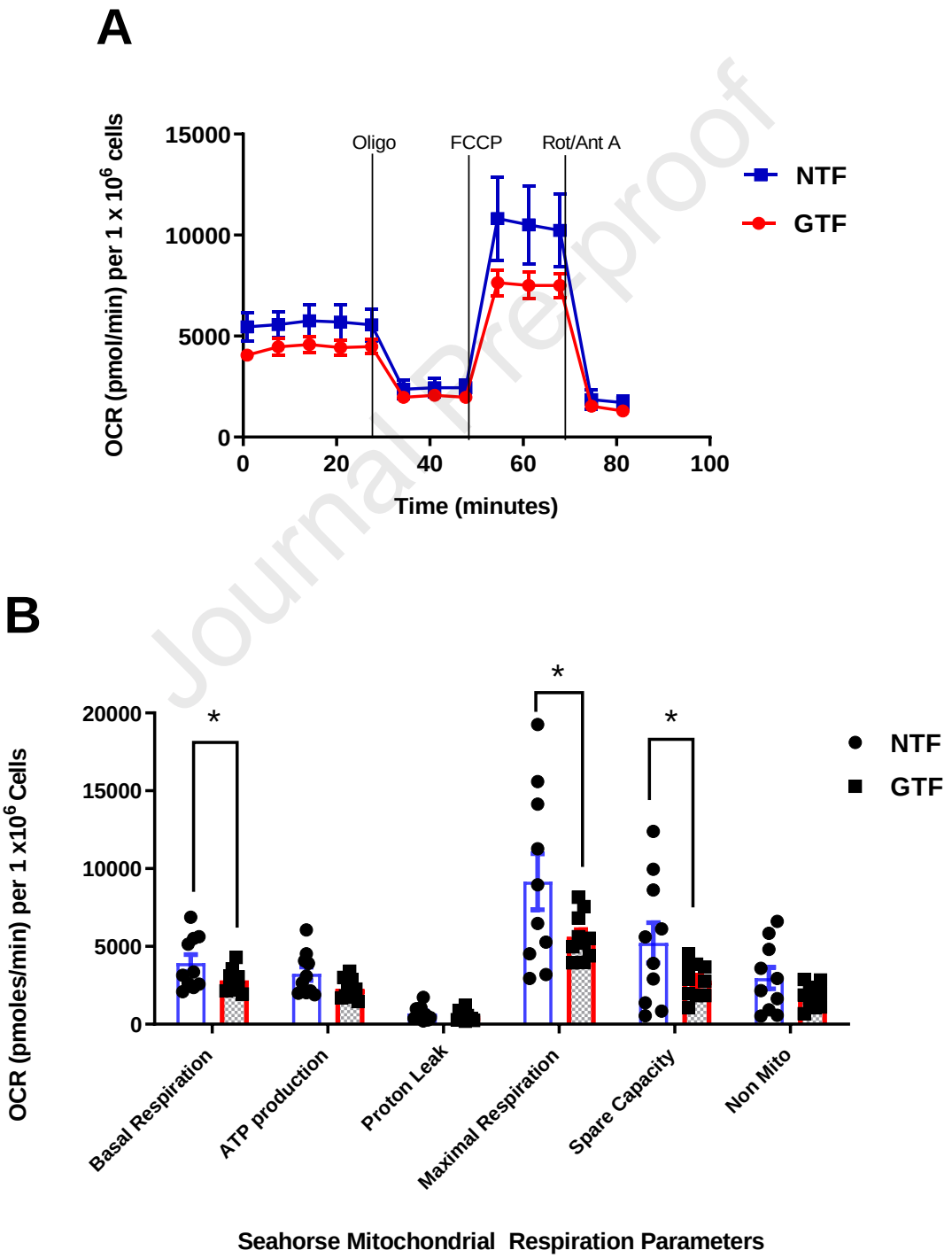


Fig 3. Effect of hydrogen peroxide (H_2O_2) induced oxidative stress on mitochondrial cellular bioenergetics in Tenon's ocular fibroblasts. (A) The oxygen consumption rate (OCR) curve of the Mito Stress Test in in disease negative non-glaucomatous Tenon's ocular fibroblasts (NTFs) (n=10) and glaucomatous Tenon's ocular fibroblasts (GTFs)) (n=10) after sequential addition of Oligo (oligomycin), FCCP and Rot/Ant A (rotenone/ antimycin A) (mean of six replicates). These graphs compare pre incubation with 0µm H_2O_2, 100µm H_2O_2 and 200µm H_2O_2 in A) control group B) glaucoma group. The mitochondrial respiration parameters were then calculated, and two-way ANOVA testing was performed to compare the differences. The respiration parameters include C) basal respiration, D) ATP production, E) proton leak, F) maximal respiration, G) spare capacity, H) non-mitochondrial respiration. The data shown is the mean ± SEM. (* = p< 0.05 * = p<0.05, **= p< 0.01, ***= p<0.005, **** = p<0.0001).

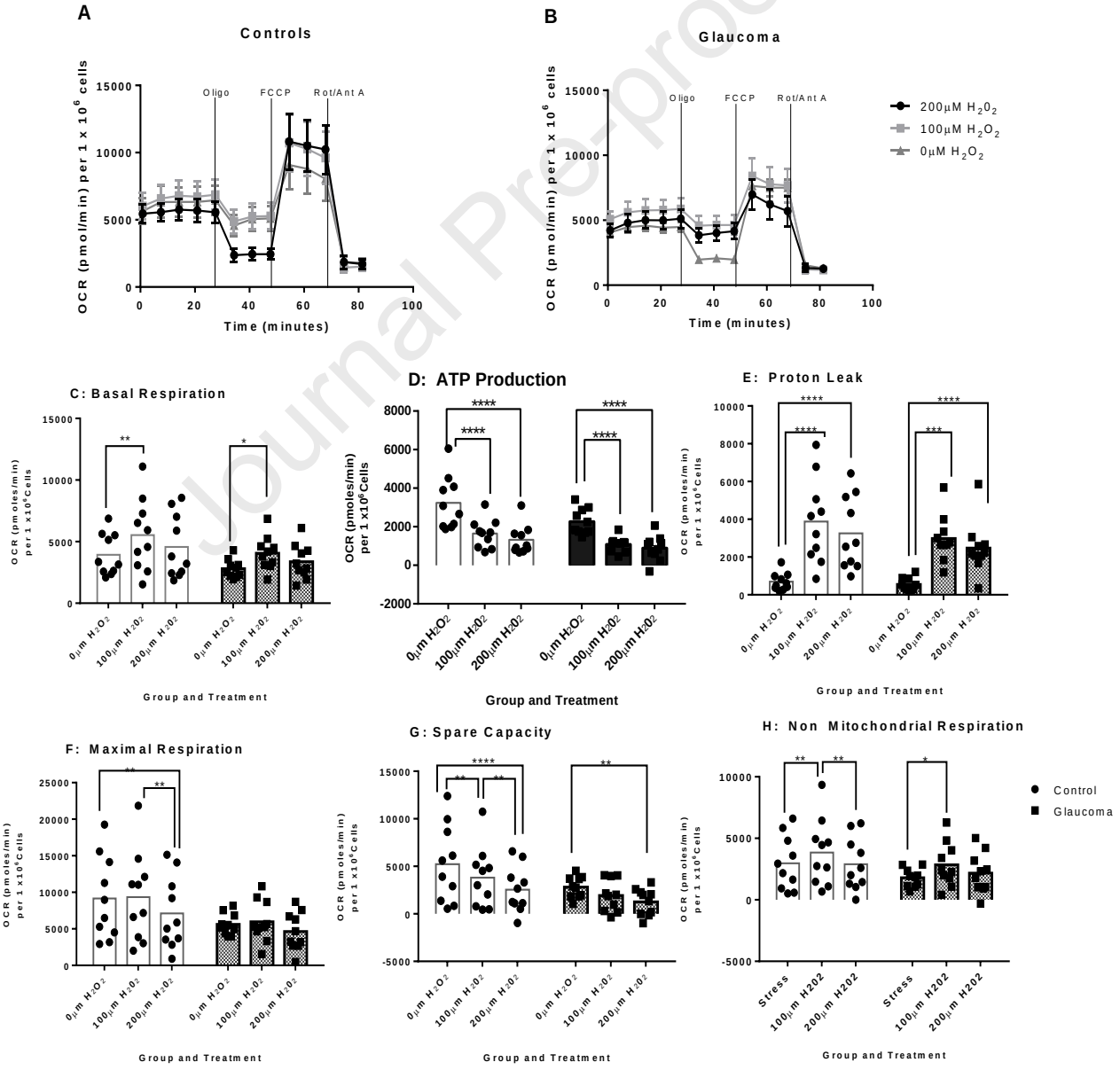
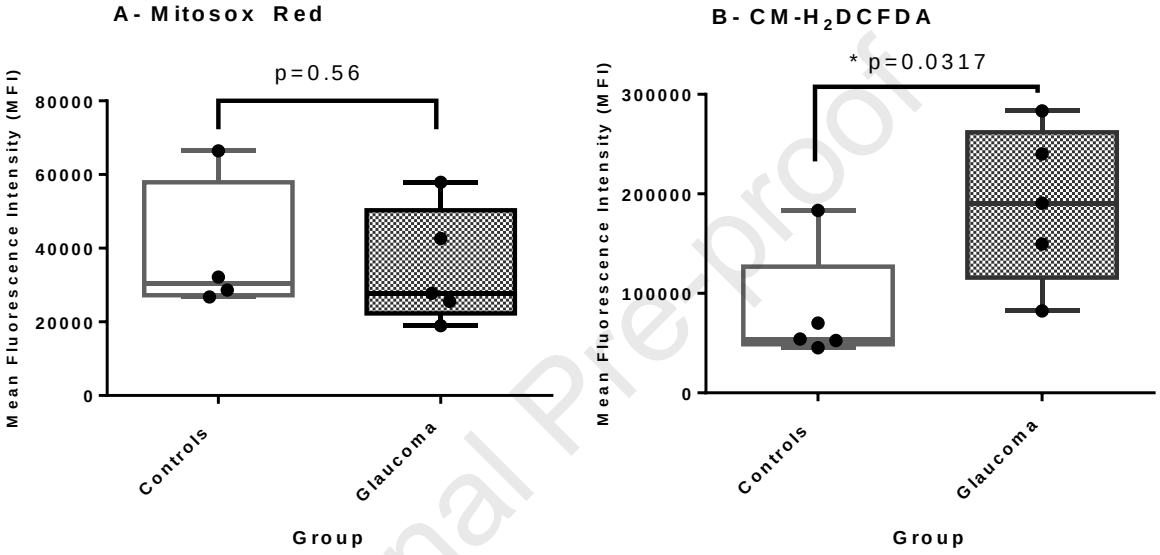


