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Article

Oxylipins in Aqueous Humor of Primary Open Angle Glaucoma Patients

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Abstract: Purpose: To investigate the oxylipin profiles in aqueous humor of primary open angle glaucoma (POAG) patients. **Methods:** Aqueous humor samples were collected from 17 POAG patients and 15 cataract subjects and subjected to the liquid chromatography-mass spectrometry (LC-MS) analysis to detect the oxylipins. The prediction potential of the differential abundant oxylipins was assessed by the receiver operating characteristic (ROC) curves. Pathway and correlation analyses on the oxylipins and clinical and biochemical parameters were also conducted. **Results:** The LC-MS analysis detected a total of 76 oxylipins, of which 29 oxylipins reached the detection limit. The multivariate analysis identified 5 differential abundant oxylipins, 15-keto-prostaglandin F2 alpha (15-kPGF2 α), Leukotriene B4 (LTB4), 12,13-Epoxyoctadecenoic acid (12,13-Epome), 15-Hydroxyeicosatetraenoic acid (15-HETE) and 11-Hydroxyeicosatetraenoic acid (11-HETE). The 5 oxylipins are enriched in the arachidonic acid metabolism and linoleic acid metabolism pathways. Pearson correlation analysis showed that 11-HETE was positively correlated with intraocular pressure and central corneal thickness and negatively with cup/disk ratio in the POAG patients. In addition, 15-kPGF2 α was positively correlated with visual field defect, and LTB4 was negatively correlated with macular thickness. **Conclusions:** This study revealed the oxylipin profile in the aqueous humor of POAG patients. Oxylipins involved in arachidonic acid metabolism pathway could play a role in POAG and anti-inflammatory therapies could be potential treatment strategies for POAG.

Keywords: primary open-angle glaucoma; aqueous humor; oxylipins; arachidonic acid

Introduction

Glaucoma is a leading cause of irreversible visual impairment and blindness, with 111.8 million individuals expected to be affected by 2040 [1]. Primary open angle glaucoma (POAG), a common subtype of glaucoma, is characterized by the elevated intraocular pressure (IOP), visual field (VF) defects and progressive loss of retinal ganglion cells (RGCs) [2,3]. Current POAG treatments limited to lowering IOP treatments [1,2]. However, a prospective cohort study [4] reported that 42 out of 179 eyes (23.5%) with well-controlled IOP exhibit continuously progressive visual field loss over a five-year follow-up period, suggesting that additional risk factors could be involved in the development of POAG.

Dysregulation in lipids has been implicated in the development of POAG [5,6]. We previously identified the association of the *CAV1* and *ABCA1* variants, the genes for lipid transfer, with POAG

[7]. We found that the POAG patients have significantly higher plasma triglycerides but lower high density lipoprotein (HDL) cholesterol levels as compared to the cataract subjects. Moreover, we identified that the low density lipoprotein (LDL) subclass, LDL3, small dense LDL, and oxidized LDL(ox-LDL) were significantly higher in the POAG patients with elevated total cholesterol and/or LDL-cholesterol levels [8]. We also demonstrated that ox-LDL can promote the expression of pro-inflammatory cytokines and increased the levels of fatty acid and sphingomyelin metabolites in microglia and macrophages [8]. Critically, we recently revealed that multiple oxylipins, 15-keto-prostaglandin F2 alpha, 13,14-dihydro-15-keto-prostaglandin D2, 11-dehydro-thromboxane B2, 8,9-epoxyeicosatrienoic acid, and arachidonic acid are significantly decreased in the plasma samples of POAG patients [9].As aqueous humor is produced by the ciliary body epithelial cells and contributes to the establishment of IOP [10]. this further study aimed to determine the profiles of oxylipins in the aqueous humor samples of the POAG patients. The metabolic pathways, the correlation with clinical and biochemical parameters, and the prediction potential of the oxylipins were also evaluated.

Materials and Methods

Study Subjects

In total, 17 POAG patients and 15 cataract control subjects were enrolled. The study protocol has been approved by the Ethics Committee for Human Medical Research at the Joint Shantou International Eye Center of Shantou University and The Chinese University of Hong Kong, which is in accordance with the tenets of the Declaration of Helsinki. Written informed consent was obtained from all study subjects after explaining the nature and possible consequences of the study.

