Proteomic profile analysis of plasma and aqueous humor from glaucoma and non-glaucomatous patients

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Abstract

Purpose: Glaucoma, a multifactorial ocular neuropathic and age associated disease, can lead to irreversible vision loss. Diagnosis involves assessing optic cupping (increased cup-to-disc ratios) and structural changes (like retinal nerve fiber layer thinning) through clinical imaging. Elevated intraocular pressure (IOP) is commonly associated with glaucoma, but not always. However, understanding disease progression is hindered by limited access to donor ocular tissue and consistent clinical data. Our study focuses on live patient samples, analyzing their proteome for potential biomarkers to enhance precise diagnosis and monitor glaucoma progression.

Methods: Aqueous humor (AH) samples were collected from 36 glaucoma patients (17 male, 19 female), and 35 non-glaucomatous control patients (16 male, 19 female) undergoing cataract surgery. The protein profile was compared using the SOMAscan® assay system for proteome profiling. From glaucomatous donors, significant correlations between IOP and cup-to-disc ratios to proteome differences were identified.

Results: Correlations in proteins between plasma and AH were identified. These proteins were enriched in pathways related to vascular integrity, inflammatory response, humoral & adaptive immune response, cell-cell & cell-matrix adhesion, and complement activation. Glaucomatous AH exhibited increased protein levels in general. Neurofilament light chain (NEFL) protein correlated with elevated IOP and inflammatory markers, but not with cup-to-disc ratios.

Conclusions: Together, our data demonstrate that the proteins identified in this study from glaucomatous donors correspond to both markers of neurodegeneration and those that may inhibit cell proliferation or disrupt vascular integrity.

Translational Relevance: Proteome analysis may lead to potential biomarker identification during disease progression.

Precis: Clinical glaucoma diagnosis is by assessing structure-function relationship which doesn't yield molecular mechanisms underlying disease. Here, we analyzed proteome from aqueous and plasma of glaucomatous patients. Proteins correspond to markers of neurodegeneration, pressure and cup-to-disc-ratios.

1. INTRODUCTION

Primary open angle glaucoma (POAG) is one of the leading causes of irreversible blindness, estimated to affect 111.8 million people worldwide by 2040 [1, 2]. Although the etiology and progression of POAG is multifactorial, the only modifiable causal risk factor for managing visual field loss is to reduce intraocular pressure (IOP), which is demonstrated to slow the progression of damage to the optic nerve and death of retinal ganglion cells [3]. IOP is largely determined by a balance between aqueous humor production and resistance to outflow [4, 5]. In the human eye, approximately 75% of all aqueous humor flows from the anterior chamber through the trabecular meshwork (TM) and Schlemm's canal (SC) [6, 7] and is thus the primary egress for aqueous humor [8]. Increased resistance to aqueous drainage due to changes in TM cells and extracellular matrix (ECM) is considered a major contributor to ocular hypertension (OHT) [9, 10]. Differential profiles and levels of soluble factors have been identified in the AH, which have the potential to affect cells in the tissues (outflow tract) that they traverse through, and thus influence OHT.

AH is secreted into the posterior chamber by the non-pigmented epithelial cells of the ciliary processes, and serves to supply critical nutrients and signaling molecules, and to remove metabolic waste from the cornea, lens, and trabecular meshwork. Though the total amount of protein in the anterior chamber is less than 1% (w/v) due to the functional blood aqueous barrier [4], the aqueous humor is enriched with proteins secreted by intraocular structures from both the anterior and posterior segments [11]. It is also thus feasible to hypothesize that systemic disorders, genetic conditions, or perturbations to ocular integrity and health may all adversely influence expression of proteins in the aqueous humor and other ocular tissues [12]. Disease associations of protein compositions of tear films, AH and vitreous humors have been observed with dry eye, glaucoma, age related macular degeneration, or other pathologies [13-18].

Several studies have demonstrated differences between metabolites or proteomes in the AH of patients with primary open angle glaucoma and have subsequently posited that these may serve as biomarkers of dysfunction in the outflow apparatus [16, 19-29]. Complementary approaches

were used to quantify protein expression in AH from glaucomatous donors, including ELISA, multiplexed immunoassays, LC-MS/MS, or antibody microarrays. A few limitations in using the above methods to comprehensively quantify proteins/metabolites are small volume of and limited protein concentration within the AH, as well as the requirement of validated antibody-based approaches, which limits the number of biomarkers that can be identified. There is thus a need to identify the AH proteins more broadly with increased sensitivity, in order to identify biomarkers of disease progression when comparing pre- and post- therapeutic intervention. Further discovery of previously unreported proteins in the AH of patients may provide new insight into the molecular mechanisms underlying outflow resistance in OHT and glaucoma. Here, we utilize the SOMAscan® assay [30], a multiplexed, aptamer-based technology, to detect and assess relative abundance of a broad range of proteins from small volumes of human blood alongside AH from patients undergoing glaucoma surgery, as compared to controls patients subject to cataract surgery.

2. MATERIALS AND METHODS

2.1 Study population

The study was approved by the ethics committee investigational review board (CEP 1.209.725) and adhered to the principles of the Declaration of Helsinki. Informed consent was obtained from all participants. Aqueous humor (AH) and plasma samples were collected from 36 glaucoma patients (17 male, 19 female), and 35 cataract control patients (16 male, 19 female) undergoing cataract surgery (**Figure 1**). Within the glaucoma patient group one underwent trabeculectomy, two were pseudophakic, and AH was collected at the beginning of the surgery. Clinical diagnosis of glaucoma was based on elevated IOP (IOP > 21 mmHg), documented progressive cupping or thinning of the neuroretinal rim. All glaucoma patients were treated with medication to control IOP. Patients with other eye diseases or associated with systemic diseases were not included.

2.2 Aqueous humor (AH) and plasma sample collection

AH samples were collected from patients following a previously validated method [31] at the beginning of the surgery. Briefly, after anesthetic and antiseptic eyedrops administration, a 1mL tuberculin syringe (27 gauge, ½ inch) was used to aspirate the AH. Approximately, 0.1 ml was collected, and transferred to two labeled Eppendorf tubes. Peripheral blood (10 mL) was collected from an arm of the patient in EDTA tubes during the surgery. The tubes were centrifuged for 10 min at 1900 x g, and plasma was collected. Total protein concentrations in the AH samples were determined using the BCA Protein Assay Kit (Pierce, Rockford, IL).

