Assesment Of Protein Profile In Vitreous Samples Of Patients With Age-Related Macular Degeneration By Proteomic Approaches

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Abstract

Background: To investigate the protein content in patients with age-related macular degeneration (AMD) with the aim of revealing the pathogenesis of the disease.

Methods: Two groups were formed: 13 patients as the AMD group and 11 patients as the healthy control group. Vitreous samples were taken from both groups under sterile conditions and sent to the proteomics laboratory for proteomics analysis. In this study, we evaluated the proteome of vitreous samples of AMD and control groups using two-dimensional gel electrophoresis (2DE) coupled with MALDI-TOF/TOF.

Results: We detected 11 proteins differentially regulated in the vitreous of AMD patients relative to healthy controls. The only protein that was up-regulated was Apolipoprotein E. We observed that Alpha 1-acid glycoprotein (AAG), Leucine-rich alpha-2-glycoprotein (LRG-1), Alpha-2-HS-glycoprotein, Haptoglobin, Alpha-Crystalline A Chain, Alpha-Crystalline B Chain, Immunoglobulin kappa constant, Beta-crystallin B2, Beta-crystallin A3, Beta-crystallin S levels were significantly decreased in patients with AMD.

Conclusion: The detected proteins are related to biological regulation, retinal protection, and regulation of inflammation and angiogenesis processes. We believe that investigating these proteins will help to reveal the pathological mechanisms of AMD.

Key Messages

- Molecular mechanisms mainly related to VEGF are involved in the pathogenesis of neovascular age-related macular degeneration.

In this study;

- Proteomic analysis of vitreous in patients with neovascular age-related macular degeneration showed changes in protein composition of these patients.
- Eleven proteins were detected, and these proteins are mainly related to protection, inflammation, and angiogenesis. Studying these proteins will help reveal the molecular mechanisms of the disease and develop improved treatments.

Introduction

Age-related macular degeneration (AMD) is the progressive degeneration of the retinal pigment epithelium (RPE), retina, and choriocapillaris observed among elderly people and is a leading cause of blindness.¹

AMD is a multifactorial disease, and many risk factors have been described. The most commonly reported environmental risk factors are aging, smoking, family history, low dietary intake of antioxidants and omega-3 fatty acids, and reduced physical activity.² Study models of AMD have shown increased VEGF-A mRNA and protein in retinal pigment epithelial (RPE) cells, which contributes to the development of choroidal neovascular membrane (CNVM).³

The vitreous structure consists of a collagen fibril network suspended in the hyaluronic acid matrix with high liquid content and, although 99% is water, its viscosity is about twice that of water. The remaining 1% consists of low molecular weight oils and inorganic salts, soluble and insoluble proteins, and hyaluronic acid.⁴ Free amino acids in the vitreous are one-fifth of the amount of serum. The proteins might change in response to many conditions, and they are dynamic. They adapt to new challenges and environments. The protein component of an organ might change in a disease context, or the disease might be the reason for a changed protein environment.

Proteomics studies allow monitoring of proteome changes during the transition from a healthy to a disease state. Proteomic approaches could help to discover disease-specific proteins involved in AMD.⁵ Currently, only a few studies have attempted to identify AMD-related proteins using proteomics approaches.^{6–8} In this study, we aim to identify AMD-related proteins. We investigated the differentially regulated proteins of the vitreous in AMD patients and matched, non-AMD cataract controls.

Material And Methods

Application of the Experiment

All work was done in accordance with the ethical standards of the institutional and/or national research committee (KOU-GOKAEK 2016/81) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This study was approved by

Kocaeli University Clinical Experiments Local Ethics Committee. Informed consent was obtained from all individuals. We received support from Kocaeli University Scientific Research Project with project number 2018/87. The sponsor had no role in the design or conduct of this research. All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript. Thirteen eyes of 13 patients were diagnosed with age-related macular degeneration among a group of patients between May 2016 and January 2018 at the Kocaeli University School of Medicine Eye Diseases Retina Department. These patients were treatment naïve. Eleven eyes of 11 patients were in the control group. These were the age-matched patients who were prepared for cataract surgery. Patients with signs of uveitis attack, glaucoma, age-related macular degeneration, retinal artery or vein occlusion, hypertensive retinopathy, diabetes, and previous vitreoretinal surgery were not included in the control group. The patients were informed about the form of sampling from the vitreous form, the application of intravitreal injections, the expected effect and possible complications, and informed consent forms were taken to perform the procedure.

