Metabolomic study of eyeball rupture and patients with cataracts in aqueous humor

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Abstract. The purpose of the present study was to identify metabolic biomarkers and study the metabolic changes in relation to cataracts and eyeball rupture in human aqueous humor. This case-control study included 3 patients with traumatic ocular rupture treated by surgery as the control group, 10 patients with severe cataracts as the severe cataracts group and 10 patients with mild cataracts as the mild cataracts group. The present study used liquid chromatography-mass spectrometry to analyze the metabolomics of aqueous humor samples. Databases including the Kyoto Encyclopedia of Genes and Genomes and MetaboAnalyst were used to find potential pathways for metabolites. Aqueous humor metabolic spectrum can competently distinguish patients with different degrees of cataracts from the control group. A total of 34 metabolites were identified as potential biomarkers that could distinguish patients with different degrees of cataracts; 36 metabolites were identified as potential biomarkers that could distinguish patients with severe cataracts from the control group; 34 metabolites were identified as potential biomarkers that could distinguish patients with mild cataracts from controls. In pathway analysis, glycerolphospholipid metabolism was highly affected, which meant that these metabolic markers serve an important role in the regulation of this pathway. The present study identified valuable metabolic biomarkers and pathways, which is helpful for an improved understanding of the pathogenesis of cataracts. This discovery has transformation value for the development of new treatment methods for cataracts.

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Introduction

Cataracts are the leading cause of blindness caused by the opacification of the lens. A total of ~95 million individuals are affected by cataracts worldwide (1). According to the etiology, cataracts can be divided into age-related cataracts, pediatric cataracts and cataracts secondary to other causes. Age related cataracts is the most common type in adults (2). The loss of lens crystal transparency may be associated with an increase of light scattering or light absorption. It is mainly affected by two main factors. First, the lens is mainly composed of fiber cells with inactive metabolism. These cells have a high concentration of structural protein e crystallin, which is evenly distributed in the cells. This provides a high refractive index lens required to focus incident light on the retina. Second, the lens has no vascular system (3,4); it supplies nutrients, such as a variety of metabolites, through the aqueous humor around the lens (5,6). The drawback of this design is that the crystallins in the lens core do not turnover (5,6). With age, it accumulates a large number of post-translational modifications, resulting in protein insolubility, aggregation and coloring (5,6). Finally, these processes lead to the formation of insoluble protein aggregates and the occurrence of age-related nuclear cataracts (5,6). The long-term integrity of the crystallin network is provided by metabolites, which are synthesized in the lens epithelium or enter the lens from the aqueous humor through the lens epithelium (5,6). However, the mechanism of metabolite transport in crystals remains to be elucidated.

Metabonomics is a widely used technology, which is often used to understand the pathophysiological processes leading to disease occurrence and progression and to provide molecular information about disease phenotype (7-9). In ophthalmic diseases, metabonomics has been successfully used to determine the metabolic characteristics of diabetic retinopathy (10). However, few studies have focused on cataracts. It is reported that the structural component of the lens is a specific protein in the aqueous humor of cataracts patients (11,12). The lens does not have its own blood supply. It is nourished by aqueous humor. Aqueous humor is secreted by ciliary epithelium and enters the posterior chamber. Then it flows around the lens, enters the anterior chamber through the pupil and finally exits through the ciliary sclera. In addition to proteins, human aqueous humor is also composed of small molecules. It contains electrolytes, oxygen, carbon dioxide, glucose,

urea, antioxidants, organic solutes, amino acids, fatty acids and lipids (11,12). The actual composition and level of small molecules can be considered as the chemical reflection of the phenotype of a specific biological system. Metabonomics extends our understanding of the metabolic components of aqueous humor (13,14). It is often used in disease diagnosis, new therapeutic targets or prognostic markers (15). However, studies on changes in human aqueous humor metabolism associated with specific eye diseases are limited (16).

Materials and methods

Study participants and sample collection. The present study included 13 participants recruited between 1 December 2021 and 1 March 2022. All procedures of this study were in accordance with the tenets of the Declaration of Helsinki. The current study was approved by the Research Ethics Committee of the Tong Ren Hospital affiliated with Shanghai Jiao Tong University School of Medicine, Shanghai, China (approval no. 2021-078-01; 17 November 2021). Samples were collected following the patients' written informed consent to participate in the study. The process of collecting aqueous humor is to puncture the anterior chamber with a 30 g needle and inhale ~50-100 μl aqueous humor, transferred to Eppendorf tube and stored at -80℃.