2.3 SOMAscan® analysis

All AH and plasma samples were analyzed in parallel using the SOMAscan® proteome profiling platform that provides a broad and relative abundant assessment of selected proteins in biological fluid. The SOMAscan® platform, using affinity-based slow off-rate modified aptamers (SOMAmers), has been described extensively elsewhere [30, 32, 33]. The custom SOMAscan® version used in our study covered 5,080 SOMAmers, including 4,785 human-specific somamers targeting 4,161 human proteins. The targeted proteins include transmembrane receptors, secreted proteins, kinases, transcription regulators and signal transducers. The samples were aliquoted into 2D barcoded tubes and were shipped on dry ice to SomaLogic for analysis.

2.4 Statistical analysis

The relative abundance of captured SOMAmers were quantified by microarray hybridization as relative fluorescence units (RFU). We focused on SOMAmers detecting human proteins in this study which focuses on 4,785 SOMAmers for the follow up analysis. Quality control and sample outlier detection was conducted using the arrayQualityMetrics[34], and by running principal component analysis (PCA) and Spearman's correlation analysis with custom R scripts. RFU were log2-transformed and normalized by smooth quantile normalization using R package qsmooth [35]. In total, 5 samples (2 non-glaucomatous, 3 glaucoma) were removed from the AH cohort and 2 non-glaucomatous samples were removed from the plasma cohort, which left 62

samples in the AH cohort and 65 samples in the AH cohort. We applied the limma R package (v3.50.3) in R 4.1.0 environment to determine differentially expressed proteins between groups[36]. Pathway enrichment analysis was performed with pre-ranked GSEA using R package fgsea v1.20.0 and Gene Ontology Biological Process category (GOBP) in MSigDB[37-40].

2.5 Correlation analysis

We conducted Elastic Net, a machine learning based method, to identify proteins correlated with the total protein concentration. For this analysis, we utilized the cv.glmnet function from the R package glmnet v4.1-3. We employed a 10-fold cross-validation approach to optimize the alpha and lambda parameters. Additionally, we performed a 1000-fold bootstrapping using the optimized alpha and lambda values to determine the frequency at which each protein was selected as a top predictor for estimating the total protein concentration. To enhance the performance of our analysis, we only included proteins with an absolute Pearson's correlation coefficient greater than 0.4 with the total protein concentration.

3. RESULTS

3.1 Donor demographics

Donor demographics for the patient samples used in this study are provided in **Figure 1**. The average age of donors with cataract was 66.9 ± 10.9 while those with glaucoma was 68.41 ± 12.84. No significant differences (p=0.60, t-test; **Figure 1**) were seen in donor ages between the groups *in toto* or when evaluated by sex. Amongst these donors, we note that one donor had primary angle closure glaucoma while the remainder had POAG. The mean IOP of glaucoma donors (IOP controlled with medication) was 17.94 ± 8.28 mmHg with no differences seen between male or female donors. Only one POAG donor had an IOP > 24 mmHg. A statistically significant difference in total protein concentration in AH was observed between cataract and glaucoma groups.

3.2 Differential analysis of SOMAScan® profiling data

Principal component analysis of the cataract and glaucoma samples revealed heterogeneity between samples, including outliers that were subsequently excluded from further analysis. Subsequent analysis revealed few differences in protein levels between non-glaucomatous and glaucomatous aqueous humor and plasma (**Supplementary Fig. 1**). Nevertheless, we observed that samples were clustered by tissue types with reasonable correlation ($r > 0.5$) between AH and plasma samples (**Figure 2a**). The histogram (**Figure 2b**) demonstrates that the distribution of Pearson's correlation coefficients between AH and plasma samples from same donor demonstrates some variability. Considering all donors, a reasonable correlation ($r > 0.5$) was observed comparing protein levels between AH and plasma samples.

Subsequent to multivariate analysis, we performed differential protein analysis for both plasma and aqueous samples. Expression values that were $|\log2(RFU)| > 0.5$ and where the adjusted p-value was < 0.05 are highlighted as significant (**Figure 3**). Within glaucomatous AH (Figure 3A), 7 SOMAmer probes representing 6 target proteins were observed to be significantly down regulated, while 49 SOMAmer probes representing 49 target proteins were upregulated. Examples of top up-regulated proteins include complement proteins (C3, C7, C1QTNF3), Apoliprotein (APOE, APOF), matrix protein (endostatin/Col18A1), macrophage associated proteins (CSF1R, CD163), while top down-regulated proteins include growth factors such as FGF9, FGF20 and CCK. We observed that in plasma samples, the majority of the differential proteins were upregulated in glaucoma. Some of the upregulated proteins of interest include ENO1, NEK7, AKT, PRKCA, PRKCB, JAK2, MAP kinase-activated protein kinase 2 (MAPKAPK2), whereas the top down-regulated proteins include APOC3, GDF2, DCP1B and EDIL3. A comprehensive list of proteins altered in their levels comparing glaucomatous and non-glaucomatous plasma and AH are provided in **supplementary table 1**. Although protein levels from AH and plasma from the same donor showed a positive correlation, we only observed SNX4 to be significantly up regulated in both AH and plasma samples from glaucomatous donors.

3.3 Pathway enrichment analysis

Next, we asked if the differentially enriched proteins were enriched in common biological pathways, by performing pathway analysis (**supplementary table 2**). We identified 51 and 559 significantly up-regulated pathways (adjusted p-value < 0.05) in AH and plasma samples from glaucoma donors, respectively. 16 pathways were up-regulated in both AH and plasma, including inflammatory response, IL6 production and immune response related pathways.

3.4 Correlation analysis of protein levels with total protein concentration

While individual protein changes may help identify signaling pathways perturbed in disease, total protein concentration of the AH may help ascertain the overall health of the blood-aqueous barrier and the outcomes of IOP lowering medications. Thus, normalizing individual proteins to total protein concentrations assists in determining if observed changes reflect pathway changes underpinning disease, or are merely a consequence of structural failure or drug treatment(s). We conducted an Elastic Net regression analysis to identify proteins that can accurately predict the total protein concentration (**Figure 4a**). Our analysis specifically focused on AH proteins, as proteins from these samples may provide a better reflection of changes in glaucoma disease compared to plasma samples. Through our analysis, we successfully identified 5 proteins (KNG1, IGFBO6, MAP2K4, H6PD, and C3) that demonstrated the highest predictive capability for the total protein concentration collected from the patients (**Figure 4a**).