The inclusion criteria of the POAG subjects included IOP > 21 mmHg at diagnosis, open anterior chamber angle by gonioscopy, cup-to-disk (C/D) ratio > 0.5 or binocular C/D differences > 0.2. retinal nerve fibre layer (RNFL) thinning, and the visual field defects. The patients with secondary glaucoma and previous glaucoma surgery were not included in this study. The age and sex-matched senile cataract control subjects without glaucoma and other eye diseases were recruited.

The demographic data and the results of the blood test were retrived from the electronic medical records.The disease course, medication history, C/D ratio, and visual field defects of POAG patients are shown in Table 1 and Supplementary Table S1.

Table 1. Demographics of the POAG and control subjects.

	POAG (n = 17)	Control (n = 15)	P
Age (years)	64.71 ± 10.31	66.47 ± 7.74	0.593 ^a
Sex (male/female)	15/2	12/3	0.645 ^b
Height (m)	1.61 ± 0.09	1.64 ± 0.05	0.307 ^a
Weight (kg)	60.90 ± 8.22	64.20 ± 7.86	0.256 ^a
BMI(kg/m²)	24.31 ± 2.50	23.51 ± 2.75	0.399 ^a
SBP (mmHg)	137.88 ± 18.97	133.60 ± 15.56	0.494 ^a
DBP (mmHg)	80.00 ± 12.83	85.73 ± 14.31	0.152 ^a
WBC (*10 ⁹ /L)	7.48 ± 2.55	7.03 ± 2.09	0.738 ^c
Neu (*10 ⁹ /L)	5.08 ± 1.57	4.65 ± 1.90	0.486 ^a
Lym (*10 ⁹ /L)	1.95 ± 0.74	2.27 ± 0.68	0.225 ^a
Mono (*10 ⁹ /L)	1.05 ± 1.91	0.54 ± 0.22	0.317 ^a
Eos (*10 ⁹ /L)	0.13 ± 0.10	0.20 ± 0.14	0.178 ^a
Baso (*10 ⁹ /L)	0.16 ± 0.24	0.07 ± 0.06	0.147 ^a
Glucose (mmol/L)	6.59 ± 2.02	6.02 ± 0.94	0.992 ^a
TC (mmol/L)	4.96 ± 1.08	5.52 ± 0.59	0.090 ^a
TG (mmol/L)	1.18 ± 0.41	1.16 ± 0.50	0.942 ^a
HDL (mmol/L)	1.41 ± 0.29	1.65 ± 0.36	0.041 ^a
LDL (mmol/L)	3.14 ± 0.94	3.37 ± 0.71	0.442 ^a
Apo-A1 (g/L)	1.36 ± 0.27	-	-

Apo-B (g/L)	1.03 ± 0.22	-	-
Apo-A1/Apo-B	0.77 ± 0.14	-	-
LPa (mg/L)	124.58 ± 93.28	-	-

^a Independent T-test; ^b Fisher’s exact test; ^c Mann-Whitney U test. BMI, body mass index; SBP, Systolic blood pressure; DBP, diastolic blood pressure; WBC, white blood cells; Neu, Neutrophils; Lym, Lymphocytes; Mono, Mononuclear cells; Eos, Eosinophils; Baso, Basophilic granulocytes; TC, total cholesterol; TG, triglyceride; HDL, high density lipoprotein-cholesterol; LDL, low density lipoprotein-cholesterol; Apo A-1, Apolipoproteins A-1; Apo B, Apolipoproteins B; LP(α), lipoproteinsα; POAG, primary open angle glaucoma. Continuous variables were presented as mean ± SD according to the normality of the data. Categorical variables were presented as proportion. Statistical test: ^a Student t test. ^b Fisher’s exact test. ^c Mann Whitney U test.

Ophthalmic Examinations and Blood Tests

All study subjects underwent comprehensive ophthalmic examinations, including the refraction, best-corrected visual acuity (in logMAR scale), tonometry, slit-lamp biomicroscopy, gonioscopy, ocular biometry, visual field, and OCT. The IOP was measured using Goldmann applanation tonometry (Haag-Streit, König, Switzerland). The anterior chamber and lens were examined with slit-lamp biomicroscopy (Haag-Streit model BQ-159 900; Haag-Streit). Non-contact partial coherence interferometry (IOL Master V3.01, Carl Zeiss Meditec AG, Jena, Germany) was employed to measure the axial length (AL), central corneal thickness (CCT), and anterior chamber depth (ACD). The visual field defect were evaluated by the Humphrey MATRIX (Carl Zeiss, Germany), and the retinal nerve fibre layer (RNFL) thickness was measured by the Cirrus HD-OCT 4000 (Carl Zeiss, Germany). Fasting peripheral blood samples were collected for routine blood and biochemical tests.