Sample Collection and Injection Technique

All interventions were done in operating room conditions. After peribulbar anesthesia in the control groups, the eyelids and around the area were wiped with 10% povidone-iodine impregnated sterile gauze. Before starting cataract surgery, a transconjunctival 23-gauge trocar was located from the patients, and a vitreous specimen (approximately 0.5–0.6 mL) was collected with the vitrectomy probe. Then, the routine surgical procedure was continued.

In the study groups, 1 cc (lidocaine + bupivacaine mixture) after peribulbar anesthesia, the eyelids and around the eyes were wiped with 10% povidone-iodine impregnated sterile gauze. The transconjunctival 23-gauge trockar was located, and a 0.5 mL vitreous sample was collected with the vitrectomy probe. The transconjunctival sclerotomy trocar was removed, and the pressure was applied with a cotton swab. Then, 0.5 mg ranibizumab or aflibercept was injected at 4 mm from the limbus.

The samples were placed in sterile Eppendorf tubes, snap-frozen in liquid nitrogen, and stored at – 80°C. Samples were always taken by the same surgeon. No complications were developed in any operation.

Sample Preparation and 2D Gel Electrophoresis

The volume of the vitreous samples was too low (50–100 µl) for 2DE experiments. Therefore, a pool from vitreous samples was formed using an equal amount of sample, and the protein concentration of the pooled sample was measured by Bradford Protein Assay. The measured protein concentration was compared with the calculated protein concentration to validate if the samples were correctly pooled. Further validation was achieved using SDS-PAGE followed by visual examination of the protein profiles. Nine hundred micrograms of protein from each protein pool were loaded onto immobilized, 11-cm, pH-gradient strips (IPG) (pH 3–10 L). The following conditions were used for isoelectric focusing: 20 min at 250 V with rapid ramp, 2 h at 10,000 V with slow ramp, and 2.5 h for 10,000 V with rapid ramp until a total of 50,000 V/h was reached at 20°C. After isoelectric focusing, strips were then washed with buffer-I and buffer-II (as mentioned in the previous study) and subjected to SDS-PAGE for 2D separation in a Dodeca gel running system (Bio-Rad, USA).⁹ After the separation, the gels were fixed and then stained with colloidal Coomassie blue G250 (Bio-Rad, USA). In the gel image analysis, protein spots that significantly differed in expression (more than 2-fold) were selected and excised from the gels.⁹

Protein Identification by Mass Spectrometry

Protein identification experiments were performed at Kocaeli University DEKART proteomics laboratory using a ABSCIEX MALDI-TOF/TOF 5800 system (Applied Biosystems®, Framingham, MA, USA). In-gel tryptic digestion of the proteins was performed using an in-gel digestion kit following the recommended protocol (Pierce, USA). Before deposition onto a MALDI plate, all samples were desalted with a 10 µL ZipTip_{C18} (Millipore®, USA). Peptides were eluted in a volume of 1 µL using a concentrated solution of α-Cyano-4-hydroxycinnamic acid (CHCA) in 50% acetonitrile and 0.1% trifluoroacetic acid in water and spotted onto the MALDI target plate. The TOF spectra were recorded in the positive ion reflector mode with a mass range from 400 to 2000 Da. Each spectrum was the cumulative average of 2000 laser shots. The spectra were calibrated with the trypsin autodigestion ion peaks *m/z* (842.510 and 2211.1046) as internal standards. Ten of the strongest peaks of the TOF spectra per sample were chosen for MS/MS analysis. The data obtained from MALDI-TOF/TOF were searched against the MASCOT database version 2.5 (Matrix Science) by using a streamlined software, Protein Pilot (ABSCIEX, USA), with the following criteria: National Center for Biotechnology Information non-redundant (NCBI nr); species restriction to *H. sapiens*; enzyme of trypsin; at least five independent peptides matched; at most one missed cleavage site; MS tolerance set to ± 50 ppm and MS/MS tolerance set to ± 0.4 Da; fixed modification being cells International carbamidomethyl (Cys) and variable modification being oxidation (Met); peptide charge of 1 + and being monoisotopic. Only significant hits, as defined by the MASCOT probability analysis (*P* < 0.05), were accepted.