The present study was conducted on aqueous humor samples from 3 patients with ocular rupture and 20 patients with cataracts surgery. Aqueous humor was collected after topical anesthesia with promecaine hydrochloride eye drops. The patients were divided into three groups: 3 patients with ocular rupture as the control group, 10 patients with mild age-related cataracts as the mild group and 10 patients with severe age-related cataracts as the severe group.

Patients were considered to be mild age-related cataracts of cataracts if the nuclear opalescence or nuclear color was < grade 3, the cortical cataracts score was < grade 3 and the posterior subcapsular cataracts score was < grade 3 in the affected eye based on the Lens Opacities Classification System (LOCS) III (17). Patients were considered to be severe age-related cataracts of cataracts if the nuclear opalescence or nuclear color was ≥ grade 3, the cortical cataracts score was ≥ grade 3 and the posterior subcapsular cataracts score was ≥ grade 3 in the affected eye based on the LOCS III.

Liquid chromatography-mass spectrometry (LC-MS) measurements. The sample was slowly thawed on ice and 1.5 ml of chloroform/methanol (2:1) solution directly added to the sample. Subsequently, 0.5 ml pure water was added, vortexed for 1 min and centrifuged (1,006.2 x g, 10 min, 4℃). The organic phase was placed into a clean test tube and blown dry with nitrogen. Following drying, 100 μl isopropanol/methanol (1:1) and 4 μl internal standard LPC (0.14 mg/ml) were redissolved, vortex mixed and centrifuged (16,099.2 x g, 10 min, 4℃). The supernatant was placed into the injection bottle. Extracted samples were randomly analyzed by an LC-MS system (Waters Corp.). The chromatographic separation conditions were as follows: Column temperature, 40℃; flow rate, 0.3 ml/min; injection volume, 1 μl (positive mode) and 3 μl (negative mode); and autosampler temperature, 4℃. The

detection parameters of mass spectrometry were divided into ESI+ and ESI-. ESI+: Heater temperature, 300℃; sheath gas flow rate, 45 arb; aux gas flow rate, 15 arb; sweep gas flow rate, 1 arb; spray voltage, 3.0 KV; capillary temperature, 350℃; S-lens RF level, 30%. ESI-: Heater temperature, 300℃; sheath gas flow rate, 45 arb; aux gas flow rate, 15 arb; sweep gas flow rate, 1 arb; spray voltage, 3.2 KV; capillary temperature, 350℃; S-lens RF level, 60%.

Statistical analysis. Peak alignment and extraction were performed using Compound Discoverer software (Thermo Fisher Scientific, Inc.). An unsupervised model of principal component analysis (PCA) was used to assess the overall trend of segregation between these samples. A supervised model of orthogonal projections to latent structures-discriminate analysis (OPLS-DA) was used to screen for significantly different metabolites between the groups. In order to improve the analysis, the variable importance in the projection (VIP) was obtained. VIP values >1 were first selected as changed metabolites. In addition, at a critical P-value of 0.05, these selected metabolites were further verified using two sided student's t-test. The area under the receiver operating characteristic curve (AUC) was calculated to evaluate the recognition ability of each metabolite marker. The pathways of metabolites was performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.genome.jp/kegg/) and MetaboAnalyst (http://www.metaboanalyst.ca/) (18,19). P<0.05 was considered to indicate a statistically significant difference.

Results

Characteristics of the participants. A total of three groups of patients underwent surgery and provided written informed consent to obtain aqueous humor samples for research purposes. All participants had no systemic diseases such as hypertension and diabetes. The average age of the control group was 40.67 years (range, 34-42 years), the average age of patients with mild cataracts was 70 years (range, 60-79 years) and the average age of patients with severe cataracts was 75.90 years (range, 66-87 years). In the control group, females accounted for 33 and 67% of the study samples, respectively; females in the mild cataracts group accounted for 20 and 80% of the study samples, respectively; females in the severe cataracts group accounted for 50 and 60% of the study samples, respectively. None of the participants was administered any preoperative drugs.

