ORIGINAL RESEARCH

Comparative Proteomic Analysis of the Aqueous Humor from Patients with Pseudoexfoliation Syndrome

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Abstract

Purpose: The goal of this study was to pinpoint potential molecular pathways that may have contributed to the onset of pseudoexfoliation syndrome (PEX), a systemic illness associated with aging that has no known cause and is brought on by the deposition of fibrillary white flaky debris in ocular tissues.

Materials and methods: Protein pools representing each group were created using two-dimensional gel electrophoresis (2DE) in conjunction with a matrix-assisted laser desorption ionization-time of flight/time of flight (MALDI-TOF/TOF) mass spectrometer. Aqueous humor (AH) from patients with PEX and cataracts was also collected for a comprehensive study of the data; ingenuity pathway analysis (IPA) was used for the discovered proteins.

Results: In comparison to controls, 2DE showed that 10 sites in PEX patients had differently altered gene expression. Two of these proteins, transthyretin (TTR) and apolipoprotein A4 (ApoA4) were significantly overexpressed in PEX patients, but the remaining proteins were only mildly altered. The liver X receptor (LXR) and the retinoid X receptors (RXR) may play a crucial role in the pathophysiology of PEX according to IPA employing these 10 proteins.

Conclusion: The altered proteins, particularly ApoA4 and TTR, may be important in revealing the molecular process behind PEX, as anticipated by IPA.

Keywords: Apolipoprotein A4, Liver X receptor, Pseudoexfoliation, Retinoid X receptor, Transthyretin.

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Introduction

The progressive deposition of fibrillary white flaky material onto ocular structures is a hallmark of pseudoexfoliation syndrome (PEX).[1] Although PEX materials are created and accumulate throughout the body, clinical symptoms are first discovered during an anterior segment slit lamp examination in ophthalmology.[1] The most frequent definite cause of open-angle is PEX, and glaucoma is the second greatest cause of blindness in the globe.[1,2] Management of glaucoma that develops as a result of PEX is more difficult than managing glaucoma that develops naturally.[1]

Unknown is the etiopathology of PEX. The majority of investigations that were conducted to clarify the etiopathology focused on identifying fresh biomarkers that might point to particular pathways connected to PEX. Endothelin-1, transferrin, clusters, and matrix metalloproteinases are discovered to be significant and have undergone extensive research among the biomarkers for transforming growth factor β.[1,3] Lysyl oxidase like-1 (LOXL1) protein is the only gene product so far connected to PEX disease. A copper-dependent amine oxidase called LOXL1 participates in the development of connective tissues. In elastin and collagen, it creates cross-links.[4]

We believe that by performing a comparative proteome study on aqueous humor (AH), it will be possible to identify unknown proteins that are more closely associated with etiopathology. In this study, we closely examined proteome alterations in AH samples from PEX patients and healthy people. This discovery is a step in the right direction toward figuring out the potential mechanisms responsible for PEX formation.

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Conflict of interest: None

Materials and Methods

Study Population

The Kocaeli University Research Ethics Committee accepted this work, and it complies with the Helsinki Declaration. All patients gave their informed consent before the samples were taken. All patients got a thorough eye examination that included taking their medical history, measuring their best corrected visual acuity (BCVA), using a slit lamp for biomicroscopy, measuring their tonometry using the Goldmann applanation, and examining their fundi under dilated pupils. The Lens Opacities Classification System III[5] was used to classify several types of lens nuclei.

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IOLMaster was used to measure axial lengths (Zeiss, Version 5.0; Carl Zeiss Meditec, Inc., Dublin, California, United States of America). The log of the minimum angle of resolution (logMAR) was used to transform BCVA records for statistical analysis (logMAR).

A total of 15 individuals with PEX and senile cataracts and 15 control patients with senile cataracts participated in this prospective cross-sectional investigation. The controls and PEX patients were both scheduled for cataract surgery. Participants were deemed to have PEX if they had pupil margin accumulation of pseudoexfoliation material, pupillary ruff loss, or patchy transillumination abnormalities at the lens (often central disk or peripheral zone). The only ocular conditions they had were cataracts and PEX. All PEX eyes had normal intraocular pressure, which is defined as <21 mm Hg, and no other ophthalmic conditions besides cataracts. None of the patients were taking any eye medicine. The age-matched control group alone had cataracts as their only ocular pathology. The following conditions were excluded: a history of prior laser and ophthalmic surgery, retinal vascular and degenerative diseases, grade 6 (adult) cataracts, and systemic illnesses such as diabetes, hypertension, and neurodegenerative disorders. A competent surgeon took AH samples from the individual's eye during cataract surgery.