Statistical and bioinformatics analysis

Quantitative results were expressed as a mean $\pm$ standard deviation. Two-sample *t*-tests assuming unequal variances were used for the statistical analyses (SPSS v.20.0, IBM® Corporation, NY, USA). P-values < 0.05 were considered to indicate statistical significance. A protein-protein interaction network of the identified proteins was constructed with the online analysis tool STRING v10.0 (<http://www.string-db.org>).¹⁰ Classifications, biological processes, and molecular functions were predicted using a freely available classification system (PANTHER, v11.0, Released 2016.07.05, <http://www.pantherdb.org>), NCBI (<http://www.ncbi.nlm.nih.gov/pubmed>), and Swiss-Prot/TrEMBL annotations (<http://www.expasy.org>).¹¹

Results

In this study, vitreous fluid samples of 13 patients diagnosed with AMD and 11 healthy individuals who constituted the control group without any systemic and retinal disease were studied. The composition of the vitreous is 95% water, 5% hyaluronic acid, and proteins. Because of high water content, the samples were precipitated by the modified TCA/acetone protocol for extraction of the proteins (Fig. 1–a). Protein pools for the patient group and the control group were created by taking equal volumes of protein from each sample, which were then combined into a single tube (Fig. 1–b). The pooled samples were subjected to SDS-PAGE. After the gel staining, sharp protein bands were observed, indicating that the protein pools were ready for 2DE experiments. By using these protein pools, well-resolved and reproducible 2D gels were produced (Fig. 2). The gel images were captured and analyzed according to two-fold change regulation criteria. On the 2D gels, high molecular weight abundant proteins located towards pH of 8.0 were detected. We omitted these abundant proteins and did not consider them as differentially regulated. In addition to those abundant proteins, 150 ± 20 well-resolved protein spots were detected and matched by comparing the gels. Analysis of the matching spots revealed the presence of differentially regulated protein spots between the control and the AMD groups. The regulated protein spots were excised from the gels and identified. The name of the identified proteins, their respective MALDI scores, and their regulation ratios are presented in Tables 1 and 2, respectively. Table 1 shows that all spots are defined with high reliability, except SSP 1103, SSP 1104, SSP 3007, SSP 7004, and SSP 9003.

More than 150 ± 20 protein spots were analyzed in each gel. The protein spot analysis revealed the presence of proteins that were either up- or down-regulated. Of these spots, 17 showed significant changes in their expression levels compared to the control group and were successfully identified (Table 2). Figure 3 shows representative close-up images of the differentially regulated protein spots whose relative quantities changed between the groups were presented.

To predict the interaction partners of the differentially regulated proteins identified in this study, we performed STRING analysis. Initial STRING analysis using high stringency with no first and second shell application created two different interactomes with ten nodes among the differentially regulated proteins (Fig. 4A). The immunoglobulin kappa constant (IGKC) protein was not included in the STRING analysis as it did not create any node. These proteins are mainly structural constituents of the eye lens. Five of the identified proteins belong to the main structural constituents of the eye lens (crystalline proteins CRYA1, CRYAB, CRYBA1, CRYBB2, and CRYGS). With a lower accuracy, proteins associated with amyloid-beta binding, antioxidant activity, and unfolded protein binding were found.¹² When stringency was lowered by expanding the first shell interacting proteins to five, we were able to create an interactome with 15 nodes among all differentially regulated proteins connected with Albumin (Fig. 4B). The STRING results reveal the presence of several statistically significant biological processes, including regulation of amyloid fibril formation, acute-phase response, platelet degranulation, regulation of plasma lipoprotein particle levels, regulated exocytosis, high-density lipoprotein particle remodeling, receptor-mediated endocytosis, and s vesicle-mediated transport. The proteins of acute phase response, namely ORM1, HP, and AHSG, specifically played roles in neutrophil degranulation, in which LRG1 also functions. Also, HP and IGKC are other major serum proteins, such as Albumin.