3.5 Correlation analysis of protein levels with IOP

We conducted Elastic net regression analysis to identify 13 proteins to be associated with IOP values reported in glaucomatous patients (**Figure 4b**). We note that a glaucoma sample with extremely high IOP measurement was excluded from the analysis. Among the proteins of interest in glaucoma, the protein with maximal correlation with IOP levels in glaucomatous patients was neurofilament light chain (NEFL; **Figure 4c, Supplementary Fig. 2**). Linear regression analysis validated this finding and demonstrated a net correlation with IOP

measurements (R^2 = 0.3067). Since NEFL is a widely accepted marker for several neurodegenerative diseases including glaucoma [41-50], we then decided to further investigate if levels of NEFL correlated with other SOMAmers identified in glaucomatous donors. We calculated the Pearson's correlation coefficient of NEFL and rest of the proteins and identified 29 proteins that are positively or negatively correlated with NEFL (|r > 0.4|), including TEAD3, CNTF, C4A, C4B and CCL27 (**Figure 5**).

3.6 Changes in protein levels as a function of cup-to-disc ratios in glaucoma

Finally, we sought to determine if changes in relative abundance of protein levels in AH correlated with cup-to-disc ratios of glaucomatous patients via Pearsons's correlation analysis (**Figure 6**). For patients where cup-to-disc ratios were available for both eyes, we used the average measurements from both eyes. We note that among the 29 glaucomatous samples obtained, cup-to-disc ratios were available for 26 donors. Figure 6 shows the top 6 positive (KLRG2, C8G, PENK, ALB, APOA1, and KNG1) and negative (IL12A/B, LRRC3, ZAP70, CTSG, DEFB115, and NSG1) correlated proteins with cup-to-disc ratios.

4. Discussion

Diagnosis and prognosis of glaucoma disease is clinically monitored by IOP, and structure function measurements (advanced imaging, and visual field testing etc). However, these require longitudinal measurements supported by patient compliance, adherence to medications and regular in-patient visits. While these are critically valuable to ascertain the stage of glaucoma, in the absence of longitudinal ocular and systemic medical history, very little information is gleaned about molecular mechanisms underlying the disease or help in identifying a soluble biomarker to ascertain therapeutic engagement and efficacy. Since obtaining intraocular tissues, such as retina and optic nerve, from live human donors is not possible, reliance on sampling biofluids (such as AH and blood) to ascertain changes in the secretome that may further our standing of homeostasis/disease, is favored. In this study, we determined the abundance of proteins in both

the AH and blood plasma from patients (with and without clinically diagnosed glaucoma) undergoing cataract surgery and correlated them with disease-relevant clinical measures: IOP and cup-to-disc ratio.

The ages and sex of glaucomatous and non-glaucomatous donors in this dataset were comparable. We observed significant changes in relative abundance of a subset of proteins identified both in AH and plasma. Protein levels from AH and plasma from the same donor sample showed a positive correlation ($r > 0.5$ for all of samples). Interestingly, when comparing protein levels and correlations across all fluids and disease states, we only observed a significant upregulation of SNX4 , a synaptic & endosomal membrane trafficking in the secretory pathway and mitochondrial recycling quality protein in aging [51-53], upregulated in glaucomatous AH and plasma when compared with those from non-glaucomatous donors. The lack of greater correlation between plasma and AH proteins may be due to several reasons: turnover rates of AH *vs* plasma, tissues & cell types involved in protein secretions, systemic *vs* local transport of proteins. It is yet unclear if glaucoma is purely a disease of the eye or if systemic factors exist that regulate its etiology and progression, although co-morbidities have been reported [54-58]. Nevertheless, in the plasma, 48 proteins were upregulated while 4 (EDIL3, DCP1B, GDF2, APOC3) were downregulated when comparing non-glaucomatous and glaucomatous donors. Gene ontology analyses of proteins altered in the plasma of glaucomatous donors were related to multiple pathways corresponding to protein & macromolecule localization, intracellular transport, kinase activity pathways, post translational / post transcriptional regulation of proteins / genes, cytoskeletal reorganization and GPCR related pathways. On the other hand, 7 (ISLR2, CCK, INSL4, FGF9, FGF20, BMPR1A) proteins were significantly downregulated and 49 were significantly upregulated in the AH of glaucomatous donors. Gene ontology analyses of proteins identified as altered in the AH of glaucomatous donors were related to multiple pathways corresponding to inflammatory response, humoral & adaptive immune response, cell-cell & cell-matrix adhesion, complement activation, and wound healing responses. These together suggest

that though overall concordance in proteins may be observed between AH and plasma, some differences exist and thus proximity of biofluid to relevant tissue being studied (i.e. ciliary process, ciliary muscle and trabecular meshwork/Schlemm's canal) may be important to consider. As such, deeper analysis in this study was focused on the ocular specific biofluid (AH) proteins.

All changes in protein levels were normalized to total protein concentration and as such these changes are independent of mean protein concentration. However, interestingly regression modeling demonstrated that total protein concentrations trended towards being significantly greater in AH of glaucomatous compared with non-glaucomatous donors. While this is suggestive of a breakdown in the blood-aqueous barrier [4], whether this is due to the chronic nature of the disease or due to IOP lowering medications, other systemic medications or other co-morbidities is unclear. Nevertheless, the changes in proteins appear to be directly related to pathways with the potential for consequential effects on IOP regulation and outflow homeostasis.

