The Constitutive Proteome of Human Aqueous Humor and Race Specific Alterations

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Abstract: Aqueous humor (AH) is the fluid in the anterior and posterior chambers of the eye that contains proteins regulating ocular homeostasis. Analysis of aqueous humor proteome is challenging, mainly due to low sample volume and protein concentration. In this study, by utilizing state of the art technology, we performed Liquid-Chromatography Mass spectrometry (LC-MS/MS) analysis of 88 aqueous humor samples from subjects undergoing cataract surgery. A total of 2263 unique proteins were identified, which were sub-divided into four categories that were based on their detection in the number of samples: High ($n=152$), Medium ($n=91$), Low ($n=128$), and Rare ($n=1892$). A total of 243 proteins detected in at least 50% of the samples were considered as the constitutive proteome of human aqueous humor. The biological processes and pathways enriched in the AH proteins mainly include vesicle mediated transport, acute phase response signaling, LXR/RXR activation, complement system, and secretion. The enriched molecular functions are endopeptidase activity, and various binding functions, such as protein binding, lipid binding, and ion binding. Additionally, this study provides a novel insight into race specific differences in the AH proteome. A total of six proteins were upregulated, and five proteins were downregulated in African American subjects as compared to Caucasians.

Keywords: aqueous humor; mass spectrometry; proteomics

1. Introduction

Aqueous humor (AH) is the fluid in the anterior and posterior chambers of the eye. It is produced by the non-pigmented ciliary body epithelium primarily through active transport of ions and solutes into the posterior chamber [1–4]. From the posterior chamber, the AH enters the anterior chamber via the lens and iris. After supporting the metabolic requirements of the avascular tissues of the anterior segment, the AH mainly exits the eye via the trabecular meshwork/Schlemm’s canal into the episcleral veins, known as conventional outflow. AH outflow also occurs via an alternative route through the ciliary muscle bundles into the supraciliary and suprachoroidal spaces, which is known as uveoscleral outflow [5].

AH is an integral component in many ocular health functions, including nutrient and oxygen supply, the removal of metabolic waste, ocular immunity, and ocular shape and refraction [6–8]. The dynamics of AH and the fine balance between production and drainage is essential in maintaining the physiological intraocular pressure (IOP) [2].
The major constituents of AH are water, electrolytes, organic solutes, cytokines, growth factors, and proteins [3,9–11]. The protein concentration in AH is in the range of 150 to 500 µg/mL [2]. Although proteins in AH are present in relatively low concentrations when compared to blood plasma, they are vital in the maintenance of anterior segment homeostasis [2,8,12–19]. Previous studies have shown significant alterations in several proteins in the AH obtained from eyes with glaucoma [12–15,20,21] and other eye disorders, including age-related macular degeneration [14,16,22–25].

Therefore, identifying the protein contents of AH is vital in understanding their physiological and pathological roles in the eye. However, given the low protein concentration and small volume, traditional low throughput approaches are not suitable for the proteomic analysis of AH. Liquid-chromatography Mass spectrometry (LC-MS/MS) has emerged as the analytical method of choice because of its high throughput nature, sensitivity, high dynamic range, and ability to identify complex mixtures even from small sample volumes [26,27]. However, many experimental and data-analytical hurdles exist, hampering the reliability and reproducibility of the data. In LC-MS/MS profiles, the proteins with high concentrations have a higher chance of detection, whereas the proteins with lower concentrations are detected only in a smaller percentage of samples due to random chance. It is exceedingly difficult to draw statistical conclusions for the proteins with a very low detection rate and should be excluded at the data analysis step. However, making such decisions that are based on the smaller sample set can lead to poor reproducibility. Therefore, a reference list of AH proteins detected reliably while using a larger sample set may be helpful in alleviating these concerns.

In this study, we identified the constitutive proteome of human aqueous humor, which may be useful as a reference for future studies, by utilizing a large sample set, state of the art technology, and revolutionary data analysis methods. Based on their abundance, the proteins were sub-divided into four (high, medium, low, and rare) categories. Interestingly, a comparison of African American and Caucasian subjects led us to the discovery of race-specific differences in the AH proteome, which are also presented in this study.

2. Materials and Methods

2.1. Human Subjects and Sample Collection

Aqueous humor samples were collected from 88 subjects undergoing cataract surgery at the Department of Ophthalmology, Medical College of Georgia at Augusta University. During these surgical procedures, a corneal incision is made, through which the aqueous humor fluid is evacuated from the anterior chamber and discarded. Instead of discarding, the AH samples were aspirated from the anterior chamber and collected in Eppendorf tubes. This method of sample collection is safe and efficient and it does not pose any risk to the subjects. The study was approved by the Institutional Review Board (IRB# 611480-13) at Augusta University, and written informed consent was obtained from all of the study participants. A chart review was conducted for all subjects to record their age, race, gender, smoking history, presence of systemic and ocular diseases, and IOP levels. Table 1 shows the characteristics of the study participants.

| Table 1. Characteristics of study participants. |
|-------------------------------|-------------|
| Characteristics               | Count       |
| Subjects, (n)                 | 88          |
| Sex: F/M                      | 55/33       |
| Age (years)                   | 67.0 ± 9.56 |
| Race: AA/Caucasian            | 66/22       |
| Hypertension, N/Y             | 41/47       |
| Smoking, N/Y                  | 56/32       |
| Cardiovascular disease, N/Y   | 81/7        |
| Cerebrovascular disease, N/Y  | 87/1        |
| Collagen vascular disease, N/Y| 75/13       |
| Intraocular Pressure (IOP)    | 19.5 ± 7.01 |
2.2. Aqueous Humor Sample Preparation

The aim of this study was to characterize all of the proteins present in the human aqueous humor and we did not utilize immunodepletion to remove abundant proteins. Aqueous humor samples (60 µL) were lyophilized and subsequently reconstituted in 30 µL of 8 M urea in 50 mM Tris-HCl (pH 8). 20 mM Dithiothreitol (DTT) was then added to the mixture in order to reduce cysteine residues, followed by alkylation with 55 mM iodoacetamide. 240 µL of 50 mM ammonium bicarbonate buffer was added in order to reduce urea concentration to below 1 M. Total protein concentration was measured while using a Bradford assay kit (Pierce, Rockford, IL, USA), according to the manufacturer’s instructions. The digestion of proteins was performed using a 1:20 ratio (w/w) of Trypsin (Pierce, Rockford, IL, USA) at 37 °C overnight. Figure 1 shows a schematic of the workflow involved in the AH sample preparation and proteomic quantification.

2.3. LC-MS/MS Analysis

The trypsin-digested samples were analyzed using an Orbitrap Fusion Tribrid mass spectrometer coupled with an Ultimate 3000 nano-UPLC system in order to perform in-depth proteomic characterization. Reconstituted peptides (6 µL) were trapped and washed on a Pepmap100 C18 trap at the rate of 20 µL/min using a gradient of 2% acetonitrile in water with 0.1% formic acid for 10 min. Subsequently, the peptide mixture was separated on a Pepmap100 RSCLC C18 column using a gradient of 2% to 40% acetonitrile with 0.1% formic acid for 120 min at a flow rate of 300 nL/min. Eluted peptides from the column were introduced into the Mass Spectrometer via nano-electrospray ionization source (temperature: 275 °C; spray voltage: 2000 V) and analyzed via data-dependent acquisition in positive mode. Orbitrap MS analyzer was used for precursor scan at 120,000 FWHM from 300 to 1500 m/z. MS/MS scans were taken while using an ion-trap MS analyzer in top speed mode (2-s cycle time) with dynamic exclusion settings (repeat count 1, repeat duration 15 s, and exclusion duration 30 s). Collision-induced dissociation (CID) was used as a fragmentation method with 30% normalized
collision energy. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [28] partner repository with the dataset identifier PXD022463.