Furthermore, we used Panther Analysis to evaluate the molecular functions, biological processes, and metabolic pathways of these proteins (Fig. 5). According to molecular function, the proteins were mostly related to binding, molecular function regulator, and catalytic function (Fig. 5a). These processes appear to be predominantly biological regulation, metabolic and developmental processes, cellular and multicellular organismal processes, as well as processes that cellular components organization and biogenesis, response to stimulus and localization (Fig. 5b). Proteins involved in these cellular processes have been identified, including Alpha-2-HS-glycoprotein, Immunoglobulin kappa constant, Leucine-rich alpha-2glycoprotein, Alpha-crystallin A chain, and Alpha-crystallin B chain. These types of proteins are called “hub proteins” since they serve as central support in many metabolic events. When we analyze the metabolic pathways in which proteins take part, the Alpha-crystallin A chain and Alpha-crystallin B chain caught our attention. Both proteins appear to function in angiogenesis and VEGF signal transduction pathways (Fig. 5c). In protein class analysis, we found that Immunoglobulin kappa constant, haptoglobin, and Alpha-2-HS-glycoprotein were included in the defense/immunity protein, protein-binding activity modulator, and protein modifying enzyme classes, respectively.

Discussion

In this study, we compared the levels of differentially regulated proteins in the AMD group to a control group using a 2DE-PAGE approach.

By evaluating the proteins individually, we found that Apolipoprotein E was the only up-regulated protein. Apolipoprotein E is produced by most of the organs in the body, especially the brain, liver, and eyes. It also has non-lipid functions, such as modulating immune regulation, cell growth, and differentiation. It can be highly expressed in the retina, Bruch's membrane, RPE, and choroid.¹³ Previous studies have reported a link between ApoE polymorphism and Alzheimer's disease and AMD.¹⁴ In our study, we found that Apo E was up-regulated (3-fold) in patients with exudative AMD.

By 2D gel analysis, we detected major changes in crystallin family proteins. The alpha crystalline A chain and alpha crystalline B chain proteins are regulated by oxidative stress. They protect the cells by increasing the effects of the antioxidants, and this protection might vary related to the type of stress stimulus.¹² The alpha crystalline A chain can be produced by many tissues, including the retina, brain, muscle, spleen, lungs, and skin. Both the alpha crystalline A chain and B chain have regulatory roles in apoptosis, angiogenesis, and production of b-amyloid fibrils. In our study, we found that the alpha crystalline A chain is 779-fold downregulated and the alpha crystalline B chain 232-fold down-regulated in AMD patients.

The regulatory effect of alpha crystallins in angiogenesis has multiple mechanisms.¹⁵ It has been shown that alpha crystalline functions as a chaperone for VEGF, fibroblast growth factor-2 (FGF-2), nerve growth factor-beta, insulin, and beta-catenin.¹⁶ Kase et al. have reported that after chemical hypoxia, αB-crystallin initially increased, followed by down-regulation.¹⁴ The relationship between ischemia and AMD is well known.¹⁷

Apoptotic stimuli caused by various factors (such as staurosporine, tumor necrosis factor, ultraviolet A irradiation, okadaic acid, H₂O₂, hypoxia, and ceramide) can be silenced by increased expression of alpha crystallins in RPE cells.¹⁸ Alpha crystallins can also silence cells that are susceptible to apoptosis. Additionally, increased alpha crystallins result in higher levels of cellular glutathione in RPE mitochondria, which increases the resistance to oxidative stress-induced cell death.¹² Deficiency in alpha-crystallin B chain has been shown to increase the levels of reactive oxygen species.¹⁹ These protective effects of alpha crystallins might be decreased with their downregulation in patients with AMD. Consistent with our findings, in the previous two proteomic studies, alpha crystalline protein levels were differentially regulated.⁶⁻⁷ The second most downregulated proteins were the beta crystallines. Beta crystallins are located in rod and cone cells, and they have been implicated in the protection of the retina from intense light exposure.¹⁶ Beta crystallins also have neuroprotective effects on the retina. Beta crystalline B2 might promote axon outgrowth.²⁰ In an animal study, it has been shown that intravitreal injection of beta-crystallin B2 reduced the retinal ganglion cell loss.²¹ Downregulation of beta crystallines might be related to decreased protection of the retina that establishes the environment suitable for the AMD formation.