Oxylipins Analysis

The oxylipins analysis was performed by Sensichip Biotechnology Co., Ltd. (Shanghai, China), according to the established procedures. The oxylipins analysis was conducted using the liquid chromatography (LC)/mass spectrometry (MS) platform (Thermo, Ultimate 3000LC, Q Exactive). Chromatographic separation utilized an ACQUITY UPLC HSS T3 column (100 mm × 2.1 mm, 1.8 μm) (Waters Corporation, Milford, MA) with a binary solvent system (solvent A: 0.05% formic acid in water; solvent B: acetonitrile).

The gradient elution program was: 0–1 min, 95% A; 1–12 min, 95% A; 12–13.5 min, 5% A; 13.5–13.6 min, 95% A; 13.6–16 min, 95% A. The column temperature was maintained at 40°C, with a flow rate of 0.3 mL/min and an injection volume of 5 μL. Full-scan mode (m/z range 7-1050) with data-dependent secondary mass spectrometry scanning (TopN = 10) was used, operating in both positive and negative ion modes. The MS parameters were set as: heater temperature = 300°C (+) and 300°C (-); sheath gas flow rate = 45 arb (+) and 45 arb (-); auxiliary gas flow rate = 15 arb (+) and 15 arb (-); sweep gas flow rate = 1 arb (+) and 1 arb (-); spray voltage = 3000 V (+) and 3200 V (-); capillary temperature = 350°C (+) and 350°C (-); and S-Lens RF level = 30% (+) and 60% (-). The compounds were identified based on the retention time, accurate mass, and fragmentation patterns as compared with the authentic standards and database entries (<http://metlin.scripps.edu>).

The oxylipins analysis was conducted using SIMCA-P software (V14.1, Sartorius Stedim Data Analytics AB, Umea, Sweden). Principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA) were applied to determine the differentially abundant oxylipins between the POAG and cataract subjects. In the OPLS-DA permutation test, R² and Q² values indicated the model’s explainability and predictability, respectively. The oxylipins with a variable importance in projection (VIP) score > ±1, P < 0.05, fold change (FC) > 1.5 or < 0.7, and area under the receiver operating characteristic (ROC) curves (AUC) > 0.7 were considered as the differentially abundant oxylipins. Hierarchical clustering maps and the scatter plots were generated using the ggplot package (v.3.3.0). KEGG (<http://www.genome.jp/kegg/>) and MetaboAnalyst (<http://www.metaboanalyst.ca/>) were utilized in the pathway analysis.

Statistical Analysis

The data was presented as mean ± standard deviation (SD). Independent T-test was used to analyze the variables with normal distribution, while non-parametric Mann Whitney U test were used to analyze the variables not following the normal distribution. Categorical data was analyzed by the Fisher’s exact test. Pearson correlation was performed between the clinical and biochemical parameters and the differentially abundant oxylipins. The AUC was calculated to assess the prediction potential of the differential abundant oxylipins. All statistical tests were conducted using IBM SPSS STATISTICS 26 (SPSS Inc., Chicago, IL). $p < 0.05$ was considered as statistically significant.

Results

Demographics and Clinical Examinations of the Study Subjects

The age, sex, height, weight, and body mass index (BMI) of the POAG subjects showed no statistically significant differences as compared to the cataract control subjects (Table 1). For the blood biochemical tests, only high density lipoprotein-cholesterol (HDL-C) of POAG patients (1.41 ± 0.29 mmol/L) was significantly lower than that of the control subjects (1.65 ± 0.36 mmol/L, $P = 0.041$).

There were no statistically significant differences in BCVA, AL, CCT, and ACD between the POAG and cataract subjects (Table 2). The POAG subjects have significantly higher IOP (23.52 ± 8.46 mmHg) than the cataract control subjects (13.53 ± 1.99 mmHg, $P < 0.001$). The retinal and visual field parameters were only recorded in the POAG patients due to the opacity of the refractive medium in the cataract subjects.

Table 2. Clinical parameters of the POAG and control subjects.

