

Metabolomics of the aqueous humor in patients with primary congenital glaucoma

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Purpose: Primary congenital glaucoma (PCG) is an autosomal recessive eye disorder, accounting for 0.01%–0.04% of blindness around the world. Unfortunately, the molecular characteristics concerning the pathogenic mechanisms of the disease remain poorly understood.

Methods: Here, for the first time, we employed gas chromatography coupled to time-of-flight mass spectrometry (GC/TOF MS) to reveal comprehensively the metabolic characteristics of PCG.

Results: First, 363 metabolites were detected in 50 aqueous humor (AH) samples from 30 patients with PCG, 10 patients with congenital cataracts (CCs), and 10 patients with aged-related cataracts (ARCs). Second, 290 metabolites in total were found in another 15 patients with PCG and 10 patients with primary open angle glaucoma (POAG). A further analysis suggested that patients with PCG had a significantly distinct metabolomics profile. Three amino acid-associated metabolites, including glycine, urea, and phenylalanine, were identified to be significantly different ($p \leq 0.05$) in relation to PCG. Meanwhile, three glaucoma-associated single nucleotide polymorphisms (SNPs), rs7114303, rs9364602, and rs2165241, were determined to be related to these three metabolites. The results here indicate that certain amino acid-associated metabolites and their metabolisms are key regulatory elements and metabolic pathways in the pathogenesis of PCG.

Conclusions: Collectively, this work not only extended our understanding of the molecular characteristics of PCG, but also presented glycine as a potential biomarker for earlier diagnosis and may provide new therapeutic strategies for the disease.

Glaucoma is one of the most common causes of global irreversible blindness. The number of glaucoma patients was estimated at 64.3 million in 2013, and it is expected to increase to nearly 80 million globally by the year 2020 [1]. Generally, there are three subtypes of primary glaucoma according to the age of onset, etiology, and anatomy of the anterior chamber: primary open angle glaucoma (POAG), primary congenital glaucoma (PCG), and primary angle closure glaucoma (PACG) [2]. Among them, POAG is the most common form, accounting for 80% of glaucomatous diseases, while PCG is recognized as another severe form of glaucoma in newborns and infants, despite its low incidence [2,3].

The disease of glaucoma, including PCG, remains poorly understood, and more importantly, there are no biomarkers for diagnosis and prognosis, as well as a small range of therapeutic options [4]. Genomic, proteomic, and metabolomics techniques have recently offered the potential to identify molecular biomarkers in glaucoma due to increased sensitivity and accuracy [5]. For example, several genetic biomarkers have been determined, including CDKN2B-AS1 genomic region polymorphisms, which display a strong association with POAG [5]. Moreover, Funke et al. (2016) collected retina samples of glaucoma and non-glaucoma control donors to uncover proteomic changes using a state-of-the-art mass spectrometry (MS) workflow [6]. The results finally identified new molecular players, including adenine nucleotide translocase 3 (ANT3), methyl-CpG binding protein 2 (MeCp2), and dense fine speckles 70 (DFS70), as significantly associated with glaucoma. González-Iglesias et al. (2014) also conducted a comparative proteomic study of POAG, pseudoexfoliation glaucoma (PEXG), and healthy controls using two-dimensional fluorescence difference gel electrophoresis (2D-DIGE), and 17 proteins were determined to be most differentially altered, presenting new perspectives in biomarker discovery for glaucoma [7].

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In addition, another advanced high-throughput approach, metabolomics, has been applied to extend our understanding of molecular changes in the pathogenesis and to provide potential biomarkers for various eye diseases, including age-related macular degeneration, high myopia, glaucoma, and diabetic retinopathy [4,8-11]. For instance, using gas chromatography-mass spectrometry (GC-MS), Rong et al. (2017) reported the targeted metabolite profiling of 86 serum samples from newly diagnosed PACG patients and controls [12]. Palmitoleic acid, γ -linolenic acid, and linoleic acid were determined to be potential biomarkers for the screening of PACG. Likewise, Ji et al. (2017) employed a non-targeted metabolomics technology, gas chromatography coupled to time-of-flight mass spectrometry (GC/TOF MS) to identify potential biomarkers in relation to high myopia [11]. In total, 242 metabolites were identified in 40 aqueous humor (AH) samples from patients with high myopia and the controls. Twenty-nine significantly changed metabolites and their regulatory aspects of the metabolic pathways were further determined to be key regulatory elements or pathways in the development of high myopia.

The AH plays a significant role in glaucoma pathogenesis, as efflux congestion of the AH mainly contributes to ocular hypertension in the disease [13]. However, limited studies on the molecular characteristics in relation to PCG have been previously published except in genetic linkage analysis. Here, taking advantage of the non-targeted metabolomics technology GC/TOF MS reported by Ji et al. (2017), for the first time, we comprehensively uncover metabolomics signatures in AH samples from patients with PCG and controls [11]. The results here may provide potential biomarkers and extend our understanding of the pathophysiology, allowing for a better understanding of the mechanisms of onset and for the determination of new therapeutic strategies for PCG.

METHODS

Subjects: Four kinds of patients were recruited in the present study (Table 1): 45 patients with PCG, 10 patients with congenital cataracts (CCs), 10 patients with aged-related cataracts (ARCs), and 10 patients with POAG. All patients were diagnosed by the same ophthalmologist according to previous reports [14-17]. Especially for patients with PCG, the diagnostic criteria were: (1) intraocular pressure (IOP) >21 mmHg, (2) corneal diameter enlargement or corneal edema, and (3) increased cup-to-disc ratio. Meanwhile, the ARC and CC patients met the inclusion criteria with no ocular disease other than cataracts. In addition, we enrolled

POAG patients with an age greater than 35 years, an open anterior chamber angle without morphological abnormalities, an IOP of more than 22 mmHg, and glaucomatous optic neuropathy upon funduscopy. All participants provided written informed consent, and the Ethics Committee of the Eye & ENT Hospital of Fudan University (Shanghai, China) reviewed and approved the study protocol. Meanwhile, all investigations were conducted in accordance with the tenets of the Declaration of Helsinki.