Along with significant changes in the crystalline family proteins, the Ig kappa chain C region protein expression was also changed. Immune globulin kappa constant globulin participates in a tissue homeostatic mechanism that regulates the inner balance of the retina. This regulation includes cell proliferation, cell death, and metabolic functions.²²

Haptoglobin is an acute-phase protein that participates in the clearance of hemoglobin after its release from red blood cells. Haptoglobin is also a potent antioxidant molecule against oxidative stress.²³ Haptoglobin can be produced in photoreceptors, the inner nuclear layer, and the ganglion layer in the neuroretina.²⁴ The effects of haptoglobin related to inflammation are inhibition of prostaglandins and nitric oxide.²⁵ Haptoglobin also participates in the immune system, altering neutrophil metabolism and the production of B lymphocytes and antibodies. Haptoglobin is a positive acute-phase reactant.²⁶ It has different phenotypes, and the distribution of these phenotypes changes with age. Angiogenesis is an important factor in AMD, and it has been reported that haptoglobin might also affect angiogenesis.²⁵ The haptoglobin phenotype 1-1 has been found to be protective against diabetic retinopathy. It was reported that it was lower in patients with diabetic retinopathy, and this effect is related to its antioxidant properties.²⁷ In our study, we found four different spots of haptoglobin. We believe that antioxidant activity and its role in inflammation might be effective in protection from AMD.

Alpha HS 2 glycoprotein and alpha 1 acid glycoprotein are acute phase reactants. Acute phase reaction is a complex mechanism, and the level of acute phase reactants may change with the inflammation period. Alpha HS 2 glycoprotein regulates speed, frequency, and extent of inflammation. It also modulates defense reaction against infection and injury caused by chemical and physical agents.²⁸ Alpha 1 acid glycoprotein is one of the main positive acute phase reactants in human plasma. It modulates the activity of the immune system during acute phase reaction. It has been reported that alpha 1 acid glycoprotein levels increase in serum during the inflammation period in severe uveitis.

Leucine-rich alpha 2 glycoprotein levels are increased in retinal models of choroidal and retinal neovascularization. It acts as a pro-angiogenic factor by activating TGF β in animal studies. Human retinal vessels also express this protein, but its expression in humans is weak. In our study, the leucine-rich alpha 2 glycoprotein is down-regulated in AMD patients. This finding is somewhat inconsistent with the proposed pathogenesis of the disease. However, this protein participates in protein-protein interactions and regulates the signaling and cell adhesion of other proteins. The neovascularization mechanism in AMD should be elucidated by investigating the presenting proteins and their interactions.

The relationships between vitreous proteins and AMD have been evaluated in recent studies. Koss et al. identified 19 proteins in the vitreous that are up-regulated in patients with AMD. Only three of these proteins were also detected in our study (Ig kappa chain C region, Alpha-2-HS-glycoprotein, and haptoglobin).²⁹ However, in our study, each of these proteins was downregulated. When we evaluated the Koss study, we saw that the mean age of the AMD patients and controls were 77.8 ± 8.9 years and 60 ± 16 years, respectively, and the groups were not demographically matched. The protein vitreous composition changes with age, so these age differences might account for the difference between the studies. In another study, the vitreous samples of patients with neovascular AMD were compared with age-matched controls.¹² The authors found that nine of the significant proteins in vitreous were up-regulated, and four of the proteins were down-regulated. Similar to our study, the Alpha crystalline A chain was downregulated. This protein was the most downregulated in our study.

Conclusion

In conclusion, we describe the changes in protein content of the vitreous in patients with AMD relative to a non-AMD group. By bioinformatics analysis, we have seen that other than VEGF and its mediators involved in the development of AMD, the proteins we mentioned could also be effective. The proteins that we have detected were mainly related to retinal protection, inflammation, and angiogenesis processes. It is thought that the selective regulation of these structural proteins will help describe the etiopathology and contribute to the development of novel treatments. Few studies have investigated the proteome changes associated with AMD. This study will make an important contribution to the literature, and the data will be useful for designing targeted studies investigating the pathology of this disease.

Declarations

Authors' contributions: All authors adhere to the guidelines for authorship that are applicable in their specific research field.

Data availability: Available.

Compliance with ethical standards

Conflict of interest: The authors declare that they have no conflicts of interest. Fund was received from Kocaeli University with the project number 2018/87.

Ethical approval: This study was approved by the institutional review board and the local ethics committee of our institution and adhered to the tenets of the Declaration of Helsinki

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Tables

Table 1. The identified proteins and their respective MALDI scores in 2DE experiments.