2.4. Protein Identification and Quantification

For the protein identification and quantification, raw MS data were processed using Proteome Discoverer software (ver 1.4; Thermo Scientific, Waltham, MA, USA) and then submitted for SequestHT search against the reviewed-manually annotated Uniprot-SwissProt human database with 20,385 entries. The following search parameters were used: 10 ppm precursor mass tolerance and 0.6 Da product ion tolerance; static carbamidomethylation (+57.021 Da) for cysteine, dynamic oxidation (+15.995 Da) for methionine, and dynamic phosphorylation (+79.966 Da) for serine, threonine, and tyrosine. Proteins that contain similar peptides, which cannot be differentiated based on MS/MS analysis alone, were grouped in order to satisfy the principles of parsimony. A report comprising the identities and spectrum counts (number of peptide-spectrum match) for each protein was then exported as a semi-quantitative measure for relative protein levels that were detected in the AH sample. Figure 2 shows an example of LC-MS/MS analysis of one AH sample.

![Figure 2](image)

**Figure 2.** Example of LC-MS/MS analysis of human aqueous humor sample. (A) LC-MS/MS total ion current chromatogram. The retention time (RT) elution of one reporter peptide indicative of A2M protein is marked for illustration purposes. (B) MS spectra of selected precursor peptide 444.78 m/z with a distinct isotopic pattern benefited from the high resolution of the Orbitrap MS analyzer. (C) MS/MS spectra using collision-induced dissociation (CID) fragmentation of A2M (444.78 m/z) precursor peptide, colored peaks (red for b ions and blue for y ions) indicate matches between experimental and theoretical/calculated values.

2.5. Statistical Analysis

The peptide-spectrum match (PSM) values from LC-MS/MS analysis were quantile normalized, and then log2 transformed to achieve normal distribution. For each protein, the detection rate
(proportion of samples in which the protein was detected) was quantified. The proteins that were detected in a majority of samples (>50%) were examined in detail to see whether certain protein families were enriched in human AH. These commonly expressed proteins were also associated with gene ontology terms, including biological processes, cellular components, and molecular functions, using the “goana” function from “limma” (ver.3.40.6) R package. Adjusting for confounding variables, including age, sex and hypertension, differential expression analyses were performed using negative binomial regression, in order to discover differences in protein levels between African American and Caucasian subjects. The p-values were adjusted for multiple testing using the FDR method. All of the statistical analyses were performed using the R Project for Statistical Computing (version 3.5.1).

3. Results

3.1. Protein Content of the Human Aqueous Humor

A total of 2263 unique proteins were identified in 88 aqueous humor samples (Table S1). These proteins were divided into four categories that were based on their detection in the number of samples: High (n = 152; detected in >75% of samples), Medium (n = 91; detected in 50–75% of samples), Low (n = 128; detected in 25–50% of samples), and Rare (n = 1892, detected in <25% of samples) (Figure 3A). Figure 3B shows the sample-to-sample variation in the levels of these proteins (the coefficient of variation). The majority of proteins in the “High” group show low sample-to-sample variation, indicating the uniformity of expression across samples. As the mean expression decreases, the coefficient of variation increases from high to rare proteins. Table 2 shows a complete list of 152 proteins found in at least 75% of AH samples.

![Figure 3](image-url)