Sample Collection

The collection of AH was carried out as previously mentioned.[6] In essence, a paracentesis was performed in the peripheral cornea at the start of the procedure, and the tip of a 30-gauge cannula was introduced into the mid-anterior chamber through the paracentesis tract. Slowly, a small amount of AH (50–100 L) was aspirated. The AH specimens were relocated to fresh tubes after the anterior portion was excised. The material was then kept at –80°C. Blood-contaminated samples were not included.

Preparation of Protein Samples from the Aqueous Humor

The collected samples were initially cleaned by being temporarily stretched at 4°C at 1500 ×g, and then the AH was transferred to LoBind tubes. The Bradford assay was used to quantify protein concentrations (BioRad, United States of America). An sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was performed to visually inspect the measured protein concentrations. The same microgram of protein from each sample was combined to create protein pools for each group (the PEX and the control groups) after verifying that the protein concentration measurements were accurate.

Two-dimensional Gel Electrophoresis (2DE)

Each protein pool's 75 gm of proteins underwent acetone precipitation cleaning. The final precipitate was dissolved in 2D rehydration sample buffer, which also contained traces of bromophenol blue and contained 4% 3-[(3-cholamidopropyl)—dimethylammonium]-1-propane sulphonate and 7 M urea, and 2 M thiourea. The materials were placed onto 11 cm pH gradient strips [pH 3–10 nonlinear (NL)] (immobilized pH gradient [IPG]). The proteins were subjected to first-dimension separation based on isoelectric points in a Protean IEF cell (Bio-Rad, United States of America) following overnight passive rehydration. The following conditions were used at 20°C: the voltage was raised to 250 and 4000 V in a 120-minute gradient, then to 10000 V in 2.5 hours. The strips were kept at 10000 V until they reached 25000 V/hour in total. The strips were subjected to isoelectric focusing before being submerged for 30 minutes in equilibrium buffer I, which contains 6 M urea, 2% SDS, 30% glycerol, and 2% dithiothreitol, along with 50 mM tris-HCl pH 8.8. The strips were then drained and placed for a further 30 minutes at room temperature in equilibrium buffer II, which contains 50 mM tris-HCl pH 8.8, 6 M urea, 2% SDS, 30% glycerol, and 2.5% iodoacetamide. 12% gels were used for the second-dimension separation, and the Dodeca gel running system was employed (Bio-Rad, United States of America). After fixing the gels, colloidal coomassie blue G250 (Bio-Rad, United States of America), a suitable stain for mass analysis (MS) analysis, was used to color them.

Quantitative and Statistical Analysis of Gel Images

The PDQuest Advanced software was used to measure the intensities of each spot, normalized by the sum of the volumes of all the matching spots in a set of gels (Bio-Rad, United States of America). When compared to any other sampling site, protein spot intensities with at least a two-fold significant change (p, 0.05) in a consistently up/down expression ratio were deemed to be differentially expressed.[7] The spots that displayed different expression patterns across the two experimental groups were then selected using one-way analysis of variance and student's *t*-test. The ExQuest Spot-cutter (Bio-Rad, United States of America) automatically excised the gel spots that had a differential expression for protein identification.

In-gel Tryptic Protein Digestion and MS Analysis

The gel pieces were trypsinized with an in-gel digestion kit according to the specified procedure (Pierce, United States of America). The methodology calls for three 20-minute destaining phases using 50% acetonitrile (ACN)/100 mM ammonium bicarbonate, followed by 20-minute washes using 25% ammonium bicarbonate/50% ACN, a 10-minute dehydration step with 100% ACN, and then drying. The spots were hydrated and digested for a whole night at 30°C with 10 L of 10 ng/L trypsin in ammonium bicarbonate. With one L of a matrix solution (2 mg/mL α-ciane-4-hydroxycinnamic acid dissolved in 50% ACN/0.1% trifluoroacetic acid [TFA]), the digested peptides were concentrated and desalted with a C18 microcolumn (ZipTip, Millipore, United States of America), eluted, and then immediately spotted onto matrix-assisted laser desorption ionization (MALDI) plates. Using matrix-assisted laser desorption ionization-time of flight/time of flight (MALDI-TOF/TOF), the peptides in each sample were subjected to MS (ABSCIEX 5800 system, United States of America). A 400–4000 m/z and a 25 kV accelerating voltage were adjusted for the m/z range. Using peptides from autodigested trypsin (m/z 842.509, 2211.104), MS spectra were internally calibrated. In order to offer information for use in establishing the peptide sequence, the 10 strongest peptide ions were then subjected to further fragmentation (MS/MS).