For example, endostatin (Col18A1), which was upregulated in AH of glaucomatous donors, is expressed ubiquitously in ocular tissues except in photoreceptors, and protein fragments that contain endostatin has been observed to accumulate in ocular fluid samples [59]. Interestingly, endostatin has been reported to promote expression and release of thrombospondin-1, implicated in outflow resistance & glaucoma [60-62], in non-ocular endothelial cells [63]. Conversely, endostatin has also been posited to crosstalk with Rho/ROCK, TGFβ, NF-κB, PDGF, and autophagy pathways to yield anti-fibrotic effects [64]. SVEP1, another overexpressed secreted ECM protein in AH & a disease modifier allele for congenital glaucoma [65], is known to be a binding partner for Tie1 and can thus regulate signaling outcomes in lymphatics and vasculature [66]. Since AH predominantly drains into the Schlemm's canal, a unique vessel with both vasculature and lymphatic properties [67], studying SVEP1 in the context of glaucoma warrants further investigation. Of particular interest, VCAM1, CDH11, and BGN were other proteins that

were overexpressed in the AH of glaucomatous patients. SVEP1, CDH11 and VCAM1 were all identified as genes relevant to POAG pathogenesis in a genome-wide meta-analysis across ancestries [68]. CDH11 can interact reciprocally with fibronectin binding protein syndecan-4 to facilitate cell migration and adhesion, partake in EMT, and modulate proliferation [69-72]. In non-ocular cells, CDH11 was shown to mediate adhesion of macrophages to fibroblasts promoting transdifferentiation into myofibroblasts and a self-sustaining profibrotic niche [73]. Biglycan (BGN) is an extracellular proteoglycan, expressed in the trabecular meshwork [74], is posited as a prognostic marker for cancer aggressiveness [75], fibrosis & inflammation [76] and is predicted to be a circulating "messenger" for triggering inflammation and/or autophagy [77]. Whether biglycan in the AH serves as a biomarker, a signaling molecule to trigger autophagy or inflammatory phenotypes in the trabecular meshwork to mediate subsequent outflow regulation requires mechanistic studies. High levels of circulatory VCAM1 in the blood/plasma was found to correlate with ventricular hypertrophy, hypertension and is suggested to thus serve as a predictive soluble biomarker for cardiovascular disease and inflammation [78-80]. In this study, whether VCAM1 expression is a result of chronic elevated IOP, drugs to lower IOP, or due to undiagnosed/unknown underlying systemic vascular conditions is unknown. Regardless, increased presence of the aforementioned proteins may serve as ‘proteins-of-interest’ in mechanistic understanding of outflow regulation for as potential biomarkers for dysregulation in outflow homeostasis.

Since proteins with associations with ‘hypertension’ were identified, we next sought to determine if protein changes were a function of IOP levels through additional regression modeling. Two proteins notably were found to be of importance: DLL4 and NEFL. DLL4, a Notch ligand, is a key regulator of vascular morphogenesis, vessel maturation, and function. Secreted/soluble DLL4 has previously been reported to significantly reduce hydraulic conductivity, vascular permeability, and disrupt endothelial barrier function in non-ocular vasculature [81, 82]. Further, DLL4 has been demonstrated to be critical in developing retinal

vasculature, increased endothelial cell proliferation, and angiogenic sprouting [83]. Interestingly, DLL4 was also shown to inhibit inflammatory choroidal neovascularization despite opposing effects seen in endothelial cells (anti-angiogenic) and macrophages (pro-angiogenic) [84]. Collectively, we speculate that DLL4, found upregulated with IOP, may act to increase outflow resistance via a yet unknown mechanism. Further, whether a dichotomous function for the DLL4 ligand or the Notch pathway exists in outflow regulation remains to be seen. A recent study suggests that Notch pathway protein expression may differ between segmental flow regions of healthy and glaucomatous donors, at least *in vitro* [85]. Thus, ligands circulating in the aqueous humor could differentially impact TM cell function necessitating further studies.

NEFL is a well-established marker of neurodegeneration [42, 44-47, 86] and similar to our finding, has previously been reported to be elevated in glaucomatous AH in humans and animal models [41, 43, 87]. In fact, NEFL had the highest correlation with IOP in all donors in this study, providing additional confidence that NEFL in the AH may indeed be a suitable biomarker for glaucomatous neurodegeneration and ocular hypertension. However, we advise caution that AH samples utilized in this study were from patients identified for glaucoma filtration surgery. Therefore, it is likely that the disease stage may be advanced and thus markers of axonal degeneration are expected within the AH. Whether NEFL would serve as an early marker of disease is unclear and thus requires comprehensive longitudinal natural history investigations. Further the source of the neurofilament protein, determined in the aqueous humor, is unclear (i.e. whether it is due to degeneration of the optic nerve, secretion from cells of neural crest origin (developmentally) in the anterior segment, or Schwann/nerve cells in the anterior segment remains unknown). Interestingly, while NEFL levels correlated with IOP, its levels did not appear to correlate with cup-to-disc ratios of glaucoma donors (**Supplementary Fig. 3**), though other proteins that did correlate were identified. Interestingly, cup-to-disc ratio was independent of IOP values as well (**Supplementary Fig. 3**). Inflammatory proteins (such as interleukins and cathepsins) corresponded with low cup-to-disc ratio, while apolipoprotein A1 (APOA1), albumin,

kininogen-1 (KNG1) and complement C8G correlated with a higher cup-to-disc ratio suggesting proteins altered in the AH may differ based on disease severity. KNG1 is an antiangiogenic molecule that has been suggested to be a marker of neurodegeneration [88] and can impair the proliferation of endothelial cells [89]. To the best of our knowledge, the role of KNG1 in POAG and/or ocular hypertension has not been studied, although its cleaved nonapeptide, Bradykinin (BK), has been the target for IOP lowering investigations [90-95]. A prior study reported a decrease in C8G [96] in glaucomatous AH conflicting with our results. However, Kim et al [97] previously reported C8G to act as a neuroinflammation inhibitor; our result correlating C8G levels with high cup-to-disc ratios may thus reflect the advanced stage of neurodegeneration/neuroinflammation in glaucoma.

Consistent with our study, elevated levels of APOA1 were reported in AH of POAG donors [98]. It is important to note that whether a direct role of APOA1 exists in IOP homeostasis is not known. However, APOA1 plays a critical role in reverse cholesterol transport pathway via direct interactions with the ABCA1 gene whose variants are implicated in POAG [99]. Cholesterol is itself a risk factor for elevated IOP [100]. Elevation in albumin levels in glaucomatous AH is also reported in several studies, though attributed to administration of IOP lowering drugs [101]. Finally, a correlation analysis of NEFL to other proteins identified in glaucomatous aqueous humor revealed that NELF levels may be associated with proteins regulating apoptosis, complement activation, proliferation, and cytoskeletal reorganization. Together, the proteins identified in this study correspond to both markers of neurodegeneration and those that may inhibit proliferation or vascular integrity.