**Figure 3.** Distribution of the mean values (A) and coefficient of variation (B) of proteins detected in the human aqueous humor samples. The proteins were subdivided into four categories, based on their detection rate. High: detected in >75%; Medium: detected in 50–75%; Low: detected in 25–50%; Rare: detected in <25% of the samples. Coefficient of variation decreases as mean protein expression increases.
| Uniprot ID | Gene Symbol | Description                               | Detected in Proportion of Samples (%) | Mean PSM Value |
|------------|-------------|-------------------------------------------|---------------------------------------|----------------|
| P02768     | ALB         | Albumin                                   | 100.00                                 | 4202.61        |
| P02787     | TF          | Serotransferrin                            | 100.00                                 | 765.31         |
| P01024     | C3          | Complement C3                              | 100.00                                 | 255.00         |
| P101009    | SERPINA1    | Alpha-1-antitrypsin                       | 100.00                                 | 233.24         |
| P02790     | HPX         | Hemopexin                                 | 100.00                                 | 176.64         |
| P01859     | IGHG2       | Immunoglobulin heavy constant gamma 2     | 100.00                                 | 168.92         |
| P10745     | RBP3        | Retinol-binding protein 3                 | 100.00                                 | 166.25         |
| P00450     | CP          | Ceruloplasmin                              | 100.00                                 | 165.20         |
| P01834     | IGKC        | Immunoglobulin kappa constant             | 100.00                                 | 161.42         |
| P02766     | TTR         | Transthyretin                              | 100.00                                 | 156.30         |
| P41222     | PTGD5       | Prostaglandin-H2 D-isomerase              | 100.00                                 | 142.25         |
| P36955     | SERPINF1    | Pigment epithelium-derived factor          | 100.00                                 | 141.33         |
| P01860     | IGHG3       | Immunoglobulin heavy constant gamma 3     | 100.00                                 | 127.65         |
| P02763     | ORM1        | Alpha-1-acid glycoprotein 1               | 100.00                                 | 119.16         |
| P02774     | GC          | Vitamin D-binding protein                 | 100.00                                 | 115.89         |
| P0DOX7     | N/A         | Immunoglobulin kappa light chain          | 100.00                                 | 108.22         |
| P10909     | CLU         | Clusterin                                 | 100.00                                 | 102.33         |
| P02647     | APOA1       | Apolipoprotein A-I                        | 100.00                                 | 96.78          |
| P01034     | CST3        | Cystatin-C                                | 100.00                                 | 82.73          |
| P02765     | AHSG        | Alpha-HS-glycoprotein                     | 100.00                                 | 73.27          |
| P01023     | A2M         | Alpha-2-macroglobulin                     | 100.00                                 | 72.77          |
| P19652     | ORM2        | Alpha-1-acid glycoprotein 2               | 100.00                                 | 68.70          |
| P06396     | GSN         | Gelsolin                                  | 100.00                                 | 62.66          |
| P01008     | SERPINC1    | Antithrombin-III                          | 100.00                                 | 62.65          |
| P02749     | APOH        | Beta-2-glycoprotein 1                     | 100.00                                 | 59.09          |
| P04217     | A1BG        | Alpha-1B-glycoprotein                     | 100.00                                 | 59.04          |
| P22352     | GPX3        | Glutathione peroxidase 3                  | 100.00                                 | 58.23          |
| Q9UBM4     | DPK3        | Dickkopf-related protein 3                | 100.00                                 | 56.91          |
| P01011     | SERPIN3     | Alpha-1-antichymotrypsin                  | 100.00                                 | 53.15          |
| P01876     | IGHA1       | Immunoglobulin heavy constant alpha 1     | 100.00                                 | 51.83          |
| Q12805     | EFEMP1      | EGF-containing fibulin-like extracellular matrix protein 1 | 100.00 | 50.79 |
| P00751     | CFB         | Complement factor B                       | 100.00                                 | 50.35          |
| Q13822     | ENPP2       | Ectonucleotide pyrophosphatase/phosphodiesterase family member 2 | 100.00 | 45.77 |
| P00747     | PLG         | Plasminogen                               | 100.00                                 | 45.45          |
| Q9UBM4     | OPTC        | Opticin                                   | 100.00                                 | 43.07          |
| P07339     | CTDS        | Cathepsin D                               | 100.00                                 | 39.10          |
| P00734     | F2          | Prothrombin                               | 100.00                                 | 38.21          |
| P05155     | SERPING1    | Plasma protease C1 inhibitor              | 100.00                                 | 35.94          |
| P10451     | SPP1        | Osteopontin                               | 100.00                                 | 35.94          |
| Uniprot ID | Gene Symbol | Description                  | Detected in Proportion of Samples (%) | Mean PSM Value |
|-----------|-------------|-------------------------------|---------------------------------------|----------------|
| P06727    | APOA4       | Apolipoprotein A-IV           | 100.00                                | 33.93          |
| P02649    | APOE        | Apolipoprotein E              | 100.00                                | 32.43          |
| P01042    | KNG1        | Kininogen-1                   | 100.00                                | 30.10          |
| P04196    | HRG         | Histidine-rich glycoprotein   | 100.00                                | 29.90          |
| P25311    | AZGP1       | Zinc-alpha-2-glycoprotein     | 100.00                                | 29.67          |
| P07998    | RNASE1      | Ribonuclease pancreatic       | 100.00                                | 29.30          |
| Q04985    | CLSTN1      | Calsyntenin-1                 | 100.00                                | 27.54          |
| P01019    | AGT         | Angiotensinogen               | 100.00                                | 26.00          |
| P02760    | AMBP        | Protein AMBP                  | 100.00                                | 24.23          |
| P02652    | APOA2       | Apolipoprotein A-II           | 100.00                                | 20.83          |
| P36222    | CHI3L1      | Chitinase-3-like protein 1    | 100.00                                | 20.03          |
| P23314    | FBLN1       | Fibulin-1                     | 100.00                                | 19.91          |
| P02750    | LRG1        | Leucine-rich alpha-2-glycoprotein | 100.00                  | 18.87          |
| P05156    | CFI         | Complement factor I           | 100.00                                | 18.82          |
| P02753    | RBP4        | Retinol-binding protein 4     | 100.00                                | 17.09          |
| P04004    | VTN         | Vitronectin                   | 100.00                                | 16.58          |
| Q16270    | IGBP7       | Insulin-like growth factor-binding protein 7 | 100.00   | 15.10          |
| P51884    | LUM         | Lumican                       | 100.00                                | 13.89          |
| P00746    | CFD         | Complement factor D           | 100.00                                | 13.10          |
| P05997    | APD         | Apolipoprotein D              | 100.00                                | 12.66          |
| O43505    | B4GAT1      | Beta-1,4-glucuronyltransferase 1 | 100.00                  | 12.09          |
| P24592    | IGBP6       | Insulin-like growth factor-binding protein 6 | 100.00   | 11.28          |
| Q26905    | PGLYRP2     | N-acetylmuramoyl-L-alanine amidase | 100.00                  | 11.19          |
| Q14515    | SPARC1      | SPARC-like protein 1          | 100.00                                | 11.01          |
| P61916    | NPC         | NPC intracellular cholesterol transporter 2 | 100.00     | 9.57           |
| Q09969    | RARE2       | Retinoic acid receptor responder protein 2 | 100.00     | 8.03           |
| P61769    | B2M         | Beta-2-microglobulin          | 100.00                                | 7.77           |
| Q09774    | HSDBH10     | 3-hydroxyacyl-CoA dehydrogenase type-2 | 100.00    | 7.61           |
| Q06481    | APLP2       | Amyloid-like protein 2        | 100.00                                | 7.12           |
| P43652    | AFM         | Afamin                        | 98.86                                 | 30.95          |
| Q819J3    | CPAMD8      | C3 and PZP-like alpha-2-macroglobulin domain-containing protein 8 | 98.86   | 17.18          |
| Q17476    | LTBP2       | Latent-transforming growth factor beta-binding protein 2 | 98.86  | 15.14          |
| Q15582    | TGBF1       | Transforming growth factor-beta-induced protein Ig-h3 | 98.86 | 14.68          |
| Q97J8S    | WIFI1       | Wnt inhibitory factor 1       | 98.86                                 | 14.39          |
| Q06969    | SPOCK1      | Testican-1                    | 98.86                                 | 8.93           |
| P07749    | PSAP        | Prosaposin                    | 98.86                                 | 7.48           |
| Q08380    | LGALS3BP    | Galectin-3-binding protein    | 98.86                                 | 6.67           |
| P00748    | F12         | Coagulation factor XII        | 98.86                                 | 6.06           |
Table 2. Cont.

| Uniprot ID | Gene Symbol | Description | Detected in Proportion of Samples (%) | Mean PSM Value |
|------------|-------------|-------------|----------------------------------------|----------------|
| P0DOY2     | IGLC2       | Immunoglobulin lambda constant 2 | 97.73 | 75.13 |
| P16670     | CPE         | Carboxypeptidase E               | 97.73 | 18.17 |
| Q9HCB6     | SPON1       | Spondin-1                        | 97.73 | 6.06  |
| P06312     | IGKV4-1     | Immunoglobulin kappa variable 4-1 | 97.73 | 5.19  |
| P51888     | PRELP       | Prolargin                         | 97.73 | 5.19  |
| P0DOX8     | N/A         | Immunoglobulin lambda-1 light chain | 96.59 | 64.10 |
| P00738     | HP          | Haptoglobin                       | 96.59 | 43.35 |
| P08603     | CFH         | Complement factor H               | 96.59 | 20.12 |
| Q8NG11     | TSPAN14     | Tetraspanin-14                    | 96.59 | 17.09 |
| Q14624     | ITIH4       | Inter-alpha-trypsin inhibitor heavy chain H4 | 96.59 | 14.89 |
| Q99972     | MYOC        | Myocilin                          | 96.59 | 8.76  |
| Q9NQ79     | CRTAC1      | Cartilage acidic protein 1        | 96.59 | 8.24  |
| P01861     | IGHG4       | Immunoglobulin heavy constant gamma 4 | 96.59 | 99.95 |
| P35555     | FBN1        | Fibrillin-1                       | 96.59 | 12.27 |
| Q9B5C5     | RTBDN       | Retbindin                         | 96.59 | 6.64  |
| P05452     | CLEC3B      | Tetranection                      | 96.59 | 6.41  |
| Q092520    | FAM3C       | Protein FAM3C                     | 96.59 | 5.67  |
| Q86U78     | GTF2IRD2    | General transcription factor II-I repeat domain-containing protein 2A | 95.45 | 5.51  |
| P02748     | C9          | Complement component C9           | 94.32 | 13.93 |
| Q14773     | TTP1        | Tripeptidyl-peptidase 1           | 94.32 | 5.24  |
| P01033     | TIMP1       | Metalloproteinase inhibitor 1     | 94.32 | 3.64  |
| Q15113     | PCOLCE      | Procollagen C-endopeptidase enhancer 1 | 94.32 | 3.45  |
| P01700     | IGLV1-47    | Immunoglobulin lambda variable 1-47 | 93.18 | 4.61  |
| P0C0L5     | C4B         | Complement C4-B                   | 92.05 | 124.58|
| P086972    | SERPINF2    | Alpha-2-antiplasmin               | 92.05 | 9.18  |
| A0A0C4DH25 | IGKV3D-20   | Immunoglobulin kappa variable 3D-20 | 92.05 | 6.28  |
| P10643     | C7          | Complement component C7           | 92.05 | 4.08  |
| P19823     | CDH2        | Cadherin-2                        | 92.05 | 3.83  |
| Q5T3U5     | ABCC10      | Multidrug resistance-associated protein 7 | 90.91 | 10.06 |
| P51693     | APLP1       | Amyloid-like protein 1            | 89.77 | 4.08  |
| P07357     | CA8         | Complement component C8 alpha chain | 89.77 | 3.88  |
| P20273     | CD22        | B-cell receptor CD22              | 88.64 | 15.02 |
| P98160     | HSPG2       | Basement membrane-specific heparan sulfate proteoglycan core protein | 88.64 | 5.82  |
| Q5T8P6     | RBM26       | RNA-binding protein 26            | 88.64 | 4.68  |
| Q9Y287     | ITM2B       | Integral membrane protein 2B      | 88.64 | 3.43  |
| P39060     | COL18A1     | Collagen alpha-1(XVIII) chain     | 88.64 | 2.36  |
| P18065     | IGFBP2      | Insulin-like growth factor-binding protein 2 | 87.50 | 3.78  |
Table 2. Cont.