	POAG (n = 12)	Control (n = 15)	P
Laterality (R/L)	6/6	10/5	0.452 ^b
BCVA (logMAR)	0.65 ± 0.85	0.24 ± 0.24	0.085 ^c
IOP (mmHg)	23.52 ± 8.46	13.53 ± 1.99	< 0.001 ^{a*}
AL (mm)	23.31 ± 1.05	23.42 ± 0.79	0.751 ^a
CCT (μm)	547.63 ± 46.01	536.46 ± 44.85	0.541 ^a
ACD (mm)	3.10 ± 0.33	3.17 ± 0.27	0.557 ^a
Macular thickness (μm)	237.40 ± 33.25	-	-
RNFL thickness (μm)	58.10 ± 7.99	-	-
C/D	0.78 ± 0.16	-	-
S-pRNFL (μm)	70.20 ± 12.24	-	-
I-pRNFL (μm)	60.50 ± 12.34	-	-
N-pRNFL (μm)	56.10 ± 8.56	-	-
T-pRNFL (μm)	48.40 ± 11.13	-	-
Disc Rim Area (mm ²)	0.69 ± 0.34	-	-
Disk area (mm ²)	1.95 ± 0.35	-	-
Cup volume (mm ³)	0.53 ± 0.43	-	-
VF-MD (dB)	-23.18 ± 5.49	-	-
VF-PSD (dB)	8.17 ± 2.16	-	-

* $P < 0.05$; a Student’s t-test; b Fisher’s exact test; c Mann-Whitney U test. BCVA, best corrected visual acuity; IOP, intraocular pressure; AL, axial length; CCT, central corneal thickness; ACD, anterior chamber depth; RNFL thickness, retinal nerve fibre layer; C/D, cup/disc ratio; S/I/N/T-pRNFL, superior/inferior/nasal/temporals peripapillary retinal nerve fibre layer; VF-MD, visual field main defect; VF-PSD, visual field pattern standard deviation;

Identification of oxylipins in aqueous humor of primary open angle glaucoma subjects

The LC/MS analysis identified a total of 76 oxylipins in the aqueous samples of the POAG and cataract control subjects (Figure 1A), of which 29 reached the detection limit, including arachidonic

acid, cyclooxygenase (COX) oxylipins, lipoxygenase (LOX) oxylipins, and cytochrome 450 (CYP450) oxylipins (Figure 1B and 1C). Compared to the control group, 20 oxylipins, including 15-keto-prostaglandin F2α (15-kPGF2α), 12,13-epoxyoctadecenoic acid (12,13-Epome), and 11-hydroxyeicosatetraenoic acid (11-HETE), were significantly increased and 9 oxylipins, including leukotriene B4 (LTB4) and 15-hydroxyeicosatetraenoic acid (15-HETE), were significantly decreased in the POAG group (Figure 1D).