AH samples: AH samples from patients were obtained after topical anesthesia and disinfection before routine cataract surgery [18]. Paracentesis was employed to ensure minimal contact with other intraocular structures and, more importantly, to prevent the potential influences on metabolites in the AH samples. The extraction of AH samples was conducted as previously reported [11]. Afterwards, AH samples were transferred to dust-free Eppendorf tubes immediately and centrifuged twice at 4 °C at 16,000 $\times g$ for 15 min. Finally, the supernatant was collected and rapidly stored at -80 °C until further metabolomics analysis.

Metabolomics analysis: Gas chromatography (Agilent 7890A, Agilent) combined with a Pegasus 4D time-of-flight mass spectrometer (LECO ChromaTOF PEGASUS 4D, LECO) was employed for the metabolite profiling of AH samples, which is a popular technique for metabolite profiling due to its higher mass accuracy and mass resolution [11]. Briefly, 1 μ l volume was injected into the Agilent DB-5MS capillary column (30 m \times 250 μ m \times 0.25 μ m, J&W Scientific, Folsom, CA), while the carrier gas, helium, was used at a constant flow rate (1 ml/min) through the column. Meanwhile, the initial temperature was 90 °C for 2 min, which then increased to 180 °C at a rate of 5 °C/min and finally to 285 °C at a rate of 15 °C/min. Temperatures for the injection, transfer line, and ion source were 280 °C, 270 °C, and 220 °C, respectively. The mass spectrometry data were obtained in full-scan mode at a rate of 100 spectra per second with an m/z range of 20–600 after a solvent delay of 492 s. Meanwhile, the resolution of the instrument was 1,000 (full width at half maximum [FWHM]) and the mass accuracy of the TOF attached to the GC was ± 0.1 Da. In addition, the Chroma TOF4.3X software of the LECO Corporation was used here to acquire mass spectrometric data, including raw peak extraction, data baseline filtering and calibration, peak alignment, and deconvolution analysis. As with previous studies, the LECO/Fiehn Metabolomics Library was used to identify the compounds, which would give a similarity value for compound identification accuracy [11,19,20]. If the similarity value was more than 700, the metabolite identification was reliable. If the similarity value was between 200 and 700, the compound was considered a

TABLE 1. SUMMARY OF HUMAN AQUEOUS HUMOR SAMPLES.

Group	Patient ID	Gender	Age (Years old)	Ethnicity	Disease
1	XB-5_1	Male	5	Han	Congenital cataract
	XB-10_1	Male	4	Han	Congenital cataract
	XB-8_1	Male	5	Han	Congenital cataract
	XB-9_1	Male	7	Han	Congenital cataract
	XB-2_1	Female	3	Han	Congenital cataract
	XB-6_1	Male	3	Han	Congenital cataract
	XB-7_1	Male	6	Han	Congenital cataract
	XB-3_1	Male	7	Han	Congenital cataract
	XB-4_1	Female	6	Han	Congenital cataract
	XB-1_1	Female	5	Han	Congenital cataract
	LB-10_1	Male	61	Han	Aged-related cataract
	LB-7_1	Female	56	Han	Aged-related cataract
	LB-4_1	Female	63	Han	Aged-related cataract
	LB-5_1	Female	66	Han	Aged-related cataract
	LB-6_1	Male	62	Han	Aged-related cataract
	LB-9_1	Male	63	Han	Aged-related cataract
	LB-3_1	Female	65	Han	Aged-related cataract
	LB-8_1	Male	61	Han	Aged-related cataract
	LB-1_1	Female	60	Han	Aged-related cataract
	LB-2_1	Male	63	Han	Aged-related cataract
	C416-1_1	Male	7	Han	Primary congenital glaucoma
	C467_1	Female	9	Han	Primary congenital glaucoma
	C420-2_1	Male	7	Han	Primary congenital glaucoma
	C412_1	Male	5	Han	Primary congenital glaucoma
	C426_1	Male	6	Han	Primary congenital glaucoma
	C473_1	Male	5	Han	Primary congenital glaucoma
	C424-1_1	Female	4	Han	Primary congenital glaucoma
	C461_1	Male	7	Han	Primary congenital glaucoma
	C401_1	Male	7	Han	Primary congenital glaucoma
	C472-2_1	Male	5	Han	Primary congenital glaucoma
	C411_1	Female	4	Han	Primary congenital glaucoma
	C413_1	Female	4	Han	Primary congenital glaucoma
	C423_1	Male	8	Han	Primary congenital glaucoma