Mascot and Pathway Analysis

Peptide mass fingerprinting was used to identify proteins, and the results were verified by MS/MS analysis. The ProteinPilot search engine, powered by the Mascot 2.5 search engine (Matrix Science, United Kingdom), was utilized to identify proteins (ABSCIEX, United States of America). The following criteria were used in the Mascot search process: Species restriction to *Homo sapiens*; one missed cleavage allowed with the chosen trypsin enzyme; at least five independent peptides matched; MS precursor mass tolerance

set to 50 ppm; MS/MS fragment tolerance set to 0.2 Da; fixed modification being cells international carbamidomethyl; variable modification being oxidation (Methionine [Met]); peptide charge of 1+ and being monoisotopic; National Center for Biotechnology Information nonredundant (only significant hits that met the Mascot probability analysis' threshold of 0.05 were considered valid. The software Ingenuity Pathway Analysis (IPA) (Qiagen, United States of America) was used to create the networks once the detected proteins' accession numbers were entered. Protein identifications with a *p*-value of <0.05 and an expression change of more than twofold were used to create the canonical pathways that were used to create the protein-protein interaction maps.

Results

The age and sex of the participants in the groups were kept comparable since the protein composition of aqueous material depends on the age and gender of the subjects and the matched controls. With a mean age of 62.17.07 years, the 15 patients who took part in the PEX group were split between seven female and eight male patients. With a mean age of 65.415.65 years among the 15 patients who took part in the control group, seven of them were female, and eight of them were male. According to the LOCS III scale, the degree of nuclear and cortical opacification was identical across all of our individuals.[5] As indicated in Table 1, we did not detect any statistically significant differences between the demographic information and preoperative clinical variables in our dataset during our analysis. Each sample's protein concentration was found to be too low to allow for 2D analysis. As a result, we were unable to do a comparison analysis on each sample separately. As a result, equal amounts of protein from each sample were combined to generate a sample and control protein pool. Since pooling reduces the quantity of information lost below the detection threshold, a pooling design may occasionally be effective.[8] The protein samples required a previous treatment to boost resolution because the first 2D gels made from the pooled samples had a fuzzy look and hazy patches. After cleaning the samples, a second 2D gel electrophoresis was performed. On the gels, 30,010 different protein spots were seen (Fig. 1). Gel image comparison between groups revealed extremely comparable protein profiles with a match rate of >90%. This high incidence of the match suggested that the protein composition of the AH was not the primary reflection of the disease condition.

Therefore, regardless of the quantity of each protein site, we looked at regulation ratios. A total of 10 protein spots were excised from the gels and identified using the twofold regulation criteria, which is a widely acknowledged criterion for determining

Table 1: The demographic data and the preoperative clinical characteristics

		PEX group	*Control group*	*p*
Gender (*n*)	Male	8	8	1
	Female	7	7	
Age (years)	Mean ± standard deviation (SD)	62 ± 17.07	65.4 ±15.65	0.58
Axial length (mm)	Mean ± SD	23.50 ± 0.70	23.62 ± 0.80	0.66
	Median	23.14	23.68	
	Range	2.12	3.06	
BCVA (logMAR)	Mean ± SD	0.98 ± 0.10	0.96 ± 0.11	0.60
	Median	1	1	
	Range	0.4	0.4	
Grades of LO (*n*)	Grade 3	6	5	0.80
	Grade 4	8	8	
	Grade 5	1	2	

BCVA, best corrected visual acuity; logMAR, log of the minimum angle of resolution; LO, Lens opacity

Table 2: The name of the identified proteins, their respective MALDI scores, and the regulation trends of the PEX group over the control group

SSP	*Swiss-Prot AC no.*	*Best protein accession*	*Best protein mass*	*Best protein score*	*Expectation score*	*Matches*	*Calc. pI*	*Seq. Cov (%)*	*Best protein description*	*PEX/control groups*
3003	P02766	TTHY_HUMAN	15877	121	1.60E-08	11	5.52	68	TTR	Upregulation
1102	P06727	APOA4_HUMAN	45371	160	2E-12	19	5.28	35	ApoA4	Upregulation
1204	P01009	A1AT_HUMAN	46707	130	2E-09	15	5.37	29	A1AT	Upregulation
2203	P02774	VTDB_HUMAN	52929	57	0.039	11	5.4	18	VDBP	Upregulation
2102	P02768	ALBU_HUMAN	69321	286	5.1E-025	23	5.92	26	Serum albumin	Upregulation
2004	P04264	K2C1_HUMAN	65999	297	4E-26	23	8.15	38	Keratin, type II cytoskeletal 1	Upregulation
2005	P02647	APOA1_HUMAN	30759	65	0.006	11	5.56	32	ApoA1	Upregulation
7507	P02787	TRFE_HUMAN	77000	384	8.1E-35	38	6.81	39	Serotransferrin	Upregulation
101	P02763	A1AG1_HUMAN	23497	90	2.3E-05	6	4.93	22	A1AG 1	Downregulation
102	P19652	A1AG2_HUMAN	23588	77	0.0004	6	5.03	20	A1AG 2	Downregulation