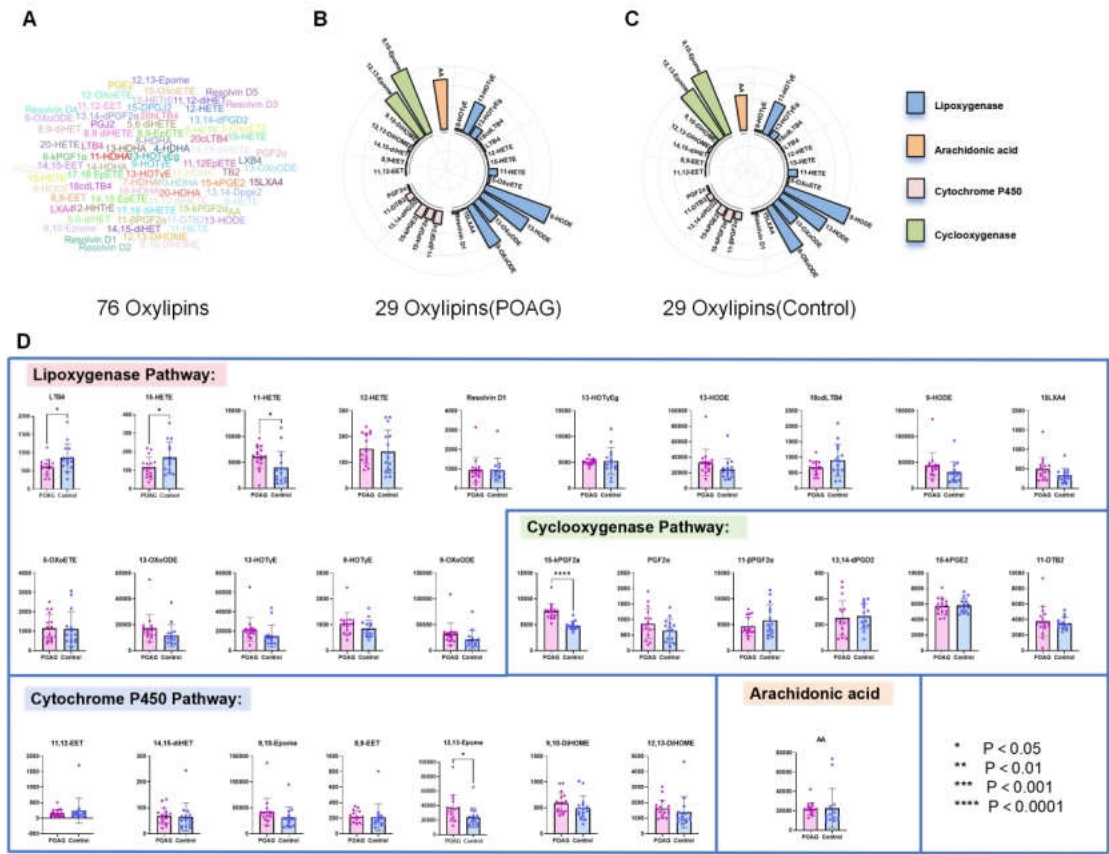


Figure 1. Univariate significance analysis. (A) Word Cloud diagram: a total of 76 oxylipins; Grouped Radiographic Histogram: Classification and specific content of 29 oxylipins in the POAG group (B) and Control (C). Blue represents Lipoxigenase, orange represents arachidonic acid, pink represents cytochrome P450, and green represents cyclooxygenase. (D)Histogram: red represents POAG, blue represents the control group, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

In order to identify the differentially abundant oxylipins, PCA model (Figure 2A) and OPLS-DA (Figure 2B) model were established through multivariate analysis. The multivariate analysis identified 5 oxylipins, 15-kPGF2α ($P < 0.05$, VIP = 2.3, FC = 1.56), 12,13-Epome ($P < 0.05$, VIP = 1.4, FC = 1.53), 11-HETE ($P < 0.05$, VIP = 1.4, FC = 1.51), LTB4 ($P < 0.05$, VIP = -1.3, FC = 0.70), and 15-HETE ($P < 0.05$, VIP = -1.6, FC = 0.68) (Figure 2C and 2D). The AUC analysis showed that 12,13-Epome (AUC = 0.78, $P = 0.033$), 11-HETE (AUC = 0.75, $P = 0.037$), 15-HETE (AUC = 0.70, $P = 0.038$), and LTB4 (AUC = 0.75, $P = 0.033$) showed good prediction performance, while 15-kPGF2α (AUC = 0.97, $P < 0.0001$) showed excellent prediction performance (Figure 2E).