Group	Patient ID	Gender	Age (Years old)	Ethnicity	Disease
	C362_1	Male	5	Han	Primary congenital glaucoma
	C456-1_1	Male	5	Han	Primary congenital glaucoma
	C393_1	Male	5	Han	Primary congenital glaucoma
	C444_1	Male	8	Han	Primary congenital glaucoma
	C469-1_1	Male	5	Han	Primary congenital glaucoma
	C454_1	Male	4	Han	Primary congenital glaucoma
	C469-2_1	Male	4	Han	Primary congenital glaucoma
	C462_1	Male	3	Hui	Primary congenital glaucoma
	C456-2_1	Female	4	Han	Primary congenital glaucoma
	C468_1	Female	4	Han	Primary congenital glaucoma
	C416-2_1	Male	4	Han	Primary congenital glaucoma
	C474_1	Male	7	Han	Primary congenital glaucoma
	C424-2_1	Male	7	Han	Primary congenital glaucoma
	C415_1	Male	3	Han	Primary congenital glaucoma
	C420-1_1	Male	3	Han	Primary congenital glaucoma
	C472-1_1	Male	3	Han	Primary congenital glaucoma
	C419_1	Male	3	Han	Primary congenital glaucoma
	2	Male	3	Han	Primary congenital glaucoma
	C478_1	Female	4	Han	Primary congenital glaucoma
	C510_1	Female	4	Han	Primary congenital glaucoma
	C506_1	Male	3	Han	Primary congenital glaucoma
	C493_1	Female	5	Han	Primary congenital glaucoma
	C481_1	Female	9	Han	Primary congenital glaucoma
	C477_1	Male	14	Han	Primary congenital glaucoma
	C485_1	Male	5	Han	Primary congenital glaucoma
	C505_1	Female	3	Han	Primary congenital glaucoma
	C513_1	Male	3	Han	Primary congenital glaucoma
	C515_1	Male	4	Han	Primary congenital glaucoma
	C476_1	Male	3	Han	Primary congenital glaucoma
	C474_1	Male	3	Han	Primary congenital glaucoma
	C520_1	Male	4	Han	Primary congenital glaucoma
	C487_1	Male	3	Han	Primary congenital glaucoma
	C507_1	Male	3	Han	Primary congenital glaucoma
	K899_1	Female	55	Han	Primary open angle glaucoma
	K2077-1_1	Female	61	Han	Primary open angle glaucoma

Group	Patient ID	Gender	Age (Years old)	Ethnicity	Disease
	K175_1	Male	44	Han	Primary open angle glaucoma
	K301_1	Male	77	Han	Primary open angle glaucoma
	K174_1	Male	77	Han	Primary open angle glaucoma
	K2085_1	Male	23	Han	Primary open angle glaucoma
	K2053_1	Male	45	Han	Primary open angle glaucoma
	K1013-2_1	Male	68	Han	Primary open angle glaucoma
	K2077-2_1	Male	68	Han	Primary open angle glaucoma
	K1013-1_1	Male	35	Han	Primary open angle glaucoma

putative annotation. If the similarity value was less than 200, the compound was defined as an “analyte.”

Data analysis: Metabolic data were normalized and analyzed as previously reported [21]. Briefly, metabolites with no value in less than 50% of the samples were first removed, and then a data normalization step was performed by registering the median level of each compound to equal to one (1.00). Missing values (if any) were assumed below the limits of detection and were imputed with the observed minimum. A hierarchical cluster analysis was then performed using the MultiExperiment Viewer (Mev) 4.8 software. Meanwhile, the SIMCA-P software (v13.0, Umetrics, Malmö, Sweden) was employed here for both the principal component analysis (PCA) and partial least-squares discriminant analysis (PLS-DA). Together with the PLS-DA, the independent *t* test (statistical product and service solutions [SPSS] 17.0 software) was used to determine significantly different metabolites. Metabolites with both variable importance in the projection (VIP) values in the PLS-DA model greater than 1 and *p* values (in *t* tests) less than 0.05 were considered significant. Regression models were further used to adjust for age and gender in comparisons among the three groups (PCG versus CC versus ARC) and between the PCG and POAG groups. In addition, both the metabolic pathway and enrichment analysis of significantly different metabolites (adjusted $p \leq 0.05$) were conducted according to the [MetaboAnalyst](#) web server. Significant pathways were judged based on false discovery rate (FDR) threshold 0.05, whose raw *p* values are less than 0.005 [22].

RESULTS

AH samples characteristics: In the present study, four kinds of patients were collected, all from the Han nationality (except one patient from the Hui nationality) in China: 45 patients with PCG, 10 patients with CCs, 10 patients with ARCs, and 10 patients with POAG. Notably, all 75 patients were then divided into two groups for two steps of metabolite profiling (Table 1): one group of 30 patients with PCG, 10 patients with CCs, and 10 patients with ARCs and a second group for the other 15 patients with PCG and 10 patients with POAG. Because it is hard to obtain normal samples for clinical use from the controls due to ethical issues, we first took two kinds of patients as the controls: patients with CCs and patients with ARCs. To investigate comprehensively the metabolic changes in patients especially with PCG, we further enrolled 15 patients with PCG and 10 patients with POAG for comparative metabolomics analyses at the second step. It should be pointed out that we analyzed the metabolic data during these two steps respectively, as the numbers of detected metabolites between them were different. We believe

that the study is sufficient to reveal the metabolic characteristics of PCG.

Metabolic changes among patients with PCG, patients with CCs, and patients with ARCs: A non-targeted metabolomics technology, GC/TOF MS, was employed here to uncover comprehensively the human AH metabolome. In total, 363 metabolites (Appendix 1) were identified in the first group of 50 AH samples from 30 patients with PCG, 10 patients with CCs, and 10 patients with ARCs, which were classified according to the database from the Kyoto Encyclopedia of Genes and Genomes (KEGG) [16]. Among them, 173 metabolites were identified as mapping to the amino acid/carbohydrate/lipid/nucleotide super pathway and 132 metabolites were identified as analytes, while the remaining 58 metabolites were determined to be unknown.