Table 1: Post hoc Tukey's Multiple Comparison Testing to analyse the findings from the significant two-way ANOVA test of the mitochondrial respiration parameters after Seahorse XF Analyzer Mito Stress testing with or without hydrogen peroxide (H_2O_2) in glaucomatous Tenon's ocular fibroblasts (GTFs) and disease negative non-glaucomatous Tenon's ocular fibroblasts (NTFs). Testing performed with 0µm H_2O_2, 100µm H_2O_2, and 200µm H_2O_2 in 10 GTFs and 10 NTFs. This demonstrates that control fibroblasts exhibit a more significant response to H_2O_2 than glaucoma fibroblasts (as shown by the response of the spare capacity and maximal respiration after pre incubation with H_2O_2. * = p<0.05, **= p< 0.01, ***= p<0.005, **** = p<0.0001.

All results per 1x 10^6 cells	Basal Respiration		ATP Production		Proton Leak		Maximal Respiration		Spare Capacity		Non Mitochondrial Respiration	
	Significant	Adjusted p Value	Significant	Adjusted p Value	Significant	Adjusted p Value	Significant	Adjusted p Value	Significant	Adjusted p Value	Significant	Adjusted p Value
Control												
0µm H_20_2 vs. 100µm H_20_2	Yes**	0.0015	Yes****	<0.0001	Yes****	<0.0001	No	0.9576	Yes**	0.0031	Yes**	0.0067
0µm H_20_2 vs. 200µm H_20_2	No	0.2940	Yes****	<0.0001	Yes****	<0.0001	Yes**	0.0061	Yes****	<0.0001	No	0.9676
100µm H_20_2 vs. 200µm H_20_2	No	0.0710	No	0.2373	No	0.3052	Yes**	0.0029	Yes**	0.0085	Yes**	0.0034
Glaucoma												
0µm H_20_2 vs. 100µm H_20_2	Yes*	0.0136	Yes****	<0.0001	Yes****	<0.0001	No	0.8342	No	0.0795	Yes*	0.0193
0µm H_20_2 vs. 200µm H_20_2	No	0.3843	Yes****	<0.0001	Yes***	0.0001	No	0.2627	Yes**	0.0012	No	0.7626
100µm H_20_2 vs. 200µm H_20_2	No	0.2379	No	0.6141	No	0.4621	No	0.0910	No	0.2419	No	0.0951

Fig 4. Measurement of intracellular reactive oxygen species in glaucomatous Tenon's ocular fibroblasts (GTFs) and disease negative non-glaucomatous Tenon's ocular fibroblasts (NTFs) was performed using A- MitoSOX Red assay to measure mitochondrial superoxide and B- CM- H2DCFDA to evaluate general reactive oxygen species**.** Oxidation of these probes by reactive oxygen species yields a fluorescent adduct Measurement of mean fluorescence intensity of TFs using flow cytometry showed a significant increase in general reactive oxygen species in GTF (n=5) compared to NTF (n=5) but no significant difference in mitochondrial superoxide**.**



Supplemental Figures:

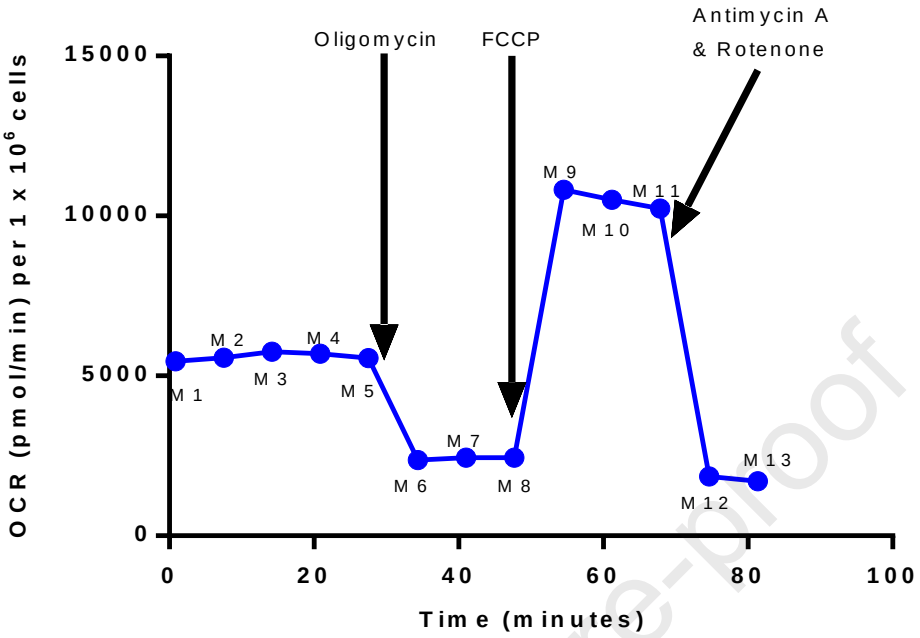


Fig S1. The Seahorse XF Mito Stress Test curve with sequential additions of treatment and the numbered measurements of oxygen consumption rates (OCR) over time, which are subsequently used for further calculations of mitochondrial respiration parameters. Measurements are labelled M1- M13. Arrows indicate time of injection for specific reagents. **Table S1** demonstrates the methods mitochondrial parameters were calculated.