5. Summary

In this study, we demonstrate the ability of the SOMAScan® proteomics platform to identify novel and previously reported proteins in the AH and plasma of glaucomatous donors. We report that proteins identified in AH in part significantly correlate directly with those identified in

plasma. However, we also note that POAG patients were treated with IOP lowering drugs, and as such the effect of medication on proteins identified cannot be delineated. Further, we identified aqueous humor proteins that correlated with either IOP elevation or with reported cup-to-disc ratios. Finally, our data comprehensively demonstrate that proteins identified in diseased AH may be indicative of markers of inflammation, neurodegeneration, and dysregulation of extracellular matrix. Together, we speculate that proteins identified in this study may be useful to the glaucoma scientific community to help identify new proteins/genes of interest and further our understanding in the identification of molecular biomarkers and the mechanisms underlying ocular hypertension and glaucoma.

6. Limitations

This study is not without limitations. One of the major limitations is that the biofluids obtained in this study were obtained at the time of ocular surgery thus representing a singular snapshot in time and no information is gleaned about the stage of the disease; although, IOP measurements and cup-to-disc ratios are available at this stage. Further a comprehensive and longitudinal history of systemic co-morbidities or ocular diseases and medications that may affect systemic or ocular hypertension including but limited to intraocular pressure is unknown. Since IOP in glaucoma patients were controlled with IOP lowering medication, the effect of drugs from disease on proteome changes cannot be adequately unraveled. Further functional measurements for visual field or additional structural deficits including longitudinal and pre-surgical fundus photography or OCT measurements (nerve fiber layer thinning, rim width, ganglion cell analysis etc.) are not known for these patients.

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Figure Legends

Figure 1: Patient demographics demonstrate study participants were age, sex matched. Intraocular pressures are reported only for glaucomatous donors.

Figure 2: Proteins identified in aqueous humor and plasma clustered by type of biofluid, and appear to correlate reasonably well as evidenced by Spearman's correlation. **(A)** Heatmap, and **(B)** histogram of Spearman's correlation index demonstrates that the correlation, while evident, is not absolute i.e. $0.5 < R^2 < 0.7$.

Figure 3: Principal component (PCA) and differential expression analysis demonstrates differences in proteins identified in **(A)** aqueous humor and **(B)** plasma comparing glaucomatous and non-glaucomatous donors. PCA demonstrates large variance between cataract (non-glaucoma) and glaucomatous donor samples with no discernible separation between the two disease groups. Volcano plots demonstrate that no significant differences were seen comparing cataract (non-glaucoma) and glaucomatous donors for most proteins. Few proteins whose levels were significantly altered are represented in the bar plot.

Figure 4: Elastic net regression analysis (aqueous humor only) demonstrated **(A)** increased protein concentration is observed in aqueous humor of donors with glaucoma, with no specific sex related differences observed, **(B)** changes in some proteins correlate significantly with IOP independent of sex of donor. **(C)** Scatter plots and linear regression of proteins ($R^2 > 0.25$) as a function of IOP indicate NEFL, DLL4, and NFE2L1 to most significantly altered proteins in the aqueous humor.

Figure 5: Heatmap demonstrates correlation of NEFL to top 30 proteins identified in the aqueous humor of glaucomatous donors. Pearson's correlation analysis comparing NEFL with rest of the proteins identified ~30 proteins that either positively (red) or negatively (blue) correlated with NEFL ($|r > 0.4|$). In general, more proteins that negatively correlated with NEFL levels were identified than those that exhibited positive correlation.

Figure 6: Heatmap identifies top 6 proteins either up or down regulated in the aqueous humor of glaucomatous donors as a function of cup-to-disc ratios.

Supplementary figure 1: Outlier and principal component analysis identifying donors to exclude from data set in an unbiased manner.

Supplementary figure 2: Scatter plots of proteins identified in glaucomatous AH as a function of IOP demonstrate large variability between samples.

Supplementary figure 3: (A) Scatter plot of NEFL expression levels as a function of cup-to-disc ratios of glaucomatous donors demonstrate no significant relationship ($R^2 = 0.037$) between the two parameters. **(B)** Cup-to-disc ratios of glaucomatous donors show no significant correlation ($R^2 = 0.008$) with intraocular pressures reported.