The hierarchical cluster analysis, an un-supervised approach, was then performed to investigate metabolic variations among the three kinds of patients (Figure 1A). Obviously, 29/30 samples from patients with PCG were clearly separated from the samples from patients with CCs and ARCs. We further employed another un-supervised approach, PCA, to determine the grouping of these 50 samples. As shown in Figure 1B, there was to some extent a separation between samples from patients with PCG and samples from the other two kinds of patients as some samples from three kinds of patients clustered together. The results here indicated that patients with PCG had a significantly distinct metabolomics profile.

The supervised statistical method, PLS-DA, was used to determine significantly different metabolites. On the one hand, samples from patients with PCG were obviously separated from those with CCs in a score plot from the PLS-DA model with high R^2Y and Q^2 values (Figure 2A). Together with a dependent *t* test, 89 metabolites were determined to be significantly different ($VIP \geq 1$ and $p \leq 0.05$), responsible for the separation (Figure 2B). On the other hand, samples from patients with PCG were also clearly separated from those with ARCs (Figure 2C). Similarly, 86 metabolites were found to be significantly changed ($VIP \geq 1$ and $p \leq 0.05$), which played a role in the separation (Figure 2D). Altogether, 38 metabolites (Appendix 2) were finally identified to be significantly different (adjusted $p \leq 0.05$) among those three kinds of patients. A further metabolic pathway analysis of these 38 significantly different metabolites revealed that the 25 most relevant pathways (Appendix 3 and Figure 3A) were enriched, among which aminoacyl-tRNA biosynthesis; valine, leucine, and isoleucine biosynthesis; arginine and proline metabolism; and valine, leucine, and isoleucine degradation were the most significantly influenced pathways (FDR <0.05).

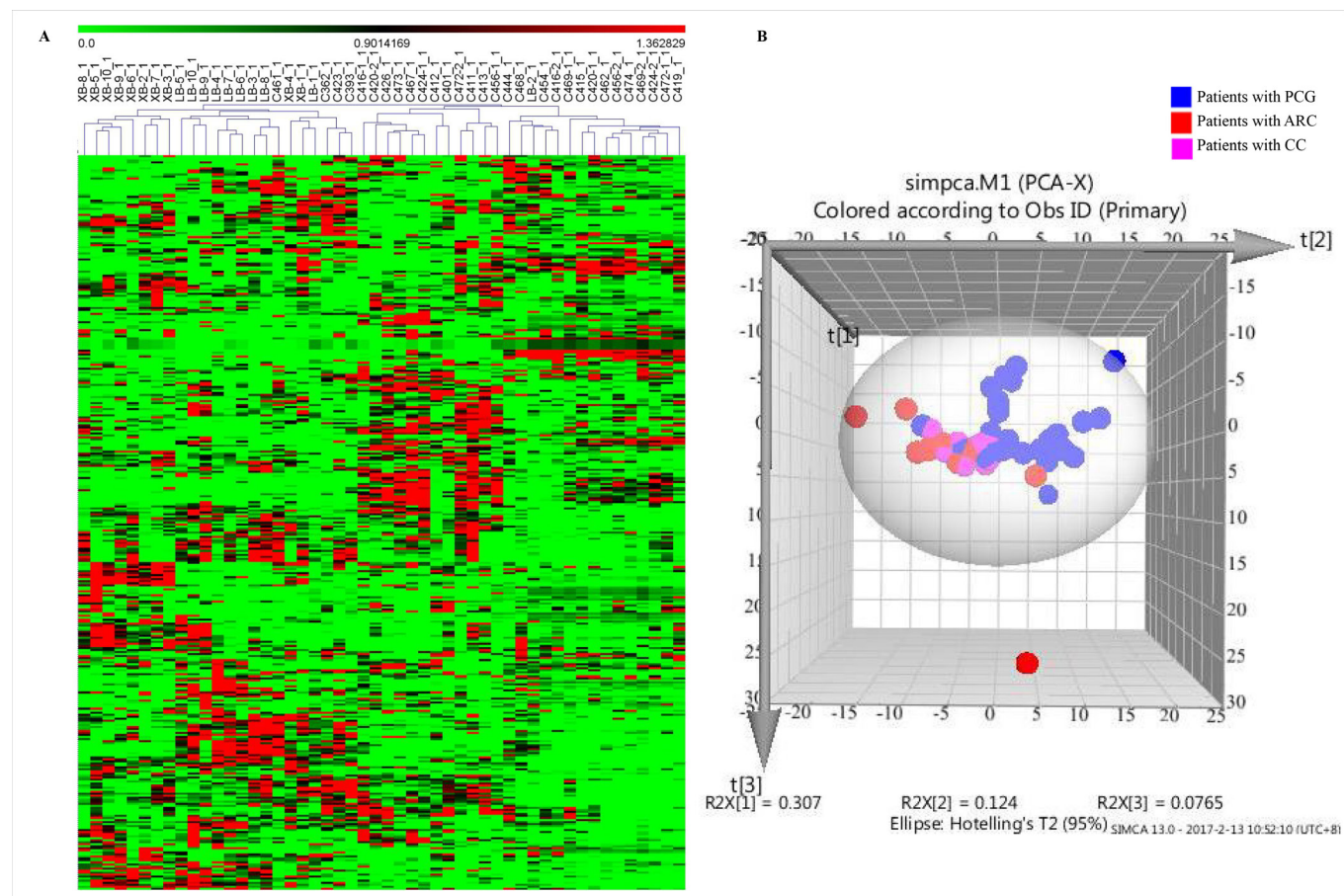


Figure 1. Metabolomics analysis of 50 AH samples, including 30 patients with PCG, 10 patients with CCs, and 10 patients with ARCs. **A:** Heat map of 363 metabolites detected in 50 AH samples by a hierarchical cluster analysis. Each line represents one metabolite. The darker the green color, the lower its content in the sample; likewise, the darker the red color, the higher its content in the sample. XB represents patients with CCs, and LB represents patients with ARCs, while C represents patients with PCG. **B:** 3D score plot for the first three components ($t[1]$, $t[2]$, and $t[3]$) in the PCA model of metabolomics data from 50 AH samples. PCG: primary congenital glaucoma; CCs: congenital cataracts; ARCs: aged-related cataracts.