Aqueous humor metabolic profiles. The results of the PCA (Fig. 1) showed one sample was separated in the control group and the other samples were closely clustered. The supervised OPLS-DA model was established to understand the holistic metabolic differences among the three groups. As shown in the OPLS-DA score plot, good separation between cataracts patients and controls could be achieved (Fig. 2). The validation plot strongly supported the validity of the model, as all permuted R2 and Q2 values on the left were lower than the original points on the right (Fig. 3).

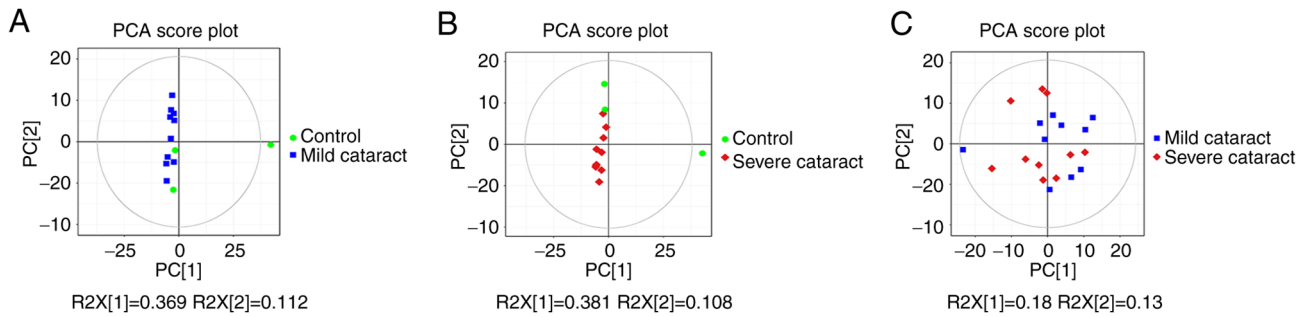*Identification of potential biomarkers*. Following the successful establishment of the OPLS-DA model, potential

Figure 1. The score plot of PCA. (A) Control vs. mild cataracts, (B) control vs. severe cataracts, (C) mild cataracts vs. severe cataracts. PCA, principal component analysis.
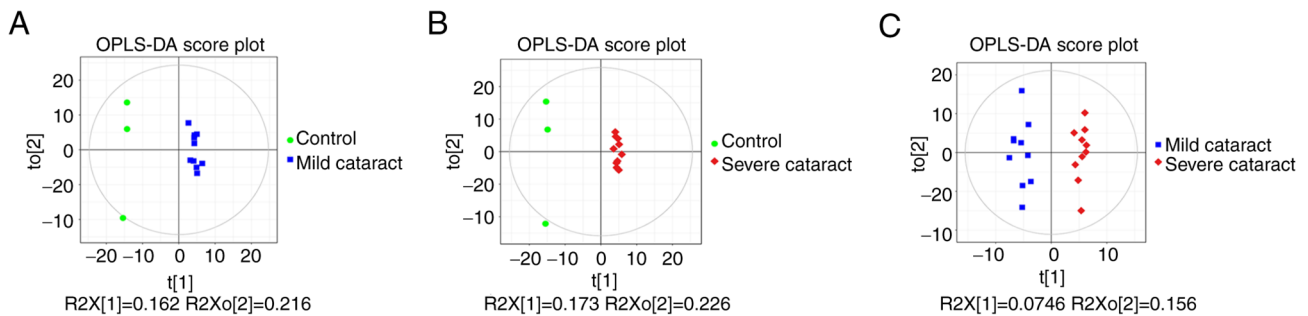

Figure 2. The score plot of the PLSDA model. (A) Control vs. mild cataracts, (B) control vs. severe cataracts, (C) mild cataracts vs. severe cataracts. PLSDA, partial least squares-discriminant analysis.
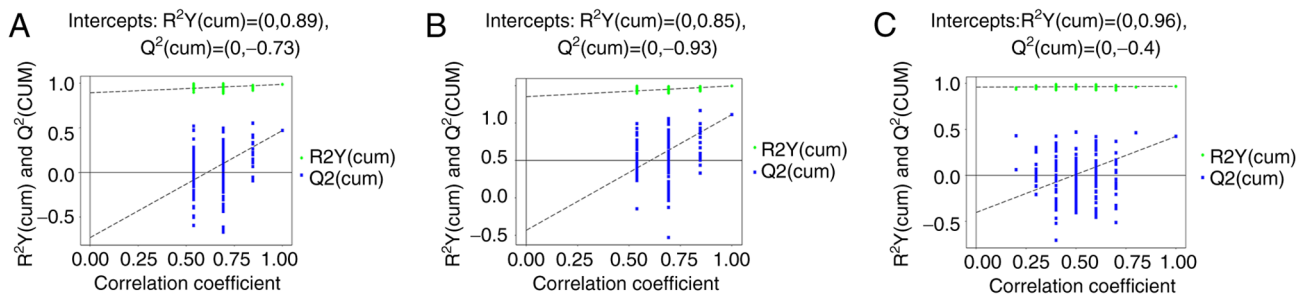