Calc. pI, calculated isoelectric point; SSP, standard spot number; Seq. Cov, sequence coverage; Swiss-Prot AC no, Swiss-Prot accession number

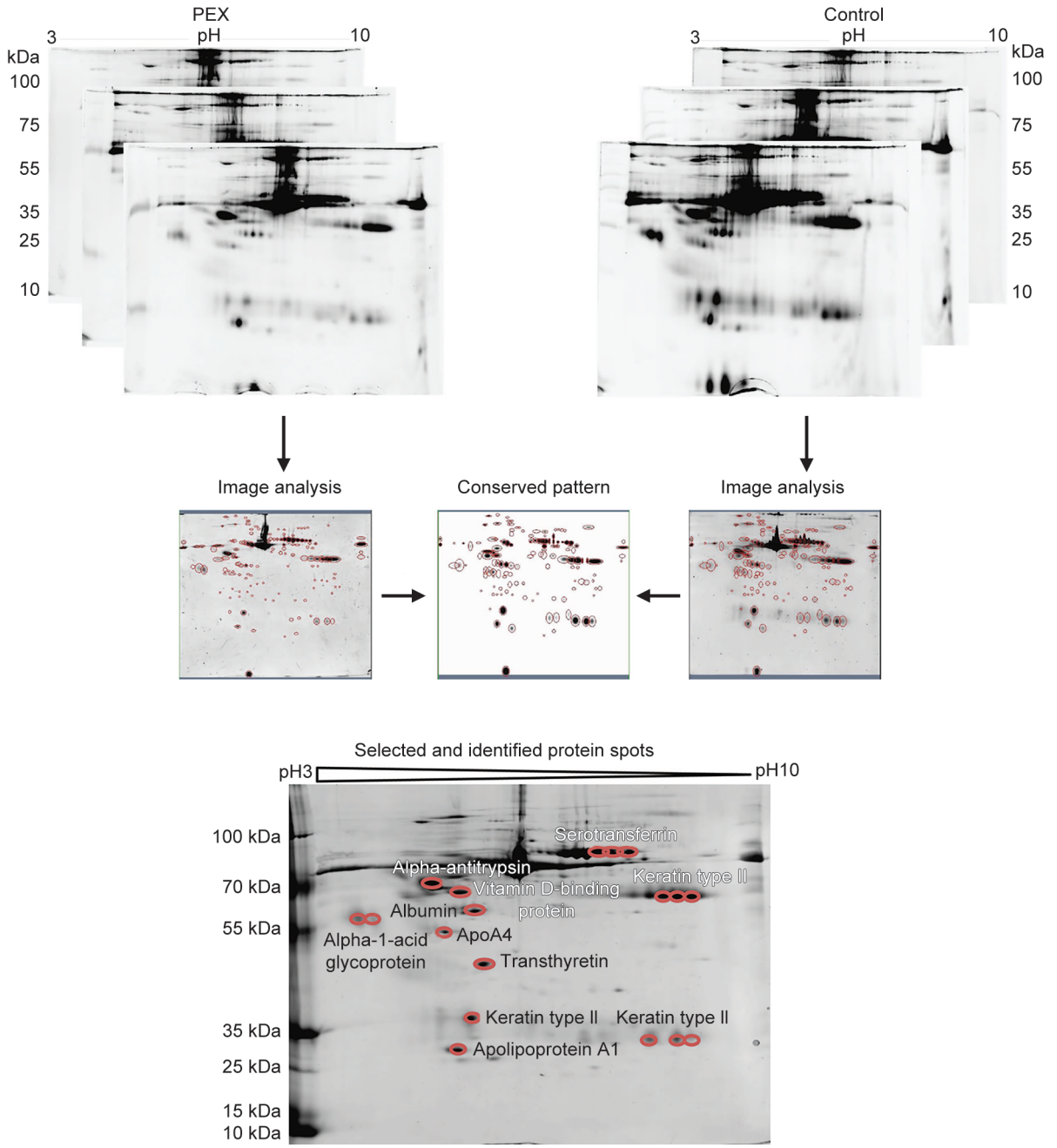

Fig. 1: Images of 2D gels and analysis made to elucidate differential expression patterns of observed protein spots; *z* total of 70, 5 µg proteins from the control ($n = 15$) and PEX ($n = 15$) protein pool were cleaned and loaded onto immobilized 11 cm, pH gradient strips (IPG) (pH 3–10 NL); the spots marked in red represent the corresponding upregulated or downregulated proteins that are listed in Table 2