Metabolic changes between patients with PCG and POAG:

Likewise, we used the same methodology for the metabolite profiling of the other 15 patients with PCG and 10 patients with POAG. The results here revealed that 290 metabolites (Appendix 4) in total were detected in all the 25 samples, which referred to the amino acid (19 metabolites)/carbohydrate (55 metabolites)/lipid (16 metabolites)/nucleotide (4 metabolites) super pathway, as well as another 196 metabolites. Likewise, a hierarchical cluster analysis of metabolic data showed that most samples (13/15) from patients with PCG were clearly separated from those with POAG (Appendix 5). A further statistical analysis showed that 54 metabolites (Appendix 6, Figure 2E,F) were significantly (adjusted $p \leq 0.05$) altered between the two groups. Among them, the levels of 17 biochemicals were increased, while the concentrations of 37 biochemicals were decreased. A

metabolic pathway analysis of these 54 metabolites suggested that the 42 most relevant pathways (Appendix 7 and Figure 3B) were enriched. Especially, the pathway of alanine, aspartate, and glutamate metabolism was the most significantly influenced one (FDR < 0.05).

Enrichment analysis of significantly changed metabolites in relation to PCG: To determine the metabolic changes of AH related to PCG development, we then identified significantly changed metabolites among the abovementioned four kinds of patients. Interestingly, only three metabolites (Table 2) were significantly changed in common between patients with PCG and the three kinds of controls (patients with CCs/ARCs/POAG). Notably, glycine 2 was synchronously upregulated and urea was synchronously downregulated in patients with PCG when compared to the other three kinds of patients. We further submitted these three metabolites to

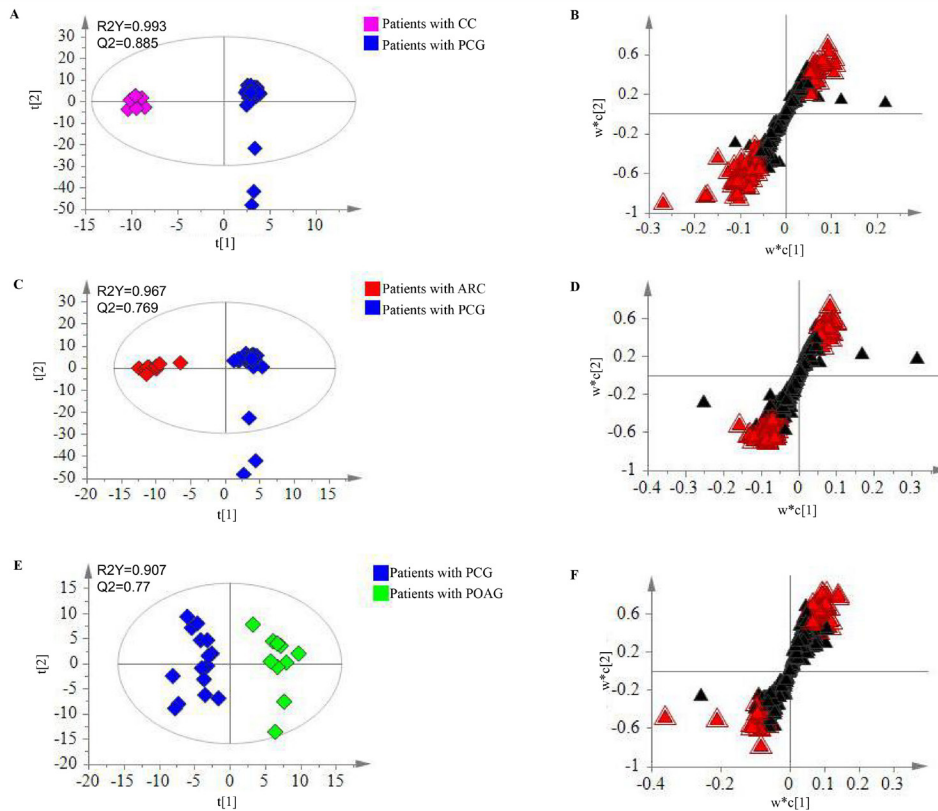


Figure 2. Metabolomic analysis for patients with PCG. **A:** Score plot for the first two components (t[1] and t[2]) between patients with CCs and PCG in the PLS-DA model. **B:** S plot for the two first components (w*c [1] and w*c [2]) between patients with CCs and PCG in the PLS-DA model. **C:** Score plot for the first two components (t[1] and t[2]) between patients with ARCs and PCG in the PLS-DA model. **D:** S plot for the two first components (w*c [1] and w*c [2]) patients with ARCs and PCG in the PLS-DA model. **E:** Score plot for the first two components (t[1] and t[2]) between patients with PCG and POAG in the PLS-DA model. **F:** S plot for the two first components (w*c [1] and w*c [2]) patients with PCG and POAG in the PLS-DA model. Significantly changed metabolites

were labeled with a red triangle. PCG: primary congenital glaucoma; CCs: congenital cataracts; ARCs: aged-related cataracts; POAG: primary open angle glaucoma.