| Uniprot ID | Gene Symbol | Description | Detected in Proportion of Samples (%) | Mean PSM Value |
|------------|-------------|-------------|---------------------------------------|----------------|
| P10645     | CHGA        | Chromogranin-A | 87.50                                 | 3.71           |
| P05067     | APP         | Amyloid-beta precursor protein | 86.36                                 | 10.08          |
| P08294     | SOD3        | Extracellular superoxide dismutase [Cu-Zn] | 86.36                                 | 5.86           |
| Q92765     | FRZB        | Secreted frizzled-related protein 3 | 86.36                                 | 4.79           |
| Q96596     | PEBP4       | Phosphatidylethanolamine-binding protein 4 | 86.36                                 | 3.76           |
| P01780     | IGHV3-7     | Immunoglobulin heavy variable 3-7 | 85.23                                 | 4.97           |
| Q9HJC0     | TNRC6C      | Trinucleotide repeat-containing gene 6C protein | 85.23                                 | 3.70           |
| O15240     | VGF         | Neurosecretory protein VGF | 85.23                                 | 2.74           |
| P02675     | FGB         | Fibrinogen beta chain | 84.09                                 | 6.75           |
| P55083     | MFAP4       | Microfibril-associated glycoprotein 4 | 84.09                                 | 2.81           |
| P22914     | CRYGS       | Gamma-crystallin S | 82.95                                 | 6.40           |
| P00441     | SOD1        | Superoxide dismutase [Cu-Zn] | 82.95                                 | 2.84           |
| P34096     | RNASE4      | Ribonuclease 4 | 82.95                                 | 1.81           |
| Q9Y6R7     | FCGBP       | IgGFc-binding protein | 81.82                                 | 6.89           |
| O75326     | SEMA7A      | Semaphorin-7A | 81.82                                 | 5.49           |
| P00736     | C1R         | Complement C1r subcomponent | 81.82                                 | 4.49           |
| Q9BZV3     | IMPG2       | Interphotoreceptor matrix proteoglycan 2 | 80.68                                 | 3.07           |
| A0A087WSY6 | IGKV3D-15   | Immunoglobulin kappa variable 3D-15 | 80.68                                 | 2.17           |
| P61260     | LYZ         | Lysozyme C | 79.55                                 | 4.83           |
| P43251     | BTN         | Biotinidase | 79.55                                 | 2.71           |
| P06733     | ENO1        | Alpha-enolase | 78.41                                 | 12.05          |
| P29622     | SERPINA4    | Kallistatin | 78.41                                 | 5.03           |
| P05546     | SERPIND1    | Heparin cofactor 2 | 78.41                                 | 5.03           |
| P08185     | SERPINA6    | Corticosteroid-binding globulin | 78.41                                 | 3.86           |
| Q7Z7G0     | ABI3BP      | Target of Nesh-SH3 | 78.41                                 | 2.72           |
| P04406     | GAPDH       | Glyceraldehyde-3-phosphate dehydrogenase | 77.27                                 | 5.63           |
| P01593     | IGKV1D-33   | Immunoglobulin kappa variable 1D-33 | 77.27                                 | 2.93           |
| P02490     | FGG         | Fibrinogen gamma chain | 76.14                                 | 4.92           |
| Q66K66     | TMEM198     | Transmembrane protein 198 | 76.14                                 | 4.40           |
| P02656     | APOC3       | Apolipoprotein C-III | 76.14                                 | 3.19           |
| P13671     | C6          | Complement component C6 | 76.14                                 | 2.96           |
| Q99574     | SERPINI1    | Neuroserpin | 76.14                                 | 2.38           |
| P04264     | KRT1        | Keratin, type II cytoskeletal 1 | 75.00                                 | 36.86          |
| Q9UHG2     | PCSK1N      | ProSAAS | 75.00                                 | 3.28           |
| P08571     | CD14        | Monocyte differentiation antigen CD14 | 75.00                                 | 2.48           |
| Q862D1     | OAF         | Out at first protein homolog | 75.00                                 | 2.12           |
| P98164     | LRP2        | Low-density lipoprotein receptor-related protein 2 | 75.00                                 | 1.84           |
3.2. Major Protein Families Detected in the Human Aqueous Humor

Five major protein families were found to be enriched in human aqueous humor, including Immunoglobulins (61 proteins), Complement proteins (25 proteins), Apolipoproteins (12 proteins), Serine Protease Inhibitors (16 proteins), and Insulin Growth Factor family (10 proteins). Table 3 lists all of the members of these five protein families detected in AH.

Table 3. Five most abundant protein families in the human aqueous humor.

| Family                     | Level | Proteins                      |
|----------------------------|-------|--------------------------------|
| Apolipoproteins            | High  | APOA1 APOE                    |
|                            |       | APOA2 APOH                     |
|                            | Medium|                                |
|                            | Low   |                                |
|                            | Rare  | APOB APOC1                    |
|                            |       | APOF APOL1                     |
| Complement Proteins        | High  | C1R C8A CFI                   |
|                            | Medium|                                |
|                            | Low   |                                |
|                            | Rare  |                                |
| Immunoglobulins            | High  | IGHV1-49 IGLV1-40             |
|                            | Medium|                                |
|                            | Low   |                                |
|                            | Rare  |                                |
| Insulin Growth Factor (IGF) Family | High | IGFBP2 IGFBP6 IGFBP7          |
|                            | Medium|                                |
|                            | Low   |                                |
|                            | Rare  |                                |
| Serine Protease Inhibitors (SERPINs) | High | SERPINA1 SERPIND1 SERPIN3 SERPIN4 |
|                            | Medium|                                |
|                            | Low   |                                |
|                            | Rare  |                                |

3.3. Gene Ontology Enrichment Analysis

A total of 243 proteins that were detected in at least 50% of the samples were considered as the constitutive proteome of human aqueous humor. Gene ontology enrichment analysis was performed in order to discover the biological processes, cellular components, and molecular functions associated with the constitutive proteome (Figure 4). The top enriched categories among the biological processes include organonitrogen metabolic process (136 proteins), protein metabolic process (127 proteins), transport...
(112 proteins), and establishment of localization (112 proteins). The most enriched cellular components are extracellular region (190 proteins), organelle (182 proteins), vesicle (161 proteins), extracellular vesicle (148), and extracellular exosome (146 proteins). Furthermore, protein binding (166 proteins), ion binding (90 proteins), molecular function regulator (56 proteins), signaling receptor binding (48 proteins), and enzyme regulator activity (47 proteins) were the top enriched molecular functions.