differential regulation. They are listed in Table 2. The PEX group had higher levels of transthyretin (TTR), apolipoprotein A4 (APOA4), α-1-antitrypsin (A1AT), ApoA1, and vitamin D binding protein (VDBP) than the control group, in addition to albumin, keratin, and serotransferrin. α-1 and α-2 acid glycoprotein (A1AG 1 and 2) levels were also found to be lower in the PEX group. Figure 2 depicts how the protein spots' relative abundance varied between the groups.

To find enriched biological pathways, we employed IPA (Fig. 3). The liver X receptor (LXR) and retinoid X receptors (RXR) activation ($p = 3.02$, 1019), the farnesoid X receptor (FXR) and RXR activation ($p = 4.4$, 1019), and acute phase response signaling ($p = 6.54 \times 10^{-13}$) are among the most significant enriched canonical pathways (Fig. 4).

Discussion

Numerous methods have been used to examine the pathophysiology of PEX. Proteomics offers the most encouraging outcomes of all the techniques tested.[1] To examine the alterations in protein expression profiles relative to healthy controls, we used 2DE combined with MALDI-TOF/TOF in the current comparative proteomics investigation. In PEX patients, the quantity of several

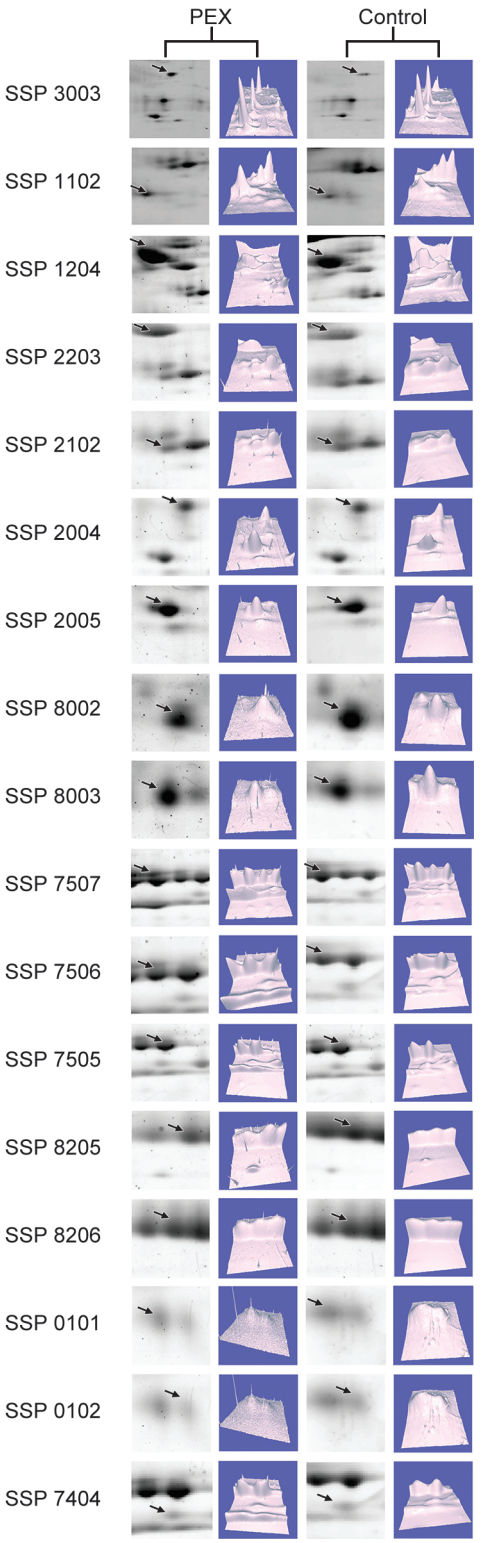

Fig. 2: Close-up images of the regulated spots and their corresponding 3D changes in protein expression with 3D images are visualized in terms of spot volume; the volume of each spot provides the basis for the comparison of protein expression between PEX and the control group; in each imagerelated protein spot was marked with an arrow

proteins changed. These proteins included A1AT, albumin, keratin, serotransferrin, and α-1 and α-2 acid glycoproteins, as well as TTR, ApoA4, ApoAI, VDBP, and TTR. Both abundant and less-abundant proteins were found in the samples, according to a review of the gel pictures. Albumin and serotransferrin, two proteins that were present in large quantities, most likely came from the serum and seeped into the anterior chamber. Our presumptions are supported by the observation that PEX affects the blood-aqueous barrier, changing the protein composition of AH.[9]