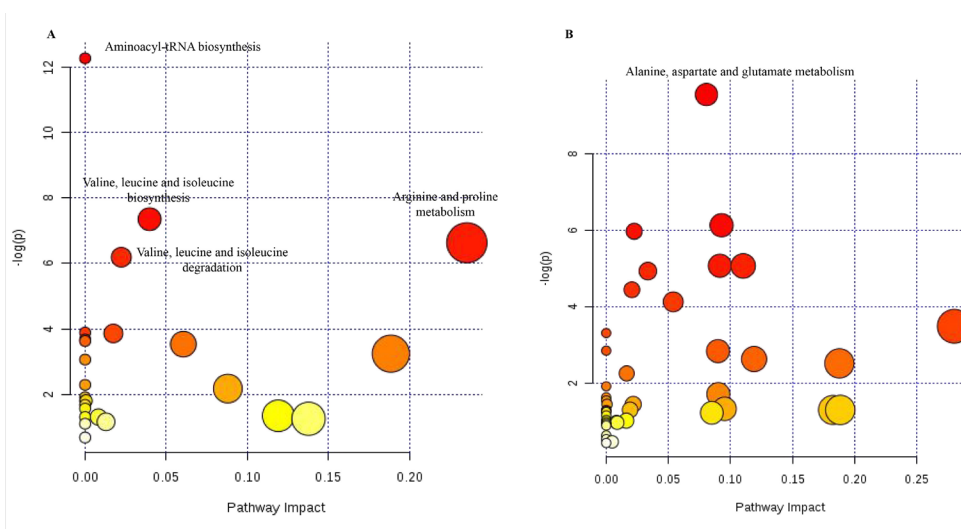


Figure 3. Metabolic pathway analysis for patients with PCG. **A:** Metabolic pathway analysis of 38 significantly different metabolites among patients with PCG, CCs, and ARCs using the MetaboAnalyst web server. **B:** Metabolic pathway analysis of 54 significantly different metabolites between patients with PCG and POAG by using the MetaboAnalyst web server. PCG: primary congenital glaucoma; CCs: congenital cataracts; ARCs: aged-related cataracts; POAG: primary open angle glaucoma. The color and size of each dot were associated

with the $-\log(p)$ value and pathway impact value, respectively, where a small p value and high pathway impact value indicate the pathway is greatly influenced.