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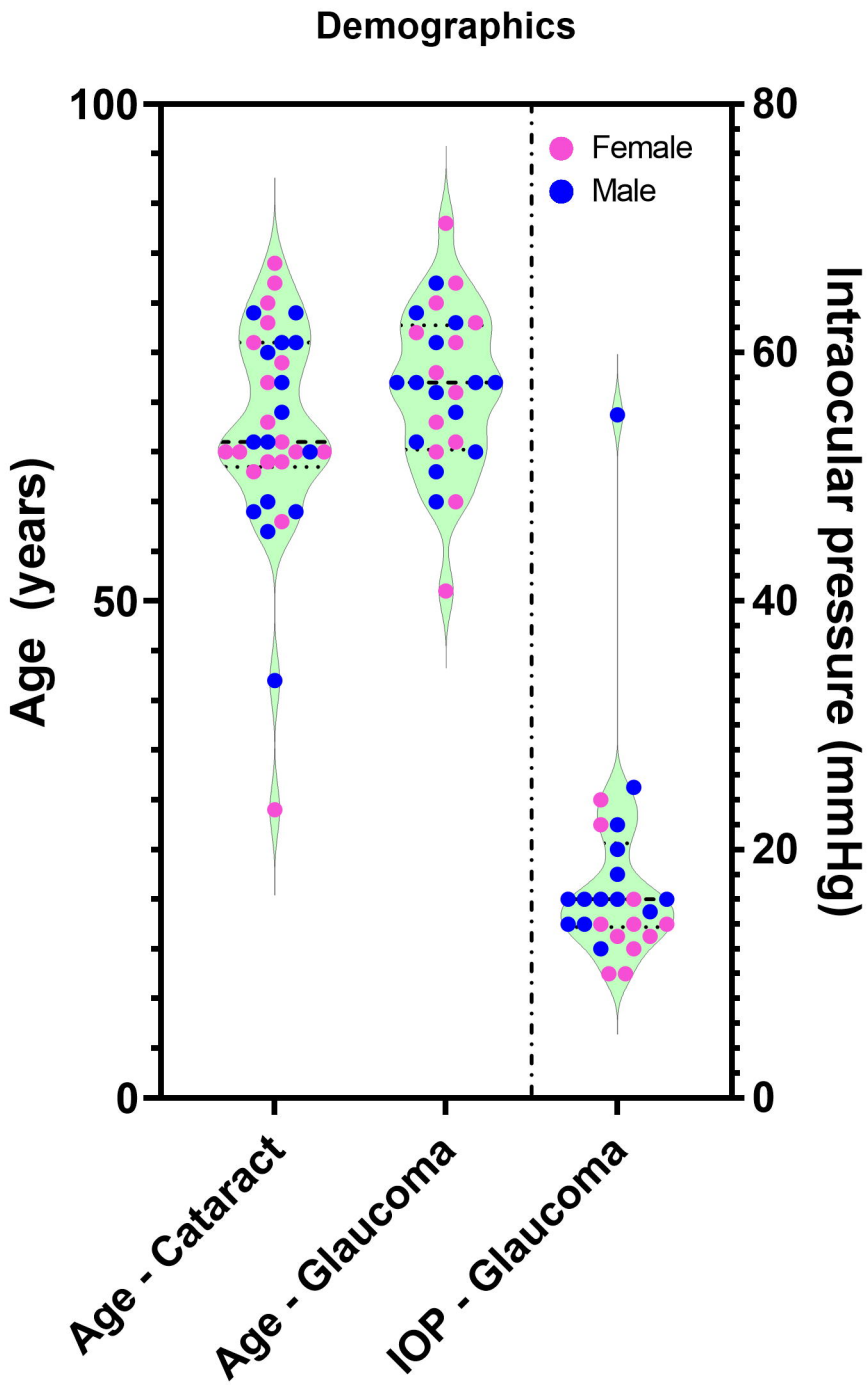
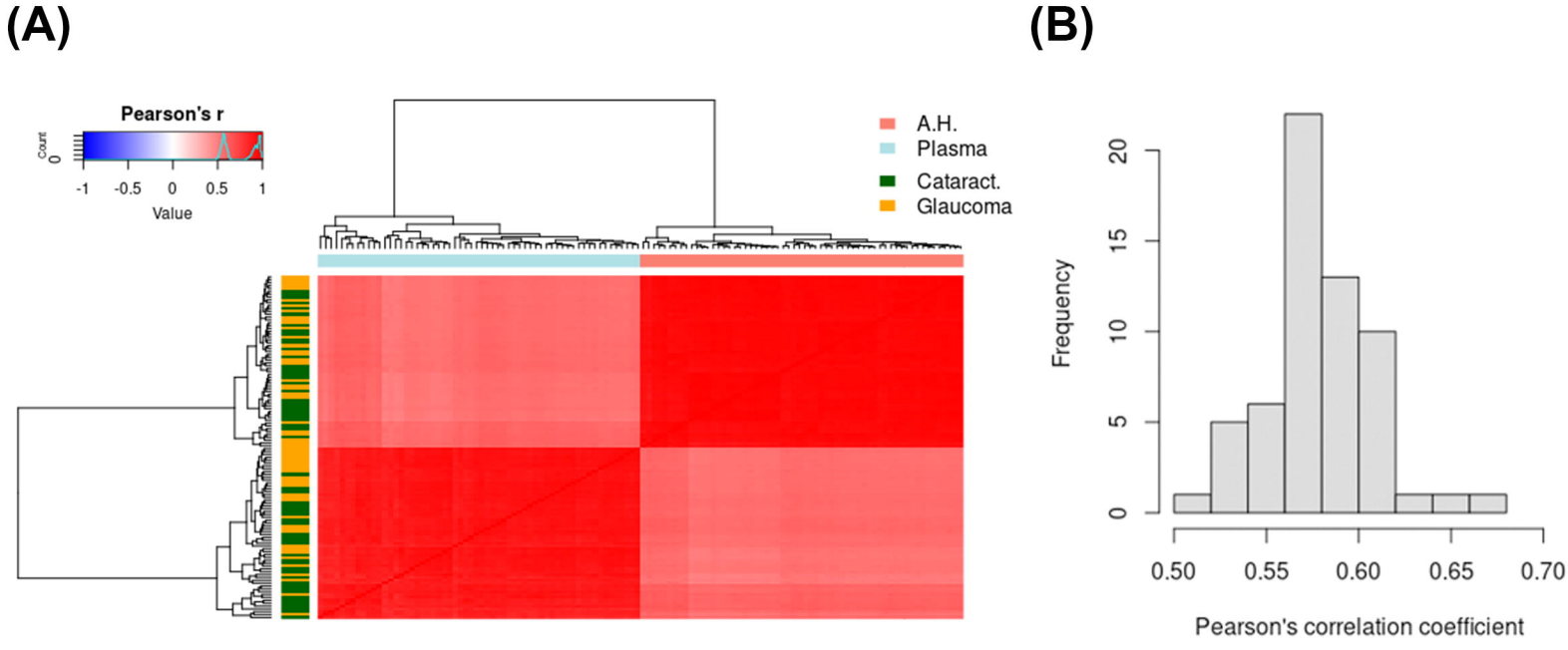