We checked the Uniprot database to see if each protein has any known ocular involvement. Thyroxin is transported from the bloodstream to the brain by the thyroid hormone-binding protein TTR.[10] The cause of TTR-related amyloidosis is thought to be TTR. Different tissues may develop protein fibrils, which might result in amyloid plugs. TTR is recently shown to be produced by the ciliary pigment epithelium as well as the retinal pigment epithelium.[11] In a different investigation, ciliary epithelial labeling was shown to be much lower in buphthalmic rabbits with ciliary body atrophy, demonstrating that the decrease in AH content and TTR was caused by lessened synthesis.[12] We believe that the dysregulated production of TTR by the ciliary body may be the cause of the rise in its concentration. Regardless of where it comes from, PEX and TTR may work together functionally. TTR and retinol binding protein can combine to form a complex that might give AH some retinoic acid (RA) in the form of retinol. Lysyl oxidase, an enzyme connected to PEX, is likewise transcriptionally regulated by RA, which is a potent regulator for several transcription factors.[4,13] The aqueous humor may provide RA to the lens epithelial cells in human eyes, according to Wakabayashi et al.[13]

Apolipoproteins (Apo) are important parts of chylomicrons and HDL.[14] In earlier studies,[15] APOA4 and A1 were also found in the aqueous humor of PEX patients. It is unclear if the presence of APOA4 and A1 in the aqueous humor is caused by a compromised blood-aqueous barrier or if PEX patients' anterior segments are responsible for producing this protein. The involvement of Apos in neurodegenerative illnesses like Alzheimer's has been clearly demonstrated in prior research.[16] Amyloid is a key factor in Alzheimer's disease, and amyloid-associated proteins co-occur with PEX fibrils. Studies have conclusively demonstrated the presence of APOA4 in amyloid material, and since it has been demonstrated to have a role in amyloid formation, it is believed that APOA4 may occasionally act as an initiator.[17,18] This discovery allowed us to hypothesize that APOA4 is similarly abundant in the PEX material. Our detection of a protein spot for APOA4 on 2DE gels lends credence to this assertion. Recent analyses of the lens capsule, however, have revealed that APOA4 and A1 are present in the PEX material.[19] These proteins have anti-inflammatory functions, but they can also impact how PEX material is made. Along this line of reasoning, there was also a rise in APOA1, indicating that alterations in Apo levels might be connected to the pathophysiology of PEX. Apos have been shown to be present in PEX material, although it is unknown at this time whether APOA4 stimulates the development of PEX material as in amyloidosis or has some other purpose.[20]

According to a study by Janciauskiene and Krakau[21] to show the existence of β-amyloid-associated protein found in AH of PEX material from PEX patients, 28.9% of the PEX patients had A1AT in their AH. We also showed that the PEX group had higher levels of A1AT than the control group did. By having a chaperone-like effect on PEX, which is referred to as age-related elastosis, A1AT may be secondarily enhanced. High levels of A1AT may limit proteolytic activity and have a protective function in elastase stabilization.

The two bands that were identified as belonging to serotransferrin in an older report may reflect transferrin isoforms