**Figure 4.** Biological processes (A), cellular components (B), and molecular functions (C) associated with the highly abundant aqueous humor proteins (detected in >50% samples). Bioinformatics analysis was performed in order to associate significantly enriched Gene Ontology (GO) terms to the constitutive aqueous humor proteome. The horizontal bars represent the number of proteins annotated to each GO term, and the black lines represent the p-value of enrichment.

### 3.4. Network and Pathway Analysis

Ingenuity Pathway Analysis (IPA) was used to discover the protein-protein interaction networks in the constitutive proteome (243 proteins) of human aqueous humor. Figure 5 presents the three top-scoring networks. Several members of the Apolipoprotein, Complement, and SERPIN families...
were part of the top-scoring network (Figure 5A). The second-highest scoring network consisted of 56 proteins, which are involved in tissue development, protein synthesis, and cellular compromise (Figure 5B). The third network includes several members of the Immunoglobulin and IGF families and other proteins that are involved in protein synthesis, humoral immune, and inflammatory responses (Figure 5C). IPA analysis also revealed that 21 canonical pathways were significantly enriched among the constitutive proteins observed in the AH (Table 4). The highly enriched canonical pathways include acute phase response signaling (40 proteins), LXR/RXR activation (33 proteins), FXR/RXR activation (32 proteins), clathrin-mediated endocytosis signaling (18 proteins), complement system (17 proteins), and coagulation system (14 proteins).

**Figure 5.** Three top-scoring interaction networks of highly abundant aqueous humor proteins. Ingenuity Pathway Analysis (IPA) was performed on the 243 proteins detected in at least half of the aqueous humor samples. (A) Network 1: includes several members of the Apolipoprotein, Complement, and SERPIN families. (B) Network 2: Network of proteins involved in tissue development, protein synthesis, and cellular compromise. (C) Network 3: Protein cluster associated with humoral immune response, inflammatory response, and protein synthesis. Each protein is represented as a node, and edges represent interactions between proteins. The intensity of color represents the relative levels of proteins (brighter red nodes indicate higher levels). Proteins are separated based on the cellular compartments.
Table 4. Canonical pathways enriched in the constitutive aqueous humor proteome.

| Canonical Pathway                                      | p-Value     | # of Proteins |
|--------------------------------------------------------|-------------|--------------|
| Acute Phase Response Signaling                         | 5.01 × 10^{-42} | 40           |
| LXR/RXR Activation                                     | 5.01 × 10^{-38} | 33           |
| FXR/RXR Activation                                     | 1.00 × 10^{-33} | 32           |
| Complement System                                      | 1.00 × 10^{-24} | 17           |
| Coagulation System                                     | 2.00 × 10^{-19} | 14           |
| Clathrin-mediated Endocytosis Signaling                | 1.58 × 10^{-12} | 18           |
| Atherosclerosis Signaling                              | 3.98 × 10^{-12} | 15           |
| IL-12 Signaling and Production in Macrophages          | 1.00 × 10^{-10} | 14           |
| Extrinsic Prothrombin Activation Pathway               | 1.15 × 10^{-10} | 7            |
| Intrinsic Prothrombin Activation Pathway               | 3.55 × 10^{-10} | 9            |
| Production of Nitric Oxide and Reactive Oxygen Species | 1.02 × 10^{-8}  | 14           |
| in Macrophages                                          |             |              |
| Hepatic Fibrosis/Hepatic Stellate Cell Activation       | 6.92 × 10^{-8}  | 13           |
| Maturity Onset Diabetes of Young (MODY) Signaling      | 1.74 × 10^{-7}  | 8            |
| Airway Pathology in Chronic Obstructive Pulmonary      | 3.63 × 10^{-6}  | 9            |
| Disease                                                |             |              |
| Systemic Lupus Erythematosus Signaling                 | 4.68 × 10^{-6}  | 12           |
| Neuroprotective Role of THOP1 in Alzheimer’s Disease    | 2.63 × 10^{-5}  | 8            |
| GP6 Signaling Pathway                                   | 2.29 × 10^{-4}  | 7            |
| Iron homeostasis signaling pathway                      | 5.37 × 10^{-4}  | 7            |
| Actin Cytoskeleton Signaling                           | 0.002        | 8            |
| Role of Macrophages, Fibroblasts and Endothelial Cells | 0.016        | 6            |
| in Rheumatoid Arthritis                                |             |              |
| Glucocorticoid Receptor Signaling                      | 0.021        | 10           |

3.5. Aqueous Humor Proteins Associated with Race

Analyses were performed in order to discover race-specific differences in the AH proteome (differentially expressed in African Americans as compared to Caucasian subjects). A total of six proteins were upregulated and 5 proteins were downregulated in African American subjects (Table 5). Proteins significantly upregulated in African Americans subjects include Immunoglobulin kappa variable 1D-33 (IGKV1-33; FC = 2.191), Extracellular superoxide dismutase (SOD3; FC = 2.190), Complement C1r subcomponent (C1R; FC = 2.182), Complement Factor H (CFH; FC = 1.865), Alpha-2-macroglobulin (A2M; FC = 1.489), and Complement C3 (C3; FC = 1.289). The proteins significantly downregulated in African American subjects include Tetraspanin-14 (TSPAN14; FC = −2.089), Retinol-binding protein 4 (RBP4; FC = −1.753), Transthyretin (TTR; FC = −1.751), Ribonuclease pancreatic (RNASE1; FC = −1.636), and Prostaglandin D2 synthase (PTGDS; FC = −1.435). Figure 6 shows the boxplots depicting the distribution of these proteins in the African American and Caucasian subjects.

Table 5. Proteins with significant differences between African American and Caucasian subjects.

| UniProt ID | Gene Symbol | Description | Fold Change | Adj. p-Value | Pathway                                      |
|------------|-------------|-------------|-------------|--------------|----------------------------------------------|
| P01593     | IGKV1D-33   | Immunoglobulin kappa variable 1D-33 | 2.191       | 0.024        | Complement activation                          |
| P08294     | SOD3        | Extracellular superoxide dismutase [Cu-Zn] | 2.190       | 0.017        | Antioxidant, Heparin binding                   |
| P00736     | C1R         | Complement C1r subcomponent | 2.182       | 0.014        | Complement activation, classical pathway       |
| P08603     | CFH         | Complement factor H | 1.865       | 0.021        | Complement activation, alternative pathway     |
| P01023     | A2M         | Alpha-2-macroglobulin | 1.489       | 0.047        | Blood coagulation                             |
| P01024     | C3          | Complement C3 | 1.289       | 0.047        | Endopeptidase inhibitor activity              |
| Q8NG11     | TSPAN14     | Tetraspanin-14 | −2.089      | 0.005        | Cellular protein metabolic process            |
| P02753     | RBP4        | Retinol-binding protein 4 | −1.753      | 0.002        | Retinol and retinol binding                   |
| P02766     | TTR         | Transthyretin | −1.751      | <0.001       | Hormone activity                              |
| P07998     | RNASE1      | Ribonuclease pancreatic | −1.636      | 0.002        | Nucleic acid binding                          |
| P41222     | PTGDS       | Prostaglandin D2 isomerase | −1.435      | 0.002        | Fatty acid binding                            |
This study provides the proteomic repertoire of human AH while using a larger sample set and highly sensitive mass spectrometry technology. The low abundant proteins have higher variation and poor reproducibility due to random nature of detection of proteins in mass spectrometry analysis. This study provides a reference AH proteome, which can be used in order to enhance the interpretation of results in future studies. We identified 243 proteins in at least 50% of samples, which we refer to as the constitutive proteome of human aqueous humor.