Table S1. The methods of calculations using different measurements from the oxygen consumption rate curve to calculate the mitochondrial respiration parameters of the Seahorse XF Mito Stress test. The measurements M1- M13 are taken over time and demonstrated in **Figure S1**.

Parameter	**Calculation**
Basal respiration	Mean (M4, M5) - Mean (M12, M13)
ATP production	Mean (M4, M5) - Mean (M7, M8)
Maximal respiration	Mean (M9, M10) - Mean (M12, M13)
Spare capacity	Mean (M9, M10) - Mean (M4, M5)
Proton Leak	Mean (M7, M8) - Mean (M12, M13)
Non-Mitochondrial respiration	Mean (M12, M13)

Table S2. A table of the phenotypic information about glaucoma patients from whom Tenon fibroblasts were extracted and used during this study

Sample phenotype was classified using the glaucoma staging system 2 (GSS2) as mild (stage 0-1), moderate (stage 2-3), advanced (stage 4-5). MOPP - Mean ocular perfusion pressure, VA - Snellen visual acuity, MD - mean deviation, PSD - pattern standard deviation, N - none, R - right, L - left, B - bilateral

Tenon sample ID	Eye	Phenotype	Other eye phenotype	Age at surgery	Ethnicity	Family history	Previous ocular surgery	Other co morbidity	MOPP (mmHg)	Max drops	VA	GSS2	MD	PSD
1	L	Advanced	Advanced	75	Caucasian	N	N	Hypertension	34.11	Xalatan, Cosopt, Alphagan	6/9-2	5	-25.58	10.25
2	L	Advanced	Normal	51	Caucasian	Mother	N	Hypertension	53.56	Cosopt, Travatan	6/5	4	-12.21	14.89
3	L	Moderate	Moderate	78	Caucasian	Mother	B blepharoplasties	Myocardial infarction and coronary artery bypass graft	27.22	Ganfort, alphagan	6/9	2	-6.56	2.67
4	R	Advanced	Advanced	75	Caucasian	Mother	N	Osteoarthritis	58.89	Ganfort, Azopt, alphagan	6/6 +3	5	-26.8	9.17
5	R	Moderate	Moderate	61	Caucasian	Grandfather and maternal uncle	N	Type 2 DM, hypertension	43.67	Xalacom, Azopt, Alphagan, Diamox	6/5	2	-3.31	6.57
6	L	Advanced	Moderate	73	Caucasian	Brother	N	Hypothyroid	43.11	Duotrav, Azopt	6/9-2	5	-22.5	7.97

7	L	Advanced	Advanced	69	Caucasian	N	N	High cholesterol	51	Cosopt, Latanoprost, Alphagan	6/5	5	-16.99	10.83
8	R	Moderate	Moderate	72	Caucasian	N	Right selective laser trabeculopla-sty	N	39.44	Lumigan, Alphagan, Cosopt	6/5-3	3	-9.27	9.24
9	L	Moderate	Moderate	89	Caucasian	None	N	Angina, High cholesterol	43.44	Travatan, Azopt	6/9	3	-5.98	6
10	L	Advanced	Moderate	61	Caucasian	Father	N	Hypertension, nasal steroid	25.67	Ganfort, trusopt	6/10-2	4	-11.39	10.53

Highlights:

- Mitochondrial bioenergetics evaluated using Seahorse Bioscience Analyser were altered in Tenon's ocular fibroblast cells from glaucoma patients compared to non-glaucomatous control patients.
- Impaired mitochondrial cellular bioenergetics was detected in glaucomatous ocular fibroblasts including basal respiration, maximal respiration and spare capacity.
- Basal oxidative stress was elevated in glaucomatous Tenon's ocular fibroblasts and hydrogen peroxide (H_2O_2) induced reactive oxygen species (ROS) simulated the glaucomatous condition in normal Tenon's ocular fibroblasts.
- Mitochondrial dysfunction observed in glaucomatous patients within this study provides further evidence for the potential of neuroprotective bioenergetic based therapies for this irreversible cause of blindness.