Figure 3. Validation plots for the OPLS-DA mode. (A) Control vs. mild cataracts, (B) control vs. severe cataracts, (C) mild cataracts vs. severe cataracts. OPLS-DA, orthogonal projections to latent structures-discriminate analysis.

metabolic biomarkers were selected using the criteria of VIP >1.0 and P<0.05. Finally, 43 metabolites were successfully selected and identified as potential biomarkers of mild age-related cataracts compared with controls. Compared with controls, six metabolites were found to be decreased in those with mild age-related cataracts. By contrast, 37 metabolites were found to be increased in participants with mild age-related cataracts compared with controls (Fig. 4A). A total of 36 metabolites were successfully selected and identified as potential biomarkers of severe age-related cataracts compared with controls. Compared with controls, five metabolites were found to be decreased in those with mild age-related cataracts. By contrast, 31 metabolites were found to be increased in participants with severe age-related cataracts compared with controls (Fig. 4B). A total of 34 metabolites were successfully selected and identified as potential biomarkers of mild age-related cataracts compared

with severe age-related cataracts. Compared with controls, 20 metabolites were found to be decreased in those with mild age-related cataracts. By contrast, 14 metabolites were found to be increased in participants with mild age-related cataracts compared with severe age-related cataracts (Fig. 4C). Among them, diglyceride (DG; 20:1e/8:0) metabolite were commonly expressed differently in the three groups. In severe and mild cataracts, DG(20:1e/8:0) increased compared with the control group; The expression of DG(20:1e/8:0) was further increased in severe cataracts compared with mild cataracts (Fig. 4D). The chromatography spectra of each sample is shown in Data S1 and the information of each sample is shown in Data S2.