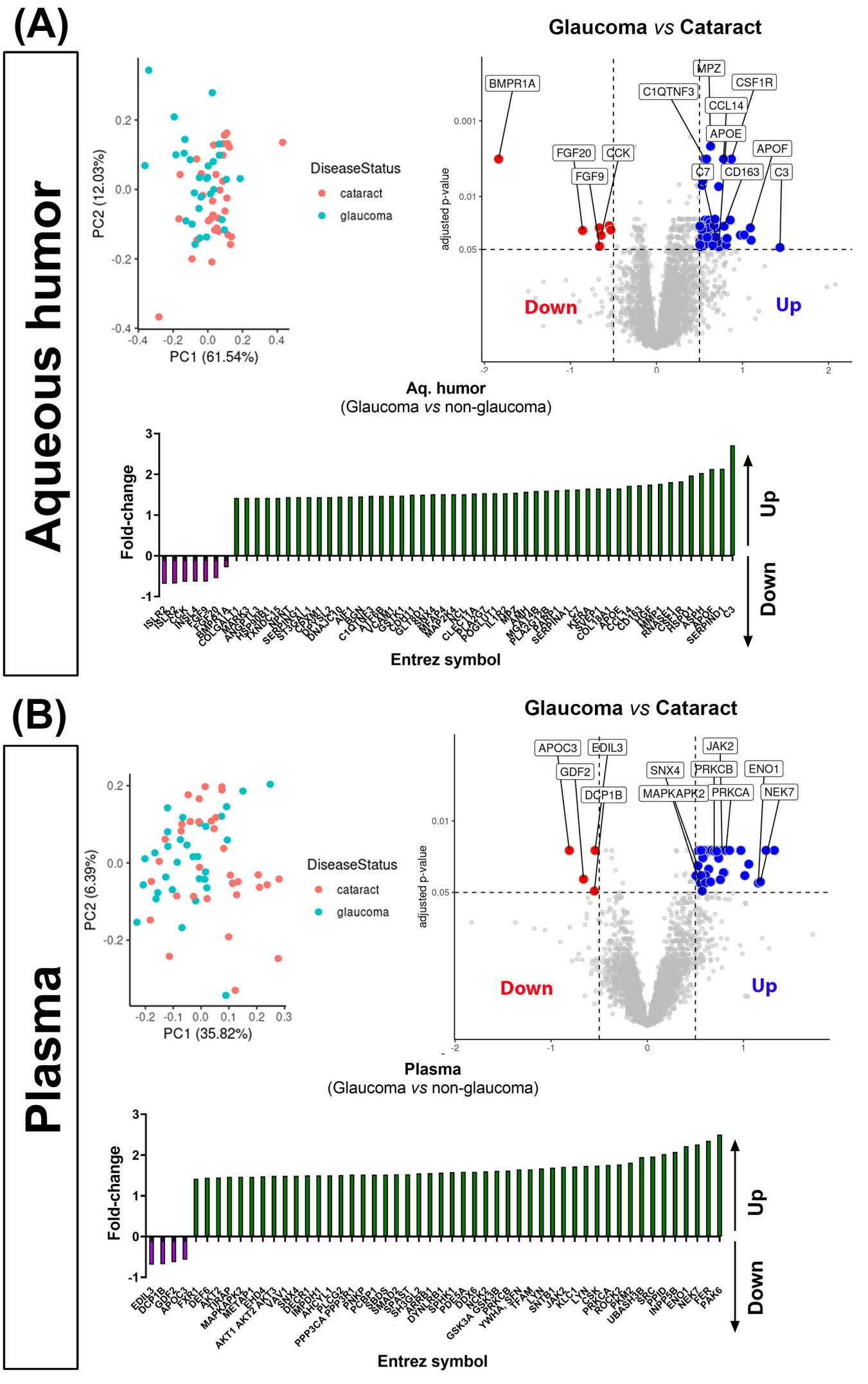
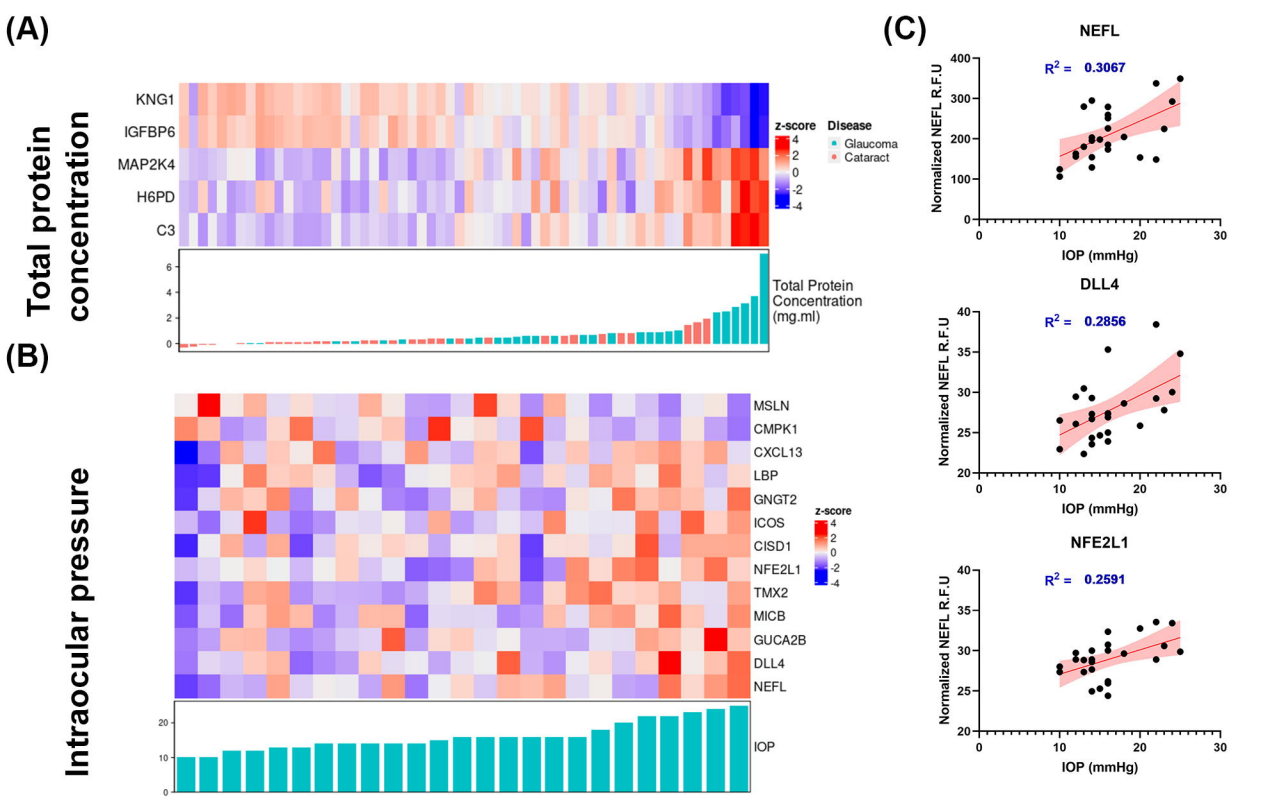
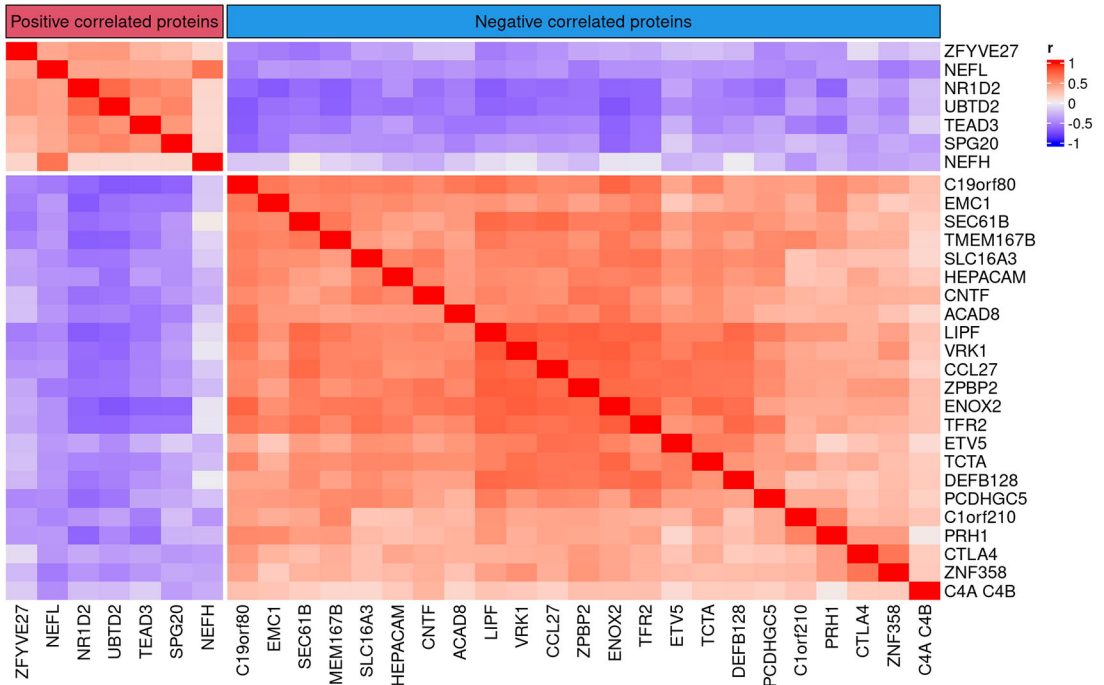