	Best Protein Acc.	Best Protein Mass	Best Protein Score	Expect	Matches	Calc pI	Seq. Cov. (%)	Best Protein Description	Ion score Observed	Mr (calc)	Ions	Peptide
'63	A1AG1_HUMAN	23497	140	2e-010	12	4.93	37	Alpha-1-acid glycoprotein 1	994.5275 1112.5270 1708.8466	993.5131 1111.5186 1707.8468	26 56 6	K.TEDTIFLR.E K.SDVVYTDWK.K K.NWGLSVYADKPETTK.E
'50	A2GL_HUMAN	38154	34	7.2	9	6.45	12	Leucine-rich alpha-2-glycoprotein	1179.6854	1178.6659	18	K.DLLLLPQPDLR.Y
'50	A2GL_HUMAN	38154	64	0.0088	14	6.45	18	Leucine-rich alpha-2-glycoprotein	968.5113	967.5127	26	R.YLFLNGNK.L
'65	FETUA_HUMAN	39300	104	8.1e-007	10	5.43	12	Alpha-2-HS-glycoprotein	802.3403 813.4344 1196.6118	801.3480 812.4432 1195.6197	24 21 37	K.QYGFCK.A K.FSVVYAK.C K.HTLNQIDEVK.V
'38	HPT_HUMAN	45177	224	8.1e-019	20	6.13	27	Haptoglobin	920.4524 980.4963 1203.6222 1345.6322	919.4552 979.4876 1202.6295 1344.6384 1706.8120	36 43 21 37 30	K.GSFPWQAK.M R.VGYVSGWGR.N K.VTSIQDWVQK.T K.SCAVAEYGVYVK.V K.YVMLPVADQDQCIR.H
'38	HPT_HUMAN	45177	136	5.1e-010	17	6.13	20	Haptoglobin	809.3663 920.4426 1345.6188 1707.8087	808.3715 919.4552 1344.6384 1706.8120	16 13 37 22	K.DYAEVGR.V K.GSFPWQAK.M K.SCAVAEYGVYVK.V K.YVMLPVADQDQCIR.H
'38	HPT_HUMAN	45177	92	1.40E-05	16	6.13	19	Haptoglobin	920.4430 1345.6157 1707.8073	919.4552 1344.6384 1706.8120	14 23 29	K.GSFPWQAK.M K.SCAVAEYGVYVK.V K.YVMLPVADQDQCIR.H
'38	HPT_HUMAN	45177	117	4.00E-08	14	6.13	14	Haptoglobin	920.4469 980.4924 1203.6094 1345.6179 1707.8086	919.4552 979.4876 1202.6295 1344.6384 1706.8120	28 16 1 6 46	K.GSFPWQAK.M R.VGYVSGWGR.N K.VTSIQDWVQK.T K.SCAVAEYGVYVK.V K.YVMLPVADQDQCIR.H
'49	APOE_HUMAN	36132	115	6.40E-08	23	5.65	44	Apolipoprotein E	899.4354 1033.5402	898.4337 1032.5352	11 22	R.FWDYLR.W R.LQAEAFQAR.L
'89	CRYAA_HUMAN	19897	133	1e-009	16	5.77	45	Alpha-crystallin A chain	1090.4823 1175.6167 1286.6127 1311.6591	1089.4839 1174.6194 1285.6303 1310.6579	5 9 16 29	R.QDDHGYISR.E R.TVLDSGISEVR.S K.VQDDFVEIHGK.H K.IQTGLDATHAER.A
'13	CRBA1_HUMAN	25134	157	4e-012	21	5.81	56	Beta-crystallin A3	1455.6920 1954.8275	1454.7041 1953.8316	34 7	K.ITIYDQENFQ GK.R R.GYQYILECDHHGGDYK.H
'14	CRBS_HUMAN	20993	143	1.00E-10	17	6.44	44	Beta-crystallin S	907.4119 915.4424 1156.6113 1517.7624 2097.8061	906.4018 914.4385 1155.6037 1516.7310 2096.7663	18 19 11 24 4	R.WMGLNDR.L K.ITFYEDK.N R.AVHLPSSGGQYK.I K.ITFYEDKNFQGR.R R.YDCDCDCADFHTYLSR.C
'13	CRBA1_HUMAN	25134	155	6.4e-012	20	5.81	54	Beta-crystallin A3	1455.6728 1847.8367 1954.8054	1454.7041 1846.8461 1953.8316	34 4 25	K.ITIYDQENFQ GK.R K.IQSGAWVCYQYPGYR.G R.GYQYILECDHHGGDYK.H
'20	CRBB2_HUMAN	23365	300	2e-026	27	6.5	66	Beta-crystallin B2	983.4629 1000.4325	982.4760 999.4345 1023.4410 1407.7398	53 13 24 36	K.GEQFVFEK.G R.DMQWHQR.G R.WDSWTSSR.R K.IILYENPNFTGK.K