Pathway analysis for potential biomarkers. Pathway analysis, including enrichment analysis and pathway topology analysis, was further performed to understand the metabolic pathways in which these potential biomarkers were involved. A total of
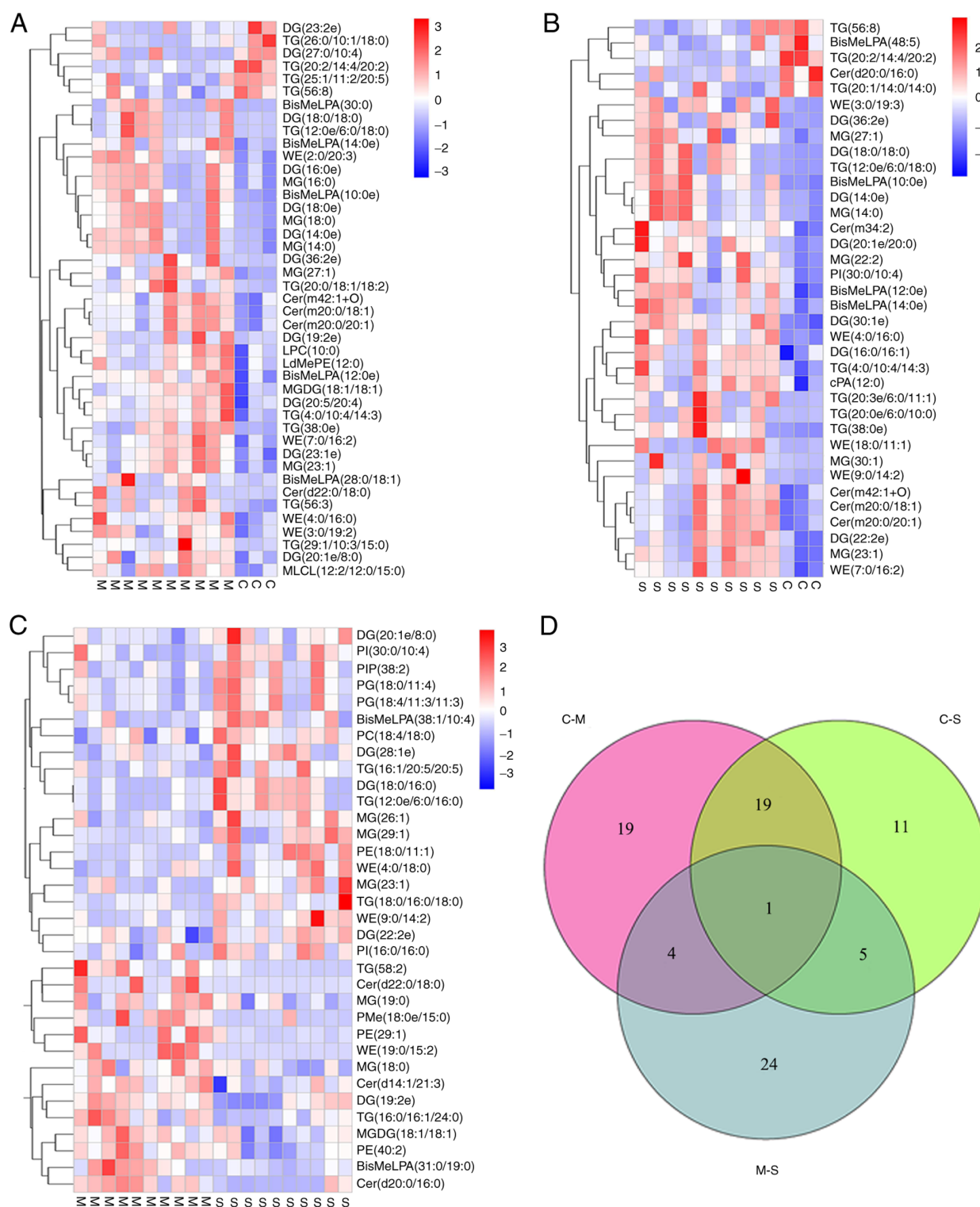
Figure 4. Heatmap indicating the concentrations of metabolite markers. (A) Control vs. mild cataracts, (B) control vs. severe cataracts, (C) mild cataracts vs. severe cataracts. (D) Metabolites were commonly expressed differently among the three groups. C, control group; M, mild cataracts; S, severe cataracts.

five pathways were significantly enriched at the significance level of 0.10, namely glycerophospholipid metabolism; linoleic acid metabolism; α-linolenic acid metabolism; glycosylphosphatidylinositol (GPI)-anchor biosynthesis; and glycerolipid metabolism (Fig. 5). In particular, the glycerophospholipid metabolism was highly affected, implying that these metabolic markers serve important roles in the regulation of this pathway.

Discussion

In clinical practice, the pathogenesis of cataracts remains to be elucidated. Therefore, there is an urgent need for in-depth understanding. The present study systematically investigated the metabolic differences in aqueous humor samples from patients with cataracts of different severity and control group.
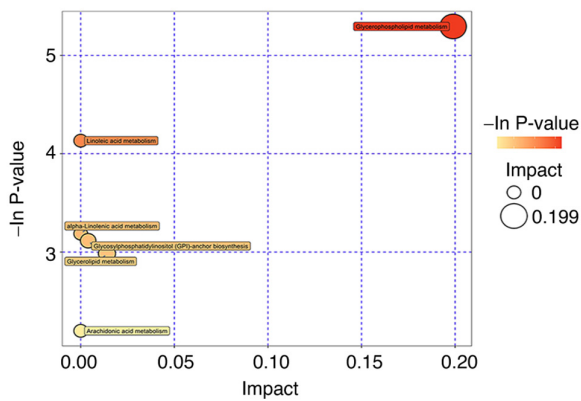
Figure 5. Enrichment analysis and pathway topology analysis for potential metabolic biomarkers.