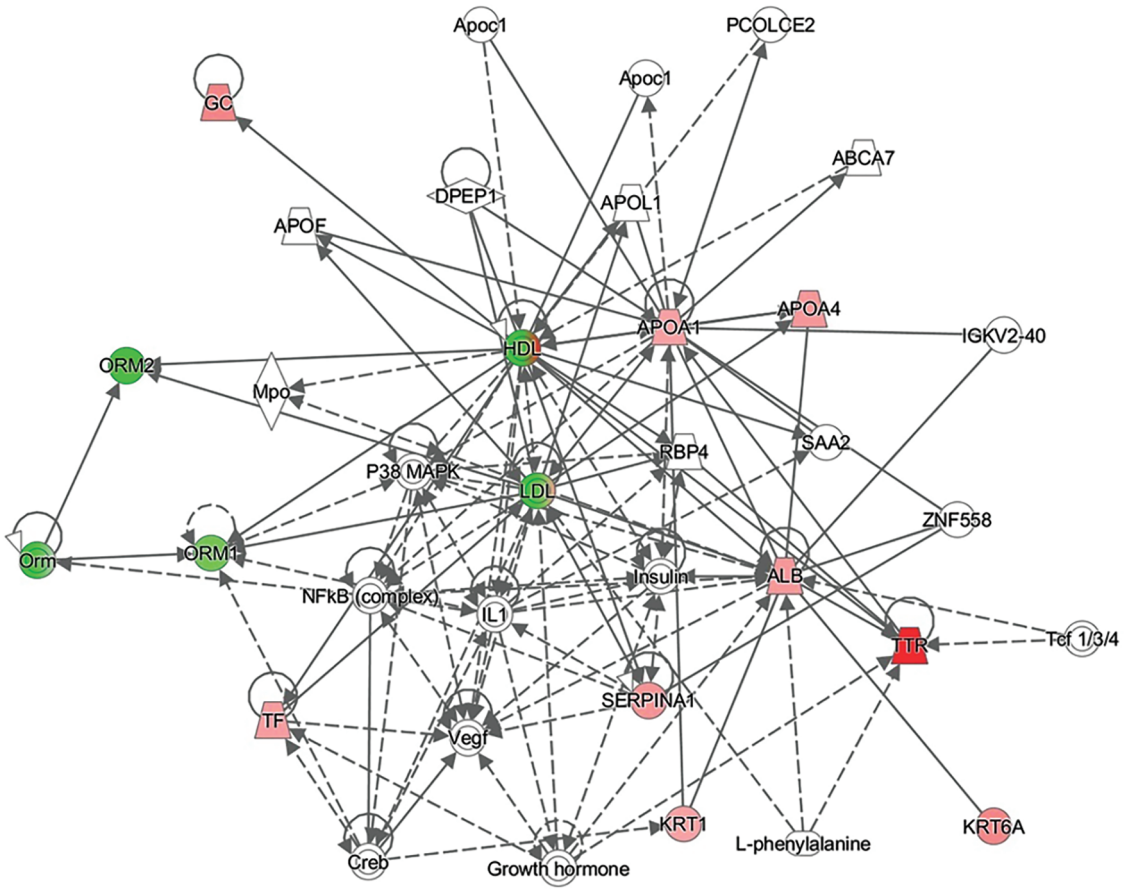

Fig. 3: Metabolic pathway analysis of the PEX group over the control group using IPA; red-marked spots show upregulated proteins and green-marked spots show downregulated proteins (orosomucoid 1 and 2); straight lines indicate direct protein–protein interactions, while dashed lines indicate indirect protein-protein interactions; colorless proteins were not detected in our study by mass spectrometry; IPA algorithm added them to the network

with various carbohydrate side chains, according to a probable link of serotransferrin with PEX.[22] At least three distinct serotransferrin isoforms with various pI values were found in our investigation. Serotransferrin and its isoforms are present in the PEX material, although their function has not been clarified. According to our hypothesis, oxidative stress, which is thought to play a role in the pathophysiology of PEX,[23] may link iron to PEX.

The primary carrier protein for 25-hydroxy vitamin D in circulation is VTDB, although this protein is also involved in other metabolic and inflammatory processes, including the binding of fatty acids and extracellular actin scavenging.[24] In agreement with Hardenborg et al., we discovered that the PEX group's VTDB level was much higher than the control group's.[15]

Acute phase proteins seen in serum include α-1-acid glycoprotein (A1AG) and α-2-AG. By lessening the tissue damage brought on by an inflammatory process, AAG's anti-inflammatory and immunomodulatory characteristics aid in maintaining homeostasis.[25] AAG has antioxidant qualities that make it protective against oxidative stress; hence, its depletion in the PEX material may speed up the development of illness.

The characterized proteins shared characteristics such as hepatic expression, systemic circulation, participation in several transport routes, participation in lipid metabolism, the existence of extracellular antioxidant capabilities, and participation in inflammation. We used IPA analysis to get a broad perspective on the proteins that were differently regulated and to build functional and metabolic viewpoints. The lipid metabolism alterations and the neurodegenerative process were the issues that were brought to our attention. IPA's canonical pathway analysis forewarned us about LXR/RXR receptor activity.