A comparison of our study with a previously published study by Chowdhury et al. [2] revealed significant overlap in the proteins identified in human AH. We detected more than 79% of the 355 AH proteins that were identified in the previous study using nano-LC-ESI-MS/MS. Also, in the previous study, the samples were divided into three matched groups and 206 proteins were found in all three groups. A comparison of these 206 proteins with the constitutive AH proteome of our study (243 proteins) revealed >70% overlap [2].

Our comprehensive proteomic analysis revealed that five protein families are highly enriched in human aqueous humor, including apolipoproteins, complement proteins, immunoglobulins, IGF family proteins, and serine protease inhibitors (SERPINs). Apolipoproteins are proteins that bind and transport lipids in biological fluids. Seven apolipoproteins, including APOA1, APOA2, APOA4, APOC3, APOD, APOE, and APOH, were highly abundant, whereas five apolipoproteins APOB, APOC1, APOLD1, APOF, and APOL1 were detected in less than 25% of samples. Consistent to our findings, APOA1, APOA2, APOA4, APOD, APOE, and APOH were also identified in previous studies [2,29,30]. Several members of this family were part of the top scoring protein interaction network identified while using IPA analysis.

The anterior chamber is immune privileged and relies on AH to maintain a pathogen-free environment. Our analysis identified 25 complement proteins from both the classical and alternative
pathways. Eleven complement proteins, including CFI, C4B, C6, C8A, and C9, were detected in more than 75% of the samples. Similar to the blood plasma, several members of the Immunoglobulin family of proteins were also identified in the AH. Immunoglobulins are involved in cell communication, defense response, and the regulation of metabolic processes. The presence of a wide array of immunoglobulins has been reported in previous studies indicating their existence in the AH of cataract and glaucoma patients [12,31,32].

Insulin-like growth factors and their binding proteins have been shown to play an important role in ocular functions. Ten IGF family proteins were identified in our analyses. Several IGFBPs in vitreous and aqueous humor have been previously reported [33]. However, the predominant serum carrier protein, IGFBP3, was present in less than 50% of AH samples, whereas IGFBP7 and IGFBP6 were highly abundant, indicating quantitative differences between the two fluids. IGFBP7 has been linked to hypertensive retinopathy and familial retinal macroaneurysms, indicating its role in retinal vascular pathology [34,35]. Furthermore, IGFBP7 was elevated in the AH of exudative age-related macular degenerative patients and is considered to be an anti-angiogenic agent in these patients [36].

The SERPIN family of proteins are ubiquitous in the body and their abnormalities are associated with ‘serpinopathies’. Ten proteins of this family, including SERPINC1, SERPINF1, SERPINA3, SERPINA1, SERPING1, SERPINF2, SERPIND1, SERPINA6, SERPINA4, and SERPINI1, were detected in at least 75% of samples. SERPINA3 is an acute phase response protein, which is involved in retinal angiogenesis and inflammation [37,38]. SERPINC1 or Antithrombin III deficiency has been associated with retinal vein occlusion, consistent with its role as an anti-clotting agent [39]. A decrease in SERPING1 was associated with neovascularization and high myopia [40,41]. Overall, this glycoprotein is known to possess beneficial effects, such as potent anti-angiogenic, anti-thrombotic, anti-tumorigenic, anti-inflammatory, and neuroprotective properties [40–47]. SERPING1, which is the largest member of the superfamily, has been associated with suppressing inflammatory conditions, fibrinolysis and blood coagulation [48,49].

Interestingly, after adjusting for confounding variables, including age, sex, and hypertension, we found 11 proteins that were differentially expressed between African American and Caucasian subjects, indicating race-specific differences in the AH proteome. Overall, six proteins were significantly upregulated, while five proteins were downregulated in African American subjects. Proteins related to immune responses such as IGKV1D-33, C6, C8A were present at elevated levels in African American subjects. Among the downregulated proteins, TSPAN-14 was at least two-fold lower in African Americans. A member of this family, TSPAN-12, was discovered as a therapeutic target for retinal vascular diseases, such as age-related macular degeneration and diabetic retinopathy [50]. Three other vision-related proteins, including RBP4, TTR, and PTGDS, were present in lower levels in the African American population. RBP4, a retinol transporter protein, is known to be involved in congenital eye disease [51]. TTR is a transport protein, which carries retinol-binding protein and is essential for the maintenance of photoreceptors, visual cycle, and perception [52]. PTGDS is a secretory retinoid transporter that is involved in the maintenance of the blood-retinal barrier. The difference in the vision-related proteins might be one of the contributing factors for increased risk of eye-related ailments in the African American population.

5. Conclusions

In conclusion, this study characterized the human aqueous humor proteome using the latest technology and a larger sample set. A total of 243 proteins, which were detected in at least half of the samples, were considered to be the constitutive proteome of human aqueous humor. Five protein families were highly enriched in the human aqueous humor proteome. Eleven proteins were significantly altered between African American and Caucasian subjects, indicating race-specific differences. The highly abundant aqueous humor proteins are involved in immune-mediated responses, transport, metabolism, and binding. The reliable characterization of the aqueous humor proteome will
provide new insights into the factors that govern anterior segment homeostasis and aid in biomarker discovery in various eye disorders.

**Supplementary Materials:** The following are available online at http://www.mdpi.com/2227-7382/8/4/34/s1, Table S1: Complete list of proteins detected in 88 aqueous humor samples.

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**References**

1. Carreon, T.; Van Der Merwe, E.; Fellman, R.L.; Johnstone, M.; Bhattacharya, S.K. Aqueous Outflow—A Continuum from Trabecular Meshwork to Episcleral Veins. *Prog. Retin. Eye Res.* 2017, 57, 108–133. [CrossRef]

2. Chowdhury, U.R.; Madden, B.J.; Charlesworth, M.C.; Fautsch, M.P. Proteome Analysis of Human Aqueous Humor. *Investig. Opthalmol. Vis. Sci.* 2010, 51, 4921–4931. [CrossRef]

3. To, C.-H.; Bsc, C.-W.K.; Bsc, C.-Y.C.; Shahidullah, M.; Do, C.; Shahidullah, M.; To, C. The Mechanism of Aqueous Humour Formation. *Clin. Exp. Optom.* 2002, 85, 335–349. [CrossRef] [PubMed]

4. Green, K.; Pederson, J.E. Contribution of Secretion and Filtration to Aqueous Humor Formation. *Am. J. Physiol. Content* 1972, 222, 1218–1226. [CrossRef] [PubMed]

5. Fautsch, M.P.; Johnson, D.H. Aqueous Humor Outflow: What Do We Know? Where Will It Lead Us? *Investig. Opthalmol. Vis. Sci.* 2006, 47, 4181–4187. [CrossRef]

6. Kramer, M.; Goldenberg-Cohen, N.; Axer-Siegel, R.; Weinberger, V.; Cohen, Y.; Monselise, Y. Inflammatory Reaction in Acute Retinal Artery Occlusion: Cytokine Levels in Aqueous Humor and Serum. *Ocul. Immunol. Inflamm.* 2005, 13, 305–310. [CrossRef] [PubMed]

7. Murthy, K.R.; Rajagopalan, P.; Pinto, S.M.; Advani, J.; Murthy, P.R.; Goel, R.; Subbannayya, Y.; Balakrishnan, L.; Dash, M.; Anil, A.K.; et al. Proteomics of Human Aqueous Humor. *OMICS* 2015, 19, 283–293. [CrossRef] [PubMed]

8. Grus, F.H.; Joachim, S.C.; Pfeiffer, N. Proteomics in Ocular Fluids. *Proteom. Clin. Appl.* 2007, 1, 876–888. [CrossRef] [PubMed]

9. Barsotti, M.F.; Bartels, S.P.; Freddo, T.F.; Kam, R.D. The Source of Protein in the Aqueous Humor of the Normal Monkey Eye. *Investig. Opthalmol. Vis. Sci.* 1992, 33, 581–595.