									1024.4380 1408.7178 1726.8201 1760.8373	1725.8071 1759.8318	23 16	K.DSSDFGAPHPQVQSVR.R R.VQSGTWVGYPGYR.G
114	CRBS_HUMAN	20993	96	5.7e-006	17	6.44	41	Beta-crystallin S	891.4082 1517.7372 1729.9080 2097.7849	890.4069 1516.7310 1728.8947 2096.7663	4 18 4 1	R.WMGLNDR.L K.ITFYEDKNFQGR.R R.KPIDWGAASPAVQSFR.R R.YDCDCDCADFHTYLSR.C
111	CRYAB_HUMAN	20146	134	8.1e-010	12	6.76	35	Alpha-crystallin B chain	1088.5059 1496.6857 1512.6663	1087.5047 1495.6766 1511.6715	21 67 (26)	R.QDEHGFISR.E R.APSWFDGLSEMR.L R.APSWFDGLSEMR.L
134	IGKC_HUMAN	11602	8.1e-007	8.1e-007	13	5.58	55	Ig kappa chain C region	1875.9359 1946.0145 1797.8800 1875.9082 1945.9818	1874.9197 1945.0197 1796.8880 1874.9197 1945.0197	8 8 15 9 13	K.VYACEVTHQGLSSPVTK.S -.TVAAPSVFIFPPSDEQLK.S K.SGTASVVCLLNNFYPR.E K.VYACEVTHQGLSSPVTK.S -.TVAAPSVFIFPPSDEQLK.S

Table 2. Regulation ratios and trends of the AMD group over the control group (no medication). Cnt/AMD: Regulation in AMD group according to control group. ↓: Down-regulation ↑: Up-regulation.

SSP No	Best Protein Description	Cnt/AMD (fold)	Regulation trends
SSP 0202	Alpha-1-acid glycoprotein 1 (A1AG1 or ORM1)	36	↓
SSP 1103 SSP 1104	Leucine-rich alpha-2-glycoprotein (A2GL or LRG1)	9 12	↓
SSP 1203	Alpha-2-HS-glycoprotein (FETUA or AHSG)	5	↓
SSP 2104 SSP 3006 SSP 3007 SSP 3008	Haptoglobin (HPT or HP)	15 4 9 5	↓
SSP 3020	Apolipoprotein E (APOE)	3	↑
SSP 4011	Alpha-crystallin A chain(CRYAA)	779	↓
SSP 8003	Alpha-crystallin B chain (CRYAB)	214	↓
SSP 5005 SSP 6003	Beta-crystallin A3(CRBA1)	13 177	↓
SSP 7003	Beta-crystallin B2(CRBB2)	232	↓
SSP 5006 SSP 7004	Beta-crystallin S (CRBS)or Gamma-crystallin S	132 216	↓
SSP 9003	Ig kappa chain C region(IGKC)	36	↓

Figures

a)

Before precipitation	After precipitation
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b)

Control	AMD
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Figure 1

Normalization of protein concentrations in protein pools. a. SDS-PAGE image of vitreous protein samples before and after precipitation. B. SDS-PAGE analysis of the protein pools of each group. AMD, age-related macular degeneration.

Figure 2

a. 2D gel images of proteins representing the study groups. b. The 2D gel image showing position of the regulated spots and the corresponding SSP. AMD = age-related macular degeneration.

Figure 3

Close-up images of the differentially regulated protein spots whose relative quantities changed between the groups. AMD = age-related macular degeneration.

Figure 4

STRING analysis of identified proteins interactions. A) The STRING analysis with high confidence and no consideration of maximum number of interactions in the first and second shells. B) The STRING analysis with decreased stringency using a maximum of five interactions in the first shells and considering all possible interaction sources provided by the database.

Figure 5

STRING Analysis of identified proteins interactions. A) The STRING analysis with high confidence and no consideration of maximum number of interactions in the first and second shells. B) The STRING analysis with decreased stringency using the settings of five (maximum number) interactions in the first shells and considering all the possible interaction sources provided by the database.