It identified 34 metabolites as possible biomarkers in aqueous humor samples between patients with different degrees of cataracts, 36 metabolites as possible biomarkers in aqueous humor samples between patients with severe cataracts and control group and 43 metabolites as possible biomarkers in aqueous humor samples between patients with mild cataracts and control group. They may distinguish patients with different degrees of cataracts from the control group. DG (20:1e/8:0) metabolite was differentially expressed in the three groups and the expression level of DG (20:1e/8:0) metabolite increased with the increase of cataracts severity, indicating that DG (20:1e/8:0) metabolite has marked significance in cataracts and classification. The identification of new metabolite markers in human cataracts aqueous humor provided insights into the potential new pathogenic pathway of this vision-threatening disease and may lead to new drug development and research routes.

Studies have shown that myo-Inositol is the most abundant lens metabolite, which mainly serves a role in maintaining cell osmotic pressure in human lens; antioxidants (the main lens antioxidant GSH, ergotathione and homocarnosine) protect the lens from oxidative stress. Nucleotides NADH, AMP and ADP serve an important role in redox reaction and participate in energy metabolism (5,20,21). One study found that the levels of glycosylated metabolites in aqueous humor samples of the control group increased compared with aqueous humor samples of cataracts (22). Most of these metabolites are synthesized in lens epithelial cells with active metabolism. They serve a crucial role in normal lens function (22). The present study found that bis-methyl lysophosphatidic acid, ceramides, DG, monoglyceride, triglyceride and wax esters metabolites were differentially expressed in aqueous humor samples of patients with different degrees of cataracts and control group. Public databases such as KEGG and MetaboAnalyst and other published studies were also searched, to attempt to find possible metabolic pathways for cataracts observed in the present study (18,19).

In the process of cell physiology, glycerolphospholipids form phospholipid bilayers, which are active participants in affecting the properties of membrane related proteins and precursors of important cell components (23). Glycerolphospholipids are the most common and abundant phospholipids in human body (23). The two hydroxyl groups of glycerol and fatty acids are converted into esters and the third hydroxyl group is phosphorylated to form phosphatidylic acid (23). They are a series of key compounds that constitute the structure of cell membrane and participate in a number of biological regulation processes (23). Studies have indicated that glycerol phospholipids are involved in the pathogenesis of asthma (24,25). Other studies have shown that there are significant changes in the metabolism of glycerophospholipid in the plasma of patients with psoriasis (26,27). The present study found that glycerophospholipid metabolism was highly affected in the pathway analysis, implying that glycerophospholipid may serve major roles in cataracts pathophysiology.

The present study had some limitations. First, the main deficiency was the small sample size, which may prevent significant changes in some metabolites. In the future, the authors will continue to collect a large number of samples for further research. In addition, the control group was patients with ocular rupture surgery, rather than 'healthy' individuals, which may distort the results of the present study. If possible, it would be ideal to obtain aqueous humor samples from healthy individuals with non-ophthalmic diseases. Moreover, the difference of some metabolites between the control group and the cataracts group was not clear. It may be that the database of biomarkers of aqueous humor metabolism is not sufficiently accurate, or that the samples were heterogeneous. It is hoped to further explore this in the future. Finally, there is a difference in the age of patients between the control group and the experimental group, which may cause errors in the experimental results. This too will be further discussed in the future.

The present study investigated metabolic markers associated with cataracts patients in human aqueous humor samples. The results showed that 34 metabolites were identified as potential biomarkers, able to distinguish patients with different degrees of cataracts; 36 metabolites were identified as potential biomarkers that could distinguish patients with severe cataracts from the control group; 34 metabolites were identified as potential biomarkers that could distinguish patients with mild cataracts from controls. Glycerolphospholipid metabolism was highly affected in pathway analysis and may serve an important role in the regulation of this pathway. Overall, the present study provided new clues for the pathogenesis of cataracts.

Availability of data and materials

All data generated and analysed during this study are included in this published article/supplementary material. Furthermore,

the full datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

QW carried out the collection of samples and was a major contributor in writing the manuscript. WQ completed the LC-MS experiment and data analysis. LL, YG and YJ conceived the experiment and revised the manuscript. QW, YG and YJ confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All procedures of the present study were in accordance with the tenets of the Declaration of Helsinki and approved by the Research Ethics Committee of the Tong Ren Hospital affiliated with Shanghai Jiao Tong University School of Medicine (Shanghai, China; approval no. 2021-078-01; 17 November 2021). Samples were collected after obtaining the patients' written informed consent to participate in the present study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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