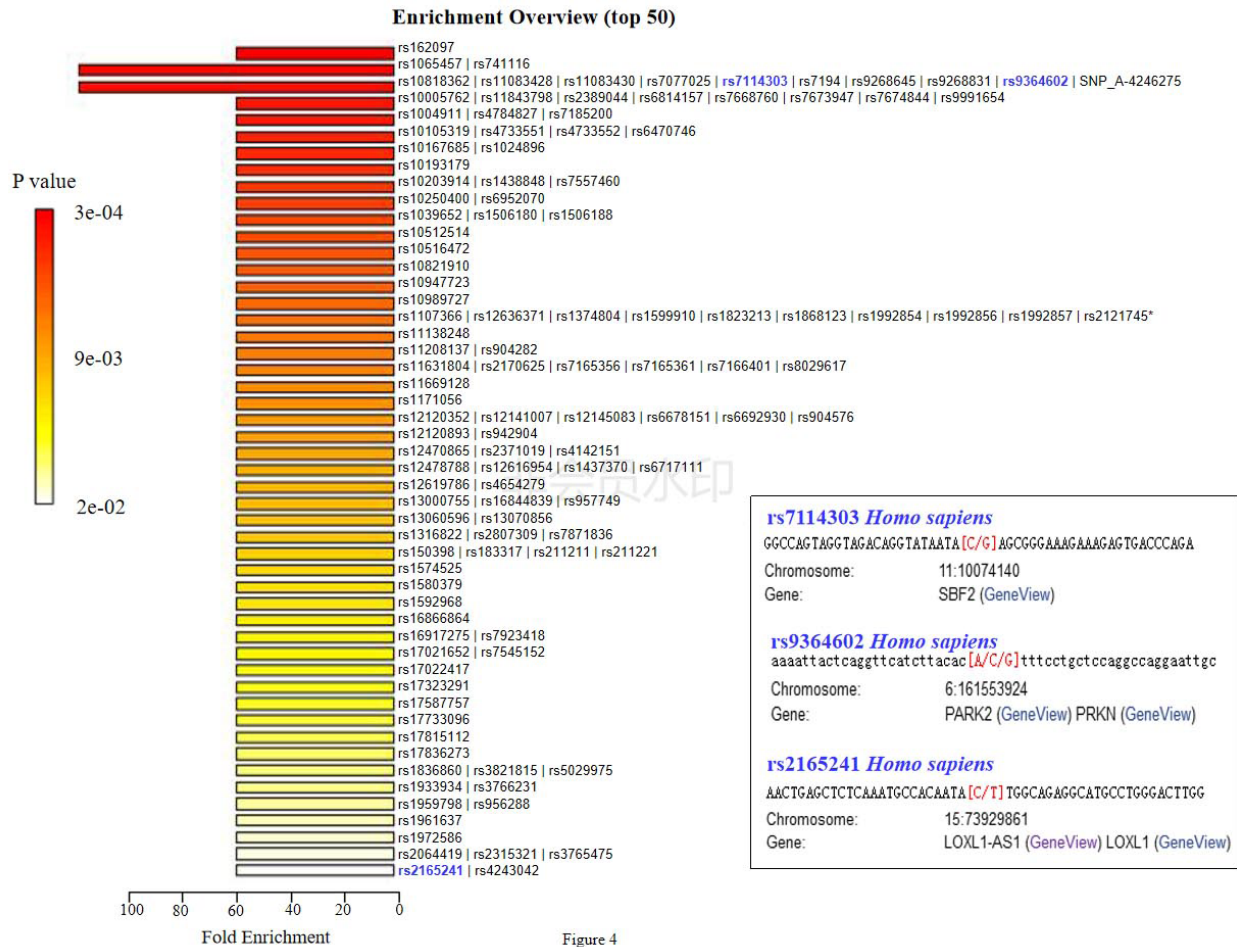


Figure 4. Top 50 groups of SNPs were highly associated with three metabolites significantly changed in common between patients with PCG and any other kind of patients (with CCs/ARCs/POAG) according to an enrichment analysis using the MetaboAnalyst web server with the SNP-associated metabolite set library (4501 SNPs). The details of three SNPs, including rs7114303, rs9364602, and rs2165241, are shown. PCG: primary congenital glaucoma; CCs: congenital cataracts; ARCs: aged-related cataracts; POAG: primary open angle glaucoma. *All SNPs in this group are shown in Appendix 8.

TABLE 2. LIST OF METABOLITES SIGNIFICANTLY CHANGED IN COMMON BETWEEN PATIENTS WITH PCG AND ANY OTHER KIND OF PATIENTS (WITH CC/ARC/POAG).

Biochemicals	Super Pathway	Ratio (PCG/POAG)	Ratio (PCG/CC)	*Adjust p value(PCG versus CC versus ARC)	Ratio (PCG/ARC)	*Adjust p value(PCG/POAG)
glycine 2	Amino Acid	3.86	8.39	0.0019	8.99	0.0018
phenylalanine 1	Amino Acid	0.91	1.84	0.0007	1.53	0.0016
urea	Amino Acid	0.94	0.65	0.0000	0.82	0.0263

*Regression models were used to adjust for age and gender in comparisons among three groups (PCG versus CC versus ARC), and between groups of PCG and POAG. PCG: primary congenital glaucoma; CC: congenital cataract; ARC: aged-related cataract; POAG: primary open angle glaucoma.

the MetaboAnalyst web server for an enrichment analysis with the single nucleotide polymorphism (SNP)-associated metabolite set library (4,501 SNPs). The results displayed the top 50 groups of SNPs that were highly associated with these three metabolites (Appendix 8 and Figure 4). Among the top 50 groups of SNPs, three SNPs, including [rs7114303](#), [rs9364602](#), and [rs2165241](#), were all reported to be involved in the development of glaucoma [23-25].

DISCUSSION

Glaucoma is the second leading cause of irreversible blindness, affecting a significant and growing proportion of the world population [26]. However, it has a small range of therapeutic options due to the pathogenic mechanisms of the disease. PCG causes great harm to visual development in infancy, though it is a rare disease [27]. As is well known, PCG manifests in the first three years of life as an autosomal recessive trait, having resulted from abnormal development of the AH drainage structures [28]. Thus, the identification of the molecular characteristics in AH of PCG may improve clinical diagnosis and extend the understanding of the pathophysiology behind the disease [4]. In the present study, for the first time, we employed metabolomics technology to study the metabolic characterization of AH samples in relation to PCG, which may not only present effective biomarkers as diagnostic and progression detection tools for PCG, but also promote new knowledge of the molecular mechanism of PCG development and further provide new therapeutic options.

Ideally, the GC-MS method has sufficient specificity and sensitivity to profile for a metabolomics analysis [11]. However, to the best of our knowledge, there have been no published reports on metabolomics for PCG. Here, we employed the non-targeted technology GC-MS to uncover comprehensively the AH metabolome of patients with PCG and controls. In total, 363 and 290 metabolites were respectively identified at two steps of metabolite profiling for all 75 patients, covering most of the central metabolism pathways, including the amino acid/carbohydrate/lipid/nucleotide super pathway. There is no doubt that our study presented thus far the broadest AH metabolome for PCG, corroborating its power in uncovering the metabolic characteristics. Though GC-TOF/MS used here is a popular technique for metabolite profiling, it has several limitations. On the one hand, it is a good choice to employ tandem mass spectrometry or MS/MS for high-confidence detection. On the other, it is indeed better to perform a metabolomics analysis in combination with LC-MS/MS, as GC-MS cannot analyze non-volatile metabolites.