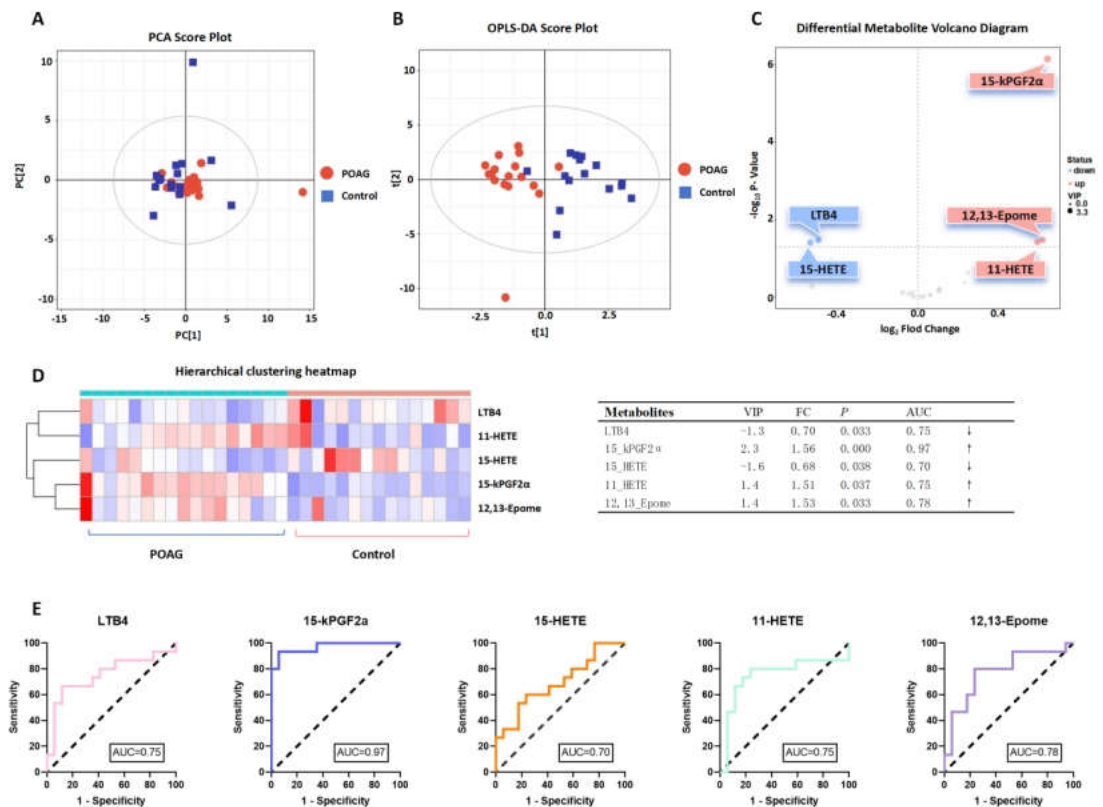


Figure 2. Metabolomic multivariate analysis. (A) Principal component analysis (PCA) plots and (B) orthogonal projections to latent structures–discriminate analysis (OPLS-DA) score plots illustrate the clustering and dispersion of the two groups. The red represents POAG, the blue represents the control group. (C) Volcano plot: Circles represent each differential oxylipins, red represents up regulation and blue represents down regulation. (D) Hierarchical clustering heatmap: demonstrates the distribution of oxylipins in POAG and control groups. Red represents up regulation and blue represents down regulation. (E) ROC curve evaluate the diagnostic performance.

We further evaluated the correlation between the 5 differentially abundant oxylipins and the clinical and biochemical parameters in the POAG subjects. The Pearson correlation showed that LTB4 was negatively correlated with macular thickness ($r = -0.51, P < 0.05$) (Figure 3). Moreover, 11-HETE was positively correlated with IOP ($r = 0.57, P < 0.05$), CCT ($r = 0.57, P < 0.05$), low density lipoprotein-cholesterol (LDL-C; $r = 0.64, P < 0.05$), and TC ($r = 0.63, P < 0.05$), and negatively correlated with C/D ratio ($r = -0.58, P < 0.05$). In addition, 15-kPGF2α was negatively correlated with Apo A1 ($r = -0.58, P < 0.05$) and mononuclear cells ($r = -0.64, P < 0.05$) and positively correlated with visual field ($r = 0.60, P < 0.05$).