10. Freddo, T.F.; Bartels, S.P.; Barsotti, M.F.; Kam, R.D. The Source of Proteins in the Aqueous Humor of the Normal Rabbit. *Investig. Opthalmol. Vis. Sci.* 1990, 31, 125–137.

11. McLaren, J.W.; Ziai, N.; Brubaker, R.F. A Simple Three-compartment Model of Anterior Segment Kinetics. *Exp. Eye Res.* 1993, 56, 355–366. [CrossRef] [PubMed]

12. Kluichnikova, A.A.; Samokhina, N.I.; Ilin, I.Y.; Karpov, D.; Pyatnitskiy, M.A.; Kuznetsova, K.G.; Toropygin, I.Y.; Kochergin, S.A.; Alekseev, I.B.; Zgoda, V.G.; et al. Human Aqueous Humor Proteome in Cataract, Glaucoma, and Pseudoexfoliation Syndrome. *Proteomics* 2016, 16, 1938–1946. [CrossRef] [PubMed]

13. Funke, S.; Perumal, N.; Bell, K.; Pfeiffer, N.; Grus, F.H. The Potential Impact of Recent Insights Into Proteomic Changes Associated With Glaucoma. *Expert Rev. Proteom.* 2017, 14, 311–334. [CrossRef] [PubMed]

14. Kaislin, M.A.; Killer, H.E.; Fuhrer, C.A.; Zeleny, N.; Huber, A.R.; Neutzner, A. Changes to the Aqueous Humor Proteome during Glaucoma. *PloS ONE* 2016, 11, e0165314. [CrossRef]

15. Izzotti, A.; Longobardi, M.; Cartiglia, C.; Saccà, S.C. Proteome Alterations in Primary Open Angle Glaucoma Aqueous Humor. *J. Proteome Res.* 2010, 9, 4831–4838. [CrossRef]

16. Duan, X.; Lu, Q.; Xue, P.; Zhang, H.; Dong, Z.; Yang, F.; Wang, N. Proteomic Analysis of Aqueous Humor From Patients With Myopia. *Mol. Vis.* 2008, 14, 370–377.

17. Semba, R.D.; Enghild, J.J.; Venkatraman, V.; Dyrdun, T.F.; Van Eyk, J.E. The Human Eye Proteome Project: Perspectives on an Emerging Proteome. *Proteomics* 2013, 13, 2500–2511. [CrossRef]
18. Chiang, S.-Y.; Tsai, M.-L.; Wang, C.-Y.; Chen, A.; Chou, Y.-C.; Hsia, C.-W.; Wu, Y.-F.; Chen, H.-M.; Huang, T.-H.; Chen, P.-H.; et al. Proteomic Analysis and Identification of Aqueous Humor Proteins With a Pathophysiological Role in Diabetic Retinopathy. *J. Proteom.* 2012, 75, 2950–2959. [CrossRef]

19. Kim, T.W.; Kang, J.W.; Ahn, J.; Lee, E.K.; Cho, K.-C.; Han, B.N.R.; Hong, N.Y.; Park, J.; Kim, K.P. Proteomic Analysis of the Aqueous Humor in Age-related Macular Degeneration (AMD) Patients. *J. Proteome Res.* 2012, 11, 4034–4043. [CrossRef]

20. Sharma, S.; Bollinger, K.E.; Kodeboyina, S.K.; Zhi, W.; Patton, J.; Bai, S.; Edwards, B.; Ulrich, L.; Bogorad, D.; Sharma, A. Proteomic Alterations in Aqueous Humor From Patients With Primary Open Angle Glaucoma. *Investig. Ophthalmol. Vis. Sci.* 2018, 59, 2635–2643. [CrossRef]

21. Duan, X.; Xue, P.; Wang, N.; Dong, Z.; Lu, Q.; Yang, F. Proteomic Analysis of Aqueous Humor from Patients with Primary Open Angle Glaucoma. *Mol. Vis.* 2010, 16, 2839–2846. [PubMed]

22. Yao, J.; Liu, X.; Yang, Q.; Zhuang, M.; Wang, F.; Chen, X.; Hang, H.; Zhang, W.; Liu, Q. Proteomic Analysis of the Aqueous Humor in Patients With Wet Age-Related Macular Degeneration. *Proteom. Clin. Appl.* 2013, 7, 550–560. [CrossRef] [PubMed]

23. Määttä, M.; Tervahartiala, T.; Harju, M.; Airaksinen, J.; Autio-Harmainen, H.; Sorsa, T. Matrix Metalloproteinases and Their Tissue Inhibitors in Aqueous Humor of Patients With Primary Open-Angle Glaucoma, Exfoliation Syndrome, and Exfoliation Glaucoma. *J. Glaucoma* 2005, 14, 64–69. [CrossRef] [PubMed]

24. Ozcan, A.A.; Ozdemir, N.; Canataroglu, A. The Aqueous Levels of TGF-2 in Patients with Glaucoma. *Int. Ophthalmal.* 2004, 25, 19–22. [CrossRef] [PubMed]

25. Baek, J.-H.; Lim, D.; Park, K.H.; Chae, J.-B.; Jang, H.; Lee, J.; Chung, H. Quantitative Proteomic Analysis of Aqueous Humor From Patients With Drusen and Reticular Pseudodrusen in Age-Related Macular Degeneration. *BMC Ophthalmal.* 2018, 18, 289. [CrossRef]

26. Baldwin, M.A. Protein Identification by Mass Spectrometry. *Mol. Cell. Proteom.* 2003, 3, 1–9. [CrossRef]

27. Di Girolamo, F.; Lante, I.; Muraca, M.; Putignani, L. The Role of Mass Spectrometry in the “Omics” Era. *Curr. Org. Chem.* 2013, 17, 2891–2905. [CrossRef]

28. Perez-Riverol, Y.; Csordas, A.; Bai, J.; Bernal-Llinares, M.; Hewapathirana, S.; Kundu, D.J.; Inuganti, A.; Griss, J.; Mayer, G.; Eisenacher, M.; et al. The Pride Database and Related Tools and Resources in 2019: Improving Support for Quantification Data. *Nucleic Acids Res.* 2019, 47, D442–D450. [CrossRef]

29. Inoue, T.; Kawaji, T.; Tanihara, H. Elevated Levels of Multiple Biomarkers of Alzheimer’s Disease in the Aqueous Humor of Eyes With Open-Angle Glaucoma. *Investig. Ophthalmal. Vis. Sci.* 2013, 54, 5353–5358. [CrossRef] [PubMed]