Condition	Gender	# of patients	Age	IOP (mmHg)
Cataract	Male	16	66.7 ± 10.2	-n/a-
	Female	19	67.7 ± 12.2	-n/a-
Glaucoma	Male	17	71.2 ± 6.3	19.6 ± 10.7
	Female	17	71.2 ± 9.9	15.4 ± 4.9


(A)
Pearson's r
Count
0
-1
-0.5
0
0.5
1
Value
A.H.
Plasma
Cataract.
Glaucoma
(B)
Frequency
0
5
10
15
20
0.50
0.55
0.60
0.65
0.70
Pearson's correlation coefficient


(A)
Aqueous humor
Glaucoma vs Cataract
PC2 (12.03%)
PC1 (61.54%)
DiseaseStatus
cataract
glaucoma
adjusted p-value
BMPR1A
MPZ
C1QTNF3
CSF1R
CCL14
APOE
APOF
FGF20
CCK
FGF9
C7
CD163
C3
Down
Up
Aq. humor
(Glaucoma vs non-glaucoma)
Fold-change
Up
Down
Entrez symbol
(B)
Plasma
Glaucoma vs Cataract
PC2 (6.39%)
PC1 (35.82%)
DiseaseStatus
cataract
glaucoma
adjusted p-value
APOC3
EDIL3
JAK2
GDF2
SNX4
PRKCB
ENO1
DCP1B
MAPKAPK2
PRKCA
NEK7
Down
Up
Plasma
(Glaucoma vs non-glaucoma)
Fold-change
Up
Down
Entrez symbol


(A)
Total protein concentration
KNG1
IGFBP6
MAP2K4
H6PD
C3
z-score
4
2
0
-2
-4
Disease
Glaucoma
Cataract
6
4
2
0
Total Protein Concentration (mg.ml)
(B)
Intraocular pressure
MSLN
CMPK1
CXCL13
LBP
GNGT2
ICOS
CISD1
NFE2L1
TMX2
MICB
GUCA2B
DLL4
NEFL
z-score
4
2
0
-2
-4
20
10
0
IOP
(C)
NEFL
R^2 = 0.3067
Normalized NEFL R.F.U
400
300
200
100
0
0
10
20
30
IOP (mmHg)
DLL4
R^2 = 0.2856
Normalized NEFL R.F.U
35
30
25
20
0
10
20
30
IOP (mmHg)
NFE2L1
R^2 = 0.2591
Normalized NEFL R.F.U
35
30
25
20
0
10
20
30
IOP (mmHg)


Positive correlated proteins
Negative correlated proteins
r
1
0.5
0
-0.5
-1
ZFYVE27
NEFL
NR1D2
UBTD2
TEAD3
SPG20
NEFH
C19orf80
EMC1
SEC61B
TMEM167B
SLC16A3
HEPACAM
CNTF
ACAD8
LIPF
VRK1
CCL27
ZPBP2
ENOX2
TFR2
ETV5
TCTA
DEFB128
PCDHGC5
C1orf210
PRH1
CTLA4
ZNF358
C4A C4B

IL12A IL12B
LRRC3
ZAP70
CTSG
DEFB115
NSG1
KLRG2
C8G
PENK
ALB
APOA1
KNG1
G.AH.26
G.AH.30
G.AH.33
G.AH.29
G.AH.31
G.AH.10
G.AH.11
G.AH.24
G.AH.27
G.AH.34
G.AH.35
G.AH.36
G.AH.37
G.AH.15
G.AH.16
G.AH.19
G.AH.25
G.AH.7
G.AH.1
G.AH.12
G.AH.13
G.AH.28
G.AH.3
G.AH.4
G.AH.8
G.AH.9
0.6
0.8
1
cup disc ratio
z-score
4
2
0
-2
-4