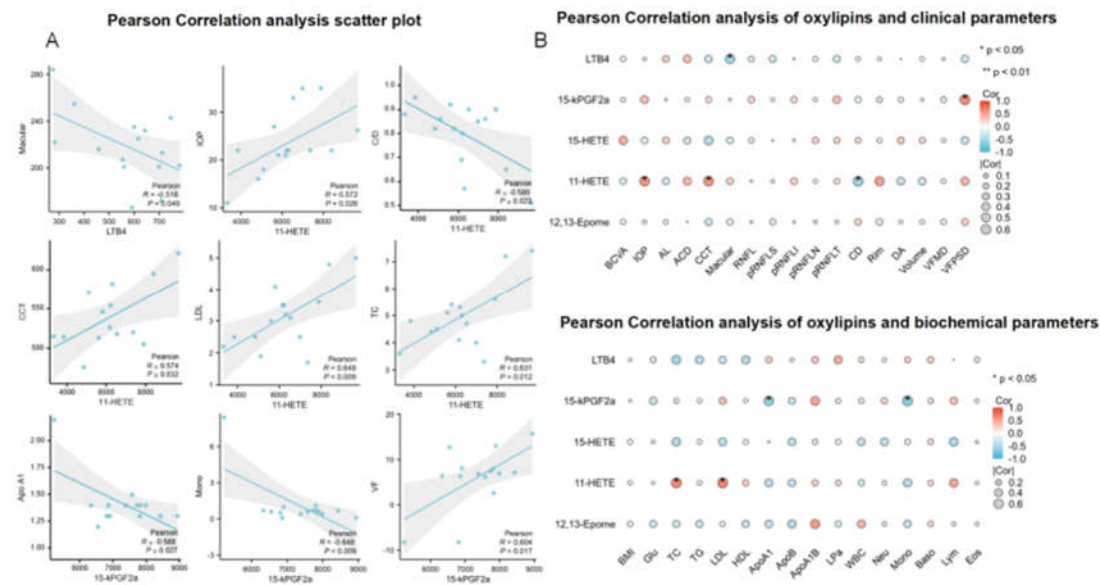


Figure 3. Pearson correlation analysis. (A) Scatter plot and (B) Pearson correlation analysis of oxylipins and clinical & biochemical parameters.

To clarify the pathways of the differentially abundant oxylipins, the Metaboanalyst and KEGG analyses demonstrated that 15-kPGF2 α , 11-HETE, 15-HETE, and LTB4 were enriched in the arachidonic acid pathway, while 12,13-Epome was enriched in the linoleic acid pathway (Figure 4).

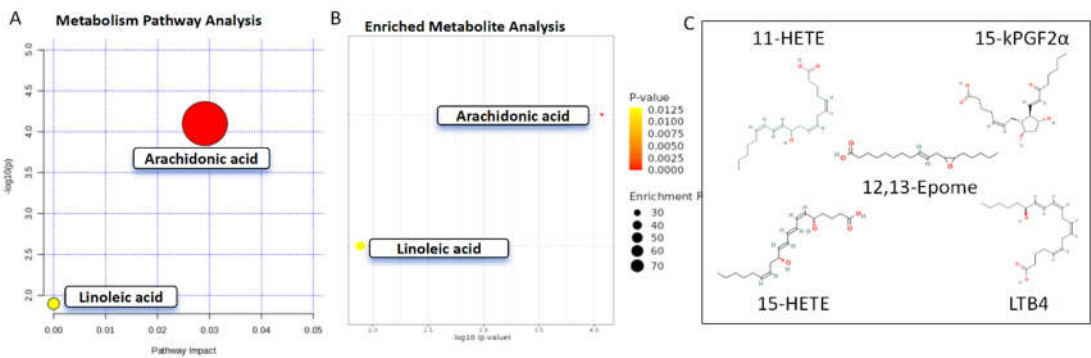


Figure 4. Pathway analysis (A) Bubble plot and (B) Enriched metabolite analysis indicate enrichment pathways in the AH between POAG and control. (C) Molecular structure and oxidation sites of 5 oxylipins.

Discussion

Results of this study demonstrated that: 1) five differentially abundant oxylipins (15-kPGF2 α , LTB4, 12,13-EpOME, 15-HETE, and 11-HETE) were identified in the aqueous humor samples of the POAG subjects; 2) the differentially abundant oxylipins were enriched in the arachidonic acid and linoleic acid pathways; 3) the differentially abundant oxylipins were correlated with the clinical and biochemical parameters in the POAG subjects. Collectively, this study, for the first time, delineated the oxylipin profile in the aqueous humor samples of the POAG patients.

Lipids are the essential cellular components, contributing to cell membrane structure, signal transduction, and regulation of immune inflammation [11]. Oxylipins, the specific lipids mediating oxidative stress and inflammation, reduce the biological activity of the lipids upon oxidation and play significant roles in cardiovascular diseases and neurodegeneration [12]. Previous studies have reported the correlation between POAG and lipids [5,6,14], and our previous study also identified the dysregulation of lipid metabolism and the changes in oxylipins in the plasma of POAG patients

[5,6,13], and our previous study also identified the dysregulation of lipid metabolism and the changes in oxylipins in the plasma of POAG patients [9]. Aqueous humor, directly involved in IOP regulation, offers the direct insights in the metabolic regulations in POAG [14].