30. Xiang, M.; Zhang, X.; Li, Q.; Wang, H.; Zhang, Z.; Han, Z.; Ke, M.; Chen, X. Identification of Proteins in the Aqueous Humor Associated With Cataract Development Using iTRAQ Methodology. *Mol. Med. Rep.* 2017, 15, 3111–3120. [CrossRef]

31. Sen, D.K.; Sarin, G.S.; Saha, K. Immunoglobulins in Human Aqueous Humour. *Br. J. Ophthalmal.* 1977, 61, 216–217. [CrossRef] [PubMed]

32. Kaur, I.; Kaur, J.; Sooraj, K.; Goswami, S.; Saxena, R.; Chauhan, V.S.; Sihota, R. Comparative Evaluation of the Aqueous Humor Proteome of Primary Angle Closure and Primary Open Angle Glaucomas and Age-Related Cataract Eyes. *Int. Ophthalmal.* 2018, 39, 69–104. [CrossRef] [PubMed]

33. Schoen, T.J.; Waldbilly, R.J.; Searcy, G.; Gaudet, S.J.; Jones, B.E.; Chader, G.J.; Moshyledi, P. Identification and Partial Characterization of a Proteinase Specific for Insulin-Like Growth Factor Binding Protein-3 in Aqueous and Vitreous Humors. *Curr. Eye Res.* 1995, 14, 127–135. [CrossRef] [PubMed]

34. Abu-Safieh, L.; Aboud, E.B.; Alkuryaya, H.; Shamseldin, H.; Al-Enzi, S.; Al-Abdi, L.; Hashem, M.; Colak, D.; Jarallah, A.; Ahmad, H.; et al. Mutation of IGFBP7 Causes Upregulation of BRAF/MEK/ERK Pathway and Familial Retinal Arterial Macroaneurysms. *Am. J. Hum. Genet.* 2011, 89, 313–319. [CrossRef] [PubMed]

35. Nguyen, D.V.; Calzi, S.L.; Shaw, L.C.; Kielczewski, J.L.; Korah, H.E.; Grant, M.B. An Ocular View of the IGF–IGFBP System. *Growth Horm. IGF Res.* 2014, 24, D442–D450. [PubMed]

36. Lu, C.-H.; Lin, S.-T.; Chou, H.-C.; Lee, Y.-R.; Chan, H.-L. Proteomic Analysis of Retinopathy-Related Plasma Biomarkers in Diabetic Patients. *Arch. Biochem. Biophys.* 2013, 529, 146–156. [CrossRef]

37. Fan, W.; Li, X.; Wang, W.; Mo, J.S.; Kaplan, H.; Cooper, N.G. Early Involvement of Immune/Inflammatory Response Genes in Retinal Degeneration in DBA/2J Mice. *Ophthalmal. Eye Dis.* 2010, 2010, 23. [CrossRef]
38. Pescosolido, N.; Barbato, A.; Pascarella, A.; Giannotti, R.; Genzano, M.; Nebbioso, M. Role of Protease-Inhibitors in Ocular Diseases. *Molecules* 2014, 19, 20557–20569. [CrossRef]
39. Kuhli-Hattenbach, C.; Jochmans, K.; Scharrier, I.; Lüchtenberg, M.; Hattenbach, L.O. Retinal Vein Occlusion Associated with Antithrombin Deficiency Secondary to a Novel G9840C Missense Mutation. *Arch. Ophthalmol.* 2006, 124, 1165–1169. [CrossRef]
40. Ogata, N.; Imaizumi, M.; Miyashiro, M.; Arichi, M.; Matsuoka, M.; Ando, A.; Matsumura, M. Low Levels of Pigment Epithelium-Derived Factor in Highly Myopic Eyes with Chorioretinal Atrophy. *Am. J. Ophthalmol.* 2005, 140, 937–939. [CrossRef]
41. Filleur, S.; Nelius, T.; De Riese, W.; Kennedy, R. Characterization of PEDF: A Multi-Functional Serpin Family Protein. *J. Cell. Biochem.* 2009, 106, 769–775. [CrossRef] [PubMed]
42. Becerra, S.; Sagasti, A.; Spinella, P.; Notario, V. Pigment Epithelium-derived Factor Behaves Like a Noninhibitory Serpin: Neurotrophic Activity Does Not Require the Serpin Reactive Loop. *J. Biol. Chem.* 1995, 270, 25992–25999. [CrossRef] [PubMed]
43. Rychli, K.; Huber, K.; Wojta, J. Pigment Epithelium-Derived Factor (PEDF) as a Therapeutic Target in Cardiovascular Disease. *Expert Opin. Ther. Targets* 2009, 13, 1295–1302. [CrossRef] [PubMed]
44. Ohno-Matsui, K.; Morita, I.; Tombran-Tink, J.; Mrazek, D.; Onodera, M.; Uetama, T.; Hayano, M.; Murota, S.-I.; Mochizuki, M. Novel Mechanism for Age-Related Macular Degeneration: An Equilibrium Shift Between the Angiogenesis Factors VEGF and PEDF. *J. Cell. Physiol.* 2001, 189, 323–333. [CrossRef] [PubMed]
45. Tong, J.-P.; Yao, Y.-F. Contribution of VEGF and PEDF to Choroidal Angiogenesis: A Need for Balanced Expressions. *Clin. Biochem.* 2006, 39, 267–276. [CrossRef]
46. Abe, R.; Shimizu, T.; Yamagishi, S.-I.; Shibaki, A.; Amano, S.; Inagaki, Y.; Watanabe, H.; Sugawara, H.; Nakamura, H.; Takeuchi, M.; et al. Overexpression of Pigment Epithelium-Derived Factor Decreases Angiogenesis and Inhibits the Growth of Human Malignant Melanoma Cells In Vivo. *Am. J. Pathol.* 2004, 164, 1225–1232. [CrossRef]
47. Funatsu, H.; Yamashita, H.; Nakamura, S.; Mimura, T.; Eguchi, S.; Noma, H.; Hori, S. Vitreous Levels of Pigment Epithelium–Derived Factor and Vascular Endothelial Growth Factor Are Related to Diabetic Edema. *Ophthalmology* 2006, 113, 294–301. [CrossRef]
48. Davis, A.E.; Mejia, P.; Lu, F. Biological Activities of C1 Inhibitor. *Mol. Immunol.* 2008, 45, 4057–4063. [CrossRef]
49. Pike, R.N.; Buckle, A.M.; Le Bonniec, B.E.; Church, F.C. Control of the coagulation system by serpins. *FEBS J.* 2005, 272, 4842–4851. [CrossRef]
50. Tomlinson, M.G. Eye-Opening Potential for Tetraspanin Tspan12 as a Therapeutic Target for Diseases of the Retinal Vasculature. *Circulation* 2017, 136, 196–199. [CrossRef]
51. Chou, C.M.; Nelson, C.; Tärli, S.A.; Pribila, J.T.; Bardakjian, T.; Woods, S.; Schneider, A.; Glaser, T. Biochemical Basis for Dominant Inheritance, Variable Penetration, and Maternal Effects in RBP4 Congenital Eye Disease. *Cell* 2015, 161, 634–646. [CrossRef] [PubMed]
52. Balaiya, S.; Zhou, Z.; Chalam, K. Characterization of Vitreous and Aqueous Proteome in Humans With Proliferative Diabetic Retinopathy and Its Clinical Correlation. *Proteom. Insights* 2017, 8, 1178641816686078. [CrossRef] [PubMed]

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