Currently, the situation with the early diagnosis of early glaucoma, including PCG, has prompted researchers to determine potential biomarkers to diagnose and monitor the progress of the disease [29]. For instance, Fraenkl et al. (2011) revealed that plasma citrate levels were significantly reduced in Caucasian patients with glaucoma, as well as indicated plasma citrate as a promising biomarker for the diagnosis of glaucoma [30]. Moreover, Castany et al. (2011) investigated the presence of dinucleoside polyphosphates in AH samples from patients with or without glaucoma and found that levels of diadenosine tetraphosphate (Ap4A) were significantly higher (almost 15 times) in glaucoma patients than in the controls, suggesting its potential application as a marker of glaucoma [29]. In this study, three metabolites, including glycine, urea, and phenylalanine, were finally determined to be significantly changed in common between patients with PCG and any other kind of patient (with CCs/ARCs/POAG). The changes in the phenylalanine concentration in patients with glaucoma were also reported at an early stage in the study by Hannappel et al. [31]. Moreover, urea may play a critical role in the pathogenesis of PCG, as urea could be used in glaucoma as an osmotic ocular hypotensive agent with a long history [32]. Notably, another metabolite, glycine, displayed high levels in patients with PCG, and it has been reported as a potential biomarker for brain tumors [33]. Likewise, previous reports have already identified the role of glycine as a neural transmitter in the retina and meanwhile revealed that vitreous levels of glycine were associated with glaucoma [34,35]. Thus, glycine likely plays a significant role in the development of glaucoma (including PCG), which is characterized by the interruption in the communication between the brain and the retinal photoreceptors. A further enrichment analysis showed that [rs7114303](#), [rs9364602](#), and [rs2165241](#) were associated with those three metabolites. PCG is a genetically heterogeneous disorder, while two candidate genes, cytochrome P450 (*CYP1B1*) and latent transforming growth factor-beta-binding protein 2 (*LTBP2*) have been reported to be responsible for congenital glaucoma [36]. Interestingly, the three identified SNPs here were all reported to be associated with glaucoma. For example, [rs7114303](#) locates in the SET binding factor 2 (*SBF2*) gene, whose mutation was reported to cause Charcot-Marie-Tooth disease type 4B (CMT4B) with juvenile onset glaucoma [23]. Furthermore, [rs9364602](#) (parkin RBR E3 ubiquitin protein ligase, *PARK2*), to some extent, is associated with both glaucoma and amyotrophic lateral sclerosis (ALS) [25]. In addition, [rs2165241](#) (located in the lysyl oxidase-like protein 1 gene), was observed to confer a risk of glaucoma [24]. It is believed that glycine may be a promising biomarker, while these three

amino acid-associated metabolites and three SNPs may play crucial roles in PCG development.

The role of amino acids has been explored in glaucomatous patients [37]. For example, Dreyer et al. (1996) showed that the level of glutamate was increased in the vitreous body of patients with glaucoma when compared with controls [38]. Meanwhile, in our study, 11 amino acid-associated metabolites, including cystine, glycine, and trans-4-hydroxy-L-proline, were significantly and greatly different in patients with PCG when compared with patients with CCs and ARCs. Especially, the above-mentioned three metabolites (glycine, urea, and phenylalanine) were also belonging to the amino acid super pathway. Moreover, a further metabolic pathway analysis revealed several significantly influenced pathways, all of which are involved in amino acid metabolism. The results here suggest amino acid metabolism may play an important role in the development of PCG, as well as in the progression of other various eye diseases, including high myopia and cataracts [11,39]. For example, aminoacyl-tRNA synthetases has been suggested to play a regulatory role in mitochondrial mutations that have reported in congenital glaucoma with low frequencies (10%–22.85%) [40,41]. This is highly consistent with our results that aminoacyl-tRNA biosynthesis was determined to be the most significantly influenced pathway in patients with PCG when compared to patients with CCs and ARCs.

Altogether, the present study fully revealed metabolic characteristics in relation to PCG. A significantly distinct metabolite profile was observed behind the disease with certain especially active amino acid-associated metabolites and their metabolisms. More importantly, glycine was supposed to be a potential biomarker for PCG screening. In the future, validation work should be done with a large number of clinical samples. In addition, three glaucoma-associated SNPs ([rs7114303](#), [rs9364602](#), and [rs2165241](#)) need further verification by genomic approaches in the next steps.

APPENDIX 1.

List of metabolites detected in the 50 AH samples from 30 patients with PCG, 10 patients with CC and 10 patients with ARC. PCG: primary congenital glaucoma; CC: congenital cataract; ARC: aged-related cataract. To access the data, click or select the words “[Appendix 1.](#)”

APPENDIX 2.

List of 38 significantly different metabolites between patients with PCG and CC/ARC. PCG: primary congenital glaucoma; CC: congenital cataract; ARC: aged-related cataract. To access the data, click or select the words “[Appendix 2.](#)”

APPENDIX 3.

Metabolic pathway analysis of 38 significantly different metabolites between patients with PCG and CC/ARC by using MetaboAnalyst web server. PCG: primary congenital glaucoma; CC: congenital cataract; ARC: aged-related cataract. To access the data, click or select the words “[Appendix 3.](#)”

APPENDIX 4.

List of 290 metabolites detected in the 25 AH samples from 15 patients with PCG and 10 patients with POAG. PCG: primary congenital glaucoma; POAG: primary open angle glaucoma. To access the data, click or select the words “[Appendix 4.](#)”

APPENDIX 5.

Heat map of 290 metabolites detected in 25 AH samples by hierarchical cluster analysis. Each line represented one metabolite. The darker the green color, the lower its content in the sample; likewise, the darker the red color, the higher its content in the sample. K represented patients with POAG, while C represented patients with PCG. To access the data, click or select the words “[Appendix 5.](#)”

APPENDIX 6.

List of 54 significantly different metabolites between patients with PCG and POAG. PCG: primary congenital glaucoma; POAG: primary open angle glaucoma. To access the data, click or select the words “[Appendix 6.](#)”

APPENDIX 7.

Metabolic pathway analysis of 54 significantly different metabolites between patients with PCG and POAG by using MetaboAnalyst web server. PCG: primary congenital glaucoma; POAG: primary open angle glaucoma. To access the data, click or select the words “[Appendix 7.](#)”

APPENDIX 8.

Enrichment analysis of three metabolites significantly changed in common between patients with PCG and any other kind of patients (with CC/ARC/POAG) by using MetaboAnalyst web server with single nucleotide polymorphisms

(SNPs)-associated metabolites set library. PCG: primary congenital glaucoma; CC: congenital cataract; ARC: aged-related cataract; POAG: primary open angle glaucoma. To access the data, click or select the words “Appendix 8.”

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