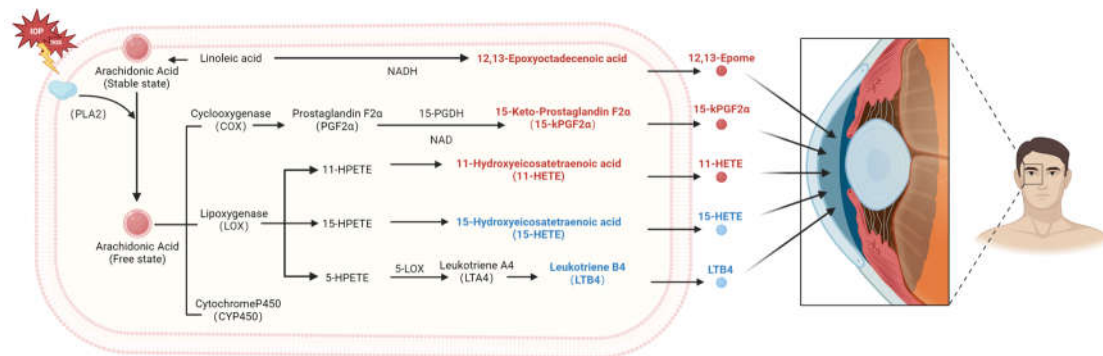


Figure 5. Metabolic pathway profiling. Metabolic pathway profiling: Linoleic acid-Arachidonic acid-Oxylipins.

15-kPGF2 α is generated from arachidonic acid via COX enzyme-mediated oxidation of prostaglandin F2 α (PGF2 α) [15]. PGF2 α binds to FP receptors, and activates the Ca²⁺/IP3 pathway [16], regulating pro-inflammation [17] and IOP [18]. PGF2 α has been reported as a marker of oxidative stress and inflammation [19]. A population study [20] on 670 elderly Swedes found that 15-kPGF2 α responds to PGF2 α levels and correlates with cardiovascular disease incidence and prognosis. In this study, we identified a negative correlation between 15-kPGF2 α and monocytes, suggesting that it may be involved in inflammation regulation. Notably, we have reported 15-kPGF2 α as a differentially abundant oxylipin in the plasma of POAG patients [9]. 15-kPGF2 α has high AUC values (aqueous humor: AUC = 0.97; plasma: AUC = 0.94), suggesting that 15-kPGF2 α could be involved in the development of POAG. However, the mechanisms of 15-kPGF2 α in POAG remain unclear and require further investigations.

Leukotriene B4 (LTB4) is produced from arachidonic acid via the 5-lipoxygenase (5-LOX) and 5-lipoxygenase-activating protein (FLAP) complex [21]. LTB4, by binding to the receptors BLT1 or BLT2, chemotaxis, and activation of neutrophils, promotes the expression of inflammatory factors and is generally considered a potent pro-inflammatory mediator [22]. However, in this study, we found a decrease in LTB4 in the aqueous humor of POAG subjects. Inhibition of LTB4 has been reported to be involved in inflammation alleviation [23,24]. In addition, the correlation analysis revealed a negative correlation between LTB4 and macular thickness.

12,13-Epome is produced from linoleic acid oxidation [25]. It induces mitochondrial dysfunction, promotes oncogene expression, and regulates inflammation [26]. Studies have found that 500 μ M 12,13-Epome significantly induces mitochondrial dysfunction and cell death [27]. In *Escherichia coli*, the inflammatory response induced by 12,13-Epome decreases with the increase of 12,13-DiHOME [28,29]. Notably, 12,13-DiHOME has also been reported in the aqueous humor samples of POAG patients [13]. In this study, we found an increase in 12,13-Epome (Figure 2C). We previously also identified the changes in linoleic acid and α -linolenic acid pathways in the plasma samples of POAG patients [9], indicating that the 12,13-Epome could be involved in POAG, potentially related to mitochondrial dysfunction, oxidative stress, and inflammation.

15-HETE and 11-HETE are conjugated tetraenoic acids produced from arachidonic acid via LOX enzymes [30,31]. 15-HETE and 11-HETE have pro-inflammatory effects, and cause cell damage and pain in arthritis and asthma [32]. In this study, we found a decrease in 15-HETE but an increase in 11-HETE. Pearson correlation analysis indicated that 11-HETE is positively correlated with IOP and CCT, and negatively correlating with C/D ratio. Its specific mechanisms require further research investigations.

Previous POAG metabolomics studies have found that amino acid metabolism [14,33], lipid metabolism [9,13,34] and mitochondrial energy metabolism [35] are involved in POAG. In this study,

we found that oxylipins are related to the inflammatory pathways, which play important roles in POAG.

There are several limitations in this study. First, the sample size is relatively small. Second, disease progression is a dynamic process, and single measurement may not fully reflect the entire metabolic status of the disease in the patients.

In summary, this study identified the oxylipins profile in the aqueous humor of POAG patients. The 5 differentially abundant oxylipins are enriched in the AA and LA pathways, implicating that the inflammation pathway may be involved in POAG.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org.

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