Allele-specific impacts on the level of LOXL1 gene regulation at transcriptional (RXR binding), cotranscriptional (pre-mRNA insertion), and posttranscriptional (mRNA degradation) levels have only been noted in one previous investigation to date. In cells and tissues with hazardous alleles, they collectively result in a downregulation (40–50%) of LOXL1 gene expression levels.[4] They propose that RXR may be a potential transcription factor, may be significant for this regulatory region's RA sensitivity, and may have RA-suppressive effects on LOXL1 expression.

Our study has a case-control ratio of 1:1, which is a constraint. If the number of samples in the control group is doubled, statistical power would further rise.

In our investigation, PEX syndrome sufferers without glaucoma were included. As the AH in glaucoma patients with PEX may differ dramatically from that in PEX patients without glaucoma, investigations in the future may include patients with PEX glaucoma in addition to PEX syndrome as a second group. In order to compare the aqueous humor of people with PEX syndrome and PEX glaucoma.

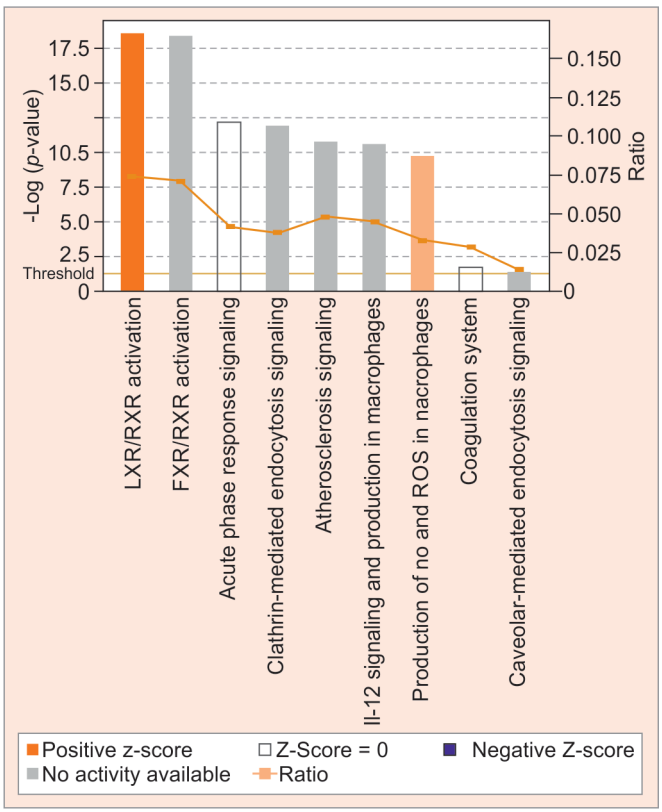

Fig. 4: Canonical pathway analysis of regulated proteins; *z*-scores associated with biological functions are estimated to be significantly activated or inhibited based on differential protein expression; the X-axis shows the name of canonical pathways identified by IPA analysis; the height of the bar against each pathway shows the negative log of the *p*-value; the strength of the effect is coordinated by the color intensity; the color of the bar shows the strength of regulation inferred from the activation *z*-score (orange, upregulation; gray, no activity pattern available; blue, downregulation; white, *z*-score = 0, indicating upregulation and downregulation of proteins)

Additionally, by using the subgroups, the impact of various degrees of glaucomatous damage in aqueous humor can be examined.

Conclusion

A total of 10 proteins were discovered to be different from the control group in this proteome analysis of aqueous humor taken from the anterior chambers of PEX patients.

Particularly, ApoA4 and TTR stand out when the proteins discovered are assessed by IPA, indicating that they may be key players in the pathophysiology of PEX syndrome.

More will be discovered about the pathology of PEX related to protein metabolism when new technologies in the field of proteomics are developed. Potential biomarkers for LXR/RXR activity in the PEX include APOA4 and TTR. Our findings and assertions will contribute to the results of further studies and, in the end, establish targets for the creation of novel PEX therapies.

Acknowledgments

The local Ethics Committee Registration number of this study is KU-KAEK Project No: 2015/35.

Informed consent was obtained from all individual participants included in the study.

Informed consent was obtained from patients regarding publishing their data and photographs.

All authors contributed to the study's conception and design. Clinical examination and evaluation were performed by Muge Toprak, Dilara Pirhan, and Busra Yilmaz Tugan; surgical procedures, material collecting, and transferring were performed by Nursen Yuksel, Muge Toprak and Busra Yilmaz Tugan; proteomics studies were performed by Murat Kasap and Gurler Akpinar. Data analysis was performed by Muge Toprak, Murat Kasap, Gurler Akpinar, and Nursen Yuksel. All authors read and approved the final manuscript.

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